INTEGRATED MANAGEMENT OF CORYNESPORA LEAF DISEASE OF RUBBER IN NURSERIES USING BACTERIAL ENDOPHYTES

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Possibility of using of endosymbionts in the management of Corynespora leaf disease of young rubber (*Hevea brasiliensis*) was studied through *in vitro* assay and field evaluation. Endophytic bacteria isolated from different parts of the rubber tree were screened for the *in vitro* antagonistic activity against *Corynespora*. A *Bacillus* isolate (8LK) showed 3.3 cm inhibition zone on the growth of the *Corynespora* in dual culture and this was selected for bioassay and nursery evaluation. Bioassay using the detached leaves of endophyte treated plants showed reduction in lesion size upon inoculation with pathogen. Compatibility study of the endophytic bacteria with recommended fungicide (Carbendazim) was conducted under *in vitro* condition and found compatible and was evaluated in susceptible clone in the nursery stage. The results showed that the integrated treatments were on par with the general fungicide recommendation in controlling the Corynespora leaf disease in nursery.

Key words: *Corynespora cassiicola*, Endophytic bacteria, *Hevea brasiliensis*, Integrated management

INTRODUCTION

Rubber (*Hevea brasiliensis*) a perennial tree crop, native to Amazon forests is widely cultivated in the humid tropics and accounts for 99 per cent of the global natural rubber production. One of the major constraints in rubber cultivation is occurrence of fungal diseases, causing considerable damage to the tree in terms of growth and yield. Among the different pathogens, *Phytophthora*, *Corynespora*, *Corticium*, *Colletotrichum* and *Oidium* cause mild to severe disease in rubber and have attained economic significance in India (Edathil *et al.*, 2000). Control of plant diseases using chemicals is the most effective short-term disease management strategy and is popular among the farmers mainly due to the immediate results. Biocontrol measures are not yet practiced for the management of diseases in rubber plantations as they were not proved effective.

Leaf disease of rubber caused by *Corynespora cassiicola* (Berk & Curt) has been reported from various rubber growing countries. Chemical control measures have been adopted for the control of this disease in rubber plantations (Edathil *et al.*, 2000; Jacob and Idicula, 2004; Manju *et al.*, 2001). Repeated use of agrochemicals is not desirable because of their adverse effect on environment. Hence, it is appropriate to

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develop alternative eco-friendly and sustainable management strategies to reduce the use of plant protection chemicals.

Endophytic bacteria have recently been a focus of interest as bio-control agents and some are capable of inducing systemic resistance in plants (Kloepper *et al.*, 1992, Philip *et al.*, 2013). A suitable bio-agent which can survive in the phyllosphere and effectively control the disease will be beneficial for environment friendly protection of rubber tree from the diseases (Joesph *et al.*, 2013). In the present study, the integrated approaches for *Corynespora* disease management was tested in bud wood nurseries in hot spot area using endophytic bacteria.

MATERIALS AND METHODS

Selection of bacterial endophytes

Eleven bacterial endophytes were selected from the microbial culture collection of RRII during the year 2016. The selected bacteria were streaked on Tryptic Soya Agar (TSA) medium and revived. The single colony was screened for *in vitro* antagonism against *Corynespora* by dual culture method on Potato Dextrose Agar (PDA). Control plates with pathogen alone were also maintained. The plates were incubated for 10 days and inhibition zones were measured. Out of the eleven bacterial endophytes showing higher inhibition zone were selected for further studies.

Biochemical studies

The selected endophytic bacterial isolates were studied for their ability to produce HCN (Wei *et al.*, 1992), Siderophore (Suryakala *et al.*, 2004) and volatile organic compound production under *in vitro* condition. The bacterial isolates were grown in Tryptic Soya Agar (TSA) medium and *C.cassicola* disc (5mm) were inoculated in the PDA plates and incubated for 10 to 12 days. Control plate with pathogen alone was also maintained. The percentage reduction in growth of the pathogen was recorded using formula:

$$\text{Percentage reduction in growth} = \frac{\text{Growth in control - Growth in antagonist inoculated plate}}{\text{Growth in control}} \times 100$$

Fungicide sensitivity/tolerance

The fungicide sensitivity/tolerance of the bacterial isolates was studied using the recommended fungicide Carbendazim at 0.05 per cent and 0.025 per cent in Tryptic Soya Broth (TSB) and Tryptic Soya Agar (TSA) media. The growth of bacterial isolates were recorded after 48 hrs incubation at room temperature and expressed as compatible (++) or incompatible (-).

Identification of endophytic bacteria isolate through molecular study

The selected antagonistic endophytic bacteria were identified using 16S rDNA sequencing. The DNA isolated from the endophytic bacteria was amplified with following conserved eubacterial 16SrDNA primers with standard PCR conditions.

8F-5 ′ -AGAGTTGATCCTGGCTCAG-3′, 1492 R-5′TACGGHTACCTTGTTAGCACTT -3′

The PCR products were size fractioned on 1.5 per cent agarose gels, stained with ethidium bromide and photographed. The PCR products were cloned and sequenced. The sequence data was blasted on Ribosomal DATA Project II of bacterial sequence to identify the bacterial group.

Bioassay study

The selected endophytic bacterial broth (TSB) containing 10^8 cfu mL^{-1} was diluted with equal quantity water (1:1) and inoculated by foliar spray on young rubber plants of a susceptible clone (RRII 105) grown in polythene bags. The light green leaves from treated plants were collected and inoculated with spore suspension of *C. cassiicola* ($1x10^4$). Lesion sizes indicating infection were recorded after five days.

Field evaluation of selected antagonist

The field evaluation of the selected antagonist was carried out in bud wood nursery in a disease hot spot area (Ulickal Nursery, Iritty, Kerala) for three years. In the first year, the effect of the antagonistic bacteria alone was studied and in the subsequent years combination of endophyte bacteria with half dose of the recommended fungicide (Carbendazim 0.025%) was also included. Broth and encapsulated formulations of the endophyte were used in the study. For encapsulation, the freeze dried antagonistic bacteria was encapsulated in gelatin capsule (500 mg). Overnight incubation of gelatin capsule in water (500 mg capsule L^{-1}) recorded a population of $3x10^6 ml^{-1}$. The standard recommended dose of Carbendazim (0.05%) was the control. The experiment was laid out in Completely Randomized Block Design (CRD) with four treatments during the first and second year and five treatments during the third year. Six round of spraying was carried out at eight days interval during the disease season. The plot size was 35 plants $treatment^{-1}$. The disease occurrence was scored visually using a 0-5 scale (Table 1).

RESULTS AND DISCUSSION

Selection of bacterial endophytes

Comparison of the effect of different bacterial isolates on growth of *C. cassiicola* showed that out of eleven isolates only three were antagonistic to *Corynespora* (Table 2). The isolate 8LK showed the highest inhibition on the fungus with a zone of 3.3 cm and the isolate 928 recorded about 2.4 cm (Fig. 1). Selection of an efficient antagonist is possible through screening of large number of bacteria in *in vitro* dual culture (Abraham *et al.*, 2013).

Biochemical studies

The production of siderophore, HCN production and growth inhibition (%) by

Table 1. **Disease score chart**

Score	Rating	Description
0	No disease	No disease
1	Very light	< 5 spots
2	Light	5-10 spots and 10-25 per cent leaf fall
3	Moderate	>10 spots and 26-50 per cent leaf fall
4	Severe	Large lesions and 51-75 per cent leaf fall
5	Very severe	Large lesions and > 75 per cent leaf fall

Table 2. **Antagonistic activity of bacterial endophytes against *C. cassiicola***

Sl. No.	Bacterial Strain	Inhibition (cm)
1	RB 66	2.0
2	RB 88	1.7
3	A1	1.7
4	RH 104	2.0
5	8LK	3.3
6	928	2.4
7	623	1.5
8	648	1.1
9	36A	1.0
10	132	1.0
11	564	2.3

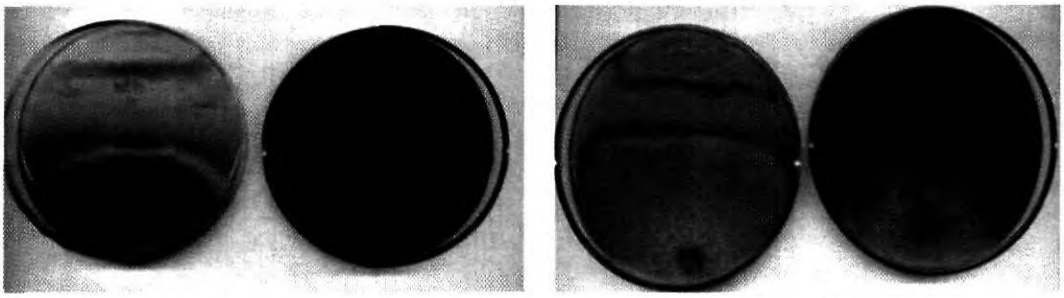

Fig. 1. Inhibition of growth of *Corynispora cassiicola* by the selected isolates

volatile organic compound production are shown in the Table 3. The isolates showed difference in the siderophore and HCN production capacity. They were grouped into three categories low,medium and high based on the colour developed in $FeCl_3$ test for siderophore and picric acid treated filterpaper for HCN. HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens (Rijavec and Lapanje, 2016). The growth inhibition percentage of pathogen by volatile organic compounds produced by different selected bacteral isolates were tested. Out of eleven bacteria tested all produced antipathogenic organic volatile compound. All eleven isolates reduced the growth of *C.cassiicola*. Highest growth reduction was shown by the isolate 8LK (74.4%).

Fungicide sensitivity/tolerance

All bacterial endophytes showed moderate to high tolerance to Carbendazim

Table 3. **Biochemical studies on endophytic bacteria**

Sl. No.	Bacterial strain	Siderophore production	HCN production	Growth inhibition (%) by volatile organic compound production
1	RB 66	+ ++	+++	35.2
2	RB 88	++	+++	50.4
3	A1	+	-	41.2
4	RH 104	+++	++	54.0
5	8LK	-	+	74.4
6	928	-	+	58.0
7	623	-	+	50.0
8	648	+	-	36.5
9	36A	+	+	55.0
10	132	-	+	43.8
11	564	-	-	41.2

+ =Low, ++ = Medium, +++ = High

Table 4. **Effect of Carbendazim on bacterial growth**

Sl. No.	Bacterial isolate	Carbendazim (0.025%) in TSA	Carbendazim (0.05%) in TSA	Carbendazim (0.025%) in broth	Carbendazim (0.05%) in broth
1	RB 66	++	+	$2x10^3$	$2x10^2$
2	RB88	+	+	$2x10^2$	$2x10^2$
3	A1	+	+	$2x10^3$	$1x10^2$
4	RH 104	+	+	$2x10^3$	$1x10^3$
5	8LK	++	++	$4x10^8$	$3x10^8$
6	928	++	++	$3x10^6$	$3x10^6$
7	623	+	+	$2x10^3$	$1x10^2$
8	648	+	+	$2x10^3$	$1x10^2$
9	36A	+	+	$2x10^3$	$1x10^2$
10	132	+	+	$2x10^3$	$1x10^2$
11	564	+	+	$2x10^3$	$1x10^2$

+ = Low ++ = Medium

both at 0.05 and 0.025 per cent concentration. However, isolates, 8LK and 928 recorded better tolerance in both the medium *viz.* TSA and TSB after 48 hrs. of incubation (Table 4). So based on *in vitro* evaluation for antagonism, growth inhibition and fungicide tolerance, the endophytic bacterial isolate 8LK was selected for further field evaluation.

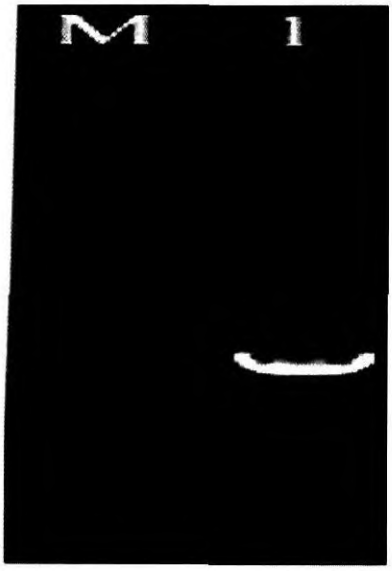

Fig. 2. 16S rDNA confirmation of *Bacillus* spp.

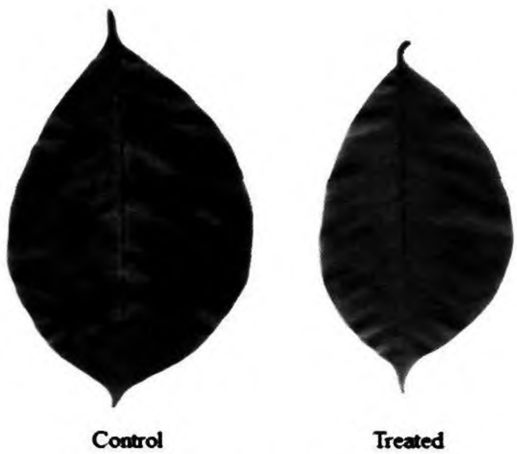

Fig. 3. Bioassay through challenge inoculation

Identification of the endophytic bacteria isolate through molecular study

Molecular characterization of the endophytic bacteria (8LK) through 16S rDNA sequencing (Fig. 2) and confirmed as *Bacillus subtilis.*

Bioassay

The bacterial antagonist (8LK) treated leaves significantly reduced the disease lesion size compared to control plants upon challenge inoculation. Lesions of high range were observed on control leaves while only very minute spots were developed on treated leaves (Fig. 3).

Field evaluation in the bud wood nursery

During 2016 the treatment with endophytic bacteria alone gave good control of the disease. However, during 2017 and 2018 observation, among the different treatments, the integrated treatment Carbendazim (0.025%) plus bacterial endophyte 8LK ($1x10^8ml^{-1}$) prepared in broth and capsule form were found effective against Corynespora leaf disease and was on par with the standard recommended fungicide (Carbendazim 0.05 %) treatment.

Endophytic bacteria are bacteria that establish an endo-symbiosis with plant where by plant receives an ecological benefit from the presence of symbiont such as increased stress tolerance (abiotic and biotic) or plant growth promotion (de Lamo and Takken, 2020). Inducing plants own defense mechanism by prior application of biological agent is a novel strategy in plant defense management. The defence mechanism include accumulaion of HCN, siderophores and volatile compounds produced by the microbes. The selected endophytic bacterial isolate (8LK) is superior in all biochemical properties,compatible with recommended fungicide. The integrated treatment of the endophytic bacteria with fungicide gave good contol of disease in the nursery.

Table 5. **Comparison of disease intensity during 2016**

Treatments	Disease intensity
Carbendazim (0.05%)	1.5
Antagonistic endophyte (RB 928) $1x10^6 ml^{-1}$	2.9
Antagonistic endophyte (8LK) $1x10^6 ml^{-1}$	2
Control	4.2
CD (P=0.05)	0.5

Table 6. **Comparison of disease intensity during 2017**

Treatments	Disease intensity
Carbendazim (0.05%)	2.4
Antagonistic endophyte (8LK) + Carbendazim (0.025%)	2.3
Antagonistic endophyte (8LK) alone	1.3
Control	4.7
CD (P=0.05)	0.2

Table 7. **Comparison of disease intensity during 2018**

Treatments	Disease intensity
Carbendazim (0.05%)	1.1
Antagonistic endophytes (8LK) broth + Carbendazim (0.025%)	0.5
(Antagonistic endophyte (8LK) alone	2
Antagonistic endophytes (8LK) encapsulated + Carbendazim (0.025 %)	0.5
Control	3.5
CD (P=0.05)	0.3

Fig. 4. Effect of integrated treatments on leaf disease

CONCLUSION

In the present study an attempt was made to control Corynespora leaf disease in nursery grown rubber plants by integrated approaches using an antagonistic endophytic bacteria with reduced concentration of the recommended fungicide carbendazim. Biochemical and antimicrobial studies proved that the isolate (8LK) of the endophytic bacteria *Bacillus spp.* produced high HCN and volatile compounds compared to other isolates. *In vitro* and nursery evaluation studies confirmed the efficiency of the selected isolate (8LK) combined with low concentration of Carbendazim (0.025%) against Corynespora leaf disease. The study showed that the integrated approaches are an excellent method for disease management and the possibility of using the antagonist which can reduce the fungicide usage and protect the ennviorment. However, more studies are required for large scale evaluation of the efficiency of the antagonist in disease contol in mature trees.

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