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Studies on survival of *Rhizobium* in the carriers at different temperatures using green fluorescent protein marker

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The present work was undertaken to use green fluorescent protein marker to monitor population of *Rhizobium* transformant in low-cost carriers in comparison with lignite. The viability of the *Rhizobium* transformant carrying the reporter gene *gfp*, was studied in four different bioinoculant carriers at various temperatures. The viable count of *Rhizobium* transformant was minimum in groundnut shell carrier and maximum in lignite, at all temperatures. Lignite was used as a standard carrier to assess suitability of three available low-cost carriers, viz. paddy husk, sawdust and crushed groundnut shells. These low-cost carriers are byproducts of agro-based industries. In this study attempts have been made to use these byproducts as carriers, thus exploiting the wastes to their full potential. Paddy husk proved to be a good protective material compared to sawdust and crushed groundnut shells at all temperatures. The reporter gene, *gfp*, can be used as marker for monitoring the bioinoculants.

SCIENTISTS have typically relied on antibiotic resistance as the marker for their introduced micro-organisms. By culturing field samples on media containing the appropriate antibiotics or other selective agents, selection and enumeration of the introduced bacterium resistant to the selective agent are often possible[1–3]. This popular technique is simple, rapid, selective, and inexpensive and has been used successfully with samples from various environments. There are, however, several drawbacks to this approach, since there frequently exists high background microbial populations resistant to the antibiotics used as selective agents, the limit of detection may be unacceptably high[4]. Researchers are trying for unambiguous metabolic and/or physical markers.

In recent years, molecular tools have been developed to identify and quantify specific microbial cells or specific microbial activities in mixed populations. These tools are especially valuable for analysis of specific bacteria in complex environmental samples. Increasingly, research has concentrated on the development of marker genes for tagging a particular bacterial species of interest, so that the cells can be specifically identified and monitored[5–7]. For example, the genes encoding bacterial Luciferase (*Lux AB*) or firefly Luciferase (*Luc*) have been found to be very useful markers, since tagged cells can be detected on the basis of their bioluminescent phenotype. Other example of genes specifically used to mark bacteria include metabolic markers, such as *Lac ZY* (β-galactosidase and lactose permease), *Gus A* (β-glucuronidase), and *xylE* (2,3-catechol deoxygenase), which are detected on the basis of unique coloured products formed after growth of the cells on specific media[8–10].

There was a tremendous burst of interest in the scientific community when the gene encoding green fluorescent protein, *gfp*, from jellyfish *Aequorea victoria*, was cloned and expressed in *Escherichia coli* and in a range of cell types. An advantage of *gfp* is that, unlike other biomarkers, it does not require any substrate or additional cofactors for fluorescence[11].

Peat and soil rich in organic matter are generally used in the preparation of legume inoculants and constitute a suitable carrier for the purpose[12]. For the execution of healthy inoculation practice, besides manufacturing efficient *Rhizobium* inoculants, it is equally important to maintain the viability of *Rhizobium* cells at a satisfactory level for a longer period of storage. Some works related to different factors affecting longevity of rhizobial cells, e.g. temperature, moisture and carriers have been carried out. Peat possesses a privileged position in the manufacturing of legume inoculants because of many advantages associated with it. However, there are no large-scale deposits of peat in India and therefore any commercial exploitation seems to be a remote possibility. Lignite is now regarded as a standard carrier, and also adopted for commercial production of legume inoculants, as well as for other nitrogen-fixing cultures in India.

The present communication deals with use of green fluorescent protein (GEP) marker to study viability of *Rhizobium* in low-cost carriers, viz. paddy husk, sawdust and groundnut shells in comparison with standard lignite carrier.

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Rhizobium sp. of cowpea group isolated from root nodules of *Dalbergia melanoxylon* was maintained on yeast extract mannitol agar (YEMA) with congo red. *E. coli* with genetically engineered GFP plasmid, procured from School of Biological Sciences, University of Surrey, UK, and the *Rhizobium* transformant obtained by transforming the rifampicin-resistant mutant of *Rhizobium* with GFP plasmid from *E. coli*, were maintained on Luria Bertani (LB) medium[13] containing 100 μg/ml ampicillin.

Transformation of *Rhizobium* sp. was carried out with a modified method of Kiss and Kalman[14]. Overnight growth (10^6 cells/ml) of *Rhizobium* sp. was inoculated into 100 ml of LB broth containing 50 μg/ml ampicillin and incubated on shaker at 200 rpm for 6–8 h at 37°C. Cells were harvested by centrifugation at 8000 rpm at 4°C and resuspended in chilled 20 ml mixture of 150 mM $CaCl_2$ and 100 mM $MgCl_2$ solutions, 10 ml each, and kept at 4°C for 10 min. The cells were harvested by centrifugation and resuspended in 1 ml mixture of 150 mM $CaCl_2$ and 100 mM $MgCl_2$ solutions, 0.5 ml each, after chilling for 30 min. 100 ml of *gfp* plasmid preparation in TE buffer was added. The preparation was incubated at 4°C for 60 min and subjected to heat shock at 42°C for 2 min in a water bath. The mixture was then incubated on ice for the next one hour. Bacteria were then plated on LB agar for scoring the total number of cells surviving the treatment. *Rhizobium* transformant was selected on LB agar containing 100 μg/ml ampicillin and 50 μg/ml rifampicin subsequently confirmed by fluorescence.

Four carrier materials, viz. lignite, sawdust, powdered groundnut shells and paddy were oven-dried, crushed and passed through a sieve of mesh size < 1 mm. The pH of lignite was neutralized with 10% $CaCO_3$. These carriers were sterilized in an autoclave (121°C, 1 h) for three consecutive days. *Rhizobium* transformant was cultured in LB with 100 μg/ml ampicillin. After growing for 24 h incubation at 37°C on a rotary shaker, the bacterial suspension was centrifuged at 7000 rpm for 20 min and resuspended in sterile demineralized water and mixed with carriers uniformly. Each carrier was stored in a polythene bag and incubated at 28, 37 and 40°C in the incubators up to 22 weeks. The viable count of *Rhizobium* was monitored periodically at all treatments by conventional viable plate count method using LB medium with ampicillin (100 μg/ml). Each data point in the figure represents average of three replicate observations. The standard deviations were all within 7% of the mean values presented.

The *Rhizobium* isolate (cowpea group) was successfully transformed with a mixture of 150 mM $CaCl_2$ and 100 mM $MgCl_2$ solutions, using heat shock at 42°C. Figure 1 indicates that the paddy husk is a good carrier at par with lignite. However, there was significant decline in the *Rhizobium* transformant population 50 days onwards in paddy husk. The groundnut and sawdust were relatively inferior carriers. The suitable temperature for survival of the transformant in paddy husk was found to be 28°C, which was similar at uncontrolled room temperature. However, there was successive decline in the counts of the cells when the temperature was raised to 37°C or 40°C (Figure 2). Figure 2 shows that the transformant remained almost at constant level for about five weeks from the date of manufacture in paddy husk carrier. From seven weeks onward, there was gradual reduction in cell number. Several indigenously available carriers have been compared with American peat for their ability to support the growth of *Rhizobium japonicum*[15]. Among the different carriers, combination of Indian peat soil, farmyard manure (FYM), compost or pressmud with charcoal gave higher rhizobial count than individual carriers. At the end of one month the population of rhizobia started declining at 30°C (ref. 16).

Roughley and Vincent[12] have reported optimum moisture levels of 40–50% for maximum survival. But in the present investigation, in case of paddy husk, groundnut shell and sawdust the moisture content was around 40%. Bowen and Kennedy[17], working on both tropical and temperate legumes, reported that temperate strains of *Rhizobium meliloti* were most tolerant to heat (35.5 to

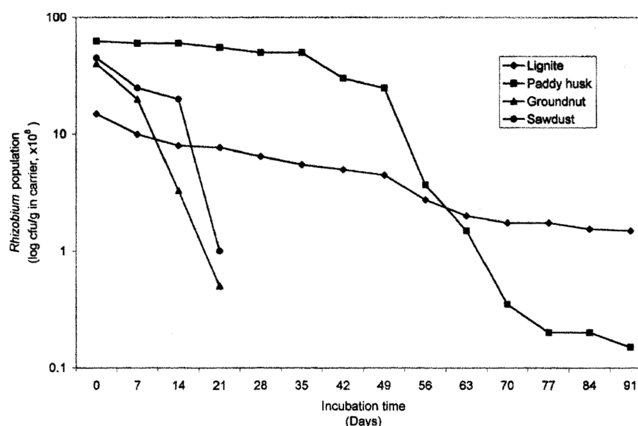

Figure 1. Survival of *Rhizobium* transformant in different carriers at 28°C.

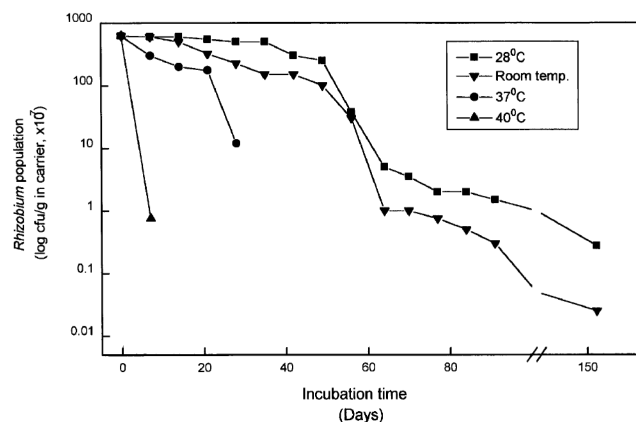

Figure 2. Survival of *Rhizobium* transformant in the carrier, paddy husk, at different temperatures.

42.5°C) than *Rhizobium leguminosarum* and *R. trifolii*. Brockwell[18] observed that up to 40°C, there was no serious mortality, but beyond this temperature mortality rate was very high. It was reported that counts of *R. japonicum* at temperature ranging from 28 to 35°C were appreciable, while at a temperature of 40°C the mortality rate was high[19]. But in present investigation, survival rate of *Rhizobium* transformant was minimum at higher temperatures, i.e. above 28°C in the case of paddy husk, but in the case of sawdust and groundnut shell the count of the cells declined after two weeks. The higher temperatures affect the longevity of transformant in all carriers as shown in Figure 2.

The viability of *Rhizobium* transformant carrying reporter gene *gfp* was found to be good, since its stability was recorded up to 154 days. The *gfp* plasmid does not require any other substrate for fluorescence like other markers. Moreover being a non-conjugal plasmid, spread of the antibiotic marker encoded on it is highly restricted. It can be concluded that GFP can be used as a marker for monitoring the survival of *Rhizobium* sp. in bioinoculants. The paddy husk is a very good alternate low-cost carrier at par with conventional lignite carrier.

Peat and lignite, though good carriers, are not easily available and are expensive in India. Paddy husk, sawdust and groundnut shells are agricultural and industrial waste products. The low cost and easily available paddy husk can be used as a carrier and reporter gene *gfp* can be used as a marker for monitoring bioinoculants.

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Plant regeneration from immature cotyledon-derived callus of *Vigna unguiculata* (L.) Walp (cowpea)

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An efficient plant regeneration protocol from immature cotyledon-derived callus was standardized for cowpea (*Vigna unguiculata* (L.) Walp). Callus cultures were initiated from immature cotyledon explants on MS + B5 medium containing IBA and Kn, each at 2.0 mg l^{-1} concentration. These cultures on transfer to a medium containing 0.1 mg l^{-1} zeatin produced adventitious shoots. The regenerated plantlets were then transferred to root induction medium containing IBA. Histological analyses were made to confirm organogenesis from callus.

GRAIN legumes in general, and *Vigna* in particular, are recalcitrant in nature. As a consequence, genetic transformation is hard to achieve. In most cases where transgenic grain legumes have been obtained with reasonable efficiency, regeneration of shoots involved a callus phase[1]. There have been several reports on plant regeneration through organogenesis in different explants of cowpea[2–11]. However, limited success has been achieved for shoot production via callus phase in cowpea. Pellegrineschi[10] reported regeneration of shoots from callus cultures of hypocotyl in the presence of 0.1 mg l^{-1} zeatin. Pandey and Bansal[3] induced organogenesis from leaf callus of cowpea in the medium containing IBA (10 μM) and Kn (10 μM). Development of reliable protocols for regeneration of grain legumes was reported in soybean[12], black gram[13], mungbean[14], common bean[1,15], tepary bean[16] and pigeonpea[17]. In the present study, a protocol for regeneration

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