Short Communication

# A cheap and farmer-friendly method for mass multiplication of *Pseudomonas fluorescens*

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**Abstract**

An easy, farmer-friendly field level multiplication method for *Pseudomonas fluorescens* in coconut water is described. *Pseudomonas fluorescens* was multiplied in boiled coconut water under non-sterile conditions and the population reached to $10^9$ cfu/ml after 48 hours. Though the inoculation of the growth medium was done without complete sterilization, there was no detectable growth of contaminants in the inoculated boiled coconut water. Since the population level of the bacterial strain in the inoculated bottles had gone up to $10^{8-9}$ cfu/ml, the same preparation could be further diluted up to 10 to 50 times and could be applied to plants in the form of spray or soil drench.

**Key words**: *Pseudomonas fluorescens*; Coconut water; Multiplication

Biological control of plant diseases is an important component in the current agricultural production system especially in the wake of increased attention towards organic agricultural practices. *Pseudomonas fluorescens* is one of the important antagonistic bacteria that is commercially exploited as a biological control agent to combat plant diseases. Production of anti-fungal, anti-bacterial antibiotics, siderophores, competition for nutrients and space, effective root colonization and induction of systemic resistance are major factors contributing to the biological control potential of fluorescent Pseudomonads (Hass and Defago, 2005). Many strains of this bacterial species have disease suppression activities against several soil borne plant pathogens and foliar pathogens including fungi and bacteria (Walsh et al., 2001). Several of them also act as effective plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009). Delivery of the biological agents into the field is one of the most important aspects in their commercial exploitation. Usually, *Pseudomonas fluorescens* strains are produced either as carrier based inoculants or as liquid bio-formulations (Manikandan et al., 2010). Several commercially available inert materials such as peat, lignite, talc etc., are being used as carrier materials for bio formulation production. Compared to the carrier based inoculants, liquid bio formulations have advantages such as easiness for application, enhanced survival and longer shelf life and improved protection against environmental stress (Vendan and Thangaraju, 2006). They are easy to apply in the field as spray solutions.

Field level augmentation and multiplication of bioagents gives the farmers several added advantages. Storage at unfavourable conditions for long periods is one major issue that reduces the viability of the biocontrol bacteria in the formulations (Manikandan et al., 2010). The cost of the input can be reduced if the farmers are able

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to multiply the bioagents in their own field without incurring additional cost. Coconut water is considered as a waste material of the copra processing industry and is rarely used for any commercial purpose. Coconut water is regarded as a rich nutritional medium for growth of many bacteria and has been used as a supplement in many bacteriological media and tissue culture media (Shivakumar and Vijendra, 2006; Survase et al., 2007; Unagul et al., 2007). It was earlier reported that coconut water is an excellent multiplication medium for plant growth promoting rhizobacteria (Anith, 2009). A cheap and farmer-friendly method for multiplication of plant growth promoting rhizobacteria within mature coconut has been reported (Anith, 2009). Here, we describe an easy, farmer-friendly field level multiplication for *Pseudomonas fluorescens* in coconut water, a waste product of the coconut processing industry.

*Pseudomonas fluorescens* PN026R, a proven biocontrol agent against fungal and bacterial diseases that showed high multiplication rate in coconut water in an earlier report, was taken as the bacterial strain (Anith, 2009). Coconut water was procured from local copra processing industry and filtered twice with muslin cloth to remove the suspended particles and debris. Two litres of the freshly collected coconut water was boiled in a covered stainless steel vessel for 15 minutes on a hot plate under non-sterile room conditions. The same was allowed to cool to room temperature (around $30^0$C). Boiled coconut water was then transferred to either plastic mineral water bottles (one litre capacity) or glass jam bottles (250 ml capacity). Before transfer of the boiled coconut water, the bottles were thoroughly rinsed 2-3 times with boiled water. The mineral water bottles and jam bottles were filled with 500 ml and 150 ml of the boiled coconut water respectively. Immediately after the transfer, bottles were capped. They were then inoculated with a talc based formulation or liquid bio-formulation of the biocontrol bacterium *Pseudomonas fluorescens* PN026R prepared according to the method described earlier (Manikandan et al., 2010). One gram of the talc or one ml of the liquid formulation of the bioagent was used for inoculation of the coconut water in each bottle. The bottles were sealed tight with the cap and mixed thoroughly by shaking 4-5 times.

All the above procedures were carried out under non-sterile room conditions. The bottles were then briefly taken to an air flow chamber and a sample of one ml was aseptically drawn for assessing the initial population of the bacteria in the coconut water. Serial dilution in sterile water was followed by plating on King's B medium, used for assessing the population of the bacteria. The colonies were identified with production of green fluorescent pigmentation. Incubation of the bottles was done under ordinary room temperature ($30 \pm 2^0$C). After 24 hours and 48 hours of incubation, the bacterial population was further analysed under aseptic conditions as described above. Five replications in each group were maintained. Boiled coconut water

***Table 1.*** Population of *Pseudomonas fluorescens* in boiled coconut water in different containers

| Inoculation method | Population of inoculated bacteria (log cfu/ml)* | | |
|---|---|---|---|
| Inoculation with talc based formulation | Initial | 24 hrs | 48 hrs |
| Jam bottle | 5.2430ab | 8.7451b | 9.2625b |
| Water bottle | 5.3541a | 8.3345b | 9.6263a |
| Inoculation with liquid formulation | | | |
| Jam bottle | 5.0086b | 9.1430a | 9.5416a |
| Water bottle | 5.6580a | 8.5809b | 8.9978b |

*Mean of five observations. Data were analysed by one way factorial ANOVA and the means compared using Duncan's Multiple Range Test (DMRT) using statistical package SAS 8.0. Digits in a column followed by the same letter do not differ significantly according to DMRT ($p = 0.05$)

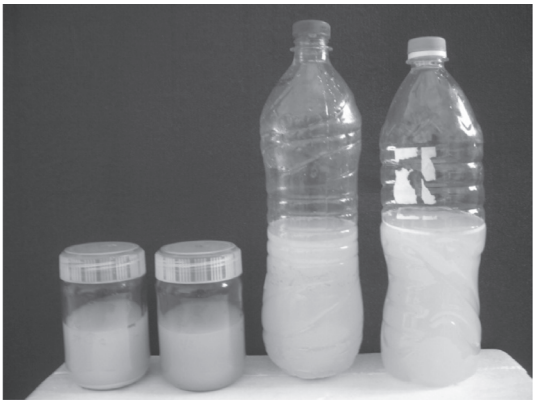

*Figure 1.* Growth of *Pseudomonas fluorescens* in boiled coconut water in different containers after 48 hours From left to right: Jam bottles with talc based inoculum and liquid inoculum; Water bottles with talc based inoculum and liquid inoculum

filled in bottles without inoculation served as control treatment.

Mean bacterial populations from four independent bottles in each treatment during the incubation period of 48 hours are given in Table 1. It was noticed that the bacterial strain was able to multiply in the medium and a high population of $10^8$ to $10^9$ cfu/ml was achieved after 48 hours of incubation. A slight greenish pigmentation of the liquid was also observed, which is a characteristic feature of many of the fluorescent Pseudomonads in liquid cultures (Fig.1). Analysis of data showed that maximum population was obtained in water bottle inoculated with a talc based formulation. Considering the cost of container and the formulation of *Pseudomonas fluorescens* available in the market, it is economical to use the water bottle-talc formulation combination for field level multiplication. Also the volume of coconut water that could be contained in the water bottles is more than that of the jam bottles.

It was also observed that there is no contamination from other organisms in the inoculated coconut water. Determination of contaminants could be done only above the dilution of $10^{-5}$, as well separated individual colonies were not obtained at less than $10^5$ dilutions due to heavy population build up. When control bottles in the experiments that received no inoculation were analysed after 24 and 48 hours, there were bacterial growth in the coconut water to the tune of around 1.35 x $10^8$ cfu/ml. However, no fluorescent colonies could be obtained from them. When native coconut water immediately after collection was analysed for presence of microorganisms especially bacteria, 5.9 X $10^3$ cfu/ml could be obtained. Though, coconut water is considered to be 'sterile' within coconut, it gets contaminated during the process of collection. However, it was found that the process of boiling coconut water for 15 minutes completely eliminated the bacterial population as evidenced by the absence of any colonies formed on nutrient agar on plating after boiling.

The method described here is very useful for farmers for field level multiplication of *Pseudomonas fluorescens*. Though, the inoculation of the growth medium was done without complete sterilization, there was no growth of contaminants in the inoculated coconut water. The absence of any contaminant in the inoculated coconut water could be due to the fact that the growing antagonistic bacteria overwhelmed the population of probable contaminants that could have been carried into the coconut water during the inoculation process. Many of the fluorescent Pseudomonads produce anti-fungal and anti-bacterial metabolites and suppress the growth of other microorganisms (Hass and Defago, 2005). This could be the reason for the fewer amounts of contaminants in the growth medium. However in the uninoculated coconut water there was growth of many different types of bacteria, the population level of which came up to $10^8$ cfu/ml after a period of 48 hours of incubation. Such contaminants get into the coconut water during the handling process. The natural contaminants proliferated in the control bottles, as there was no competition from the inoculum. Since the population level of the bacterial strain in the inoculated bottles has gone up to $10^{8-9}$ cfu/ml, the same preparation could be further diluted to a tune

of 10 to 50 times and could be applied to plants in the form of spray or soil drench. It has been earlier reported that the growth of rhizobacteria in coconut water resulted in increased seed/root colonization due to the production of mucoidal colonies that in turn improved plant growth (Anith, 2009). It was also suggested that the growth promotion could be due to extra auxins provided by the bacteria growing in coconut water (Yong et al., 2010). The coconut water based bacterial suspension could also be used for seedling dip and vegetatively propagated planting materials such as black pepper cuttings. It was earlier reported that treating black pepper cuttings by coconut water based bacterial suspension significantly increased root development, that too, at an early stage in the nursery (Anith, 2009).

The above method can also be tested in case of other PGPR strains and biocontrol bacterial strains. However, in the case of those PGPR strains that do not have biocontrol activity through the production of anti-microbial agents, contaminating bacteria and fungi may be one problem that could be encountered, as they may not be eliminated or suppressed as in the case of biocontrol Pseudomonads, due to lack of antagonistic activity in the boiled coconut water. This has to be verified before the same method is recommended for other such bacterial strains.

## References

Anith, K.N. 2009. Mature coconut as a bio-fermentor for multiplication of plant growth promoting rhizobacteria. Curr. Sci., 97:1647-1653.

Hass, D. and Defago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonas. Nature Rev. Microbiol., 3: 307-319.

Lugtenberg, B. and Kamilova, F. 2009. Plant-Growth-Promoting rhizobacteria. Annu. Rev. Microbiol., 63:541-556.

Manikandan, R., Saravanakumar, D., Rajendran, L., Raguchander, T. and Samiyappan, R. 2010. Standardization of liquid formulation of *Pseudomonas fluorescens* Pf1 for its efficacy against *Fusarium* wilt of tomato. Biol. Control, 54: 83-89.

Shivakumar, S. and Vijendra, S.V.N. 2006. Production of exopolysaccharides by *Agrobacterium* sp. CFR-24 using coconut water- a by product of food industry. Lett. Appl. Microbiol., 42:477-482.

Survase, S.A., Saudagar, P.S. and Singhal, R.S. 2007. Use of complex media for the production of scleroglucan by *Sclerotium rolfsii* MTCC 2156. Biores. Tech., 98: 1509-1512.

Unagul, P., Assantachai, C., Phadungruengluij, S., Suphantharika, M., Tanticharoen, M. and Verduyn, C. 2007 Coconut water as a medium additive for the production of docosahexaenoic acid by *Schizochtrium mangrovei* sk 02. Biores. Tech., 98:281- 287.

Vendan, R. and Thangaraju, M. 2006. Development and standardization of liquid formulations for *Azospirillum* bioinoculant. Indian J. Microbiol., 46: 379-387.

Walsh, U.F., Morrissey, J.P. and O'Gara, F. 2001. Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr. Opinion Biotechnol., 12: 289-295.

Yong, J.W.H., Tan, S.N., Ge, L. and Ng, Y.F. 2010. Mature coconut as a bio-fermentor. Curr. Sci., 98:881-882.