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Biochemical and Molecular Characterization of fluorescent *Pseudomonas* from Vindhyan region

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ABSTRACT

Microbial diversity of vindhya region was explored especially of fluorescent *Pseudomonas* for their ability to produce different hydrolytic enzymes, inhibit phytopathogens and promote plant growth. Thirty one isolates of fluorescent *Pseudomonas* were isolated from *rhizospheric* soils of different plants from different locations of the Vindhya region on the basis of different parameters namely colony characteristic like colony shape, size, surface, margin and colour. Out of the 31 isolates, 30 isolates produced cellulase and amylase, 20 produced lipase, 28, 27, 21 isolates produced protease, phosphatase and pectinase, respectively in the medium. Out of the 31 isolates of fluorescent *Pseudomonas* 8 isolates showed antagonistic property against the pathogen *Rhizoctonia solani* and the percentage of inhibition ranged from 60.00-77.77%, whereas 6 isolates against the pathogen *Sclerotium rolfsii* and the percentage of inhibition ranged from 55.55-74.44%. Selected isolates of fluorescent *Pseudomonas* [BM-4(i), BM-5(iii), BC-9(i), BO-11(iv), BO15-(ii)] were examined for growth promotion activity on Moong (*Phaseolus aureus*) and a marked increase in shoot and root length was observed. Genomic DNA of 5 bacterial isolates were isolated from these found to be antagonistic against *Rhizoctonia solani* or *Sclerotium rolfsii*. The DNA samples (against a 100bp ladder sequences) were run in the agarose gel and the samples 2, 3, 4 and 5 showed clear bands in agarose gel. The samples showed clear visualizing bands containing adequate amount of DNA.

Keywords: Biochemical and Molecular Characterization, Pseudomonas, Vindhyan region

Introduction

A major problem in agriculture is the difficulty in managing different diseases caused by pathogens specially root pathogens and even when chemical pesticides are available, questions arise concerning their efficacy when applied to soil in nurseries or field. The use of chemical pesticides in agriculture though have helped in maintaining a low cost, high quality food supply, their positive contributions has been challenged by individuals and groups suggesting that the negative environmental effects of these pesticides far outweigh their social benefits. Bio-control of plant pathogens through antagonistic microorganism is a promising alternative to the use of chemicals. Fluorescent *Pseudomonas* spp. makes up a diverse group of bacteria that can generally be visually distinguished from other *pseudomonads* by their ability to produce a water soluble yellow-green pigment. They are typically gram negative, chemoheterotrophic motile rods with polar flagella and are grouped in rRNA homology group I, as defined by Palleroni *et al*. (1973).They are known to produce a large number of secondary metabolites, which may affect the growth, and health of plants. Fluorescent *pseudomonads* have simple nutritional requirements, and this is reflected by the relative abundance of these organisms in nature. They are found in soils, foliage, fresh water, sediments, and seawater, and the type species of the group, *Pseudomonas aeruginosa*, is a classified secondary pathogen of animals. As a group, the fluorescent *pseudomonads* are of primary significance in such diverse areas as medical pathogen city, plant pathogencity, food spoilage, and biological control. Although some fluorescent *pseudomonads*, e.g.,

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Pseudomonas syringae, are well-known plant pathogens, it is now generally recognized that members of this group can be beneficial to plants. During the last 25 years, research has illustrated the latent potential of exploiting certain bacteria for the bio-control of root crop diseases. Beneficial or plant growth-promoting rhizobacteria have been isolated and demonstrated to protect the roots of certain root crop plants (Burr and Caesar, 1984; Schroth and Hancock 1981). For any disease suppressive mechanism to be effective, it is important that the antagonist first be able to efficiently establish itself in the rhizosphere of that particular plant (Kloepper *et al*., 1988). Many workers documented that inadequate colonization leads to decreased PGP activities (Antoun *et al*., 1998). Root colonization is an important step in the interaction of antagonist group of bacteria with host plant. It has been reported that antagonistic microorganisms are ideal bio-control agents because front line defense is provided by the rhizosphere against the pathogenic infection. *Pseudomonas* spp. was prominent in the rhizosphere, rhizoplane and ectorhizosphere because of their siderophore complexes (Baker *et al*., 1986) and production of antibiotic compound (Howell and Stipanovic, 1980).

Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant growth promoting rhizobacteria (PGPR). Although significant control of plant pathogens or direct enhancement of plant development has been demonstrated by PGPR in the laboratory and in the greenhouse, results in the field have been less consistent. Because of these and other challenges in screening, formulation and application, PGPR have yet to fulfill their promise and potential as commercial inoculants. Recent progress in our understanding of their diversity, colonization ability, and mechanisms of action, formulation, and application should facilitate their development as reliable components in the management of sustainable agricultural systems. Plant growth-promoting rhizobacteria (PGPR) were first defined by Kloepper *et al.* (1988) to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. PGPR enhance plant growth by direct and indirect means. Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogen or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPR to reduce the deleterious effects of plant pathogens on crop yield.

Molecular techniques are major tools for the characterization of bacteria from food and other biological substances. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), DNA Amplification (PCR), Agarose gel electrophoresis and Restriction Fragment Length Polymorphism (RFLP) are the molecular techniques used for the characterization of bacterial macromolecules and are of significance importance. SDS-PAGE is an important molecular technique used for the identification of total proteins and it has the advantage of being fairly simple and rapid to perform.

Materials and Method

The soil samples were collected up to 15 cm from the surface of the soil and stored in sterile polythene bags from the experimental fields of Rajiv Gandhi South Campus Barkachha, Banaras Hindu University, Mirzapur and some rural areas and villages of Mirzapur district.

Isolation of fluorescent *Pseudomonas*

After serial dilution of fluorescent *Pseudomonas*, 1 ml concentrate from 10^{-4}, 10^{-5} and 10^{-6} dilutions and transferred in a plate which contained cheek hot KBM by pour plate method. Bacteria were grown in incubator for 2-3 days at 25±2ºC. After incubation when colonies of bacteria were seen on plates, then picked and streaked on other KBM poured plate for obtaining pure culture. These strains were preserved on slant of KBM. The test organisms namely

Rhizoctonia solani and *Sclerotium rolfsii* against which the bio-control agent is to be screened for its antagonistic potentialities, were isolated from the infected plant tissues of rice and chick pea respectively after surface sterilization with chemicals such as $HgCl_2$ (0.01%) solution or $NaCl_2$ solution. Sample with clear visible symptoms were washed thoroughly sterilized with distilled water. A small portion of diseased tissues along with a portion of adjacent healthy tissue was cut into small pieces around 1 mm in length. Then surface sterilized with $HgCl_2$ (0.01%) solution for 2-3min. The pieces were then rinsed thrice with sterilized distilled water and inoculated aseptically on sterilized PDA plates. The inoculated petriplates were incubated at 25°C ± 2 °C. When the fungal colony developed, a small cut of single mycelium was transferred to another petriplate containing PDA medium to obtain the pure culture. The pure culture of pathogen was maintained in PDA slants and after full growth the slants were stored in refrigerator at 4°C for further studies.

Antagonistic assay by dual culture technique

Colony interaction between the soil fungi, bacteria and the individual species of test pathogen can be studied by adopting Dual Culture Experiment. *Pseudomonas* isolated from soil sample was evaluated against *Rhizoctonia solani:* and *Scleroteum rolfsii* in laboratory by dual culture technique to screen out the most efficacious one. Dual culture assay was done by placing the inoculum of test fungi in the centre of petriplates poured with PDA. Then the bacterial inoculants were streaked parallel on opposite sides of fungal inoculums. Inoculated plates were incubated at 25±1°C in BOD. The radial growth of pathogen was measured at interval of 24 hrs up to 7 days after incubation. Controls without bacteria were maintained and each treatment was replicated twice. Observations were recorded up to 7 days of inoculation on area covered by pathogen Inhibition of mycelia growth of pathogen fungi by each strain was recorded. Percentage growth inhibition was calculated as per formula given below:

% growth inhibition = r-r'/r × 100
Where r = Colony growth in control plate,
r'= Colony growth in intersecting plate

Biochemical potential of fluorescent *Pseudomonas* isolates

For phosphatase test, first NBRI-BPB media was prepared and poured into the petriplates after autoclaving in test tubes with 25 ml media each. Fresh cultures of *Pseudomonas, was* streaked in the centre of each plate of around 1-2 cm in diameter. The plates incubated at 28±1°C for 2-3 days. After incubation, a clear zone was checked around bacteria inoculated. If zone present, it designated that isolate is positive for phosphate solubilization. For amylase test, firstly amylase media was prepared and poured into the petriplates after autoclaving in test tubes with 25 ml media each. Fresh culture of *Pseudomonas was* streaked in the centre of each plate of around 1-2 cm in diameter. The plates incubated at 28±1°C for 3-5 days. After incubation, the plates were flooded with an iodine solution and a yellow zone around colony was noticed. If the yellow zone was present, the isolates were designated as positive for amylase activity. For lipase test, firstly lipase media was prepared and poured into the petriplates after autoclaving in test tubes with 25 ml media each. Sterilized separately each by autoclaving, 1 ml added per 100 ml of sterilized basal cooled medium before pouring. Fresh culture of *Pseudomonas* was streaked in the centre of each plate of around 1-2 cm in diameter. The plates were incubated at 28±1°C for 3-5 days. After that crystals formation was seen in the plates. The species with presence of crystals is positive to test. For pectinase test, firstly pectinase media was prepared and poured into the petriplates after autoclaving in test tubes with 25 ml media each. Fresh cultures of *Pseudomonas* were streaked in the centre of each plate of around 1-2 cm in diameter. The plates incubated at 28±1°C for 3-5 days. After incubation, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. Transparent circle around inoculums seen is positive indication of pectinase. Chitinase activity was

determined with a medium composed of 500 ml of mineral solution, 500 ml of distilled water, 0.002% of yeast extract, 15 g of agar and 2.4% of purified chitin. The chitin was prepared by treatment of chitin (Poly-N-acetyl glucosamine from crab shells, sigma) as described by Campbell and Williams. The chitin was dissolved in 50% sulfuric acid and the solution was quickly poured into15 volumes of distilled water. The precipitated chitin was collected by centrifugation. The washing and centrifugation was continued until the acid was removed. Finally the chitin was suspended in water and was sterilized by autoclaving. The solid content of sterile solution was determined by moisture analysis and the amount needed to give 2.4% in the basal medium was calculated. In practice, plate were prepared with an under layer of 1.5% agar and 3 ml over layer of test medium. After 5-8 days of incubation clear zone were seen in the opaque agar around colonies able to degrade chitin. Test tube containing 5 ml of nutrient broth were incubated with bacterial strains and incubated at 30^0 C in rotator shaker operated at 180 rpm for 24 hrs 10 µl of suspension were spotted on the cellulolytic media plate containing substrate of enzyme to be tested and incubated at 30^0C in BOD incubator for 48 hrs. For visualization of β-D glucan hydrolysis the medium plates were flooded with an aqueous solution of congored (1mg/ml) for 15 minute followed by NaCl (5.8%). These plates were observed for clear zone around colonies exhibiting the corboxy methyl cellulose activity.

Protease test media contains agar and 0.4% gelatin. 28 g nutrient agar was dissolved by boiling in distilled water and 8% solution of gelatin in water was sterilized separately and added to nutrient agar at the rate of 5 ml/100 ml of medium. Bacterial isolates were inoculated on this medium by putting spot. After incubation (5-8 days) due to degradation of gelatin clearance is seen around colonies. Plates were flooded with an aqueous solution of ammonium sulfate, precipitates were formed which make agar more opaque and enhance the clearance.

Plant growth promotion activity of isolates on Moong

Broth of selected isolates were prepared in nutrient broth media, harvested after 48 hour by centrifugation at 8000 rpm for 10 minute and washing with autoclaved distilled water, repeated this process three times. Then pellet was collected and dissolved in 1 ml autoclaved distilled water by vortex. Seeds of moong were taken and soaked in water for 2-3 h after that sterilized properly with Sodium hypochlorite or 0.1% Mercuric chloride for 30 sec, then three times washed with autoclaved distilled water. Sterilized seeds were soaked in 2% CMC for 10 minute after that seeds were transfer on sterilized Whatmann filter paper for 1 h.

Moisture chamber were prepared by placing two sterilized Whatmann filter papers in petriplate and sprinkled with sterilized distilled water. Seeds dried with CMC were treated with prepared suspension of *Pseudomonas*, and then transferred in moisture chamber. These moisture chambers were incubated for seven days at 28±2°C. The moisture content was maintained by sprinkling water on the filter paper. The plant growth promotion activity was analyzed after seven days by measuring the root growth with respect to the control plant which was not treated with *Pseudomonas*.

Extraction and SDS-Polyacrilamide Gel Electrophoresis of protein

Root was taken from germinating seed treated with *Pseudomonas* inside moisture chamber after 5-6 days of germination. 1g of root was weighed. The seedling was transferred into the mortar and 5 ml of 0.2 M Tris.Cl (pH 8) was added. The mortar pestle was kept in ice bucket and seedling was crushed with the help of pestle, till fine slurry was made. The slurry was transferred in centrifuge tubes and centrifuged at10,000 rpm at 4^0 C for 10 min. The supernatant was transferred in fresh tubes. The supernatant was stored at 4^0C for further use.

Isolation and Purification of genomic DNA

Two ml of 24 h old broth culture of bacteria was centrifuged at 10,000 rpm at 28^{0}C for 5 minute and the pellet was collected by discarding the supernatant.The pellet was washed by TE buffer pH 8.0 (10 mM Tris-HCl pH-8.0) and 1 mM EDTA pH - 8.0, this process was repeated twice. The pellet was resuspended in 0.5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8.0, 20 mM Tris HCl, pH 8.0). 0.1 volume of 10 % SDS and 10 µl of poteinase K (10 mg/ml) was added to above solution and incubated at 55^{0}C for 60 min. 0.3 volume of 5M NaCl and equal volume of Tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) was added and incubated at room temperature for 30 min followed by gentle vortexing and centrifuged at 5000 rpm for 15 min at room temp.

To the equal phase 0.1 volume of the sodium acetate (pH-4.8) and 1 volume of chilled absolute ethanol were added by following gentle extraction and incubated at room temperature for 30 min. The mixture was incubated at 10,000 rpm for 5 min at 4^{0}C. The pellet was washed with 70% ethanol and again centrifuged at 10,000 rpm for 4^{0}C for 5 minutes (repeated this step twice). The pellet was dried and dissolved in TE buffer and store at 4^{0}C. 2µl RNase-A was added to the eppendorf tube containing 200 µl of extracted DNA and then incubated for 3 hour at 37^{o}C in a water bath. The DNA was further extracted with equal volume of Phenol: Chloroform: isoamyl: alcohol (25:24:1 v/v) and centrifuged at 10,000 rpm for 12 min. at 10^{o}C. Supernatant was taken into a fresh eppendorf tube. 0.6 volume of ice cold isopropanol and 0.1 volume of ice cold sodium acetate (3 M) were added and the mixture was kept at -20^{o}C for at least 2 hour. The mixture was then centrifuged at 10,000 rpm at 10^o C for 12 minute. Supernatant was removed using a micropipette and pellet was washed with 70% ethanol and dried completely. The DNA pellet was redissolved in minimum amount of TE buffer and store at 4^{0}C. 0.8% agarose gel was prepared and it was loaded with 5µl sample (4µl DNA+1µl loading dye). Gel documentation was done after 3 hour of running of DNA in agarose gel at 50 mV.

Results and Discussion

Conventional agriculture is heavily dependent on the application of chemical inputs, including fertilizers and pesticides, to maintain consistently high yields. However, this has heavy impact on the natural and human environment, through the pollution of soils, waters, and the whole food supply chain. To reduce these problems the sustainable agriculture has pointed many approaches and techniques. One of those strategies is the utilization of the soil micro biota to induce plant growth, control of plant diseases and biodegradation of xenobiotic compounds. Studies on the relationship between bacteria and roots are essential to achieve viable agricultural applications. Root colonizing and plant growth promoting bacteria referred to as PGPR affect plant growth by increasing nutrient cycling, suppressing pathogens and producing biologically active compounds (Khalid *et al.*, 2004). There is currently a considerable interest in rhizobacteria, which are being exploited for better crop production by virtue of their quick colonization, production of growth-promoting substances, and prevention of pathogen establishment in the rhizosphere through antibiosis, siderophore production or secretion of other hydrolytic enzymes (Weller, 1988; Bakker et al.1991).

Keeping these facts in view the soil samples were collected up to 15 cm from the surface of the soil and stored in sterile polythene bags from the experimental fields of RGSC barkaschha and some other villages of Mirzapur . Out of number of collections finally thirty isolates were screed for further study. These thirty one isolates of fluorescent *Pseudomonas* isolated from different soil sample on the basis of different parameters like colony shape, size, surface, margin and colour. All fluorescent *Pseudomonas* isolates were found to be Gram negative. Out of 31 isolates of fluorescent *Pseudomonas* 8 isolates of fluorescent

Pseudomonas viz. BM-4(i), BG-7(i), BG-7(ii), BG-7(iii),BW-8(i),BO-15(i), BO-15(iii), BO-15(iv) showed antagonistic property against the pathogen i.e. *Rhizoctonia solani*. Among these BO-15(i) have maximum % of inhibition whereas BG-7(iii), minimum activity. Similarly out of 31 isolates tested against *Sclerotium rolfsii* only six isolates of fluorescent *Pseudomonas* namely BM-4 (i), BG-7 (i), BG-7(ii), BO-15(ii), BO-15(iii), BO-15(iv)exhibited antagonistic property. Among these isolate BO-15(iii) had maximum percentage of inhibition whereas BG-7(i) had the minimum inhibition. Similar studies were made in past by several workers like Bakker et al. 1986, 1991; Khalid *et al.* 2004; Kloepper et al. 1988 and Palleroni *et al.* 1973

Dual culture of isolate BO-15(ii) against *S. rolfsii*

One percent aqueous solution of Hexa decyltrimethyl ammonium bromide, precipitate the intact pectin in the medium. Thus clear zones around colony or opaque medium indicated degradation of pectin.10 isolates of fluorescent *Pseudomonas* did not show any activity towards pectinase. Fourteen isolates showed best result, whereas 7 isolates showed good pectinase activity. The blue colour of medium was because of dissolving of phosphate by enzyme phosphatase secreted by bacteria.. Out of 31 isolates only 17 isolates showed good result, whereas 10 isolates showed average result and 4 isolates did not show phosphatase activity. After incubation, due to degradation of gelatin (a protein) in the medium, clearance was seen around the colonies. 4 isolates showed good result, 24 isolates showed minimum result and 3 isolates did not show any protease activity.

Yellow zone around colonies or blue medium was indicated the amylolytic activity. Among 31 isolates of fluorescent *Pseudomonas* tested, maximum activities were found in 16 isolates. Fourteen isolates showed good result and 1 isolate showed negative activity. Visible precipitate or clearing of such precipitate around a colony exhibited the lipolytic activity. Among 31 isolates, 20 isolates showed maximum activity whereas 11 isolates did not produce good lipolytic activity. Isolates were shown transparent zone around colony on medium. 30 isolates showed positive result and 1 isolate showed negative result. Among 30 isolates, 1 isolate was found to be excellent, 16 isolates were good and 13 isolates were average producer in cellulose activity. In this study all the fluorescent *Pseudomonas* isolates were found with negative chitinase test. Among 6 isolates of fluorescent *Pseudomonas*, BM-4(i) was found to show maximum plant growth promotion activity compare to rest of 5 isolates.

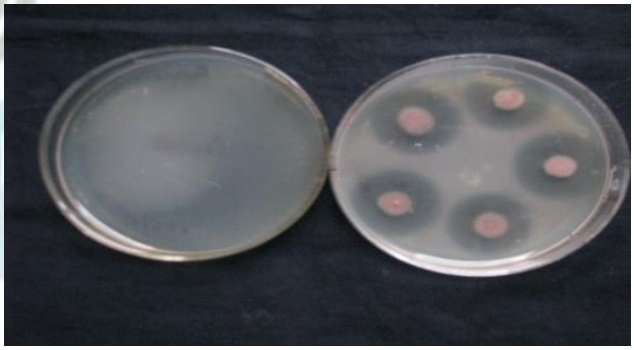
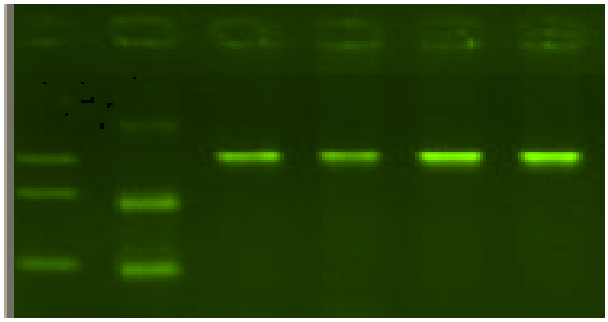


Figure 1 Showing isolates BM-4(i) pectolytic activity

We have isolated genomic DNA of 5 bacterial isolates in which we found antagonistic strains against *Rhizoctonia solani* or *Sclerotium rolfsii*. After loading 5 DNA samples (against a 100bp ladder sequences), the samples were run in the Agarose Gel Electrophoresis system for 3 hr at 50V. The sample 1 was not visualized clearly and shows multiple bands. It means the sample may contain any kind of possible contamination, whereas 2, 3, 4, 5 show clear bands.



Note: Lane 1=Ladder, Lane 2=BM-5(iii), Lane 3=BM-4(i), Lane 4=BC-9(i), Lane 5=BO-15(iii), Lane 6=BO-11 (iv).

The samples showing clear visualizing bands contained adequate amount of DNA. Increase in total protein content was observed in the plant treated with isolate BM-4(i).

Among the all isolates, twenty nine isolates were observed as phosphate solubilizers. Phosphate solubilization potential of *Pseudomonas* isolates might be responsible for their PGP activity.

Table 1 Best five strains showing Plant growth promotion test.

Isolates	Root length (cm)	Shoot length (cm)
BM-4(i)	7.10	2.10
BM-5(iii)	1.00	2.00
BC-9(i)	5.00	2.10
BO-11(iv)	3.80	2.16
BO-15(ii)	1.80	2.10
Control	1.20	1.00

The bio control agents provide protection against plant diseases either by direct action against the pathogen (i.e. antagonism) and/or indirectly by reducing host susceptibility towards the pathogen. In the present study, the production of well-defined inhibition zones by strains of fluorescent *Pseudomonas* indicates that there was a secretion of some antibiotic compound that may have checked the growth of mycelia of *S. rolfsii* as well as *R. solani* confirmed the finding of Anand *et al.*, (2002). In this study, 8 fluorescent *Pseudomonas* isolates namely. BM-4(i), BG-7(i), BG-7(ii), BG-7(iii),BW-8(i),BO-15(i), BO-15(iii), BO-15(iv) were found to inhibit the growth of *Rhizoctonia solani* and 6 isolates namely BM-4(i),BG-7(i),BG-7(ii),BO-15(ii),BO-15(iii),BO-15(iv) were found to inhibit the growth of *Sclerotinia sclerotium*.

Growth inhibition of these pathogens might be due to production of antibiotics, siderophores, ammonia, cyanide and hydrolytic enzymes like, cellulose, pectinase, amylase, lipase, protease etc which induce host plant defense mechanisms and eliminate plant signals that trigger pathogen development. This finding confirmed the finding of Howell and Stipanovic (1980), Paulitz et al. (1992) and Benhamou et al. (1996), where they had found the ability to reduce the development of various soil borne plant pathogens, production of different antimicrobial compounds, competition and induction of the plant defense mechanisms. In addition, 21 isolates showed pectinase activity, all 31 isolates showed proteolytic activity, 30 isolates showed amylolytic activity, all 31 isolates showed lypolytic activity and 30 isolates showed cellulose activity. These activities of fluorescent *Pseudomonas* might be used for suppressing the growth of pathogens. In SDS- PAGE analysis it was found that there is increase in the total content of protein in the fluorescent *Pseudomonas*-treated Moong seeds when compared to untreated control seeds. The increase in the protein content of treated plant might be due to induction of the gene responsible for synthesis of either defense related enzymes (protein) or plant growth related proteins.

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