



ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS FLUORESCENS* STRAINS FROM THE DIFFERENT REGIONS OF CHAKRATA, JAUN SAR-BAWAR, UTTARAKHAND

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Abstract:

Chakrata, Jaunsar-Bawar region is conferred by diverse wealth in terms of flora. The flora is so wealthy just because of the microorganism which inhabits the soil. *Pseudomonas fluorescens* belongs to the PGPR (plant-growth promoting rhizobacteria), which plays a major role in plant growth promotion and reduce severity of various diseases, bio-control agent as well as antagonist, and a potential biosurfactant producer. A total of six soil samples were collected from different villages of chakrata. Dilutions of soil sample were made and isolated on the nutrient agar medium followed by identification through Gram's staining and different biochemical analysis. Different tests were performed for each isolated bacteria from different soil samples. Out of which four samples showed the presence of *Pseudomonas fluorescens* strains according to the Bergey's manual of determinative bacteriology. Seeds of black-eyed peas (lobia) and okra (bhindi) were treated with isolated strains of *P. fluorescens*. After treating them, seeds were sown in the pots. A pot as a control is taken for the comparison of growth between inoculated and uninoculated seeds. The inoculated seeds showed positive growth than control. The present study indicates that the presence of *Pseudomonas* species in soils of chakrata is selective in nature and supports the well growth of selected plants and this is one of the main reasons for the well productivity of different varieties of crops and medicinal plants in chakrata, jaunsar-bawar areas.

Keywords- Biofertilizers, PGPR, Chakrata, *Pseudomonas fluorescens*

Introduction:

The bacteria which colonize the roots of the plants and enhance plant growth are referred to as plant growth promoting rhizobacteria (PGPR). *Pseudomonas fluorescens* is one of the PGPR that inhabits soil, plants and water surfaces. This rhizobacterium is well adapted to grow in the rhizosphere. It is a Gram-negative rod shaped bacteria with optimum growth temperature between 25-30⁰C. The Pf-5 strain resides in the plants rhizosphere and produce a variety of secondary metabolites including antibiotic against some soil borne pathogens while PFO-1 strain is well adapted to the soil where it was first isolated in agricultural species that contributes greatly to the turnover of organic matter and while present in soil, is abundant on the surfaces of plants they get nutrients and environmental protection. In exchange, they also destroy things that might be potentially harmful to the plants. These things include toxins and pollutants like TNT, styrene, and polycyclic hydrocarbons. *P. fluorescens* can multiply very quickly, this means they can rapidly colonize a space and indirectly protect a crop. Certain members of *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the seeds and roots from fungal infections (Hoffland et al. 1996, Wei et al. 1996). This effect is the result of the antibiotics, siderophores and hydrogen

cyanide (O'Sullivan & O'Gara 1992). Of the plant colonizing strains, some isolates are known to positively affect plant health and nutrition. It usually affects the patients which are immuno-compromised and they also produces phenazine, phenazine carboxylic acid, 2,4-diacetylphloroglucinol and the MRSA (methicillin resistant staphylococcus aureus) active antibiotic mupirocin.

Materials and Methods

Experiment location and soil sample collection

This experiment was conducted at USERC (Uttarakhand Science Education and Research centre) and SGRR University, Dehradun, Uttarakhand. The soil samples were collected from the different sites of Chakrata – Kanasar, Haiya, Sahiya, Jutapa, Kalsi and Ragula. The dilutions of different soil samples were made for isolation of microorganism.

Morphological Characterization

Samples of different dilutions were spreaded on the nutrient agar and then isolated colonies were streaked on nutrient gar petri-plates separately for colony development. Streaked plates were observed under UV transilluminator for fluorescence produced by bacteria.

Identification by Gram's Staining

This method is used to differentiate among Gram- Negative (pink) and Gram-Positive (purple) bacteria. It is based on the ability of bacteria to retain the color of the stain used in Gram reaction. Gram-Negative bacteria were decolorized by alcohol, losing the purple color of crystal violet, a counter stain safranin is used to impart a pink color to the bacteria. But Gram-Positive bacteria were not decolorized by alcohol and remans as purple (Fig.2).

Biochemical test for *Pseudomonas fluorescens*

For identification of *P.fluorescens* , certain biochemical test were conducted according to the Bergey's manual for determinative bacteriology.

Catalase test

Bacteria produce catalase enzyme to protect themselves from lethal effect of hydrogen peroxide which is accumulated as end product of aerobic carbohydrate metabolism. Use a sterile loop to transfer a small amount of colony growth in the surface of a clean, dry glass slide. Add a drop of 3% hydrogen peroxide in the glass slide (Fig. 5). Evolution of bubbles shows positive catalase while no bubble formation shows catalase negative.

Oxidase test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase enzyme. Soak the filter paper (Whatman's #1) in a 1% tetramethyl-p-phenylene-diamine dihydrochloride solution. Dry for about half a minute. With the help of loop (platinum material or plastic stick), pick the colony and rub the culture on the filter paper (Fig. 4). Inoculated area of paper changes color from blue to dark purple within 10-30 seconds showing positive oxidase test while no appearance of color shows oxidase negative.

Indole Test

It is used to determine the ability of an organism to split amino acid tryptophan to form compound indole. Inoculate the tryptophan broth with broth culture. Incubate at 37⁰ C for 24-48 hours in ambient air. After incubation, add 1-2 drops of Kovac's Reagent to the broth culture. Development of cherry-coloured ring shows indole positive.

Citrate Test

Citrate utilisation test is used to check the ability of an organism to utilize sodium citrate as a sole carbon source and ammonium salts as a sole source of nitrogen. It is indicated by change in color of bromothymol blue indicator from green to blue (Fig.6). Inoculate and incubate the Simmons citrate agar by touching colony that is 18-24 hours old with an inoculating loop.

OF(Oxidation-Fermentation) Test

Certain Gram-Negative bacteria metabolizes glucose by aerobic respiration (oxidatively). The high concentration of acid produced during aerobic respiration will turn the bromothymol blue indicator in OF media from green to yellow in the presence or absence of oxygen. Inoculate two test-tubes of OF medium with test organism by stabbing with the help of straight wire and covering one tube of each pair with 1 cm of sterile liquid paraffin wax or sterile mineral oil, while leave the other tube open to the air. Development of yellow colouration in open tube shows oxidative utilisation of carbohydrate while fermentative utilisation of carbohydrates shows development of yellow colouration in both open and closed tubes (Fig.10).

Gelatin Hydrolysis Test

Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy the gelatin. Inoculate a heavy inoculum of test bacteria (18-24 hours old) by stabbing 4-5 times (half inch) on the tube containing nutrient gelatin medium. Incubate the inoculated tube along with an uninoculated medium at 35^o C, or at the test bacterium's optimal growth temperature for up to 2 weeks. Remove the tube daily from the incubator and place in ice bath or refrigerator (4^o C) for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction (Fig.9). Gelatin normally liquefies at 28^o C and above, so to confirm that liquefaction was due to gelatinase activity (the tubes are immersed in an ice bath or kept in refrigerator at 4^o C).

Blood Haemolysis Test

Blood agar contains general nutrients and 5% sheep blood is added to it after autoclaving. It is useful for cultivating fastidious organisms and determining their haemolytic capabilities. Some bacteria produce different exoenzymes that lyse red blood cells and degrade haemoglobin and are called haemolysins. Bacteria producing β -haemolysin break down red blood cells and haemoglobin completely by forming a clear zone around the bacterial growth. α -haemolysins producing bacteria partially breaks down red blood cells and leaves a greenish color around the colony while γ -haemolysins does not break any blood cells (Fig.8).

Motility test by Hanging drop method

Motility is the ability of an organism to move itself by unique flagella or cilia. The ability of the bacteria to move has been used as a means of classification and characterization. Place a small drop of freshly prepared suspension of 24 hours old culture over a clean coverslip. Put a little balls of paraffin wax on the corners of coverslip and place the coverslip on the concave slide so that the drop of suspension will be hanging upside down from the under surface of coverslip. Place a drop of immersion oil on the coverslip and focus the edges of the drop under 100X objective lens. The cells will look like either dark or slightly greenish, very small rods or spheres.

Result & Discussion

This study revealed that all the six soil samples were collected and analysed, out of which four showed the presence of *Pseudomonas fluorescens* strains. For culturing bacteria, Nutrient agar was prepared which further isolated by Quadrant streaking (Fig.1). Morphological identification done by Gram Staining (Fig.2). Characterization of different bacterial isolates were done by performing different biochemical tests (Table No.2) such as catalase, oxidase, citrate utilization, carbohydrate test (TSI agar), MSA (mannitol salt agar), Indole test, haemolysis test, gelatin hydrolysis test and motility test. From biochemical analysis it is shown that all bacterial isolates are catalase positive whereas haemolysis is shown by all bacterial isolates except Kalsi (Table No.2). Soil microorganisms may be an important source of producing chemicals (antibiotics and enzymes) having biochemical and pharmacological importance. In this study, the screened bacterial isolates showed the presence of enzymes such as catalase, cytochrome c oxidase, proteolytic enzymes and haemolysins.

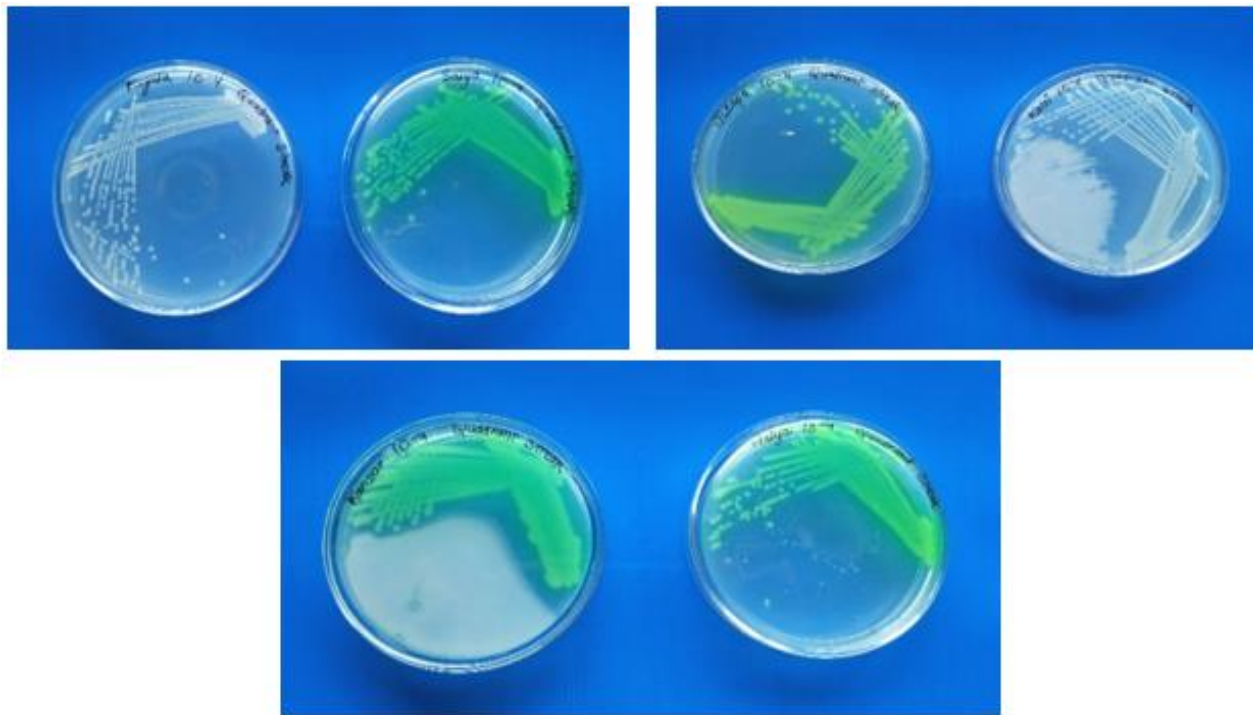


Fig 1. Isolated colonies by Quadrant streaking on Nutrient Agar

Table No.1 Morphological study of bacteria on Nutrient Agar plates and Gram staining

S.NO	SAMPLE	ELEVATION	COLOR	GRAM STAIN TYPE	ILLUMINATION
1	Kanasar	Convex	Light-Green	-	+
2	Haiya	Convex	Green	-	+
3	Sahiya	Convex	Light-Green	-	+
4	Jutapa	Convex	Yellowish-Green	-	+
5	Kalsi	Raised	Shiny white	+	-
6	Ragula	Raised	Off-white	+	-

(+ = positive, - = negative)

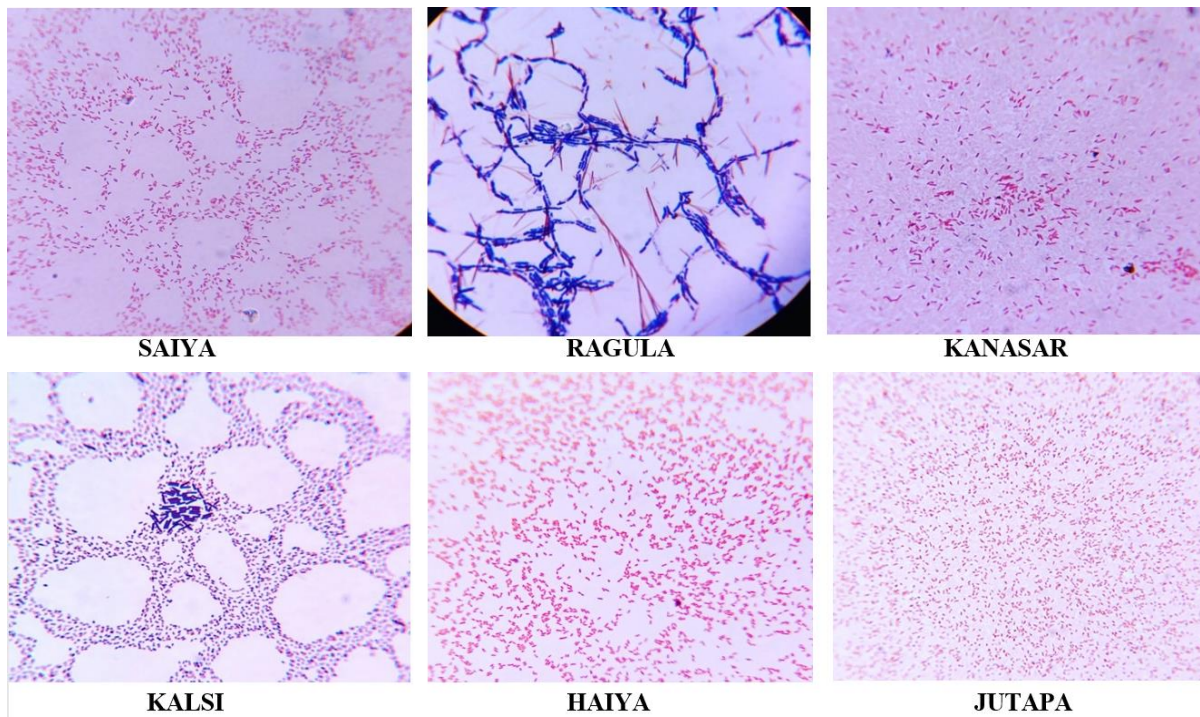


Fig 2. Gram staining images of different soil samples

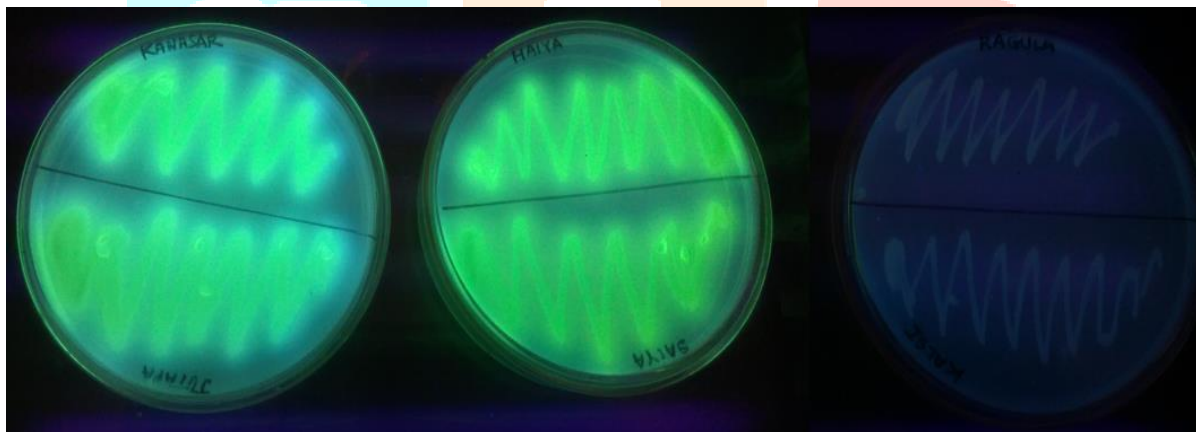


Fig 3. Streaked plates under UV Transilluminator

Table No. 2 Biochemical Analysis

S.NO	SAMPLES	OXIDASE	CATALASE	CITRATE	TSI	MSA	INDOLE	HEMOLYSIS	GELATIN HYDROLYSIS	MOTILITY
1	Kanasar	+	+	+	-	-	-	+(α)	+	+
2	Ragula	+	+	-	+	-	-	+(β)	+	-
3	Jutapa	+	+	+	-	-	-	+(α)	+	+
4	Sahiya	-	+	+	-	-	-	+(α)	+	+
5	Haiya	-	+	+	-	-	-	+(α)	+	+
6	Kalsi	-	+	+	-	-	+	-(γ)	-	-

(+ = positive, - = Negative, α = alpha hemolysis, β = beta hemolysis, γ = gamma hemolysis)

Table No. 3 OF (Oxidative and Fermentative) Test

S.NO	SAMPLES	OPEN (AEROBIC)	TUBE	CLOSED TUBE(ANAEROBIC)	METABOLISM
1	Kanasar	yellow		Green	Oxidative
2	Ragula	yellow		yellow	Fermentative
3	Jutapa	yellow		Green	Oxidative
4	Sahiya	yellow		Green	Oxidative
5	Haiya	yellow		Green	Oxidative
6	Kalsi	yellow		Green	Oxidative

(Yellow = Acid, Green = Alkaline)

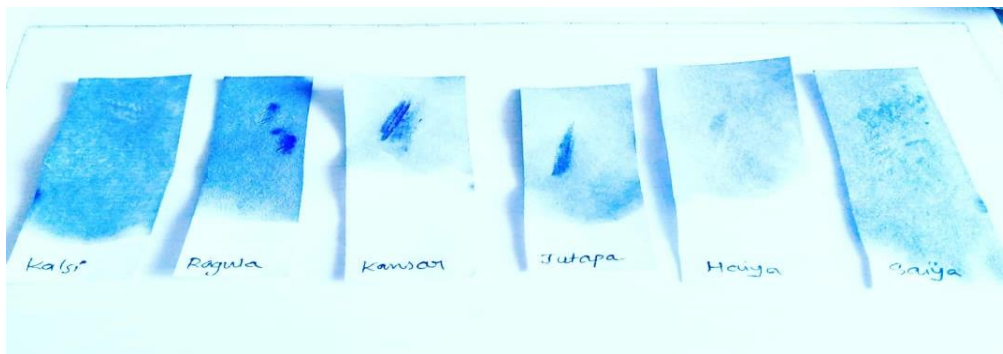


Fig 4. Oxidase test

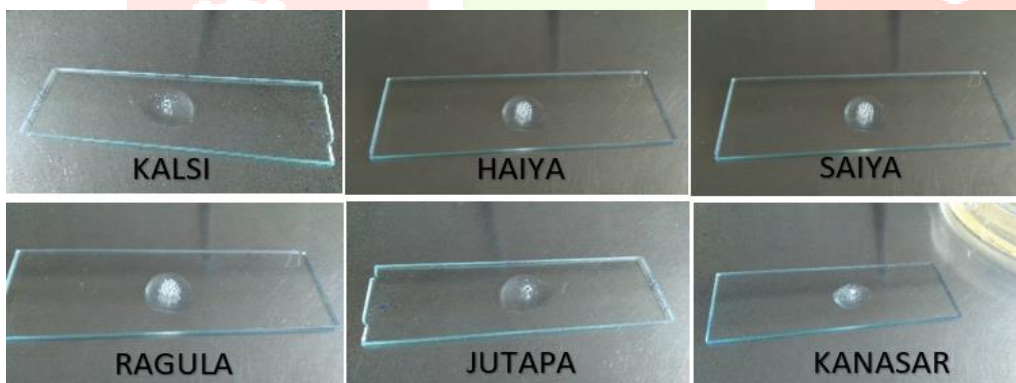


Fig 5. Catalase test

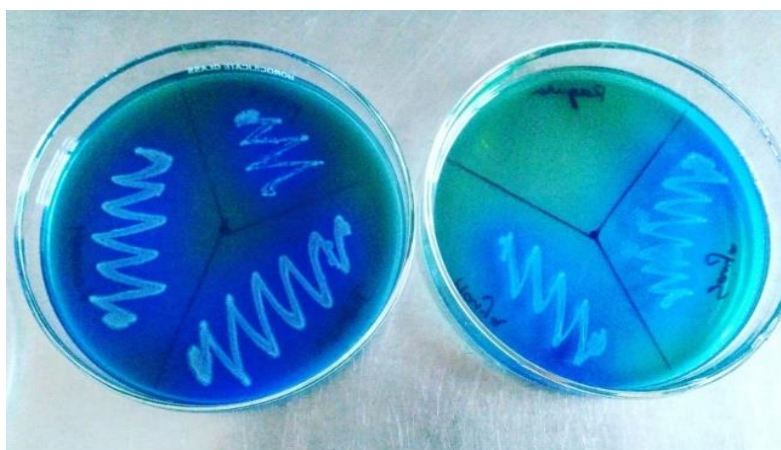


Fig 6. Citrate test

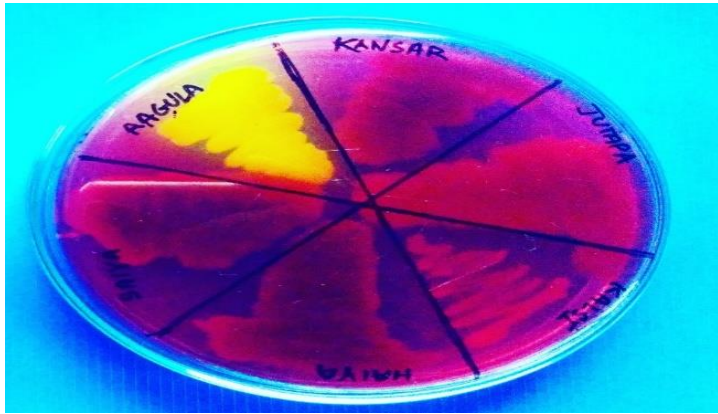


Fig 7. TSI plate

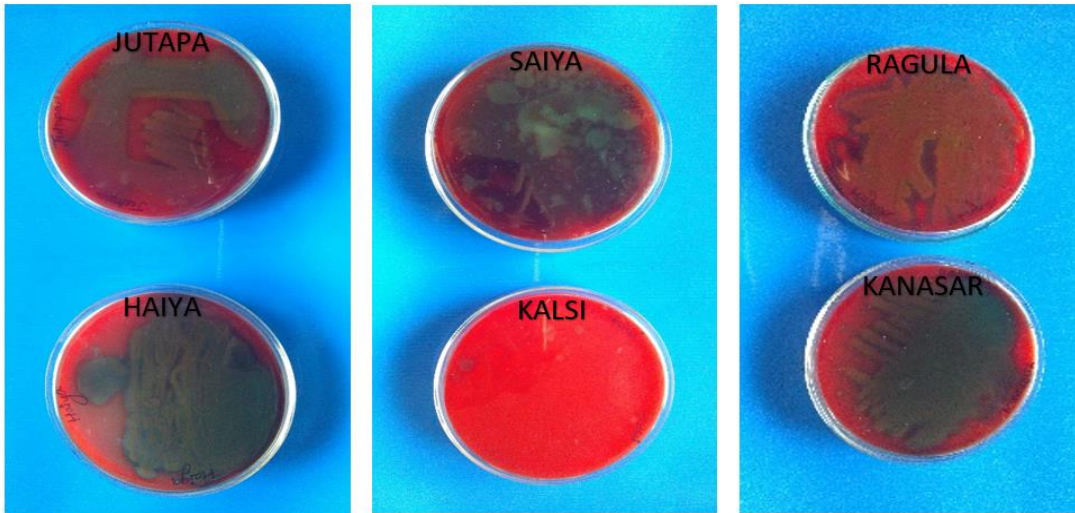


Fig 8. Blood hemolysis by bacteria

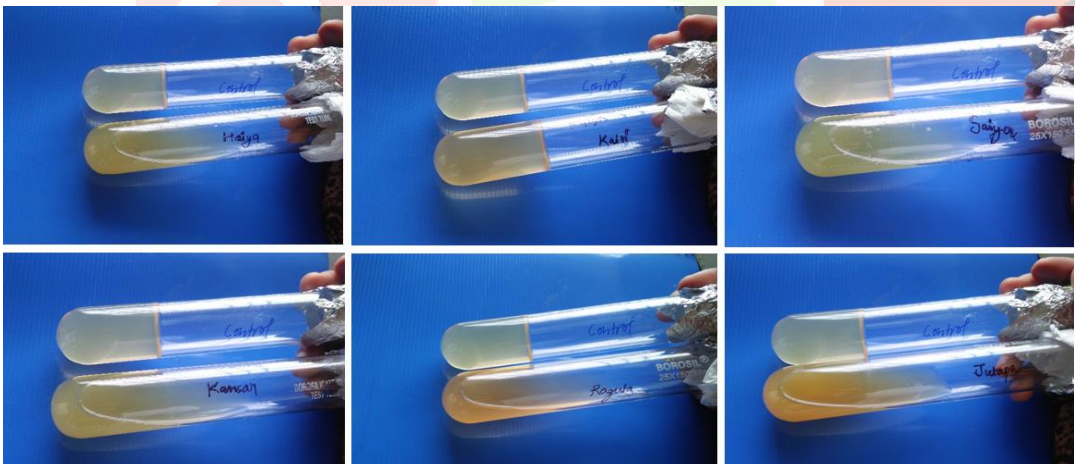


Fig 9. Gelatin Hydrolysis



Fig 10. OF test

Effect of Microorganism on seeds of Black-eyed peas and Okra

Potential for application of bacteria in soil. There are vast possibilities in the application of bacteria for beneficial purposes; the potential for tailoring organism to specific tasks using genetic engineering techniques has certainly contributed to this. Many different bacteria, in particular *Pseudomonas* and *Bacillus* strains, are known to be able to control pathogenic agents especially fungi. Microbial inoculants applied as seeds treatments deliver microorganism directly to plants rhizosphere – the narrow zone of soil that surrounds the root where plants interact directly with microorganism (Philippot et al.2013). It is a zone of intense microbial activity with growth of plants and microorganism dependent on reciprocal provision of nutrients and a wide range of other compounds including plant growth regulators and antibiotics.

Normal soil for pot is collected and autoclaved at 121⁰C and then incubated overnight and next day again autoclaved. This is done to kill the other microbes and endospores in the soil which affect the results and efficiency of isolated microbes (Fig. 1).

Seeds of black-eyed peas and okra is inoculated with *P.fluorescens* isolated strains from different sites of Chakrata. 5 ml of each broth culture of bacteria is poured in 5 test tubes. In 4 tubes, 4 different bacterial sample was inoculated and one tube is taken as control(uninoculated). 2 gm of Okra and Black-eyed peas was weighed respectively and put it into each tubes containing different bacterial culture. Seeds were soaked in bacterial culture for 2-3 hours. Remove the seeds and put it into pots and labelled well.

Table No. 4 Comparison of plant growth

S.NO	SAMPLES	HEIGHT OF PLANT AFTER 7 DAYS FROM ROOT TO TIP		HEIGHT OF PLANT AFTER 15 DAYS	
		Black-eyed peas	Okra	Black-eyed peas	Okra
1	Haiya	12 cm	8 cm	27 cm	11 cm
2	Sahiya	-	9 cm	-	17 cm
3	Jutapa	11 cm	-	26 cm	-
4	Kanasar	15 cm	-	38 cm	-
5	Control	17cm	-	33cm	-

(- = No growth)



Fig 11. Growth after 7 days (captured by Saif Ali)



Fig. 12 Growth of plants after 15 days (captured by Saif Ali)



Fig. 13 Growth of Okra and black-eyed peas after 28 days (captured by Babali Semwal)

Conclusion

By studying the different soil samples, we conclude that soil of Jutapa, Haiya, Sahiya and Kanasar have the *Pseudomonas fluorescens* strains, while Ragula and Kalsi may not have those useful bacterial strains as compared by the results (Table No.4). Ragula and Kalsi have the most abundant gram-positive long bacilli which may be a useful species for humans, but for other samples it is cleared by the above results that it has the *Pseudomonas sp.* which is useful for agricultural fields.

P.fluorescens is a very wide spread bacteria and not harmful to humans, inhabits soil and water. It multiplies very quickly, this means they can rapidly colonize a space and indirectly protect a crop. It also acts as a bio-control agent, means it can kill other pathogens in the soil which may harm the crop. In short, we will say that it is a multi-tasking bacteria which is fully compatible with soil and helpful to human beings.

These four samples were tested for the growth of plants and control is taken for the comparison of growth. Kanasar showed the maximum growth of black-eyed peas and Okra where as control only showed the growth of black-eyed peas. Jutapa showed the least growth of plants. Haiya and Sahiya showed the growth of Okra only. However, some seeds were not grown.

From the above research it is cleared that Chakrata soil is rich in microflora. Different sites do not support the growth of all plants but support growth of selected plants which shows a large bio-diversity.

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