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Study of characterization of tamarind associated *Rhizobium* spp. and phosphate solubilizing bacteria and their potency for germination of tamarind seeds

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Abstract
The present study was undertaken to isolate and characterize *Rhizobium* and phosphate solubilizing bacteria from rhizosphere soils of tamarind and determination of their potency for germination of Tamarind seeds in nursery. Pure cultures of *Rhizobium* and PSB strains were isolated on PCA (Plate Count Agar) of Yeast Extract Mannitol (YEMA) and Pikovaskya media respectively. Tamarind-*Rhizobium* and PSB isolates showed positive reaction for starch hydrolysis, catalase test and are Gram – ve bacteria. TSI test showed glucose, lactose and sucrose fermentation occurred with tamarind-*Rhizobium* whereas in PSB only glucose fermentation was there. Their assessment on potency for germination performance of tamarind seeds in nursery was carried out in glass house condition with six treatments consisting of T_1 - Control (Seed soaking), T_2 - Seed soaking +*Rhizobium* + 25% N + 50%P, T_3 - Seed soaking + PSB + 50%N+ 25% P, T_4 - Seed soaking + *Rhizobium* + PSB+ 25% NP, T_5 - Seed soaking + 50% NP and T_6 - Mechanical scarification + 50% NP. These treatments were replicated four times in tamarind (*Tamarindus indica*) to study the influence of microbial inoculants on seed germination. The combined inoculation of *Rhizobium* and PSB along with 25% NP showed significant increase in germination percentage over control (Uninoculated seeds). Further decrease in number of days taken (8.25 days) for initiation of germination (14.5 days in Uninoculated seeds) was noticed in seeds subjected bioinoculants. It was concluded that the treatment *Rhizobium* + PSB + 25% NP was most effective and may be adopted to improve germination of tamarind in nursery condition.

Keywords: Tamarind, *Rhizobium*, PSB, characterization and germination percentage

Introduction
Tamarind (*Tamarindus indica* L.) is a very popular indigenous leguminous fruit crop which is commonly known as *Imli* in India. It is evergreen multipurpose tropical fruit tree primarily used as fruits but also a valuable timber species. Tamarind belongs to the dicotyledonous, family Fabaceae, and subfamily Caesalpiniaceae. In India, the tree is found abundantly in Chhattisgarh, Madhya Pradesh, Telangana, parts of Maharashtra, Tamil Nadu, Orissa, Bihar and Bengal. Tamarind is an important cash crop of India and includes 6th position in terms of export earnings (Muzaffar and Kumar, 2017) [21]. In India, it is cultivated in 59.6 thousand ha area with the production of 206.3 thousand MT and productivity of 3.5 MT/ha (Anonymous, 2011) [3].

Tamarind pulp can be processed into number of products including tamarind juice, concentrate, powder, pickles and paste (Caluwe, De *et.al*. 2010) [12], (T, Jyothirmayi *et.al.* 2006) [15]. Tamarind pulp also claims some medical uses and is regarded as a digestive, carminative, laxative, expectorant and blood tonic (Lee, PL. *et.al*.1975). The pulp of the fruit contains tartaric acid, reducing sugars, pectin, protein, fiber and cellulosic materials. Fruit pulp is a good source of minerals especially potassium, calcium, phosphorous magnesium and sodium (Almeida, M.M.B. *et.al*. 2009) [1]. Tamarind is traditionally propagated from seed; produces relatively large seeds that average about 11-12.5 mm in diameter with a hard impermeable seed coat. On an average, tamarind seeds begin to germinate about 13 days after sowing, but may take a month to complete. The main disadvantage of seed propagation is freshly harvested seeds of tamarind exhibit poor germination percentage even if exposed to favorable conditions of germination owing to seed dormancy. It may be due to morphological factor such as hard, thick testa or due to incorrect storage or handling (secondary dormancy). Such seeds may require special treatments like scarification, soaking in water, growth regulators, biofertilizers etc., for overcoming dormancy. Pre-sowing seed treatment with bio fertilizers may improve the seed germination and seedling growth through producing several

Growth regulators substances like Indole Acetic Acid (IAA), Gibberlic Acid (GA) and vitamins besides fixation of atmospheric nitrogen. Biofertilizers are the carrier based formulations of beneficial microorganisms are more effective and inexpensive source of plant nutrients. *Rhizobium* is the most well-known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. Phosphate solubilizing microorganisms (PSM) are capable of bringing insoluble P into soluble form and they provide an effective alternate to the P availability in the rhizosphere of the crop plants. The use of biofertilizers should receive the considerable attention for improving germination. Therefore the present study was carried out with the objective of characterization of Tamarind associated *Rhizobium* and PSB and their inoculation effects on germination of Tamarind seeds.

Materials and Method

The present investigation was conducted in glass house of Department of Agricultural Microbiology, College of Agriculture, Raipur, Chhattisgarh during 2017-18. The experiment was laid out in complete randomized design with 6 treatments, replicated 4 times taking Tamarind (*Tamarindus indica*). Treatments were T1 = Control (Seed soaking), T2= Seed soaking +*Rhizobium* inoculation + 25% N + 50%P, T3 = Seed soaking + PSB inoculation + 50%N+ 25% P, T4 = Seed soaking + *Rhizobium* + PSB+ 25% NP, T5 = Seed soaking + 50% NP and T_6 = Mechanical scarification + 50% NP.

Isolation of Rhizobium and P-solubilizers, preparation of inoculums

The soil samples at a depth of 15cm from rhizosphere soil of tamarind, naturally grown in the nearby Agriculture College campus of IGKV, Raipur, Chhattisgarh were collected for the purpose of isolation of *Rhizobium* and PSB by following serial dilution and spread plate method. For isolation of *Rhizobium*, 0.1ml of each 10^{-3} and 10^{-4} dilution was spread on surface of YEMA media plate (Yeast Extract Mannitol Agar Media) as described by Vincent 1970 and incubated the plate at 28 ^{0}C for 2-3 days. Loopful of growth of bacterial colony was again restreaked in YEMA plate until a pure culture was obtained. A single discrete colony was transferred to YEMA slant to maintain the isolate.

For isolation of PSB, 0.1ml of 10^{-4} dilution was spread on Pikovskaya's agar medium (PVK) plate and incubated the plate at 27 – 30 ^{0}C for 2-3 days. Plates showing some clearing zones around the growth of bacterial colony were selected, the isolated PSB culture was purified by repeated culturing. The pure isolates capable to form clearing zone, were maintained by streaking on Pikovskaya's agar slants. To prepare the culture suspension for experimentation, the isolate was inoculated in sterilized concerned broth in conical flask incubated at 28 ± 20 ^{0}C for 48 hours and then were kept on a rotary shaker for 7 days. This culture was then used for seed inoculation treatment.

Seed treatment method, Seed sowing and after care:

A well mixed 4 kg mixture of soil, sand and compost FYM in 3:1:1 ratio was filled in each polythene bag (12"x10"). Seeds of Tamarind were collected from naturally grown area of village Dumali (Block: kanker, Distt: Kanker of Chhattishgarh) and were allowed to germinate by pretreating seeds by soaking in distilled water for 24 hour except T_6 where mechanical scarification was done. Uniform size seeds were selected for seed inoculation treatment.

Soaked Seeds of Tamarind were inoculated with *Rhizobium* and PSB as per treatment description before sowing in polybags, so that a single healthy seedling was maintained in each polybag. Biofertilizers were also given as soil application @ 10 g/pot as per treatment description. For inorganic fertilization, Nitrogen and Phosphorus were applied to polybags (@ 200:150g N: P_2O_5 per plant per year) in liquid form in water as per treatment at 8 days after sowing of inoculated seeds. Uniform irrigation to all pots was given as and when required. and the seedlings were allowed to grow up to 4 months.

Characterization of isolates

Tamarind associated *Rhizobium* and PSB isolates were characterized by Gram staining, colony morphological characters, Starch Hydrolysis Test, Catalase test and Triple Sugar Iron Agar test (TSI test).

The Gram's reaction was observed and bacteria were classified as gram positive and / or gram negative. Purple for Gram positive and pink or red for Gram negative cells (Aneja, 2003) [2]. The colony characters *viz,* margin, elevation, size and colour were observed on agar medium and recorded. 1 ml of appropriate dilution of Tamarind-*Rhizobium* was transferred into the petri plates containing YEMA with congored medium and PSB on Pikovaskya medium. The plates were incubated at room temperature for 2-3 days. The phenotype and growth pattern were observed.

Starch hydrolysis test is used to detect the enzyme amylase. Isolates were single streaked on starch agar media and incubated at 28±2 °C for 48 hrs. After required incubation, few drops of Iodine solution was added. A yellow zone around the colony indicated starch hydrolysis, while a blue/black area around the indicated presence of starch. For catalase test, small amount of bacterial colony was transferred to surface of clean, dry glass slide using a loop or sterile wooden stick. A drop of 3% H_2O_2 was put on to the slide and was mixed, rapid evolution of oxygen (within 5- 10 sec) as evidenced by bubbling indicated the positive result and no bubbles or only a few scattered bubbles signifies negative result (Schmidt &Caldwell, 1967) [23]. Triple Sugar Iron agar media is used for the differentiation of microbes by ability to determine carbohydrate fermentation and hydrogen sulfide production. Triple sugar iron agar medium was prepared by dissolving 16.13 g TSI medium in 1000 ml of distilled water and slants were prepared to set in sloped form with a butt about 1 inch long slants were streaked, incubated at 28±2ºC for48 hrs and results were observed.

Germination studies

The data on **Seed** germination studies were recorded from the four labeled seedlings in each treatment were averaged. Germination of tamarind seeds was observed and days to start germination and germination percentage were recorded.

The germination in each treatment was recorded at 30 days after sowing. Number of seedlings were counted and expressed as germination percentage.

$$\text{Germination (\%)} = \frac{\text{Total number of seed germinated}}{\text{Total no. of seeds sown}} \text{x} 100$$

The data were statistically through F-test at 5% level of significance. The standard error of means (SEm±) and CD were calculated where F-test was significant for comparing treatment means (Panse and Shukhatme, 1978) [22].

Results and disscussion

The study comprised of isolation of Tamarind *Rhizobium* and PSB from rhizosphere soils of Tamarind. The results of the studies pertaining characterization of these isolates and the effect of these microbial inoculants on germination of tamarind seedlings were discussed.

Isolation and characterization of Tamarind – *Rhizobium* and PSB

A survey was conducted in the months July- August of the year 2017 in Raipur district of Chhattisgarh for collection of rhizosphere soil samples of tamarind with the purpose of isolation of native *Rhizobium* and PSB isolates of tamarind. The collection of soil inoculation from native location for isolation was felt necessary to achieve the objectives of this investigation. This view is in line with several researchers who mentioned that even though, we have national strains identified as efficient strains, there is still scope for improving the efficiency or for identifying a location specific better ones (Bergersen, 1970; and Halliday, 1984) [5, 14].

Table 1: Colony characterization & Gram staining of isolated *Rhizobium* and PSB isolates from tamarind tree root regions

Isolates	Morphological Test	
	Colony Morphology	Gram's reaction & cell Shape
Tamarind-*Rhizobium*	On YEMA, produced Medium Round, White translucent, Circular with Entire margin, Convex (raised) and Smooth Surface, Slime Producing	Gram –ve bacteria Short rod
PSB	On Pikovskaya′s agar media it produced smooth, round with entire margin and creamy to yellowish. Showing moderate clear zone surrounding the colony growth	Gram –ve bacteria rod shaped

Colony morphology & Gram staining of Tamarind – *Rhizobium* and PSB isolates

Rhizobium isolate required for inoculation purpose was isolated from Tamarind root region. It was tested for cultural and biochemical reaction. *Rhizobium* isolate from tree legume, Tamarind produced translucent, nearly round and gummy colonies on YEMA medium. Results showed the isolate was gram negative and on YEMA media produced colonies found convex (raised) and Smooth Surface and Slime Producing.

PSB isolate required for the study was isolated from rhizospheric soils of tamarind tree. The selection of PSB was made after screening the bacterial colonies developed on Pikovskaya′s agar plates for their morphological similarities to *Bacillus* and *Pseudomonas* genera, as it was reported by Komy (2005) [17] and also other researchers that Strains from the genera *Pseudomonas* and *Bacillus* are among the most powerful phosphate solubilizers. This work is in line with Krishnaveni M.S. (2010) [18]. Isolated PSB capable of forming a clear zone on solid Pikovskaya′smedia. On Pikovskaya′s agar media it produced smooth, round with entire margin and creamy to yellowish. Showing moderate clear zone surrounding the colony growth After the gram- staining the bacteria assumed a red colour which indicated that it was a Gram -ve stain. Gram staining of the isolate was done to provide information as presumptive tests of the isolates. (Table 1)

The *Rhizobium* and PSB isolated from rhizosphere soils of tamarind are characterized based on biochemical tests. It was observed that both the isolate Tamarind *Rhizobium* and PSB were positive with respect to Amylase and catalase test. As per triple sugar iron test showed fermentation of glucose, lactose and sucrose occurs in tamarind *Rhizobium* whereas PSB isolate showed only glucose fermentation. Also gas production and H_2S evolution was observed in tamarind *Rhizobium* only (Table 2). Also the starch hydrolysis test and TSI agar test of tamarind associated *Rhizobium* and PSB isolates were depicted in Figure 1 and 2 respectively. Based on the biochemical and morphological tests, PSB isolate was found to be belonged to *Pseudomonas sp.* The isolated bacteria were identified in consultation with Bergey"s Manual of Systematic Bacteriology Pseudomonas sp. act as an efficient solubilizers is in line with the findings of Komy (2005) [17]. The isolation and identification studies are also in coordination with the works done by Kelel M. (2014) [16] studied that PSB colonies isolated from the Acacia plant rhizosphere soil demonstrated high phosphate liberating activity and survived at temperatures to 47oC over a pH range of 4.5 to 8.5. These results provide a strong baseline source for application in agriculture when a biofertilizers required. Also the characterization of tree legume rhizobium was carried out by Surange *et al.* (1997) [25].

The authentication of the isolates was performed using sub culturing method. For preparation of liquid culture, loopful of pure isolate of Tamarind - *Rhizobium* and PSB were transferred to sterilized YEMA and Pikovskaya′s broth respectively as inoculants of seed as per experimental study.

Table 2: Biochemical characterization of isolated *Rhizobium* and PSB isolates from tamarind tree root Regions

Isolates	Biochemical Test						
	Starch hydrolysis	Catalase test	Triple Sugar Iron (TSI) test				
			Glucose fermentation	Lactose fermentation	Sucrose fermentation	Gas production	H2S production
Tamarind *Rhizobium*	+	+	+	+	+	+	+
PSB	+	+	+	-	-	-	-

Inoculation effects alone and along with different fertilizer doses on germination characteristics of tamarind seeds

Results on germination characteristics of tamarind seeds treated with different treatments are presented in Table 3.

The data pertaining to days taken to initiate germination were recorded and statistically analyzed.

Data revealed that the effect of biofertilizer had shown significant effect on germination of tamarind seed. Germination initiated earlier in inoculated ones followed followed by mechanical scarification treatment. The minimum days of 8.25 were taken to start germination of tamarind seed under T4 (at Seed soaking + *Rhizobium* + PSB+25% NP) which showed superiority over rest of the treatments and maximum days of 14.50 was taken to start

germination under uninoculated control treatment T1 i.e. seed soaked in water only.

Results depicted in Table 3 clearly show that influence of different Inoculation and mechanical scarification of seeds with inorganic fertilizer had significant effect over only inorganic fertilizer application and control on days taken regarding initiation of germination of tamarind seeds. Significantly minimum days taken for start of germination was seen under treatment T4 i.e. 8.25 which was statistically at par with T3 (9.25) and T2 (8.75) followed by mechanical scarification treatment (T6). The seeds subjected to mechanical scarification were recorded earliest germination (10.50 days) as compared to T1and T5. All the treatments showed superiority over control. The increase in germination due to involvement of growth regulator secretion due to microbial inoculation along with increase in cell wall plasticity and better water absorption.

These findings are supported by Dhankhar and Singh (2008) [13] and Chiranjeevi *et al*. (2018) [8, 9] which are close to the conformity of the findings.

Germination percentage

Data (Table 3) revealed that effect of biofertilizer had significant effect in germination at 30 days after sowing. The maximum value of 100 per cent germination was recorded when seed soaked in distilled water along with *Rhizobium* and PSB inoculation alone and both, as treatments T2,T3 and T4 while minimum value of 81.25 per cent germination was recorded under treatment T1 (only Seed soaking in distilled water). Further significant effect in germination percentage 93.5% was recorded under mechanical scarification, T6 over T5 and T1. Whereas, the lowest germination was noticed in control (81.25%). The seed germination in tamarind is erratic due to the possession of various degrees of physical dormancy caused due to hard seed coat, which is impermeable to water and oxygen (Bewley and Black, 1982) [6]. The mechanical scarification treatment removed the seed coat there by increased the permeability of air and water through seed which favors the earliest germination. The exogenous application of biofertilizer antagonizes the ill effect of inhibitors and the higher seed germination percentage was due to secretion of the proteolytic enzymes like amylase. Amylases in turn hydrolyses starch in the endosperm, providing the essential sugars for the initiation of growth processes (Copeland and Mc Donald, 1995) [11]. The highest seed germination was seen due to microbial inoculation, it might be due to secretion of beneficial hormones which would have triggered the activity of specific enzymes that promoted early germination, such as α- amylase, which have brought an increase in availability of starch assimilation. (Chiranjeevi *et al*. (2018) [8, 9].

The applied inoculants might have attributed for creating favourable condition through secretions of vitamins and growth promoting substances. The treatment with bioinoculants on soaked seeds proved to be good treatment. The results are in line with those obtained by Aseri and Rao (2004) [4] in aonla seedlings and Thoke *et al.*, (2011) [26] in Jamun seedlings.

Table 3: Effect of inoculation on germination attributes in Tamarind

Treatments	Treatment details	Initiation of germination (Days)	Germination percentage (%)
T1	Control (Seed soaking*)	14.50	81.25
T2	Seed soaking +*Rhizobium* + 25% N + 50%P	8.75	100
T3	Seed soaking + PSB + 50%N+ 25%P	9.25	100
T4	Seed soaking + *Rhizobium* + PSB+ 25% NP	8.25	100
T5	Seed soaking + 50% NP	12.25	87.5
T6	Mechanical scarification + 50% NP	10.50	93.5
SEm(±)		0.454	1.273
CD (5%)		1.362	

Conclusion

Co-inoculation of tamarind seeds with *Rhizobium* + PSB along with application of less NP dose (25%) was significantly influenced the germination of tamarind. The present study has clearly shown that germination of tamarind seeds could be enhanced effectively by inoculating with the combined microbial inoculation which may result healthy tamarind seedlings for planting in the main field.

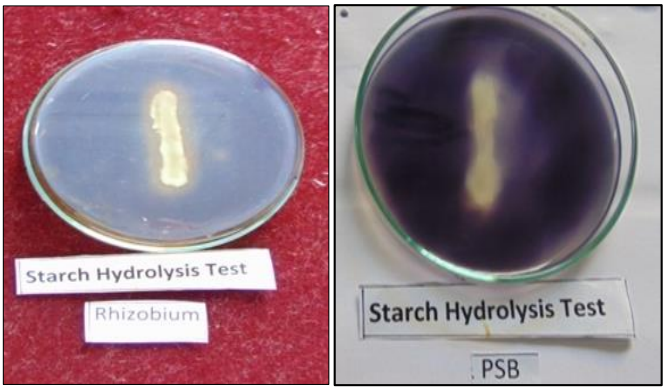
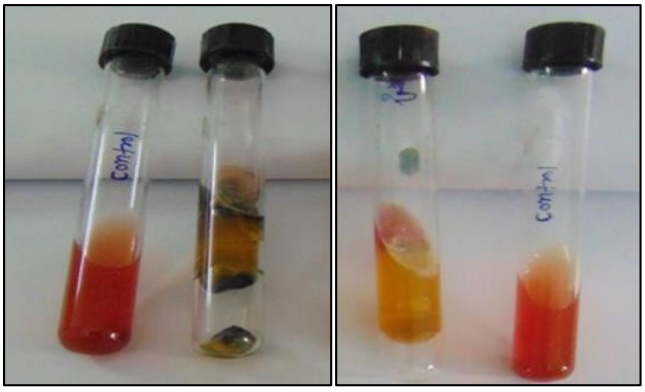


Fig 1: Starch Hydrolysis test



Rhizobium PSB

Fig 2: Triple sugar iron agar test

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