Research Article

Effect of Free-Living Nitrogen Fixing and Phosphate Solubilizing Bacteria on Growth of *Gossypium hirsutum* L.

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ABSTRACT

Phosphorus and nitrogen are very much crucial for growth of Plant. Nitrogen fixing bacteria and phosphate solubilizing bacteria (PSB) promote plant growth and enhance various cellular processes like root elongation, proliferation and changes of root architecture, seed development and normal crop maturity. In present study the inoculation effect of phosphate solubilizing and Nitrogen fixing bacteria, isolated from rhizosphere and non-rhizosphere soil of North Gujarat, India are investigated. The isolated bacteria were tested for indole acetic acid (IAA), ammonia production and phosphate solubilization *in vitro*. Various parameters like root and shoot height, number of root hair, length, width and weight of leaves and chlorophyll content under greenhouse condition was measured. The study shows highest IAA production 0.1822 µg mL^{-1} by isolate N8 and highest phosphate production 0.029 µg mL^{-1} by isolate P6. The DNA of effective bacterial isolates was amplified using 16S r-DNA primers. Amplified PCR product were purified and sequenced to identify the isolates up to species level using BLAST. The isolates were identified as *Azotobacter vinelandii* and *Bacillus niabensis*, respectively (Accession number: JX564632 and KF535156).

Key words: Ammonium production, Cotton, IAA production, Nitrogen fixing bacteria, Phosphate solubilization.

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INTRODUCTION

There are 16 necessary plant nutrients which are required for normal growth and development of plant. Analysis of soil generally indicates that most of nutrients are present in soil. Plants take their carbon and oxygen from air through photosynthesis. Hydrogen is obtained from water but several other elements like Nitrogen, Phosphorus, Potassium, Magnesium, Calcium and Sulfur are obtained from soil and they require in larger amount than other elements. Plants cannot utilize Nitrogen directly from air but they can easily take up Nitrogenous salts from soil, which is provided by different Nitrogen fixing microorganism.[1] Various types of N_2 fixing bacteria, including symbionts, root nodulating *Rhizobium* spp[2] and different free living Rhizobacteria, such as *Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas, Serratia* and *Streptomyces*[3-5] have been described for plant growth promotion. Phosphates are probably one of the least available plant nutrients found in the rhizosphere. Total soil phosphorus occurs either in organic phosphorus (P0) or inorganic phosphorus (Pj) forms.[6] Soil phosphates are made available either by plant roots or by soil microorganisms by the activity of phosphatases and secretion of organic acids. Phosphorus cycle in the biosphere can be described as open or sedimentary because there is no change with the atmosphere. Species of genus *Bacillus, Pseudomonas, Rhizobium, Aspergillus* and *Penicillium* are the most capable Phosphate solubilizes commonly present in soil.[7]

Nitrogen fixation and Phosphate solubilization mechanism provide two major elements (Nitrogen

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and Phosphorus) to plant are most necessary for plant growth and development. In the agriculture field microbes which that have function as decomposer, will breakdown organic matters, form humus and release useful nutrients (N, P, K, S and trace elements etc). *Bacillus* species used as biofertilizers may have direct effect on plant growth through synthesis of plant growth hormones.[8]

PGPR influenced the plant growth by direct or indirect method.[9] The direct growth promoting mode includes nitrogen fixation, Solubilization of minerals, production of plant hormones. Indirect mode includes production of antibiotic and/or inhibitory substances for pathogenic organism. Plant hormones enhances several cellular and physiological system of working like cell division, cell enlargement, flowering, fruit ripening, seed germination and leaf abscission. Indole 3- acetic acid (IAA) is one of the natural auxin.[10] In this research cotton (*Gossypium hirsutum*) plant were grown under the application of nitrogen fixing bacteria and phosphate solubilizing bacteria under the nursery condition and growth was monitored and compared with controls.

MATERIALS AND METHODS

Collection of soil samples

Free living nitrogen fixing bacteria and Phosphate solubilizing bacteria were isolated from rhizospheric and non-rhizospheric area of plant from selected sites of Mehsana District, North Gujarat region, india.

Isolation of Free living Nitrogen fixing and Phosphate solubilizing bacteria

Nitrogen free medium Ashby's mannitol medium was used for isolation of free living nitrogen fixing bacteria. Enrichment of rhizospheric soil sample was done by inoculating 1 g of soil sample. All the flasks were incubated at 30°C for 3-4 days (until turbidity of organism was observed) in rotary shaker at 120 rpm. A loop full culture medium from each flask was streaked on the sterile AMA (Ashby's Mannitol Agar) plate by four flame method. All the plates were incubated at 28°± 2°C for 48-72 h. Pikovskaya's medium was used for isolation of Phosphate solubilizing bacteria.[11] Enrichment of Rhizospheric soil sample was done by inoculating 1 g soil sample. All the flasks were incubated at 28°± 2°C in rotary shaker at 120 rpm for 48-72 h (until turbidity of organism was observed). A lapful of culture medium from each flask was streaked on sterile Pikovskaya's agar plate in aseptic condition. All the plates were incubated at 30°C for 48-72 h.

Characterization of free living Nitrogen fixing and Phosphate solubilizing bacteria

Morphological characterization of isolated bacteria was determined by Gram staining which was developed by Christian Gram in 1884. Colonial characterization of was done by observing size, shape, Margin, Texture, Elevation, Consistency, Opacity, Pigmentation etc. Genetic characterization of isolates was done by 16s-rRNA sequencing method.

Detection of Phosphate solubilization

All isolated phosphate solubilizing bacteria were detected for solubilization on PVK agar plate. Single colony of each isolated PSB were picked from pure plate and placed on PVK agar plate. All the plates were incubated at 30°C for 5-7 days. After seven days diameter of halo zone surrounding the colony were measured. Solubilization efficiency (SE) and solubilization index (SI) were calculated using the formula.[12]

$$SE = \frac{solubilization\ diameter}{growth\ diameter} \times 100$$

$$SI = \frac{Colony\ diameter + halo\ zone\ diameter}{colony\ diameter} \times 100$$

IAA and Ammonia production

The quantitative analysis of indole-3-acetic acid (IAA) was performed as per the method suggested by Bric *et al*.[13] All the flasks containing Luria Burtani (LB) broth were inoculated with 0.1 ml of active bacterial culture and control was remaining uninoculated. All the flasks were incubated at 28±2°C on rotary shaker at 125 rpm for 24 hrs. After incubation 5 ml of broth from each flasks were centrifuged at 10000 rpm for 15 min. 2 ml of supernatant was taken from each tube and 2 ml of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added and incubated at 28±2°C in the dark for 1 hr. After incubation O.D. of each sample was measured in spectrophotometer at 540 nm. The IAA concentration was determined from standard curve. Isolated bacteria were tested for ammonia production by using peptone water.[14]

Plant experiment

Cotton (*Gossypium hirsutum*) plant was selected for the study of effect of nitrogen fixing and phosphate solubilizing bacteria. Effect of single culture inoculation and dual culture of selected bacteria were studied. For plantation polythene bags were selected due to its easy transportation and cost. Cotton seeds were coated with the culture for 2 hand sawn in each bags. All the bags were placed in controlled condition of nursery where

sufficient sunlight was available. Growth was observed until 50 days. Various parameters were determined e.g, Root height, Shoot height, Number of root hairs, Number of leaves, Length and Width of leaves and Fresh weight of leaves.

Estimation of chlorophyll

Chlorophyll content was estimated using Holden protocol.[15] 100 mg weight of leaves was homogenized by mortar and pestle separately in presence of 80% acetone. To prevent pheophytin formation pinch of $CaCO_3$ was added. All the samples were centrifuged at 5000 rpm for 10 min. After centrifugation supernatant was collected from each sample and final volume made 10 ml using 80% acetone. All the test tubes were wrapped with black paper to protect degradation of chlorophyll. The optical density of each sample was measured at 663 nm and 645nm.

RESULTS

Isolation and Identification of Nitrogen fixing and Phosphate solubilizing bacteria

Free nitrogen fixing bacteria were isolated from different soil samples. For isolation purpose Ashby's Mannitol Agar plate and Pikovskaya's agar plate were used respectively.

Determination of Phosphate solubilization on PVK plate

All Isolated bacteria were inoculated on Pikovskaya's medium for determination of phosphate solubilization efficiency. Isolated colonies gave clear zone by which it was confer that they can solubilize phosphate (Figure 1). Phosphate solubilization determined by studying various criteria includes zone diameter, colony diameter, solubilization efficiency and solubilization index which are shown in Table 1.

All the selected isolated bacteria were able to solubilize phosphate and produce halo zone surrounding the colony. According to study, Isolate P6 is more potential for solubilizing water insoluble phosphate then other isolates. As shown in Table 2 all the isolated bacteria have ability to produce phosphate either in less or more amount. From this Isolates P6 could produce highest amount of phosphate (0.029µg/ml) after 5 days of incubation. Isolate P7 and P8 is moderate effective and produces 0.024 µg/ml and 0.026 µg/ml phosphate production respectively whereas isolate P4 is less effective.

Quantitative estimation of phosphate by Fiske Subbaraw method

Fiske Subbaraw method was performed for estimation of phosphate liberated in liquid broth. Inorganic phosphate concentrations were measured after 2 to 5 days are shown in Table 2.

Characterization of bacteria

Morphological characterization

Morphological characterization of Phosphate solubilizing bacteria and N_2 fixers are shown in Table 3.

Cultural characterization

The Colonial characterization of NFB and PSB are shown in Table 4.

Genomic charcterization

The genomic DNA extracted by Madox Bio Kit is shown in Figure 2 and amplified PCR product on Agarose Gel is shown in Figure 3. The sequence submitted to NCBI Gene bank and the isolates were identified as *Azotobacter vinelandii* and *Bacillus niabensis* and the accession number provided by NCBI is JX564632 and KF535156 respectively.

IAA and Ammonia production

Production of Indole 3-acetic acid was determined using method given by Brick *et al.* 1991. Absorbance and concentration of Indole 3-acetic acid produced

Table 1: Determination of phosphate solubilization.

Isolate	Diameter of zone (cm)	Diameter of colony (cm)	SE (solubilization efficiency)	SI (solubilization index)
P1	1.4	0.5	280	3.8
P2	1.7	0.5	340	4.4
P3	1.5	0.6	300	3.5
P4	1.8	0.6	108	4
P5	1.9	0.6	316.66	4.16
P6	3.1	0.8	387.5	4.875
P7	2.1	0.9	233.33	3.33
P8	2.3	0.7	328.57	4.28

by isolated N_2 fixing bacteria are shown in Table 5. In Ammonia production test after addition of Nessler's reagent yellow color was produced in all of peptone water containing flasks. Therefore it has been detected that all the isolated bacteria can produce ammonia *in vitro*[14] and generally all NFB have ability to produce ammonia.

Pot experiment

On *Gossypium hirsutum*, the effect of free living nitrogen fixing and phosphate solubilizing bacteria and mixed culture of both the bacteria were found to be statistically significant. Root height, number of root hair, shoot height, length and width of leaves, number of branches of shoot were increased in inoculated plant compared to control plant (Figure 4).

Estimation of Chlorophyll

Chlorophyll content was estimated using Holden protocol.[15] The amount of chlorophyll a, chlorophyll b and total chlorophyll were estimated. The estimated data for chlorophyll content are summarized in Table 7.

DISCUSSION

This present study reveals total of 25 free living N_2 fixers and 28 Phosphate solubilizing bacteria were isolated from rhizosphere and non-rhizosphere soil of North Gujarat, India. Out of these isolates 8 isolates were selected for further study. For the study of phosphate solubilization various criteria measured includes zone diameter, colony diameter, solubilization efficiency and solubilization index.

It has been investigated that 80% of free living N_2 fixers isolated from the rhizosphere can produce the plant hormone IAA.[16] After 72 hr of inoculation, Isolate N8 was produced high amount of Indole 3-acetic acid (IAA) than other isolates, whereas isolate N4, N5, N6, N7 could produce moderate amount of IAA andisolate N2 and isolate N1 could produce less amount of IAA. The hormone produced by most of the N_2 fixers is usually indole – 3- acetic acid (IAA)[17] Frietas and Germida investigated inoculation effect of *P. aeroginosa, P. cepacia, P. Putida and P. fluorescens* strains on winter wheat increases the plant height, root and shoot mass and number of tillers in growth chamber. Isolate 5, 9 and 7 can produce more amount of IAA, so they can

Table 2: Phosphate liberated by isolated bacteria.

ISOLATES	Concentration of phosphate (µg/ml)			
	After 2 days	After 5 days	After 2 days	After 5 days
P1	1.236	1.534	0.015	0.019
P2	1.439	1.67	0.018	0.021
P3	1.239	1.491	0.015	0.018
P4	0.906	1.131	0.011	0.014
P5	1.453	1.893	0.018	0.023
P6	2.012	2.328	0.025	0.029
P7	1.678	1.944	0.021	0.024
P8	1.878	2.13	0.023	0.026

Table 3: Morphological characterization of PSB and N_2 fixers.

Organism	Size	Shape	Arrangement	Gram's reaction
P1	Small	Rod	Single or in chain	Gram negative
P2	Small	Cocci	Single or in bunches	Gram positive
P3	Big	Rod	Single or in chain	Gram positive
P4	Small	rod	Single	Gram negative
P5	Small	Rod	Single	Gram negative
P6	Small	Rod	Single	Gram negative
P7	Big	Rod	Single or in chain	Gram positive
P8	Big	Rod	Single or in chain	Gram positive
N1	Small	Rod	Single or in pair	Gram negative
N2	Small	Rod	Single or in pair	Gram negative
N3	Small	Rod	Single or in pair	Gram negative
N4	Small	Rod	Single or in pair	Gram negative
N5	Small	Rod	Single or in pair	Gram negative
N6	Small	Rod	Single or in pair	Gram negative
N7	Small	Rod	Single or in pair	Gram negative
N8	Small	Rod	Single or in pair	Gram negative

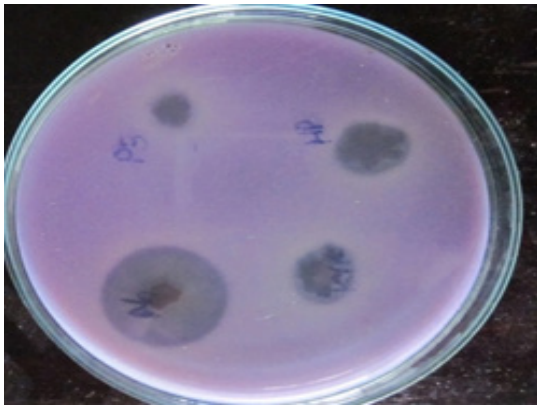

Figure 1: Phosphate solubilization by isolate P6.

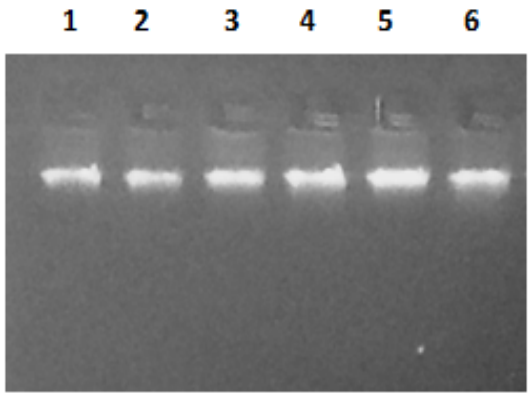

Figure 2: P8 Genomic DNA Loaded on 1% Agarose Gel. Lane Description: 1. Isolate P5 2. IsolateP7 3. IsolateN8 4. solateN4 5. IsolateN6 6. IsolateP6

Table 4: Colonial characteristics of NFB and PSB.

Isolates	Size	Shape	Margin	Texture	Elevation	Consistency	Opacity	Pigmentation
P1	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Brownish black
P2	Big	Round	Irregular	Rough	Flat	Dry	Opaque	None
P3	Intermediate	Round	Irregular	Rough	Convex	Dry	Opaque	None
P4	Small	Round	Entire	Smooth	Pin pointed	Moist	Transparent	None
P5	Small	Round	Entire	Smooth	Raised	Moist	Transparent	None
P6	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Yellowish white
P7	Big	Round	Undulate	Rough	Raised	Dry	Opaque	Pink
P8	Big	Irregular	Lobate	Rough	Flat	Dry	Opaque	Pink
N1	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Clear White
N2	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Dull white
N3	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Yellowish white
N4	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Clear white
N5	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Dark brown
N6	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Creamy white
N7	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Clear white
N8	Big	Round	Entire	Smooth	High convex	Moist	Translucent	Light brown

Table 5: Production of IAA and Ammonia by isolated bacteria.

Isolates	Concentration of IAA (µg/ml)						Ammonia production
	After 24 hrs	After 48 hrs	After 72 hrs	After 24 hrs	After 48 hrs	After 72 hrs	
N1	0.005	0.032	0.055	0.0013	0.0081	0.0139	+
N2	0.045	0.148	0.157	0.0114	0.0037	0.0040	+
N3	0.007	0.053	0.077	0.0018	0.0134	0.0195	+
N4	0.008	0.164	0.189	0.0020	0.0415	0.0478	+
N5	0.004	0.032	0.056	0.0010	0.0081	0.0142	+
N6	0.051	0.121	0.142	0.0129	0.0306	0.0359	+
N7	0.007	0.025	0.081	0.0018	0.0063	0.0205	+
N8	0.023	0.067	0.72	0.0058	0.0170	0.1822	+

Table 6: Comparative study of cotton plant growth.

Name of isolates	Root height (cm)	No. of root hair	No. of leaves	length of leaves (cm)	Width of leaves (cm)	Shoot height (cm)	No. of Branches of shoot	Fresh weight of leaves (gm)
CONTROL	14±1.05	39	8	4.2±0.26	3.53±0.84	18±1.18	9	1.66±0.21
N1	24.5±2.18	47	8	4.86±0.49	5.4±0.52	21.6±0.53	11	2.49±0.17
N4	24.46±1.75	49	8	4.96±0.70	4.93±0.47	22.53±0.45	10	2.25±0.35
N2	23.83±1.36	57	8	4.56±0.65	4.96±0.60	24.66±0.50	9	2.68±0.23
N8+P6	26.36±1.27	66	9	6.26±0.38	5.63±0.15	26.06±3.52	11	2.93±0.23
N3	22.73±1.10	66	8	5.06±0.23	4.83±0.25	22.16±0.91	10	2.43±0.12
N2	24.63±1.46	61	8	4.9±0.26	4.73±0.68	21.86±1.91	11	2.66±0.20
N5	22.56±1.16	66	7	4.9±0.10	4.7±0.17	20.6±0.87	10	2.34±0.11
P3	23.13±1.05	52	8	5.83±0.57	5.6±0.46	21±1.56	9	2.42±0.55
P4	21.93±0.80	70	9	5.53±0.32	5.2±0.30	22.63±2.10	11	2.81±0.55
N8	22.7±1.48	67	8	5.66±0.15	5.56±0.42	24.43±1.50	9	3.12±0.54
P5	22.8±1.30	57	8	5.66±0.29	5.73±0.25	21.06±1.68	10	2.57±0.51

Data are average values of three replicates ± SD.

Table 7: Estimation of chlorophyll.

Sr. No.	O.D. at 663 nm	O.D. at 645 nm	Total chlorophyll (mg/l)	Chlorophyll 'a' (mg/l)	Chlorophyll 'b' (mg/l)
control	1.512	1.113	34.6	16.2	18.41
N8+P6	2.248	2.134	61.14	22.81	38.34
N4	2.207	2.037	58.85	22.55	36.96
N2	1.947	1.394	43.77	20.98	22.81
N1	2.134	1.958	56.67	21.83	34.85
N8	2.248	2.11	60.65	22.87	37.7
P3	2.102	2.052	58.31	21.18	37.15
P4	2.207	1.998	58.06	22.65	35.42
N3	2.248	2.114	60.73	22.86	37.89
P5	1.064	0.843	25.6	11.24	14.37
N5	2.169	2.009	57.98	22.14	35.86
P6	1.817	1.308	40.99	19.56	21.45

induce the growth of plant by producing phytohormone IAA which promotes the growth of cotton plant.

It has been recorded that the out turn of chickpea plants was better on inoculation of N_2 fixers and phosphate solubilizers.[18] After 50 days in nursery condition growth of cotton plants were measured. Co-inoculation with free living nitrogen fixer N8 and Phosphate solubilizer isolate P6showed significant effect on plant growth. The data of plant growth promotion is shown in Table 6. It was experimentally determined co-inoculation of NFB and PSB was more effective than single inoculation of culture that providing a more balanced nutrition for plants.[19] Growth of Cotton plant by combined inoculation free living nitrogen fixing bacteria and phosphate solubilizing bacteria can be improved. Carrier based biofertilizer having beneficial effects which overcome the limitations of use of chemical fertilizers.[20]

It was determined that cotton plant inoculated with isolate P8 shows more amount of chlorophyll (61.14

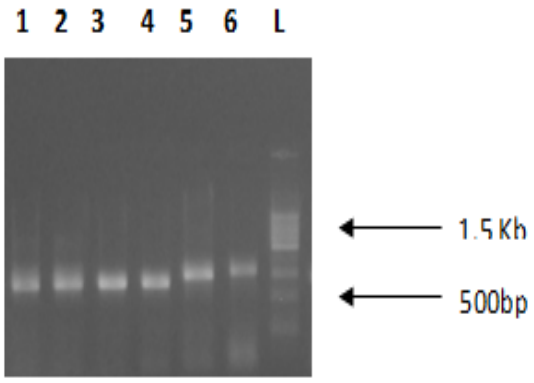

Figure 3: PCR Product loaded on 1% Agarose Gel. Lane Description: 1. Isolate P5, 2. IsolateP7, 3. IsolateN8, 4 IsolateN4, 5. IsolateN6, 6 IsolateP6 and 7. 500 bp ladder

Figure 4: Inoculation effects on Cotton Plant growth.

mg/l) than all of others. While isolate 9 (60.73 mg/l) and isolate 6 (60.65 mg/l) were moderately effective. It can provide more nitrogen content and nitrogen fixation to the plant due to high chlorophyll content[21] and N_2 fixation.[22] Isolate 4 was less effective than others (43.77 mg/l). Co-inoculation with isolate 3 and 7 is more efficient (57.98 mg/l).

CONCLUSION

This study revealed that inoculation of free living nitrogen fixing bacteria and phosphate solubilizing bacteria either alone or in combination, benefited in increasing growth of *Gossypium hirsutum*. They could effectively increase shoot height and root height. Combination of nitrogen fixer and phosphate solubilizer show more beneficial effect on root growth of cotton plant. All bacteria were isolated from rhizospheric soil and they have ability to produce plant growth promoting substances like phytohormone. So plant growth was induced by the exerted phytohormone.

From all isolated bacteria most effective one is isolate 5. Therefore it can be used as a potent biofertilizer. It will be beneficial in sustainable agriculture and effective in Semi-arid tropical area.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ABBREVIATIONS

PSB: Phosphate solubilizing bacteria; **IAA:** Indole 3-acetic acid; **Po:** Organic phosphorus; **Pi:** Inorganic phosphorus; **g:** Gram; **rpm:** Rotation per minute; **AMA:** Ashby's Mannitol Agar; **h:** Hour; **PVK:** Pikovskaya's medium; **SE:** Solubilization efficiency; **SI:** solubilization index; **LB broth:** Luria Burtani broth; **hrs:** Hours; **O.D.:** Optical density; **M:** Molar; **$FeCl_3$:** ferric chloride; **nm:** Nanometer; **mg:** Milligram; **$CaCO_3$:** Calcium Carbonate; **ml:** Milliliter; **µg:** Microgram.

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