

ISOLATION AND CHARACTERIZATION OF PGPR ASSOCIATED WITH CAULIFLOWER ROOTS AND ITS EFFECT ON PLANT GROWTH

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ABSTRACT

Four PGPR isolates of bacteria, designated as AK₁, AK₂, AK₃ and AK₄ were successfully isolated and characterized. To investigate the effects of PGPR isolates on the growth of cauliflower, a pot culture experiment was conducted. Isolates AK₁, AK₂, AK₃ and AK₄ showed the production of indole acetic acid (IAA), whereas the isolates AK₂, AK₃ and AK₄ isolate were able to solubilize phosphorus. All the isolates resulted in a significant increase in plant height, root length and dry matter production of shoot and root of cauliflower plant compared to the control. PGPR were characterized based on its cultural, morphological and biochemical characters. Pot experiments showed that the PGPR treated seeds grew better than the untreated seeds. The present work suggests that the use of PGPR isolates AK₁, AK₂, AK₃ and AK₄ as inoculants biofertilizers might be beneficial for cauliflower cultivation as they enhanced growth of cauliflower by inducing IAA production and phosphorus solubilization.

INTRODUCTION

Cauliflower is one of the several vegetables in the species *Brassica oleracea*, in the family Brassicaceae. It is an annual plant that reproduces by seed. Typically, only the head (the *white curd*) of aborted floral meristems is eaten, while the stalk and surrounding thick, green leaves are used in vegetable broth or discarded.

It is low in fat, but high in dietary fiber, foliate, water and vitamin C, possessing a high nutritional density. It contains several phytochemicals, common in the cabbage family that may be beneficial to human health. Sulforaphane, a compound released when cauliflower is chopped or chewed, may protect against cancer. Other glucosinolates, Carotenoids, Indole-3-carbinol, a chemical that enhances DNA repair and acts as an estrogen antagonist, slowing the growth of cancer cells. A high intake of cauliflower has been associated with reduced risk of aggressive prostate cancer.

PGPR have been subjected to numerous investigations focused on biotechnological applications in agriculture, horticulture, forestry and environmental protection (Zahir *et al.*, 2004). Early studies in the 1950's began with a focus on nitrogen fixing bacteria. Since then, a large number of PGPR belonging to different bacterial classes and genera with multifunctional traits have been described (Rodríguez-Díaz *et al.*, 2008). PGPR strains are broadly distributed among many taxa including *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* (Tilak *et al.*, 2005), such that

determination of the background population size and activity of PGPR in resident microbial communities is difficult to assess based on analysis of microbial community structure or abundance of a particular taxonomic group. The main aim of biotechnological development based on PGPR has been to develop soil inoculants that can contribute to sustainable agriculture, there by diminishing the need for use of chemical fertilizers and pesticides (Adesemoye and Kloepper, 2009).

PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, and some have been commercialized (Dey *et al.*, 2004; Herman *et al.*, 2008). Under salt stress, PGPR have shown positive effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots and roots, yield, and plant growth (Kloepper *et al.*, 2004). Another major benefit of PGPR is to produce antibacterial compounds that are effective against certain plant pathogens and pests.

The chemical fertilizer is the most important input required for cauliflower cultivation. In order to make it's cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR that can biologically fix nitrogen, solubilize phosphorus and induce some substances like Indole acetic acid (IAA) that can contribute to the improvement of cauliflower growth. Recently, there is a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops (Thakuria *et al.*, 2004).

Thus the aim of this study was to determine the effect of PGPR

strains that are compatible with cauliflower.

MATERIALS AND METHODS

Isolation of PGPR from cauliflower rhizosphere

Sample collection

Soil samples were collected from the rhizosphere of two month-old cauliflower plants. The rhizosphere was dug out with intact root system. The samples were placed in plastic bags and stored at 4°C.

Bacterial isolation

Ten grams of rhizosphere soil were taken into a 250mL of conical flask and 90mL of sterile distilled water was added to it. The flask was shaken for 10min on a rotary shaker. One milliliter of suspension was added to 10mL vial and shaken for 2min. Serial dilution technique was performed upto 10⁻⁷ dilution. An aliquot (0.1mL) of this suspension was spread on the plates of Luria Bertani (LB) agar medium. Plates were incubated for 3 days at 28°C to observe the colonies of bacteria. Bacterial colonies were streaked to other LB agar plates and the plates were incubated at 28°C for 3 days. Typical bacterial colonies were observed over the streak. Well isolated single colony was picked up and re-streaked to fresh LB agar plate and incubated similarly.

Identification of isolates

The bacterial strain was studied for cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology (Hoit *et al.*, 1989).

Cultural characteristics

All the isolates were streaked on LB agar plates. After 3 days of incubation, different characteristics of colonies such as growth, form and colour.

Morphological characteristics

The suspected organisms were subjected to Gram's staining (Vincent, 1970). The bacteria which retained the primary stain called gram + ve while those that lost the crystal violet and counter stained by safranin were referred as gram – ve.

Biochemical characterization

Methyl Red test

Tubes containing the sterilized MR –VP broth were inoculated with isolated bacterial strains and one tube as uninoculated comparative control. Then all the inoculated and uninoculated tubes were incubated at 37°C for 48h. After 48h of incubation 5 drops of methyl red indicator was added to each tube. When methyl red was added, it remained red which was indicative of positive test while turning of methyl red to yellow indicated the negative test.

Hydrogen sulfide production test

The inoculum was stab inoculated onto the SIM agar medium and incubated at 37°C for 48h. Blackening of the culture medium indicated a positive test while no change in colour of the medium indicated a negative test.

Catalase test

The hydrogen peroxide solution (2%) was added to the culture on a slide. The release of free oxygen bubbles indicated a

positive test.

Casein Hydrolysis test

The casein agar plate was streaked with the isolated organism in sterile condition. After that the plates were incubated at 37°C for 24-48h in an inverted position (Seeley and Vandemark, 1970). A clear zone surrounding the bacterial growth indicated the positive reaction for extracellular caseinase secretion while absence of clear zone indicated the negative reaction.

Gelatin Hydrolysis test

Gelatin agar medium was melted and cooled to 45-50°C to pour into four sterile petri dishes. All the inoculated and uninoculated (control) plates were incubated at 37°C for 4 -7 days. After incubation, plates were placed in refrigerator at 4°C for 24h. The incubated agar plates were flooded with mercuric chloride solution and the plates were allowed to stand for 5 to 10 minutes. Then plates were examined for clearing zone around the line of growth (Blazevic and Ederer, 1975).

Indole test

The isolated organism was inoculated into tryptone broth the inoculated and uninoculated (control) tubes were incubated at 37°C for 48h. After incubation, Kovac's reagent was added to inoculated and control tubes. Development of cherry red colour at the top layer in the form of ring indicated the positive test while its absence indicated the negative test.

Ammonia production test

Fresh culture were inoculated into 10mL peptone water and incubated for 48-72h at 37°C. Nessler's reagent (0.5mL) were added to each tube and development of yellow to brown colour was considered as positive test while no change in colour showed negative test (Cappuccino and Sherman, 1992).

Phosphate solubilization test

The bacterial isolates were inoculated into plates with sterilized Pikovskaya medium containing tricalcium phosphate and incubated at 30°C for 72h. Formation of clear zone around the colony indicated the phosphate solubilization by the bacteria (Pikovskaya, 1948).

Growth under different temperature condition

Cultures (24h old) of the isolated bacterial strains were streaked on LB Agar plates and incubated for 24-48h at 4°C, 12°C, 28°C and 37°C. The change in growth and colour were observed.

Pot experiment

A soil sample was collected from the plots where cauliflower was produced for the past several years. The soil was sieved and mixed with thoroughly washed and dried sand in a proportion of 3:1 (soil: sand). The soil and sand mixture was then autoclaved for 15 mins at 121°C. A hand full seeds of cauliflower were surface sterilized with 0.5% (w/v) HgCl₂ at room temperature and thoroughly washed with sterilized distilled water (Siriskandarajah *et al.*, 1993). Then 24h fresh isolated bacterial cultures were inoculated into 10mL LB broth and incubated for 24h at 37°C and 12 sterilized seeds were added to each tube. After 24h of incubation, 5 seeds were sown to each of the 4 pots and control seeds were incubated

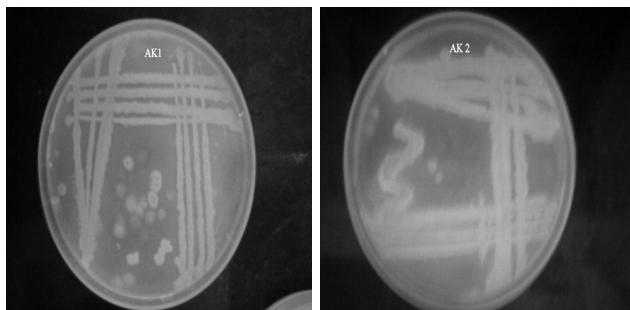


Figure 1: Gelatin hydrolysis of the Isolate AK1

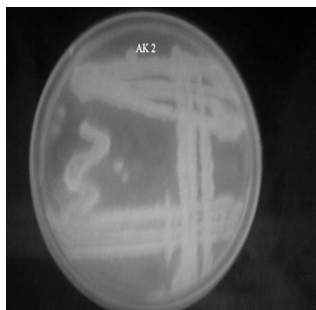


Figure 2: Gelatin hydrolysis of the Isolate AK2

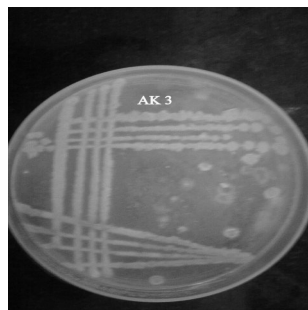


Figure 3: Gelatin hydrolysis of the Isolate AK3

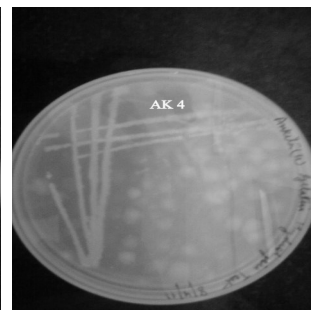


Figure 4: Gelatin hydrolysis of the Isolate AK4



Figure 5: Effect of PGPR isolates on growth of Cauliflower plants (A: Control, B: AK1 treatment, C: AK2 treatment, D: AK3 treatment, E: AK4 treatment)

into sterilized distilled water. All pots were watered daily. Harvest occurred 4-5 weeks after beginning of the experiment.

RESULTS

Isolation of PGPR strains from cauliflower rhizosphere

To isolate the PGPR strains from cauliflower field soil samples were collected and inoculated on LB agar media. They were designated as AK₁, AK₂, AK₃ and AK₄ and were subjected to cultural, morphological, biochemical characterization.

Cultural characteristics

The isolates AK₁, AK₃ and AK₄ were having smooth, irregular

and yellow colour colonies. AK₂ isolate was regular, smooth and light yellow colour colony. All the isolates were odourless (Table 1).

Morphological characteristics

The morphological characteristics of PGPR isolates widely varied. All the isolates produced rod shaped and were gram negative in reaction (Table 2).

Biochemical characteristics

Methyl- red tests

In MR test, on addition of methyl red indicator the isolate AK₂ and AK₄ showed positive test. While the isolate AK₁ and AK₃ showed negative test.

Hydrogen sulphide production test

All the isolates AK₁, AK₂, AK₃ and AK₄ indicated the positive test.

Catalase test

All the isolates AK₁, AK₂, AK₃ and AK₄ showed the catalase positive test.

Casein hydrolysis

The AK₁ isolate followed by AK₃ and AK₄ isolates were capable of producing a clear zone surrounding the bacterial growth *i.e.* positive reaction for extracellular caseinase secretion while absence of clear zone was observed in the AK₂ isolate *i.e.* negative test for casein hydrolysis.

Hydrolysis of gelatin, a protein (production of gelatinase)

A clear zone was observed around the bacterial growth in presence of mercuric chloride solution in the inoculated petri plates demonstrating the proteolytic hydrolysis of gelatin *i.e.* positive reaction was shown by all the isolates (Fig.1- 4).

Indole test

Isolates AK₃ was found to be good producer of IAA. On the contrary, AK₂ and AK₄ were found to be medium producers of IAA in comparison to the weak producer isolate AK₁.

NH₃ production test

The isolates AK₁, AK₂, AK₃ and AK₄ were capable of producing ammonia.

Phosphate solubilization test

The isolates AK₂, AK₃ and AK₄ were capable of solubilizing the phosphorus while AK₁ was not capable of solubilizing the phosphorus.

Growth under different temperature conditions

The growth of isolates on LB agar plates varied in temperature

Table 1: Cultural characteristics of the Isolates

Isolate	Elevation	Margin	Colour	Odour
AK ₁	Raised	Smooth	Yellowish	Odourless
AK ₂	Raised	Smooth	Yolk yellowish	Odourless
AK ₃	Raised	Smooth	Yellowish	Odourless
AK ₄	Raised	Smooth	Yellowish	Odourless

Table 2: Morphological characteristics of the Isolates

Isolate	Shape	Arrangement	Gram reaction
AK ₁	Rod	Staphylo	-ve
AK ₂	Rod	Strepto	-ve
AK ₃	Rod	Staphylo	-ve
AK ₄	Rod	Staphylo	-ve

(Table 4). The growth of all isolates was good in the temperature ranges of 28°C to 40°C. In addition, AK₃ and AK₄ isolates showed maximum growth at 40°C.

Pot experiment

The highest root length (4 cm) was recorded in AK₄ among the four isolates. The PGPR

isolates significantly affected the shoot length of cauliflower seedlings. Results reveal that shoot length increased in PGPR treated plants over uninoculated control (Fig. 5). The highest shoot length (11.8cm) was recorded in AK₃ among the four isolates. The highest fresh weight (0.067gm) was recorded in AK₃ among the four isolates.

A significant increase in dry matter of cauliflower seedlings was observed in response to PGPR isolates. The highest dry matter was recorded in isolate AK₃ (Table 5).

Table 3: Biochemical characteristics of PGPR isolates

Isolates	AK1	AK2	AK3	AK4
Methyl red test	-	+	-	+
H ₂ S production test	+	+	+	+
Catalase test	+	+	+	+
Casein hydrolysis test	+++	-	+	++
Gelatin hydrolysis test	+	+	+	+
Indole tests	+	++	+++	++
Phosphate sol.test	-	+	+	+
NH ₃ test	+	+	+	+
Identification	<i>Azospirillum Pseudomonas Azotobacter</i>			

Table 4: Growth under different temperature conditions

Isolates	Temperature			
	4°C	12°C	28°C	40°C
AK ₁	-	-	++	++
AK ₂	-	-	++	+
AK ₃	-	-	++	+++
AK ₄	-	-	++	+++

Table 5: Effect of PGPR isolates on growth of cauliflower plant

Isolate	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
Control	1.0	4.0	0.032	0.003
AK ₁	2.0	7.3	0.041	0.004
AK ₂	1.5	8.0	0.043	0.006
AK ₃	3.0	11.8	0.067	0.008
AK ₄	4.0	10.0	0.058	0.007

DISCUSSION

The isolates AK₁, AK₂, AK₃ and AK₄ induced the IAA production. On contrary, AK₂ and AK₄ were found to be medium producer of IAA in comparison to the weak producer isolate AK₁. It has been reported that IAA production by PGPR can vary among different species and it is also influenced by culture condition, growth stage and substrate ability (Mirza *et al.*, 2001).

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability. Reports were available on *Azotobacter spp.* isolated from different sources showed IAA production (Gonzalez-Lopez *et al.*, 2008; Jagnow, 1987; Nieto and Frankenberger, 1989). In the present study IAA production in *Azotobacter* isolates were in agreement with earlier reports. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Arshad and Frankenberger, 1993; Glick, 1995). Higher level of IAA production by *Pseudomonas* was recorded by other workers (Xie *et al.*, 1996).

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Pradhan and Sukla, 2006). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria was commonly found in the rhizosphere (Raghu and MacRae, 1966). The isolates AK₂, AK₃ and AK₄ were able to solubilize phosphate in the rhizosphere soil.

The PGPR isolates remarkably affected the germination of cauliflower seeds. The highest seed germination was recorded when seeds were pretreated with AK₃ isolate. A large body of evidence suggests that PGPR enhance the growth, seed emergence and crop yield (Dey *et al.*, 2004; Kloepper *et al.*, 2004; Kokalis-Burelle *et al.*, 2006; Herman *et al.*, 2008).

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