vation of Röst[4] that air canals per unit area in the central part of the blades is the only reliable structural character for identification of cytotypes in *Acorus*. A remarkable feature of early autumn senescence and late spring growth was found in all the three accessions with low β-asarone contents irrespective of their ploidy levels, as compared to other accessions with high β-asarone contents growing under identical conditions at our campus.

In conclusion, most of the accessions from Indian subcontinent were predominantly triploids with very high β-asarone contents and not tetraploids as reported earlier. Only one triploid accession was found to be with low β-asarone content. Two diploids were also detected with very low β-asarone contents from temperate western Himalayas. All the low β-asarone accessions were collected from western Himalayas and appear to be more akin to European *A. calamus* with respect to β-asarone contents. Amongst the phenotypic characters, lower SLW values and quicker senescence and late emergence in a population may help in preliminary quick screening of accessions for low β-asarone contents.

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Mature coconut as a bio-fermentor for multiplication of plant growth promoting rhizobacteria

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A cheap and farmer-friendly multiplication method for plant growth promoting rhizobacteria (PGPR) in mature coconut is described here. Uncontaminated coconut water naturally available within the coconut was used as medium for multiplication of two PGPR strains, *Pseudomonas* sp. PN026R and *Bacillus pumilus* SE34. Bacterial strains were separately innoculated in aseptically collected coconut water as well as coconut water in mature intact coconut and the growth pattern was studied. Growth of PGPR strains with coconut water as a sole nutrient source was comparable to that in conventional liquid medium. Both the strains multiplied in the coconut water to the tune of 10^8 cfu ml^{-1} within a period of 24 h. The bacterial strains developed mucoidal colonies on coconut water agar medium as a result of increased polysaccharide production. Seed colonization and plant growth pro-

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motion in tomato was more when cultures grown in coconut water were used for seed treatment. Treatment with bacterial cultures grown in coconut water improved root initiation and increased the number of roots of black pepper cuttings in the nursery both under sterile and non-sterile soil conditions.

Keywords: Black pepper, coconut water, colonization, mature coconut, plant growth promoting rhizobacteria, rooting.

PLANT growth promoting rhizobacteria (PGPR) are rhizosphere bacteria that exert a positive influence on the plant growth especially under stress conditions[1]. They can influence plant growth directly either by providing specific compounds that help plant growth or by facilitating uptake of nutrients from the soil, and indirectly by suppressing the phytopathogenic organisms in the rhizosphere[2]. The interaction between rhizobacteria and the plant host depends on the intimate association between the two partners brought about by 'effective' root colonization. Root colonization by the rhizobacteria is an important aspect that determines the efficacy of plant growth promotion, pathogen suppression and disease control[3]. PGPR usually colonize the rhizosphere around the roots, the rhizoplane, i.e. the root surface and often within the roots as well. The root colonization efficiency is determined by several factors and the medium in which the bacteria are grown prior to crop application is also one of the important determinants[3,4].

Search for cheap and locally available materials for bacterial cultivation and as carriers for formulations has resulted in use of several naturally occurring plant derived organic substrates. Coconut water (CW) present in the intact coconut is free from any microbial contamination and is highly nutritious, rich in many amino acids, vitamins and minerals[5]. CW is a rich source of nutrients for the multiplication of microbial agents also[6–8]. It has been shown that CW also acts as a good nutritional supplement in many of the bacteriological media[9,10]. A cheap and ecofriendly method for multiplication of the mosquitocidal agent, *Bacillus thuringiensis* var. *israelenis* within mature coconut for releasing into the breeding sites of mosquitoes has also been reported[11]. CW also has plant growth promoting ability and is routinely used as a supplement in plant tissue culture media[12]. It is traditional knowledge among the farmers, that dipping the cut ends of black pepper cuttings in tender CW, prior to planting, improves rooting and growth of the plantlets in the nursery. Scientific information is also available on the ability of CW to promote rooting in black pepper cuttings[13].

A simple method for multiplication of PGPR in CW and within mature coconut is described here. This method is expected to help farmers easily multiply PGPR, for usage in nurseries of transplanted and vegetatively propagated crops.

Plant growth promoting rhizobacterial strains *Pseudomonas* sp. strain PN026R and *Bacillus pumilus* strain SE34 were obtained from the Department of Agricultural Microbiology, College of Agriculture, Vellayani and Auburn University, Alabama, USA respectively. *Pseudomonas* and *Bacillus* strains were grown on King's medium B (KB) and nutrient agar (NA), respectively, at 28°C.

As an initial step to exploit the potential of CW as a medium for multiplication of the PGPR strains, the ability of the bacterial strains to use CW as a sole source of nutrients was assessed. Ten-month-old mature coconut was de-husked and the eye of the coconut bearing the embryo was surface sterilized with 70% alcohol. The eye of the coconut was cut open and the embryo removed with the help of a sterile blade. CW was then aseptically collected and transferred to a sterile conical flask with the help of a sterile 20 ml syringe fitted with an 18 gauge needle by inserting it through the opened eye of the coconut in a laminar air flow chamber. Twenty ml of the CW was further filter sterilized with a 0.2 µ disposable syringe filter and collected in a 100 ml sterile conical flask. A single colony of the bacterial culture was suspended in 1 ml of sterile distilled water and 100 µl of the suspension transferred to the CW and incubated without shaking at 28°C for 24–48 h in an incubator. Samples were drawn at regular time intervals and the population of the *Pseudomonas* and *Bacillus* strains was monitored using viable plate counts on KB and nutrient agar plates respectively.

For assessing the ability of the bacterial strains to multiply within coconut, the embryo from the eye of the coconut was removed, as described above, in a laminar flow chamber. A single colony of the bacterial culture was suspended in 1 ml of sterile distilled water and 100 µl of the suspension was injected into the coconut with the help of a sterile syringe. The opened eye of the coconut was covered with a band of sterile cotton. Samples from the coconut were drawn with the help of a sterile syringe at regular intervals and the population of the bacteria was monitored by viable plate counts. In both the above cases, *Pseudomonas* strain incubated in KB broth and *Bacillus* strain in nutrient broth served as control.

To understand the colony morphology and growth pattern of the PGPR strains on solid medium, when grown with only coconut water as a nutrient source, both the strains were grown on coconut water agar (CWA) plates. Plain agar (20 ml) with double the agar concentration (agar @ 30 g/l) in 100 ml conical flasks was prepared with double distilled water as the base and sterilized by autoclaving. 20 ml of filter sterilized coconut water collected aseptically as described above was added to the sterile molten plain agar and poured into plates. Bacterial cultures were streaked on the plates, incubated at 28°C and observations were made on the colony morphology and growth characteristics. Growth characters were compared with bacterial cultures grown on KB agar and

nutrient agar plates, respectively, for the *Pseudomonas* and *Bacillus* strains.

As the medium for multiplication of the PGPR cultures has a direct effect on the colonization ability, cultures grown in CW were compared for the ability to colonize tomato with that grown in KB broth for the *Pseudomonas* and nutrient broth for *Bacillus*. Seeds of tomato variety 'Shakthi' were surface sterilized with 1% sodium hypochlorite for 3 min and washed in sterile distilled water twice in a laminar air flow chamber. Seeds were spread on sterile tissue paper to drain the excess water. The surface sterilized seeds were then separately soaked in *Pseudomonas* culture grown in CW and KB broth and *Bacillus* grown in CW and NB for 30 min. The population density in the bacterial cultures was adjusted to equality by spectrophotometrically determining the turbidity and by diluting the suspension with the corresponding sterile liquid medium[14]. The treated seeds were kept in sterile petri plates at room temperature. The population of bacteria on the seeds was determined 30 min and 24 h after the seed treatment by serial dilution and dilution plating in KB agar and nutrient agar, respectively, for the *Pseudomonas* and *Bacillus* strains. Four independent observations were made in each treatment and the population was expressed as cfu/seed and cfu/g of the seed.

Plant growth promoting ability of the bacterial cultures grown in different media was assessed under glass-house experimental condition. Bacterial strains were grown in conventional broth as well as coconut water within mature de-husked coconuts. Innoculation of the bacteria into coconut was done as described earlier. Seeds of the tomato variety Shakthi were surface sterilized and soaked in bacterial culture for 30 min after adjusting the population density of the suspension as described. Soaking seeds in coconut water without innoculation of the bacterial cultures and sterile distilled water served as controls. Seeds were then sown in sterile sand-soil (1 : 1) mixture taken in plastic trays of dimension $18 \times 16 \times 5$ cm. The planting medium was sterilized by autoclaving at 121°C for 20 min for three consecutive days. Sixty seeds were planted in each tray and four replications having four trays each were kept in a glass house. After emergence, the plant population was thinned to 30 seedlings per tray. The plants were watered regularly with sterile water. No external application of chemical fertilizers was done. After 21 days of planting, the seedlings were uprooted carefully, washed under running tap water and biometric observations were taken.

Coconut water is an essential component in many of the tissue culture media as it provides several growth factors and enhances plant growth. With the knowledge that it also has some root initiation property in black pepper cuttings, the effect of CW and PGPR strains that were grown in CW on the rooting of black pepper was tested both under sterile and non-sterile soil conditions in a glass house. The PGPR strains, *Pseudomonas* sp. PN026R and *B. pumilus* SE34 tested in this experiment also had better root initiating ability in black pepper (data unpublished). Same treatments as in the growth promotion experiment with tomato were imposed in the rooting experiment with black pepper also. Disease free three-node cuttings of black pepper cv. Karimunda selected from the gardens of College of Agriculture, Vellayani, were surface sterilized with 0.1% sodium hypochlorite solution for 3 min, rinsed with sterile water twice and used for planting. Before planting, the basal 5 cm cut-end portion of the planting material was dipped for 30 min separately in bacterial suspension grown in the conventional medium and in CW within coconut. The population density of the bacterial cells in the suspension was adjusted to the same level before dipping the cuttings. Control treatments received dip in freshly collected sterile coconut water or sterile distilled water for the same period. Planting medium consisted of sand and soil in the ratio of 1 : 1. Experiments were performed both in sterile and non-sterile planting medium. Five hundred grams each of the potting mixture was filled in polythene bags of size 15×10 cm and five cuttings were planted in each bag. Four replications, each having 10 cuttings, were kept under glass house conditions. The plants in sterile medium were watered with sterile distilled water and those in non-sterile soil with tap water regularly. No external application of chemical fertilizers was done. After 30 days of planting, the cuttings were uprooted and root growth measured.

Both the PGPR strains, *Pseudomonas* sp. PN026R and *B. pumilus* SE34 were found to multiply well in coconut water utilizing it as a sole source of nutrients. When they were grown in CW collected in conical flask as well as within intact coconut, the growth pattern was comparable to that in conventional liquid medium (Figures 1 and 2). The growth pattern of both the bacterial strains showed a similar trend. In CW, the maximum population was attained within a period of 24 h and then

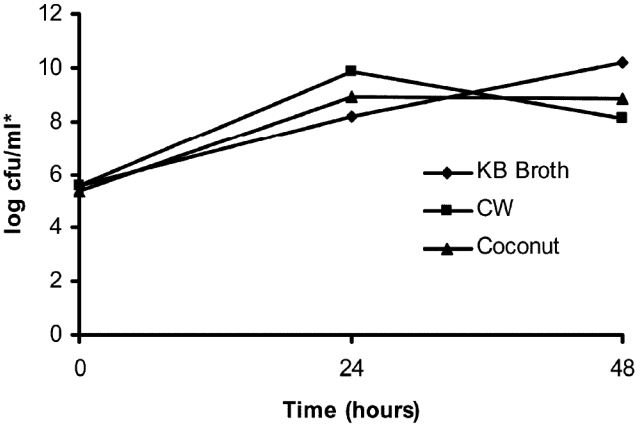

Figure 1. Multiplication and growth of *Pseudomonas* sp. PN026R in different media. KB, King's Medium B; CW, Coconut water in flask. *Mean of four independent observations.

Table 1. Bacterial population on tomato seeds

Source	Initial population in the suspension (cfu/ml)*	cfu/seed*		cfu/g of seeds*	
		30 min	24 h	30 min	24 h
PN026R in KB	1×10^9	7.388×10^6	3.81×10^6	7.39×10^8	4.705×10^8
PN026R in CW	0.95×10^9	3.68×10^8	6.737×10^6	1.369×10^9	7.234×10^8
SE34 in NB	1.32×10^9	6.025×10^6	2.4×10^6	5.738×10^8	2.21×10^8
SE34 in CW	1.28×10^9	1.265×10^7	8.7×10^6	1.29×10^9	8.5×10^8

*Mean of four independent observations. KB, King's B broth; NB, Nutrient broth; CW, Coconut water.

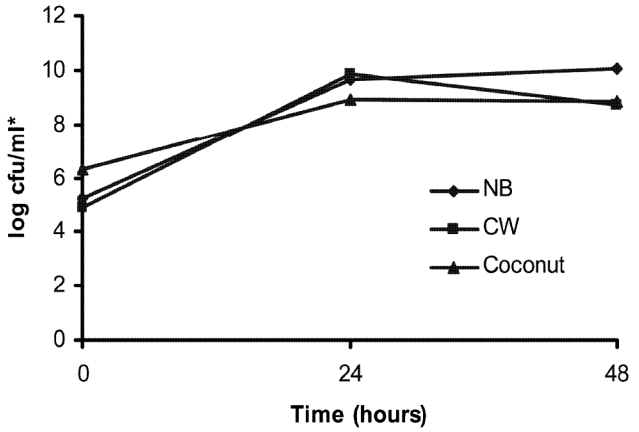

Figure 2. Multiplication and growth of *Bacillus pumilus* SE34 in different media. NB, Nutrient broth; CW, Coconut water in flask. *Mean of four independent observations.

it declined. However, with conventional liquid medium the growth continued and achieved a count of approximately 10^{10} cfu/ml at 48 h. The population of both the bacterial strains did not increase beyond 10^9 cfu/ml in the case of CW in flask and 10^8 cfu/ml in the case of CW within coconut. This could be due to a substantial change in pH of coconut water during the bacterial multiplication. The mean initial pH of coconut water before inoculation was 5.5 and that after 24 h and 48 h of innoculation was 4.8 and 4.2 respectively. This acidic condition might have adversely affected further bacterial population build-up. There are many reports about using coconut water as a medium for multiplication of bacteria as a sole source of nutrient and as a component[6–10]. In all such cases, CW was autoclaved and used. In the present study, either filter sterilized or uncontaminated CW that is naturally available within coconut in an unaltered form was used for multiplication of PGPR strains. This helps in preserving the integrity of any heat labile microbial/plant growth promoting constituents present in CW.

When the bacterial strains were grown on CWA, which actually had only half the strength of the naturally available CW, growth of both the bacterial strains was comparable with that on the conventional solid media. The colony morphology of both the bacterial strains on CWA was different from that on the conventional medium with the colonies showing mucoidal appearance in CWA (Figure 3 *a–d*). Colonies of PN026R on CWA were mucoidal, round, raised and sticky whereas those on KB were round, flat and without mucoidal appearance. Colonies of SE34 on CWA were flat, spreading with mucoidal appearance and those on NA were small, well separated and having dry appearance. The presence of sucrose in CW must have favoured an increase in production of polysaccharides by the bacterial strains. The mucoidal nature of the colonies is supposed to have a positive influence on colonization. The seed and root colonization efficiency is highly influenced by the type and amount of bacterial polysaccharides[15,16].

Assessment of the bacterial population on the treated tomato seeds showed that cells from the cultures grown in CW were present in more numbers on the seeds as compared to those treated with cultures grown in conventional broth, though the initial populations of the cultures used for the treatment were almost the same (Table 1). The mucoidal nature of the colonies must have helped in better adherence and agglutination of the cells to the seed surface. When the population present on seeds was analysed, it was found that though there was a decline in population for both the treatments over a period of time, those seeds receiving the cultures grown in CW had a higher bacterial population. The decline in population in the *in vitro* test could be attributed to insufficient environmental conditions for bacterial survival. The quantum of rhizobacteria on the seeds or in the rhizosphere at the time of delivery highly influences the population dynamics of the same over the cropping period[17–19]. Such assessments are in progress in our laboratory at present. Initial observations showed that the population of *Pseudomonas* sp. PN026R on roots of the tomato seedlings was almost 10 times more when the seeds were treated with cultures grown in CW compared to that treated with bacteria grown in KB medium (unpublished data).

It is postulated that for plant growth promotion and biocontrol to occur, there should be a minimum threshold number of bacterial cells to be present in the rhizosphere[17]. In the present study, a high population of the PGPR strains on the tomato seeds at the time of seed treatment had positive effect on the growth promotional ability of the strains as evidenced in the plant growth promotion experiment under sterile soil conditions. Seed

Table 2. Plant growth parameters of tomato seedlings under sterile soil conditions

Treatment	Germination (%)*	No. of leaves**	Height (cm)**	Fresh shoot weight (g)**	Fresh root weight (g)**
CW	64.98^{ab}	3.7013^{a}	8.1915^{ab}	0.27888^{ab}	0.019225^{b}
PN026R in CW	68.30^{ab}	3.7363^{a}	8.1825^{ab}	0.27535^{ab}	0.025725^{ab}
SE34 in CW	63.28^{ab}	4.0463^{a}	9.2250^{a}	0.33973^{a}	0.042250^{ab}
PN026R in KB	71.63^{a}	3.5438^{a}	8.0278^{ab}	0.24755^{ab}	0.036850^{ab}
SE34 in NB	42.98^{ab}	3.6875^{a}	7.8655^{ab}	0.26000^{ab}	0.036675^{ab}
Control	33.13^{b}	3.7415^{a}	6.9618^{b}	0.22500^{b}	0.025600^{ab}

*Mean of four replications having 60 seeds each. **Mean of four replication having 30 seedlings each. CW, Coconut water; KB, King's B broth; NB, Nutrient broth. Figures followed by same letters in each column do not differ significantly according to Duncan's multiple range test ($P = 0.05$).

Table 3. Root initiation and growth in black pepper cuttings

	Sterile soil			Non-sterile soil		
Treatment	Rooting (%)*	No. of roots/ cutting*	Root length/ cutting (cm)*	Rooting (%)*	No. of roots/ cutting*	Root length/ cutting (cm)*
CW	66.6^{bcd}	2.75^{cd}	0.836^{cd}	31.25^{d}	2.43^{e}	2.17^{ab}
PN026R in CW	54.5^{e}	3.61^{bc}	1.326^{abc}	26.6^{e}	3.00^{c}	1.76^{b}
SE34 in CW	70.6^{abc}	4.42^{a}	1.88^{a}	42.10^{c}	5.656^{a}	2.23^{a}
PN026R in KB	72.7^{ab}	2.75^{cd}	1.046^{bcd}	52.63^{a}	4.0^{b}	2.15^{ab}
SE34 in NB	75.0^{a}	3.375^{b}	1.564^{ab}	50.0^{ab}	2.57^{cd}	1.90^{ab}
Control	57.1^{e}	2.25^{e}	0.605^{d}	31.25^{d}	1.40^{f}	1.95^{ab}

*Mean of four replications having 10 cuttings each. CW, Coconut water; KB, King's B broth; NB, Nutrient broth. Figures followed by same letters in each column do not differ significantly according to Duncan's multiple range test ($P = 0.05$).

treatment with bacterial cultures, irrespective of the medium in which they were grown, improved the germination percentage of the seeds (Table 2). Treatment of seeds with PN026R grown in KB showed the maximum germination and the untreated control the minimum. There was no significant difference among the treatments with respect to the number of leaves produced by the seedlings as well as the fresh root weight. However there was statistically significant difference with respect to the height and fresh shoot weight of seedlings between the untreated control and seeds treated with SE34 grown in CW. Comparison of the treatments revealed that in all the cases, seeds treated with PGPR grown in CW had more growth promotional ability than those treated with bacteria grown in conventional media (Figure 3 *e–m*). Such a benefit was more pronounced in the case of the *Bacillus* strain SE34. The PGPR strain SE34 has been reported to have plant growth promoting ability in tomato[14] as well as other crops[20]. Seed treatment with CW alone also improved plant growth which was comparable with that observed in other bacterial treatments. The combination of PGPR and CW as a one-step application method assumes importance as many of the PGPR are growth promoters as well as efficient biocontrol agents. Studies on efficiency of biocontrol as well as root colonization in such treatments are to be done to understand any further synergistic benefits of the application method.

Very little work has been done on plant growth promotion and biological control of diseases in vegetatively propagated crops. Black pepper is mainly propagated by vegetative means using runner shoot cuttings. It is a regular practice of farmers in pepper growing tracts of Kerala to treat the planting material of black pepper with tender coconut water prior to planting in the nursery. Several personal accounts suggest that such a treatment improves the overall establishment of the plantlets. In the nursery, black pepper cuttings take a period of 30–45 days to develop proper root system. Treatment of basal end of the black pepper runner shoot cuttings for 24 h in 50% CW is reported to increase the root initiation and root system formation[13]. Several microbial agents also help in root initiation in vegetatively propagated crops[21,22]. The *Pseudomonas* sp. strain PN026R, an isolate from black pepper rhizosphere, is an efficient root growth promoting bacterium as well as biocontrol agent against *Phytophthora capsici*, the causal agent of root rot of black pepper[23,24]. The synergistic effect of both CW and PGPR strains on rooting in black pepper could therefore be studied by combining them. Experimental results both under sterile and non-sterile soil conditions in the present study showed that PGPR strains when grown in CW had positive influence on the root formation in vegetatively propagated black pepper (Table 3, Figure 3 *n* and *o*). The number of roots per cutting and the mean root length per cutting were more in the case of planting material treated with bacterial suspension of SE34 grown in CW. In sterile and non-sterile soil conditions, the number of roots per cutting was statistically significant in cuttings treated with SE34 grown in CW compared to all other treatments. A higher number of roots is expected to

Figure 3. ***a***, *Pseudomonas* sp. PN026R on King's B agar medium; ***b***, *Pseudomonas* sp. PN026R on CWA medium; ***c***, *Bacillus pumilus* SE34 on nutrient agar medium; ***d***, *Bacillus pumilus* SE 34 on CWA medium; ***e***, Growth of tomato plants treated with coconut water (1), PN026R in CW (2), SE34 in CW (3), PN026R in KB (4), SE34 in NB (5), untreated control (6); ***f***, Growth of tomato plants treated with coconut water (1), PN026R in CW (2), SE34 in CW (3), PN026R in KB (4), SE34 in NB (5), untreated control (6); ***g***, Growth of tomato plants treated with coconut water (1), PN026R in CW (2), PN026R in KB (3), untreated control (4); ***h***, Growth of tomato plants treated with coconut water (1), SE34 in CW (2), SE34 in NB (3), untreated control (4); ***i***, Pairwise comparison of growth of tomato plants. Coconut water (1) vs untreated control (2); ***j***, Pairwise comparison of growth of tomato plants. PN026R in CW (1) vs untreated control (2); ***k***, Pairwise comparison of growth of tomato plants. SE34 in CW (1) vs untreated control (2); ***l***, Pairwise comparison of growth of tomato plants. PN026R in KB (1) vs untreated control (2); ***m***, Pairwise comparison of growth of tomato plants. SE34 in NB (1) vs untreated control (2); ***n***, Root initiation and growth in black pepper cuttings treated with coconut water (1), PN026R in CW (2), PN026R in KB (3), untreated control (4); ***o***, Root initiation and growth in black pepper cuttings treated with coconut water (1), SE34 in CW (2), SE34 in NB (3), untreated control (4).

improve the establishment of the plants. Attachment of bacterial cells to the cuttings is an important aspect for establishment of rhizobacteria in black pepper as the root system development is very much delayed unlike seed propagated crops[23]. The high amount of polysaccharides produced when bacterial cells were grown in CW must have helped in better attachment of the bacterial cells to the cuttings. Population of bacterial cells on the developed roots and rhizosphere was more in the case of cuttings treated with bacterial cultures grown in CW than those grown in conventional liquid media, both in the case of PN026R and SE34 under sterile soil conditions (data not presented).

To test the feasibility of farmers carrying out this bacterial multiplication technique for seed treatment in the field, an improvized test was also performed. Instead of carrying out the innoculation in a laminar air flow chamber, the same was done in a room in front of a candle flame. De-husked coconut, after cutting open the eye of the coconut with a flame sterilized knife, was innoculated with the bacterial suspension stored in an injection vial with the help of a commercially available sterile disposable syringe. The innoculated coconut, after covering the pierced eye with a piece of cotton and adhesive tape, was then stored at room temperature for 24 h. Thereafter, samples from the coconut were drawn as mentioned earlier and dilution plated on agar plates under laboratory conditions for assessing the extent of contamination with external bacteria. Five independent samplings each with the *Pseudomonas* and *Bacillus* strains showed little to negligible amount of contamination occurring within the innoculated coconut even when the innoculation was performed under ordinary room conditions.

It is therefore proposed that farmers could carry out inoculation of mature coconut without contamination and multiply PGPR, if cultures are provided in stable liquid formulations in containers similar to injection vials, from which cell suspension could be drawn with a syringe without exposing them to the external environment. The procedure also requires no sophisticated instruments and methods as there is avoidance of sterile glassware and the lengthy procedure of sterilization by autoclaving.

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