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Impact on the productivity of preparation on rhizobial inoculant carriers

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ABSTRACT: Selection of a suitable carrier material for rhizobial inoculants is essential for biofertilizers production. Locally available wastes or by-products as carrier material will increase the cost effectiveness of the inoculants preparation. Here, were evaluated four such waste materials from local ground *viz.* charcoal, saw dust, garden soil and sugarcane bagasse with carrier based inoculums (10^8 viable cells/ml) and kept at room temperature (30 ± 2^0C). The colony forming unit (CFU) count of each strain in different carriers was monitored every month. The charcoal, garden soil and saw dust resulted to allow a better survival of the inoculums. The viable counts in charcoal, soil, saw dust and sugarcane bagasse after 240 days of storage was recorded as 10^7, 10^6, 10^5 and 10^3 for MPR_8 and 10^7, 10^5, 10^5 and 10^3 for TFR_3 strains respectively. The effects of storage of carrier on plant productivity showed better plant biomass accumulation and nodulation in cases of charcoal, sawdust and garden soil. However it was insignificant with the sugarcane bagasse based inoculants.

Keywords: Carrier material; Inoculants; Biofertilizers; Strain; Biomass; Nodulation.

1. INTRODUCTION

Rhizobia form symbiotic root nodules with various legume plants and have ability to fix atmospheric nitrogen. These bacteria, although present in most soil types, vary in number and beneficial effectiveness of a subsequent crop, which is one of the scientific basis in the agricultural practice of crop rotation. Rhizobia are cultured in the laboratory and mixed with a suitable carrier material, such as peat, charcoal or other locally available materials to formulate an inoculant [1]. A high quality formulation of the inoculum before its application must be maintained and should allow its delivery in a convenient and economy way for obtaining high population of effective rhizobia [2]. Cost effective biofertilizers using optimised media for *Rhizobium* and formulated with coal powder was found highly effective in improving nitrogen content in the soil along with other potential parameters for plant growth in a NPK deficient soil that supports the growth and development of new legume plants [3]. Improving crop productivity through

the practice of transferring productive soil from one field to other dates back to ancient times [4]. After the famous Hellriegel et al. [5] concerning the nitrogen nutrition of leguminous plants, the practice of 'soil transfer' became a recommended method of legume inoculation. *Rhizobium* inoculants for legumes consist of root nodule forming bacteria usually mixed with solid based carrier materials.

In Nepal, the production of bio-fertilizers, started on a very small scale at the National Agricultural Research Council (NARC, Khumaltar, Kathmandu) in 1960. Since then not much has been further achieved if one considers the significance of the technology. Though, peat is universally considered a good inoculant carrier material, it is also not available in Nepal. Kalimati soil (locally available alluvial soil) mixed with charcoal at 3:1 ratio and other soils rich in organic matters are used as carriers. Among the different inputs, chemical fertilizers play an important role in supplying crop nitrogen needs. However, leguminous plants by virtue of their nitrogen fixing ability, when growing in association with proper rhizobia, need very little nitrogen for growth. In the hills or at high altitudes where small farmers can hardly afford to buy chemical fertilizers, legume cultivation is practiced to supply part of the nitrogen needs for the crop.

The excessive use of nitrogenous fertilizer in developing countries created hazards to such an extent that the ground water at several places has been reported unfit for drinking purpose [6]. Increasing population compelled many nations to take necessary steps to increase organic food production by using alternate means. Bio-fertilizers not only augment and increase the nutrient availability but also make the soil vital [7]. Such fertilizers' effect can provide permanent benefits to the soil without any associated problems and can increase soil fertility. The cost involved is quite low and imparts better crop management and provision of additional major nutrients for the plants and inoculum. It has been proved that the bio-fertilizers are cost effective, cheap and renewable source of supplements than chemical fertilizers [8].

Several attempts have been made to improve the quality of soil based inoculants. Sterilisation of the carrier material is important to eliminate competition from fungi and other bacteria, and hence to obtain high numbers of rhizobia. This may be achieved through autoclaving, γ-irradiation, chemical sterilisation or flash drying [9]. The most effective method for sterilisation, through γ-irradiation, is limited due to unavailability of a radiation source in many countries [10]. Autoclaving is perhaps the most effective common method of sterilisation, but requires a packaging material capable of withstanding high temperature conditions while allowing subsequent conservation of moisture and passage of gases [11]. Moisture content affects the ability of a carrier to maintain rhizobial numbers. Rhizobial populations decline more rapidly during storage with decreasing moisture content [12, 13].

The objective when considering inoculation with beneficial bacteria is to find the most potent bacteria available [14, 15] and then a study of the specific inoculant formulation is generally undertaken. In practical terms, the formulation chosen determines the potential success of the inoculant [16]. Many potentially useful bacteria reported in the scientific literatures never appear on the commercial market, perhaps due to inappropriate formulation. During the present study, attempts were made for the formulation of the inoculant with carrier materials available locally. Four different carrier materials available locally. Four different carrier materials were tested charcoal, sawdust,garden soil and sugarcane bagasse during the present investigation. The survival percentage of two rhizobia strains, MPR_8 and TFR_3 was evaluated in the aforementioned four carrier materials during their storage at room temperature for 8 months.

2. MATERIALS AND METHODS

2.1. Rhizobial strains

Strains MPR_8 and TFR_3 were maintained at 4^0C and used for inoculum preparations.

2.1.1. Carrier materials and preparation of inoculants and storage

Late log phase broth cultures of both MPR_8 and TFR_3 strains were prepared (contained 10^8 viable cells ml^{-1} of liquid medium) and injected aseptically into sterilised carriers with the help of a syringe. The colony forming units (CFU) were counted by serial dilution technique on YEMA plates. Charcoal was inoculated with 60 ml/bag; sawdust 120 ml/bag; garden soil 24 ml/bag; and sugarcane bagasse 180 ml/bag. The amounts of the liquid cultures were added on the basis of the water holding capacities of the individual carriers (about ½ of the WHC). The bags were thoroughly kneaded to ensure absorption of the liquid culture into the carrier. The inoculants so prepared were stored at room temperature (30 ± 2^0C) up to 240 days.

2.1.2. Enumeration of rhizobia

Rhizobia in each of the inoculant containing bags were enumerated by plating serially diluted samples of the inoculants on congo red YMA (0.0025% congo red) using spread plate method in triplicate with proper control. The CFU count was done on inoculums stored at room temperature, 30 days after inoculation and then every 30 days up to 8 months. Finally, the identities of the isolates were confirmed by plant infection test on the respective hosts [17]. The rhizobial counts were then transformed (log_{10}) for statistical analysis.

2.2. Effect of inoculant carriers on plant productivity

The impacts on productivity of the carrier-based inoculants were determined in earthenware pots of approximately 1kg soil capacity. The pots were filled with sterilized garden soil. Surface sterilized seeds of *Mucuna pruriens* and *Trigonella foenumgraecum* were sown in the earthenware pots. The 8 month stored inoculants were used to inoculate the plants. The inoculated plants were grown for 45 d and then were uprooted very carefully to measure plant biomass, nodule number and nodule fresh weight. The experiments were carried out in three replicates for each treatment. The results obtained were analysed statistically according to Gomez et al. [18].

3. RESULTS

Four locally available carrier materials (charcoal, sawdust, garden soil and sugarcane bagasse) were tested for their ability to sustain survivability up to 240 days at room temperature (Table 1, Fig. 1). The strains MPR_8 and TFR_3 contained 10^8 viable cells/ml of the broth culture was used as inoculum with each carrier materials. The viable counts in different carrier materials at room temperature increased initially up to 30 days, but on further storage it decreased. In case of sugarcane baggase the reduction was faster than any other carrier. The via after 24 days with respect to the initial microorganisms load. In charcoal, sawdust and garden soil the reduction was by 22%, 44% and 45.0% for TFR_3 and 24%, 38.5% and 35% ble count of MPR_8 was decreased by 66% in sugarcane baggase and 61% in case of TFR_3 and 24%, 38.5% and 35% for MPR_8 strains, respectively. Charcoal resulted to have the highest survival rate at the end of the storage period. In comparison to charcoal, it was lowered by 48% in sugarcane baggase, 29% in garden soil and 26.5% in sawdust in case of the strain TFR_3. The strain MPR_8 showed a decrease of viable counts compared to charcoal by 55% with sugarcane baggase, 16% with the garden soil and 19% with sawdust. The final concentration of viable counts/g of the carrier materials recorded were 10^7 cells/g in charcoal, 10^5 cells/g in sawdust and in garden soil (10^6 cells/g in the strain MPR_8) and 10^3cells/g in sugarcane bagasse in both the strains MPR_8 and TFR_3.

There was a gradual decline in the number of viable counts during increasing length of storage period at room temperature A cfu count of 10^8 viable cells/g was obtained in charcoal up to 180 d in both the strains MPR_8 and TFR_3. Garden soil (MPR_8) and sawdust (TFR_3) also supported similar viable counts per gram but with one order of magnitude less, to about 10^7 cells/g in garden soil (TFR_3) and sawdust (MPR_8) after 180 d of storage. Instead, a sharp decline of the viable counts was observed with sugarcane bagasse, to 10^6 cells/g, for both strains after 180 d (Fig. 1).

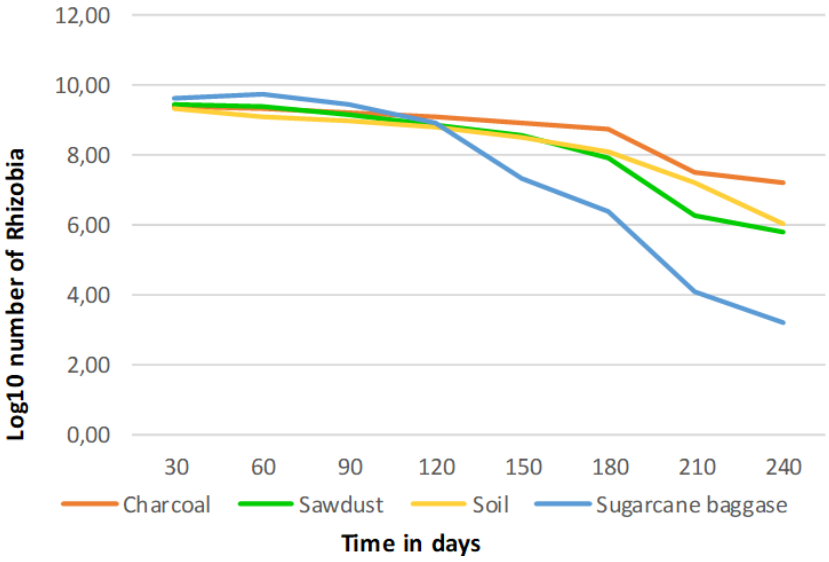

Figure 1. Survival of *Rhizobium* in different carriers MPR_8 and TFR_3.

Table 1. Physical properties of the carrier's materials.

Name of the carrier	pH	Moisture content (%)	Water holding capacity (%)
Charcoal	7.2	5.0	180
Saw dust	6.8	5.15	375
Soil	7.0	0.53	60
Sugarcane baggase	6.9	0.7	625

The application of bio- inoculants induced an increase in plant biomass, nodule number and nodule fresh weight (Table 2 and 3). The increase using MPR_8 on *Mucuna* was 69%, 45% and 47% for charcoal, sawdust and garden soil, respectively, in comparison to that of control. However, the sugarcane bagasse inoculant showed a 23% decreased biomass accumulation with respect to control (Table 2). Similarly, the nodule number per plant in *Mucuna* showed no significant differences in case of charcoal, sawdust and soil based inoculants. *R. meliloti* TFR_3 inoculated to *T. foenumgraecum* induced an increase in biomass by 54%, 29%, and 21% for charcoal, sawdust and garden soil, respectively, compared to control. However, 37% reduction of biomass accumulation was observed in case of sugarcane bagasse based inoculant as compared to control. The nodulation by charcoal, sawdust and soil based inoculants were found to be almost similar which was better than the control.

Table 2. Effect of application of 240-old bio inoculant of MPR_8 on plant biomass, nodule number and nodule fresh weight after 45 d of plant growth.

Inoculant	Plant biomass (g)*	Average nodule No./Pl*	Avarage fresh wt. of nodule/pl (g)*
Control	2.34±0.256	0	0
Charcoal	3.95±0.207	32	1.17
Saw Dust	3.38±0.177	27	0.994
Soil	3.42±0.269	29	0.899
Sugarcane baggase	1.73±0.094	13	0.312

Each value is mean of 3 replicates ±SD. *Results are significant at p≤0.01 level of probability.

Table 3. Effect of application of 240 d stored bioinoculant of TFR_3 on plant biomass, nodule number and nodule fresh weight after 45 d of plant growth.

Inoculant	Plant biomass (g)*	Average nodule No./pl*	Avarage fresh wt. of nodule/pl (g)*
Control	0.45±0.131	0	0
Charcoal	0.695±0.012	19±1.41	0.589±0.036
Saw Dust	0.586±0.012	16±2.16	0.468±0.066
Soil	0.541±0.017	16±1.63	0.465±0.051
Sugarcane baggase	0.28±0.041	7±2.16	0.196±0.044

Each value is a mean of 3 replicates ±SD. *Results are significant at p≤0.01 level of probability.

4. DISCUSSION

The viable cell counts of both the strains MPR_8 and TFR_3 remained more than 10^8 cells/g of the carrier up to 180 d but there was a dramatic reduction after 210 d. Among all materials tested, charcoal proved to be the most suitable carrier, holding the maximum number of viable counts up to 240 d. Similar results were obtained for *Rhizobium phaseoli* [19]. To obtain the maximum benefits from legume inoculation technology, the inoculum must contain high populations of viable rhizobia [20]. Nair, et al. [21] explained that as regards the influence of different carriers on the survival of rhizobia [22].

It has been reported that rhizobia survive better under refrigeration than at room temperatures [23, 24]. But the facility of refrigeration is not easily available in the developing countries including Nepal, therefore, good survival of the inoculant strain at room temperature constitute a desirable property. The carrier material should have a rhizobial cells number of at least 5×10^8/g [25], but minimum standards for viable rhizobia vary in different countries. During the present investigation the viable cell count was higher than 10^8 viable cells/g for up to 150 days in all four carriers taken. A similar result was observed by Muniruzzaman and Khan [26]. However, in many countries like Thailand or Russia [27], 10^7 viable cells/g or more is taken as a standard. In the western countries, peat was commonly used as a carrier of *Rhizobium* sp. for commercial legume inoculants production. However, its unavailability has prompted the use of alternate materials [27-30]. Wastewater sludge, a worldwide recyclable waste, has shown good potential for inoculant production as a growth medium and as a carrier (dehydrated sludge) which usually contains nutrient elements at concentrations sufficient to sustain rhizobial growth and heavy metals are usually below the recommended level [31]. The capacity of soil to support the survival rhizobia implies that mineral soils, could substitute for peat if amended with organic carbon [32]. The fact that charcoal supported acceptable numbers of viable cells of *R. phaseoli* CIAT 75 and 650 R at 25^0C but not at 4^0C contradicted previous reports where in the survival of rhizobia under refrigeration was better than at or near room temperature. In the

present study two indigenous rhizobial strains *R. meliloti* MPR_8 and *R. meliloti* TFR_3 isolated from *Mucuna pruriens* and *Trigonella foenumgraecum* respectively survived at room temperatures up to 240 d in charcoal which proved the best among the four carriers tested on the basis of periodical viable counts. The sequence of treatment success with different carrier on both the strains tested was found to be charcoal > sawdust = mgarden soil > sugarcane bagasse (LSD = 1.59; P = 0.01 for MPR_8 and LSD = 2.10; P = 0.01 for TFR_3).

During the present study, it was observed that charcoal supported better survival of both the strains MPR_8 and TFR_3 throughout the storage period. In case of MPR_8 the viable count was 10% more in charcoal than that of sawdust after 180 d but it was 8% less for the strain in garden soil. When charcoal, garden soil and sawdust carriers were compared with each other it was observed that all the three carriers showed almost similar viable counts throughout the experimental period. It was revealed that there was only 11% less viable counts in sawdust compared to charcoal for the strain MPR_8 and 10% less in case of the strain TFR_3. Similarly, in garden soil and sugarcane bagasse the reduction was 8% and 27% for the strain MPR_8 and 22% and 11% for the strain TFR_3 respectively. The genetic superiority and better adaptability of rhizobial strains to a particular soil type are also considered as significant parameters that influences inoculant performance.

The major challenge in the inoculant industries at present is to develop the improved carrier materials that can sustain a high shelf life for comparatively longer duration of time, protection against hostile soil environments, easy to use and cost effectiveness [33]. Since a century, the bio-inoculants have been in the market but their present availability with respect to chemical fertilizer is still very low [34].

Long-term rhizobial survival in the carrier inoculant preparations includes lignite that promoted rhizobial population [33]. Sugarcane bagasse could not hold the good survival of rhizobial cells probably due to high contamination with fungi and their competition with the rhizobial cells. The inoculants should contain a minimum of 10^8 viable cells/ml within 15 days of manufacture and 10^7 viable cells/ml within 15 days before expiry i.e. after 6 months.

Various workers [35-37] found high count of rhizobial cells in inoculants at temperature range between 28-32^{0}C. The carriers with inoculum in the present study were stored at room temperature (30 ± 2^{0}C). In the present study, the effect of carrier based inoculants after storage on the productivity of *Mucuna pruriens* and *Trigonella foenumgraecum* was determined *in vivo*. Plant biomass, nodule number and nodule fresh weight were reported to be maximum with inoculants formulated with charcoal, sawdust and garden soil. Several workers have reported an increase in yield of the legumes when inoculated with carrier-based inoculants [38, 39]. Very recently, biochar, a charcoal produced from plant matter, positively affected plant growth metrics, root characteristics, and the chemical composition of plants supplied with N-free nutrient solution [40]. Similar increase in soybean yield when inoculated with peat-based inoculants was observed by [41, 42]. Arora et al. [43] emphasized the importance of specific rhizobia bioinculants for the legume crops.

5. CONCLUSION

The selection of suitable strains of *Rhizobium* is the basis to the process of inoculant production and commonly demands specific cultures for species, groups of species or even varieties in the one-inoculum group. However, there is very little information explaining their superiority at the genomic level. Presently, some works in this line explaining how their genomes may influence t Increasing rhizobia cells concentration per unit seed up to ×3 (cowpea) and ×4 (bean) improves response to inoculation and grain productivity suggesting a need to change product formulation or increase inoculation rate inoculant performance are ongoing. Legume inoculants should contain sufficient viable rhizobia so that the intended host is satisfactorily

nodulated in a *Rhizobium*-free soil, or the inoculant rhizobia can effectively compete with indigenous rhizobia in soils where the crop has previously been grown.

Insufficient knowledge and understandings exist concerning the responses of the micro-symbiont and host to select an inoculant standard that would achieve successful nodulation by the inoculum strain under all conditions. The development of more effective and specific inoculant with specific strain of *Rhizobium* targeting on specific soil type, environment and host plant are the new lines of research needed for this technology.

The present study reports the development of bio-inoculants that can be used in increasing the legume productivity, restoration of soil fertility, reclamation of the barren lands as well as the use of the inoculants for the high altitude legumes where the soil nitrogen content in low due to leaching by surface runoff water resources.

Authors' Contributions: SPP designed, conducted and interpreted the experiment, BDD and NP interpreted the results, statistical analysis and wrote the manuscript, and VRP help for correction of manuscript. All authors read and approved the final manuscript.

Conflict of Interest: The author has no conflict of interest to declare.

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