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Enhance plant health by using of phosphate solubilizing *Bacillus sp.* as ecofriendly Bio fertilizer

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Abstract:

In the rhizosphere, bacterial population promote plant health by acquisition of essential micro-macro nutrients, production of growth hormones, phosphate solubilization (PS), ammonia production and inhibition against soil borne pathogens. In our present study, we have isolated (Ari_D) and characterized a plant growth promoting rhizospheric (PGPR) Bacillus sp. bacterium from Occimintum sanctum rhizospeheric zone. This articles aims to summarized, the overall performance of inoculated treatments reveled that soil treatments of Corchoruscapsularis and Cicer arietinum L., with Ari_D could give significantly better results (seeds germination, root-shoot: height-weight and leaves) in respect of control. Ari_D solubilized phosphate at a concentration of 170 µg/mL in PKV broth after 5 days of incubation. The biochemical test, growth under stress condition (temperature and pH) were also looked into. In other study focused, IAA, cellulose-protease (Ep), ammonia production and antibiotic test (aT) were highly performed by Ari_D. Its potentiality as in developing bio fertilizer and a good phosphate solubiliser in Agriculture.

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Keywords:

Bacillus sp.; PGPR; PS; aT; Ep; *Occimintum sanct*

Introduction

Plant growth promoting rhizobacteria (PGPR) accounts for about 2-5% of total the rhizobacteria involved in plant growth promotion (Antoun*et al.,* 2001;Joshi *et al.,* 2011) . Such PGPR use one or more direct or indirect mechanisms to improve the growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth. Among these, phosphate solubilization, biological nitrogen fixation, improvement of other plant nutrients uptake, and phytohormone production like, indole-3-acetic acid are some of the regulators that profoundly influence plant growth (Keyser *et al.,* 1997). PGP affect plant growth in two different ways, indirectly or directly. The direct promotion of plant growth by PGP entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Vessey, 2003; Glick, 1995). Biofertilizers with new concept of PGP have an important role in crop nourishment as well as enhancement of soil fertility by means of several mechanisms like biological nitrogen fixation, solubilization of native phosphorus, acquisition of essential macro and micronutrients with mineralization of organic manures-organic matter, production of plant growth promoting substances, disease control by suppression of soil borne phytopathogens and acceleration of other microbial activities in the soil (Elkoca*et al.,* 2008; Verma*et al.,* 2010). The immediate response to soil inoculation with associative, nonsymbiotic PGPB (but also for rhizobia) varies considerably depending on the bacteria, plant species, soil type, inoculant density and environmental conditions. In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively (Bashan *et al.,* 1995; Button, 1976; Carrillo *et al.,* 1997). Chen, 2006; Note that phosphorus (P) is an essential macronutrients often limiting the plant growth due to its low solubility and fixation in the fertile land. Improving soil fertility by releasing bound phosphorus by microorganism's inoculants is an important aspect for increasing crop yield (Ahlawat*et al.,* 2005; Cattelan*et al.,* 1999). Phosphorus deficiency is a major constraint to crop production due to rapid binding of the applied phosphorus into fixed forms not available to the plants (Ho¨flich*et al.,* 1994). Phosphorous release from insoluble phosphate reported for several soil microorganisms has been attributed mainly to the production of organic acids and their chelating capacity (Goldstein, 1995; Kloepper*et al.,* 1980).The aim of this preliminary study was to isolate and identify bacteria from tall fescue roots and select isolates that have the ability to produce IAA, release ammonia and solubilize Phosphate Selected bacterial isolates harboring all three traits will be considered for seed treatment to improve establishment of Agriculture and Biotechnology.

Materials and Methods

Isolation and biochemical characterization of Ari_D

Ari_D isolated from rhizospheric zone of *Occimintum sanctum*, were characterized on the basis of morphological and biochemical test describe by Dubey, 2007. Soil sample is added in double distilled water (0.50gm/10mL dH_2O) and shaking at 250 rpm. The soil sample was serially diluted and 100µl of dilution sample (10^{-6}) was plated onto PYD medium (Bacterial Peptone: 2.00gm, Yeast extract: 2.00gm,

Dextrose: 5.00gm, Agar: 15.00gm, Distilled water: 1000mL). For the confirming, *Bacillus sp., Ari*$_D$was streaked onto *Bacillus* isolation medium. Then preservation for future added 20% glycerol in pure culture and store at -20^{0}C.

To determine the carbohydrate utilization, assays were performed with sucrose, dextrose, maltose with phenol red as indicator. The ability to use glucose, lactose, and sucrose was measured using Triple Sugar Iron Agar (TSIA) slant, the production of H_2S and gas were also observed (Dubey, 2007).

The utilization of citrate as only carbon source was measured using Simmons Citrate Agar slants. To detect the conversion of pyruvic acid in acetone, the Methyl Red (MR) test was used and to see the fermentation of glucose and its transformation to pyruvic acid, we used Voges-Proskauer (VP) test (Aneja, 2003).

The purpose is to see if the microbe has catalase, a protective enzyme capable of destroying the dangerous chemical hydrogen peroxide. Growth from an overnight culture of the microbe is smeared on a microscope slide. A drop of 3% hydrogen peroxide is added (Rollins, 2009). If copious bubbles are observed, the microbe is positive for catalase.

Phenylalanine deaminase production test is positive, prepare phenylalanine agar plate and streak with culture and incubate at 37^{0}C for 48 hours. Add 4-5 drops of 10% $Fecl_3$, after 1 minute examine the colour.

Ari$_D$was analyzed for its ability for PGPR

Indol and IAA production

After incubation (at 28^{0}C for 48 hrs.) of Ari$_D$, added 1 mL of Kovac's reagent, 3 mints. Result is appeared (MacFaddin, 1980). For confirm, indol is diffused, and a impregnated with oxalic acid white paper strip into the tube. IAA production by Ari$_D$was assayed calorimetrically using $Fecl_3$ reagents.

Enzyme production (Ep)

Protease activity (casein degradation) was determined from clear zone in skim milk agar (skim milk powder-100.0g, peptone-5.0g, agar-15.0g, P^H-7.2) (MacFaddin, 1980) (fig. 3. A). Colonies were screened for cellulose activity by plotting on CMC (Carboxy Methyl Cellulose) agar and Czapek-mineral salt medium ($NaNO_3$-2.2g, K_2HPO_4-1.0g, $MgSO_4.7H_2O$-0.7g, KCl-0.5g, CMC-5.0g, peptone-2.5g, agar-18.0g, distilled water-1000ml, P^H-6.0) (Cattelan *et al.,* 1670-1680).

Screening of the Ari$_D$screening phosphate solubilizing isolates

Phosphate solubilizing ability of the Ari$_D$ was evaluated on Pikovskaya (PVK) medium incorporated with tri-calcium phosphate (TCP) [Ca_3 $(PO_4)_5$] as insoluble phosphate. Quantitative phosphate solubilization was estimated by Fiske and Subbarow method.

For confirming, The isolates was tested in vitro for their phosphate solubilization activity following the method described by Donate-Correa et al. (2004) on Pikovskaya agar medium containing the ingredients : Glucose :5gm, $Ca_3(PO_4)_2$:5gm, $(NH_4)_2SO_4$:0.5gm, Yeast Extract :0.5gm, $MgSO_4.7H_2O$:0.2gm, NaCl :0.1gm, $MnSO_4$:0.002gm, $FeSO_4$:0.002gm and Agar :15gm (Zhang *et al.,* 2002). The medium was autoclaved at 121°C & 15lb/inch^{2}for 15 minutes. About 20 ml of the molten agar medium was

poured into each petridish and allowed to solidify before inoculating the isolates. A 24 hrs. Broth culture was spot inoculated on the petridishes in triplicate using sterile loop and incubated at 30 ± 2°C for 5-7 days. Bacterial colonies that formed clear zones (haloes) were considered as phosphate solubilizers and clear zone diameters were measured in cm.

Ammonia production

Detection of ammonia production was done by adding 1.5 ml Nessler's reagent to a 48hours culture in 4% peptone broth and recording the presence of the yellowish brown color (Glick, 1991).

Plant growth promoting by Ari_D

Seeds collection and sterilization

All seeds are collected and used for PGP were obtained from PTcL, OIST, Paschim Medinpur, West Bengal-721102 India. The seed were surface sterilization in 70% ethanol for 20 sec and 2% sodium hypochloride for 5 min., allows to 5 times wash by sterile distilled water (Zhang *et al.,* 2002).

Seeds summary

We selected two different types of seeds for the PGP that's are *Cicer arietinum L.* and*Corchoruscapsularis.*

Chick pea (*Cicer arietinum L.*): Plant Description (PD)

The chickpea or chick pea (*Cicer arietinum*) is a legume of the family *Fabaceae*, subfamily *Faboideae*. It is also known as gram, (Encyclopædia Britannica, 1880; USDA GRIN Taxonomy) or Bengal gram, garbanzo (Garbanzobean) or garbanzo bean and sometimes known as Egyption pea, (USDA GRIN Taxonomy-2014)ceci, cece or channa. Its seeds are high in protein.

Chickpeas are a type of pulse, with one seedpod containing two or three peas. It has white flowers with blue, violet or pink veins ('Garbanzobean'). Chickpeas are grown in the Mediterranean, western Asia, the Indian subcontinent, Australia, the Palouse region, and the Great Plains (Maulik, 2001).

PD: WhiteJute (*Corchoruscapsularis*)

Jute is a long, soft, shiny vegetable fiber that can be spun into coarse, strong threads. It is produced from plants in the genus *Corchorus*, which was once classified with the family *Tiliaceae*, more recently with *Malvaceae*, and has now been reclassified as belonging to the family S*parrmanniaceae* (Moses, 2004). "Jute" is the name of the plant or fiber that is used to make burlap, Hessian or gunny cloth (Jute-Retrieved 13 June 2007).

The plants are tall, usually annual herbs, reaching a height of 2–4 m, unbranched or with only a few side branches. The leaves are alternate, simple, lanceolate, 5–15 cm long, with an acuminate tip and a finely serrated or lobed margin (International Jute Study Group –IJSG, 2013).

The flowers are small (2–3 cm diameter) and yellow, with five petals; the fruit is a many-seeded capsule. It thrives almost anywhere, and can be grown year-round (Roy, 2002). Jute fiber is 100% bio-degradable and recyclable and thus environmentally friendly. Jute has low pesticide and fertilizer needs (Srinivasan, 1999). It is a natural fiber with golden and silky shine and hence called The Golden Fiber. It is the cheapest vegetable fiber procured from the bastor skin of the plant's

stem (Shenai, 2003). It is the second most important vegetable fiber after cotton, in terms of usage, global consumption, production, and availability(Srinivasan, 1999).

Soil treatments and incubation

The soil sample was autoclave at 15 lb/inch^2 pressure for 15 mints and bacterized separately in sterilized 50ml culture tubes for 10 days. 250ml of bacteria inoculums, containing 3×10^8c.f.u/ml, are separately centrifuged at 7500rpm for 10 mints (cooling centrifuged, REMI C-24 BL, India) supernatant are discarded (Zehnder*et al.,* 2000; [2]Zehnder et al., 2000).

Bacterial pallets were washed four times with carboxy-methylcellulose (MERCK-MF8M581422) solution (1mg CMC /100ml sterile distilled water) (Ramamoorthy*et al.,* 2002). Then the bacterial solution is added separately with the sterilized soil sample kept in culture tubes (Abdul *et al.,* 1973).

After 5days incubation, all plant PGP activities by Ari$_D$ are assessed and results were calculated for this all set.

Stress condition growth

ARI$_D$growth under temperature

In order to find out the optimal conditions for growth of the bacterial isolate, the effect of different temperatures levels on the development and antagonism of antagonistic bacteria was examined. Three temperatures (28, 37, and 60°C) were mainly used (Al-Hinai*et al.,* 2010).A bacterial suspension (200 µl) was placed in a tube containing 5 ml Kings's B broth. The tube was incubated at the predetermined temperatures for 48 hours after which bacterial growth was determined by measuring the optical density (O.D.) at 660 nm in UV/Visible Spectrophotometer (Ghosh *et al.,* 1996).

Ari$_D$growth under pH

The ability of the strain to adapt to pH, stress may be important for the several of the bacteria during drought and different physiological condition (Wei *et al.,* 1996). In order to find out the optimal condition for growth of the Ari$_D$, the effect of pH levels on the development of growth was examined (Yan *et al.,* 2003).

Antibiotic test (aT)

Drug/Antibiotic test of Ari$_D$ to various antibiotic (HiMedia, India) was assayed by Kirby-Bauer disc diffusion method on Muller-Hinton agar (HiMedia, India) plates describe by Bauer *et al.,* 1966. This test results and antibiotic concentration are given in table- 2.

Results and Discussion

Morphological

Biochemical characterization of Ari$_D$

The isolate was gram-positive, rods shaped and non-endospore forming. By biochemical characterization, the Ari$_D$ was identified as belonging to the genus *Bacillus sp*. Biochemical test in order to determine the physiology of the isolated strain, a series of biochemical tests were performed (Tab-1).

Morphology: gram-positive	**Shaped: rods**
Endospore Formation: *-ve*	*Aerobic Bacteria*

BIOCHEMICAL TEST

MR-VP: +/+	Ammonia: +	Catalase: +
Oxidase: +	Phe-Ala: +	Citrate utilization: +
Production:		**Degradation:**
Lactose: ±	Maltose: +	Starch: +
Glucose: AG	Frucose: ±	Cascin: +
Sucrose: +	Dextrose: +	Urea: +

AG: acid+ gas, +: positive, -: negative, ±: variation result, Phe-Ala: Phenylalanine deaminase

Table 1. Taxonomic Characteristics

Anti-bio gram study of Ari_D

The susceptibility of these Ari_D tested against 8 different types of antimicrobial agents and resistance show that on 3 different types of antibiotic (Tab-2)

Anti-microbial Agents	*Ampicillin*	*Amoxicillin*	*Cephalothin*	*Vancomycin*	*Tetracycline*	*Methicillin*	*Chloramphenicol*	*Penicillin*	*Clindamycin*	*Azithromycin*	*Erythromycin*
Result	†	†	†	†	†	†*†	†	†*†	†	†*†	†

†: Sensitive, ††: Resistance*

Table 2. Antibiotic test Performed by Ari_D

Phosphate solubilization

Solubilization of tri-calcium phosphate was detected in Pikovskaya s Agar (Johri*et al.,* 1999). Ari_D was streaked on the surface of Pikovskaya agar medium and phosphate solubilizing activity was estimated after 5 days at a concentration of 170 µg/mL of incubation at room temperature [fig-2]. For confirm, Phosphate solubilization activity was determined by the development of the clear zone around bacterial colony.

Ari_Dgrowth under temperature and pH

To determine the effect of temperature (0-60^{0}C) and pH (6-9), the extent of growth of the Ari_D was determined by measuring at $O.D._{660}$ in UV/Visible Spectrophotometer, spent culture at the aforementioned conditions. Results are shown in fig 1. A & 1.B

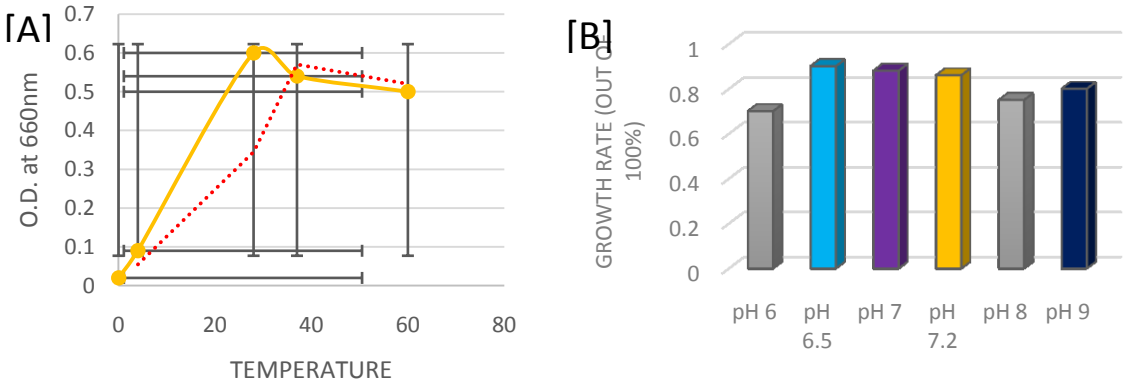

Figure 1. [A]: Ari_D growth in temperature**Figure 1.[B]:** Growth under pH performed by Ari_D

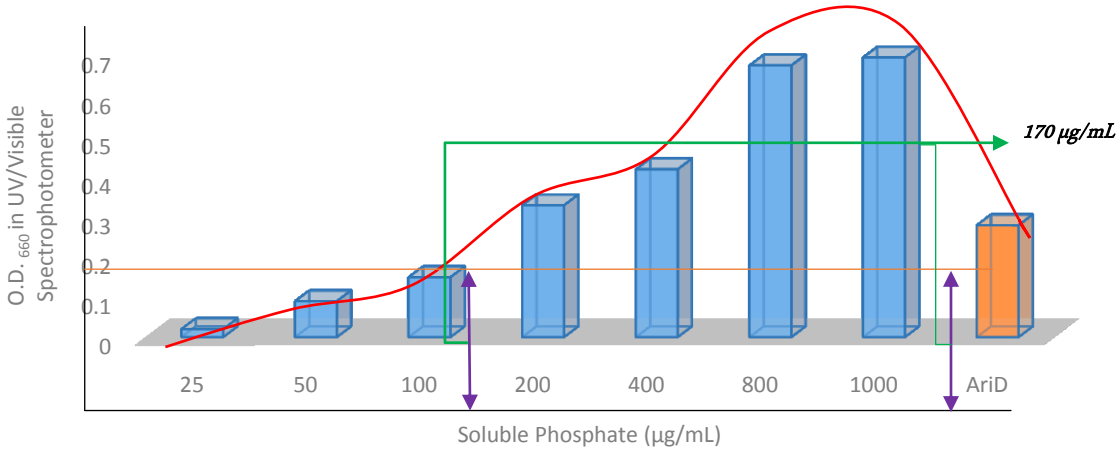

Figure 2. Phosphate solubilization by Ari_D

After 5 days plant growth promotion of *Cicer arietinum L.* ByAri_D

The overall performance of inoculated treatments revealed that soil treatment of chickpea (Cicer arietinum L.) with inoculant Ari_D, could give significantly better performance in respect of control. Results are shown in Table-3

Sl. No.	Seed sample name	*Shoot Height (cm)*	*Shoot Weight (mg)*	*Root Height (cm)*	*Root Weight (mg)*
1.	Control	3.60±.0.20	0.870	1.80±0.20	0.190
2.	Ari-C -I	7.80±0.10	0.980	2.90±0.20	0.240
3.	Ari-C-II	6.90±0.20	0.960	2.70±0.10	0.210
4.	Ari-C-III	7.00±0.20	0.970	2.90±0.00	0.220
5.	Ari-C-IV	7.50±0.20	0.980	2.50±0.20	0.240

Control, Ari-C-I, II, III, and IV: seed sample of Cicer arietinum L.

Table 3. Plant Growth Promoting treatments result in chickpea (Cicer arietinum L.)

PGP activity by ARI_D on whitejute (*Corchoruscapsularis*):

In, anotherexperiment on White Jute (*Corchoruscapsularis*) by inoculant Ari_D, we take 5 most well plant from jar, then could give a unique results in respect of on White Jute (*Corchoruscapsularis*) control. Results are shown in Table 4.

Sl. No.	Seed sample name	*Shoot Height (cm)*	*Shoot Weight (mg)*	*Root Height (cm)*	*Root Weight (mg)*
1.	Control	2.10±0.00	0.490	1.70±0.20	0.170
2.	Ari-J-I	5.10±0.20	0.880	3.20±0.00	0.240
3.	Ari-J-II	5.00±0.00	0.860	3.10±0.10	0.220
4.	Ari-J-III	4.90±0.20	0.890	3.40±0.30	0.250
5.	Ari-J-IV	5.10±0.10	0.880	3.20±0.10	0.250

Control, Ari-J-I, II, III, and IV: seed sample of Corchoruscapsularis

Table 4. PGP treatments results in on White Jute (*Corchoruscapsularis*)

***Figure 3. A**: Clear zone in casein hydrolysis agar for Protease activity by Ari_D, B: Control of Jute, C: Ari_D Apply in Jute*

Conclusion

In our study, isolated bacterium, Ari_D is good phosphate solubilizer with an unbillable plant growth promoting bacterium. The biochemical characterization and morphological study of the isolate shows that its Bacillus sp. Bacterial, which have a protease activity show in CH agar. The fact that the *Occimintum sanctum*rhizosphere bacteria to be positive for various PGPR characteristics suggests that the isolates have better potential for agriculture , field testing and application in improving of plant health.

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