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Factors Determining the Variability of Performance of Bio-Control Agents against Root-Knot Nematodes in Vegetable Plants

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Abstract: The application of management strategies against plant-parasitic nematodes (PPNs), an alternative to the use of toxic nematicides, has become of paramount importance due to the recognized environmental impact. Pre-treatments with bio-control agents (BCAs), such as bio-control fungi (BCF, *Trichoderma* spp.) and arbuscular mycorrhizal fungi (AMF), have been proved to protect many crop plants from endoparasitic sedentary nematodes (ESNs), the most damaging PPN group. However, the use of commercial BCA formulates is not always successful because of an array of variables that influence their performance. One AMF-based and 2 BCF-based commercial formulates were used as soil-drench pre-treatments to protect tomato, egg, and pepper plants from ESN attack. High variability of performance occurred according to the growth stage of treated plants and the amounts of formulates provided per plant. All formulates were highly effective in reducing both root-knot (RKN) and potato cyst (PCN) nematode infection when plants had reached an intermediate growth stage (3.5–5 g plant weight at treatment). However, only specific ranges of doses had to be used. Lower doses were ineffective against nematode attack; higher doses were often toxic to plants. When plants were grown from seeding in BCA-enriched soil, priming against RKNs was even more active. If plants were not challenged by nematodes, BCAs had a low bio-fertilizer effect.

Keywords: AMF; BCF; bio-control agents; resistance induction; sedentary endoparasitic nematodes

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1. Introduction

Plant-parasitic nematodes (PPNs) are small animal parasites of almost all crops worldwide. They all have a protrusible stylet in their mouth apparatus by which they suck cell sap and inject an array of digestive compounds. These compounds are produced by three to five pharyngeal glands and act as effector molecules that alter gene expression and metabolism of the host [1]. PPNs can be divided into two broad categories by their feeding habit. Ectoparasites feed from outside the root at the expense of external cortical cells and live in the root rhizosphere; conversely, sedentary endoparasites enter the root and migrate up to the vascular cylinder where they establish their feeding site, become sedentary, develop into gravid females and reproduce [2]. The most damaging sedentary endoparasitic nematodes are root-knot (RKNs), *Meloidogyne* spp., and potato cyst nematodes (PCNs), *Globodera* spp. Chemical control by using toxic nematicides, which has been the most reliable management strategy against RKNs and PCNs up to the recent past, is gradually being phased out by EU regulations (EC No1107/2009) because of the high impact on human and animal health and environment safety. Therefore, the search for sustainable alternatives to nematicides by the scientific community has become of paramount interest [3].

One of the most promising management strategies is biological control (or bio-control) of nematodes through the action of living organisms defined as bio-control agents (BCAs). BCAs can directly act as antagonists through antibiosis and competition for nutrients or

space or indirectly as inducers of resistance by the activation of the plant immune system [4,5]. The most studied BCAs are: (i) bio-control fungi (BCF), including the well-studied *Trichoderma* spp., a class of opportunistic fungi that may colonize roots of most plants and promote highly effective defense in nematode-infected plants [6,7]; (ii) arbuscular mycorrhizal fungi (AMF) are obligate root symbionts, diffused in most of the soils, that improve plant growth and can alleviate plant damage due to nematode attack [5,8]; (iii) plant growth-promoting rhizobacteria (PGPR), such as some species of *Pseudomonas* spp., *Bacillus* spp., and *Serratia* spp., have been reported to be efficient BCAs against *M. javanica* [9].

In particular, AMF are being tested as bio-fertilizers in sustainable agriculture [10]. The industrial production and large application in sustainable agricultural practices of AMF as bio-fertilizers are gaining credit because of the negative impacts of the overuse of chemical-based fertilizers [11]. At present, there is a large availability of commercial BCA formulates, which, opportunely used, have most recently shown suitable performances against RKNs [5,6]. Variability of performance is an actual concern among specialists and has been associated with the isolate used, the pathogen/parasite to be controlled, different responses of the plant species analyzed, and the different environmental conditions [12]. In this study, three different commercial BCA formulates, two *Trichoderma*-based and one AMF-based, have been used as resistant inducers against RKNs and PCNs in tomato and against RKNs in tomato, eggplant, and pepper grown under glasshouse controlled conditions. Under these conditions, variability of performance against RKNs has been proved to be associated mainly with the growth stage of the treated plants and the amount of formulate provided to plants. Therefore, many reported failures may be simply consequences of the wrong dosage used.

2. Materials and Methods

2.1. Preparation of Plants and Nematode Inoculation

Seeds of the commercial cultivars of tomato (*Solanum lycopersicum* L.) Roma VF, eggplant (*Solanum melongena* L.) Black Beauty, and pepper (*Capsicum annuum* L.) Theos, all susceptible to RKNs, were used in the experiments. Rossol was used as the tomato cultivar resistant to RKNs. Seeds were sown in a sterilized mixture of peat and soil at 23–25 °C in a glasshouse. Groups of seeds were sown in soils enriched with BCA-containing formulates, according to the methods described below. Plantlets were transplanted to clay pots of different sizes according to plantlet age; pots were filled with an autoclaved mixture of loamy soil and sand (1 + 1 by volume). Pots were put in temperature-controlled benches (soil temperature 23–25 °C) located in a glasshouse and provided with a regular regime of 12 h light/day. Plants were regularly watered with Hoagland's solution.

In another set of experiments, plants at different stages of growth were soil-drenched with BCA formulates in pots. The tested growth stages are indicated according to the corresponding plant weights, as follows:

- Seedlings (1.0–2.5 g);
- Young plants (3.5–5 g);
- Adult plants (8.5–10 g).

Field populations of the root-knot nematode *Meloidogyne incognita* (Kofoid et White) Chitw, grown on susceptible tomato in a glasshouse, were used for plant inoculation. The virulent isolate *SM2V* was used to parasitize the resistant tomato cv. Rossol. Nematode populations were species-identified by isozyme electrophoretic patterns of esterase and malate dehydrogenase before being used as inoculum [13]. Living and active second-stage juveniles (J2s) were obtained by incubation of egg masses in tap water at 25 °C. At the third day of incubation, J2s were collected and concentrated by filtering through 500 mesh sieves. Plants were inoculated with 300 J2s each by pouring a few milliliters of a stirring J2 suspension into 2 holes made in the soil at the base of plants. A week before inoculation, plants at different growth stages were soil-drenched with BCAs in pots (100 cm^3 vol.). In the other set of experiments, young plants developed from seeds sown in BCA-enriched soils were transplanted from seedbeds to clay pots (100 cm^3 vol.), which were filled with

untreated soil. After few days needed for recovering from transplanting, young plants were inoculated with nematodes. In few experiments, tomato young plants were inoculated with 300 living J2s of different pathotypes of the cyst nematode *Globodera rostonchiensis*. J2s were recovered from cysts, collected from soils of infected potato, incubated at 22 °C in water.

2.2. Treatments of Plants by BCAs

Three commercial BCAs were used as resistance elicitors against RKNs: (1) Micosat F® (named Myco in the text) was purchased from C.C.S. Aosta, Italy; it is constituted by 40% roots hosting arbuscular mycorrhiza-forming fungi (AMF) and also contains *Trichoderma harzianum* TH 01, *Pochonia chlamydosporia* PC 50 and rhizobacteria, such as *Agrobacterium radiobacter* AR 39, *Bacillus subtilis* BA 41, *Streptomyces* spp., and yeasts (*Pichia pastoris* PP 59); (2) Tusal® was a gift by Certis Europe B.V; it contains *T. asperellum* T25 and *T. atroviride* T11 (1×10^8 CFU/g, each). (3) Tellus™ was a gift by Syngenta Italia S.p.A; it contains *T. asperellum* ICC012 and *T. gamsii* CC080 (3×10^7 CFU/g, each). All BCAs are commercially available as powder formulates. Before use, they were dissolved in a peptone-glucose suspension (0.5 g L^{-1}) and incubated in an orbital shaker at 25 °C for 1–3 days in the dark. Then, they were provided as soil drenches by adding appropriate amounts of stirring aqueous solutions to the potting soil. Average doses of BCA formulates effective as resistance elicitors and producing acceptable fitness costs were set for young plants, as follows:

- Tomato: 1, 0.04, 0.18 g/plant for Myco, Tellus and Tusal, respectively;
- Egg plant: 1, 0.6, 0.4 g/plant;
- Pepper: 1, 0.04, 0.08 g/plant.

Soils for seeding were enriched with 1.2 g kg^{-1} for both Myco and Tellus and 0.6 g kg^{-1} soil for Tusal. In this case, groups of plants were left un-inoculated to test the effect of BCAs on the growing seedlings in the absence of nematode challenge.

After treatment and nematode inoculation, pots were arranged in a randomized complete block design and placed on temperature-controlled benches in a glasshouse.

2.3. Measurements of Plant Growth and Nematode Infection Factors

Plants were harvested 40 or 60 days after inoculation (DAI). BCA effects on nematode infection at 40 DAI refer to the first invasion by the inoculated J2s, which, in the experimental time adopted, are able to develop in gravid females and reproduce by egg laying in gelatinous egg masses (EMs); in addition, when plants were harvested at 60 DAI, information is available also on the second and heavier invasion from the J2s hatched in pot soil from the eggs produced by the first invasion. However, in this case, J2s do not have the time to reproduce, although their massive development into sedentary forms (SFs) produces heavy damage to plants.

BCA effects on plant growth and nematode infection were recorded by means of different measurable factors. The elicitation of highly effective plant defense against nematodes by BCAs may determine plant fitness costs, due to the energy expenditure for defense response, as it has been reported for other biotic attacks [14]. Plant fitness costs were determined as changes in plant weight (PW, expressed in grams) and length (PL, expressed in cm) with respect to not elicited control plants. These measurements were performed as soon as plants were uprooted and roots washed free of soil debris. BCA doses were considered as phytotoxic if some plant mortality occurred and/or significant decrease ($\geq$20% with respect to the average weight of control plants) was recorded in treated plants.

Two root systems were chopped together to have 1 sample for detection of infection factors. Three different sub-samples were obtained and weighed. One sub-sample was used to detect the number of egg masses (EMs); the other ones were used for egg and extractions of sedentary forms (SFs: J3s, J4s and swollen females), respectively. For EM detection, root samples were immersed in a solution (0.1 g L^{-1}) of the colorant Eosin Yellow for at least 1 h in a refrigerator. Red-colored EMs were manually separated from

the roots by forceps under a stereoscope (×6 magnification) and counted. Eggs were extracted by sodium hypochloride, according to the methods described in the work of [15]. Eggs were then counted (1 mL samples) under a stereoscope (×25 magnification). SF extraction was carried out by incubation with pectinase and cellulase enzyme mixture at 37 °C in an orbital shaker to soften the roots. After a brief homogenization in physiological solution, sedentary forms were collected on a 90 µm sieve and counted under a stereoscope (×12 magnification).

Some parameters were calculated according to these counts to determine damage and reproductive potential of nematode infection in BCA-treated plants with respect to untreated control plants. Reproduction rates are shown as (i) numbers of EMs g^{-1} root fresh weight; (ii) the fraction of sedentary forms that were able to reproduce (repr./devel.); (iii) female fecundity (FF, n. eggs/EMs); (iv) reproduction potential (RP, n. eggs/inoculated J2s), that indicates the number of times the initial population multiplied after a crop season. To have a measurable index of the damage caused to plants, the numbers of SFs g^{-1} root fresh weight were measured, which is a more objective and measurable parameter than the commonly used gall index.

Infection level for cyst nematodes was determined as numbers of cysts per 100 g soil.

2.4. Experimental Design and Statistical Analysis

Six different experiments were performed using Myco as an activator, while 3 experiments each were performed with Tellus and Tusal. In each experiment, 6 treated and 6 control untreated inoculated young plants were used. Means of plant growth and infection factors are the results of 9 to 18 replicates. In the experiments in which seeds were sown in BCA-enriched soils, one experiment was performed by using 36 treated and 36 control young plants for tomato and 24 for eggplants and pepper.

Means ± standard deviations of control and treated plants were separated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$), using Excel Software.

3. Results

3.1. Effects of Myco on Plant Fitness and Nematode Infection in Tomato Plants at Different Growth Stages

The same dose of Myco (1 g/plant) produced different effects on plant fitness and nematode infection in tomato, depending on the growth stage of treated plants (Figure 1).

It was toxic to seedlings (approximately 25% reduction in plant weight and length with respect to untreated seedlings) and did not reduce nematode reproduction. Conversely, the same dose reduced both nematode development and reproduction in young plants and slightly increased plant weight (+8%). No evident effects were recorded in adult plants.

3.2. Effects of Myco on Fitness and Nematode Infection on Young Plants of Different Vegetable Species

Since only young tomato plants showed a consistent reduction in nematode infection when treated with Myco 1 g/plant, the same dose was tried with eggplants, pepper, and resistant tomato plants at the same growth stage (Table 1). Resistant tomato plants were infected by a resistant-breaking population of *M. incognita*. Plants were harvested 60 DAI. Generally, Myco reduced nematode reproduction rates (25–40%) on all tested species, although it left unchanged female fecundity, with the exception of pepper. Plant damage, as indicated by the SFs developed in the roots, was almost halved in susceptible tomatoes; however, a consistent reduction was also observed in the other tested species. Apparently, Myco-mediated priming and activated defense reactions did not cause plant fitness costs; rather, in tomato, weight at harvest of Myco-treated plants resulted slightly enhanced, possibly due to the relief of symptoms of nematode parasitism.

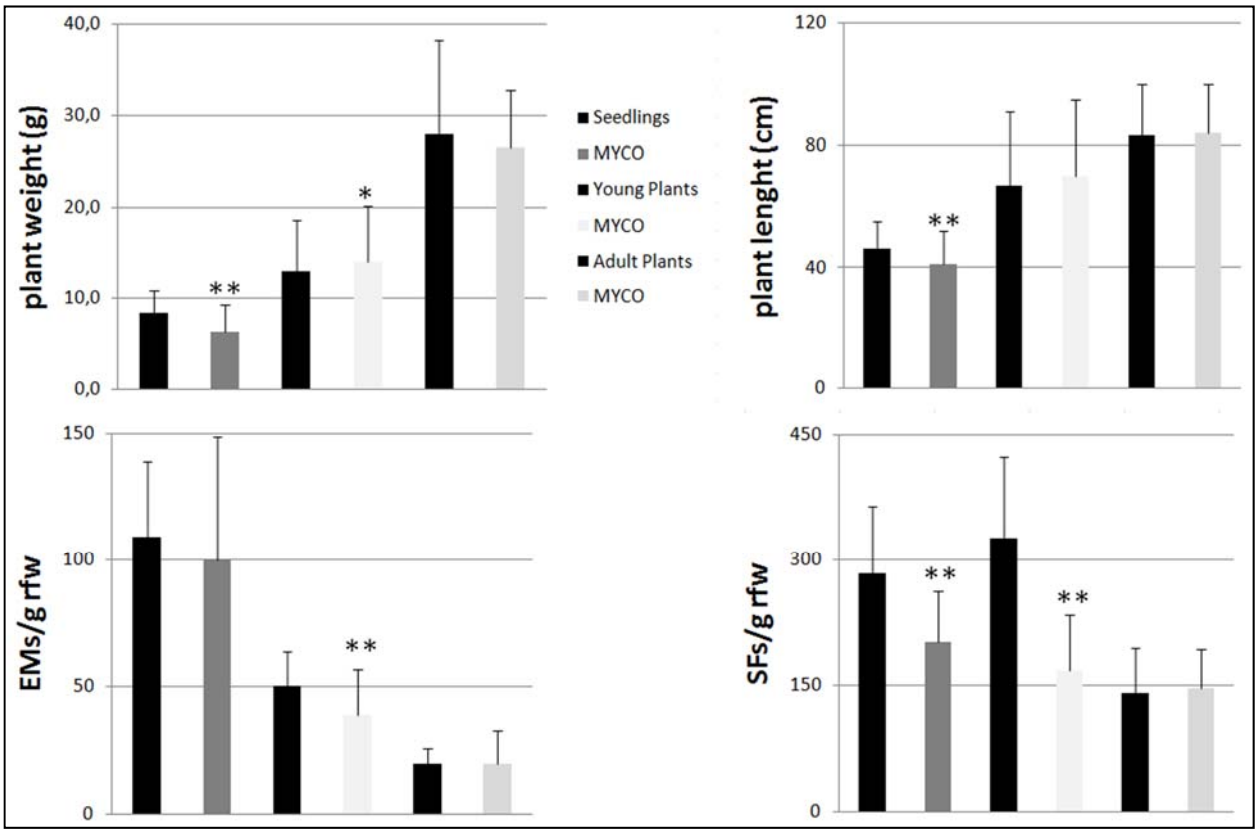

Figure 1. Plant growth and nematode infection factors of tomato seedlings (weight 1.0–2.5 g), young (3.5–5 g), and adult (8.5–10 g) plants untreated and treated with 1 g/plant Myco. Plants were inoculated with RKN J2 and harvested after 60 days. Plant growth was evaluated by plant weight and length, nematode infection by the numbers of egg masses (EMs), and sedentary forms (SFs) per gram of root fresh weight. Values are expressed as means ± standard deviations; means of untreated plants factors were separated from those of Myco-treated plants by a *t*-test (* $p < 0.05$; ** $p < 0.01$).

Table 1. Plant growth and infection factors of RKN-inoculated susceptible and resistant tomato, eggplant, and young pepper plants untreated and treated with 1 g/plant of Myco. Data were taken 60 DAI. Plant growth factors are plant weight in gram (PW) and plant length in cm (PL); infection factors are numbers of egg masses per gram root fresh weight (EMs/g rfw), numbers of sedentary forms per gram root fresh weight (SFs/g rfw), female fecundity (FF), and reproduction potential (RP). Values are expressed as means ± standard deviations that are separated by a *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, the effect of treatments on plant growth and nematode infection, if significant, is expressed as a percentage with respect to the untreated controls (100%).

	PW	PL	EMs/g rfw	SFs/g rfw	FF	RP
Untreated sus. tomato	13.0 ± 5.7	67 ± 24	50 ± 14	326 ± 98	422 ± 91	128 ± 65
Myco-treated	14.1 ± 6.1 * (+8)	70 ± 25	39 ± 18 ** (−22)	167 ± 67 ** (−49)	489 ± 130	82 ± 33 ** (−36)
Untreated res. tomato	14.2 ± 4.6	78 ± 26	42 ± 28	166 ± 79	386 ± 96	116 ± 56
Myco-treated	16.3 ± 4.8 ** (+14)	80 ± 18	32 ± 23 * (−23)	116 ± 57 * (−30)	399 ± 105	72 ± 36 ** (−38)
Untreated eggplant	10.5 ± 4.1	42 ± 6	61 ± 26	263 ± 154	330 ± 159	243 ± 115
Myco-treated	10.7 ± 4.7	44 ± 8	46 ± 20 ** (−25)	207 ± 135 ** (−21)	327 ± 169	168 ± 106 ** (−31)
Untreated pepper	10.3 ± 5.4	48 ± 9	48 ± 23	244 ± 113	264 ± 104	182 ± 102
Myco-treated	10.6 ± 5.0	48 ± 7	31 ± 26 ** (−35)	161 ± 115 ** (−34)	443 ± 186 * (+68)	143 ± 95 * (−21)

3.3. Effects of BCF-Based Formulates on Plant Fitness and Nematode Infection

The commercial formulates Tellus and Tusal were used to test the effect of *Trichoderma* spp. in reducing nematode infection. Young plants of susceptible tomato, eggplant, and pepper, in these experiments, were exposed to soil drenches of opportune BCA doses before nematode inoculation. The most effective dose for each BCA was established after testing

2–3 different dose ranges per species (Table S1). Plant growth and nematode infection data, in this case, were taken 40 DAI, and reproduction rates expressed as the fraction 13of developed sedentary individuals that were able to reproduce (repr./devel., Table 2). Tellus and Tusal treatments were highly effective in reducing nematode infection in tomato and pepper, much less effective in eggplants. Normally, a very consistent reduction in developing nematodes in roots induces an augment of the fraction of reproducing females because of reduced competition for food. This is why decreases in EMs generally appear to be less consistent than those in SFs. Reduction in infection in pepper treated with Tellus was so consistent (approximately 70%) to produce significant fitness costs. Otherwise, less consistent plant responses did not affect plant growth or even increased it due to the lower impact from nematode infection with respect to untreated plants.

Table 2. Plant growth and infection factors of RKN-inoculated susceptible tomato, egg, and pepper young plants untreated and treated with different amounts per plant of Tellus®, and TusalTM. Data were taken 40 DAI. Plant growth factors are plant weight in gram (PW), and plant length in cm (PL); infection factors are numbers of egg masses per gram root fresh weight (EMs/g rfw), numbers of sedentary forms per gram root fresh weight (SFs/g rfw), and the fraction of developed nematodes that reproduced (repr./devel.). Values are expressed as means ± standard deviations that are separated by a *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, the effect of treatments on plant growth and nematode infection, if significant, is expressed as percentage with respect to the controls (100%).

BCA-Plant Interaction	PW	PL	EMs/g rfw	SFs/g rfw	Repr./Devel.
Untreated sus. tomato	8.1 ± 2.6	53 ± 8	37 ± 14	94 ± 49	0.3 ± 0.1
Tellus-treated (0.04 g/plant)	8.1 ± 2.6	50 ± 9 * (−6)	25 ± 15 * (−34)	39 ± 13 ** (−59)	0.5 ± 0.3 * (+51)
Untreated eggplant	8.8 ± 2.5	44 ± 9	38 ± 20	111 ± 50	0.3 ± 0.1
Tellus-treated (0.6 g/plant)	10.5 ± 3.7 ** (+20)	49 ± 11 ** (+12)	32 ± 22 * (−16)	87 ± 47 ** (−21)	0.3 ± 0.1
Untreated pepper	8.9 ± 2.8	43 ± 5	32 ± 12	112 ± 41	0.4 ± 0.1
Tellus-treated(0.04 g/plant)	7.5 ± 2.6 ** (−15)	41 ± 6	12 ± 10 ** (−63)	35 ± 31 ** (−69)	0.5 ± 0.3 * (+23)
Untreated sus. tomato	9.9 ± 3.6	48 ± 11	34 ± 19	108 ± 55	0.35 ± 0.2
Tusal-treated (0.18 g/plant)	11.1 ± 3.7 * (+12)	50 ± 11 * (+6)	24 ± 18 ** (−31)	48 ± 30 ** (−56)	0.55 ± 0.4 * (+56)
Untreated eggplant	7.2 ± 1.5	44 ± 8	54 ± 29	99 ± 43	0.7 ± 0.4
Tusal-treated (0.4 g/plant)	8.0 ± 2.8 * (+12)	47 ± 11 * (+7)	36 ± 17 ** (−34)	74 ± 37 * (−25)	0.6 ± 0.3
Untreated pepper	8.1 ± 2.4	44 ± 5	37 ± 25	103 ± 48	0.4 ± 0.2
Tusal-treated (0.08 g/plant)	8.4 ± 3.1	44 ± 5	24 ± 18 * (−35)	44 ± 31 ** (−57)	0.6 ± 0.2 * (+50)

The effect of Tusal in reducing infection by the other main ESN group, i.e., cyst nematodes, was compared to that of Myco (Figure 2). In this case, the tomato was inoculated by freshly hatched J2s of the cyst nematode *Globodera rostochiensis.* Both formulates were effective in partially reducing final populations of the nematodes, in terms of numbers of cysts in 100 g soil, with respect to untreated control plants.

3.4. Effects on Plant Fitness and Nematode Infection in Plants Grown from Seeds Germinated in BCA-Enriched Soils

Time of colonization allowed in experiments in which BCAs were soil-drenched in pots before nematode inoculation was relatively short (1 week). To test the effect of a more durable relationship between plants and BCA microorganisms, seeds of the tested plants were sown in BCA-enriched soils, and seedlings were allowed to grow in the same media for approximately one month. Then, seedlings were transplanted in pots filled with standard soil and let grow to the young plant stage. At this stage, plants were inoculated with RKNs. Plants from seedlings grown in BCA-enriched soil showed reduced infection factors compared with control plants (Table 3).

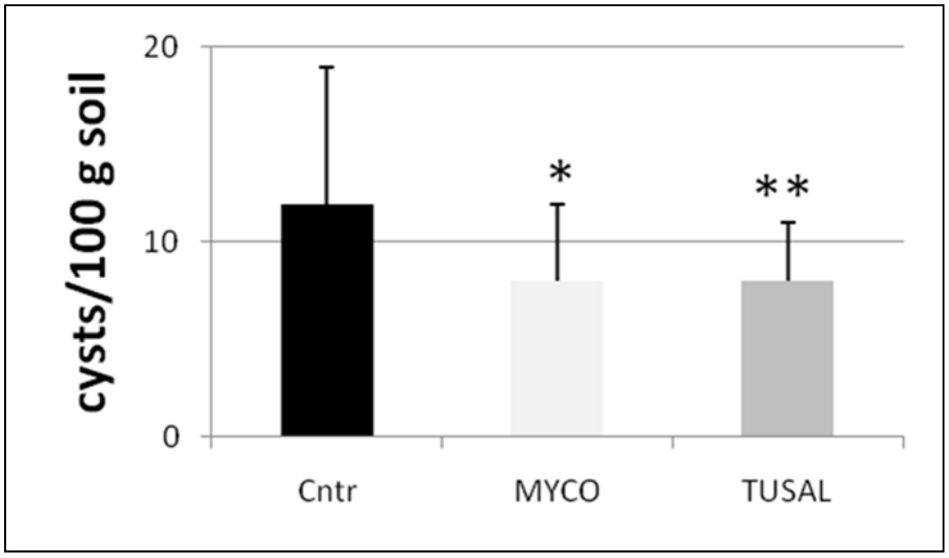

Figure 2. Effect of soil-drench treatments of Myco and Tusal on the numbers of cysts per 100 g soil of tomato plants inoculated with different pathotypes of the potato cyst nematode *Globodera rostochiensis*. Means of Myco- and Tusal-treated plants were separated from that of untreated control plants (Cntr) by a *t*-test (* $p < 0.05$; ** $p < 0.01$).

Table 3. Plant growth and infection factors of RKN-inoculated susceptible tomato, egg, and pepper young plants. Seeds were sown, and seedlings were grown in soils untreated and treated with 0.6 g/Kg soil Tusal and 1.2 g/Kg soil Myco and Tellus. Plants were harvested 60 DAI. Plant growth factors are plant weight in gram (PW) and plant length in cm (PL); infection factors are numbers of egg masses per gram root fresh weight (EMs/g rfw), numbers of sedentary forms per gram root fresh weight (SFs/g rfw), female fecundity (FF), and reproduction potential (RP). Values are expressed as means ± standard deviations that are separated by a *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, the effect of treatments on plant growth and nematode infection, if significant, is expressed as a percentage with respect to the controls (100%).

	Untreated	Myco-Treated	Tellus-Treated	Tusal-Treated
Tomato				
PW	11.1 ± 5.3	9.9 ± 4.0 ** (−11)	9.5 ± 4.3 ** (−14)	10.6 ± 6.5
PL	48 ± 9	48 ± 8	45 ± 7 ** (−7)	45 ± 10 ** (−7)
EMs/g rfw	39 ± 25	25 ± 15 ** (−36)	23 ± 15 ** (−41)	23 ± 16 ** (−41)
SFs/g rfw	239 ± 112	158 ± 52 ** (−34)	110 ± 70 ** (-54)	188 ± 49 ** (−22)
FF	232 ± 136	212 ± 111	234 ± 152	230 ± 114
RP	87 ± 35	51 ± 20 ** (−42)	43 ± 25 ** (−50)	53 ± 33 * (−40)
Eggplant				
PW	7.4 ± 3.0	6.0 ± 1.5** (−20)	8.0 ± 3.2	7.8 ± 2.1
PL	40 ± 7	39 ± 5	42 ± 8 ** (+7)	43 ± 7 ** (+8)
EMs/g rfw	47 ± 26	34 ± 23 * (−26)	23 ± 14 ** (−51)	36 ± 25 ** (−23)
SFs/g rfw	168 ± 45	106 ± 31 ** (−37)	122 ± 36 ** (−27)	102 ± 25 ** (−39)
FF	248 ± 70	268 ± 90	301 ± 105 * (+21)	243 ± 56
RP	139 ± 50	75 ± 33 ** (−46)	73 ± 38 ** (−48)	119 ± 26 * (−15)
Pepper				
PW	9.4 ± 2.2	8.2 ± 0.9* (−13)	7.7 ± 1.1 * (−18)	8.5 ± 1.0
PL	38 ± 4	36 ± 3	35 ± 3	31 ± 3 ** (−19)
EMs/g rfw	52 ± 8	57 ± 16	21 ± 14 ** (−61)	41 ± 14 * (−21)
SFs/g rfw	238 ± 72	139 ± 74 ** (−42)	72 ± 24 ** (−70)	77 ± 26 ** (−68)
FF	201 ± 58	208 ± 56	453 ± 107 ** (+126)	257 ± 110 *
RP	87 ± 38	94 ± 32	75 ± 26	69 ± 24 * (−21)

However, performance was different for different species and formulates. In plants grown in Myco-enriched soil, nematode development was evenly reduced in all tested species (approximately 40%), although the reproduction rate was not lowered in treated pepper. Tomato and pepper plants grown in soils enriched with the *Trichoderma*-containing

formulates, Tellus and Tusal, were generally highly effective in reducing nematode symptoms and reproduction. Eggplants responded less actively to treatments. Fitness costs associated with energy expenditure due to the more active defense response were apparently low; however, they might have been higher considering that treated plants showed much less damage; growth stimulation in healthier plants was probably counterbalanced by the shift of growth metabolism for defense. When the formulates were used as biofertilizers to tomatoes, without nematode challenge, there was no growth increase, except for Tusal; plants grown in Tusal-enriched soil showed a 24% increase in plant weight and 9% increase in plant length at the end of experimental time (Table 4).

Table 4. Plant growth of susceptible tomato young plants. Seeds were sown and seedlings grown in soils untreated and treated with 0.6 g/Kg soil Tusal and 1.2 g/Kg soil Myco and Tellus. Plant growth factors are plant weight in gram (PW) and plant length in cm (PL). Values are expressed as means ± standard deviations that are separated by a *t*-test (** $p < 0.01$). In parentheses, the effect of treatments on plant growth, if significant, is expressed as percentage with respect to the controls (100%).

	Untreated	Myco-Treated	Tellus-Treated	Tusal-Treated
PW	11.2 ± 3.0	12.4 ± 3.2	10.8 ± 2.6	13.9 ± 3.7 ** (+24)
PL	45 ± 7	47 ± 7	46 ± 10	49 ± 8 ** (+9)

4. Discussion

Data presented here confirm that AMF and *Trichoderma*-containing BCAs are able to restrict endoparasitic sedentary nematode infection and reproduction, although this capability is subjected to an array of factors that determine the high variability in performance. As it concerns AMF, variability in performance has been the reason why they have not been considered yet as a routine agricultural practice [12]. It has already been reported that AMF performance as biological control agents depends on the AMF isolate, pathogen/parasite, and plant species involved [16]. According to the results obtained in this study, variability in performance significantly depended on the growth stage of the treated plant and the provided dose of formulate. Given a specific growth stage of plants to test, doses should carefully be screened for the highest protective effect with the lowest fitness costs. High doses can directly be toxic to plants, whereas low doses are ineffective to reduce nematode infection in relation to plant age. Generally, young plants (3.5–5 g) of each tested species responded better to treatments; therefore, most of the data shown here refer to them.

The AMF-containing formulate Myco reduced nematode development in roots of the vegetable species tested. In tomatoes, particularly, numbers of developed sedentary forms were found almost halved with respect to untreated plants. In a previous study, restriction of nematode development was found to be up to approximately 80% in earlier stages of nematode infection [5]. Conidial suspensions of two accessions (T908 and T908-5) of a *Trichoderma harzianum* wild strain were used as soil drenches to induce resistance to RKNs in tomatoes [7]. SFs in roots of treated plants were found to be approximately 25% less than in control plants, in that case. In this study, two commercial formulates containing *T. asperellum* T25 and *T. atroviride* T11 (Tusal), and *T. asperellum* ICC012 and *T. gamsii* CC080 (Tellus) were used. Both formulates were very effective (approximately 60%) in reducing nematode development in tomato and pepper treated plants and less effective (approximately 20%) in eggplants. Tomato and pepper seem to respond to BCA resistance eliciting effect better than eggplants, probably because they can host resistance genes against RKNs [17], and the relative SA-dependent metabolic machinery, which could not be found in eggplant so far. The mechanisms by which nematode infection is limited may be different in tomato and pepper on one hand and eggplants on the other hand. It is important to note that both AMF- and *Trichoderma*-containing BCAs were effective in reducing infection of both RKNs and PCNs.

The involvement of an induced systemic immune response against RKNs, in tomato plants previously treated with Myco, has already been proved and likely triggered at the

contact with the invading juveniles [5]. This type of response is SA-mediated in that it was accompanied by major changes in *PR*-gene expression and seems to have many analogies with mycorrhiza-induced resistance (MIR) [18,19]. The activation of the immune system, with intense protein synthesis and secondary metabolite production, normally conveys energy and solutes from growth to defense. The priming of plants for fast and active defense response to biotic stresses and execution of immunity is commonly associated with fitness costs [14]. In this study, a trade-off between reduced nematode infection by BCA treatments and plant fitness costs did apparently not occur when suitable doses were used. Probably, fitness costs were counterbalanced by the benefits in terms of plant growth elicitation, deriving from the general relief of symptoms shown by infected BCA-treated plants.

Seedlings were grown for one month in soils enriched by BCAs, in order to prolong the time of root colonization before nematode challenge. Then, they were transplanted in pots containing BCA-free soil, where they were inoculated. Actually, the longer duration of symbiosis between AMF/BCF and host roots resulted in a more effective defense reaction against RKNs. Infection factors were even lower than those obtained by soil-drench treatments. However, this relevant effort spent by the colonized plants for defense resulted in significant fitness costs; such costs would probably have been higher if they had not been counterbalanced by the fitness benefits that prolonged colonization with beneficials had brought in terms of restriction of infection and plant damage. The higher the infection suppression, the higher costs of fitness were monitored. Moreover, the same plants grown in BCA-enriched soils did not suffer plant growth limitation if not challenged with nematodes; when BCAs were used just as bio-fertilizers, no change in plant growth was observed except for a positive effect by Tusal.

Although primed plants contain from 30% to 70% fewer developing sedentary stages of nematodes than not primed plants, this reduction is not as correspondingly relevant in terms of reproduction rates and numbers of eggs produced. Fewer females developing in one gram of root means that those females compete less for food and are able either to reach in higher numbers the reproductive state or to augment their fecundity and egg production. For this adaptive mechanism, performance in reducing nematode accumulation in the roots by BCA treatments is hardly achieved in terms of reproduction reduction, although the detected reductions of 30%–50% in reproductive potentials are an acceptable result.

5. Conclusions

Despite the high number of studies reporting a protective effect of both *Trichoderma* and Mycorrhizal fungi against plant-parasitic nematodes [4], none has pointed out the crucial importance of the growth stages of the treated plants and the opportune dose ranges with which each stage should be provided. In this study, it is described that certain dose ranges may be ineffective to assure a detectable protective effect or may even support nematode infection; the highest doses may require too elevated costs of fitness as an exchange to protection or be directly toxic to growing plants. Previous screening of the plant-BCA-nematode interactions is mandatory for obtaining optimal performance from BCAs used as actors of nematode bio-control. A suitable performance may depend on a series of variables, such as plant and beneficial microorganism species and strains; however, the correct amounts of the BCA formulate to provide to the growing plants can be a discriminating factor and should previously be determined. Despite the many reports of the effective protective role of AMF, their use as BCAs in the field is still not a routine practice [20]. As it concerns commercial formulates, in this study, it is suggested that their variable performance is mainly a consequence of unsuitable dosages. Finally, such dosages and variables may be different when provided to potted plants in a glasshouse or to plants growing in open and protected fields or in not potted soil. Determination of the variables and opportune dosages in BCA-treated plants under latter conditions will be the topic of the next investigation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081602/s1, Table S1: Plant growth and infection factors of RKN-inoculated susceptible tomato, egg, and pepper young plants untreated and treated with different amounts per plant of Tellus, and Tusal. Plant weight is expressed in gram (PW) and the determined infection factor was the numbers of sedentary forms per gram root fresh weight (SFs/g rfw). Values are expressed as means ± standard deviations that are separated by a *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, the effect of treatments on plant growth and nematode infection, if significant, is expressed as percentage with respect to the controls (100%). Doses chosen for further investigation are in bold.

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