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Endophytic and Rhizospheric Bacteria are Equally Effective in Promoting Seed Germination in Upland Rice

Madhusmita Borah[1], Pompi Das[2], Robin Ch. Boro[3] and Madhumita Barooah[4*]

[1]Senior Research Fellow, [2]PhD Scholar, [3]Assistant Professor, [4]Professor

Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat-13, Assam, India

*Corresponding Author E-mail: m17barooah@yahoo.co.in

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ABSTRACT

This paper reports the effect of growth-promoting bacteria of endophytic and rhizospheric origin on the seed germination process of upland rice variety Ilongkari. Seed germination is the most crucial event in the plant life cycle which determines how the seedling will establish relates to grow and yield. We aimed to determine the effect of Plant Growth Promoting Bacteria (PGPB) isolated from rice plant and the rhizosphere and evaluate their efficiency in stimulating the seed germination process. The experiment was designed in completely randomized design (CRD) with three replicates and carried under laboratory condition in perti dishes with filter paper. Endophytic bacteria Bacillus subtilis RHS 01, Microbacterium testaceum MK LS01 and rhizospheric bacteria Bacillus cereus ABT J11, Bacillus sp. ABT J45 were selected for the study. While the individual treatments with both endophytic bacteria (T1, T2, T3) and rhizospheric bacteria (T4, T5, T6) enhanced the germination process in rice seeds and significantly increased the seedling vigor over that of the control (TW and T0) , it was the combined effect of both (T7) that had significantly greater effect over all the other treatments.

Key words: *Endophytes, Rhizospheric bacteria, Plant growth promotion, Seed germination, Seedling vigor.*

INTRODUCTION

Microorganisms, a major group of biotic factors of an ecosystem are constantly involved in interactions with plants. Plant–bacterial interactions are the determinants of plant health and soil fertility. Plant Growth Promoting Bacteria (PGPB) are bacteria that improve plant growth when introduced onto seeds, seedling, roots, or into soil. They prevail in different strata of an agro-ecosystem such as in the rhizosphere or reside within the plant tissues as endophytes. The PGPB, encompassing bacteria from diverse groups are found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. Equally, important are the endophytic bacteria that reside within the plant tissues and help in promoting plant growth.

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The PGPB either of endophytic or rhizospheric bacteria confer benefits to plant communities through various mechanisms such as secretion of phytohormones, solubilize minerals, fix assimilable nitrogen, produce siderphores enhance nutrient uptake and suppress phytopathogens[23,13,9,21]. Plant growth promoting bacteria when used as inoculants can lead to increase yield of the crops[24]. Treating the seeds with PGPB is an efficient mechanism for not only to improve germination process but to help deliver microorganisms directly to the plant rhizosphere and facilitates their re-entry to the plant if, of endophytic origin[15]. This promotes PGPB proliferation and enhances the colonisation of the seedling roots and help the crop to establish. The use of PGPB for enhancing seed germination process is increasingly being considered as in sustainable agricultural production system[10].

Rice is the staple diet for the entire population of the Northeast India and mostly cultivated in the uplands through direct sowing. Direct seeding of rice is an ancient method of sowing seeds and still practiced for most of the upland rice varieties. During the recent years this method is regaining popularity due to its low-input demand. Unfortunately, direct seed sowing is constrained by low seed germination and poor crop establishment which has limited its potential. It was towards this aim that this research was undertaken to increase the germination process through treatment with selected efficient PGP strains based on their ability to produce phytohormones (IAA and GAs) and solubilise minerals (phosphate, potassium, zinc).

METHOD AND MATERIALS

Isolation of endophytic and rhizospheric bacteria from rice

Paddy varieties Mosuri and Miatong were collected from Jorhat (26°43′03.8″N 94°11′40.2″E) and Tinsukia (27°20'34.5"N 95°42'33.2"E) districts of Assam. The whole plant was uprooted along with soil adhered to its roots. After collection from the rice field, healthy and disease free plant samples were immediately transported to the laboratory in ice boxes.The plant samples were thoroughly cleaned with running water to remove the attached debris. After that, leaves, stems, and roots were separated and cut into thin sections of 2-3 cm long and washed thoroughly with double distilled water. Samples were rinsed in 70% ethanol then sterilized with 0.1% $HgCl_2$ and further washed with sterile distilled water for several times to remove the surface sterilizing agents[22]. One gram of the samples was homogenized in 10 ml of distilled water to prepare a stock solution of tissue homogenate. To prepare the homogenate for rhizosphere sample, 1 gm of the soil adhering to the roots were mixed with 10 ml of distilled water. After appropriate serial dilution, both endophytic and rhizosphic samples were inoculated in Tryptic Soya Agar (TSA) plates and incubated at 30^{o} C for 48 hrs. From these plates, pure colonies were isolated by streak plate method. To confirm that the sterilization process was successful, the aliquot of the sterile distilled water used in the final rinse was platted on TSA medium. The plates were observed for bacterial growth after incubation at 30°C for 3 days.

Twenty morphologically different bacterial colonies were picked from each of endophytic and rhizospheric sample. The isolates were screened *in vitro* for plant growth promoting activities (data not shown) and 2 best performing isolates possessing multiple plant growth promoting traits were selected from each category for in this study.

Characterization of endophytic and rhizospheric bacteria for PGP traits

Phytohormone Production

IAA production

Quantitative estimation of IAA was performed by the method as described by Patten *et* al, 2002. Isolates were cultured overnight in Dworkin and Foster minimal salt (DFMS) media. 20µlof culture aliquots were transferred into 5 ml of DFMS media amended with filter sterilized L-tryptophan (1000µg/ml). After incubation at 30^{o}C for 72 hrs, the culture was centrifuged at 5,500 x g for 10 minutes. For

measuring the amount of IAA produced, 1ml of culture supernatant was mixed with 4 ml of Salkowski reagent (150ml concentrated H_2SO_4, 250ml of distilled H_2O,7.5 ml 0.5 M $FeCl_3.6H_2O$ solution). Development of pink color indicates the production of IAA. After 20 minutes optical density was taken at 535 nm by UV-VIS spectrophotometer (Spectroquent Pharo300, Merck Millipore, India). The concentrationof IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 10–100 µ/ml.

GA Production

Quantitative estimation of GA was determined by the method as described by Vikram *et al*.[5]. Fresh bacterial cultures (1 ml of 0.6 OD) were inoculated intonutrient broth modified withL-tryptophan (1000µg/ml) and incubated at 30^o C for 72 hrs. 5 ml of the supernatant was taken in a test tube to which 0.4 ml of zinc acetate was added. After 2 min of incubation at room temperature, 0.4 ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 min.The supernatant was collected and 3 ml of 30% HCl was added and incubated at 20°C for 75 min. The blank sample was treated with 5% HCl and the absorbance of the sample as well as blank was measured at 254 nm in a spectrophotometer. The amount of GA present in the extract was calculated from the standard curve of GA and expressed as µg/ml of the medium.

Mineral Solublization

Phosphate solubilisation

The isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur 1990.Inoculated plates were incubated at 30^o C for 72 hours forthe formation of clear zones around the colonies. Quantitative analysis of solubilisation of tri-calcium phosphate in liquid medium was done by the method as described by Jackson, 1973. The test isolates were inoculated in 25 ml Pikovskaya's broth and incubated for 2 days at 30^{o}C. The bacterial cultures were centrifuged at 10,000 rpm for 10 min. 1 ml of supernatant was mixed with 10 ml of chloromolibidic acid and the volume was made up to 40 ml with distilled water. 1 ml cholorostannous acid was added and the volume was made up to 50 ml with distilled water. The absorbance of the developing blue color was measured at 600 nm wavelength with UV- VIS spectrophotometer. The amount of soluble phosphorus was detected from the standard curve of KH_2PO_4.

Potassium Solubilization

The isolates were first screened for potassium solubilization on Aleksanderov's medium with 0.2% (w/v) potassium aluminium silicate[12]. Isolates showing positive results in plate were subjected to quantitative estimation by inoculating into 40 ml of Aleksanderov's broth culture media. The flasks were incubated for 5 days at 30°C with continuous shaking at 140 rpm. After incubation broth was centrifuged at 10000 rpm for 10 minutes. 5ml supernatant of each sample was taken in a test tube and 5ml sodium cobaltinitrite solution was added. The reaction mixture was incubated at 37°C for 45 mins to precipitate to settle down in the tube. The supernatant was decanted, precipitated and collected and washed twice with distilled water and once with absolute alcohol. After careful washing, 10 ml of conc HCL was added to precipitate and incubated at 37 C for 15 mins for colour development and absorbance was measured at 623 nm. Different concentrations of KCl solution, ranging from 0-100 µg/ml were used for the preparation of standard curve.

Zinc Solubilization

For detection of zinc solubilisation, freshly cultured bacterial isolates were spot inoculated on tris minimal agar medium modified with Zinc oxide (ZnO) at a concentration of 0.1% and incubated at 30^{O}C. After 72 hours of incubation, clear halo zone formation was observed in the positive isolates capable of solubilizing zinc. The solubilization efficiency (SE) was calculated by the method as described by Ramesh *et* al.[3] as,

$$SE = \frac{\text{diameter of solubilization halo} \times 100}{\text{Diameter of the colony}}$$

The isolates with the highest SE are considered to be effective zinc solubilizers.

Characterization and identification by 16S rRNA gene sequencing

Genomic DNA was extracted from bacteria as per standard phenol–chloroform method. The 1500 bp region of the 16S rRNA gene was amplified from the extracted genomic DNA using the following universal forward primer 5′-AGAGTTTGA TCCTGGCTC -3′ and reverse primer 5′-AAGGAGGTGATCCA GCCG-3′. The amplification was carried out in a reaction with a final volume of 25 µl containing 1 µl (0.5–10 ng) of total DNA, 1µl (20 picoM) of the forward primer, 1 µl (20picoM) of the reverse primer, 2.5 µl (2.5mM of each) dNTP mix, 2.5 µl of 10x PCR buffer, 1µl (1U) of Taq DNA polymerase. A negative control (PCR mix without DNA) was included in all PCR experiments. The PCR reaction conditions were set for 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 1 min and extension at 72ºC for 2 min, before a final extension at 72ºC for 7 min. The PCR products thus obtained were sequenced. The forward and reverse sequences obtained were assembled using the Codon Code Aligner software. Nucleotide sequence identities were determined using the BLAST tool from the National Center for Biotechnology Information (NCBI). Partial sequence data for the 16S rRNA genes were deposited in the Gen Bank nucleotide sequence data libraries and Gene Bank accession number obtained.

Selection of PGPB and inocula preparation for seed germination trial

Efficient plant growth promoting endophytic and rhizospheric bacteria were selected for formulation of the bioinoculum. These include an inoculum of endophytes *Bacillus subtilis* RHS 01 (Accession No. KF957735), *Microbacterium testaceum* MK LS01 (Accession No. KF953537) and rhizospheric bacteria *Bacillus cereus* ABTJ11 (Accession No. KY646308), *Bacillus* sp. ABTJ45 (Accession No. KY646309).

The rice variety *Inglongkiri* was selected for *invitro* seed germination test. Selected bacterial strains were cultured in NB for 24 hours and the exponentially growing cells were used as inocula. Rice seeds were washed with double distilled water and rinsed in 70% ethanol, followed by sterilization with 0.1% $HgCl_2$ and further washed with sterile distilled water for several times to remove the surface sterilizing agents[22]. After that, seed treatments were given by soaking the seeds in inoculum suspension (10^8cfu/ml) of different isolates. Seed soaked in sterile water (TW) was treated as control and sterile Nutrient broth (T0) was taken to test the media effect if any. Seeds were given treatments (Table 2) containing endophytic bacteria, EB (*Microbacterium testaceum* MKLS01and *Bacillus subtillis* RHS01), rhizospheric bacteria, RB (*Bacillus cereus* ABTJ11and *Bacillus* sp. ABT J45) and combination of EB and RB. Twenty five seeds from the treatments (T3, T4, T5, T6 and T7) and controls (TW, T0) were put in sterilized petri dishes containing filter paper (Whatman # 1) and kept in an incubator at 30˚ C The experiment was design in completely randomized (CRD) with three replicates. The number of germinated seeds was evaluated daily after every 24 hours from the second day after the start of the experiment continuing upto seven days (168 hours).Various parameter such as Total Germination percentage, Length of plumule, length of radicle, germination index and Vigor Index were evaluated by following standard methods as described by Elouaer *et al*.[17].

Table 2: Details about bio-innoculum used in rice seed as treatments.

Treatments	Source	Bio-innoculum using Plant growth promoting bacteria
TW	Control	Sterile water
T0	Control	Sterile Nutrient Broth
T1	Endophytic Bacteria(EB)	*Microbacterium testaceum*
T2		*Bacillus Subtilis*
T3		*Microbacterium testaceum*+ *Bacillus subtilis*
T4	Rhizosperic Bacteria (RB)	*Bacillus cereus*
T5		*Bacillus* sp.
T6		*Bacillus cereus* + *Bacillus* sp.
T7	Endophytic Bacteria (EB) + Rhizosperic Bacteria(RB)	*Microbacterium testaceum* + *Bacillus subtilis*+ *Bacillus cereus*+ *Bacillus* sp.

Data Analysis: All the data were subjected for analysing the variance using MS Excel and SPSS 22.0 software and the difference between means were compared by LSD tests ($P<0.05$). The Pearson correlation matrix was carried out to study the correlation between the treatments given in rice seeds and germination percentage.

RESULTS

Plant growth promoting traits of the isolates

Isolates were screened for multiple PGP traits such as phytohormone production (IAA and GA) and mineral solubilization (phosphate, potassium and zinc). Among the isolates tested for phytohormone production, 3 isolates were found to secrete both phytohormones IAA and GA. The isolate ABTJ 45 and ABTJ11 produced highest amount of IAA (38.79 µg/ml) and GA (169.1 µg/ml) respectively when compared to other strains. Maximum phosphate solubilization activity was observed in isolate RHS 01 (81.7µg/ml). MK LS01 showed highest solubilization of potassium (81.28µg/ml) and zinc (125.53 %) (Table1).

Table 1: Plant growth promoting trait of the isolates

Category	Isolate	IAA (µg/ml)	GA (µg/ml)	P (µg/ml)	K (µg/ml)	Zn (%)
Endophytic bacteria	RHS 01	2.88	35.1	81.7	-	111.62
	MK LS01	-	-	-	81.28	125.33
Rhizopheric bacteria	ABTJ11	22.14	169.1	36.38	43.42	-
	ABTJ45	38.79	33.6	13.39	73.42	-

Characterization and identification by 16S rRNA gene sequencing

On the basis of 16S rRNA gene sequencing endophytic isolates were identified as *Bacillus subtilis* (RHS 01) and *Microbacterium testaceum* (MK LS01) and rhizospheric isolates as *Bacillus cereus* (ABTJ11) and *Bacillus* sp. (ABTJ4). Partial sequence data for the 16S rRNA genes were deposited in the Gen Bank nucleotide sequence data libraries and Gene Bank accession numbers have been obtained (Table 2).

Table 2: Microorganisms with their gene-bank accession number

Category	Isolate	Organism match	Accession No.
Endophytic bacteria	RHS 01	*Bacillus subtilis*	KF957735
	MK LS01	*Microbacterium testaceum*	KT380682
Rhizopheric bacteria	ABTJ11	*Bacillus cereus*	KY646308
	ABTJ45	*Bacillus* sp.	KY646309

Germination trial in rice seeds

The treatment T7 containing combination of both endophytic and rhizosphereic bacteria (*Microbacterium testaceum* + *Bacillus subtilis* + *Bacillus cereus* + *Bacillus* sp.) and T6 (*Bacillus cereus* + *Bacillus* sp.) significantly enhanced the germination process in the treated rice seed variety *Ilongkari* in comparison to both the controls. Seeds germinated after 72 hours of treatment. The T6 treatment containing rhizospheric bacteria showed consistently higher total seed germination percentage starting from 72 hrs and recorded a maximum of 94.7 % after 168 hours. However, the T7 treatment which had lower total seed germination percentage when compared to T6 showed 92.0 % total seed germination at the end of the 168 hrs which was statistically at par ($p\leq0.05$) with that of the T6 treatment. Thus, both the treatments were equally effective in promoting seed germination

The gradual increase in the length of plumule and radicle in all the respective treatments and control was observed after 72 hours. The increase in the length of plumule

was recorded after every 24 hours and at the end of 168 hours, the T7 treatment had the highest (48.33±0.57 mm) followed by T6 (39.33±1.52 mm). The T2 showed the least (33.66±1.52) effect. The length of plumule was significantly higher in the T7 over all the other treatments ($p \leq 0.05$). There was no marked difference among the other treatments. All the treatments significantly enhanced the length of radicle over both the controls (TW and T0) at the end of 168 hours. The treatments did not show any statistically significant difference between themselves.

Vigor index was calculated to be the highest (828.00) in seeds treated with both the endophyitic and rhizosphereic bacteria (T7) over all the other treatments. Graphical representation of Germination index and Vigour Index is shown in Fig. 1 (d) and (e).

The Pearson correlation analysis revealed a positive correlation between the treatments and germination performance (Table 4).

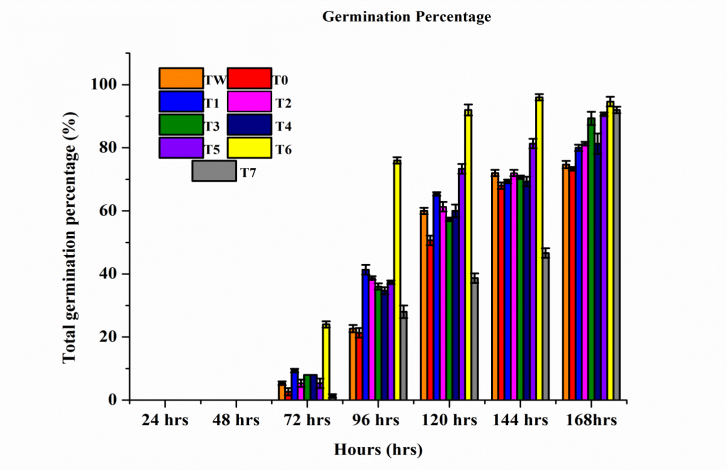
Table 3: Mean comparison of the treatments at the end of 168 hours

Treatment	Germination %	Length of the Plumule	Length of the Radicle
Tw	74.67 a	2.57 a	3.53 a
T0	73.33 a	2.43 a	3.33 a
T1	80.00 ab	3.57 b	3.67 a
T2	81.33 ab	3.37 b	4.20 b
T3	89.33 bd	3.73 bc	4.13 b
T4	81.33 ab	3.67 b	3.80 a
T5	90.67 bc	3.77 b	4.20 b
T6	94.67 bd	3.93 c	4.13 b
T7	92.00 cd	4.83 d	4.17 b

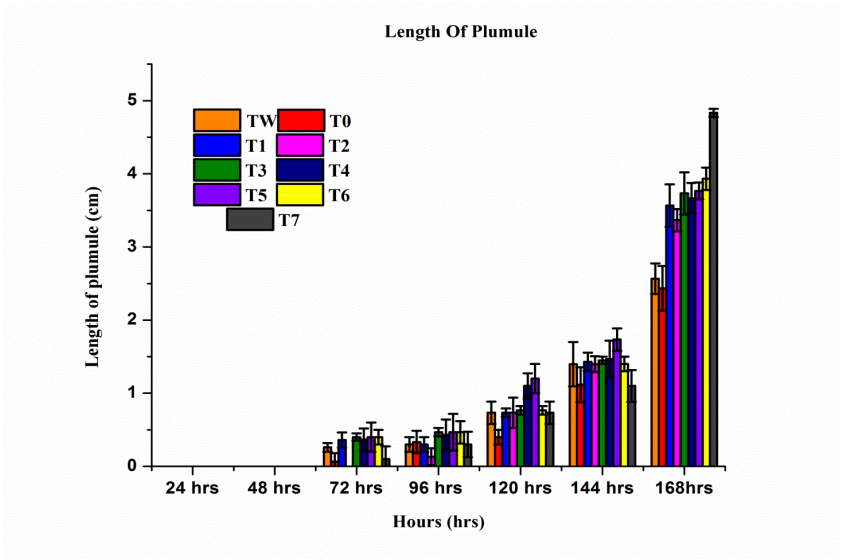
*Means with the same letters in each column are not significantly different at 0.05 according to LSD test

Table 4: Correlation matrix of different treatments in rice seeds

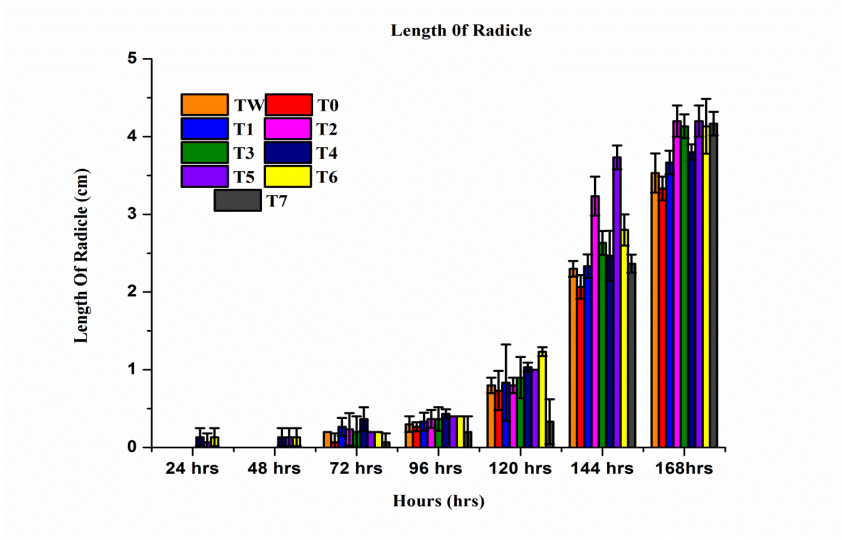
	Tw	T0	T1	T2	T3	T4	T5	T6	T7
Tw	1								
T0	0.99	1							
T1	0.97	0.97	1						
T2	0.98	0.98	0.99	1					
T3	0.97	0.98	0.98	0.99	1				
T4	0.98	0.98	0.99	0.99	0.99	1			
T5	0.99	0.98	0.99	0.99	0.98	0.99	1		
T6	0.91	0.89	0.96	0.95	0.92	0.94	0.94	1	
T7	0.88	0.91	0.90	0.91	0.95	0.92	0.90	0.81	1



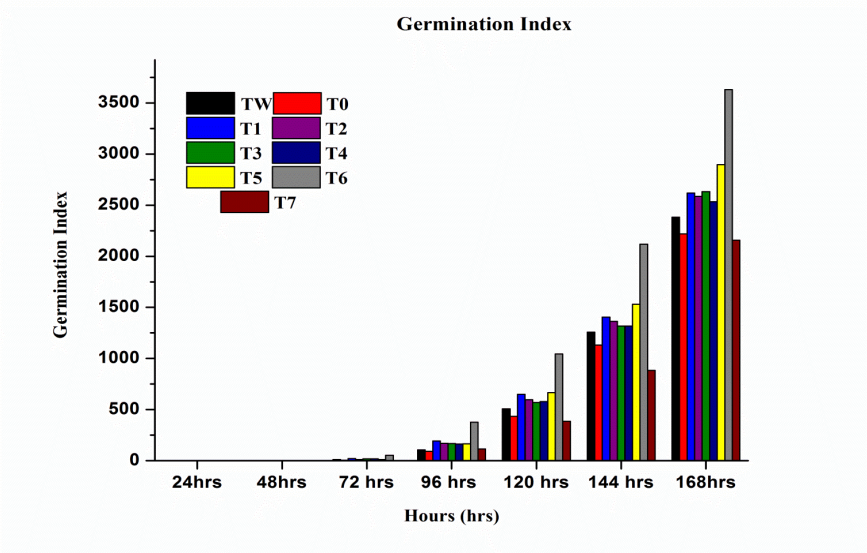
(a)


Length Of Plumule
Length of plumule (cm)
TW
T0
T1
T2
T3
T4
T5
T6
T7
0
1
2
3
4
5
24 hrs
48 hrs
72 hrs
96 hrs
120 hrs
144 hrs
168hrs
Hours (hrs)

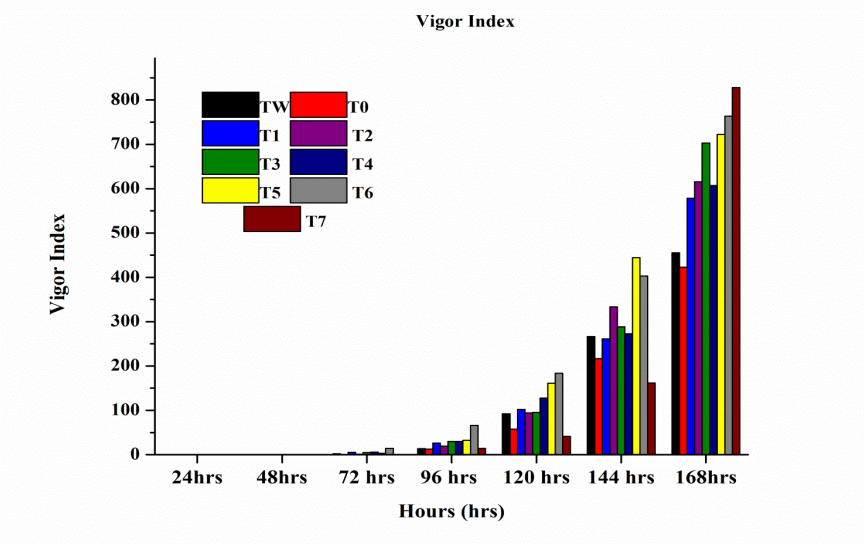
(b)


Length 0f Radicle
Length Of Radicle (cm)
TW
T0
T1
T2
T3
T4
T5
T6
T7
0
1
2
3
4
5
24 hrs
48 hrs
72 hrs
96 hrs
120 hrs
144 hrs
168hrs
Hours (hrs)

(c)


Germination Index
Germination Index
TW
T0
T1
T2
T3
T4
T5
T6
T7
0
500
1000
1500
2000
2500
3000
3500
24hrs
48hrs
72 hrs
96 hrs
120 hrs
144 hrs
168hrs
Hours (hrs)

(d)



(e)

Fig: Effect of bio-innoculum using Plant growth promoting bacteria on (a) Germination percentage (b) Length of plumule (c) Length of radicle (d) Germination Index and (e) Vigor Index of rice seeds in 168 hrs

DISCUSSION

The present study revealed that seed treatment of upland rice variety *Inglongkiri* with selected endophytes and rhizospheric bacteria with growth promoting property, separately or in combination improved seed germination and increased vigor index. Both the endophytic and rhizopheric bacteria applied alone or in combination, performed equally well over untreated control, without any significant difference between their performances. However, the combined effect of both endophytes and rhizospheric bacteria (T7) significantly increased the germination index over all other treatments. Seed germination is a critical step in committing the plant to initiating it's life cycle starting with imbibitions followed by increased metabolic activity finally, leading to initiation of growth[18]. Higher germination percentage and vigor index are indicators of improved seedling establishment. Three of the selected isolates *viz*., *Bacillus subtilis* RHS 01of endophytic origin and *Bacillus cereus* ABTJ11and *Bacillus* sp. ABTJ45 both of rhizospheric origin had phytohormone biosynthesis capacity which might have stimulated the seed germination[7]. Gibberellin (GA) is known to enhance the action of α-amylase and other germination specific enzymes like protease and nuclease involved in hydrolysis and assimilation of the starch[1]. The endophyte *Bacillus subtilis* RHS 01 (35.1 µg/ml) and rhizobacteria *Bacillus cereus* ABTJ11 (169.1 µg/ml) produced high amount of GAs which might have helped in the germination process. Increase in the length of radicle and plumule may have been stimulated by other growth promoting attributes of the isolates. The secretion of IAA by the isolates, *Bacillus subtilis* RHS 01, *Bacillus cereus* ABTJ11 and *Bacillus* sp. ABTJ45might have aided in improving root development while GA in promoting shoot growth. Seed vigor index is one of the most essential component for influencing seedling establishment and crop improvement[16]. Our result demonstrated that the combination of endophytic and rhizopheric bacteria (T7) enhanced the seed vigor index when compared to other treatments which may be due to P, K and Zn solubilising activity of the bacterial isolates under study. Phosphorus and potassium are important macronutrients required during the seed germination process. Phosphorus is the second most important nutrient for plants, after nitrogen. It stimulates root development and increased stalk and stem strength. Potassium plays greater role in root development as it helps maintaining cell turgor, enhances photosynthesis, reduces respiration, helps in transport of sugars and starches, helps in

nitrogen uptake and is essential for protein synthesis. Potassium activates at least 60 different enzymes involved in plant growth[26]. It also increases disease resistance and helps the plant better to withstand stress. *Microbacterium testaceum* MK LS01, *Bacillus cereus* ABTJ11 and *Bacillus* sp. ABTJ45used in this study were able to solubilise potassium. Zinc is another important element which plays a crucial role during seed germination and early seedling growth[14]. *Bacillus subtilis* RHS 01 and *Microbacterium testaceum* MK LS01 had good zinc solubilising activity that might have aided the process of seed germination. The efficiency of endophytes and rhizobacteria in germination of rice seeds has been reported by Etesami *et* al.[11] and Pradhan and Mishra[2]. The co-inoculants (*Microbacterium testaceum + Bacillus subtilis+ Bacillus cereus+ Bacillus* sp) increased the seed germination index (3158.33), vigor index (828.00), radicle (17%) and plumule (92%) lengths of germinated seeds of Illongkari rice variety over the non-inoculated. Our result corroborate the earlier findings of Rocheli *et al.*[24] in which co-inoculants with growth promoting microbes *Nitrosomonas europaea*, *Rhodopseudomonas palustris* and *Acinetobacter sp*. significantly increased percent of radicle and plumule length on two lowland rice varieties (Mahsuri and Sri Malaysia 1) and two upland rice landraces (Panderas and SK-1). Similar increase in germination rate of seeds and improve seedling emergence through inoculation of both endophytic and rhizobacteria have been observed by several researchers in other crops like maize[4], sorghum[19] and pearl millet[25].

CONCLUSION

The present study reports significant increase in seed germination, radicle and plumule length the vigour index in the upland rice variety, Illongkari when co-inoculated with both endophytic and rhizospeheric bacteria (*Microbacterium testaceum + Bacillus subtilis+ Bacillus cereus+ Bacillus* sp.) over all the other treatments applied. Among the inoculants, isolates belonging to the genus Bacillus were more effective than *Microbacterium testaceum* in enhancing seed germination parameters. The effectiveness of our isolates indicate that they can be ideal candidates to develop bioformulation to improve the germination process in direct seeded upland rice varieties which will reduce the dependence on commercial fertilizers.

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