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Preparation of Novel Rhizoazophos Biofertilizer Consortium and Evaluation of Their Shelf Life in Rice Bran, Coir Pith and Sugarcane Trash

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ABSTRACT

Nitrogen fixing and phosphate solubilizing microbes were isolated from legume nodules and rhizosphere region of legumes respectively. The distribution of phosphate-solubilizing microbes was more in the rhizosphere region. The solubilization efficiency of the microorganisms ranged from 46.67 to 75.00 % on the Pikovaskaya medium. The Symbiotic effectiveness of isolated Rhizobialisolates no 2 was 162.50 %. A novel Rhizoazophos consortium was prepared using the microbial inoculants isolated from Virudhunagar. The application of Rhizoazophos biofertilizer prepared with rice bran significantly increased the shoot and root dry matter, survived better and increased the number of nodules and dry matter production

Key words: *Rhizoazophos, Biofertilizer, Consortium, Microbial inoculant, Rice bran*

Introduction

In developing countries, the use of fertilizers to increase crop production is highly essential. Biofertilizers, more commonly known as microbial inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility, crop productivity and sustainability. *Azolla, Azospirillum, Azotobacter* and *Rhizobium* have established biofertilizers and are referred to as inoculants (Verma and Bhattacharya, 1994). The beneficial effects of legumes in improving soil fertility were known since ancient times and their role in biological nitrogen fixation was discovered more than a century ago. Biologically fixed nitrogen is a source that can supply an adequate amount of nitrogen to plants. Biologically fixed nitrogen consumes about 25 to 30% less energy than is normally used in chemical processes. Phosphate Solubilizing Microorganisms (PSMs) have the potential to increase available P for plants, especially in soils with large amounts of precipitated phosphate. Phosphate solubilizing bacteria (PSB) release bound phosphate by secreting a number of organic acids. PSMs convert these insoluble phosphates into soluble forms through the process of acidification, chelation, exchange reactions and production of gluconic acid (Chung *et al*., 2005). Biofertilizers are activating the soil biologically and add nutrients (Nitrogen) to the soil / making them (Phosphorus) available to the crop. Microorganisms convert complex organic material into simple compounds so that plants are easily taken up and secrete certain growth-promoting substances. Under certain conditions, biofertilizers exhibit anti-fungal activities and thereby protect plants from pathogenic fungi. Biofertilizers are easy to produce in abundance and are available at low cost to marginal farmers. Application of

biofertilizers increases yields up to 45 percent and the leftover biofertilizers in the soil increase yield as long as the biofertilizer remains in the soil for up to 3 to 4 years. Symbiotic nitrogen-fixing *Rhizobium* adds 50 to 150 kg of nitrogen to the soil per hectare. *Azospirillum* is a plant growth-promoting rhizobacteria and has beneficial effects on plant growth and crop yields. *Azotobacter* and *Azospirillum* secrete antibiotics that act as biopesticides. It is a non-hazardous way of increasing soil fertility. Based on the importance of biofertilizers the objectives of the present study is to isolate effective strain of nitrogen-fixing *Rhizobium* sp., *Azospirillum* sp. and PSB, to prepare Rhizoazophos biofertilizer (Rhio – *Rhizobium* biofertilizer, azo –*Azospirillum* sp. and Phos – phosphate solubilizers), and to evaluate the viability of the consortium in rice bran, coir pith, and sugarcane trash as carrier materials and to check the effect of rhizoazophos biofertilizer consortium on the growth of cowpea plant.

Materials and Methods

Isolation of phosphate solubilizing microorganisms

Phosphate solubilizing bacteria were isolated from the rhizosphere soil of leguminous plants from V.V. Vanniaperumal College for Women, Virudhunagar, Tamil Nadu. For the isolation of rhizosphere bacteria, the adhering soil on the root was gently shaken to collect the rhizosphere soil (Johri *et al*., 1999). A serial dilution of the rhizosphere soil sample was individually plated on Nutrient Agar (NA), and Pikovskaya Agar Medium (PVK) as described by Gaur (1990). The solubilization activity, solubilization zone (cm) produced by the organisms, the culture diameter (cm) and Solubilization efficiency (E) (%) of the microorganisms were observed on PVK solid medium after 7 days of incubation. Screening of microorganisms for phosphate solubilization in a PVK broth assay after 15 days of incubation, maximum P solubilized as P_2O_5 (mg), solubilization of total P in the medium (%) and Final pH of the medium was also observed.

Isolation of *Azospirillum* sp.

Azospirillum sp. isolated from the rhizosphere soil samples. 25 ml test tubes with 5 ml of NFb semisolid medium were inoculated with one gram rhizosphere soil inoculated with malate semisolid medium (Baldani and Dobereiner, 1980).

Isolation of *Rhizobium* from root nodules

The root nodules were collected from *Vigna unguiculata,* for isolation of *Rhizobium* sp. They were isolated from root nodules following the technique of Vincent (1970). The white, translucent, glistering and elevated colonies were transferred to YEMA slants. Authentication of *Rhizobium* isolates carried out with biochemical tests like Growth in Congo red medium, Hofer's alkaline broth (Hofer, 1935), Glucose – Peptone Broth, 3-Ketolactose Production (Bernaerts and Delay,1963), Production of acid or alkali and Growth characteristics of *Rhizobium* sp. and Leonard jar assembly method, (1944) for plant infection test.

Identification of microorganisms

The bacterial cultures were identified up to the genus level by cultural, morphological and biochemical characteristics in accordance with Bergey's Manual of Determinative Bacteriology (2001).

Rhizoazophos medium preparation and mass multiplication of rhizoazophos

A novel media was formulated to support the growth of all three microorganisms as a biofertilizer consortium. After sterilization of rhizoazophos medium, *Rhizobium* sp. was first inoculated in the medium and incubated for 24 hrs to 48 hrs. After 48 hrs of incubation, *Azospirillum* sp. incubated and left for a maximum of 24 hrs. After 24hrs *Bacillus* sp. was inoculated and incubated for a maximum of 4 to 5 days at 30°C. After incubation, 10 ml of the inoculum was transferred to 1000 ml of rhizoazophos broth and kept in a shaking incubator for mass multiplication.

Preparation of biofertilizer

Carrier materials such as rice bran (RB), coir pith (CP) and sugarcane trash (ST) were prepared separately. The carrier materials were sterilized under 121°C in an autoclave for 3 hours and allowed to cool at room temperature. Each carrier material (165 g) was mixed with 35 ml of mass multiplied rhizoazophos cultures holding 1×10^9 inoculants per gram of carrier material. The carrier material with inoculum was packed under the sterile condition. Each carrier material with inoculum was kept at room temperature in sealed bags with 3 replicates. The viability of the Rhizoazophos was analyzed by

using the spread plate technique. The viable count of the organisms in stored carrier material was individually analyzed by spread plate technique once in 15 days for a total period of 60 days.

Influence of rhizoazophos on growth parameters of cowpea under sterilized pot culture condition

Ten uniform-size seeds of cowpea was surface sterilized with 0.5 percent sodium hypochlorite solution for 45 min, rinsed in sterilized water and soaked overnight in the liquid microbial consortium. Then seeds were sown in a garden soil mixture. After germination, seedlings were thinned to five per pot. For each treatment, there were three replications. The pots were watered on alternate days to maintain a 60 % maximum water-holding capacity (WHC). The seeds, which did not soak overnight in the microbial consortium, served as a control. Germination percentage (%) was taken after ten days of sowing. Shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, nodule fresh weight, nodule dry weight, and nodule numbers on taproot and lateral roots of *Vigna unguiculata* were observed on 30 days of sowing.

Results and Discussion

Isolation

Phosphate solubilizing microbes (PSM) are isolated from the rhizosphere soil of *Vigna unguiculata*. The total number of bacterial colonies varied in the nutrient agar and Pikovskaya medium. In the present study, the observations indicate that the distribution of PSM was more in the rhizosphere region. A large number of PSM observed in the rhizosphere might be due to the favorable influence of root exudates, containing amino acids, organic acids, sugars, growth-promoting substances, etc., and certain environmental factors (Kumar and Nerula, 1999). Widawati (2011) observed that the highest bacterial population was observed in the rhizosphere region of *Ipomoea aquatic* and also stated that more population of PSB was observed in terrestrial rhizosphere soil than in the sea, coastal areas, offshore areas and mangroves.

The isolated microorganisms were further screened for their ability to solubilize phosphate. The production of clearing zones around the colonies of the organism is an indication of PSM. The solubilization zone produced by the organisms varied from 0.5 cm to 0.9cm. The colony diameter of the isolates ranged between 0.7 to 1.5 cm. Solubilization efficiency (E) (%) of the microorganisms was observed from 46.67 to 75.00 on the PVK medium (Table 1). In the present study, the selected microorganisms were able to form a solubilization zone in the Pikovskaya (PVK) agar medium containing tricalcium phosphate (TCP) and others were able to grow but not able to produce a solubilization zone. Solubilization of insoluble phosphate sources depends on the type of acid secreted. The kind and concentrations of organic acids produced varied with P sources employed.

Isolated microorganisms were screened for their ability to solubilize insoluble phosphate sources quantitatively in liquid medium. The solubilization efficiency of each microorganism varied with the reduction of pH in the medium during the incubation period. In the Screening of microorganisms for phosphate solubilization, maximum P solubilized as P_2O_5 (mg) by *Bacillus* sp. (2) i.e., 71.24 ± 3.15, and solubilization of total P in the medium (%) was 31.75 in a broth assay after 15 days of incubation (Table 2). The pH of the medium turned acidic after 15 days by all the inoculants (Table 2). The phosphate solubilization (PS) activity in a liquid medium has a wider difference from the control. Production of organic acid by the isolated organisms in the liquid medium coupled with the decrease of the pH value

Table 1. Screening of microorganisms for phosphate solubilization and evaluation of their solubilization efficiency (E) by plate assay method

Organism	Solubilization activity	Solubilization zone (cm)	Colony diameter (cm)	Solubilization efficiency (E) (%)
Bacillus sp. (B1)	+++	0.7	1.5	46.67
Bacillus sp. (B2)	++++	0.9	1.2	75.00
Pseudomonas sp.(P1)	+	0.5	0.7	71.42
Pseudomonas sp.(P2)	++	0.6	1.0	60.00
Proteous sp.	++	0.5	0.9	55.56
Azospirillum sp.	+++	0.8	1.1	72.73

of the medium thereby resulting in phosphate solubilization.

Identification of isolated Microorganisms

The bacterial isolates were identified on the basis of morphological and biochemical tests as described in Bergey's manual (Bergey, 1984). Isolates 2 and 6 were identified as *Bacillus* sp. and *Azospirillum* sp.

***Bacillus* sp.**

The isolate is gram-positive, rod-shaped, 0.4-0.6 µm wide and 2-4 µm in length and was arranged in small chains. It is also motile. The strain produces pinpoint colonies in nutrient agar after 3 days and gives vigorous growth in nutrient broth. It ferments carbohydrates and mannose. The isolate gave positive tests to nitrate and ammonia production but does not produce H_2S. Starch hydrolysis, catalase test and methyl red tests were also positive.

***Azospirillum* sp.**

It is a gram-negative rod-shaped bacteria, 0.9-1.2 µm in width. It is motile. The isolate gave positive results to the oxidase test. It has a single flagellum at one pole and lateral flagella found when grown on agar media. When they were grown on potato agar the colony type was white, flat with raised margins. They can utilize glucose as a carbon source and they can thrive in the pH 6.0-7.3.

***Rhizobium* sp.**

An efficient indigenous symbiotic diazotroph, *Rhizobium* sp. was isolated from leguminous plants. Rhizobial isolates produced white, translucent, glistening, and elevated colonies on Congo red containing YEMA medium. The isolates did not show growth in Hofer's alkaline broth and glucose peptone broth. In lactose agar, the isolates did not produce any yellow coloration, when Benedict's reagent was added. Isolates produced acid in YEMA medium incorporated with BTB. All these tests confirmed that the isolates belonged to the genus *Rhizobium.* Two isolates are slow growers of *Rhizobium* sp., and one isolate is a fast grower i.e., an alkali producer (Table 3). Norris (1965) suggested that rhizobia could be broadly differentiated into acid and alkali-producing strains. Zablotowitcz and Facht (1981) while evaluating fast and slow-growing strains of *Rhizobium* found that levels of nodulation and nitrogen fixation were better for fast-growing strains.

Authentication of rhizobial strains by plant infection test in green gram

The efficiency of root nodule rhizobial isolate was tested with *V. unguiculata* as a test plant under Leonard jar and tube method. The *Rhizobium* isolate No. 2 was able to form nodules after 25 days on the green gram (Table 4). It reveals that isolate 2 has

Table 2. Screening of microorganisms for phosphate solubilization in a broth assay after 15 days

Organism	Maximum P solubilized as P_2O_5 (mg)	Solubilization of total P in the medium (%)	Final pH
Control	3.50±0.21	1.56	6.4 ±0.4
Bacillus sp. (B1)	48.72 ±2.25	21.72	4.3 ±0.2
Bacillus sp. (B2)	71.24 ±3.15	31.75	4.1 ±0.1
Pseudomonas sp.(P1)	38.41 ±2.08	17.12	4.6 ±0.2
Pseudomonas sp.(P2)	32.68 ±1.38	14.57	5.1 ±0.3
Proteous sp.	14.17 ±0.69	6.32	4.7 ±0.3
Azospirillum sp.	61.71 ±3.53	27.51	4.2 ±0.2

Table 3. Authentication of rhizobial strains by biochemical tests

Rhizobial isolates	Biochemical test				
	Congored Test	BTB Test	Hofer's alkaline Test	Glucose peptone Test	Growth in lactose agar
Rhizobium sp. 1	Slimy White	Slow Growth	No Growth	No Growth	No yellow coloration
Rhizobium sp. 2	Slimy White	Fast Growth	No Growth	No Growth	No yellow coloration
Rhizobium sp. 3	Slimy White	Slow Growth	No Growth	No Growth	No yellow coloration

better symbiotic efficiency. The Symbiotic effectiveness (S.E.) % of Control N $^{+}$ and *Rhizobium* isolate no 2 were 111.09 and 162.50 respectively (Tables 5 and 6). The study on the efficiency of the three-rhizobial isolates on the growth, dry matter production and nodulation in green gram plants indicated that only one rhizobial isolate significantly increased the plant growth parameters and nodulation over the uninoculated control in the plant infection test. Inoculation with the *Rhizobium* sp. 2 resulted in maximum plant growth and dry matter production of cowpea in the Leonard jar method. The highest symbiotic effectiveness (162.50%) was recorded in cowpea inoculated with *Rhizobium* sp. 2 Based on its symbiotic effectiveness *Rhizobium* sp. 2 was chosen for rhizoazophos preparation.

Microbial inoculant

A novel liquid (Rhizoazophos) medium is used to mass culture the isolated three different microorganisms as a consortium. Colony forming units (CFU) of each microorganism in the consortium was counted in the respective medium after incubation. The results of the *Rhizobium* sp. count was $4X10^8$, the *Azospirillum* sp. count was $3X10^8$ and the *Bacillus* sp. the count was $2X10^8$. Rice bran, coir pith and sugarcane trash were used as a carrier material for the preparation of microbial inoculant. Rice bran was a control carrier material used in the present study. At the end of 2 months of incubation period viable cells of microorganisms were observed. Viable microbial cells as inoculant in different carrier materials showed decline towards the end of incubation period. At the end of 2 months of incubation period $1x10^4$ $g^{-1}$of viable cells of *Bacillus* sp. was observed. At the end of 2 months of incubation period $21x10^7g^{-1}$of viable cells of Rhizoazophos was observed in rice bran (Table 7). There were no viable counts observed after six months of incubation.

Pot culture studies

In the present study, the maximum percentage of seed germination on Rhizoazophos fertilizer was recorded in rice bran after ten days of sowing i.e., 93.33 %. The application of Rhizoazophos biofertilizer prepared with rice bran significantly increased the shoot and root dry matter and survived better and increased the number of nodules and dry matter production understerilized pot culture conditions after 30 days (Tables 8 and 9). This may be due to the ability of the inoculated microbes to make available nutrients to the plants. There is firm evidence that plant growth-promoting substances like IAA, GA and cytokinins are produced by a number of rhizospheric microorganisms and a proper concentration of the hormones is essential to induce successful nodulation (Palzinski and Rolfe, 1985). *Rhizobium* sp. is reported to synthesize a vari-

Table 4. Authentication of rhizobial strains by plant infection test in green gram plant

Rhizobial isolates	Plant infection test method	Observation for nodule formation
	Leonard jar method	
Control N $^{-}$	Negative	Not Deducted
Control N $^{+}$	Positive	After 25 days
Rhizobium sp. 1	Negative	Not Deducted
Rhizobium sp. 2	Positive	After 25 days
Rhizobium sp. 3	Negative	Not Deducted

Table 5. Testing the symbiotic ability of the isolated rhizobial strains by the Leonard jar method in cowpea

Rhizobial isolates	Plant infection test - Leonard jar method					
	Shoot length (cm)	Root length (cm)	Shoot fresh wt (g)	Root fresh wt (g)	Shoot dry wt (g)	Root dry wt (g)
Control N $^{-}$	14.7 ±0.07	12.5 ±0.07	0.89 ±0.03	0.41 ±0.02	0.42 ±0.01	0.26 ±0.01
Control N $^{+}$	18.5 ±0.06	20.8 ±0.73	1.34 ±0.05	0.61 ±0.02	0.59 ±0.02	0.35 ±0.01
Rhizobium sp. 1	16.2 ±0.06	14.8 ±0.07	1.05 ±0.05	0.46 ±0.01	0.50 ±0.02	0.29 ±0.01
Rhizobium sp. 2	18.9 ±0.07	16.2 ±0.99	1.76 ±0.06	0.69 ±0.03	0.85 ±0.05	0.47 ±0.02
Rhizobium sp. 3	15.1 ±0.06	15.2 ±0.95	1.08 ±0.06	0.48 ±0.03	0.49 ±0.02	0.28 ±0.01

ety of cell surface polysaccharides like capsular polysaccharides and ß-glucans, which play an important role in the attachment of rhizobia to root hair and formation of infection thread (Leigh and Walker, 1994).

The population of PSM was generally higher in the inoculated treatments than in uninoculated treatments during the crop growth period. Anthoniraj *et al.* (1994) reported that there was the multiplication of inoculated PSB up to 30 DAS and a decline at harvest time. It indicates clearly that the population of potential PSB was more dependent on available P than other soil characteristics.

The use of these PSB as microbial inoculants will increase the available P in soil, help to minimize the P fertilizer application, reduce environmental pollution and promote sustainable agriculture. It is evident from the present study that rice bran as a carrier material supports the survival of viable cells for nearly 6 months. Rice bran alone or in high proportion with rice bran are suitable materials that can be used as carrier material for the long time survivability of the biofertilizers. The application of biofertilizer is helpful to reduce the salinity of soil by neutralization phenomenon because these microorganisms release the acid in very minute quantities during phosphate solubilization. Soil fertility management by biofertilizers is one of the basic components of sustainable agriculture.

Table 6. Testing the symbiotic ability of the isolated rhizobial strains by the Leonard jar method in cowpea

Rhizobial isolates	Plant infection test - Leonard jar method					Symbiotic Effectiveness (S.E.) %
	Nodule fresh wt (g)	Nodule dry wt (g)	No. of nodules per plant			
			Taproot	Lateral root	Total	
Control N $^{-}$	-	-	-	-	-	
Control N $^{+}$	0.15 ±0.01	0.08 ±0.01	5	4	9.0 ±0.3	111.09
*Rhizobium*sp. 1	-	-	-	-	-	
Rhizobium sp. 2	0.37 ±0.02	0.13 ±0.01	6	7	13.0 ±0.8	162.50
Rhizobium sp. 3	-	-	-	-	-	

Table 7. Testing the shelf life of Rhizoazophos consortium in various carrier material (CFU × $10^7 g^{-1}$)

Carrier materials	15th day	30th day	45th day	60th day
Rice bran	13	19	26	21
Coir pith	10	16	24	19
Sugarcane trash	7	12	18	14

Table 8. Influence of Rhizoazophos fertilizer on growth parameters of cowpea under sterilized pot culture condition after 30 DAS in the different carrier material.

Treatments	Shoot length (cm)	Root length (cm)	Shootfresh wt (g)	Rootfresh wt (g)	Shootdry wt(g)	Rootdry wt(g)
Control	13.52	11.24	03.17	0.23	0.96	0.09
Rice bran	24.15	18.63	16.25	0.51	5.08	0.20
Coir pith	19.94	16.47	14.33	0.39	4.11	0.17
Sugarcane trash	16.55	13.03	7.94	0.29	2.53	0.11

Table 9. Influence of Rhizoazophos fertilizer on root nodule parameters of cowpea under sterilized pot culture condition after 30 days of sowing (DAS) in different carrier material

Treatments	Nodule fresh wt (g)	Nodule dry wt(g)	No. of nodules per plant		
			Tap Root	Lateral Roots	Total
Control	-	-	-	-	-
Rice bran	0.556	0.162	9	12	21
Coir pith	0.429	0.145	6	9	15
Sugarcane trash	0.315	0.118	5	5	10

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