Short communication

Population dynamics and efficiency of coconut water based liquid formulation of *Pseudomonas fluorescens* AMB-8

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Abstract

Liquid bio-formulations are more acceptable than solid bioformulations as they have improved shelf life and better field performance. Among the limited choice of naturally available liquid carriers, coconut water stands out as an efficient one. The population dynamics of *Pseudomonas fluorescens* AMB-8 in a coconut water based liquid formulation along with nutrient broth (NB) and King's B broth (KB) in the presence of different preservatives, and their effect on the seedling growth of chilli and tomato were studied. It was observed that the rate of decline in population was less in coconut water amended with PVP (2% w/v) and glycerol (2% v/v) during six months of storage. Even in the raw autoclaved coconut water. (ACW), the maintenance of the population of the bacterial strain was significantly higher than in all the synthetic media except nutrient broth amended with glycerol (2% v/v). In the plant growth promotion experiments, all biometric parameters had higher values when freshly grown bacterial strain in KB with population density of 10^9cfu ml^{-1} was used for seed bacterization. High bacterial population density in the formulations had a positive effect on the chilli and tomato seedlings. The results of our study showed that a liquid formulation of *Pseudomonas fluorescens* AMB-8 could easily be made from an agricultural waste product, coconut water.

Keywords: Coconut water, Liquid formulation, Plant growth promotion, *Pseudomonas fluorescens*

Increasing human population throughout the world demands high growth output in food production. This has resulted in dependence on intensive agriculture that relies on higher input use in the form of chemical fertilizers and pesticides. However there is alternative thinking on the use of such chemical inputs. Sustainable agricultural practices that minimize use of chemical inputs and depend more on naturally occurring alternatives are gaining popularity. One of the emerging and highly researched areas is the use of microbial agents as pest and disease management alternatives. Integrated disease management (IDM) strategies advocate need based, timely and eco-friendly use of biological agents as an integral part in combating plant diseases.

Though microbial agents have many advantages over conventional chemical inputs, one of the major problems with them is their inconsistency in field performance. This has been attributed to several factors and one among them is the poor formulations involved in their production. Many, especially the bacterial bio agents, are formulated in solid carriers like talc, perlite, vermiculite, peat, compost etc. The bulkiness, high application rate, shorter shelf life of the organisms in the carrier materials, non-sterile nature and comparatively low field performance of solid formulations limit their popularity among farmers. Moreover, large particle size of carrier materials results in clogging of spray nozzle causing difficulty in application at the field level. In addition, solid formulations have little use when it

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comes to modern techniques like hydroponics, bio-hardening of tissue culture plants and horticultural plantlets (Manikandan et al., 2010). Liquid formulations overcome these limitations and serve as a better alternative (Bashan et al., 2014).

Microbial cultures or suspensions amended with compatible substances to improve viability, stickiness, stability, surfactant and dispersal ability are in general considered as liquid bioformulation (Singleton et al., 2002). Unlike solid carrier-based formulations, in liquid formulations it is possible to incorporate sufficient amount of nutrients, cell protectants and inducers responsible for spore/cyst formation that enhance the field performance (Bashan et al., 2014). Other advantages of liquid bio-formulations are high cell count in the formulated product, low application rate, minimal contamination, improved shelf life, better stability and increased field efficacy (Hegde, 2002; Vendan and Thangaraju, 2006).

Despite having many advantages there are certain limitations that preclude the use of liquid formulations. One of the major problems is that many of the liquid formulation without proper amendments tend to lose their viability quickly (Albareda et al., 2008; Bashan et al., 2014). This issue could be solved to some extent by the addition of certain preservatives like sucrose, glycerol, gum arabic, carboxymethyl cellulose (CMC), polyvinyl pyrrolidone (PVP), trehalose etc. These substances are added into the media with the objective of providing a protective environment to the microbial cells and thereby improving the inoculant quality through better adhesion to seeds, better stability of products, inactivation of toxins or enhancement of the strain survival during storage and after exposure to extreme environmental conditions (Albareda et al., 2008; Bashan et al., 2014).

Generally, liquid formulations are based on aqueous broth cultures, mineral or organic oils, oil-in-water or polymer-based suspensions. High cost involved in the preparation of such aqueous or oil based suspensions calls for a cheaper and efficient liquid carrier. Naturally available substances have an upper hand over synthetic liquid carriers when cost effectiveness and safety are concerned. Among the limited choice of naturally available liquid carriers, coconut water stands out as an efficient one. Reports reveal that coconut water free of microbial contamination and rich in nutrients like amino acids, vitamins and minerals can be used as an efficient and cheap media for the multiplication of microorganisms especially bacteria (Survase et al., 2007; Unagul et al., 2007; Anith et al., 2014). Our group had earlier reported the multiplication of plant growth promoting rhizobacterial (PGPR) strain of *Pseudomonas fluorescens* in intact mature coconut (Anith, 2009). A farmer friendly method for field level multiplication of the same bacterium in boiled coconut water has also been reported (Anith et al., 2014).

Here, the population dynamics of a plant growth promoting rhizobacterial strain, *Pseudomonas fluorescens* AMB-8 in a coconut water based liquid formulation and conventional culture media such as nutrient broth (NB) and King's B broth (KB) in presence of different preservatives, and their effect on the seedling growth of two vegetable crops, chilli and tomato, in a transplant system are being reported.

Plant growth promoting rhizobacterial strain *Pseudomonas fluorescens* AMB-8 available with the Department of Agricultural Microbiology, College of Agriculture, Vellayani was used for the preparation of liquid formulations. The bacterial strain has already been reported to multiply in autoclaved coconut water (ACW) with the same growth rate as in other conventional media like nutrient broth (NB) or King's medium B broth (KB) (Anith, 2009). For the preparation of ACW, fresh coconut water was procured from a local copra processing facility and filtered through muslin cloth to remove the debris and suspended matter. 100 ml each of the coconut water was transferred to 500 ml Erlenmeyer flasks after adjusting the pH to 6.5

with required quantity of a five percent KOH solution and was sterilized by autoclaving at 121° C for 20 min. Amendment with either PVP (2% w/v) or glycerol (2% v/v) was done prior to autoclaving whenever required. NB and KB broth were prepared using synthetic chemicals, and preservatives were separately added before autoclaving. While NB was amended with PVP and glycerol separately, glycerol amendment was not done in KB as it already had glycerol as a component in it. A single colony of the bacterial culture was suspended in 1 ml of sterile distilled water (population density of 10^9 cfu ml^{-1}) and 100 µl of culture suspension was added to each of the liquid broths. Four flasks each medium with and without the amendment were maintained for monitoring the population of bacterial strain over a period of six months. Initial population of the bacterium in each of the medium was observed by serial dilution and spread plating on KB. The inoculated broth cultures were incubated in an incubator cum shaker at 28± 2°C at 100 rpm for 48 hours. Bacterial population buildup at 24 h intervals for two days was also monitored. Thereafter the formulations were kept at room temperature (25°C) and samples were drawn at monthly intervals up to a period of 180 days, and bacterial count in each formulation was estimated using viable plate count method on KB agar plates. The data were analysed using Duncan's multiple range test (DMRT) for comparing the means using the statistical package SAS version 8.1 (SAS Institute Inc., Cary, NC, USA).

The ability of the bacterial strain preserved in different culture broth formulations to promote plant growth was assessed under *in vivo* condition using the formulation preserved till 4th month. Fresh bacterial culture grown in KB (with a population density of 10^9cfu ml^{-1}) was also included as a treatment in addition to all the other treatments. Seeds of tomato variety Anagha and chilli variety Ujwala were surface sterilized with 1% sodium hypochlorite for 3 minutes and washed in three changes of sterile distilled water in a laminar air flow chamber. Seeds were spread on sterile tissue paper to drain excess water. The surface sterilized seeds were soaked separately in bacterial cultures grown in freshly inoculated KB and other formulations for 30 minutes. Control treatment received no seed bacterization and the seeds were soaked in sterile water for 30 min. The planting medium, vermiculite: perlite (1:1), was sterilized in an autoclave at 121°C and 15 lbs pressure for one hour for 3 consecutive days. Two treated seeds each were then dibbled in each of the cavity filled with sterile planting medium in plastic protrays. The population was thinned out to one in each cavities after one week of germination. Each treatment contained three replications having 10 plants each. The experiment was laid out as a completely randomized design (CRD). The protrays were kept in a green house and the seedlings were watered with sterile water twice a day. Hoagland's nutrient solution was provided to the seedlings at a rate of 10 ml per protray cavity, once in 10 days, starting one week after sowing. On the 21st day of sowing, the seedlings were carefully uprooted, the root portions were washed in running tap water and biometric observations were recorded. Statistical analysis of all the parameters was performed using one way analysis of variance (ANOVA) and the means were compared by DMRT using the statistical package SAS version 8.1.

Results of the population dynamics of the bacterial strain in different liquid bio-formulations are given in Table 1. Though the initial population was almost the same in all the formulations, the bacterial strain grew at a faster rate in KB broth for the first 48 hours. KB has been found to be a better medium for the support of the bacterial growth at least for the initial period of growth. There was a steady decline in population in all the formulations which was more pronounced in the case of conventional media except in NB-GLY. The reduction in population was the minimum in NB-GLY followed by ACW-PVP and ACW-GLY. As previously reported, nutrient broth amended with glycerol supported the viability of *Pseudomonas fluorescens* strain Pf1 to the tune of 10^7cfu ml^{-1} up to 180th day

Table 1. Population of *Pseudomonas fluorescens* in different liquid formulations (log cfu ml^{-1})*

Formulation	0 hrs	24 hrs	48 hrs	1st month	2ndmonth	3rd month	4th month	5th month	6th month
KB	6.20bc	10.04a	10.12a	7.96d	7.19d	7.15e	6.66f	5.91e	5.32e
KB-PVP	6.17bc	9.85b	10.10a	7.90d	7.05e	8.42c	7.78c	5.90e	5.64d
NB	6.34a	9.82b	9.83c	7.58e	6.32g	6.85f	6.88e	6.20d	5.78d
NB-PVP	6.25ab	9.82b	9.91b	7.29f	6.85f	6.72f	6.73f	6.08d	5.31e
NB-GLY	6.17bc	9.88b	9.95b	8.72c	8.17c	8.56b	8.62b	8.33b	7.39a
ACW	6.22b	9.69c	9.75d	9.13a	8.30b	7.72d	7.31d	6.78c	6.25c
ACW-PVP	6.11c	9.61d	9.78cd	9.17a	9.11a	9.48a	8.98a	8.68a	7.15b
ACW-GLY	6.23b	9.56d	9.63e	8.89b	9.05a	8.47bc	8.68b	8.36b	7.08b

*Mean of population from four independent observations. Figures in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test (DMRT) $p<0.05$.

KB: Kings medium B broth, KB-PVP: Kings medium B broth amended with 2% polyvinylpyrrolidone, NB: Nutrient broth, NB-PVP: Nutrient broth amended with 2% polyvinylpyrrolidone, NB-GLY: Nutrient broth amended with 2% glycerol, ACW: Autoclaved coconut water, ACW-PVP: Autoclaved coconut water amended with 2% polyvinylpyrrolidone, ACW-GLY: Autoclaved coconut water amended with 2% glycerol

(Manikandan et al., 2010). In the present study the population level of the strain AMB-8 came down to about 10^7cfu ml^{-1} on 180th day in NB-GLY and PVP amended nutrient broth showed more decline in population over a period of six months. Our observation was however contradictory to that of Manikandan et al. (2010) as far as the population dynamics of AMB-8 in KB as a nutrient base was concerned. They observed very poor survival of the strain Pf1 in KB, whereas the strain AMB-8 was able to maintain a population of up to 10^5cfu ml^{-1} in KB or PVP amended KB. Such differences of survival ability of different strains of the same bacterial species in different liquid media on storage have been reported earlier (Albareda et al., 2008). In the current experiment, it has been observed that the rate of decline in population was less in coconut water amended with PVP and glycerol. Even in the

Table 2. Biometric observations on tomato seedlings as affected by seed bacterization with different liquid formulations of *Pseudomonas fluorescens* AMB8

Treatment*	Leaf number (per plant)	Shoot length (cm)	Shoot fresh weight (g/plant)	Shoot dry weight (mg/plant)	Root fresh weight (g/plant)	Root Dry weight (mg/plant)
Fresh bacterial culture in KB	5.85a	19.80ab	1.84a	136.45a	0.43a	21.46a
KB	5.28de	15.28e	1.6cd	106.82c	0.23d	14.80cd
KB PVP	5.28de	17.44d	1.67abc	118.43bc	0.31c	16.34dcd
NB	5.39cd	18.73bcd	1.74abc	89.75d	0.22d	15.34cd
NB PVP	5.14e	18.15cd	1.65bcd	90.34d	0.23d	13.39d
NB GLY	5.28de	20.78a	1.75abc	122.73b	0.35b	15.72bcd
ACW	5.25de	18.78bcd	1.64bcd	107.2c	0.32bc	17.09bc
ACW PVP	5.71ab	19.77ab	1.80ab	118.8bc	0.42a	17.52bc
ACW GLY	5.21de	19.56abc	1.74abc	136.33a	0.33bc	18.95ab
Control	5.57bc	14.25e	1.49d	69.0e	0.23d	13.19d

*Observations taken 21 days after planting. Mean of three replications having 10 seedlings each. Digits in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test (DMRT) $p<0.05$. *a:* seed bacterization was done with four month old formulated product. KB: Kings medium B broth, KB-PVP: Kings medium B broth amended with 2% polyvinylpyrrolidone, NB: Nutrient broth, NB-PVP: Nutrient broth amended with 2% polyvinylpyrrolidone, NB-GLY: Nutrient broth amended with 2% glycerol, ACW: Autoclaved coconut water, ACW-PVP: Autoclaved coconut water amended with 2% polyvinylpyrrolidone, ACW-GLY: Autoclaved coconut water amended with 2% glycerol.

Table 3. Biometric observations of chilli seedlings as affected by seed bacterization with different liquid formulations of *Pseudomonas fluorescens* AMB8

Treatment *	Leaf number (per plant)	Shoot length (cm)	Shoot fresh weight (g/plant)	Shoot dry weight (mg/plant)	Root fresh weight (g/plant)	Root Dry weight (mg/plant)
Fresh bacterial culture in KB	5.14ab	10.13a	0.68a	76.59a	0.21ab	17.16a
KB	5.14ab	9.01de	0.56d	73.33abc	0.16d	11.29d
KB-PVP	4.85cde	9.65abc	0.62c	69.31bcde	0.18c	14.18c
NB	4.63e	8.59e	0.50e	65.45de	0.12e	13.87c
NB-PVP	4.79de	9.11cde	0.53de	67.21cde	0.13e	12.22d
NB-GLY	4.96bcd	9.82ab	0.63bc	64.38e	0.18c	14.42c
ACW	4.08abc	9.47bcd	0.63bc	71.59abcd	0.19c	14.61c
ACW-PVP	5.29a	9.74ab	0.66ab	77.35a	0.22a	16.26ab
ACW-GLY	5.07abc	9.83ab	0.69a	74.56ab	0.20bc	15.12bc
Control	4.71e	7.81f	0.54d	69.50bcde	0.13e	11.26d

*Observations taken 21 days after planting. Mean of three replications having 10 seedlings each. Digits in a column followed by same letter do not differ significantly according to Duncan's Multiple Range Test (DMRT) $p<0.05$. *a:* seed bacterization was done with four month old formulated product. KB: Kings medium B broth, KB-PVP: Kings medium B broth amended with 2% polyvinylpyrrolidone, NB: Nutrient broth, NB-PVP: Nutrient broth amended with 2% polyvinylpyrrolidone, NB-GLY: Nutrient broth amended with 2% glycerol, ACW: Autoclaved coconut water, ACW-PVP: Autoclaved coconut water amended with 2% polyvinylpyrrolidone, ACW-GLY: Autoclaved coconut water amended with 2% glycerol.

raw ACW, the maintenance of the population of the bacterial strain was significantly higher compared to synthetic media other than NB-GLY.

Pseudomonas fluorescens AMB-8 has been reported to have plant growth promoting ability along with biocontrol potential and is classified as a plant growth promoting rhizobacterium (PGPR) (Anith et al., 2002; 2003; 2015). Generally, solid carrier based formulations are expected to have a shelf life of three months period and therefore we had taken 4th month as a bench mark for testing the efficacy of the liquid formulations. All biometric parameters had higher values when freshly grown bacterium in KB with population density of 10^9cfu ml^{-1} was used for seed bacterization (Table 2 and 3). High bacterial population density in the formulations had a positive effect on the plant growth, both in the case of chilli and tomato seedlings. At the 4th month period, NB-GLY, ACW-PVP and ACW-GLY had a population density of 10^8cfu ml^{-1} of the formulation. Direct co-relation between seed colonization and plant growth promotion has been earlier reported with the strain AMB-8 in tomato seedlings (Anith, 2009).

The importance and need for research on the development of liquid biopesticides in India for sustainable agricultural production has been stressed recently (Rao et al., 2015). The results of our study showed that a value added product with high potential could easily be made from an agricultural waste product like coconut water. The input cost of one litre of nutrient broth and King's medium B broth comes to Rs. 50.00 and Rs. 220.00 respectively, whereas it is almost no cost (if the transportation cost is not accounted) in the case of coconut water. Addition of the amendments further increases the cost by Rs. 18.00 and 108.00 per litre in the case of glycerol and PVP respectively. The abundant availability of coconut water, especially in south India makes it a viable option for commercial production of liquid bioformulation of PGPR and other biocontrol bacteria locally. However, strain to strain difference with respect to their ability to survive in coconut water has to be evaluated before the same formulation strategy is recommended for commercial production of other bacterial biological agents.

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