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Antagonistic Effects of Bacteriocin and Bacteriocinogenic Lactobacillus on stem rot causing *Sclerotium rolfsii* in Betel

Malay Mandal and Subhas Chandra Jana*
P.G. Department of Microbiology, Bidhannagar College (Govt. of West Bengal), EB 2, Salt Lake City, Kolkata – 700064, West Bengal, India.

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Abstract: Economic potentiality of betel vine (*Piper betle* L.) is always under threat of fungal diseases caused by various soil borne phytopathogenic fungi. Betel vine production accounts for extensive economic loss due to collar rot caused by *Sclerotium rolfsii*. With a view to search an eco-friendly biocontrol system for the management of *S. rolfsii*, we have studied the antagonistic effect of a bacteriocinogenic Lactic Acid Bacteria (LAB) strain, *Lactococcus lactis* JC10 and produced bacteriocin on this pathogen. Significant decrease in mycelia growth of Sclerotium in dual culture plate experiment was observed. There was also noteworthy amount of decrease in growth rate of mycelium in presence of viable *L. lactis* JC10 in medium. Crude bacteriocin (25% v/v) in a PDA plate can totally seize the mycelia growth. It was also found that crude bacteriocin treatment of Sclerotium spore can delay germination considerably.

Key words: Betel; Biocontrol; Collar Rot; *Sclerotium rolfsii*

INTRODUCTION

Betel vine (*Piper betle* L.) is an important cash crop for not only West Bengal but at national level in India. This crop provides a national income of Rs. 60,000- 70,000 million per annum and Rs. 800-1,000 million for the state of West Bengal (Guha, Proshanta, 2006). In-spite of having such vast economic potentiality, its yield is always under threat of fungal diseases caused by various soil borne fungal pathogens. The shady and humid environmental conditions of betel farming enhance the growth of phytopathogenic fungi (Shahzad, Saleem, 2001). One of the main obstacles in the effort to increase in betel vine production is collar rot disease caused by *Sclerotium rolfsii,* a wide spread phytopathogenic fungus. *S. rolfsii* is responsible for considerable economic loss in a variety of cultivated crops in tropical and sub-tropical countries due to its over 500 botanical species host range (Khanzada *et al.*, 2006).The mycelia of *S. rolfsii* spread rapidly and can remain active in soil for long period as sclerotia.

To meet the ever increasing demand of food, indiscriminate practice of chemical fungicides sacrifices environment and results in continuous pollution of ground water, food-stuffs and other environmental degradation (Rakh *et al.*, 2011). In a good many cases of fungal disease management, chemical fungicide does a pretty good job. But management of collar rot by *S. rolfsii* is not feasible only with fungicides, mainly because this fungus displays strong ability to sustain chemical fungicides by developing dark brown spherical sclerotia. Usual disease control methods like soil solarization (Mello *et al.,* 2005), crop rotation are also not much of use as sclerotia displays strong resistance towards biological degradation (Khanzada *et al.,* 2006). Apart from that, as the betel vine is directly consumed by mankind, use of hazardous chemical should be restricted immediately. In this context we can sense the urge of an alternative management system for protection of betel vine from *S. rolfsii*.

Our aim is to search an eco-friendly biocontrol system for *S. rolfsii.* For that, we have studied the effect of bacteriocin and bacteriocinogenic Lactic Acid Bacterial strain on live *S. rolfsii* and on spore germination as well.

MATERIALS AND METHOD

Bacterial strains, Growth medium and Growth conditions

The bacteriocinogenic strain, *Lactococcus lactis* JC10 was isolated in our own laboratory from papaya. MRS media (Hi-media, India) was used for its sub-culturing. With 1% inoculums load, it grows and produces highest level of bacteriocin within 5 hrs of incubation (37 °C) at static condition. *S. rolfsii* (NCIM No. - 1084) was obtained from NCIM, Pune, India. Potato Dextrose media (Hi-media, India) was used for sub-culturing as well as for all the experiment.

Study the antagonism of Lactococcus lactis JC10 *on Sclerotium rolfsii*

L. lactis JC10 was grown in MRS broth media in static condition at 37 °C for 5 hrs and *S. rolfsii* was grown on PDA plates. One loop full of cell suspension of *L. lactis* JC10 was taken and inoculated on one half of overnight dried PDA plate. Three days old *S. rolfsii* culture in disc (0.5 mm in diameter) was inoculated on the other side of the plate. Experimental set up was carried out in triplicate replica. All the plates were incubated at 30 °C and observed on daily basis for comparative decrease in mycelia growth of *S. rolfsii* in presence of antagonist. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control and percentage inhibition was calculated by the following equation (Riungu *et al.,* 2008).

$$\text{Percentage Inhibition (\%)} = \frac{\text{Colony diameter of pathogen alone (control)} - \text{colony diameter of pathogen + antagonist}}{\text{Colony diameter of pathogen alone}} \times 100$$

***Corresponding Author:**
Dr. Subhas Chandra Jana,
Officer-in-Charge,
Bidhannagar College (Govt. of West Bengal)
EB 2, Salt Lake, Kolkata 700064.,
West Bengal, India.

Determine the antagonism of viable Lactococcus lactis* JC10 *on Sclerotium rolfsii

50 ml of PDA media was sterilized in several conical flasks. All the PDA media in flasks were melted and after cooling down, different amount (50 µl, 100 µl, 250 µl, 500 µl, 1000 µl) of *L. lactis* JC10 cell suspension (grown at 37°C, static condition, 5 hours) was added to each 50 ml molten PDA and mixed thoroughly. 3 plates were made from each set of 50 ml molten PDA media. After solidification, *S. rolfsii* disks (diameter – 0.5 mm) from 3 days old culture were inoculated at the center of PDA plate. Inoculated *S. rolfsii* disk on a normal PDA plate used as positive control. All the plates were incubated a 30 °C and observed daily to compare the growth inhibition with control. Percentage inhibition for each case was calculated by the following equation

$$\text{Percentage Inhibition (\%)} = \frac{100\,(C - T)}{C}$$

C = Radial growth of fungus in control plates, **T** = Radial growth of fungus in test plates

Study the inhibitory effect of different amount of crude bacteriocin on Sclerotium rolfsii

L. lactis JC10 was grown in MRS broth media at desired growth condition with 1% inoculum load till 5th hour of incubation. The broth culture was collected and centrifuged at 10,000 rpm for 15 minutes at 4 °C. Different amount (250 µl, 500 µl, 1 ml, 2 ml, 3 ml, 4 ml, 5 ml) of crude bacteriocin was then mixed with PDA plates containing 20 ml media as total volume in one plate. All those plates were kept for solidification. After solidification, *S. rolfsii* disks (diameter – 0.5 mm) from 3 days old culture were inoculated at the center of specially prepared PDA plates. As control, one *S. rolfsii* disk was inoculated on normal PDA plate. All the plates were incubated at 30 °C and observed regularly. Percentage inhibition for each case was calculated by the equation stated above.

Study the effect of crude and neutralized bacteriocin on S. rolfsii spore germination

L. lactis JC10 was grown in MRS broth media at desired growth condition with 1% inoculum load till 5th hour of incubation. The broth culture was collected and centrifuged at 10,000 rpm for 15 minutes at 4°C. Crude bacteriocin was taken in two different test tubes. Crude bacteriocin of one of the test tube was neutralized by pre sterilized 1 N NaOH. *S. rolfsii* spores were collected from 7 days old culture. Spores were mixed with crude and neutralized bacteriocin respectively and incubated at 30°C in shaker. After regular interval spores were inoculated on PDA plates. As control, non-treated spores were also inoculated on PDA plates. All the plates were then incubated at 30°C and study the germination time in each case.

RESULTS AND DISCUSSION

Antagonism of Lactococcus lactis* JC10 *on Sclerotium rolfsii

It was revealed from the result of dual culture plate experiment that the test strain, *L. lactis* JC10 was able to inhibit *S. rolfsii* to a certain extent. It was found from our results that at the end of day four, test strain caused visible amount of decrease in mycelia growth of *S. rolfsii* (Fig. 1).

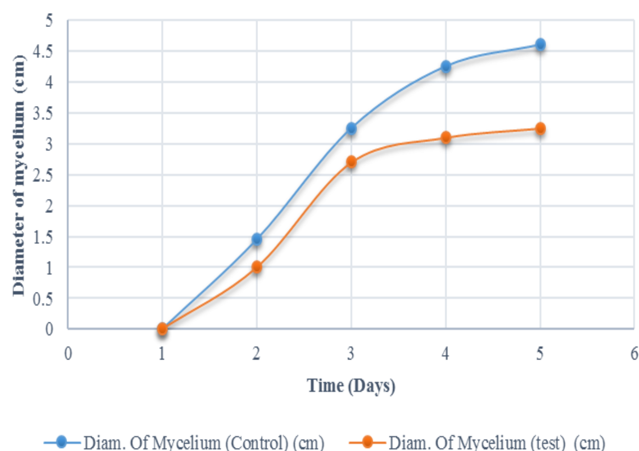

Fig. 1: Antagonism of *L. lactis* JC10 on *Sclerotium rolfsii* in dual culture technique

Antagonism of viable Lactococcus lactis* JC10 *on Sclerotium rolfsii

The effect of different amount of vaiable *L. lactis* JC10 in PDA media was significant regarding *S. rolfsii* mycelial growth rate. When compared with the control, increased amount of viable *L. lactis* JC10 exterts significantly more inhibitory effect on mycelial growth of *S. rolfsii* under in vitro condition. While 50 µl *L. lactis* JC10 cell suspension in 50 ml PDA media causes 2.89% inhibition in mycelia growth on the 4th day of growth, 1000 µl cell suspension in 50 ml PDA media causes 32% inhibition (Table 1).

Table 1: Antagonism of viable *Lactococcus lactis* JC10 on *S. rolfsii*

Experiment set up	Age of culture	Colony diameter (cm)[a]	Parentage inhibition[b]
Normal PDA	Day 1	1.4033±3.333E-03	0%
	Day 2	3.1667±1.667E-02	0%
	Day 3	4.1833±1.667E-02	0%
	Day 4	4.4833±1.667E-02	0%
50 ml PDA + 50µl cell suspension	Day 1	1.2233±3.333E-03	14.28%
	Day 2	3.0433±6.667E-03	5.31%
	Day 3	4.0500±2.887E-02	3.33%
	Day 4	4.3733±1.453E-02	2.89%
50 ml PDA + 100µl cell suspension	Day 1	1.1533±3.333E-03	17.14%
	Day 2	3.0167±1.667E-02	6.25%
	Day 3	3.8433±6.667E-03	8.8%
	Day 4	4.2100±1.000E-02	6%
50 ml PDA + 250µl cell suspension	Day 1	0.9100±1.000E-02	33.57%
	Day 2	2.0167±1.667E-02	37.5%
	Day 3	3.7567±3.333E-03	10.47%
	Day 4	3.9367±1.856E-02	12%
50 ml PDA + 500µl cell suspension	Day 1	0.8667±1.667E-02	39.28%
	Day 2	1.8167±1.667E-02	43.75%
	Day 3	3.0100±1.000E-02	27.85%
	Day 4	3.7167±8.819E-03	17.11%
50 ml PDA +1000µl cell suspension	Day 1	0.6400±1.000E-02	55.71%
	Day 2	1.1267±1.453E-02	64.68%
	Day 3	2.6133±6.667E-03	37.61%
	Day 4	3.0333±1.667E-02	32%

[a]Each value is an average of 3 replicate samples ± standard deviation
[b]Result significant at $P \leq 0.05$

Effect of different amount of crude bacteriocin (in solid growth media) on Sclerotium rolfsii

As the result suggested, presence of crude bacteriocin in solid growth media inhibited the mycelial growth of *S. rolfsii*. Rate of inhibition of mycelial growth of *S. rolfsii* increasesed with the increasing amount of crude bacteriocin in specially prepared solid PDA plate. PDA media supplemented with 15% crude bacteriocin caused 80.43% inhibition in mycelia growth on the end of 4th day. Likewise 20% crude bacteriocin in PDA media caused 95.65% inhibition. More importantly 25% crude bacteriocin supplemented media can cause of 100% inhibition of *S. rolfsii* (Table 2).

Table 2: Effect of different amount of crude bacteriocin (in solid growth media) on *S. rolfsii*

Experiment set up	Age of culture	Colony diameter (cm)[a]	Parentage inhibition[b]
Normal PDA	Day 1	1.4367±6.667E-03	0%
	Day 2	3.2167±1.667E-02	0%
	Day 3	4.2167±1.667E-02	0%
	Day 4	4.5833±1.667E-02	0%
Normal PDA + 250µl crude bacteriocin	Day 1	1.1833±1.667E-02	17.24%
	Day 2	2.8667±1.667E-02	9.37%
	Day 3	3.7167±1.667E-02	11.9%
	Day 4	4.1167±6.009E-02	8.69%
Normal PDA + 500µl crude bacteriocin	Day 1	0.8167±1.667E-02	44.82%
	Day 2	1.4067±6.667E-03	56.25%
	Day 3	1.8667±1.667E-02	54.48%
	Day 4	3.5833±6.667E-02	21.73%
Normal PDA + 1ml crude bacteriocin	Day 1	0.5833±1.667E-02	58.62%
	Day 2	1.1167±1.667E-02	65.62%
	Day 3	1.6333±1.667E-02	61.9%
	Day 4	2.0667±3.333E-02	54.34%
Normal PDA + 2ml crude bacteriocin	Day 1	0.2333±3.333E-02	86.2%
	Day 2	0.8833±1.667E-02	76.78%
	Day 3	1.0333±1.667E-02	76.19%
	Day 4	1.1767±1.453E-02	73.9%
Normal PDA + 3 ml crude bacteriocin	Day 1	0	100%
	Day 2	0	100%
	Day 3	0.3933±6.667E-03	90.46%
	Day 4	0.8833±1.667E-02	80.43%
Normal PDA + 4 ml crude bacteriocin	Day 1	0	100%
	Day 2	0	100%
	Day 3	0	100%
	Day 4	0.2167±1.667E-02	95.65%
Normal PDA + 5 ml crude bacteriocin	Day 1	0	100%
	Day 2	0	100%
	Day 3	0	100%
	Day 4	0	100%

[a]Each value is an average of 3 replicate samples ± standard deviation
[b]Result significant at P ≤0.05

Effect of crude and neutralized bacteriocin on Sclerotium spore germination

It was very much evident from the result that crude bacteriocin from *L. lactis* JC 10 could significantly inhibit spore germination of *S. rolfsii*. Incubation of *S. rolfsii* spore for merely 1 hour with crude bacteriocin caused significant delay of 3 days in spore germination. More interestingly, 2 hours treatment of spore with crude bacteriocin caused delay of 4 days in germination. Treatment of 3 hours and 4 hours with crude bacteriocin totally seized spore germination. Treatment of spore with neutralized crude bacteriocin showed nearly same result (Table 3).

Table 3: Effect of crude and neutralized bacteriocin on *S. rolfsii* spore germination

Experiment set up	Germination of the spores occurs on the day			
A) Incubation time after crude bacteriocin treatment	Day 1	Day 2	Day 3	Day 4
1 hr	(–)	(–)	(+)	(++)
2 hrs	(–)	(–)	(–)	(+)
3 hrs	(–)	(–)	(–)	(–)
4 hrs	(–)	(–)	(–)	(–)
15 hrs	(–)	(–)	(–)	(–)
B) Incubation time after neutralized bacteriocin treatment	Day 1	Day 2	Day 3	Day 4
1 hr	(–)	(–)	(+)	(++)
2 hrs	(–)	(–)	(+)	(+)
3 hrs	(–)	(–)	(–)	(+)
4 hrs	(–)	(–)	(–)	(–)
15 hrs	(–)	(–)	(–)	(–)
C) Incubation time after non-treated spores germinate	(+)	(++)	(+++)	(++++)

(-) No visible mycelia growth occurs
(+) Mycelia growth occurs
Addition of each '+' within (+) denotes further mycelia growth.

Discussion

Four different experiments were set up to evaluate the efficiency of bacteriocinogenic Lactobacillus strain itself and bacteriocin as antifungal agent against *S. rolfsii*. Effect on growth rate of *S. rolfsii* was determined by measuring the deviation in mycelia growth. The readings obtained from each experiment, except germination delay experiment, were transformed into percentage inhibition to indicate the ability of the Lactobacillus strain and bacteriocin to inhibit the growth of the pathogen.

Significant decrease in mycelia growth of *S. rolfsii* in dual culture plate technique experiment was observed. Inhibition was probably occurred due to production of volatile antifungal compound by *L. lactis* JC10. Results also suggested the decrease in growth rate of *S. rolfsii* mycelium in presence of viable *L. lactis* JC10 in solid medium. Growth rate gradually decreased with increasing amount of viable *L. lactis* JC10 cells in each plate. We also observed major decrease in mycelia growth of *S. rolfsii* in crude bacteriocin mixed PDA plate. More importantly, 25% crude bacteriocin in a PDA plate can totally seized the mycelia growth. It was also found that crude bacteriocin treatment of *S. rolfsii* spore can delay germination significantly. In case of neutralized crude bacteriocin, it was found to be slightly less effective comparatively in delaying spore germination. Results revealed that *L. lactis* JC10 and crude bacteriocin from *L. lactis* JC10 may be identified as potentially active against *S. rolfsii,* known to be the most destructive soil borne pathogen of *Piper betel.* In each experiment we used bacteriocin in crude form in order to get rid of the extra cost for purification purpose of bacteriocin. As we know purification needs a lot more effort in terms of labour and money, bacteriocin, if can be used in crude form commercially, will be a cheaper alternative biocontrol agent against *S. rolfsii.*

Conclusion

Bacteriocinogenic *L. lactis* JC10 can effectively inhibit the growth of *S. rolfsii* under laboratory conditions. These results suggest possible promising application of Lactic Acid Bacterial (LAB) strains against phytopathogenic fungi of betel vine (*Piper betle* L.).

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