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Salt tolerant endophytic and diazotrophic strain of *Proteus mirabilis* PD25 and its effect on the growth of wheat under saline conditions

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Abstract

In the present investigation, an endophytic diazotroph PD25 with novel properties was isolated from roots of wheat growing in saline soil (EC~10.6 dS m^{-1}). The isolate was chosen for further experimental work based on plant growth promoting (PGP) attributes and identified as *Proteus mirabilis* by 16S rRNA sequence homology. The work presented in this article reports the salt tolerant bacterium *P. mirabilis* PD25 as effective diazotrophic endophyte and the isolate was also found to be positive for other PGP attributes such as siderophore, indole acetic acid (IAA), accompanied by the solubilization of phosphorus (P), zinc (Zn), and potassium (K). The data signifies the prominent involvement of plant growth promoting endophyte (PGPE) in agriculture and suggests that novel bioformulations developed by them can be a potential development strategy in boosting plant growth and development in saline soils in the future.

Keywords: Plant growth promoting endophytes, Wheat, Bioformulations, Soil salinity, *Proteus,* Nitrogen fixation.

1. Introduction

The effects of climate change on the environment have been very significant over the years and have caused various abiotic stresses, negatively affecting crop quality and productivity. Salt stress is one of the major abiotic threat to agriculture that has impacted yield of plants in many areas of the world due to increased use quality of water for irrigation and soil salinization [1]. Reduced growth of roots and shoots, slow germination rate, decreased or no development of seedling, stomatal closure, and deterioration of photosynthetic activity are some of the key responses of plants to stress [2, 3]. Nitrogen metabolism and phytohormones production play significant roles in improvisation of growth and yield of various plants and is regulated an complex enzyme known as nitrogenase [4]. Nitrogenase is mainly responsible for biological nitrogen fixation (BNF) and synthesis is regulated by oxygen (O_2) and ammonia (NH_3) [5]. Microorganisms present in soil are able to fix nitrogen from the atmosphere with the help of various genes reported to be involved in the process of nitrogen fixation and most commonly reported genes for synthesis of nitrogenase comprise of *nifH*, *nifD*, and *nifK* [6]. Endophytes are the microbial endosymbionts including bacteria, fungi, and actinomycetes that reside in the microenvironment of the host plant [7]. Diazotrophic endophytes possessing the nitrogen fixing genes play beneficial role in increasing uptake by plants which further results in promotion of various attributes ultimately improving the health and productivity of plants. Thereby this work was conducted to study the effect of salt tolerant bacterial endophytes on growth and vigor index of one of the most important cereal crop i.e, wheat in saline soils along with the assessment of their potential of nitrogen fixation in such condition. Understanding diversity of these beneficial endophytes and their application as bioinoculants in agricultural sector can be a suitable bio-approach to reclaim salt-stressed soils and increase the plant biomass production of wheat and other cereal crops.

2. Material and Methods

2.1 Sampling sites and isolation of endophytes

Fresh and undamaged wheat samples were collected from agricultural areas of Kanpur Dehat region (26.52° N, 79.82° E) in Uttar Pradesh, India. Procured roots were washed with sterile deionized water and were further processed for surface sterilization. The external surface of the root was exposed to 70% ethanol for 3 minutes followed by three to four times washing with distilled water. Roots were afterward treated with mercuric chloride for 8-10 minutes and washed up with sterile water many times [8]. To know the sterilization efficacy, the scrubbed external surface of the root was incised with help of a sterile blade and was directly mounted on the surface of the nutrient agar medium for 24-48 h at 28°C to observe the bacterial growth.

The surface sterilized roots were aseptically weighed, segmented into small pieces, and homogenized using sterilized mortar pestle. A 100 µl sample of root extracts and their subsequent dilutions were spreaded on plates containing nutrient agar, Jenson's agar, yeast extract mannitol agar (YEMA). Plates were incubated aerobically at 28°C for 48-72 hours. All the discrete bacterial colonies were expressed as colony forming units per gram (CFU g^{-1}) and were refined by following the quadrant streak method until pure bacterial cultures were obtained.

2.2 Morphological, biochemical, and PGP characterization of endophytic isolates

All the endophytic isolates were presumptively distinguished on the basis of physiological and biochemical characters according to Bergey's Manual of Systematic Bacteriology [9].

Plant growth promoting characters were checked by adding the log phase cultures of all the isolates to the respective medium. Phosphate solubilization was checked using Pikovskaya's medium and the clear zone around bacterial colonies were observed [10]. Siderophore production was performed on both Chrome-Azurol S (CAS) agar plates as well as in CAS broth using microtiter plates [11, 12]. Zn solubilization and K solubilizing activity were determined through plate assays on zinc medium and Aleksandrov medium (by using acid-base phenol red as indicator dye) [13, 14]. Production of IAA was checked by using Salkowski's reagent and measuring the color development at 530 nm as described by [15].

2.3 Nitrogen fixation assay

The ability of bacterial endophytes to fix nitrogen was examined by using Jensen's medium and bromothymol blue (BTB) as color indicator. Freshly grown cultures of all the isolates were inoculated in the test medium and the plates were further placed for incubation at 28^{o}C for 48 hours. Nitrogen fixing activity was assessed by color alteration of medium to dark blue.

2.4 Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

Polymerase chain reaction (PCR) amplification of selected endophytic bacterial isolate i.e, PD25 was done using universal primers and 243F (5'-GGATGAGCCCGCGGCCTA-3') and 1378R (3'-CGGTGTGTACAAGGCCCGG-5') were used as forward and reverse primers respectively. The amplification primers reactions were performed in 100 µl including initial denaturation for 2 minutes at 95°C followed by 35 cycles with a denaturation step for 30 seconds at 95°C, annealing for 30 seconds at

52°C, extension for 2 minutes at 72°C and final extension for 15 minutes at 72°C. The amplified products were electrophoresed on 1% (w/v) agarose gel along with molecular weight marker, visualized under UV light, and the PCR products were further sequenced by outsourcing. Nucleotide sequences were analyzed based upon the closest match to sequences available through the National Center for Biotechnology Information Blast (www.ncbi.nlm.nih.gov/BLAST) using the Basic Local Alignment Search Tool (BLAST) search algorithm. Phylogenetic tree was constructed by the neighbor joining method and using MEGA software version 6 [16, 17].

2.5 Determination of nitrogenase activity

Acetylene reduction assay (ARA) was used to quantitatively examine the nitrogen fixation efficiency of the selected isolate. Freshly grown pure culture (48 h) of bacterial isolate PD25 was taken and cell pellets were formed in order to remove the nitrogen traces. The obtained pellets were further inoculated into Modified Burk's nitrogen free medium (50 ml) and incubated for 5 days on a rotary shaker at 28^{o}C. Aliquots (0.1 OD at 610 nm) were obtained after incubation till exponential phase; 20 ml was transferred into the assay vials with an air tight lid equipped with a suba seal. The gas in headspace of the vials was replaced with acetylene (10% v/v) and were allowed to incubate for 24 hours at optimum growth temperature After incubation, gas was withdrawn from the vials and injected into a gas chromatograph (Agilent 7890A GC System) fitted with a HP-5 column and FID detector. The oven temperature was adjusted to 45°C and ethylene production was assayed.

2.6 PCR amplification of *nifH* genes

The presence of *nifH* in the nitrogenase complex of the selected bacterial isolate was detected by PCR using a Veriti Thermal Cycler and the genomic DNA extraction was done according to the chloroform/isopropyl alcohol method [18]. The primers 19F (5'-GCIWTYTAYGGIAARGGIGG -3') and 407R (5'-AAICCRCCRCAIACIACRTC-3') were used in the study to amplify a fragment (~390 bp) of the *nifH* gene. The condition maintained included 40 cycles of initial denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, 30 sec at 72 °C, followed by a final extension at 72°C for 5 min. Afterward, the PCR products were stained with ethidium bromide and analyzed by 1.5% agarose gel electrophoresis to separate the fragments and confirm their sizes.

2.7 Halotolerance assay

The log phase culture of the selected endophytic isolate was added to the nutrient broth medium amended with different concentrations of NaCl (0-6%). Tubes were incubated at 28°C in a shaking incubator for 48 h at 120 rpm. Optical density (OD) was measured at 610 nm by using Thermo Scientific™ Evolution 201 UV–Vis spectrophotometer and the experiment was conducted taking five replicates [19]. A growth curve of bacteria under salinity stress was made.

2.8 Endophytic nature of bacterial endophytes in plantae

Colonization of PD25 in wheat roots was checked to determine the endophytic nature of the endophyte isolate [20]. Wheat seeds (Annapurna PBW-343) were surface sterilized and made endophyte free by treating them with NaOCl solution for 5 min. Thereafter the seeds were dipped in the freshly grown bacterial culture of the selected isolate and raised on water agar medium in petri dishes for two weeks [21]. The roots were taken from the germinated wheat plants, adhering soil was removed by washing, and sections of root tissue were processed for the re-isolation of endophytes

using the same procedure as described above. The identification of obtained bacterial colonies was further confirmed by 16S rRNA sequencing according to [22] and was matched with the lab culture of *P. mirabilis*.

2.9 Pot trials

The experiments were performed to study the PGP ability of selected bacterial isolate by taking wheat as the test crop. It was conducted during the months of November-February for two consecutive years (2019 and 2020) in earthen pots sized 15 × 11 × 11 cm. The pots were filled with sterilized and non-sterilized saline soil (EC 10.6 dS/m) which was collected from adjoining rural areas of Lucknow, India (26.72°N, 80.84°E). Each treatment was conducted in triplicates and pots were grown in an open greenhouse [23]. The soil was wetted properly before sowing the seeds and subsequently irrigated with normal water two times a day. Physicochemical parameters of the soil including pH, EC, available nitrogen, P, K, and organic carbon were also analyzed according to standard methods of [24]. Wheat variety Annapurna-PBW 343, which is a salt-tolerant variety and commonly used by farmers of the region, was selected for the study and water agar plate assay was performed to check the salt tolerance level prior to pot experiments. Wheat seeds were surface sterilized with 70% ethanol for 2 min and 2% sodium hypochlorite for 10 min followed by thorough rinsing with sterilized distilled water. The autoclaved aqueous solution of 1% carboxymethyl cellulose (CMC) was used as a binding agent (4 ml CMC with 1 g seed) to effectively coat the sterilized seeds [25]. Bacterial cell suspension of PD25 was prepared by growing the bacterium in nutrient broth in a rotary shaker at 27±2°C at 150 rpm and 450 ml of culture suspension from log phase was added to CMC solution. The bacterial population density was measured by adding 1 ml of the resultant mixture into distilled water (10 ml) which was then serially diluted up to 10^{-6} and 10^{-7} [26]. Surface sterilized seeds were immersed in the bioformulation, kept overnight (10-12 h), and air-dried for 3-4 h in order to adjust moisture content to ~35%. Treated and untreated seeds were sown in pots (10 in each) and observed further for germination and growth. The seeds without any bacterial treatment were taken as the test control. Experiments were categorized with the following sets of treatments: (i) untreated control (ii) seeds + PD25. Plants were uprooted carefully after 60 days of sowing and plant growth parameters such as shoot length, root length, fresh and dry weight, no. of spikes, and grain yield were measured for harvested wheat plants.

2.10 Data analysis

Microsoft Office Excel had been used to create data base. The data on growth parameters were analyzed statistically and means were compared using the student's t-test at a probability of α=0.05. The analysis was performed using the statistical package software for social sciences (SPSS, 2016) for Windows.

3. Results and Discussion

The present study focused on exploration of diazotrophic PGPE and their role in mitigation of salinity stress was also elucidated. In total twelve isolates were obtained and PD25 was chosen for further experimental work on the basis of PGP traits, and nitrogen fixation activity on Jensen's medium. Morphological and biochemical characterization of the isolate was done and colonies were found to be colorless on nutrient agar plates, Gram-negative, motile and rod-shaped. Biochemical traits such as gelatin hydrolysis, catalase production, citrate utilization, methyl red, nitrate reduction, lipase enzymatic reactions were also detected in the selected isolate. Identification of PD25 was done on

the basis of partial 16S rRNA gene sequencing (~1200 bp) (Fig 1). The gene sequence data showed 99.93% similarity with *P. mirabilis* strain ATCC 29906 through BLASTn and was submitted to NCBI GenBank with the accession number MW405826.

Qualitative and quantitative assays confirmed the PGP attributes of PD25 displaying IAA, and siderophore production (Table 1). The nutrient chelation properties were also checked and it was found that the isolate was able to solubilize P, Zn, and K. Phytohormone IAA directly plays an integral role in regulating various aspects of plant growth and development [27]. PD25 produced significant amount of IAA (27.18 µg ml−1) in the presence of tryptophan as precursor in the medium. Iron is another one of the nutrients essential for many biological functions and siderophores are iron-chelating molecules produced by bacteria to support iron uptake in plants [28, 29]. The siderophore assay revealed that isolate PD25 managed to produce significant amount of siderophore (38.9 psu) and helped the plants in obtaining iron from the soil. [30] reported siderophore production ability in *P. mirabilis* strain isolated from tomato though the isolate was found to be negative for traits such as hydrogen cyanide and lipase. PD25 also played noteworthy role in increasing the bioavailability of soil phosphates by forming a clear halo zone (2.39 psi) around the colonies due to solubilization of P. P is the second most important macronutrient after nitrogen required for metabolism and its deficiency may result into alterations in the membrane lipid composition of plants [31, 32]. Zn is another key constituent of many enzymes and proteins responsible for driving various metabolic reactions in plant growth and development. Carbohydrate, protein, and chlorophyll formation is significantly reduced if these specific enzymes were not present and the stress caused by Zn deficiency may result in several abnormalities of the plants [33]. In the present study, the selected isolate PD25 formed a clear transparent zone around the colonies and exhibited the trait of Zn solubilization. Potassium solubilizing microorganisms offer one of the environmentally sustainable approach to make potassium available for uptake by plants and the same attribute was also detected in the case of isolate PD25. *P. mirabilis* has previously been reported as a plant growth promoter with diverse spectrum of beneficial traits profiting the host plants under various conditions. *P. mirabilis* inhabiting cattle dung and its role in improving K solubilizing efficiency along with advantageous effect on the growth and yield of fennel was also demonstrated by [34]. Isolate PD25 was also able to grow in the presence of NaCl and could tolerate up to 6 % NaCl. Bacterial growth curve under salinity stress was plotted as depicted in Fig 2**.** There are few reports discussing genus *Proteus* for their salt tolerant properties however previous reports have confirmed them as known plant growth promoting rhizobacteria (PGPR) but mostly under hospitable conditions. *P. mirabilis* was studied as one of the significant isolate procured from the rhizosphere of maize and was further screened for their plant growth promoting abilities as reported by [35]. Similarly, PGP activities along with alleviation of Zn toxicity were also seen in the strains of *P. mirabilis* ZK1 isolated from contaminated soil [36].

The plant endosphere contains a diverse group of microbial communities and endophytes inhabit the interior of plants without causing any apparent negative effects [37, 38]. The root infectivity assay of PD25 was performed to know the endophytic nature of the isolate and it was found that the bacterium was able to effectively reinfect and colonize the roots of host plant i.e., wheat. Rhizospheric or endophytic colonization was not evident in the experimental control which rules out probability of cross contamination. [39] characterized bacterial endophytes from seeds of rice (*Oryza sativa*) for different PGP traits and it was found that the seeds were dominated by *Pseudomonas* sp., *Flavobacterium* sp., *Microbacterium* sp. and *Xanthomonas* sp. which promoted growth during early seedling development. Endophytic colonization of plant growth promoting *Pseudomonas fluorescens*

isolated from *Pisum sativum* was also reported by [40] and the endophytes were further demonstrated to stimulate the growth of pea plants under soluble phosphate limiting conditions.

Nitrogen is a crucial macronutrient required for the proper functioning of plants and has been a major constraint for crop productivity [41]. Biological nitrogen fixation is an efficient source of fixed nitrogen in biogeochemical cycles and is carried out by group of microorganisms known as diazotrophs [42, 43]. The *nifH* gene encodes the iron protein subunit of nitrogenase and considered to be the most widely sequenced marker used to classify nitrogen-fixing microorganisms [44, 45, 46]. Amplification of *nifH* fragments yielded the product of expected size (390bp) from DNA template in (Fig 3) and this indicated that the selected isolate have the ability to fix nitrogen. Further ARA also confirmed the presence of nitrogenase activity and production of ethylene was further calculated and expressed as 29.9598 nmole C_2H_4 h^{-1}. *P. mirabilis* has been studied by several workers as bioremediators and PGPR [47, 48, 49]; it is observed that its role in nitrogen fixation has not been elucidated yet. Therefore findings of this study can be considered as novel illustrating the expression of *nifH* gene and production of ethylene by salt tolerant endophyte *P. mirabilis* PD25.

Salt stress affects the growth of plants at both physiological and cellular levels through ionic toxicity and osmotic imbalance, and thereby negatively impacts the agricultural yield [50]. To alleviate the negative effects of soil salinity, pot experiment was conducted and wheat plants were inoculated with PD25. Saline soil with EC of 10.6 dS m^{-1} and a pH of 8.0 was used in pot trials. Further physicochemical properties of the soil were: available nitrogen 0.11 g/kg, available phosphorus 41.3 mg/kg, available K 150 mg/kg, organic carbon content 3.9 g/kg, microbial biomass 100.7 mg/kg, and soil organic matter 5.1 g/kg. All the plant growth parameters were found to be significantly increased by inoculation with endophytic bacterium (PD25) and yield components were investigated as depicted in Table 2. In the present study, significant increase in root length (101.4%), shoot length (56.91%), fresh weight (52.75%), dry weight (105.3%), and spike length (48.54%) were reported for the plants inoculated with PD25 (as compared to untreated control)**.** The augmented plant growth parameters upon inoculation clearly showed the beneficial role of this endobacterium (PD25) under saline conditions. Improvement might be attributed to the exhibition of growth-promoting traits as well as N_2-fixing capacity by selected endophytic isolate i.e., PD25. Previously, [51] procured two bacterial endophytes from *Piper nigrum* and the investigation showed that the isolates i.e., *Klebsiella* sp. and *Enterobacter* sp. considerably stimulated mung bean growth and increased biomass production compared to uninoculated plants. In another study, *P. mirabilis* BUFF14 was isolated and found to improve the vegetative and reproductive parameters of *Foeniculum vulgare* [34].

In the present study, the mutualistic associations of endophytes with its host plant and their role in growth and productivity of wheat was explored. The work reports a novel salt tolerant diazotrophic endophytic isolate of *P. mirabilis* PD25, alongwith other beneficial PGP attributes.

4. Conclusion

The study provides insight into the occurrence and colonization of novel multi-trait nitrogen fixing novel endophyte *P. mirabilis* PD25 from the root tissues of wheat from saline agro-ecosystems of northern India. It can be concluded from the study that inoculation of endophytic isolate PD25 considerably enhanced the growth of wheat simultaneously mitigating deleterious effects of salinity. To fully understand the possible mechanisms by which the bacterial endophyte reduced salinity

stress requires further research. However, endophytic plant growth promoting bacteria can be sustainable solution for improving crop productivity in agro-ecosystems.

5. Declarations

Authors's contributions

NKA conceptualized the idea and approved the manuscript for publication. SV did the experiments and collected the data. All authors analyzed the data and contributed in writing of the manuscript. NKA supervised the whole study.

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Conflicts of interest/Competing interests

All authors declare that they have no conflict of interest.

Ethnic's approval

This article does not contain any studies with human or animal subjects.

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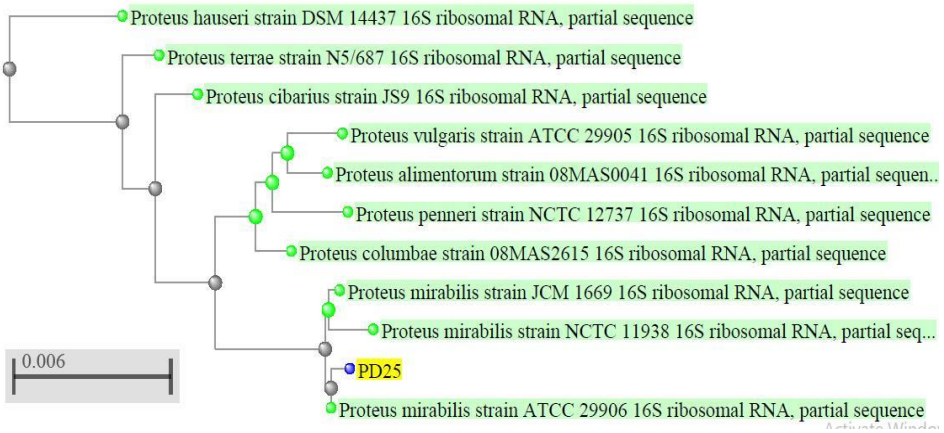

Figure 1. Phylogenetic tree of 16S rRNA gene of endophytic isolate *P. mirabilis* PD25

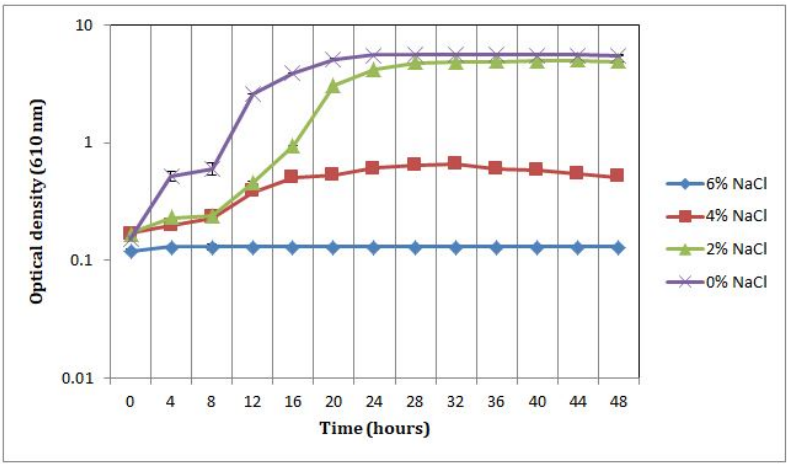

Figure 2. Effect of salt stress conditions on growth of PD25

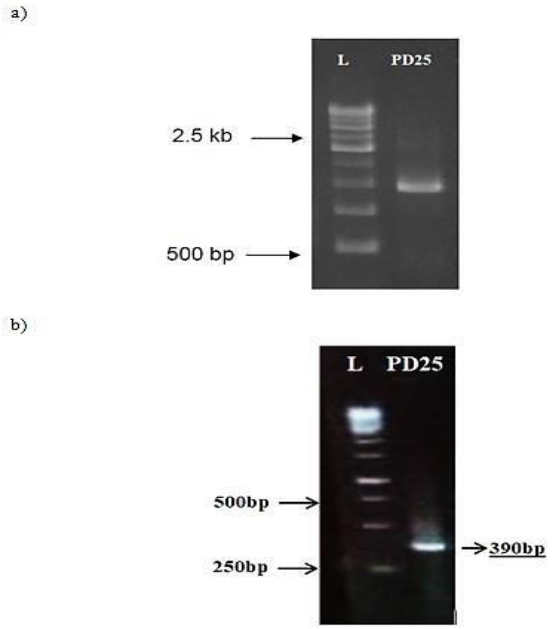

Figure 3. Agarose gel electrophoresis of PCR amplification products a) 16S rRNA, b) *nifH* gene

Table 1. Plant growth promoting attributes of all the endophytic isolates

Isolates	IAA production	Phosphate solubilization	Zinc solubilization	Siderophore production	K solubilization	HCN production	Nitrogen fixation
KB1	+	-	-	-	+	-	-
KB2	-	-	-	-	-	+	-
KB3	-	-	+	-	-	-	-
KB4	-	+	-	+	-	-	-
MP1	-	-	+	-	+	+	-
MP2	+	+	-	-	-	-	-
MP3	-	-	+	-	-	-	-
PD11	+	-	-	-	-	-	-
PD25	**++**	**+**	**+**	**+++**	**+++**	**-**	**+**
PD27	-	-	-	+	+	-	-
PD29	+	-	-	-	-	+	-
PD30	-	-	-	+	-	+	-

+ = positive for test, +++ = more positive for test, - = negative for test

Table 2. Effect of *P. mirabilis* PD25 on growth and productivity of wheat under saline conditions (pot study)

Treatments	Root length (cm)		Shoot length (cm)		Fresh weight (g/plant)		Dry weight (g/plant)		Tiller numbers		Spike length (cm)		No. of grains per spike		Germination (%)	
	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil	Sterilized soil	Non-sterilized soil
Untreated control	4.73± 0.20[a]	3.54± 0.14[a]	17.50± 0.70[a]	15.34± 0.72[a]	14.86± 0.55[a]	11.73± 0.29[a]	1.49± 0.35[a]	0.96± 0.81[a]	1.66±0 .57[a]	1.33± 0.57[a]	4.80± 0.36[a]	2.15± 1.10[a]	25±1.0 [a]	20.33± 2.08[a]	73.33	66.66
PD25	9.53± 0.50[b]	8.95± 0.32[b]	27.46± 0.56[b]	24.92± 0.39[b]	22.70± 0.45[b]	18.55± 0.78[b]	3.06± 0.40[b]	1.99± 0.95[a]	3±1.0 [a]	2.66± 0.57[b]	7.13± 1.15[b]	5.10± 0.99[b]	36.66±1 .52[b]	32.33± 1.15[b]	80	76.66

Data is shown as the mean of two years.

Results expressed as mean ± SD (n = 3 pots).

Values with different letters are significantly different by Student's t-test at ($P \leq 0.05$).