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A Role of the Gelatinous Matrix in the Resistance of Root-Knot Nematode (*Meloidogyne spp.*) Eggs to Microorganisms

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Abstract: The survival of eggs of the root-knot nematode *Meloidogyne javanica* was studied in a series of experiments comparing the infectivity of egg masses (EM) to that of separated eggs (SE). The EM or SE were placed in the centers of pots containing citrus orchard soil and incubated for 24 hours, 10 days, or 20 days. Following each incubation time, 10-day-old tomato plants were planted in each pot, and 3 to 4 weeks later the plants were harvested and the galling indices determined. In the EM treatments, galling indices of ca. 4.0 to 5.0 were recorded after all three incubation periods; in the SE treatments, the infectivity gradually declined to trace amounts by 20 days. Incubating EM and SE for 2 weeks in four different soil types showed the same pattern in all the soil types: EM caused heavy infection of the test plants while the infection rate from the SE was extremely low. Incubating EM and SE in soil disinfested with formaldehyde resulted in comparable galling indices in most treatments. In petri dish experiments, 100 mg of natural soil was spread at the perimeter of a Phytagel surface and EM or SE of *M. incognita* were placed in the center. Light microscopy revealed that within 5 to 10 days the SE were attacked by a broad spectrum of microorganisms and were obliterated while the eggs within the EM remained intact. Separated eggs placed within sections of gelatinous matrix (GM) were not attacked by the soil microorganisms. When selected microbes were placed on Phytagel surfaces with EM of *M. incognita,* electron microscopy demonstrated that at least some microbes colonized the GM. As the major difference between the EM and the SE was the presence of the GM, the GM may serve as a barrier to the invasion of some microorganisms.

Key words: biological control, *Burkholderia cepacia,* egg, electron microscopy, gelatinous matrix, *Meloidogyne, Mortierella* sp., nematode, root-knot nematode.

The survival of plant-parasitic nematode eggs in soil, particularly within egg masses (EM) of root-knot and other nematodes, is a fascinating chapter in the ecological adaptation of organisms to a hostile environment. As microorganisms are the major factor responsible for the decomposition of organic matter in the soil, survival of nematode eggs likely depends on an antimicrobial defense mechanism. The root-knot nematode gelatinous matrix (GM) is synthesized by female rectal glands (Maggenti and Allen, 1960). Because it is secreted in voluminous amounts around the nematode eggs, one speculation is that the GM provides some protection against predators or microbial antagonists in the soil (Bird, 1971; Eisenback, 1985). Another view is that the function of the GM is unclear; it could have no role or merely function as a waste product of a high-carbohydrate diet (Geraert, 1994).

Direct experimental evidence for an antimicrobial role of the GM has been limited to studies revealing that the GM can agglutinate or inhibit reproduction of certain bacteria (Orion and Kritzman, 1991; Sharon et al., 1993). The purpose of the present study was to further investigate the putative role of the GM in protection of eggs against microorganisms by comparing the infectivity and survival of free eggs vs. eggs surrounded by gelatinous matrix.

Materials and Methods

Pot experiments: For experiments comparing infectivity of eggs in EM with the infectivity of separated eggs (SE), the inoculum source was infected tomato roots containing galls and EM of the root-knot nematode *Meloidogyne javanica,* obtained from 2-month-old nematode cultures in the greenhouse. Separated eggs were obtained by agitating root pieces 1.0 to 2.0 cm long for 2 minutes in 1.0% sodium hypochlorite solution and then rinsing three times in tap water over 60- and 30-µm sieves. The SE were transferred from the 30-µm sieve to a 250-ml beaker to form an egg suspension. Inoculum units were 100 mg of chopped galled roots per pot for the EM treatment and the quantity of SE obtained from 100 mg of chopped galled roots (8,000–12,000 eggs) for the SE treatment.

Soil samples were collected from the four agricultural locations specified in Table 1. The samples were taken from a depth of 15 to 40 cm, sieved, and kept moist in plastic buckets at room temperature (20–30 °C). Disinfected soil was prepared by mixing 38% formaldehyde solution with soil in a 10-liter plastic bucket with a perforated bottom at a concentration of 1.5 ml 38% formaldehyde per kg soil, rinsing the soil three times with 5 liters of tap water, and aerating it for 10 days (Sinha et al., 1988).

To examine the relative infectivity of SE and EM and the effects of the soils on infectivity, 250-ml plastic pots were filled with soil and inoculum units of EM or SE were placed in the centers of the pots at a depth of 3 to 4 cm. The inoculated pots were incubated in a screen house (24–28 °C) with natural lighting for 24 hours, 10 days, or 20 days. Following incubation, one 10-day-old *M. javanica*-susceptible tomato (*Lycopersicon esculentum*

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The *Mortierella* sp. was isolated through an activity negotiated by ARS as part of the Sino-American Biological Control Laboratory Program.
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TABLE 1. Characteristics of four Israel soil types used in the pot experiments.

Location	Region	Soil type	Annual rainfall (mm)	Crop	Sand (%)	Silt (%)	Clay (%)	Organic matter (%)
Zemach	Jordan Valley	Calcerous Serozem	350 + irrigation	Banana	27.0	33.2	39.8	0.2
Hazore'a	Yizre'el Valley	Alluvial brown Gramusol	600 + irrigation	Cotton	12.3	33.5	54.2	0.2
Ra'anana	Coastal Plain	Sandy loam	500 + irrigation	Citrus	71.0	5.7	23.4	0.4
Be'eri	Northern Negev	Loess	250, dry farming	Wheat	51.1	32.0	16.9	0.0

cv. 144) seedling was planted in each pot and grown for 3 to 4 weeks under screen-house conditions, allowing the development of one generation of *M. javanica*. Plants were fertilized weekly with a 0.1% solution of a commercial 20-20-20 N-P-K mixture. Upon harvesting, the fresh weight of each plant shoot and the galling index on a 0-to-5 scale of each root system were determined. The galling index scale was defined as follows: 0 = no visible galls, 1 = up to 10% of the roots galled, 2 = 11 to 25%, 3 = 26 to 50%, 4 = 51 to 75%, 5 = 76 to 100%. Each treatment within the pot experiment was replicated 8 times.

Data were subjected to Duncan's multiple-range test.

Petri dish experiments: Five hundred mg of natural garden soil (46% sand, 30% silt, 24% clay; 8.6% organic matter) collected in Columbia, Maryland, was spread over 0.5% Phytagel medium (Sigma Chemical Co., St. Louis, MO) in 9-cm-diam. petri dishes. Either 1 EM or 200 SE, obtained from monoxenic cultures of *M. incognita* on excised tomato roots (*L. esculentum* cv. Rutgers), were placed at the center of each dish. The dishes were sealed and kept in the dark at 25 °C. Light microscopic observations were made twice weekly for 3 weeks. Each treatment in the petri dish experiments was replicated 12 times.

In an experiment designed to investigate the resistance to microorganisms of SE surrounded by GM, artificial EM were prepared by first cutting sections of GM from 4 to 7-day-old EM by means of a sharp-ended length of microcapillary tube under a dissecting microscope. Separated eggs were placed on top of these sections with a pipet.

Scanning electron microscopy was used to examine interactions between GM and (i) a nematode-associated microbe and (ii) a known antagonist of nematodes. For the former, *Mortierella* sp. (Nematology Laboratory #L196, originally isolated from *Heterodera glycines* in the People's Republic of China) was cultured on PDA and transferred to a Phytagel plate containing EM *M. incognita* from 2-month-old monoxenic cultures on tomato roots. Egg masses were examined via field emission scanning electron microscopy 10 to 14 days later. Similar experiments were performed with the nematode antagonist *Burkholderia cepacia* strain Bc-1, previously cultured on nutrient agar.

Scanning electron microscopic observations were made with a Hitachi S-4100 field emission scanning electron microscope equipped with an Oxford (Eynsham, UK) CT-1500HF Cryotrans System. Specimen preparation consisted of dissecting 7 × 7-mm Phytagel squares containing the EM of interest and then gently placing them in a small volume of methyl cellulose on the surface of flat copper specimen holders. The contents of each holder were cryofixed by placing them on the surface of a liquid nitrogen cooled brass tube (–196 °C); the holders were then transferred to the Oxford pre-chamber and etched for 10 minutes at –90 °C. After recooling, the samples were sputter-coated with platinum and moved to the cryostage for observation. Accelerating voltages of 2–10 kV were used to observe the specimens or record images onto Polaroid type 55 P/N film.

RESULTS

Incubating EM or SE in pots containing Ra'anana citrus orchard soil for periods of 24 hours and 10 and 20 days resulted in hampered growth and chlorotic appearance of the subsequently transplanted tomato seedlings in the EM treatments but normal growth of the tomato seedlings in the SE treatments. The difference in plant vigor was evident 1 week following transplanting of the tomato seedlings and became greater until the plants were harvested. The galling index of the 24-hour SE treatment was barely half that of the 24-hour EM treatments, and declined even further during the two longer incubation periods (Fig. 1). In contrast, the galling index of the EM treatments remained quite high and was hardly affected by the incubation time.

Incubation of EM or SE in formaldehyde-treated or natural Ra'anana citrus orchard soil affected both the growth of the tomato seedlings and the numbers of galls that developed on them. The greatest shoot fresh weight was observed in the treatments with SE incubated in natural soil; the lowest was found in the treatment with EM in natural soil. Shoot weight was intermediate for plants grown in the formaldehyde-disinfested soil containing EM or SE (Table 2). The galling index found for the SE in natural soil was about half that for the other treatments (Table 2).

In experiments comparing the four soil types listed in Table 1, the difference between the fresh shoot weights of the SE and the EM treatments was significant in Ra'anana sandy loam but not significant in the three other soil types (Fig. 2). For each soil type, use of EM

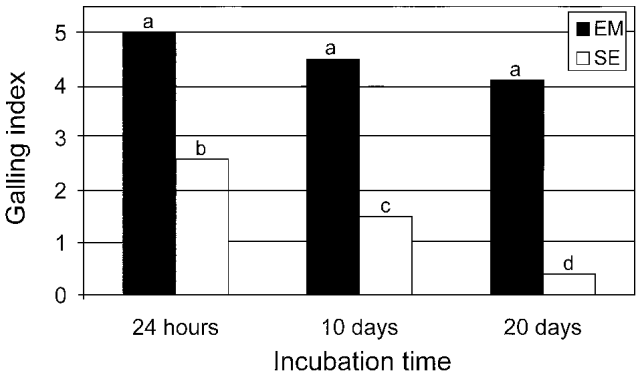

FIG. 1. Mean galling indices of tomato (*Lycopesicon esculentum* cv. 144) seedlings transplanted into soil previously treated with egg masses (EM) or separated eggs (SE) of *Meloidogyne javanica;* EM and SE were incubated in Ra'anana citrus orchard soil for various periods before transplanting of the tomato seedlings into treated soil. Means with the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Galling index scale: 0 = no visible galls, 1 = up to 10% of the roots galled, 2 = 11 to 25%, 3 = 26 to 50%, 4 = 51 to 75%, 5 = 76 to 100%.

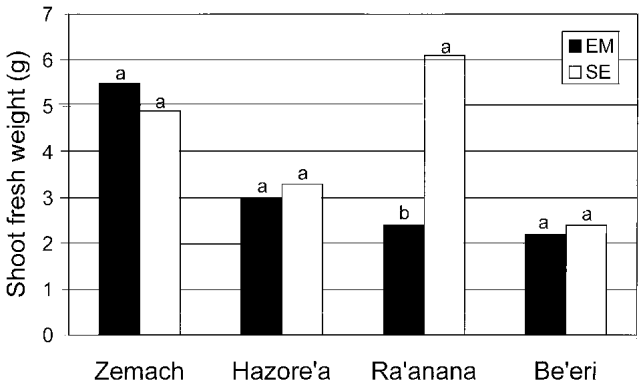

FIG. 2. The effect of inoculation with egg masses (EM) or separated eggs (SE) of *Meloidogyne javanica* on the fresh shoot weight of tomato (*Lycopersicon esculentum* cv. 144) plants grown in four soil types. Means with the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

and SE resulted in significant differences in galling indices, all of which were lower in the SE treatments (0.5 to 1.5 vs. 1.8 in a heavy soil to 4.6 in the lightest one for the EM treatment; Fig. 3).

Observations of SE and EM exposed to natural garden soil in petri dishes revealed that, 48 hours later, various unidentified bacteria, fungi, nematodes, mites, and other arthropods attacked both the SE and the EM. Within 5 to 9 days the SE were almost completely destroyed by the soil microorganisms while the eggs in the EM remained basically intact (Fig. 4A-D). Microorganisms were observed in dense populations around the EM but did not invade them in some cases. When SE were inserted within sections of GM dissected from EM and examined via the same methods, the SE remained intact for at least 13 days (Fig. 4E).

Electron microscopic examination revealed *B. cepacia* and *Mortierella* sp. within the GM (Fig. 5). Numerous hyphae of *Mortierella* were also seen inside hatched eggs but not unhatched eggs (Fig. 5C).

DISCUSSION

Two approaches were employed to investigate the putative role of the GM in protecting the eggs against the soil microflora: (i) infectivity determinations in pot experiments and (ii) microscopy of the interactions between eggs and microorganisms. In the pot experiments, SE and intact EM of *M. javanica* had vastly different infectivities after incubation in natural soil, although there might be a difference in the infection intensity of the two inoculation methods. The longer that SE were exposed to the soil environment, the fewer the eggs that remained viable; the same treatment hardly affected eggs in EM. After the longest incubation period (20 days), SE had lost their infection potential almost completely, whereas EM retained their infectivity and produced many second-stage juveniles (J2) that invaded the tomato roots to form galls. Thus, SE may have been vulnerable to a soil factor that attacked the eggs and consumed their contents, while eggs embedded in GM remained viable and thereby retained their infectivity. Eliminating or greatly reducing the original soil microflora and microfauna with formaldehyde treatment partly reversed the destructive effect of the soil on SE-evidence that the factor affecting the SE was

TABLE 2. The effects of inoculation with either egg masses (EM) or separated eggs (SE) of *Meloidogyne javanica* in Ra'anana citrus orchard soil or soil treated with formaldehyde (for) on the mean galling indices and on the fresh shoot weight of tomato (*Lycopersicon esculentum* cv. 144). Galling index scale: 0 = no visible galls, 1 = up to 10% of the roots galled, 2 = 11 to 25%, 3 = 26 to 50%, 4 = 51 to 75%, 5 = 76 to 100%. In each row, means with the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

	EM	EM + for	SE	SE + for
Galling index	3.4a	3.6a	1.5b	2.6a
Fresh shoot weight (g)	0.6c	2.0b	3.5a	2.6b

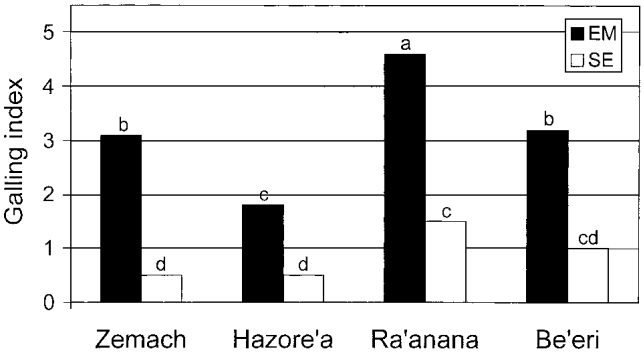

FIG. 3. Mean galling indices of tomato (*Lycopersicon esculentum* cv. 144) seedlings planted in four soil types inoculated with either egg masses (EM) or separated eggs (SE) of *Meloidogyne javanica.* Within each soil type, means with the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Galling index scale: 0 = no visible galls, 1 = up to 10% of the roots galled, 2 = 11 to 25%, 3 = 26 to 50%, 4 = 51 to 75%, 5 = 76 to 100%.

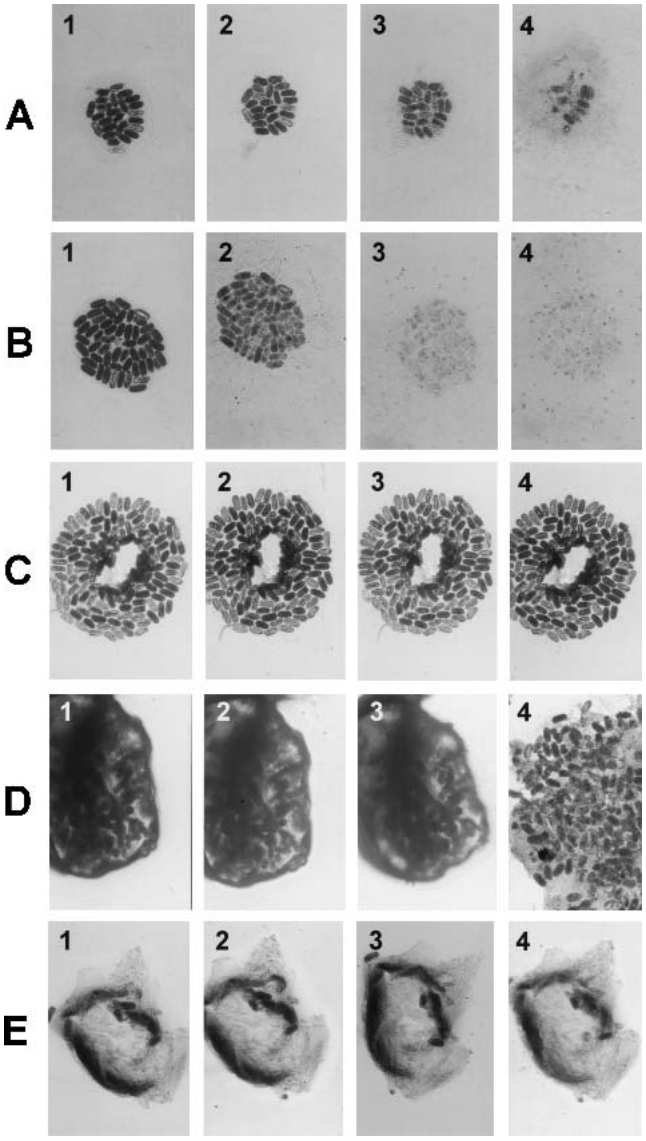

FIG. 4. *Meloidogyne incognita* eggs photographed at 1 (column 1), 5 (2), 9 (3), and 13 (4) days after transfer to Phytagel plates. A, B) Separated eggs placed on medium containing 500 mg soil. C) Separated eggs placed on sterile medium without soil. D) An egg mass placed on medium containing 500 mg soil; D4 is a smear of the egg mass showing the intact eggs. E) Separated eggs placed on section of gelatinous matrix on medium containing 500 mg soil.

the population of soil microorganisms. The susceptibility of the SE to soil microorganisms was evident in four soil types differing in physical, chemical, and biological properties.

The growth of the tomato plants was related to nematode infection resulting from the presence of SE or EM; in natural Ra'anana sandy loam, the EM had a devastating effect on the young tomato seedlings, whereas SE did not affect their growth. In the heavier soils (Zemach, Hazore'a, and Be'eri), the high galling indices did not affect plant growth, possibly reflecting the frequently higher severity of *Meloidogyne*-induced crop damage in lighter soils (Van Gundy, 1985).

Light microscopic observations of the interactions between soil microorganisms and EM and SE clearly demonstrated that the SE were vulnerable to a broad spectrum of unidentified fungi and bacteria that penetrated and digested the eggs. In contrast, although dense populations of various microorganisms were observed in close contact with the GM or colonizing it, the

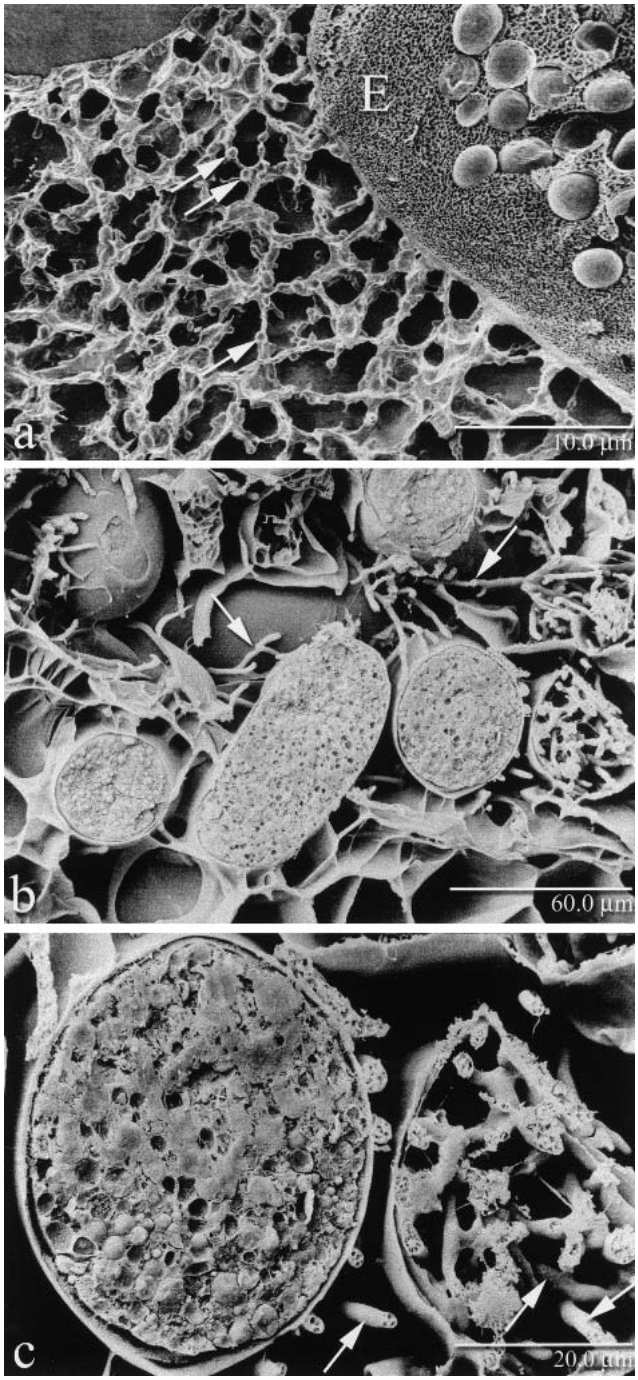

FIG. 5. Scanning electron micrographs of fractured, frozen egg masses of *Meloidogyne incognita*. a) Gelatinous matrix colonized by the bacterium *Burkholderia cepacia* isolate Bc-1 (arrows). Bacteria were not observed within the fractured egg (E). b) Egg mass colonized by the fungus *Mortierella* sp. Hyphae (arrows) are growing within the gelatinous matrix. c) Higher magnification of an unhatched egg (left) and a hatched egg (right). Hyphae (arrows) are not present within the unhatched egg.

nematode eggs and the embryos within generally remained intact. These observations suggest that the GM may retard microbial invasion of the eggs. However, some of the microorganisms were capable of colonizing the GM, as has been described for fungal biocontrol agents of nematodes (Dunn et al., 1982; Meyer and Wergin, 1998; Oka et al., 1997; Stirling and Mankau, 1979). Stirling (1991) stated that, although dry GM may provide protection against antagonists, when it hydrated it may have served as a food source for some parasites. Recently, Papert and Kok (2000) detected greater numbers of bacteria inside the GM of *M. hapla* than within the adjacent rhizosphere and speculated that these bacteria could be the source of the antimicrobial activity of the GM.

The different results observed with the SE and EM may have occurred because the SE had been obtained by treating EM with sodium hypochlorite solution, which can diminish egg viability (Ehwaeti et al., 1998). However, in the formaldehyde-treated soil, the high infectivity of the SE indicates that the hypochlorite treatment in our study did not reduce egg viability and infectivity. Another possibility is that the removal of the GM with hypochlorite solution removed a protectant compound in the eggshell but SE placed on a section of GM remained intact in the presence of soil microorganisms, suggesting that the GM itself has a role in protection against soil microorganisms. Whether this likely protective role of the GM against microorganisms is mechanical, biochemical, or microbial remains to be determined.

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