SUITABILITY OF DIFFERENT FORMULATED CARRIERS FOR SUSTAINING MICROBIAL SHELF LIFE

Tosif Tabassam*, Tariq Sultan**, M.Ehsan Akhtar**, M.Mahmood-ul-Hassan** and Arshad Ali**

ABSTRACT:- Non-availability of a suitable carrier for bioinnoculant is a serious constraint for dissemination of biofertilizer technology in Pakistan. Present study was designed to formulate a suitable carrier from locally available cheap material and evaluate for shelf life by using locally isolated plant growth promoting rhizobacteria (PGPR) strains from maize rhizosphere. Different combinations of material were prepared using clay soil (35-50%), fly-ash (30-45%), press mud (5-15%) and lignite (5-15%). Clay soil (53% clay) was used for adhesion purpose but considering free of lump formation an important property of a good carrier, mixing 40% of soil with other material was found suitable. Using 40% of soil, six different treatments were formulated and physico-chemical characteristics were determined. Four combinations in the range of 40% clay, 30-40% fly-ash, 10-15% press mud and 10-15% lignitic coal were selected which had good adhesion capacity, moisture holding capacity, nutrient contents and investigated for microbial shelf life. Significant difference regarding microbial survival was observed between different formulations as well as between different incubation intervals. Among different carrier tested the FC-4 supported the maximum population of $33.5x10^8$ - $10.8x10^8$ cfu g^{-1} for MR-8 and $32.6x10^8$ - $7.2x10^8$ cfu g^{-1} for MR-5. Results showed that the required population of PGPR was sustained in all the formulation tested up to six months of storage period.

Key Words: Biofertilizer; Carrier Development; Storage; Survival Efficiency; Pakistan.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) promotes plant growth by diverse mechanisms, and often the beneficial influence is due to a combination of mechanisms (Bashan et al., 2004). These diverse mechanisms include nitrogen fixation, phosphorous solubilization, phytohormones production and biocontrol, which enhance plant growth directly (Noumavo et al., 2013). To protect the environment from excessive use of chemical fertilizer and reducing nitrogen and phosphate fertilizers, application of biofertilizer to the field under cultivation is essential.

Biofertilizers are carrier based formulations containing viable cells of efficient strains of N-fixing, P-solubilizing or cellulolytic microorganisms used for application to seed or soil, intended to improve soil fertility. Assimilation of microorganisms in carrier materials enables sustain-

* *PARC Institute of Advanced Studies in Agriculture, National Agricultural Research Centre, Islamabad, The University of Agriculture, Peshawar, Pakistan.*
** *Land Resources Research Institute, National Agricultural Research Centre, Islamabad, Pakistan.*
Corresponding author: tauseefrwp@gmail.com

ability issues like easy-handling, long term storage and high effectiveness of biofertilizers (Trevors et al., 1992). The carrier is any material which can be used as a delivery vehicle of viable micro-organisms from the laboratory to the field. The quality of carrier is an important factor to determine the microbial population density and shelf life of biofertilizer. Properties of a good carrier material are; good moisture holding capacity, available in adequate quantity, inexpensive, easy to process and sterilize, non-toxic to inoculated bacterial strains and plants (Samasegaran and Hoben, 1994). Naturally, no single carrier can have all these qualities, but a good one should have as many as possible (Bashan, 1998). Since the beginning of biofertilizers use at large scale, several carrier materials like farm yard manure, compost, peat soil, coal, charcoal, cellulose powder, lignite, talc, bagasse, sedge peat, press mud, teak leaf meal, coconut shell powder etc. have been tried. However, no universal carrier or formulation is presently available for the production of biofertilizer (Trevors et al., 1992).

Peat-partially decomposed plant matter, is the most effective carrier but this material, however is not readily available in many developing countries including Pakistan. Various earlier investigations have suggested that coal has potential as a carrier, but the value of these studies has been limited by the restricted scope of the experimentation (Paczkowski and Berryhill, 1979). Lignite powder is being used as carrier material for most of the bioinoculant production (Saravanakumar and Gandhi, 2009), improving it with cheaper nutrient sources could increase the efficiency and potential life of inoculums (Menaka and Alagawadi, 2007). Problematic fly-ash waste can be utilized in carrier formulations in a useful manner but investigations will have to be conducted, to evaluate the survival / shelf-life of bio-fertilizers (Gaind and Gaur, 2004). As fly-ash is basic in nature and it has good mineral composition, use of fly-ash in soil as bio-formulation increases pH of soil, converts the nutrients in available form and furnish to soil (Kumar, 2014). Sugarcane press-mud suitability as a carrier for the production of bacterial inoculants was studied in various countries and found not a perfect carrier for bacterial inoculant production, its effectiveness can be enhanced by amending it with soil and charcoal. The survival of Rhizobium and Azotobacter in various press mud amended samples was studied, press mud/charcoal combination was found the best (Jauhri, 1990).

Clay is a widely distributed and abundant mineral resource that has high adsorption power, good colloidal properties and high amending capability that make it the most suitable material for solid carrier formulations. Clay minerals got negative charges due to isomorphous substitution. Ion-exchange property of clay minerals can possibly influence microbial metabolism through trapping and release of cations. The carrier materials and the type of formulation may vary, but a good carrier should have one essential characteristic: the capacity to deliver the right number of viable cells in good physiological condition at the right time (Bashan, 1998). In Pakistan, bioinoculant technology has tremendous potential for the

country's economy but non-availability of a suitable carrier material has seriously limited the mass production of inoculant (Khalil et al., 1991). Present study was conducted to formulate a suitable carrier from locally available cheap material with capability of sufficient and acceptable bacterial population at standard level and use of it for bio-fertilizer production.

MATERIALS AND METHOD

Formulation of Carriers

The present study was conducted at National Agricultural Research Centre, Islamabad during 2013 to formulate carriers for bioinoculant from locally available agricultural and industrial waste product and to select a suitable carrier for sustainable biofertilizer production. Carriers were formulated by different combinations of press mud (pH, 6.04; OC, 40%; N, 3.48%; P, 1.87%; K, 1.18%), fly-ash (pH, 8.25; N, 0.12%; P, 0.04%; K, 0.10%), clay soil (pH, 7.45; organic matter, 0.76%, clay, 53%) and lignitic coal (pH, 2.75; OC, 34%; N, 0.64%, P, 0.17%; K, 0.06%). Press mud and fly-ash were collected from sugar mill, high clay content soil was collected from Nandipur, Gujranwala and lignitic coal was collected from Chakwal area.

Carrier Material Preparation

Material collected from different sources was dried, grinded and sieved through 116 mesh size sieve. Quality of materials was determined by estimating soil texture, pH, organic matter, total nitrogen, total phosphorus and micronutrients (Fe, Zn, Cu, Mn) etc. using standard methods (Ryan et al., 2001). Collected materials were mixed with different combinations i.e., clay soil (35-50%), fly-ash (30-45%), press mud (5-15%) and lignitic coal (5-15%). Clay soil (53% clay) was used for adhesion purpose but considering; free of lump formation an important property of carrier, different percentages (35-50) of clay soil were used in combination of other material. It was observed that mixing 40% clay soil with other materials, the formulation was free of lumps. Six different treatments developed were:

T_1 = 40% clay soil +35% fly-ash +15% press mud + 10% lignitic coal,

T_2 = 40% clay soil + 40% fly-ash + 10% press mud + 10% lignitic coal,

T_3 = 40% clay soil + 45% fly-ash + 5% pressmud + 10% lignitic coal,

T_4 = 40% clay soil + 35% fly-ash + 10% press mud + 15% lignitic coal,

T_5 = 40% clay soil + 30% fly-ash + 15% press mud + 15% lignitic coal

T_6 = 40% clay soil + 40% fly-ash + 5% press mud + 15% lignitic coal.

Adhesion with the seed, requirement of $CaCO_3$ for pH adjustment and water holding capacity was estimated according to Samasegaran and Hoben (1994) method. Mineral composition and heavy metals were analyzed using standard methods (Ryan et al., 2001). The experiment was conducted with complete randomized design using three replications. The data recorded was statistically analyzed with Analysis of Variance technique using Statistix 8.1 software. For significant F-value Tukey HSD was used for means comparison at 5% level (Steel et al., 1997).

On the basis of water holding capacity (WHC), adhesion with the seeds and nutrients contents, following four best treatments were selected and designated as formulated carrier (FC):

FC-1 = 40% clay soil + 35% fly ash + 15% press mud + 10% lignitic coal.

FC-2 = 40% clay soil + 40% fly ash + 10% press mud + 10% lignitic coal.

FC-3 = 40% clay soil + 35% fly ash + 10% press mud + 15% lignitic coal.

FC-4 = 40% clay soil + 30% fly ash + 15% press mud + 15% lignitic coal.

Microbial Shelf Life Investigations in Formulated Carriers

The survival of PGPR was investigated in the selected formulated carriers and biozote carrier (reference carrier). Biozote carrier is a mineral soil (pH, 7.70; clay, 15.82%; silt, 25%; sand, 54%; organic matter, 4.6%) collected from northern areas of Pakistan (Khalil et al., 1991), which is being used as a carrier for biofertilizer production in Land Resources Research Institute, National Agricultural Research Centre, Islamabad. The 50 g carriers were taken in the autoclave-able cellophane bags and sealed with electric sealer. Packets were auto-claved at 121°C with pressure of 1.34 atm for one hour (two consecutive days) and dried in oven at 70°C for 3 h to dry out the material. Before starting the experiment, the carriers were checked for sterility, by plating aliquots from serial dilutions on Luria Bertani (LB) agar plates and monitoring any growth. The experiment was conducted with factor factorial, complete randomized design in triplicate with five treatments; four formulated carriers (FC-1, FC-2, FC-3, FC-4) and one biozote carrier (BC). All the treatments were inoculated with broth containing 10^{10} cfu ml^{-1} of locally isolated PGPR strains (MR-5 and MR-8) obtained from the culture collection of Soil Biology and Biochemistry lab. National Agricultural Research Centre, Islamabad. Non-inoculated controls were used to check any contaminations during the study. Moisture of carriers was maintained at 40-50% level and temperature was maintained at 29°C in an incubator. Colony forming units (cfu g^{-1}) were calculated under sterilized conditions after 15, 30, 60, 90, 120, 150 and 180 days of incubation and controls were checked for sterility, by plating aliquots from serial dilutions on agar plates and monitoring any growth. The bacterial count was taken by serial dilution and drop plate methods (Motsara and Roy, 2008). The cfu was calculated according to following formula:

$$\text{Cfu} = \frac{\text{No. of colonies x Dilution factor}}{\text{Volume of inoculum}}$$

RESULTS AND DISCUSSION

Formulation of Carriers

Development of suitable carrier material is essential for successful field application of any biofertilizer. Based on the physico-chemical properties of the respective carriers, the suitable bio-inoculants can be tested and produced. Potential life of inoculum in the carrier material can be increased by supplementing it with some amendments. Several research workers utilized different materials like garden soil (Madhok,

1934), peat (Graham et al., 1974), sugarcane press mud (Kumar et al. 1982), coal (Dube et al., 1975), cellulose powder (Pugashetti et al., 1971), charcoal and vermiculite (Sparrow and Ham, 1983) as carrier for the preparation of inoculant.

Moisture holding capacity (MHC), adhesion with the seeds, both macro and micro-nutrient were found significantly ($P \leq 0.05$) different but non-significantly different for heavy metals composition in different treatments (Table 1). It is evident from the data presented that suitability of different treatments regarding physico-chemical properties was in the following order $T_5>T_1>T_4>T_2>T_3>T_5$. As T_1, T_2, T_4 and T_5 were found better, these four best combinations were designated as FC-1 (T_1), FC-2 (T_2), FC-3 (T_4) and FC-4 (T_5) and selected for microbial shelf life investigation.

Microbial Viability in the Formulated Carriers

Effectiveness of microbial inoculation to enhance growth of plant is vastly influenced by the number of introduced viable cells into the soil. Therefore, determination of the bacterial survival duration in the respective carrier materials is paramount to ensure that desired level of bacterial population remains viable for the inoculants (Arora et al., 2014). Different carrier formulations as well as Biozote carrier showed a signi-

Table 1. Physico-chemical characteristics of different treatments

Characteristics	Treatment						Tukey's HSD ($P \leq 0.05$)
	T_1	T_2	T_3	T_4	T_5	T_6	
WHC (%)	106^{a}	97^{bc}	92^{d}	100^{b}	105^{a}	93^{cd}	4.14
Adhesion (%)	42.22^{b}	40.06^{b}	36.19^{c}	41.56^{b}	53.88^{a}	35.04^{c}	2.56
P (%)	0.24^{a}	0.20^{ab}	0.12^{c}	0.26^{a}	0.23^{ab}	0.17^{bc}	0.07
N (%)	0.65^{b}	0.62^{b}	0.60^{b}	0.64^{b}	0.89^{a}	0.59^{b}	0.07
K (%)	0.97^{a}	0.83^{b}	0.65^{c}	0.73^{c}	0.92^{a}	0.51^{c}	0.08
Na (%)	0.33^{b}	0.43^{a}	0.43^{a}	0.28^{bc}	0.22^{c}	0.34^{b}	0.08
Cu (mg kg^{-1})	17.17^{ab}	19.67^{a}	16.00^{b}	17.20^{ab}	18.20^{ab}	16.27^{b}	2.30
Mn (mg kg^{-1})	240.2^{a}	210.8^{b}	127.1^{c}	153.2^{c}	229.3^{ab}	153.2^{c}	28.25
Zn (mg kg^{-1})	75.73^{a}	75.20^{a}	44.77^{b}	45.80^{b}	66.63^{a}	42.50^{b}	9.20
Fe (mg kg^{-1})	434.8^{a}	398.4^{b}	370.8^{b}	435.3^{a}	466.1^{a}	390.5^{b}	220.21
Cd (mg kg^{-1})	0.40	0.53	0.30	0.33	0.50	0.40	_
Cr (mg kg^{-1})	13.33	14.13	14.23	15.40	15.23	15.80	_
Ni (mg kg^{-1})	12.23	13.07	14.67	14.57	14.96	14.95	_
Pb (mg kg^{-1})	7.46	11.23	9.80	10.60	10.63	10.63	_

Means followed by same letter do not differ significantly at 5% level of probability

ficant ($P \leq 0.05$) decline in micro-bial population after 30 days, towards the end of incubation period (Table 2 and 3). The population density of bacteria dropped with the passage of time due to lack of moisture and nutrients (OM, N, P, K, etc.) of the carriers, bacterial activities and storage conditions while transitioning from logarithmic to stationary phase during incubation period (Phiromtan et al., 2013). However all the carriers retained more than 10^7 cfu g^{-1} viable propagates up to 180 days. Different carriers also found significantly different regarding survival of bacteria. The results indicated that FC-4 was the most suitable carrier for production of PGPR inoculums with higher viability i.e., $32.6x10^8$ to $7.2x10^8$ cfu g^{-1} of MR-5 and $35.5x10^8$ to$10.8x10^8$ cfu g^{-1} of MR-8 during the storage period, followed by FC-1, FC-3 and FC-2. Better physico-chemical properties due to presence of high OC contents of press mud and lignitic coal and especially high nutrient contents of press mud supported the higher survival of tested organisms. Non-inoculated controls which were used to check any contamination showed a negligible bacterial growth on agar plate in serial dilution (10^{-1}) during the study.

Kandaswamy and Prasad (1971) described lignite as a good alternate for peat as a carrier material in which *Rhizobium* propagated well. Raja Sekar and Karmegam (2010) used different vermicast and lignite ratio (0:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 1:0) as carrier materials for biofertilizers (*Azotobacter chroococcum, Bacillus megaterium* and *Rhizobium leguminosarum*). Viability of cells of these strains was more than 1×10^7 g^{-1} in 4:1, 5:1, 6:1 and 1:0 (vermicast: lignite) at the end of 10^{th} month. In lignite alone, no viability was observed in 10^7 g^{-1} at the end of 6^{th} month for *A. chroococcum* and *R. leguminosarum* and 5^{th} month for *B. megaterium*. Kumar and Gupta (2010) formulated carriers for *A. chroococcum* in fly-ash alone and in combination with lignite (1:0, 1:1 and 0:1) and evaluated the shelf-life of PGPR on wheat and viability of *A. chroococcum* up to 9 months. Viability was in the order: fly-ash > lignite and fly-ash > lignite (1:1) > lignite. Sugarcane press mud carrier based inoculants have lower tolerance for physical stress during storage for

Table 2. Population density (1×10^8 cfu g^{-1}) of MR-5 in different formulated carriers

	Incubation (Days)							
Treatment	15	30	60	90	120	150	180	Mean
FC-1	30.6^{a-c}	31.0^{a-c}	30.3^{a-c}	26.5^{c-c}	20.4^{fg}	10.0^{ij}	5.5^{j-l}	22.0^{b}
FC-2	29.3^{a-c}	29.3^{a-c}	29.7^{a-c}	21.4^{f}	18.0^{f-h}	6.7^{jk}	0.8^{lm}	19.2^{c}
FC-3	29.5^{a-c}	29.6^{a-c}	28.3^{a-c}	22.7^{d-f}	16.5^{gh}	7.5^{jk}	1.1^{lm}	19.3^{c}
FC-4	32.4^{ac}	32.6^{c}	31.7^{ab}	31.6^{ab}	27.5^{b-d}	13.6^{hl}	7.2^{jk}	25.2^{a}
BC	29.6^{a-c}	31.5^{ab}	28.7^{a-c}	22.3^{ef}	16.4^{gh}	4.8^{k-m}	0.5^{m}	19.1^{c}
Mean	30.3^{a}	30.3^{a}	31.1^{a}	24.9^{b}	19.7^{c}	8.5^{d}	3.0^{e}	-

** Each value is an average of three replicates*
** Tukey's HSD (≤ 0.05)= Trt., 0.46; Days, 1.66; Trt* Days, 4.87*
** SE= Trt., 0.46; Days, 0.55; Trt.*Days, 1.22*
Means followed by same letter do not differ significantly at 5% level of probability

Table 3. Population density (1×10^8 cfu g^{-1}) of MR-8 in different formulated carriers

	Incubation (Days)							
Treatment	15	30	60	90	120	150	180	Mean
FC-1	33.5^{ab}	33.3^{ab}	29.9^{b-d}	27.3^{d-f}	21.5^{h-k}	15.5^{lm}	10.2^{p}	24.5^{b}
FC-2	31.1^{a-d}	29.9^{b-d}	24.0^{e-l}	23.5^{f-l}	18.2^{j-l}	12.6^{n-p}	5.4^{qr}	20.7^{d}
FC-3	32.4^{a-c}	31.2^{a-d}	28.4^{c-e}	26.7^{d-g}	20.3^{j-k}	13.0^{m-p}	9.5^{pq}	23.1^{c}
FC-4	35.5^{a}	35.4^{a}	33.5^{ab}	27.5^{d-f}	22.5^{g-j}	14.8^{r-o}	10.8^{op}	25.7^{a}
BC	31.1^{a-d}	30.1^{b-d}	25.0^{e-h}	23.2^{f-l}	17.3^{k-m}	09.2^{pq}	3.0^{r}	19.8^{d}
Mean	32.7^{a}	32.0^{a}	28.2^{b}	25.7^{c}	20.0^{d}	13.0^{e}	7.8^{f}	-

** Each value is an average of three replicates*
** Tukey's HSD (≤ 0.05)=Trt. 1.21, Days 1.55, Trt *Days 4.55*
**SE= Trt. 0.43, Days 0.51, Trt.*Days 1.44*
Means followed by same letter do not differ significantly at 5% level of probability

bacteria. Sugarcane press-mud provides organic material and often suppresses the unwanted contamination which reduces the shelf life of the inoculants (Nagesh et al., 2013). As no single material has ability for maintaining the required population of viable cells for long time, therefore, it can be concluded that a suitable carrier for PGPR can be formulated from different combinations in the range of 40% clay, 30-40% fly-ash, 10-15% press mud and 10-15% lignitic coal with capability of sufficient and acceptable population of bacteria at standard level for longer period of time.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

S. No	Author Name	Contribution to the paper
1.	Mr. Tosif Tabassam	Conducted research and prepared write up
2.	Dr. Tariq Sultan	Conceived the idea, Technical input at every step
3.	Dr. M. Ehsan Akhtar	Conceived the idea, Finalizing the

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4.	Dr. M. Mahmood-ul-Hassan	Data analysis and script writing
5.	Dr. Arshad Ali	Overall management of the article, Results and Discussion

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