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Antifungal and Plant Growth Promoting Characters of Siderophore Producing Bacteria

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Abstract

The experiment was conducted during January (2021–2022) at Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Surat, Gujarat, India to study antifungal and plant growth promoting activities of siderophore producing bacteria isolated from cotton rhizosphere. Siderophore producing bacteria, coded as NAU-RPJ-54 was isolated from the cotton rhizospheric soil at Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU). It showed 0.94 of zone index (ZI) and 66.24% siderophore unit (PSU) with Chrome Azurol Sulfonate (CAS) agar and liquid assay, respectively at 30±2°C for 72 hrs. Csaky's assay and Thin Layer Chromatography (TLC) revealed that it possessed hydroxamate type of siderophore. Confrontation assay revealed 84.04% and 61.53% mycelium inhibition of *Sclerotium rolfsii* at 72 hrs and *Fusarium oxysporium* f.sp. *Lycopersicon* at seven days, respectively. *In vitro* studies of plant growth promoting activities showed that NAU-RPJ-54 produced indole acetic acid, cellulase, protease and HCN; it solubilized zinc, potash and phosphate. Further, salt tolerance test using nutrient agar prepared with different concentration *viz*. 100, 200, 400 and 600 mM of NaCl, $CaCl_2$ and $MgCl_2$ mixture at ratio of 3:2:1, respectively showed NAU-RPJ-54 was resistant to salt upto 600 mM. Microbiological characterization indicated that NAU-RPJ-54 might belong to *Statphylococcus* genus. NAU-RPJ-54 showed potential attractive attributes like siderophore production, antifungal activity and plant growth promoting traits. Further, comprehensive studies required to explore this bacterium in field conditions.

Keywords: Cotton rhizosphere, siderophore, plant growth promoting, protection activities

1. Introduction

Phytopathogens are one of the major threats that significantly affect the yield and quality of crop products. The use of synthetic pesticides leads to realize protection of the crops from insect pest, quality product with increased production with an impact on environment. Indeed, prolonged use of these pesticides caused adverse effects on environment including human health (Ayilara et al., 2023; Deb and Tatung, 2024). Furthermore, this practice has rendered fertile soil infertile, imbalanced microflora composition in the soil and leaching of chemicals into water bodies that caused extensive damage to the ecosystems which rising concerns for the future (Chincholkar et al., 2000; Mir et al., 2022). Sustainable agriculture focuses on the crop production and protection practices that are environmentally safe. Thus, sustainable agriculture warrants an urgent need of the alternative environment friendly approach for crop management practices.

In this regard microflora of rhizosphere known to play crucial role in plant growth and protection through various direct and indirect mechanisms (Hayat et al., 2010; Hussein and Joo, 2012; Kumari et al., 2020). Herlihy et al. (2020) reported that in iron-stressed soil, plant itself also produced phytosiderophore to maintain the iron level required for plant metabolism but is does not attain the perfect level of sufficient iron. In this situation, microbes of plant rhizosphere produce siderophore that chelate and solubilize iron from minerals under stress condition and translocate it to the plant. Siderophore is the Greek word meaning 'iron carrier' and was first isolated during 1949-1952 by Hider and Kong (2010). Further, the crystalline form of siderophore was isolated by Neilands (1952) that triggered research activities for microbial siderophore (Chincholkar et al., 2000). Siderophore producing microbes act as biocontrol agent was first time reported by Klopper et al. (1980). Few studies reported that such siderophore producers have potential to suppress phytopahogens, solubilize minerals, produces hormones, synthesize plant protecting

lytic enzymes that protect the plants against stress condition (Sati et al., 2023; Deb and Tatung, 2024). Further, production of siderophore is closely related to cyanide production, and the absence of siderophore production can impact the biocontrol activity of the microbes (Ho et al., 2021). Many bacteria like *Alcaligenes faecalis*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Azotobacter* sp., *Bacillus cereus*, *Bacillus simensis*, *Bacillus subtilis*, *Brevundmonas diminuta*, *Brevibacillus brevis*, *Enterobacter* sp., *Pseudomonas fluroscens*, and *Providencia* sp. are siderophore producers.

Cotton crop grown worldwide and as a glycophyte it shows tolerance to biotic and abiotic stress as compare to wheat, rice and maize (Yu et al., 2016). Thus, cotton rhizosphere might possess potential microbes that can be useful as biopesticide and/or biofertilizers. Thus, an attempt made to study antifungal and plant growth promoting activities of siderophore producing bacteria isolated from cotton rhizosphere.

2. Materials and Methods

A total of 18 bacteria isolated from rhizospheric soil of cotton crop from Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Surat, Gujarat, India and screened for siderophore production in the year 2021–22. Bacteria coded as NAU-RPJ-54 was one of the potent siderophore producer with antifungal activities against two plant pathogenic fungi *viz*., *Sclreotium rolfsii* and *Fusarium oxysporum* f.sp. *lycopersici* and also showed plant growth promoting activities, described in this research paper. The bacterial and fungal cultures maintained on nutrient agar and potato dextrose agar slants, respectively at 4°C.

2.1. Chrome azurol sulphonate (CAS) method

Qualitative and quantitative CAS method was performed to screen siderophore production by the bacteria as per method of Schwyn and Neilands (1987). Bacterial culture enriched in the succinic acid medium was spot inoculated on CAS agar plate for qualitative assay; while 10 ml culture was inoculated in 90 ml of succinic acid medium in 250 ml of Erlenmeyer flask for quantitative assay. Plates and flasks were incubated at 30±2°C for 72 hrs. Zone index (ZI) based on yellow to orange color formation was calculated as per formula; Zone Index (ZI) = Diameter of zone (mm) – Diameter of colony (mm)/ Diameter of colony (mm). For CAS liquid assay, percent siderophore unit (% SU) was calculated based on color change of CAS dye that was measured through spectophotometrically at 630 nm. Siderophore unit (%) was calculated using the formula: PSU (%)=[(Absorbance of reference-Absorbance of sample)]/ Absorbance of reference×100.

2.2. *Thin layer chromatography, arnow's and csaky's tests*

Detection of siderophore was carried out by Thin Layer Chromatography (TLC) as per method of Kumari et al. (2021); while identification of type of siderophoredone by Arnow's and Csaky's test (Arnow, 1937; Csaky, 1948). Hydroxylamine hydrochloride and 2, 3-dihydrobenzoic acid used as positive control for hydroxamate and catechol type of siderophore, respectively.

2.3. Antifungal activity

Antifungal activity was performed through confrontation assay against two soil borne pathogenic fungi *viz*. *Sclreotium rolfsii* and *Fusarium oxysporum* f.sp. *lycopersici* as per method of Kotasthane et al. (2017). Supernatant of enriched bacterial culture prepared in succinic acid medium was inoculated surrounding the pre-inoculated fungi block on nutrient agar plate. The percent mycelia growth inhibition was calculated using the formula; Growth of pathogen in control (mm)– Growth of pathogen in test (mm)/Growth of pathogen in control (mm)×100.

2.4. Plant growth promoting assay

Minerals solubilization characters *viz*., zinc, potassium and phosphate of the bacteria detected using method of Di Simine et al. (1998), Singh and Sindhu (2013) and Pikovskaya (1948), respectively. Indole-3-acetic acid (IAA) and Gibberellic acid (GA3) production was studied using method of Bric et al. (1991) and Henderson and Graham (1962), respectively. Chitinase, protease and hydrogen cyanide (HCN) traits determined using chitin agar plate (Kuddus and Ahmed, 2013),skim milk agar plate (Patel and Patel, 2009) and nutrient agar plate embedded with glycine (Lorck, 1948), respectively. The tolerance ability of the bacteria with salt stress was studied using nutrient agar plates prepared with varied concentrations (100, 200, 400 and 600 mM) of NaCl, $CaCl_2$ and $MgCl_2$ in the ratio of 3:2:1, respectively as per method of Pirhadi (2016).

2.5. Microbiological characters of the bacteria

Microbial characterization *viz*., morphological, cultural and biochemical of the bacteria was done through gram reaction, colony appearance on nutrient agar plate; KB003 Hi25 Identification kit and KB009 Hi-carbohydrate kit, respectively.

2.6. Statistical tools

Completely Randomized Design (CRD) was used to test the level of significance. Critical difference (CD) between variance was calculated as $p \leq 0.05$.

3. Results and Discussion

3.1. Siderophore production, detection and characterization

Microbial activity is 10 to 1000 fold higher near plant roots (Ownleyet al., 2004), presenting both pathogenic and antagonistic microbes attributing plant growth promoting (PGP) properties. Among the diversified secondary molecules produced by microorganisms, siderophores is a versatile biomolecule with both antagonistic and PGP activities. Bacterial strain NAU-RPJ-54, isolated from the cotton rhizosphere was identified as a potent siderophore producer. It produced ZI of 0.94 and 66.24% siderophore unit (PSU) production with Chrome Azurol Sulfonate (CAS) agar and liquid assay, respectively at 30±2°C for 72 hrs (Figure 1(a)

and 1(b)). Previously, Hussain and Jho (2012) reported *Staphylococcus* species with 91.2% production of siderophore. Mokrani and El-hafid (2020) identified potent siderophore producers from the *Phaseolus vulgaris* L and *Allium cepa* L rhizosphere.

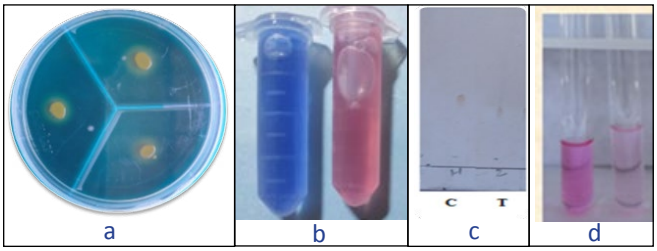

Figure 1 : Detection of siderophore through (a) CAS agar (b) CAS liquid assay (c) TLC and (d) Csaky's test

Siderophores are iron binding ligands and the iron ligation groups are mainly classified into hydroxamate, catechol and mixed types. Arnow's, Cskay's and TLC are the techniques used to detect and characterize the type of siderophore. Thus, the siderophore producer NAU-RPJ-54 was characterized using TLC with Arnow's and Csaky's tests. Pink spot on TLC along with Rf value of test 2.0 that close to theRf value of control 2.3 (Figure 1 (c)); development of pink color in Cskay's test (Figure 1(d)) revealed the presence of hydroxamate type of siderophore. Watteau and Berthelin (1994) observed that *Baillus granulatus* produced hydroxamate siderophores that played key role in the solublization of iron minerals. Radhakrishnan et al. (2014) reported hydroxamate type of siderophore by *Bacillus* Sp.

3.2. Antifungal and plant growth promoting traits

Siderophore is an important biomolecule that required to constituent proteins for microbial growth during stress or iron limiting condition. Iron uptake through siderophore producing bacteria, create iron depletion in the rhizosphere that cause inhibition of fungal or other phytopathogens. Confrontation assay was performed to detect the antifungal activity of NAU-RPJ-54 against two phytopathogenic fungi *viz*., *Sclerotium rolfsii* and *Fusarium oxysporum* f.sp. *lycopersici.* The fungal mycelium inhibition was found 84.04% and 61.53% with *S. rolfsii and F. oxysporum* f.sp. *lycopersici* at 72 hrs and seven days, respectively (Figure 2). Ghazyand Nahrawy (2021) identified potent siderophore producing strains *Bacillus*

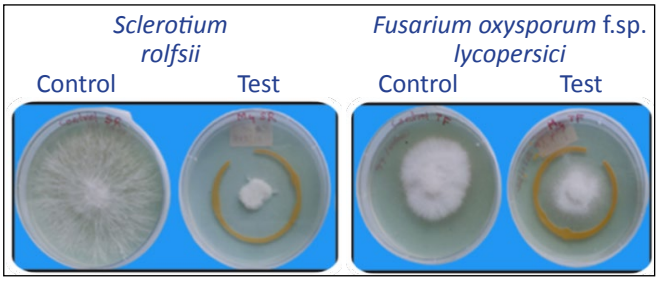

Figure 2: Antifungal activity of NAU-RPJ-54

subtilis and *Pseudomonas koreensis* that showed inhibition of pathogenic fungi *Cephalosporium maydis*. Recently, Shen et al. (2022) reported *Bacillus simensi* with antifungal activity of 68.8% against *Fusarium oxysporum* f.sp. *lycopersici* with 56.43% siderophore unit production.

Moreover, many siderophore producers are used as plant growth promoters due to its PGP traits. *In vitro* studies of PGP revealed that NAU-RPJ-54 significantlysolubilizedzinc;produced protease and IAA; while moderately solubilized potash and phosphate and produced cellulase, chitinase and HCN (Figure 3 and Table 1) at 72 hrs at 30±2°C except chitinase that produced after ten days of incubation. Further, NAU-RPJ-54 was found inept for gibberellic acid.

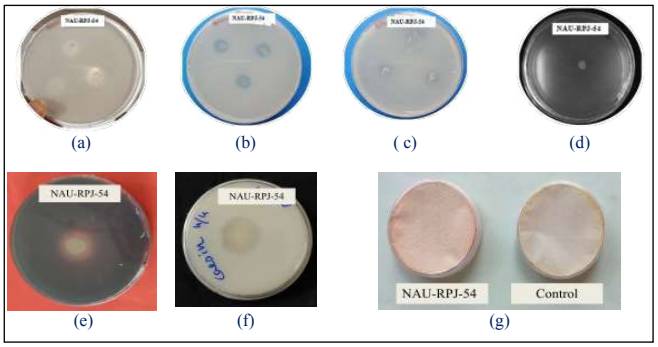

Figure 3: PGP activities: solubilization of zinc (a), potash (b), phosphate (c); production of chitinase (d), cellulase (e), protease (f) and HCN (g)

Rhizospheric microbes may benefit plants through various multifunctional traits including plant stress tolerance to salinity. Salt tolerance test of NAU-RPJ-54 on nutrient agar embedded with 100, 200, 400 and 600 mM of NaCl, $CaCl_2$ and $MgCl_2$ mixture at ratio of 3:2:1, respectively showed NAU-RPJ-54 was resistant salt upto 600 mM. Our results were also supported by the wok of Sarwar et al. (2020), they screened 20 siderophore producing bacteria from groundnut rhizosphere with multiple plant growth promoting traits.

3.3. Microbiological characters

Morphological, cultural and biochemical characters of the bacteria NAU-RPJ-54 was done by gram staining, colony characters and KB003 Hi25; KB009 Hi-carbohydrate kit, respectively. Gram staining revealed that the bacteria was gram positive cocci and shown round, small, convex, shiny and yellow pigmented colonies on nutrient agar medium. Biochemical test showed majority of the tests were negative while few were positive (Table 2). Based on microbial characters, the isolate NAU-RPJ-54 might belong to genus *Staphylococcus*.

Recently, Bhattacharyya et al. (2020) and Sati et al. (2023) reported the strain belongs to *Staphylococcus* genus owning the traits of siderophore production along with plant growth promoting activities.

Table 1: PGP characters of NAU-RPJ-54

Plant growth promoting characters								Salt tolerance assay			
Mineral solubilization (ZI)			Hormone production (ug ml^{-1})	Lytic enzyme (ZI)			HCN*	% inhibition of bacterial growth			
Z	K	P	IAA	Protease	Cellulase	Chitinase		100 mM	200 mM	400 mM	600 mM
2.7	0.7	0.4	18.2	2.8	0.8	0.3	+	22.3	33.2	33.4	49.2

*Indicating qualitative production of HCN

Table 2 : Biochemical tests of the NAU-RPJ-54

KB0003 Hi25 Identification kit		KB009 Hi-carbohydrate kit			
Tests	Results	Tests	Results	Tests	Results
ONPG	+	Lactose	-	Cellobiose	-
Lysine utilization	-	Xylose	+	Melezitose	-
Ornithine utilization	-	Maltose	-	α-Methyl-D-mannoside	-
Urease	+	Fructose	-	Xylitol	-
Phenylalanine deamination	-	Dextrose	-	ONPG	-
Nitrate reduction	+	Galactose	+	Esculin hydrolysis	-
H2S production	-	Raffinose	-	D-Arabinose	-
Citrate utilization	-	Trehalose	-	Citrate utilization	+
Vogesproskauer`s	-	Melibiose	+	Malonate utilization	-
Methyl red	+	Sucrose	+	Sorbose	-
Indole	-	L-Arabinose	-		
Malonate utilization	+	Mannose	-		
Esculin hydrolysis	-	Insulin	-		
Arabinose	-	Sodium gluconate	-		
Xylose	+	Glycerol	-	"+" : indicates positive test	
Adonitol	¬-	Salicin	+	"-" : indicates negative test	
Rhamnose	-	Dulcitol	-		
Cellobiose	-	Inositol	-		
Melibiose	-	Sorbitol	-		
Sachharose	-	Mannitol	-		
Raffinose	-	Adonitol	-		
Trehalose	-	Arabitol		-	
Glucose	+	Erythritol	-		
Lactose	-	α-Methyl-D-glucoside	-		
Oxidase	-	Rhamnose	-		

4. Conclusion

Our present study identified NAU-RPJ-54 bacteria as potential siderophore producer with hydroxamate type of siderophore and that inhibited fungal pathogens like *S. rolfsii* and *F. oxysporum* f.sp. *lycopersici*. Further, it showed plant growth promoting traits like indole acetic acid, cellulase, protease and HCN; solubilized zinc, potash and phosphate along with salt tolerance ability. Microbial characters revealed that it might belong to *Staphylococcus* genus. However, molecular characterization and extensive studies required to explore the strain in agriculture.

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