SCREENING OF ENDOPHYTES FROM RUBBER TREES (*HEVEA BRASILIENSIS*) FOR BIOLOGICAL CONTROL OF *CORTICIUM SALMONICOLOR*

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(Received: February 06, 2018; Revised: May 03, 2018; Accepted: May 14, 2018)

ABSTRACT

28 leaves and living-tissue samples of rubber tree (*Hevea brasiliensis*) were collected from Ho Chi Minh City, Binh Phuoc province and Binh Duong province (Viet Nam). We isolated and screened endophytes that have potential application as agents for biocontrol of *Corticium salmonicolor*, the agent of Pink Disease in rubber trees. As a result, 21 strains of endophytic bacteria and 14 strains of endophytic fungi were isolated. Antagonistic activity of the endophytes towards *C. salmonicolor* was checked by using a dual culture. Testing results showed that: T9, T15 and T16 strains have inhibited *C. salmonicolor*. T9 and T16 strains showed result that 100% of inhibiting *C. salmonicolor* at the concentration of 1:1. In the test of ability to kill *C. salmonicolor*, T9 and T16 strains showed that they could kill *C. salmonicolor* after 3 sprays of bacterial filtrate. T9 and T6 strains, which were identified by biochemical methods, have similar characteristics to *Bacillus thuringiensis.*

Keywords: Antagonistic activity test; Biocontrol; *Corticium salmonicolor;* Endophtytes; *Hevea brasiliensis.*

1. Introduction

Rubber tree (*Hevea brasiliensis*) is the native tree of South American in Amazone rainforest. Although it was tamed from wild type recently, it is significantly important for the economy, commerce and society in many countries. However, Rubber tree is usually attacked by pink disease (*Corticium salmonicolor*) that causes serious consequences. Pink disease can attack sapling and mature tree. Using too many chemical fungicide can lead to land degradation and insecticide resistance of insects. To cope with this problem, controlling disease by biological methods are catching more attention and some studies have recently been done (Narayanan et al., 2012). Endophytes are considered to be one of the most important target organisms that were isolated and screened for the bioproducts, using for the prevention of fungal diseases. Taking advantage of endogenous life characteristic in plant tissue should shorten the adaptation time of the biological product. It can create many antibiotics for fungal diseases, stimulates to growth of plants, and it does not effect to the

environment, public health (Khan, 2007; Strobel, 2003). The objective of this study was to find endophytes in rubber tree that have potential application as agents for biocontrol of *Corticium salmonicolor*.

2. Materials and Methods

2.1. Materials

Endophytes were isolated from 28 samples of healthy rubber trees in Cu Chi District – Ho Chi Minh City, Ben Cat District – Binh Duong Province, Chon Thanh and Dong Xoai District – Binh Phuoc Province. *Corticium salmonicolor* was provided from Plant Protection Department of Rubber research institute of Viet Nam.

2.2. Methods

2.2.1. Isolation of endophytes

Endophytes were isolated from leaves and living-tissues of healthy rubber trees, without disease. The samples were isolated within 6 hours since they were collected. Tissues and leaves were washed under strong jet of water, cutting into small section 2 – 4 cm for easy manipulation, surface disinfection of samples were done step by step, respectively: ethanol 70% (5 minutes), sodium hypochlorite 2% (5 minutes), ethanol 70% (30 seconds). Washing with sterile distilled water for 5 times. Using sterilized equipments to cut/grind these samples and isolate on TSA (Trypticase Soy Agar), incubate at 37^{o}C for several days to allow growth of endophytic bacterial; on PDA (Potato Dextrose Agar) with Chloramphenicol 0.05% and sealed tightly paraffin, incubated at 27 ± 2^{0}C for from several days to 2 months to allow growth of endophytic fungi (Arnold et al., 2001; Gazis and Chaverri, 2010; Khan, 2007).

2.2.2. Resistance testing between endophytes and Corticium salmonicolor

Using dual testing method, *Corticium salmonicolor* and endophytes were cultured 3 cm apart on PDA (petri dish 90mm) at 27^{o}C for 6 days. If endophytes have ability to inhibit fungal disease, mycelium does not grow or grow weakly around the endophytes colonies. If endophytes have not ability to inhibit growth of fungal disease, *Corticium salmonicolor* grow normally (Gong et al., 2006).

2.2.3. Identification of percentage of Corticium salmonicolor inhibition by concentration

Bacteria were cultured in 50mL PD (Potato Dextrose Broth), shaking at 150 rpm, 37^{o}C for 48 hours. The culture medium was centrifuged at 9000 rpm for 15 minutes at 4^{0}C. Then, filtered through membrane filter 0.2µm. The culture medium was mixed with PGA (45^{o}C) at 5 ratios: 1:1, 1:2, 1:4, 1:8, 1:16 and pouring onto petri dish (90mm). Taking in 20mL disease fungal solution 10^6 CFU/mL pumped to center area of the prepared petri. Incubate at room temperature for 6 days and identify percentage of inhibition according to the formula: I(%)=(C-E)/C x100%, in it : I – percentage of inhibition; C – diameter of fungi on the control petri disk (mm); E – diameter of fungi on petri dish contain bacterials (mm); The bacteria are able to inhibit fungi at I ≥ 20%. The bacteria are not valuable in resistance fungi at I < 20% (Chang et al., 2007).

2.2.4. Identification of ability of Corticium salmonicolor killing under growth conditions

The process of preparing bacterial culture is similar to the experiment percentage of inhibition *Corticium salmonicolor*. Bacterial culture was mixed with sterile distilled water at concentrations: 1, 1:2, 1:4, 1:8 và 1:16. Spraying the bacterial concentrations onto the Pink Disease dish which were cultured on PDA for 4 days. The volume of bacterial to be sprayed is 2mL. The diameter of fungi group was measured after 2 days spraying. The spraying process was carried out 3 times, each time 2 days apart. After reading out the third result, the fungi which from the plates were sprayed bacteria were transplanted into PDA, incubated at 27 ±

20°C for 5 days, to test the viability of fungi. If the fungi do not grow, the bacteria are capable of destroying the fungi.

2.2.5. Identification of endophytic strains

The strains that strongly inhibited *C. salmonicolor* were identified by biochemical methods according Cowan and Steel (Cowan and McFaddin, 1993).

3. Results and discussion

3.1. Isolation of endophytes

From 28 samples foliage and sapwood of rubber tree (*Hevea brasiliensis*), we isolated 21 strains of endophytic bacterial and 14 strains of endophytic fungi.

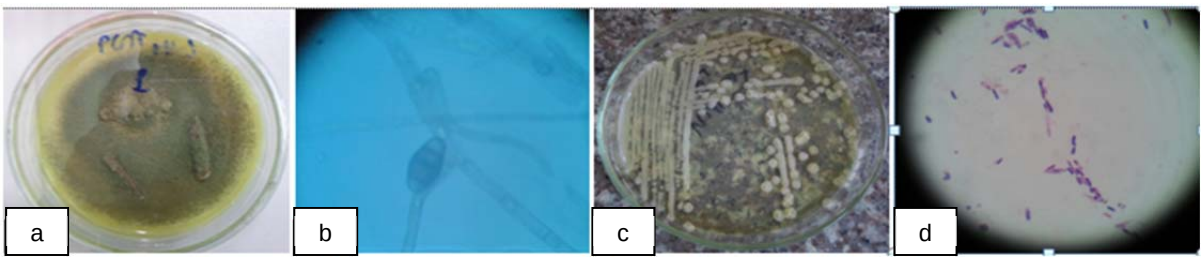

Figure 1. *Macroscope (a) and microscope (b) endophytes fungi N4*
Macroscope (c) and microscope (d) endophytes bacterial T9

3.2. Result of Resistance testing between endophytes and Corticium salmonicolor

After 6 days of testing, among 21 of strains endophytic bacterial and 14 strains of endophytic fungi, T9, T15, T16 endophytic bacterial strains can resist to *Corticium salmonicolor*. T9 and T16 showed the strongest resistance among them (Figure 2a). The remaining strains have no resistance ability. The T9 and T16 were used for subsequent testings. Studying of Philip (2004) (Philip, 2004) showed similar result that bacteriaisolated from rubber tree can resist to *Corticium salmonicolor.*

3.3. Result of identification of percentage of Corticium salmonicolor inhibition by concentration

After 6 days of testing, percentage of *Corticium salmonicolor* inhibition by concentration 1:1, 1:2, 1:4, 1:8 and 1:16 of T9 strain were 100 ± 0.00 % (Figure 2b), 98.89 ± 0.56%, 93.77 ± 2.12%, 72.44 ± 0.77%, 45.88 ± 2.41%, respectively; and that of T16 strain were 100 ± 0.00%, 76.66 ± 5.09%, 67.0 ± 2.45%, 55.55 ± 3.37% and 43.67 ± 4.09%, respectively. This testing showed that concentration of bacteria decline, the ability to inhibit fungi also decline.

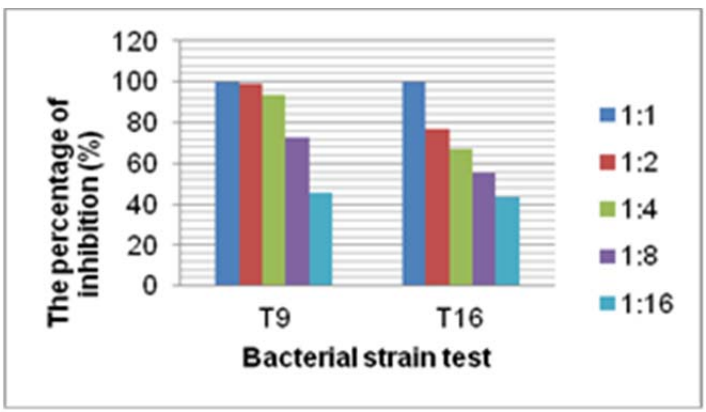

Chart 1. The graph show the percentage of inhibition C. salmonicolor of the strains in the concentrations

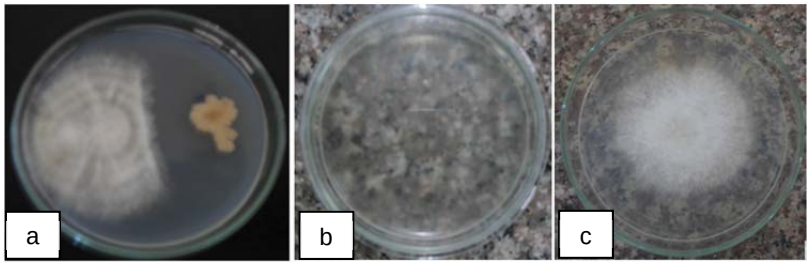

Figure 2. *(a) - Ability of endophytic bacterial T9 resistance to C. salmonicolor; (b) – Percentage of inhibition C. salmonicolor of T9 in 1:1 concentration. (c) – Control sample*

3.4. The result of spraying endophytic culture fluid to C. salmonicolor

After 10 days of testing, combining observation and measurement of fungal growth diameter, we collected the result. At the test of both T9 strain and T16 strain with 1 and 1:2 concentration, mycelia were dry and no signal of growth. The remaining concentrations 1: 4, 1: 8, 1:16 showed signal of mycelial growth but limited (Table 1, 2).

Table 1

The results of spraying endophytic T9 strain to C. salmonicolor

Dilute concentration	Result of fungal diameter (mm)						
	Before spraying (4th day)	First (6th day)	Growth diameter	Second (8th day)	Growth diameter	Third (10th day)	Growth diameter
1:1	52,33±1,45	54,33±2,19	1,00±0,74^{d}	55,33±1,20	1,00±0,46^{c}	55,67±1,33	0,00±0,00^{b}
1:2	57,67±2,33	64,33±2,72	6,66±0,39^{c}	65,00±2,51	0,67±0,21^{c}	65,33±2,67	0,00±0,00^{b}
1:4	54,00±1,00	65,00±0,57	11,00±0,43^{b}	66,00±0,57	1,00±0,00^{c}	66,33±0,57	0,33±0,00^{b}
1:8	51,67±0,33	63,00±0,58	11,33±0,25^{b}	65,67±0,67	2,67±0,09^{b}	66,00±0,57	0,33±0,10^{b}
1:16	53,67±2,33	65,00±1,00	11,33±1,33^{b}	67,00±0,10	2,00±0,90^{b}	68,33±0,67	1,33±0,57^{b}
Control	50,00±2,89	63,00±2,08	13,00±0,81^{a}	66,67±1,08	3,67±0,73^{a}	76,00±4,70	9,33±3,62^{a}

Table 2

The results of spraying endophytic T16 strain to C. salmonicolor

Dilute concentration	Result of fungi diameter (mm)						
	Before spraying (4th day)	First (6th day)	Growth diameter	Second (8th day)	Growth diameter	Third (10th day)	Growth diameter
1:1	52,33±2,33	60,00±1,52	7,67±0,81^{c}	60,67±1,20	0,67±0,32^{c}	62,00±1,52	0,00±0,00^{b}
1:2	54,67±1,45	60,00±1,52	5,33±0,07^{d}	61,00±2,00	1,00±0,48^{bc}	61,00±2,00	0,00±0,00^{b}
1:4	48,00±3,05	55,67±3,84	7,67±0,79^{c}	56,67±3,33	1,00±0,51^{bc}	57,00±3,51	0,33±0,18^{b}
1:8	53,33±1,33	64,33±1,45	11,00±0,12^{b}	66,00±1,52	1,67±0,07^{b}	67,00±2,00	1,00±0,48^{b}
1:16	53,00±4,04	63,67±1,86	10,67±2,18^{b}	66,67±2,02	3,00±0,16^{a}	68,67±1,86	2,00±0,16^{b}
Control	50,00±2,89	63,00±2,08	13,00±0,81^{a}	66,67±1,08	3,67±0,73^{a}	76,00±4,70	9,33±3,62^{a}

Notes: Table 1, 2, in the same column, the average values following the same letter are not different at 95%.

Examining the survial of fungal disease, results obtained as follows: plates have spray T9 and T16 strain at 1:1 and 1:2 concentration, mycelium did not grow, the plate control has growth fungi with 63,33±1,33 mm diameter. From this result, we can conclude: at 1:1 and 1:2 concentration, T9 and T16 can kill *C. salmonicolor.* If concentration of bacteria decline (1:4, 1:8, 1:16), the ability to inhibit fungi also decline.

3.5. Result of identification of T9 and T16 strain

With the identification according to Cowan and Steel, the T9 and T16 strain have characteristics similar to *Bacillus thuringiensis*. *Bacillus thuringiensis* can resist to diseased fungi.

4. Conclusion

With the experimental results above, we isolated 21 endophytic bacterial strains and 14 endophytic fungal strains. T9 and T16 strains can inhibit to *Corticium salmonicolor* 100% at concentration 1:1 and kill fungi at concentration 1:1 and 1:2 after spraying 3 times. The identification result showed that 2 strains T9 and T16 have characteristics similar to *Bacillus thuringiensis*■

Acknowledgements

This work was funded by Ho Chi Minh Open University.

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