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Potential use of plant colonizing *Pantoea as* generic plant growth promoting bacteria for cereal crops

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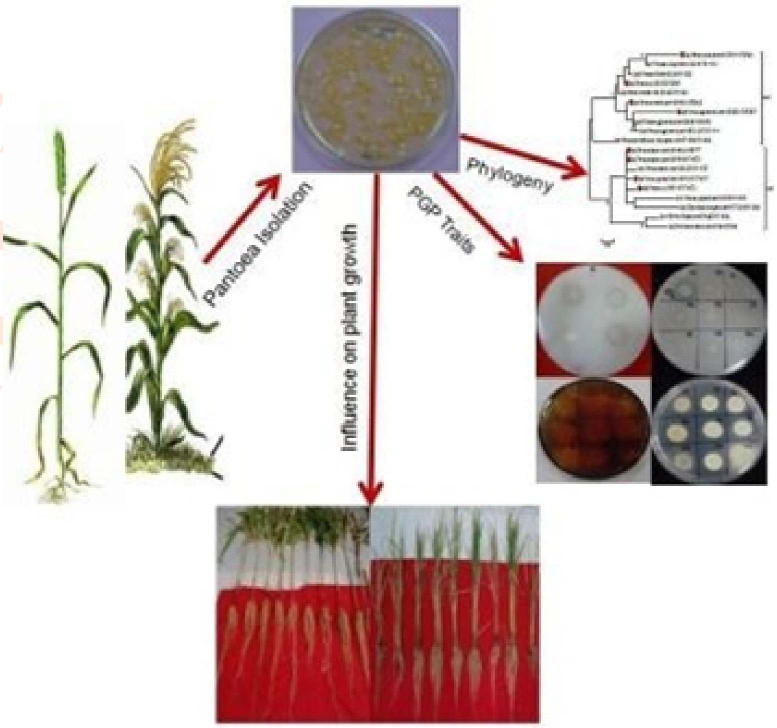
Abstract

Aim: To investigate *Pantoea* isolates for multifarious plant growth promoting activities, their phylogenetic positioning and inoculation impact on plant growth of cereal crops to develop as generic PGP biofertilizer for different crops

Methodology: Eight *Pantoea* sp. isolated from wheat and maize growing in different agro-climatic conditions were used for studying cultural characteristics (pH, Salt and Temp tolerance) PGP traits (nutrient solubilization, siderophore, IAA and hydrolytic enzyme production) and 16SrRNA gene phylogenetic analysis. *In vivo* evaluation of *Pantoea* inoculants was studied with 3 cereal test crops in pot house studies.

Results: *Pantoea* isolates identified through 16S rRNA gene sequencing *viz. Pantoea cyperipedii*, *Pantoea dispersa, Pantoea* sp. (endophytes) from maize and *Pantoea agglomerans, Pantoea ananatis* (rhizospheric), *Pantoea eucalypti, Pantoea* sp., and *Pantoea dispersa* (endophytes) from wheat, possessed major and micronutrient solubilization activities with solubilization Index for P (PSI), K (KSI) and Zn (ZSI) varied from 2.7 to 5.3, 0 to 5.3 and 3.7 to 6.0, respectively. Production of siderophore (SPI) and IAA varied from 3.3 to 5.7 and 9.0 to 131.8 μg mg^{-1} proteins. Isolates of *P dispersa and P agglomerans* possessed extracellular activities of hydrolytic enzymes cellulase, amylase, lipase and protease. *The* inoculation effect of *Pantoea* isolates in pot house studies indicated positive influence on plant growth of maize, wheat and rice crops.



Interpretation: *Pantoea* isolates, due to their plant growth improving capabilities and wide colonization capabilities, may be used for preparing a new safe biofertilizer.

Key Words: Bioinoculants, Endophytes, Nutrient solubilization, *Pantoea*, PGPB

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Introduction

Beneficial effects of plant associated bacterial communities have been well recognized in natural and managed ecosystems for their important roles in supporting plant health, growth and productivity. Compant *et al*. (2016) described endophytes as neutral, commensal, beneficial and dormant saprobe microflora that can be isolated from different plant tissues not showing any visible symptoms. Endophytic microflora develops microbial communities in close associations within different tissues/organs of plant endosphere ((Chanway, 1996; Suman *et al*., 2001). These endophytic microorganisms benefit host plant directly by promoting nutrient availability, biological nitrogen fixation, and the production of phytohormones (Adesemoye and Egamberdieva, 2013; Suman *et al.*, 2005). Indirectly, they may act as biological control agents to reduce microbial pathogens, through antibiosis, competition and/or systemic resistance induction (Lugtenberg and Kamilova, 2009).

The members of *Enterobacteriaceae* (*Gamma proteo bacteria*) are frequent colonizers of plant endo- and rhizosphere of grasses and other crops (Coleman-Derr *et al.*, 2016). The bacterial genus *Pantoea,* comprises many versatile species that have been isolated from a multitude of environments. These have been reported as rhizospheric and endophytic colonizers of corn and wheat plant, potato stems, rice seeds and citrus leaves. Several studies have reported the potential of *Pantoea* spp. as plant growth promoter by solubilizing phosphorus, stimulating phytohormone production, induction of systemic resistance and protection against pests and plant-pathogenic microorganisms (Sergeeva *et al*., 2007; Dastager *et al*., 2009). *Pantoea* group being a ubiquitous and versatile group of genetic tractability is apt for exploring niche-specific adaptation and opportunism. These are also being explored for the development of agricultural, medical and environmental products (Walterson and Stavrinides, 2015).

Identification of new plant growth promoting bacterial strains having abilities of wider adaptation to colonize different crops is gaining importance in sustainable agriculture and their commercial applications as microbial technologies for improved and profitable agriculture is expanding quite rapidly (Backer *et al*., 2018).

Therefore, the present study was planned to investigate diverse *Pantoea* isolates for multifarious plant growth promoting activities and their inoculation effect on plant growth of different graminaceous crops.

Materials and Methods

***Pantoea* isolates, Phylogenetic analysis and Cultural characteristics :** Eight *Pantoea* sp. isolated from wheat and maize growing in different agro-climatic conditions were used in the present study (Table 1) Isolates were cultured and maintained on nutrient agar growth medium. The sequences of 16S rRNA gene of *Pantoea* isolates (present study) and eleven of different species of *Pantoea* downloaded from NCBI GenBank database as reference for phylogenetic analysis were used to construct a phylogenetic tree. Sequences of *Erwinia tasmaniensis, Erwinia billingiae* and *Enterobacter aerogenes were used* as outgroups for phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method, based on the Tamura-Nei model and the tree with the highest log likelihood as conducted in MEGA7 software. *Pantoea* isolates were screened by plate spot assay (10µl ≈ 10^3cells) on nutrient agar plates for temperature tolerance by incubation at temperature ranging from 4 to 40 ºC for 72-96 h. For pH tolerance, nutrient agar with pH ranging from 3 to 11 with an increment of one was plate spotted and incubated at 30 ºC for 72-96 hrs. The isolates were screened for salt tolerance by spot inoculating each *Pantoea* isolate onto nutrient agar plates supplemented with variable NaCl concentrations of 5, 10, 15, 20 and 25 % (w/v) and incubating at 30 ºC for 72 hrs. Bacterial growth on spots was considered positive after incubation.

Hydrolase activity of *Pantoea* isolates : *Pantoea* isolates were screened qualitatively for extracellular hydrolytic enzyme activities for which 10µl (≈ 10^3cells) suspension of different *Pantoea* isolates were spotted onto minimal media (Mm) agar plates (0.1 % $NaNO_3$, 0.1 % K_2HPO_4, 0.05 % $MgSO_4$, 0.1 % KCl, 0.05 % yeast extract and 1.5 % Bacto agar) pH 7.0, supplemented

Table 1 : *Pantoea* isolates from different crop resources

Pantoea isolates	Isolate No	Genbank accession	Endophytic/ Rhizospheric	Source crop/ Variety	Location
P. agglomerans	IARI-BHD-15	KF054874	Rhizospheric	Wheat-HD1563	NEPZ
P. eucalypti	IARI-DV-11	KF054839	Endophytic	Wheat-HD3043	NWPZ
Pantoea sp.	IARI-DV-65	KF054847	Endophytic	Wheat-HD2987	NWPZ
P. ananatis	IARI-IHD-30	KF054905	Rhizospheric	Wheat-IWP5007	CZ
P. dispersa	IARI-HHS2-3	KF054777	Endophytic	Wheat-HS507	NHZ
P.cyperipedi	IARI-PC4-37	KT149747	Endophytic	Maize- PC4	NWPZ
P dispersa	IARI-PC4-49	KT149750	Endophytic	Maize- PC4	NWPZ
Pantoea sp.	IARI-PC4-87	KT149752	Endophytic	Maize- PC4	NWPZ

NEPZ: North Eastern Plain Zone of India; NWPZ: North Western Plain Zone of India; CZ: Central Zone of India; NHZ: North Hill Zone of India with appropriate substrate for extracellular hydrolytic enzyme detection. For estimation of cellulase activity minimal medium was supplemented with 1% CMC (w/v) and after incubation at 30^{0}C for 72 hrs, CMCase activity was detected by staining the plates using 0.2 % Congo red for 15 min and then de-stained by washing twice with 1 M NaCl for 15 min for measuring the zone of clearance around the bacterial colony (Teather and Wood, 1982). For amylolytic activity, minimal medium was supplemented with 1% soluble starch (w/v) and after incubation for 72 hrs at 30^{0}C, iodine solution was flooded in the plates for zone of clearance (Hankin and Anagnostakis 1975). Minimal media plates containing 1% gelatine (w/v) were used for proteolytic activity of the isolates as produced zone of clearance around protease producing bacteria (Nigam and Singh, 1995). Similarly, lipase activity of the *Pantoea* isolates was screened on tributyrin minimal media plates containing 1% (v/v) tributyrin.

Plant growth promoting attributes of *Pantoea* isolates : *Pantoea* isolates were screened qualitatively for plant growth promoting attributes by estimation of solubilization of nutrients (phosphorus, potassium and zinc) and production of phytohormone indole-3-acetic acid and siderophores. Spot plate assays were conducted for phosphorous solubilization using Pikovskaya agar medium supplemented with 0.5% tricalcium phosphate (Pikovskaya, 1948), Aleksandrov medium supplemented with 0.2% potassium aluminosilicate for potassium solubilization (Hu *et al.*, 2006) and 0.1% insoluble zinc compound ZnO for Zn solubilization (Fasim *et al.*, 2002). For siderophore production, Chromeazurol 'S' agar (Schwyn and Neilands, 1987) plates were spot inoculated with actively grown culture and analysed for the appearance of orange-halo zones against blue background after 72 hrs of incubation at 28°C. All assays were done in triplicate. After incubation period, diameter of the bacterial colony, the clearing zone on P, K and Zn plates and orange halo zone on siderophore plates were recorded. IAA production was detected by the modified method of Brick et al. (1991) using Salkowski reagent.

Plant growth promotion assay : Effect of *Pantoea* isolates on the growth parameters of 3 crops viz. maize (PEHM5), wheat (HD2967) and rice (P1121) was studied in three pot experiments in a net house located at Division of Microbiology, Indian Agricultural Research Institute, New Delhi, during respective weather conditions. Seeds treated with *Pantoea* culture suspension were used. There were 10 treatments that included 8 *Pantoea* inoculants, positive control (recommended dose of nitrogen, phosphorous and potassium (NPK)) and negative control (no recommended NPK and bacterial inoculants) and were replicated 3 times. Pots were completely randomized and 60-70% available soil moisture was maintained throughout the growth cycle by watering pots as and when required. At two month stage each pot was washed carefully with running water to take out plants with intact root system. Plant growth promotion was evaluated by measuring plant shoot and root length and their fresh weight. A portion of washed shoot and root was dried in the oven at 80 ºC for 96hrs to record dry weights.

Statistical analyses : Plant biometric data was subjected to analysis of variance with one way classification to find treatment differences and interaction effects (p=0.05) (Snedecor and Cochran, 1967). Correlation among *Pantoea* PGP traits and their influence on plant biometric parameters was drawn using MS Excel Data Analysis.

Results and Discussion

Use of beneficial microbial inoculants for plants is a promising partial alternative for hazardous chemical fertilizers. Genus *Pantoea* are highly adaptive for colonization in different hosts due to its ability to utilize diverse compounds. Nearly, 20 species *of Pantoea* have been isolated from diverse hosts over last few years (Walterson and Stavrinides, 2015). Most of the early research on *Pantoea* has focused on their parasitic association with plant hosts, but lately their potential role as plant growth promoter is realized and are being researched more and more. In this study, 8 *Pantoea* isolates from rhizo-endosphere of diverse ecotypes of wheat and maize plants were identified using

Table 2 : Cultural characteristics and PGP indicators of *Pantoea* isolates

Pantoea Isolates	Cultural characteristics				PGP index				
	Colour	Temp (°C)	NaCl (%)	pH	PSI	KSI	ZnSI	SPI	IAA
P. agglomerans	Cream	4-30	5	7-10	4.3	5.0	6.2	5.8	131.8
P. eucalypti	Cream	20-40	5	3-6	5.3	4.7	4.3	4.7	91.7
Pantoea sp.	White	10-30	5	5-6	2.7	-	3.7	3.3	64.7
P. ananatis	Cream	4-30	5	5-8	4.0	5.3	4.3	4.7	44.8
P. dispersa	Yellow	10-30	15	3-7	5.3	3.0	5.3	5.0	78.6
P.cyperipedi	Yellow	10-30	5	5-7	5.0	4.0	6.0	5.0	9.0
P dispersa	Yellow	20-30	5	4-8	4.3	4.3	5.0	4.7	86.4
Pantoea sp.	Yellow	10-30	5	3-7	5.3	5.0	6.0	5.3	83.3

Solubilization Index for Phosphorous (PSI), Potassium (KSI), Zinc (ZnSI) and Production Index for Siderphore (SPI) calculated as (colony diameter + solubilization zone)/Colony diameter. Indole Acetic Acid (IAA): $\mu g\ mg^{-1}$ protein

16SrRNA gene as *Pantoea cyperipedii*, *Pantoea dispersa, Pantoea* sp. (endophytes) from maize and *Pantoea agglomerans, Pantoea ananatis* (rhizospheric), *Pantoea eucalypti, Pantoea* sp., and *Pantoea dispersa* (endophytes) from wheat plants. On phylogenetic analysis, all isolates were grouped in two major clades. Clade I had five species of *Pantoea* viz; *P. eucalypti*, *P. allii*, *P. ananatis*, *P. agglomerans* and *P. stewartii*. The isolate number IARI-DV-65 was close to *Pantoea allii,* and IARI-PC4-87 was identified as *Pantoea cypripedii.* Clade II have *P. dispersa*, *P. cypripedii* and outgroup isolates. Brady *et al.* (2008) evaluated multi locus sequence analysis (MLSA) using four housekeeping genes, gyrB, rpoB, atpD and infB as a means for rapid classification and identification of *Pantoea* strains.

Different *Pantoea* isolates exhibited white to yellowish circular colonies of 2-3 mm diameter on nutrient agar medium plates at 30 ºC incubation. Probably owing to their isolation from the host growing in different environmental conditions, these isolates possessed variable cultural characteristics as they could grow on variable pH, temperature and salt levels (Table 2). Similar reports indicate that cold tolerant *P. dispersa* and *P. agglomerans* isolated from soils of Himalaya could grow from 4º to 42 ºC with maximal growth at 30 ºC, at pH 3–10 and at NaCl concentrations up to 8% (Selvakar *et al*., 2008; Gupta and Vakhlu, 2015). After rhizosphere and rhizoplane colonization, some microorganisms enter roots and establish as endophytic microorganisms through passive diffusion from cracks and root tips and by secreting cell-wall degrading enzymes (Hallman *et al*., 2001, Lodewyckx *et al*., 2002, Krause *et al*., 2006). In the tested *Pantoea* isolates, good cellulase activity and moderate activities of amylase, lipase and protease enzymes were present. *P. dispersa and P. agglomerans* possessed all 4 tested hydrolytic enzymes (Table 3). The

Table 3 : Extracellular Hydrolytic Activities of *Pantoea* isolates

***Pantoea* Isolates**	**Hydrolytic Enzyme Production**			
	Cellulase	**Amylase**	**Lipase**	**Protease**
P. agglomerans	++	+	-	+
P. eucalypti	+	+	+	-
Pantoea sp.	-	+	-	+
P. ananatis	+++	-	+	+
P. dispersa	+	+	+	+
P.cyperipedi	+	+	-	-
P dispersa	++	+	+	+
Pantoea sp.	+	-	+	+

–, no activity; +, moderate activity; ++, high activity; +++, very high activity

Table 4 : Correlation among bacterial PGP traits and plant biometric parameters

	PSI	***KSI***	***ZnSI***	***SPI***	***IAA***
PSI	1.00				
KSI	0.62	1.00			
ZnSI	0.58	0.51	1.00		
SPI	0.69	0.81	0.86	1.00	
IAA	0.04	0.18	0.15	0.35	1.00
MSL	0.22	-0.47	0.16	-0.05	0.43
MRL	0.16	0.48	0.43	0.40	-0.36
MSW	0.56	0.42	0.93	0.76	0.11
MRW	0.11	-0.49	0.29	-0.18	-0.59
RSL	-0.35	-0.38	-0.41	-0.50	-0.49
RRL	0.11	-0.02	-0.19	-0.13	-0.49
RSW	-0.25	-0.24	-0.29	-0.35	-0.52
RRW	-0.18	0.39	0.08	0.32	0.23
WSL	0.19	0.13	0.64	0.54	0.52
WRL	0.15	0.27	0.67	0.58	0.08
WSW	0.11	-0.06	0.59	0.37	0.30
WRW	0.29	0.41	0.59	0.55	0.27

Solubilization Index for Phosphorous (PSI), Potassium (KSI), Zinc (ZnSI) and Production Index for Siderophore (SPI), Maize shoot length (MSL), root length (MRL) shoot weight (MSW), root weight (MRW); Rice shoot length (RSL), root length (RRL) shoot weight (RSW), root weight (RRW); Wheat shoot length (WSL), root length (WRL) shoot weight (WSW), root weight (WRW)

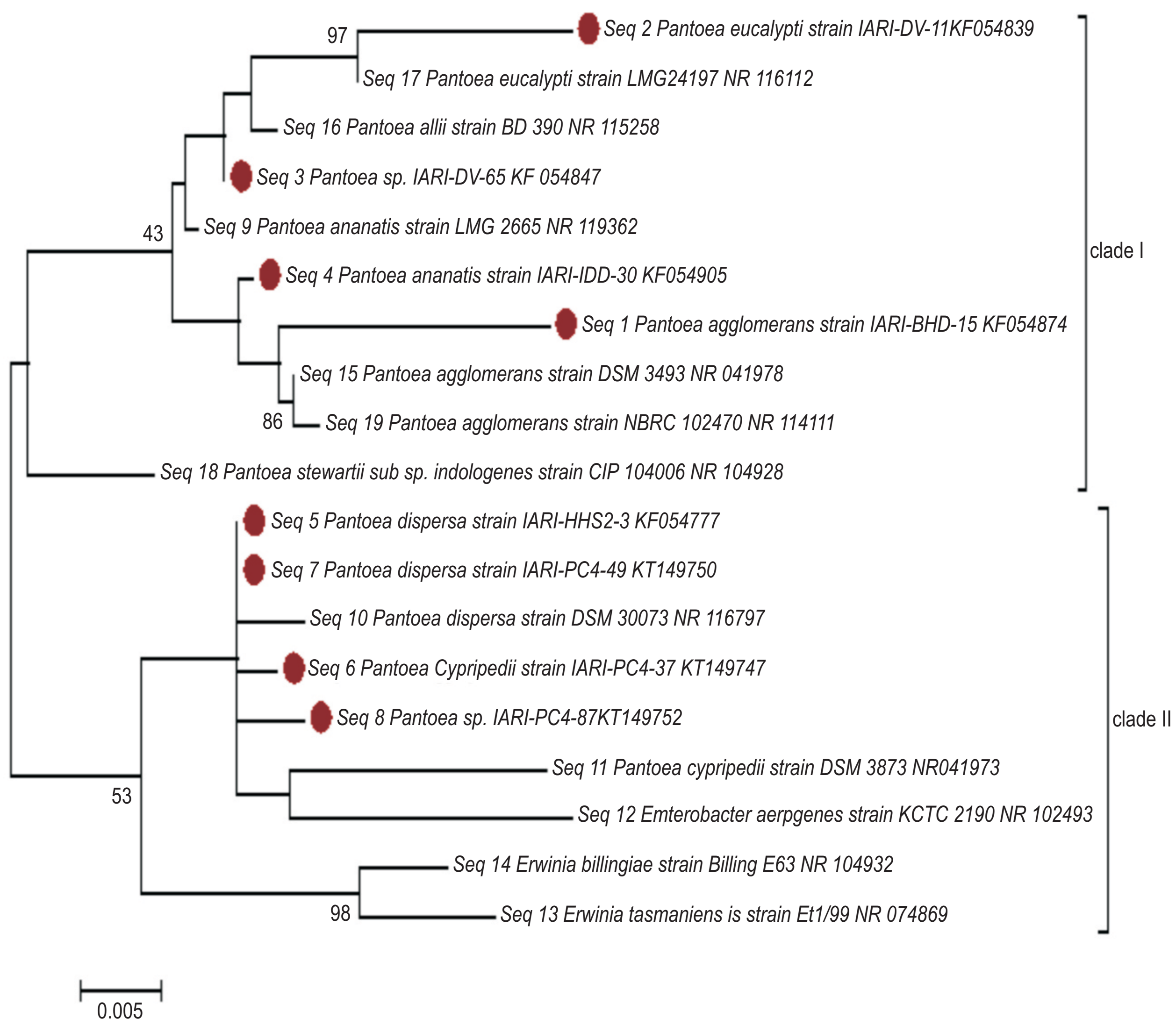

Fig. 1 : Phylogenetic analysis of *Pantoea* isolates

hydrolytic enzyme complex of amylase, protease, lipase, phosphatase and gelatinase produced extracellularly play a crucial role in mineralization of nutrients by hydrolyzing complex organic matter. NabiJatt *et al*. (2015) had reported a link between QS signals and extracellular hydrolytic enzymes in *P.ananatis* isolated from marine environment.

All *Pantoea* isolates showed variable magnitude of plant growth promoting attributes for 3 types of indicators viz. solubilization of different nutrients, production of siderophore and phytohormones IAA (Table 2). Among plant growth promotion indices, PSI was positively correlated with KSI, ZnSI and SPI (Table 4). Earlier many PGP strains from *Pantoea* have been reported for P solubilization and phytohormones production (Sergeeva *et al*., 2007; Dastager *et al*., 2009). Reports about K solubilization by *Pantoea* are meagre. Zang and Kong (2014) isolated K solubilizing bacteria from tobacco rhizosphere; wherein, majority were members of enterobacteriaceae. To the best of our information, Zn solubilization by *Pantoea* has been reported for the first time in this study, as all isolates were solubilizing Zn. Functional expression of these plant growth promoting traits along with several others make *Pantoea* a competent bacteria for colonization in diverse hosts.

The beneficial effect of these *Pantoea* isolates was assessed *in planta* with maize, wheat and rice crops. *Pantoea* inoculated maize plant shoot length varied between 112 to146 cm with overall average of 130.5 cm compared to 124 and 92 cm in positive control and negative treatments, with improvement of 5 and 41.8%, respectively (Fig 2). The improvement was highest in *P. agglomerans* and *P. sp.* and minimum in *P. ananatis* and *P. dispersa*. Shoot fresh weight was at par with positive control but

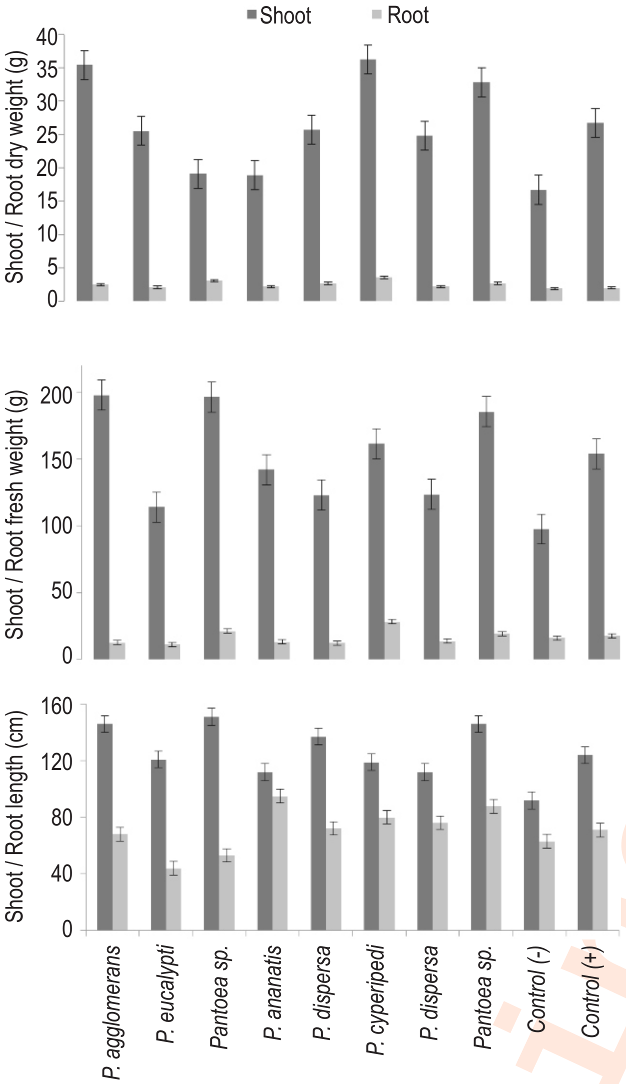

Fig. 2 : Effect of *Pantoea* isolates on maize plant biometric parameters.

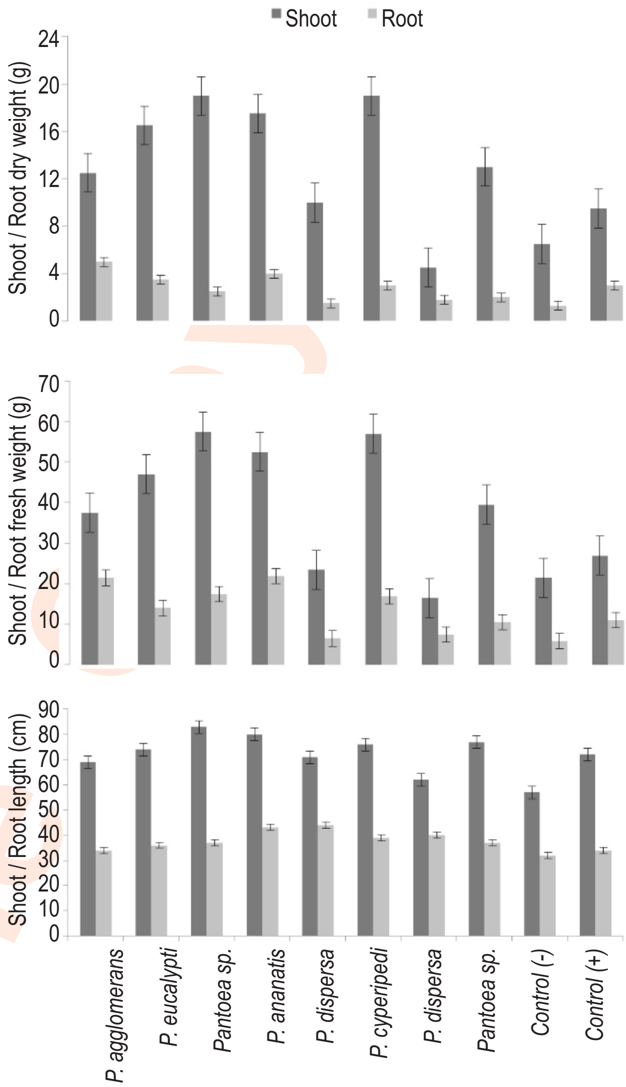

Fig. 3 : Effect of *Pantoea* isolates on rice plant biometric parameters.

was 59% more than negative control treatment. Similar reflection was observed in shoot dry weight as it was at par with positive control but was 63.5% higher than negative control treatment. *Pantoea* inoculated plant roots varied from 44 to 95cm with mean length of 72 cm which was at par with positive control treatment (71cm) and 14.3% more than negative control (63cm) treatment. Similarly root fresh and dry weight was 2.8 and 38% higher than both the respective control treatments.

In rice plant assay, overall effect of *Pantoea* isolates on shoot length was at par with positive control treatment and 29.8% higher than the negative control treatment (Fig 3). Individually, each *Pantoea* isolate improved shoot length above negative control by 8.8 to 45.6% with highest in *Pantoea sp* followed by *P ananatis* and *P cyperipedi*. *Pantoea* inoculation improved shoot fresh weight by 44 and 80.9% as compared to positive and negative control, respectively, with highest in *Pantoea sp* and *P cyperipedi* . Similar trend was recorded in shoot dry weight also. Root length was almost at par in both control treatments but in negative control, roots were very fragile and fresh weight was near half of the positive control treatment. *Pantoea* inoculation improved root length by 21% and fresh weight by 39.6% as compared to negative control, whereas, these were 14 and 26% more as compared to positive control treatment.

Pantoea inoculated wheat shoot and root length was at par with positive control but was 46.8 and 62% more as compared to negatively control, respectively (Fig. 4). Shoot fresh weight due to *Pantoea* inoculation was 6.5 and 145% more than positive and negative control, respectively. Similarly root fresh weight increased by 27 and 50% compared with both controls, respectively. Individually all plant biometric parameters were better due to *Pantoea* inoculation with highest shoot length in *P. agglomerans* and lowest in *P. annanatis* and *P. eucalypti*. The

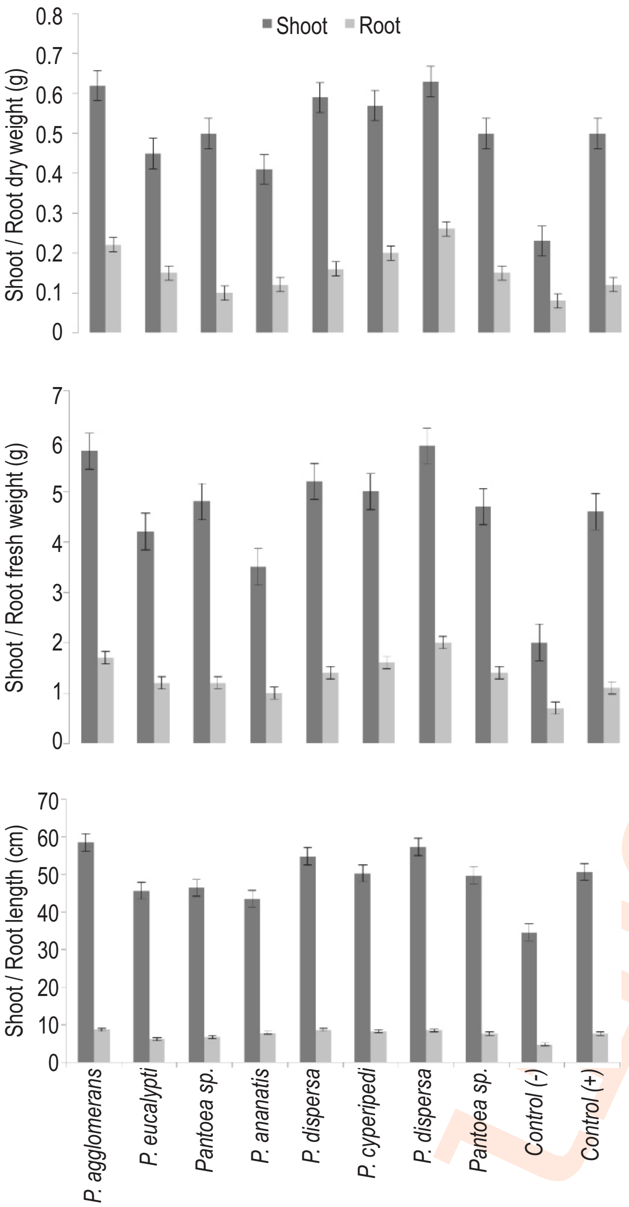

Fig. 4 : Effect of *Pantoea* isolates on wheat plant biometric parameters.

shoot fresh weight was highest in *P. agglomerans* and *P.dispersa* (195%) and lowest in *P. annanatis* (75%) compared to positive control. Similar trend was observed in root dry weight also.

On correlating plant growth promoting indices of *Pantoea* isolates with plant biometric parameters of maize, rice and wheat, PSI and ZNSI were positively correlated with maize and wheat parameters, but overall poor correlation was observed with rice (Table 4).

Plant growth promoting abilities of *Pantoea sp*. has been reported for different crops. Selvakumar *et al*. (2008) have demonstrated the beneficial effect of a cold tolerant *P.dispersa* isolate on wheat growth at low temperatures. Mishra *et al.* (2011) demonstrated the positive effect of *P. agglomerans* strain for improving maize and chickpea growth. The promotion of sugarcane growth by the endophytic *Pantoea agglomerans* strain 33.1 was studied under gnotobiotic and greenhouse conditions using the green fluorescent protein (GFP)-tag (Quecine *et al*., 2012). Khalimi *et al*. (2012) has demonstrated the positive influence of combined inoculation of two isolates of *P. agglomerans* isolated from groundnut rhizosphere on the growth and yield of rice crop. Kim *et al*. (2012) have successfully demonstrated the high endophytic colonization of *P. annanatis* in different tissues of pepper and growth promotion in the pepper plantations. Singh *et al*. (2014) have reported *P. cypripeddi* in consortium with *Enterobacter aerogens* and *Rhizobium ciceri* for enhancing the performance of chickpea crop. Cherif-Silini *et al*. (2019) reported that *Pantoea agglomerans* strain Pa induced stress tolerance in durum wheat and strongly promoted this as an efficient biofertilizer for wheat plants in arid and salinity-impacted regions.

Pantoea isolates, owing to their diverse colonization capabilities and abilities to function in phosphate, potash and zinc solubilization, siderophore and IAA production, belong to a class of generic plant growth promoting bacteria (PGPB), which can promote growth of several plant species. Since these PGPB inoculants exhibited multiple traits beneficial to the host plants at lower chemical inputs, they may be applied for preparing a new, safe, and effective product for partial replacement of chemical fertilizer.

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