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Original article (Orijinal araştırma)

Optimization of a *Bacillus*-based bioproduct for *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) control using response surface methodology[1]

Meloidogyne incognita (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) kontrolü için *Bacillus* bazlı bir biyoürünün yanıt yüzeyi metodolojisi kullanılarak optimizasyonu

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Abstract

Root-knot nematodes (RKNs) cause significant yield losses in agriculture. Environmentally friendly bioproducts are important components of sustainable nematode management. This study evaluated the efficacy of two commercial *Bacillus* bioproducts, *Bacillus amyloliquefaciens* MBI 600 (Bioproduct-I) and *Bacillus subtilis* QST 713 (Bioproduct-II), against *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae), a major RKN species, on tomato plants. The trial, conducted in 2024 at Bolu Abant İzzet Baysal University, Faculty of Agriculture, Department of Plant Protection, assessed root gall index, number of second-stage juveniles (J2) and number of egg masses in pot experiments at seed and seedling stages. The dose of Bioproduct-I was optimized using response surface methodology (RSM) and design of experiments (DOE), with results visualized using Pareto and normal plots. The 1000 ml/ha dose of Bioproduct-I was characterized by the lowest root gall index (2.37 in seed, 2.75 in seedling) and the lowest number of J2 (382.5 in seed, 415.0 in seedling). However, higher doses showed reduced efficacy, indicating that increasing concentrations did not increase biological activity. This study highlights the potential of *Bacillus* spp. for biological control and demonstrates the usefulness of statistical tools in optimizing Bioproduct applications against RKN.

Keywords: *Bacillus*, bioproduct, response surface method (RSM), root-knot nematode, tomato

Öz

Kök-ur nematodları tarımda önemli verim kayıplarına yol açmaktadır. Çevre dostu biyolojik ürünler, sürdürülebilir nematod yönetiminde önemli bir rol oynamaktadır. Bu çalışmada, iki ticari *Bacillus* tabanlı biyolojik ürünün, *Bacillus amyloliquefaciens* MBI 600 (Bioproduct-I) ve *Bacillus subtilis* QST 713 (Bioproduct-II), domates bitkilerinde önemli bir kök-ur nematodu türü olan *Meloidogyne incognita*'ya (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) karşı etkinliği değerlendirilmiştir. Bolu Abant İzzet Baysal Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü'nde 2024 yılında yapılan denemelerde, tohum ve fide aşamalarındaki saksı deneylerinde kök ur indeksi, ikinci dönem larva (J2) sayısı ve yumurta paketi sayısı değerlendirilmiştir. Bioproduct-I dozu, yanıt yüzeyi metodolojisi (YYM) ve deney tasarımı (DT) kullanılarak optimize edilmiş ve sonuçlar Pareto ve normal plot grafikleri ile görselleştirilmiştir. Bioproduct-I'in 1000 ml/ha dozu, en düşük kök gal indeksi (tohumda 2.37, fidede 2.75) ve en düşük ikinci dönem larva (J2) sayısı (tohumda 382.5, fidede 415.0) ile belirlenmiştir. Daha yüksek dozlar, biyolojik aktiviteyi artırmamış, daha düşük etkinlik göstermiştir. Bu çalışma, *Bacillus* spp. türlerinin biyolojik mücadele potansiyelini ve kök-ur nematodlarına karşı biyolojik ürün uygulamalarının optimize edilmesinde istatistiksel araçların yararlılığını ortaya koymaktadır.

Anahtar sözcükler: *Bacillus*, biyolojik ürün, yanıt yüzey metodu (YYM), kök-ur nematodu, domates

[1] This study is derived from the MSc thesis of the first author.

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Introduction

Nematodes are found in every ecosystem and are one of the most common in animal taxa being thought to number over 250.000 species (Mitreva et al., 2005). In addition, over 4100 species of nematodes have been identified that are associated with plants (Decraemer et al., 2006). Plant-parasitic nematodes (PPNs) are one of the most destructive soil-borne pathogens, causing devastating economic losses in agriculture (Imren et al., 2017; Pires et al., 2022). They cause significant losses to agriculture worldwide, estimated at more than $80 billion per year (Nicol et al., 2011; İmren et al., 2019; Abd-Elgawad, 2024).

Among PPNs, the most important nematodes are the root-knot nematodes (RKNs; *Meloidogyne* spp.), which are responsible for most of the major agricultural losses worldwide (Elling, 2013). RKNs are found in almost all regions of the world and parasitize all vascular plants in greenhouses and in the field. They are among the most economically important nematodes at the genus level, as there are approximately 100 species in the genus *Meloidogyne* (Sikandar et al., 2020). *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 and *Meloidogyne hapla* Chitwood, 1949 (Tylenchida: Meloidogynidae) are the most common species of RKNs (Wesemael et al., 2011; Jones et al., 2013; Coyne et al., 2018).

Meloidogyne incognita is the most important *Meloidogyne* species due to its economic importance. Although common in the tropics, it is typically restricted to greenhouses in temperate regions (Karssen & Moens 2006). *Meloidogyne incognita* placed in as eggs or second-stage juveniles (J2) in the soil, and J2s infect roots and creates feeding sites. This results in root gall and reduced water and nutrient uptake, resulting in loss of quality and yield (Talwana et al., 2016). Yield losses due to *M. incognita* infection range from 20% to 60% in tomato (Li et al., 2020).

Current RKN control practices are predominantly based on chemical nematicides. Unfortunately, indiscriminate use of these chemicals and prolonged exposure lead to critical adverse effects on both human health and the environment (Riga, 2011). Therefore, it became essential to develop alternative means using a multi-pronged and environmentally friendly strategy, such as biological control strategies, to reduce nematode damage without causing previous adverse effects (Singh & Mathur 2010).

Biological control of PPNs is a promising area for nematode control in sustainable agriculture. To date, rhizosphere microorganisms have been identified as very good biological control agents for PPN management (Ye et al., 2022; Shi et al., 2024). Among antagonistic bacteria, *Bacillus* spp., have been shown to be effective against RKNs due to their ability to rapid colonize the rhizosphere and direct the nematicidal production of secondary metabolites (Tian et al., 2022; Bhat et al., 2023). The bionematicidal activity of *Bacillus* is based on its ability to induce a systemic plant defense mechanism of nematode resistance. Proteases, chitinases, antibiotics, crystalline proteins and several secondary metabolites are formed by *Bacillus* species to perform this function (Engelbrecht et al., 2018).

Design of experiments (DOE) is one of the main tools used in plant biology, providing plant scientists with a systematic method to study various aspects of plant biology, plant ecology, and even plant agronomy (Timmusk et al., 2017). DOE provides an organized means of initiating and conducting research on plants and the environment in terms of growth, development, and other responses. The use of modern DOE techniques, such as factorial design, response surface methodology (RSM), and Taguchi techniques, provides further information on plant physiology and plant adaptation (Swain et al., 2021). RSM is a statistical technique that uses designed experiments to analyze the behavior of complex systems (Nwabueze, 2010). This method addresses the problem of parameter optimization in a variety of processes driven by more than one input variable, which can be optimized through a series of statistically validated model estimates (Baçaoui et al., 2001). RSM provides an efficient approach to optimizing parameters by approximating relationships using a quadratic surface. In addition, RSM helps us to analyze the interaction between several parameters (Azargohar & Dalai, 2005).

Although many studies have been conducted on the efficacy of bioproducts against nematodes, there are very few comparative studies on the efficacy and dose optimization of *Bacillus*-based bioproducts against the root-knot nematode *M. incognita*. In this context, dose optimization comparisons with RSM are an important tool to better understand the efficacy of bioproduct applications and to determine the most efficient treatment conditions. Therefore, the aim of this study was to investigate the effects of *Bacillus*-based bioproducts on *M. incognita* and to optimize bioproduct dosages using RSM. In this way, the efficacy of bioproducts can be increased and the most appropriate application conditions can be determined.

Materials and Methods

Nematode population

The RKN species *M. incognita* was used for the experiment. RKN pure cultures were established from single egg masses on tomato cultivar in a growth room (25±2ºC, > 60% humidity) at the Bolu Abant Baysal University. Infected roots were carefully cleaned to remove soil particles. Egg masses from the infected plants were collected with care and immersed in distilled water and then placed in a BOD incubator at 28±2°C to obtain the J2. The juvenile suspension was calibrated to a final concentration of 100 juveniles per milliliter of distilled water.

Bioproduct and nematicides

The following active ingredients were used in the study: Abamectin 20 g/l, abbreviated as 'Abamectin'; Bioproduct-I containing *Bacillus amyloliquefaciens* MBI 600; and Bioproduct-II containing *Bacillus subtilis* QST 713. The commercial bioproducts and nematicides used in the study are listed in Table 1.

Table 1. Bioproducts and nematicides used in the experiment

Code	Active substance	Trade name of product	Company
Nematicide	Abamectin 20 g/l	TERVIGO 20 SC	Syngenta Agriculture Ind. & Trade JSC
Bioproduct-I	*Bacillus amyloliquefaciens* MBI 600	SERIFEL WP	BASF Türk Chemistry Ind. Ltd. Co.
Bioproduct-II	*Bacillus subtilis* QST 713	SERENADE SC	Bayer Türk Chemistry Ind. Ltd. Co.

Experimental design and set up

The experiment was established as a completely randomized design, including six treatments with four replications each. All experiments were performed twice to ensure consistency and reliability of results. The experiments were conducted in 500 cc plastic pots. The soil mixture, composed of 75% sand and 25% peat, was sterilized in an autoclave at 121°C. Three-week-old susceptible tomato seedlings were planted in each pot for every treatment.

In the study of investigating the effect of some bioproducts on the reproduction of the RKN species *M. incognita*, the treatments were applied at different times to assess their effect on various stages of the nematode's life cycle (Table 2). The J2s of RKN were inoculated into two wells near the roots immediately prior to treatments on the planting days for treatment code A at a rate of 1000 J2 per pot using a 5 ml micropipette according to the application times listed in (Table 2). Simultaneously with the J2, 10 g of infected roots were embedded within the soil in each pot.

Table 2. Application code timing

Code	Time
A	at seeding or transplanting by drench
B	28 days after at seeding or transplanting by drench

The commercial bioproducts and nematicides were applied at the recommended doses, considering that tomato seedlings must be planted at a density of 1500 plants per decare. After application, the pots were irrigated to enhance the effect. Table 3 presents the doses and experimental design.

Table 3. Application treatments and rates used in trials

No	Treatments	Application on seed or seedling	Formulation Type	Rate (ml/ha or ml/da)	Time
1	Bioproduct-I	Seed- seedling	WP	250 mL/ha	AB
2	Bioproduct-I	Seed- seedling	WP	500 mL/ha	AB
3	Bioproduct-I	Seed- seedling	WP	750 mL/ha	AB
4	Bioproduct-I	Seed- seedling	WP	1000 mL/ha	AB
5	Bioproduct-I	Seed - seedling	WP	1250 mL/ha	AB
6	Bioproduct-I	Seed- seedling	WP	1500 mL/ha	AB
7	Bioproduct-II	Seed- seedling	SC	1000 mL/ha	AB
8	Nematicide	Seed- seedling	SC	400 ml/da	AB
9	Control (+)	Seed- seedling	NA	NA	NA
10	Control (-)	Seed- seedling	NA	NA	NA

* Control (+) refers to the condition with nematodes applied; NA refers to the condition with no application.

** Bioproduct-I, recommended at a dosage of 50 g/100 L, is in WP formulation and prepared for application at different dosages (250, 500, 750, 1000, 1250 and 1500 ml) for 1 ha area.

Evaluation of the trial

Overall, 56 days after the start of the experiment, tomato plants were ready for harvesting. The plants were harvested by cutting at soil level, the roots were carefully removed from the soil and gently washed under running water to remove adhering soil particles. The fresh and dry weights of the roots were then carefully measured and recorded. For dry weight measurement, the roots were dried in an oven at a constant temperature of 60°C.

The severity of root galling indices was assessed using the 0-10 scale described by Zeck (1971) and the degree of damage caused by *M. incognita* infestation was determined. The modified Baermann funnel technique (Hooper, 1986) was applied to determine the nematode population density and the J2 were counted under Zeiss light microscope with coverslip under 100x magnification.

Statistical analysis

The experimental data were evaluated using Minitab software, version 21.1.0. Analysis of variance (ANOVA) was used to analyze the significance of variance in different parameters of the experiment. Post hoc analysis was performed using the Tukey test at a 95% confidence level. In addition, RSM was used to optimize the experiment and study the dependencies and interactions between the independent and dependent variables, in order to obtain the optimum values of the different factors affecting the root gall index and the population of J2s. The variables were Pareto charts and normal plots, which revealed the effects of the variables and deepened the trustworthiness of the results.

Results

The treatments effect on nematode reproductive parameters

The analysis of the effect of different bioproduct and nematicide treatments on root gall index found the lowest values in Bioproduct-II and Nematicide treatments. Extremely low root gall index was recorded for both the seed 2.25 and seedling 2.37 treatments at the 1000 ml/ha dose of Bioproduct-II. The nematicide treatments had similarly low root gall index values of 2.37 for seed and 2.25 for seedling. In the case of the Bioproduct-I treatment, the root gall index was found to be low with increasing dose. The minimum root gall index was found to be 2.37 for seed at 1000 ml/ha dose and 3.00 for seedling at 750 ml/ha dose. However, an increase in root gall index was observed at 1250 ml/ha and 1500 ml/ha. The maximum root gall index of the positive control remained at 7.00 and 7.12 in seed and seedling respectively. The above results indicate that Bioproduct-I and Bioproduct-II inhibit the growth of root gall index (Table 4).

Table 4. The effects of treatments on reproduction parameters in *Meloidogyne incognita*-infested plants

Run No.	Treatment	Root gall index Mean±SD (95% CI)*	Second-stage juveniles (J2) Mean±SD (95% CI)	Number of egg masses Mean±SD (95% CI)
1	Bioproduct-I (Seed, 250 ml/ha)	6.62±0.47^{abc} (6.067; 7.183)	1725.0±64.5^{ab} (1628.3; 1821.7)	187.0±9.83^{e} (174.65; 199.35)
2	Bioproduct-I (Seedling, 250 ml/ha)	7.00±0.70^{ab} (6.442; 7.558)	1637.5±179.7^{b} (1540.8; 1734.2)	275.25±9.14^{bd} (262.90; 287.60)
3	Bioproduct-I (Seed, 500 ml/ha)	4.25±0.50^{efg} (3.692; 4.808)	1062.5±110.9^{d} (965.8; 1159.2)	171.25±11.12^{ef} (158.90; 183.60)
4	Bioproduct-I (Seedling, 500 ml/ha)	5.62±0.47^{bcde} (5.067; 6.183)	1362.5±110.9^{c} (1265.8; 1459.2)	230.25±13.33^{d} (217.90; 242.60)
5	Bioproduct-I (Seed, 750 ml/ha)	2.75±0.95^{hi} (2.192; 3.308)	707.5±29.9^{e} (610.8; 804.2)	33.50±10.79^{h} (21.15; 45.85)
6	Bioproduct-I (Seedling, 750 ml/ha)	3.00±0.81^{ghi} (2.442; 3.558)	652.5±114.7^{ef} (555.8; 749.2)	70.25±12.28^{g} (57.90; 82.60)
7	Bioproduct-I (Seed, 1000 ml/ha)	2.37±0.47^{i} (1.817; 2.933)	382.5±104.4^{gh} (285.8; 479.2)	41.50±9.33^{gh} (29.15; 53.85)
8	Bioproduct-I (Seedling, 1000 ml/ha)	2.75±0.50^{hi} (2.192; 3.308)	415.0±50.0^{fgh} (318.3; 511.7)	51.25±9.81^{gh} (38.90; 63.60)
9	Bioproduct-I (Seed, 1250 ml/ha)	5.12±0.25^{def} (4.567; 5.683)	1052.5±38.6^{d} (955.8; 1149.2)	186.5±21.5^{e} (174.2; 198.8)
10	Bioproduct-I (Seedling, 1250 ml/ha)	5.25±0.28^{cdef} (4.692; 5.808)	1145.0±137.0^{cd} (1048.3; 1241.7)	246.25±12.53^{cd} (233.90; 258.60)
11	Bioproduct-I (Seed, 1500 ml/ha)	4.12±0.85^{fgh} (3.567; 4.683)	630.0±235^{efg} (533; 727)	151.75±16.01^{f} (139.40; 164.10)
12	Bioproduct-I (Seedling, 1500 ml/ha)	5.87±0.25^{abcd} (5.317; 6.433)	1092.5±69.9^{d} (995.8; 1189.2)	291.25±7.97^{b} (278.90; 303.60)
13	Bioproduct-II (Seed, 1000 ml/ha)	2.25±0.50^{i} (1.692; 2.808)	255.0±31.1^{h} (158.3; 351.7)	28.25±3.10^{hi} (15.90; 40.60)
14	Bioproduct-II (Seedling, 1000 ml/ha)	2.37±0.47^{i} (1.817; 2.933)	292.5±58.0^{h} (195.8; 389.2)	28.00±6.88^{hi} (15.65; 40.35)
15	Nematicide (Seed)	2.37±0.47^{i} (1.817; 2.933)	285.0±23.8^{h} (188.3; 381.7)	22.50±2.65^{hi} (10.15; 34.85)
16	Nematicide (Seedling)	2.25±0.50^{i} (1.692; 2.808)	267.5±17.08^{h} (170.82; 364.18)	24.75±4.03^{hi} (12.40; 37.10)
17	Control (+) (Seed)	7.00±0.81^{ab} (6.442; 7.558)	1735.0±107.5^{ab} (1638.3; 1831.7)	287.0±21.9^{b} (274.7; 299.3)
18	Control (+) (Seedling)	7.12±0.62^{a} (6.567; 7.683)	1905.0±38.7^{a} (1808.3; 2001.7)	369.3±24.9^{a} (356.9; 381.6)
19	Control (-) (Seed)	0.00±0.00^{j} (-0.00; 0.00)	0.00±0.00^{i} (-0.00; 0.00)	0.00±0.00^{i} (-0.00; 0.00)
20	Control (-) (Seedling)	0.00±0.00^{j} (-0.00; 0.00)	0.00±0.00^{i} (-0.000; 0.000)	0.00±0.00^{i} (-0.000; 0.000)

* Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different at the 0.05 probability level, according to Tukey's HSD test.

When the number of J2 was analyzed, the lowest values were recorded in Bioproduct-II and Nematicide treatments. The 1000 ml/ha dose of Bioproduct-II provided the lowest values with 255.0 J2 counts in seed treatment and 292.5 J2 counts in seedling treatment. Nematicide treatment gave similarly low J2 numbers (285.0 in seed and 267.5 in seedling). In Bioproduct-I treatments, a decreasing trend in J2 numbers was observed as the dose increased. At 1000 ml/ha dose, 382.5 and 415.0 J2 numbers were recorded in seed and seedling applications, respectively. However, at 1250 ml/ha and 1500 ml/ha doses, J2 numbers increased again. In the positive control group, J2 numbers were quite high and recorded as 1735.0 in seed and 1905.0 in seedling. These results indicate that Bioproduct-II and Nematicide are effective in suppressing J2 population (Table 4).

The effects of different bioproduct and nematicide doses applied in the trial on number of egg masses were clearly observed. The nematicide treatments gave the lowest number of egg masses, 22.50 in the seed treatment and 24.75 in the seedling treatment. Similarly low number of egg masses were recorded in

the Bioproduct-II treatments, with values of 28.25 in the seed treatment and 28.00 in the seedling treatment. Seed application of Bioproduct-I at a dose of 750 ml/ha gave a low value of 33.50 number of egg masses, while this number was measured as 70.25 in the seedling treatment. The highest number of egg masses were recorded in the positive control groups; 287.0 number of egg masses in the seed treatment and 369.3 number of egg masses in the seedling treatment. These data indicate that Nematicide and Bioproduct-II are effective in preventing egg mass formation (Table 4).

The impact of the treatments on plant growth parameters

When analyzing the plant height data, the highest value of 34.63 cm was measured in the negative control group in the seedling treatment. Bioproduct-II applications had a positive effect on plant height, reaching a value of 28.38 cm in the seedling treatment. In Bioproduct-I applications, the plant height reached 32.97 cm in the seedling application at a dose of 1000 ml/ha and provided a remarkable development (Table 5).

Table 5. The effects of treatments on plant development parameters in *Meloidogyne incognita*-infested plants

Run No.	Treatment	Plant height* Mean±SD (95% CI)	Root fresh wt. Mean±SD (95% CI)	Root dry wt. Mean±SD (95% CI)
1	Bioproduct-I (Application on seed) 250 ml/ha	14.75±1.19^{d} (12.76; 16.74)	17.65±0.47^{i} (15.99; 19.31)	1.79±0.10^{g} (1.61; 1.97)
2	Bioproduct-I (Application on seedling) 250 ml/ha	26.38±2.06^{d} (24.39; 28.36)	26.78±1.67^{def} (25.12; 28.44)	2.64±0.19^{de} (2.46; 2.81)
3	Bioproduct-I (Application on seed) 500 ml/ha	15.23±1.34^{d} (13.24; 17.21)	19.21±1.17^{hi} (17.55; 20.87)	1.97±0.09^{fg} (1.79; 2.14)
4	Bioproduct-I (Application on seedling) 500 ml/ha	26.88±2.25^{c} (24.89; 28.86)	28.34±1.80^{cd} (26.67; 30.00)	2.75±0.19^{de} (2.57; 2.93)
5	Bioproduct-I (Application on seed) 750 ml/ha	18.90±3.76^{d} (16.91; 20.89)	23.95±3.42^{efg} (22.29; 25.61)	2.37±0.34^{ef} (2.20; 2.55)
6	Bioproduct-I (Application on seedling) 750 ml/ha	26.70±2.90^{c} (24.71; 28.69)	32.54±1.02^{bc} (30.88; 34.20)	3.27±0.15^{bc} (3.09; 3.44)
7	Bioproduct-I (Application on seed) 1000 ml/ha	18.38±0.95^{d} (16.39; 20.36)	19.74±1.18^{ghi} (18.08; 21.40)	1.92±0.17^{fg} (1.74; 2.09)
8	Bioproduct-I (Application on seedling) 1000 ml/ha	32.98±1.61^{ab} (30.99; 34.96)	31.75±1.91^{bc} (30.09; 33.41)	3.23±0.16^{bc} (3.05; 3.40)
9	Bioproduct-I (Application on seed) 1250 ml/ha	16.63±0.75^{d} (14.64; 18.61)	17.13±1.13^{i} (15.46; 18.79)	1.78±0.07^{g} (1.60; 1.95)
10	Bioproduct-I (Application on seedling) 1250 ml/ha	26.75±2.63^{c} (24.76; 28.74)	26.05±2.44^{def} (24.39; 27.71)	2.71±0.28^{de} (2.54; 2.89)
11	Bioproduct-I (Application on seed) 1500 ml/ha	16.75±0.65^{d} (14.76; 18.74)	17.63±1.67^{i} (15.97; 19.29)	1.84±0.13^{g} (1.66; 2.01)
12	Bioproduct-I (Application on seedling) 1500 ml/ha	25.38±2.25^{c} (23.39; 27.36)	28.25±0.86^{cde} (26.59; 29.91)	2.92±0.16^{cd} (2.74; 3.09)
13	Bioproduct-II (Application on seed) 1000 ml/ha	17.75±1.85^{d} (15.76; 19.74)	18.56±0.79^{i} (16.90; 20.22)	1.86±0.09^{g} (1.68; 2.03)
14	Bioproduct-II (Application on seedling) 1000 ml/ha	28.38±1.11^{bc} (26.39; 30.36)	33.73±1.33^{b} (32.07; 35.39)	3.43±0.14^{b} (3.26; 3.61)
15	Nematicide (Application on seed)	18.75±2.78^{d} (16.76; 20.74)	22.94±1.37^{fgh} (21.28; 24.60)	2.34±0.15^{ef} (2.16; 2.52)
16	Nematicide (Application on seedling)	33.88±2.50^{a} (31.89; 35.86)	33.30±2.62^{b} (31.63; 34.96)	3.45±0.31^{b} (3.27; 3.62)
17	Control (+) (Application on seed)	14.63±1.11^{d} (12.64; 16.61)	16.62±1.97^{i} (14.96; 18.28)	1.75±0.17^{g} (1.57; 1.93)
18	Control (+) (Application on seedling)	25.38±1.70^{c} (23.39; 27.36)	25.84±1.25^{def} (24.18; 27.50)	2.64±0.11^{de} (2.46; 2.81)
19	Control (-) (Application on seed)	18.25±0.87^{d} (16.26; 20.24)	18.34±0.98^{i} (16.67; 19.99)	1.92±0.12^{fg} (1.75; 2.10)
20	Control (-) (Application on seedling)	34.63±1.97^{a} (32.64; 36.61)	45.57±1.20^{a} (43.91; 47.23)	4.65±0.11^{a} (4.48; 4.83)

* Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different at the 0.05 probability level, according to Tukey's HSD test.

The nematicide applications supported plant growth with a plant height of 33.88 cm in the seedling application. In the positive control groups, plant height values were lower and measured as 14.62 cm in seed and 25.37 cm in seedling. When evaluating the seed treatments, it was observed that some bioproduct doses had the potential to increase plant height. In particular, the seed treatment with 1250 ml/ha dose of Bioproduct-I achieved a plant height of 18.75 cm, which showed a positive effect compared to the seedling treatments. These results showed that bioproducts can have different effects on plant growth depending on the method of application (Table 5).

In terms of root fresh weight, the highest value was obtained in the seedling application of the negative control group and this value was measured as 45.57 g. Seedling application of Bioproduct-II at 1000 ml/ha showed positive effects on root development and root fresh wt. was recorded as 33.73 g. Seedling application of nematicide also gave a high value in root development and reached 33.30 g root fresh wt. In Bioproducts-I seedling applications supported root development; in particular, seedling application at 750 ml/ha dose stood out with 32.54 g root fresh wt. Although root growth was generally lower in seed treatments than in seedling treatments, significant results were obtained at some doses. For example, 23.95 g root fresh wt. was measured in the seed treatment with the 750 ml/ha dose of Bioproduct-I and this value stood out among other seed treatments (Table 5).

In root dry weight measurements, the highest value was observed in the seedling treatment in the negative control group and this value was recorded as 4.65 g. In Bioproduct-II applications, the seedling application gave favorable results in terms of root dry wt. and a value of 3.43 g was obtained. The nematicide seedling application also increased the root dry wt. and reached a value of 3.45 g. In the Bioproduct-I applications, the seedling application at a dose of 750 ml/ha gave a root dry weight of 3.26 g. In the seed treatments, the root dry weights remained at lower levels compared to the seedling treatments. However, the root dry wt. of 2.37 g obtained with the 750 ml/ha seed treatment of Bioproduct-I was higher than the other seed treatments. These results showed that seed treatments can be effective especially in the early development of the root system (Table 5).

Response surface analysis (RSM)

The experimental data were analyzed using the surface regression model (Table 6), which is an important component of RSM and allows 10 independent variables to be optimized simultaneously (Ali and Aasim 2024). For the nematode reproduction parameters, the square effect of dose (p<0.001), application method (p<0.001) and dose (p<0.001) on root gall index and number of J2 were significant at 1% level. This indicates a non-linear dose-response relationship, suggesting that bioproduct efficacy increases at certain dose intervals, but this effect may tend to decrease at extreme doses. However, the method of application to seed or seedling showed no significant effect on root gall index (p= 0.386) and J2 number (p= 0.123). This result indicates that the type of application and dose are the main determinants of bioproduct efficacy, not the method of application (Table 6).

When examining the interaction analyzes, although the Dose ml/ha × Application on seed or seedling interaction was not significant overall, a trend approaching significance at the 5% level was observed for the J2 number (p= 0.072). This finding suggests that different treatment combinations may have potential effects on nematode control and warrants further research in the future.

The effect of factors on plant growth was more pronounced. Plant height, fresh weight and dry weight parameters were significantly affected by treatment type (p<0.001), application on seed or seedling (p<0.001) and the square effect of dose (p<0.001) at the 1% level. This indicates that the method of application has a direct effect on plant growth and that seedling treatments generally favored plant growth more. Furthermore, the interaction of treatment type × application on seed or seedling was significant at the 1% level for plant height (p= 0.006), fresh weight (p<0.001) and dry weight (p<0.001), indicating that different treatment combinations can have strong effects on plant growth (Table 6). In general, bioproduct dose and application method were the most critical factors in nematode control, while application method was the

main factor directly affecting plant growth. These results highlight the importance of strategic application plans in biological control and provide guidance for determining optimal application combinations that both reduce nematode pressure and support plant growth. In this context, it is recommended that interactions, especially those of borderline significance, should be re-examined with a larger sample in the future.

Table 6. Response surface regression analysis of *Bacillus*-based bioproducts on *Meloidogyne incognita*

Source	F-Value	*p*-Value	F-Value	*p*-Value
	Root gall index		Second-stage juveniles (J2)	
Model	43,08	0,000**	51,41	0,000**
Linear	84,69	0,000**	101,32	0,000**
Dose ml/ha	24,06	0,000**	80,18	0,000**
Treatment	123,37	0,000**	128,83	0,000**
Application on seed or seedling	0,76	0,386	2,44	0,123
Square	104,45	0,000**	83,86	0,000**
Dose ml/ha×Dose ml/ha	104,45	0,000**	83,86	0,000**
2-Way Interaction	0,77	0,576	1,01	0,421
Dose ml/ha×Application on seed or seedling	0,45	0,506	3,33	0,072
Treatment×Application on seed or seedling	0,89	0,476	1,12	0,354
	Number of egg packs		Plant height	
Model	39,37	0,000**	50,88	0,000**
Linear	72,21	0,000**	65,69	0,000**
Dose ml/ha	2,01	0,161	2,51	0,118
Treatment	100,71	0,000**	15,57	0,000**
Application on seed or seedling	6,19	0,015*	325,48	0,000**
Square	101,51	0,000**	14,72	0,000**
Dose ml/ha×Dose ml/ha	101,51	0,000**	14,72	0,000**
2-Way Interaction	2,12	0,074	3,31	0,010**
Dose ml/ha×Application on seed or seedling	0,77	0,382	0,89	0,350
Treatment×Application on seed or seedling	2,65	0,041*	4,00	0,006**
	Fresh wt. (g)		Dry wt. (g)	
Model	77,02	0,000**	75,92	0,000**
Linear	110,47	0,000**	110,54	0,000**
Dose ml/ha	0,02	0,877	0,92	0,341
Treatment	40,01	0,000**	39,41	0,000**
Application on seed or seedling	502,76	0,000**	505,12	0,000**
Square	26,40	0,000**	20,45	0,000**
Dose ml/ha×Dose ml/ha	26,40	0,000**	20,45	0,000**
2-Way Interaction	24,97	0,000**	24,74	0,000**
Dose ml/ha×Application on seed or seedling	0,66	0,418	2,50	0,118
Treatment×Application on seed or seedling	27,47	0,000**	28,74	0,000

* Significant at p=0.05; ** significant at p=0.01.

Pareto charts and normal plots analysis

The Pareto charts from the surface regression (Figure 1) rank the factors in order of importance: dose (A), treatment (B), and application method (C). The quadratic effect of dose (AA) significantly reduced root gall development, indicating a non-linear effect. Treatment (B) also had a notable impact, while treatment method (C) had minimal effect. Dose (A), the quadratic effect of dose (AA), and treatment (B) had significant effects on the J2 population, while treatment method (C) was insignificant (Figure 1).

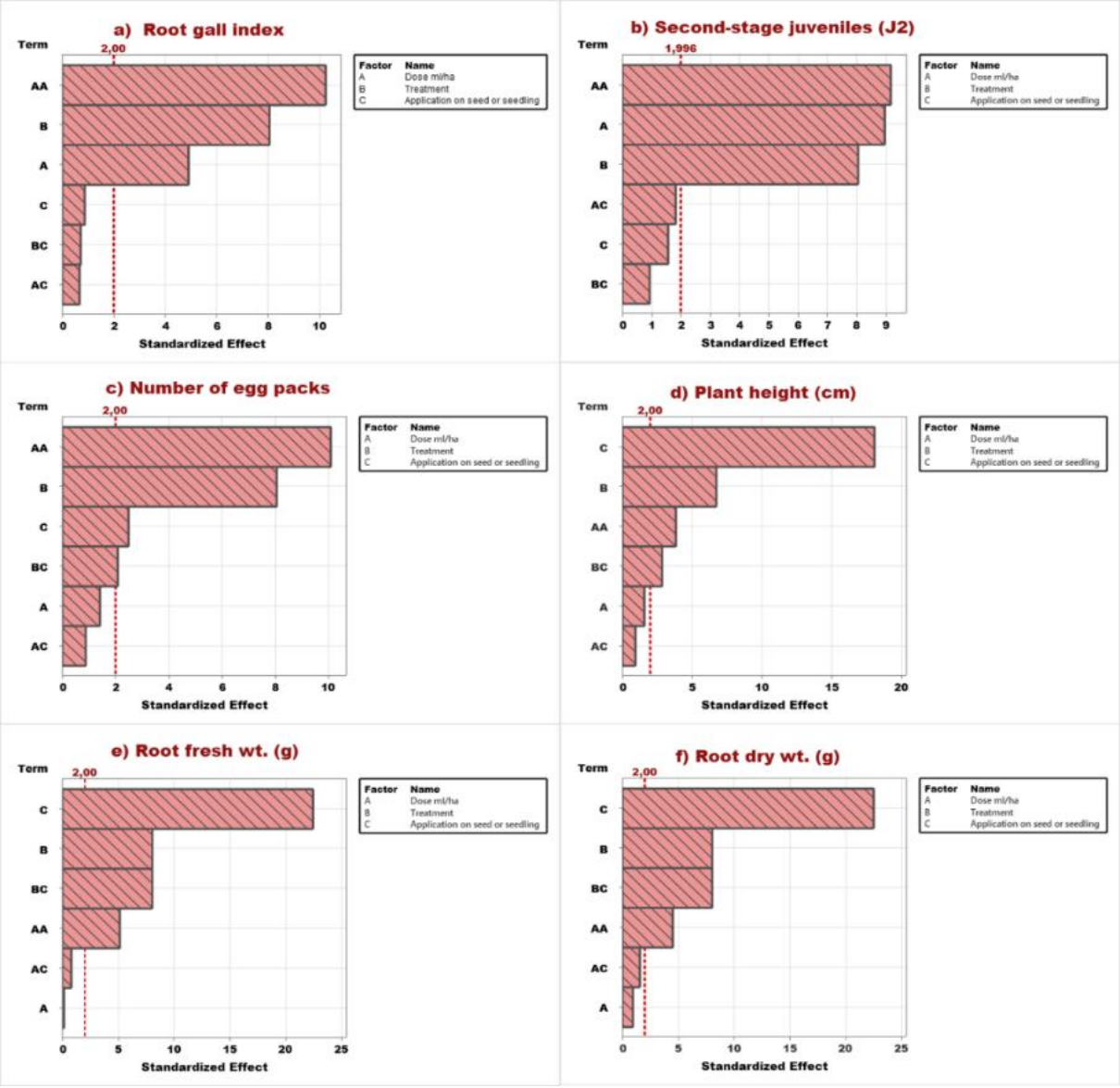

Figure 1. Pareto charts analysis of *Bacillus*-based bioproduct effects on *Meloidogyne incognita*.

AA was the most influential factor on egg production, with both high and low doses affecting it differently. Treatment (B) and method (C) also reduced egg production. Application method (C) had the greatest effect on plant height and root weight, with treatment (B) also contributing. Overall, dose (AA) was key for nematode control, and method (C) was crucial for plant growth, underscoring the need for optimal dose, application method, and timing for effective results (Figure 1).

In addition, normal plots indicate significant input factors with red squares and insignificant ones with blue circles. Factors to the right of the median line show a positive relationship with output (Xu et al., 2019). The dose (A) significantly reduced root gall index and J2 highlighting the importance of dose level. Application to seed or seedling (C) negatively impacted root weight, while treatment (B) benefited plant height and root growth, with no effect on J2 population. The BC interaction showed optimal results for plant growth. These findings suggest the need to optimize dose rates and application methods for effective nematode control and plant growth (Figure 2).

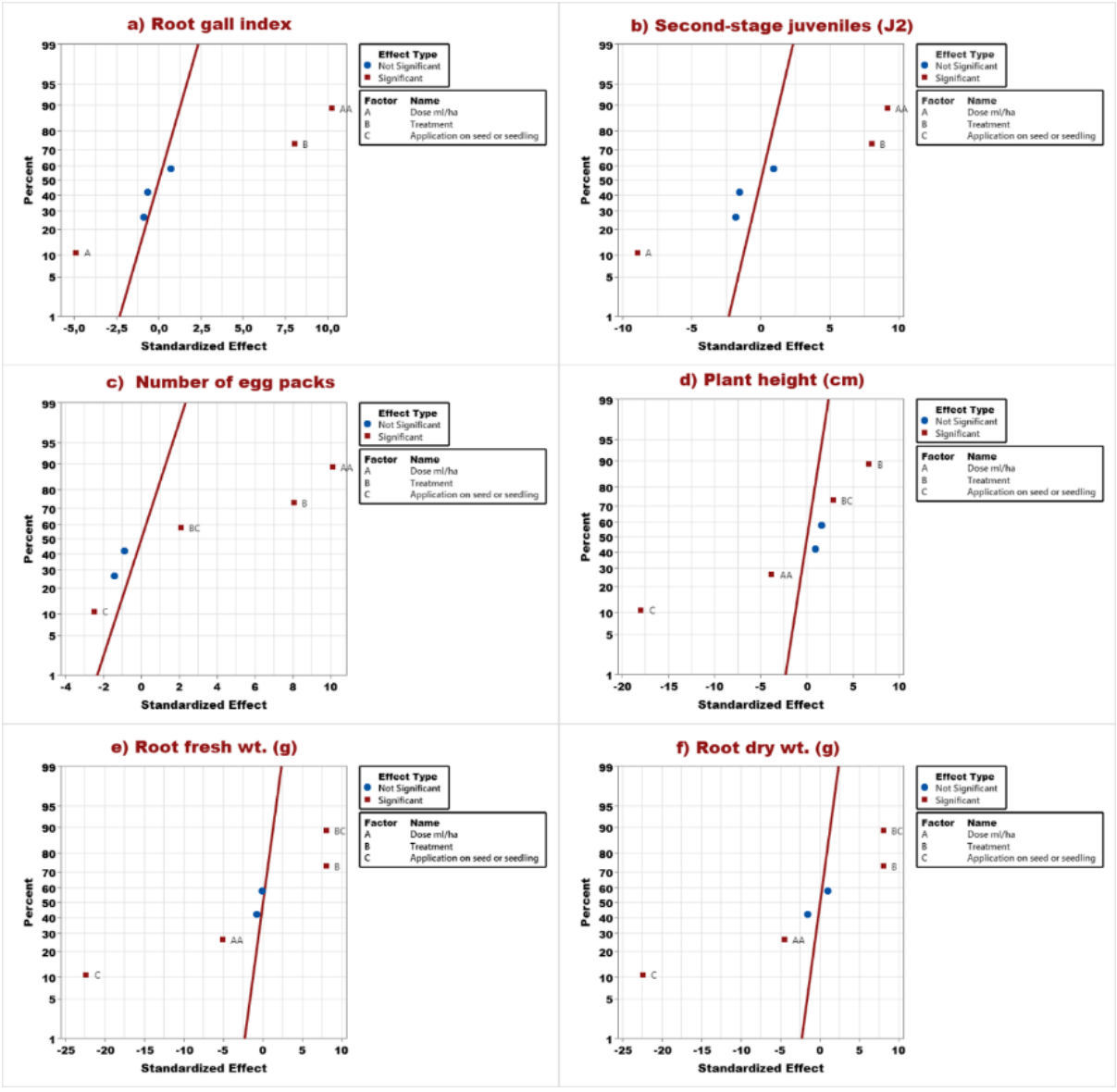

Figure 2. Normal plots analysis of *Bacillus*-based bioproduct effects on *Meloidogyne incognita.*

Discussion

The RKN species *M. incognita* causes significant soil-borne diseases by attacking a wide range of hosts (Kavitha et al., 2012). Although conventional chemical pesticides are widely used, their environmental damage and adverse effects on human health have increased interest in biological control (Yılmaz et al., 2025). Biocontrol agents colonize the rhizosphere, suppress pathogens through mechanisms such as antibiosis, competition, mycoparasitism and cell wall disruption, develop resistance in plants and promote growth (Junaid et al., 2013). Against plant-parasitic nematodes (PPNs), antagonistic bacteria provide an important line of defense in the rhizosphere, protecting roots from nematode attack (Yang et al., 2013). *Bacillus* species are particularly noted for their ability to colonize the rhizosphere, promote plant growth and reduce nematode populations. These bacteria produce toxins and enzymes that inhibit nematode reproduction, suppress egg hatching and reduce juvenile survival (Siddiqui & Mahmood, 1999).

In the current study, the efficacy of two bioproducts, *B. amyloliquefaciens* MBI 600 and *Bacillus subtilis* QST 713, as commercial formulations, in suppressing *M. incognita* infection was evaluated in pot tests in a growth chamber. In addition, the effect of different bioproduct doses on root gall index, J2 population and egg masses was approximated using Design of Experiment (DOE) and Response Surface Methodology (RSM). These statistical tools allowed the performance of the bioproducts to be analyzed in detail. Such as pareto and normal plots were used to improve the accuracy of the results and identify the variables that made the greatest contribution to the outcomes.

The results show that the application of *B. subtilis* QST 713 (Bioproduct-II) was the most effective biological agent on root gall index, J2 population and egg masses. The lowest root gall index (2.25 in seed, 2.37 in seedling) and J2 numbers (255.0 in seed, 292.5 in seedling) were recorded with Bioproduct-II, especially at the 1000 ml/ha dose. *B. amyloliquefaciens* MBI 600 (Bioproduct-I) also performed well, with the lowest root gall index (2.37) observed at 1000 ml/ha, although a slight increase was noted at 1250 ml/ha and 1500 ml/ha. Similarly, J2 populations decreased at moderate doses but increased again at the highest doses (Table 4), indicating that while the bioproduct suppresses nematode populations up to a point, additional dosage offers no further benefit. This finding highlights the importance of applying the correct dose when using bioproducts.

The ability of *Bacillus* species to control nematode infestations has been recognized in several previous studies. For example, *B. subtilis* has been considered a preventive agent against soil-borne infections, with demonstrated efficacy against nematodes and fungal species (Abd-Elgawad et al., 2010). Khalil et al. (2012) and Khalil (2013) found that root gall formation and egg masses of *M. incognita* in cotton were inhibited by *B. subtilis*, reducing nematode density in the soil. Xiang et al. (2017) similarly demonstrated that *B. subtilis* strains reduced cotton egg numbers by 73.63% to 80.72% and increased cotton yield in microplot studies. In pot trials, *B. subtilis* DTBS 5, *Pantoea agglomerans*, and *B. amyloliquefaciens* DSBA 11 significantly reduced root galls, egg masses, the egg/egg mass ratio, and reproductive factor (RF) following soil irrigation with PGPR isolates (Aballay et al., 2020). Gattoni et al. (2023) also reported over 75% mortality of second-stage juveniles (J2) of *M. incognita* with *B. firmus* I-1582 and its metabolite extracts.

In greenhouse trials, *B. amyloliquefaciens* QST 713 and *B. firmus* I-1582 were as effective against *M. incognita* as the chemical nematicide fluopyram. However, the efficacy of *Bacillus* species is strongly influenced by external factors such as temperature, light, and humidity (Brar et al., 2006). Differences between pot trials and field performance can be attributed to variations in soil structure, climatic conditions and microbial competition (Meyer, 2003; Tian et al., 2007). Therefore, developing stable and effective bioproduct formulations with long shelf-life is crucial for sustainable agriculture (Zhang et al., 2023).

Process and dose optimization is critical for enhancing efficiency and ensuring stability of promising microorganisms in bioproduct development (Kumar & Banerjee, 2013). Optimizing the rate and timing of application can influence the growth stage at which the target pest is most vulnerable (Hynes & Boyetchko, 2006). Factors such as nutrients, pH, temperature, inoculum volume, and inducers significantly affect the production of secondary metabolites and enzyme output (Kumar & Banerjee, 2013; Fayad et al., 2022). Overall, production efficiency is controlled by physicochemical parameters like temperature, carbon, nitrogen, aeration and pH (Kumar & Banerjee, 2019; dos Santos et al., 2024).

In this study, Bioproduct-I (*B. amyloliquefaciens* MBI 600) was optimized using DOE and RSM. The results showed a dose-dependent decrease in J2 population and root gall index, with the lowest values observed at 1000 ml/ha (2.37 in seed, 2.75 in seedling for root gall index; 382.5 in seed, 415.0 in seedling for J2 population). However, a significant increase in these parameters was noted at higher doses (1250 and 1500 ml/ha), once again indicating that higher concentrations do not necessarily enhance performance. RSM provided valuable insights into optimal application rates based on precise modelling of bioproduct effects.

These results underscore the importance of robust statistical methods such as DOE and RSM in developing biological control strategies. Furthermore, validation of laboratory results with field data will improve the success rate of bioproducts under real agricultural conditions. The market success of bioproducts relies not only on their efficacy in controlled environments but also on their consistency in the field. Future research should focus on evaluating the performance of bioproducts under various environmental conditions and refining their formulations accordingly.

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