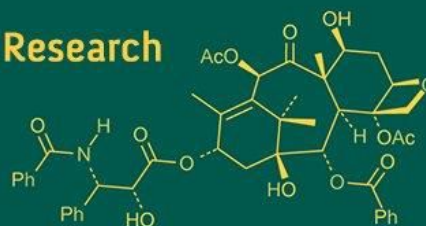


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Crop specific plant growth promoting effects of ACCd enzyme and siderophore producing fluorescent *Pseudomonas* on okra and pumpkin

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Abstract

The use of microbial technologies in agriculture is currently expanding quite rapidly with the identification of new bacterial strains, which are more effective in promoting plant growth. The plant rhizosphere is an important soil ecological environment for plant microbe interactions. It involves colonization by a variety of micro-organisms in and around the roots which may result in symbiotic, associative, neutralistic or parasitic relations within the plant, depending on the type of microorganism, soil nutrient status, and plant defense system and soil environment. A substantial number of bacterial species, mostly those associated with rhizosphere, may exert a beneficial effect on plant growth and development. This group of bacteria has been termed "plant growth promoting rhizobacteria" or PGPR. In the present study 55 strains of *Pseudomonas* were isolated from soil sample collected from different geographical locations of Chhattisgarh. Among 55 isolated *Pseudomonas* strains 20 isolates exhibited biocontrol activities against soil borne phytopathogenic fungi were selected on the basis of their antifungal activity to explore its potential as plant growth promotion. Isolates showed multiple plant growth promoting attributes such as phosphate solubilization activity, indole acetic acid (IAA), siderophore, and HCN production.

Keywords: Biological control, fluorescent *Pseudomonas*, PGPR, seed bacterization siderophores

Introduction

The plant rhizosphere is an important soil ecological environment for plant microbe interactions. It involves colonization by a variety of micro-organisms in and around the roots which may result in symbiotic, associative, neutralistic or parasitic relations within the plant, depending on the type of microorganism, soil nutrient status, plant defense system and soil environment. Aerobic gram-negative *Fluorescent Pseudomonas spp.* has emerged as the largest and potentially most promising group of plant growth promoting rhizobacteria involved in the bio-control of plant diseases (Weller *et al.* 2002; Fravel 2005) [17, 4]. A large number of secondary metabolites, growth hormones, antibiotics and chelating compounds such as siderophores (Choudhary *et al.* 2009; Beneduzi *et al.* 2012) [3, 2] are known to be released by these fluorescent pseudomonads. This group of bacteria has been termed "plant growth promoting rhizobacteria" or PGPR. Plant growth promoting rhizobacteria (PGPR) can stimulate growth by one or more different mechanisms. The direct mechanisms include production of growth hormones like IAA, phosphate-solubilization and uptake of iron, whereas indirect mechanisms include check on phytopathogens by the release of HCN, antibiotics and siderophores (O'Sullivan and O'Gara, 1992) [6]. Antagonistic rhizobacteria, more specifically, fluorescent pseudomonads, are well known to suppress fungal root diseases of agronomic crops, such as seedling disease of cow pea and wheat caused by *S. rolfii*. Considerable progress has been made over the past decades to elucidate the mechanisms by which fluorescent pseudomonads suppress disease. It is apparent that a large variety of antifungal metabolites are produced by fluorescent pseudomonads. Hydrogen cyanide, phloroglucinol, pyoluteorin and pyrrolnitrin are produced by many of these bacteria, and attempts have been made to quantify the importance of each in disease suppression. Characteristics, especially biochemical characteristics, of fluorescent *Pseudomonas* has an important role to determine its potential level in inhibition of plant pathogens but the characterization never been done. The characteristics should be tested in order to know the

field application of antagonist. The aim of this research was to know biochemical characteristics of fluorescent *Pseudomonas* isolates and assessment of potential PGPR agents. The present study evaluated local isolates of phosphate solubilizing bacteria (*Pseudomonas* spp.) from different geographical locations of Chhattisgarh on growth parameters of Okra (Var. ADITYA) and Pumpkin (var. HARITA) in the field condition.

Materials And Methods

The present investigation was carried out at Microbial Biotechnology Laboratory at the Department of Plant Molecular Biology and Biotechnology, R.H. Ricchharia Research Laboratory, College of Agriculture, IGKV, Raipur characterise siderophore producing fluorescent *Pseudomonas* and to screen the isolates for phosphate solubilization, siderophore and at Research cum Instructional farm, College of Agriculture, IGKV to carry out pot experiments. The experimental material consisted of purified 55 isolates of fluorescent *Pseudomonas* spp. from soils (rhizospheric and non-rhizospheric) of different geographical locations of Chhattisgarh. Isolation of fluorescent pseudomonads was done by adopting serial dilution method on King's B (KB) medium. Isolates were characterized on the basis of biochemical tests as per the procedures outlined in Bergey's Manual of Systematic Bacteriology (Sneath 1986) [11]. Selected 21 candidate potential isolates were then used to screen the isolates for phosphate solubilization, siderophore producing and plant growth promoting ability. These isolates were isolated from soil samples of different geographical locations of Chhattisgarh. During the course of study, King's B medium was used for maintaining the cultures of *Pseudomonas*.

Biochemical characterization of bacterial isolates

The biochemical characterization of fluorescent *Pseudomonas* isolates will be carried out to characterize all the isolates of Fluorescent *Pseudomonas*. A rapid antibiotic sensitivity test was used to distinguish different species of fluorescent *Pseudomonas*. Isolates will be then characterized based on biochemical tests as per the procedures outlined in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1984) [12]. The siderophore production was determined qualitatively by CAS agar plate method and specific tests were carried out for identification of hydroxamate and Catecholate types of siderophores following the standard methods (Arnow, 1937) [1]. Qualitative screening of phosphate solubilizing fluorescent *Pseudomonas* was performed on Pikovskaya's agar medium (Hi media) containing tricalcium phosphate as a phosphate source and Bromo Cresol Purple (0.1 g/l) as a pH indicator for acidification (Vazquez *et al.* 2000) [15]. HCN production was determined on KM plates supplemented with 4.4 g L⁻¹ glycine as described by Wei *et al.* (1991) [16]. ACC deaminase activity was determined by measuring the production of α-ketobutyrate and ammonia generated by the cleavage of ACC by ACC deaminase (Honma and Shimomura 1978) [5]. Based on the production of siderophores, HCN and phosphate solubilization candidate isolates were selected for experiments and therefore was also characterized for other plant growth promoting potentials.

In vitro Screening for antagonistic activity against soil borne phytopathogens

The twenty isolates were tested for their efficacy as biocontrol agent against the phytopathogens *Sclerotium rolfsii*. Equal volume of sterilized potato dextrose agar (PDA) and King's B medium was mixed and poured in sterilized petridishes. A heavy inoculum from an actively growing fluorescent *Pseudomonas* was streaked at 1 cm away from the edges of the plate and the mycelia disc of the pathogens were placed at the centre of petriplates. Control plates were inoculated only with phytopathogens but not with *Pseudomonas* isolates. Percent inhibition of pathogens by *Pseudomonas* isolates over control was calculated by using the formula:

$$[(\text{Growth of pathogen in control} - \text{Growth of pathogen with } Pseudomonas \text{ isolate}) / \text{Growth of pathogen in control}] \times 100$$

Host seeds

Seeds of Okra (Var. ADITYA) and Pumpkin (var. HARITA) were obtained from Research cum Instructional farm, IGKV, Raipur, India.

Seed bacterization

Twenty isolates of fluorescent *Pseudomonas* were tested for their ability to promote plant growth in pot experiments. The bacterial cultures were inoculated in 25ml King's B broth and incubated at 28±2°C for 48 hrs. For seed soaking inoculums of all the isolates were diluted to final concentration of 10⁸-10⁹ CFU mL⁻¹ (600 nm). Seeds of chick pea variety were soaked in the diluted suspension for 1 hour. Seeds soaked in water served as control.

Pot experiment

The treated seeds were planted in pots containing soil mixed with sand and compost in the ratio of 3:1:1. Each treatment was replicated twice and the experiment was repeated two times. For taking measurement on growth parameters, three plants were randomly uprooted from each pot at 30 days after sowing (DAS). The following observations were taken - shoot and root length (cm), shoot and root fresh weight (gm) and dry weight (gm). Shoot and root dry mass was recorded after drying the samples in oven at 60 °C.

Results and Discussion

All the twenty isolates of fluorescent *Pseudomonas* showed positive response for siderophore production and phosphate solubilization (in Pikovskaya's agar medium with and without BCP indicator). Isolates showed variability for traits such as gelatin liquefaction, casein hydrolysis, lipolytic activity, nitrate reduction and antibiotic sensitivity test. In antibiotic sensitivity test, all twenty isolates were resistant to antibiotic carbenicillin amended medium. Siderophores are also thought to facilitate bio control by sequestering iron from pathogens, thus limiting their growth and for the solubilization of extracellular ferric iron by most bacteria and fungi. CAS assay is the universal assay for detection of siderophores. The principle of this assay is based on a colour change of CAS from blue to orange resulting from siderophoral removal of iron from the chrome azurol dye. All the isolates showed positive response for siderophore production on CAS agar plate i.e. orange colour halo was

observed in all the isolates. All isolates exhibited positive response for its ability to produce HCN (Table 1). Overall, these isolates were found to be more efficient against *S. rolfii* indicating that these isolates could therefore be exploited as potential candidates for the development of bio pesticides more specifically against these most devastating pathogens of crops.

Plant growth promotion activity of fluorescent *Pseudomonas* on Okra (Var. ADITYA) and Pumpkin (var. HARITA)

Efficacy of different isolates of *Pseudomonas* for mungbean okra plants varied to induce shoot and root length ranging from $9.05i\pm0.1$ to $21.45a\pm0.9$ cm and $14.025k\pm0.825$ to $36.175a\pm0.975$ cm respectively. Maximum shoot length ($21.45a\pm0.9$ cm) and root length ($36.175a\pm0.975$) were recorded when seeds were treated with P-11 and P-12, respectively as compared to control (Table 3, Fig. 1 and Fig. 2). Plants treated with P-30 and P-11 recorded highest shoot and root fresh weight ($4.915a\pm0.017$ and $1.173a\pm0.018$ gm, respectively) and maximum shoot and root dry weight ($1.385a\pm0.015$ and $0.137a\pm0.015$ gm, respectively).

Similarly in case of pumpkin efficacy of different isolates of *Pseudomonas* for pumpkin plants varied to induce shoot and root length ranging from $7.375h\pm0.125$ to $17.425a\pm0.00$ cm and $28.075i\pm5.575$ to $76.9a\pm0.650$ cm respectively. Maximum shoot length ($17.425a\pm0.00$ cm) and root length ($76.9a\pm0.650$ cm) were recorded when seeds were treated with P-15 and P-2, respectively as compared to control (Table 4, Fig. 1 and Fig. 2). Plants treated with P-1 and P-9 recorded highest shoot and root fresh weight

($20.07a\pm1.073$ and $4.255a\pm0.215$ gm) whereas P-1 and P-6 recorded maximum shoot and root dry weight ($2.29a\pm0.107$ and $1.52a\pm0.0403$ gm, respectively).



Fig 1: Plant growth promotion activity of fluorescent *Pseudomonas* in Pumpkin and Okra

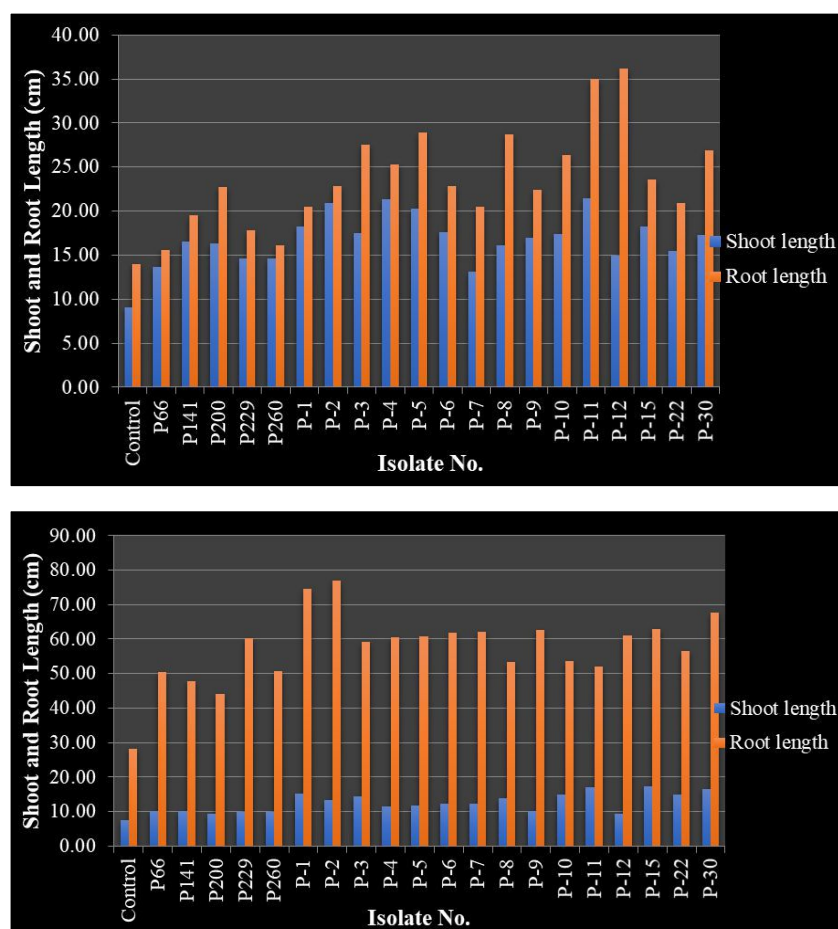


Fig 2: Effect of fluorescent *Pseudomonas* on Shoot & Root length of Okra (*Abelmoschus esculentus* var. ADITYA) and Pumpkin (*Cucurbita pepo* var. HARITA)

Table 1: *In vitro* Phosphate solubilisation and HCN production by Fluorescent *Pseudomonas*

S. No.	Isolate no.	pH of the Pikovskaya's medium 7 DAI	Phosphate solubilized (µg/ml)	Phosphate solubilization in Pikovskaya's agar medium (with Bromocresol purple)	Phosphate solubilization in Pikovskaya's agar medium (without Bromocresol purple)	HCN production
1	P66	3.66	916cd±31.0	+++	++	Negative
2	P141	3.82	616.5e±0.5	+++	++	Negative
3	P200	4.22	793cd±6.0	++	+	Negative
4	P229	3.55	919cd±4.0	++	+	Negative
5	P260	3.76	846cd±1.0	++	+++	Negative
6	P-1	3.66	789.5d±21.5	++	++	Negative
7	P-2	3.72	950.5c±6.5	+++	++	Positive
8	P-3	3.41	867.5cd±4.5	++	++	Negative
9	P-4	4.11	947.5cd±4.5	++	+++	Negative
10	P-5	3.48	1453.5a±49.5	+++	+++	Negative
11	P-6	3.67	909.5cd±5.5	+++	+++	Positive
12	P-7	4.12	809.5cd±13.5	+++	++	Positive
13	P-8	3.71	919cd±6.0	+++	+	Negative
14	P-9	3.33	1524.5a±8.5	+++	+	Positive
15	P-10	3.82	941cd±12.0	++	+	Positive
16	P-11	3.98	595e±32.0	+	+	Negative
17	P-12	3.78	1393a±45.0	+	+++	Negative
18	P-15	3.77	1217.5b±222.0	+++	+++	Negative
19	P-22	3.58	898cd±21	++	++	Negative
20	P-30	3.66	1413a±15	+++	++	Positive
	Max		1524.5a±8.5			
	Min		595e±32.0			
	CV		7.709			
	CD0.01		216.092			
	CD0.05		158.444			
	Fcal		25.032			

Table 2: Quantification of Indole Acetic acid and siderophores production by Fluorescent *Pseudomonas*

S. No.	Isolates	IAA production(µg/ml)	pH of the minimal medium 3DAI	Siderophore units (%)	Arnow's test
1	P66	16.727	7.26	82.85 ^{defg} ±0.250	Negative
2	P141	17.636	7.49	88.35 ^{ab} ±0.117	Negative
3	P200	10.909	6.68	80.96 ^{gh} ±0.9	Negative
4	P229	17.818	7.74	79.016 ^h ±4.283	Negative
5	P260	11.091	7.2	85.35 ^{abcdef} ±0.483	Negative
6	P-1	41.455	7.49	87.033 ^{abc} ±0.733	Negative
7	P-2	57.727	7.79	86.33 ^{abcd} ±0.067	Negative
8	P-3	46.909	7.44	82.85 ^{defg} ±0.117	Negative
9	P-4	52.818	7.33	82.03 ^{fgh} ±0.283	Negative
10	P-5	68.636	7.69	84.916 ^{bcd} ±0.150	Negative
11	P-6	75.091	7.72	82.383 ^{efgh} ±0.350	Negative
12	P-7	71.455	7.46	82.61 ^{defgh} ±2.850	Negative
13	P-8	80.090	7.59	82.88 ^{defg} ±0.169	Negative
14	P-9	80.630	6.88	86.13 ^{abcde} ±0.617	Negative
15	P-10	69.182	7.84	88.65 ^{ab} ±2.0	Negative
16	P-11	79.182	7.62	83.86 ^{cdefg} ±0.169	Negative
17	P-12	81.000	7.79	86.9 ^{abc} ±0.165	Negative
18	P-15	41.909	7.49	86.1 ^{abcde} ±0.165	Negative
19	P-22	77.818	7.74	86.1 ^{abcde} ±0.165	Negative
20	P-30	81.909	7.23	88.85 ^a ±0.165	Negative
	Max.	81.909	7.84	88.85 ^a ±0.165	
	Min.	10.909	6.68	79.016 ^h ±4.283	
				2.148	
				5.161	
				3.79	
				4.441	

Table 3: Plant growth promotion activity of fluorescent *Pseudomonas* in Okra (Var. ADITYA)

Treatments	Isolate No.	Average value					
		Shoot length (cm)	Root Length (cm)	Shoot fresh weight (gm)	Root fresh weight (gm)	Shoot dry weight (gm)	Root dry weight (gm)
T-1	Control	9.05i±0.100	14.025k±0.825	0.545i±0.125	0.198l±0.043	0.165g±0.047	0.015l±0.000
T-2	P66	13.65gh±0.900	15.6jk±0.500	1.57efgh±0.120	0.652hij±0.061	0.81cde±0.020	0.081ef±0.001
T-3	P141	16.575bcd±0.225	19.55hi±0.950	1.265ghi±0.345	0.674hij±0.098	0.725def±0.002	0.088f±0.003
T-4	P200	16.295cdef±0.895	22.75fg±0.750	0.845hi±0.015	0.673hij±0.058	0.61ef±0.075	0.074g±0.001
T-5	P229	14.65fgh±0.550	17.825ij±0.975	1.25ghi±0.030	0.623jk±0.043	0.665ef±0.015	0.037k±0.002
T-6	P260	14.6fgh±0.000	16.15jk±0.450	0.83hi±0.020	0.808f±0.055	0.83cde±0.100	0.089f±0.010
T-7	P-1	18.25b±0.500	20.5gh±0.250	1.665defg±0.195	0.949c±0.002	0.68def±0.070	0.078g±0.002
T-8	P-2	20.95a±0.050	22.825fg±0.575	1.34fgh±0.360	1.131a±0.015	0.93cd±0.150	0.088g±0.003
T-9	P-3	17.5bc±0.200	27.55bcd±0.850	1.795defg±0.112	0.911cd±0.048	1.24ab±0.030	0.096d±0.003
T-10	P-4	21.3a±0.125	25.325de±0.225	2.62bc±0.230	0.7gh±0.025	1.475a±0.185	0.073g±0.001
T-11	P-5	20.275a±0.350	28.925b±0.775	2.425cd±0.445	1.054b±0.010	1.045bc±0.085	0.095d±0.002
T-12	P-6	17.65bc±0.475	22.825fg±0.925	1.485efgh±0.188	0.872de±0.033	0.71def±0.040	0.077g±0.006
T-13	P-7	13.125h±1.400	20.525gh±0.625	1.725defg±0.272	0.682hi±0.045	0.705def±0.088	0.067h±0.004
T-14	P-8	16.15cdef±0.300	28.675bc±0.225	1.915cdefg±0.012	0.852ef±0.055	0.845cde±0.205	0.074g±0.001
T-15	P-9	16.95bcd±0.525	22.425fg±0.475	1.545efgh±0.085	0.711gh±0.035	0.71def±0.060	0.053hi±0.005
T-16	P-10	17.425bc±0.400	26.325cd±1.225	3.28b±0.280	0.635ijk±0.050	1.35a±0.020	0.043j±0.008
T-17	P-11	21.45a±0.900	34.95a±0.650	1.54efgh±0.300	1.173a±0.018	0.73def±0.100	0.137a±0.015
T-18	P-12	14.95efgh±0.900	36.175a±0.975	2.25cde±0.110	1.055b±0.063	0.555f±0.045	0.106g±0.005
T-19	P-15	18.2b±0.050	23.575ef±0.425	2.03cdefg±0.420	0.746g±0.023	0.675ef±0.060	0.084ef±0.008
T-20	P-22	15.45defg±0.200	20.95gh±1.950	2.065cdefg±0.655	0.597k±0.033	0.615ef±0.005	0.056i±0.003
T-21	P-30	17.3bcd±0.225	26.85bcd±0.100	4.915a±0.017	0.74g±0.012	1.385a±0.015	0.124b±0.007
	Max	21.45a±0.900	36.175a±0.975	4.915a±0.017	1.173a±0.018	1.385a±0.015	0.137a±0.015
	Min	9.05i±0.100	14.025k±0.825	0.545i±0.125	0.198l±0.043	0.165g±0.047	0.015l±0.000
	CV	5.45	4.857	20.309	3.233	14.821	4.109
	CD0.01	2.59	3.233	1.065	0.076	0.348	0.009
	CD0.05	1.89	2.373	0.781	0.056	0.252	0.005
	Fcal	21.10	51.161	12.586	150.825	12.956	147.714

Table 4: Plant growth promotion activity of fluorescent *Pseudomonas* in Pumpkin (var. HARITA)

Treatments	Isolate No.	Average value					
		Shoot length (cm)	Root Length (cm)	Shoot fresh weight (gm)	Root fresh weight (gm)	Shoot dry weight (gm)	Root dry weight (gm)
T-1	Control	7.375h±0.125	28.075i±5.575	4.215k±0.035	1.16h±0.390	0.355k±0.025	0.14f±0.030
T-2	P66	10.25g±0.000	50.35fg±1.450	9.98hi±0.800	2.22def±0.530	0.865hij±0.025	0.25ef±0.010
T-3	P141	10.2g±0.200	47.725gh±2.975	7.86ij±0.760	1.66gh±0.000	0.94hij±0.070	0.28ef±0.067
T-4	P200	9.275g±0.475	44.15h±3.650	5.7425jk±0.202	1.7825fg±0.007	0.685j±0.015	0.25ef±0.037
T-5	P229	9.775g±0.025	60.25cd±0.500	8.165ij±0.725	1.87efg±0.110	0.895hij±0.065	0.32ef±0.050
T-6	P260	9.9g±0.100	50.7fg±1.050	7.085j±0.625	1.735fg±0.005	0.775ij±0.045	0.45de±0.050
T-7	P-1	15.25b±4.250	74.6a±0.150	20.0725a±1.073	2.06defg±0.132	2.29a±0.107	0.525cde±0.080
T-8	P-2	13.3de±0.500	76.9a±0.650	12.685efg±1.275	2.38cde±0.227	1.795cd±0.190	0.60bcd±0.028
T-9	P-3	14.3bcd±0.125	59.075cd±0.575	13.875cde±2.125	1.875efg±0.050	1.735cde±0.145	0.715bcd±0.050
T-10	P-4	11.5f±0.050	60.4cd±0.950	15.775bc±1.025	2.81c±0.057	2.1ab±0.005	0.74bc±0.028
T-11	P-5	11.825f±0.700	60.775cd±0.325	12.62efgh±0.523	3.44b±0.053	1.88bcd±0.053	0.86b±0.035
T-12	P-6	12.3ef±0.200	61.775c±0.825	10.02ghi±0.280	3.88ab±0.295	1.02ghi±0.030	1.52a±0.0403
T-13	P-7	12.225ef±0.500	62.225c±0.975	10.915egh±0.635	2.60cd±0.227	0.87hij±0.022	0.885b±0.055
T-14	P-8	13.85cd±1.675	53.35ef±0.550	12.96def±0.700	3.48b±0.017	1.1gh±0.070	0.68bcd±0.027
T-15	P-9	10.175g±0.049	62.725bc±0.775	16.075bc±1.375	4.255a±0.215	1.685de±0.190	1.26a±0.030
T-16	P-10	15bc±0.375	53.725ef±0.975	15.425bcd±1.125	2.22def±0.110	1.66de±0.023	0.68bcd±0.017
T-17	P-11	17.1a±0.300	52.075efg±0.425	17.85ab±0.050	1.96efg±0.033	1.98bc±0.138	0.43de±0.053
T-18	P-12	9.45g±0.800	61.1cd±1.350	12.5efgh±0.450	2.19defg±0.040	1.12gh±0.053	0.45de±0.078
T-19	P-15	17.425a±0.000	62.825bc±0.875	16.9b±0.000	3.42b±0.170	2.09ab±0.023	0.775bc±0.045
T-20	P-22	14.85bc±0.150	56.425de±1.275	11.16fgh±0.383	1.935efg±0.005	1.28fg±0.117	0.69bcd±0.022
T-21	P-30	16.5a±0.300	67.7b±0.201	12.82def±1.475	2.19defg±0.052	1.485ef±0.055	0.87b±0.038
	Max	17.425a±0.000	76.9a±0.650	20.072a±1.073	4.255a±0.215	2.29a±0.107	1.52a±0.0403
	Min	7.375h±0.125	28.075i±5.575	4.215k±0.035	1.16h±0.390	0.355k±0.025	0.14f±0.030
	CV	4.71	4.378	10.621	11	9.212	21.767
	CD0.01	1.66	7.118	3.641	0.75	9.212	0.399
	CD0.05	1.22	5.23	2.681	0.556	0.265	0.284
	Fcal	47.68	34.944	20.314	18.45	38.627	11.798

Conclusion

This study illustrates the significance of rhizobacteria under *in vitro* conditions for multiple PGPR traits. This led to the selection of effective PGPR Isolates which as a result of their multiple PGPR traits, could prove effective in improving the seed germination rate, plant growth and vigor index of plants. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers and these PGPR isolates may be used as biofertilizers to enhance the growth and productivity of for commercially grown plants under local agro-climatic conditions of Chhattisgarh.

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