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Biocontrol agents against pathogens of coconut leaf rot disease

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Abstract

The Leaf rot (due to a fungal complex - *Colletotrichum gloeosporioides, Exserohilum rostratum* and *Fusarium solani* as major pathogens) in association with root (wilt) is economically important limiting coconut production in Kerala and Tamil Nadu. The control of leaf rot is an integral part in the management of root (wilt) complex. Prevalence of antagonistic bacteria (*Bacillus subtilis, Pseudomonas fluorescens*) and fungi (*Trichoderma viride, T. harzianum*) in the coconut rhizosphere was brought out. *T.viride* invariably affected growth of all the three pathogens heavily due to its faster growth over the pathogens' growth. However, inhibition zones developed by bacterial antagonists were higher and more effective than the inhibition zones developed by the fungal antagonist-antifungal substances secreted by bacterial isolates lead to development of distinct inhibition zones against the pathogens in the medium. Investigations with one effective isolate of each antagonist for compatibility among them and interaction with pathogens also yielded useful information *viz.*, compatibility of *B. subtilis* with *P. fluorescens* as well as *T. viride*; non-compatibility of *T. viride* with *P. fluorescens*; consistent inhibition of all pathogens with inhibition zones by bacterial and fungal species; heavy suppression of growth of pathogens by *T.viride* individually (with less inhibition zone) and in combination with bacterial bioagents (with more inhibition zone); synergism phenomenon in inhibition of pathogens with consortia - *B. subtilis* + *P. fluorescens*, B. *subtilis* + *T. viride*, *B. subtilis* + *P. fluorescens* + *T. viride* etc.

Key words : Biological control, coconut, rhizosphere, leaf rot disease, pathogens, *Bacillus subtilis, Pseudomonas fluorescens, Trichoderma viride*

Introduction

The Leaf rot in association with root (wilt) is a major disease of coconut in Kerala and Tamil Nadu. It is economically important as more than 65% of root (wilt) affected palms are superimposed with leaf rot and the affected palms deteriorate fast (Srinivasan, 1991, 2002). Leaf rot is a disease of fungal complex wherein *Colletotrichum gloeosporioides, Exserohilum rostratum* and *Fusarium solani* are the major pathogens. Biocontrol agents isolated from coconut rhizosphere and phylloplane is found to be effective for management of leaf rot disease in the field (Alka Gupta *et al.*, 2000; Srinivasan and Gunasekaran, 2000; Srinivasan, 2003; Srinivasan *et al.*, 2006). In the application of biocontrol agents in control strategy, when a single antagonist is introduced some inconsistencies may arise due to interaction of biotic and abiotic factors leading to limited effectiveness of the organism (Weller, 1988), which can be overcome by a mixture of two or more antagonistic organisms provided they are compatible with each another. The ability of antagonistic organisms to suppress the pathogens besides eliciting plant growth promoting activity and induced systemic resistance against diseases has been proved in several other crops also (Elad and Chet, 1987; Mukhopadyay, 1987; Rajan *et al.*, 2002). In the present study bacterial and fungal isolates derived from coconut rhizosphere that are antagonistic against leaf rot pathogens were screened *in vitro* and studied their compatibility.

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Materials and Methods

Cultures of leaf rot pathogens and isolation of antagonists:

C. gloeosporioides, E. rostratum and *F. solani* were isolated afresh from leaf rot affected coconut palms on Potato dextrose agar (PDA) medium by conventional method (Srinivasan, 2002) and pure cultures of them utilized for screening/assessment of microorganisms in various experiments. Soil samples from rhizosphere of coconut palms from different locations of disease endemic region were collected at 10-20 cm depth and isolations made for bacterial (Nutrient agar (NA) and King's B agar media) and fungal antagonists (Potato dextrose agar and *Trichoderma* selective media) with special thrust on *B. subtilis*, *P. fluorescens* and *Trichoderma* spp. Each one-gram rhizosphere soil sample was suspended - mixed in 100 ml sterile distilled water, serially diluted (up to 10^{-8} dilution) and 0.1 ml from 10^{-5} to 10^{-8} dilutions inoculated into various media, and a pool of isolates of bacteria and fungi made out by following conventional steps - the organisms representing *B. subtilis*, *P. fluorescens*, *T. viride* and *T. harzianum* were identified (Rifai, 1969; Hildebrand *et al.*, 1992; Van Loon *et al.*, 1998) and assessed *in vitro* for their antagonistic potential against leaf rot pathogens, and other related investigations.

Evaluation of bioagents against pathogens:

Bacterial and fungal isolates were tested in vitro for antagonism against the major pathogens of leaf rot (*C. gloeosporioides, E. rostratum* and *F. solani*) on PDA by dual culture method and a systems approach was adopted (Srinivasan *et al.*, 2006). Accordingly isolates of bacteria and fungi made out from rhizosphere of coconut were initially screened against major pathogens and from the pool of isolates those bearing perceptible antagonistic reactions were sorted out. In the preliminary observations *B. subtilis* and *P. fluorescens* and were *T. viride* found consistently inhibitory to all three pathogens. These isolates were further tested for eliciting their relative antagonistic potential. A loopful of 48 hr. old bacterial culture (from stock cultures) was streaked in the centre of the petri plates containing PDA and mycelial discs (5 mm diameter) of each pathogen were placed on two sides of the streak in the petri plates. In the case of *T. viride* 5-day-old mycelial disc (5 mm diameter) was placed in centre of petri plate in place of bacterial streak. Independent inoculations with each isolate of bioagent and also each pathogen in petri plates were adopted (six replications). Controls were maintained for comparison. The plates inoculated in that manner were incubated at 30 ± 2° C and growth of pathogens and inhibition zone developed due to antagonists were recorded in different (3, 6, and 9) days after inoculation in comparison with control. The percent inhibition of growth of fungi in different days of observations was arrived at (Srinivasan and Gunasekaran, 1998). Mean growth, mean inhibition percent and mean inhibition zone of the fungi were also calculated. Out of three isolates of each species of antagonist one effective isolate representing each organism was selected for further investigations.

Evaluation of compatibility among antagonists and their effect on pathogens:

The selected isolates of *B. subtilis*, *P. fluorescens* and *T. viride* were subjected to evaluations - individually and in combinations of *B. subtilis* + *P. fluorescens*, *B. subtilis* + *T. viride*, *P. fluorescens* + *T. viride* and *B. subtilis* + *P. fluorescens* + *T. viride* (with and with out inoculation of pathogens) - for eliciting the status of compatibility besides their effect on the pathogens by generally following the described method with modifications wherever required. For evaluating the compatibility of *B. subtilis* with *P. fluorescens* the bacterial cultures were streaked one near another (side by side - approx. one cm. gap) in center of petri dish (NA) and for evaluating interaction of *B. subtilis* with *T. viride* and *P. fluorescens* with *T. viride* the mycelial discs of T. viride was placed on two sides of relevant bacterial streak in the petri plates containing PDA. Similarly mycelial discs of *T. viride* were placed on two sides of double bacterial streaks in the petri plates for evaluating the combined interaction of *B. subtilis* and *P. fluorescens* with the fungal antagonist. The interaction among *B. subtilis, P. fluorescens* and *T. viride* was further confirmed in a sub experiment by pouring bacterial cultures (each 2 ml) in to petri plates, followed by adding PDA, mixing the organisms and then by placing mycelial disc of *T. viride* in a triangular pattern. In parallel experiments mycelial discs of pathogens were inoculated into petri dishes containing PDA where the antagonists introduced previously - individually and in combinations as detailed previously. For combined effect of *B. subtilis, P. fluorescens* and *T. viride* on pathogens, the bacterial cultures were streaked first one near another in center of petri dish and then mycelial disc of T. viride placed centrally on the streaks, followed by inoculation of concerned pathogen on sides. Independent inoculation of each bioagent and combination of bioagents, as the case may be, against each pathogen in petri plates were adopted (six replications), and controls maintained for comparison. The petri plates inoculated in that manner were incubated at 30 ± 2° C and compatibility trend among antagonists as well as their effect on pathogens,

development of inhibition zone etc. recorded in different (3, 6, and 9) days after inoculation in comparison with control, and mean growth / mean inhibition percent/mean inhibition zone of the fungi arrived at.

Results and Discussion

The data evolved from experiments of the effect of bacterial (*B. subtilis*, *P. fluorescens*) and fungal (*T. viride*) antagonists from coconut rhizosphere - individually and in combinations as the case may be - on growth / inhibition of growth of the of leaf rot pathogens and development of inhibition zones against the pathogens are presented in Fig. 1 and Table 1.

The results revealed that all isolates of bacterial and fungal antagonists affected the growth thereby exerting inhibition of leaf rot pathogens *in vitro* in different (3, 6 and 9) days of incubation and variable extent of inhibition effects occurred among isolates. The over all mean diameter (for three isolates and three stages of observation) of *C. gloeosporioides* stood in the levels of 25.3 mm, 22.5 mm and 9.1mm due to *B. subtilis*, *P. fluorescens* and *T. viride*, respectively as compared to its growth of 31.2 mm in control. The mean percent inhibition of the pathogen worked out to be 19.6, 27.9 and 65.4 due to the corresponding antagonists indicating clearly the growth suppressive effect of antagonists. The mean growth of *E. rostratum* – 37.8 mm, 30.1 mm, 18.7 mm– (mean inhibition per cent levels: 9.4, 26.4 and 55.2) and *F. solani* – 29.2 mm, 26.3 mm, 12.7 mm - (mean inhibition per cent levels: 16.2, 25.7 and 62.4) also stood lesser due to the effect of the corresponding bioagents in comparison with their growth in control plates (41.5 mm and 35.0 mm respectively for the pathogens). The isolate 1 of *P. fluorescens* lead to maximum inhibition (mean for three days of observations) of *C. gloeosporioides* (34.9 %), *E. rostratum* (31.5 %) and *F. solani* (28.2 %). Similarly, the isolate 1 of *B. subtilis* also recorded maximum inhibition of *C. gloeosporioides* (20.8 %), *E. rostratum* (12.3 %) and *F. solani* (19.0 %); the isolate 1 of *T. viride* lead to mean minimum growth of *C. gloeosporioides* (8.3 mm), *E. rostratum* (10.2 mm) and *F. solani* (9.2 mm) with corresponding mean percent inhibitions of 68.6, 75.1 and 72.6 as compared to the effect of other isolates of the antagonist. As such, isolates of *T.viride* invariably lead to lesser growth of all the three pathogens in comparison with bacterial antagonists. This is due to faster growth of the antagonist over the pathogens' growth in the medium (thereby reducing space availability to pathogens) in comparison to growth of bacterial antagonists.

Further in these studies all isolates of the antagonists in different days of incubation developed inhibition zones against all the pathogens and also variable levels of inhibition by isolates of each antagonist evident. The over all mean inhibition zones (for three isolates and three stages of observation) against *C. gloeosporioides* stood in the levels of 12.9 mm, 16.3 mm, 2.6 mm due to *B. subtilis*, *P. fluorescens* and *T. viride*, respectively. Similarly the mean inhibition zones developed against *E. rostratum* (4.7 mm, 8.5 mm, 2.4 mm) and *F. solani* (9.8 mm, 12.8 mm, 2.8 mm) due to corresponding bioagents could be recorded. Among isolates of *P. fluorescens* the isolate 1 developed maximal inhibition zone against *C. gloeosporioides* (19.5 mm), *E. rostratum*

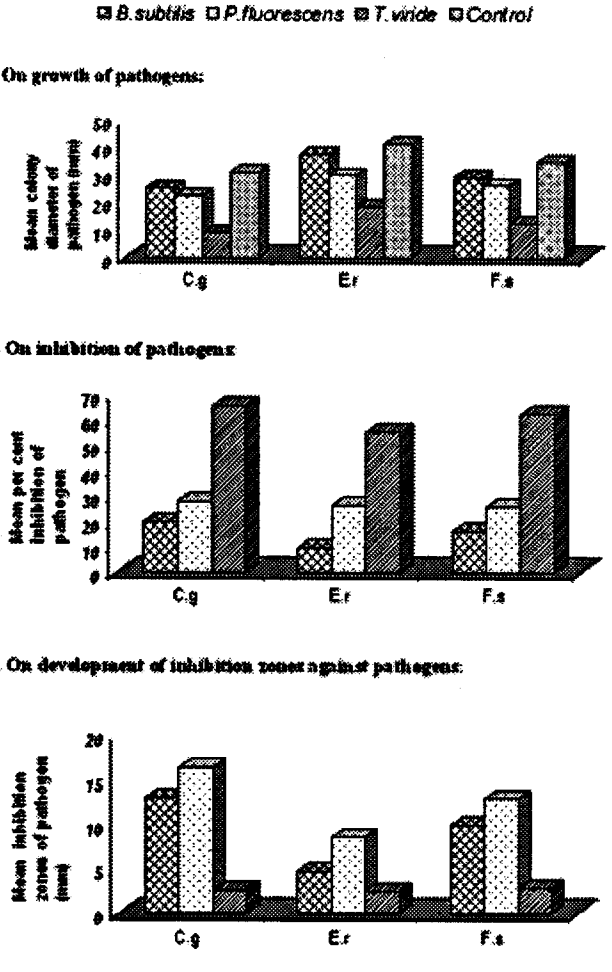
Table 1. Effect of bacterial (*Bacillus subtilis* and *Pseudomonas fluorescens*) and fungal (*Trichoderma viride*) antagonists, individually and in combination, on major pathogens of leaf rot *in vitro*

Antagonist and combination	Mean colony diameter of pathogen (mm)*			Mean per cent inhibitionof pathogen*			Mean inhibition zone of Pathogen (mm)*		
	C.g	E.r	F.s	C.g	E.r	F.s	C.g	E.r	F.s
B. subtilis	26.6	37.2	32.8	12.6	4.3	9.5	10.4	4.5	6.9
P. fluorescens	23.8	27.2	27.0	22.1	31.1	27.8	14.0	11.1	11.7
T. viride	8.0	9.6	7.5	67.2	74.4	78.2	2.9	2.8	3.1
B. subtilis + *P. fluorescens*	23.6	28.6	28.5	23.6	26.8	21.5	14.5	10.3	10.8
B. subtilis + *T. viride*	9.6	11.1	9.1	63.1	70.7	73.8	5.9	6.4	5.3
P. fluorescens + *T. viride*	10.0	11.6	8.9	60.3	69.3	74.3	5.8	6.0	5.4
B. subtilis + *P. fluorescens* + *T. viride*	10.3	11.9	8.2	61.5	69.0	76.3	5.1	5.9	5.9
Control	30.3	39.1	36.6	-	-	-	-	-	-
C. D. P= 0.05	12.8	14.3	13.4	24.6	26.3	27.4	2.1	2.4	2.2

*Mean of 3rd, 6th and 9th days of observations - averaged out of 6 replications for each day. C.g: *Colletotrichum goleosporioides*; E.r: *Exserohilum rostratum*; F.s: *Fusarium solani*

(11.0 mm) and *F. solani* (14.6 mm). The isolate 1 of *B. subtilis* also recorded considerable inhibition zone against *C. gloeosporioides* (14.2 mm), *E. rostratum* (5.4 mm) and *F. solani* (11.5 mm). All isolates of the fungal antagonist, *T. viride* invariably developed lesser inhibition zones against all the pathogens, as for example, the mean inhibition zones developed by isolate 1 against *C. gloeosporioides* (3.3 mm), *E. rostratum* (2.7 mm) and *F. solani* (4.1 mm) stood less than the fore mentioned levels of inhibitions by bacterial antagonists (Fig. 1).

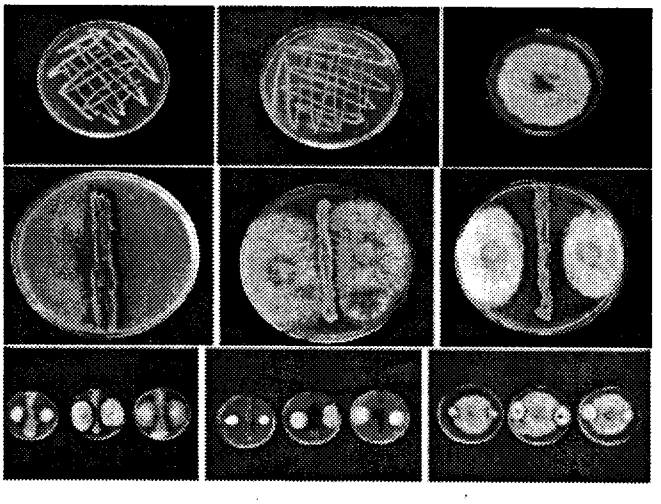
From the results it is evident that the inhibition zones developed by bacterial antagonists were invariably higher (and also more effective) than the inhibition zones



developed by the fungal antagonist. This may be due to stronger antifungal substances/compounds secreted by the bacterial isolates into the growth medium leading to development of distinct inhibition zones between the antagonists and pathogens. Elaboration of inhibitory substances, secondary metabolites particularly by plant growth promoting rhizobacteria (PGPRs) that playing significant role in biological control of plant pathogens has been widely reported (Ramamoorthy *et al.*, 2001; Srinivasulu *et al.*, 2004 a) and current results with antagonists from coconut system is generally in confirmatory with previous observations ((Alka Gupta *et al.*, 2000; Srinivasan and Gunasekaran, 2000).

One effective isolate of each antagonist (B. subtilis-isolate 1, *P. fluorescens*- isolate 1 and *T. viride*- isolate 1) was experimented in further studies of compatibility among antagonistic bacteria and fungus in vitro. During the course of these investigations on ability of growth of *B.subtilis*, *P. fluorescens* and *T. viride* individually and in their combinations the following results were observed depending upon the antagonist species: Both *B.subtilis*, *P. fluorescens* showed no inhibitory effect of one over the other and hence are co-cultivable in a common medium (compatible); both *B. subtilis* and *T. viride* also compatible in a common medium (colonies able to merge with no inhibition zone developed between them); *P. fluorescens* and *T. viride* not compatible in a common medium (presence of *P. fluorescens* in the medium affected the growth of *T. viride* and distinct inhibition zone developed); in the triple combinations of *B. subtilis*, *P. fluorescens* and *T. viride* in a common medium the growth of *T. viride* affected by *P. fluorescens* (incompatibility confirmed) even as positive compatibility in combinations of *B. subtilis* + *P. fluorescens* and *B. subtilis* + *T. viride* consistently prevailed; in a sub experiment involving isolates of each species of bioagent - *B. subtilis*, *P. fluorescens* and *T. viride* existence of compatibility among isolates found. (Fig. 2).

In a further replicated experiment, the bacterial and fungal antagonists were grown both independently and in combinations with major pathogens of leaf rot and



the resultant effect on pathogens was studied. Inhibition of all the pathogens by bacterial and fungal species, both individually and in combinations, was consistently evident at different (3, 6 and 9) days of incubation and

also in mean for stages of observation. As per the mean colony diameter for the stages of observations T.viride individually (7.5 mm – 9.6 mm) and in combination (8.2 mm – 11.6 mm) with bacterial bioagents invariably lead to lesser growth of all the three pathogens (mean per cent inhibitions: 67.2-78.2 and 61.5-76.3 respectively) in comparison with either of the bacterial antagonist alone. The mean diameters (for three stages of observation) of *C. gloeosporioides*, *E. rostratum* and *F. solani* ranged in the levels of 23.8-26.6 mm, 27.2-37.2 mm, 27.0-32.8 mm respectively due to bacterial antagonists (mean per cent inhibitions: 12.6-22.1, 4.3-31.1 and 9.5-27.8, respectively) as compared to these pathogens' growth of 30.0 mm, 39.1 mm and 36.6 mm in control. Even as the bacterial and fungal species independently inhibited the pathogens positive impact by way of reduced growth of pathogens was noticed in various combinations of the antagonists. Further, bacterial and fungal antagonists independently and in combinations also developed inhibition zones against all the pathogens. The fungal antagonist, *T.viride* individually developed lesser inhibition zones against all the pathogens - *C. gloeosporioides* (2.9 mm), *E. rostratum* (2.8 mm) and *F. solani* (3.1 mm) than the levels of inhibition zone developed by bacterial antagonists (10.4-14.0 mm, 4.5-11.1 mm and 6.9-11.7 mm against the corresponding pathogens) - indicating intrinsic effect of bacterial agents in inhibition of the leaf rot pathogens. The bacterial consortium generally leads to a synergism in inhibition of *C. gloeosporioides* (14.5 mm), *E. rostratum* (10.3 mm) and *F. solani* (10.8 mm). *T.viride* in combination with a bacterial antagonist also generally lead to a higher inhibition zone than that of it's alone (Table 1; Fig. 2). This finding is in agreement with earlier observations of bioagents' combination of B. subtilis and P. fluorescens in the greater suppression of leaf rot pathogens (Srinivasan et al., 2006) and also on par with the results of application of *B. subtilis* and *P. fluorescens* on chilli fruit rot pathogen - *C. capsici* where combined treatment of antagonistic bacteria suppressed the pathogen to a greater extend than the individual treatments alone (Bharathi, 2001).

The efficacy of consortium approach is gaining more attention in biocontrol of plant diseases. A similarity of inhibition trend of all pathogens by the antagonists' combination could be noted with interest in the study. The in vitro test of *B. subtilis* culture with *T. viride* showed that both the antagonists were also synergistic but *P. fluorescens* culture with *T. viride* upon dual inoculation resulted in clear inhibition zone directed against *Trichoderma*. This finding is in similarity with the observations of Duffey *et al.* (1996) that *P. fluorescens* exhibited antagonistic effect on *Trichoderma spp.* In the triple combinations of *B.subtilis, P. fluorescens* and *T. viride* also in a common medium, growth of T. viride was affected by *P. fluorescens* (incompatibility confirmed) even as positive compatibility in combinations of *B.subtilis* + *P. fluorescens* and *B. subtilis* + *T. viride* consistently prevailed. Therefore, caution should be exercised in mixing *T. viride* particularly with *P. fluorescens* in consortium approach. As the combined inoculation of *B. subtilis* and *P. fluorescens* (with an accurate confirmation on their compatibility) invariably lead to maximal inhibitory effect on leaf rot pathogens the current results assume even greater significance for evolving commercial formulation of consortium of such antagonists in managing leaf rot where efficiency and broad spectrum activity could function together.

Microorganisms from rhizosphere have been implicated elsewhere in the biocontrol of plant pathogens. Prevalence of biocontrol potential among rhizosphere microbial agents of coconut roots has been confirmed in the present study. Various other workers have also reported individual biocontrol potential of *B. subtilis*, *P. fluorescens* and *T. viride* against pathogens of a wide spectrum of plant diseases (Jeyalakshmi *et al.*, 1998; Srinivasulu *et al.*, 2004 b). The PGPRs, particularly *B. subtilis* and *P. fluorescens* draws added importance in view of their ability to induce systemic resistance in plants against diseases (Sible *et al.*, 2003). Mixtures of plant growth promoting rhizobacteria would be more useful to enhance biological control of diseases like leaf rot where multiple pathogens are involved. The search for eco-friendly control of leaf rot disease in coconut through use of antagonists has thus exhibited encouraging results that may be exploited in biopesticide formulations. Field experiments (combining the efficiency and ease of application) in assessing the efficacy of consortium of bio agents and for promoting a delivery system are in progress for management of leaf rot. The technology of mass production of biocontrol agents with efficient isolates is also being standardized and knowledge on use of biocontrol agents in integrated management of leaf rot is disseminated through large-scale awareness programmes.

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