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Isolation and characterization of plant growth promoting rhizobacteria from rhizospheric soil of selected pulses and their effect on *Coriandrum sativum* plants

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Abstract. *Pirapak W, Siriphap A, Inprasit T, Phuangsri C, Kraivuttinun P. 2022. Isolation and characterization of plant growth promoting rhizobacteria from rhizospheric soil of selected pulses and their effect on* Coriandrum sativum *plants. Biodiversitas 23: 5765-5770.* Plant growth-promoting bacteria (PGPB) are recognized as efficient biofertilizers, biocontrol agents and microbial inoculants. In the present study five best plant growth-promoting rhizobacteria were isolated from the root zone of healthy and vigorously growing plants in an agricultural farm in the Uttaradit Province, Thailand. All the isolates were selected on the basis of nitrogen fixation, indole-3-acetic acid (IAA) production and phosphate solubilization. Based on morphological traits and 16S rRNA sequence analysis, isolated bacteria were distributed into the following six genera: *Burkholderia cepacian* (A05, A06), *Agrobacterium deltaense* (A13), *Pseudomonas plecoglossicida* (A39), *Paenarthrobacter aurescens* (A45) and *Stenotrophomonas pavanii* (A64). All the selected bacterial isolates boosted coriander growth. *Paenarthrobacter aurescens* (A45) showed the best results, it increased plant height (up to 1.38 times), dry weight (up to 1.63 times), stem diameter (up to 2.05 times) and biomass (up to 1.65 times). This is the first report of plant growth-promoting ability of *Paenarthrobacter aurescens*, which could be used as a biological agent instead of chemical fertilizers in agroforestry production to reduce environmental pollution and increase the yield of coriander.

Keywords: *Coriandrum sativum*, nitrogen-fixing bacteria, *Paenarthrobacter aurescens*, plant growth-promoting bacteria, phosphate solubilizing bacteria

INTRODUCTION

The agricultural sector is very important in Thailand. However, most farming processes use many chemicals and pesticides, resulting in a deterioration of the soil quality. Soil is a unity of subsistence that includes a variety of microorganisms that are important to the composition of soil particles. The type and activity of these microorganisms influence the physical and chemical properties of soil, climate and vegetation (Jha et al. 1992). Apart from microorganisms, soil contains plants and animals of many species, which form a complex relationship.

In the rhizosphere, found around the roots of plants, a group of metabolically versatile microorganisms have been observed. Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria that are beneficial to plants (Triwidodo and Listihani 2020; Agustiyani et al. 2021). Microbial inoculants or biofertilizers are used to improve the availability of plant nutrients by fixing atmospheric nitrogen and rendering insoluble phosphate soluble, thus increasing biological activity (Mehta et al. 2015). Plants need many essential nutrients for growth. Two of these are nitrogen and phosphate, both of which are important nutrients that affect plant growth and metabolism. They are a limited natural resource, something which is gradually being recognized as a new challenge for global sustainable development (Chen et al. 2021). Insufficient available phosphorus is available to meet the plant's need for growth (Hedley and Nye 1982). The subtropical forest soil in particular is severely deficient in phosphorus (Kizilkaya 2009), which greatly limits the development and productivity of plants. About 5.23 ten billion tonnes of phosphorus fertilizer are used annually to maintain soil fertility. However, the recovery of P fertilizer from the soil is quite low (15-20%), and residual P is fixed in soil. Therefore, improving the absorption and use of P by crops is of great significance from ecological (Chen et al. 2021). Nitrogen is an important plant nutrient. Every year, hundreds of millions tonnes of nitrogen are removed from the soil by crop harvesting. Humans return more than 40% of the lost nitrogen to the soil by fertilization; the remaining nitrogen deficit is replenished by the activity of microorganisms (Ha and Chu 2020).

A group of nitrogen-fixing bacteria (NFB), such as *Azotobacter*, *Azospirillum* and *Rhizobium*, are commonly found in soil and play an important role in agricultural production. The free-living nitrogen-fixing bacteria group *Azotobacter* not only provides nitrogen nutrients but also stimulates germination by producing plant growth stimulants; the commensal nitrogen-fixing bacteria group *Azospirillum* is found on the roots of herbaceous plants, cotton and vegetables. A symbiotic-living nitrogen-fixing bacteria group, *Rhizobium*, lives in the roots of legumes and creates nodules. Rhizobium group plays the most important and well-known role in N_2 fixation. The presence of nitrogen-fixing bacteria in soil is considered to be an indicator for assessing soil quality (Kizilkaya 2009).

Phosphate solubilizing bacteria (PSB) are a group of microorganisms capable of solubilizing insoluble phosphate, fixing nitrogen and secreting auxin, which promotes plant growth. In recent years, research on PSB has mainly focused on plant growth. It has been observed that inoculation of seeds or soil with phosphate solubilizing bacteria improves the solubilization of fixed soil phosphorus and applied phosphates, boosting plant performance (Chen et al. 2021). In this study, nitrogen-fixing bacteria and phosphate solubilizing bacteria were isolated from the root zone of healthy and vigorously growing plants in an agricultural farm in the Uttaradit Province, Thailand. Plants were also screened for their ability to nitrogen fixation, indole-3-acetic acid (IAA) production and phosphate solubilization. The most promising strains were used to assess growth promotion in *Coriandrum sativum* L. plants *in vitro*.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from the root zone of healthy and vigorously growing plants in an agricultural farm in the Uttaradit Province, Thailand (sea level +63 m, latitude: 17°37′33″N and longitude: 100°5′48″E). Soil samples were collected from the root zone of Thai jasmine rice, sunn hemp and mung bean at a depth of about 20 cm using an auger (Yanai et al. 2003).

Isolation and screening of nitrogen-fixing bacteria and indole-3-acetic acid (IAA) production

Ten grams of each soil sample was suspended in 90 mL of sterile water, mixed thoroughly, and allowed to settle. The samples were then diluted to achieve appropriate dilutions (10^{-2}, 10^{-3}, 10^{-4}) by serial dilution technique. Next, samples were spread on Norris Glucose Nitrogen Free Medium (NFM) containing10 g dextrose (glucose), 1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 0.005 g Na_2MoO_4, 0.1 g $FeSO_4.7H_2O$, 2 ml 0.5 % bromothymol blue (BTB) and 15 g agar (pH 7.0) per liter) (HiMedia) and incubated at 30°C for 7 days. The strains that changed the color of BTB-containing medium to blue were found to be nitrogen fixers, as the color change indicates excretion of ammonia, increasing the pH of the medium (Latt et al. 2018). Indole-3-acetic acid (IAA) production was measured by colorimetric assay. Bacterial isolates were cultured for 3 days in tryptone yeast (TY) broth (without L-tryptophan or supplemented with 500 µg/mL of l-tryptophan) in the dark at 30°C. Bacterial cells of all isolates were transferred from the culture medium by centrifugation at 13,000 rpm for 10 min and then, 1 mL of supernatant was mixed vigorously with 2 mL of Salkowski’s reagent (4.5 g of $FeCl_3$ per L in 10.8 M H_2SO_4). Samples were incubated for 30 min at room temperature and optical density of IAA was estimated at 600 nm by comparison with a standard curve prepared from known concentrations of IAA (Chen et al. 2021).

Isolation and screening of phosphate solubilizing bacteria

All the selected isolates were screened for their phosphate solubilizing ability by culturing at 30°C on Pikovskaya (PVK) solid medium. A spot inoculation procedure was used for screening the phosphate solubilizing bacteria. After 7 days, colonies showing a clear phosphate solubilizing zone were selected for further characterization and the size of phosphate solubilizing zone was determined for each colony. The diameter of halo zones was used to calculate the solubilization index (SI) using the following formula (Nathiya et al. 2020):

Solubilization Index (SI) = (colony diameter + halo zone diameter) / colony diameter

Effect of PGPB on the growth of *Coriandrum sativum*

Based on the solubilizing ability of bacterial isolates, six isolates with a strong P solubilizing ability (A05, A06, A13, A39, A45 and A64) and IAA production were selected to test their effect on the growth of coriander (*Coriandrum sativum* L.). A pot experiment was conducted in a greenhouse that included the following 7 treatments: control: no bacterial isolate (CK), A1: isolate A05, A2: isolate A06, A3: isolate A13, A4: isolate A39, A5: isolate A45, and A6: isolate A64. Each treatment was performed four times. 42-day-old seedlings of coriander were selected from a nursery according to plant stem diameter and height and grown in the pots. Bacterial isolates were transferred to flasks containing 100 mL of meat broth and then incubated at 30°C on a shaker at 180 rpm for 18 h. The bacterial suspension was centrifuged and washed five times with sterile distilled water to minimize the effect of culture medium. A 10 mL bacterial suspension (10^8 CFU mL^{-1}) was applied to coriander roots. At the same time, 10 mL sterile distilled water was inoculated to the control treatment.

Bacterial identification based on 16S rDNA gene sequence analysis

The partial 16S rDNA gene sequences of the six isolates were obtained by the Thailand Institute of Scientific and Technological Research. The sequences were first affiliated to bacterial taxa using the GenBank database. An OUT based taxonomic assignment of sequences was further performed by clustering sequences at 5% partial 16S rDNA gene dissimilarity level. The sequences were submitted to the GenBank with accession number.

Statistical analysis

Statistical analysis of the data was performed by using one-way ANOVA (difference among treatments) and Tukey's HSD (to compare means).

RESULTS AND DISCUSSION

Isolation of free nitrogen fixing bacteria and IAA production

Free-living nitrogen-fixing bacteria)NFB(were isolated from twenty-three soil sources from an agricultural farm in the Uttaradit Province, Thailand. Seventy strains were detected on Norris Glucose Nitrogen Free Medium. Sixty-four strains of putative nitrogen-fixing bacteria showed color changes in BTB containing medium and all strains produce IAA. The IAA concentration ranged from 0.39 to 50.44 µg/mL at 600 nm by comparison with a standard curve prepared from known concentrations of IAA (Figure 1). Strains A39, A64 and A45 showed the highest IAA production, with 50.44, 49.69 and 47.90 µg/mL, respectively.

Isolation and screening of phosphate solubilizing bacteria

All 64 nitrogen-fixing strains were spot-inoculated on Pikovskaya solid medium, with 23 isolates showed phosphate solubilizing activity. The Pikovskaya solid medium showed phosphate solubilization around the colonies)Figure 2(with a solubilization index (SI) ranging from 1.4 to 7.6 (Figure 3). Strains A05, A06 and A13 showed the highest solubilization index (SI) i.e. 7.6, 7.6 and 3.6, respectively.

Promotional effects of PGPB on *Coriandrum sativum* growth

The six bacterial strains exhibiting the most promising free-nitrogen fixation, indole 3-acetic acid production and phosphate solubilization (A05, A06, A13, A39, A45 and A64) were further examined for their growth-promoting activity in coriander (*Coriandrum sativum* L.). Selected bacterial isolates affected the development of coriander root system and stem integrity. Inoculation with PGPB significantly promoted coriander growth (Figure 4) as compared to uninoculated plants used as a control (CK). Different strains produced significantly different increases in plant height ($p < 0.05$). The values of dry weight, root length and ground diameter were significantly higher than the control seedlings in all treatments. Among inoculated seedlings, A4 seedlings exhibited highest increase in plant height (27.67 cm), which was about 1.38 times compared to the control group. A2 and A5 seedlings increased plant height by 1.32-fold and 1.31-fold, respectively, as compared to control seedlings. Moreover, different treatments showed different growth-promoting effects on the dry weight of coriander seedlings ($p < 0.05$). A4 seedlings showed the highest increase in dry weight (1.44 g), with a 1.63-fold increase compared to the control group. Furthermore, root length of A4 and A2 seedlings increased significantly ($p < 0.05$) compared to the control group, with a 1.47-fold and 1.41-fold increase, respectively, compared to the control group. A4 seedlings had the largest ground diameter, with an increase of 2.05-fold compared to the control group (Figure 4).

Bacterial identification based on 16S rDNA gene sequence analysis

The nucleotide sequences of 16S rRNA genes of the six selected strains (A05, A06, A13, A39, A45 and A64), approximately 1350 bp long, were amplified by PCR and sequenced. These sequences were compared by BLAST with those in the GenBank and EzBioCloud databases, and results are shown in Table 1. The results showed that six strains of growth-promoting bacteria belonged to six genera. In particular, two identical strains (A05 and A06) displayed high sequence similarity with *Burkholderia cepacian, Burkholderia territorii* and *Burkholderia anthina* (99.78-99.93%). Moreover, the similarity between 16S rRNA gene sequence of strain A13 and A39 was 100% with that of genus *Agrobacterium deltaense* and *Pseudomonas plecoglossicida,* respectively. In addition, 16S rRNA gene sequence of strain A45 was almost identical to that of *Paenarthrobacter aurescens* (99.11%). This is the first report on the ability of *Paenarthrobacter aurescens* to promote plant growth. Finally, strain A64 showed high sequence (99.56 and 99.41%) similarity with *Stenotrophomonas pavanii* and *Pseudomonas geniculate* (Table 1).

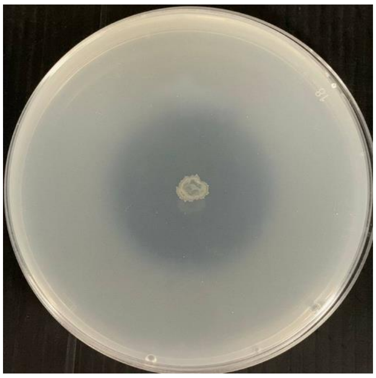

Figure 2. Clear zone of phosphate solubilization of bacterial isolate A05 on PVK solid medium after 7 days of incubation

Table 1. Identification of PGPB based on their 16S rRNA gene sequences

Strains no.	Strains type	Identity)%)	GenBank acc. no.	References
A05, A06	*Burkholderia cepacia*	99.93	OM301952	(Bagyalakshmi and Balamurugan 2017)
	Burkholderia territorii	99.93		(Júnior et al. 2020)
	Burkholderia anthina	99.78		(Tapia-García et al. 2020)
				(Lau et al. 2020)
A13	*Agrobacterium deltaense*	100	OM368496	(El Attar et al. 2019)
A39	*Pseudomonas plecoglossicida*	100	OM368446	(Pankaj et al. 2020)
A45	*Paenarthrobacter aurescens*	99.11	OM368478	(Not previously reported)
A64	*Stenotrophomonas pavanii*	99.56	OM368497	(Lau et al. 2020)
	Pseudomonas geniculata	99.41		(Gopalakrishnan and Samineni 2017)

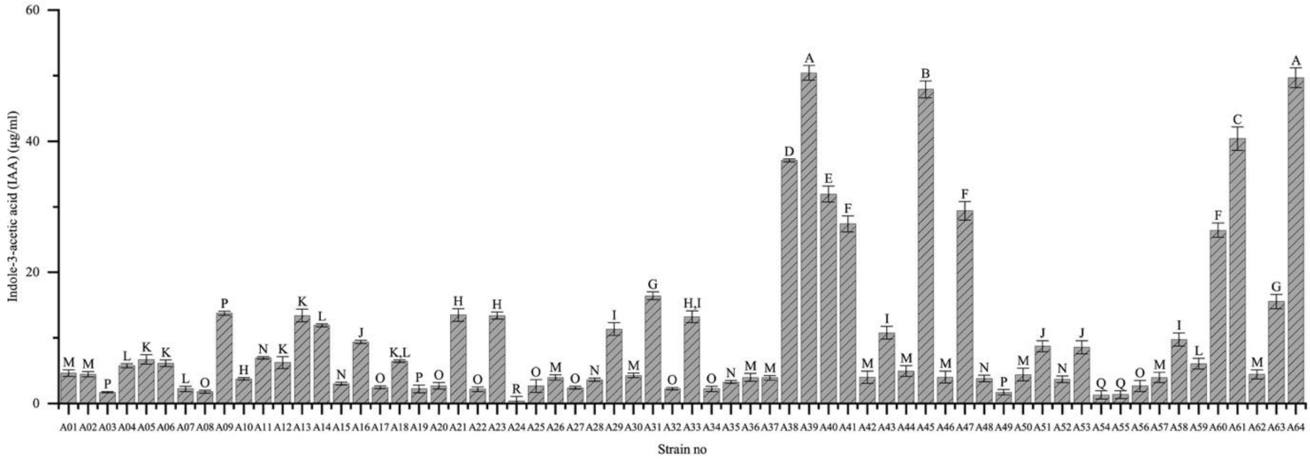

Figure 1. Comparison of IAA production by the bacterial isolates in tryptone yeast (TY) broth (n= 3*)

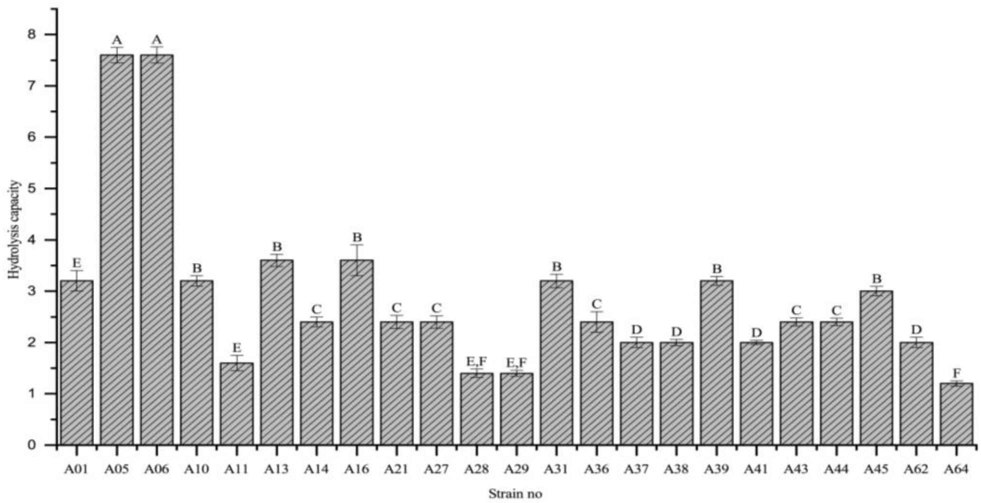

Figure 3. Comparison of phosphate solubilization index (SI) of 23 isolates in Pikovskaya solid medium

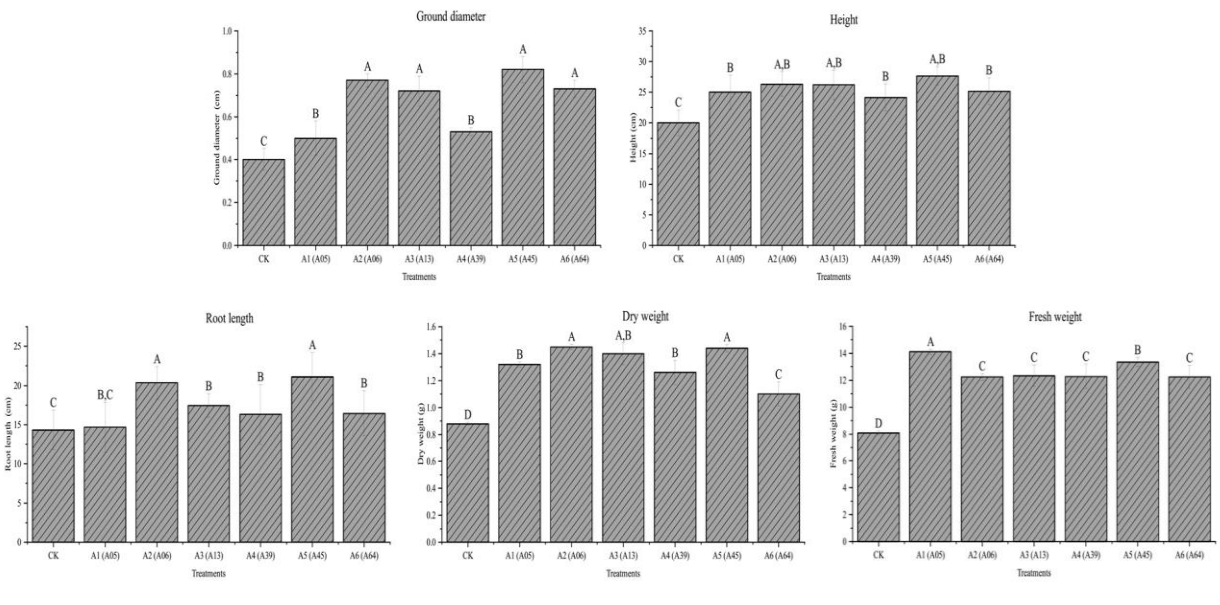

Figure 4. Effect of PGPB on different aspects of *Coriandrum sativum* growth

Discussion

Different soil samples were collected from the root zone of healthy and vigorously growing plants in an agricultural farm in the Uttaradit Province, Thailand. IAA is an important plant hormone that participates in many important physiological processes in plants, including cell growth and division and tissue differentiation, thereby promoting the growth of plant roots and shoots (Chen et al. 2021; Karcz and Burdach 2002). Results of present study showed that three strains, namely A39, A64 and A45 produced higher amount of IAA at 47.90 - 50.44 µg/mL, consistent with the results reported by Kizhakedathil and Devi (2018) and Puri and Chanway (2020). Phosphorus is one of the major elements in plant mineral nutrition. It is required in large amounts for adequate growth and development of plants. It plays an important role in all major metabolic processes occurring in plants, including photosynthesis, energy transfer, macromolecular biosynthesis and respiration (Hamim et al. 2019). However, its use by plants is quite limited, because phosphorus is present in an insoluble, immobilized and precipitated form (Debabrata et al. 2017). In this study, strains A05, A06 and A13 had the highest solubilization index (SI), with values of 7.6, 7.6 and 3.6, consistent with the results reported by Beneduzi et al. (2013). This was higher than the results reported by Andy and Gour (2020).

Pot experiments with coriander showed that the most promising inoculants)A05, A06, A13, A39, A45 and A64(improved almost all plant growth parameters considered. One possible reason is that all inoculants solubilized P and produced significant concentrations of IAA and nitrogen. The response of plants to different isolates was variable, which may be explained by their individual traits and rhizosphere competence (Iyer and Rajkumar 2017).

The increase in plant height, root length, ground diameter and biomass is likely due to the ability of PSB to dissolve phosphorus and nitrogen, which promotes efficient absorption and use of nutrients in order to synthesize organic matter and increase plant biomass. Similarly, Chen et al. (2021) and Pereira and Castro (2014) observed that inoculation of PSB significantly promoted plant height, ground diameter and biomass, which may be due to organic acid production (such as gluconic, formic and citric acids) by these strains. In this study, the plant height and biomass of seedlings treated with bacteria were significantly higher than those of control seedlings. Altogether, these results indicate that plant growth-promoting bacteria can promote plant growth within a certain range of concentrations. It has been reported that *Burkholderia cepacia*, *Burkholderia territorii* and *Burkholderia anthina* have great potential as plant growth-promoting bacteria (Bagyalakshmi and Balamurugan 2017; Júnior et al. 2020; Lau et al. 2020; Tapia-García et al. 2020). El Attar et al. (2019) isolated *Agrobacterium deltaense* from root nodules of *Phaseolus vulgaris*. Earlier studies have reported the potential of *Pseudomonas plecoglossicida*, *Stenotrophomonas pavanii* and *Pseudomonas geniculate* as plant growth-promotors (Gopalakrishnan and Samineni 2017; Lau et al. 2020; Pankaj et al. 2020). Among the tested strains, this is a first report that *Paenarthrobacter aurescens* promotes vigorous growth in coriander and is suitable for application as a plant growth-promoting bacterium.

In conclusion total of sixty-four bacteria were isolated from twenty-three soil sources from an agricultural farm in the Uttaradit Province, Thailand. These bacteria were screened for their putative beneficial characteristics as PGPB. Following nucleic acid analysis and in silico nucleotide similarity search analysis, six isolated bacteria were selected for further investigation. The results of present investigation revealed that three bacterial isolates (strains A39, A64 and A45), belonging to various species, produced high levels of IAA production. Four isolates (strains A05, A06 and A13) showed remarkable phosphate solubilizing activity and six isolates showed plant growth-promoting effects on *Coriandrum sativum* L. when compared with control seedlings. Morphological differences among coriander plants treated with PGPB isolates clearly demonstrated a positive effect on plant growth and development. In addition, this is the first report of *Paenarthrobacter aurescens* capable of growth-promoting in plants and thus can be considered as PGPB.

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