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Isolation and Diagnosis of Bacillus sp From The Rhizosphere of Halophytic Wild Plants and Evaluation of The Efficiency of Isolates in Dissolving Tri-Calcium Phosphate

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Abstract. *Bacillus* sp was isolated and diagnosed from the rhizosphere of halophytic wild plants (*Schanginia aeguptiace* plant, *Atriplex halimus* plant, and the comparison soil far from the effect of the roots) and during three stages of plants growth (germination stage, vegetative growth stage, stage post flowering), and for three locations that included (Diyala University, Muqdadiya , Baladrooz) during the season of 2020. The diagnosis included studying the phenotypic, microscopic and biochemical features of these isolates and testing their efficiency in dissolving Tri-calcium phosphate $Ca_3(po_4)_2$ as a source of phosphate after growing them in liquid and solid Pikovskayas media and estimating its solubility factor, and its efficiency in changing the degree of the medium reaction. The results of isolation and diagnosis showed the spread of *Bacillus* sp in saline environments, as 7 isolates out of 27 isolates that isolated during the growth stages of different halophytic wild plants were belonged to *Bacillus subtilis*, viz (B1, B5, B6, B7, B11, B15, B18, B19, B22, B23, B26), while the rest of the isolates belonged to *Bacillus megaterium*, whereas the isolates B1 and B2 were recorded the highest solubility factor reached (1.40 and 1.50) respectively, the isolate B26 was able to reduce the pH value of the inoculated medium to 4.81.

Keywords. Bacillus subtilis**,** Bacillus megaterium**,** Schanginia aeguptiace , Atriplex halimus.

I. INTRODUCTION

Bacillus species spread in different environments and generally in soils because of their physiological capabilities that allow them to flourish in ecosystems and compete with other organisms within the environment, also due to their ability to form endospores that are highly resistant to environmental conditions such as heat, humidity, drought and salts [1,2]**.** The genus Bacillus was discovered early by the scientist Ehrenberg in 1835 and called it *Vibrio suptilis*, then it was changed to Bacteridium by Davaine in 1864, but the scientist Cohn proposed in 1872 the term Bacillus as a new nomenclature for this genus [3]. The cells of genus Bacillus are described as slightly curved rod-shaped cells, singly or in pairs or chains, sometimes resembling long filaments, Gram-positive in the early stages of growth, forming endospores that are motile by peritrichous flagella, facultatively aerobic or anaerobic, some of their species resemble anaerobe bacteria [4], do not need complex media for their growth, as they grow on normal media such as Nutrient agar and Pikovskaya medium (1948) that is modified by [5]. The dimensions of its cells range between 0.3-2.2 µm in width and 1.2-7 µm in length, producing the lactase enzyme , and some of its types are positive for the production of oxidase, spread in most environments, water, soil, air, food, plants, animals and medical samples. Its spores are resistant to drought, radiation, antiseptics and heat, due to they contain Dipicolinic acid ranging from 5 to 15% of the dry weight [6], and they are used in agricultural biological applications such as *B.megaterim* and the use of *B.cereus* in the production of polyhydroxybutyrate with industrial and medical uses [7]**.** Others cause food poisoning, such as *B.cereus* and *B.subtilis* [8]**.** [4] indicated during the isolation and diagnosis of *B. subtilis* and testing its efficiency in dissolving Tri-Calcium Phosphate in liquid cultures, as 25 samples were collected from the rhizosphere soil of different plants (barley, tomatoes, alfalfa, wheat, beans and maize), and the results showed the presence of ten isolates, which was diagnosed based on phenotypic, agronomic and biochemical characteristics, it has the ability to dissolve solid Tri-Calcium Phosphate in Nutrient Broth medium, the highest rate of soluble phosphorus for the two best isolates, B11 and B6, was 53.6 and 50.3 mg $p.L^{-1}$, while the values of change in pH after incubation for 24 hours for the isolates were 6.5 and 6.9 respectively, which were superior on the other isolates, [9] showed that species of Bacillus genus have the ability to dissolve calcium phosphate at a rate of 50 µg ml^{-1}, while the amount of phosphorous dissolved by a type of Streptomyces was 29 µg ml^{-1}.

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II.MATERIALS AND METHODS

Twenty-seven samples were collected from the rhizosphere soil of *Schanginia aeguptiace* plant, *Atriplex halimus* plant, and the comparison soil far from the effect of the roots for three locations that included (University of Diyala, Muqdadiya, Baladrooz) and during three stages of growth of halophytic wild plants. The samples were placed in sterile polyethylene bags with alcohol and kept until use. Dilutions were made for each soil sample by adding 10 g of soil samples to 90 ml of sterile distilled water in a beaker 250 ml and mixing well, then sequential dilutions were made 10^{-1} to 10^{-7} by transferring 1 ml of soil suspension to test tubes containing 9 ml of sterile distilled water for each soil sample. Dishes containing solid Pikovskaya agar media and sealed tubes containing Pikovskaya liquid medium were inoculated with 0.1 ml from dilutions 10^{-5}, 10^{-6}, 10^{-7} , then the dishes were incubated at 30 °C for 48 hours, the developing colonies were counted, while the tubes were incubated in the vibrating incubator at the same temperature and for the same period of time in order to be used in biochemical tests [10].

- *Diagnosis of Bacillus spp.*

It relied on the diagnostic diagram of Bacillus, as stated in [11] and as showed in (Figure 1).

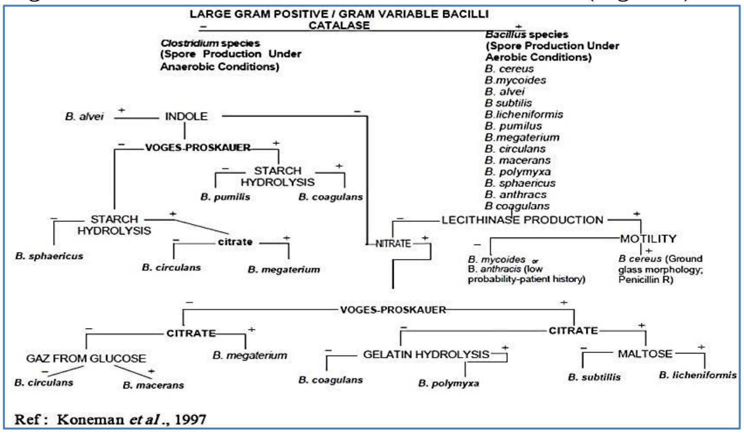

FIGURE 1. Diagnostic diagram of *Bacillus* spp.

- *Phenotypic features*

The phenotypic features of the growing colonies on solid Pikovskaya agar medium were recorded as reported [10].

- *Microscopic features*

A part of a colony was transferred by a sterile loop to a glass slide, then fix it and stain it with gram dye in order to observe the shapes and sizes of cells, the method of their assembly, formation the cover and the interaction with the dye as mentioned by [12].

- *Motility test*

The motility test was carried out after growing *Bacillus* sp on Nutrient Broth by using the hanging drop technique as mentioned in [12].

- *Biochemical tests*
- *Starch hydrolysis test*

Starch hydrolysis medium was prepared from 10 g starch, 3 g beef extract, 12 g agar, 1000 ml sterile distilled water by adjusting pH at 7, and poured in dishes, then inoculated with all bacterial isolates separately and incubated at 32 °C for 48 hours. The starch hydrolysis was observed after adding iodine solution with amount that covered the growing colony, the dishes were left for 30 seconds, then the iodine solution was poured, and the dishes were left for 2-3 minutes to dry. The appearance of a transparent halo around the colony is a positive test result [13]. A dilute iodine solution was prepared in a test of starch hydrolysis by dissolving 2 g of potassium iodide in 300 ml of sterile distilled water.

- *Citrate consumption test*

The dishes containing Simmon citrate medium were inoculated with the different bacterial isolates and incubated at 32°C for 48 hours, then the dishes were checked, where if the color of the medium was changed from green to blue is a positive result of consuming the citrate, as the only carbon source [14].

- *Gelatin liquefaction test*

The gelatin 12% was added to the Nutrient Broth medium and the pH was adjusted at 7.2, and the medium was distributed into test tubes with add 5 ml for each tube, then the tubes were inoculated from the different isolates by stabbing method with bacterial culture, the tubes were incubated at a temperature of 32° C for 7 days, then placed in the refrigerator for an hour, where it was noticed that the liquefaction of gelatin is evidence of bacterial production to gelatinase enzyme and hydrolysis of gelatin [15].

- *Oxidase enzyme test*

The dishes containing Pikovskaya agar medium were inoculated with the different bacterial isolates and incubated at 32° C for 24 hours, then drops of Oxidase reagent were added to the growing colonies, an appearance of the violet color of the colonies is a positive result of this test [16].

- *Catalase test*

A drop of the different isolates was placed on a sterile glass slide and a drop of hydrogen peroxide solution 3% was added. The appearance of air bubbles on the surface of the colony is a positive result of this test [17].

- *Growth test in the saline concentration NaCl 7%*

The concentration 7% of sodium chloride was added to the solid Pikovskaya agar medium and the pH was adjusted at 7, then the dishes were inoculated with the different isolates and the dishes were incubated at 32 °C for 48 hours. The appearance of the colonies is a positive result of tolerance to the level of salinity [16].

- *Acidity tolerance test*

Nutrient Broth medium with pH numbers (5, 7 and 10) was used and distributed in dishes, then were inoculated with the different isolates and incubated at 32°C for 48 hours, the appearance of the colonies is a positive result [13].

- *Growth in temperature 55°C*

The tubes containing nutrient broth medium were inoculated with the different bacterial isolates and incubated at 55°C for 48 hours. The appearance of membranes near the surface of the medium is a positive result of the isolates' ability to grow at high temperatures [18].

- *Carbohydrate fermentation test (glucose and maltose)*

The medium was prepared by dissolving 1 g peptone, 0.1 g Beef extract, 0.5 g NaCl, and 0.0018 g Phenol red in 1000 ml sterile distilled water, and adjusted pH to 7.4, carbohydrates were added separately and sterilized through a fine filter, then added to the medium, the medium was distributed into test tubes with add 5 ml for each tube, then inoculated with the different isolates and incubated at 32° C for 2-3 days. Changing the color of the medium to yellow is an indication of carbohydrate consumption and acid production [16].

- *Testing the efficiency of Bacillus sp isolates in dissolving phosphate in the solid culture medium*

The dishes containing solid Pikovskaya agar medium with $Ca_3(PO4)_2$ as a source of phosphorous were inoculated with the different isolates and incubated at 28° C for 48 hours, the solubility coefficient was estimated by applying the equation proposed by [19] that the appearance of a transparent halo around the colony is evidence of dissolving phosphate.

Solubilization coefficient = (total diameter of the colony + diameter of the transparent halo / diameter of the colony only).

- *Testing the efficiency of Bacillus sp isolates in changing the pH value of the medium*

The liquid Pikovskaya medium was prepared and sterilized with an autoclave device at a temperature of 121 °C for a period of 15 minutes and a pressure of 15 pounds / $inch^2$, the medium was distributed into test tubes and inoculated by adding 1 ml of the different isolates separately, and the tubes were incubated at 32°C for 5 days, the pH of the medium was estimated with the device of a pH meter [18].

III.RESULTS AND DISCUSSION

- *Isolation of Bacillus spp*

The results of the isolation showed that all the soil samples and during the different age stages of the halophyte wild plants were contained *Bacillus* spp. Twenty seven bacterial isolates were obtained, where Table (1) shows the symbol of the isolates and their collection locations, which were isolated during the stages of plant growth.

TABLE 1. The symbol of isolates *Bacillus* sp and their collection locations during the growth stages of halophytes wild plants.

	Isolation symbol	Collection region	Growth stage	Collection location
1	B1	Rhizosphere of *S. aeguptiace*	Germination	Diyala university
2	B2	Rhizosphere of *A. halimus*	Germination	Diyala university
3	B3	A comparison soil	Germination	Diyala university
4	B4	Rhizosphere of *S. aeguptiace*	Germination	Muqdadiya
5	B5	Rhizosphere of *A. halimus*	Germination	Muqdadiya
6	B6	A comparison soil	Germination	Muqdadiya
7	B7	Rhizosphere of *S. aeguptiace*	Germination	Baladrooz
	Isolation	**Collection region**	**Growth stage**	**Collection**

	symbol			location
8	B8	Rhizosphere of *A. halimus*	Germination	Baladrooz
9	B9	A comparison soil	Germination	Baladrooz
10	B10	Rhizosphere of *S. aeguptiace*	Vegetative growth	Diyala university
11	B11	Rhizosphere of *A. halimus*	Vegetative growth	Diyala university
12	B12	A comparison soil	Vegetative growth	Diyala university
13	B13	Rhizosphere of *S. aeguptiace*	Vegetative growth	Muqdadiya
14	B14	Rhizosphere of *A. halimus*	Vegetative growth	Muqdadiya
15	B15	A comparison soil	Vegetative growth	Muqdadiya
16	B16	Rhizosphere of *S. aeguptiace*	Vegetative growth	Baladrooz
17	B17	Rhizosphere of *A. halimus*	Vegetative growth	Baladrooz
18	B18	A comparison soil	Vegetative growth	Baladrooz
19	B19	Rhizosphere of *S. aeguptiace*	Post flowering	Diyala university
20	B20	Rhizosphere of *A. halimus*	Post flowering	Diyala university
21	B21	A comparison soil	Post flowering	Diyala university
22	B22	Rhizosphere of *S. aeguptiace*	Post flowering	Muqdadiya
23	B23	Rhizosphere of *A. halimus*	Post flowering	Muqdadiya
24	B24	A comparison soil	Post flowering	Muqdadiya
25	B25	Rhizosphere of *S. aeguptiace*	Post flowering	Baladrooz
26	B26	Rhizosphere of *A. halimus*	Post flowering	Baladrooz
27	B27	A comparison soil	Post flowering	Baladrooz

- *Diagnosis of Bacillus spp.*

Table (2) showed the phenotypic, microscopic and biochemical characteristics of isolates grown in liquid and solid Pikovskayas media at a temperature of 30 ° C and an incubation period for three days [10], where the colonies were characterized by a gradation of colors from white and transparent white to yellow and creamy yellow, medium to large in size, circular, irregular, with a circular edge, lobed, serrated, slightly convex, smooth and shiny, as shown in Figure (2). The results of the microscopic test showed *Bacillus* spp is rod-shaped, gram-positive, single and some of them are in the form of chains and rarely found in pairs, mobile, forming a transparent halo around the colonies growing on solid Pikovskayas medium containing triple calcium phosphate as a source of phosphate of varying diameters, evidence of the bacteria's ability to dissolve phosphates, as well as their ability to change the pH value of the liquid culture medium pH (7.0) to the acidic medium due to its production of many organic acids. It also has the ability to grow in media with different pH (10, 7, 5), in addition to its growth in the medium containing NaCl 7%, positive for oxidase and catalase test and some of its types are negative for it, and based on what was mentioned, these characteristics correspond with the microscopic and phenotypic characteristics of the genus *Bacillus* spp, and this is consistent with [20,4,7,18].

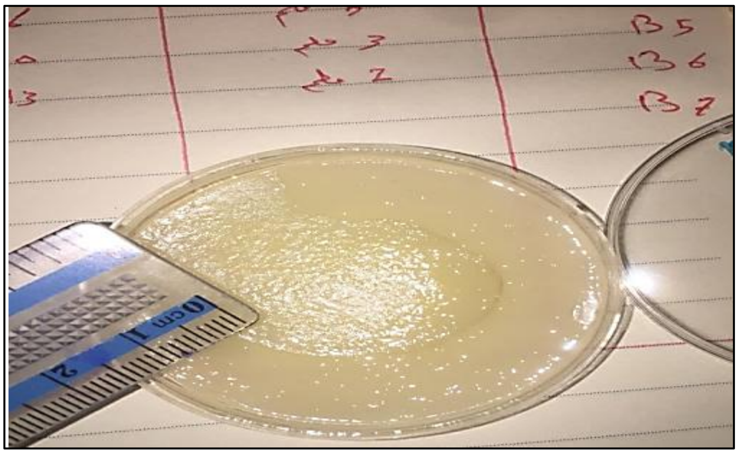

FIGURE 2. *Bacillus* spp colonies in the solid Pikovskayas medium that isolated from the comparison soil during the germination stage.

It relied on the diagnostic diagram shown in Figure (1) in diagnosing *Bacillus* spp as mentioned in [11] and the taxonomic keys in most of the previous studies [21]. The results of Tables (2) showed that the isolates (B1, B5, B6, B7, B11, B15, B18, B19, B22, B23, B26) isolated during the growth stages of halophytes wild plants belong to *Bacillus subtilis*, whose cells were characterized as rod-shaped and gram-positive as shown in Figure (3), non-producing for dyes, it is mobile, positive for citrate, catalase and starch hydrolysis tests, and has the ability to consume glucose, negative for the oxidase test, and it does not have the ability to consume maltose, growing at a temperature of 55 ° C [2], also in the medium containing NaCl 7% and the medium with pH (7) and its inability to grow in the medium with pH (5 and 10), these characteristics correspond with the morphological and biochemical characteristics of *Bacillus subtilis* [4,9,21]. As for the rest of the isolates, they belonged to *Bacillus megaterium*, and this is consistent with [22,7]**,** who confirmed the possibility of isolating this bacterium from the rhizosphere of Iraqi soils, as it is efficient in the tolerance the problems of most the Iraqi soils with high levels of salinity, which affects significantly on the activity of microorganisms in soils [23]**.**

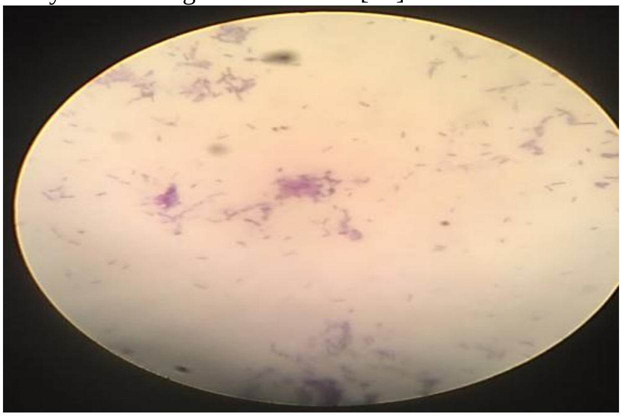

FIGURE 3. *Bacillus* spp at magnification power 40X.

- *Efficiency of Bacillus spp isolates in dissolving tri-calcium phosphate Ca3(Po4)2 and changing the pH of the liquid medium after inoculation*

Table (2) showed the variation in the ability of the isolates to dissolve phosphate and the change in the reaction degree of the liquid medium after incubation, as isolate B2 and isolate B1 recorded the highest solubility coefficient of (1.50, 1.40), respectively, and the pH of the medium changed to (5.08, 5.40) of the two types *B. megaterium* and *B. subtilis*, respectively, and this reflects an inverse relationship between the solubility coefficient (the dissolved amount of phosphate) and the pH value of the medium in which the dissolution occurs. Its efficiency in dissolving phosphate is estimated by the ability of biochemical microorganisms to produce the quantity and quality of secreted organic acids, this was confirmed by [18,4] that *Bacillus* spp produces a different group of organic acids, which works to reduce the pH of the rhizosphere and the most important are lactic acid, succinic acid and citric acid. Also, there was a clear variation between the isolates in the values of the solubilization coefficient and pH values of medium after incubation, as the isolate B26, belonging to the species *B. subtilis*, gave a solubilization coefficient of 1.34 and reduced pH value to 4.81, this variation between organisms can be attributed to their genetic difference in the coding for the production of different organic acids, especially lactic acid and 2-ketogluconic acid, which proved their effectiveness in reducing the value of the degree of reaction of the medium and the dissolution of phosphates. The dissolution of phosphate by these types of bacteria, which made them a candidate to be one of the most important growth-promoting organisms and as an effective bio-fertilizer to improve plant nutrition with

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phosphorous and as a vital pollinator to increase the availability of phosphorous in the soil and to reduce the addition of mineral fertilizers, which reduces the impact of pollution and costs of agriculture [21,23].

IV.CONCLUSION

The findings of isolation and diagnosis showed the spread of Bacillus sp in the Iraqi saline environments that belonged to *Bacillus subtilis* and *Bacillus megaterium*, which they proved their effectiveness in dissolving phosphate and changing the pH of the liquid medium.

TABLE 2. Some phenotypic, microscopic and biochemical characteristics of *Bacillus* spp.

Isolation number	Microscopic features			Phenotypic features		Carbon consumption			Biochemical tests								Ph medium after inoculation	Solubilization coefficient	
	Gram dye	Motility	Cell shape	Colony color	Colony shape	Starch hydrolysis	Maltos	Glucose	oxidase	catalase	Citrate	pH10	pH7	pH5	55 °C	NaCl 7%			
B1	+	+	Rod	White	Circular	+	-	+	-	+	-	-	+	-	+	+	5.40	1.40	*B.su*
B2	+	+	Rod	Brown	Lobed	+	-	-	+	-	+	+	+	+	-	+	5.08	1.50	*B.me*
B3	+	+	Rod	Brown	Spherical	+	-	-	+	-	+	+	+	+	-	+	5.25	1.28	*B.me*
B4	+	+	Rod	White	Circular	+	-	-	+	+	+	+	+	+	-	+	5.18	1.37	*B.me*
B5	+	+	Rod	Brown	Spherical	+	-	-	-	+	+	+	+	-	+	+	5.51	1.30	*B.su*
B6	+	+	Rod	Brown	Circular	+	-	-	-	-	+	+	+	-	+	+	5.26	1.33	*B.su*
B7	+	+	Rod	White	Spherical	+	-	-	-	+	+	+	+	-	+	+	5.05	1.23	*B.su*
B8	+	+	Rod	Brown	Serrated	+	-	+	+	-	+	+	+	+	-	+	5.51	1.22	*B.me*
B9	+	+	Rod	White	Spherical	+	-	-	+	+	+	+	+	+	-	+	5.40	1.33	*B.me*
B10	+	+	Rod	Yellow	Circular	+	-	+	+	+	+	+	+	-	-	+	5.61	1.25	*B.me*
B11	+	+	Rod	White	Circular	+	-	-	+	+	+	-	+	-	+	+	5.12	1.23	*B.su*
B12	+	+	Rod	Yellow	Spherical	+	-	+	+	+	+	+	+	+	-	+	5.23	1.26	*B.me*
B13	+	+	Rod	Yellow	Convex	+	-	-	+	+	+	+	+	+	-	+	4.90	1.31	*B.me*
B14	+	+	Rod	White	Serrated	+	-	-	+	+	+	+	+	-	-	+	4.91	1.26	*B.me*
B15	+	+	Rod	Yellow	Convex	+	-	-	+	+	+	-	+	-	+	+	5.09	1.15	*B.su*
B16	+	+	Rod	Yellow	Flat	+	-	+	+	+	+	+	+	+	-	+	5.54	1.09	*B.me*
B17	+	+	Rod	Yellow	Spherical	+	-	+	+	+	+	+	+	+	-	+	5.03	1.22	*B.me*
B18	+	+	Rod	White	Circular	+	-	-	+	+	+	-	+	+	+	+	5.45	1.10	*B.su*
B19	+	+	Rod	White	Convex	+	-	+	-	+	+	+	+	-	+	+	5.65	1.13	*B.su*
B20	+	+	Rod	Yellow	Convex	+	-	-	+	+	+	+	+	+	-	+	5.60	1.188	*B.me*
B21	+	+	Rod	White	Lobed	+	-	+	+	+	+	-	+	+	-	+	5.53	1.25	*B.me*
B22	+	+	Rod	Brown	Serrated	+	-	+	-	+	+	-	+	-	+	+	5.71	1.28	*B.su*
B23	+	+	Rod	Yellow	Serrated	+	-	+	-	+	+	+	+	-	+	+	5.47	1.22	*B.su*
B24	+	+	Rod	Yellow	Convex	+	-	-	+	+	+	+	+	+	-	+	5.35	1.16	*B.me*

B25	+	+	Rod	Yellow	Lobed	+	-	-	-	+	+	-	+	+	-	+	5.48	1.20	*B.me*
B26	+	+	Rod	Brown	Serrated	+	-	+	-	+	+	+	+	-	+	+	4.81	1.34	*B.su*
B27	+	+	Rod	Yellow	Convex	+	-	-	+	+	+	+	+	+	-	+	5.63	1.21	*B.me*

B.su = Bacillus subtilis, B.me= Bacillus megaterium

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