

# Biostimulant and Nematicidal Effect of *Trichoderma harzianum* Rifai and Aqueous Extract of *Azadirachta indica* A. Juss. in *Solanum lycopersicum* L.

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**Abstract** The experiment was carried out with the objective of evaluating the biostimulating and nematicidal effect of *Trichoderma harzianum* Rifai "strain A-34" and aqueous extract of *Azadirachta indica* A. Juss. (neem) in *Solanum lycopersicum* L. (tomato) cv. "PR-92". Two trials were developed, the first in the seed germination process and the second in potting conditions for the treatments interaction and the inoculation of *Meloidogyne* spp. with tomato seedlings. The results showed that tomato seeds cv. "PR-92" treated with *T. harzianum* "A-34" and aqueous extract of neem leaves did not affect the percentages of germination, however, the combined use of the preparations promote the seedlings growth. It was also corroborated that the seedlings infested with *Meloidogyne* spp. showed lower means for stem length, total fresh mass and dry mass variables, compared to non-infested ones. As for the regulation of *Meloidogyne* spp., a reduction of the gill index was achieved in 53.5%, 38.5% and 61.5% for treatments where *T. harzianum*, neem and their combination were respectively applied with significant differences respect to control.

**Keywords** *Meloidogyne*, Tomato, *Trichoderma*, Neem

## 1. Introduction

The production and fruit quality in tomato crops depends on an adequate control of fungi, phytopathogenic bacteria and insects that damage foliage and fruits, also the root-knot nematodes and weeds that compete with plants for moisture and nutrients [1]. Phytopathogenic nematodes are at the forefront of pests affecting this crop in the world, especially

galls formers (*Meloidogyne* spp.). Moosavi [2] states that cause 12.3% losses in agriculture in the world, and around 5% of losses are attributed to *Meloidogyne* spp.; considered as important pests of many cultivated plants [3]. The root nematode (*Meloidogyne incognita*) is one of the major limiting factors affecting growth and yield, causing an estimated \$ 100 billion loss per year worldwide [4].

At present, the use of biological mediums and botanical preparations are booming as non-chemical alternatives in the management of *Meloidogyne* spp., because they reduce the economic-environmental impact and frequently promote the plants development [5].

One of the biological alternatives for the *Meloidogyne* spp. management is the use of antagonist fungi of the genus *Trichoderma*. Several species of *Trichoderma* have been evaluated as biological control agents against nematodes in various crops and experimental conditions, emphasizing among the mechanisms involved the parasitism, enzymatic lysis, antibiosis and induced resistance [6]. The increase of plant growth, yield and other parameters could be attributed to the release of growth promoting substances by *Trichoderma* spp. and other biological agents studied in the control of *M. incognita* [7].

The use of plants with properties or characteristics that promote the regulation of populations of *Meloidogyne* spp., constitutes a very promissory practice for the management of this pest, especially for the development reached in Cuba urban agriculture. An example of a plant with nematicidal activity is *Azadirachta indica* A. Juss., plant belonging to the family *Meliaceae*, with biological activities widely used in agriculture for the management of insect pests [8] and nematodes [9]. Several authors reported reduction in gill index by *Meloidogyne* spp. through biodesinfection with foliage of *A. indica* [10] and different doses of *A. indica*

wastes [11], although they recommend to deepen the effect over plant growth.

Taking into account the above, the research was carried out with the objective of evaluating the biostimulating and nematicidal effect of *T. harzianum* "strain A-34" and aqueous extract of *A. indica* in *S. lycopersicum*.

## 2. Materials and Methods

The research was carried out at the Microbiology Laboratory and Experimental Areas of the Forestry and Agriculture Sciences Faculty in University of Pinar del Río, Cuba, located at 22° 24'48"N and 83° 41'16' "W, between September and December 2014.

### 2.1. Description of the Experiment I

The effect of *T. harzianum* "strain A-34" and aqueous extract of *A. indica* on germination of tomato seeds was evaluated, considering the treatments described in table 1.

**Table 1.** Description of the treatments in the germination experiment

Number	Treatments	Tag
1	Seeds immersed for two hours in conidial solution of <i>T. harzianum</i> "strain A-34" at a concentration of 2.5% (w/v) of the solid bioprepared with distilled water.	T
2	Seeds immersed for two hours in aqueous extract of leaves of <i>A. indica</i> at 5% (w/v).	N
3	Seeds submerged for two hours in distilled water.	C

The plant material used was *S. lycopersicum* cv. "PR-92". After treatment, the seeds were placed on filter paper for drying in laboratory conditions at 25.6°C temperature. Six hours later they were randomly distributed into Petri dishes of 10 cm diameter, using five plates per treatment and 50 seeds per plate. They were subjected to a dark chamber for 12 hours and subsequently to a light and dark regime at the same period of time up to 94 hours after sowing, with evaluations at six hour intervals.

Germination was quantified at six-hour intervals and the germination percent (GP), germination rate index (GRI) [12] and mean germination time (MGT) [13] were evaluated. Calculations were made by the formulas described below.

$$GP (\%) = \frac{X}{S} * 100 ;$$

$$GRI = \frac{x_1}{t_1} + \frac{x_2}{t_2} + \dots + \frac{x_n}{t_n} ;$$

$$MGT (\text{hours}) = [(x_1 t_1) + (x_2 t_2) + \dots (x_n t_n)] / X_n$$

Legend: (X) number of germinated seeds, (S) total seed, (x1, x2 ... xn) number of germinated seeds in the count corresponding to t1, t2 ... tn, (t) time (hours) elapsed since sowing until the count, (Xn) total germinated seeds at the last count.

### 2.2. Description of the Experiment II.

The experiment was developed in climatic conditions characterized by temperatures between 18.2 and 28.9 °C, with an average of 22.6 °C, and average relative humidity of 79%. The plant material used was *S. lycopersicum* cv. "PR-92". Four treatments were established in *S. lycopersicum* plants with and without inoculation of *Meloidogyne* spp., following a completely randomized design with factorial arrangement (Table 2).

**Table 2.** Treatments evaluated in experiment II.

Number	Treatments	Tag
1	Applications of <i>T. harzianum</i> in <i>S. lycopersicum</i> seedlings.	T
2	Applications of aqueous extract of leaves of <i>A. indica</i> at 5% (w/v) in <i>S. lycopersicum</i> seedlings.	N
3	Applications of <i>T. harzianum</i> and aqueous extract of leaves of <i>A. indica</i> at 5% (w/v) in <i>S. lycopersicum</i> seedlings.	T+N
4	Seedlings of <i>S. lycopersicum</i> without treatment.	C

Twenty pots with a capacity of 900 g of substrate were used in each treatment, 10 with inoculation of nematodes (*Meloidogyne* spp.) in the rhizosphere and the remaining ones without inoculation of nematodes. As a substrate, a mixture of Ferralitic Yellowish Leached Soil [14], peat and rice husk, was used to obtain a ratio of 70: 20: 10, respectively. In soil disinfection, 4% formaldehyde was used for 72 hours, whereas peat and rice husk were sterilized at a temperature of 121°C in an oven during four hours.

Sowing was done manually, placing four seeds per bag, leaving two seedlings per bag after germination with uniformity in the choice of the same.

The evaluations were carried out 35 days after germination, at which time 10 seedlings were randomly extracted from each treatment and processed in the Laboratory of Microbiology at Pinar del Río University. It was determination the galls index [15], shoot length and diameter, leaf number, total fresh mass and total dry mass, foliar and root mass.

### 2.3. Obtaining, Preparation and Application of *Trichoderma* and Neem.

The *T. harzianum* "strain A-34" bioprepared was obtained at the Reproduction of Entomophages Center of "La Conchita", in Pinar del Río municipality. It had a concentration of  $1.9 \times 10^9$  cfu ml<sup>-1</sup>, 100% purity and 96% viability. The applications were carried out three days before planting and two others every seven days, using a dose of 10 ml/pot of conidial solution, obtained at a concentration of 2.5% (w/v) of the solid bioprepared with distilled water.

In the preparation of the aqueous extract was used neem leaf collected from trees located at 22° 25'11 "N 83° 41'17" W, in organoponics of the municipality of Pinar del Río. The foliage was subjected to a drying process under ambient conditions, in the shadow, until losing between 90 and 95%

humidity. The preparation was carried out in a glass vessel, with a proportion of 50 g l<sup>-1</sup> of crushed foliage in distilled water during 8 hours, later the extract obtained was filtered. Three applications were carried out at a rate of 15 ml / pot from five days after germination with a seven day interval.

**2.4. Preparation and Application of the Inoculum of *Meloidogyne* spp.**

Samples of Ferralitic Yellowish Leached Soil [14] were collected from the experimental area of the Pinar del Rio University, where infestation by *Meloidogyne* spp. in *S. lycopersicum* was detected. The population of *Meloidogyne* spp. was extracted through bioassay method by indicator plant, using *Cucurbita maxima*. The analysis of the species composition showed the presence of *M. incognica* and *Meloidogyne* sp. to a less extent. The application of the inoculum was carried out in the rhizosphere five days after germination, guaranteeing 0.5 J<sub>2</sub>-eggs per gram of soil in each pot.

**2.5. Statistical Analysis Employed**

A simple and multivariate analysis of variance was performed using the Duncan Multiple Rank test for mean

comparison, with a confidence level of 95% (p≤0.05). Linear regression analysis was also applied for germination dynamics. The statistical program SPSS version 21.0 for Windows was used.

**3. Results**

In experiment I, germination was initiated 30 hours after sowing for all treatments, with a linear increase up to 66 hours expressed in coefficients of determination (R<sup>2</sup>) higher than 97%, although the germination percent increased significantly at 48, 54 and 60 hours with *T. harzianum* "strain A-34", whereas it was reduced in seeds treated with neem aqueous extract. The germination percent at 66 hours was 91.7%, 86.5% and 84.5% in treatments T, C and N, respectively.

Table 3 shows similar values of germination rate index (GRI) in treatments T, N and C (1.5, 1.29 and 1.41), although the mean germination time (MGT) was affected in the treatment N (51.13), with significant differences regarding T and C (47.2 and 48.8). In addition, similar results were achieved in percentages of germination at 96 hours, with values above 96%.

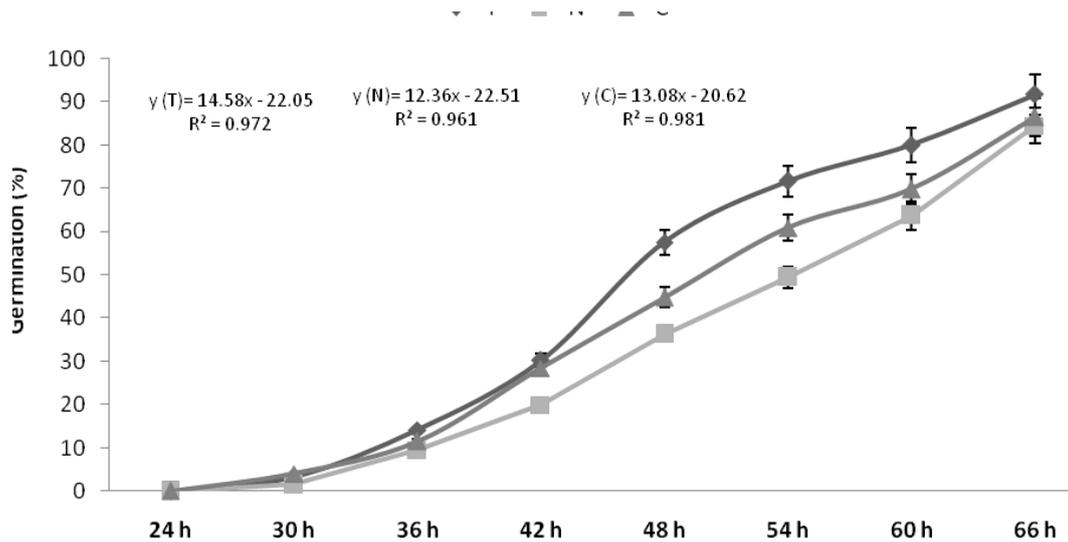


Figure 1. Effect of the treatments in the germination experiment

Table 3. Germination rate index (GRI), mean germination time (AGP) and germination percentage (GP) in treatments evaluated.

Treatments	GRI	AGP (hours)	GP (96 hours)
T	1.50	47.19 b	98.67
N	1.29	51.13 a	96.17
C	1.41	48.78 b	97.05
SE	0.112 ns	0.061 *	0.743 ns

ns: not significant, \* significant for p≤0.05

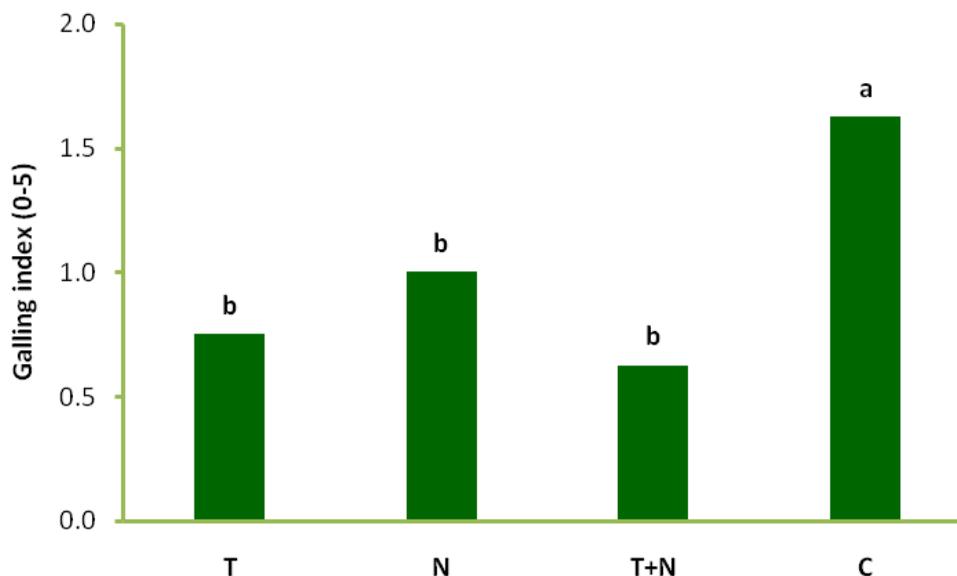


Figure 2. Effect of the treatments in the galls index

In experiment II the galls index was reduced by *Meloidogyne* spp. in 53.5%, 38.5% and 61.5% for T, N and T+N treatment, respectively, with significant differences over control (figure 2), where a galls index of 1.6 grade was determined.

The analysis of variance for the effect of the evaluated factors and their interaction on the growth variables of *S. lycopersicum* cv. "PR-92" is presented in table 4. Except for shoot diameter, leaf number and leaf dry mass, the other variables showed significant differences between infested plants and those not infested with *Meloidogyne* spp. For the treatment factor, there was a significant effect over all variables evaluated. The interaction was only significant for shoot length, indicating strong influence of the factors evaluated on this variable.

Table 4. Analysis of variance (F value) for growth variables (experiment II)

FACTORS	Shoot length (cm)	Shoot diameter (mm)	Leaf number	Total fresh weight (g)	Total dry weight (g)	Leaf dry weight (g)	Root dry weight (g)
Nematode	13.47**	1.95ns	1.12ns	4.21*	5.98*	3.84sn	22.85**
Treatments	14.97**	4.68*	5.12*	10.34**	8.64**	8.71**	6.84**
Nem.*Treat.	2.97*	0.50ns	0.13ns	2.21ns	0.81ns	0.59ns	2.66ns
SE	.323	.005	.079	.144	0.011	0.009	0.002

\*\* and \*: significant differences for  $p \leq 0.01$  and  $p \leq 0.05$ , ns: not significant

Table 5 shows the average values of the growth variables in *S. lycopersicum* cv. "PR-92" with and without nematode inoculation (*Meloidogyne* spp.). It should be noted that all variables reached lower means in infested plants (with nematodes) than in non-infested plants (without nematodes), although the greatest reduction in growth was expressed in shoot length (10.4%), total fresh weight (12.9%), dry weight (17.3%), dry leaf weight (14.2%) and dry weight (36.4%).

Table 5. Effect of nematode inoculation on the growth of *S. lycopersicum* cv. "PR-92"

Growth Variables	With nematodes	Without nematodes	Sig.
Shoot length (cm)	12.790	14.275	.001
Shoot diameter (cm)	.337	.349	.172
Leaf number	3.350	3.500	.297
Total fresh weight (g)	2.905	3.335	.048
Total dry weight (g)	.196	.237	.020
Leaf dry weight (g)	.175	.204	.059
Root dry weight (g)	.021	.033	.000

Table 6 shows the effect of treatments on the growth of *S. lycopersicum* cv. "PR-92". Higher means could be observed in seedlings treated with *Trichoderma* (T) and their combination with neem (T + N), which showed significant differences in reference to those treated with neem (N) and untreated (C). Application of aqueous neem extract did not affect the growth of *S. lycopersicum* cv. "PR-92", since it shows means similar to the control in the evaluated variables.

**Table 6.** Effect of treatments on the growth variables of *S. lycopersicum* cv. "PR-92"

Treatments	Shoot length (cm)	Shoot diameter (cm)	Leaf number	Total fresh weight (g)	Total dry weight (g)	Leaf dry weight (g)	Root dry weight (g)
T	14.26 a	.35 ab	3.70 a	3.61 a	.256 a	.225 a	.030 ab
N	12.46 b	.32 b	3.20 b	2.43 b	.170 b	.151 b	.019 c
T+N	15.38 a	.37 a	3.70 a	3.78 a	.263 a	.228 a	.034 a
C	12.03 b	.33 b	3.10 b	2.66 b	.179 b	.154 b	.025 bc
SE	.323	.005	.079	.144	.011	.009	.002

## 4. Discussion

Seed treatment of *S. lycopersicum* cv. "PR-92" with *T. harzianum* "strain A-34" and aqueous extract of 5% (w/v) neem leaves has caused a linear growth in the germination dynamics, without affecting the germination percentage, even though between 42 and 60 hours after sowing significantly lower means were manifested when the seeds were treated with neem leaf aqueous extract. Germination rate and mean germination time indexes were also verified with little variability, although in the last one it was observed a reduction in seeds treated with *Trichoderma* and an increase of 2.35 hours with aqueous extract of neem leaf. The use of *T. harzianum* "A-34 strain" promote the germination, a result that could be related to the reports on the production of growth factors (auxins, gibberellins and cytokinins) by *T. harzianum*, which are released into the medium and stimulate germination and plant development [16-17], whereas the results with the aqueous extract of neem leaf suggest that the uniformity of germination is not affected. The effect of *T. harzianum* during 15 days after inoculation on passion fruit seeds (*Passiflora adulos* Degener), showed significant increases in germination after four days after inoculation [18]. Recent study on the effect of *T. harzianum* "strain A-34" inoculated in seeds of *S. lycopersicum* cv. "Vyta", reports absolute germination percent higher than control [19].

The use of *T. harzianum* "strain A-34" and aqueous extract of neem leaves evidenced a reduction in the agglutination index by *Meloidogyne* spp., although the infestation provoked with the level of inoculum used is considered low, however it is be observed that agglutination index  $\leq$  to grade 2 caused significant reductions in shoot length, total fresh mass and total and radical dry mass of *S. lycopersicum* cv. "PR-92", which corroborates the phytoparasite action of *Meloidogyne* spp., Although it would be opportune to evaluate different levels of inoculum.

It was also possible to observe higher growth of *S. lycopersicum* cv. "PR-92" in seedlings treated with *Trichoderma* and its combination with aqueous extract of neem, an aspect that is favorable in the search of alternatives that can be integrated in the management of *Meloidogyne* spp. and that at the same time can promote, or not affect, the growth of plants. The results corroborate the biostimulating effect of *Trichoderma* and suggest that aqueous extract concentrations of neem  $\leq$  5% do not affect the growth of *S.*

*lycopersicum* cv. "PR-92".

In the treatments where *T. harzianum* was used, it was reduced the number of galls compared with the control [20]. Several authors report a significant reduction of the agglutination index by *Meloidogyne* spp. with different forms of use of neem preparations in *S. lycopersicum* [2, 11, 21]. Applications of neem cake extract and *T. harzianum* caused a reduction in the number of galls and egg masses in 70.3% and 59.3%, respectively [22].

*Trichoderma* species may induce changes in plant morphology and development, with increasing root and/or foliar biomass being the most common expression of growth promotion [23]. In treatments where *T. harzianum* was applied, increases were reported in relation to the control for the total fresh and dry mass of the plants [7]. There was also an increase in shoot length with different forms of use of neem preparations in tomato plants inoculated with *M. javanica* [21], although there were no significant differences for this variable in *S. lycopersicum* cv. "Campbell 28" when used in biodesinfection of soil infested with *Meloidogyne* spp. [11].

## 5. Conclusions

The results evidenced biostimulating effect of *T. harzianum* "strain A-34" on the germination and growth of *S. lycopersicum* cv. PR-92, whereas the aqueous extract of 5% neem leaf does not limit these processes, although it causes a delay in the seed germination without affecting the germination percentage. A regulatory effect on *Meloidogyne* spp. with the use of *Trichoderma*, neem and their combination suggests deepening the use of these biopreparates as alternatives that offer different benefits when used in the management of plant parasitic nematodes.

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