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Evaluation of rhizospheric and endophytic *Bacillus* spp. and fluorescent *Pseudomonas* spp. isolated from *Theobroma cacao* L. for antagonistic reaction to *Phytophthora palmivora*, the causal organism of black pod disease of cocoa

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Abstract

Biological control has assumed significance in the research programmes on disease management of cocoa due to the increasing concern on the detrimental effect of agrochemicals on environment and the presence of residues in food products. This study was conducted to evaluate the potential of *Bacillus* spp. and fluorescent *Pseudomonas* spp. isolated from cocoa roots and rhizosphere for their antagonistic reaction against *Phytophthora palmivora* (Butl.) Butl., causing black pod disease of cocoa. A total of 519 isolates obtained from the rhizosphere and roots of cocoa trees in different locations in South India were evaluated for their antagonistic reaction to *P. palmivora*, under *in vitro* conditions on Carrot Dextrose Agar (CDA). Out of the 519 cocoa isolates (359 *Bacillus* spp. and 160 fluorescent *Pseudomonas* spp.), 95 isolates (44 rhizospheric and 45 endophytic *Bacillus* spp; and 6 rhizospheric fluorescent *Pseudomonas* spp.) were found to be antagonistic to *P. palmivora*. Four *Bacillus* spp., one from Pollachi (*Bacillus* sp. PSB6) and three from Kasaragod (*Bacillus* sp. KGSB5, *Bacillus* sp. KGSB11 and *Bacillus* sp. KGSB26) effected a maximum of 57 % mycelial growth reduction of the fungal pathogen. None of the endophytic fluorescent *Pseudomonas* spp., showed antagonism against the black pod pathogen. *Bacillus* spp. isolated from Tamil Nadu showed higher antagonistic potential (48 % of rhizospheric and 76 % of endophytic *Bacillus* spp.) than bacterial isolates obtained from other states. Further studies with these antagonists showed that 35 %, 18 %, and 56 % of the isolates produced siderophore, HCN and antibiotics respectively. The isolates showing antagonistic activity (89 *Bacillus* spp. and 6 *Pseudomonas* spp.) against the fungal pathogens can be used in developing bio-control agents.

Keywords: Antagonism, *Bacillus* spp., Cocoa, fluorescent *Pseudomonas* spp., *Phytophthora palmivora*

Introduction

Cocoa (*Theobroma cacao* L.) was introduced in India as a mixed crop of arecanut (*Areca catechu* L.) and coconut (*Cocos nucifera* L.). Black-Pod Rot (BPR), caused by several species of the *straminipile* (formerly oomycete) genus *Phytophthora*, is the main disease affecting the cocoa crop (*Theobroma cacao* L.) worldwide (Hanada *et al*., 2009). *Phytophthora palmivora* (Butl.) Butl. is the predominant species causing BPR. Phytosanitation, chemical fungicides and genetically resistant varieties are the main methods of controlling pod infection by *Phytophthora* spp. However, chemical control frequently requires several applications of sprays and is often not efficient (Holderness, 1992). Though the removal of diseased pods has shown some efficiency in reducing secondary inoculum, it is labor intensive, expensive and economically viable only when market prices of cocoa are remunerative. Although genetic resistance is the most cost-effective control measure, a standardized methodology to evaluate cocoa germplasm for resistance against *Phytophthora* spp. is lacking in several cocoa-producing countries (Hanada *et al*., 2009).

In this context and from the perspective of Integrated Pest Management (IPM), biological control

is an additional method that can help in reducing the disease to economically viable levels, with a concomitant decrease in the use of chemicals (Hanada *et al*., 2009). Biological control agents isolated from the rhizosphere and roots of cocoa (resident antagonists) can interfere with the growth of the pathogen. Epiphytic microorganisms, especially bacteria, are capable of inhibiting the growth of *P. palmivora* (Bong *et al*., 1998). The humid conditions in which cocoa is cultivated provide a favorable environment for the development and survival of epiphytic microorganisms antagonistic to *P. palmivora*. The discovery and development of antagonistic endophytes offers more promise. Recent evidence suggests that antagonistic endophytes re-introduced into cocoa persist and protect the tree against *Phytophthora* (Arnold *et al*., 2003). In cocoa, *Trichoderma* species are primarily being studied for their ability to control disease (Bailey *et al*., 2006). Several authors have reported the use of *Trichoderma* species as a biological control agent (BCA) against *Phytophthora* infection in cocoa (Hanada *et al*., 2009). Endophytic colonization of cocoa seedling by *Trichoderma* spp. has been reported to activate plant defense cascades in cocoa seedlings (Bailey *et al*., 2006). Promising results for the control of diseases of cocoa have been obtained using epiphytic mycoparasitic fungi. Furthermore, recent evidence shows that in some cases endophytic fungi restrict cocoa pathogen growth or damage *in vitro* and *in vivo* (Arnold *et al*., 2003; Mejía *et al*., 2008) highlighting their status as a new source of biological control agent for combating cocoa pathogens. Studies on the potential of *Trichoderma* based biological control against *P. palmivora* in Peru and the aggressive *Phytophthora megakarya* Brasier & Griffin in cocoa-producing regions of Cameroon (Deberdt *et al*., 2008) indicate promise for this approach.

Previous research on biological control agents for cocoa diseases has focused mainly on fungi. Very limited attempts have been made to control *phytophthora* infection in cocoa using *Bacillus* and fluorescent *Pseudomonas* species, as a biological agent. The present study is aimed to isolate fluorescent *Pseudomonas* spp. and *Bacillus* spp. from the rhizosphere and roots of cocoa and investigate their ability to control black pod pathogen under *in vitro* conditions.

Materials and Methods

Isolation of *Bacillus* and *Pseudomonas* spp.

Rhizosphere soil and root samples of cocoa were collected from different locations in four states - Kerala, Karnataka, Tamil Nadu and Andhra Pradesh. Three districts (5 places) were covered in Kerala, two in Karnataka (3 places), two in Tamil Nadu and one in Andhra Pradesh. Five soil samples were collected from each place and stored at 4 degree Celsius till analysis. The fluorescent *Pseudomonas* spp. were isolated from the rhizosphere soil and roots samples by standard procedures using the serial dilution technique in King's B (KB) medium (King, 1954), and S2 medium (Gould *et al*., 1985). Nutrient Agar was used for isolation of *Bacillus* spp.

Isolation and purification of *Phytophthora palmivora*

A virulent *P. palmivora* isolate was obtained from infected cocoa pods collected from farmer's garden at Vata, Kasaragod. Baiting technique was adopted for isolation of fungal pathogen on Carrot Dextrose Agar medium.

The pods manifesting symptoms of the disease were surface sterilized using 70 % alcohol. From the advancing margins of the infected parts of the pods, 5mm tissues were excised. The tissues were rinsed with ethyl alcohol, soaked in distilled water for one minute and then dried on a sterile filter paper. The surface sterilized tissue pieces were placed on carrot dextrose agar (CDA) medium and incubated at 24 °C temperature. When growth of the pathogen was observed, the advancing portion of its mycelium was transferred on CDA plates and then maintained on CDA slants for further studies.

Plate inhibition assays

Carrot dextrose agar was used for *in vitro* antagonism tests. Bacterial cultures were streaked at four equidistant points along the periphery of the CDA plates. Mycelial discs of *Phytophthora palmivora* (5 mm diameter) were cut out from the edge of an actively growing colony with the help of a sterile 5mm diameter cork borer and placed upside down at the center of the assay plates. Plate with *P. palmivora* mycelial disc alone served as control. The plates were incubated for three to five days at 30^0C and the colony radius of *P. palmivora* was measured when the radial growth of the colony in the control plate reached the periphery of the plate. The mycelial growth between each of the four bacterial spots was measured. The percentage of mycelial inhibition (%) was calculated using the equation:

$$R1-R2/R1x100$$

where, R1 is maximum radius of mycelial growth on the control plate and R2 is radius of mycelial growth directly opposite to the bacterial growth (Fernando and Pierson., 1999).

Analysis for siderophore, HCN and antibiotics production

Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands, (1987). Bacterial strains (24 h old cultures) were spotted on CAS medium plates and incubated at 28 ± 1 °C for 48 h. Formation of orange to yellow halo around the colonies showed the production of siderophore. HCN production was determined by modified method of Bakker and Schippers(1987). For demonstration of HCN production, exponentially grown cultures (10^8 cells ml^{-1}) were streaked on solid agar plates with simultaneous addition of filter paper soaked in 0.5% picric acid in 1 % Na_2CO_3 in the upper lid of plates. Plates were sealed with parafilm and after incubation at 28 ± 1°C, development of colour from yellow to light brown, moderate brown or strong brown was examined for putative HCN production. For detecting the production of antibiotics, supernatants of the individual isolates grown in appropriate broth were transferred to wells that were made on TSA (Tryptic Soya Agar) plates seeded with soil dilution and incubated for 24 h. Presence of inhibition zone around the colonies was observed.

Results and Discussion

The approaches to biological control of plant diseases involve the use of antagonists selected by testing their ability *in vitro* and *in vivo* (Raupach and Kloepper, 1998). Sharifuddin (2000) identified nine potential antagonistic bacteria against *P. palmivora* and *P. nicotianae* from cocoa rhizosphere based on *in vitro* screening with dual culture technique. These were *Enterobacter* sp; *Pseudomonas aeruginosa*, *Serratia marescens*, *Burkholderia cepacia* and five isolates of *Bacillus* spp.

The bacterial isolates taken for this study belonged to rhizosphere region and roots of cocoa trees growing in four southern states namely Kerala, Karnataka, Tamil Nadu and Andhra Pradesh of India. They were isolated from different places in each state. A comparative study of the antagonistic behavior of the isolates was done as part of the work. A total of 198 isolates from Kerala, 212 bacterial isolates from Karnataka state, 63 isolates from Tamil Nadu and 46 bacterial isolates from Andhra Pradesh were tested (Table 1). Out of the total of 519 bacterial isolates, 160 belonged to *Pseudomonas* spp. and the remaining 359 to *Bacillus* spp. Both endophytic as well as rhizosphere inhabitants were taken for the study.

Results indicated that 44 *Bacillus* spp. from the rhizosphere, 45 *Bacillus* spp. from the roots of cocoa and 6 fluorescent *Pseudomonas* spp. from rhizosphere inhibited the growth of *Phytophthora palmivora*, where as none of the endophytic fluorescent *Pseudomonas* spp. showed antagonism against the black pod pathogen (Table 2). *Bacillus* spp. isolated from Tamil Nadu showed higher antagonistic potential (48 % rhizospheric and 76% endophytic *Bacillus* spp.) than bacterial isolates from other states (Fig. 1). *Bacillus* spp. were found to be more antagonistic to *Phytophthora palmivora* than fluorescent *Pseudomonas* spp. In 2008, Rachel *et al.*, reported that some *Bacillus* spp. from vegetable crops were capable of long-term colonization of cocoa leaves and subsequent disease reduction. *Bacillus* spp. inhibited mycelial growth of *Phytophthora palmivora* in the range of 7-57 percent (Fig. 2) where as fluorescent *Pseudomonas* spp. were able to inhibit 38-50 % of mycelial growth on CDA. Mejia

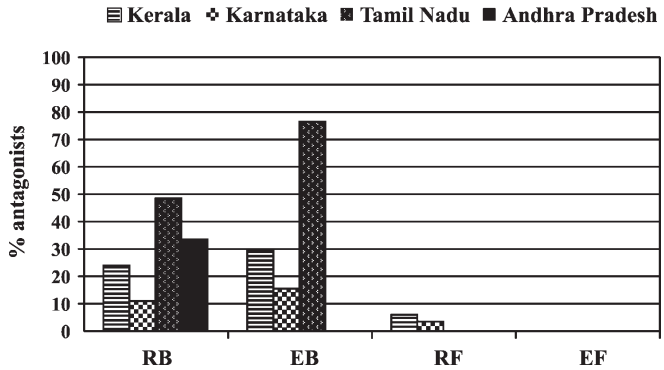

Fig.1. State wise distribution of cocoa isolates showing antagonism against *Phytophthora palmivora.* RB- Rhizospheric *Bacillus* spp., EB- Endophytic *Bacillus* spp., RF- Rhizospheric fluorescent *Pseudomonas* spp., EF- Endophytic fluorescent *Pseudomonas* spp

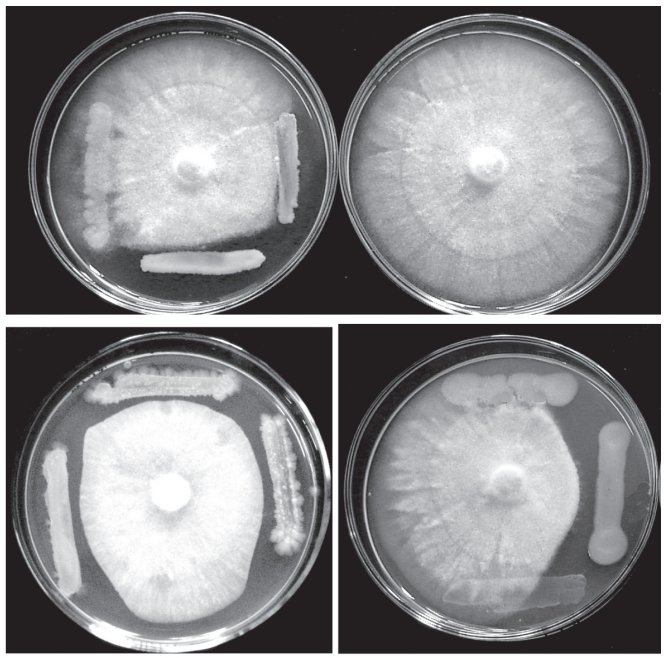

Fig.2. Endophytic *Bacillus* spp. from cocoa showing antagonism against *Phytophthora palmivora* (fungal control on upper right corner

Table 1. Details of *Bacillus* spp. and fluorescent *Pseudomonas* spp. isolated from cocoa rhizosphere and roots

State	Place	Sample used for isolation	Organism	No. of isolates obtained
	Kasaragod	Rhizospheresoil	*Bacillus* spp.	29
			Pseudomonas spp.	28
		Roots	*Bacillus* spp.	45
			Pseudomonas spp.	3
Kerala	Wayanad	Rhizosphere soil	*Bacillus* spp.	12
			Pseudomonas spp.	20
		Roots	*Bacillus* spp.	17
			Pseudomonas spp.	4
	Kozhikode	Rhizospheresoil	*Bacillus* spp.	13
			Pseudomonas spp.	18
		Roots	*Bacillus* spp.	9
			Pseudomonas spp.	0
	Tumkur	Rhizosphere soil	*Bacillus* spp.	46
			Pseudomonas spp.	17
		Roots	*Bacillus* spp.	27
			Pseudomonas spp.	0
Karnataka	Kidu	Rhizospheresoil	*Bacillus* spp.	17
			Pseudomonas spp.	25
		Roots	*Bacillus* spp.	24
			Pseudomonas spp.	2
	Vittal	Rhizospheresoil	*Bacillus* spp.	17
			Pseudomonas spp.	16
		Roots	*Bacillus* spp.	20
			Pseudomonas spp.	1
Tamil Nadu	Coimbatore	Rhizospheresoil	*Bacillus* spp.	21
			Pseudomonas spp.	3
		Roots	*Bacillus* spp.	12
			Pseudomonas spp.	1
	Pollachi	Rhizospheresoil	*Bacillus* spp.	12
			Pseudomonas spp.	6
		Roots	*Bacillus* spp.	5
			Pseudomonas spp.	3
Andhra Pradesh	Ambajipetta	RhizosphereSoil	*Bacillus* spp.	18
			Pseudomonas spp.	11
		Roots	*Bacillus* spp.	15
			Pseudomonas spp.	2

et al. (2008) reported that 65 % of the tested fungal endophytes isolated from healthy *Theobroma cacao* tissues showed *in vitro* antagonism against *P. palmivora.* In our study, sixteen isolates showed significant (>50 %) reduction in the mycelial growth of fungal pathogen, resuls of which are presented in Table 3. All the sixteen antagonists belonged to *Bacillus* spp. Out of these, eleven *Bacillus* spp. were obtained from Kerala, three from Karnataka, one isolate each from Tamil Nadu and Andhra Pradesh.

Test for the antagonists to produce secondary metabolites showed that 35 %, 18 %, and 56 % of the antagonistic isolates produced siderophores, HCN and antibiotics, respectively. Attributes like HCN, chitinase, antibiotics and siderophore production reduce plant mortality and disease severity by suppressing root pathogens and thus enhancing plant growth. About 70 % of the antagonists produced either siderophores or HCN or antibiotics. Siderophore and antibiotic production were found to be the most frequently occurring traits among *Bacillus* spp. obtained from the rhizosphere. State wise distribution of antagonistic *Bacillus* spp. producing secondary metabolites is detailed in Fig.3 and 4. Among the 6 antagonistic fluorescent *Pseudomonas* spp., all isolates showed siderophore production under *in vitro* conditions whereas, only two isolates produced HCN and one isolate was found to be positive for antibiotic production. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction (O'Sullivan and O'Gara, 1992). Since all antagonists did not show production of siderophores/ HCN/ antibiotics, involvement of some other mechanism(s) for growth reduction of fungal mycelia is a possibility.

Table 2. Growth inhibition of *Phytophthora palmivora* by bacterial isolates from cocoa rhizosphere and roots

State	Origin	Organism	Total no. of isolates	No.of isolates showing antagonism	Range of antagonism (%) 1-10	11-20	21-30	31-40	41-50	51-60
Kerala	Rhizospheresoil	*Bacillus* spp.	54	13	0	0	0	2	6	5
		Pseudomonas spp.	66	4	0	0	0	1	3	0
	Roots	*Bacillus* spp.	71	21	2	2	0	2	9	6
		Pseudomonas spp.	7	0	0	0	0	0	0	0
Karnataka	Rhizospheresoil	*Bacillus* spp.	80	9	0	0	0	6	2	1
		Pseudomonas spp.	58	2	0	0	0	2	0	0
	Roots	*Bacillus* spp.	71	11	0	0	0	2	7	2
		Pseudomonas spp.	3	0	0	0	0	0	0	0
Tamil Nadu	Rhizospheresoil	*Bacillus* spp.	33	16	0	0	5	6	4	1
		Pseudomonas spp.	9	0	0	0	0	0	0	0
	Roots	*Bacillus* spp.	17	13	0	0	2	7	4	0
		Pseudomonas spp.	4	0	0	0	0	0	0	0
Andhra Pradesh	Rhizospheresoil	*Bacillus* spp.	18	6	0	0	0	2	3	1
		Pseudomonas spp.	11	0	0	0	0	0	0	0
	Roots	*Bacillus* spp.	15	0	0	0	0	0	0	0
		Pseudomonas spp.	2	0	0	0	0	0	0	0
	Total		**519**	**95**	**2**	**2**	**7**	**30**	**38**	**16**

Among *Bacillus* spp., four isolates, one from Pollachi (*Bacillus* sp. PSB6) and three from Kasaragod (*Bacillus* sp. KGSB5, *Bacillus* sp. KGSB11 and *Bacillus* sp. KGSB26) caused the highest inhibitory effect (57 %) on mycelial growth. Further studies with these isolates revealed the antibiotic production ability of *Bacillus* spp. PSB6, KGSB11 and KGSB26. *Bacillus* spp. KGSB5 and KGSB11 were found to produce siderophores under *in vitro* conditions (Table 3). In case of fluorescent *Pseudomonas* spp., one isolate from the cocoa rhizosphere from Kannipady area of Kasaragod (*Pseudomonas* sp. KGSF26) and another from Kozhikode (*Pseudomonas* sp. KZSF6), showed 43 % antagonism against *P. palmivora*. Endophytic *Bacillus* spp., in general, were found to be better antagonists than their rhizospheric counter parts, with the exception of the isolates from Andhra Pradesh. Presented data offer further support for the potential of endophytic microbes in the management of cocoa diseases as suggested by Arnold *et al*. (2003), Bailey *et al*. (2006) and Rachel *et al*. (2008). Previous studies reported that *Trichoderma longibrachiatum* and *Trichoderma stromaticum* reduced witches' broom and *Trichoderma virens* reduced black pod when evaluated under field trials conducted on neglected cocoa farms in eastern Peru. (Ulrike Krauss and Whilly Soberanis, 2002)

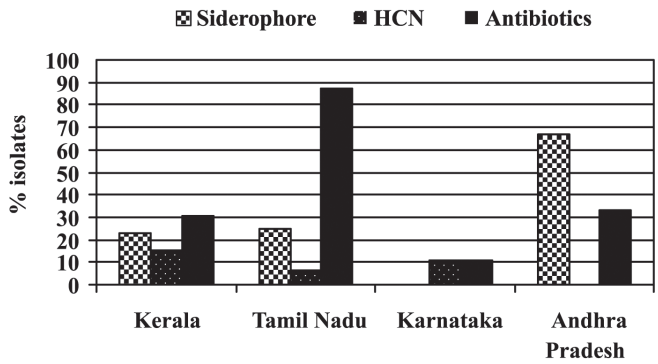

Fig. 3. Production of secondary metabolites by antagonistic *Bacillus* spp. isolated from the rhizosphere of cocoa

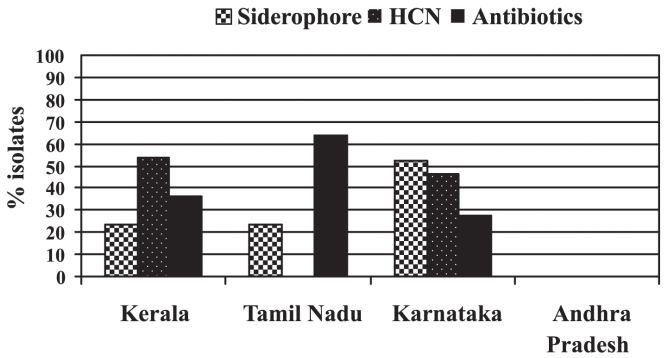

Fig. 4. Production of secondary metabolites by antagonistic *Bacillus* spp. isolated from the roots of cocoa

In vitro and *in vivo* studies (Arnold *et al*., 2003; Hanada *et al*., 2009) suggest that different endophytic fungi associated with *T. cacao* reduce the damage associated with pathogens in a variety of different ways *in planta*. Our results support these findings by showing that endophytic and rhizospheric microbes isolated from healthy roots and rhizospheric soil of *T. cacao* restrict *in vitro* growth of the most common and economically important pathogen of cocoa, *P. palmivora*. Our results suggest that the diverse assemblage of microbial species

Table 3. Details of cocoa isolates showing significant reduction in the mycelial growth of *Phytophthora palmivora*

PGPRs	Isolated from	Zone of inhibition	% of mycelial growth reduction	Sidero -phore production	HCN production	Antibiotic production
Kerala						
Bacillus sp.KGSB 5	Rhizospheresoil	1.9	57	++	-	-
Bacillus sp.KGSB11	,,	1.9	57	++	-	+
Bacillus sp.KGSB26	,,	1.9	57	-	-	+
Bacillus sp.KGSB1	,,	2.1	52	-	-	-
Bacillus sp.KGSB 4	,,	2.1	52	-	-	-
Bacillus sp.KGEB5	Roots	2.1	52	++	-	-
Bacillus sp.KGEB6	,,	2.1	52	+	-	+
Bacillus sp.KGEB15	,,	2.1	52	-	-	-
Bacillus sp.KGEB41	,,	2.1	52	++	-	+
Bacillus sp.KZEB1	,,	2.1	52	-	++	+
Bacillus sp.KZEB3	,,	2.1	52	-	-	+
Tamil Nadu						
Bacillus sp. PSB6	Rhizosphere soil	1.9	57	-	-	+
Karnataka						
Bacillus sp.TSB 15	Rhizosphere soil	2.1	52	-	+	+
Bacillus sp.VEB5	Roots	2.0	55	+	-	-
Bacillus sp.VEB8	,,	2.0	55	-	-	-
Andhra Pradesh						
Bacillus sp.ASB 4	Rhizospheresoil	2.0	55	++	-	-

'+' = positive; '-' = negative
'+' and '++' indicates low and high levels of production, respectively

associated with *T. cacao* play an integral role in the resistance of their hosts to pathogen damage and that these organisms can potentially be used as effective biocontrol agents.

Conclusion

The present study indicates that 89 isolates of *Bacillus* spp. and 6 isolates of fluorescent *Pseudomonas* spp. have the potential for the management of *Phytophthora* disease of cocoa. However they would have to be evaluated further under field conditions to assess their suitability as bioagents for the management of black pod disease of cocoa.

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