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Pseudomonas sp.: A promising biocontrol agent against selected phytopathogens

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Abstract: The biological control of plant pathogenic microorganisms by *Pseudomonas* sp. is attracting increasing attention in the agricultural world, since the indiscriminate use of toxic chemicals constitutes a potential threat to both human and animal health, as well as the environment. A total of 27 bacterial strains were isolated from the rizhospheric soil of the tomato (*Solanum lycopersicum*) plant, and their antagonistic activities against a few of the selected pathogenic bacteria and fungi were evaluated using dual-culture assays, biochemical tests for the production of Hydrogen Cyanide (HCN), Indole Acetic Acid (IAA), Iron Chelating Agent (siderophore), and phosphate solubilisation. Furthermore, the separated antagonists were examined for the generation of hydrolytic enzymes such as protease, amylase, ß-1, 3 glucanase, cellulose, chitinase, and pectinase. In all 27 strains isolated, the highest antagonistic activity was shown by only two bacterial strains *Pseudomonas* (VK1and VK2), against *Klebsiella pneumoniae, Salmonella typhi*, *Xanthomonas campestris*, *Bacillus sp*., *Alternaria alternata*, *Aspergillus niger*, *A. flavus* and *Fusarium sp.* Both the strains were identified as *Pseudomonas sp.* on the basis of morphological, physiological and biochemical characterization. Comparatively, strain VK1 was more effective against above pathogens than VK2 strain which was confirmed by dual culture assay. Both the strains of *Pseudomonas sp.* further were characterized fortheir antagonistic traits likeproduction of HCN, IAA, siderophore, insoluble phosphate solubilising ability and hydrolytic enzymes properties.Similarly, VK1 strain showed positive test for all above biochemical tests, while VK2 strain was not able to produce IAA and showed negative test for phosphate solubilizing activities. So, VK1 strain of *Pseudomonas* was able to produce all above hydrolytic enzymes and found to be efficient antagonistic PGPRs. These findings indicated that isolated *Pseudomonas* sp. has a promising natural, eco-friendly, bio-safe and cost-effective approach to cope against both phytopathogenic bacteria and fungi.

Key words: *Pseudomonas* • Biocontrol • Phytopathogen • Siderophore • IAA • HCN • Phosphate solubilisation • Dual culture

Introduction

Extensive use of hazardous chemicals as fungicide, pesticide and fertilizers causes irreparable loss of our environment. To overcome such type of irreparable harm to our environment, it is necessary to adopt alternatives of chemical agents like, pesticides, herbicides, fungicides etc, for eliminating the phytopathogenic microbes. In the present scenario, it is also mandatory to use eco-friendly methods in the management of agricultural practices. Indiscriminate use of chemicals has deteriorated the environment and destroyed the ecological balance.The biological solution forcontrolling different phytopathogens is the use of biocontrol plant growth-promoting rhizobacteria (PGPR), which is capable of suppressing or preventing the plant disease causing organismlike *Azospirillum, Azotobacter, Pseudomonads, Bacillus, Streptomyces, Enterobacter, Clostridium, Burkholderia* (Devi 2022, Tapia 2020).

Biological control is the practice or process by which plant pathogens are controlled by means of another beneficial microorganisms which are termed as antagonists. It involves the use of an organism or organisms to inhibit the pathogen and reduce disease (Cook and Baker, 1983). The increased interest in bio-control is due to its eco-friendly effect, however some of the antagonists also been found to show direct growth promoting effect on infected plants (Glick et.al 1995). *Pseudomonas* bacteria are

the best characterized biocontrol PGPR organism (Upadhyay and Mishra 1998), and they are important group of bacteria that play a major role in the plant growth promotion, induced systemic resistance, biological control of pathogens etc (Ganeshan and Kumar, 2007). Bacterium *Pseudomonas* produces various compounds which are responsible for biocontrol. These important inhibitory compounds are HCN, IAA, siderophores, degradative extracellular enzymes such as chitinase, protease, cellulose, β-1,3 glucanase and antibiotics such as pyrrolnitrin, pyoluteorin, phenazine (Haas and Defago, 2005). Pyoluteorin is a polyketide metabolite, which is well known for its fungicidal, bactericidal and herbicidal activity, was also reported from fluorescent *Pseudomonas* (Pellicciaro et.al 2022). Similarly, natural pyrazolotriazine pseudoidinine which was also isolated from *Pseudomonas mosselii*, can also be used to control plant diseases (Yang et.al 2023).

Pseudomonads are strong competitors in the rhizosphere and on organic matter in the soil due to their metabolic versatility (Bolton et.al 1993). Moreover, *Pseudomonas* also possesses plant growth-promoting traits such as nitrogen fixation, phosphate solubilization, iron chelation and phytohormone production (Panpatteet.al 2016). Dwivedi and Johri, (2003) reported the role of antifungal metabolites produced by *Pseudomonad*s in disease suppression. Antibiotics such as, pyrrolnitrin, diacetylphloroglucinol and phenazines have been found to inhibit the growth of many plant pathogens (Oni et.al 2015; Upadhyay and Srivastava 2008). The roles of lytic enzymes produced by *Pseudomonas* PGC2 in inhibiting the growth of *Rhizoctonia solani* and *Phytopthoracapsici* have been studied by Arora et.al. (2008). Similarly, the antagonistic effect of *Pseudomonas* on six diseases causing fungi*Pyriculariaoryzae*,*Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata* and *Erysiphe cruciferarum* have been reported by Pandey and Chandel (2014). Nagajothi and Jayakumararaj (2020) showed the potential of *Pseudomonas* sp. as rhizosphere microbiome against selected pathogenic fungal strains *Pyriculariaoryzae*, *Rhizoctonia solani*and *Fusarium oxysporum* that causes devastating loss in rice crop yield as a proto-typical from Madurai and Sivagangai Districts, TamilNadu, South India. So, the objective of present investigation was to isolate and screen the biological control potential of bacteria *Pseudomonas* against some selected plant pathogens.

Material and Methods

Isolation and Preliminary Screening: Twenty-seven bacterial strains were isolated from rhizospheric region of Tomato plant fromgarden soilof our college campus at Pauri Garhwal districtby serial dilution methods (Fig. 1A). The isolated bacteria were grown on LB medium and plates were incubated for 48 hrs at 30±2 ^{o}C (Fig. 1B). All the microbial pathogens, *Klebsiella pneumoniae*, *Salmonella typhi*, *Xanthomonas campestris*, *Bacillus sp*., *Alternaria alternata*, *Aspergillus niger*, *A. flavus* and *Fusarium sp.* which were used in these experiments were procured from Applied Microbiology and Biotechnology Lab, Department of Botany, University of Delhi.

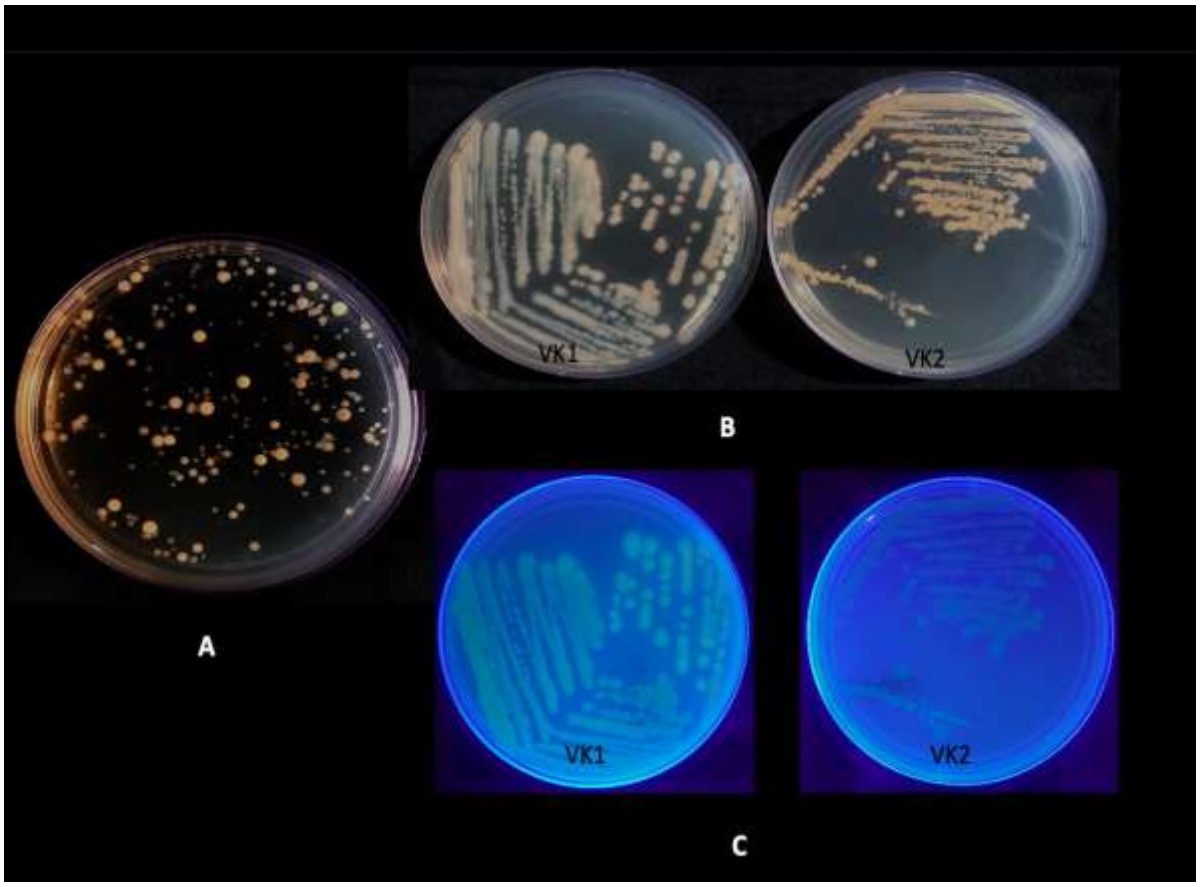

Figure 1. Isolation of rhizospheric microbes by serial dilution method (A), purification of isolated microbes (VK1 and VK2) by streaking plate method on LB media (B) and fluorescent pigment production under the UV light (C).

Bacterial identification using biochemical and physiological characterization: Both the rhizospheric bacterial strains were characterised biochemically and physiologically (Garrity 1984). Morphological features were observed on LB medium. Crystal violet safranin and reaction with 3.0% KOH were used to observe the Gram character (Gregersen 1978, Suslow et al., 1982). Gram negative character of both the strains were confirmed based on their growth on MacConkey agar media (MacConkey 1905). Further Gram -ve character was reconfirmed based on their lactose metabolism. Nutrient broth medium was used to observe the motility of both the strains under the compound microscope at 400X magnification. Spore formation and its characteristics were observed by staining the heat fixed bacterial smear with 0.5% malachite green (Shaeffer and Fulton, 1933). Mixed acid fermentation pathway was detected by Methyl-Red test or MR test. Butanediol fermentation pathway was detected by performing Voges-Proskauer test (Barry et al 1970). Lactose utilization was observed on nutrient broth medium supplemented with 0.5% lactose at pH 7.0, after 24 h pH and red colour changeof the medium was observed (Red to yellow). Oxidase activity, nitrate reduction, urease activity, catalase reaction, gelatin and starch hydrolysis were tested based on the procedure given by Lelliott et al (1966). Triple sugar iron (TSI) agar test was used to determine the ability to ferment glucose, lactose and sucrose based on the procedure given by (Hajna, 1945). Utilization of citrate as sole carbon source was detected by measuring the growth of both the strain on Simmons citrate agar medium supplemented with bromothymol blue as indicator (Simmons 1926). Colonies of VK1 and VK2 growing on

King's B medium were subsequently use to detect the yellow fluorescent pigment production under the UV light (Manjunatha 2017).

In vitro antagonastic activity: In dual-culture assay, antagonistic activity of isolated *Pseudomonas* sp. (VK1 to VK2) was tested against *Klebsiella pneumoniae*, *Salmonella typhi*, *Xanthomonas campestris*, *Bacillus sp*., *Alternaria alternata*, *Aspergillus niger*, *A. flavus* and *Fusarium sp.* Selection of best medium for this assay was done and it was observed that the bacterial colonies grew well on Czapek-Dox Agar (CDA) media, while the fungal colonies grew well on Potato-Dextrose Agarmedia. It was considered necessary to select the best medium to support the growth of the potential antagonist and other selected bacteria and fungi. The distance between the inoculum points of each phytopathogen and potential antagonist was 5 cm. The inoculated plates were incubated in a BOD incubator at 30±2 ºC. Observations on growth of antagonists and the tested pathogens from centre of inoculums were recorded after three days of incubation (Kedia and Singh 2013). The growth inhibition zone and percentage inhibition were calculated by using the formula:

r1 – r2 / r1 X 100

where, r1 – denotes the radial growth of test microbes away from the antagonist.

r2 – denotes the radial growth of test microbes towards the antagonist.

Antimicrobial and Plant Growth Promoting molecules characterization: Isolated *Pseudomonas* sp. (VK1 to VK2) were tested for further characterization by standard biochemical tests (Nithya et.al 2019).

Hydrogen Cyanide (HCN) Production ability - Kings B Medium supplemented with glycine (4.4 g/l) was used to screen the HCN producing properties. Sterile picrate paper was placed inside the lid of King's B medium petri plates inoculated with both the strain and incubated in a BOD incubator at 30±2 ºC (Bakkers and Schippers 1987). Picrate papers wereprepared using Whatman filter paper No.1 shocked in solution containing 0.5% picric acid in 2% sodium carbonate). Change in colour from yellow to orange or brown was observed after 10 days of inoculation as an indication for the production of HCN.

Phosphate solubilization activity - Both the isolateswere inoculated on the turbid Pikovskaya's agar supplemented with Ca_3PO_4 (insoluble phosphate) for 3 days at 30±2 ºC (Pikovskaya, R.I. 1948). Clearing zone around the colonies of *Pseudomonas* sp. (VK1 to VK2) was observed as positive for solubilization of insoluble phosphate compared to control.

Production of Iron Chelating Molecule (Siderophore) - Universal Chrome Azurole S (CAS) assay given by Schwyn and Neilands, 1987 were used to screen siderophore activity under deferrated condition. Iron free MM9 medium supplemented with Chrome Azurole S were used to grow the bacterial strain VK1 and VK2 and incubated at 30±2 ºC for 3-4 days. The production of siderophores was indicated by change in the colour from blue to brownish orange.

Production of growth hormone (Indole acetic acid) -Method given by Bric et al in 1991 were used to screen IAA production. Both the isolates were inoculated in a Patri plate containing King's B medium supplemented with L-tryptophan and incubated for 24 hr 30±2 ºC. The Salkowski reagent (1ml of 0.5 $FeCl_3$ in 50 ml of 35% $HClO_4$) was poured in the inoculated Petri plate. The production of IAA was indicated by change in the colour from light yellow to reddish brownish due to formation of complex between IAA and Fe^{3+}(Kamnev et.al 2001).For further quantification of IAA production, King's B broth with L-tryptophane were inoculated with VK1 strain IAA positive for 24 h. Cell culture were centrifuged at 10,000 rpm for 15 min. 2 ml of cell free culture filtrated was treated

with 4 ml of Salkowski reagent and kept for 20-30min at room temperature in dark and observed the optical density at 536 nm.Colour change from light yellow to reddish brownish indicates the production of IAA during L-tryptophane metabolism (L-Trp-IAM -IAA). Uninoculated broth served as control. Standard curve was prepared with 5-150 µg/ml of IAA for the quantification (Fig. 02C, D).

Production of hydrolytic enzymes: Both antagonistic bacterial strains were tested for hydrolytic enzyme production of protease, amylase, ß-1, 3 glucanase, cellulose, chitinase and pectinase on nutrient agar (NA) medium with 1% of respective substrates for hydrolytic enzymes (Ayyadurai et al.2006 and Kedia 2010). The formation of a halo zone surrounding the bacterial colony was considered to be a positive test for hydrolytic enzyme production by both antagonists.

Results

Isolation and morphological characterization: A total of 27 bacterial strains were isolated from rhizospheric region, out of which only 02 bacterial strains were found to be effective antagonist against selected pathogens. Based on biochemical and morphological tests, effective isolates strains were identified as *Pseudomonas* sp. (VK1 to VK2). Both *Pseudomonas* sp. were taken for further morphological and physiological studies. Both isolates were Gram-negative, rod-shaped, motile and positive for catalase, oxidase and citrate utilization and show yellow colour florescent production under UV light (Table 1, Figure 1 C).

Table 1: Biochemical and physiological characterizationof isolated rhizospheric antagonistic *Pseudomonas* sp. (VK1 to VK2).

Isolated strains	Gram's reaction	Shape	Motile	Spore	Fluorescent	MR	VP	Oxidase	Citrate utilization	Urease	H2S	Gelatin Hydrolysis	Catalase	Nitrate Reduction	Lipase	ONPG Test	Peroxidase	Lactose utilization
VK1	-	Rod	+	Non-Sporing	+	-	-ve	+	+	-	-	+	+	+	+	-	-	+
VK2	-	Rod	+	Non-Sporing	+	-	-ve	+	+	-	-	+	+	+	+	-	-	+

Screening of antagonastic activity: CDA medium was found to be the best medium supporting the growth of *Pseudomonas* sp. (antagonist) and the test organisms both. In dual-culture assay, a *Pseudomonas* sp. VK1 strain was shown to have more growth inhibition of plant pathogen on the CDA medium than *Pseudomonas* sp.VK2 isolates (Table 2). Observations on dual-culture assays revealed that *Pseudomonas* sp. VK1 strain showed maximum percentage (80%) of inhibition against*Alternaria alternata* followed by *Aspergillus niger* (78%), *Aspergillus flavus* (77%) and *Fusarium sp.* (69%).

However, antibacterial activity was observed slightly low than antifungal activity. *Pseudomonas* sp. VK1 strain showed maximum percentage (72%) of inhibition against *Xanthomonas campestris*. Similarly, the percentage inhibition of growth of the *Pseudomonas* sp.VK2 strain was determined maximum (71%) against *Aspergillus niger*. It

was observed that the *Pseudomonas* sp.VK2 strainshowed almost same level of antibacterial and antifungal activity, but as compared to the *Pseudomonas* sp. VK1 strain, the earlier strain was less effective for antagonistic activity (Table 2).

Table 2: Percentage inhibition of growth of various bacterial and fungal species by *Pseudomonas* sp. (VK1 to VK2).

Sr. No.	Microorganism	Growth in control (cm)	Percentage inhibition of growth	
			VK1	VK2
Bacteria				
01	*Klebsiella pneumoniae*	4.3 ± 0.2	71%	64%
02	*Salmonella typhi*	3.7 ± 0.3	67%	59%
03	*Xanthomonas campestris*	2.8 ± 0.2	72%	67%
04	*Bacillus sp.*	2.2 ± 0.2	65%	62%
Fungi				
05	*Alternaria alternata*	5.1 ± 0.2	80%	59%
06	*Aspergillus niger*	5.2 ± 0.4	78%	71%
07	*Aspergillus flavus*	5.9 ± 0.3	77%	68%
08	*Fusarium sp.*	5.7 ± 0.2	69%	61%

Biochemical assay for secondary metabolites: The biochemical assay for secondary metabolites production results indicated that both the strainsof *Pseudomonas* sp. (VK1 and VK2) produced HCN, siderophores and were able to solubilise phosphate. Furthermore, *Pseudomonas* sp. VK1 isolates produced more siderophores than *Pseudomonas* sp. VK2 strain (Fig. 02 A). IAA detection test was performed with both *Pseudomonas* sp. strains, in which the change in colour of medium indicated IAA production by VK1 strain only, while isolated strain VK2 showed negative response for IAA production (Fig. 02 C&D), and phosphate solubilisation ability was shown by VK01 strain of *Pseudomonas* sp. (Fig. 02 B).

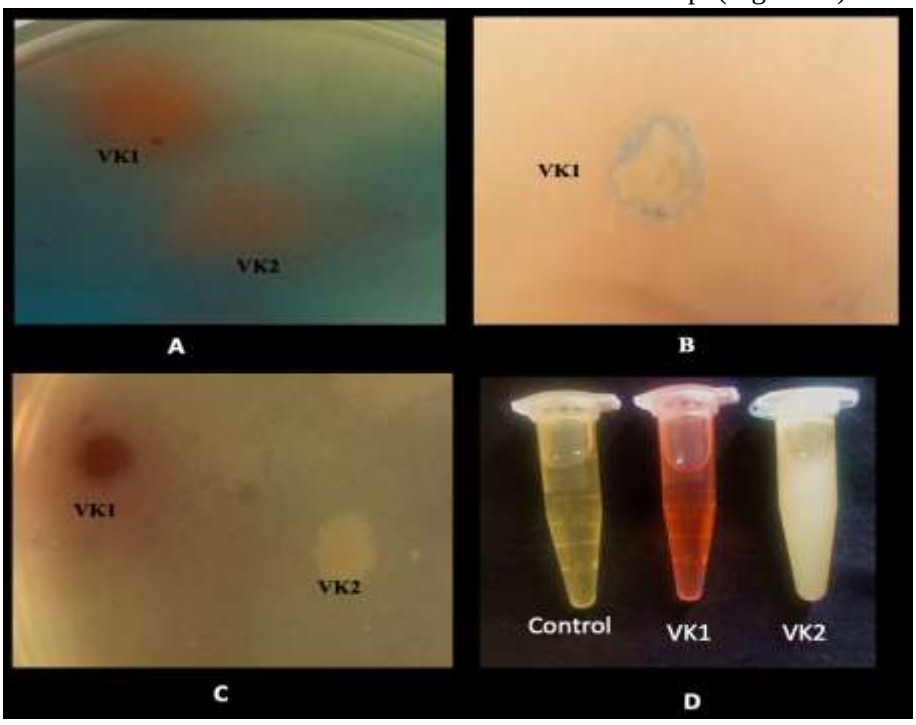

Figure 2: Culture plate showing the production of siderophore (A), phosphate solubilizing ability (B), Indole Acetic Acid production on culture plate (C) and IAA production in culture broth of both the strains VK1 and VK2 (D).

Hydrolytic Enzyme assay: Hydrolytic enzymes assay revealed that both isolates (VK1 and VK2) were able to produce protease, amylase, ß-1, 3 glucanase, cellulose, chitinase and pectinase enzymes (Table 3). These lytic enzymes play crucial role in suppression of phytopathogens, therefore the production of hydrolytic enzymes was conducted during this study.

Table 3: Assay of various hydrolytic enzymes from soil isolated *Pseudomonas* sp. (VK1 to VK2).

Isolated strains	Protease	Amylase	ß-1,3 glucanase	Cellulose	Chitinase	Pectinase
VK1	+	+	+	+	+	+
VK2	+	+	+	+	+	+

Discussion

Nowadays, much research is aimed at understanding, at the molecular level, the mechanisms that enable *Pseudomonas* strains to act as efficient biological control agents (Walsh et.al 2001). *Pseudomonas* sp. that can colonise the roots of crop plants and produce antifungal metabolites represent a real alternative to the application of chemical fungicides (Kumar et.al 2005). Beneficial rhizobacteria are known to colonize rapidly and aggressively the root system, suppress pathogenic microorganism, and enhance plant growth and development. Haas and Keel (2003) studied the PGPR *Pseudomonas* for regulation of antibiotic production and its relevance for biocontrol of plant diseases. Even most of the studies revealed that *Pseudomonas* is the most efficient biocontrol agent in the soil (Haas and Defago 2005; Bakker et al 2007).

In present investigation, the isolated rhizospheric strains VK1 and VK2 exhibited antagonistic effects against selected pathogens like *Klebsiella pneumoniae*, *Salmonella typhi*, *Xanthomonas campestris*, *Bacillus sp*., *Alternaria alternata*, *Aspergillus niger*, *A. flavus* and *Fusarium sp.* Similarly, many scientists have also reported that rhizobacteria have shown antagonistic activity against various fungal and bacterial phytopathogens (Elsayed et.al., 2020; Suresh et.al., 2021). The production of secondary metabolites, hydrolytic enzymes, HCN, siderophores and phosphate solubilisation ability play important role in the suppression of fungal and bacterial phytopathogens (Antoun and Kloepper, 2001; Nithya et.al 2019). In this context, results from our investigation showed the production of hydrolytic enzymes like protease, amylase, ß-1, 3 glucanase, cellulose, chitinase and pectinase by isolated strains VK1 and VK2. Chitin and ß-1,3-glucan are the main structural components of fungal cell-wall. Thus, chitinases and ß-1,3-glucanases secreted by these antagonists have been considered as the key enzymes in the lytic process of soil-borne phytopathogenic fungal cell-wall during mycoparasitic action (Lorito et al., 1994). Similarly, production of various metabolites like HCN, siderophores and phosphate solubilisation ability were shown by isolated *Pseudomonas sp.* VK1. Accordingly, *Pseudomonas* VK1 was found to be the most suitable candidate as it exhibited the capacity to inhibit maximum growth of fungal pathogen by producing various lytic enzymes.

Harikrishnan et.al., 2014 revealed that many rhizobial microbes like *Trichoderma, Pseudomonas, Bacillus* and *Streptomyces* were effectively enhance the growth of various crop plants. Similarly, our antagonist strain VK1 was also able to secretion of IAA. These isolated *Pseudomonas sp.* may act as a PGPR and able to produce different plant growth promoting substances like IAA as reported by Ricci et.al., 2019. Spaepen et.al., 2007

suggested that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phyto-stimulation and circumvention of basal plant defence mechanisms (Rathore et al 2020).

We must consider the use of biocontrol agents for disease management and plant growth when practising sustainable agriculture. *Pseudomonas fluorescens* is a PGPR that can aid in plant growth as well as the control of several plant diseases. It has been used in agriculture for decades as a biofertilizer as well as a biocontrol agent. It has enormous promise in terms of plant nutrient satisfaction, phytopathogen control, bioremediation, and so on. As a result, it has enormous potential as an alternative to agrochemicals because it is less expensive, more efficient, environmentally benign, and an effective PGPR in crop yield enhancement. Aside from that, it lowers input costs and pollution (Bhetwal 2021).

Conclusion

In present studies, *Pseudomonas* strain VK1 has been suggested as a potential biological control agent due to its ability to prevent the growth of harmful fungi and bacteria by the production of antifungal and anti-bacterial compounds. The soil isolate VK1 may prove the capacity to produce hydrolytic enzymes, secondary metabolites and plant growth promoting substances. Thus, PGPR *Pseudomonas* is an important component of integrated pest management system, which reduces the use of agrochemicals and it is a way for economical and eco-friendly agriculture.

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