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Optimum Concentrations of *Trichoderma longibrachiatum* and Cadusafos for Controlling *Meloidogyne javanica* on Zucchini Plants

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Abstract: A factorial experiment was established in a completely randomized design to verify the effect of different inoculum levels of an Iranian isolate of *Trichoderma longibrachiatum* separately and in combination with various concentrations of cadusafos against *Meloidogyne javanica* in the greenhouse. Zucchini seeds were soaked for 12 hr in five densities (0, 10^5, 10^6, 10^7, and 10^8 spores/ml suspension) of the fungus prior to planting in pots containing four concentrations of cadusafos (0, 0.5, 1, and 2 mg a.i./kg soil). The data were analyzed using a custom response surface regression model and the response surface curve and contour plots were drawn. Reliability of the model was examined by comparing the result of new experimental treatments with the predicted results. The optimal levels of these two variables also were calculated. The interactive effects of concentrations of *Trichoderma* and cadusafos were insignificant for several responses such as the total number of eggs per gram soil, the number of intact eggs per gram soil, nematode reproduction factor, and control percent. Closeness of experimental mean values with the expected values proved the validity of the model. The optimal levels of the cadusafos concentration and *Trichoderma* concentration that caused the best plant growth and lowest nematode reproduction were 1.7 mg a.i./kg soil and 10^8 conidia/ml suspension, respectively.

Key words: biological control, cadusafos, *Cucurbita pepo*, Javanese root-knot nematode, management, nematicide, nematophagous fungi.

Plant-parasitic nematodes damage more than 12% of worldwide agricultural products (Nicol et al., 2011), which is equal to 157 billion U.S. dollars in monetary terms (Singh et al., 2013). About 5% of all plant losses are caused by root-knot nematodes either as yield loss or quality loss (Agrios, 2005). *Meloidogyne javanica* (Treub) Chitwood has a widespread distribution in Iran (Moosavi, 2012) and often causes considerable losses to many crops including zucchini (*Cucurbita pepo* L.) (Ghaderi et al., 2012).

The current management method for *M. javanica* is the use of chemical nematicides, which can have adverse effects on the environment and human life (Moosavi and Zare, 2015). Cadusafos (Rugby 10 G; FMC Corporation, Philadelphia, PA) is one of the most effective nematicides against root-knot nematodes in Iran that is widely used at the dose of 50 to 60 kg/ha in zucchini farms. There is a need to reduce the application of these harmful compounds by finding some alternative methods like biological control (Davies and Spiegel, 2011; Moosavi and Askary, 2015). Efficacy of various biocontrol agents for controlling nematodes is dissimilar to chemical control, because none of them can suppress phytonematode populations rapidly (Cumagun and Moosavi, 2015). Perhaps one approach to nematode control would be the integration of chemicals with biocontrol agents where the nematicide prevents initial nematode injury and the biocontrol agent provides long-term protection (Moosavi and Zare, 2012). Knowledge on the use of chemical nematicides combined with fungal biocontrol agents is limited (Hildalgo-Diaz and Kerry, 2008; Timper, 2014) and more studies are essential.

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Trichoderma species are an important group of biocontrol agents that can antagonize a wide range of economically important plant pathogens including plant-parasitic nematodes (Sharon et al., 2011). *Trichoderma longibrachiatum* Rifai is a cosmopolitan soil fungus (Samuels et al., 2012) that recently has shown good potential in management of *M. javanica* (Al-Shammari et al., 2013) and *Heterodera avenae* Wollenweber (Zhang et al., 2014).

This research was designed (i) to determine the effect of different inoculum levels of an indigenous isolate of *T. longibrachiatum* against *M. javanica* on zucchini plants and (ii) to optimize the concentrations of fungus and nematicide when they are applied simultaneously for controlling *M. javanica*.

Materials and Methods

Fungal isolate and inoculum preparation: An indigenous strain of *T. longibrachiatum* (MIAU 143 C), obtained from the fungi culture collection of the Plant Pathology Department, Marvdasht Branch, Islamic Azad University, was selected for this experiment. The fungus was previously isolated from a *M. javanica* infested farm (Estahban, Fars, Iran). The fungus was grown on potato dextrose agar at 25°C for 6 d in darkness. The conidia were collected by adding 3 ml sterile distilled water to each petri dish and shaking for a few minutes, followed by scraping the agar surface with a sterile glass rod. After filtering the suspension through a Whatman paper No. 3 and 0.22-mm Millipore membranes (Zhang et al., 2014) the conidial concentration in the suspension was counted using a haemocytometer (Precicolor HBG, Lützellinden, Gießen, Germany) by averaging three counts. The final conidial concentration was adjusted to 10^5, 10^6, 10^7, and 10^8 spores/ml and stored at 4°C.

Preparing nematode inoculum: A population of *M. javanica* (Moosavi, 2014), which originated from a single nematode egg mass, was used in this experiment.

Galled roots of tomato plants (*Solanum lycopersicum* cv. Early-Urbana) were cut into 0.5- to 1-cm pieces and agitated for 2 to 3 min in 0.5% sodium hypochlorite solution. The suspension was rinsed over 60- and 20-μm-pore sieves (Hussey and Barker, 1973). Eggs were transferred to a 250-ml beaker with 100 ml of sterilized distilled water. The number of nematodes were counted and adjusted to 100 total nematodes (combination of eggs and J2) per milliliter.

Experimental design, plant material, and inoculation: A factorial experiment was established in a completely randomized design investigating five different conidial concentrations (0, 10^5, 10^6, 10^7, and 10^8 conidia/ml) of *T. longibrachiatum* and four different concentrations (0, 0.5, 1, and 2 mg a.i./kg soil equal to 0, 12.5, 25, and 50 kg/ha) of cadusafos in controlling *M. javanica* on zucchini plants.

Appropriate amounts of cadusafos were thoroughly mixed with sterile sandy loam soil (sand 67.3%, clay 12.1%, silt 20.6%, and organic matter 3.5% with pH 7.5) before introduction into the 1-kg plastic pots (18 cm diameter, 25 cm depth). Seeds of a highly susceptible local zucchini cultivar (Ghalami) were surface sterilized with 1% NaOCl for 5 min and soaked for 12 hr in different conidial concentrations of *T. longibrachiatum*. One zucchini seed was planted in each pot and after 1 mon, each pot was inoculated with 20 ml of the nematode inoculum. Each treatment had five replications and the pots were kept in a greenhouse (27 ± 3°C).

Pot experiment: The plants were harvested 45 d after inoculation with *M. javanica* and their shoots and roots were weighed. After drying at 70°C for 48 hr, the dry weight of the shoots was also determined. The number of galls on roots from each pot was counted. Soil from each pot was mixed thoroughly and nematodes were extracted from a 100 g soil subsample by the Baermann funnel technique (Baermann, 1917). The J2 were collected on 25-μm-pore sieves and counted at ×100 magnification.

The proportion of infected eggs was estimated by taking 35 egg masses at random (7 egg masses from each pot). Each egg mass was placed in a drop of 1% NaOCl between a sterilized glass slide and a coverslip, and crushed to release the eggs. The number of infected eggs was counted using a microscope at ×100 magnification. Infected eggs contained fungal mycelium inside and over their surface. The embryos were colonized by the fungus and no embryogenesis occurred in the infected eggs. Percentage of egg infection was calculated by dividing the number of infected eggs by total eggs, and multiplying the result by 100. To determine the nematode population on the roots, eggs were extracted from the root system using blender maceration in 1% (v/v) NaOCl solution for 4 min as described by Bourne et al. (1996). Number of eggs was expressed per gram of soil. Root dry weight was determined by recovering the root fragments from the sieves and drying them at 70°C for 48 hr.

The number of intact (not infected) eggs was calculated considering the percentage of egg infection of each pot. The nematode final population (P_f) was calculated by adding the soil-extracted J2 to the number of intact eggs. The reproduction factor (P_f/P_i) for each treatment was calculated by dividing the final population density at the end of experiment (P_f) by the beginning inoculum level (P_i). The percent control of each treatment was calculated by the following formula where X = eggs + J2 per g soil of the treatment without either fungus or nematicide and Y = intact eggs + J2 per g soil of treatment (Sholevarfard and Moosavi, 2015).

$$\text{Percent control} = \frac{X-Y}{X} \times 100$$

Statistical analysis: Statistical analyses were performed using SPSS software (version 15 for Windows, SPSS Inc., Chicago, IL). An analysis of variance was carried out to determine the effects of biocontrol agent and nematicide concentrations and their interactions in the factorial experiment and the mean values were separated by Tukey's studentized range test.

Because a factorial experiment had been designed with two continuous variables whose interactions were significant, the data were reanalyzed with a custom surface response regression to see whether there was any significant trend in the responses with the changes in the concentrations. The response surface curve and contour plots were drawn by the model to show the relationship of each response and the concentrations of fungal inoculum and nematicide (Minitab 16, Minitab Inc., State College, PA).The probability of significance allowed for the model and each term of the model was $P \leq 0.05$.

Response surface model validation: Reliability of the model was examined by comparing the results of two new experimental treatments with five replications (0.4 mg a.i. cadusafus/kg soil and 10^6 fungal conidia/ml suspension; 1.2 mg a.i. cadusafus/kg soil and 10^4 fungal conidia/ml suspension) with the results that were predicted by the model. The same methods as described for "pot experiment" were used for test establishment, inoculation, harvest, and processing of the second experiment. The optimal levels of these two variables were calculated by solving the regression equation for the best plant growth, the highest fungus efficacy, and the lowest nematode reproduction and by analyzing the response surface contour plots (Minitab 16).

Results

The weight of both aerial and underground parts of zucchini plants were significantly affected by the nematicide and conidial concentrations and their interaction. Shoot fresh and dry weights were higher for plants that had been treated with 1 or 2 mg a.i. of the nematicide per kilogram soil as a main factor than at

0.5 or no nematicide per kilogram soil (Table 1). As well, shoot weights (fresh and dry) were higher when 10^7 or 10^8 conidia (as a main factor) of fungus were applied per milliliter suspension than at 0, 10^5, or 10^6 conidia/ml suspension (Table 1). There was no significant difference between the plant shoot weight in the pots that had been treated with 10^7 and 10^8 conidia per milliliter suspension (Table 1). As cadusafos concentration increased, root fresh and dry weights decreased; the lightest roots were from plants treated with 2 mg a.i./kg soil (Table 1). The highest *T. longibrachiatum* concentration of 10^8 conidia/ml resulted in significantly smaller roots than those observed from plants receiving 0 or 10^5 conidia/ml (Table 1).

Nematicide alone and nematicide and fungus significantly affected all nematode-related measurements. The fungal density alone affected all nematode-related measurements except total number of eggs per gram soil. Considering the nematicide application concentration as a main factor, the lowest mean values in number of galls, total number of eggs per gram soil, number of intact eggs per gram soil, number of J2 per gram soil, and reproduction factor were recorded when 2 mg a.i. of nematicide was added per kilogram soil (Table 2). The second best dosage for nematicide application was 1 mg a.i./kg soil. The effect of 10^7 and 10^8 conidia of fungi per milliliter suspension as a main factor on the number of galls, number of J2 per gram soil, number of intact eggs per gram soil, and reproduction factor was statistically similar and more effective against the nematode than lower dosages of the fungus. The concentration of conidia had little effect on the total number of eggs (intact and parasitized) per gram soil, and no statistical differences were observed among different concentrations of fungal conidia (Table 2).

The highest percent of parasitized eggs was observed when the pots had 2 or 1 mg a.i. cadusafos per kilogram soil or concentrations of 10^8 or 10^7 conidia per milliliter suspension (Table 2). The best nematode percent control was attributed to the treatments with 2 mg a.i. cadusafos per kilogram soil (as a main factor).

Roots with poor nematode control were heavily galled and weighed more than roots with better nematode control. The highest root weights (fresh and dry) were associated with those treatments with no cadusafos and no or 10^5 conidia per milliliter (Table 3). As a result, these combinations also resulted in the poorest shoot weights (Table 3). The root weight of untreated control plants (i.e. not inoculated with *M. javanica*) was not significantly different than plants from pots that had 2 mg a.i. cadusafos in combination with all concentrations of fungal conidia or with the pots that had 1 mg a.i. cadusafos in combination with 10^6, 10^7, or 10^8 conidia/ml (Table 3). Shoot dry weights comparable to those from the untreated control plants we observed from pots treated with 10^7 or 10^8 conidia/ml, regardless of cadusafos concentration (Table 3). Fresh weights of shoots from pots that had 2 or 1 mg a.i. cadusafos in combination with 10^7 or 10^8 conidia/ml and from pots treated with 10^8 conidia/ml but no nematicide were similar to those from the untreated control plants (Table 3).

There were some promising treatments in management of *M. javanica* and in reducing the level of nematode reproduction factor when the nematicide and fungus were applied simultaneously (Table 4). The highest decrease in the nematode reproduction factor was achieved when 2 mg a.i. of cadusafos was applied to soil regardless of fungal conidia concentrations or when 1 mg a.i. of cadusafos was applied in combination with 10^7 or 10^8 conidia. When the concentration of conidia was 10^7 or 10^8 conidia per milliliter suspension, increasing the concentration of cadusafos from 1 to 2 mg a.i. had no significant effect on decreasing the number of nematode intact eggs (Table 4).

At 1 and 2 mg a.i. cadusafos/kg soil, the percent of parasitized eggs was statistically similar when the conidial concentration was 10^7 or 10^8 (Table 4). Concurrent use of 1 mg a.i. cadusafos with 10^7 or 10^8 of conidia

TABLE 1. The effect of different concentrations of cadusafos (mg a.i./kg soil) and *Trichoderma longibrachiatum* (conidia/ml suspension) as main factors on the growth parameters of zucchini plants 45 d after being inoculated with *Meloidogyne javanica*.

Main factors and concentrations[a]		Shoot[b]		Root[b]	
		Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Cadusafos	0	51.3 b	17.3 c	32.5 a	11.7 a
	0.5	54.8 b	18.5 bc	24.5 b	8.9 b
	1	59.6 a	20.5 a	20.5 c	7.5 c
	2	57.3 a	19.7 ab	16.2 d	5.8 d
Fungus	0	49.9 C	16.7 C	27 A	8.7 A
	10^5	51.5 BC	16.9 C	25.7 A	9.8 A
	10^6	56.3 B	19.1 B	22.1 AB	12 AB
	10^7	61.1 A	20.9 A	21.5 AB	13.6 AB
	10^8	61.6 A	20.9 A	20.8 B	13.4 B

[a] Each treatment had five replications.
[b] The mean values of each main factor (cadusafos and fungus) were analyzed separately. Small letters were used to separate the mean values related to cadusafos, whereas capital letters were used for *T. longibrachiatum*. Values followed by different letters (small or capital) are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

TABLE 2. *Meloidogyne javanica* reproduction on zucchini plants and *Trichoderma longibrachiatum* parasitism rates at different concentrations of cadusafos (mg a.i./kg soil) and *T. longibrachiatum* (conidia/ml suspension) as main factors 45 d after the plants were inoculated with the nematode.

Main factors and concentrations[a]		No. of galls[b]	Total no. of eggs/g soil[b]	No. of intact eggs/soil[b]	No. of J2/g soil[b]	Reproduction factor[b]	Parasitized eggs %[b]	Percent control[b,c]
Cadusafos	0	138 a	11.7 a	10.7 a	0.47 a	5.6 a	8.2 c	8.4 d
	0.5	95.3 b	7.5 b	6.6 b	0.45 a	3.5 b	12.5 b	42.4 c
	1	43.6 c	2.7 c	1.7 c	0.41 b	1.1 c	39.1 a	82.7 b
	2	25.2 d	1.3 d	0.8 d	0.32 c	0.5 d	40.3 a	91.1 a
Fungus	0	94.7 A	6.2 A	6.2 A	0.47 A	3.4 A	–	45.1 C
	10^5	86 B	5.9 A	5.4 B	0.43 B	2.9 B	12.4 C	52 B
	10^6	69.5 C	6 A	5.1 C	0.41 B	2.7 C	25 B	55.3 B
	10^7	63.9 D	6 A	4.4 D	0.38 C	2.4 D	45.3 A	61.1 A
	10^8	63.7 D	6 A	4.5 D	0.38 C	2.4 D	42.4 A	60.4 A

[a] Each treatment had five replications.
[b] The mean values of each main factor (cadusafos and fungus) were analyzed separately. Small letters were used to separate the mean values related to cadusafos, whereas capital letters were used for *T. longibrachiatum*. Values followed by different letters (small or capital) are significantly different at $P \leq 0.05$ (Tukey's studentized range test).
[c] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where X stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and Y stood for intact eggs + J2 per gram soil of treatment.

per milliliter suspension could control the nematode as efficiently as when 2 mg a.i. cadusafos was used (Table 4). Cadusafos at 0.5 mg a.i./kg soil did not reduce the number of galls, intact eggs per gram soil, and J2 per gram soil as well as higher rates of cadusafos, regardless of *T. longibrachiatum* conidia concentration, though nematode control was improved as fungal concentration increased. Use of *T. longibrachiatum* alone resulted in some nematode suppression. For example, plants treated with 10^7 conidia/ml treated plants had 31% fewer galls, 14% fewer intact eggs/g soil, 22% fewer J2/g soil, and a 15% lower nematode reproduction factor than in the absence of cadusafos and fungal treatment (Table 4). However, cadusafos at 2 mg a.i./kg soil or at 1 mg a.i./kg soil + 10^7 to 10^8 fungal conidia/ml resulted in approximately 88% fewer galls, 97% reduction in intact eggs/g soil, 46% fewer J2/g soil, and a 94% reduction in the nematode reproduction factor.

In the custom response surface analysis, second-order regressions explained the role of each independent variable and their interactions in the responses. The significance of each estimated regression model and its terms was evaluated by Student's *t*-test (Table 5) and insignificant terms whose *P* values were more than 0.05

TABLE 3. The effect of different combinations of chemical nematicide (cadusafos; mg a.i./kg soil) and *Trichoderma longibrachiatum* (conidia/ml suspension) concentrations on the growth parameters of zucchini plants 45 d after inoculation with *Meloidogyne javanica*.

		Shoot[a]		Root[a]	
Cadusafos concentration	Fungal concentration	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
0	0	40.18 i	13.39 j	36.62 a	13.17 a
	10^5	45.42 h	15.15 ij	39.46 a	14.17 a
	10^6	51.39 fg	17.56 fgh	30.22 b	11.09 b
	10^7	59.07 cd	20.23 bcd	27.59 bc	10.09 b
	10^8	60.54 bc	20.28 bcd	28.64 bc	10.22 b
0.5	0	48.1 gh	16.03 hi	29.39 bc	10.61 b
	10^5	51.44 fg	17.13 gh	26.2 cd	9.44 bcd
	10^6	55.48 def	18.75 defg	22.44 ef	8.26 de
	10^7	59.59 cd	20.21 bcd	23.03 de	8.36 cde
	10^8	59.25 cd	20.26 bcd	21.29 efg	7.85 def
1	0	52.96 f	17.79 fgh	27.31 bc	9.97 bc
	10^5	54.38 ef	18.04 efg	21.85 ef	8.07 def
	10^6	59.88 cd	21.37 abc	19.19 fgh	7.07 efg
	10^7	65.59 a	22.85 a	17.57 ghi	6.46 fgh
	10^8	65.31 a	22.59 a	16.57 hi	5.94 gh
2	0	58.51 cde	19.76 cde	14.53 i	5.22 h
	10^5	59.17 cd	19.05 def	15.39 i	5.62 gh
	10^6	58.38 cde	18.8 defg	16.75 hi	6.03 gh
	10^7	60.23 bc	20.46 bcd	17.76 ghi	6.42 fgh
	10^8	61.47 abc	20.37 bcd	16.63 hi	5.92 gh
Untreated plants[b]		64.79 ab	21.98 ab	15.52 hi	5.67 gh

[a] Each treatment had five replications. Values in the same column followed by different letter(s) are significantly different at $P \leq 0.05$ (Tukey's studentized range test).
[b] No nematode, no nematicide, and no fungus were added to the untreated plants.

TABLE 4. The effect of combinations of *Trichoderma longibrachiatum* (conidia/ml suspension) and nematicide (cadusafos; mg a.i./kg soil) concentrations on fungal percent control and nematode reproduction on zucchini plants 45 d after inoculation with *Meloidogyne javanica*.

Cadusafos concentration	Fungal concentration	No. of galls[a]	Total no. of eggs/g soil[a]	No. of intact eggs/g soil[a]	No. of J2/g soil[a]	Reproduction factor[a]	Parasitized egg %[a]	Percent control[a,b]
0	0	167.25 a	11.7 ab	11.7 a	0.54 a	6.13 a	–	–
	10^5	157.75 a	11.2 b	10.77 b	0.48 b	5.62 b	4.2 d	8.2 h
	10^6	131.25 b	11.7 ab	10.69 b	0.47 bc	5.58 b	8.6 d	8.9 h
	10^7	114.75 c	11.8 ab	10.03 b	0.42 cde	5.22 b	14.7 cd	14.7 h
	10^8	119 bc	12.2 a	10.54 b	0.46 bcd	5.5 b	13.6 cd	10.2 h
0.5	0	123 bc	7.7 c	7.7 c	0.5 ab	4.11 c	–	32.9 g
	10^5	112.75 c	7.5 c	7.08 cd	0.48 b	3.78 cd	6.3 d	38.3 fg
	10^6	81.25 de	7.3 c	6.37 de	0.45 bcd	3.41 de	13.1 cd	44.3 ef
	10^7	81.5 d	7.8 c	6.14 e	0.41 def	3.28 e	21 c	46.5 e
	10^8	78.25 de	7.4 c	5.74 e	0.41 de	3.08 e	22.1 c	49.7 e
1	0	68.25 e	3.3 d	3.29 f	0.46 bcd	1.88 f	–	69.4 d
	10^5	44.75 f	2.5 d	2.07 g	0.45 bcd	1.26 g	19.5 c	79.5 c
	10^6	38.75 fg	2.8 d	1.65 gh	0.41 de	1.03 gh	41.2 b	83.1 bc
	10^7	34.5 fgh	2.6 d	0.82 hi	0.37 efg	0.59 i	69.6 a	90.3 a
	10^8	32 fghi	2.5 d	0.72 i	0.38 ef	0.55 i	71.3 a	91 a
2	0	20.2 i	1.1 e	1.08 hi	0.3 h	0.69 hi	–	88.8 ab
	10^5	28.75 ghi	1.3 e	1.04 hi	0.31 gh	0.68 hi	21.6 c	88.9 ab
	10^6	26.75 ghi	1.4 e	0.88 hi	0.32 gh	0.60 hi	37.7 b	90.2 ab
	10^7	24.75 hi	1.6 e	0.49 i	0.35 fg	0.42 i	69 a	93.1 a
	10^8	25 hi	1.2 e	0.40 i	0.29 h	0.35 i	67.2 a	94.3 a

[a] Each treatment had five replications. Mean values in the same column followed by different letter(s) are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

[b] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where X stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and Y stood for intact eggs + J2 per gram soil of treatment.

were deleted from the models. The modified equations for uncoded units were demonstrated in Table 6. The regressions have a maximum of six terms and contain an intercept, two linear terms, two quadratic terms, and a factorial interaction. The coefficient of determination (R^2) is a reliable indicator for evaluating the goodness of fit of the data in the regression models. In the current study, the coefficients of determination were between 0.82 and 0.97 that indicate the credibility of the computed quadratic surface response regressions (Table 6).

The results of three-dimensional response surfaces and two-dimensional contour graphs were plotted to describe the main and interaction effect of cadusafos and *Trichoderma* concentrations on plant growth characteristics (Fig. 1) and nematode population responses (Figs. 2,3). The interactive effects of *Trichoderma* and cadusafos concentrations were insignificant in several responses such as the total number of eggs per gram soil, the number of intact eggs per gram soil, nematode reproduction factor, and percent control (Tables 5,6; Figs. 2,3). The total number of eggs per gram soil was not affected by fungal concentration (Table 5).

The new experimental mean values from the response surface validation experiment were close to the expected values that were predicted by the original model so the validity was proved (Table 7). The calculated optimum

TABLE 5. The total and separate significance of the terms for the surface response regression equations that were calculated by Student's *t*-test.

	P value						
Responses	Equation[a]	Intercept	X_1[b]	X_2[b]	X_1^2	X_2^2	X_1X_2
Shoot fresh weight (g)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Shoot dry weight (g)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Root fresh weight (g)	0.0001	0.0001	0.0001	0.0001	0.0001	0.013	0.0001
Root dry weight (g)	0.0001	0.0001	0.0001	0.0001	0.0001	0.008	0.0001
Total eggs/g soil	0.0001	0.0001	0.0001	0.881	0.0001	0.333	0.874
J2/g soil	0.0001	0.0001	0.0001	0.0001	0.008	0.015	0.0001
Intact eggs and J2/g soil	0.0001	0.0001	0.0001	0.0001	0.0001	0.146	0.115
Reproduction factor	0.0001	0.0001	0.0001	0.0001	0.0001	0.142	0.114
Percent parasitized eggs	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Percent control[c]	0.0001	0.0001	0.0001	0.0001	0.0001	0.142	0.114

[a] This column shows the total *P* value of terms for each regression model.

[b] X_1 = cadusafos concentration (mg a.i./kg soil); $X_2 = \log_{10}$ *Trichoderma longibrachiatum* concentration (conidia/ml suspension).

[c] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where X stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and Y stood for intact eggs + J2 per gram soil of treatment.

TABLE 6. Regression model of zucchini plant growth parameters, *Meloidogyne javanica* reproduction, and *Trichoderma longibrachiatum* parasitism rates to cadusafos and *T. longibrachiatum* inoculation concentrations.

Responses	Regression model[a]	R^{2b}
Shoot fresh weight (g)	$Y = 39.7 + 16.84\ X_1 + 0.23\ X_2 - 3.55\ X_1^2 + 0.29\ X_2^2 - 1.03\ X_1X_2$	0.87
Shoot dry weight (g)	$Y = 13.06 + 6.66\ X_1 + 0.013\ X_2 - 1.68\ X_1^2 + 0.11\ X_2^2 - 0.38\ X_1X_2$	0.82
Root fresh weight (g)	$Y = 39.23 - 20.34\ X_1 - 0.536\ X_2 + 4.23\ X_1^2 - 0.12\ X_2^2 + 0.74\ X_1X_2$	0.88
Root dry weight (g)	$Y = 14.09 - 7.09\ X_1 - 0.145\ X_2 + 1.44\ X_1^2 - 0.048\ X_2^2 + 0.25\ X_1X_2$	0.87
Total eggs/g soil	$Y = 12.12 - 12.04\ X_1 + 3.32\ X_1^2$	0.98
J2/g soil	$Y = 0.55 - 0.084\ X_1 - 0.006\ X_2 - 0.02\ X_1^2 - 0.001\ X_2^2 + 0.008\ X_1X_2$	0.86
Intact eggs and J2/g soil	$Y = 12.84 - 12.63\ X_1 - 0.12\ X_2 + 3.52\ X_1^2$	0.97
Reproduction factor	$Y = 6.42 - 6.31\ X_1 - 0.06\ X_2 + 1.76\ X_1^2$	0.97
Percent parasitized eggs	$Y = -3.41 + 19.75\ X_1 - 4.42\ X_2 - 10.86\ X_1^2 + 0.89\ X_2^2 + 3.79\ X_1X_2$	0.82
Percent control[c]	$Y = -4.78 + 103.12\ X_1 + 0.99\ X_2 - 28.77\ X_1^2$	0.97

[a] Y = response; X_1 = cadusafos concentration (mg a.i./kg soil); $X_2 = \log_{10}$ *Trichoderma longibrachiatum* concentration (conidia/ml suspension).
[b] R^2 shows the coefficient of determination for fitting data in each regression model.
[c] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where X stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and Y stood for intact eggs + J2 per gram soil of treatment.

levels for the combination of input variables, which led to the most desirable predicted responses (maximum or minimum values according to corresponding responses) are provided in Table 8. Based on these calculations, the optimal cadusafos and *Trichoderma* were 1.7 a.i./kg soil and 10^8 conidia/ml suspension, respectively.

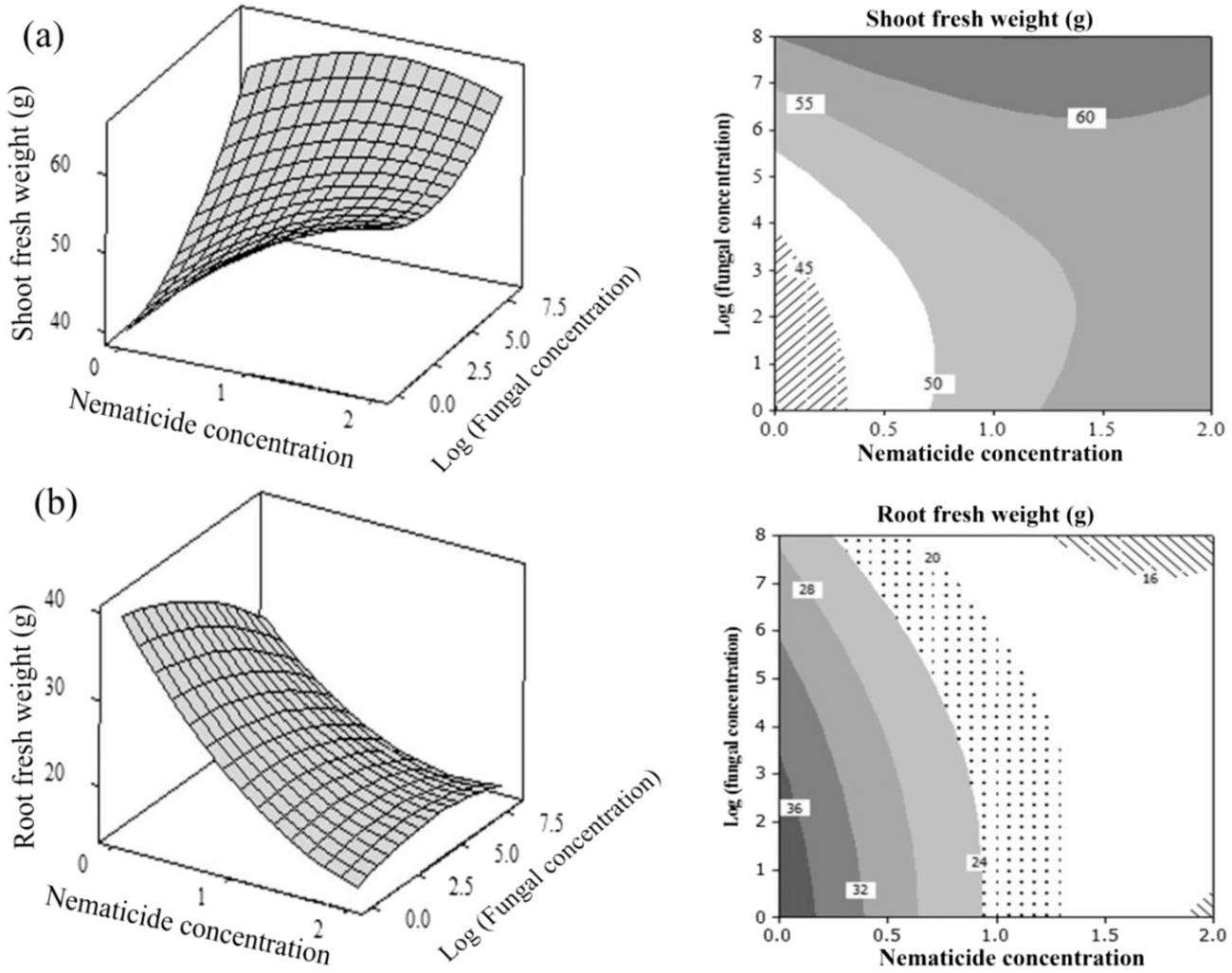

FIG. 1. Response surface curve and contour map of *Meloidogyne javanica*–infected zucchini plants at different concentrations of nematicide (cadusafos; mg a.i./kg soil) and *Trichoderma longibrachiatum* ($\log_{10}$ of conidia/ml). A. Shoot. B. Root fresh weight. The plants were kept in greenhouse for 45 d after inoculation with the nematode.

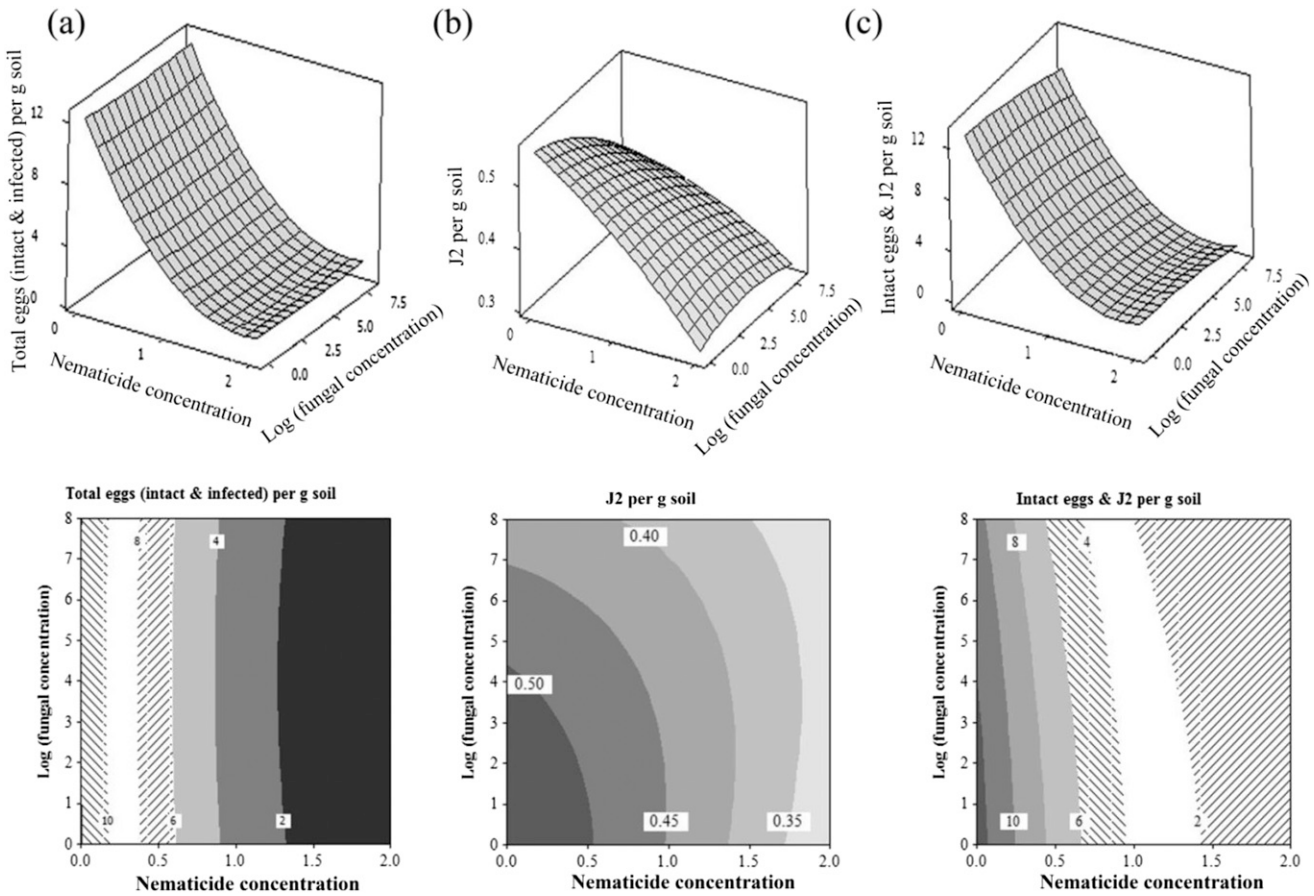

FIG. 2. Response surface plots and contour plots showing the combined effects of nematicide (cadusafos; mg a.i./kg soil) and fungal biocontrol agent (*Trichoderma longibrachiatum*; $\log_{10}$ of conidia/ml) concentrations on *Meloidogyne javanica* populations 45 d after inoculation of zucchini in a greenhouse test. A. Number of eggs (intact and infected) per gram soil. B. Number of J2 per gram soil. C. Total number of intact eggs and J2 per gram soil.

DISCUSSION

About 5% of global agriculture production is destroyed due to root-knot nematodes (Agrios, 2005). Management of this pest has been principally based on the application of chemical nematicides, but there is a need to decrease the amount of utilized chemicals because of their destructive effect on the environment (Moosavi and Askary, 2015). Integrating chemical nematicides with biological control could prevent initial nematode injury and the biocontrol agent could provide long-term protection (Moosavi and Zare, 2012).

The selected fungus for this experiment was isolated from the soil of a *M. javanica* infested farm and we expected that it could efficiently antagonize the nematode. There were several reports on the potency of *T. longibrachiatum* against *M. javanica* (Al-Shammari et al., 2013), *H. avenae* (Zhang et al., 2014), J2 of *Heterodera* sp. and *Meloidogyne* sp., and adults and juveniles of *Xiphinema* sp. and *Pratylenchus* sp. (Djian et al., 1991). Our results are in contrast with the previously published ones. The fungus was not very effective in suppressing *M. javanica* when applied alone. Variation in biological control ability of different isolates of a species, even those collected from similar soils, was frequently reported (Kerry, 2000; Moosavi et al., 2010).

Trichoderma longibrachiatum concentrations had little effect on several responses including the total number of eggs per gram soil and percent control. When interpreting the models, the smaller *P* values of each term in the surface response regression equations indicate the more meaningful coefficients. Quadratic response surface regression models are a combination of fractional factorial regression and polynomial regression designs that include the characteristics of both techniques. These models have the traits of polynomial regression as well as the two-way interactive effects of the independent variables (Myers et al., 2009).

The higher the R^2, the more accurately the regression model can predict the response in accordance with the experimental factors and their interactions. The R^2 of the experiment indicated the reliability and accuracy of the generated regressions are valid and reasonable. However, the validity was also confirmed by comparing the results of extra treatments examined in a separate validation test with predicted results.

Root fresh weight was heaviest when nematode control was poorest because of the many galls that were

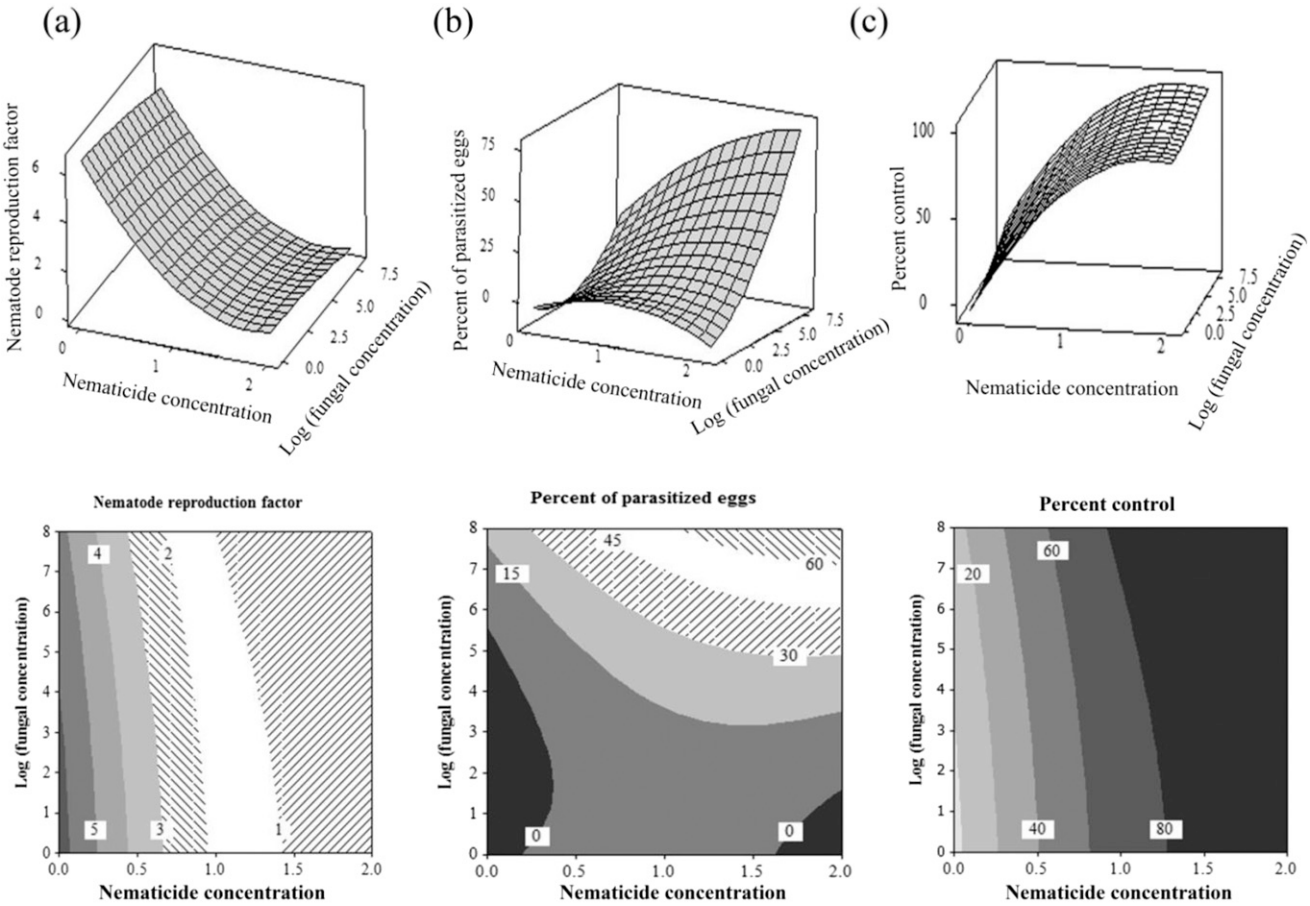

FIG. 3. Response surface curves and contour plots of *Meloidogyne javanica* reproduction factor. A. Percent of parasitized eggs. B. Percent control of nematode. C. Under the combined influence of different concentrations of nematicide (cadusafos; mg a.i./kg soil) and *Trichoderma longibrachiatum* ($\log_{10}$ of conidia/ml) on zucchini in a greenhouse test. The plants were harvested 45 d after inoculation with the nematode. The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where *X* stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and *Y* stood for intact eggs + J2 per gram soil of treatment.

produced on the infected roots. More nematode suppression caused lighter root weight. Some species of *Trichoderma* can induce plant innate defense mechanisms against phytonematodes (Sharon et al., 2011; Contreras-Cornejo et al., 2014; Saldajeno et al., 2014). This ability was also reported for *T. longibrachiatum* where the fungus could increase the activities of some defense-related enzymes (phenylalanine ammonia lyase,

TABLE 7. Comparison of the experimental mean values (±SE) from a response surface model validation experiment with the expected values that were predicted by the original model. Conidial concentrations indicate the spore concentration of *Trichoderma longibrachiatum* in which the zucchini seeds were soaked for 12 hr.

	0.4 mg a.i. cadusafos/kg soil and 10^6 conidia of fungus/ml		1.2 mg a.i. cadusafos/kg soil and 10^4 conidia of fungus/ml	
Responses	Predicted	Experimental	Predicted	Experimental
Shoot fresh weight (g)	55.216	53.73 (±1.4)	55.412	54.52 (±1.47)
Shoot dry weight (g)	19.91	19.09 (±0.98)	18.6208	18.54 (±0.94)
Root fresh weight (g)	26.01	25.15 (±1.14)	20.4012	19.34 (±0.81)
Root dry weight (g)	9.49	8.85 (±0.67)	7.5076	7.198 (±0.75)
Total eggs/g soil	7.8352	7.94 (±0.15)	2.4528	2.28 (±0.2)
J2/g soil	0.4964	0.48 (±0.01)	0.4348	0.44 (±0.01)
Intact eggs and J2/g soil	7.6312	7.11 (±0.16)	2.2728	2.3 (±0.17)
Reproduction factor	3.8176	3.56 (±0.08)	1.1424	1.15 (±0.09)
% parasitized eggs	17.3684	16.57 (±0.95)	19.4036	18.71 (±0.91)
Percent control[a]	37.8048	41.95 (±1.29)	81.4952	81.23 (±1.4)

[a] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where *X* stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and *Y* stood for intact eggs + J2 per gram soil of treatment.

TABLE 8. The optimal levels of cadusafos and *Trichoderma longibrachiatum* concentrations which led to the best plant or nematode responses. Higher plant growth or lesser nematode reproduction rates were considered as the best responses.

Responses	Optimal concentrations of variables		Best response
	Cadusafos (mg a.i./kg soil)	*T. longibrachiatum* (spore/ml suspension)	
Shoot fresh weight (g)	1.2	10^8	Max = 65.15
Shoot dry weight (g)	1.07	10^8	Max = 22.44
Root fresh weight (g)	1.7	10^8	Min = 15.03
Root dry weight (g)	1.76	10^8	Min = 5.4
Total eggs/g soil	1.82	9.12×10^3	Min = 1.02
J2/g soil	2	0	Min = 0.31
Intact eggs and J2/g soil	1.72	10^8	Min = 0.15
Reproduction factor	1.72	10^8	Min = 0.08
% parasitized eggs	2	10^8	Max = 75.27
Percent control[a]	1.72	10^8	Max = 98.75

[a] The percent control of each treatment was calculated by the formula $\left(\frac{X-Y}{X} \times 100\right)$ where *X* stood for eggs + J2 per gram soil of the treatment without either fungus or nematicide and *Y* stood for intact eggs + J2 per gram soil of treatment.

polyphenol oxidase and peroxidase) in wheat roots (Zhang et al., 2014). It has been also suggested that *T. longibrachiatum* can induce defense enzymes and probably other defense compounds in tomato plants leading to systemic resistance against *M. javanica* (Al-Shammari et al., 2013). The results of this study have not supported this conclusion because the total number of nematode eggs (intact and parasitized) per gram soil did not significantly change in accordance with different conidial concentrations. In other words, at a constant concentration of cadusafos, the fungus could not decrease the total number of eggs even when its concentration was increased. This means that *T. longibrachiatum* isolate MIAU 143 C did not have the potential for stimulation of plant defense against this nematode population. Profound variations among different isolates or species of fungi in suppressing phytonematodes could be the consequence of differences in fungal aggressiveness and ecological (biotic and abiotic) factors (Moosavi and Zare, 2015).

The most appropriate rate of *T. longibrachiatum* for *M. javanica* control was determined as 10^8 conidia/ml suspension. Al-Shammari et al. (2013) demonstrated significant suppression of *M. javanica* on tomato by dipping the roots for 5 min to 10^6 and 10^7 CFU/ml concentration of *T. longibrachiatum*. Zhang et al. (2014) found the best concentration of the fungus for decreasing the population of *H. avenae* on the wheat roots was 30×10^8 spores added to each pot. Despite differences in fungal application method, our result is supported by other studies. Variation in *M. javanica* (Sahebani and Hadavi, 2008; Al-Hazmi and TariqJaveed, 2016) and *Meloidogyne enterolobii* Yang and Eisenback (Jindapunnapat et al., 2013) suppression because of different concentrations of *T. harzianum* Rifai were also reported.

Notwithstanding the unsatisfactory results of *M. javanica* suppression by solitary application of MIAU 143 C isolate of *T. longibrachiatum*, its combining with cadusafos could result in decreasing the amounts of needed nematicide. The best combination to achieve the maximum control percent (98.75 %) was 1.72 mg a.i. cadusafos/kg soil and 10^8 conidia of *T. longibrachiatum*/ml suspension. Integration of 10^8 conidia/ml with cadusafos could decrease the required amount of nematicide from 50 to 42.5 kg/ha (from 2 mg a.i. to 1.7 mg a.i./kg soil). However, when the desired control percent was 80%, the level of required cadusafos in combination with 10^8 conidia/ml suspension was less than 1 mg a.i./kg soil, which is equal to less than 25 kg/ha.

The two-dimensional contour plots and the corresponding three-dimensional response surface plots can provide useful information on the main and interaction effects of the variables. The elliptical shape of the contour lines reflects the significant interactions of the variables, whereas the straight lines indicate that the interactions were insignificant (Myers et al., 2009).

The results of this study suggest that MIAU 143 C isolate of *T. longibrachiatum* cannot be used singly as a reliable biocontrol agent for suppression of *M. javanica* on zucchini plants. The optimum combination of cadusafos and *T. longibrachiatum* concentrations for gaining the best plant growth and lowest nematode reproduction was 1.72 mg a.i. cadusafos/kg soil and 10^8 conidia of *T. longibrachiatum*/ml suspension. However, the lesser levels of cadusafos concentration could provide promising responses when integrated with 10^8 or even 10^7 conidia of *T. longibrachiatum*/ml suspension.Further study on different hosts and under field conditions is required to confirm the results of this experiment.

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