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SHORT COMMUNICATION

Pre-inoculation with arbuscular mycorrhizal fungi suppresses root knot nematode (*Meloidogyne incognita*) on cucumber (*Cucumis sativus*)

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Abstract A pot experiment was conducted to evaluate the influence of pre-inoculation of cucumber plants with each of the three arbuscular mycorrhizal (AM) fungi *Glomus intraradices*, *Glomus mosseae*, and *Glomus versiforme* on reproduction of the root knot nematode *Meloidogyne incognita*. All three AM fungi tested significantly reduced the root galling index, which is the percentage of total roots forming galls. Numbers of galls per root system were significantly reduced only in the *G. intraradices*+*M. incognita* treatment. The number of eggs per root system was significantly decreased by AM fungus inoculation, no significant difference among the three AM fungal isolates. AM inoculation substantially decreased the number of females, the number of eggs g^{-1} root and of the number of eggs per egg mass. The number of egg masses g^{-1} root was greatly reduced by inoculation with *G. mosseae* or *G. versiforme*. By considering plant growth, nutrient uptake, and the suppression of *M. incognita* together, *G. mosseae* and *G. versiforme* were more effective than *G. intraradices*.

Keywords *Glomus* spp. · Nematode reproduction · Cucumber

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Introduction

Cucumber is one of the economically most important vegetables in China, the cultivated area of which (9.85×10^5 ha) accounts for 5.4% of the total vegetable cultivation area (Chinese Ministry of Agriculture 2006). In recent years yield losses have been strongly associated with root knot nematode (RKN) caused by *Meloidogyne* spp. together with soil pathogens such as *Fusarium oxysporum, Phytophthora melonis*, and *Pseudoperonospora cubensis* (Qu et al. 2003). These soil pathogens are widespread and may become the major constraint in many cucumber production areas, particularly in greenhouse systems in which continuous cropping is practiced. A 3-year survey conducted in Shandong province, northeast China, found RKN disease in 67.6% of greenhouses investigated and approximately 50% of the plants infected (Dong et al. 2004). After four or more crops, the rate of occurrence of RKN was up to 95% (Dong et al. 2004) and RKN has been associated with fruit yield losses of 20–30% and even 100% in some cases (Peng 1998). Unlike *F. oxysporum, P. melonis* and *P. cubensis* which can be controlled to some extent by means of grafting or the use of resistant cultivars, there is still no satisfactory control measure for RKN disease in continuous cropping of cucumber in China. Chemical nematicides are prohibited due to their potentially detrimental effects on the environment and human health even though they have been

shown to be effective in inhibiting nematode infestations. Agronomic practices such as crop rotations and the use of fallow and cover crops could be employed but have not been introduced in China because of economic factor. Biological control of nematodes is therefore an attractive option with the aim of maintaining current cultivation practices and minimizing damage to the environment.

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil organisms that can form mutualistic associations with the roots of the majority of vascular plant species (Lindermann 1988). The establishment of AM association is often beneficial for plant nutrition and AM fungi may also confer tolerance of and resistance to various abiotic and biotic stresses to the host plant (Smith and Read 1997; Colla et al. 2008). It has even been suggested that many root pathogens can be regarded as pathogens of mycorrhizae (Rhodes 1980), implying that AM fungi are closely associated with soil-borne pathogens, including nematodes (Graham 2001). Since AM fungi and RKN are all indigenous soil organisms and therefore co-exist in plant roots, the potential role of AM fungi as biocontrol agents and their protective effects on plants against RKN have been well documented (Hussey and Roncadori 1982; Diedhiou et al. 2003). Studies have shown that inoculation with AM fungi can significantly reduce RKN infestation and reproduction in some plant–nematode systems (Hol and Cook 2005) such as papaya with *Meloidogyne incognita* (Jaizme-Vega et al. 2006), olive planting stocks with *M. incognita* and *Meloidogyne javanica* (Castillo et al. 2006), tomato with *M. incognita* (Talavera et al. 2001; Siddiqui and Akhtar 2007), pyrethrum with *Meloidogyne hapla* (Waceke et al. 2001), Prunus rootstocks with *M. javanica* (Calvet et al. 2001), and banana with *M. javanica* (Rodriguez and Jaizme-Vega 2005). In addition to enhancement of plant nutrition, especially P nutrition, establishment of arbuscular mycorrhizae may exert beneficial effects on plant growth though direct competition with RKN for infection sites and space, alteration of the composition of root exudates, or through activation of plant defense reactions and other mechanisms (Smith et al. 1986; Azcón-Aguilar and Barea 1996; Harrier and Watson 2004; Li et al. 2006). However, the positive response in interactions between AM fungi and nematodes can vary and negative responses and lack of any response have also been reported (Smith et al. 1986; Carling et al. 1989).

Inoculation of cucumber plants with AM fungi has been shown to increase plant growth (Trimble and Knowles 1995), protect plants from salt stress (Rosendahl and Rosendahl 1991), and improve plant resistance to Fusarium wilt (Hao et al. 2005) and *Pythium ultimum* (Rosendahl and Rosendahl 1990). It may therefore be feasible to use AM fungi in the control of RKN in cucumber production. The present study was conducted to assess the possible effects of three *Glomus* species on cucumber growth and nematode infection and reproduction.

Materials and methods

Host plants and soil

Cucumber seeds (*Cucumis sativus* L. cv. Zhongnong 16) were surface sterilized with a 10% (*v*/*v*) solution of H_2O_2 for 10 min and then rinsed thoroughly with deionized water. The seeds were placed on autoclaved filter papers soaked with sterile distilled water and incubated at 25°C for 24 h. Four seedlings were planted in each pot and were thinned to two per pot after the emergence of the second leaf.

Soil was collected from Daxing county in the suburbs of Beijing and was a low phosphorus loamy sand with the following physico-chemical properties: total N 0.08%, available P (Olsen-P) 7.72 mg kg^{-1}, available K (NH_4OAc-K) 33.6 mg kg^{-1}, pH (H_2O, soil 2.5:1, *v*/*v*) 8.44, and total organic matter 0.30%. Soil and river sand were sieved (<2 mm) and sterilized by autoclaving at 121°C for 2 h. After drying at room temperature, the soil and river sand were mixed at a ratio of 1:1 (*v*/*v*). Before use the substrate was amended with a mixture of nutrients as basic fertilizers: N 200 mg kg^{-1} (NH_4NO_3), P 50 mg kg^{-1} (KH_2PO_4), K 200 mg kg^{-1} (K_2SO_4), Mg 100 mg kg^{-1} ($MgSO_4$ $7H_2O$), Zn 5 mg kg^{-1} ($ZnSO_4 \cdot 7H_2O$), Mn 5 mg kg^{-1} ($MnSO_4$ $7H_2O$) and Cu 5 mg kg^{-1} ($CuSO_4 \cdot 5H_2O$).

Arbuscular mycorrhizal fungal isolates

Three AM fungal species, *Glomus intraradices* Schenck & Smith (BEG141), *Glomus mosseae* (Nicol & Gerd) Gerd & Trappe (BEG167), and *Glomus versiforme* (Karsten) Berch were propagated on maize and white clover host plants in a growth chamber at 30°C/18°C with a 16 h/8 h light/dark regime and 50–75% relative humidity. The plants were harvested after growth for 4 months. Soils containing spores, external mycelium and roots of maize and white clover were used as inocula.

The experimental containers were plastic pots 16 cm in diameter×14 cm in height. Soil and sand mixture (1.5 kg) and 150 g inoculum were placed in each mycorrhizal pot. The inoculum was placed about 2 cm under the soil surface. Non-mycorrhizal pots received an equivalent amount of soil and sterilized inoculum together with 10 ml soil filtrate (0.45 μm pore size) to provide a similar soil microbial community (Calvet et al. 1993).

Nematode isolates

M. incognita was kindly provided by Professor Z. P. Cao of the College of Resources and Environmental Sciences, China Agricultural University, Beijing. The nematodes were multiplied on tomato (*Lycopersicon esculentum* cv. 'Hezuo 908') using single egg mass inoculation. Plants

were placed in a greenhouse at the Department of Plant Nutrition, China Agriculture University and grown for 3 months. Severely infected roots were harvested and washed with deionized water. The egg masses were hand-picked using sterilized tweezers and the nematodes were then collected according to the method of Hussey and Barker (1973). Briefly, the detached eggs from the roots were extracted in 1.5% NaClO. The eggs were added to deionized water by using a 25-μm sieve and stirred to stimulate the development of J2 juveniles. The separation of eggs and juvenile nematodes then followed the method of Baerman (Oostenbrink 1960). The concentration of the suspension of nematodes was adjusted to 400 J ml^{-1}. After growth for 30 days, cucumber plants were inoculated with 2,000 (J2) nematodes per pot by injecting the suspension into five holes of 3-cm depth. The holes were formed in a circle about 2 cm away from the stem base of the seedlings.

Experimental treatments and plant growth conditions

There were eight treatments: (1) non-inoculated control (-AMF-Mi); (2) non-inoculated control, inoculated 30 days later with *M. incognita* (−AMF+Mi); (3) inoculated with *G. intraradices* (+Gi); (4) inoculated with *G. intraradices*, inoculated 30 days later with *M. incognita* (+Gi+Mi); (5) inoculated with *G. mosseae* (+Gm); (6) inoculated with *G. mosseae*, inoculated 30 days later with *M. incognita* (+Gm+Mi); (7) inoculated with *G. versiforme* (+Gv); (8) inoculated with *G. versiforme*, inoculated 30 days later with *M. incognita* (+Gv+Mi).

Each treatment was replicated eight times. *M. incognita* inoculum was applied 30 days after planting when the fourth leaf had emerged. Seedlings were grown for a further 5 weeks and then harvested. The pots were arranged in a completely randomized design in a greenhouse at China Agriculture University in Beijing. Seedlings were grown from April to June at a temperature regime of approximately 35°C/23°C (day/night) with a 16 h/8 h (light/dark) photoperiod and 50–75% relative humidity. Seedlings were irrigated daily with deionized water and weekly with P-free Hoagland nutrient solution (Liu and Li 2000) after inoculation with *M. incognita*.

Harvest and analysis

At harvest shoot length, leaf numbers, shoot dry weight, root fresh weight, number of galls, reproduction of nematodes, mycorrhizal infection, and shoot concentrations of nutrient elements were determined. Fresh roots were divided into three parts: a portion was used to record the egg masses and eggs, a portion to count the females, and the remainder to measure root length and the percentage of root length colonized by AM fungus. The severity of RKN disease was denoted by root galling index which was assessed on a rating scale of 0–4 according to the number of roots forming galls expressed as a proportion of the total root system: 0=no galls,1=1–25%, 2=26–50%, 3=51–75%, and 4=76–100% (Krusberg and Nelson 1958). The number of galls on the roots was recorded using an arithmometer. Egg masses were stained in 0.015% phloxine B for 20 min, rinsed in sterilized distilled water and then counted under a stereomicroscope. Eggs were estimated according to Hussey and Barker (1973). The egg suspension was adjusted to 25 ml and 1 ml of the suspension was taken and used to record the number of eggs. The number of female nematodes within the roots was counted using a dissecting microscope (magnification, ×40) and staining by the NaOCl-acid fuchsin technique (Byrd et al. 1983). The percentage of root length colonized by AM fungi was determined using the grid line intersect method (Giovannetti and Mosse 1980) under a stereoscopic microscope. Plant shoots were dried in an air-forced oven at 70°C for 48 h. The dried samples were milled with a high-speed micro-pulverizer (Whirl Type, ModelY-60, Hebei, China) prior to elemental analysis. Sub-samples (about 0.3 g) were wet digested with HNO_3 and H_2O_2 in a microwave digester (MARS CXPress, CEM Corporation) and the concentrations of the elements were measured by ICP-MS (Optima 3300DV, Perkin Elmer).

Statistical analysis

Analysis of variance was carried out using the SAS software package version 6.12 (SAS Institute, Inc., Cary, NC, USA). Duncan's multiple range test or Fisher's LSC test was used to test for significant differences between treatment means at the 5% level.

Results and discussion

No AM fungus colonization was observed on the roots of uninoculated plants. Roots of inoculated plants were extensively mycorrhizal and the mean percentage of root length colonized ranged from 52% to 68% (Table 1). Root colonization rates in the absence of *M. incognita* were significantly higher in plants inoculated with *G. mosseae* or *G. intraradices* than in those inoculated with *G. versiforme* and in the presence of *M. incognita* were higher in plants inoculated with *G. intraradices* than in those inoculated with either of the other two fungi. Inoculation with *M. incognita* decreased root colonization rates significantly in plants inoculated with *G. mosseae* (by 16%) or *G. versiforme* (by 11%). Changes in AM fungus colonization in the presence of nematodes has been observed in other studies (Carling et al. 1989; Waceke et al. 2001; Castillo et al. 2006) and has been attributed mainly to competition between AM fungi and

Table 1 Shoot length, shoot dry weight, root fresh weight and root length of mycorrhizal and non-mycorrhizal cucumber plants inoculated with or without Mi

Inoculation treatment	Root colonization rate (%)	Shoot length (cm)	Shoot dry weight (g)	Root fresh weight (g)	Root length (m)
Without *M. incognita*					
Non-mycorrhizal	0	27.5±3.6b	2.51±0.35b	5.56±0.53b	6.49±0.89c
G. intraradices	68±2a	24.3±1.7b	2.26±0.13b	5.32±0.24b	8.44±0.46c
G. mosseae	68±3a	51.9±3.7a	4.63±0.20a	12.1±0.63a	12.82±0.86b
G. versiforme	64±3b	53.2±3.2a	4.82±027a	14.08±0.94a	15.67±1.25a
With *M. incognita*					
Non-mycorrhizal	0	31.0±3.0c	2.74±0.25c	9.16±0.48b	4.55±0.29c
G. intraradices	62±3a	34.4±4.8c	2.82±0.45c	10.16±0.92ab	6.10±0.63c
G. mosseae	52±4b	57.3±2.4b	4.52±0.26b	14.04±0.64ab	9.74±0.65b
G. versiforme	53±4b	73.7±2.8a	5.66±0.16a	17.54±0.79a	11.58±0.81a
Significance[a] due to					
Mi inoculation	***	***	NS	***	***
AMF inoculation	***	***	***	***	***
Interaction	*	NS	NS	NS	NS

Data are the means of eight replicates±SE and were compared by Duncan's multiple range test. Within each group of four values any two means sharing a lower case letter are not significantly different within a *M. incognita* treatment

NS Not significant

***$P<0.001$; **$P<0.01$; *$P<0.05$

[a] By analysis of variance

RKN for feeding sites and carbon substrates from host photosynthesis (Smith 1998; Hol and Cook 2005).

Inoculation with *M. incognita* significantly increased shoot length and root fresh weight but decreased root length (Table 1) and shoot dry weight remained unaffected. Galls in the roots might have caused the increase of root fresh weight in *M. incognita* infested plants. Inoculation with the RKN had significant effects on shoot Mg, Cu, and Zn concentrations (Table 2) and frequently increased shoot Mg and Cu concentrations but decreased Zn concentrations irrespective of the AM fungal isolate present. Root knot nematode infection often reduces plant growth and yield

Table 2 Elemental concentrations in shoots of mycorrhizal and non-mycorrhizal cucumber with or without *M. incognita*

Inoculation treatment	P (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})
Without *M. incognita*								
Non-mycorrhizal	4.86±0.19b	33.50±0.91b	29.43±0.79ab	11.16±0.35b	150.23±6.18b	26.99±1.11b	21.54±0.64b	27.34±1.37a
G. intraradices	4.17±0.13c	35.99±1.42ab	30.57±0.66a	12.82±0.19a	172.36±7.46a	28.46±0.98ab	17.94±0.16c	28.63±1.16ab
G. mosseae	5.25±0.17b	35.46±0.78ab	29.10±0.60ab	12.86±0.24a	158.13±5.87ab	29.44±0.74ab	24.45±0.45a	28.23±0.59ab
G. versiforme	5.77±0.15a	38.65 ±1.33a	27.85±0.90b	12.07±0.45ab	152.11±6.26b	30.89±1.61a	25.80±0.65a	31.47±1.35a
With *M. incognita*								
Non-mycorrhizal	4.46±0.19c	31.61±1.54b	29.45±0.57a	12.34±0.17b	146.10±6.69b	28.69±0.92 bc	23.20±0.40d	23.15±1.20c
G. intraradices	4.31±0.06c	36.34±1.76b	28.13±0.60a	12.75±0.25ab	156.91±6.58b	24.99±1.30c	18.00±0.17c	27.33±1.39ab
G. mosseae	5.40±0.17b	34.64±1.34b	28.56±0.71a	13.10±0.23ab	160.75±9.59b	32.37±1.18ab	25.29±0.44b	24.89±0.96bc
G. versiforme	6.25±0.14a	42.19±0.86a	30.05±0.99a	13.37±0.35a	194.12±6.85a	32.94±0.95a	28.31±0.61a	30.12±1.01a
Significance[a] due to								
Mi inoculation	NS	NS	NS	**	NS	NS	***	**
AMF inoculation	***	***	NS	***	*	***	***	***
Interaction	NS	NS	NS	NS	**	NS	NS	NS

Plants were pre-inoculated with one of three *Glomus* species or remained non-mycorrhizal for 30 days and were then inoculated with *M. incognita* for an additional 5 weeks. Data are the means of eight replicates±SE and were compared by Duncan's multiple range test. Within each group of four values any two means sharing a lower case letter are not significantly different within a *M. incognita* treatment

NS Not significant

***$P<0.001$; **$P<0.01$; *$P<0.05$

[a] By analysis of variance

(Sasser and Freckman 1987; Williamson 1998) and decreases nutrient uptake (Patel et al. 1988), and infested plants show deficiencies of N, Mg, Fe, B, Cu, and Zn (Good 1968) due to root damage by RKN and subsequent prevention of water and nutrient uptake by the roots (Gaillaud et al. 2008). The increased shoot length in infested plants could be a temporal response of plants to *M. incognita* infestation which might be driven by the carbon drain due to the RKN infestation. Similarly shoot dry weight of tomato plants inoculated with *M. incognita* was increased at week 9 and was unaffected at week 12, while decreased shoot dry weight was only observed when tomato was infested with *M. incognita* after 15 weeks of growth (Diedhiou et al. 2003). The significant decrease in root length in the present experiment implies that in the long term plants may suffer from RKN infection, as indicated by the higher root fresh weight in infested plants compared to non-RKN plants an indication of root damage due to gall formation (Table 3).

Different published studies have reported decreases, increases, or no effects of AM fungal inoculation on nutrient uptake and growth of plants in the presence of nematodes (Jaizme-Vega et al. 1997; Kesba and Al-Sayed 2005) and inoculation with AM fungi has often eliminated the negative effects of RKN on plant growth and nutrient uptake. For example, decreased uptake of N, P, K, Ca, Zn, and Mn by tomato plants due to *M. incognita* was mitigated by inoculation with *Glomus fasciculatum* (Bagyaraj 1984). AM fungal colonization was shown to alter the uptake of Zn, Cu, and B in coffee (de Souza 1979). The effect of *Glomus macrocarpus* on the NPK content of grape leaves depended on the species of associated nematode (Kesba and Al-Sayed 2005). Although we did not observe negative effects of *M. incognita* on plant growth or nutrient uptake in the present experiment, inoculation with AM fungi greatly simulated plant growth and enhanced nutrient uptake in both the presence and absence of *M. incognita* and the extent of the effect differed among the three fungi tested. Shoot length, shoot dry weight, root fresh weight, and root length were often significantly higher in plants inoculated with *G. mosseae* or *G. versiforme* than in uninoculated controls or plants inoculated with *G. intraradices* irrespective of RKN inoculation (Table 1). Inoculation with *G. mosseae* generally produced the largest shoot biomass among the three AM fungal isolates. In general, inoculation with AM fungi tended to affect shoot concentrations of P, K, Mg, Fe, Mn, Cu, and Zn but not Ca concentrations irrespective of treatment with *M. incognita* (Table 2). Plants inoculated with *G. versiforme* had the highest nutrient concentrations. Enhanced plant growth and nutrient uptake can consequently enhance plant resistance to RKN infection.

One of the prerequisites for managing AM fungi as biological control agents against root knot nematodes is the selection of a broad spectrum of AM fungal isolates that are capable of suppressing the parasites and conferring tolerance of plants to the RKN. Numerous studies have shown the existence of differences among AM fungi in their interactions with RKN and that prior establishment of the mycorrhiza often impairs the development and reproduction of the nematodes (Stroble et al. 1982; Habte et al. 1999; Forge et al. 2001; Waceke et al. 2001). All of the three AM fungi tested significantly reduced the galling index. The galling indexes were 4.0, 3.0, 2.4, and 2.0 for non-mycorrhizal and plants inoculated with *G. intraradices*, *G. mosseae*, and *G. versiforme* respectively. Inoculation with *G. versiforme* decreased the number of galls g^{-1} root by 45% and inoculation with either of the other two fungi showed a similar tendency but the trend was not significant (Table 3). Numbers of egg masses and females per root system were not significantly affected by AM fungal inoculation, but the number of eggs per root system was significantly decreased to similar extents by inoculation with the AM fungi. Inoculation with AM fungi substantially decreased the number of females g^{-1} root and no significant difference was observed among the three fungal isolates (Table 3). Similarly, AM inoculation decreased the number of eggs g^{-1} root and the number of eggs per egg

Table 3 Infection and reproduction of *Meloidogyne incognita*

Mycorrhizal status	Root system				Per gram root				Number of eggs/egg masses
	Number. of galls	Number of females	Number of egg masses	Number of eggs	Number of galls	Number of females	Number of egg masses	Number of eggs	
Non-mycorrhizal[a]	426±40ab	706±91a	130±2a	89752±19968a	97±9a	160±17a	32±4a	19602±3171a	694±136a
G. intraradices	357±20b	537±86a	147±11a	21191±2614b	84±14a	106±8b	29±5a	4049±949b	146±16b
G. mosseae	511±36a	587±57a	122±17a	41295±13899b	76±6ab	84±3b	17±1b	4707±313b	334±96b
G. versiform	448±31ab	698±60a	120±8a	15346±3693b	53±5b	83±8b	18 ±2b	2186±394b	151±45b

Plants were pre-inoculated with one of three *Glomus* species or remained non-mycorrhizal for 30 days and were then inoculated with *M. incognita* for an additional 5 weeks. Within each group of four values any two means sharing a lower case letter are not significantly different

[a] Data are the means of eight replicates±SE and were compared by Fisher's LSC test at $P \leq 0.05$

mass. The number of egg masses g^{-1} root was not significantly affected under inoculation with *G. intraradices* but was reduced by about 45% with inoculation with *G. mosseae* or *G. versiforme* (Table 3).

Our results suggest that the three fungi tested can to some extent inhibit the development and reproduction of the RKN and thus may be considered to control RKN disease in cucumber. The protective effect of AM fungi may be due to systematic or local (Cordier et al. 1998; de la Pena et al. 2006) operating mechanisms which may mediate the physical and physiological plant response to the RKN. Alternatively, AMF may have a direct suppressive effect on nematodes as both organisms might compete for root space and feeding sites (Francl 1993). *G. intraradices* was the least effective of the three fungi when plant growth and nutrient uptake and the suppression of RKN are taken together (Tables 1, 2, and 3). Similarly, Habte et al. (1999) reported that *G. mosseae* was superior to *G. intraradices* in terms of reducing the reproduction of *M. incognita* and increasing the growth of red clover. However, Castillo et al (2006) found that establishment of *G. intraradices*, *G. mosseae*, or *G. visocum* could increase the growth of olive plants and decrease the severity of root galling as well as reproduction in both *M. incognita* and *M. javanica*.

In conclusion, our results indicate that pre-inoculation with AM fungal may be beneficial for growth and nutrient uptake of cucumber by suppressing the development and reproduction of root knot nematodes. Pre-planting is a common practice in greenhouse cucumber production in China and it is therefore feasible to incorporate the AM fungi during the greenhouse growth phase before transplanting to control RKN disease. As *Glomus* species are regarded as more resistant to disturbances than other taxa of AM fungi (Dodd 2000) and our preliminary field investigation also showed that *Glomus* species are the dominant AM fungi in the soil used for cucumber production (data not shown), this genus may be a promising candidate to control RKN under field conditions.

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