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Antagonistic activity of coconut rhizospheric and endophytic *Bacillus* spp. against *Ganoderma applanatum* and *Thielaviopsis paradoxa*

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Abstract

A screening study was carried out to detect the antagonistic potential of *Bacillus* spp. against *Ganoderma applanatum* and *Thielaviopsis paradoxa*, fungal pathogens of coconut. A total of 327 heat resistant, endospore producing bacilli were isolated from the rhizospheric soil and roots of coconut growing in Kerala, Tamil Nadu, Karnataka, Andhra Pradesh and Maharashtra. All the isolates were tested for antifungal activity against *G. applanatum* and *T. paradoxa* by dual cultural technique on nutrient agar medium. The zone of inhibition was measured and percentage of inhibition was calculated. More than 90 % of the rhizospheric and root endophytic isolates were found to effectively inhibit the mycelial growth of *G. applanatum*, with a maximum inhibition zone of 12 mm and percentage inhibition ranging from 44 to 91. About 86 % of the isolates inhibited the mycelial growth of *T. paradoxa*, with a maximum inhibition zone of 14 mm and percentage inhibition ranging from 42 to 93. Further tests of potent antagonists revealed that more than one mode of mechanisms like production of chitinase, siderophores, HCN, antibiotics, ammonia, β-1,3- glucanase and salicylic acid may be involved in the antagonistic activities. The results of this study revealed 13 *Bacillus* spp. having potential for use as biocontrol agents against *G. applanatum* and *T. paradoxa*, fungal pathogens of coconut.

Keywords: Antagonistic activity, Antifungal metabolites, *Bacillus* spp., Coconut, *Ganoderma applanatum, Thielaviopsis paradoxa*

Introduction

The coconut (*Cocos nucifera* L.) palm is one of the most economically important tree crops of the humid tropical regions in the world. India accounts for 22.34 per cent of the world's coconut production and is one of the major players in the world's coconut trade. In India, it is grown in 1.903 million ha with an annual production of nearly 14,744 million nuts as per 2008-09 statistics. Coconut palm is affected by a number of diseases, some of which are lethal while others gradually reduce the vigor of the palm causing severe loss in the yield. Among the fungal diseases that affect coconut production in India, basal stem rot (*Ganoderma* wilt) caused by *Ganoderma applanatum* (Pers) Pat. and *G.lucidum* (Leys) Karst. and stem bleeding disease caused by *Thielaviopsis paradoxa* (de Seynes) Von Hohnel are important diseases of coconut other than bud rot (Bhaskaran *et al*., 1994, Srinivasulu and Rao, 2007). The non-judicial use of chemical pesticides or fungicides to cure or prevent plant diseases has caused soil pollution and detrimental effects in humans. Additionally it eliminates the beneficial soil and biocontrol microorganisms. A better strategy to avert the development of epidemics is to treat the pathogen when its level in the field is low and to prevent further increases over the growing season. Effective options include employing the pathogen's natural enemies as biological control agents, as less destructive or more environmentally friendly than chemical treatments (Bashan and de-Bashan, 2005).

Bacillus spp. have been identified as potent antagonists against *Macrophomina phaseolina* (Muhammad and Amusa, 2003), *Fusarium* spp. (Chan *et al*., 2003), *Rhizoctonia solani* (Jensen *et al*., 2002) and *Pythium ultimum* (Muhammad and Amusa, 2003) due to the production of antifungal compounds, antibiotics and proteases, and hence are extensively used in agricultural systems. The potential increased use of these microorganisms afforded by their multifaceted beneficial effects may further help in reducing problems associated with the use of synthetic chemicals in agriculture (Avis *et al*., 2008). Berg *et al*. (2001) selected efficient bacteria using three different screening methods such as *in vitro*

antagonism toward *Verticillium dahliae* and other plant-pathogenic fungi, production of fungal cell wall-degrading enzymes and plant growth–promoting effects on strawberry seedlings. Hence, the screening study is the preliminary step towards the identification of a potent biocontrol agent. The objectives of this study were to screen the *Bacillus* spp. isolated from the rhizospheric soil and roots of coconut palm growing in different agro climatic regions of India for their antagonistic effect on the coconut fungal pathogens *viz.*, *Thielaviopsis paradoxa* and *Ganoderma applanatum* and to elucidate the possible role of *in vitro* production of biocontrol mechanisms like production of chitinase, siderophores, HCN, ammonia, antibiotics, β-1,3- glucanase and salicylic acid by the antagonists.

Materials and Methods

Collection of soil and root samples

Rhizospheric soil samples and root bits of healthy and yielding coconut palms were collected from different locations (five samples from each location) in Kerala, Karnataka, Tamil Nadu, Andhra Pradesh and Maharashtra states of India. The places covered were Kasaragod, Palakkad, Alappuzha and Ernakulam districts of Kerala, Tumkur and Dakhshina Kannada districts of Karnataka, Coimbatore and Pollachi districts of Tamil Nadu, East Godavari district of Andhra Pradesh and Ratnagiri district of Maharashtra.

Isolation and identification of *Bacillus* spp.

Ten gram of the collected rhizosphere soil samples were each added to 90 ml of sterile water blanks and shaken for 20 min at 180 rpm at 30 °C (INNOVA 4335; USA). They were heated on water bath at 80 °C for 20 min for the selective isolation of *Bacillus* spp. and 10-fold dilutions were made. 100 µL of the suspension from 10^{-3} and 10^{-4} dilution were spread plated on to nutrient agar (NA) and incubated at 30 °C for 48-96 h. For the isolation of endophytic *Bacillus* spp., 10 g of each root sample was taken and washed in running tap water for 10 min. Surface sterilization was carried out by dipping in 0.1 % $HgCl_2$ for 1 min. Excess $HgCl_2$ was removed by washing five times in sterile water. One microlitre of the final washing was transferred to 9 ml nutrient broth to serve as sterility check. Samples were discarded if growth was detected in the nutrient broth after 24 h incubation. Root bits were then ground in sterile mortar and pestle and added to 90 ml sterile water blank to give rise to 10^{-1} dilution. It was heated at 80 °C for 20 min and then plated on NA. After incubation, Colony forming units (CFU) were counted and morphologically distinct colonies were randomly selected from each sample and further purified to establish pure cultures. Colony morphology, cell morphology and physiological characters were determined using standard procedures and *Bacillus* species were identified according to Claus and Berkeley (1986). All the *Bacillus* spp. were maintained on NA slants.

Isolation and identification of fungal pathogens

Thielaviopsis paradoxa and *Ganoderma applanatum* were isolated from diseased portions of the stem (stem bleeding) and roots (basal stem rot) of coconut palm. Pathogens were identified by morphological, cultural characteristics and pathogenicity tests (Nambiar *et al*., 1986, Bhaskaran *et al*., 1994). These pathogens were grown and stored in Potato Dextrose Agar (PDA) medium.

Selection of suitable medium for antagonistic studies

Twenty two randomly selected *Bacillus* spp. along with the fungal pathogens were inoculated on different media like PDA, soil extract agar (SEA), Nutrient agar (NA) and Waksman agar (WA) to select an appropriate medium which would allow both bacteria and fungi to grow well for antagonistic studies.

In vitro evaluation of antagonistic activity of *Bacillus* spp. against *Thielaviopsis paradoxa* and *Ganoderma applanatum*

Dual culture technique was followed for evaluating the efficacy of *Bacillus* spp. against *T. paradoxa* and *G. applanatum.* Fungal cultures were grown on PDA plates and *Bacillus* spp. were raised in nutrient broth. 24 h old *Bacillus* isolates were streaked towards the periphery of the NA plates and 48 h old fungal discs (7 mm) were placed at the centre of the plate concurrently. Control plate was kept without bacterial inoculation. All the plates were incubated at 30 °C. After 72 h incubation, radial growth of fungus was measured and percent inhibition over control was calculated by the formula Kr – ri / Kr x 100, where Kr is the radius of the control pathogen growth and 'ri' is the radius of the pathogen's growth towards the antagonist (Skidmore and Dickinson, 1976).

In vitro production of antifungal metabolites

The isolates that showed higher inhibition per cent towards *Thielaviopsis paradoxa* or *Ganoderma applanatum* were tested for the *in vitro* production of antifungal metabolites such as ammonia, HCN, siderophore, antibiotics, chitinases, β-1, 3- glucanases and salicylic acid.

Ammonia production: Bacillus spp. were qualitatively tested for ammonia production by looking for development of brown to yellow colour of peptone water on addition of Nessler's reagent (Cappuccino and Sherman, 1992).

HCN production: HCN production by *Bacillus* spp.was determined by looking for colour change of filter paper, soaked in 2 % sodium carbonate and 0.5 % picric acid, from yellow to orange (Bakker and Schippers, 1987).

Antibiotic production: The capability of isolates for antibiotic production was detected. Supernatant of the culture was added to the wells on TSA plates, which already been spread with soil dilution (10^{-1}) and incubated. Presence of inhibition zone around the well was observed.

Siderophore production: The CAS assay of Schwyn and Neilands (1987) was followed for the detection of siderophores. Isolates were spotted on CAS plates and observed for orange halo around the colony.

Chitinase activity: Chitinase activity was estimated as described by Lim *et al*. (1991). Colloidal chitin was prepared freshly according to the procedure of Shimahara and Takiguchi (1988). One unit (U) of chitinase activity was defined as the amount required for releasing one µg N-acetyl glucosamine from chitin per hour per mg protein. Protein content in the cell pellet of all the samples was determined as described by Bradford (1976) using bovine serum albumin as the standard.

Salicylic acid (SA) *production:* The quantity of SA in the culture filtrate was estimated as given by Meyer and Abdallah (1978) and expressed as µg SA/ml of the culture.

β - 1, 3 - glucanase activity: Cultures were inoculated in peptone-laminarin medium (Lim *et al*., 1991) and incubated for 96 h on a rotary shaker. β- 1, 3-glucanase activity was assayed by incubating 0.25 ml supernatant solution, 0.3 ml 0.1 M phosphate buffer and 0.5 ml 0.2 % laminarin (Sigma) at 40 °C for 2 h. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid (DNS) reagent and the mixture was heated in a boiling water-bath for 10 min. Reducing sugar equivalents were measured in the solutions by the spectrophotometric method of Miller (1959), with glucose as standard at 540 nm. β- 1, 3- glucanase activity was determined as µg glucose released per minute per mg of protein. Protein content in the cell pellet of all the samples was determined as described by Bradford (1976) using bovine serine albumin as the standard.

Results and Discussion

The enumeration studies in rhizosphere of coconut palms growing in several parts of Kerala, Karnataka, Tamil Nadu, Andhra Pradesh and Maharashtra under different soil and ecological conditions clearly indicated that the *Bacillus* spp. occur in good numbers in the rhizosphere of this perennial plantation crop (Table 1). Population of *Bacillus* spp. in the rhizospheric sample was found to be higher (6.29 $\log_{10}$ value of cfu g^{-1} soil) in Tumkur, Karnataka and root endophytic *Bacillus* spp. were also found to be higher in numbers in the roots from the same region (Table 1). A total of 327 morphologically distinct isolates were selected and purified from the rhizospheric soil (206 isolates) and roots (121 isolates)

Table 1. Details of *Bacillus* spp. isolated from rhizosphere and roots of coconut from different locations in Southern India

State (s)	Place (s)	Rhizospheric soil		Roots	
		log cfu/g soil	Number of *Bacillus* spp. isolated	log cfu/g root	Number of *Bacillus* spp. isolated
Kerala	Kasaragod - (HDMSCS(CPCRI)	4.65	9	3.39	18
	Kasaragod-Kunnamkai	5.08	30	2.36	6
	Palakkad - (Vadakkenchery)	3.53	13	1.87	4
	Alappuzha - (Chengannur)	4.14	13	2.11	5
	Ernakulam - (Thoppumpady)	4.92	15	2.14	4
Tamil Nadu	Coimbatore	5.18	10	3.19	8
	Pollachi	5.15	10	2.39	5
Karnataka	Tumkur	6.29	21	4.30	22
	Kidu (CPCRI plot)	6.08	34	3.38	33
Andhra Pradesh	Ambajipetta	4.70	29	3.40	11
Maharashtra	Ratnagiri - (Bhatye)	5.25	22	2.95	5

of healthy coconut palm (Table 1). All of them were endospore producing, catalase positive, gram positive rods and identified as *Bacillus* spp. according to Claus and Berkeley (1986). The spore forming nature of *Bacillus* and its sustainable crop protection in agriculture is well documented (Leclere *et al*., 2005).

All the 327 *Bacillus* spp. isolated from coconut rhizosphere and roots were individually screened for their ability to suppress the growth of two soil borne pathogens *viz.*, *T. paradoxa* and *G. applanatum* of coconut in dual culture test. In our experiments, it was found that the *Bacillus* spp. and the fungal pathogens exhibited satisfactory growth on nutrient agar (NA) at 30 °C. Whereas, PDA and WA though favoured the growth of fungal pathogens did not suit the growth of many of the bacterial isolates. Similarly, fungal pathogens and bacterial isolates showed very poor growth on SEA (data not given). The *in vitro* antagonistic studies therefore, were carried out using NA as a standard medium. Most of the bacilli were found to be antagonistic towards both coconut pathogens tested and produced its maximum antagonistic activity on NA medium. 95 % of the *Bacillus* spp. (97 % of rhizosphere and 93 % of endophytic isolates) inhibited *G. applanatum* growth on NA (Fig. 1) and per cent inhibition ranged from 44 to 91(Table 2). Similarly, 87 % bacilli (88 % and 86 % of rhizosphere bacilli and endophytic bacilli) of coconut were found to antagonize for *Thielaviopsis paradoxa* with per cent inhibition ranging from 42 to 93 (Table 2, Fig. 2). Greater levels of antagonism on the nutrient agar medium could be related to more suitable conditions for synthesis of antagonistic bioactive molecules. It is reported that peptone is a key nutrient for the production of antifungal compounds by *B. amyloliquefaciens* RC-2 (Yoshida *et al*., 2001). Two types of fungal growth inhibitions were observed when screenings were performed on agar media.

Table 2. *In vitro* antagonistic activity of *Bacillus* spp. isolated from rhizosphere and roots of coconut

Inhibition range (%)	No. of isolates antagonistic to *G. applanatum*	No. of isolates antagonistic to *T. paradoxa*
40-59	179	190
60-79	126	88
80-93*	6	7
Total	311	285

*Highest antagonism showed by the isolates tested

1) Bacterial inhibition of fungal growth in a visibly clear zone between the bacterial colony and fungal mycelia and 2) inhibition of fungal growth upon contact of the mycelium to the bacterial colony. From this test, 264 isolates were found to produce inhibition zones by inhibiting the mycelial growth of *G. applanatum* to a varying degree and 47 isolates inhibited the growth of fungi on contact. Eighty one isolates were found to produce inhibition zones by inhibiting the mycelial growth of *T. paradoxa* to a varying degree and 204 isolates inhibited the growth of fungi on contact. Among 311 (95 %) antagonists of *G. applanatum*, 199 were from rhizosphere and 112 from roots of the coconut palm of different states and181 isolates from rhizosphere and 104 endophytic isolates were found antagonistic to *T. paradoxa*. Six *Bacillus* spp. showed strong antagonistic activity against *G. applanatum* and seven isolates against *T. paradoxa* (≥ 80 %). In the case of *G. applanatum*, *Bacillus* sp. TSB 1 showed the strongest antagonistic activity. This isolate suppressed the mycelial growth by 91 % over control. The maximum inhibition zone produced was 12 mm by four *Bacillus* spp. isolates. Likewise, the maximum per cent inhibition (93) towards *T. paradoxa* was shown by *Bacillus* sp. REB 1 from Ratnagiri and *Bacillus* sp. RSB 21 from the same location. The maximum zone size was 7 mm and produced by many isolates. Large inhibition zones suggested a

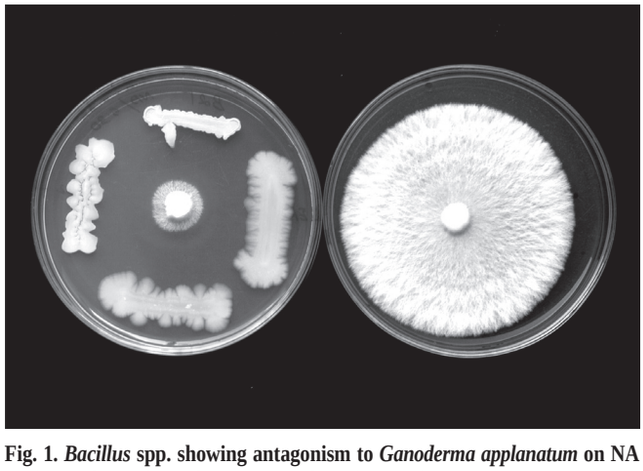

Fig. 1. *Bacillus* spp. showing antagonism to *Ganoderma applanatum* on NA medium after 3 days of incubation (fungal control on right side)

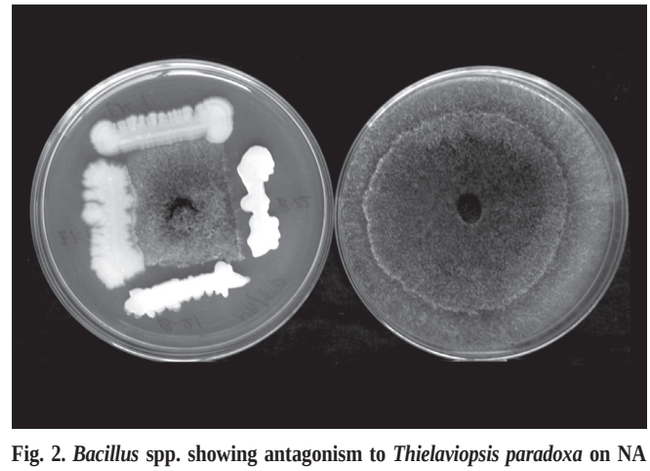

Fig. 2. *Bacillus* spp. showing antagonism to *Thielaviopsis paradoxa* on NA medium after 3 days of incubation (fungal control on right side)

presence of a metabolic compound in the medium that restricted mycelial growth in that area.

In this study, the *Bacillus* spp. with antagonistic activity against both of the pathogens, *Ganoderma applanatum* and *Thielaviopsis paradoxa* could be isolated from the rhizosphere and roots of coconut. Using individual strains for biocontrol of multiple targets would facilitate the management of both diseases caused by soil-borne fungal pathogens. We got several isolates (86 %) with antagonistic potential towards both pathogens. Similarly, a potent endophytic antagonist, *Bacillus vallismortis* zz185 was reported to inhibit the growth of several phytopathogenic fungi, including *F. graminearum, A. alternate, P. capsici* and *R. solani* (Zhao *et al*., 2010).

Antagonistic *Bacillus* spp. were categorized as potent antagonists if they showed an inhibition capacity greater than 80 % towards any of the pathogen tested. Six *Bacillus* spp. showed strong antagonistic activity against *G. applanatum* and seven isolates against *T. paradoxa*. These thirteen isolates were further tested for the production of antifungal metabolites that may be involved in the antagonistic activities. The details of antagonistic properties are given in Table 3. Knowledge of the mechanism of antagonism may be used to improve biocontrol (Spurr, 1981). An antagonist may have more than one mode of action. Exploiting all modes of action will increase the efficacy of the biocontrol agent (Alvindia and Natsukai, 2009). Production of ammonia was positive for 12 isolates out of total of 13. Brimecombe *et al*. (2001) reported the important role of volatile compounds like ammonia and HCN in biocontrol. HCN production was detected in only one of our isolates. Presence of siderophores was noticed in 8 of the 13 isolates tested. They formed an orange halo around the colony on CAS medium. The role of siderophore in biocontrol of several fungal phytopathogens has been reported. It is reported that siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit growth of other microorganisms with release of their antibiotic molecule and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron siderophore complex (Solano *et al*., 2008). Antibiotic production was showed by only three isolates. They produced inhibition zones around the well on TSA plates containing soil dilution. There is now unequivocal evidence that antibiotics play a key role in the suppression of various soil-borne plant pathogens by antagonistic microorganisms (Raaijmakers *et al.*, 2002). In the present study, seven bacilli produced extracellular chitinase and *Bacillus* sp. RSB 14 showed the maximum activity in liquid medium (137 µg N-acetyl glucosamine released per hour). β-1, 3 glucanase production was detected in 10 isolates with a maximum production of 611 µg glucose/minute/mg protein. Production of extracellular enzymes by biocontrol bacteria is a well-documented phenomenon that is involved in lysis of the cell wall of phytopathogenic fungi (Nautiyal *et al*., 2006). The role of lytic enzymes appears to be significant because chitin and laminarin are present in the cell wall of *G. applanatum* and *Thielaviopsis paradoxa*. Therefore, it is likely that inhibition of pathogens occurred by the concerted action

Table 3. Antagonistic properties of *Bacillus* spp.

Isolate (s)	% inhibition		Biocontrol traits						
	GA	TP	Ammonia	HCN	Siderophore	Antibiotics	Chitinase*	β-1, 3-glucanase**	Salicylic acid (µg/ml)
Bacillus sp. KdSB 25	80	74	+	-	-	-	31 (±0.2)	-	5 (±0.1)
Bacillus sp. ASB 22	80	73	+	-	-	-	-	503 (±3.0)	_
Bacillus sp. TSB 1	91	78	+	-	-	-	-	44 (±2.0)	_
Bacillus sp. REB 4	83	66	-	-	+	-	1 (±0.5)	-	_
Bacillus sp. CEB 7	80	64	+	-	-	+	28 (±2.0)	35 (±0.5)	3 (±0.3)
Bacillus sp. CEB 4	80	64	+	-	+	+	-	132 (±0.0)	_
Bacillus sp. RSB 21	57	93	+	-	+	+	48 (±0)	611 (±1.5)	_
Bacillus sp. RSB 14	57	80	+	-	+	-	137 (±3.8)	600 (±6.0)	4 (±0.0)
Bacillus sp. CSB 8	57	80	+	-	-	-	_	249 (±4.0)	2 (±0.1)
Bacillus sp. ESB 3	63	80	+	-	+	-	_	81 (±0.5)	_
Bacillus sp. ESB 15	59	80	+	+	+	-	1 (±0.5)	-	1 (±0.0)
Bacillus sp. KnSB 20	72	80	+	-	+	-	_	126 (±0.3)	2 (±0.0)
Bacillus sp. REB 1	67	93	+	-	+	-	3 (±0.5)	16 (±0.1)	9 (±0.2)

GA- *Ganoderma applanatum*, TP - *Thielaviopsis paradoxa*

*Chitinase activity was expressed as µg N-acetyl glucosamine released per hour per mg of protein.

**β- 1, 3- glucanase activity was expressed as µg glucose released per minute per mg of protein.

Values in parentheses represent standard error of mean

of chitinase as well as β-1, 3 glucanase. β-1, 3-glucanases and chitinases produced by *Trichoderma longibrachiatum* responsible for the degradation of *Thielaviopsis* hyphae had been reported (Sanchez *et al*., 2007). Seven bacilli produced salicylic acid, with a maximum yield of 9 µg/ml in optimized culture conditions (pH 7.0, temperature 30 °C, at 180 rpm rotary condition) in succinate broth. Extensive studies have shown that salicylic acid (SA) plays a central role in plant defense against pathogens. The exogenous application of SA can induce a set of pathogenesis-related (PR) genes and establish systemic acquired resistance (SAR) (Uknes *et al*., 1992). The present studies suggested that more than one mechanism might be involved in the suppression of fungal pathogens by *Bacillus* spp. Avis *et al*. (2008) suggested that plant growth promoting microorganisms would promote plant growth and productivity (primary effect) but could equally play a role in reducing disease (secondary effect) regardless of plant growth and nutritional status. Findings of this research suggest that the potent *Bacillus* spp. may be good candidates for biocontrol of *G. applanatum* and *T. paradoxa,* coconut pathogens. Although promising results were obtained from *in vitro* tests, field studies must be done to confirm their efficacy *in vivo.*

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