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AN ISOLATE OF *BACILLUS SUBTILIS* FROM SRI LANKA INHIBITORY TO *RHIZOCTONIA SOLANI*

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Abstract: A deep purple pigmented isolate of *Bacillus subtilis* obtained from paddy soil inhibited the growth of *Rhizoctonia solani*, the causative agent of sheath blight of rice. The rod shaped bacterium hydrolysed starch and gelatin, reduced nitrate, was aerobic, formed acetyl methyl- carbinol, catalase positive and spore forming. The inhibitory action was due to the secretion of three inhibitory compounds by the bacterium. The inhibitory compounds are not composed of amino acids and are highly thermostable.

Key words: *Bacillus subtilis, Rhizoctonia solani,* sheath blight inhibitor.

INTRODUCTION

Rhizoctonia solani Kuhn. causes sheath blight of rice. Sheath blight has become increasingly important in recent years in most rice growing regions of the world including Sri Lanka. The disease at present is mainly controlled by the use of expensive and often harmful fungicides since rice varieties showing high resistance to the fungus has not been developed yet.[1] Hence, the development of biologically oriented control methods as an alternative to the fungicides becomes increasingly important.

Soil bacteria, especially bacilli, from many localities in different countries have been shown to have inhibitory activity against several plant pathogenic fungi including *R. solani*, and in some instances the active chemical has been identified[2,3]; *Bacillus subtilis* and several other *Bacillus* species produce polypeptides inhibitory to *R. solani*.[2] Similar information that would be useful in developing new methods to control plant diseases especially those like the sheath blight of rice, is not available in Sri Lanka.

Here we report the isolation of a soil bacterium, *Bacillus subtilis,* inhibitory to the rice sheath blight fungus from Sri Lanka, the characteristics of the inhibitory bacterium and preliminary studies on the inhibitory compounds.

METHODS AND MATERIALS

The Fungus: The fungus *Rhizoctonia solani* was isolated from infected plants of rice obtained from the Agriculture Research Station at Bombuwela, Kalutara. Pathogenicity of the fungus was confirmed by following the Koch's postulates. The isolated fungus was maintained on potato dextrose agar (PDA) at 25°C during the period of study.

Isolation of Bacteria: Bacteria were isolated from the soil of a paddy field close to Makandura in the North Western Province. From this field (about 0.2 ha in area) six soil samples (each weighing 10g) were collected at random during both the wet and dry seasons. Serial dilutions (upto 10^{-5}) were made using sterile distilled water. The respective dilutions were plated on peptone sucrose agar (PSA) and nutrient agar (NA) plates and incubated at 30° C. Pure cultures were obtained from single colonies of the resulting mixed cultures. The pure cultures were maintained on PSA at 30°C.

Growth of Bacteria for Initial Screening: To initially screen the bacterial isolates for secretion of inhibitory compounds, each isolate was grown in 50 ml of peptone sucrose liquid medium in 150 ml Erlenmyer flasks. Each liquid medium was inoculated with a 1 cm diameter agar disc obtained from the centre of a 3-day old bacterial culture on PSA. The inoculated media were incubated at 30° C and harvested at 1, 3 and 5 d after inoculation by filtration through a membrane filter (0.22 μ, Millipore). The culture filtrates after concentration (x10) by freeze drying were used to determine inhibitory activity by the filter paper disc method described below. In the control experiments similarly concentrated liquid medium was used instead of the culture filtrate.

Test for Inhibition: The inhibitory tests were carried out on PDA plates at 30°C. At the periphery of 9.0 cm diameter petri dishes having the medium, 5 mm diameter mycelial discs of *R. solani* were kept equidistant to each other and about 3.0 cm from the centre of the plate. (The mycelial discs were obtained from the periphery of 7-day old cultures of the fungus on PDA at 30°C). A sterile filter paper disc (5 mm diameter) dipped in 0.1 ml of the test solution were placed at the centre of the plates containing the mycelial discs. The inoculated plates were incubated at 30°C and examined for inhibitory zones. The diameter of any inhibitory zones were measured.

Extraction of the Inhibitor: To obtain the inhibitor in large quantities the bacterial isolate was grown in 500 ml flasks having 200 ml peptone sucrose liquid medium. The liquid medium was inoculated with 2 ml of a 3-day old culture of the bacterium in peptone sucrose liquid medium. The inoculated media were incubated at 30°C and harvested 3 days after inoculation by filtration through a bacterial filter (0.22μ, Millipore). The resulting filtrate was extracted with methylene dichloride (200 ml x 3) followed by ethyl acetate (200 ml x 3) based on a method described by Roy *et al.*[4] The extracts were evaporated to dryness in a rotary

evaporator at 30°C, re-dissolved in 2 ml of the respective solvents and examined for inhibitory activity using the filter paper disc method. In the control equal amounts of the respective solvents were used to treat the filter paper discs.

Chromatography (Thin Layer Chromatography tlc): Ascending tlc using the methylene dichoiride extract on silica gel 60 (Merck) glass plates (20x20 cm and 0.2 mm thick) were developed with methylene dichloride : methanol (96:4) for 5-6 h in tanks equilibrated with the solvent system. Inhibitory compounds were detected by the *Cladosporium cladosporioides* bio-assay[5] and a modified *Cladosporium* bio-assay method where instead of the *Cladosporium* spore suspension a similarly prepared hyphal suspension of *R. solani* were used. To prepare the hyphal suspension a 5-day old culture of *R. solani* on PDA was used. Both the spore and hyphal suspensions were prepared in a Czapek-dox nutrient medium.

The areas corresponding to the inhibitory zones observed in the bio- assays were eluted 3 times each with 10 ml methylene dichloride, evaporated to dryness at 30°C and re-dissolved in 1 ml of the solvent. The inhibitory activity of the re-dissolved extracts were examined by the filter paper disc method. In the control methylene dichloride was used to treat the filter paper discs.

In a seperate experiment, the developed plates were sprayed with 0.2 g of ninhydrin per 100 ml of 95% ethanol and heated to 100° C for 5-10 min to detect ninhydrin positive compounds.

Column Chromatography: The dried methylene dichloride extract (1.2 g) dissolved in 1.0 ml of methylene dichloride was placed on a silica gel (Merck 20g) glass column (2.5 cm diameter). The column was eluted by gravity with a methylene dichloride, methanol mixture of increasing polarity. The flow rate was 2 ml/min and 8 ml fractions were collected and examined for inhibitory activity by the filter paper disc method.

Characterization of the Bacterial Isolate: Morphological and biochemical characters of the bacterial isolate were examined as described by Kiraly.[6]

Thermal Stability: Equal amounts of the inhibitory compounds eluted from thin layer chromatography as described above were kept at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90° and 100°C in glass test tubes in a water bath for 30 min. At the end of the time period the tubes were rapidly cooled to room temperature. Afterwards the samples were adjusted to their starting volumes using methylene dichloride and the samples were examined for inhibitory activity. The % decrease in activity was calculated as follows:

$$\% \text{ decrease} = \frac{X - Y \times 100}{X}$$

X = Activity after exposure to 30° C, Y = Activity after exposure to the required temperature. (No decrease in activity is observed after exposure to 30°C).

RESULTS

Isolation of Bacteria and Initial Screening: A large number of bacteria (95 forms) were isolated from the soil samples. The culture filtrates (harvested at 1,3 and 5 d) of all isolates were screened for inhibitory activity. A few isolates (12 forms) had a inhibitory effect on the growth of the fungus. The inhibitory effect of only one isolate was persistent - in all other 11 forms the inhibitory effect was temporary (lasting from 12 - 24 h). No inhibitory effects were seen in the control experiment.

In the isolate which showed persistent inhibitory activity, the inhibition was highest in the culture filtrate harvested 3 d after inoculation. This isolate which showed persistent inhibition was chosen for further study. We were unable to isolate the bacterium showing persistent inhibition from soil samples collected during the dry season.

Characters of the Inhibitory Bacterium: The characters observed are listed in Table 1. The colonies on both PSA and NA were small, circular and flat with an entire margin. The colonies had an intense purple colour and the bacterial cells were rod shaped. The bacterium also had the following characters: hydrolysed starch and gelatin, catalase positive, reduced nitrate to nitrite, formed acetyl-methylcarbinol, aerobic and spore forming.

Table 1 : Characteristics of the *B. subtilis* isolate.

Character	Description
1. Colonies on NA and PSA	Small, circular and flat. Deep purple pigment present.
2. Anaerobic growth	-
3. Catalase	+
4. Hydrolysis of	
Starch	+
Gelatin	+
5. Nitrate reduced to nitrite	+
6. Formation of acetyl methyl carbinol	+
7. Spore formation	+

+ Character present, - Character absent.
NA- Nutrient agar, PSA- Peptone sucrose agar.

When the bacterium was grown in peptone sucrose liquid medium it turned light purple within 24 h after inoculation and the colour intensified 48 h after inoculation. When the liquid culture was filtered using a membrane filtre (0.22 μ, Millipore) a clear colourless filtrate free from the pigment was obtained.

Charcterization of the Inhibitor: The methylene dichloride extract of the culture filtrate showed persistant inhibition. Inhibition was totally absent in the ethyl acetate extract. In the control experiments where discs dipped in the respective solvents were used no inhibitory effects were observed.

When the methylene dichloride extract was subjected to tlc and the *Cladosporium* bio-assay 3 inhibitory zones were obsereved. The R_f values were 0.66 (CI), 0.56 (CII) and 0.31 (CIII). Similar inhibitory zones were also observed in the modified *Cladosporium* bio-assay. All three zones fluoresced under UV (254 nm) and were ninhydrin negative.

The three zones when examined by the filter paper disc method after elution, inhibited the growth of the fungus. Inhibition was highest in CII followed by CI and CIII. The inhibition was persistent. No inhibition was seen in the control experiments.

Column chromatography confirmed the existence of more than one inhibitory compound. Three peaks of inhibition was also obtained when the fractions from the silica gel column were examined for inhibition. Two peaks were in the fractions eluting with methylene dichloride:methanol (98:2) and the other eluting with methylene dichloride:methanol (98:1).

Temperature Stability: The inhibitory compounds showed tolerance to temperatures upto about 60°C. At this temperature they retained about 85% of activity. Thereafter, a rapid decline in activity was seen (Table 2).

Table 2: Temperature stability of the inhibitor.

	% Decrease in Activity*		
Temperature (°C)	C I	CII	C III
40	3.0	2.5	2.2
50	5.0	6.0	4.5
60	14.0	10.0	11.0
70	59.0	65.0	70.0
80	81.0	78.0	100.0
90	100.0	100.0	-

* Activity at 30° C was taken as 100%
Mean of 3 replicates.

DISCUSSION

The morphological characters observed indicate that the inhibitory bacterium is a *Bacillus* species. The ability to hydrolyse starch and gelatin, formation of acetyl methyl-carbinol and reduction of nitrate strongly suggest that the bacterium is an isolate of *Bacillus subtilis*.[7] The characteristic deep purple colour was due to an intra-cellular pigment. The colour was always absent in the cell free culture filtrates.

The characters of *B. subtilis* isolates as given in the Bergeys' manual[8] show that the formation of pigments is not a common occurrence. Brown, red, orange and black are the pigment colours reported. It does not mention a deep purple pigmentation. Hence, the present isolate has a very characteristic pigment.

The inhibitory action was due to 3 compounds secreted by the bacterium. The ninhydrin test indicates that the 3 compounds do not have any amino acid or protein residues. The inhibitory compounds of *B. subtilis* reported by Loeffler *et al.*[2] are all different forms of peptides. Therefore, the inhibitory compounds of this isolate of *B. subtilis* are different from those reported.

The activity of the inhibitory compounds detected were persistent and they show a high degree of temperature tolerance - they retain most of their activity even after exposure to 60° C. They are, therefore, well suited to Sri Lankan environmental conditions. Hence, the bacterium or the individual compounds have the potential to be developed as useful bio-control agents against the sheath blight fungus.

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References

1. Wickremasingha D.L. & Mithrasena Y.J.P.K. (1989). Sheath blight disease of rice in the low country wet zone of Sri Lanka; Effect on yield and factors influencing spread and distribution. *Tropical Agriculture Research Series* **22:** 38-48.

2. Loeffler W., Tschen J.S.M., Vanittanakom N., Kugler M., Knorpp P., Hsiech T.G. & Wu T.G. (1986). Anti-fungal effects of Bacilysin and Fengymycin from *Bacillus subtilis* F-29-3. A comparison with other *Bacillus* antibiotics. *Journal of Phytopathology* **115:** 204-213.

3. Mckeen C.D., Rielley C.C. & Pusey L.L. (1986). Production and partial characterization of antifungal substances antagonistic to *Monilinia fructigena* from *Bacillus subtilis*. *Phytopathology* **76**: 136-139.

4. Roy K., Mukhopadhyay T., Reddy G.C.S., Desikan K.R., Rupp R.H. & Ganguli B.N. (1988). Aranorosin, a novel antibiotic from *Pseudoarachniotus roseus*. *Journal of Antibiotics* **41**: 1780-1784.

5. Smith D.A . (1982). Toxicity of Phytoalexins. In *Phytoalexins* (Eds. J.A. Bailey & J.W. Mansfield). pp. 218-252. Blackie, London.

6. Kiraly Z., Klement Z., Solymosy F. & Voros J. (1974). *Methods in Plant Pathology pp. 215-225. Elsevier Scientific Publishing Co., London.*

7. Burrows W. (1973). *Text Book of Microbiology*. pp. 607-615. Saunders, Philadelphia.

8. Bergeys' Manual of Systematic Bacteriology Vol 2. (1986). List of species of the genus *Bacillus*. pp. 1127- 1130. Williams and Wilkins, Baltimore.