https://doi.org/10.33451/florafauna.v28i1pp03-10

FLORA AND FAUNA ISSN 2456 - 9364 (Online)

2022 Vol. 28 No. 1 PP 03-10 ISSN 0971 - 6920 (Print)

Biochemical characterization and synergistic effects of plant growth promoting rhizobacteria against *Fusarium oxysporum* in pigeonpea, *(Cajanus cajan)*

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Received **:** 25.02.2022; ***Accepted*** : 28.03.2022

ABSTRACT

Soil management today is dominated by toxic chemical fertilizers that pose a serious health and environmental risk. Beneficial soil microbes play a promising role in sustainable agriculture when used as biofertilizers. Pigeonpea (*Cajanus cajan)* is an ancient protein-rich leguminous pulse in India. Twelve different locations in Punjab (India) provided rhizospheric soil samples and the growth rates of 35 isolates were assessed at various temperatures. To identify potential plant growth-promoting traits with antifungal properties, morphological and biochemical characterization were carried out. Compared to uninoculated pigeonpea seeds, inoculated seeds with rhizoisolates showed a significant increase in growth parameters in greenhouse pot conditions. Plant growth-promoting rhizobacteria (PGPRs) are a good way to increase crop yields while also controlling disease outbreaks.

Figures : 02 References : 28 Tables : 03

KEY WORDS : Antagonistic, Antifungal, PGPRs, Pigeonpea, Rhizobacteria.

Introduction

Commonly farmers use chemical fertilizers, which have detrimental environmental effects. Reduced soil fertility, the release of greenhouse gases and the acidification of soil are all results of the massive use of chemical fertilizers. Nitrates and phosphorus in fertilizers cause eutrophication, which reduces the amount of oxygen available to aquatic animals. The environment and human health are both harmed as a result of its excessive use of chemical fertilizers. Aldrin, nitrofen, DDT and sodium cyanide, among others, have been banned by the Indian government[24]. Biofertilizers can be used instead of chemical fertilizers because they are good for the environment, help with sustainable farming and cut down on pollution[25].

Pigeonpea (*Cajanus cajan*) is a versatile and valuable legume crop that can be used for a variety of purposes, including food, fodder and ethanol production. Vegetarian foods are essential for the majority of the world's population in developing countries, where they are the primary source of protein (17.9 to 24.39/100 g) [4]. It is extremely difficult to control a soil-borne disease. Plant growth promoting rhizobacteria (PGPR), which naturally occur as soil bacteria capable of acting as biocontrol agents for soil-borne pathogens, should be abandoned in light of the harmful effects of agrochemical fertilizers. In many integrated management practices, PGPR is used to reduce the use of agrochemicals and to promote organic farming. This potential is due to antifungal plant growth promoting traits that reduces disease and increases yields. To chelate iron from soil-borne pathogens and synthesize volatile antifungal metabolites like ammonia, aldehydes and ketones, these siderophore production traits are essential [7]. The goal of the study was to find biocontrol agents that could work in greenhouse conditions, boost organic farming and cut down the damage done to the environment due to using chemical fertilizers by improving long-term farming development.

ACKNOWLEDGEMENTS : The authors would like to thank the Department of Plant breeding and genetics and the Department of Microbiology, Punjab Agricultural University, Ludhiana (Punjab) for providing the necessary facilities.

Materials and Methods

Isolation of rhizobacteria

An investigation to the isolation of rhizobacteria from twelve different pigeon pea growing fields in the Punjab region, using three different media, the 35 rhizobacteria found in rhizosphere soil, were all isolated. *Pseudomonas* spp., *Bacillus* spp., and *Rhizobium* spp. Samples were kept in the incubation chamber at 28°C + 2°C[12] and stored at agar slants on low temperature[28].

Characterization of pigeonpea rhizobacteria

To determine the strain characteristics, a subset of rhizobacterial isolates was morphologically and biochemically characterized. Testing for Gram's reaction and urease as well as the standard biochemical methods

TABLE-1: Morphological, Physiological and Biochemical characters of rhizobacteria from Pigeonpea rhizosphere

Characteristic of test organism	S-2	S-4	S-18
Gram's reaction	-	+	-
Shape	Rods	Rods	Rods
Pigment	+	na	na
Pigment color	Fluorescent green	White	White
Elevation	Flat	Raised	Raised
Consistency	Smooth	Smooth	Smooth
Margin	Entire	Entire	Undulated
Endospore formation	-	-	-
Starch hydrolysis	+	+	+
Catalase production	+	+	+
Methyl red test	Na	na	na
Nitrate reduction	+	+	+
Ammonia Production	+	+	+
Urease test	+	+	-
Indole production	+	+	+
Lactose fermentation	-	-	+
Citrate utilization	+	+	+

Abbreviations: + = Positive; - = Negative; na = not assessed; S-2 = *Pseudomonas*; S-4 = *Rhizobium*; S18 = *Bacillus.*

provided[13, 15] as well as catalase and lactose fermentation, nitrate reduction and indole production, starch hydrolysis and the methyl red and citrate utilization tests.

***In vitro* antagonistic activity**

The dual culture technique [22] and three replications were used to test the antagonistic properties of rhizobacteria isolates against *Fusarium oxysporum*. Calculating the percentage of growth inhibition of the test fungus was done using the following formula:

$$\text{Inhibition (\%)} = (R - r)/R \times 100$$

r is the fungus's radius opposite the bacterial colony and R is the fungus's maximum radius without the bacterial colony present.

Determination of plant growth Promoting traits and screening for biological control agents

Various plant growth promoting traits isolated from the soil of pigeonpea were evaluated, including IAA production[9], plate assay for zinc solubilization on Tris-minimal medium[8], siderophores production using blue agar plates containing the dye chrome-azurol S (CAS)[21], ammonia production[4] and hydrogen cyanide production[3].

Evaluation for bioantagonistic potential under greenhouse conditions

A pot assay was conducted in a greenhouse condition during the Kharif season of 2017 to test the efficacy of selected antagonists for three rhizobacterial traits. The Department of Plant Breeding and Genetics at Punjab Agricultural University, Ludhiana, India, provided the PAU 881 variety of pigeonpea (*Cajanus cajan*) seeds. Rhizobium of pigeonpea (LAR-06) was recommended by PAU to be used in combination with selected three antagonistic strains before sowing the seeds. The pots of sterile soil were sterilized using autoclaved water and 0.1 percent mercuric chloride. In addition to the PAU-recommended rhizobacteria (LAR-06), treatments

TABLE-2: *In vitro* production of plant growth promoting rhizobacteria and biological antifungal traits

PGP traits		Rhizobacterial isolates		
		S-2	S-4	S-18
Growth O.D (600 nm)	35°C	2.43	2.22	2.22
	40°C	2.83	2.04	2.56
	45°C	2.09	1.79	1.96
IAA at 40°C 5th day (µg/ml)		19.25	18.63	20.56
	24hr	1.1	1.0	1.9
Siderophore production (dia in cm)	48hr	1.6	1.3	1.6
	72hr	2.6	1.8	2.3
Catechol siderophore (µg/ml)		81.2	56.2	68.1
Mycelial Growth Inhibition (%)		48.6	44.4	36.8
Zn-solubilization (mm)		41.8	40.6	43.6
HCN (%)		100	100	100
NH_3 (µg/ml)		82.4	79.3	81.5

included isolated rhizobacteria (S-2, S-4, and S-18) and the negative control (pathogen-treated). The pots were irrigated on a regular basis, which helped to keep the trails at a consistent temperature. A variety of growth and disease indicators were measured after 45 days and 60 days, respectively. In order to conduct this experiment, CPCS1 software was used for statistical evaluation. The following formula was used to determine the seedling vigour index[16] (SVI):

SVI = Healthy survivals × (mean shoot length + mean root length)

Isolation, screening, and characterization of rhizobacteria

There were found rhizobacterial strains in pigeonpea rhizosphere soil in 12 different Punjab (India) locations. The morphological and biochemical characteristics of rhizobacteria isolated from different media were analyzed. Morphological analysis was used to identify all of the pigeonpea bacteria, including colony color, size and border. This strain was chosen for testing in the greenhouse pots because it was found to grow well at 35°C, 40°C and 45°C. All three isolates selected for experimentation were positive for nitrate reduction, citrate utilization, ammonia and indole production based on biochemical attributes. Lactose fermentation, found to be present in S-18. S-2 and S-18, on the other hand, tested positive for urease (Table-1). It was based on this evidence that *Bacillus, Pseudomonas* and *Rhizobium* were three rhizobacterial genera. It turned out that this investigation was similar to one done earlier[20].

Results and Discussion

***In vitro* screening for antagonistic rhizobacteria**

The pigeonpea pathogenic fungus *Fusarium oxysporum* was inhibited in dual culture on agar plates at 30° C by potential rhizobacteria. All isolates had the same inoculum load, but inhibition potentials varied. Rhizobacteria S-2 (48.6%), S-4 (44.4%), and S-18 (368%) all inhibited mycelial growth against the test fungus S-2 (Table 2). The growth of *F. oxysporum* was clearly inhibited by the potential antagonists on the fifth day of incubation at 30°C. Studies[6] found that *Pseudomonas* and *Bacillus* rhizobacteria (NS 1 and NS 22) were effective against *Fusarium oxysporium in vitro.*

TABLE-3: *In vivo* assessment of bioantagonist on plant growth to examine the control of infectivity due to *Fusarium oxysporum* f. sp. *udum* in Pigeonpea

Treatments	Seedling emergence (%)	Shoot length (cm)	Root length (cm)	Incidence of wilt (%)	Seedling index
Negative control (pathogen)	60.63	67.80	8.37	77.67	4618.24
Control	71.80	76.77	11.00	65.87	6301.65
Rhizobium	78.90	81.43	14.27	56.00	7550.73
S-2	81.83	84.50	15.70	48.47	8199.70
S-4	80.50	82.30	14.50	49.83	7792.40
S-18	83.03	83.93	15.37	44.77	8245.21
Rhizobium+ S-2	87.07	86.17	16.07	39.37	8901.12
Rhizobium+ S-4	84.07	84.70	15.53	39.10	8426.28
Rhizobium+ S-18	87.37	85.33	17.10	38.93	8949.26
CD (p=0.05)	3.85	1.96	1.69	4.07	4.16

Estimation of plant growth promoting traits for rhizobacterial isolates

IAA production

PGPRs release a hormone called Indole-3-acetic acid (IAA) that stimulates plant growth. Roots and shoots contribute to a plant's overall growth. Plant growth and expansion are a direct result of its role in activating cell elongation and cell division [26]. At a temperature of 40°C, these rhizobacteria were examined for the presence of IAA. Rhizobacteria selected for pot experimentation showed a range of 7.98-9.56 g/ml in the absence of tryptophan and 11.31-12.56 g/ml in the presence of tryptophan after 3 days of incubation. After 5 days of incubation at 40°C, IAA production increased from 8.62-10.89 g/ml in the absence of tryptophan to 18.63-20.56 g/ml when tryptophan was present. The most prominent IAA producers were S-18 (20.56 g/ml), S-2, S-4, and S-30 (19.5 and 18.6 g/ml, respectively) (Table 2).

Synergistic effect between *P. flurosence* AK1 and *P. aeroginosa* AK2 increased IAA production, as demonstrated[10]. When L-tryptophan was present, these isolates produced IAA and performed better than uninoculated seeds.

Siderophore production by rhizobacterial isolates

Bacteria and fungi produce high-affinity iron chelators known as siderophores. Microbes benefit from siderophore because it aids in growth, oxidative stress, sporulation and colonization during both sexual and asexual development. When pathogens are prevented from attacking, it activates the plant defense mechanism. The salicylic and jasmonic acid are also stimulated, which aids in the growth of a plant. After 24 hours of incubation with rhizobacteria inoculation on CAS medium, the color of the siderophores changed from blue to golden yellow. After 72 hours of incubation, it reached its peak. Rhizobacteria from 35 isolates were selected for experimentation because of their distinct orange halo on CAS plates (Fig. 2). S-2 (2.6 cm) produced the largest siderophore halo zone on a CAS plate, followed by S-18 (2.3 cm) and S-4 (1.8 cm) (Table 2).

After three days of incubation, these rhizobacterial isolates were retested for the quantitative type of siderophores they produce. Three rhizobacteria were found to produce catechol-type siderophores, which ranged from 56.2-81.2 g/ml when treated with arnow's reagent. According to report [1] *Pseudomonas sp.* produced a siderophore on CAS medium and these findings are in agreement with the previous findings. The rhizosphere of chickpeas was used to determine the assay's quantitative properties.

Zinc solubilization by rhizobacterial isolates

Rhizobacteria bacteria that controlled plant growth had a Zn solubilizing trait. Leaf chlorophyll and protein content increased as a result of zinc. Zinc deficiency in plants causes leaf discoloration, while enzyme deficiency aids seedling growth. Zinc was solubilized by the rhizobacteria used in the greenhouse pot experiment. After S-18 (43.6 mm) and S-2 (41.8 mm), S-4 (40.6 mm) showed the highest solubilization (Table 2). As evidenced by similar literature, isolates MDSR7 and MDSR14 from rhizosphere soybean and wheat, respectively, produced high levels of zinc soluble[18].

Production of hydrogen cyanide (HCN) and ammonia (NH_3)

Rhizobacteria that promote plant growth are capable of producing a wide range of antifungal and biocontrol agents. Potential biofertilizers and biocontrol

(a) (b)

Fig. 1: Dual culture assay against *F. oxysporum* at 30ºC (a) Control (b) Antagonistic culture S-2 and S-4

agents based on HCN-producing rhizobacteria have been identified for their ability to increase crop production. The intensity of color variation from yellow to light brown, moderate brown or reddish-brown was used to measure HCN production. According to research (Table 2), all three of rhizobacterial isolates, S-2, S-4, and S-S-18, were found to be highly productive HCN producers. According to previous studies[2] HCN has antimicrobial properties that aid in the biological control of root diseases with the assistance of rhizobacteria. It was found that three *Pseudomonas* from the pigeonpea rhizosphere were very good at producing HCN, while eleven others were also good at producing HCN.

Rhizobacteria releases ammonia into the soil, which aids plant growth. Light energy is converted into chemical energy, which increases seed and fruit production. For pot experimentation, all three isolates were found to be ammonia producers. The strength and stability of color development were used to estimate the level of expertise in the production of ammonia. There was a high concentration of ammonia in the rhizoisolates S-2, S-4, and S-18. More than 90% of rhizobacterial isolates from rice fields produce ammonia, which improves the plant's growth characteristics [19].

Assessment of treatments on plant growth parameters and fusarium wilt control under greenhouse conditions

Assessment of rhizobacteria on seedling emergence

In this study, we looked at the effects of seed treatment with PGPRs and Rhizobium co-inoculation with PGPRS on the pigeonpea variety PAU 881. As evidenced by emergence, S-2 (81.83 percent), S-4 (80.5 percent), and S-18 (83.03 percent) revealed a synergistic interaction with the inoculants. It was found that when treatments with *Rhizobium* and PGPRs were compared to treatments with only PGPRs, there was an increase in the percentage of seeds that germinated (Table-3). When compared to the recommended *Rhizobium* strain, almost all of the rhizobacterial isolates improved seedling emergence. Furthermore, when *Rhizobium* and PGPR were inoculated together, the symbiotic parameters significantly improved when compared to the uninoculated control. In contrast, a combination of antagonists and *Rhizobium* significantly increased seedling emergence in S-2 (87.07 percent), S-4 (84.07 percent), S-18 (84.07 percent), and S-20 (84.07 percent) (87.37 percent). Many plant hormones, like IAA and gibberellins made by rhizobacteria, are linked to more seeds sprouting [11].

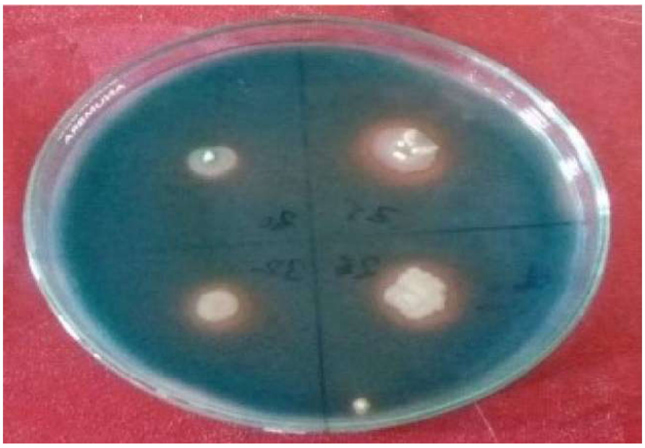

Fig. 2 : Siderophore production by rhizobacterial isolates (S-2, S-4 andS-18) indicated by the orange halo zone

Assessment of rhizobacteria on incidence of disease and to control the infectivity due to *Fusarium wilt*

There is strong evidence that pigeonpea wilt can be suppressed by the S-2, S-4, and S-18 antagonists for greenhouse pot experiments. As xylem vessels fail, *Fusarium* wilt causes dry leaves with inter-venial chlorosis, as well as a purple band along the stem. Co-inoculation of PGPRs and *Rhizobium* reduced *Fusarium* wilt because of the antagonistic effect of PGPRs. In comparison to *Rhizobium* alone, *Rhizobium* inoculation with S-18 (38.93 percent), S-4 (39.1 percent), and S-2 (39.37 percent) resulted in the greatest reduction in *Fusarium* wilt percentage (Table 3). For example, antifungal metabolites such as siderophores and antifungal phenolic compounds may be produced [14]. S-18 showed a 44.77% decrease in disease incidence, followed by S-2 (48.47%), and S-4 (48.57%). (49.83% of the total).77.67% of the incidence of *Fusarium* wilt was observed in the negative control. *Rhizobium*-treated seeds, on the other hand, saw a 56% reduction in disease severity.

Plant SVI values (P 0.05) indicated that the bacterial isolates had a positive effect on plant growth[15]. *Rhizobium* inoculation with S-18 (8949.26 percent) showed the highest SVI, followed by S-2 (8901.12 percent) and S-4 (8426.28 percent) (Table-3). Thus, *in vitro* experiments against the test fungi can be used to determine the antifungal potential of *Rhizobacteria* strains. Consequently, the biocontrol agents S-2 and S-18 may be effective.

Conclusion

Rhizobacteria isolated and screened for plant growth-promoting traits were isolated from various locations in Punjab. As demonstrated in this study, rhizobacteria improved plant growth while protecting it from pathogens. Plant growth under controlled conditions is promoted by the S-2 and S-18 strains of rhizobacteria. Rhizobacteria inoculants are used for plant growth promotion based on greenhouse pot evaluation. The use of these antagonistic combinations for environment friendly disease management is highly effective. In order to prove this hypothesis, a lot of field trials and detailed studies will need to be done.

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