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EFFECT OF CULTURE CONDITIONS ON NITROGEN-FIXING ACTIVITY OF BACTERIA ISOLATED FROM CASSAVA CULTIVATED SOILS OF VIETNAM

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ABSTRACT

The bacteria capable of fixing atmospheric nitrogen were isolated from cassava cultivated soils of Vietnam. The potential isolates were identified by analyzing the 16S rRNA gene and by morphological, biochemical, cultural characteristics. The selected isolates were assigned to the species *Bacillus* sp. DQT2 M17, *Bacillus subtilis* DTAN6 M17, and *Bacillus megaterium* DSHB I8. The effect of culture conditions on the nitrogen-fixing activity of three selected isolates were studied and the obtained results showed that the highest amount of accumulated ammonia was detected after 6 days of incubation at 35 ^{o}C, pH 7.0 with sucrose as a carbon source. The selected strains could be exploited as inoculants for microbial fertilizer production.

Keywords: Nitrogen-fixing, nitrogen, nitrogenase, biological nitrogen fixation.

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INTRODUCTION

In agroecosystems, agricultural productivity is often limited by available soil nutrients, especially nitrogen, because nitrogen is one of the major nutrients limiting plant growth. Although nitrogen is the most abundant gas in Earth's atmosphere, it is extremely unreactive and is not present in soil (Mahmud et al., 2020). Nitrogen is an essential element that affects the yields of crops by influencing leaf area development and photosynthetic efficiency (Fang et al., 2018; Hedin et al., 2009). The plant's nitrogen input and crop productivity largely depend on synthetic fertilizer applications and biological nitrogen fixation (BNF) (Vitousek et al., 2013). Nitrogenous fertilizer production currently represents a significant expense for the efficient growth of various crops in the developed world (Santi et al., 2013). More than 50% of the applied nitrogen-based fertilizer is used by the plants and the remaining can be subjected to losses like surface runoff and leaching, leading to nitrate contamination of soils and groundwater. In terms of energy efficiency, moreover, manufacturing nitrogen-based fertilizers requires six times more energy than that needed to produce either phosphorous or potassium-based fertilizers (Da et al., 1978). Therefore, reducing dependence on nitrogenous fertilizers in agriculture in the developed world and developing countries may lead to potential gains in an agricultural setting. Biological nitrogen fixation (BNF) in economically important food and forage crops (Sulieman et al., 2016) has drawn attention to achieve sustainable agricultural goals of the world (Lindström et al., 2010). I would mention more about the cassava plant and the soils for cultivating this plant. The biological nitrogen fixation (BNF) process transforms atmospheric nitrogen into the forms available for plants through nitrogen-fixing microorganisms (Singh et al., 2015). The nitrogen-fixation process is catalyzed by a complex of enzymes known as nitrogenase complex (Choudhary & Varma, 2017). Structurally, the nitrogen-fixing system has variations among different bacterial genera. Most BNF is catalyzed by the activity of molybdenum nitrogenase, which is found in all diazotrophs (Mus et al., 2018). The genes responsible for nitrogen fixation called nif genes are found in both symbiotic and free-living nitrogen-fixing microorganisms. In a symbiotic nitrogen fixation system, plant growth-promoting rhizobacteria (PGPR) colonizing internal tissues of the plant provide a limiting oxygen environment for activation of nif genes and transfer the fixed nitrogen to the host plants (Wongdee et al., 2018; Nyoki & Ndakidemi, 2018). BNF represents an alternative to chemical fertilizers due to its economic and environmental advantages. In this study, the culture conditions of some selected nitrogen-fixing bacterial strains were optimized to improve their nitrogen fixation capacity for their further application in microbial fertilizer production. I would introduce more about specific nitrogen-fixation bacteria and their application in biofertilizer production.

MATERIALS AND METHODS

Isolation and characterization of nitrogen-fixing bacteria

The bacteria capable of fixing atmospheric nitrogen were isolated on a nitrogen-free medium and 3 isolates with higher nitrogen-fixation activity were selected. The morphology, color, and size of the colonies on Burk's solid medium (sucrose 20.0 g, K_2HPO_4 0.64 g, KH_2PO_4 0.16 g, $MgSO_4.7H_2O$ 0.20 g, NaCl 0.20 g, $CaSO_4.2H_2O$ 0.05 g, $Na_2MoO_4.2H_2O$ (0.05%) 5.0 ml, $FeSO_4.7H_2O$ (0.3%) 5.0 ml, 15 g agar in 1000 ml, pH=7) were recorded. The selected isolates were identified based on the analysis of morphological characteristics (Bacterial colonies, Gram stain) and 16S rRNA sequences. The effects

of temperature, pH, carbon sources and incubation time on the growth and nitrogen fixation capacity of the three selected strains were surveyed.

Effect of the cultural temperature on the nitrogen-fixing activity

Three selected isolates were grown in Burk's broth, shaken at 150 rpm at temperatures ranging from 25−40 ºC, with pH ranging from 7.0 to 7.2. The concentration of ammonia produced in the medium was determined by the colourimetric method using Nessler's reagent at the wavelength of 630 nm (Page et al., 1982).

Effect of incubation time on the nitrogen-fixing activity

To test the effect of incubation time on the nitrogen-fixing capacity of bacteria, the bacteria were cultured in liquid Burk's medium, shaking at 150 rpm, with the optimal temperature, pH, and carbon source. Samples were taken at intervals every 24 hours. The concentration of produced ammonia in the medium was calculated by the colourimetric method using Nessler's reagent at 630 nm.

Effect of the cultural pH

The influence of the medium's pH on the nitrogen-fixing activity of bacteria was studied by inoculating the bacteria in Burk's broth, shaking at 150 rpm, optimal temperatures and pH of the media was ranging from 6.0 to 8.0. The concentration of ammonia produced in the medium was calculated by the colourimetric method using Nessler's reagent at 630 nm after 6 days of incubation.

Effect of the cultural carbon sourceson nitrogen-fixing activity

The bacteria were cultured in liquid Burk's medium with neutral pH (7.0 to 7.2), shaking at 150 rpm, optimal temperatures. Different carbon sources such as glucose, sucrose, maltose, and mannitol were added to the culture media at a concentration 20 g/L. The concentration of produced ammonia was determined by the colourimetric method using Nessler's reagent at the wavelength of 630 nm after 6 days of incubation.

Identification of selected isolates

Primers 27F (5'-TAACACATGCAAG TCGAACG-3') and 1492R (5'-GGTTACC TTGTTACGACTT-3') (Tran et al., 2017) are used to amplify the 16S rRNA sequences from the DNA of the three selected bacterial strains. Aliquots (10 µl) of PCR products were analyzed on 1% agarose gel using standard electrophoresis procedures. 16S rRNA gene of selected isolates was sequenced by the 1st BASE company (Malaysia). Finally, 16S rRNA gene sequences of the investigated bacteria were compared to these quences from GenBank to ascertain the relationship between strains (Tamura et al., 2011), and phylogenetic trees were thereby constructed by the neighbour-joining method using MEGA software version 6.06 based on 1000 bootstraps.

RESULTS

Identification of 3 selected isolates

Nitrogen-fixing bacteria can live and grow in a nitrogen-free medium. From the cassava cultivated soil samples the collected in the three regions of Vietnam (the North of Phu Tho province, the South of Tay Ninh province and the Central region of Quang Tri province), 17 strains of nitrogen-fixing microorganisms were isolated on Burk's medium lacking a nitrogen source. Among them, three isolates with the highest nitrogen fixation activity were selected. The colonise of the isolates are a whitish cream colour, smooth, irregular, and shiny. The cells of the isolates were Gram-positive (Fig. 1). The selected isolates were identified to be genus *Bacillus* based on the analysis of the 16S rRNA sequences. The selected isolates were assigned to *Bacillus sp*. DQT2 M17, *Bacillus subtilis* DTAN6 M17, *Bacillus megaterium* DSHB I8.

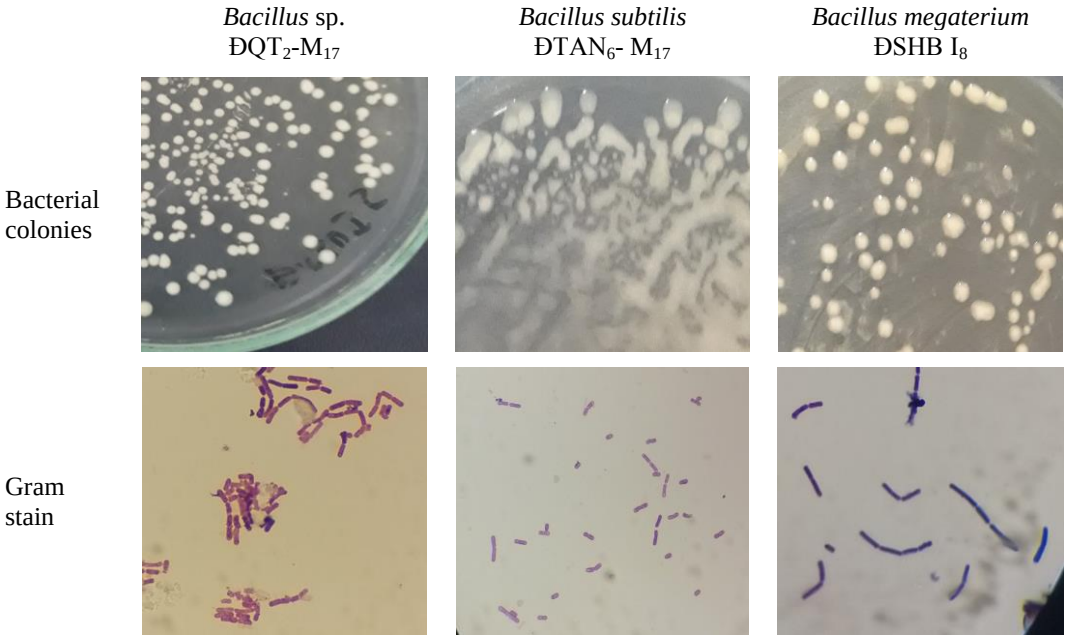

Figure 1. Morphological characteristics of three isolates

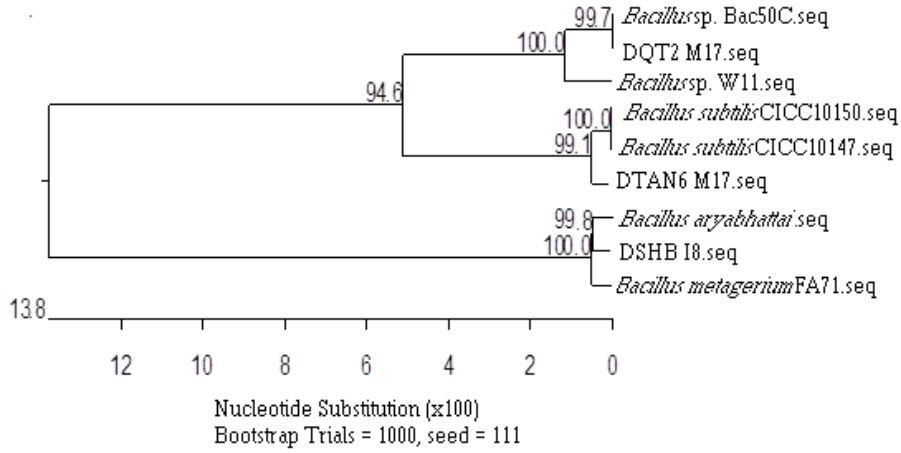

Figure 2. The phylogenetic tree showing the relative position of three strains using the neighbor-joining method and the complete 16S rRNA sequence

Effect of the temperatures on nitrogen fixation

Using the colourimetric method at 630 nm with Nessler's reagent, the nitrogen-fixation capacity of the selected isolates was evaluated. The ammonium concentration in the culture medium at different culture temperatures was calculated based on the standard curve (Fig. 3). The results show that the three strains can fix nitrogen at temperatures between 20 ºC and 40 ºC. These strains have the highest nitrogen-fixation capacity at a temperature 30 ºC. The nitrogen-fixation ability of the investigated bacteria ranged from 0.56 mg/L to 3.83 mg/L. The *Bacillus megaterium* DSHB I8 strain could fix the highest amount of nitrogen (3.83 mg/L); the *Bacillus* sp. DQT2 M17 strain fixed the least (2.77 mg/L) and *Bacillus subtilis* DTAN6 M17 (2.98 mg/L) (Fig. 3).

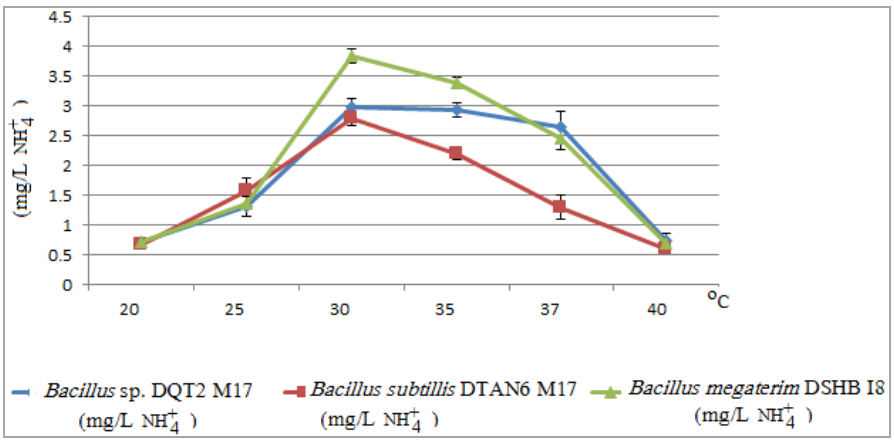

Figure 3. Effect of the temperatures on nitrogen-fixation activity of selected isolates

Effect of the pH medium on nitrogen fixation

One of the most important parameters for the growth and metabolic activity of nitrogen-fixing organisms is pH. In our investigation, the maximum ammonium production was observed at pH 7.0 (Fig. 4). *Bacillus* sp. among other rhizobacteria such as Azospirillum, Azotobacter and Paenibacillus are some of the symbiotic nitrogen fixers in plant rhizospheres (Ahemad & Kibret, 2014; Goswami et al., 2015). The nitrogen-fixing *B. megaterium* has previously also been isolated from the rhizosphere of maize (Liu et al., 2006). Maximum ammonium production of the *Bacillus megaterium* DSHB I8 was observed at pH 7.0 (3.8 mg/L). *Bacillus subtilis* DTAN6 M17 and *Bacillus* sp. DQT2 M17 produced 3.51 mg/L and 2.94 mg/L ammonium at this pH point.

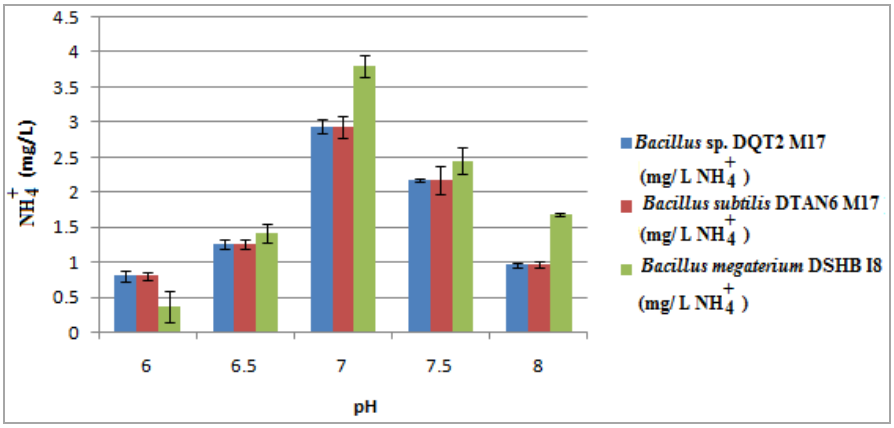

Figure 4. Effect of the pH on nitrogen fixation activity of selected isolates

Effect of incubation time on nitrogen fixation

The three bacterial strains were cultured in Burk's liquid medium, shaken at 150 rpm, at 30 °C. The ammonium production was measured after 48 hours interval of incubation (Fig. 5). The obtained result suggests that ammonium production depends on culture time. The ammonium production increased with prolonged culture time and reached maximum at day 6, then gradually decreased (Fig. 5). *Bacillus megaterium* DSHB I8 produced 4.76 mg/L ammonium, *Bacillus subtilis* DTAN6 M17

produced 4.58 mg/L and *Bacillus* sp. DQT2 M17 produced 3.27 mg/L ammonium after 6 days of incubation. After 8 days of incubation, *Bacillus megaterium* DSHB I8 produced 3.85 mg/L, *Bacillus subtilis* DTAN6 M17 produced 3.66 mg/L, and *Bacillus* sp. DQT2 M17 produced 2.92 mg/L ammonium.

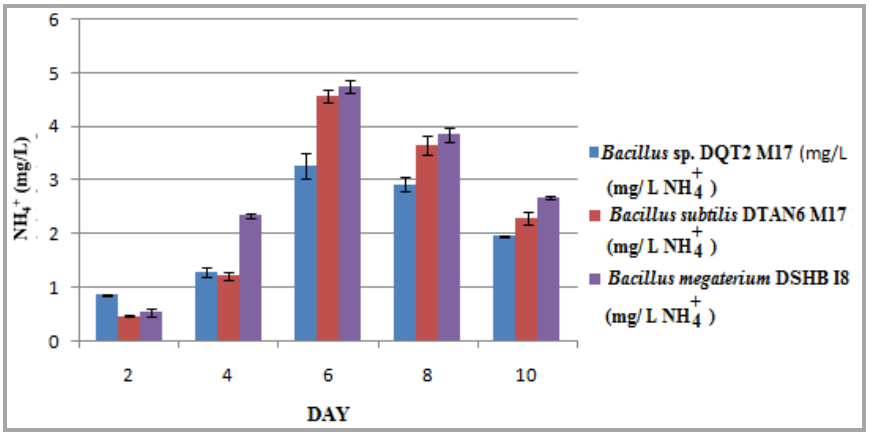

Figure 5. Effect of incubation time on nitrogen fixation activity of selected isolates

Effect of carbon Source on nitrogen fixation

The three selected strains *Bacillus* sp. DQT2 M17, *Bacillus subtilis* DTAN6 M17, *Bacillus megaterium* DSHB I8 were cultured in Burk's liquid medium at 30 ºC, neutral pH, and four different sugars (glucose, sucrose, maltose, and mannitol) were added externally in the production medium in order to evaluate their effect on nitrogen fixation activity of the strains. Sucrose at concentration 2% was found to be the best carbon source for selected isolates to fix nitrogen. The *Bacillus megaterium* DSHB I8 produced 6.83 ± 0.13 mg/L ammonium, *Bacillus subtilis* DTAN6 M17 produced 6.58 ± 0.126 mg/L and *Bacillus* sp. DQT2 M17 produced 6.38 ± 0.098 mg/L ammonium when sucrose was used, followed by glucose (produced 5.85 ± 0.135 mg/L; 5.78 ± 0.09 mg/L, and 5.58 ± 0.45 mg/L ammonium, respectively). Contrary, mannitol exhibited a negative effect in nitrogen fixation activity of the strains, *Bacillus megaterium* DSHB I8 produced 4.7 ± 0.05 mg/L ammonium, *Bacillus subtilis* DTAN6 M17 produced 4.46 ± 0.18 mg/L ammonium and *Bacillus* sp. DQT2 M17 produced 4.89 ± 0.078 mg/L (Fig. 6).

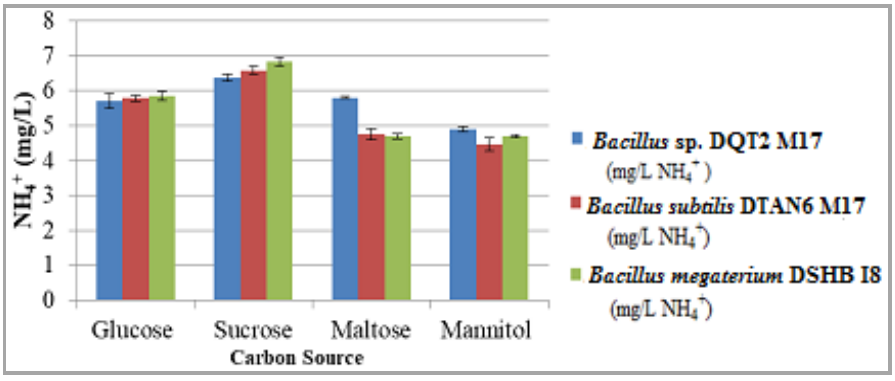

Figure 6. Effect of carbon source on nitrogen fixation activity of selected isolates

DISCUSSION

More than 80% of nitrogen in the atmosphere is inert gas which is not available to plants (Patel & Minocheherhomji, 2018). To supply this important nutrient to plants, nitrogenous fertilizers are often applied during crop production. Recent reports indicate that less than half of applied nitrogen is effectively absorbed by plants with the rest being lost through volatilization or leaching, resulting in environmental pollution (Le Mire et al., 2016). For instance, nitrous oxide (N_2O) which is one of the gases evolved during the application of nitrogenous fertilizers is one of the most important greenhouse gases (Adesemoye et al., 2009). These problems can be adequately solved by exploiting the biological nitrogen-fixing microorganisms (Calvo et al., 2014). Biological nitrogen fixation (BNF) is the process through which atmospheric nitrogen is reduced to ammonia which can be taken up by plants (Gothwal et al., 2007). *Bacillus* sp. among other rhizobacteria such as *Azospirillum*, *Azotobacter*, and *Paenibacillus* are some of the symbiotic nitrogen fixers in plant rhizospheres (Ahemad & Kibret, 2014; Goswami et al., 2015). In a study by Ding et al. (2005) investigating nitrogen-fixing strains from plant rhizospheres in the Beijing region, the presence of nitrogen-fixing genes in *Bacillus* was reported. The nitrogen-fixing B. *megaterium* has previously also been isolated from the rhizosphere of maize (Liu et al., 2006). *B.subtilis* UPMB10 is also reported to have the capacity to fix nitrogen (Gouda et al., 2018). Older studies also reported the nitrogen-fixing ability of *Bacillus* spp. including *B.megaterium*, *B.cereus*, *B.pumilus*, *B.circulans*, *B.licheniformis*, *B.subtilis*, *B.brevis* and *B.firmus* which contain nitrogenase activities (Xie et al., 1998). Three bacterial strains capable of fixing nitrogen were isolated from cassava cultivated soil of Vietnam, *Bacillus* sp. DQT2 M17, *Bacillus subtilis* DTAN6 M17, *Bacillus megaterium* DSHB I8, exhibited the highest nitrogen fixation activity among other isolates. Interestingly, quite recent reports indicate that some Bacilli rhizobacteria can be involved in symbiotic nitrogen fixation (Bhattacharyya & Jha, 2012; Ikeda et al., 2013). Szilagyi-Zecchin et al. (2014), also report on three endophytic *Bacillus* spp. isolated from corn roots with nitrogen-fixing capacity, evaluating through acetylene reduction assay and identification of nitrogen fixation genes. All three investigated strains showed the highest nitrogen fixation activity after 6 days of incubation and with a prolonged incubation period, nitrogen fixation started to decrease. This may be due to the decrease in the growth of the isolate. Most of the studies reported that the highest nitrogen fixation enzyme production was between 4 days and 6 days of incubation. In this study, three bacterial strains were revealed to have maximal nitrogen fixation activity at pH 7.0. The supplement of carbon sources in either monosaccharide or polysaccharide form may induce nitrogen fixation activity. In our present study, the influence of sucrose was more positive than the other carbon sources tested. Glucose was the second-best supplementary carbon source. Mannitol induced the lowest nitrogen-fixing activity. The obtained result showed that the different carbon source differently influences the nitrogen fixation activity of the investigated bacteria. Most symbiotic nitrogen fixation, even Bacilli rhizobacteria, has been reported in leguminous plants; it will also be immensely important to investigate the ability of Bacilli rhizobacteria to symbiotically fix nitrogen in non-leguminous plants (Gouda et al., 2018). Nitrogen fixation is an important trait of PGPRs as it directly provides nitrogen to the plant and nitrogen-fixing rhizobacteria have been marketed as biofertilizers for over 20 years (Goswami et al., 2015). For instance, co-inoculation of *Azospirillum lipoferum* and *B. megaterium* was reported to improve both nitrogen and phosphorus nutrition in wheat plants (El-Komy, 2005). The culture conditions for our nitrogen-fixing isolates

were conducted and these strains will be promising candidates for application in biofertilizer production and in improving soil quality and cassava yield.

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