



Field management of coconut leaf rot disease with biological control agents, their bioformulation and viability in organics

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Abstract

Leaf rot disease is an integral part of root (wilt) disease of coconut and hence its control a great importance. Potential of biological control agents against leaf rot pathogens have been established. Field performance of talc-based *B. subtilis* and *P. fluorescens* against leaf rot (individually and in consortium mode), growth of bioagents in coconut water-based media for enriching bioformulations and viability of the bioagents in organic carriers based formulations were evaluated. A field experiment conducted with treatments of *B. subtilis*, *P. fluorescens*, *B. subtilis* + *P. fluorescens* and Phytosanitation + *B. subtilis* + *P. fluorescens* in comparison with control showed a decline of disease index in newly emerged leaves of treated palms (higher disease ameliorative effect through consortium of *B. subtilis* and *P. fluorescens*). Multiplication of bacterial (*P. fluorescens*) and fungal (*Trichoderma viride*) bioagents could be achieved in coconut water-based media - moderate-good growth of *P. fluorescens* in coconut water (pH adjusted to 7.0), coconut water amended with peptone at 1 % or jaggery at 5 % or 10 % levels comparable with its growth in King's B broth; moderate-good mycelial yield of *T. viride* in coconut water-based media with or without jaggery amendment comparable to its growth in potato dextrose broth. The bacterial and fungal biocontrol agents as grown in coconut water-based media could be processed into talc-based bioformulations (quality comparable with such products evolved using conventional media). The talc-based formulations admixed with organics viz., neem cake, vermicompost and coir pith-(1: 1w/w) that were individually packed also assured reasonable period shelf-life (six months) of bioagents, compared well to corresponding other media. Thus mass production of popular biological control agents in coconut water based medium followed by their bioformulations through fortifications with popular organics has been successfully evolved.

Keywords: Bio agents, biological control, coconut, leaf rot disease, mass production, organics

Introduction

Leaf rot disease is an integral part of coconut root (wilt) disease widely prevalent in southern districts of Kerala and in Tamil Nadu (Theni district). Leaf rot is important as its superimposition in root (wilt) affected palms leads to a sharp decline in the condition of the palms. Even as no therapeutic measures are available for controlling root (wilt), leaf rot control is of great importance in the management of root (wilt) disease. The leaf rot is due to a complex of fungal pathogens wherein *Colletotrichum gloeosporioides*, *Exserohilum rostratum* and *Fusarium solani* are the major pathogens (Srinivasan and Gunasekaran, 1996a). A simple technique of applying fungicide on to well of the spindle leaf of coconut crown for amelioration of leaf rot and

such a technique has been standardized which received wide spread attention in the disease affected region (Srinivasan, 2008). Biological control agents are widely utilized as a component in the integrated management of plant diseases. This technology has gained popularity in view of eco-friendly nature with environmental protection by way of avoiding costly pesticides. Bacterial antagonistic agents like *Bacillus subtilis*, *Pseudomonas fluorescens* and fungal antagonistic agent like *Trichoderma* spp. is most commonly used world wide against plant diseases (Srinivasan and Bharathi, 2006). The advantages of consortium of biological control agents in the diseases control are also exploited. Srinivasan *et al.* (2006) strongly established antagonistic potential of such organisms against leaf rot pathogens

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and also synergism (*B. subtilis* and *P. fluorescens*). Amelioration of leaf rot affected palms in the field with talc-based preparation of *P. fluorescens* has been also found. The need of field evaluation of such bioagents in combination in comparison with their individual impact on the disease was felt necessary. 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 *P. fluorescens*, *B. subtilis* and fungal bioagent like *Trichoderma* spp. are grown in certain defined media. A variety of substances are found as useful substrates for the mass production of biocontrol agents (Suseela Bhai *et al.*, 1994). The ability of coconut water, supporting the growth of biocontrol agents for possible mass culture has been reported (Mathew, 2003). Although coconut water is commonly available that is rich in nutrients the scope of its utilization as a mass multiplication medium for biocontrol agents needed to be expanded. Srinivasan (2007) investigated coconut water from various stages of nuts for evolving bioformulations and accomplished economic utilization of coconut water especially from matured nuts. However, enriched coconut water media for mass multiplication of bioagents, bioformulations and exploitation of organics as possible carriers of bioagents suitable for field application etc. are also felt necessary. Accordingly experiments were conducted with reference to field utility of talc-based formulations of *B. subtilis* and *P. fluorescens* against leaf rot disease (individually and in consortium mode); multiplication of bacterial and fungal bioagents in coconut water based media for evolving enriched bioformulations; evaluation of viability/persistence of the bioagents in formulations with certain popular organics as carriers.

Materials and Methods

Field efficacies of biological control agents on leaf rot:

A field trial was conducted with talc based formulations of *B. subtilis* and *P. fluorescens* in 30 year old leaf rot affected palms and the experiment designed based on single palm plot size: Treatments - *B. subtilis*, *P. fluorescens*, *B. subtilis* + *P. fluorescens*, Phytosanitation + *B. subtilis* + *P. fluorescens* in comparison with Control @ 10 palms per treatment. After pre-treatment observations of disease status talc-based bioagents of *B. subtilis* and *P. fluorescens*, singly or in combination (2.5×10^8 cfu/g) as the case may be, was suspended in water (50g/500 ml per palm) and poured into the axil of the spindle of palms. The phytosanitation measure - cut and removal of leaf rot affected portions of spindle/young leaves - was adopted in relevant

treatment. The treatments were imposed twice per year (April/May and September/October) and the trial continued for about three years. Disease index for emerged leaves of palms at each round was evolved in a cumulative manner. The data of treated palms including mean disease indices for various rounds were compared for assessing the effect of treatments over disease amelioration (Srinivasan and Gunasekaran, 1996b).

Multiplication of biological control agents in coconut water based media:

As economic utility of coconut water drawn especially from developed (matured) nuts of coconut was evidenced multiplication of each one bacterial (*P. fluorescens*) and fungal (*T. viride*) biocontrol agents were individually effected in coconut water based media. Treatments for multiplication of *P. fluorescens* were: Coconut water - in natural state of pH, coconut water - pH adjusted to 7.0, coconut water + peptone (1 %) - pH 7.0, coconut water + jaggery (5 %) - pH 7.0, coconut water + jaggery (10 %) - pH 7.0, jaggery solution alone (5 %) - pH 7.0 and jaggery solution alone (10 %) - pH 7.0 (King's B broth - for comparison). Each medium in culture flasks (200 ml/flask) in sterile state served as test growth medium. Stock inoculum of the bioagent was prepared by transferring a loopful of culture from solid medium into 10 ml of sterile distilled water. From the stock cultures 0.1 ml of inoculum was inoculated into each medium in flasks and incubated at $30 \pm 1^\circ\text{C}$ with periodical shaking. After 48 hrs. of incubation 0.1 ml of the grown culture was subjected to serial dilutions (upto 6th stage) with sterile distilled water and from the last dilution 0.1 ml of inoculum transferred to sterile petridishes. Sterile Nutrient agar (NA) was added into the Petri dishes, mixed and the petri dishes incubated. Bacterial colonies in the petri dishes (six replications per treatment) were counted after 48 hrs. of incubation. Treatments for multiplication of *T. viride* were: Coconut water - in natural state of pH, coconut water - pH adjusted to 7.0, coconut water + jaggery (5 %) - pH 7.0, coconut water + jaggery (10 %) - pH 7.0, jaggery solution alone (5 %) - pH 7.0 and jaggery solution alone (10 %) - pH 7.0 (potato dextrose broth - for comparison). 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 potato dextrose agar) was individually inoculated into culture flasks @ one disc per flask and incubated at $30 \pm 1^\circ\text{C}$ for 18 days with periodical observations. The mycelia mass of *T. viride* was harvested weighed on dry weight basis (six replications per treatment).

Biological control agents: Bioformulations - viability - shelf life in organics:

Bacterial and fungal bioagents grown independently in coconut water medium and each at least in one conventional medium were prepared as bioformulations using neutral talc powder as carrier. The test inoculum of *P. fluorescens* were 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 for approximately 72 hrs. as per the standard procedure and C.f.u. of test organisms calculated. The talc-based formulations in combination with previously steam sterilized organics (neem cake, vermicompost and coir pith - (1:1w/w) individually were packed in opaque polythene covers, sealed and stored in room temperature. Viability and shelf life of bioagents in the talc-organic mixture were assessed at monthly intervals upto six months of storages by enumeration of colony-forming units (three replications per treatment).

Results and Discussion

Field efficacies of biological control agents on leaf rot:

The effect of talc based preparations of *B. subtilis* and *P. fluorescens* - individually and in consortium mode - on leaf rot disease in field palms are presented in Table 1- treatment of palms with these bacterial bioagents lead to decline of disease index in newly emerged leaves of coconut in various rounds of observations, whereas in control palms the disease index increased. The mean disease index among treatments (5.59 - 6.76) in treated palms (pre-treatment level) declined steadily over the rounds of treatments imposition to the level of 3.35 - 4.90 at the final round. Thus the treatment of bacterial antagonists individually and in consortium mode lead to a decline of disease index in newly emerged leaves in various rounds. In control palms, the disease index increased steadily from the pre-treatment disease index of 4.70 to 12.56 at the final round of observation. At the last round of observation the mean disease index in the experimental palms was less in the range of 23.91 - 46.49 % among treatments over the pre-treatment and also less by 60.99 - 73.33 % in comparison with control palms. In the control, the mean disease index increased to 167.23 % from pre-treatment level and consequently the disease level stood at 274.93 % in comparison with the highest performed treatment of phytosanitation + consortium of *B. subtilis* and *P. fluorescens*. Comparisons

of disease index of palms for all rounds of observations also revealed constant effect of *B. subtilis* and *P. fluorescens*, individually and in combination in amelioration of leaf rot affected palms. The mean disease index of various treatments for the rounds (after pre-treatment) for treated (4.10-5.45) and control palms (8.95) were also evidenced. Therefore, reduced levels of disease in treated palms over pre-treatment (15.37 % - 34.50 %) and also control palms (39.11 % - 54.19 %) besides increased levels of disease in control palms over its own pre-treatment (90.43 %) and treated palms with highest performed treatment (118.29 %) are brought out. In over all, *B. subtilis* and *P. fluorescens* individually were able to ameliorate the disease in field palms; however, their combination along with adoption of phytosanitation poised for a higher impact on disease reduction. Hence, improvement in condition of bio treated palms and constant deterioration of palms under the untreated control could be found besides the clear evidence of higher ameliorative effect of consortium of bioagents along with phytosanitation. Bacterial antagonists such as *B. subtilis* and *P. fluorescens* have been used as potential biocontrol agents for the management of diverse group of plant diseases (Reddy *et al.*, 2003) and utility of commercial preparation of the bioagent proved (Rajbir Singh and Sinha, 2004). Srinivasan *et al.* (2006) evidenced inhibition of pathogens of leaf rot by bacterial (*B. subtilis* and *P. fluorescens*) and fungal (*Trichoderma* sp.) bioagents, effectiveness of consortium of *B. subtilis* and *P. fluorescens* and also disease amelioration in field palms with talc-based *P. fluorescens*. Biological control agents inducing systemic resistance in plants against diseases has been widely reported elsewhere. Utility of various biocontrol agents in inducing systemic resistance/ production of value added coconut seedlings through biopriming with various biocontrol agents against leaf rot disease have been also shown (Srinivasan, 2010). Further work on lines of demonstration of field management of leaf rot disease with consortium of biological control agents in comparison with fungicide, integrated approach for large scale penetration of the disease management technology into farmers' gardens, production of bioprotected coconut seedlings using bioagents etc. are in progress.

Multiplication of biological control agents in coconut water based media

Both *P. fluorescens* and *T. viride* were found to multiply in coconut water based media, comparable with relevant conventional media (Tables 2 and 3). The c.f.u. of *P. fluorescens* was 31.8 in coconut water in natural

Table 1. Effect of application of talc-based preparations of *Bacillus subtilis* and *Pseudomonas fluorescens* - individually & consortium mode on leaf rot disease on coconut palms*

S. No.	Treatment	Pre-treatment Disease Index**	Leaf rot Disease Index in emerged leaves in different rounds **					Mean of rounds
			I	II	III	IV	V	
1	Application of <i>Bacillus subtilis</i>	6.44	6.10	5.82	5.32	5.10	4.90	5.45
	Improvement over pre-treatment (Decrease in DI) - %		(-) 5.28	(-) 9.63	(-) 17.39	(-) 20.81	(-) 23.91	(-) 15.37
	Improvement over control (Decrease in D I) - %		(-) 2.87	(-) 10.46	(-) 37.85	(-) 52.91	(-) 60.99	(-) 39.11
2	Application of <i>Pseudomonas fluorescens</i>	6.76	5.98	5.70	5.10	4.80	4.50	5.22
	Improvement over pre-treatment (Decrease in DI) - %		(-) 11.54	(-) 15.68	(-) 24.56	(-) 28.99	(-) 33.43	(-) 22.78
	Improvement over control (Decrease in D I) - %		(-) 4.78	(-) 12.31	(-) 40.42	(-) 55.68	(-) 64.17	(-) 41.68
3	Combined application of <i>B. subtilis</i> and <i>P. fluorescens</i>	5.59	4.86	4.80	4.25	3.75	3.63	4.26
	Improvement over pre-treatment (Decrease in DI) - %		(-) 13.06	(-) 14.13	(-) 23.97	(-) 32.92	(-) 35.06	(-) 23.79
	Improvement over control (Decrease in D I) - %		(-) 22.61	(-) 26.15	(-) 50.35	(-) 65.37	(-) 71.10	(-) 52.40
4	Phytosanitation + Combined application of <i>B. subtilis</i> and <i>P. fluorescens</i>	6.26	4.90	4.75	4.00	3.50	3.35	4.10
	Improvement over pre-treatment (Decrease in DI) - %		(-) 21.73	(-) 24.12	(-) 36.10	(-) 44.09	(-) 46.49	(-) 34.50
	Improvement over control (Decrease in D I) - %		(-) 21.97	(-) 26.92	(-) 53.27	(-) 67.68	(-) 73.33	(-) 54.19
5	Control palms	4.70	6.28	6.50	8.56	10.83	12.56	8.95
	Deterioration over pre-treatment (Increase in DI) - %		(+) 33.62	(+) 38.30	(+) 082.13	(+) 130.43	(+) 167.23	(+) 090.43
	Deterioration over treatment (Increase in DI) - %		(+) 28.16	(+) 36.84	(+) 114.00	(+) 209.43	(+) 274.93	(+) 118.29

*Mean of 10 palms in each category.

**For leaves in whole crown, at the time of Experiment initiation.

***For leaves emerged subsequent to pre-treatment - Cumulatively emerged leaves.

state of pH (5.5-6.1) and the bacterial growth slightly higher (32.6) in coconut water media where pH adjusted to neutral state. The mean bacterial growth in peptone or jaggery solution enriched-pH adjusted coconut water medium was also higher (32.2-33.1) as compared to less cell yield in jaggery solution alone (10.3-10.5). As such *P. fluorescens* showed moderate-good growth in coconut water (pH adjusted to 7.0), coconut water amended with peptone at 1 % or Jaggery at 5 % or 10 % levels-comparable with its growth in King's B broth. Earlier observations of enhanced bacterial cell yield in coconut water from developed nuts, increased harvest of the microbe by alteration of medium pH to the desired level and enrichment with peptone are conformed (Mathew, 2003; Srinivasan, 2007). As jaggery enrichment with coconut water seemed to be useful that can be exploited along with coconut water in bioagents' multiplication. Mycelial growth of *T. viride* on coconut water was closely comparable with its growth on potato dextrose broth. As such, coconut water in natural state of acidic pH had an edge for the growth of the fungus (12.5 g). The jaggery enriched coconut water along with its pH adjustment to neutral state by and large favoured the fungal yield (12.2-12.4 g) whereas, jaggery solution alone as a medium poorly supported the growth of *T. viride* (2.8-2.9 g). As such moderate-good mycelial yield of *T. viride* could be seen from coconut nut water media with or without Jaggery amendment - comparable to yield in potato dextrose broth. The present results further validated use

Table 2. Growth of *Pseudomonas fluorescens* in coconut water based and other media after two days of incubation (Mean of six replications)

S. No.	Medium of growth	Mean No. of bacterial colonies (Cfu/ml at 10 ⁻⁶ dilution)
1	Coconut water - in natural state of pH	31.8
2	Coconut water - pH adjusted to 7.0	32.6
3	Coconut water + Peptone (1%) - pH 7.0	33.1
4	Coconut water + Jaggery (5%) - pH 7.0	32.8
5	Coconut water + Jaggery (10%) - pH 7.0	32.2
6	Jaggery solution alone (5%) - pH 7.0	10.3
7	Jaggery solution alone (10%) - pH 7.0	10.5
8	King's B broth	33.5
	CD (P=0.05)	4.2

Table 3. Growth of *Trichoderma viride* in coconut water based and other media after 18 days of incubation (Mean of six replications)

S. No.	Medium of growth	Mean mycelial weight of <i>Trichoderma viride</i> (gm)
1	Coconut water - in natural state of pH	12.5
2	Coconut water - pH adjusted to 7.0	11.8
3	Coconut water + Jaggery (5%) - pH 7.0	12.4
4	Coconut water + Jaggery (10%) - pH 7.0	12.2
5	Jaggery solution alone (5%) - pH 7.0	2.9
6	Jaggery solution alone (10%) - pH 7.0	2.8
7	Potato Dextrose Broth	17.2
	CD (P=0.05)	5.5

of coconut water from matured coconuts for multiplication biocontrol agents (Srinivasan, 2007) besides quantifying the growth of *T. viride*.

Biological control agents: Bioformulations - viability - shelf life in organics

Bacterial (*P. fluorescens*) and fungal (*T. viride*) biocontrol agents grown in coconut water and conventional medium could be processed into talc-based bioformulations in the current study. Bioformulations of bacterial and fungal biological control agents grown exclusively in coconut water based media are reported elsewhere. Reasonable level of high populations of bioagents in the talc-based formulation sustained for a period of six months of storage and equally in talc-neem cake mixture bioformulation (Srinivasan, 2007) opened ground for further exploration of other popular organics (vermicompost, coir pith etc.) for their possible source as effective carrier material. The initial C.f.u. of *P. fluorescens* (multiplied in coconut water/King's B broth) ranged from 125×10^{12} - 130×10^{12} in talc-organic (neem cake/vermicompost/coir pith) carrier increased for one (152×10^{12} - 180×10^{12}) to two months (167×10^{10} - 179×10^{10}) and there after declined (Table 4). The population of *T. viride* (multiplied in coconut water/potato dextrose broth) with initial population level of 2.5×10^8 - 2.9×10^8 also increased for one (2.8×10^8 - 3.7×10^8) to three months (2.4×10^8 - 3.3×10^8) followed by incremental decline. The population of *P. fluorescens* (originally grown in coconut water medium) in talc-organic mixture till 6th month of storage was in the range of 39 - 52×10^8 - closer to the level with King's B medium (42 - 53×10^8).

A similar trend in *T. viride* could also be observed: C.f.u. of coconut water based - 1.9×10^8 - 2.0×10^8 ; potato dextrose broth based - 2.0×10^8 - 2.2×10^8 . Therefore, the population levels of *P. fluorescens* and *T. viride* (grown in coconut water media) in the fortified bioformulations (talc-organic fortifications) during six months of storage compared well with the corresponding bioformulations evolved through conventional media; data revealed shelf life of the fortified bioformulations and importance of organics as carriers for biocontrol agents. (Srinivasan, 2007). Neem cake is an accepted organic material for biocontrol agents; utilization of other organics such as vermicompost and coir pith also possible. Since vermicompost is commonly produced in farm level units and coir pith widely available as a by-product of coconut industry their utilization as carriers in bioformulations could be well exploited.

Current study has strengthened biological control of leaf rot disease in field level; mass production of popular biological control agents in coconut water based medium followed by their bioformulations through fortifications with popular organics successfully evolved. Large-scale production of value added coconut seedlings for disease affected regions is needed (Srinivasan, 2010) and therefore utilization of bioagents through fortification in organics for evolving seedlings with induced systemic resistance is receiving consideration.

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

Biocontrol agent	Nature of talc-organic mixture carrier	Medium of growth	Initial population in talc-organic mixture carrier (x 10 ¹²)	Population of bacterial bioagent (Cfu/gm) in talc-organic mixture carrier at different months of storage					
				1 st x 10 ¹²	2 nd x 10 ¹⁰	3 rd x 10 ¹⁰	4 th x 10 ⁹	5 th x 10 ⁸	6 th x 10 ⁸
<i>Pseudomonas fluorescens</i>	Neem cake	Coconut water	128	176	178	120	70	65	52
		King's B Broth	130	180	179	142	72	67	53
	Vermi compost	Coconut water	126	174	176	118	68	62	44
		King's B Broth	129	176	177	132	71	65	48
	Coir Pith	Coconut water	125	152	167	108	65	56	39
		King's B Broth	128	161	171	121	69	59	42
			(x 10 ⁸)	Population of fungal bioagent (Cfu/gm) in talc-organic mixture carrier at different months of storage (x 10 ⁸)					
<i>Trichoderma viride</i>	Neem cake	Coconut water	2.7	3.1	3.1	2.8	2.5	2.3	2.0
		Potato Dextrose Broth	2.9	3.7	3.5	3.3	2.9	2.6	2.2
	Vermicompost	Coconut water	2.6	2.9	2.9	2.6	2.5	2.3	2.0
		Potato Dextrose Broth	2.7	3.0	2.9	2.8	2.7	2.5	2.2
	Coir Pith	Coconut water	2.5	2.8	2.6	2.4	2.3	2.1	1.9
		Potato Dextrose Broth	2.8	2.9	2.8	2.5	2.5	2.2	2.0

*Mean of 3 replications

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