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Induction of Systemic Resistance against *Phytophthora* Blight in Pigeonpea Through the Interaction of Plant Growth Promoting Rhizobacteria: *In vivo* and *in vitro* Study

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ABSTRACT

Background: *Phytophthora* blight (PB), caused by *Phytophthora drechsleri* f. sp. cajani, is soil borne disease of pigeonpea (*Cajanus cajan* L.). The use of plant growth promoting rhizobacteria (PGPR) in agriculture leads to alternative disease management of crop in a sustainable ecofriendly way.

Methods: *In vitro* and *in vivo* experimentation was conducted at fields of Punjab Agricultural University, Ludhiana during *kharif* season of 2017-18 to study the percentage of blight inhibition by rhizobacteria and production potential in pigeonpea. The present study consisted of 6 treatments of dual inoculation of antagonistic bacteria with recommended rhizobium of PAU (LAR-06).

Result: The selected rhizoisolates were studied against *Phytophthora drechsleri* caused stem blight of pigeonpea. The allelochemicals and plant growth promoting rhizobacteria traits involved in antagonistic behavior significantly inhibited the growth of the test fungus. Scanning electron microscopy between potential rhizoisolates and test fungus revealed intumescent hyphae with irregular cell surface morphology. The synergistic effects of LAR-06 + S-2 and LAR-06+ S-18 were found to be the most potent ones in inhibiting the radial growth of the test pathogen with increased yield production in the field as well as greenhouse conditions.

Key words: Antagonistic, Dual inoculation, Pigeonpea, Siderophore, Synergistic.

INTRODUCTION

Current soil management strategies are mainly dependent on inorganic chemical-based fertilizers, which cause a serious threat to human health as well as the environment. The use of PGPR in agriculture is steadily increasing and it offers an appealing alternative to artificial fertilizers and pesticides. The beneficial microbes in biofertilizer have transformed agriculture because it encourages sustainable crop production.

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a versatile and profitable legume crop with several applications as food, fodder and fuel. With 20 to 22% protein, it has been identified as a rich source of protein (Saxena *et al.,* 2002) particularly in underdeveloped nations where a large population relies on vegetarian diet. Phytophthora blight of pigeonpea caused by *Phytophthora drechsleri* Tucker f. sp. cajani is a soil-borne disease that is difficult to manage. *Phytophthora drechsleri* causes seedlings to die suddenly as in damping-off disease. Its outbreak in the Deccan Plateau region of India was reported with 11.0-31.5% disease incidence (Naik *et al.,* 2020). In contrast with chemical fertilizers, plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria which have capabilities to act as biocontrol agents against soil-borne pathogens. Therefore, co-inoculation with antagonistic efficient strains of *Rhizobium* with plant growth promoting rhizobacteria (PGPR) shows effective plant growth promotion and resistance to disease. The antagonistic potential of PGPR is due to antifungal plant growth-promoting traits *i.e.,* siderophores having the efficiency to chelate iron from soil-borne pathogens in soil, synthesis of volatile antifungal metabolites such as ammonia, aldehydes and ketones (Attia *et al.,* 2020).

The present study was undertaken to isolate rhizobacteria from pigeonpea rhizosphere with potential antagonistic activity to control *Phytophthora* blight disease and the effect of symbiotic efficiency of *Rhizobium* with isolated PGP traits under greenhouse pot and field conditions.

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MATERIALS AND METHODS

Isolation and characterization of rhizobacteria

Phytophthora drechsleri was isolated from the infected main stem of pigeonpea as per the method given by (Sosa *et al.,* 2015)

and pure culture was maintained on V8 juice agar slants. All the isolates were isolated from 12 different locations of Punjab by the standard isolation methods given by (Kumar *et al.,* 2010).

Morphological, physiological and biochemical characteristics of isolated rhizobacteria were determined as per the standard techniques given by Cappuccino and Sherman, (1992); Holt *et al.* (1994).

In vitro antagonistic activity of rhizoisolates and effect of culture filtrates against *Phytophthora drechsleri*

Antagonistic properties of rhizobacterial isolates were tested against *Phytophthora drechsleri* on V8 juice agar plates using the dual culture technique (Sharma *et al*. 2006). Radial growth and percentage growth inhibition were calculated using the formulae:

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

Where

r = Radius of the fungal colony opposite the bacterial colony.

R = Maximum radius of the fungal colony in the absence of the bacterial colony.

Growth inhibition of *Phytophthora drechsleri* by culture filtrates was recorded as per the method given by (Kumar *et al.,* 2010).

Characterization of antagonistic rhizobacteria for Multifarious PGP traits

Siderophore production of isolates was done both qualitatively and quantitatively as per the method described by (Schwyn and Neilands, 1987) and (Arnow, 1937) respectively. Hydroxamate siderphores were evaluated by the method of (Csaky, 1948). Characterization of isolates for the production of IAA was determined as per the method given by (Bakker and Schippers, 1987). Qualitative and quantitative phosphate solubilizing activity was measured according to the method described by (Jackson, 1973) and (Nautiyal, 1999) (incubated at 28±2°C for 15 days). Zinc solubilization efficiency using Zinc Oxide (ZnO) supplemented in Tris-minimal medium was assayed (incubated in dark at 28±2°C for 7 days) according to (Batool *et al.* 2021). The selected antagonistic bacterial isolates were also characterized for indirect multifunctional PGP traits, production of volatile antifungal metabolite Hydrogen Cyanide (HCN) production (Bakker and Schippers, 1987), production of ammonia in peptone water (Cappuccino and Sherman, 1992). Bacterial strains were assayed for the production of hydrolytic enzymes such as chitinase (El-Katatany *et al.* 2003). cellulase (β-1-4-gluconase) (Ariffin *et al.* 2006) and protease (Chaiharn *et al*. 2008) were tested for all the isolates.

Scanning electron microscopy (SEM) of the interaction between rhizobacteria and test fungus

SEM mycelia fixation preparation for topography visualization was given by Kang *et al.* (2010). Two random fields of view as per sample were photographed.

***In vitro* biocompatibility interaction of potential antagonistic rhizoisolates**

The compatibility of antagonistic rhizoisolates was tested on the soybean digest agar disc plate as per the method given by Subramanian *et al.* (2015).

Evaluation of bioantagonistic potential under greenhouse conditions and field study

To study the disease suppression capability of the 4 selected antagonists *i.e.,* S-2, S-4, S-18 and S-30 pot assay was conducted as per the method given by (Ghassemi-Golezani *et al.* 2008). Seedling vigor index (SVI) was calculated by shoot and root lengths of dried seedlings using the formulae:

SVI =

Healthy survivals × [Mean shoot length + Mean root length]

Field experiments were conducted during the Kharif season of 2017 on pigeonpea at the research farms of Punjab Agricultural University, Ludhiana, Punjab, India (30°54′5″N 75°47′53″E)(Table 1). This experiment was performed as per the recommended practices (Daspute *et al.,* 2014).

Per cent disease incidence, (PDI%) =

$$\frac{\text{No. of plants infected by disease}}{\text{Total no. of plants}} \times 100$$

Growth parameters

Different growth parameters such as nodule number, nodule dry weight, the number of pods per plant, the number of seeds per pod and plant height were determined given by methods (Singh *et al.,* 2011). Chlorophyll content was estimated by the method of Witham *et al.* (1971) and leghemoglobin content by the method of (Wilson *et al.,* 1963). Grain yield from each plot (g/plot) was recorded at the final harvest and was expressed in kg/ha. Nitrogen content in the grains was determined by using the method given by (Nwokolo, 1987). The grain protein content was calculated by multiplying grain N content with 6.25 and expressed in %. Harvest index calculating by (Thanki *et al.,* 2010).

$$\text{Harvest index (\%)} = \frac{\text{Grain yield (Kg ha}^{-1})}{\text{Biological yield (Kg ha}^{-1})} \times 100$$

RESULTS AND DISCUSSION

Morphological pathogen identification

On V8 tomato juice agar, the fungus forms the circular, slightly petaloid colonies with compact hyphae. The structure of sporangium varied in different isolates from broadly ovoid, obpyriform to elongate and nonpiliated. The identity of morphological characters of the pathogen was done as described in the manual on phytophthora (Gallegly and Hong, 2008).

Isolation and biochemical characterization of rhizobacterial isolates

Isolated seven (S-2, S-4, S-18, S-28, S-30, S-32 and S-34) antagonistic rhizobacteria on different respective media were

assessed. The morphologically and biochemical characteristics of rhizobacteria given at (Table 2). Based on these tests, the isolates were tentatively placed into three genera: *Bacillus*, *Pseudomonas and Rhizobium*. The outcomes were coinciding with previous investigations documented by new (Kumar *et al.,* 2010).

In vitro screening for antagonistic rhizobacteria against *Phytophthora dreschsleri*

The per cent growth inhibition was found to range between 17.8-39.6% in dual culture against *P. drechsleri* as compared to control. Out of 7 antagonists S-18 (39.6%) isolate showed the highest mycelial growth inhibition against the test fungus as compared to others (Table 3). Similar studies were conducted by (Anjum *et al.,* 2019) reported the bioactivity of biocontrol agents against *Phytophthora drechsleri* infection was studied *in vitro. T. asperellum* (47.3%) showed the highest growth rate of *Phytophthora drechsleri* in potato dextrose.

Table 1: Experimental details.

Test crop	Pigeonpea (*Cajanus cajan* L.)
Treatments	Six
Replications	hree
Total number of plots	6 × 3=18
Plot Size	3×2 m^2
Statistical design	Duncan's multiple range test (DMRT)
Variety	PAU 881
Rhizobium strain	*Rhizobium* (LAR-06) of PAU, Ludhiana
Fertilizers and irrigation	As and when required
Date of sowing	June 7, 2017
Date of harvesting	Nov 2, 2017

Quantitative evaluation of antagonism

In vitro, broth-based dual cultures offer a better method for the evaluation of antagonistic efficiency of the biocontrol agents. The maximum per cent biomass inhibition on a dry weight basis was recorded after 5 days of incubation by S-2 (79.3%) followed by isolates S-18 (71.4%) and were highest as compared to others (Table 3). Mathur and Mathur, (2021) reported similar results of inhibition fungus biomass in broth-based dual culture was revealed by 11 antagonists in chickpea.

Antagonism of siderophore producing bacteria against *P. drechsleri*

All seven isolates showed a distinct orange halo on CAS plates indicating siderophore production. The highest amount of catechol and hydroxamate type siderophore was produced by S-18 (78.2 µg/ml^{-1} and 70.8 µg/ml^{-1}) respectively as compared to other isolates after 6 days of incubation (Table 3). Siderophore production from rhizobacteria has been reported by several researchers, Gupta *et al.* (2020) found that under the iron-deficient condition all the isolated

Table 2: Morphological, physiological and biochemical characteristics of antagonistic bacterial isolates from pigeonpea rhizosphere.

Characteristic of test organism	S-2	S-4	S-18	S-28	S-30	S-32	S-34
Gram's reaction	-	+	-	+	-	-	-
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Pigment	+	na	na		+	+	
Pigment color	Fluorescent green	Semitran slucent	White	White	Fluores cence	Semitran slucent	White
Elevation	Flat	Raised	Raised	Flat	Umbonate	Raised	Pale
Consistency	Smooth	mucilaginous	Smooth	Smooth	Smooth	Mucoid	Smooth
Margin	Entire	Entire	Undulated	Entire	Entire	Entire	Entire
Endospore formation	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	-
Catalase production	+	+	+	+	+	+	+
Methyl red test	na	na	na	na	na	na	na
Nitrate reduction	+	+	+	+	+	+	+
Ammonia production	+	+	+	+	+	+	+
Urease test	+	+	-	+	-	+	-
Indole production	+	+	+	+	+	-	+
Lactose fermentation	-	-	+	-	+	-	+
Citrate utilization	+	+	+	+	+	+	+
Malonate	-	-	-	-	-	-	-
pH tolerance	7-10	7-11	7-10	7-10	7-11	7-10	7-10
Temperature tolerance	45±48°C	45±50°C	50±55°C	45±50°C	40±45°C	45±50°C	45±50°C

Abbreviations: + = Positive; - = Negative; na = not assessed; S-2 = *Pseudomonas*; S-4 = *Rhizobium*; S-18 = *Bacillus,* S-28= *Bacillus,* S-30= *Pseudomonas,* S-32= *Rhizobium,* S-34= *Bacillus.*

rhizobacteria inhibited the vegetative growth of *P. drechsleri* and also benefited the growth of heterologous microbes in the soil.

Characterization of antagonistic rhizobacteria for multifarious PGP traits

All the seven antagonistic rhizobacteria solubilized inorganic phosphate on pikovskya's agar, after 24 hrs. of incubation. The maximum solubilization index was showed by S-18 (21.5 mm) as compared to others (Table 3). IAA as evidenced by the development of pink color without the addition of tryptophan into the culture media. Rhizobacteria S-2 (10.8) produced maximum IAA without the addition of tryptophan as compared to other rhizoisolates (Table 3). A similar pattern of IAA production and phosphate solubilization has been reported by (Tewari *et al.,* 2021). All seven strains of rhizobacteria produced ammonia and HCN which is indicated by the change of filter paper color from yellow to brown and reddish-brown. Aggarwal *et al.* (2010) reported the role of HCN and ammonia in inhibiting the growth of *P. drechsleri*. A marked variation in the ability to produce ammonia was observed amongst the isolates indicated by the intensity of color developed.

Cell wall degrading enzymes produced by rhizobacteria

All the antagonistic isolates produced cellulase and protease on CMC and skim milk agar media, respectively (Table 3). Clear halo on skim milk agar medium with a diameter ranging from 1.96 to 5.02 cm for protease enzyme production. It has been demonstrated that cellulase and protease synthesized by rhizobacteria digest and lyse the mycelium of *P. dreschsleri* (Panth *et al*., 2020).

Scanning electron microscopic (SEM) observation of post-interaction abnormalities between rhizoisolates and test fungal mycelia

Scanning electron micrographs depicted the morphological abnormalities in the hyphae of *P. dreschsleri* obtained from the zone of interaction during dual culture. Loss of structural integrity of conidia of *P. dreschsleri*, hyphal perforations and swelling were clearly observed (Fig 1). The SEM results complied with Kumar *et al.* (2010) reported that allelochemicals (volatile and non-volatile) HCN, antibiotics and enzymes produced by antagonistic rhizobacteria resulting in the lysis of mycelial structure and hence curbing the growth of *Phytophthora dreschsleri*.

Biocompatibility of potential microbial consortium in pigeonpea

Four potential antagonistic rhizoisolates (S-2, S-4, S-18 and S-30) were found compatible without producing any zone of inhibition on tryptone soy agar plate assay. These isolates with positive compatibility were assessed spectrophoto metrically for mutual interaction in Luria broth. Dual inoculation enhanced the growth as compared to monoculture treatment. The highest growth in terms of optical density (OD at 600 nm) was recorded with LAR06+

Table 3: *In vitro* antagonistic activity of plant growth promoting rhizobacterial isolates against *Phytophthora drechsleri* f. sp. *cajani* and production of antifungal traits.

Isolates	Mycelial growth Inhibition (%)	Reduction in dry weight (%)	Catechol type siderophore (µg/ml)	Hydroxamate type siderophore (µg/ml)	HCN Production	Ammonia Production	IAA TRP (µg /ml) at 40°C	(L- (-) at Zn solubilization index (mm) 40°C	Phosphate solubilization (mm) index at 40°C	Cellulase	Protease diameter (cm)
Control	-	-	-	-	-	-	-	-	-	-	-
S-2	39.5±1.34	79.3±2.86	68.1± 0.87	62.4±1.92	+++	+++	10.89±2.86	41.8±2.87	18.5±3.28	+++	4.26 ±0.12
S-4	29.3±0.67	68.4±0.36	65.2±1.76	60.5±1.07	+++	++	9.86±0.38	40.6±0.38	19.2±1.36	++	3.07±0.03
S-18	39.6±2.09	71.4±0.98	78.2±2.76	70.8±2.87	++	+++	8.62±3.27	43.6±3.19	21.5±2.50	+++	5.02±0.37
S-28	23.3±0.26	57.1±2.56	53.1±1.76	49.3±0.87	+	+++	8.26±0.38	34.7±0.28	17.4±2.65	+++	2.89±0.86
S-30	32.5±1.78	63.8±1.86	61.2±2.65	58.4±0.64	++	++	9.56±2.18	36.9±3.28	13.4±0.38	++	2.34±1.43
S-32	17.8±0.45	51.5±0.38	49.4±1.37	-	+	+++	3.56±1.58	33.5±1.73	12.3±1.35	+++	1.96±1.33
S-34	26.2±0.22	39.8±0.71	-	46.2±1.30	+	++	7.58±0.57	-	-	+++	2.91±1.04

Abbreviations: +++ = Strongly producers; ++ = moderately producers; + = Low producers; - = Negative.

S-2 treatment (1.31) followed by LAR06+S-18 (1.05), LAR06+S-4 (0.95) and S-18 (0.91) as compared to respective single inoculants S-2, S-18, S-4 and S-30 (0.82 and 0.81, 0.74 and 0.64 respectively) at 9th day of incubation. These combinations showed a sustained population of bacterial growth at different incubation periods. Subramanian *et al.* (2015) reported the positive interaction of rhizobacteria done on soybean digest agar disc plate with pre-seeded *rhizobacteria* in *in-vitro* conditions. This protocooperation is due to the release of non-reactive metabolites during co-inoculation of rhizobacteria.

In vitro effect of antagonistic rhizobacteria on the incidence of *Phytophthora* blight under pathogen stress conditions

Antagonists S-2, S-4, S-18 and S-30 further authenticated *in vitro* tests, providing a strong confirmation efficiency of these isolates in suppressing *Phytophthora* blight in pigeonpea. Maximum seed vigor index (SVI) shown by dual inoculation of *Rhizobium* with S-2 (8678.67) followed by S-18 (8518.51), S-4 (8057.17), S-30 (8262.68) as compared to recommended *Rhizobium* alone under pathogen stress conditions (Fig 2). Treatment with bio antagonist S-18 alone, showed the highest SVI (7907.72) as compared to others and recommended *Rhizobium* (7250.4). In the case of negative control, SVI (4770.44) was recorded.

Similar findings have been reported by Anjum *et al.* (2019) revealed that reduction in *Phytophthora* blight incidence was due to their synergistic nitrogen fixation and antagonistic effect treatment with PGPR strains and improved the germination rate of seeds as compared to others.

In vivo effects of compatible rhizobacteria on incidence of blight and symbiotic growth parameters

Based on strong *in vitro* antagonistic PGP activities, strains S-2, S-4, S-18 and S-30 were selected for *in vivo* experimentation. Percentage of incidence of blight was found to range between (27.7-41.6%) as compared to others. Co-inoculation of seeds with recommended rhizobium enhanced various plant growth parameters (plant height, number of nodules, nodule dry weight, number of pods and number of seeds compared to control (Table 4). After 30 DAS, Chlorophyll content was enhanced in dual inoculation of S-2 (2.73 mg/g) and Leghemoglobin in dual inoculation S-18 (1.65 mg/g). Similar trend was observed in both chlorophyll leghemoglobin content after 60 DAS (Table 3). These results were coinciding with Bhowmik and Das, (2018) reported that co-inoculation of *rhizobacteria* with a recommended dose of biofertilizer significantly improved plant growth parameters as compared to un-inoculated control treatment in pigeonpea.

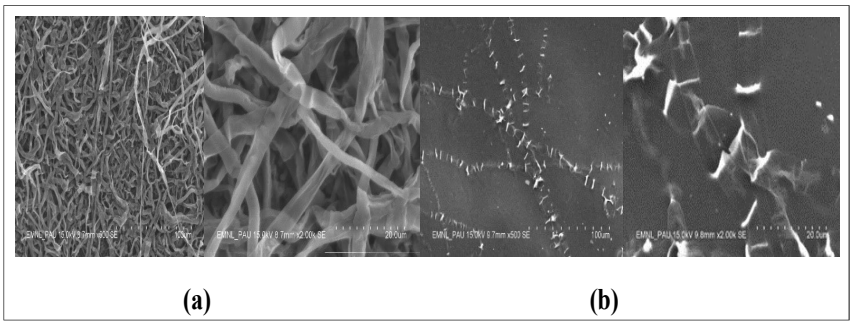

Fig 1: Scanning electron microscopic photographs of mycelial and conidial destruction by antagonistic effects of rhizobacterial isolates; (a) Test fungus (*Phytophthora dreschsleri)* (b) Hyphal and conidial destruction by rhizobacteria.

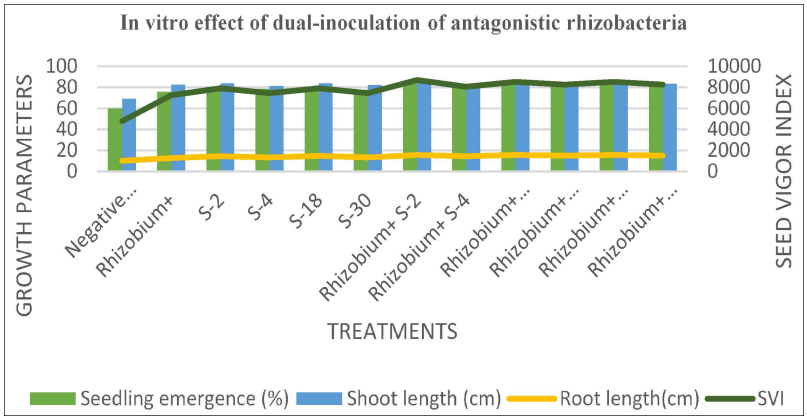

Fig 2: *In vitro* effect of dual-inoculation of antagonistic rhizobacteria under pathogenic stress (*P. drechsleri*) on seedling vigor index (SVI) of pigeonpea.

Table 4: *In vivo* assessment of bioantagonist on yield attributing traits and plant growth to examine the control of infectivity due to *Phytophtora drechsleri* Tucker var. *cajani* in pigeonpea.

Treatments	No. of nodule / plant		Nodule dry weight (mg/plant)		Incidence of blight (%)	Plant height (cm) at harvesting	Chlorophyll content (mg/g fresh weight of leaves)		Leghemoglobin content (mg/g fresh weight of nodules)		Protein content % in grains	No. of pods/ plant	No. of seeds/ pod	Biological yield (kg ha^{-1})	Grain yield (kg ha^{-1})	Harvest index (%)
	30 DAS	60 DAS	30 DAS	60 DAS			30 DAS	60 DAS	30 DAS	60 DAS						
Control	3.3^{f}	4.3^{f}	19.5^{f}	48.1^{f}	82.6^{a}	158.3^{f}	2.02^{f}	2.54^{f}	0.94^{f}	2.53^{f}	3.6^{f}	109.8^{f}	2.37^{f}	5730^{c}	1037^{d}	18^{e}
Rhizobium	4.2^{e}	8.1^{e}	35.2^{de}	71.3^{c}	54.6^{b}	160.7^{e}	2.27^{e}	3.05^{e}	1.12^{e}	2.91^{e}	3.9^{e}	112.1^{e}	2.66^{e}	5723^{d}	1062^{c}	18.5^{c}
Rhizobium+S-2	6.5^{a}	10.6^{a}	52.8^{a}	86.3^{a}	28.5^{f}	163.6^{c}	2.73^{a}	3.53^{b}	1.34^{c}	3.24^{c}	4.5^{a}	115.0^{ab}	2.94^{b}	5709^{e}	1086^{b}	19^{a}
Rhizobium+S-4	4.9^{d}	8.9^{cd}	35.2^{de}	62.4^{e}	41.6^{c}	164.0^{b}	2.35^{d}	3.34^{c}	1.43^{b}	3.03^{d}	4.1^{cd}	116.7^{c}	2.80^{c}	5787^{b}	984^{f}	17^{f}
Rhizobium+S-18	5.4^{b}	9.2^{b}	43.6^{b}	85.2^{a}	29.7^{e}	165.3^{a}	2.66^{b}	3.92^{a}	1.65^{a}	3.37^{ab}	4.4^{ab}	115.0^{ab}	3.02^{a}	5790^{a}	1091^{a}	18.8^{b}
Rhizobium+S-30	5.1^{c}	8.8^{c}	42.7^{bc}	68.7^{d}	35.7^{d}	161.9^{d}	2.64^{c}	3.08^{d}	1.25^{d}	3.38^{a}	4.2^{c}	113.4^{d}	2.74^{d}	5409^{f}	1000^{e}	18.4^{cd}

Abbreviations: DAS= Days after sowing.

* Values with same alphabet are non-significant to each other and S- Significant at P 0.05; NS- Non-Significant at P>0.05.

Post-harvest analyses indicated maximum grain yield wasfound in the combination treatment of rhizobium and S-18 (1091 kg/ha), followed by the other combination S-2 (1086 kg/ha) as compared to control (1037 kg/ha) and recommended rhizobium (LAR-06) ((1062 kg/ha). The protein content of seeds in pigeonpea (Table 4) showed that co-inoculation of S-2 + Rhizobium exhibited maximum protein content (4.5%) compared to control (3.6%) and Rhizobium alone (3.9%). A similar trend was observed in other treatments too. Earlier also, Tewari *et al.* (2020) reported that combined inoculation of biofertilizers increased protein content and seed yield by 1.2- and 2.2-fold increments respectively in comparison with control treatment in pigeonpea.

CONCLUSION

The results of this study strongly suggest that potential rhizobacteria (S-2 and S-18) can be investigated as potent inoculants for pigeonpea biofertilization, in addition to the recommended Rhizobium, PAU (LAR-06), in the future. Moreover, it promoted systemic resistance to soil pathogens under low-input technology for increasing pulses production in sustainable agriculture.

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Conflict of interest

All authors declared that there is no conflict of interest.

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