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Research Article

Isolation, Characterisation and Identification of Plant Growth Promoting Rhizobacteria from Cauliflower (*Brassica oleracea*)

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Abstract

Bio-fertilizers are currently being considered as effective and environmentally-safe alternatives to synthetic fertilizers. In recent years, the use of many Rhizobia in the growth of many plants has found commercial utilization in developing countries especially as growth promoters. This study was conducted to isolate Rhizobacteria from the rhizosphere, rhizoplane and endorhizosphere of cauliflower (*Brasicca oleracea*) collected from ChattaBakhtawar, Islamabad, Pakistan. Extraction of rhizobacteria was done and the composition was maintained accordingly as for biofertilizers. Luria-Bertini (LB) media was used to isolate rhizobacterial strains and the identification of rhizobacteria was done by studying many morphological characteristics of colonies growing on the media. These characteristics include Form, Elevation, Margin, Opacity and Colour; these were recorded periodically. For the confirmation of rhizobacteria; biochemical tests were performed. Gram staining was done for 14 strains CF-ES-4, CF-ES-5A, CF-ES-5B, CF-ES-5C, CF-ES-6A, CF-ES-6B, CF-RP-4C, CF-RP-4B, CF-RP-6, CF-RP-5A, CF-RP-4, CF-RS -4, CF-RS-5, and CF-RS-6: Among these, 11 strains were Gram negative while the 3 strains CF-RP-5A, CF-ES-5A and CF-RP-4 were Gram positive. The strains which were phosphate negative were: CF-RP-4C, CF-RS-8, CF-RP-4A, CF-ES-4, CF-RS-7, CF-ES-5C, CF-RP-6 while the CF-RP-5A CF-RP-5B, CF-ES-5B, CF-ES-5A, CF-RP-4B, CF-ES-6B, CF-RS-4 and CF-RS-6 strains were phosphate positive; these confirmed qualities of rhizobacteria. Taken together, the present investigation adds more information to current understanding of Nitrogen-fixing by rhizobacteria; and will be useful for the commercialization of these strains to improve the yield of economic plants like cauliflower. Thus, identification of strains to species level and field studies are recommended before adoption by farmers in agricultural field practices.

Key Words: *Rhizobacteria, Cauliflower, Luria-Bertini media, Gram staining, Phosphate*

INTRODUCTION

The rising global population coupled with the steady degradation of land resources, currently places a great strain on food production, hence the need to seek for alternate means to improve agricultural production systems. Synthetic fertilizers are employed as inputs to boost productivity however their use is been undermined due to their negative impacts on the soil such as increasing soil acidity (Turan *et al*. 2007); reduction of beneficial soil organisms population and interference with plant growth (Asuming-Brempong & Aferi, 2014). Also, the growing cost of synthetic fertilizers and the increasing demand for chemical-free foods have all necessitated the search for effective alternatives to boost food production.

Bio-fertilizers are currently being considered as effective and environmentally safe alternatives to synthetic fertilizers; as they have the potential to provide efficient suppression of plant diseases (Gerhardson, 2002), are inducers of disease resistance in plants (Cattelan et. al., 1999, Bergabus et. al. 2002 and Bais *et al*., 2004), control plant pathogens and also increase plant growth (Liu *et al*. 2007, Chen *et al* 2009, El Sayed et. al. 2014).

Phosphorus is among the major macronutrients that are essential for plant growth and development. However, its deficiency is wide spread due to various reasons including its fixation in soil leading to non-availability of soluble phosphate forms to plant roots; thus, resulting in symptoms such as severe stunting in plants. Phosphorus-solubilizing bacteria are able to convert insoluble forms of phosphorus into soluble phosphate forms. The use of these P- soluble bacteria are important for crop improvement in enhancing productivity through efficient utilization of soluble P-forms for use by plants and other organisms in soil; thus, their consideration as biofertilizers for commercial usage.

Rhizobacteria that are beneficial to plants are called Plant Growth Promoting Rhizobacteria (PGPR); they act by stimulating growth and suppressing disease incidence in plant (Kloepper et. al. 1980) and may be used to augment plant growth and development (Illmer & Schinner, 1992, Glick *et*

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al. 1999, Tripura *et al*. 2007, Liu *et al*. 2007, Gerhardt *et al*. 1999 and El Sayed et. al. 2014).

The objectives of this study were to isolate bacterial strains from the rhizosphere of Cauliflower (Brassica oleracea) rhizosphere; to identify the PGPR strains from the rhizosphere, rhizoplane and endorhizosphere of the Cauliflower and then characterize the isolates based on morphological properties.

MATERIALS AND METHODS

Collection of plant: The cauliflower plants were collected from ChattaBakhtawar Islamabad, Pakistan. The cauliflower plants at vegetative stage were uprooted with the whole root system along with adhering soil particles. Three samples were made i.e. Rhizosphere, rhizoplane and endorhizosphere and these samples were collected in aseptic plastic bags and stored at 40C till further processing.

Isolations: For the preparation of the rhizospheric sample, adhering soil was suspended in 1ml sterile distilled water and dilutions were made.

For rhizoplane sample, adhering soil was shed off by hand jerk so that only attached particles of soil remain on roots. one gram of attached soil was taken and dilutions were made.

For preparation of endorhizosphere sample, one gram roots were taken and were surface sterilized with ethanol. These roots were then crushed in a mortar and pestle and 9ml water was added in it to make 10x dilution. Then 1ml of this was taken to make further required dilutions.

For isolation of rhizobacterial strains the Luria–Bertini medium was used. All the above preceding ingredients were weighed according to 250 ml of distilled water for the preparation of 250ml LB medium. Conical flask of 500 ml volume was used. Salts were dissolved in distilled water and stirred using a magnetic stirrer. The pH was then adjusted to 7 by the addition of NaOH. When salts were completely mixed, flask was plugged tightly and then placed in autoclave for 30 minutes at 1210C and 15 psi pressure. After autoclaving, flask containing medium was taken out of autoclave and left it to cool down for some time. Then medium was poured into the pre-autoclaved Petri Plates and allowed to solidify; then these plates were inverted so the condensed water does not drop on the medium surface.

Preparation of serial dilution: For serial dilution, 9 screw capped McCartney bottles with 9 ml distilled water were autoclaved, numbered and were brought into the laminar flow hood for further processing. All the bottles were marked in a sequence from 1 to 9; the stored 1 gram of rhizosphere soil sample was added to bottle No.1 and was mixed vigorously using the vortex mixer to allow proper mixing of soil sample and microorganisms, then 1 ml of the solution from test tube no.1 was taken using a micro pipette and poured in into bottle No. 2, then the test tube No.2 was shaken vigorously and 1ml solution from this bottle was added to bottle No.3. Similar procedure was repeated for the remaining bottles to give 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8and 10-9 serial dilutions for use in the study.

Isolation by Spread Plating Method: For getting bacterial colonies, serial dilution No. 1, 2, 3, 4, 5, 6, 7, 8 and 9 were used. 0.1ml of solution from already shaken bottle No.1 was taken by micro pipette having a tip of 0.1ml volume and dropped on the media plate and then spread with the glass spreader. The plate was marked as Cauliflower Rhozoshere (CF-RS) 10-1, after each spreading the spreader was sterilized and this procedure was repeated for the rest of bottles and plates 10-2-10-9.

Incubation

After spreading, all the plates were placed in an incubator in inverted form at 280C for 2 days.

Colony Counting

After two days, counting was done with the aid of a colony counter. Number of colonies present in the plate was used to determine the number of cells present in the dilution.

Purification of pure culture by streaking

When colonies are formed on the plates then distinct and single colonies were picked with the help of sterilized loop and streaked. This loop on already prepared media plate before and after each streak the loop was washed with sprit and made red hot using spirit lamp for sterilization; after streaking these plates were placed inverted in the incubator at 280C for 2 days.

Gram staining

The Gram staining procedure was done using the method described by Mudili, (2007) and Cappuccino & Natalie, (2011). A drop of the algae broth was placed on a microscope slide and heat-fixed. The slide was then stained with crystal violet as a primary stain for 1 minute and rinsed with water. The slide was then treated using iodine solution as a mordant for 1 minute and rinsed with water. The slide was then decolorized rapidly with alcohol and rinsed with water. After this the slide was then counterstained with SafraninO for 1 minute and rinsed with water and examined under the microscope.

Phosphate solubilizing test

Media Preparation: The isolates were checked for phosphate solubilizing ability on Pikovskaya (PVK) medium (Pikovskaya, 1948) incorporated with tricalcium phosphate (Ca3(PO4)5). Composition of all mediums used for this study is given in Table 1. Formation of a clear halo zone around the growth after 5 days of incubation indicates phosphate solubilizing ability. This media was prepared for PSB isolation, Pikovskaya's medium (Pikovskaya, 1948) used.

Table 1:
Ingredients and its concentration for Pikovskaya medium

Chemicals	Quantity (g/L)
Glucose	10.0
$Ca_3 (PO_4)_2$	5.0
$(NH_4)2SO_4$	0.5
NaCl	0.2
$MgSO_4.7H_2O$	0.1
KCl	0.2
Yeast extract	0.5
$MnSO_4$	Trace
$FeSO_4.7H_2O$	Trace
Agar	15.0

All the above preceding ingredients except Tri calcium Phosphate were dissolved in 200ml distilled water in a flask; Tri calcium phosphate was mixed in 50ml distilled water in a separate flask. Both flasks were then autoclaved for 40 minutes at 121oC temperature and 15 psi pressure. After autoclaving, Tri calcium phosphate was mixed with the media, this was then poured in the plates. The plates were allowed to cool for few minutes and thereafter placed inverted to save the media from condensed water drops.

Preservation of the colonies: For the preservation of the microbial colonies; the respective media were prepared and the media poured in the slants and cooled for some time to allow the media to solidify. After that, microbial colonies were picked from the pure culture and streaked colony in the slant and other colonies were also streaked in the similar way and marked the slants. The slants were incubated for three days. After growth, these slants were preserved in the refrigerator

RESULTS AND DISCUSSION

A total of 19 strains, [CF-RS -4, CF-RS-5, CF-RS-6, CF-RS-7, CF-RS-8, CF-RP-4A, CF-RP-4B, CF-RP-4C, CF-RP-5A, CF-RP-5B, CF-RP-6, CF-RP-7, CF-ES-4, CF-ES-5A, CF-ES-5B, CF-ES-5C, CF-ES-6A, CF-ES-6B, CF-ES-7B] were isolated from rhizosphere, rhizoplane and endorhizosphere of cauliflower. It was found that all the colonies have different morphological characteristics.

Those strains which had irregular form were CF-RS -4, CF-RS-7, CF-RP-4C, CF-RP-5B, CF-ES-5A, CF-ES-5B, and CF-ES-7B. While the form of these CF-RS-6, CF-RS-8, CF-RP-7, CF-ES-4, CF-ES-5C, CF-ES-6A strains was circular, two strains CF-RS-5, CF-RP-5A were found filamentous, two strains CF-RP-6, CF-ES-6B were in rhizoid form and the CF-RP-4B was found punctiform.

On the basis of elevation, the strains were divided into 5 groups. It was found that the CF-RS -4, CF-RS-8, CF-RP-4C, CF-RP-5A, CF-ES-4, CF-ES-5C, and CF-ES-6A were raised. The CF-RS-5, CF-RS-6, CF-RP-7, CF-ES-5A, CF-ES-5B, CF-ES-6B were found flat. While the CF-RS-7, CF-RP-4B, CF-RP-5B, CF-ES-7B have pulvinate elevation. CF-RP-4A and CF-RP-6 were found convex and umbunate respectively. The strains with undulate margin were CF-RS-7, CF-RS-8, CF-RP-4A, CF-RP-4B, CF-RP-5A, CF-RP-7, CF-ES-5A, strains with entire margin were CF-RS-6, CF-RP-5B, CF-ES-4, CF-ES-5C, CF-ES-6A and the four isolates CF-RS-4, CF-RS-5, CF-RP-4C, CF-ES-7B were observed with erose margin. While only two strains CF-RP-6, CF-ES-5B were found lobate.

The strains with opaque opacity were CF-RS -4, CF-RS-5, CF-RS-6, CF-RS-7, CF-RP-4A, CF-ES-5A, CF-ES-5B, CF-ES-5C, CF-ES-6B and CF-ES-7B. While the CF-RP-4B, CF-RP-4C, CF-RP-5A, CF-RP-5B, CF-RP-6, CF-RP-7, CF-ES-4 and CF-ES-6A strains have translucent opacity.

The results demonstrate that the strains CF-RS -4, CF-RS-6, CF-RP-5A, CF-ES-5C, CF-ES-6A, CF-ES-6B and CF-ES-7B appeared white in colour. The strains CF-RS-5, CF-RP-4B, CF-RP-7 and CF-ES-5A were off white, CF-RS-8, CF-RP-4C, CF-RP-5B and CF-ES-4 were yellowish in colour, CF-RS-7 full white, CF-RP-4A orange, CF-RP-6 milky white and CF-ES-5B were deep red as shown in Table 2.

Phosphate test

Phosphate solubilizing activity of sixteen PGPR strains was checked. Out of these sixteen PGPR strains, eight strains were Phosphate positive because they formed clear zone in the Pikovskaya medium while the remaining eight strains were Phosphate negative. The strains which were phosphate negative were CF-RP-4C, CF-RS-8, CF-RP-4A, CF-ES-4, CF-RS-7, CF-ES-5C, CF-RP-6 while the CF-RP-5A CF-RP-5B, CF-ES-5B, CF-ES-5A, CF-RP-4B, CF-ES-6B, CF-RS-4 and CF-RS-6 strains were phosphate positive (Table 3).

Table 2:
Colonies with different morphological characteristics

S. No	Name	Form	Elevation	Margin	Opacity	Colour
1	CF-RS -4	Irregular	Raised	Erose	Opaque	White
2	CF-RS-5	Filamentous	Flat	Erose	Opaque	Off white
3	CF-RS-6	Circular	Flat	Entire	Opaque	White
4	CF-RS-7	Irregular	Pulvinate	Undulate	Opaque	Full white
5	CF-RS-8	Circular	Raised	Undulate	Translucent	Yellowish
6	CF-RP-4A	Circular	Convex	Undulate	Opaque	Orange
7	CF-RP-4B	Punctiform	Pulvinate	Undulate	Translucent	Off white
8	CF-RP-4C	Irregular	Raised	Erose	Translucent	Yellowish
9	CF-RP-5A	Filamentous	Raised	Undulate	Translucent	White
10	CF-RP-5B	Irregular	Pulvinate	Entire	Translucent	Yellowish
11	CF-RP-6	Rhizoid	Umbunate	Lobate	Translucent	Milky white
12	CF-RP-7	Circular	Flat	Undulate	Translucent	Off white
13	CF-ES-4	Circular	Raised	Entire	Translucent	Yellowish
14	CF-ES-5A	Irregular	Flat	Undulate	Opaque	Off white
15	CF-ES-5B	Irregular	Flat	Lobate	Opaque	Deep red
16	CF-ES-5C	Circular	Raised	Entire	Opaque	White
17	CF-ES-6A	Circular	Raised	Entire	Translucent	White
18	CF-ES-6B	Rhizoid	Flat	Filamentous	Opaque	White
19	CF-ES-7B	Irregular	Pulvinate	Erose	Opaque	White

Keys of table 2: CF= Cauliflower, RS= Rhizosphere, ES= Endorhizosphere, RP= Rhizoplane. 4, 5, 6, 7= Strains.

Table 3:
Phosphate solubilization ability of Cauliflower rhizobacterial strains

S. No.	Name	Phosphate test
1	CF-RP-4C	Negative
2	CF-RS-8	Negative
3	CF-RP-4A	Negative
4	CF-RP-5A	Positive
5	CF-ES-4	Negative
6	CF-RS-7	Negative
7	CF-RP-5B	Positive
8	CF-ES-5B	Positive
9	CF-ES-5A	Positive
10	CF-ES-5C	Negative
11	CF-RP-6	Negative
12	CF-ES-6A	Negative
13	CF-RP-4B	Positive
14	CF-ES-6B	Positive
15	CF-RS-4	Positive
16	CF-RS-6	Positive

In several soils, Phosphate is one of the major nutrients limiting plant growth; due to formation of insoluble complexes. This is a result of excessive use of synthetic fertilizers including the phosphate fertilizers which lead to soils deficient in available phosphorus but containing high levels of total phosphate (Borie and Rubio, 2003). A study conducted by Woyessa and Assefa, (2011) reported that three isolate out of four showed phosphate solubilization in soils.

Table 4:
Gram staining and microscopy of rhizobacterial strains of cauliflower

S. No.	Name	Gram staining	Colour
1	CF-ES-4	Negative	Pink
2	CF-ES-5A	Positive	Violet
3	CF-ES-5B	Negative	Pink
4	CF-ES-5C	Negative	Pink
5	CF-ES-6A	Negative	Pink
6	CF-ES-6B	Negative	Pink
7	CF-RP-4C	Negative	Pink
8	CF-RP-4B	Negative	Pink
9	CF-RP-6	Negative	Pink
10	CF-RP-5A	Positive	Violet
11	CF-RP-4	Positive	Violet
12	CF-RS -4	Negative	Pink
13	CF-RS-5	Negative	Pink
14	CF-RS-6	Negative	Pink

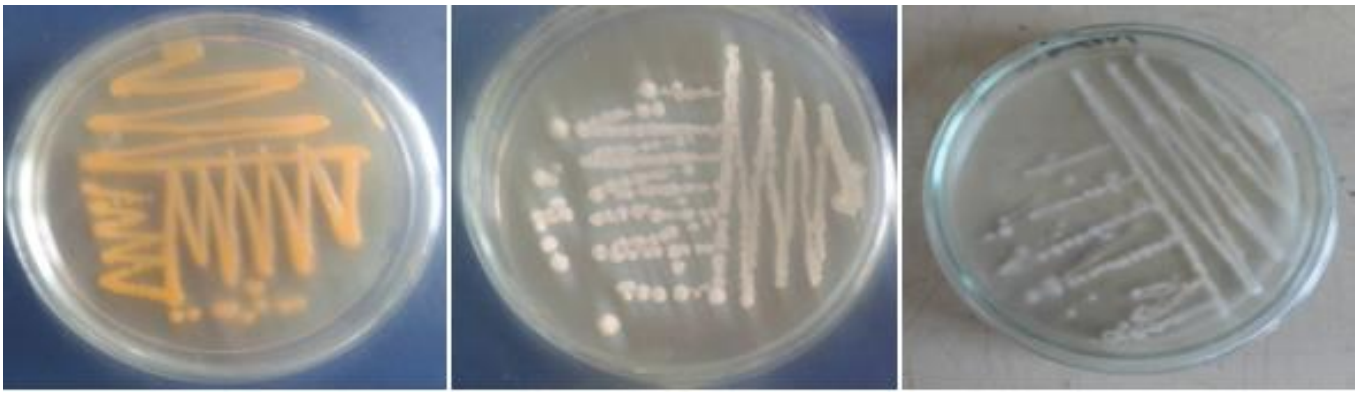

Plate 1:
Streaking view of strains CF-RP-5B, CF-RS-7, CF-RP-5A respectively

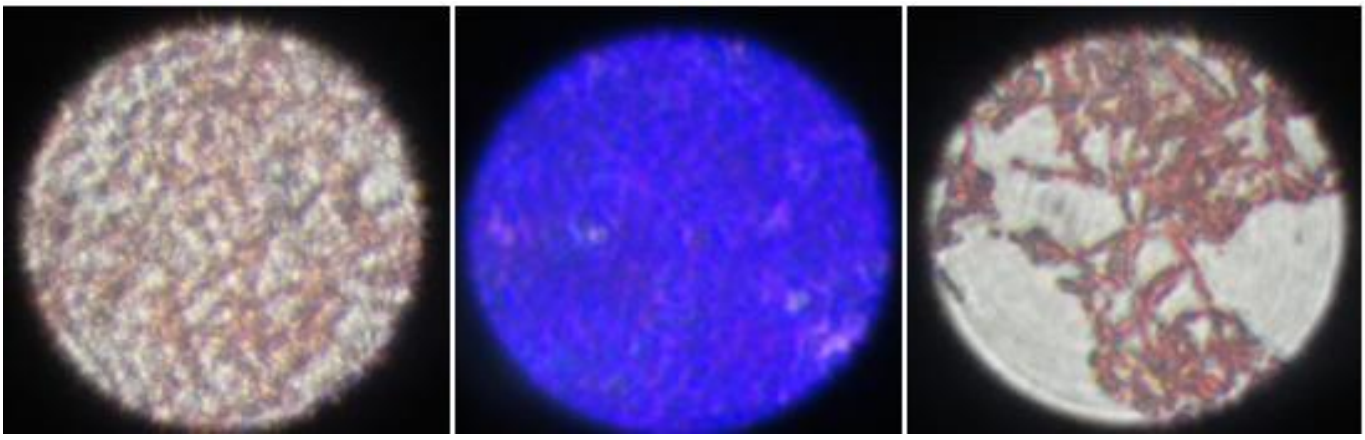

Figure 2:
Microscopic view of rhizobacterial strains after Gram staining

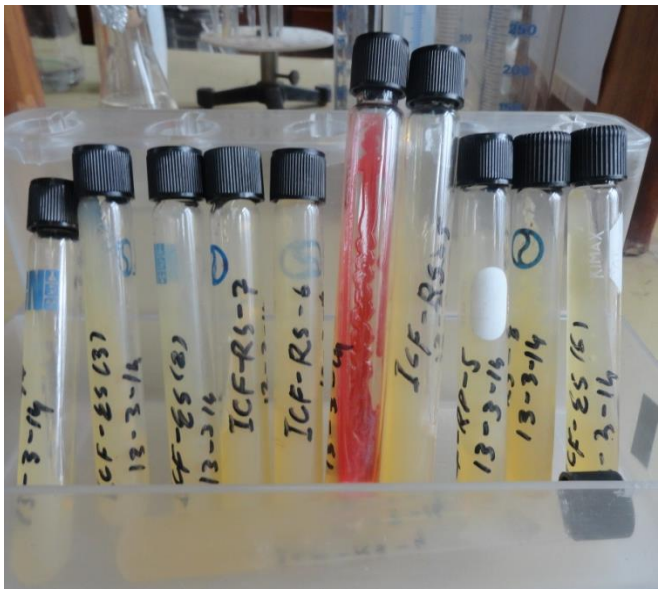

Figure 3:
Slants preparation

Gram staining: Gram staining was done for 14 strains CF-ES-4, CF-ES-5A, CF-ES-5B, CF-ES-5C, CF-ES-6A, CF-ES-6B, CF-RP-4C, CF-RP-4B, CF-RP-6, CF-RP-5A, CF-RP-4, CF-RS -4, CF-RS-5, and CF-RS-6. The result shows that the 11 strains were Gram negative while the 3 strains CF-RP-5A, CF-ES-5A and CF-RP-4 were Gram positive (Table 4). After microscopy different shapes of the strains were observed. From the result, most of the isolates were Gram negative and this is in tandem with previous reports that the rhizosphere of many plants provide conducive and favorable environment for Gram negative bacteria. (Johansen & Olsson, 2005). Most of these Gram negative bacteria are motile and according to Johansen & Olsson (2005) are stimulated by rhizodeposition whereas Gram positive bacteria are inhibited.

In the present study it was revealed that the PGRP play an important role in the growth of plants. The present investigation was carried out in Islamabad and total of nineteen species were identified. It has been studied that the colony morphology of bacterial strains isolated from cauliflower was found in different manner and it was revealed that CF-RS-4, CF-RS-7, CF-RP-4, CF-RP-5B, CF-ES-5A, CF-ES-5B, and CF-ES-7B were irregular in shape. Furthermore, it was also studied that the effect of different environmental factors plays an important role in the raising of morphological characteristics. The elevation of these pure strains varies, two of them CF-RS-4, CF-RP-4C were raised, three CF-RS-7, CF-RP-5B, CF-ES-7B were Pulvinate while the other two were flat. The morphological features also indicate the presence of certain conditions in rhizospheres of many legumes as these plants use plenty of nitrogen for the proper utilization of nitrogen compounds.

These strains have variation in colour, opacity and margins but most strains are found opaque and white. Some strains like CF-RS-6, CF-RS-8, CF-RP-4A, CF-RP-7, CF-ES-4, CF-ES-5C, CF-ES-6A, were found circular and all of them were translucent except CF-RS-6 which was opaque. Most of these mentioned PGPR strains were noticed in raised elevation with varying margins. Out of nineteen PGPR strains only two were in filamentous and two were in rhizoid form, while only one was in Punctiform having white color and undulate margins.

In recent years, it has been reported that many nitrogen-fixing bacteria may be utilized as biofertilizers in many plants especially serving as growth promoters (Babalola and Akindolire, 2011). Especially, as many Rhizobia are capable of mobilizing phosphorus into available forms to promote the growth of many plants; this has potential for commercial utilization in developing countries, particularly for sustainable agriculture. The isolation and then characterisation of Rhizobia as a plant growth promoter calls for more research with respect to solution preparation of inoculum and then its direct use on the field to evaluate its impact on boosting food production.

In conclusion, this research indicates the presence of Rhizobia in the roots of Cauliflower from the growing region of ChattaBakhtawar Islamabad, Pakistan. The morphological characteristics also indicate the presence of conditions in the rhizospheres that can enable the plant use plenty of nitrogen for proper utilization of nitrogen compounds. In a developing country like Pakistan it is very important to increase the yield of pulses and many other crops by utilizing the results of such kinds of research. Hence, further studies including identification of strains to species level and field studies are recommended before adoption by farmers in agricultural practices.

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