



## A simple, quick and contamination-free method for mass-multiplication of plant-beneficial microbes by small and marginal farmers using coconut water and rice gruel medium

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### ABSTRACT

A simple method for mass-multiplication of microbial inoculants is reported which can empower small and marginal farmers, or group of farmers, to mass-multiply by themselves plant-beneficial microbial inoculants of bacterial plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas* spp., *Bacillus* spp., free-living nitrogen-fixers like *Beijerinckia* spp., *Azotobacter* spp. and phosphate solubilizing bacteria like *Bacillus megaterium*) and fungal antagonists/entomopathogens such as *Trichoderma* spp., *Metarhizium anisopliae*) origin. The mass-multiplication medium is based on mixture of rice gruel and mature coconut water in a set ratio, along with microgram quantities of biochar filled in pressure cooker. The method yields contamination-free liquid bioinoculant containing sufficient viable cells (as per BIS norms) within 48 hr for bacteria and 4-7 days for fungal bioinoculant for immediate application (seed or seedling treatment and soil application) by the farmers. The method requires supply of proven contaminant-free and pure bioinoculant culture to farmers in pre-filled and stoppered syringes with long needle (5 or 10 ml volume) or pre-filled ink-filler from reliable and responsible agencies for mass-multiplication. The procedure requires little technical expertise and could to a large extent emancipate farmers from depending on bioinoculants available in agriculture stores in markets, which often suffer from contamination and below par viable inoculant population.

**Key words:** Biochar, Bioinoculant, Farmer, Ink filler, Mass-multiplication, Mature coconut water, PGPR, Plant-beneficial microbes, Pressure cooker, Rice gruel, Syringes

The biofertilizer or bioinoculant technology containing plant beneficial microbes such as nitrogen-fixers, phosphate solubilizers, plant growth promoting rhizobacteria (PGPR), arbuscular mycorrhizae, antagonistic and entomopathogenic fungi mixed with solid or liquid carrier are becoming an important input for practising safe and ecological agriculture (Vessey 2003, Bhardwaj *et al.* 2014, Sinha *et al.* 2014). The demand for biofertilizer has increased day by day and the production level of biofertilizer in 2012-13 had jumped to 65527 Mt, almost triple when compared to 25005 Mt in 2008-09 (Indian Fertilizer Scenario 2014, Dept of Fertilizers, GOI). Biofertilizer/bioinoculants are currently available as powder or liquid formulations (Bashan *et al.* 2014) from counters selling agriculture-related products. The plant-beneficial bacteria or fungi are usually mixed with carriers such as lignite, talc or appropriate liquid medium and sold to

farmers for their use. Many issues hamper the performance of the biofertilizers (Raychaudhuri 2004, Rupela 2004); two most critical ones being- i) contamination of carrier- based bioinoculant with other bacteria/or fungi and ii) lower levels of population of viable inoculant bacteria or fungi present in the carriers than the prescribed at the time of use by the farmers.

These problems arise because of the following reasons: i) lack of aseptic environment while preparing or mixing the inoculum, ii) atmospheric variables such as temperature and humidity affecting the viability of the inoculants, iii) improper packaging, handling, transporting and storage of the bioinoculants, etc. Farmers are the main sufferers because of these problems as it leads to substantial fall in the efficacy of the bioinoculant resulting in their below par performance. This also affects the economy of the farmers to a large extent, particularly those of small and marginal ones who constitute bulk of the farming community in India. Moreover, with emphasis on organic farming increasing with each passing day, spurious bioinoculants are flooding the markets that undermine this important agricultural biotechnology. To overcome these issues and empower the small and marginal farmers for mass-producing bioinoculant

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themselves, a simple, quick and contamination-free method using household kitchen wastes as liquid substrates, pressure cooker, etc. is reported in this communication.

The methodology involves productive utilization of plant-based wastes such as rice gruel (starchy solution decanted after cooking rice with extra amount of water, which usually is thrown out) and mature coconut water (which goes as waste after coconut kernel is taken out) produced in farm households. To provide large surface area in this liquid medium for the bacteria/fungi to grow, biochar produced from tender or immature coconut husk (Gopal *et al.* 2016) was also added in the liquid medium. Perusal of the Indian Fertilizer Scenario 2014 (Dept. of Fertilizers, GOI) document clearly indicated that almost 50% of the biofertilizer production and consumption is in the southern states of India. Besides them, Maharashtra and West Bengal are two other states with substantial biofertilizer production and consumption. Incidentally all these states have large areas under coconut cultivation and rice is the staple food for the population there. This means, availability of rice gruel and mature coconut water will not be a limiting factor for this technology. Use of coconut water for production of PGPR (Anith 2009, Anith *et al.* 2014) and entomopathogenic fungi such as *Metarhizium anisopliae* (Dangar *et al.* 1999, Gopal *et al.* 2006), *Beauveria bassiana*, *Verticillium lecanii* and *Paecilomyces fumarosus* (Sahayaraj and Namasivayam, 2008) and *Nomuraea rileyi* (Tin Cilley *et al.* 2004) is well documented. Similarly, the use of rice gruel as liquid substrate for production of mostly entomopathogens such as *Nomuraea rileyi* (Ramegowda *et al.* 2007) and biofertilizers (Princy *et al.* 2014) and regeneration of phosphobacteria (Gomathy *et al.* 2007) is reported as well. On the other hand, biochars which are a recent development have also been found to be a good solid carrier for biofertilizers (Beck 1991, Saranya *et al.* 2011, Hale *et al.* 2015).

#### MATERIALS AND METHODS

The following materials are required for the methodology- i) pressure cooker, ii) rice gruel which is a waste produced from parboiled rice cooked in traditional Indian method, iii) mature coconut water which is a waste produced from mature coconut in homes using coconut for preparing daily food, iv) powdered biochar or charcoal commonly available in farm households, v) bioinoculants, bacteria or fungi or entomopathogen in pre-filled 5-10 ml plastic syringe or ink-filler, vi) LPG cylinder, vii) gas stove, and viii) candle or bunsen burner. Supply of bioinoculant culture to farmers in pre-filled syringe or ink-filler (5-10 ml capacity) having a stoppered clog-free nozzle or blunt tip needle can be done by reliable certified agencies. The culture must have at least  $10^5$  viable cells/ml. The syringes/ink-fillers can be prepared, stored and transported in cool-box similar to that used for vaccination medicines employed during mass vaccination drive in children.

This simple, farmer-friendly method to mass-multiply plant-beneficial microbes has the following procedure: Add appropriate volume of the fresh rice gruel + coconut water

mix (1:1 ratio, v/v) into pressure cooker (say 1.0 l medium in a 2.5 l cooker or 2.5 l medium in 5.0 l cooker). Biochar or any other charcoal is added @ 0.1% (w/v) to the liquid medium. Sterilize the contents for 15 min. in pressure cooker following three whistles. Remove the pressure cooker from heat and allow the contents to cool. Do not open the cooker for the purpose of cooling the contents. Add the bioinoculant culture into the pressure cooker as mentioned in the method given as follows. A clean place that is away from living area, particularly kitchen, must be identified for this part of the work. A candle or bunsen burner can be lighted and the flame is brought near the weight of the cooker. The weight is removed while the flame from candle or bunsen burner is near it. Five to ten ml of microbial culture (in liquid form) present in pre-filled syringe or ink-filler is then injected or pumped into the cooker through the cooker nozzle opening. The length of the needle must be such that it easily extends into the full length of the cooker nozzle. Care should be taken to prevent any spillage of the inoculants outside the cooker. The transfer of the inoculum into the cooker is done with the candle/bunsen burner flame near the mouth of the nozzle. The weight must be immediately replaced. Now the candle/bunsen burner flame can be extinguished. The cooker is then slowly rotated (clockwise and anticlockwise, 3 times each) so that the microbial inoculum mixes well and the internal aeration is improved. The cooker is then kept under room temperature conditions in a clean area. The contents are mixed well once in 4 to 5 hr interval by rotating the cooker gently if biochar is not added. If biochar is added, mixing twice on the day of inoculation would suffice. Then the cooker can be left undisturbed for the rest of the incubation period. The incubation period for bacterial mass-multiplication is 24-72 hr and for fungi is 5-7 days. After incubation, the mass-multiplied microbial inoculants will be ready for application. A minimum of  $10^7$  to  $10^9$  colony forming units (cfu) of the inoculants will be present in per ml of rice gruel-coconut water medium prepared in this way. It will be devoid of any contaminant bacteria or fungi too. The bioinoculant can now be used for seed treatment, seedling root dip or soil application.

#### RESULTS AND DISCUSSION

Coconut water is acidic (pH 4.5 to 5.2), rich in inorganic ions such as potassium and sodium, contains up to 20 mg/ml of sugars (sucrose, glucose and fructose), phytohormones such as auxin, cytokinin and gibberellins, vitamins like ascorbic acid, lipids, amino acids, organic acids, and to some extent dietary fibres (Yong *et al.* 2009, Appaiah *et al.* 2014). The rice gruel on the other hand is rich in starch (amylase and amylopectin) and proteins (containing lysine, glycine, leucine and isoleucine) but is low in electrolytes (Mehta and Subramaniam 1986). Thus, the rice gruel-mature coconut water mixture forms an ideal physiological basis for supply of all the necessary growth chemicals needed for the quick multiplication of the bacteria/fungi. The addition of micronized biochar provides extensive surface area for the microbes to reside (Schnee *et al.* 2016) and proliferate

using the nutrients provided by the medium. In this study, we used biochar produced from tender or immature coconut husk using simple charring kiln. Besides providing surface area to microbes, the tender or immature coconut husk biochar could also have helped in buffering of the medium owing to its high pH range of 8.1-9.4 (Gopal *et al* 2016). The plant-beneficial bacterial and fungal cultures were sourced from the microbial culture collection available in the Institute. The bacteria *Bacillus megaterium*, *Bacillus licheniformis*, *Pseudomonas putida*, isolated from coconut and cocoa rhizosphere (Thomas *et al.*, 2011a,b; George *et al.* 2012a,b, 2018; Gupta *et al.* 2014), have proven plant growth promoting properties. *Beijerinckia* sp. isolated from coconut soils is a proven nitrogen fixing bacterium (Merilyn and Thomas 1991) and *Trichoderma* sp. is a proven antagonist (Chandra Mohanan *et al.* 2013) and *M. anisopliae* proven for pest management (Gopal *et al.* 2006).

The selected microbes, initially, were multiplied in small volumetric flasks using the coconut water+rice gruel+biochar medium and ascertained for optimum days of incubation for bacteria and fungi by taking samples every 24 hr and pour plating for colony counts. Based on these results, the mass-multiplication was carried out in small pressure specified cooker (2.5 l). After the specific incubation period, the cooker was opened inside laminar air-flow chamber and samples were drawn, serially diluted up to  $10^{-12}$  dilution and pourplated on nutrient agar medium for bacterial counts and potato dextrose agar medium for fungal counts. Inoculated plates were incubated for 48 hrs and up to 7 days for bacteria and fungi, respectively. Each plant-beneficial microorganism was tested minimum three times. Results of different plant-beneficial bacteria and fungi tested by this method are furnished in Table 1. Among the different plant-beneficial bacteria tested, *Bacillus* spp. grew the fastest to the tune of  $10^7$  to  $10^9$  cfu/ml medium within 48 hrs incubation at  $30^\circ\text{C}$ , whereas, *Pseudomonas putida* grew to a higher population of  $10^{11}$  cfu/ml by 72 hr of incubation. The antagonistic and entomopathogenic fungi took about 6-7 days to grow and reach the stipulated

standards. No contaminating microbe was encountered in any of the samples and only the inoculated ones grew on artificial media. Counts of the bacteria and fungi inoculated in standard nutrient and potato dextrose broth, respectively, gave slightly better cfu counts (data not given). Our results are superior in terms of populations than those of Srinivasan (2007), who reported close to  $10^6$  to  $10^7$  cfu/ml of *Bacillus subtilis* and *Pseudomonas fluorescens* and their mixture when cultivated in coconut water alone after 48 hrs of incubation. On the other hand, *Bacillus pumilus* SE34 and *Pseudomonas* sp. PNO26R were found to multiply to similar population we report (about  $10^8$  cfu/ml) in mature coconut water medium within 24 hr (Anith 2009). Anith *et al* (2014) also reported a simple, farmer-friendly method in which PGPR *Pseudomonas fluorescens* was mass-multiplied in boiled coconut water kept in plastic water or jam bottle and achieved contamination-free culture to the tune of  $10^9$  cfu/ml by 48 hr of incubation. However, we feel that boiled coconut water could be prone to contamination and hence proper sterilization is essential considering the fact that coconut water is rich in nutrients and can quickly allow growth of contaminants.

Thus, it was seen that all the tested microorganisms grew favourably in the coconut water- rice gruel-biochar medium and plating them after the respective incubation period indicated presence of no contaminants. The colony counts were found to satisfy the BIS stipulated standards for liquid bioinoculants such as i) pH range of 5 to 8 for liquid media, ii) a minimum population of  $10^7$  to  $10^8$  cfu/ml for plant-beneficial bacteria or fungi, iii) excellent suspensibility for the multiplication of the microorganisms and iv) absence of external bacterial or fungal contamination (Yadav and Chandra 2012).

However, the farmers need to exercise certain precaution while carrying out mass-multiplication in their farm premises: i) the cooker must be cleaned with boiling water post bioinoculant production and use. The nozzle, weight and rubber gasket must be cleaned with more precaution, ii) use clean dry cooker for next round of mass-multiplication, iii)

Table 1 Population count and pH of the medium (rice gruel+mature coconut water in 1:1 ratio, v/v, with 0.1%, w/w, biochar) for the following plant-beneficial microbes mass-multiplied by this farmer-enabled technology

Organism	pH of the medium	Population range (cfu/ml)	Incubation period
<i>Beijerinckia</i> sp. (N-fixer)	5.1 - 5.6	$20 \times 10^8$ to $26 \times 10^9$ (9.3 to 10.4)	72 hr
<i>Bacillus licheniformis</i> (PGPR)	5.2 - 5.4	$7 \times 10^8$ to $16 \times 10^9$ (8.9 to 10.2)	48 hr
<i>Bacillus megaterium</i> (PGPR)	5.5 - 5.7	$3 \times 10^7$ to $17 \times 10^7$ (7.5 to 8.2)	48 hr
<i>Pseudomonas putida</i> (PGPR)	5.3 - 5.8	$9 \times 10^{10}$ to $19 \times 10^{11}$ (11 to 12.3)	72 hr
<i>Trichoderma</i> sp. (Antagonistic fungus)	5.5 - 5.9	$7 \times 10^7$ to $15 \times 10^7$ (7.9 to 8.2)	6 -7 days
<i>Metarhizium anisopliae</i> (Entomopathogenic fungus)	5.2 - 5.9	$12 \times 10^8$ to $34 \times 10^8$ (9.1 to 9.5)	6-7 days

use separate cookers for bacterial and fungal inoculants to prevent cross contamination, iv) boil the inoculant syringe/ink filler before its disposal and keep the syringe and ink-filler out of the reach of the children and v) strictly avoid the cooker for preparation of any food.

Thus, our communication reports a unique minimal process technology that is repeatable, that utilizes locally available food-and plant-based aqueous by-products in synergistic manner admixed with biochar for on-farm production of contaminant-free bacterial and fungal inoculants using a pressure cooker by the farmers. Supply of the inoculants via reliable certified agencies is an important external need in this method.

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