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Mass production of biocontrol agents in coconut water based media

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Biocontrol agents are widely utilized for management of plant diseases in different crops as a component in the integrated management of diseases (Srinivasan, 2003; Srinivasan *et al.*, 2006; Srinivasulu *et al.*, 2004; Velusamy *et al.*, 2003). Commercial formulations of the biocontrol agents as biopesticides, suitable for field application have also become available, though only to a limited extent. Bacterial bioagents like *Pseudomonas fluorescens, Bacillus subtilis* and fungal bioagent like *Trichoderma* spp. are grown in certain defined media involving costly chemical components. Various workers have tried a variety of substances, agricultural wastes/byproducts etc. and found them as successful substrates for the mass production of biocontrol agents (Ramanujam *et al.*, 2004; Suseela Bhai *et al.*, 1994). Like wise, the ability of coconut water, supporting the growth of biocontrol agents for possible mass culture has been reported (Anandaraj and Sarma, 1997; Mathew, 2003). Although coconut water is commonly available that is rich in nutrients despite changes happening in developmental stages of nuts (Jeyalekshmy *et al.*, 1988; Nathanael, 1996) the scope of its utilization as a mass multiplication medium for biocontrol agents needed to be expanded. Coconut water from various developmental stages of nuts was investigated for multiplication of popular biocontrol agents, their viability status in formulations evolved and the results are presented in the paper.

Experiment I: Coconut water was tested in comparison with certain other media for growth of *B. subtilis* and *P. fluorescens* as shown in Table 1. Each medium in culture flasks (200 ml/flask) in sterile state served as test growth medium for the antagonists. Stock inoculums of bioagents were independently prepared by transferring a loopful of cultures from solid medium into 10 ml of sterile distilled water. Mixture of inocula of two species was also appropriately prepared. From the stock cultures 0.1 ml of inoculum was inoculated into each medium in flasks and incubated at 30 ± 1°C with periodical shaking. After 48 hrs. of incubation 0.1 ml of the grown culture was subjected to serial dilutions (up to 6th stage) with sterile distilled water and from the last dilution 0.1 ml of inoculum transferred to sterile petri dishes. Sterile Nutrient agar (NA) was added into the petri dishes, mixed and the petri dishes incubated. Bacterial colonies in the petri dishes were counted after 48 hrs. of incubation.

Experiment II: Coconut water was enriched independently with Glucose and Peptone, each at 1% level in sterile state and such enriched media on growth of *B. subtilis* and *P. fluorescens*, was assessed in different combinations as given in Table 1. Colonies of *B. subtilis*, *P. fluorescens* and *B. subtilis* + *P. fluorescens* in combination in NA solid medium were counted after due transfers from coconut water liquid cultures and incubation. No. of C.f.u was recorded in each case after 48 hrs. of incubation.

Experiment III: The media were prepared under two categories – coconut water medium in natural state (of acidic pH) and medium adjusted to the level of around neutral (pH: 7 ± 0.1) in different combinations as shown in Table 2 and appropriately inoculated with *B. subtilis* and *P. fluorescens* - their growth assessed and colonies counted.

Experiment IV: Coconut water media under two categories –in natural state (of acidic pH) and where pH

adjusted to the level of 6.0-6.5 were assessed for growth of the fungal antagonist, *T. viride* in comparison with its growth in Potato dextrose broth (PDB). Each medium in culture flasks (200 ml/flask) served as test growth medium. Mycelial discs (5 mm diameter) of five-day-old culture of *T. viride* (previously grown in PDA) was individually inoculated into culture flasks @ one disc per flask and incubated at 30 ± 1°C for 20 days with periodical observations. The growth status of *T. viride* was qualitatively compared under grades of + - Low, ++ - Moderate, +++ - High and ++++- Very high growth.

Table 1. Mean growth of bacterial antagonists in coconut water, enriched coconut water liquid media besides various other media after two days of incubation

S. No.	Type of liquid medium*	Mean No. of bacterial colonies(C.f.u. /ml at 10^{-6} dilution) **			Mean
		Bacillus subtilis	*Pseudomonas fluorescens*	*B. subtilis* +*P. fluorescens*	
	Conventional medium				
01	Nutrient broth	31.3 (5.6)	30.0 (5.5)	31.3 (5.6)	31.0 (5.6)
02	King's B broth	33.3 (5.8)	32.3 (5.7)	32.7 (5.7)	32.8 (5.7)
03	Glucose Peptone broth	29.7 (5.4)	28.7 (5.3)	30.3 (5.5)	29.6 (5.4)
04	Sabouraud dextrose broth	18.3 (4.3)	17.3 (4.1)	18.7 (4.3)	18.1 (4.2)
	CD at 5%	**7.5**	**NS**	**6.5**	**4.1**
	Mean	**28.2**	**27.2**	**28.3**	
	Coconut water medium				
01	WCT – I	21.3 (4.6)	20.7 (4.5)	21.7 (4.6)	21.2 (4.6)
02	WCT – II	32.0 (5.6)	32.0 (5.7)	32.7 (5.7)	32.2 (5.7)
03	WCT – III	33.7 (5.8)	32.0 (5.7)	33.0 (5.7)	32.9 (5.7)
04	COD – I	20.7 (4.5)	18.3 (4.3)	20.7 (4.5)	19.9 (4.4)
05	COD – II	29.7 (5.4)	28.7 (5.3)	30.0 (5.5)	29.4 (5.4)
06	COD – III	29.7 (5.4)	28.7 (5.3)	30.3 (5.5)	29.6 (5.4)
	CD at 5%	**8.8**	**6.2**	**9.2**	**4.3**
	Mean	**27.8**	**26.7**	**28.1**	
	Enriched coconut water medium				
01	WCT – I + G	20.0 (4.5)	18.3 (4.3)	21.0 (4.6)	19.8 (4.4)
02	WCT – II + G	30.3 (5.5)	31.0 (5.6)	31.7 (5.6)	31.0 (5.6)
03	WCT – III + G	32.0 (5.6)	30.3 (5.5)	31.0 (5.5)	31.1(5.6)
04	COD – I + G	18.7 (4.3)	17.0 (4.1)	19.0 (4.4)	18.2 (4.2)
05	COD – II + G	28.3 (5.3)	26.0 (5.1)	28.3 (5.3)	27.6 (5.2)
06	COD – III + G	28.0 (5.3)	27.3 (5.2)	29.0 (5.4)	28.1 (5.3)
07	WCT – I + P	23.7 (4.9)	21.7 (4.6)	23.0 (4.8)	22.8 (4.8)
08	WCT – II + P	34.7 (5.9)	33.7 (5.8)	34.7 (5.9)	34.3 (5.9)
09	WCT - III + P	35.7 (6.0)	34.0 (5.8)	34.7 (5.9)	34.8 (5.9)
10	COD - I + P	24.3 (4.9)	20.7 (4.5)	23.7 (4.9)	22.9 (4.8)
11	COD - II + P	32.7 (5.7)	30.7 (4.5)	32.0 (5.6)	31.8 (5.6)
12	COD - III + P	33.3 (5.8)	31.7 (5.6)	32.7 (5.7)	32.6 (5.7)
	CD at 5%	**06.3**	**07.2**	**07.3**	**3.9**
	Mean	**28.5**	**26.9**	**28.4**	

*WCT: West Coast Tall; COD: Chowghat Orange Dwarf; **Mean of 3 replications

I – III: Stages of coconut nuts development in months (I: 6-8, II: 8-10, III: 10-12) G – Glucose 1%, P – Peptone 1%

Figures within the parentheses are square root transformed values

Bioformulations, viability and shelf life of bioagents: Both bacterial and fungal antagonists grown independently in coconut water medium and each at least in one another (conventional) medium were prepared as bioformulations using neutral talc powder as carrier. The test inoculums of each bioagent was mixed thoroughly with talc powder @ 400 ml of liquid culture per kg. of talc powder containing 5 g of carboxy methyl cellulose as adjuvant in clean conditions. In the case of *T. viride* the fungal biomass along with broth was transferred from flasks and mixed thoroughly in the talc powder. The mixed product was air-dried at room temperature as per the standard procedure for approximately 72 hrs. and C.f.u. of test organisms calculated. The talc-based formulations were packed in opaque polythene covers either individually or in combination with previously steam sterilized neem cake (1 : 1w/w) sealed and stored in room temperature for varying durations. Viability and shelf life tests were done at monthly intervals upto six months of storages.

Both *B. subtilis* and *P. fluorescens* were able to multiply in coconut water from various stages of nuts of

both varieties and the growth comparable with that of conventional media in Experiment I. The C.f.u. of *B. subtilis*, *P. fluorescens* and *B. subtilis* + *P. fluorescens* in combination were 27.8, 26.7 and 28.1, respectively, among coconut water media from different stages of nut while in other media, the counts for the corresponding treatments were 28.2, 27.2 and 28.3, respectively. However, the growths of the bacterial species were higher (29.4 - 32.9) in nut water derived from developed nuts (above 8 months) as compared to their growth in nut water from younger nuts (19.9 - 21.2). Therefore, the bioagents can be multiplied with relatively higher yield in nut water from matured nuts (Table 1). *B. subtilis* and *P. fluorescens* were slightly depressed in glucose enriched coconut water, whereas their growth enhanced with peptone in Experiment II - the C.f.u. of *B. subtilis*, *P. fluorescens* and *B. subtilis* + *P. fluorescens* combinations for various treatments were 28.5, 26.9 and 28.4, respectively. The range of mean bacterial growth in peptone-enriched media was higher (22.8 – 34.8) as compared to that in glucose-enriched media (18.2 – 31.1) that is in conformity with earlier observations that peptone enrichment of coconut water aids higher cell yield of *P. fluorescens* (Mathew, 2003). As nut water from developed nuts seemed to consistently yield more cells the peptone enrichment of coconut water drawn especially form matured nuts can be exploited.

The coconut water medium, which is basically of acidic pH adjusted to neutral pH supported the growth of the bacteria to a higher extent in Experiment III - the C.f.u. of *B. subtilis*, *P. fluorescens* and *B. subtilis* + *P. fluorescens* in combination were higher in coconut water media with neutral pH (29.7, 28.4 and 30.1) while that in acidic pH was 28.3, 26.8 and 28.8, respectively (Table 2). The mean bacterial colonies also stood at a higher range (21.0 – 34.9) in media where pH was adjusted to neutral state as compared to the control (19.7 – 33.4). The possibility of increased harvest of the microbes by alteration of medium pH to desired level besides their co-cultivability thus exists (Srinivasan *et al.*, 2006). During the study the bacterial bioagents could be grown in coconut water medium within Bioreactor of a Fermentor system also.

Mycelial growth of the fungal antagonist, *T. viride* on coconut water liquid medium was comparable to its growth on PDB. However, its growth was higher in coconut water drawn from fully matured nuts as compared to other sources and pH adjustment of nut water (to 6.0-6.5) also positively influenced the bioagent's growth. Reported utility of mature coconut water for multiplication of fungal bioagents, *Trichoderma* and *Gliocladium* (Anandaraj and Sarma, 1997) supports the present results and therefore *T. viride* can also be mass multiplied in coconut water preferably drawn from matured nuts.

Table 2. Mean growth of bacterial antagonists in coconut water liquid media under natural state of acidic pH and pH adjusted to neutral after two days of incubation

S. No.	pH status of coconut water liquid medium*	Mean No. of bacterial colonies(C. f. u. /ml at 10^4 dilution) **			
		Bacillus subtilis	*Pseudomonas fluorescens*	*B.subtilis + P.fluorescens*	Mean
	Natural – acidic pH				
01	WCT – I	21.7 (4.6)	21.0 (4.6)	22.7 (4.7)	21.8 (4.7)
02	WCT – II	31.3 (5.6)	32.3 (5.7)	33.3 (5.8)	32.3 (5.7)
03	WCT – III	34.7 (5.9)	32.0 (7.7)	33.7 (5.8)	33.4 (5.8)
04	COD – I	21.7 (4.6)	16.0 (4.0)	21.3 (4.6)	19.7 (4.4)
05	COD – II	30.7 (5.5)	28.3 (5.3)	31.0 (5.6)	30.0 (5.5)
06	COD – III	30.0 (5.5)	31.0 (5.6)	31.0 (5.6)	30.7 (5.5)
	CD at 5%	**7.3**	**6.0**	**9.3**	**4.0**
	Mean	**28.3**	**26.8**	**28.8**	
	pH adjusted to neutral				
07	WCT – I	23.0 (4.8)	22.3 (4.7)	24.3 (4.9)	23.2 (4.8)
08	WCT – II	32.7 (5.7)	34.0 (5.8)	34.3 (5.9)	33.7 (5.8)
09	WCT - III	35.7 (6.0)	34.3 (5.9)	34.7 (5.9)	34.9 (5.9)
10	COD - I	23.7 (4.8)	16.7 (4.1)	22.7 (4.7)	21.0 (4.6)
11	COD - II	32.0 (5.7)	31.0 (5.6)	32.0 (5.6)	31.7 (5.6)
12	COD - III	31.3 (5.6)	32.3 (5.7)	32.3 (5.7)	32.0 (5.6)
	CD at 5%	**7.1**	**4.7**	**NS**	**3.9**
	Mean	**29.7**	**28.4**	**30.1**	

*WCT: West Coast Tall; COD: Chowghat Orange Dwarf; **Mean of 3 replications

I – III: Stages of coconut nuts development in months (I: 6-8, II: 8-10, III: 10-12)

– pH of nut water of respective stage: 4.6 - 5.8; 5.2 - 5.9; 5.5 - 6.1

Figures within the parentheses are square root transformed values

The bioformulations of *B. subtilis*, *P. fluorescens* and *T. viride* individually- developed through growth of bioagents in coconut water medium was comparable to that of conventional media grown preparation (Ramamoorthy *et al.*, 2001; Pramod and Sivaprasad, 2003). The population of bioagents in talc formulation and in talc-neem cake mixture - grown in coconut water media - was comparable correspondingly with population noticed in the case of conventional media. The initial C.f.u. of bacterial antagonists ranged from 248 x 10^{12} to 256 x 10^{12} in talc powder carrier. The population of *B. subtilis* and *P. fluorescens* in talc increased for one (340 x 10^{12} - 355 x 10^{12}) - two months (328 x 10^{10} - 345 x 10^{10}) and thereafter slightly declined. The population (C.f.u.) of *T. viride*, initially stood at the level of 4.8 x 10^{8} – 5.3 x 10^{8} also increased for one (5.8 x 10^{8} - 6.5 x 10^{8}) - two months (5.5 x 10^{8} – 6.2 x 10^{8}) and thereafter declined. However, reasonably high population of the bioagents stood available in the formulations till the end of 6 months of storage and with only marginal differences between coconut water and other media for corresponding bioagents (Table 3). The initial C.f.u. of bacterial antagonists ranged from 125 x 10^{12} to 130 x 10^{12} in talc powder – neem cake mixture carrier that increased for one (172 x 10^{12} - 179 x 10^{12}) - two months (171 x 10^{10} - 175 x 10^{10}) and declined. The population (C.f.u.) of *T. viride*, which stood at the level of 2.6 x 10^{8} – 2.8 x 10^{8} also increased for one (3.2 x 10^{8} - 3.6 x 10^{8}) - three months (2.9 x 10^{8} – 3.2 x 10^{8}) and thereafter declined. The population of the bioagents available in the mixture till 6th month of storage were - bacteria: 46 x 10^{8} - 53 x 10^{8} and fungus: 1.8 x 10^{8} - 2.1 x 10^{8} - compared well to the population of corresponding bioagents grown in other media (Table 4). Utility of neem cake for growing biocontrol agents has been widely reported (Ramanujam *et al.*, 2004). As neem is acceptable to the bioagents (Anishkumar *et al.*, 2004), its importance for fortification stands validated.

Table 3. Viability of biocontrol agents - grown in coconut water and conventional media - in talc powder carrier in different months of storage*

Biocontrol agent	Medium of growth	Initial population (Cfu/gm) in talc carrier (X 1012)	Population of bacterial bioagent (Cfu/gm) in talc carrier at different months of storage					
			1 (X 1012)	2 (X 1010)	3 (X 1010)	4 (x 109)	5 (X 108)	6 (X 108)
Bacillus subtilis	Nutrient Broth	256	352	345	230	112	100	85
	Coconut water	254	355	328	200	114	95	81
Pseudomonas fluorescens	King's B Broth	250	350	340	210	100	90	81
	Coconut water	248	340	345	205	110	85	79
		(X 108)	Population of fungal bioagent (Cfu/gm) in talc carrier at different months of storage (X 108)					
Trichoderma viride	Potato Dextrose Broth	5.3	6.5	6.2	5.6	4.8	4.0	3.7
	Coconut water	4.8	5.8	5.5	5.3	4.2	3.8	3.5

*Mean of 3 replications

Table 4. Viability of biocontrol agents - grown in coconut water and conventional media - in talc powder-neem cake mixture carrier (1:1 ratio - w/w) in different months of storage*

Biocontrol agent	Medium of growth	Initial population (Cfu/gm) in talc-neem cake mixture carrier (X 1012)	Population of bacterial bioagent (Cfu/gm) in talc-neem cake mixture carrier at different months of storage					
			1(X 1012)	2(X 1010)	3(X 1010)	4(X 109)	5(X 108)	6(X 108)
Bacillus subtilis	Nutrient Broth	130	179	175	122	65	60	53
	Coconut water	128	178	171	113	63	57	51
Pseudomonas fluorescens	King's B Broth	126	176	174	117	62	58	48
	Coconut water	125	172	173	110	60	51	46
		(X 108)	Population of fungal bioagent (Cfu/gm) in talc-neem cake mixture carrier at different months of storage (X 108)					
Trichoderma viride	Potato Dextrose Broth	2.8	3.6	3.5	3.2	2.7	2.3	2.1
	Coconut water	2.6	3.2	3.1	2.9	2.4	2.2	1.8

*Mean of 3 replications

This study has strengthened utilization of coconut water for mass production of bacterial and fungal biocontrol agents and consequently bioformulations with reasonably high population/shelf-life (3-6 months) of bioagents possible. Coconut water from matured nuts could be economically utilized for mass production of biocontrol agents.

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