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BIOEFFICACY AND SHELF LIFE OF *Rhizobium leguminosarum* LOADED ON DIFFERENT CARRIERS

M.S. Argal*, A.K. Rawat, S.B. Aher[1] and P. S. Rajput[1]

Department of Soil Science and Agriculture Chemistry, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur - 482 004, Madhya Pradesh (India)
[1]*Indian Institute of Soil Science, Bhopal - 462 038, Madhya Pradesh (India)*
*e-mail: *mohakam@rediffmail.com*

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ABSTRACT

The shelf life and bioefficacy of *Rhizobium leguminosarum* loaded on various carriers was assessed under laboratory and field conditions at JNKVV, Jabalpur (India). The carriers tested were lignite, coal, vermicompost, poultry manure, dolomite, Azolla, lignite + coal, lignite + vermicompost, lignite + poultry manure, lignite + dolomite and Azolla + lignite. *R. leguminosarum* culture isolate G_4 was multiplied in YEM broth under aseptic conditions and after specified growth, the rhizobial liquid broth was mixed with eleven carriers, each separately, in a ratio to obtain 40% moisture in the finished product. The initial rhizobial population in the finished product was estimated before final packing and storage. For shelf-life assessment, the samples were drawn from the stored products at 30 days intervals upto 180 days and rhizobial population assessed. The bioefficacy of carrier loaded *R. leguminosarum* biofertilizers were studied in field on chickpea variety JG-74. The nodule number plant^{-1}, nodule dry weight plant^{-1} and nitrogen content in nodules were recorded 45 days after sowing (DAS) while grain yield, total biomass, nitrogen content in seed and straw and total nitrogen uptake were studied at harvest. Lignite proved best carrier followed by coal, while other indigenous carriers failed to support rhizobia. Use of these carrier materials in conjunction with lignite (1:1) did not yield satisfactory results. It may be concluded that vermicompost, poultry manure, dolomite and Azolla cannot substitute lignite as a carrier for rhizobial inoculants, although coal proved safe for this purpose.

Key words: Bio-efficacy, coal, indigenous carrier, lignite, *Rhizobium leguminosarum*, shelf life

INTRODUCTION

Biofertilizers, applied onto either seed or plant surfaces or soil, colonize rhizosphere or the interior of plant and promote growth by enhancing the availability of essential nutrients to the host plant (Vessey, 2003). Biofertilizers are prepared as carrier-based inoculants which enable easy handling, long-term storage and effectiveness. The carrier material used plays vital role in efficiency and effectiveness of a biofertilizer. The carrier material must be non-toxic to bacterial strain, retain good moisture, have high absorption capacity, easy to process and free of lump forming materials, easy to sterilize by autoclaving or γ-irradiation, easily available in adequate amounts, inexpensive and must have pH buffering capacity (Somasegaran and Hoben, 1994). Among the various biofertilizers, bacterial inoculant is a major group which includes N_2 fixing rhizobacteria that fix atmospheric nitrogen symbiotically in leguminous plants (Brown and Walker, 1970). Around 2000 tons of commercial rhizobial inoculants are produced annually in the world (Ben Rebah *et al*., 2007) and applied on seed or in soil to improve crop productivity (Lupwayi *et al*., 2006). Mostly, peat materials are used as a carrier for rhizobia but its limited availability in some part of world has made it expensive (Chao and Alexander, 1984). Since 1971, lignite was used as a carrier material instead of peat for biofertilizer production in India (Saranya *et al*., 2011). Alternative materials such as sawdust (Arora *et al*., 2008), rice husk (Kaljeet *et al*., 2011), fly ash (Kumar *et al*., 2010) and biochar (Saranya *et al*., 2011) have been tested for bio-inoculants preparation. Present study was aimed to examine the shelf life of *Rhizobium leguminosarum* on different carrier materials and assess their performance in chickpea.

MATERIALS AND METHODS

The present work was carried out at JNKVV, Jabalpur which lies $23^{0}10'$N, $79^{0}57'$E and 393 m masl. The site has semi-arid and sub-tropical climate with characteristics dry summer and cool winter.

Preparation of rhizobial inoculants

Eleven carriers used for the preparation of *Rhizobium leguminosarum* inoculant biofertilizer were lignite, coal, vermicompost, poultry manure, dolomite, Azolla, lignite + coal (1:1), lignite + vermicompost (1:1), lignite + poultry manure (1:1), lignite + dolomite (1:1) and Azolla + lignite (1:1). Carrier materials (lignite, coal, vermicompost, poultry manure, dolomite, and Azolla) were obtained from the Biofertilizer Production Centre, JNKVV, Jabalpur (India). The physico-chemical properties of carrier materials were assessed and analyzed according to the standard methods. The pH and EC were determined (1:2 carrier: water suspension) on potentiometer (Page *et al*., 1982). The water holding capacity was determined gravimetrically. The organic carbon content was determined according to wet oxidation method (Walkley and Black, 1934).

Proven isolate of chickpea rhizobial G_4 *(R. leguminosarum)* was procured from Culture Collection of Agriculturally Beneficial Microorganisms, Department of Soil Science and Agricultural Chemistry, JNAU, Jabalpur (CCAM-JNAU). Mother culture prepared by multiplying the strain on YEM broth (Subba Rao, 1977). The quality of incubated broth was tested under ensured aseptic conditions. Rhizobial population in broth was determined by plate count method (Schmidt and Coldwell, 1967). Contaminant, if any, was tested by streaking the incubated broth on Hoffer’s alkaline media. After quality check the incubated broth was mixed with sterilized carriers in a ratio to obtain 40% moisture in the finished product. After mixing, the trays along with impregnated carrier materials, were incubated for 48 h and the finished products packed in low density polythene bags. The packets were sealed with the help of electric sealer. For aeration, 4-5 pin holes were made in each packet.

Assessment of shelf life

To assess the shelf life of *R. leguminosarum* in prepared inoculants, the final products were stored in corrugated boxes at room temperature. The samples were drawn at 30 days interval from inoculants-preparation upto 6 months to determine rhizobial population. The population of rhizobia was determined by dilution plate count method using YEM broth (Hahn, 1966; Subba Rao, 1977).

Bioefficacy of inoculants

The bioefficacy of *R. leguminosarum* loaded on different carriers was studied on chickpea cv. JG-74 under field conditions at Livestock Farm, Department of Soil Science and Agricultural Chemistry, JNKVV, Jabalpur during *rabi* 2009 in a randomized block design with 4 replications per treatment. The nodule number plant^{-1}, nodule dry weight plant^{-1} and nitrogen content in nodules were recorded 45 days after sowing (DAS) while grain yield, total biomass, nitrogen content in seed and straw and total nitrogen uptake were assessed at harvest. For nodulation 5 plants $plot^{-1}$ were uprooted at 45 DAS and number of nodules counted. The separated nodules were dried in hot air oven at 60°C for 3-4 days and weights recorded. For total nitrogen estimation, oven-dried plant materials (grain/straw) were digested in sulphuric acid and suitable digestion mixture ($CuSO_4$, K_2SO_4 and salicylic acid) at temperature between 360 to 410ºC. After complete digestion, the samples were cooled and diluted, to be added to sulphuric acid digest for distillation. The distilled ammonia was quantitatively absorbed in boric acid and titrated against standard sulphuric acid (Parkinsons and Allen, 1975; Bremner and Mulvaney, 1982). The data was analyzed statistically and treatment means compared using LSD techniques at 5% probability level (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Physico-chemical properties of carrier materials

The physico-chemical properties of carrier materials used for the preparation of rhizobial inoculant revealed that the pH of the materials evaluated ranged between 4.4 and 8.4 (Table 1). The pH of lignite was acidic while dolomite was alkaline. The coal, vermicompost, Azolla and poultry manure were neutral in reaction. The electrical conductivity (EC) of materials ranged between 0.7 and 2.25 dS m^{-1} with highest EC in Azolla followed by vermicompost and coal. The lowest EC (0.7 dS m^{-1}) was in dolomite. The organic carbon content ranged between 0.06 and 3.17% with highest organic carbon in vermicompost, poultry manure and Azolla. The water holding capacity (WHC) of carrier materials ranged between 12.6 and 83.9%.

Table 1: Some physico-chemical properties of different carrier materials

Carriers used	Physico-chemical properties			
	pH	Electric conductivity (dS m^{-1})	Organic carbon (%)	Water holding capacity (%)
Lignite	4.4	1.90	2.23	12.6
Coal	7.3	1.95	1.82	23.9
Vermicompost	6.8	2.20	3.17	67.9
Poultry manure	7.6	1.65	3.17	83.9
Dolomite	8.4	0.70	0.06	31.3
Azolla	6.7	2.25	3.17	79.7

Rhizobial shelf life

Shelf life of *R. leguminosarum* loaded on different carrier materials revealed that each standard inoculum initially had a load of 10^8 cell mL^{-1} and the rhizobial population gradually decreased in all the carrier materials on 180 days of storage (Table 2). Out of the 6 carriers and their combinations, coal + lignite (1:1) supported rhizobial population most favourably yielding highest cell count at all stages of shelf life, except at 0 day where it was inferior to lignite. The rhizobial population in individual carriers, irrespective of stage of storage, revealed coal and lignite to be most favourable carriers for rhizobia whereas other carriers like vermicompost, poultry manure, dolomite and Azolla did not retain enough population of rhizobia. When lignite or coal were combined to other carriers, the rhizobial counts were comparatively better. It was surprising to note that the population at 0 day varied in carriers tested when all carriers received the same quantity of broth with similar cell counts. No definite trend could be seen with different treatments at different stages of shelf life.

Rhizobial count in all the carriers tested decreased gradually from the date of inoculant preparation to 180 days. High water holding capacity of charcoal has also been observed by Sudhakar *et al.* (2004).

Table 2: Shelf life of *Rhizobium leguminosarum* ($x10^5$ cfu g^{-1}) inoculated on different carrier materials during 180 days storage

Carrier materials	Storage time (days)						
	0	30	60	90	120	150	180
Lignite	79.31	61.63	18.8	14.45	13.61	1.04	0.75
Coal	77.41	74.87	49.6	7.31	6.48	1.87	1.12
Vermicompost	14.49	9.31	3.78	3.60	1.75	1.19	0.38
Poultry manure	24.62	4.58	4.46	4.23	2.36	0.92	0.78
Dolomite	36.64	6.38	1.46	1.37	1.02	0.34	0.00
Azolla	38.27	22.43	8.83	5.46	2.47	1.16	1.12
Coal + lignite (1:1)	74.87	58.92	15.74	14.70	10.60	3.79	2.59
Vermi compost + lignite (1:1)	15.40	8.95	8.81	7.04	2.18	2.10	1.45
Poultry manure + lignite (1:1)	63.69	28.57	7.87	7.54	4.89	1.60	1.43
Dolomite + lignite (1:1)	33.33	16.81	10.36	2.99	1.87	1.16	0.79
Azolla + lignite (1:1)	43.71	38.77	8.42	8.33	5.77	5.31	1.50
SEm±	14.61	9.88	4.78	3.20	2.28	1.41	0.68
$CD_{0.05}$	46.04	31.19	15.07	10.10	7.19	4.46	2.17

Pandher *et al.* (1993) have reported that lignite and charcoal favoured higher growth and survival of *Rhizobium* as compared to other carriers. The higher population in charcoal and lignite may also be due to their ability to absorb toxic compounds and improve ventilation of inoculant in a better way than other sources (Lal and Mishra, 1998). Coal and lignite yielded satisfactory population of rhizobia and also transmitted satisfactory population on the seed at normal rate application of inoculant (Dubey *et al*., 1975; Singh, 1986). Individually maximum population was supported by coal followed by lignite and their shelf life was better than other carriers which indicated that coal based inoculants are more protective to rhizobia (Crawford and Berryhill, 1983; Pandher *et al*., 1993). Longer storage time of inoculants reduce the inoculum survival (Singh *et al*., 1992). Survival of rhizobia in inoculant is affected by the type of carrier, storage temperature and storage period and their interactions (Robert and Harold, 1983). Johar (1971) evaluated several indigenous materials and observed that after 40 days of storage under sterilized conditions at ambient temperature, decline in population was generally noticed which was comparatively less in lignite. The results of our study are in agreement with Kukreja *et al*. (2004), Kumar and Gupta (2010) and Abd El-Fattah *et al*. (2013).

Bioefficacy of Rhizobium leguminosarum loaded in different carriers

Nodulation: The plants inoculated with lignite and coal based rhizobia showed significant increase in the number of nodules $plant^{-1}$ over uninoculated control while other carrier materials or their combinations failed to respond significantly. Highest nodule number was recorded in case of lignite followed by coal and their combination while numerically there was no difference between dolomite and control. Vermicompost and poultry manure were found better than Azolla. With regard to nodules dry weight, lignite and coal proved statistically superior over uninoculated control while other treatments were at par with control. Dolomite was a little better over control. Among other individual carriers lignite, coal, vermicompost and poultry manure were better than Azolla.

Maximum nitrogen content in nodules was observed in the plants treated with lignite followed by coal, vermicompost + lignite and coal + lignite, while insignificant difference in nitrogen content was noticed within the treatments of vermicompost, poultry manure, poultry manure + lignite, dolomite + lignite. In other cases insignificant differences in nitrogen content were seen within the treatments of control, Azolla and dolomite. The results are in agreement with Khattak *et al*. (2006), Shahzad *et al*. (2008) and Rokhzadi and Toashih (2011). Effective nodulation is dependent not only on the presence of a large number of viable rhizobia on the seed but also on the conditions which favour growth and development of rhizobia on the planted seed in soil (Gupta, 2005; Jadhav *et al*., 1988).

Table 3: Growth parameters of chick pea as influenced by the application of *Rhizobium leguminosarum* loaded on different carriers

Carrier materials used	No. of nodules plant^{-1}	Nodule dry weight (mg plant^{-1})	N content in nodule (%)	Yield (kg ha^{-1})		N content (%)		N uptake (kg ha^{-1})
				Grain	Straw	Straw	Grain	
Control	11.2	1.59	1.64	1413	2575	0.63	2.76	54
Lignite	19.0	5.62	4.71	2000	3588	0.89	3.58	103
Coal	18.1	5.43	4.66	1925	3375	0.89	3.50	97
Vermicompost	16.8	4.47	3.76	1925	3188	0.83	3.25	89
Poultry manure	15.6	3.84	3.60	1875	3138	0.82	3.25	88
Dolomite	11.5	2.35	1.75	1650	2588	0.64	2.89	62
Azolla	12.3	2.46	1.90	1675	2688	0.66	2.97	70
Coal + lignite (1:1)	17.4	4.59	4.24	1938	3325	0.88	3.28	92
Vermicompost + lignite (1:1)	15.4	3.64	4.44	1813	3013	0.75	3.23	82
Poultry manure + lignite (1:1)	14.8	3.40	3.18	1750	2925	0.75	3.15	78
Dolomite + lignite (1:1)	14.6	3.22	3.07	1731	2813	0.71	3.11	73
Azolla + lignite (1:1)	13.0	3.01	2.12	1688	2725	0.71	3.10	71
SEm±	2.06	1.17	-	291.6	186.6	0.08	0.19	5.2
$CD_{0.05}$	6.52	3.71	-	575.5	918.8	NS	0.61	25.2

Straw and grain yield: Maximum straw yield of 3588 kg ha^{-1} was observed in the plants treated with lignite based rhizobia followed by coal-based inoculums, however only lignite was significantly superior over uninoculated control while rest treatments were at par with control (Table 3). Azolla, dolomite and their combinations with other material showed poor performance. Only lignite proved significantly superior over control by yielding 2000 kg seed ha^{-1} while rest treatments were at par with control. Maximum yield was obtained in plants treated by lignite-based rhizobia followed by coal + lignite. The largest benefits of biological nitrogen fixers to agriculture are derived from the symbiosis between legume and species of *Rhizobium*. The field response of *R. leguminosarum* inoculation was quite variable and chickpea reportedly exhibited significant improvement in grain yield by 14-30% over control due to rhizobial inoculation (Biswas *et al*., 2001; Khattak *et al*., 2006, Shahzad *et al*., 2008; Rokhzadi and Toashih, 2011).

Nitrogen content in straw and seed and total nitrogen uptake by crop: All treatments failed to significantly improve nitrogen content in straw. However, maximum value was obtained in plants inoculated with lignite based rhizobia followed by coal, coal + lignite, vermicompost and poultry manure. The other treatments were numerically inferior to these treatments (Table 4). The seed nitrogen content was significantly more in lignite and coal treatments as compared to control, while other treatments were at par with coal. Considering the total nitrogen uptake by crop, it may be concluded that lignite-based rhizobial inoculant gives highest nitrogen uptake followed by coal, coal + lignite, vermicompost, poultry manure and vermicompost + lignite and all these were significant over control. The remaining five treatments formed another group of non-significant status. Lowest nitrogen uptake by crop was recorded in case of dolomite. Different carrier materials have shown variable potential for the survival of inoculated bacteria. Lignite is a widely accepted carrier for inoculant production. Several attempts have been made to find out alternative carriers but lignite maintained its superiority with respect to shelf life and ultimate response on concerned legumes (Jadhav *et al*., 1988; Singua and Hudnall *et al*., 1991; Kaushal *et al*., 1996).

Conclusion: The overall trend indicated that lignite is a best carrier followed by coal, while other indigenously available carriers failed to support *Rhizobium leguminosarum* inoculants. Even when mixing these carriers material (1:1) with lignite did not show much satisfactory results. It may be concluded that vermicompost, poultry manure, dolomite and azolla can not serve as substitute for lignite or coal as a carrier for rhizobial inoculants. Some other indigenously available by-products need to be explored as carriers for microbial preparations.

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