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Effects of carrier, sterilisation method, and incubation on survival of *Bradyrhizobium japonicum* in soybean (*Glycine max* L.) inoculants

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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: perlite with pH adjusted with calcium carbonate or charcoal, 1:4 mixtures of perlite and malt residue, sugarcane bagasse, coal, and rice husk. We also contrasted sterilisation procedures (autoclaving or gamma irradiation) and incubation after injection (with or without initial two weeks incubation at 28 °C) for these various carriers. Survival of *Bradyrhizobium japonicum* strain CB1809 was monitored over a period of 6 months upon storage at 4 °C. Most carriers evaluated, were able to maintain rhizobial populations of more than 1×10^9 rhizobia per gram of inoculant over that time period, with mixtures of perlite with either sugarcane bagasse or malt residue supporting the largest rhizobial populations and a mixture of perlite and rice husk the lowest. All carriers supported rhizobial growth over the 6 months period. Initially, rhizobial populations were greater with gamma irradiation than autoclaving, however after 6 months, this response was significant only with the perlite and sugarcane bagasse mixture. The incubation of the inoculant after injection also ultimately did not benefit rhizobial levels for any of the carriers, tested. Using simple sterilisation procedures and without incubating after injection, perlite based carriers can produce high quality inexpensive inoculants, maintaining bacterial populations of more than 1×10^9/g rhizobia for at least 6 months.

Keywords: *Bradyrhizobium japonicum*; Carrier; Incubation; Soybean (*Glycine max* L.) inoculant; Sterilisation

1. Introduction

Rhizobium inoculants, with a high number (10^9/g) at the right time and for a 3–6 month storage period, and a suitable carbon source, are necessary for biological nitrogen fixation, with 2000 tonnes of inoculants sufficient to inoculate 20 mha of agricultural lands, worldwide [1]. Inoculants are commercially available as solid products, powder, produced from peat, or as granular form, or liquid inoculants using broth medium [1,2].

For laboratory usage the standard medium including a carbon source (manitol), a nitrogen source (yeast extract), growth parameters and mineral salts is used, which is expensive at a large or industrial scale [1]. In many countries, the local development of commercial rhizobial inoculants is limited by technological limitations or the scarcity of local sources of peat, the most commonly used rhizobial carrier in countries including the USA, Canada, Australia, and Russia [3,4], currently limiting the production of soybean inoculants in several developing countries, and hence they should be imported.

The number of rhizobia, required for inoculation is dependent on plant seed size and also the rate of inoculation, suggested by the manufacturer [1]. The development of locally produced inoculants is desirable, due to adaptation to local conditions. To do so, it is necessary to find carriers and preparation methods that are widely available and accessible locally.

A suitable rhizobial carrier should have a good water holding capacity, good aeration characteristics, support bacterial growth and survival, be non-toxic, easily sterilized, manufactured, and handled in the field, environmentally friendly, and have good storage quality. In addition, it is important for its production and utilisation to be easily converted to powder, mixable, packageable, adhere to seeds, be available as powder or granule, easily release rhizobia in the soil, and be inexpensive [4–7].

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Briefly, three general categories of alternative materials are used: (i) soil materials including coal or mixtures of coal and other materials, mineral soils and mixtures of mineral soils and other materials; (ii) plant materials including bagasse, rice husk, and other plant composts; and (iii) neutral ingredients including vermiculite, mixtures of vermiculite and other materials, perlite, rock phosphate, calcium sulphate, and some polyacrylic gel synthetics [1,4].

The goal of our study was to develop inexpensive and simple soybean rhizobial inoculants for developing countries. We tested the potential carriers of various materials, widely available in several developing countries including perlite, coal, sugarcane bagasse, rice husk, and malt residue. We also investigated the potential of simplifying the inoculant preparation by using autoclaving instead of gamma-ray irradiation, and by eliminating the incubation period after injection of rhizobia in the sterilised carriers.

2. Materials and methods

2.1. Carrier preparation

Perlite was used as the core carrier material and evaluated in various combinations with coal, sugarcane bagasse, rice husk, and malt residue. Perlite is an organic stone with volcanic origin and made of aluminimm silicate with little hydration. For commercial use it is sterilized using exfoliation at high temperatures. If necessary, sterilization process can be easily performed without producing any unfavourable substances Perlite (Rose Granite Co., Tehran, Iran; http://www.Macraesbluebook.com) contained (%) 70–80 SiO_2, 12–16 Al_2O_3, 2–5 Na_2O, 2–5 K_2O, 0–1 MgO, 0–1 FeO_3, 0–1.5 S and SO_3 [8]. With respect to the use of little inoculants per hectare (500–1000 g/ha, depending on the quality of the inoculants and the amount of seed) and the calcareous soils of developing countries (High CEC), it would not affect soil pH.

Materials were first powdered and passed through a 100-mesh sieve for physico-chemical characterization before being evaluated (Table 1). The main criteria used for their selection were possible pH adjustment to neutral value, maximum water holding capacity, cost, and availability. Six different mixtures were tested, namely: (1) perlite with pH adjusted to 7.0 using calcium carbonate ($CaCO_3$), (2) perlite with pH adjusted to 7.0 using charcoal, to adjust pH, 0.15 g of charcoal was thoroughly mixed with 5 g of perlite, then 10 ml of distilled water was added, and was completely shaken and eventually the pH values were measured. It is worth mentioning that the values used for charcoal and perlite had been previously established using different experiments. The 1:10 pH of charcoal was 8.59, (3) a 1:4 mixture of perlite and malt residue, (4) a 1:4 mixture of perlite and sugarcane bagasse, (5) a 1:4 mixture of perlite and coal, and (6) a 1:4 mixture of perlite and rice husk.

2.2. Carrier sterilisation procedure

Fifty grams of each of the six carriers were put into 50 cm × 16 cm polypropylene plastic bags with a thickness of 0.08 mm. Packages were sterilised with gamma irradiation (5 Mrad) or autoclaving. Autoclaving was done by first saturating the carriers with yeast extract mannitol broth [9] to 40% of the water holding capacity. The open end of each bag was folded back to produce a narrow flap, 4 cm in width, which was held in place by two no. 1 paper clips. The bags were arranged in rectangular wire baskets with sufficient space for steam circulation between bags. The carriers were sterilised for 40 min at 121 °C and 1.05 kg cm^{-2}. Bags were removed for sealing in a laminar flow hood after overnight cooling in the autoclave.

2.3. Inoculant preparation, incubation, and storage

The *Bradyrhizobium japonicum* strain CB1809 (USDA136) obtained originally from the CSIRO collection (CSIRO, Canberra, Australia) was used in this study. Previous research has shown this strain to be suitable for soybean inoculant production, and to be highly effective for the soil and climatic conditions prevailing in several developing countries, as well as with soybean varieties used locally (e.g., [10–12]). It is thus the strain currently recommended for soybean inoculant production in several countries including, among others, Australia and Iran [12]. The strain was cultured in a yeast extract mannitol broth to the late log-phase culture. The culture was injected aseptically into sterilised bags containing the carriers to be tested using an automatic syringe injector [9]. Bags were then thoroughly kneaded and incubated at 28 °C (except in treatments where incubation was to be omitted) for 2 weeks before being stored at 4 °C.

2.4. Experimental design

In a first experiment, the capacity of the six carriers to support adequate populations of *B. japonicum* was evaluated. To do so, number of rhizobia in inoculants was determined using enumeration by plating serially diluted samples of each inoculant on Congo red YMA (25 μg Congo red dye ml^{-1}) using the spread plate method [9]. Number of rhizobia was determined at nine-time periods: 0, 7, 14, 30, 60, 90, 120, 150, and 180 days after rhizobial injection. Each treatment was replicated three times in a completely randomised design. In this initial experiment, carriers were sterilised using gamma irradiation and were incubated after rhizobial injection.

In a second experiment, methods of sterilisation of carrier materials were compared. Each of the six carriers was either sterilised by gamma irradiation or autoclaving. Treatments were evaluated by determining number of rhizobia at nine-time periods as described above. Each treatment was replicated three times in a factorial experiment combining carriers and sterilisation methods. All carriers were here incubated after rhizobial injection.

Finally, in a third experiment the requirement for incubation following injection was determined for each carrier. Each of the six carriers was either incubated or not, following injection of the rhizobia as described earlier. Treatments were evaluated by determining number of rhizobia at nine-time periods as described above. Each treatment was replicated three times in a factorial experiment combining carriers and incubation. All carriers were here sterilised using gamma irradiation.

2.5. Statistical analyses

For each experiment, at each time period, treatments effects and interactions were determined using the General Linear Model procedure of SAS [13].

Table 1
Chemical and physical characteristics of materials used as carriers

Material	N (%)	P (%)	K (%)	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})	EC (dS m^{-1})	pH	OM (%)	WHC (%)
Perlite	0.02	0.01	0.05	190	9	1	2	0.37	5.70	0.1	400
Coal	0.00	0.10	0.12	3455	55	6	30	1.04	7.51	38.2	20
Malt residue	2.17	0.21	0.05	912	300	18	218	2.30	5.10	54.2	80
Rice husk	0.45	0.04	0.20	810	275	2	19	4.20	5.20	42.1	60
Sugarcane bagasse	0.42	0.04	0.16	2370	44	5	26	1.62	7.34	53.0	200

EC: electrical conductivity; OM: organic matter; WHC: water holding capacity.