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Isolation of antagonistic bacteria from chickpea rhizosphere (*Cicer arietinum* L.)

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Abstract
India is the world's leading chickpea producer. It covers roughly 38% of the entire pulse crop area in India and accounts for about 50% of overall pulse production. By reducing the activity of plant diseases, biological control provides a low-cost, environmentally acceptable alternative to the use of expensive and harmful pesticides. Rhizobacteria is among the plant growth promoter and antagonistic organism against soil-borne pathogens. Many different places at random were chosen and soil samples and root samplings of chickpea rhizospheric soil were collected at Agriculture Research Station, Ummedganj-Kota and College of Agriculture, Kota. Total 10 isolates were obtained from different soil samples before and after flowering from different investigation areas. Isolation of rhizobacteria was done on nutrient agar. Rhizobacterial isolates were designated as PR 1 to PR 10 on the basis of biochemical characterization. The results of biochemical tests performed for the identification of effective rhizobacterial isolates showed that all isolates showed similar results with regard to gram staining (negative), catalase test (positive), H_2S test (positive) and KOH test (positive). PR 1, PR 2, PR 5, PR 7, PR 8, PR 9 and PR 10 showed positive results in starch hydrolysis test. Out of 10 isolates, 7 isolates showed positive results for casein hydrolysis, 6 isolates showed positive results for indole production and only 2 isolates PR 6 and PR 7 showed positive results for urease test.

Keywords: Rhizobacteria, biological control, biochemical tests, characterization

1. Introduction

India is the world's leading producer of chickpea. Chickpea occupies about 38% of area under pulse crop and contributes to about 50% of total pulse production in India (Yadav *et al*., 2020) [9]. It is a rich source of protein (21.1%), with high quality of albumins and globulins, along with smaller quantity of glutens and prolamins (Wallace *et al*., 2015) [8]. Various plant diseases caused by pathogenic microorganisms causes destruction of plant tissues and reduces yields varying from 25% to 100% (Iqbal *et al.,* 2019) [3]. Due to the increased demand of high yielding crops under the green revolution, agricultural practices has shifted more towards the usage of chemical fertilizers which results in higher productivity. However, the widespread use of pesticides is a subject of public concern as it may cause harmful effects on the environment. Moreover, the pathogen develops resistance against the repetitive application of pesticides. The traditional cultural practices would not be feasible on large scale as they are time consuming and costly (Mustafa *et al*, 2019) [6]. Moreover, the development of disease resistant varieties is a year's long process and is a most challenging breeding objective. To solve this issue, we need to develop some innovative technologies with more emphasis on the use of indigenous soil microbes, particularly biocontrol agents for sustainable food production. Biological control thus offers an alternative approach to the use of costly and harmful chemicals and provides low cost, environmental friendly control measures to reduce the activity of plant pathogens (Manczinger *et al,* 2002) [5]. For plant-microbe interactions, the plant rhizosphere is an essential ecological ecosystem in soil. Pathogenic microorganisms cause a variety of plant diseases that weaken or kill plant tissues, resulting in crop yield reductions ranging from 25% to 100%. Root diseases are estimated to cause 10-15% yield losses annually in the world (Dua and Sindhu, 2012) [2]. Biocontrol agents include all classes of organisms like fungi, bacteria, virus, nematodes,

protozoa, viruses and seed plants that inhibit or arrest the growth of other living organisms. The rhizosphere of plant is an important ecological environment in soil for plant- microbe interactions. Besides functioning as biocontrol agents, PGPR protect plants against pathogens by eliciting biochemical and molecular defense responses within the plant (Lugtenberg and Kamilova, 2009) [4]. Among the various biocontrol agents, antagonistic rhizobacteria is being opted due to its abundant presence in rhizosphere which serve as an energy source, promoting growth and influencing the root system for the rhizobacteria, they also have products that inhibits the growth of soil borne pathogens which provide additional advantage to the plant roots (Shaikh and Sayyed, 2015) [7]. Antagonistic rhizosphere microorganisms without disrupting the ecological balance, inhibits the growth of pathogenic microrganisms and thus biological control strategies are highly compatible with the sustainable agriculture. The collection of the soil samples from chickpea rhizospheres in the field gives information about the extent of diseases affecting the crop in different locations.

2. Materials and Methods

2.1 Isolation of rhizobacteria from chickpea rhizosphere

Samplings of root and soil system was done from chickpea rhizosphere, before flowering and after flowering stage. Different sites were demarcated in chickpea growing fields at Agricultural Research Station, Ummedganj-Kota and College of Agriculture, Kota. The plants were carefully uprooted, labelled, put in poly bags and brought to laboratory for isolation of rhizobacteria.

The soil adhering loosely to the roots was washed thoroughly under running tap water. Root samples were crushed in the mortar with the help of pastle and shaken with 100 ml sterilized distilled water for 10-20 minutes to obtain bacterial suspension. Different dilution of this bacterial suspension was obtained through serial dilution technique and was processed for soil samples collected from rhizosphere. Suitable dilution of both rhizoplane and rhizosphere solutions was then plated on appropriate culture medium (Aneja, 2002) [1]. The culture plates were then incubated in an incubator at appropriate temperature for 24-48 h and colony growth was observed.

100 ml of liquid nutrient agar medium was inoculated with 5.0 g of soil samples and incubated for 7 days at 30°C. From these enriched samples isolation was performed following serial dilution method. Well separated individual colonies with light yellow, yellow and white pigments were marked and detected by viewing under U.V. light. The individual colonies were picked up with sterilized loop and transferred on fresh nutrient agar medium. The plates were incubated at 28±2 °C for 24 h the single colonies developed were subsequently transferred in King's B medium slants and the pure cultures so obtained were stored in refrigerator at 4 °C till further processing. Further the isolates were subjected to biochemical characterization to distinguish the isolates among themselves. The rhizobacterial isolates were designated as PR 1 to PR 10.

3. Results and Discussion

3.1 Isolation of rhizobacteria from chickpea rhizosphere

In this study, a total of ten rhizobacterial isolates were procured from rhizosphere soil samples collected from chickpea plants at various investigation sites, both before and after the flowering stage. The soil samples were cultured on solid nutrient agar (NA) plates and incubated at temperatures ranging from 28 °C to 30 °C for 3 to 4 days (as shown in Plate 1). This initial incubation period allowed for the growth of rhizobacteria present in the soil samples. To enhance the chances of isolating specific rhizobacterial strains, the initial cultures were further enriched. This enrichment process involved transferring the isolates to nutrient agar (NA) medium and incubating them for an additional 7 days. Enrichment in nutrient agar provides optimal growth conditions and allows for the proliferation of bacterial populations, making it easier to isolate and identify specific rhizobacterial strains. After the enrichment phase, the samples were serially diluted in a range from 10^{-2} to 10^{-6}. This serial dilution helps in reducing the concentration of microbial cells, which allows for better separation and isolation of individual bacterial colonies when spread on the surface of fresh NA medium. By spreading these diluted samples on NA plates, distinct colonies could be obtained and selected for further analysis. The enriched and isolated bacterial cultures were then designated as PR 1 through PR 10. This designation provides a systematic way to refer to the different isolates throughout the study. The process of enrichment and serial dilution significantly increases the likelihood of isolating specific rhizobacterial strains, thereby improving the efficiency and reliability of the subsequent characterization and analysis. This methodical approach ensures that the isolates are representative of the rhizobacterial diversity present in the rhizosphere of chickpea plants and provides a solid foundation for further study of their properties and potential applications.

These isolates were first distinguished based on colony colour in visible light and fluorescence produced under U.V. On nutrient agar medium in visible light, isolates PR 2, PR 3 showed yellow pigment, PR 5 showed light yellow pigment and the rest of the isolates showed white pigment. PR 3 and PR 4 showed fluorescence in U.V. light at 365nm while others were non fluorescent (Table 1).

All the isolates of rhizobacteria showed gram reaction negative. Further the isolates were subjected to biochemical characterization to distinguish among themselves and results revealed catalase test positive ranging from high activity (PR 3) to low activity, and KOH test positive. Rhizobacterial isolates PR 1, PR 2, PR 5, PR 7, PR 8, PR 9 and PR 10 showed positive results for starch hydrolysis by forming clear halo zone around the colony. All the isolates (except PR 7, PR 8 and PR 9) showed positive test for casein hydrolysis by forming clear halo around the colony. In H_2S test all the rhizobacterial isolates showed positive test by developing black colour along the stab. For indole production all the rhizobacterial isolates, excluding PR 3, PR 4, PR 5 and PR 10 showed positive results. Out of all 10 rhizobacterial isolates only PR 7 and PR 10 showed positive result for urease test which was confirmed by formation of pink colour in test tubes (Table 2).

Plate 1: Sampling and collection of soil samples from chickpea rhizosphere

Table 1: Pigmentation of isolates on nutrient agar media under visible and U.V. light

S. No.	Name of isolate	Pigmentation	
		Visible light	U.V light
1.	PR 1	White	Non fluorescence
2.	PR 2	Yellow	Non fluorescence
3.	PR 3	Yellow	Fluorescence
4.	PR 4	White	Fluorescence
5.	PR 5	Light yellow	Non fluorescence
6.	PR 6	White	Non fluorescence
7.	PR 7	White	Non fluorescence
8.	PR 8	White	Non fluorescence
9.	PR 9	White	Non fluorescence
10.	PR 10	White	Non fluorescence

Table 2: Biochemical characterization of rhizobacterial isolates

Rhizo-bacterial Isolates	Catalase test	KOH test	Starch hydro-lysis	Casein hydro-lysis	Indole produc-tion	H_2S produc-tion	Urease test
PR 1	+	+	+	+	+	+	-
PR 2	+	+	+	+	+	+	-
PR 3	+++	+	-	+	-	+	-
PR 4	+	+	-	+	-	+	-
PR 5	++	+	+	+	-	+	-
PR 6	+	+	-	+	+	+	+
PR 7	+	+	+	-	+	+	+
PR 8	+	+	+	-	+	+	-
PR 9	+	+	+	-	+	+	-
PR 10	+	+	+	+	-	+	-

+ Positive; - Negative; Catalases: +++ High, ++ Moderate, + Low activity

4. Conclusion

In this study, a total of ten rhizobacterial isolates were obtained and designated as PR 1 through PR 10 based on initial characterization. These isolates were further differentiated using morphological observations, color appearance under ultraviolet (UV) and visible light, and a series of biochemical tests. All isolates shared consistent results in several key biochemical tests: they were all Gram-negative, indicating they do not retain the crystal violet stain used in Gram staining. They were catalase-positive, meaning they produce the enzyme catalase that breaks down hydrogen peroxide into water and oxygen. Additionally, all isolates tested positive for hydrogen sulfide (H2S) production and the potassium hydroxide (KOH) test, both of which provide insights into their metabolic capabilities. Further differentiation among the isolates was achieved through additional starch hydrolysis

Further differentiation among the isolates was achieved through additional biochemical tests:

- **Starch Hydrolysis:** PR 1, PR 2, PR 5, PR 7, PR 8, PR 9, and PR 10 were positive for starch hydrolysis, indicating their ability to produce enzymes that break down starch into simpler sugars.
- **Casein Hydrolysis:** Seven isolates demonstrated positive results for casein hydrolysis, which shows their capability to degrade casein, a major protein in milk, into smaller peptides and amino acids.
- **Indole Production:** Six isolates were positive for indole production, signifying their ability to convert the amino acid tryptophan into indole.
- **Urease Test:** Only PR 6 and PR 7 showed positive results for urease activity, indicating that these isolates can hydrolyze urea to produce ammonia and carbon dioxide.

This detailed biochemical profiling reveals the functional diversity among the rhizobacterial isolates. While they share some common characteristics, such as Gram-negative staining and catalase activity, their varied abilities to hydrolyze starch and casein, produce indole, and hydrolyze urea suggest a range of metabolic capabilities. Such diversity can be important for understanding their potential applications in agriculture and biotechnology.

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