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The Use of *Trichoderma longibrachiatum* and *Mortierella alpina* Against Root-Knot Nematode, *Meloidogyne javanica* on Tomato

Turki A. AL-Shammari[1], Ali H. Bahkali[2], Abdallah M. Elgorban[1], Maged T. El-Kahky[3] and Basheer A. Al-Sum[2]*

[1]Center of Excellence in Biotechnology Research, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia,
[2]Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.
[3]Plant Pathology Department, College of Agriculture, Mansoura University, Mansoura 35516, Egypt.

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The anti-nematode activity of *Trichoderma longibrachiatum* and *Mortierella alpina* was studied against *Meloidogyne javanica*. Treatment of eggs with *T. longibrachiatum* and *M. alpina* infected 89.3 and 90.3% eggs, respectively at 10^7cfu/ml. The both fungi decreased hatching rate of eggs by 8.9% (*T. longibrachiatum*) and 5.7% (*M. alpina*) and. Otherwise, the culture filtrates of *T. longibrachiatum* and *M. alpina* caused mortality of *M. javanica* second stage juveniles (J_2) with 64.5 and 54.3% after 72 h of exposures, respectively. Under greenhouse conditions, all treatments reduced the disease severity and enhanced plant growth compared to untreated control. Both fungi reduced galls number/plant, number of egg masses/plant and the number of eggs/eggmass. Plant height, root length, fresh and dry weight/plant were significantly reduced because of infection with *M. javanica*, however the application of biocontrol agents recovered this reduction. Furthermore, they enhanced the growth parameters compared with the control. Our results proved that the application of different biocontrol agents not only has a toxic effect on *M. javanica*, but also enhances the plant growth, supplying many nutritional elements and induction the systemic resistance in plants. We demonstrated that application at a concentration of 10^6 or 10^7cfu/ml to soil is needed for sufficient biocontrol of *M. javanica* by *T. longibrachiatum* and *M. alpina*.

Key words: *Meloidogyne javanica*, *T. longibrachiatum*, Tomato.

Plant-parasitic nematodes cause major economic losses to agricultural crops worldwide[1]. Because of the problems caused by agrochemicals, evolution of alternative method measures is of considerable importance. *Meloidogyne* species are sedentary endoparasites and are through the most deleterious agricultural pests, attacking a wide range of crops[2].

* To whom all correspondence should be addressed.
E-mail: basheeralsum@gmail.com

Tomato (*Lycopersicon esculentum* Mill.) is considered as one of the most important, favorable and economic vegetable in Saudi Arabia. The root-knot disease is one of most disease attracting tomato under greenhouse conditions tomato[3]. *Trichoderma* species have been used as biocontrol agents against foliar, soilborne and postharvest diseases[4,5]. These fungi may also promote plant growth[6] and have the ability to root colonization and the cortex[7,8].

Several attempts have been made to utilize *Trichoderma* spp. to management *Meloidogyne* species. Windham *et al*.[9] reported

that the eggs production in *Meloidogyne arenaria* was reduced following soil treatments with *T. harzianum* and *T. koningii*.. Among several other plant-based formulations of *T. harzianum* that were evaluated against *M. incognita*, castor cake extracts showed the best biocontrol activity[10]. Depression of *M. javanica* infection with some isolates of *T. harzianum* and *Arthrobotrys oligospora* has been reported[11].

Different mechanisms have been approached for the biocontrol activity of *Trichoderma* spp. against pathogenic fungi: competition, antibiosis, mycoparasitism, and enzymatic hydrolysis[12,13]. Enzymes such as glucanases, chitinases, and proteases appear to be very important in the mycoparasitic process[14]. A mechanism of induced resistance is currently being examined, and guide for defense responses induced by *T. harzianum* has been provided[8]. All mechanisms, except competition, can potentially be involved in the nematode biological control process. Information about the possible mechanisms of this fungal activity against nematodes is very limited; understanding these processes could lead to the development of improved biocontrol application methods and selection of active isolates. According to Saifullah and Thomas[15] direct interactions between *T. harzianum* and the potato cyst nematode *Globodera rostochiensis* were shown *in vitro* . This fungus penetrated the cysts and the eggs in those cysts, resulting in larval death. The effect of *T. viride* metabolites on nematodes was demonstrated by implementing root-dipping in the fungal culture filtrate[16]. In the present study, we study the nematicidal activity of *T. longibrachiatum* and *M. alpina* against *M. javanica in vitro* and *in vivo*.

MATERIALS AND METHODS

In vitro experiments

Nematode inoculum preparation

Samples were collected from a tomato greenhouse in Riyadh region (Saudi Arabia), and a single eggmass was used to obtain a population on a Sultana tomato variety for the experiments. The Eggs were extracted from infested tomato roots by 1% NaOCl. The extracted eggs were washed with tap water to remove NaOCl[17]. The specie of the nematode was identified as *M. javanica* according to morphological and morphometrical characters[18].

Fungi isolation and identification

Females, eggs were randomly selected from each soil sample and investigated for fungi colonization. Females were surface-treated with 0.5% NaOCl for 3 min, rinsed with sterile deionized water, and treated with an antibiotic solution (SC) (100-ppm streptomycin and 50 ppm chlortetracycline). Females were placed on water agar (WA) in 9 cm. diameter Petri plates containing SC, five females per plate. The plates were incubated at room temperature (25± 2 °C) and examined for fungal growth at 3-5 day intervals up to 15 days of incubation. The Mycelial of antagonistic fungi growing on females were transferred to potato dextrose agar (PDA) plates and next transferred to other media to identification.

For isolation from eggs, were carefully rinsed with sterile deionized water and suspended in sterile water at 5eggs/ml. A total of 1ml of the egg suspension was spread on the surface of each 10-cm. diameter WA plate containing the above antibiotics, and 10 plates per soil sample were used. Mycelium growth of antagonistic fungi from eggs was examined using a microscope (40-100× magnification) at 1-2 day intervals from 3 days to 3 weeks after inoculation. The mycelium growing from the eggs was transferred to PDA, cornmeal agar (CMA) and oatmeal agar (OA). Identification was based on colony morphology and microscopic characters[19,20,21,22].

Effect of *T. longibrachiatum* and *M. alpina* on egg hatching of *M. javanica*

The effect of *T. longibrachiatum* and *M. alpina* on egg colonization and egg hatching were studied. Five concentrations from antagonistic fungi were prepared (0, 10^4, 10^5, 10^6 and l0^7cfu/ml) in hatching cups (sterilized by 5% NaOCl); supplemented with SC. Egg masses were dissolved in a 1% sodium hypochlorite solution, and then calculated. A total 2000 eggs were added to hatching cups. All cups were incubated at 25±2 °C for 2 weeks in the dark, with four replicates. Number of infected eggs, uninfected eggs and empty eggs were counted using a microscope at 40x and 100x magnifications. The eggs infection percentages were calculated[23,24]. In case of eggs hatching,

number of emerged J_2 in cups was calculated by using a stereomicroscope[25].

In order to re-isolate the fungi, infected eggs were washed three times with sterile distilled water and spread on the surface of Kerry's semi-selective media[26] and incubated at 25±2°C. The eggs were inspected daily (20x magnification) and fungi that grew from the eggs were isolated by aseptic transfer of hyphal tips to cornmeal agar (CMA) supplemented with 200 ppm of streptomycin sulfate and fungal colonies were identified[27]. The Eggs hatching rate were determined by counting all eggs and J_2 in each cup under the microscope and calculated according to Sun *et al.*[28], the following formula: Egg hatch rate = 100 x J_2/ (eggs + J_2).

Effect of culture filtrates of *T. longibrachiatum and M. alpina* on mortality of *M. javanica* J_2.

The effect of culture filtrates of *T. longibrachiatum* and *M. alpina* was studied against *M. javanica in vitro*. Culture filtrates were obtained by growing *T. longibrachiatum* and *M. alpina* on potato dextrose broth (100 ml in 250 ml flasks). The medium was autoclaved; each flask was then inoculated with four discs (8 mm. diameter) from a vigorously growing culture on the PDA, and incubated at 25± C for two weeks. The cultures were passed through Whatman filter paper No. 1 to remove the mycelial mats. The filtrates thus obtained were designated as a 100% concentration. Further concentrations (0, 25, 50, 75 and 100%) were made by adding the requisite amount of sterilized distilled water. 5 ml of the culture filtrate from each concentration was poured into each Petri plate and about 80 freshly J_2 of *M. javanica* in 0.2 ml distilled water were added to each Petri plate. Each treatment was replicated five times. The number of dead (unmoved) J_2 in each Petri plate were counted after 24, 48 and 72 h and their percentages were calculated.

***In vivo* experiment**

Effect of various concentrations of *T. longibrachiatum* and *M. alpina* on *M. javanica* infection

Seeds of tomato var. Sultana were surface sterilized with 1% NaOCl for 5 min and sown in pots (4kg) containing a pasteurized soil mix (sandy loam: sand: Peat moss, 2:1:1). Tomato seedlings at the four-leaf stage were inoculated with different extract concentrations of *T. longibrachiatum* and *M. alpina* (at 10^4–10^7cfu/ml) by root dipping (5 min.), respectively. After 3 days, the seedlings were inoculated with 2000 *M. javanica* J_2 per individual seedling. Seedlings that were inoculated with the nematode or not treated with nematode and fungi were used as controls. Four weeks after seedling inoculation with nematode, number of gall/plant, egg masses/plant and number of eggs/single egg mass were calculated. In addition, the growth parameters were recorded.

Statistical analysis

The data collected from all experiments were statistically analyzed using the Statistic Analysis System Package (SAS Institute, Cary, NC, USA). The differences between the treatments were studied using Fisher's Least Significant Difference (LSD) test and Duncan's Multiple Range Least[29]. All analysis were performed at P 5 % level.

RESULTS

Effects of *T. longibrachiatum* and *M. alpina* on egg hatching and egg infection of *M. javanica in vitro*

The Results in Table 1 show that *M. alpina* caused the lowest hatching rate (5.7%) and gave the highest eggs infection with 90.3 when compared with control. While, *T. longibrachiatum* were less effective than *M. alpina* in reduction of eggs hatching (8.9% hatching rate) and eggs infection (89.3%). On the other hand, it was observed that there was a significant difference between the concentrations in eggs hatching inhibition and eggs infection, but there were non-significant difference between 10^6 and10^7cfu/ml (Table 2).

Effect of culture filtrates on J_2 mortality of *M. javanica.*

The Data in Table 2 reveal that the maximum mortality of J_2 (64.5%) was observed in 100% concentration of *T. longibrachiatum* filtrate of 72 h from exposure. While, *M. alpina* produced 54.3% mortality of J_2 at 100% concentration (Figure 1). The mortality was a little in 25% concentration, which was statistically similar to that in 0% concentration.

Mean individual mortality (50.6%) was observed in 100% concentration of culture filtrate after 72 h followed by 75% concentration which

caused 37.3% mortality in case of *T. longibrachiatum*. While, *M. alpina* produced 42.3% mean of mortality at100 concentration after 72 h of exposure to culture filtrate and 33.1% mortality mean at 75% concentration. A progressive an increase in the concentrations of the culture filtrate resulted in increase in the percent mortality of J_2. The relationships between the concentrations and percent mortality at different time intervals were noticed.

Effect of *T. longibrachiatum* and *M. alpina* on disease severity

The results of pot experiment indicated that all treatments significantly reduced the number of galls/plant, eggmasses/plant and eggs/single eggmass compared with controls. The highest reduction in gall number/plant (48.0), number of eggmasses/plant (30.8) and number of eggs/single eggmass (398.8) were produced when *T. longibrachiatum* was applied at 10^7cfu/ml concentrate. However, *M. alpina* reduced the galls number/plant (55.8), the number of eggmasses/ plant (35.0) and the number of eggs/single eggmass (413.3), when compared with controls (Table 3). Conversely, it was observed that there were significant differences between these

Table 1. Effects of *T. longibrachiatum* and *M. alpina* on egg hatching and egg infection *in vitro*

Treatment	10^4 cfu/ml				
	Total egg	J_2	Hatching rate%	Egg infection	Infection %
T. longibrachiatum	2000	409.3^b	17.0^b	1463.5^a	73.2^a
M. alpina	2000	276.0^c	12.1^c	1143.0^b	57.2^b
Control	2000	895.2^a	30.9^a	8.6^c	0.43^c
10^5 cfu/ml					
T. longibrachiatum	2000	311.3^b	13.5^b	1603.0^a	79.9^a
M. alpina	2000	194.4^c	8.9^a	1531.0^b	76.6^a
Control	2000	895.2^a	30.9^c	8.6^c	0.43^b
10^6 cfu/ml					
T. longibrachiatum	2000	203.7^b	9.2^b	1776.2^a	88.9^a
M. alpina	2000	123.2^c	5.8^c	1804.6^b	90.2^a
Control	2000	895.2^a	30.9a	8.6^c	0.43^b
10^7 cfu/ml					
T. longibrachiatum	2000	192.4^b	8.9^b	1785.0^a	89.3^a
M. alpina	2000	121.0^c	5.7^c	1805.8^b	90.3^a
Control	2000	895.2^a	30.9^a	8.6^c	0.43^c

Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P≥0.05)

Table 2. Effects of different concentration from *T. longibrachiatum* and *M. alpina* on egg hatching and egg infection *in vitro*

Treatments	*T. longibrachiatum*				*M. alpina*		
	Total egg	J_2	Hatching rate%	Egg infection	J_2	Hatching rate%	Egg infection
Control	2000	895.2^a	30.9^a	8.6^c	895.2^a	44.6^a	8.6^c
10^4cfu/ml	2000	409.3^b	17.0^b	1463.5^b	276.0^b	12.1^b	1143.0^c
10^5cfu/ ml	2000	311.3^c	13.5^c	1603.0^{ab}	194.4^c	8.9^c	1531.0^b
10^6cfuml	2000	203.7^d	9.2^d	1776.2^a	123.2^d	5.8^d	1804.6^a
10^7cfu/ ml	2000	192.4^d	8.9^d	1785.0^a	121.0^d	5.7^d	1805.8^a

Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P≥0.05)

Table 3. Effect of biocontrol agents on growth parameter and disease severity of tomato plants under greenhouse conditions

Treatment	10^4 cfu/ml						
	PH (cm.)	RL (cm.)	FW/P (gram)	DW/P (gram)	NG/P	NEM/P	NE/E
T. longibrachiatum	46.3^b	12.9^b	311.7^b	27.5^b	71.0^b	41.7^b	412.2^b
M. alpina	43.2^b	10.4^c	297.4^b	24.1^b	75.8^b	46.3^b	427.5^b
Non-infested	64.3^a	16.5^a	337.5^a	32.5^a	0.0^c	0.0^c	0.0^c
Infested control	41.7^b	8.0^d	151.8^c	17.1^c	340.3^a	156.3^a	599.3^a
10^5 cfu/ml							
T. longibrachiatum	51.9^b	15.1^a	318.6^b	31.0^a	61.0^b	35.2^a	414.5^b
M. alpina	45.5^{bc}	12.8^b	300.8^b	26.5^b	56.5^b	35.8^b	407.3^c
Non-infested	64.3^a	16.5^a	337.5^a	32.5^a	0.0^c	0.0^c	0.0^d
Infested control	41.7^c	8.0^c	151.8^c	17.1^c	340.3^a	156.3^a	599.3^a
10^6 cfu/ml							
T. longibrachiatum	62.4^a	16.0^a	347.8^a	33.1^a	51.3^b	32.5^b	400.5^c
M. alpina	52.9^a	13.5^b	303.0^b	28.9^b	55.5^b	35.5^b	414.3^b
Non-infested	64.3^a	16.5^a	337.5^a	32.5^a	0.0^c	0.0^c	0.0^d
Infested control	41.7^b	8.0^c	151.8^c	17.1^c	340.3^a	156.3^a	599.3^a
10^7 cfu/ml							
T. longibrachiatum	64.3^a	16.7^a	349.8^a	34.2^a	48.0^c	30.8^b	398.8^c
M. alpina	58.4^a	13.4^b	303.3^b	29.0^b	55.8^b	35.0^b	413.3^b
Non-infested	64.3^a	16.5^a	337.5^a	32.5a	0.0^d	0.0^c	0.0^d
Infested control	41.7^b	8.0^c	151.8^c	17.1^c	340.3^a	156.3^a	599.3^a

PH= Plant height RL=Root length FW/P= Fresh weight DW/P=Dry weight/plant
NG/P= NO. of Galls/Plant NEM/P=No. of Eggmasses/Plant NE/E=No. of egg/eggmass
Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (Pe≥0.05)

concentrations. The best concentrate in all treatments was 10^7cfu/ml (Table 4).

Effect of *T. longibrachiatum* and *M. alpina* on growth parameter of tomato plants

The data in Table 3 show that the application of the two bio-agents resulted in improved plant growth parameters. The results showed that plant height, root length, fresh and dry weight of plants infected with *M. javanica* were significantly lower than those of other treatments at Pe"0.05. The maximum increase in plant growth parameters was recorded in plants treated with *T. longibrachiatum*. It was noticed that application of *T. longibrachiatum* improved the plant height,

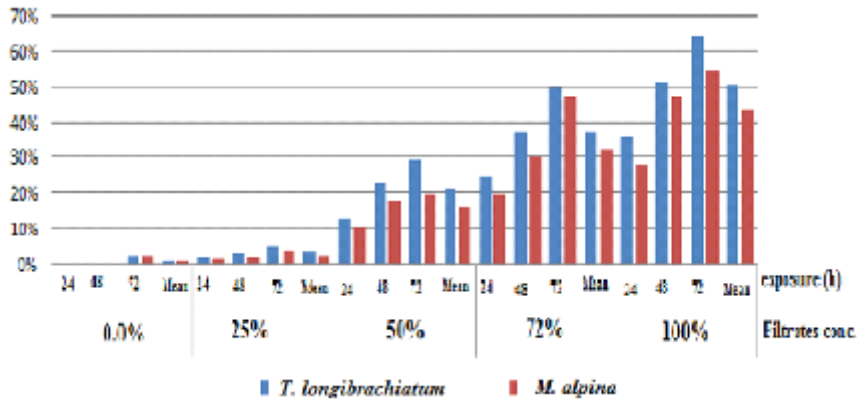

Fig. 1. Effect of culture filterates ov *T. longibrachiatum* and *M. alpina* on mortality of *M. javanica* J_2

Table 4. Effect of different concentrations from biocontrol agents on growth parameter and disease severity of tomato plants under greenhouse conditions

Treatment	*T. longibrachiatum*						
	PH (cm.)	RL (cm.)	FW/P (gram)	DW/P (gram)	NG/P	NEM/ P	NE/E
Non-infested control	64.3[a]	16.5[a]	337.5[a]	32.5[a]	0.0[d]	0.0[c]	0.00c
Infested control	41.7[d]	8.0[c]	151.8[d]	17.1[c]	340.3[a]	156.3[a]	599.3[a]
10^4 cfu/ml	46.3[c]	12.9[b]	311.7[c]	27.5[b]	71.0[a]	41.7[b]	412.25[b]
10^5 cfu/ml	51.9[b]	15.1[ba]	318.6[b]	31.0[a]	56.5[b]	35.2[c]	407.3[b]
10^6 cfu/ml	62.4[a]	16.0[a]	347.8[a]	33.1[a]	51.3c[b]	32.5[c]	400.5[b]
10^7 cfu/ml	64.3[a]	16.7[a]	349.8[a]	34.2[a]	48.0[c]	30.8[c]	398.8[b]
M. alpina							
Non-infested control	64.3[a]	16.5[a]	337.5[a]	32.5[a]	0.0[d]	0.0[d]	0.0[c]
Infested control	41.7[e]	8.0[d]	151.8[c]	17.1[c]	340.3[a]	156.3[a]	599.3[a]
10^4 cfu/ml	58.4[b]	10.43[c]	297.4[c]	24.1[b]	75.8[b]	46.3[b]	427.5[b]
10^5 cfu/ml	52.9[c]	12.8[b]	300.8[bc]	26.5[b]	61.0[c]	35.8[c]	414.5[b]
10^6 cfu/ml	45.5[d]	13.5[b]	303.0[b]	28.9[b]	55.5[c]	35.5[c]	414.3[b]
10^7 cfu/ml	43.2[d]	13.8[b]	303.3[b]	29.0[b]	55.8[c]	35.0[c]	413.3[b]

PH= Plant height RL=Root length FW/P= Fresh weight DW/P=Dry weight/plant
NG/P= NO. of Galls/Plant NEM/P=No. of Eggmasses/Plant NE/E=No. of egg/eggmass
Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P≥0.05)

root length, fresh and dry by 64.3, 16.7, 348.9 and 34.2, respectively. In case of effect of different concentration, it was cleared that 10^7 cfu/ml the best concentrate affecting growth parameters of tomato plants in all treatments (Table 4).

DISCUSSION

In vitro treatments, we noticed that *T. longibrachiatum* able to colonize egg and J_2 of *M. javanica*. Sharon *et al*.,[30] showed direct parasitism of *T. harzianum* on *M. javanica* under *in vitro* condition. The fungus parasitism of eggs and larva through the increase in chitinase and protease activities. This would be indicators of eggs infection capability. They showed extracellular protease enzyme secreted by the fungus, which play an important role for the penetration cuticle of J_2 and gelatin matrix of eggs. Because of *Trichoderma* takes place in the soil, out of plant roots or in the cortex tissue[4,31] and has no direct relationship with nematode in the host tissue, so an induced resistance cascade can probably be excluded or fungal metabolites with anti-nematode activity.

Our results suggest that *T. longibrachiatum* caused direct and indirect effect on nematode eggs, J2 and inducing resistance in the plant, and it can reduce nematode penetration, nematode feeding and egg hatching. Chitinases, which have been assumed, required for hyphal growth[32], are a kind of inducible enzyme catalyzing chitin that is one of the important components of fungal cell wall, nematode and insect eggshell and insect cuticle.

The role of chitinase in infecting nematode eggs were reported in *Paecilomyces lilacinus* and *Pochonia* spp. isolated from infected nematode eggs[33,34]. Because of the proteinaceous and chitinous nature of nematode eggshell, *T. longibrachiatum* like other nematophagous fungi must be able to produce extracellular chitinase and protease enzymes. It is also possible that other lytic enzymes be involved in egg penetration. Our results indicate that *T. longibrachiatum* have significant potential as a bio-control agent against the root-knot nematode *M. javanica* in greenhouse experiment.

Application of tomatoes with *T. longibrachiatum* can significantly decrease disease severity. Appropriate rate of *T. longibrachiatum* or reducing nematode activities such as number of gall, eggmasses production and number of egg/ eggmass was observed in both 10^6

and 10^7cfu/ml concentrations. Results also showed that *T. longibrachiatum* significantly increased growth parameters compared to control and this suggest that *T. longibrachiatum* can induce such defense enzymes and probably other defense compounds leading to systemic resistance in plants. A number of studies have revealed that root colonization by *Trichoderma* spp. increased level of defense related plant enzymes as well as various peroxidase, chitinase, b-1, 3-glucanases, lipoxigenase and phenylalanine ammonia lyase[8,35,36]. Also, *Trichoderma harzianum* mutant promotes cucumber growth by enhanced production of indole acetic acid and plant colonization[37]. The results of our study showed that *in vitro* application of *M. alpina* significantly increased the mortality of J_2 of nematode compared to the untreated control. In addition, *T. longibrachiatum* produced the greatest inhibition of eggs and infection rate of eggs of *M. javanica* compared with treatment with *T. longibrachiatum* and control. A few studies have focused on the use of this fungus to control root-knot nematode. Daniel *et al*.,[38] revealed that under electron microscopic examination *Mortierella* sp. within the gelatinous matrix of *Meloidogyne* spp. and observed numerous hyphae of *Mortierella* was seen inside hatched eggs but un-hatched eggs. Under greenhouse condition, our results proved that treatment with *M. alpina* increased the growth parameters of tomato compared with controls. It was mentioned that *M. alpina* plays a role in growth promotion by production of plant hormones and other growth-promoting substances such as auxins[39].

CONCLUSION

The results of our study suggest that *T. longibrachiatum* and *M. alpina* can be used as a bioagents for the sustainable management of root knot nematodes on selected crops and in particular circumstances.

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