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THE ROLE OF MYCORRHIZATION HELPER BACTERIA IN THE ESTABLISHMENT AND ACTION OF ECTOMYCORRHIZAE ASSOCIATIONS

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ABSTRACT

More than 95 % short roots of most terrestrial plants are colonized by mycorrhizal fungi as soon as they emerge in the upper soil profiles. The establishment of mycorrhizal association involves profound morphological and physiological changes in root and fungus. It is affected by other rhizospheric microorganisms, specifically by the bacteria. Bacteria may have developed mechanisms of selective interaction with surrounding microorganisms, with neutral or positive effects on mycorrhizal associations, but negative effect on root pathogens in general. Because of the beneficial effect of bacteria on mycorrhizae, the concept of Mycorrhization Helper Bacteria (MHB) was created. Five main actions of MHB on mycorrhizae were proposed: in the receptivity of root to the mycobiont, in root-fungus recognition, in fungal growth, in the modification of rhizospheric soil and in the germination of fungal propagules. MHB appear to develop a gradation of specificity for the mycobiont, but little or no specificity for the host plant in symbiosis. One of the main groups of MHB is the fluorescent *Pseudomonas*, well represented in diversity and cell density studies of mycorrhizal associations. This review covers the activity of MHB in the establishment of ectomycorrhizae, taking as model the effects of *Pseudomonas* sp. described in scientific literature.

Key words: Mycorrhization Helper Bacteria, MHB, ectomycorrhizal fungi, *Pseudomonas*

INTRODUCTION

The root-soil interface is a dynamic environment, a microcosm where microorganisms, plant roots and soil constituents interact (31), and develop what is known as rhizosphere. The rhizosphere, therefore, is the zone of influence of plant roots on the associated microbiota and soil components, characterized by an altered microbial diversity with increased activity and number of microorganisms (27). It is clearly an environment which is physically, chemically and biologically different from the bulk soil (6). Actually, the structure and diversity of root-associated fluorescent pseudomonads were shown to differ significantly from those of bulk soil populations (4). Rhizospheric and non-rhizospheric populations could be discriminated on the basis of their ability to use specific organic compounds, to mobilize ferric iron and to reduce nitrogen oxides (29). The microbial activity in rhizosphere is under direct influence of plant roots, which release organic material, mainly as root exudates. These exudates serve as substrates for the indigenous microorganisms

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(43). On the other hand, microorganisms associated with plant roots, both free or symbiotically living, would help the host plant to adapt to stress conditions concerning water and mineral nutrition and soil-borne plant pathogens (31).

The association between soil fungi and plant roots is called mycorrhiza. The establishment of mycorrhiza implies profound morphological and physiological changes in the root, which operates in an integrated manner with the fungus, thus promoting gains in adaptability and survival of symbionts (8). According to Wang and Qiu (41), out of a total of 3,617 species belonging to 263 families of terrestrial plants analyzed, 80% of the species and 92% of the families are associated to mycorrhizae. Among the angiosperms, 85 and 94% of the species and families, respectively, are mycorrhizal. The establishment of mycorrhizal association is affected by other microorganisms of the rhizosphere, specifically by bacteria. Bowen and Theodorou (5) demonstrated *in vitro* that some bacteria are able to affect the growth of the ectomycorrhizal fungi *Rhizopogon luteoliis* in symbiosis with *Pinus radiata,* positively or negatively, depending on the bacterial strain present. Although most of the interactions are described as competition, some may benefit the process of plant infection by the mycobiont.

From studies of isolation and identification of bacterial species present in mycorrhizal fungi and analysis of the bacterial action on the symbiosis, Duponnois and Garbaye (13) proposed for the first time the term Mycorrhization Helper Bacteria (MHB), referring only to bacteria that promoted the establishment of the root-fungus symbiosis. This concept was reinforced and clarified latter by Garbaye (22). Since then, much progress was made in the research of this interaction among bacteria, fungus and plants. Frey-Klett *et al.* (21) proposed two functional MHB categories from the knowledge about the bacterial action: the first, *Mycorrhization Helper Bacteria*, strictly referring to those that stimulate the process of mycorrhiza formation (in the applied context of mycorrhizal inoculation, a technique referred to as "controlled mycorrhization"); and the second, *Mycorrhiza Helper Bacteria,* for those that interact positively with the functioning of the already-established symbiosis. However, both the categories can be represented by different groups or by overlapping groups of microorganisms, and the term MHB is used to represent both groups.

In ectomycorrhizae studied so far, the stimulation of fungal growth appears to be the main effect of MHB. For this reason, one of the practical applications suggested for the MHB is the production of inocula containing the fungus and bacteria, what could increase the efficiency of inoculation of plant seedlings with selected ectomycorrhizal fungi in order to stimulate plant growth.

This review covers the activity of the MHB, taking *Pseudomonas* sp. as a model on the establishment of ectomycorrhizae.

Occurrence of ectomycorrhizae

Fossil records indicate that ectomycorrhizal associations emerged at least 50 million years ago (30) although there is evidence of this emergence dated to more than 180 million years ago (33). Ectomycorrhizae are the most common type of associations formed by ascomycetes and basidiomycetes fungi, although, in general, ectomycorrhizal associations are much rarer than arbuscular mycorrhizae in terrestrial plants. From the perspective of plant phylogeny, the distribution of ectomycorrhizae suggests many independent origins of this symbiosis, since its occurrence is sporadic in terrestrial plants, and it is mostly found in derived lineages in the main plant clades (30, 42).

In this association, the fungal symbionts produce extensive nets of mycelium that extend the scope of exploratory roots of plants (32). The mycelium provides to the host soil minerals through solubilization, particularly of phosphorus and nitrogen, while the plants provide photoassimilates to the mycobiont. This fungal net is capable of connecting one plant to another, and even to transfer nutrients between them (32, 24). Consequently, mycorrhizal fungi alter the physical, chemical and microbiological characteristics of the surrounding soil and create a special environment called mycorrhizosphere in which the microbial communities differ from those in the rhizosphere

and in other portions of the soil (24).

Ectomycorrhizal associations are characterized by presenting mantle, a layer of hyphae surrounding the root cells of the epidermis; the Hartig net, a structure that results from the hyphal growth in intercellular spaces of epidermis and cortex; and a net of mycelial filaments that mediate the connection of mycorrhizae to the soil and to fructification bodies. The formation of ectomycorrhizae inhibits the formation of root hairs, which are functionally replaced by the fungal hyphae. This inhibition involves the secretion of indole compounds by the fungus, such as indolacetic acid and hypaphorine, responsible for regulating the root morphogenesis (11, 12).

MHB - Mycorrhization Helper Bacteria

The lineages of MHB identified so far belong to many groups and bacterial genera, such as Gram-negative Proteobacteria (*Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella* and *Rhizobium*), Gram-positive Firmicutes (*Bacillus, Brevibacillus,* and *Paenibacillus*) and Gram-positive actinomycetes (*Rhodococcus, Streptomyces,* and *Arthrobacter*) (21).

In a study, Garbaye and Bowen (23) found approximately 10^6 bacterial colony-forming units per gram (fresh weight) of mycorrhiza. Among these colonies, the majority corresponded to the fluorescent *Pseudomonas* group and 80% had a positive effect on the establishment of mycorrhizae, while only 20% were neutral or inhibitory (22). Based on available information, Garbaye (22) suggested a definition for MHB: bacteria associated with roots and mycorrhizal fungi that selectively promote the establishment of mycorrhizal symbiosis. According to the authors, the MHB are probably very common, being found wherever sought, under very different conditions and various plant-fungus combinations.

According to Garbaye (22), the MHB are not plant-specific, but are clearly selective about the fungal species, and the term fungus-specific can be used. Among ectomycorrhizal fungi, only basidiomycetes have been described to be interacting with MHB (21). Studies have shown that the ectomycorrhizal symbiosis has an indirect positive effect on the selective pressure of bacterial communities. Frey-Klett *et al.* (20) demonstrated that the ectomycorrhizal symbiosis determines the composition of *Pseudomonas fluorescens* populations and selects strains potentially beneficial to the symbiosis and to the plant.

According to Freitas and Vildoso (17), strains of fluorescent *Pseudomonas, Bacillus* and other rhizospheric bacteria may act as growth promoters of citric plants. Then, the question arises of whether the MHB are rhizobacteria occasionally acting as auxiliary to mycorrhization, if present by chance near a symbiotic fungus, or are dependent on the fungus and persist in its stages of development. Garbaye (22) suggests that the second hypothesis is supported by the fact that sporocarp of some ectomycorrhizal fungi, as *Laccaria*, *Tuber, Suillus*, *Hymenogaster* and *Cantharellus* are usually inhabited by large bacterial populations. Furthermore, many isolates of MHB described in the literature have been collected from mycorrhizospheres, fructification bodies of ectomycorrhizal fungi and fungi spores of arbuscular mycorrhizal (21).

Many MHB are considered nowadays as Plant Growth Promoting Rhizobacteria (PGPR), such as *Pseudomonas* sp. (39, 25, 34). As reported by Fitter and Garbaye (16), these classifications may overlap, due to the prominence of *Pseudomonas* and *Bacillus* in both groups. Another factor that complicates the distinction of the two terms (PGPR and MHB) is that studies with PGPR generally exclude the evaluation of mycorrhization (35). However, it is interesting to note that some fungal signaling pathways are mutually regulated by different rhizobacteria, while others are specific to some MHB (10).

The effect of MHB on ectomycorrhizal associations

Five possible ways of action of MHB on mycorrhiza were proposed by Garbaye (22): in the receptivity of the root to the mycobiont, in root-fungus recognition, in fungal growth, in the modification of the rhizospheric soil and in germination of fungal propagules. In the ectomycorrhizae studied so far, the stimulus to fungal growth appears to be the primary MHB

effect. The germination of spores and the mycelial growth can be stimulated by MHB through the production of growth factors, detoxification of antagonistic substances or inhibition of competitors and antagonists (21). The stimulus to growth represents an adaptive advantage to the fungus, which becomes heavily associated to the host plant and acquires more competitive capacity against other mycobionts in the planting area (15). Currently, the contribution of each of these effects has not been fully established, and further studies are needed to elucidate these issues.

One of the features also observed in MHB is the stimulus to the formation of lateral roots in mycorrhizal plants. This fact, associated to the stimulus to fungal growth, could lead to an increase in the number of possible interaction sites between the plant and the fungus (38) and, consequently, promote greater plant mycorrhization by the mycobiont. Furthermore, apparently, different MHB may develop different helper mechanisms, even for the same pair of mycorrhizal symbionts. For example, Poole *et al.* (36) observed that the MHB *Burkholderia* sp. EJP67 isolated from *Pinus sylvestris*-*Lactarius rufus* ectomycorrhizae stimulated both first- and second-order mycorrhizal roots, while *Paenibacillus* sp. EJP73 isolated from the same ectomycorrhizae only promoted the formation of second-order mycorrhizal roots.

Aspray *et al.* (1) demonstrated that the contact between MHB cells and the symbionts is necessary for the helper effect to be exerted. The MHB can improve the nutrition of the fungus, for example, through the provision of nitrogen in the case of diazotrophic bacteria, or contribute to the solubilization of minerals by the secretion of protons and complexing agents, such as organic anions of low molecular weight or siderophores. It is possible that the MHB stimulate the production of phenolic compounds by the fungus, such as hypaphorine, and thus enhance the aggressiveness of the mycobiont (15).

Some strains of MHB are capable of competing with bacteria that inhibit mycorrhization (22) and, consequently, reduce the concentration of anti-fungal metabolites in mycorrhizosphere. The fungus favors the MHB by releasing exudates that serve as nutrients for the bacteria. An interesting fact is that the fungus *Amanita muscaria* secret substances (organic acids or protons) that can modulate the spectrum of antibiotics production by MHB (21). Keller *et al.* (26) reported that the metabolite auxofuran, produced by *Streptomyces* sp. AcH505, seems to stimulate the pre-symbiotic growth of *A. muscaria* but inhibit the growth of pathogenic fungi.

The researches available so far suggest that MHB may have developed selective mechanisms of interaction with surrounding microorganisms, with neutral or positive effects on mycorrhizal associations, but negative effects on the root pathogens that threaten its habitat (21). However, there are data concerning MHB stimulating phytopathogenous fungi and this should be considered in the biotechnological applications of MHB, for instance, as inoculum for plants. Further researches are necessary to determine whether MHB could promote the colonization of the roots by pathogenic fungi and development of disease.

Specificity of the interaction between MHB and ectomycorrhizal symbiosis

MHB are fungus-specific but not plant-specific (22). Many studies have been carried out in order to explore the specificity of the interaction between MHB and the fungi and between MHB and the symbiont plant, and diverse results have been obtained (2, 3, 15, 21, 22).

Frey-Klett *et al.* (21) reported that the MHB *Streptomyces* sp. AcH505 is capable of promoting growth of *A. muscaria* and *Suillus bovinus* and increase the formation of ectomycorrhizae between *A. muscaria* and *Picea abies*, but the growth of *Hebeloma cylindrosporum* and pathogenic fungi is inhibited. Bending (3) observed that the production of the metabolite auxofuran by *Streptomyces* sp. AcH505 and its selective effect on the growth of *A. muscaria* may support the hypothesis of specificity between some MHB and mycorrhizal fungi, since it is a specific interaction between these microorganisms.

The results of Duponnois and Plenchette (15) support the evidence from Garbaye (22) that the effect of the MHB is not plant-specific. This was demonstrated in an experiment in

which *Pseudomonas fluorescens* BBc6 promoted the formation of mycorrhiza by *Laccaria laccata* in four species of conifers (*Picea abies, Pinus nigra, Pinus sylvestris* and *Pseudotsuga menziesii*) and in the angiosperm *Quercus robur*. However, Duponnois and Plenchette concluded that the effect of the MHB was not fungus-specific, as *Pseudomonas monteilii* HR13 isolated from *Pisolithus alba* stimulated the development of mycorrhiza in *Acacia holosericea* with two species of *Scleroderma* and, more surprisingly, with the arbuscular mycorrhizal fungus *Glomus intraradices*.

In general, it is noted that MHB exhibit a degree of specificity with the mycobiont, with some strains apparently specific to certain ectomycorrhizal fungi (14) and other capable of stimulating the mycorrhization by different ectomycorrhizal fungi (2).

MHB Effect of *Pseudomonas*

The genus *Pseudomonas* is included in several groups of microorganisms in association with fungi and plants, as MHB, PGPR and EMAB (Ectomycorrhiza Associated Bacteria) (28). In Brazil, the first researches with bacteria promoting the growth of plants, tested the ability of fluorescent *Pseudomonas* to increase the growth of tomato and coffee plants in nurseries. Since then, many studies have considered the positive effect of these bacteria. Fluorescent *Pseudomonas* spp. promoted better growth of beans seedlings and the mycorrhization rate was increased when they were co-inoculated with the fungus *Glomus etunicatum*, which refers to the *Pseudomonas* role as MHB (40).

One of the most studied strains of *Pseudomonas* is BBc6R8. Frey-Klett *et al.* (21) demonstrated that, in nurseries, the survival of *P. fluorescens* BBc6R8 is significantly enhanced by the presence of the ectomycorrhizal strain *Laccaria bicolor* S238N, from which the bacterium was isolated. This effect did not occur in the presence of non mycorrhized roots of the conifer *Pseudotsuga*, suggesting that this strain of *P. fluorescens* depends more on the presence of the fungus than on the plant. Likewise, the fungus of arbuscular mycorrhiza *Glomus mosseae* promoted longer survival of *P. fluorescens* 92rk in the rhizosphere of tomato plants (*Lycopersicon esculentum*).

P. fluorescens BBc6R8 promoted the pre-symbiotic survival, the growth of *L. bicolor* S238N in soil, and the establishment of symbiosis between *Pseudotsuga* and the fungi (10). However, using the same organisms, the results obtained by Brule *et al.* (7) showed that the bacteria did not significantly modify the fungal survival. The authors suggest that the beneficial MHB effect on the mycobiont depends on the condition in which the fungus is, and that the greatest benefit occurs when the fungus is under unfavorable conditions. Further researches are needed to validate this hypothesis.

Pseudomonas acts as MHB not only with ectomycorrhizae, as there is evidence of its effect on the establishment of association between *Acacia holosericea* and the endomycorrhizal fungus *Glomus intraradices* (15). Furthermore, not all strains of *Pseudomonas* act as MHB. *P. fluorescens* Pf29A, for example, is a rhizospheric non-MHB bacteria (10), used as a biocontrol agent.

Antagonism against phytopathogens by this genus has been observed *in vitro*. Frey-Klett *et al.* (21) showed that the proportion of *Pseudomonas* that inhibited the growth of seven fungal root pathogens in ectomycorrhizae of *L. bicolor* was significantly higher than in surrounding soil. Many *Pseudomonas* strains produce antimicrobial metabolites, such as phloroglucinols, fenazines, pyoluteorin and pirrolnitrine (9).

Duponnois and Plenchette (15) studied the effect of *Pseudomonas monteilii* HR13 in the formation of ectomycorrhizae between combinations of the *Acacia* species *A. mangium* and *A. auriculiformis*, and strains of *Pisolithus* and *Scleroderma*. *P. monteilii* promoted mycorrhization of both species of *Acacia,* from 45.8 % in *A. mangium* to 70.3 % in *A. auriculiformis*. The stimulation of mycorrhization was observed for all fungal isolates.

Many authors suggested that the MHB effect of *P. fluorescens* is due to the stimulation of fungal growth, thus increasing the possibility of interaction between root and mycelium (10, 19). The possibility of BBc6R8 to act directly on the receptivity of the root to the fungus is considered low,

and Frey-Klett *et al.* (19) were of the view that BBc6R8 does not act on mycorrhizae already in formation.

Morphophysiological changes in mycobiont caused by *Pseudomonas*

Deveau *et al.* (10) compared *P. fluorescens* BBc6R8 to six other rhizobacteria (*Collimonas fungivorans* Ter331, *Paenibacillus* sp. EJP73, *Pseudomonas fluorescens* Pf29A, *Bacillus subtilis* MB3, *Burkholderia* sp. EJP67 and *Paenibacillus* sp. F2001L) and found that *P. fluorescens* BBc6R8 was the only one that induced increase in survival, in the apex density of the hyphae, in the branching angle and radial growth of the fungus *Laccaria bicolor* S238N. The morphological modifications were associated with changes in the transcriptome of *L. bicolor* that varied throughout the interaction. The authors reported that some responsive genes were partially specific to the interaction with *P. fluorescens* BBc6R8, which provides evidence of the specificity of the relationship between MHB and the mycobiont. In general, the data suggest that the effect of MHB involves changes in the fungal anabolism and catabolism of lipids that could cause increased lipids synthesis, required for higher growth rates.

According to Deveau *et al.* (10), the morphological changes of the mycelium *in vitro* may be beneficial to the host root infection by the fungus, representing a transition from the saprophytic to the pre-symbiotic state. It is interesting to note that not all bacterial strains are able to promote such changes in the fungus. Results suggest that additional mechanisms, not limited to the increase of growth rate, are involved in stimulation of mycorrhization. *P. fluorescens* Pf29A, a non-MHB strain, was also able to induce changes in growth and morphology of *L. bicolor* S238N, although only *P. fluorescens* BBc6R8 increased the diametral growth of the colony, the density of the hyphae apex and angle of branching at the pre-contact stage.

Much remains to be clarified about the consequences of the interaction between MHB and associated fungi. An interesting information that suggests the intensity of interaction is that the MHB *Streptomyces* sp. ACH505 is capable of altering the regulation of the actin cytoskeleton organization in *A. muscaria* (38).

The interaction *Pseudomonas*-fungi-plant

Frey-Klett *et al.* (21) demonstrated in nurseries that the survival of *Pseudomonas fluorescens* BBc6R8 is significantly enhanced by the presence of the ectomycorrhizal strain *Laccaria bicolor* S238N, from which the bacterium was isolated. The authors observed that *P. fluorescens* BBc6R8 shows adherence to hyphae of different ectomycorrhizal fungi and is also able to develop biofilm-like structures in hyphae of *L. bicolor in vitro*. This proposition is consistent with previous hypothesis that, after inoculation, the population of *P. fluorescens* BBc6R8 decreases in soil, but concentrates on target niches, such as the fungal cell wall (18).

Izumi *et al.* (24) classified *Pseudomonas* as an endobacterium. In this context, the term endobacterium is defined as bacteria that exist within the compartments of the fungus or the host of mycorrhiza, or still within the cells of one of the symbionts. The study (24) covered four morphotypes of ectomycorrhizae of *Pinus sylvestris*: *Suillus flavidus, Suillus variegatus, Russula* sp. and *Russula paludosa.* After superficial sterilization of mycorrhizal roots, the culturable bacteria were analyzed by RFLP (Restriction Fragment Length Polymorphism) of the rDNA intergenic spacer regions and 16S rRNA genes. The results showed the presence of *Pseudomonas* in more than one ectomycorrhizal morphotype and about 50% of the isolates belonged to the genera *Pseudomonas* and *Paenibacillus*, suggesting that these two genera should be widely distributed in different ectomycorrhizae of *Pinus sylvestris*.

One of the suggested mediators of the attraction between *P. fluorescens* BBc6R8 and the fungus is the disaccharide trehalose, produced by fungi from the carbon compounds received from the phytobionts. This bacterial strain presents a chemical attraction both to the pure disaccharide and to the hyphae of *L. bicolor*, which accumulate trehalose (21).

The presence of the low molecular weight fraction from the supernatant of *Pseudomonas putida* cultures promoted a

significant increase in the rates of fungal growth and mycorrhization by *Glomus fistulosum*, similarly to that caused by the co-inoculation of the cells. This fact suggests that effective substances were present in this fraction (41).

The physical and chemical interactions between ectomycorrhizal fungi and soil can significantly change the structure of *P. fluorescen*s populations, selecting strains potentially beneficial to the symbiosis and to the plant, as described by Frey-Klett *et al.* (20). This study showed that populations of *Pseudomonas* are quantivative and qualitatively regulated in the symbiosis bacteria-fungus, as the genetic diversity of cultivable *P. fluorescens* was higher in mycorrhizosphere of *L. bicolor-Pseudotsuga menziesii* than in bulk soil. Most of the *Pseudomonas* isolated from the mycorrhizosphere was able to solubilize inorganic phosphate, and this characteristic was not found in the majority of soil bacteria. This ability probably favors the growth of plants in symbiosis. The mycorrhizosphere also contained isolates of *P. fluorescens* presenting a greater spectrum of antagonism against phytopathogens than other isolates from the rest of the soil. The proportion of *P. fluorescens* capable of fixing nitrogen did not differ significantly between the mycorrhizosphere and bulk soil, indicating that the symbiosis *L. bicolor-P. menziesii* did not select this feature. This fact contrasts with the study of Rózycki *et al.* (37) which showed an increase in nitrogen fixing bacteria, mainly *Pseudomonas*, in the mycorrhizosphere of pine and oak. Frey-Klett *et al.* (21) suggested that the presence of nitrogen fixing bacteria in various ectomycorrhizal interactions indicating the potential of MHB to assist the nutrition of the associated plant.

Conclusions and prospects

The studies concerning the action of MHB on the establishment and development of ectomycorrhizae may generate an interesting comprehension about the interaction between these organisms and the other components of the environment. More specifically, the study of MHB is essential in promoting the knowledge of how mixed microbial communities stimulate the formation of mycorrhizae. In Brazil, there are few studies with MHB and, in general, those involving *Pseudomonas* cover only their role as promoting the growth of plants without assessing the effect on the establishment of mycorrhizae. MHB could be very useful in techniques of controlled mycorrhization in forest management, through its application to soil in nurseries. Co-inoculation with the mycobiont enables the saving of fungal inoculum and may improve the quality of the mycorrhizal association in seedlings (22). Although bacteria with the potential to act as MHB apparently occur everywhere, the activities of most of the MHB have been demonstrated in laboratories or greenhouses, and the extension of these results to natural conditions of cell density and patterns of location *in situ* remains to be elucidated. Selective pressure of ectomycorrhizal symbiosis on bacterial communities should be considered in *in situ* studies (20), which, as demonstrated for *Pseudomonas*, is able to select the components of mycorrhizosphere. Little is known about the molecular mechanisms induced by MHB and involved in promoting growth of mycobiont. A deeper study on MHB could generate a model for genomic analysis of bacteria-fungus interactions, that may benefit other research areas in which these interactions have a central role, such as protection of plant species and medicine (10).

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