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The effect of plant growth promoting bacteria both in vitro and hydroponic culture of barley *(Hordeum vulgare L.)* growth

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Abstract: Plant growth promoting bacteria (PGPB) boost plant development and promote soil bioremediation by secreting a variety of metabolites and hormones. Several important bacterial characteristics, such as biological nitrogen fixation, phosphate solubilization, ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, and production of siderophores and phytohormones, can be assessed as plant growth promoting (PGP) traits. In the current study, three *Bacillus megaterium* strains and one *Pseudomonas fluorescens* strain have been investigated for their plant growth promoting abilities in vitro and in a hydroponic culture on barley. Firstly, these bacterial strains have been investigated for their salt (NaCl) tolerances and then phosphate solubilization, nitrogen fixation and the production of ammonia and indole-3-acetic acid. These bacterial strains were also inoculated with *Hordeum vulgare* in a hydroponic culture. The investigated bacterial strains were found to have different plant growth promoting activities even within same species (*B. megaterium*). In the hydroponic culture experiments, it was determined that comparing to the control group, the growth rates (total weight) of the plants enhanced with *B. megaterium* (FDG108), *B. megaterium* (FDG161), and *B. megaterium* (FDG2) ranged from 3.91, 43.45, and 73.73, respectively. Even these three bacteria were *B. megaterium,* the best result regarding the total plant weight was obtained with *B. megaterium* (FDG2). These results showed that even if many bacteria enhance plant growth, the use of better one is of vital importance in agriculture practice for production of food.

Key words: *Bacillus megaterium,* barley, PGPB, *Pseudomonas fluorescens*

1. Introduction

The human population is growing very fast and expected to be 10 billion by 2050. In order to supply the need of human population food demand, novel solutions in plant growth are required. According to the Centre for Study of Carbon Dioxide and Global Change, more than 70–100 % increase in plant production can fulfill the food demand of the increasing human population. The awareness of the increase in human population and decrease in agriculture lands and soil quality leads the human to search new alternatives. In an attempt to produce more food, one of the solutions is the enlargement of the agricultural lands. The agricultural lands, on the contrary, are reduced due to excessive synthetic chemicals usage including herbicides, fertilizers, and pesticides. For the adequate plant growth, the nutrients in the soil should be existing in a sufficient amount and in a form useful for plants. As known, soil contains nutrients largely in the forms unavailable to plants and must be processed either by microorganisms or chemically. It is clear that the chemical fertilizers can increase the plant growth only when chemical is not available in the soil. In case the synthetic chemicals are over applied to the soils, they can pollute water; reduce the soil microorganisms and beneficial insects for the sustainability of agriculture and increase the susceptibility of the plant to the disease (Chen, 2006).

Besides the application of synthetic chemicals, the application of microbe-based strategies in order to enhance the plant growth is in use. Among microbe-based strategies, the application of plant growth promoting rhizobacteria (PGPR) has begun about 100 years ago in many countries such as United State, China, Soviet Union, and Europe. According to the available literature, the application of PGPR onto roots and seeds leads these microorganisms to colonize onto the root system and enhance the plant growth by producing certain phytohormones and breaking down organic matters (Valencia-Cantero et al., 2007). In this regard, the application of PGPR on different crops has

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been shown to increase growth of plants up to 50% and yield up to 57% (Kloepper et al., 1980; Asghar et al., 2004; Khalid et al., 1997).

The mechanisms in which PGPR enhance the growth of plants are usually divided into two groups as direct and indirect mechanisms. Either with direct or indirect mechanisms, the abiotic and biotic components of the rhizosphere community are changed by PGPR or the secondary metabolites of PGPR in order to enhance the growth of plants (Gray and Smith, 2005; Lugtenberg and Kamilova, 2009; Kloepper and Schroth, 1978). More clearly, as a general rule, indirect mechanism occurs outside the plant, while direct mechanism happens inside the plant and therefore directly affects the metabolism of the plant (Lugtenberg and Kamilova, 2009). Whereas the direct mechanism involves biological processes such as solubilization of complex matters (organic and inorganic), mobilization of Fe, production of plant regulators (gibberellins, cytokines, and indole acetic acid) and nitrogen-fixation (Bhardwaj et al., 2014), the indirect mechanism involves the production of different antibiotics and lytic enzymes to prevent proliferation of pathogens (Akhgar et al., 2014). In this study three *Bacillus megaterium* strains and one *Pseudomonas fluorescens* strain have been investigated for their plant growth promoting abilities in vitro and in a hydroponic culture, a soil-free method of growing barley in water, on barley.

2. Materials and methods

2.1. Determination of the indole acetic acid production

Salkowski's colorimetric method was used to determine the indole acetic acid (IAA) concentration produced by each isolate (Ehmann, 1977). The pure culture of each strain was grown in a nutrient broth medium containing 0.1 mg/mL tryptophan and 5% NaCl and was incubated at 30 °C for 2–4 days. After incubation, the broth was centrifuged at 12.000 rpm, the supernatant was retained, and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) and kept in the dark for minimum 30 min. Subsequently, the optical density (OD) was measured at 530 nm.

2.2. Determination of ammonia potential

To test the ammonia production activity, the bacterial strains were added to peptone water (Peptone 20.0 g/L and NaCl 30.0 g/L) with constant shaking at 140 rpm for 5 days at 30 °C. After incubation, 0.2 mL of the culture supernatant was mixed with 1 mL Nessler's reagent. The OD of the mixture was measured at 450 nm using a spectrophotometer (Marques et al., 2010) and an endpoint of a brown to yellow color was evaluated as ammonia production.

2.3. Determination of the nitrogen fixation potential

The nitrogen fixing (N-fixation) ability was determined using Burk's modified N-free medium, which contained the following ingredients per liter: sucrose, 10.0 g; glucose, 10.0 g; K_2HPO_4, 0.64 g; KH_2PO_4, 0.16 g; $MgSO_47H_2O$, 0.20 g; NaCl, 30.0 g; $CaSO_42H_2O$, 0.05 g; $Na_2MoO_42H_2O$, (0.05 %) 5.0 mL; $FeSO_47H_2O$, (0.3 %) 5.0 mL and agar, 15 g (Wilson and Knight, 1952). The lightening of the blue color was evaluated as positive.

2.4. Determination of phosphate solubilization ability

The bacterial strains were incubated at 30 °C for 7 days with Pikovskaya's modified medium to determine the phosphate solubilization ability. Pikovskaya's modified medium contained the following per liter: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_47H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4H_2O$, 0.002 g; $FeSO_47H_2O$, 0.002 g and NaCl, 30.0 g (Surange and Kumar, 1993). The lightening of the blue color was evaluated as positive.

2.5. Determination of the plant growth promoting bacteria (PGPB) potential of strains

Strains and culture condition:

The pure bacterial cultures were grown in a nutrient agar for experiments. A single colony from each strain was transferred to a 50-mL flask containing the nutrient broth. The colonies were aerobically grown in the flasks overnight on a rotating shaker (200 rpm) at 30 °C before application.

Plant materials and bacterial inoculation:

Barley seeds (*H. vulgare* cv. Olgun) were obtained from East Anatolian Agricultural Research Institute. The barley seeds were surface sterilized with 3% sodium hypochlorite for 5 min and then washed 5 times with sterilized distilled water. Following the sterilization, the seeds were allowed to germinate in net cups filled with hydrotons at 30 ºC for 4 days. The seedlings were sown at a planting density of 20 seeds/net cup (4 net cups/pot) containing half-strength Hoagland's medium (pH 6.0) for 10 days. The medium contains KNO_3, 18.05 mg/L; K_2SO_4, 146.5 mg/L; $CaCl_22H_2O$, 73.5 mg/L; $MgSO_47H_2O$, 51.5 mg/L; NaH_2PO_42H2O, 2.51 mg/L; $FeSO_47H_2O$, 1.66 mg/L; H_3BO_3, 0.47 mg/L; $MnCl_24H_2O$, 0.19 mg/L; $ZnSO_47H_2O$, 0.04 mg/L; $MnCl_24H_2O$, 0.19 mg/L; $CuSO_45H_2O$, 0.015 mg/L; and H_2MoO_4, 0.11 mg/L (Hoagland and Arnon, 1950). In order to eliminate the effect of nutrient broth medium on plant growth, the same volume of bacteria-free nutrient broth medium was added to the control and salt application groups. The experiments were designed as follows: Control: Hoagland's medium and 5 mL of bacteria-free nutrient broth medium. Bacterial application: Hoagland's medium and 5 mL of nutrient broth containing each bacterial strain (the concentration of each strain was 1×10^8 colony forming units/mL). To provide a homogeneous distribution of nutrients and oxygen for the bacterial strains and plant roots, the hydroponic systems were continuously aerated with an air pump during the experiments. Water lost by

evapotranspiration was supplied with the same Hoagland's medium. Each treatment was replicated thrice. On day 10 after the germination period, the plants were analyzed for the root and shoot length and total fresh weight.

2.6. Statistical analysis

The results are presented as the average means and standard error (SE) of triplicate. The data were further analyzed for statistical significance using analysis of variance (ANOVA), and the difference between means was compared by a high-range statistical domain using Tukey's test. A p-value < 0.05 indicated statistical significance. The data were discussed in terms of percentage variation, with respect to the control plants.

3. Results and discussion

In our previous study the investigated bacterial isolates have been identified and characterized by conventional (morphology, physiology, and biochemical tests) and molecular techniques (16 rDNA). In the current study, the salt tolerance (NaCl), IAA, and ammonia production, N-fixation and phosphate solubilization activities of 4 bacterial strains (three of these strains were *B. megaterium* and one strain was *P. fluorescens*) were investigated. According to the result obtained, all bacterial strains have different salt tolerances. All strains have grown well in 5% NaCl. In 10% NaCl, while two *B. megaterium* strains (FDG2 and FDG108) grew well, *P. fluorescens* grew slightly and one *B. megaterium* isolate (FDG161) did not grow. In 15% NaCl only two *B. megaterium* strains (FDG2 and FDG108) slightly have grown. Similar to the salt tolerances, all strains have different IAA, ammonia production and phosphate solubilization capacities. While *P. fluorescens* (FDG37) has the highest IAA production, *B. megaterium* (FDG161) had the lowest IAA production. The highest phosphate solubilization and N-fixation activities were obtained with *B. megaterium* (FDG161) followed with *P. fluorescens* (FDG37), *B. megaterium* (FDG2) and *B. megaterium* (FDG108) (Table).

After determining PGPB activities, these bacterial isolates were used to study plant growth in a hydroponic culture. The results showed that all the strains inoculated with plant have plant growth promoting properties. Namely, most of the PGPB bacterial strains significantly increased the root and shoot length and total fresh weight of the plants. The highest total weight (g) of the plants was obtained with a *B. megaterium* isolate (FDG108), followed by *P. fluorescens* (FDG37), *B. megaterium* (FDG161), and *B. megaterium* (FDG2) which were 71.2, 70.6, 69.1, and 64.7 (Figure 1).

The highest total length of the plants was obtained with a *B. megaretium* isolate (FDG2), followed by *B. megaterium* (FDG108), *P. fluorescens* (FDG37), and *B. megaterium* (FDG161) which were 37.12, 31.77, 31.13 and 22.55 g (Figure 2).

Considering the plants total length, the promoting rates (%) caused by PGPB bacterial strains compared to control were 0.9, 7.8, 10.14, and 11.04 for *B. megaterium* (FDG161), *P. fluorescens* (FDG37), *B. megaterium* (FDG108) and *B. megaterium* (FDG2), respectively. Similar to the total length of the plants, the total weight of the plants has been increased by PGPB bacterial strains. The promoting rates (%) in total weight caused by PGPB bacterial strains compared to control were 3.91, 43.45, 46.40, and 73.73 for *B. megaterium* (FDG2), *B. megaterium* (FDG161), *P. fluorescens* (FDG37), and *B. megaterium* (FDG2), respectively.

Table. Plant growth promoting traits and salt tolerances of bacterial strains.

		***Bacillus megaretium* isolates**			***Pseudomonas fluorescens* isolate**
		FDG2	**FDG108**	**FDG161**	**FDG37**
Salt tolerances (%)	**5**	++	++	++	++
	10	+	+	–	Slight
	15	Slight	Slight	–	–
IAA production		+	+	+	+
NH3 production		+	+	+	+
P solubilizing		+	+	+	+

* –: Negative, +: Positive, **IAA**: indole-3-acetic acid, **NH_3**: ammonia, **N**: nitrogen, **P**: phosphate.

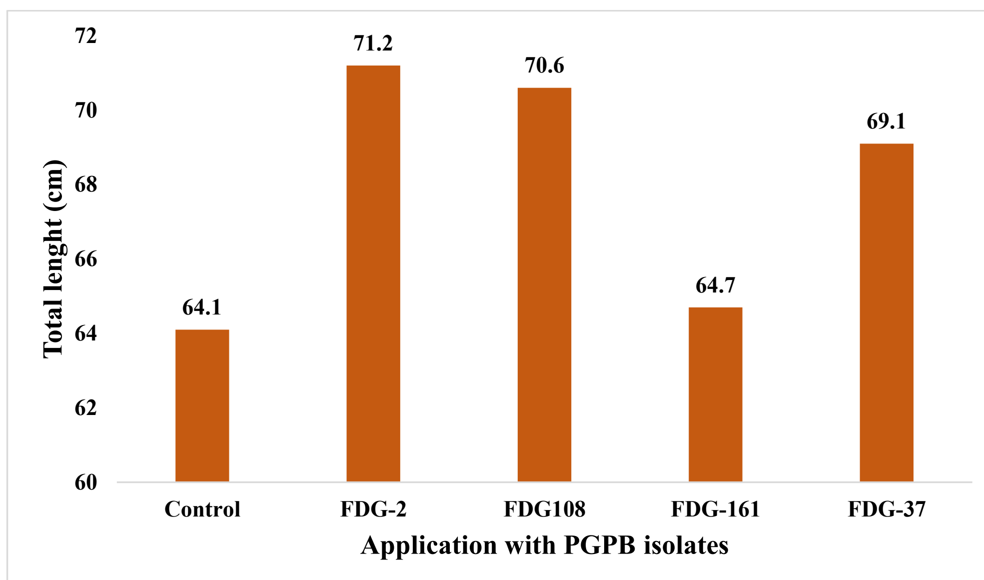

Figure 1. Application with PGPB isolates.

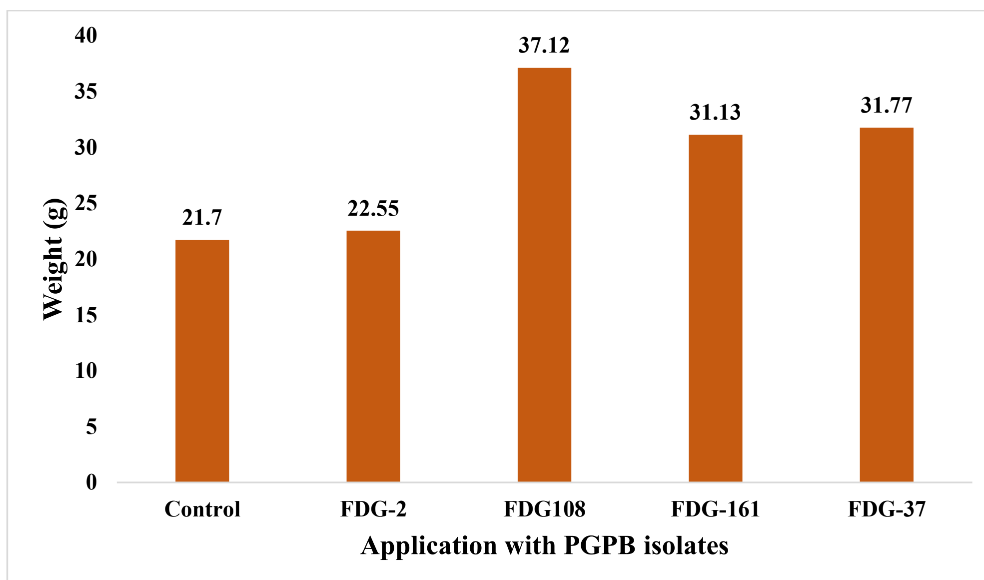

Figure 2. Application with PGPB isolates.

4. Conclusion

Soil contains a huge amount of microbiotic life forms as algae, *Actinomycetes*, fungi, protozoa, and bacteria. Among these microorganisms bacteria are more than others and their number can vary according to the soil conditions and the plants existed in the soil (Glick et al., 1999; Timmusk et al., 2011). Around of plant roots the number of these bacteria is high due to the compounds produced and secreted by plants. These bacteria living around the plant roots can be harmful, neutral or beneficial (Lynch, 1990). When a bacterium causes a disease or inhibits plant growth it can be considered harmful (or plant pathogen). In fact, a beneficial bacterium can have no effect on plant when the conditions are optimum for the plant growth. On the other hand, when a bacterium produces and secretes compounds upregulating plant growth or inhibits plant pathogens the relationship can be considered beneficial. Another classification of plant

growth promoting bacteria is their relation with plants regarding they live outside or inside the plants. While the former is called nonsymbiotic bacteria, the later is called symbiotic bacteria. The members of nonsymbiotics include *Azoarcus*, *Azospirillum*, *Burkholderia*, *Gluconacetobacter*, *Pseudomonas*, *Azotobacter*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Klebsiella*, and *Acetobacter*, the members of symbiotics include *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium* (Gray and Smith, 2005). Regardless of that these bacteria are nonsymbiotic or symbiotic, they have been proven to enhance the growth of plants. The phenomena using soil bacteria to prompt plant growth dates to 372–287 BC. Afterwards, Hellriegel and Wilfarth (1888) reported that rhizobia in root nodules of legumes can convert atmospheric nitrogen into ammonia to be used by plants (Bhattacharyya and Jha, 2012; McNear and David, 2013). Between 1895 and 1909, Russian researchers showed that different beneficial bacteria have positive effects in improve plant growth and that work led to the industrial production and general use of different cultures of *Bacillus* species and *A. chroococum* to improve plant growth (Kloepper, 1996). Since 1909, these bacteria are increasingly continued being used in agriculture practice.

In this study we have investigated three *B. megaterium* strains and one *P. fluorescens* strain for their plant growth promoting abilities in vitro and in a hydroponic culture on barley. According to the data obtained, the strains were shown to have different proportion promoting activities in producing IAA and ammonia, fixing nitrogen and solubilizing phosphate. On the other hand, regardless of these differences, the bacterial strains have increased both the plant's total length and weight. The results showed that the promoting effect of bacterial strains in total plant weight was higher than in total plant length.

Among the *B. megaterium* strains, the highest production in IAA was obtained with *B. megaterium* (FDG108), the highest ammonia production with *B. megaterium* (FDG2), the highest phosphate solubilization and N-fixation with *B. megaterium* (FDG108). The difference in plant growth promoting activities between *B. megaterium* strains has been demonstrated in previous study. In the study of Gunes at al. (2015), it has been reported that 10 different bacterial isolates [*B. megaterium* (KBA10), *B. megaterium* (TV3D), *B. megaterium* (M3), *B. megaterium* (TV17C), *Hafnia alvei* (TV34A), *P. fluorescens* (FDG37), *B. megaterium* (TV60D), *B. megaterium* (TV91C), *Pantoea agglomerans* (RK92), *B. megaterium* (TV87A)] including 7 *B. megaterium* have different plant growth activities (IAA, salicylic acid, acid phosphatase, alkaline phosphatase, etc.). In a study performed by Sezen at al. (2016) 16 strains [*Cellulomonas turbata* (AS1), *B. megaterium* (AS2), *P. putida* (AS3), *Enterobacter cloacae* (AS6), Unknown (AS7), *B. mycoides* (AS9), *E. cloacae* (AS10), *Vibrio furnissii* (AS11), *B. cereus* (AS12), *B. cereus* (AS14), *B. megaterium* (AS16), *B. cereus* (A4), *Neisseria mucosa* (A5), *B. megaterium* (A8), *B. cereus* (A13), *B. megaterium* (A15)] have been shown to be different than each other both in plant growth promoting activities (nitrogen fixation, phosphate solubilizing, IAA and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production) and in increasing plant (wheat) weight and length (shoot and root). Fan et al. (2016) reported that tomato under salt stress inoculated with *Arthrobacter* (TF1), *Arthrobacter* (TF4), *Arthrobacter* (TF4), *Arthrobacter* (TF4), *Arthrobacter* (TF7), *B. megaterium* (TF2) and *B. megaterium* (TF3) strains shown to possess different plant growth promoting activities (phosphate solubilizing) and at least one plant growth promotion (PGP)-related gene.

In a recent study, Perez-Fernández and Alexander (2017) reported that low and high IAA producer of two *B. megaterium*, *P. putida* and *Mesorhizobium ciceri* have been tested on chickpea and shown to have different plant growth promoting potential (dry shoots and roots) than each other. Moreover, it has been reported that while the low IAA producer strains of *B. megaterium* and *P. putida* have no ACC deaminase activity, the high IAA producer strains of *B. megaterium* and *P. putida* have the ACC deaminase activity. On the other site, while the low IAA producer strain of *M. ciceri* has ACC deaminase activity, the high IAA producer strain of *M. ciceri* has no ACC deaminase activity. This means that these traits (IAA and ACC deaminase) are encoded by different genes, and thus, these genes can exist/be upregulated alone or exist/be upregulated at the same time. As *B. megaterium*, *P. fluorescens* strains have also been reported to possess different plant growth promoting activities than each other and enhance plant growth. For example, in the study of Subramanian and Satyan (2014) 144 fluorescent *Pseudomonas* isolates from rhizosphere soil samples have been isolated and then tested for their plant growth promoting abilities such as production of IAA, siderophore, ammonia, hydrogen cyanide, phosphate solubilization, root growth promotion, and biofilm forming abilities, along with two known control strains of *Pseudomonas*. According to their findings among 144 isolates, 100 isolates have biofilm formation, 41 isolates produce siderophore, 84 isolates enhance plant root growth, 44 isolates solubilize phosphate, 38 isolates produce IAA, 52 isolates produce HCN and 100 isolates produce ammonia. In the study of Aponte et al. (2017) five strains of *P. fluorescens* and one of *Azospirillum sp*. strain have been inoculated on seed germination and vegetative development of lettuce (*Lactuca sativa* L.) and shown to have different plant

growth promoting activities than each other. Similarly, the growth of *Brassica napus* inoculated with four plant growth promoting *P. fluorescens* strains [*P. fluorescens* (L228), *P. fluorescens* (L321), *P. fluorescens* (F113), *P. fluorescens* (L111)] has been investigated in a study of Lally et al. (2017). According to their results, these *P. fluorescens* strains have different plant growth activities than each other in both greenhouse and field experiments. In a more recent study, Abdelkerim et al. (2018) showed that twelve bacterial strains including *Rhizobium leguminosarum* (M5), *R. leguminosarum* (M6), *R. leguminosarum* (M12), *R. leguminosarum* (M4), *Sinorhizobium meliloti* (M7), *B. megaterium* (K5), *B. simplex* (K14), *Variovorax sp*. (K17), *Luteibacter sp.* (K20), *P. fluorescens* (K23), *Pseudomonas sp*., *P. fluorescens* (M11) have different level of plant growth promoting activities such as production of IAA, siderophore, HCN and solubilize phosphate and plant biomass on *Lathyrus sativus*.

The results of both *B. megaterium* and *P. fluorescens* strains have been determined to be different than each other in possessing PGPB traits and enhancing plant growth which may arise from their gene(s) regulation. In conclusion, even if both *B. megaterium* and *P. fluorescens* strains have PGPB potentials, the use of proper/better strains in agriculture practice is of vital importance for crop production as their potential can differ even within the same strains. Finally, in the view of this information, the other PGPB bacterial strains should also be investigated in obtaining better results in food production.

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