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Isolation and Characterization of Phosphate Solubilizing Bacteria from Rhizosphere of Coffee Plant and Evaluating Their Effects on Growth and Development of Coffee Seedlings

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Abstract

Soil samples from the rhizosphere of 6 months old coffee seedling were collected. Phosphate Solubilizing Bacteria (PSB) were selected based on the quantitative ability to solubilize phosphate by measuring the clear zone surrounding the colonies in Pikovskaya's agar. In total, 26 PSB were isolated. Best 8 isolates (PSB 1, PSB 2, PSB 8, PSB 10, PSB 11, PSB 14, PSB 19, and PSB 20) were selected and phenotypic and biochemical characteristics were carried out. Best four isolates were selected for germination assay, seedling growth experiment and greenhouse experiment. The effect of inoculation of PSB on germination of coffee seeds in vitro was evaluated and the result showed that all isolates (PSB 1, PSB 10, PSB 11, and PSB 20) enhanced germination by 51.72% - 58.82% over uninoculated water control. In seedling growth experiment, isolate PSB 20 isolates significantly enhanced shoot length and shoot fresh weight. In the greenhouse experiment conducted over 5 months, isolates PSB 20 and PSB 1 significantly enhanced coffee seedling shoot length, fresh shoot biomass and fresh root biomass over uninoculated water control plants. PSB 1 and PSB 10 significantly increased chlorophyll content and protein content in the coffee leaves. So, this study suggests bio-inoculants are able to stimulate plant growth, and recommends further experiments at field conditions.

***Keywords**: Coffee; Phosphate solubilization; Phosphate solubilizing bacteria; Pikovskaya's media; Rhizosphere*

Introduction

Phosphorus (P) is an essential element required for plant growth and is involved in many plant metabolic functions [1,2]. Compared with the other major nutrient, P is by far the least mobile due to the large reactivity of phosphate ions relative to numerous soil constituents, and therefore frequently a limiting factor for plant growth [3]. P in soil is present in mineral forms such as apatite, hydroxyapatite and oxyapatite, and also in organic forms such as inositol phosphate, phosphoesters, which cannot be directly assimilated by plants [4]. A large portion of soluble inorganic phosphatic fertilizer is mined from non-renewable rock phosphate sources. The phosphatic fertilizer that is applied to agricultural soil to reduce P deficiency, however, is immobilized rapidly and becomes unavailable to plants and also increases the acidity of the soil which reduces the soil's beneficial organism population [5]. Furthermore, transfer of soil P derived from fertilizer is a major cause of P-induced eutrophication of surface water [6]. With ever increasing demand for food production, there is a huge challenge for

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sustainability of current agricultural practices, and phosphorus security is emerging as a global sustainability challenge. So, there is an imperative need for a sustainable phosphorus use in agriculture [5,7].

One of the cheap and environmentally safe alternatives for improving the deficiency of phosphate nutrition is the manipulation of rhizospheric Phosphate Solubilizing Bacteria (PSB) [8]. PSB are able to release soluble P from inorganic and organic sources in soil through solubilization and mineralization, thus promote plant growth [9]. Besides increased P release, PSB also plays an important role in plant growth promotion through production of Indole Acetic Acid (IAA), ACC deaminase siderophore, antibiotics, Hydrogen Cyanide (HCN) and exopolysaccarides. Many PSB including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium* and *Serratia* have been reported to enhance plant growth in many commercially important crops [10].

Coffee is an emerging cash crop in mid-hills of Nepal and economically more profitable than any other cash crop or cereal crop. Nepalese coffee is organic - grown without the use of chemical fertilizers and pesticides [11]. The ease of production and low input requirement means even marginal lands are suitable for coffee production, and particularly suitable for farmers deprived of ample resources. Currently, it is grown in more than 40 districts of Nepal in 1,760 hectares, and more than 27,000 households are involved in coffee farming. It is estimated that 1.1 m hectares of land is suitable for coffee farming [12]. Despite immense potential, commercialization is lacking and productivity of Nepalese coffee is still poor compared to other countries [13].

Coffee rhizosphere is associated with large number of beneficial microorganisms including PSB which may contribute to nutritional requirement of the plant. The objective of this study is to isolate, characterize and determine the ability of phosphate solubilizing bacteria associated with the rhizosphere of coffee plant and to access the effect of PSB on coffee seedling growth.

Materials and Methods

Sampling site

Rhizospheric soil samples were collected from 6 month old coffee seedling grown at Himalayan Organic Coffee, Khawa, Kavre, Nepal.

Soil sampling, isolation of PSB and maintenance

The roots were carefully uprooted along with their rhizospheric soil intact. Bulk soil was removed from plants by shaking vigorously by hand for 10 min. The rhizospheric soil was collected by handshaking roots for 10 min in one liter of a sterile 0.9% NaCl solution [14]. The soil suspension was incubated on a shaker for 25 minutes on an orbital shaker at 100 rpm 30°C. The subsequent suspension was serially diluted. 1ml of the solution was then diluted up to 10-6 dilution and 0.2 ml from each dilution was placed on Pikovskaya's Agar (PKA) plates and incubated for 5 days at 30°C. The presence of phosphate solubilizing bacteria was indicated by the formation of transparent (halo) zone around the colonies in PKA agar plates. Colonies with large halo zone and different morphology were selected and purified to obtain pure culture. Isolates were maintained in 40% (v/v) glycerol at -20°C for long term storage.

Evaluation of phosphate solubilizing bacteria (PSB)

PSB isolates were further screened for their Tri-Calcium Phosphate (TCP) and Rock Phosphate (RP) solubilizing activity in agar plates. Pure cultures of phosphate solubilizing bacteria were spot inoculated at the center of Pikovskaya's agar medium, NBRIP agar media and NBRIP-RP (5g/L Morocco RP) [15,16]. The plates were incubated at 30°C for 10 days and zones of

phosphate solubilization formed around the colonies were recorded. The analysis of the phosphate solubilizing ability was made by measuring the zone of solubilization around the colony growth as Solubilization Index (SI) [17]. The experiments were performed in triplicates.

PSB isolates were further analyzed for their ability to solubilize phosphate in broth medium. 250 µL of inoculum isolates were inoculated in Pikovskaya's broth, NBRIP broth and NBRIP-RP (5g/ L Morocco RP) broth (100 ml) in conical flask and were incubated on a shaking incubator at 120 rpm for 10 days at 30°C. Sterile uninoculated medium served as control. The cultures were harvested by centrifugation at 10000 rpm for 10 min, using centrifuge. The total soluble phosphate in culture supernatant was measured by UV-visible spectrophotometer at 420 nm following the vanadomolybdophosphoric acid colorimetric method [18].

Morphological and biochemical characterization

The efficient PSB were identified on the basis of morphological and biochemical characteristics. For identification of PSB isolates based on colony morphology, PSB isolates were grown in nutrient agar plates for 24 hours at 30°C. Gram reaction was done using the HiMedia Gram Staining kit (K001-1KT) according to the manufacturer's instructions. Indole test, MR-VP test and citrate test was performed according to Hemraj et al. [19].

Effect of PSB on coffee seed germination

Effect of PSB on seed germination was studied by inoculating seeds of *Coffea arabica* with best four isolates. *C. arabica* seeds were collected from local coffee cooperative. Endocarp (parchments) was manually removed [20]. The seeds were then surface sterilized with 1% sodium hypochlorite for 1 min and washed three times with double distilled water [21]. For the preparation of bacterial inoculum, isolates were first streaked on nutrient agar plates and incubated at 30°C for 48 h. Single colonies on NA plates were picked with an inoculating loop and transferred to 250 ml nutrient broth and grown aerobically in a rotary shaker at 160 rpm at 30°C for 24 h. The bacterial suspension was maintained at OD550=0.5-1

For bacterilization, the seeds were soaked in bacterial suspension. The bacterial suspension was drained and seeds were dried overnight in laminar hood [22]. Seeds were then placed in sterile filter paper in petri-plate and incubated for 7 days at 30°C in incubator. Seeds immersed in distilled water and media (HiMedia Nutrient Broth) were used as a control treatment. Germination percentage of the seeds was recorded. The experiments were performed in triplicates.

Effect of PSB on coffee seedling growth

For the evaluation of effect of PSB on coffee seedling growth, *C. arabica* seeds with endocarp (parchments) intact were surface sterilized with 1% sodium hypochlorite for 1 min and washed three times with double distilled water and soaked in bacterial suspension (OD550=0.5-1) for 24 hours. The bacterial suspension was drained and seeds were dried overnight in laminar hood. Seeds immersed in distilled water and media (HiMedia Nutrient Broth) only were used as a control treatment. For preparation of sterile soil, field soil was autoclaved twice for 20 min at 120°C with a 24 h interval. Sterile soil was filled in plastic square plug tray. There were 10 seed per treatment. Treatments were arranged based on completely randomized design and placed in standard laboratory condition at 25°C. The experimental set-up was watered daily, and no additional fertilizer was added. After 70 days of sowing seeds, shoot length, root length, shoot fresh weight, root fresh weight, chlorophyll content and protein content of the coffee seedlings were measured. Chlorophyll was extracted from the leaves and estimated as described by Arnon, 1949 [23] while protein content of leaves and seeds was estimated by while protein content of leaves and seeds was estimated as described by Lowry et al. [24].

Effect of PSB on coffee plant growth under greenhouse condition

Plant growth promoting ability of the best four PSB isolates was assessed in *C. arabica* seedlings in pots under greenhouse conditions. Roots of four months old seedlings of *C. arabica* were uprooted and cleaned, and root surface sterilized using alcohol. Bacterial inoculum was prepared as described above. The roots were then dipped in bacterial suspension for 10 minutes, and transferred to pot with 12 cm height and 9 cm diameter. The pots contained 600 g of twice autoclaved soil and 50 g wood ash. The experiment was performed in a completely randomized design with five replications per treatment. Uninoculated plants were used as negative control and plants treated with commercial grade single super phosphate fertilizer (16% water soluble P205, 11% sulphur, 21% calcium) was used as positive control. The coffee plants were placed in greenhouse conditions and were watered every alternate day. Observation were made on shoot length, root length, shoot fresh weight, root fresh weight, chlorophyll content, protein content at the end of five months.

Statistical analysis

Data generated from in vitro and green house experiments were analyzed using MS-Excel. The effects of the treatments in seedling growth experiment and greenhouse experiments were analyzed by ANOVA and means were compared using Tukey's test, at 5% significance level.

Results and Discussion

Isolation and biochemical characterization of PSB

Plant rhizosphere due to its rich nutrient availability is a hot spot of various soil bacteria. A total of 26 PSB isolates were obtained from the rhizosphere of coffee when plated on Pikovskaya's agar media (Table 1).

TABLE 1. **Solubilization index (SI) of PSB isolates isolated from rhizosphere of coffee plant in Pikovskaya's agar plate, calculated as the ratio of diameter of halo (mm)/diameter of colony(mm).**

Bacterial Isolates	Solubilization index (SI)
PSB 1	1.61 ± 0.13
PSB 2	1.88 ± 0.08
PSB 3	1.48 ± 0.1
PSB 4	1.69 ± 0.07
PSB 5	1.2
PSB 6	1.17
PSB 7	1.19 ± 0.01
PSB 8	1.6
PSB 9	1.27 ± 0.03
PSB 10	1.79 ± 0.07
PSB 11	1.62 ± 0.04
PSB 12	1.35 ± 0.02
PSB 13	1.2
PSB 14	1.71
PSB 16	1.73 ± 0.08
PSB 17	1.57
PSB 18	1.88 ± 0.08
PSB 19	1.66 ± 0.11
PSB 20	1.75 ± 0.07

PSB 21	1.46 ± 0.03
PSB 22	1.53 ± 0.09
PSB 23	1.30 ± 0.06
PSB 24	1.18 ± 0.04
PSB 25	1.26 ± 0.01
PSB 26	1.58 ± 0.07
*Results are presented as means of three replicates ± standard error	

TABLE 2. **Morphological and biochemical characteristics of PSB isolates isolated from rhizosphere of coffee plants.**

	Isolates							
Parameters	**PSB 1**	**PSB 2**	**PSB 8**	**PSB 10**	**PSB 11**	**PSB 14**	**PSB 19**	**PSB 20**
Cell shape	rod	rod	rod	coccus	rod	rod	rod	rod
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Colony shape	circular	circular	circular	circular	circular	circular	circular	circular
Indole Test	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-
Methylene Red test	-	-	-	-	-	-	-	-
Citrate Test	+	+	+	+	+	+	+	+
+/- showed positive and negative test results respectively								

The PSB isolates were further characterized by a series of biochemical reactions (Table 2). The biochemical analysis revealed all bacteria as Gram negative. Gram negative bacteria dominated the rhizosphere of coffee plant in the studies of [25,26]. Further biochemical test shows greater similarity of the PSB isolates with Pseudomonas species. Pseudomonas species are free living and most abundant in soil and can be cultured with ease in vitro and thus are more frequently encountered.

Phosphate solubilization index of the isolates on Pikovskaya's agar plate

The solubilization index of each PSB isolate on Pikovskaya's agar plate is presented in Table 1. The result shows a varying degree of phosphate solubilization ranging from 1.20 to 1.88. Formation of halo in Pikovskaya's agar media is attributed to production of different organic acids and acid phosphatase production by the PSB isolates [9].

Evaluation of PSB on different insoluble P sources

Phosphate solubilizing bacteria have shown to enhance the solubility of diverse insoluble P sources [27]. In our study, only 8 isolates with higher phosphate solubilizing abilities on Pikovskaya's agar (SI>1.60) were selected for evaluation of solubilization efficiency in NBRIP agar media and NBRIP-RP agar (5 g/L Morocco RP), and Pikovskaya's broth, NBRIP broth and NBRIP-RP broth (5 g/L Morocco RP).

All 8 isolates showed clear halo zone around their colonies. PSB 2, PSB 8 and PSB 10 showed maximum solubilization index in Pikovskaya's agar (1.88 ± 0.08), NBRIP agar (2.01 ± 0.10) and NBRIP-PR agar (2.79 ± .54) media respectively (Table 3).

TABLE 3. **Solubilization index (SI) of selected PSB isolates on different insoluble P sources, which was calculated as the ratio of diameter of halo (mm)/diameter of colony (mm).**

Bacterial Isolates	**Solubilization index**		
	Pikovskaya's	**NBRIP**	**NBRIP-RP**
PSB 1	1.61 ± 0.13	1.71 ± 0.04	2.11 ± .05

PSB 2	1.88 ± 0.08	1.80 ± 0.11	1.55 ± .02
PSB 8	1.6	2.01 ± 0.10	2.01 ± .01
PSB 10	1.79 ± 0.07	1.54 ± 0.23	2.79 ± .54
PSB 11	1.62 ± 0.04	1.87 ± 0.06	2.76 ± 0.20
PSB 14	1.71	1.70 ± 0.05	2.07 ± 0.06
PSB 19	1.66 ± 0.11	1.48 ± 0.07	1.44 ± 0.05
PSB 20	1.75 ± 0.07	1.66 ± 0.08	3.71 ± 0.19
Results are presented as means of three replicates ± standard error			

The amount of soluble P released after 10 days of incubation are presented in Table 4. The levels of solubilization of different insoluble P sources with different isolates were varied.

TABLE 4. **Phosphate solubilization by different PSB isolates in different insoluble P sources in broth media.**

Bacterial Isolates	**Phosphate solubilization (µg/ml) after 10 days**		
	Pikovskaya's	**NBRIP**	**NBRIP-RP**
PSB 1	356.67 ± 11.73	262.91 ± 6.22	209.58 ± 6.67
PSB 2	318.33 ± 27.93	174.58 ± 5.42	222.92 ± 5.79
PSB 8	82.92 ± 4.23	484.17 ± 2.73	244.58 ± 5.83
PSB 10	407.5 ± 2.50	213.75 ± 3.82	263.33 ± 2.92
PSB 11	298.5 ± 6.29	529.17 ± 6.14	216.25 ± 6.96
PSB 14	287.08 ± 4.70	142.08 ± 7.95	180.83 ± 3.63
PSB 19	187.92 ± 11.42	343.75 ± 6.29	205.42 ± 2.73
PSB 20	90.83 ± 2.32	277.08 ± 4.10	216.25 ± 5.73
Results are presented as means of three replicates ± standard error			

After incubation for 10 days in broth medium, isolate PSB 10 showed maximum phosphate solubilization in Pikovskaya's (407.5 ± 2.50 µg/ml) and NBRIP-RP (263.33 ± 2.92 µg/ml) whereas isolate PSB 11 maximum solubilization index in NBRIP agar (529.17 ± 6.14 µg/ml).

Many researchers have reported the incubation period of more than 10 days and even up to 15 days to be optimum for phosphate solubilization by various bacterial isolates [28,29]. Formation of halo zone on agar plate and P solubilization in the liquid culture can be contradictory [15].

Effect of PSB on coffee seed germination

All four PSB isolates had a positive effect on coffee seed germination compared to water and media control. PSB 1 (68.00 ± 2.00) and PSB 20 (63.33 ± 9.89) showed best result on coffee seed germination. Bacterilization of the coffee seeds leads to greater establishment of these PSB isolates in this niche as they have the competitive advantage to utilize the large amount of metabolic exudates, and thus provide protection against potential pathogens [30,31]. Similar positive effect on seed germination by phosphate solubilizing bacteria was observed in faba beans and *Cicer arietinum* [32,33] (Table 5).

TABLE 5. **Effect of PSB isolates on the germination of *Coffea arabica* seeds after 10 days.**

Bioassay	**Germination %**
Water Control	28.00 ± 2.00
Media Control	36.67 ± 2.11

PSB 1	68.00 ± 2.00
PSB 10	58.00 ± 2.00
PSB 11	56.67 ± 6.15
PSB 20	63.33 ± 9.89
Results are presented as means of three replicates ± standard error	

Effect of PSB on coffee seedling growth

TABLE 6. **Effect of PSB isolates on *Coffee arabica* seedlings after 70 days of sowing.**

Treatment	Shoot Length (cm)	Root Length (cm)	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Chlorophyll content (mg/g)	Protein Content (mg/g)
PSB 1	5.43 ± 0.15bc	3.90 ± 0.11	0.3106 ± 0.0081c	0.0493 ± 0.0023	0.520 ± 0.54	0.553 ± 0.048
PSB 10	5.37 ± 0.20bc	4.19 ± 0.27	0.4041 ± 0.0164ab	0.1056 ± 0.0503	0.578 ± 0.054	0.219 ± 0.008
PSB 11	5.92 ± 0.07ac	3.38 ± 0.11	0.3973 ± 0.007abc	0.0549 ± 0.0029	0.533 ± 0.064	0.224 ± 0.018
PSB 20	6.12 ± 0.06a	4.04 ± .012	0.437 ± 0.0146b	0.0603 ± 0.0025	0.597 ± 0.071	0.209 ± 0.007
Media Control	5.40 ± 0.17bc	4.20 ± 0.12	0.3467 ± 0.0335ac	0.0405 ± 0.0019	0.509 ± 0.047	0.237 ± 0.018
Water Control	5.31 ± 0.18bc	3.95 ± 0.13	0.3454 ± 0.0302ac	0.0438 ± 0.0014	0.530 ± 0.031	0.266 ± 0.018
Numbers in the same column followed by the same letter did not differ significantly at 5% Tukey test						

ANOVA test revealed mean values of root length, root fresh weight, chlorophyll and protein content were not significantly different ($p < 0.005$). Seedling growth experiment showed isolate PSB 20 significantly increased shoot length (6.12 ± 0.06 cm) and shoot fresh weight (0.437 ± 0.0146 cm) (Table 6).

Effect of PSB on coffee plant growth under greenhouse condition

TABLE 7. **Effect of PSB isolates on different growth parameters in *Coffea arabica* under greenhouse condition after 5 months.**

Treatment	Shoot Length (cm)	Root Length (cm)	Fresh Shoot Biomass (g)	Fresh Root Biomass (g)	No. of Leaves	Chlorophyll content (mg/g)	Protein Content (mg/g)
PSB 1	15.94 ± 0.42a	19.24 ± 1.37	5.2191 ± 0.1353c	3.5697 ± 0.1914b	14.2 ± 0.37	0.594 ± 0.04c	0.205 ± 0.01bc
PSB 10	15.5 ± 0.66a	19.22 ± 0.84	5.4352 ± 0.2188c	3.4804 ± 0.2689b	14.4 ± 0.75	0.492 ± 0.05bc	0.232 ± 0.03c
PSB 11	15.62 ± 0.71a	17.58 ± 0.39	5.5660 ± 0.1379a	3.4719 ± 0.1235b	13.4 ± 0.6	0.442 ± 0.49ab	0.166 ± 0.008ab
PSB 20	16.1 ± 0.71a	16.5 ± .095	5.6335 ± 0.1999c	4.3154 ± 0.3322c	14.4 ± 0.24	0.509 ± 0.02bc	0.154 ± 0.01a
Chemical Control	14.7 ± 0.62a	18.50 ± 1.24	5.5397 ± 0.1334a	3.5104 ± 0.2663b	14.2 ± 0.37	0.467 ± 0.03b	0.177 ± 0.008ab
Water Control	12.16 ± 0.32b	17.26 ± 1.00	2.4766 ± 0.1918b	1.7877 ± 0.0972a	13 ± 0.45	0.327 ± 0.03a	0.183 ± 0.02ab
Numbers in the same column followed by the same letter did not differ significantly at 5% Tukey test							

ANOVA test revealed mean values of root length and no. of leaves were not significantly different ($p<0.005$). The greenhouse experiment result after 5 months showed that inoculation with phosphate solubilizing bacteria significantly improved shoot length, fresh shoot biomass, fresh root biomass and chlorophyll content over the uninoculated coffee seedlings. Their effect was comparable or greater than the chemical fertilizer control. All PSB isolates significantly increased the shoot length and fresh shoot biomass of coffee seedling. Isolate PSB 20 showed the best effect on shoot length (16.1 ± 0.71 g), fresh shoot biomass (5.6335 ± 0.1999 g) and fresh root biomass (4.3154 ± 0.3322 g) on the coffee seedling (Table 7). Isolate PSB 1 and PSB 11 showed second and third best effect on shoot length and fresh shoot biomass of coffee seedling respectively. Similar increase in stem dry weight and leaf dry weight in coffee plants inoculated with PSB isolates has been reported in coffee [25]. Increase in biomass due to treatment with phosphate solubilizing bacteria has been reported in maize [34] and mugabeen [35]. This could be attributed to the activity of PSB in rhizosphere by releasing soluble P and also through release of IAA, ACC deaminase, siderophore, antibiotics and HCN [10]. The photosynthetic pigment, chlorophyll content was enhanced in the coffee plants whose roots were treated with PSB inoculum. PSB 1 and PSB 20 showed significant increase in chlorophyll content, and it may be indicative of interactions that trigger the chlorophyll related enzymes for increased chlorophyll synthesis [36]. Similarly, PSB 10 significantly increased the protein content (0.232 ± 0.03 mg/g) in coffee leaves.

Coffee seedling growth and promotion can be attributed to many mechanisms, direct and indirect, through which rhizospheric bacteria contribute to sustainable plant growth promotion. Generally, beneficial rhizospheric bacteria contribute through synthesizing particular compounds for plants or facilitating the uptake of particular nutrient from the soil or preventing and protecting the plants from pathogens [37].

Conclusion

Coffea arabica rhizosphere is a hotspot for many phosphate solubilizing bacteria. These PSB can solubilize different insoluble form of P sources and thus can act as biofertilizers in sustainable and organic farming systems. In the present, 26 phosphate solubilizing bacterial isolates were isolated from rhizosphere of coffee plant. These isolates have shown varying degree of phosphate solubilizing ability. Results from in vitro and greenhouse experiment indicate a significant improvement in coffee seedlings treated with PSB isolates. One of the isolate, PSB 20 has shown unique characteristics of phosphate solubilization and plant growth promotion, and thus has a great potential for use as biofertilizer in coffee seedling growth. The use of indigenous rhizospheric phosphate solubilizing bacteria can be a reliable alternative in low capital agriculture and sustainable management of P use.

Conflicts of Interest

The authors declare no conflicts of interest.

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