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Influence of arbuscular-mycorrhizal fungi, *Rhizobium meliloti* strains and PGPR inoculation on the growth of *Medicago arborea* used as model legume for re-vegetation and biological reactivation in a semi-arid mediterranean area

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Abstract

Medicago arborea can be used for re-vegetation purposes under semiarid conditions. These woody legumes have the ability to form an association with arbuscular mycorrhizal (AM) fungi and rhizobial bacteria, which can be maximised by microorganisms producing certain stimulating metabolites acting as plant growth promoting rhizobacteria (PGPR). The effects of single and combined inoculations using microorganisms with different and interactive metabolic capacities, namely three *Glomus* species, two *Rhizobium meliloti* strains (a wild type, WT and its genetically modified derivative GM) and a plant growth promoting rhizobacterium, (PGPR), were evaluated. All three inoculated AM fungi affected *Medicago* growth in different ways. Differences were maintained when soil was co-inoculated with each of the rhizobial strains (WT or GM) and the PGPR. Mycorrhizal fungi were effective in all cases, but the PGPR only affected plant growth specific microbial situations. PGPR increased growth of *G. mosseae*-colonised plants associated with *Rhizobium* WT strain by 36% and those infected by *G. deserticola* when associated with the rhizobial GM strain by 40%. The most efficient microbial treatments involved mycorrhizal inoculation, which was an indication of the AM dependency of this plant species. Moreover, PGPR inoculation was only effective when associated with specific mycorrhizal endophytes (*G. mosseae* plus WT and *G. deserticola* plus GM rhizobial strain). The reduced root/shoot (R/S) ratio resulting from PGPR inoculation, was an indication of more effective root function in treated plants. AM colonisation and nodule formation were unaffected by the type of AM fungus or bacteria (rhizobial strain and/or PGPR). AM from natural soil were less infective and effective than those from the collection. The results supported the existence of selective microbial interactions affecting plant performance. The indigenous AM fungi appeared to be ineffective and *M. arborea* behaved as though it was highly dependent on AM colonisation, which implied that it must have a mycorrhizal association to reach maximum growth in the stressed conditions tested. Optimum growth of mycorrhizal *M. arborea* plants was associated with specific microbial groups, accounting for a 355% increase in growth over nodulated control plants. The beneficial effect of PGPR in increasing the growth of a woody legume, such as *M. arborea* under stress, was only observed with co-inoculation of specific AM endophytes. As a result of the interaction, only shoot biomass was enhanced, but not as a consequence of enhancing of the colonising abilities of the endophytes. The growth stimulation, occurring as a consequence of selected microbial groups, may be critical and decisive for the successful establishment of plants under Mediterranean climatic and soil conditions.

Introduction

Medicago arborea is a woody species and, woody legume plants, can be used for re-vegetation of ecosystems with low availability of N, P and other nutrients (Olivares et al. 1998). In general plants are very dependent on mycorrhiza to achieve maximum growth (Barea and Azcón-Aguilar 1983). The effectiveness of arbuscular mycorrhizal (AM) fungi and rhizobial bacteria in improving growth of this plant was shown in a semiarid Mediterranean ecosystem in the Southeast of Spain (Herrera et al. 1993a, 1993b); microbial activities affected the biogeochemical cycling of essential plant nutrients (Jeffries and Barea 1994). AM fungi occur naturally in most soils and improve the uptake and transport of phosphorus in roots of many plants under a variety of stress situations (Azcón and El-Atrash 1997; Ruiz-Lozano et al. 1995). The mechanisms of stress tolerance may involve promotion of root extension, improved mineral nutrition and water uptake (Bethlenfalvay and Linderman 1992; Ruiz-Lozano et al. 1995). Thus, an alternate plant strategy for coping with environmental deficiencies is the establishment of an AM symbiosis with an appropriate fungus (Azcón and Barea 1997; Estaún et al. 1997; Herrera et al. 1993a).

New biotechnological approaches are generating genetically modified microorganisms which enhances the availability and success of microorganisms as inoculants in agriculture. Previous studies using *G. mosseae*, as a representative AM fungus, demonstrated that genetically modified *Rhizobium meliloti* increased AM colonisation and nutrient acquisition by *Medicago sativa* compared to the wild type (Barea et al. 1996; Tobar et al. 1996). Nevertheless, differences among the rhizobial strains in their reaction and functional compatibility with specific AM fungal species have been reported previously (Azcón et al. 1991).

For effective growth of *M. arborea* an interaction between efficient microorganisms and host plants is necessary for the optimal utilisation of plant assimilates or microbial metabolites, respectively, which means a functional compatibility has to be established between the associated organisms (Hoflich et al. 1994).

Efforts have been made to increase the plant growth stimulating effect by combining inoculant microbial groups possessing different characteristics. With this aim in mind a combined inoculation of selected N_2-fixing bacteria, AM fungi and microorganisms producing certain stimulating metabolites, appears promising for enhancing survival of plants used in re-vegetation programmes.

The rhizosphere PGPR bacteria used in this study have shown repeatedly their effectiveness on various crops and in different growing media (Azcón 1993; Azcón et al. 1991). Thus, the compatibility of GM rhizobial strains with other AM fungi (besides *G. mosseae*), using *M. arborea* as a host plant needed to be tested.

To maximize growth and nutrition of *Medicago arborea* a selection of endophyte was required. The tripartite symbiotic efficiency can be improved by the use of a plant growth-promoting rhizobacteria (PGPR) (Azcón 1993).

In this paper, we report results from a microcosm that mimics interactions that occur in living soil-plant situations. The experiment was designed to determine the effects of a soil bacterium in association with mycorrhiza formed by endophytes from natural soil or with three endomycorrhizal *Glomus* species from a collection and the interaction with two *Rhizobium meliloti* strains [a wild-type (WT) and its genetically modified derivative (GM)]. *Medicago arborea* growth was measured to determine the effect of each bacterial-*Glomus* strain combination. The effectiveness of the symbiotic relationship was measured, along with the development of symbiotic parameters, in a degraded low-nutrient mediterranean soil from a semiarid area used for rehabilitation purpose.

Materials and methods

Host legumes and test soil

The woody legume species *Medicago arborea* was used. Twenty-day old seedlings were transplanted (1 plant per pot) into pots containing 500 g of experimental soil-sand mixture.

Soil for the experiment was from the province of Alicante (Spain), used in a re-vegetation programme. It was sieved (2 mm), diluted with quartz sand (1/1, v/v) and part of it autoclaved (100 °C; 1 h on each of three consecutive days). Natural soil did not received this treatments.

The soil used was a loam soil classified as an Eutric Regosol. This soil (47.3% sand, 43.8% silt and 8.9 clay) had a pH (water) of 8.49; contained 1.8 ppm. available P (Olsen), 0.085% total N content (Kjeldhal) 52.5 ppm K and 1.86% organic matter.

Mycorrhizal and bacterial inoculations

The AM species were *G. fasciculatum* (Thaxter Sensu Gerd.), *G. mosseae* (Nicol. and Gerd.) Gerd and Trappe and *G. deserticola* (Trape, Blosson and Menge). Native endophytes were also maintained in the experimental soil for comparison with those from the collection. Mycorrhizal fungal inoculum from each endophyte was multiplied in open pot cultures of *Lactuca sativa* L. and consisted of soil, spores, hyphae and AM roots fragments. Five grammes of each inoculum, having similar characteristics (on average of 30 spores g^{-1} and a mean of 75% mycorrhizal colonisation) were placed in each pot. Non-mycorrhizal treatments received the same amount of autoclaved inoculum.

Rhizobium strains were obtained from the collection of the Department of Microbiology of Soil and Symbiotic Systems, Estación Experimental del Zaidín. Two strains were used: Gr4B, a wild type (WT) and a genetically modified type to improve competitiveness, Gr4(pck3) (GM) developed by Sanjuan and Olivares (1991). Rhizobial isolates were grown for three days in Ty liquid medium (WT) and the same medium with tetracycline (GM).

The bacterium PGPR used was an *Enterobacter* sp. (Azcón 1987, 1993) isolated from local soil. It was grown in a nutrient broth medium (8 $g\,l^{-1}$) in shaking culture at 28 °C for 3 days. One mL of bacterial culture (10^8 CFU) was added at sowing and also after 15 days.

Plant growth conditions

Medicago arborea seeds were surface-sterilised and then germinated on filter paper. After germination, uniform seedlings were transplanted, one per pot.

The plants were grown in a temperature controlled greenhouse under a 16-h light (21 °C) and 8-h dark (15 °C) cycle, with 50% relative humidity and a photosynthetic photon flux density of 700 $\mu mol\,m^{-2}\,s^{-1}$ for the compensating photophase. Throughout the experiment, pots were weighed every day and the water lost was replaced by top watering to maintain soil moisture close to 80% field capacity.

Growth measurements

Plants were harvested after 10 weeks and shoot and root dry weights were recorded after drying the plant material at 70 °C.

Nodulation was checked visually, and the percentage of mycorrhizal root length was estimated by a microscopic examination of stained samples (Phillips and Hayman 1970) using the grid-line intersect method of Giovannetti and Mosse (1980).

Mycorrhizal dependency (based on dry matter) was calculated using the following equation:

$$\frac{\text{DW of AM plant} - \text{DW of non-AM plant}}{\text{DW of AM plant}} \times 100$$

Statistics

Data were subjected to analysis of variance. When the main effects were significant ($P < 0.05$) differences among means were evaluated for significance by Duncan's multiple range test.

Results

Mycorrhizal colonisation by inoculated endophytes positively affected the growth of *Medicago arborea* (Tables 1 and 3), an effect opposite to that of microorganisms existing in the natural soil. The three inoculated AM fungi showed different effectiveness on shoot and root biomass production when they were co-inoculated with *Rhizobium* strains and/or PGPR. In general, the GM strain of *Rhizobium* was less effective (non-significant differences in some cases) on *Medicago arborea* growth than the WT strains and plants growing in natural soil did not survive, even when inoculated with the GM rhizobial strain. The growth of mycorrhizal plants was optimal when associated selectively with specific groups of microorganisms. *G. mosseae*-colonised plants grew best when grown with *Rhizobium* WT plus PGPR culture. The beneficial effect of PGPR inoculation resulted in an increase of about 36% in shoot growth. Another positive specific response of this bacterium was found in plants infected with *G. deserticola* plus the *Rhizobium* GM, an increase in about 40% ocurring due to the beneficial effect of this dual symbiotic colonisation.

Increases in shoot weight by the most effectives mycorrhizal treatments ranged from 326% (*G. fasciculatum*) and 452% (*G. deserticola* plus PGPR) in *Rhizobium* WT co-inoculated plants and from 483% (*G. fasciculatum*) and 620% (*G. deserticola* plus

Table 1. Effects of PGPR (*Enterobacter* sp) and *Rhizobium meliloti* strains (WT or GM) on shoot dry weight (mg per plant) of *Medicago arborea* grown in natural soil or in sterilised soil, non-inoculated, or inoculated with AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti			
	WT strain		GM strain	
	—	PGPR	—	PGPR
Control	163.0a	122.4a	107.3a	95.3a
G. mosseae	319.0bc	434.0de	311.6bc	287.6b
G. deserticola	500.0def	553.0f	412.0cd	578.6f
G. fasciculatum	531.0f	529.0f	498.0def	396.6cd
Natural soil	65.6a	53.0a	—	—

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

Table 2. Mycorrhizal dependency (%) of *Medicago arborea* as affected by *Rhizobium* strains (WT or GM), PGPR (*Enterobacter* sp.) and AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti			
	WT strain		GM strain	
	—	PGPR	—	PGPR
G. mosseae	49.0a	71.8bc	65.6b	66.8b
G. deserticola	67.4b	77.9c	73.9c	83.5d
G. fasciculatum	63.4b	76.8c	78.4c	75.9c

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

Table 3. Effect of PGPR (*Enterobacter* sp) and *Rhizobium meliloti* strains (WT or GM) on root dry weight (mg per plant) of *Medicago arborea* grown in natural soil or in sterilised soil, non-inoculated, or inoculated with AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti			
	WT strain		GM strain	
	—	PGPR	—	PGPR
Control	129ab	73a	87a	69a
G. mosseae	216cd	210cd	238cde	185bc
G. deserticola	299ef	284dc	247cde	246cde
G. fasciculatum	310efg	255cde	382g	255cde
Natural soil	79a	62a	—	—

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

PGPR) in plants nodulated by the GM *Rhizobium* strain.

The dependence of *Medicago arborea* on AM colonisation was considerable but it was related to the colonising fungus involved, the *Rhizobium* strain used and the presence of PGPR (Table 2). Inoculation with PGPR enhanced the dependence of *M. arborea* on AM colonisation and resulted in optimum growth under the experimental conditions used. In the multiple-inoculated plants, the greatest shoot biomass produced was not correlated with the more developed root system (Table 3). Thus, PGPR inoculation decreased root/shoot ratio (Table 4), which indicated a more effective root system was formed. The effectiveness of root function is normally related to the amount of mycorrhizal colonisation, but in the present study the colonisation rate was not sufficient to indicate a direct correlation between colonisation rate and growth response. The percentage of infectivity reached in roots inoculated with AM fungi remained similar irrespective of the endophyte and bacterium (PGPR or *Rhizobium*) involved. The total amount of AM-colonised root was not changed by PGPR inoculation, with the *exception* of *G. fasciculatum* plus the GM strain that was enhanced by the bacterium. Native endophyte-colonised plants grown in natural soil were less infective and grew less than when AM inocula were applied (Table 6). Nodule numbers formed were affected by AM colonisation (Table 5). In control and natural soil, the nodulation was much less than in AM inoculated plants. No important differences in nodule formation was observed in plants colonised by exotic AM fungi and *Rhizobium* WT. Nevertheless the number of nodules formed by *Rhizobium* GM were increased in the presence of *G. deserticola*.

Table 4. Effect of PGPR (*Enterobacter* sp) and *Rhizobium meliloti* strains (WT or GM) on root shoot/ratio of *Medicago arborea* grown in natural soil or in sterilised soil non-inoculated or inoculated with AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti			
	WT strain		GM strain	
	—	PGPR	—	PGPR
Control	0.79c	0.60b	0.81c	2.8e
G. mosseae	0.68b	0.48a	0.76c	0.64b
G. deserticola	0.60b	0.51ab	0.60b	0.42a
G. fasciculatum	0.70bc	0.48ab	0.77c	0.64b
Natural soil	1.20d	1.17d	—	—

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

Discussion

Table 5. Effect of PGPR (*Enterobacter* sp) and *Rhizobium meliloti* strains (WT or GM) on nodule number formed in *Medicago arborea* roots growing in natural soil or in sterilised soil, non-inoculated, or inoculated with AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti			
	WT strain		GM strain	
	—	PGPR	—	PGPR
Control	2.2a	5.4ab	3.0ab	7.4b
G. mosseae	25.0de	23.0de	21.0cd	15.6c
G. deserticola	24.0de	31.0fg	31.4fg	34.4g
G. fasciculatum	28.0ef	24.0de	18.0c	24.0de
Natural soil	6.3ab	2.6ab	—	—

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

Soil erosion and disturbance results in a reduction of AM propagules which can be critical for plant growth because AM symbiosis is a key biological component in a desertified semiarid mediterranean ecosystem (Herrera et al. 1993b; Jeffries and Barea 1994). A low density of AM propagules normally limits the establishment of native plants. A low, infective AM inoculum potential, as was evidenced in the present study, is inadequate to support plant growth. Regarding *M. arborea* growth, it was necessary to use effective an infective AM fungi, in order to enhance the ability of the plant to become established and to cope with stress situations such as nutrient limitation and/or imbalance. These results agree with those reported previously (Herrera et al. 1993b; Requena et al. 1996; Toro et al. 1997). In fact, mycorrhizae have been found, in general, to increase legume performance, since woody legumes usually exhibit a high degree of mycorrhizal dependence in order to thrive in stressed situations (Herrera et al. 1993a; Osonubi et al. 1991). Specific rhizosphere microorganisms are also important and can play a relevant role in promoting root growth and mycorrhizal development, facilitating plant performance in a semiarid ecosystem. This could be critical for optimal establishment of plants in these areas (Requena et al. 1996, 1996; Toro et al. 1997). Nevertheless, there are few reports describing the beneficial effect of PGPR on performance of woody legumes under stress situations.

Under the experimental conditions reported here, indigenous AM fungi appeared to be ineffective and plant growth in natural soil was less (NS) as compared with the sterile control treatment. The fact that spores present in natural soil may be parasitised or that deleterious microorganisms may be present could explain such results. Conversely, the exotic endophytes used here may have been very effective in promoting plant growth in spite of differences in microbial combinations involving *Glomus* sp., PGPR or *Rhizobium* strains.

In preliminary studies (Barea et al. 1996; Tobar et al. 1996; Toro et al. 1998), the GM rhizobial strain did not interfere negatively with AM spore germination and mycelial growth of *Glomus mosseae*. Moreover, it enhanced the number of mycorrhizal entry points in the alfalfa root system in comparison to the WT strain. In the present study involving *M. arborea* as the host plant and other AM fungi (beside *G. mosseae*), the GM rhizobial strain showed no significant positive effect on the development of AM colonisation in plants inoculated with this strain and shoot biomass was even less (NS in most of the cases) than in treatments involving the WT strain. In fact, the genetically modified *Rhizobium* strain showed no superior traits as compared to the WT rhizobial strain in *M. arborea* under the present experimental conditions. However, studies are in progress to assess the real ecological impact of such a new microbial strains which in previous experimental approaches proved useful as an inoculant, (Barea et al. 1996; Tobar et al. 1996; Toro et al. 1997).

The efficiency of the symbiosis depends on the particular combination of *Rhizobium* strain and *Glomus* sp. involved, which showed specific compatibilities in interaction with PGPR (Azcón 1993; Azcón et al. 1991). The interaction between the symbionts and PGPR ranges from very compatible in co-inoculation with *G. deserticola* and GM strain (enhancing plant weight of non-mycorrhizal plants in a 539%) to less compatible, when associated with *G. mosseae* and GM. Thus, the effectiveness of microbial inoculations depends on specific combinations of endophytes and functional compatibility with the host, as previously demonstrated (Ruiz-Lozano and Azcón 1993, 1994), as well as on associated bacteria. There seemed to be no relationship between AM colonisation rate or nodule formation and growth response.

Lower differences between treatments were observed in root dry weight, which was enhanced by *G. fasciculatum* and reduced by PGPR (particularly in *G. fasciculatum* colonised plants); Similar trends were observed previously in lavender plants, (unpublished results). However the results were unexpected since phytohormone production is one of the mechanisms

Table 6. Effect of PGPR (*Enterobacter* sp) and *Rhizobium meliloti* strains (WT or GM) on mycorrhizal infection (% and total length) formed on *Medicago arborea* roots growing in natural soil or in sterilised soil, non-inoculated or inoculated with AM fungi (*G. mosseae*, *G. deserticola* or *G. fasciculatum*).

	Rhizobium meliloti							
	WT strain				GM strain			
	——		PGPR		——		PGPR	
	%	Total	%	Total	%	Total	%	Total
G. mosseae	82de	177.0b	78c	164.0b	83.5de	199.0bc	85c	157b
G. deserticola	83de	248.0c	84de	238.6c	81cd	200.0bc	84de	207bc
G. fasciculatum	85e	263.5c	89f	227.0c	92f	351.0d	93f	233c
Natural soil	17.7a	13.9a	48b	30.0a				

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P < 0.05$).

described for the rhizobacteria stimulating effect (Gutierrez Mañero et al. 1996).

Microbial metabolites may also affect acetylene reductase activity (ARA) and nodular CO_2, as well as nitrogen content in PGPR-inoculated plants (Probanza et al. 1997). Physiological changes in root and/or nodule functioning could be involved in the specific effects obtained from the various microbial combinations.

It is assumed that when the plant and AM population inhabiting the same rhizosphere are not developed together any association between them may not be functionally compatible. However, the present study supports the view that non-native microbial species promoted plant growth and were able to colonise plant roots to a greater extent than did native species. These results suggest that it is possible to use mycorrhizal technology to accelerate the natural process of re-vegetation in a region of a desertified, semiarid ecosystem, such as in the Southeast of Spain. In fact, the low density of AM propagules could limit the re-establishment of plants showing a large degree of mycorrhizal dependency. Thus, the ineffectiveness of a natural AM inoculum highlight needs to consider inoculation strategies, and the appropriate selection of microorganisms must be done under local environmental conditions using native plants. In the case of *M. arborea*, the most suitable AM endophytes for improving plant growth were not isolated locally from the disturbed site. However non-native fungi appeared to be adapted to the particular ecosystem selected for re-vegetation since the inoculated microsymbionts improved the establishment and growth of these tree legumes. The survival and growth of native plants, as a consequence of microsymbiont inoculation is of interest for re-vegetation strategies in desertified areas (Barea et al. 1990a, 1990b; Herrera et al. 1993a, 1993b; Requena et al. 1996, 1996).

In the present study, PGPRs did not behave as mycorrhizal helper bacteria (Garbaye 1994) because they did not promote root AM colonisation, but operated in interaction with native endophytes. Perhaps the enhancement effect on colonisation was relevant only in the case of reduced-coloniser conditions. There was no conclusive effect of PGPR on nodule formation.

The main influence of PGPR was in inducing changes in root/shoot ratio. Our findings show that not only AM colonisation, but also PGPR, reduced this relationship and the dual association resulted in the greatest reductions. In fact, the positive changes in shoot biomass as a consequence of microbial inoculations were greater than those observed in roots.

An improvement of nodule formation in GM-inoculated plants was expected (Sanjuan and Olivares 1991), but results from the present study did not show a greater colonising ability of GM, compared with WT. The lack of plant growth in natural soil inoculated with GM did not allow us to test the reported enhanced nodulating ability of this strain when there were natural *Rhizobia* in the growing medium.

Ours results indicated that in order to obtain optimum growth *M. arborea* needed to be associated with a mycorrhizal fungus, under the prevailing fertility conditions of their natural habitats (Brundrett 1991).

Mycorrhizal dependence is a plant property which refers to the degree of plant responsiveness to mycorrhizal colonisation. Mycorrhizal dependency is the results of morphological and physiological plant traits modulated by the effectiveness of the mycorrhizal fungus involved. Results supported the view that such a dependence was affected also by associated micro-

organisms, which may enhance the mycorrhizal effect under limiting conditions.

The greater growth rates achieved in mycorrhizal plants is important, not only for contributions to the re-establishment of vegetation, but also for protection against soil erosion. In fact, the plant growth-promoting effect of selected microbial groups is particularly critical under mediterranean climatic conditions and can be decisive for the successful establishment of plants in re-vegetation strategies (Azcón and Barea 1997; Requena et al. 1996, 1996).

A major objective of soil microbiological studies is the practical application of rhizosphere microorganisms. More investigation is required to evaluate the compatibility of microbial groups involved in plant nutrition and factors that are responsible for maximum plant growth in bacterial-AM interactions. The study reported shows that it is not possible to generalise on microbial interactions, because each microbial partner needs a specific study. Under the present experimental conditions, the most effective AM inocula were formed by non-native endophytes. Contrary data reported previously, (Monzón and Azcón 1996) showed that indigenous AM endophytes were more effective in improving growth and nutrient uptake by *Medicago* species. The host plant genotype was relevant to the effectiveness of the tripartite symbiosis.

The application of combined microbial groups can be used for the biological reactivation of devastated soils. To achieve this requires the testing of selected microorganisms, with specific soils and plants in mind. The growth-stimulating effect of combined microbial inoculations can be much greater than that of individual applications. However, in future it may be necessary to characterise the metabolites which play a relevant role in the stimulation effect.

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