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Effect of Molasses Application Alone or Combined with *Trichoderma asperellum* T-34 on *Meloidogyne* spp. Management and Soil Microbial Activity in Organic Production Systems

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Abstract: The effect of molasses alone or combined with *Trichoderma asperellum* T34 Biocontrol® was assessed on *Meloidogyne* reproduction, disease severity, and density and activity of soil microorganisms in pot and field experiments. Firstly, molasses application at 1 mL m^{-2} was assessed in four different textured soils. Secondly, molasses application at 5, 10, 20, and 40 mL m^{-2}, alone or combined with T34, was assessed in pot and field experiments at 10 mL m^{-2} in two different textured soils. The application of 1 mL m^{-2} of molasses was effective in reducing nematode reproduction in the loam textured soil but not in sandy clay loam, sandy loam, or clay loam textured soils. Increasing molasses dosage reduced the tomato dry shoot and fresh root weights, producing phytotoxicity at 40 mL m^{-2}. The disease severity and nematode reproduction were reduced between 23% and 65% and 49% and 99%, respectively. In the field experiment, molasses applied at 10 mL m^{-2} reduced the disease severity and the nematode reproduction in the loam textured soil. The soil microbial density and activity did not increase in sites where the nematode reproduction and the disease severity were reduced by molasses application, irrespective of T34.

Keywords: *Lactuca sativa* L.; lettuce; organic amendments; root-knot nematodes; *Solanum lycopersicum* L.; tomato

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1. Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are one of the most damaging biotrophic parasites of wild and cultivated plants around the world [1]. Among most of the 100 *Meloidogyne* species described until now, *M. arenaria*, *M. incognita*, and *M. javanica* are responsible for the majority of vegetable crop yield losses caused by this genus [2]. Maximum vegetable yield losses cultivated in plastic greenhouses have been estimated at 37%, 39%, 62%, 88%, and 95% in watermelon, zucchini-squash, tomato, cucumber, and melon, respectively [3–7]. Plant-parasitic nematode (PPN) control has been mainly based on the use of chemical nematicides [8,9]. Nonetheless, the number of chemical active substances available in Europe has been progressively reduced because of its harmful effects on environmental, human, and animal health [10], and its use has been limited to strictly necessary circumstances according to the European Directive 2009/128/CE for the sustainable use of pesticides. Moreover, in some sustainable agricultural production systems, such as organic farming, the use of synthetic pesticides is forbidden and only biologically based pesticides and plant extracts are allowed (Council Regulation (EC) No. 834/2007). In Europe, the area cultivated under organic standards increased by 32% between 2012 and 2020, representing 8.5% of the total agricultural land [11], but the problems caused by PPNs are similar to

or higher than in other agricultural production systems [12]. The main reason for this is that in organic farming there is a constant presence of plants, which prevents nutrient leaching, but leads to maintaining or increasing RKN densities [13]. The application of soil organic amendments, such as animal and plant byproducts is recommended to improve soil fertility and structure, and to enhance soil microbial activity and/or release toxic compounds against PPNs [14–17]. Among the available organic amendments, molasses, a byproduct from the sugar beet or sugarcane industry, has been assessed alone or combined with urea against plant-parasitic nematodes, including *Meloidogyne* spp. [18–23], with variable results. Molasses has also been used as carbon source combined with composted poultry litter in anaerobic soil disinfestation [24]. Moreover, molasses has been used as a substrate for producing microorganisms by liquid fermentation [25], and as a carrier component of formulations of fungal and bacterial antagonists, providing greater multiplication and survival of the biocontrol agents, including *Pochonia chlamydosporia* and *Trichoderma harzianum* [26]. In Spain, some *Trichoderma* species are the active ingredient of commercial formulates registered to be used against plant diseases caused by soil-borne fungi and oomycetes. However, it has been demonstrated that some strains of this fungal species can affect nematode motility, development, reproduction, and egg hatching, and are able to induce resistance in tomato against RKN [27–30]. However, the effect of different molasses dosage applications combined or not with *Trichoderma* to assess the optimal dosage against RKN without affecting the plant health is unknown. Therefore, the aim of the present study was to determine the effect of molasses dosage application alone or combined with *Trichoderma asperellum* T34 on *Meloidogyne* spp. management, the disease severity, and the soil microbial activity and density in pot and field under organic production standards.

2. Materials and Methods

2.1. Effect of Molasses on Meloidogyne spp. Reproduction and Soil Microbial Density

A pot experiment was carried out in a glasshouse located in Viladecans (41°17′21.8″ N 2°02′41.1″ E), from June to September 2016, using previously collected soil from four vegetable organic production sites: Sant Vicenç dels Horts (41°23′21.8″ N 2° 00′56.2″ E), Torrelles (41°21′14.5″ N 1°58′04.8″ E), Begues (41°20′29.4″ N 1°56′16.6″ E), and Martorell (41°28′01.4″ N 1°54′34.8″ E), carrying out field experiments at each site. The physicochemical soil characteristics of each site are summarized in Table 1.

Table 1. Physicochemical soil characteristics of the organic vegetable production sites.

	Sites				
Soil Characteristics	**Sant Vicenç Dels Horts**	**Torrelles**	**Martorell**	**Castellbisbal**	**Begues**
pH	8.2	7.9	7.9	8.1	8.0
E.C (dS m^{-1})	0.44	0.43	0.61	0.22	0.33
Organic Matter %	1.8	2.5	3.0	3.3	3.9
Texture (USDA)	Loam	Sandy clay loam	Sandy loam	Loam	Clay loam
N-NO_3 (mg kg^{-1})	20	11	11	12	34.7
P (mg kg^{-1})	21	88	83	142	50
K (mg kg^{-1})	303	369	474	395	488
Mg (mg kg^{-1})	166	197	285	199	281
Ca (mg kg^{-1})	3088	2332	2814	1679	2429
Na (mg kg^{-1})	152	84	229	87	59

A pot experiment was carried out with soil taken from each of the four sites. At each site, soil samples were taken with a hoe from 0 to 30 cm deep in a zig-zag pattern. Each soil sample was passed through a 4 mm sieve screen to remove roots and stones, homogenized, and mixed with sterilized river sand (1:1 v:v). Afterwards, each soil mixture was placed into 3 L pots, and five 500 cm^3 subsamples were placed in Baermann trays [31] to determine the nematode population density and to adjust it to a 1 second-stage juvenile (J2) cm^{-3} soil mixture (initial population: Pi) per pot 1 day before transplanting a susceptible tomato

cultivar. The nematode inoculum used to achieve the specified Pi consisted of J2 of *M. incognita* obtained from nematode eggs produced in tomato roots. Nematode eggs were extracted from roots by maceration in a 5% of commercial bleach solution (40 g L^{-1} NaOCl) [32] and placed in Baermann trays. J2 hatched during the first 24 h were discarded, and subsequent ones were collected daily and stored at 9 °C until use. The J2 suspension was applied in two opposite holes, 3 cm deep and 6 cm apart, which were covered with the soil mixture, and then 20 mL of water was added on the soil mixture surface. A day after nematode soil inoculation, one three-leaf stage susceptible tomato cv. Durinta per pot was transplanted, and beet molasses (invert sugar: 1.4%; sucrose: 50.4%) (ED & F MAN Liquid products ltd. Ireland) was applied at a rate of 1 mL m^{-2}, also at 19 and 45 days after transplanting. An untreated control receiving the same amount of water was included for comparison. Each soil-molasses application combination was repeated 10 times. Tomato plants were fertilized with a slow release fertilizer [15N-10P-12 K + 2 MgO + trace elements; Osmocote Plus (ICL Specialty, St. Louis, MO, USA)] at a rate of 2 g L^{-1} of soil, and drip irrigated as needed. Soil temperature at 8 cm depth was recorded at 1 h interval using soil probes 5TM (Decagon devices, Inc, Pullman, USA). At the end of the first and the second nematode generation (Tb = 10 °C; K = 600 °C) [33], the aboveground part of five plants were detached from roots and placed in an oven for 2 days at 60 °C to determine the dry shoot weight (DSW). After that, roots were carefully washed, air dried, and weighed to determine the fresh root weight (FRW), and the disease severity was estimated using the galling index in a 0–10 scale [34]. The number of eggs + J2 per plant was determined by extracting them from roots according to the Hussey and Barker procedure [32]. In addition, egg parasitism was assessed from five egg masses per plant using the protocol described by Giné et al. [35]. Briefly, five egg masses were handpicked from the roots of each plant and placed in a watchglass containing sterile distilled water. The egg masses were placed in a 1.5 mL centrifuge tube containing 1 mL of sterile distilled water after removal of the outer part of the gelatinous matrix. Afterwards, eggs were dispersed from the egg masses using a pestle, and 333 µL aliquots of the eggs' suspension were spread onto each of three replicated Petri dishes (9 cm ø) containing a growth-restricting medium [36]. Petri dishes were incubated at 25 ± 0.5 °C. The number of parasitized eggs was recorded after 24 h and 48 h under a dissecting microscope, and the percentage of egg parasitism was calculated as the number of parasitized eggs per plate/number of total eggs per plate. Moreover, the culturable soil microbial density was assessed at the end of each nematode generation. The number of fungal colony-forming units (CFU) was determined by spreading 100 µL of 10^{-3} and 10^{-4} serial dilutions of 10 g of soil onto Rose Bengal + chloramphenicol agar media (VWR International, Leuven, Belgium) supplemented with streptomycin (50 mg L^{-1}) and chlortetracycline (50 mg L^{-1}) incubated at 25 °C in the dark, counting them after 2–5 days. The number of bacterial CFU was determined by spreading 100 µL of 10^{-4} and 10^{-5} serial dilutions onto Luria Bertoni agar (Scharlab, Barcelona, Spain) incubated at 30 °C in the dark, counting them after 2–3 days. The same parameters were assessed at the end of the experiment from the five remaining replications.

Field experiments were conducted at each of the four sites. Plots of 10 m^2 with historical problems caused by root-knot nematodes were selected for soil sampling. Composite soil samples consisting of 10 soil cores were taken from the first 30 cm of soil with a soil auger (2.5 cm diameter). Soil cores were mixed, sieved through a 4 mm sieve screen, and homogenized. Nematodes were extracted from a 500 cm^3 soil subsample placed in Baermann trays [31] for a week and then counted. At each site, plots with similar Meloidogyne spp. densities before transplanting (Pi) were selected to carry out the experiment. The experiment consisted of molasses application at a rate of 1 mL m^{-2} at transplanting after 19 and 45 days, and an untreated control for comparison. Each treatment was repeated five times. At each plot, 30 lettuce cv. Paraday plants, spaced with 30 cm between them, were transplanted. In addition, four susceptible tomato cv. Durinta were transplanted between lettuce plants for assessing the effect of molasses application at the end of the first nematode generation. Molasses was applied by drip irrigation using a Venturi injector. The

non-treated control was irrigated with the same amount of water used for the molasses applications. Soil temperature and water content of soil from each site were recorded daily at 1 h intervals with digital temperature probes 5TM (Decagon devices, Inc, Pullman, WA, USA) placed at 15 cm depth. At the end of the first nematode generation in tomato, plants were uprooted and nematode reproduction (eggs + J2) per plant was assessed. At the end of the lettuce crop, the soil was sampled and the nematodes were extracted from the soil, as previously described, to determine the nematode densities (final population: Pf). The galling index, the percentage of egg parasitism, and the density of culturable bacteria and fungi were assessed as previously stated. To assess the nematode reproduction, the eggs + J2 produced in two groups of 20 g of homogenized lettuce roots were extracted by the Hussey and Barker procedure [32], counted, and expressed as number of eggs + J2 g^{-1} root.

2.2. Effect of Molasses Alone or Combined with T34 Biocontrol on Meloidogyne spp. Reproduction and Soil Microbial Activity

A pot experiment using soil from an organic vegetable production site, Castellbisbal (41°28′19.8″ N 1° 57′39.5″ E), was conducted from June to July 2017. The experiment was carried out in the same place and following the same procedure described previously and before starting field experiments at two sites, Castellbisbal and Martorell (41°28′01.4″ N 1°54′34.8″ E). The physicochemical soil characteristics of each site are summarized in Table 1.

The pot experiment consisted of three treatments: molasses, molasses and T34 Biocontrol® (*T. asperellum* isolate T34; 1×10^{12} CFU kg^{-1}), and an untreated control. Molasses was applied at 0, 5, 10, 20, and 40 mL m^{-2} just after transplanting the susceptible tomato cv. Bodar and then weekly. T34 Biocontrol was applied at 0.5 g m^{-2} to the substrate of the tomato plants in the polystyrene tray 1 day before transplanting and just after transplanting at 0.01 g L^{-1} of soil mixture. Each treatment was repeated 10 times. At the end of the experiment, 6 weeks after nematode inoculation, the dry shoot weight (DSW), the galling index, and the nematode reproduction were determined as previously described. In addition, the soil microbial activity was measured by fluorescein diacetate (FDA) hydrolysis, following the Fernández et al. [37] procedure.

Field experiments were carried out at the Martorell and Castellbisbal sites following the same criteria described previously. The experiment was conducted from August to October 2017 and consisted of three treatments: molasses application at a rate of 10 mL m^{-2} at weekly intervals, molasses application at a rate of 10 mL m^{-2} at weekly intervals and T34 Biocontrol applied to the plants 1 day before transplanting in the polystyrene tray at 0.5 g m^{-2} and after transplanting at 500 g ha^{-1} by drip irrigation, and an untreated control. Each treatment was repeated four times at Martorell and five at Castellbisbal. The plot size, the plant number and distribution of both lettuce cv. Paraday and tomato cv. Bodar, the molasses or T34 Biocontrol application system, the assessment procedures of nematode densities in soil and in tomato and lettuce roots, and disease severity were the same as those described in the previous field experiment. The assessment of soil microbial activity was performed as described in the pot experiment.

2.3. Statistical Analyses

Statistical analyses were performed using nonparametric analysis. Parameters were compared between treatments with the Wilcoxon rank test for pairwise comparisons or the Krustal–Wallis test for more than two treatments. In addition, the relationship between molasses dosage alone or combined with T34 Biocontrol and the tomato dry shoot weight, fresh root weight, galling index, and number of eggs per plant were submitted to regression analysis and compared.

3. Results

3.1. Effect of Molasses on Meloidogyne spp. Reproduction and Soil Microbial Density

The daily minimum, maximum, and average soil temperatures during the 13 weeks of the experiment were 14.1, 37.5, and 25.9 °C, respectively. The molasses application onto the Begues, Martorell, or Torrelles soil mixtures did not significantly affect ($p < 0.05$) the nematode reproduction (number of eggs + J2 per plant), the disease severity, or the percentage of egg parasitism, but it did in the Sant Vicenç dels Horts soil mixture. At the end of the first nematode generation, the nematode reproduction significantly increased in plants cultivated in pots treated with molasses, and also significantly increased the tomato FRW, the disease severity, and the nematode reproduction at the end of the experiment. Soil microbial density was not influenced by molasses treatment, irrespective of the soil mixture ($p < 0.05$) (Table 2).

Table 2. Effect of 1 mL m^{-2} of molasses application at transplanting the tomato cv. Durinta and after 19 and 45 days of cultivation on the plant dry shoot weight (DSW), fresh root weight (FRW), galling index (GI), number of eggs + J2 per plant, egg parasitism (%), and soil bacterial and fungal density (CFU) after the completion of 1 and 2 nematode generations in 3 L pots with a soil mixture (soil from the organic fields and sterile river sand 1:1 (v:v)) infested with 1 J2 of *M. incognita* cm^{-3} of soil, maintained in a glasshouse.

		Site							
Parameter	**Treatment**	**Sant Vicenç Dels Horts**		**Torrelles**		**Martorell**		**Begues**	
		Genera 1	Genera 2	Genera 1	Genera 2	Genera 1	Genera 2	Genera 1	Genera 2
DSW (g)	Control	6.5 ± 0.2	11.9 ± 3.2	9.3 ± 1.0	18.2 ± 1.4	9.9 ± 0.8	19.6 ± 0.9	12.4 ± 1.0	16.6 ± 0.7
	Molasses	7.0 ± 0.6	12.3 ± 1.2	10.1 ± 0.7	18.1 ± 0.9	11.3 ± 0.3	19.6 ± 0.7	9.8 ± 0.8 *	17.2 ± 0.5
FRW (g)	Control	2.4 ± 0.3	7.3 ± 1.0	4.4 ± 1.3	25.0 ± 12	3.6 ± 0.6	12.0 ± 2.1	6.4 ± 1.1	21.0 ± 5.2
	Molasses	3.6 ± 0.7	11.7 ± 1.1 *	6.7 ± 1.6	26.9 ± 6.0	5.9 ± 0.9 *	10.5 ± 1.6	9.5 ± 2.2	18.2 ± 1.8
GI	Control	3.0 ± 0.0	3.8 ± 0.6	2.8 ± 0.2	4.4 ± 0.4	2.8 ± 0.2	3.6 ± 0.2	3.8 ± 0.2	5.0 ± 0.3
	Molasses	3.2 ± 0.2	5.4 ± 0.2 *	3.4 ± 0.3	4.6 ± 0.4	2.4 ± 0.2	3.4 ± 0.2	3.2 ± 0.2	5.4 ± 0.2
Eggs + J2 ($\times 10^4$) $plant^{-1}$	Control	3.4 ± 0.3	14 ± 6.4	8.5 ± 0.6	56 ± 43	7.6 ± 0.8	47 ± 13	13 ± 1.3	88 ± 38
	Molasses	6.0 ± 1.1 *	60 ± 10 *	11 ± 2.6	144 ± 22	8.0 ± 1.4	26.0 ± 5.3	10 ± 1.6	134 ± 26
Egg parasitism (%)	Control	0 ± 0	2.3 ± 1.3	5.3 ± 1.9	0.4 ± 0.4	0 ± 0	0 ± 0	0 ± 0	2.9 ± 1.5
	Molasses	2.7 ± 0.6	0.8 ± 0.8	5.0 ± 0.5	0 ± 0	2.9 ± 1.5	1.9 ± 1.1	0 ± 0	0 ± 0
CFU bacteria ($\times 10^4$)	Control	36 ± 5.0	72 ± 24	95 ± 26	79 ± 2.6	47 ± 0.1	77 ± 11	55 ± 26	27 ± 23
	Molasses	34 ± 0.5	46 ± 7.0	48 ± 3.0	133 ± 56	30 ± 2	52 ± 23	29 ± 6	43 ± 7
CFU fungi ($\times 10^3$)	Control	1.4 ± 0.6	3.6 ± 0.2	5.0 ± 0.4	8.8 ± 0.5	4.0 ± 0.3	7.9 ± 1.5	12 ± 0.01	8.8 ± 0.8
	Molasses	3.0 ± 0.2	4.8 ± 0.2	3.3 ± 0.6	7.4 ± 0.6	7.8 ± 0.7	8.3 ± 3.2	11 ± 0.01	10 ± 3.7

Data are presented as mean ± standard error of 5 repetitions. Data of each parameter followed by * indicates differences between treatments according to the Wilcoxon test ($p < 0.05$).

In the field experiments, the application of 1 mL m^{-2} of molasses did not affect ($p < 0.05$) the disease severity of the intercropped tomato cv. Durinta at the end of the first nematode generation. However, nematode reproduction was significantly reduced in plants grown in treated plots by 36% and 86% at the Sant Vicenç dels Horts and Martorell sites, respectively. In contrast, nematode reproduction was higher in molasses-treated soil at Torrelles (Table 3). Egg parasitism, between 0.1 and 6.5%, was detected at all sites where enough egg mass were produced, irrespective of the treatment. At the end of the lettuce crop, the disease severity and the nematode reproduction were significantly lower in treated plots at Sant Vicenç dels Horts, but 50% higher at Torrelles ($p < 0.05$). Egg parasitism did not significantly differ between soil treatments at the majority of sites, except at Martorell, where fungal egg parasitism was not detected in treated plots. The percentage of fungal egg parasitism ranged from 0 to 9.4% and 2.4 to 8% in treated and untreated plots, respectively. The density of culturable bacteria in the soil was not affected by the molasses treatment at any site. However, the density of culturable fungi significantly differed at Martorell, being lower ($p < 0.05$) in the treated than in the untreated control plots (Table 4).

Table 3. Effect of 1 mL m^{-2} of molasses application just after crop transplantation and 15 and 45 days later on nematode densities in soil (Pi; Pf) and in roots (nematode reproduction), disease severity (galling index), and egg parasitism (%) in the intercropped tomato cv. Durinta at the end of the first nematode generation, and the lettuce cv. Paraday at the end of the crop in four organic vegetable production sites.

		Parameter							
Field	**Treatment**	**Nematode Density (J2 250 cm^{-3} soil)**		**Galling Index**		**Nematode Reproduction ($\times 10^3$) †**		**Egg Parasitism (%)**	
		Pi	Pf	Tomato	Lettuce	Tomato	Lettuce	Tomato	Lettuce
Sant Vicenç dels Horts	Control	349 ± 10.2	651 ±79 *	4.0 ± 0.1	3.5 ± 0.1 *	1983 ± 132 *	11 ± 1 *	5.6 ± 1.1	8.0 ± 1.4
	Molasses	378 ± 14.8	434 ± 49	3.7 ± 0.1	3.0 ± 0.1	1284 ± 103	6 ± 0.4	6.5 ± 1.0	9.4 ± 1.6
Torrelles	Control	627 ± 162	164 ± 41	4.8 ± 0.3	2.5 ± 0.5	66 ± 23 *	3 ± 0.4 *	3.9 ± 1.5	2.4 ± 0.9
	Molasses	591 ± 116	188 ± 64	4.9 ± 0.3	3.1 ± 0.3	221 ± 57	6 ± 1	4.9 ± 1.1	6.8 ± 1.8
Martorell	Control	154 ± 32	74 ± 35	3.8 ± 0.3	2.6 ± 0.2	254 ± 83 *	13 ± 4	3.7 ± 1.2	4.4 ± 1.1
	Molasses	147 ± 90	11 ± 5	2.8 ± 0.5	3.3 ± 0.7	35 ± 11	28 ± 13	0.1 ± 0.1	0 ± 0
Begues	Control	23 ± 8	1305 ± 368	2.4 ± 0.4	5.6 ± 0.7	7 ± 3	32 ± 3	nd	2.4 ± 0.8
	Molasses	14 ± 4	427 ± 185	2.0 ± 0.2	4.2 ± 0.6	3 ± 1	20 ± 4	nd	4.4 ± 1.4

Data are presented as mean ± standard error of 5 repetitions for nematode densities, 20 for galling index and nematode reproduction in tomato, 40 for galling index and 5 for nematode reproduction in lettuce, and 5 for nematode egg parasitism. Data of each parameter in the same column followed by * indicates differences between treatments according to the Wilcoxon test ($p < 0.05$). nd—not determined. † Tomato: Eggs + J2 $plant^{-1}$; Lettuce: Egg + J2 g $root^{-1}$.

Table 4. Effect of 1 mL m^{-2} of molasses application just after crop transplantation and 15 and 45 days later on soil bacterial and fungal densities (CFU) at the beginning and at the end of the lettuce cv. Paraday crop in four organic vegetable production sites.

		Parameter			
Field	**Treatment**	**Bacterial Density (CFU $\times 10^5$)**		**Fungal Density (CFU $\times 10^3$)**	
		Initial	Final	Initial	Final
Sant Vicenç dels Horts	Control	22.7 ± 5.4	51.8 ± 29.1	10.5 ± 4.3 *	41.3 ± 4.4
	Molasses	29.5 ± 6.3	29 ± 5.6	34.8 ± 3.0	31.7 ± 3.2
Torrelles	Control	12.5 ± 3.3	13 ± 3.2	24.5 ± 2.2 *	16.5 ± 2.0
	Molasses	20.0 ± 2.0	10.4 ± 4.0	46.0 ± 6.8	11.0 ± 6.6
Martorell	Control	2.7± 0.8	2.0 ± 0.8	2.7 ± 1.4 *	10.3 ± 1.0 *
	Molasses	3.1 ± 1.6	1.9 ± 0.8	10.5 ± 2.1	2.0 ± 0.8
Begues	Control	4.1 ± 1.0	3.0 ± 1.0	8.0 ± 1.4	18.0 ± 3.7
	Molasses	2.3 ± 0.6	5.8 ± 1.8	9.9 ± 2.7	15.5 ± 1.0

Data are presented as mean ± standard error of 5 repetitions. Data of each parameter in the same column followed by * indicated differences between treatments according to the Wilcoxon test ($p < 0.05$).

3.2. Effect of Molasses Alone or Combined with T34 Biocontrol on Meloidogyne spp. Reproduction and Soil Microbial Activity

During the 6 weeks of the pot experiment, the daily minimum, maximum, and average soil temperatures were 21.4, 28.8, and 25.5 °C, respectively. Increasing molasses dosage significantly reduced tomato plant biomass, regardless of the T34 Biocontrol application, being dramatically reduced at 40 mL m^{-2}, at which plants showed acute symptoms of phytotoxicity. Therefore, this molasses dosage was excluded from the regression analysis to determine its influence on both disease severity and nematode reproduction. The tomato dry shoot and fresh root weight, disease severity, and nematode reproduction were inversely related to the increasing molasses dosage, irrespective of T34 Biocontrol application (Figure 1). The tomato dry shoot weight was significantly higher when T34 Biocontrol was combined with the molasses application (Intercept $p = 0.0009$; Slope $p = 0.0472$), but the fresh root weight was not influenced (Intercept $p = 0.4873$; Slope $p = 0.9416$). The disease severity was significantly reduced between 23% and 65% with molasses application from 5 to 20 mL m^{-2}, respectively, regardless of the T34 Biocontrol applications (Intercept $p = 0.7508$; Slope $p = 0.2190$). The nematode reproduction was significantly reduced between 49 and 99% with the molasses applications of 5 and 20 mL m^{-2}, and between 83 and 99%,

respectively, when the treatment was combined with T34 Biocontrol (Intercept $p = 0.0358$; Slope $p = 0.3410$). The soil microbial activity was significantly higher in the molasses-treated than in the untreated soil mixture, irrespective of T34 Biocontrol application. In the soil mixture treated with T34 Biocontrol, the microbial activity was generally significantly lower compared to the untreated ones ($p < 0.05$) (Figure 2).

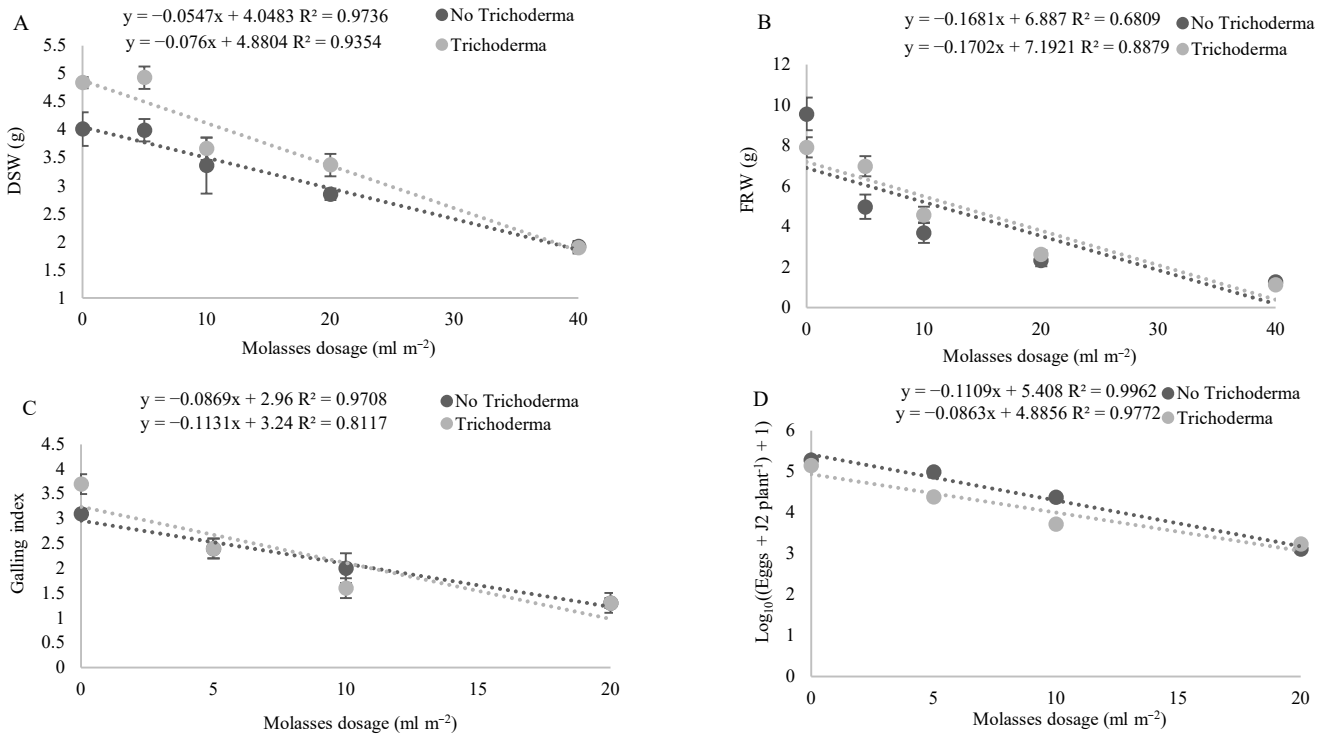

Figure 1. Relationship between the dry shoot weight (DSW) (**A**), fresh root weight (FRW) (**B**), galling index (**C**), and number of eggs + J2 plant^{-1} (**D**) with increasing molasses dosage (0, 5, 10, and 20 mL m^{-2}) alone or in combination with *Trichoderma asperellum* T34 inoculated before and at transplanting the susceptible tomato cv. Bodar in 3 L pots and inoculated with 1J2 cm^{-3} of *Meloidogyne incognita*.

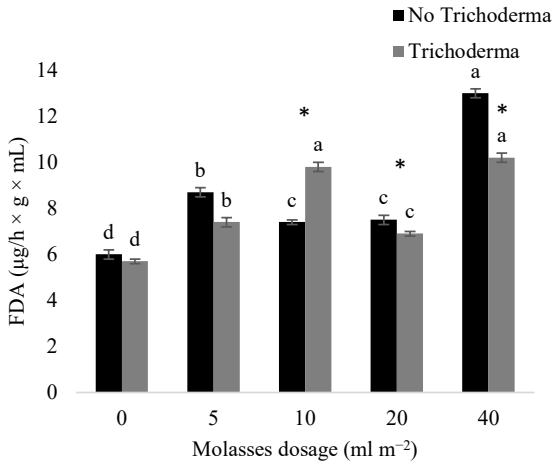

Figure 2. Effect of increasing molasses dosage (0, 5, 10, 20, and 40 mL m^{-2}) alone or in combination with *Trichoderma asperellum* T34 inoculated before and at transplanting the susceptible tomato cv. Bodar in 3 L pots and inoculated with 1J2 cm^{-3} of *Meloidogyne incognita* on the soil microbial activity (FDA). Data are presented as mean ± standard error of 10 repetitions. Data of the different dosages followed by different letters and between *Trichoderma* applications followed by * indicates differences according to the Krustal–Wallis test or the Wilcoxon rank test, respectively ($p < 0.05$).

In the field experiments, the disease severity and nematode reproduction in tomato plants grown at Castellbisbal in plots treated with molasses at a rate of 10 mL m^{-2} alone were significantly lower ($p < 0.05$) in comparison with the untreated control, but not when combined with T34 Biocontrol. However, in the lettuce crop, the nematode significantly reproduced less ($p < 0.05$) in the molasses alone- or combined with T34 Biocontrol-treated plots than in the untreated control ones (Table 5). Significantly lower ($p < 0.05$) soil microbial activity was recorded at the beginning of the experiment in the untreated control plots, but did not differ ($p < 0.05$) between treatments at the end of the lettuce crop (Figure 3). At Martorell, the application of molasses alone or combined with T34 Biocontrol did not affect ($p < 0.05$) the disease severity and nematode reproduction in either tomato or lettuce (Table 5). At the end of the lettuce crop, the application of molasses alone or combined with T34 Biocontrol significantly increased ($p < 0.05$) the microbial activity (Figure 3).

Table 5. Effect of weekly applications of 10 mL m^{-2} of molasses alone or combined with *Trichoderma asperellum* T34 on the nematode densities in soil (Pi; Pf) and in roots (nematode reproduction) and the disease severity (galling index) in the intercropped tomato cv. Bodar at the end of the first nematode generation and the lettuce cv. Paraday at the end of the crop in two organic vegetable production sites.

		Parameter					
Field	**Treatment**	**Nematode Density (J2 250 cm^{-3} soil)**		**Galling Index**		**Nematode Reproduction ($\times 10^3$) ***	
		Pi	Pf	Tomato	Lettuce	Tomato	Lettuce
Castellbisbal	Control	46 ± 13a	513 ± 182a	4.9 ± 0.5a	4.0 ± 0.1a	1455 ± 249a	133 ± 8a
	Molasses	61 ± 17a	10 ± 4b	3.4 ± 0.4b	3.2 ± 0.4b	556 ± 196b	63 ± 10b
	T34-molasses	80 ± 40a	178 ± 103ab	4.3 ± 0.5ab	4.1 ± 0.3a	765 ± 208ab	60 ± 9b
Martorell	Control	111 ± 50a	57 ± 14a	2.5 ± 0.4a	2.4 ± 0.3a	105 ± 38a	8 ± 1a
	Molasses	158 ± 38a	16 ± 9a	2.0 ± 0.4a	2.5 ± 0.2a	102 ± 29a	7 ± 2a
	T-34-molasses	117 ± 60a	26 ± 16a	2.9 ± 0.2a	2.1 ± 0.3a	124 ± 30a	6 ± 3a

Data are presented as mean ± standard error of 5 repetitions for nematode densities, 20 for galling index and nematode reproduction in tomato, and 40 for galling index and 5 for nematode reproduction in lettuce in Castellbisbal and 4 for nematode densities, 16 for galling index and nematode reproduction in tomato, and 36 for galling index and 4 for nematode reproduction in lettuce in Martorell. Data of each parameter in the same column followed by different letters indicates differences between treatments according to the Krustal–Wallis test ($p < 0.05$). * Tomato: Eggs + J2 $plant^{-1}$; Lettuce: Egg + J2 g $root^{-1.}$

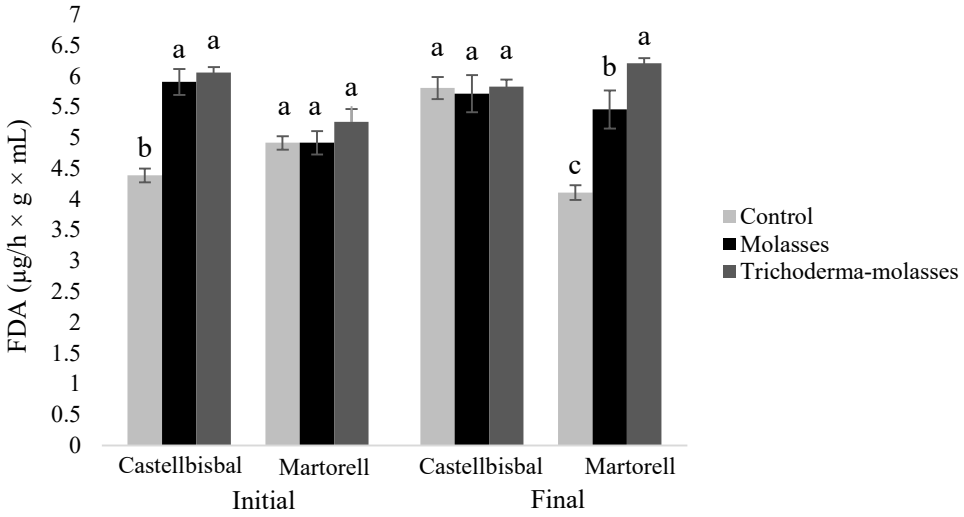

Figure 3. Effect of weekly applications of 10 mL m^{-2} of molasses alone or combined with *Trichoderma asperellum* T34 on the microbial activity (FDA) in the soil, at the beginning and at the end of the crop in two organic vegetable production sites. Data are presented as mean ± standard error of 5 repetitions in Castellbisbal and 4 in Martorell. Data within each site and period followed by different letters indicates differences between treatments according to the Krustal–Wallis test ($p < 0.05$).

4. Discussion

This study provides new insights regarding the effect of molasses application alone or in combination with a commercial formulate of *T. asperellum* on *Meloidogyne* reproduction in tomato and lettuce, disease severity, soil microbial activity and density, and nematode egg parasitism, and its optimal dosage to affect root-knot nematode reproduction without affecting plant growth.

It is hypothesized that the application of molasses can inhibit nematode reproduction to some extent and can increase soil microbial density and activity, improving the antagonistic capability of the soil against plant-parasitic nematodes. The results of our study have shown that the effect of applying molasses alone on nematode reproduction and disease severity is dependent on the site. Indeed, according to the results of the first pot experiment, applying molasses at 1 mL m^{-2} of soil at transplanting and after 15 and 45 days did not reduce either parameter, irrespective of the soil, but it did under field conditions in one out of the four sites in both tomato and lettuce crops. In the second field experiment, in which molasses was applied weekly at 10 mL m^{-2}, the nematode reproduction and the disease severity were reduced in one out of two sites. The soil texture of both sites where molasses application had a *Meloidogyne* inhibitory effect was loam. Therefore, the soil texture could be a factor affecting the effect of molasses application against RKN. In fact, Walker [20] assessed the effect of molasses applied at a rate of 10 mL kg^{-1} of sandy soil on *M. javanica* reproduction, disease severity, and tomato yield, finding no effect. Pattison et al. [21] did not find any effect from applying molasses at a rate of 300 L ha^{-1} on *Radopholus similis* in banana in three soils types: clay, silty clay, and sandy clay textured. Vawdrey and Stirling [19] reported that the weekly application of 10 g of molasses L^{-1} for 12 weeks in a field experiment reduced the disease severity caused by *M. javanica* in tomato, but did not affect either nematode density in a clay loam textured soil or plant biomass. Baños et al. [23] found that the application of 10 L ha^{-1} of molasses 5 days before transplanting a tomato crop in a sandy soil and 21 and 45 days after transplanting reduced the disease severity caused by *Meloidogyne* spp. and increased the tomato yield compared to the untreated control. Thus, further experiments should be carried out to understand the effect of the soil texture on the efficacy of molasses against RKN.

Regarding the effect of molasses on the enhancement of the antagonistic capacity of soil against *Meloidogyne* spp., the results of our study show that neither soil microbial density and activity nor nematode egg parasitism were increased in sites where the nematode reproduction and the disease severity were reduced.

Molasses dosage is poorly standardized and can be misinterpreted, resulting in a lack of nematode control at low doses or leading to phytotoxicity problems at high doses. In our study, the effect of increasing molasses dosage on nematode reproduction, disease severity, and plant biomass was assessed in pot conditions to determine the optimal doses to control the nematode without causing phytotoxicity to the plant. We calculated the dosage taking into account the pot diameter. Under our conditions, the effect on nematode reproduction, disease severity, and plant biomass was inversely related to the molasses dosage. At doses higher than 10 mL m^{-2}, both shoot and root biomass were drastically reduced, and severe symptoms of phytotoxicity were observed at 40 mL m^{-2}. Then, 10 mL m^{-2} (100 L ha^{-1}) was selected to be applied under field conditions. The results obtained with this dosage in the field experiment in the loam textured soil was consistent with those obtained with this soil in the pot experiment.

The combination of molasses with T34 Biocontrol did not increase the effect of the molasses alone against *Meloidogyne* in either pot or field experiments. Some *Trichoderma* strains can act as nematode antagonists affecting egg hatching and nematode motility, as well as nematode reproduction by inducing resistance in tomato against RKN [27–30]. The *T. asperellum* strain T34 induced resistance in tomato against *M. incognita* when it was applied 1 week before transplanting [30], but according to our results, it seems that it was not able to induce it when it was applied at transplanting. However, it promoted the

aboveground tomato plant biomass, conferring tolerance to molasses at dosages lower than 40 mL m^{-2}.

In summary, molasses application reduced *Meloidogyne* reproduction in tomato and lettuce crops when conducted under organic standards in loamy soil, but not in sandy clay loam, sandy loam, or clay loam textured soils. The optimal dose of molasses application for *Meloidogyne* management without affecting the tomato productivity was 10 mL m^{-2}, irrespective of T34 Biocontrol application.

Author Contributions: A.E., N.E., A.G. and F.J.S. conceived and designed the experiments. A.E., S.G., A.L. and M.P. performed the pot and field experiments; A.E. and S.G. analyzed the data and wrote the draft of the manuscript. N.E. and S.H. performed the soil microbial density and activity analyses. F.J.S. supervised the experiments, the data collection, and analyses. A.E., A.G. and F.J.S. wrote the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Jones, J.T.; Haegeman, A.; Danchin, E.G.J.; Gaur, H.S.; Helder, J.; Jones, M.G.K.; Kikuchi, T.; Manzanilla-López, R.; Palomares-Rius, J.E.; Wesemael, W.M.L.; et al. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol.* **2013**, *14*, 946–961. [CrossRef]
2. Hallmann, J.; Meressa, B.H. Nematode parasites of vegetables. In *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*; Sikora, R.A., Coyne, D., Hallmann, J., Timper, P., Eds.; CABI International: Wallingford, UK, 2018; pp. 346–410. [CrossRef]
3. López-Gómez, M.; Giné, A.; Vela, M.D.; Ornat, C.; Sorribas, F.J.; Talavera, M.; Verdejo-Lucas, S. Damage functions and thermal requirements of *Meloidogyne javanica* and *Meloidogyne incognita* on watermelon. *Ann. Appl. Biol* **2014**, *165*, 466–473. [CrossRef]
4. Vela, M.D.; Giné, A.; López-Gómez, M.; Sorribas, F.J.; Ornat, C.; Verdejo-Lucas, S.; Talavera, M. Thermal time requirements of root-knot nematodes on zucchini-squash and population dynamics with associated yield losses on spring and autumn cropping cycles. *Eur. J. Plant Pathol.* **2014**, *140*, 481–490. [CrossRef]
5. Giné, A.; López-Gómez, M.; Vela, M.D.; Ornat, C.; Talavera, M.; Verdejo-Lucas, S.; Sorribas, F.J. Thermal requirements and population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation. *Plant Pathol.* **2014**, *63*, 1446–1453. [CrossRef]
6. Giné, A.; Sorribas, F.J. Quantitative approach for the early detection of selection for virulence of *Meloidogyne incognita* on resistant tomato in plastic greenhouses. *Plant Pathol.* **2017**, *66*, 1338–1344. [CrossRef]
7. Expósito, A.; Pujolá, M.; Achaerandio, I.; Giné, A.; Escudero, N.; Fullana, A.M.; Cunquero, M.; Loza-Alvarez, P.; Sorribas, F.J. Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. *Front Plant Sci.* **2020**, 1742. [CrossRef]
8. Djian-Caporalino, C. Root-knot nematodes (*Meloidogyne* spp.), a growing problem in French vegetable crops. *EPPO Bull.* **2012**, *42*, 127–137. [CrossRef]
9. Talavera, M.; Sayadi, S.; Chirosa-Rios, M.; Salmeron, T.; Flor-Peregrin, E.; Verdejo-Lucas, S. Perception of the impact of root-knot nematode-induced diseases in horticultural protected crops of south-eastern Spain. *Nematology* **2012**, *14*, 517–527. [CrossRef]
10. Sorribas, F.J.; Djian-Caporalino, C.; Mateille, T. Nematodes. In *Integrated Pest and Disease Management in Greenhouse Crops*; Gullino, M.L., Albajes, R., Nicot, P.C., Eds.; Springer: Cham, Switzerland, 2020; pp. 147–174. [CrossRef]
11. EUROSTAT. Available online: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Organic_farming_statistics (accessed on 3 March 2022).

12. Briar, S.S.; Wichman, D.; Reddy, G.V. Plant-parasitic nematode problems in organic agriculture. In *Organic Farming for Sustainable Agriculture*; Nandwani, D., Ed.; Springer: Cham, Switzerland, 2016; pp. 107–122. [CrossRef]
13. Hallmann, J.; Frankenberg, A.; Paffrath, A.; Schmidt, H. Occurrence and importance of plant-parasitic nematodes in organic farming in Germany. *Nematology* **2007**, *9*, 869–879.
14. Akhtar, M.; Malik, A. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: A review. *Bioresour. Technol.* **2000**, *74*, 35–47. [CrossRef]
15. Oka, Y. Mechanisms of nematode suppression by organic soil amendments—A review. *App. Soil Ecol.* **2010**, *44*, 101–115. [CrossRef]
16. McSorley, R. Overview of organic amendments for management of plant-parasitic nematodes, with case studies from Florida. *J. Nematol.* **2011**, *43*, 69.
17. Renco, M. Organic amendments of soil as useful tools of plant parasitic nematodes control. *Helminthologia* **2013**, *50*, 3–14. [CrossRef]
18. Rodriguez-Kabana, R.; King, P.S. Use of mixtures of urea and blackstrap molasses for control of root-knot nematodes in soil. *Nematropica* **1980**, *10*, 38–44.
19. Vawdrey, L.L.; Stirling, G.R. Control of root-knot nematode (*Meloidogyne javanica*) on tomato with molasses and other organic amendments. *Australas. Plant Pathol.* **1997**, *26*, 179–187. [CrossRef]
20. Walker, G.E. Effects of organic amendments, fertilisers and fenamiphos on parasitic and free-living nematodes, tomato growth and yield. *Nematol. Mediterr.* **2007**, *35*, 131–136.
21. Pattison, A.B.; Badcock, K.; Sikora, R.A. Influence of soil organic amendments on suppression of the burrowing nematode, *Radopholus similis*, on the growth of bananas. *Australas. Plant Pathol.* **2011**, *40*, 385–396. [CrossRef]
22. Khan, M.; Saifullah, I.A.; Abbas, A.; Khan, R.A. Effect of sugarcane molasses and ash on the organic management of root-knot nematode *Meloidogyne javanica* in tomato. *J. Entomol. Zool. Stud.* **2016**, *4*, 178–183.
23. Baños, Y.S.; Concepción, A.D.B.; Lazo, R.C.; González, I.A.; Morejón, L.P. Efecto de enmiendas orgánicas y *Trichoderma* spp. en el manejo de *Meloidogyne* spp. *Rev. Bras. Agroecol.* **2010**, *5*, 224–233.
24. Butler, D.M.; Kokalis-Burelle, N.; Muramoto, J.; Shennan, C.; McCollum, T.G.; Rosskopf, E.N. Impact of anaerobic soil disinfestation combined with soil solarization on plant–parasitic nematodes and introduced inoculum of soilborne plant pathogens in raised-bed vegetable production. *Crop Protec.* **2012**, *39*, 33–40. [CrossRef]
25. Papavizas, G.C.; Dunn, M.T.; Lewis, J.A.; Beagle-Ristaino, J. Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopathology* **1984**, *74*, 1171–1175. [CrossRef]
26. Khan, M.R.; Majid, S.; Mohidin, F.A.; Khan, N. A new bioprocess to produce low cost powder formulations of biocontrol bacteria and fungi to control fusarial wilt and root-knot nematode of pulses. *Biol. Control* **2011**, *59*, 130–140. [CrossRef]
27. Sharon, E.; Chet, I.; Spiegel, Y. Improved attachment and parasitism of *Trichoderma* on *Meloidogyne javanica* in vitro. *Eur. J. Plant Pathol.* **2009**, *123*, 291–299. [CrossRef]
28. Medeiros, H.A.D.; Araújo Filho, J.V.D.; Freitas, L.G.D.; Castillo, P.; Rubio, M.B.; Hermosa, R.; Monte, E. Tomato progeny inherit resistance to the nematode *Meloidogyne javanica* linked to plant growth induced by the biocontrol fungus *Trichoderma atroviride*. *Sci. Rep* **2017**, *7*, 1–13. [CrossRef]
29. Martínez-Medina, A.; Fernandez, I.; Lok, G.B.; Pozo, M.J.; Pieterse, C.M.; van Wees, S.C. Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by *Trichoderma* protects tomato against the root knot *nematode Meloidogyne incognita*. *New Phytol.* **2017**, *213*, 1363–1377. [CrossRef]
30. Pocurull, M.; Fullana, A.M.; Ferro, M.; Valero, P.; Escudero, N.; Saus, E.; Gabaldón, T.; Sorribas, F.J. Commercial formulates of *Trichoderma* induce systemic plant resistance to *Meloidogyne incognita* in tomato and the effect is additive to that of the Mi-1.2 resistance gene. *Front. Microbiol.* **2020**, *10*, 3042. [CrossRef]
31. Whitehead, A.G.; Hemming, J.R. A comparison of some quantitative methods for extracting small vermiform nematodes from soil. *Ann. App. Biol.* **1965**, *55*, 25–38. [CrossRef]
32. Hussey, R.S.; Barker, K.R. Comparison of methods of collecting inocula of *Meloidogyne* spp.; including a new technique. *Plant Dis. Rep.* **1973**, *57*, 1025–1028.
33. Ferris, H.; Roberts, P.A.; Thomason, I.J. Nematodes. In *Integrated Pest Management for Tomatoes*; University of California Statewide Integrated Pest Management Project, Ed.; Division of Agriculture and Natural Resources, University of California: Oakland, CA, USA, 1985; pp. 60–65.
34. Zeck, W.M. Rating scheme for field evaluation of root-knot nematode infestations. *Pflanzenschutz Nachr.* **1971**, *24*, 141–144.
35. Giné, A.; Bonmatí, M.; Sarro, A.; Stchiegel, A.; Valero, J.; Ornat, C.; Fernández, C.; Sorribas, F.J. Natural occurrence of fungal egg parasites of root-knot nematodes, Meloidogyne spp. in organic and integrated vegetable production systems in Spain. *BioControl* **2013**, *58*, 407–416. [CrossRef]
36. Lopez-Llorca, L.V.; Duncan, J.M. New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica* **1986**, *32*, 486–489. [CrossRef]
37. Fernández, C.; Rodrıguez-Kábana, R.; Warrior, P.; Kloepper, J.W. Induced soil suppressiveness to a root-knot nematode species by a nematicide. *Biol. Control* **2001**, *22*, 103–114. [CrossRef]