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Effects of Irradiated Carriers, Storage Temperatures, on Rhizobium Bioinoculant at Different Intervals

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ABSTRACT

The production and quality of rhizobial inoculants in many developing countries is limited by the availability of suitable carriers or technological limitations. Experiments were conducted to evaluate the potential of various inexpensive and widely available carrier materials. The carriers, evaluated, were Vermicompost, Vermiculate, Lignite, and Sodium Alginate, the study contrasted on two effective factors carrier materials and storage temperatures, a total of ten Rhizobial bacterial isolates collected from different sources. These cultures were studied morphologically and biochemically for purity confirmation then screened for PGPR properties, among all Rhizobial isolates, GNR-1 showed best plant growth promoting abilities in in-vitro conditions. The selected efficient PGP Rhizobium (GNR-1) and isolate was multiplied in large quantities in appropriate culture broth by incubating at 28±2°C in an incubator shaker at 120 rpm till they attained log phase with a cell load of 1×10^{9}cfu ml^{-1}. The carriers sterilized by gamma irradiation at a dose rate of 5.0 kGy for 1h then carriers were used for bioinoculant preparation and stored at different temperatures i.e. 4ºC and 28ºC. The bacterial population highest in 1st month log_{10} value 9.76 in sodium alginate carrier based biofertilizer stored at 4^{0}C and at 8th month the survival rate of rhizobial cells was very highest in sodium alginate log_{10} 8.94 stored at 4ºC less in lignite log_{10} value 5.50 stored at 28±2°C.

Key words: *Rhizobium, carriers, irradiated sterilization, different temperatures, sodium alginate.*

INTRODUCTION

Organic farming has emerged as an important priority area globally in view of the growing demand for safe and healthy food and long term sustainability and concerns on environmental pollution associated with indiscriminate use of agrochemicals. Though the use of chemical inputs in agriculture is inevitable to meet the growing demand for food in world, there are opportunities in selected crops and niche areas where organic production can be encouraged to tape the domestic export market.

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Bio-fertilizers are being essential component of organic farming are the preparations containing live or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing or cellulolytic micro-organisms used for application to seed, soil or composting areas with the objective of increasing number of such microorganisms and accelerate those microbial processes which augment the availability of nutrients that can be easily assimilated by plants. Biofertilizers play a very significant role in improving soil fertility by fixing atmospheric nitrogen, both, in association with plant roots and without it, solubilise insoluble soil phosphates and produces plant growth substances in the soil. They are in fact being promoted to harvest the naturally available, biological system of nutrient mobilization[14].

Biofertilizers are low cost, environment friendly and economically viable technology which improve plant growth and development. These are integral part of organic farming system as they have several advantages, high cost benefit ratio, enhance plant growth and yield by increasing soil fertility and nutrient availability, reduce the environmental pollution caused by chemical fertilizers and protect plants against many soil-borne pathogens and help the plant to grow under stress condition[5].

Integrated use of chemical and biofertilizer is the need of the hour. In an experiment conducted on dwarf pea, the integrated use of biofertilizer along with chemical fertilizer showed maximum yield of dwarf peas. Combined application of biofertilizers along with recommended dose of fertilizers increased the grain yield up to 21.27%[9].

Selection of carrier material is very important while preparing biofertilizers. Although, there are no clear cut criteria for the selection of carrier materials but some general characteristics should be present in the material which is going to be used as a carrier for biofertilizer such as it should be cost effective, contain non-toxic compounds and high organic content, easy to process, more than 50% water holding capacity, high buffering capacity, sticky in nature and available in bulk quantity. A variety of materials can be used as carrier but the need of hour is to find out the most suitable carrier which fulfils all the above stated properties[4].

The main function of carrier material is to provide the suitable micro-environment to enhance the shelf life of introduced bacteria. Most of the carrier materials containing high organic matter increase bacterial survival and enhance the efficacy of bacterial inoculum.

Carrier based biofertilizers proved to be the best over the agrochemicals and are showing the tremendous effect on the global agricultural productivity, from the last two decades. Rectifying the disadvantages of the carrier based bio fertilizer liquids biofertilizer are developed which would be the alternative for the cost effective sustainable agriculture. Biofertilizer production in India during the period 2013-14, is 51870.67. MT, in Andhra Pradesh is 2137.14 MT.

The knowledge of applied microbial inoculums is long history which passes from generation to generation of farmers. It started with culture of small scale compost production that has evidently proved the ability of biofertilizer. This is recognize when the cultures accelerate the decomposition of organics residues and agricultural by products through various processes and gives healthy harvest of crops[1].

MATERIAL AND METHODS

The present study was carried out in the Department of Agricultural Microbiology & Bioenergy, College of Agriculture, Rajendranagar, PJTSAU, and Hyderabad. Pure cultures of Plant Growth Promoting *Rhizobium* isolates collected from different laboratories. Screening and characterization of isolates were done with multiple beneficial properties then the efficient PGPR isolate was selected for preparation of carrier based biofertilizers.

COLLECTION OF *Rhizobial* ISOLATES FROM DIFFERENT SOURCES

Promising bacterial isolates are collected from different laboratories and these isolates were

tested for their purity and preservation in Dept. of Agricultural Microbiology & Bioenergy, College of Agriculture, PJTSAU Rajendranagar, Hyderabad.

IDENTIFICATION OF BACTERIAL ISOLATES PURITY CHECKING.

Morphological and Biochemical Characterization

The isolated bacteria were studied for their morphological like gram reaction, pigmentation, cultural characteristics and biochemical characteristics like Indole production, methyl red, voges-praskaure's test, citrate utilization test, oxidase, catalase and sugar fermentation tests.

Screening for plant growth promoting properties

Screening will be carried out for different plant growth promoting properties such as mineral solublization like Phosphorus Solubilisation[10], Zinc Solubilization[12], Potassium releasing[11], Plant growth promoting substances such as IAA production[7], biocontrol activity such as HCN production[6] and Siderophore production[13] and antifungal activity with soil born plant pathogens all ten isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and gram reaction were also recorded as per the standard procedures given by Barthalomew and Mittewar[3].

Gram's staining.

A loopful of inoculum from young culture was taken, mixed with water, and placed in the center of the slide. The suspension was spread out on slide using the tip of inoculation needle to make a thin suspension. The smear was dried in air and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with Crystal violet solution for 1 min and washed gently with flow of tap water. Then the slide was flooded with Iodine solution. After incubation at room temperature for 1 min, Iodine solution was drained out followed by washing with 95% decolorizer. After that, it was washed with water within 15 to 30 sec and blot carefully. The smear was incubated with Saffranin solution for 1 min. The slide was washed gently in flow of tap water and dried in air. The slide was examined under microscope at 100X power with oil immersion and data was recorded.

Cultural Characterization

Morphological characteristics of the colony of each isolate were examined on specialized medium. Cultural characterization of isolates observed by different characteristics of colonies such as shape, size, elevation, surface, margin, colour, odour, pigmentation etc were recorded.

Yeast Extract Mannitol Agar with Congo red test (YEMAC)

The isolates were streaked on YEMAC media plates and incubated at 28 ± 2°C for 48-72h. *Rhizobial* colonies do not absorb colour and remain white in colour.

Collection of Carrier Materials

Sodium alginate collected from Department of Agricultural Microbiology and Bioenergy College of Agriculture Rajendranagar, Hyderabad. Vermiculate collected from Navaratna Crop Science Pvt Ltd, Cherlapally, Hyderabad. Lignite collected from Department of Agricultural Microbiology and Bioenergy College of Agriculture Rajendranagar, Hyderabad. Vermicompost collected from NIRD Rajendranagar, Hyderabad.

Physico-chemical properties of carriers

For the preparation of bioformulation, the collected different carriers such as lignite, vermicompost, sodium alginate and vermiculite were tested for their moisture content[2] (Aeron *et al.,* (2011) and pH at initial and end of the experiment.

Irradiation Sterilization

Lignite, vermicompost, vermiculate, and sodium alginate was sterilized by gamma irradiation at a dose rate of 5.0 kGy for 1h. (Gamma irradiation was carried out at the Irradiation unit PJTSAU, Hyderabad).

Preparation of Biofertilizers

a. **Preparation of rhizobium culture broth.**

   Yeast Mannitol Broth is used for cultivation of *Rhizobium* species.

b. **Composition**

c. Ingredients	**Gms / Litre**
Yeast extract	1.000
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Calcium carbonate	1.000
Final pH (at 25°C)	6.8±0.2

DIRECTIONS

Suspend 12.8 grams in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense into sterile test tubes. The selected isolate was multiplied in large quantities in culture broth by incubating at 28±2°C in an incubator shaker till they attained log phase with a cell load of $l \times l0^9$cfu ml^{-1} and were used for inoculant preparation. The individual carrier materials were powdered and the pH was brought to neutral by adding $CaCO_3$ then sterilized by gamma irradiation at a dose rate of 5.0 kGy for 1h after then mixed with the log phase culture ($l \times l0^9$cfu ml^{-1}) of the selected plant growth promoting bacterial isolate viz., *Rhizobium* (GNR1) in separate quantities of sterile carrier in shallow trays. The optimum moisture content was adjusted to (30-40%) prior to preparation, followed by curing in shallow trays for 24 hours in aseptic rooms and then packed in high density opaque polythene bag (12g) at the rate of 100g bag^{-1} and sealed. Individual inoculant was prepared by mixing with lignite (1:3v/w), vermicompost (1:2v/w), vermiculite (1:2v/w) volumes of each culture broth with sterile carrier materials. The populations of individual Plant Growth Promoting Rhizobacteria in the inoculant carriers were assessed at monthly intervals upto 8 months at different storage temperature at 4ºC 28 ± 2°C.

b. Preparation of Alginate based inoculant

Rhizobium spp was grown in respective medium to get a population of $lxl0^9$cfu ml^{-1} Sodium alginate beaded inoculant was prepared as per the methods described by Hegde and Brahmaprakash[8]. Two gram of sodium alginate was added to 100 ml of culture broth of PGPR and mixed for 30 minutes in a magnetic stirrer. The mixture was added drop wise through a 10 ml syringe into 100 ml sterile 0.1N $CaCl_2$ to obtain uniform Alginate beads. One gram of material contained 16 to 17 beads, each bead approximately weighing 60mg. The beads were washed twice in sterile distilled water and incubated for seven days in a psychrotherm (model environ shaker) incubator at 28±2°C to allow PGPR to multiply inside the beads. The beads were again washed in sterile distilled water and air dried in Laminar air flow chamber under aseptic condition. The Alginate beads were then stored in polythene bags at room temperature (28±2ºC) and refrigerator (4ºC) upto 8 months.

Treatments

T 1: $S_1C_1O_1$(Irradiated Vermicompost with *Rhizobium spp*)

T 2: $S_1C_2O_1$(Irradiated Sodium alginate with *Rhizobium spp)*

T3: $S_1C_3O_1$ (Irradiated Lignite with *Rhizobium spp).*

T4: $S_1C_4O_1$ (Irradiated Vermiculite with *Rhizobium spp),*

Determination of viable bacterial population in the carrier based inoculants by serial dilution and plating technique. Influence of storage temperature on the survival of the inoculants as consortium in different carrier materials .The carrier based microbial inoculants prepared with different carrier material was kept in different temperature levels *viz.,* Room temperature (28±2ºC) and Refrigerator (4°). The surviving populations of PGPB at different temperatures were determined and population was enumerated by dilution plate technique at different intervals i.e monthly upto 8 months.

RESULTS AND DISCUSSION

At 4^0C storage temperature, Irradiation sterilized vermicompost, sodium alginate, lignite, vermiculite based rhizobial inoculants, rhizobial bacterial population showed log_{10} value 9.65, 9.76, 9.64, 9.67 respectively in first month. But from 1st month to 8th month rhizobium population gradually decreased

table no.1. At 28 ± 2^{0}C storage temperature irradiation sterilized all carrier based rhizobial inoculants, showed rhizobial bacterial population $\log_{10}$ value 9.72, 9.74, 9.70, 9.73 respectively in first month and when compared to 4^{0}C storage 28 ± 2^{0}C having more $\log_{10}$ value in first month except sodium alginate and rhizobial population at 28 ± 2^{0}C also decreased from 1st month to 8th month table no.2. But survival rate of rhizobial cells was more upto 8th month at 4^{0}C compared to 28 ± 2^{0}C. This result revealed that 4^{0}C storage temperature is best suitable for storage of carrier based inoculants because of low level of moisture content in the carrier inoculants stored at 28 ± 2^{0}C temperature compared to 4^{0}C.

As per specification of biofertilizers, carrier should be minimum 5×10^7cfu g^{-1}($\log_{10}$ 7.6) viable count of powdered form of carrier based biofertilizer. So that the above results revealed that at 4^{0}C, irradiation sterilized lignite carrier based rhizobial bioinoculants supported and maintained optimum viable count $\log_{10}$ 8.2 more than 5×10^7cfu g^{-1}($\log_{10}$ 7.6) viable count of powdered form of carrier based biofertilizer upto 7th month only, but shows the viable count $\log_{10}$ 7.1 at 8th month, whereas sodium alginate, vermiculite and vermicompost based carrier bioinoculants supported and maintained optimum viable count $\log_{10}$ 8.94, 7.87, 7.72, respectively upto 8th month fig no. 1. Which is more than the minimum 5×10^7cfu g^{-1}($\log_{10}$ 7.6) viable count of powdered form of carrier based biofertilizer.

At 28 ± 2^{0}C storage temperature irradiated sterilized vermicompost and lignite carrier based rhizobial inoculants supported and maintained optimum viable count $\log_{10}$ 8.33 and $\log_{10}$ 8.16 upto 6th month only, but sodium alginate and vermiculite based carrier bioinoculants supported and maintained optimum viable count $\log_{10}$ 8.40 and $\log_{10}$ 7.65. more than 5×10^7cfu g^{-1} ($\log_{10}$ 7.6) upto 7th month fig no.2. When comparing all carrier based bio inoculants shelf life sodium alginate rhizobium carrier based biofertilizer showed highest viable count more than 5×10^7cfu g^{-1} ($\log_{10}$ 7.6) upto 8th moths at both storage temperatures *viz.*, Room temperature (28±2ºC) and Refrigerator (4°).

Table 1: Rhizobium Biofertilizer Irradiation Sterilization

MONTH	VERMI COMPOST	SODIUM ALGINAT	LIGNITE	VERMICULITE
	4 ºC	4 ºC	4 ºC	4 ºC
1	9.65	9.76	9.64	9.67
2	9.64	9.72	9.58	9.64
3	9.57	9.60	9.46	9.58
4	9.30	9.54	9.32	9.50
5	9.12	9.45	9.02	9.30
6	8.99	9.40	8.91	9.05
7	8.51	9.20	8.20	8.80
8	7.72	8.94	7.10	7.87

Table 2: Rhizobium Biofertilizer Irradiation Sterilization

MONTH	VERMI COMPOST	SODIUM ALGINATE	LIGNITE	VERMICULITE
	(28±2ºC)	(28±2ºC)	(28±2ºC)	(28±2ºC)
1	9.72	9.74	9.70	9.73
2	9.62	9.70	9.62	9.62
3	9.53	9.65	9.46	9.52
4	9.06	9.44	9.02	9.23
5	8.80	9.25	8.55	8.90
6	8.32	8.95	8.16	8.53
7	7.52	8.40	7.10	7.65
8	6.50	7.82	5.50	6.90

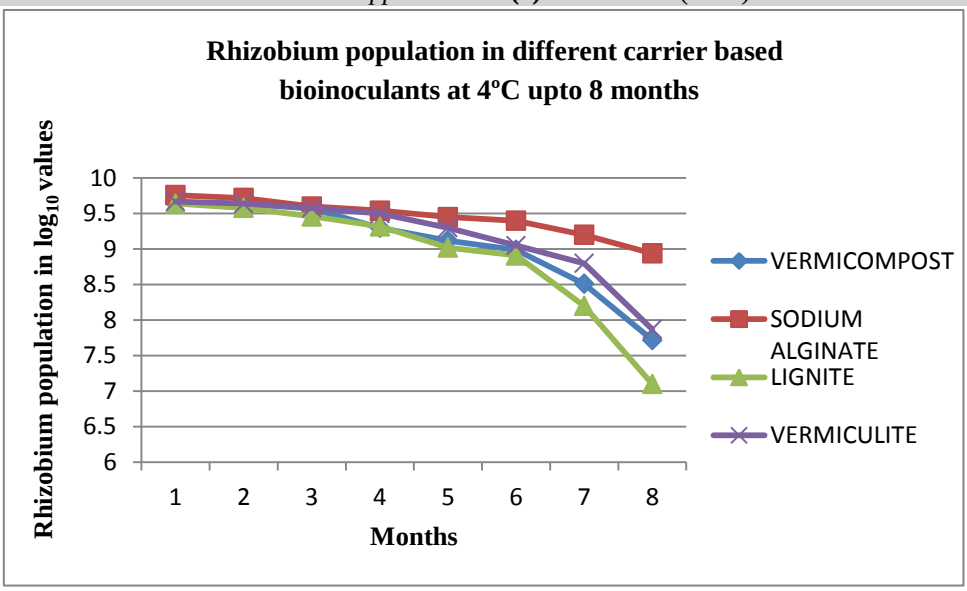

Fig. 1:

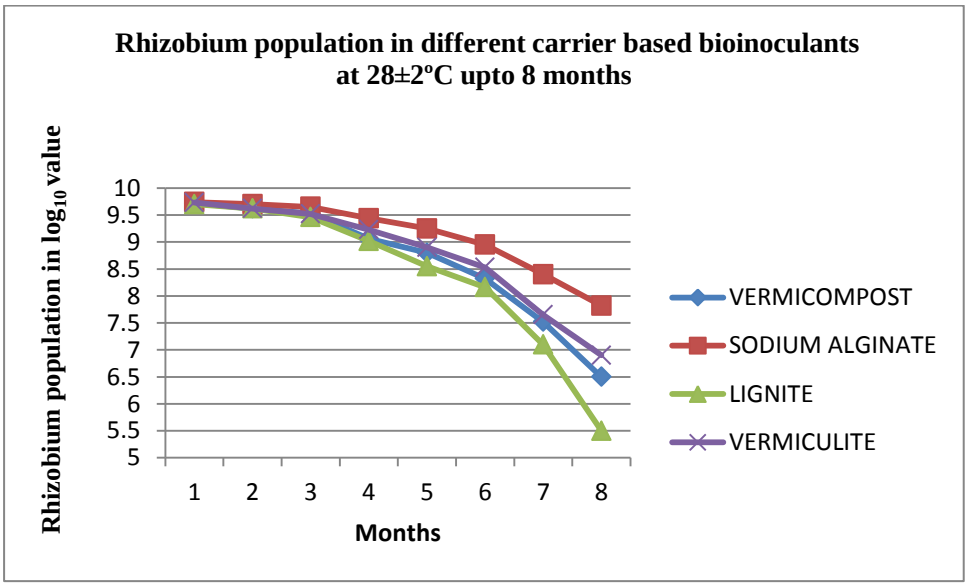

Fig. 2

CONCLUSION

The inoculation of legumes with rhizobia is used to maximise nitrogen fixation and enhance the plant yield without using N fertilisers. For this reason many inoculant types were developed and optimised. For the increasing the shelf life of rhizobial carrier based biofertilizers should be store at 4° temperature then Room temperature (28±2ºC), and also for best carrier based biofertilizer production sodium alginate and vermiculite carriers were showed highest population with long time storage capacity at both temperatures *viz.*, Room temperature (28±2ºC) and Refrigerator (4°).

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