

**BIOLOGICAL CONTROL OF *MELOIDOGYNE JAVANICA*  
AND *RHIZOCTONIA SOLANI* ON SOYBEAN BY  
FORMULATION OF *BACILLUS THURINGIENSIS*  
AND *TRICHODERMA HARZIANUM***

[27]

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**ABSTRACT**

Preparation of either *Bacillus thuringiensis* or *Trichoderma harzianum* were used as seed coating or soil application for management of root-knot and root rot disease complex caused by the root-knot nematode, *Meloidogyne javanica* and the fungus *Rhizoctonia solani* on soybean plants. Number of galls, root galling, egg masses and disease severity were reduced sharply on plants treated with both biocontrol agents, either as seed or soil application compared with non-treated plants. Soil application with both biocontrol agents was the best method in reducing nematode and fungus disease severity. Plant growth parameters i.e. fresh shoot and root weight, dry weight and number of pods and bacterial nodules were markedly increased with all treatments of biocontrol agent either with seed or soil treatment compared to plants grown in infested soil with either pathogens alone or combined. Results show that plant survival was more affected when *R. solani* was alone or combined with *M. javanica*. Seed coating was considered the effective method in increasing survival plants. The biochemical analysis of treated plants with both biocontrol agents were affected compared to non-treated plants grown in pathogen infested soil. The biochemical parameters i.e. chlorophyll A and B, carotein, phenols and amino acids were enhanced in bioagent treated plants compared to non-treated plants.

**Keywords:** Biological control, *Trichoderma harzianum*, *Bacillus thuringiensis*, Formulation, *Meloidogyne javanica*, *Rhizoctonia solani*, Soybean

**INTRODUCTION**

Soybean (*Glycine max* L. Merr) is one of the most important legume crops because it is considered a chief source of protein. In Egypt, a great interest has

been focused towards this crop. There are several constraints in the successful cultivation of many leguminous crops.

Root-knot nematodes, *M. javanica* and *M. incognita* were reported in peanut, broadbean, cowpea and many leguminous

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crops cultivated in sandy soils of newly reclaimed areas in Egypt. A great attention has been given to the interaction between the root-knot nematodes, *M. javanica* and the soil-borne fungus, *Rhizoctonia solani* on different crops *i.e.* cotton, pea, tomato, tobacco and soybean (Hillocks, 1985; Mousa & Hague, 1988 and Mousa *et al* 1989). They concluded that the interaction between the root-knot nematodes and soil-borne pathogenic fungi increased the incidence and severity of the major soil-borne diseases.

The use of nematicides and fungicides for controlling nematodes and soil-borne fungi, respectively is not currently appropriate for food and fruit production in several countries because of their high costs, extreme toxicities and environmental pollution. Biological pesticides have been developed since they are environmentally safe, highly desirable alternative to chemicals and have therefore received considerable attention.

The hyperparasite fungus, *Trichoderma harzianum* Rifai, has been reported to be one of the most promising biocontrol agents against plant-parasitic nematodes (Parvatha Reddy *et al* 1996). The most widely used microbial pesticides worldwide are the bacterium, *Bacillus thuringiensis*, which produce a wide variety of toxins (Schnepf *et al* 1998). It has been shown that some crystal proteins made by *B. thuringiensis* are toxic to a wide range of nematodes (Aroian *et al* 2002). During the last decade, intensive studies of the nematicidal effects of *Bacillus thuringiensis* have been carried out against phytoparasitic nematodes such as *Globodera pallida*, *Heterodera cajani* and *M. incognita* (Mozgovaya *et al* 2002).

This research aimed to use alternative safe control method against disease

complex caused by root-knot nematodes, *M. javanica* and root-rot fungus, *R. solani* on soybean.

## MATERIAL AND METHODS

### Pathogens and inoculum preparation

Root-knot nematode, *M. javanica* inoculum was obtained from heavily infected black nightshade roots (*Solanum nigrum* L.). Stock culture of nematode were multiplied under greenhouse condition at 25°C on tomato plants (*Lycopersicon esculentum* cv. Castle Rock) grown in 30 cm diameter plastic pots filled with a soil-sand mixture (1:2, v/v). Eggs of *M. javanica* were extracted from heavily galled tomato roots using sodium hypochlorite (NaOCl) method as described by Hussey and Barker (1973).

*Rhizoctonia solani* was isolated from soybean roots and maintained on potato dextrose agar (PDA). Fungus inoculum was prepared by culturing the isolate in potato dextrose broth medium for 15 days at 25°C. Mycelium was collected on sterilized filter paper to remove excess nutrients. One hundred gram of mycelium was macerated in 1000 ml distilled water and 10 ml of this suspension containing 1 g fungus inoculum added to soil in each pot (Siddiqui and Mahmood, 1995).

### Source of bioagents

Two biocontrol agents were used in this work: *i.e.* the bacterium *Bacillus thuringiensis* and the fungus *Trichoderma harzianum*. Both bioagents were obtained from the Agricultural Botany Department Collection, Faculty of Agriculture, Menoufiya University, Shebin El-kom, Egypt. Culture of *B. thuringiensis* and *T. harzi-*

*anum* were maintained on T3 medium (Travers *et al* 1987) and Gliotoxin fermentation Medium Agar [GFMA] (Brian and Hemming, 1945) in petri dishes (6 cm in diam.).

### Formulation of bioagents

The biomass of each bioagent was produced by inoculation of 500 ml flasks containing 200 ml of T3 broth medium with bacterial loop of *B. thuringiensis* and GFMB medium with equal disks of *T. harzianum*. Flasks were incubated on a rotary shaker at 250 rpm at 28°C for 3 and 15 days, for *B. thuringiensis* and *T. harzianum* respectively.

The biomass of *B. thuringiensis* was collected by centrifugation at 5000 rpm for 10 minutes and the supernatant was discarded. The resulting pellet was resuspended in 400 ml of phosphate buffer pH 7.0 as a final volume and stored in refrigerator until use.

The biomass of *T. harzianum* was collected by filtration through sterilized filter papers (Whatman No.1), homogenized and stored in refrigerator until use. Mixture of 10 gm of carboxymethyl cellulose (CMC) and 1 kg of talc powder were used to prepare the talc powder formulation as described by Vidhyasekaran and Muthamilan (1995).

Both bacterial suspension (400 ml) and fungus homogenized biomass (400 ml) were added to the carrier (1 kg talc powder for each bioagent) and mixed well under sterilized conditions to form a pasta. The pasta was air dried in laminar flow hood for 24 hrs. The dried product was powdered using a blender, sieved and packed in polyethylene bags. One gram sub-samples of each biocontrol agent powder was taken to count the colony

forming unites (cfu/g) of bacterial and fungal population by using dilution plate technique on T3 and GFMA media.

### Evaluation of bioagents

An experiment was carried out under greenhouse conditions at Faculty of Agriculture, Menofiya University, Shebin El-kom, Egypt. Powder formulations of both bio-control agents were used either as seed or soil treatment.

Plastic pots (25 cm diam.) containing autoclave-sterilized sand-soil mixture (2:1 v/v) was prepared and infested by *R. solani* and/or *M. javanica*. Soil was infested with both bioagents at the same time with both pathogens at sowing time.

Eggs of *M. javanica* were extracted as mentioned previously and was inoculated by pipetting 1500 eggs under the soybean seeds.

### Seed Treatment

Soybean seeds cv. Giza 21 were surface sterilized with 2.4% sodium hypochlorite solution for 2-3 min., rinsed in sterile distilled water and air dried overnight under laminar flow hood. Seeds were treated with the powder formulation at the rate of 1 g/100g seeds as slurry treatment according to Weller and Cook (1983). The slurry was prepared by mixing 10g powder in 40 ml of sterilized water.

### Soil Treatment

Soil treatment was carried out by mixing the powder formulation of each bioagent thoroughly with sand-soil mixture at the rate of 1% of soil weight (10g/1kg soil). Five treated seeds with both bio-

agents and non-treated were sown in each pot and each treatment was replicated three times.

Non-treated seeds and soil served as control and plants were arranged in a completely randomized block design in the greenhouse at approximately 25°C. Plants were fertilized weekly with a nutrient solution. Pre- and post-emergence damping-off were recorded after 15 and 30 days of sowing respectively.

Eight weeks after nematode inoculation, plant growth parameters *i.e* shoot and root fresh weight, dry weight, number of pods and number of bacterial nodules as well as root galling in 0-5 scale according to **Taylor and Sasser (1978)**, number of galls, egg masses and disease severity index were determined. Egg masses were stained prior to counting with 0.015% Phloxine B for 20 minutes as described by **Daykin and Hussey (1985)**. Disease severity index was calculated according to **Koberiger et al (1998)** where:

$$DI = \frac{[\text{Sum of (disease class } \times \text{ number of plants in class)} \times 100]}{[(\text{total plants}) \times 4]}$$

Changes in chemical composition in plants associated with *M. javanica* and/or *R. solani* as well as biocontrol agents treatment were determined for chlorophyll A and B, carotenoids (**Wettstein, 1957**), phenol (**Snell and Snell, 1953**) and total amino acids (**Rosen, 1957**).

### Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) using the software Statgraphics Version 3.1 for windows. Duncan's Multiple Test was

used to test for significant differences among means at  $P < 0.05$ .

### RESULTS

Data in Table (1) revealed that adding *B. thuringiensis* and/or *T. harzianum*, either as seed coating or soil application significantly reduced the nematode severity on soybean plants compared with non-treated. Soil treatment was better than seed coating in reducing nematode infection severity parameters where number of galls, root galling and number of egg masses per root system were lower than those produced due to seed coating treatment.

The reduction in number of galls was 92% for *T. harzianum* against *M. javanica* alone and *M. javanica* plus *R. solani*. For *B. thuringiensis*, the rate of galls reduction was 83% against *M. javanica* alone and 89% against *M. javanica* plus *R. solani*. As for number of egg masses, the rate of reduction was 97 and 94% for *T. harzianum* either against *M. javanica* alone or *M. javanica* plus *R. solani* respectively. Treatments with *B. thuringiensis* showed 92% and 99% reduction respectively. In general, all treated plants with both biocontrol agents showed significant reduction in nematode parameters especially as soil treatment compared with non-treated plants.

Results in Table (1) showed also that the treated plants with both biocontrol agents decreased the disease severity index caused by *R. solani* alone or combined with *M. javanica*. The lowest disease severity (60%) was observed with seeds treated with *T. harzianum* against *M. javanica* alone followed by the

Table 1. Biological control of root-knot and root-rot disease complex caused by root-knot nematode, *M. javanica* and root-rot fungus, *R. solani* on soybean.

Treatments*	Average No. of galls/root	Gall index (0-5)	Average No. of Egg masses/ root	Seedling damping-off		Plant Survival %	Disease severity index
				Pre-emergence	Post-emergence		
<b>Seed Coating</b>							
Th + Mj	26.3defg	3.3bcd	10.7defgh	1.7defg	1.0bc	46bc	60.0efgh
Th + Rs	0.0h	0g	0.0h	2.7abcde	1.0bc	26cdef	78.3abcdef
Th + Mj +Rs	53.7bc	4ab	22bcdef	3.0abcd	0.3c	34bcde	75.0abcdef
<b>Soil Application</b>							
Th + Mj	12fgh	2.3de	3.3gh	3.0abcd	0.0c	40bcde	70.0bcdefg
Th + Rs	0h	0g	0.0h	3.0abcd	0.3c	34bcde	75.0abcdef
Th + Mj +Rs	12.7 efg	2.7cd	6.3fgh	3.3abc	0.3c	26cdef	80.0abcde
<b>Seed Coating</b>							
Bt + Mj	27def	3.3bcd	13.0defgh	3.0abcd	0.3c	34bcde	76.7abcdef
Bt + Rs	0h	0g	0.0h	2.0cdef	0.7bc	46bc	65defg
Bt + Mj + Rs	18efgh	2.3bc	12.0defgh	3.0abcd	1.0bc	20def	86.7abcd
<b>Soil Application</b>							
Bt + Mj	18efgh	3.0bcd	8.7defgh	2.7abcde	0.3c	40bcd	70bcdefg
Bt + Rs	0.0h	0.0g	0.0h	3.3abc	1.0bc	14ef	91.7ab
Bt + Mj + Rs	2.3gh	7.0fg	0.7h	4.0a	0.7bc	06f	95a
<b>Non-treated</b>							
Mj alone	157.7a	5.0a	107.3a	1.7defg	1.6b	34bcde	75abcdef
Rs alone	0.0h	0.0g	0.0 h	1.0fgh	3.7a	06f	95a
Mj + Rs	32.7cdef	1.3ef	23.7bcd	1.0fgh	3.7a	06f	95a
<b>Control</b>							
	0.0h	0.0g	0.0h	0.0h	1.0bc	80a	35i
<b>Non-infested</b>							

\* Th = *T. harzianum*Rs = *R. solani*Bt = *B. thuringiensis*Mj = *M. javanica*

\*\* Number in each column followed by the same letter are not significantly different (P&lt;0.05) according to Duncan's multiple range test

treatment with *B. thuringiensis* as seed coating against *R. solani* (65%) compared with 95% on bioagents non-treated control.

Data presented in Table (1) show that the treatment with both biocontrol agents either as seed or soil treatments were enhanced slightly the percentage of survival plants compared with the bioagents non-treated plants. Results revealed that the highest percentage of survival plants were showed with plants treated with *T. harzianum* as seed coating.

Results in Table (1) showed that no significant differences in root-rot disease severity when *M. javanica* was combined with *R. solani* compared to plants grown in soil infested with *R. solani* alone.

Data presented in Table (2) revealed that adding both biocontrol agents, either as seed or soil application, enhanced markedly the plant growth parameters *i.e* shoot and root fresh weight, dry weight, number of pods and bacterial nodules compared with biocontrol agents non-treated plants grown in pathogen infested soil. The lowest values were recorded with plants grown in soil infested with *M. javanica* and *R. solani* alone and combined.

Results in Table (3) show remarkable changes in chemical composition of infected plants with both pathogens, *R. solani* and *M. javanica* alone or together compared with the infected plants treated with both biocontrol agents. Treatment with bioagents either as seed coating or soil treatment reduced the harmful effects of both pathogens on plant growth. Chlorophyll and carotein content in leaves of treated plants were slightly higher compared with non-treated control. Phenol content in treated plants with either bio-

agent was higher than non-treated control plants and also non-infested control. Total amino acid content was also affected (Table 3).

Generally, it could be conducted that adding the biocontrol agents as seed or as soil application to infected plants positively improved the chemical composition *i.e* chlorophyll (a & b), carotein, phenol and amino acids.

## DISCUSSION

Results of the present study showed that adding both biocontrol agents, *B. Thuringiensis* or *T. harzianum* to infected soybean plants with *M. javanica* or *R. solani* enhanced plant growth parameters. These results may be due to the antagonistic effect of the biocontrol agents by inhibiting parasitic root pathogens, producing biologically active substances or by transforming unavailable mineral and organic compounds into forms available to plants (**Broadbent *et al* 1977; Chet, 1987; Parvatha Reddy *et al* 1996 and Rao *et al* 1997**).

Nematode severity *i.e* number of galls, root galling and number of egg masses was reduced significantly with both *B. thuringiensis* and *T. harzianum* either as seed or soil application, although soil application was better. These findings may be due to the bio-control activity during the infection process (**Mahdy *et al* 2000 & 2001**) as *Trichoderma* (**Papavizas, 1985 and Cherif & Benhamou, 1990**) and *B. thuringiensis* (**Zuckerman *et al* 1995 and Wei *et al* 2001 & 2003**) produced various toxins and enzymes for *Trichoderma* and toxin crystals proteins for *B. Thuringiensis* that may be effective nematicides and play an important role to

Table 2. Effect of treatment with biocontrol agents on certain growth characters of soybean plants grown in soil infested with *M. javanica* and *R. solani*

Treatments*	Fresh shoot weight (g)/plant	Fresh root weight (g)/plant	Dry weight (g)/plant	Average No. of Pods/plant	Average No. of bacterial nodules/plant
<b>Seed Coating</b>					
Th + Mj	12.1bcde	3.5abcde	2.5a	7.3bcdef	18.0 a
Th + Rs	6.5efghij	1.9efghij	1.8a	4.0efghij	2.3efg
Th+Mj+Rs	10.1cdef	3.5abcde	2.1a	3.3efghij	9.3bcd
<b>Soil Application</b>					
Th + Mj	10.2cdef	2.9cdefg	1.2a	4.7efghij	2.7efg
Th + Rs	7.4defghi	2.2defghi	2.8a	6.0cdefgh	7.3bcdef
Th+Mj+Rs	6.0efghij	2.3defghi	2.1a	5.0efghij	11.3b
<b>Seed Coating</b>					
Bt + Mj	7.4defghi	4.1abc	2.6a	7.0bcdefg	10.3bc
Bt + Rs	10.5cdef	2.5defgh	2.6a	10.0abcd	11.0bc
Bt+Mj+Rs	6.3efghij	2.2defghi	1.4a	5.7defgh	10.0bcd
<b>Soil Application</b>					
Bt + Mj	7.9defgh	3.0cdefg	1.9a	10.7abc	11.3b
Bt + Rs	4.4fghij	1.5fghij	2.5a	2.7fghij	3.7defg
Bt +Mj+ Rs	4.5fghij	0.4hij	0.4a	0.3j	2.7 efg
<b>Non-treated</b>					
Mj alone	5.0fghij	1.7efghij	1.1a	2.3ghij	4.4 d
Rs alone	0.8 j	0.1j	0.01a	0.7ij	2.9 h
Mj + Rs	1.4 ij	0.3ij	0.3a	0.7ij	1.8 i
<b>Control Non-infested</b>	18.9a	4.8ab	2.7a	13.0a	2.2 hi

\* Th = *T. harzianum*      Rs = *R. solani*      Bt = *B. thuringiensis*      Mj = *M. javanica*

\*\* Number in each column followed by the same letter are not significantly different (P&lt;0.05) according to Duncan's multiple range test

Table 3. Effect of treatment with biocontrol agents on some chemical components changes in soybean plants grown in soil infested with *M. javanica* and *R. solani*.

Treatments*	Chlorophyll a+b (mg/g dry weight)	Carotenoids (mg/g dry weight)	Phenol (mg/g dry weight)	Amino Acid (mg/g dry weight)
<b>Seed Coating</b>				
Th + Mj	2.9h	1.6 c	3.2 b	37.4de
Th + Rs	4.4d	1.4 d	2.3 d	43.2bc
Th + Mj + Rs	3.3fg	1.7 b	2.5cd	20.9 I
<b>Soil Application</b>				
Th + Mj	2.1 k	0.7ij	3.2 b	38.2de
Th + Rs	4.6 c	1.2efg	2.6 c	33.1ef
Th + Mj + Rs	4.4 d	1.6 c	2.7 c	37.5 e
<b>Seed Coating</b>				
Bt + Mj	3.2 g	1.2efg	3.6 a	44.3bc
Bt + Rs	6.0 a	0.8hi	3.3 b	39.0cd
Bt + Mj + Rs	4.1 e	2.7 a	3.6 a	21.1 I
<b>Soil Application</b>				
Bt + Mj	2.3ijk	0.9 h	2.7 c	40.5cd
Bt + Rs	5.2 b	2.7 a	2.6 c	22.3 I
Bt + Mj + Rs	3.4 f	1.7 b	2.8 c	27.4gh
<b>Non-treated</b>				
Mj alone	1.3 m	0.4kl	1.5ef	62.4 a
Rs alone	2.9 h	1.1fg	1.7 e	47.7 b
Mj + Rs	1.8 l	0.7ij	1.4 f	31.4fg
<b>Control</b>	2.2jk	0.9 h	2.8 c	27.8gh
<b>Non-infested</b>				

\*Th = *T. harzianum* Rs = *R. solani* Bt = *B. thuringiensis* Mj = *M. javanica*

\*\* Number in each column followed by the same letter are not significantly different (P<0.05) according to Duncan's multiple range test

retardation the egg hatching in soil and also affect the movement of hatched infective juveniles towards the host roots. Results showed that soil application was the best application method in reducing the nematode severity; this may be referred to the good distribution of the bioagents in soil, consequently the good colonization around roots which protect the roots towards by nematode juveniles.

Results showed also that adding both biocontrol agents, as seed coating treatment, led to decreased disease severity index caused by *R. solani* alone or when combined with *M. javanica*. These results may be due to the biological activity of the biocontrol agents against pathogens and where the bioagents were able to grow along with germinating seeds and elongating roots and protect the roots against infection with pathogens (Klopper and Schroth, 1981).

Results revealed that *M. javanica* don't play any role to increase root-rot disease severity when compared to the treated plants with *R. solani* alone. These results may be refer to that *R. solani* doesn't need wounds caused by nematodes to colonize the cortex, as it has the ability to penetrate and colonize these tissues without wounds.

Remarkable changes in chemical components of infected plants treated with both biocontrol agents compared with untreated plants were recorded. Results showed remarkable decrease in chlorophyll (a & b) and carotein contents in plants infected with *M. javanica* and *R. solani*. These results are agreement with those obtained by Loveys & Bird (1973) and Wallace (1974) who reported that, *Meloidogyne* infection of roots decreases the rate of photosynthesis in leaves. They reported also that high inoculum levels of

*M. javanica* on tomato plants caused a decline in the net photosynthesis rate within two days after inoculation. Infection with both pathogens increased the total phenol and amino acids. The increase in free amino acids may be due to the proteolysis of host proteins in response to fungal and nematode attack. The increase might be also due to the synthesis of free amino acids in fungal mycelium within the host tissues (Mousa, 1979). Results also showed enhancement in total phenol in plants and that is it may be due to the infection by both pathogens Mousa (1979).

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## المقاومة البيولوجية لنيماتودا ميلودوجينا جافانिका و فطر ريزوكتونيا سولاني على فول الصويا بواسطة مستحضرات بكتريا باسيلس ثورينجنسس و فطر ترايكودرما هارزيانم

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الخضري و الجذري و الوزن الجاف للمجموع الخضري وعدد القرون وكذلك عدد العقد البكتيرية مقارنة بالنباتات المعاملة و المعدية بالكائنات الممرضة فقط. و كان من أهم النتائج المتحصل عليها أيضا هو أن النباتات القائمة قد تأثرت بشدة عند نموها فى التربة المعدية بفطر الريزوكتونيا سواء بمفرده أو مع النيماتودا و كانت معاملة البذرة بكائنات التضاد الحيوي من اكثر الطرق فاعلية في خفض شدة الإصابة بكلا الممرضين على النباتات الحية القائمة.

أظهرت نتائج التحليل الكيماوي للنباتات المعاملة بكلا كائني التضاد الحيوي مع الكائنات الممرضة أن النباتات قد تأثرت مقارنة بالنباتات الغير معاملة و النامية في التربة المعدية بالكائنات الممرضة فقط، و عموما يمكن القول أن المعاملة بكلا كائني التضاد الحيوي سواء معاملة البذرة أو التربة ضد الكائنات الممرضة قد اظهر زيادة في محتوى النباتات المعاملة من كلوروفيل ( ا ) ،

تم استخدام مستحضرات لكل من البكتريا باسيلس ثورينجنسس والفطر ترايكودرما هارزيانم سواء كمعاملة البذور أو التربة وذلك لمقاومة المرض المركب المتسبب عن كل من نيماتودا تعقد الجذور ميلودوجينا جافانिका و فطر الريزوكتونيا سولاني على نباتات فول الصويا. أظهرت النتائج أن استخدام كلا كائني التضاد الحيوي في صورة مركب حيوي سواء كمعاملة بذرة أو تربة قد أدى لخفض عدد العقد و عدد أكياس البيض النيماتودية و كذلك درجة الإصابة باعفان الجذور و ذلك بالمقارنة بالنباتات الغير معاملة بأي من المركبين الحيويين . أظهرت النتائج أيضا أن معاملة التربة بكلا كائني التضاد الحيوي كانت افضل طريقة في خفض الإصابة النيماتودية و الفطرية معا. أدت المعاملة بكل من البكتريا باسيلس ثورينجنسس والفطر ترايكودرما هارزيانم سواء كمعاملة البذور أو التربة إلي زيادة معنوية في الصفات الخضرية للنباتات مثل الوزن الطازج للمجموع

ب) و الكار وتين والفينولات والأحماض  
الأمينية مقارنةً بالنباتات  
الغير معاملة و النامية في تربة معدية بالفطر  
الممرض والنيماتودا أو كلاهما.

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