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Isolation, Identification, and Biocontrol Potential of Plant Growth Promoting Rhizobacteria *Pseudomonas fluorescens*

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Abstract: ***The indiscriminate use of chemical pesticides and fertilizers caused incredible harm to the environment and ecosystem, including animals and humans. To replace such hazardous inputs added to the agricultural system, biological solutions are provided by nature in the form of microorganisms that have the capacity to promote plant growth without harming the environment. One solution for the control of different phytopathogenic agents is the use of biocontrol in the form of plant growth - promoting rhizobacteria (PGPR). The important group of bacteria Pseudomonas fluorescens belongs to the plant growth - promoting rhizobacteria that play a major role in the ability to adhere to soil particles and to the rhizoplane, motility, prototrophy, induced systemic resistance, and synthesis of antibiotics. Three Pseudomonas fluorescens species were isolated from Rhizospheric soil and produced fluorescent pigments on King's B medium and Nutrient Agar medium. By using the dual culture technique, the efficacy of bacterial antagonists in controlling the fungal pathogen Fusarium solani was determined. The present study refers to the isolation, identification, and antagonistic activities of selected isolates. The isolate 1 strains could serve as promising bioagents, although further in situ investigations are needed.***

Keywords: *Pseudomonas fluorescens*, Plant growth - promoting rhizobacteria (PGPR), Bio control agent, Antagonistic activity.

1. Introduction

The application of chemicals in agriculture affects the soil environment, soil fertility, and the development of fungicide resistance in pathogens. Solutions to chemical control in agriculture have been the focus of scientific research. Biological control is a potential non - chemical way of managing plant diseases that lessens a parasite or pathogen's negative effects by using other living things. Utilising a plant's natural defense system is a stimulating field of research that can be systematically triggered when plants are exposed to strains of plant - growth - promoting rhizobacteria (PGPR) or get infected with the plant pathogen (DavidB et. al., 2018).

Rhizospheric bacteria have been given the name Plant Growth - Promoting Rhizobacteria (PGPR) because they have a number of characteristics that are important for the health and growth of plants. PGPR promotes plant growth in a number of ways, whether directly or indirectly. (Santana - Fernández, A et al; 2021, Harshada S. et al; 2019) Increased nutrient availability, phytohormone production, the development of shoots and roots, protection against a number of phytopathogens, and a decrease in disease are among the many benefits that these bacteria can offer. (Bhattacharyya P. et al; 2012) The PGPR can create enzymes that detoxify plants of heavy metals and help them tolerate abiotic challenges like salt and drought (Alemu F, 2013) (Shahzaman S, 2016).

Due to its ability to decrease the application of synthetic fertilizers and pesticides, promote plant growth and health, and improve soil quality, PGPR has become recognized as a key tool for sustainable agriculture. The literature contains a large number of studies on PGPR. Today, PGPR is being used in a practical method for sustainable production, making it easy to decrease the usage of phosphate and nitrogen fertilizers, as well as fungicides, and to boost nutrient uptake (Manasa K et. al., 2017).

Bacteria of the genus *Pseudomonas* are commonly found among the predominant genera in plant microbiomes, both in the rhizosphere (Bakker, P. Aet al.2013, DonnS et al.2015) and in the phyllosphere (VorholtJ. A. et. al., 2012; Bulgarelli D et. al., 2013; Rastogi G et. al., 2013) irrespective of the host plant species. Due to its important ecological functions, *Pseudomonas fluorescens*is considered as a helpful bacteria. It is frequently present in rhizospheric soil and is essential for soil fertility and nutrient cycling. This bacterium participates in the cycling of carbon, nitrogen, and phosphorus in ecosystems and aids in the breakdown of organic matter. By fostering plant growth, improving nutrient uptake, and offering protection from diseases, *Pseudomonas fluorescens*can colonise the rhizosphere, the area of soil around plants. This bacterium creates a variety of chemicals that encourage plant growth, including as phytohormones and enzymes that help in plant development (Müller T et. al., 2018).

Pseudomonas fluorescens has gained attention for its potential as a biocontol agent against pant pathogens. It produces a variety of antibacterial substances and enzymes that can stop the development of harmful organisms, defending plants against disease (OjhaSK et. al., 2016). It is a useful tool for integrated pest management and sustainable agriculture because of this property. Secondary metabolites having antibiotic activity are known to be produced by *Pseudomonas fluoresces*. Some *P. fluorescens* strains produce substances with antibacterial properties against various diseases, such as pyrrolnitrin and phenazines. This antibiotic contribute to the competitive advantages of *P.*

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*fluorescens*in its ecological niche (Meera T and BalabaskarP, 2012).

Mechanisms of Biocontrol

Plant growth - promoting rhizobacteria (PGPR) regulate plant pathogens such as fungi, bacteria, viruses, nematodes, and others that cause many diseases in agricultural plants. Although the induction of systemic resistance by these rhizobacteria in the host plant is the most researched phenomena, the mechanisms of biocontrol may involve competition or antagonistic relationships. (Yadav J et. al., 2014) PGPR regulates the pathogen - induced damage to plants through a number of mechanisms, such as physical displacement of the pathogen, the secretion of siderophores to stop the spread of pathogens nearby, the synthesis of antibiotics, bacteriocins, and other small molecules that inhibit pathogen growth, the production of enzymes that inhibit the pathogen, and the induction of systemic resistance in the plants.

Pseudomonas fluorescens as non - pathogenic saprophytes that colonize soil, water and plant surface environments. By producing a variety of secondary metabolites, including antibiotics, siderophores, and hydrogen cyanide, *Pseudomonas fluorescens* prevent plant diseases. This microbe has a unique ability to survive in the vascular system of the plant, access the numerous plant organs, and function as a systemic bio - control agent against various bacterial and fungal diseases. Another crucial element in disease prevention and treatment may be the competitive exclusion of pathogens as a result of *Pseudomonas fluorescens*' fast colonization of the rhizosphere (MauryaMet. al., 2016).

2. Materials and Methods

Isolation of *P. fluorescens from Rhizospheric soil*

Samples for the isolation of *P. fluorescens* were collected from the rhizospheric soils of different regions of agricultural soil. One gram of soil from each rhizospheric sample was mixed with 10 ml of sterile water and vortexed for 10 minutes to obtain a standard soil suspension. Isolation of *P. fluorescens* was made by serial dilutions up to 10^{-1} to 10^{-8} using King's B medium. (Sharma H et. al., 2022)

Gram Staining for Identification

Took a clean slide and prepared thin smear of old culture be created and heat fixed. For about 1 minute, add 1 - 2 drops of crystal violet reagent on the smear. Using running tap water, washed the slide and then used Gram's iodine to flood the smear. Let it sit for 2 minutes. Add decolorize solution drop wise, till the violet color fails to come out from the smear. Rinse the smear with water. Counter stain with safranin for 45 - 60 seconds. Washed the slide in faucet water and mount it in oil emulsion or glycerin before taking a gander at it under a magnifying lens (microscope) (NagpalS et. al., 2021; Meera T and Balabaskar P, 2012).

Biochemical tests

For the identification of *P. fluorescens*, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology (Nepali B, 2018).

1) Starch Hydrolysis Test

Spread 0.1 ml of test cultures on the starch agar plates and incubated at 28–30°C for 48 hours. Observe the zone of utilization surrounding the colony. Plates with healthy bacteria were flooded with Lugol's iodine and read immediately because the blue colour fades rapidly (Sigmon J, 2008).

2) Gelatin liquefaction

Filter paper discs were dipped in a day - old culture suspension and placed on Petri dishes containing gelatin - nutrient agar medium. The Petri dishes were incubated at 300 °C for two days and then flooded with a 12.5% HgCl2 solution. The development of a yellow halo around the growth indicates the utilization of gelatin.

3) CT (Catalase Test)

Slide test: place one or two drops of hydrogen peroxide solution on a glass slide. Pick up a well - isolated colony of P. fluoresces and transfer it into the drop of hydrogen peroxide; observe for the production of bubbles. Tube test: streak a loopful of the test culture on the nutrient agar slant or inoculate into the broth tube. Incubate the medium at 37 °C for 24 hours. After incubation, add 1 ml of hydrogen peroxide over the growth on an agar slant or in broth. Observe the effervescence of oxygen.

4) Oxidase test

Grow the test organism freely under aerobic conditions on nutrient agar medium for 18–24 h. Take a filter paper strip and moisten it with 3–4 drops of teramethyl - p - phenylenediamine dihydrochloride solution. With the help of platinium wire, pick up a colony and make a compact smear on moistened filter paper. Wait for 10–15 seconds and observe for the formation of a violet colour.

5) IP (Indole Production) Test

Inoculate tryptone broth with a loopful of test culture and incubate at 37 °C for 24 hours. After incubation, add 3–4 drops of xylene to the medium and shake it well. Allow the two layers to separate. Add slowly 1 ml of Ehrlich's reagent so as to form a layer on the surface of xylene. Observe the formation of a pink - coloured ring at the lower surface of the xylene layer.

6) MR (Methyl Red) Test

Inoculate GPB (glucose phosphate broth) with the test culture and incubate the broth at 37 °C for 48–72 hours. After incubation, add about 5 drops of methyl red indicator to the medium. Observe the development of the red colour.

7) VP (Voges proskauer) Test

Inoculate the test culture into GPB and incubate the medium at 37 °C for 24–48 hours. After incubation, add 0.6 ml of - naphthol and 0.2 ml of KOH solution per ml of culture broth. Shake well after the addition of each reagent, and slope the tube to increase the aeration. Observe the result after 15–60 minutes.

8) CU (Citrate Utilization) Test

Simmon's citrate agar slants were formed, and the bacterial culture was streaked on them. For 36–48 hours, the tubes

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were incubated at 28–32 °C. Record the colour change of the slant after incubation.

9) Urease Production Test

Inoculate a loopful of test culture in urea broth and incubate at 37 °C for 24 hours, then observe the change in colour of the broth after the incubation period.

10) CFT (Carbohydrate Fermentation Test)

First, we took the tubes of broth that had been sweetened with 4 different types of monosaccharides (0.5% of each, for example, glucose, fructose, mannose, and galactose); one Durham tube had been submerged in each tube. Test culture tubes were cultured for 24–26 hours at 28–30 °C. Signs of acid or gas generation, such as a change in colour or the creation of bubbles, were examined in the tubes.

11) Ammonia Production

Inoculate a loopful of test culture in peptone nitrate broth. Place a red litmus paper strip in the mouth of the culture tube in such a position that ¼ to ½ of the strip projects below the cotton plug. Incubate the medium at 37 °C for 24 hours. After incubation, observe for the change of red litmus to blue.

12) Phosphate Solubilization

The ability of isolates to solubilize phosphate was tested using Pikovskaya's agar plates. Plates were checked for the appearance of clearing zones surrounding the colonies after 4 days of incubation at 28–30 °C (appropriate for solubilization of inert phosphate by producing macrobiotic acid by microorganisms) (Subba Rao, 1999).

13) Zinc Solubilization.

Zinc solubilization by microbes was carried out according to (Sayyed's method 2005). Bacteria were identified using Tris - minimal media plates containing zinc phosphate and a pH indicator called bromophenol blue.

14) HCN Production.

A nutrient - sucrose agar medium was used to detect the production of HCN by the antagonistic bacteria. The production of HCN was determined by the change in colour of the picric acid - saturated filter paper from yellow to red - brown.

15) Antagonistic Activities of P. *fluorescens*

A dual culture approach was used to study the antagonistic activities of *P. fluorescens*. Common fungus pathogens were used for the dual culture technique. Such as *Fusarium solani*, *Fusarium moniliforme*, *F. oxysporum*, *Rhizoctoniasolani*, and *Alternaria* alternate. We are using *Fusarium solani* for the dual culture technique, which causes root rot.

Bacterial isolates *P. fluorescens* was streaked on one side of the Petri dish (one cm away from the edge) containing PDA.9 mm mycelial discs from a seven - day - old PDA culture of *F. solani* MTCC 3871 were placed on the opposite sides of Petri dishes perpendicular to the bacterial streak, respectively, and incubated at 28 °C for 5 - 7 days. Petri dishes inoculated with fungal discs alone served as controls. Observations on the width of the inhibition zone and mycelial growth of the test pathogen were recorded, and the percent inhibition of pathogen growth was calculated using the below formula. (Maurya M K et. al., 2016)

Per cent inhibition (I) = C - T/C ×100

Where, C - mycelial growth of pathogen in control

T - Mycelial growth of pathogen in dual culture plate

Another method for dual culture is to insert agar blocks (5mm in diameter) in the center of the assay plate from the margin of a 5 - day - old fungal pathogen culture. One 24 - hour - old isolated strain culture was founded 02 cm away from the pathogen. Plates were incubated for 3–7 days at 28°C. Zone was determined by using the following method: inhibition zone (rate) =100C−T/C,

Where C addresses spiral development in charge T addresses outspread development in double culture. (AarabS et. al., 2015)

3. Result and Discussion

Isolation of *P. fluorescens from Rhizospheric soil*

A well - separated individual colony with fluorescent yellowish - green and fluorescent green and blueish - green pigments was marked and used for further identification test. The individual colony was picked up and streaked on to fresh KMB slants. The slants were covered with parafilm tape and preserved at 4°C for further use.

Colony characteristics

Here, we studied three colonies. All Colonies had a circular convex elevation with a smooth surface and a moist and viscous consistency. The optical characteristics of colonies were opaque or translucent and fluorescently pigmented under UV light. The colony characteristics of all three isolates are represented in Table 1.

Table 1: Colony characteristics of selected isolates

No.	Colony characteristics	Colony 1	Colony 2	Colony 3
1	Size	Intermediate	Intermediate	Intermediate
2	Shape	Round	Round	Round
3	Margin	Entire	Entire	Entire
4	Elevation	Convex	convex	Flat
5	Surface	Smooth	smooth	smooth
6	Consistency	Moist	viscous	Moist
7	Optical character	Opaque	opaque	Translucent
8	Pigmentation	Fluorescent green	Bluishgreen fluorescent	Yellowish green fluorescent

Gram staining and Morphological identification

Morphologically, *P. fluorescens* are gram's negative short rods with a variation of size from 0.6 to 0.8µm × 1.7 to 1.9µm. Here, Gram negative and rod - shaped, signally arranged isolates were discovered in every case, as shown in Table 2. Pure cultures of the selected isolates were streaked on King's B agar and Nutrient agar Petri plates separately for colony development and further biochemical tests. Own growth of isolate 1 *Pseudomonas fluorescens* showed in figure 1.

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Table 2: Morphologic characteristics of selected isolates

No.	Morphological characteristics	Colony 1	Colony 2	Colony 3
1	Size	large	Small	Large
2	Shape	Short rod	Short rod	Short rod
3	Arrangement	Single	Single	Single
4	Gram Reaction	Gram negative	Gram negative	Gram negative

Biochemical test

1) Starch hydrolysis
Starch hydrolysis for each isolate was performed. Zones of hydrolysis were not observed for any of the isolates.

2) Gelatin liquefaction
After two days of incubation, each isolate was flooded with a 12.5% $HgCl_2$ solution. The development of a yellow halo around the growth is visible, which indicates the utilization of gelatin.

3) Catalase test
Slide test: In the slide test, production of bubbles was observed for all the isolates.
Tube test: In the tube test, effervescence of oxygen was observed for all three isolates.

4) Oxidase test
All three isolates appear violet on moist filter paper, which indicates that the organism possesses cytochrome oxidase.

5) Indole production
The development of a bright red colour at the interface of the reagent indicates the presence of indole. In indole production, all three isolates showed negative results.

6) MR (Methyl Red Test)
After adding 5 drops of methyl red indicator to the medium, the development of a red colour was observed in all three isolates.

7) VP (Voges Proskauer)
The development of a red color within 15 minutes indicates the presence of diacetyl. Here, after adding reagents, there is no change in all of the isolates.

8) CU (Citrate Utilization) Test
A positive test is indicated by the development of a deep blue colour within 24 - 48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, but there is no change in colour. It indicates negative results.

9) Urease Production Test:
All three isolates showed a change in colour of the broth into a purple - red colour, which indicates alkalinization and urea hydrolysis, which means all isolates produce urease enzyme.

10) Carbohydrate fermentation test
Colour changes in test tubes indicate acid production and the production of gas collected in Darham vials. All isolates showed acid and gas production.

11) Ammonia production test
Out of three isolates, two showed a change from red litmus to purple, which indicates ammonia production. One isolate showed a negative result.

12) Phosphate Solubilization
On Pikovskaya's agar plate, all of the isolates did not show a zone of solubilization, which indicates that the organism does not have the ability to phosphate solubilize.

13) Zinc Solubilization
Out of three isolates, two showed a zone of solubilization, which indicates zinc solubilization activity. The remaining isolates do not pass zinc solubilization activity.

14) HCN production
Out of three isolates, two showed a change in filter paper colour from yellow to red - brown, whichindicatesHCN production. The remaining isolates do not passes HCN production activity.

15) Antagonistic Activities of *P. fluorescens*
The results of the dual culture technique for *P. fluorescens* against inhibited the growth of *Fusarium solani* fungi significantly. In the case of *Fusarium solani*, an inhibition of 55.8% was recorded by isolate 1. In the case of isolate 2, the inhibition of 44.33 % was measured. Where, in the case of isolate 3, 44.30% inhibition was recorded.

Table 3: Biochemical tests for selected isolates

No.	Biochemical test	Colony 1	Colony 2	Colony 3
1	Starch hydrolysis	Negative	Negative	Negative
2	Gelatin liquefaction	positive	positive	Positive
3	Catalase test	positive	positive	positive
4	Oxidase test	positive	positive	positive
5	IP (Indole Production)	Negative	Negative	Negative
6	MR (Methyl Red)	positive	positive	positive
7	VP (Voges proskauer)	Negative	Negative	Negative
8	CU (Citrate Utilization)	Negative	Negative	positive
9	Urease Production Test	positive	positive	positive
10	Carbohydrate Fermentation Test	positive	positive	positive
11	Ammonia production test	Positive	Positive	Negative
12	Phosphate Solubilization	Negative	Negative	Negative
13	Zinc solubilization	Positive	Positive	Negative
14	HCN production	Positive	Positive	Negative

4. Conclusion

Due to environmental and ecological concerns, the development of biological control agents as an alternative to the development of plant growth - promoting rhizobacteria, *Pseudomonas fluorescens*, has increased. An environmentally acceptable strategy for safe farming of agricultural and horticultural crops against fungal pathogens has generated interest. *Pseudomonas fluorescens* is a Germ - negative, rod - shaped. Bacterium commonly found in Rhizospheric soil. *Pseudomonas fluorescens* Bio fertilizer can help reduce soil health problems and increase crop health. The presented work is a novel approach towards protection and control of root rot caused by fungi diseases in plants. Three isolates were chosen based on their gram staining, morphological characteristics, cultural characteristics, and biochemical tests. Three isolates were

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tested for antagonistic activity using the dual culture technique.

5. Future Scope

In the future, the study of *Pseudomonas fluorescens*as a plant growth promoting rihizobacteria (PGPR) holds great promise for agricultural and environmental application. Research can focus on developing advanced formulations of *Pseudomonas fluorescens* that optimize its survival, efficacy, and delivery to plant roots. This could lead to improved bio control capabilities against various plant pathogens and enhanced plant growth promotion.

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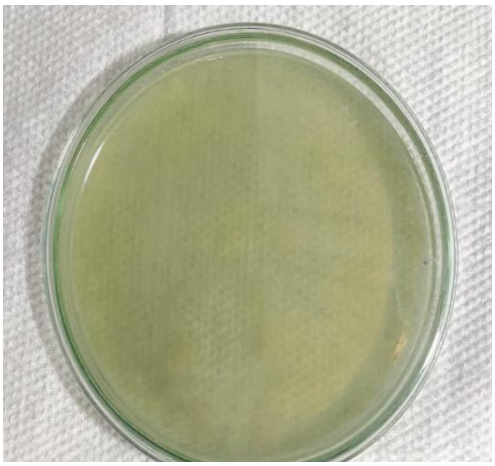

Figure 1: Lown growth of Pseudomonas fluorescens on Nutrient agar plate

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