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Production of Bacteriocins by Root Nodule Bacteria

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Abstract: Twenty *Rhizobium* species were isolated from root nodules of 20 legume hosts and studied for their ability to produce bacteriocins. Among the 20 species, the *Rhizobium* sp. isolated from root nodules of *Crotalaria alata*, *C. juncea* and *C. laburnifolia* produced bacteriocins after 72 h of growth. It was observed that *Rhizobium* sp. from *C. alata* produced small type and highly effective bacteriocin against *Rhizobium* sp. from *Arachis hypogaea*, *Cajanus cajan*, *Clitorea ternatea*, *Dolichos lablab*, *Indigofera linnaei* and *Vigna mungo*. The bacteriocin was isolated and partially purified by chloroform extraction and ammonium sulphate fractionation. The bacteriocin was sensitive to protease and insensitive to DNase and RNase. It was stable up to temperature 80°C for 15 min. The bacteriocin fraction consistently migrated as a 29 kDa polypeptide on SDS-PAGE.

Key words: Bacteriocin production, root-nodule bacteria, SDS-PAGE

INTRODUCTION

The *Rhizobium*-legume symbiosis is the most promising plant bacterium association so far known. Inoculated *Rhizobium* sp. strains often fail to compete with indigenous rhizobia and do not increase nodulation (Hafeez *et al*., 2005). Thus the successful use of rhizobial inoculants requires the knowledge of factors affecting the effectiveness and competitive ability of the rhizobia. One of the major factors reported to be affecting competition among rhizobia are bacteriocins (Oresnik *et al*., 1999).

Bacteriocins constitute a heterogenous group comprising protein complexes or peptides with antibiotic effect against closely related species and strains (Tagg *et al*., 1976). Root nodule bacteria have been shown to produce bacteriocins, which have been grouped as small, medium and large based on their size and diffusion characteristics. The small bacteriocins are heat tolerant, chloroform soluble and are less than 2000 kDa molecular weight, medium is generally bactericidal, heat labile and retained by cellophane and large are defective bacteriophages (Hirsch, 1979).

Oresnik *et al*. (1999) found that the bacteriocins appear to play a major role in determining competitiveness for nodulation when assayed against some strains. So, the successful preparation of mixed inoculum requires the knowledge of bacteriocin producing ability of inoculating strains as well as their effect on related rhizobia. The present research was taken up to study the bacteriocin producing ability of 20 *Rhizobium* spp. isolated from root nodules of 20 legume hosts.

MATERIALS AND METHODS

Microorganism, Medium and Growth Conditions

Twenty *Rhizobium* spp. were isolated from root nodules of 20 different legume hosts (*Arachis hypogaea*, *Cajanus cajan*, *Cassia absus*, *Clitorea ternatea*, *Cowpea*, *Crotalaria alata*,

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C. juncea, *C. laburnifolia*, *C. retusa*, *C. verrucosa*, *Indigofera hirsuta*, *I. linnaei*, *I. tinctorea*, *I. trita*, *Macrotyloma uniflorum*, *Sesbania procumbens*, *S. rostrata*, *Vigna mungo*, *V. radiata* and *V. trilobata*) using Yeast Extract Mannitol Agar (YEMA) medium. The isolated bacteria were identified as *Rhizobium* sp. by Bergey's Manual of Determinative Bacteriology (Jordan, 1984) and plant infection test (Vincent, 1970).

Bacteriocin Activity Assay

The bacteriocin producing ability of the strains was bioassayed by simultaneous (direct) antagonism method (Tagg *et al*., 1976). Bacteriocin activity was examined by adding 1 mL of each diluted, sterile filtered sample on to the wells made on Tryptone Yeast extract (TY) medium (0.6% w/v agar) seeded with log phase indicator strains (0.5 µL/100 mL of the medium). Activity was quantified by two fold serial dilution (Barefoot and Klaenhammer, 1983) and is expressed in arbitrary units mL^{-1} (AU mL^{-1}).

Purification and Estimation of Bacteriocin Protein

Purification of bacteriocin was carried out by using procedure of Yang *et al*. (1992) and Cell Free Supernatant (CFS) was used to carry out protein extractions. Twenty percent chloroform was added to CFS in a separatory funnel. The aqueous phase was saturated with cold ammonium sulphate from 20-100% (w/v) saturation and was kept overnight at 4°C. The precipitate was collected by centrifugation at 15,000 g for 30 min. The solid pellet dissolved in distilled water and dialyzed against distilled water at room temperature for 24 h. The suspension obtained was designated as proteinaceous fraction or crude bacteriocin fraction. All the different dialyzed material of 0.01 g was added in 100 µL Tris HCl (pH 6.5) buffer and tested for inhibitory activity. The quantity of protein concentration was done by the Bradford method (Thimmaiah, 1999). Bovine Serum Albumin (BSA) was used to construct the standard curve.

Bacteriocin Properties

The concentrated bacteriocin was treated with protease, DNase and RNase with a final concentration of 5, 10, 15, 20 and 25 µg mL^{-1} in 10 mM Tris HCl, pH 7.0 for 4 h at 37°C and residual activity was determined. Sensitivity to different temperatures (40, 50, 60, 70, 80 and 90°C) was determined by incubating up to 30 min. After incubation, the samples were cooled and residual activity was determined (Nirmala *et al*., 2001).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of 10% Sodium Dodecyl Sulphate (SDS) was performed (Laemmli, 1970). Electrophoresis was conducted at a constant current of 30 mA for 12 h at 30°C. The gel was stained with coomassie blue. A 10 kDa protein ladder was used as protein standard.

RESULTS AND DISCUSSION

The 20 *Rhizobium* spp. isolated from 20 legume hosts were fast growers (colony diameter greater than 2.0 mm) and produced acid in YEM broth. Among the twenty spp., three species from *C. alata*, *C. juncea* and *C. laburnifolia* produced bacteriocins. *Rhizobium* sp. from *C. alata* produced bacteriocins against *Rhizobium* spp. from *A. hypogaea*, *C. ternatea*, *D. lablab*, *I. linnaei* and *V. mungo*. The *Rhizobium* sp. from *C. juncea* produced bacteriocins against *Rhizobium* spp. from *V. mungo* while the *Rhizobium* sp. from *C. laburnifolia* produced bacteriocins against *Rhizobium* spp. from *D. lablab* and *I. linnaei*. The *Rhizobium* sp. isolated from *C. alata* showed highest activity than other species of

Table 1: Activity spectrum of bacteriocin producing *Rhizobium* species

Indicator strains (*Rhizobium* sp.)	Producer strains (*Rhizobium* sp.)					
	C. alata		*C. juncea*		*C. laburnifolia*	
	I.Z*	Potency	I.Z*	Potency	I.Z*	Potency
A. hypogaea	4.5	+	-	-	-	-
C. cajan	7.2	++	-	-	-	-
C. ternatea	4.9	+	-	-	-	-
D. lablab	6.2	++	-	-	4.9	+
I. linnaei	8.6	++	4.2	+	4.0	+
V. mungo	13.9	+++	4.0	+	-	-

- : Ineffective; +: Less effective; ++: Moderately effective; +++: Highly effective. All the results are means of triplicates; *I.Z: Diameter of inhibition zone

Table 2: Purification of isolated bacteriocin protein from *Rhizobium* sp. from *C. alata*

Sample material	Potency	Diameter of inhibition zone (mm)	Inhibition area (mm^2)	Arbitrary units ($AU\ mL^{-1}$)	Protein concentration ($mg\ mL^{-1}$)
Chloroform extract	+++	13.9	151.6	415	5.95
Ammonium sulphate concentration (%)					
20	++	8.1	51.5	194	8.20
30	+	5.0	19.6	105	6.40
40	+	5.5	23.7	109	7.20
50	++	8.9	62.1	110	6.80
75	+++	15.6	191.8	431	10.60
80	++	6.2	30.1	196	7.00
85	-	-	-	-	-

- : Ineffective; +: Less effective; ++: Moderately effective; +++: Highly effective. All the results are means of triplicates

Rhizobium and it also showed the broad spectrum of activity (Table 1). Thus the activity spectrum varied from strain to strain was reported earlier in *Rhizobium legumunosarum* bv. *viciae* (Hafeez *et al*., 2005). An auto-antagonism relationship was not observed; no test strains inhibited its own growth, which is characteristic of bacteriocin producers (Hardy, 1975; Nirmala and Gaur, 2000). As marked bacteriocin production was observed in *Rhizobium* sp. from *C. alata*, further studies were carried out for this strain.

The bacteriocin production was appeared after 48 h of incubation and reached maximum after 96 h of incubation. Further incubation does not affect the zone size, therefore 96 h of growth of the producer strains at 30°C was considered as optimum conditions for bacteriocin production in this study. That the production of bacteriocin is closely related with bacterial growth of producing organism and bacteriocin activity decreases more or less sharply at the end of the growth phase as a result of degradation by proteases was reported earlier in *Micrococcus* sp. (Kim *et al*., 2006).

Present results showed that when the sample is successively diluted, inhibition zone decreased until critical dilution was achieved, where no inhibition of the sensitive organism was observed. When the purified bacteriocin was tested against indicator strains, it showed highest activity at 75% ammonium sulphate saturated pellet. The activity does not depend on the quantity of the protein produced (Table 2).

When the concentrated bacteriocin was treated with protease, DNase and RNase, it was observed that it is sensitive to protease at the concentration of 25 $\mu g\ mL^{-1}$ indicating its proteinaceous nature. The activity gradually decreases, when the concentration of protease increased (Fig. 1). That the bacteriocins from *Cicer-Rhizobium* were also sensitive to proteases reported earlier (Nirmala *et al*., 2001). The bacteriocin was insensitive to DNase and RNase indicating it is not a nucleoprotein. This bacteriocin is like those of *R. leguminosarum* bv. *trifolii*, which is insensitive to DNase and RNase (Schwinghamer, 1975).

Temperature also affects the activity of bacteriocins. Optimum temperature range for bacteriocin activity was found to be 30-70°C and above 70°C, activity decreases slowly (Fig. 2). The activity

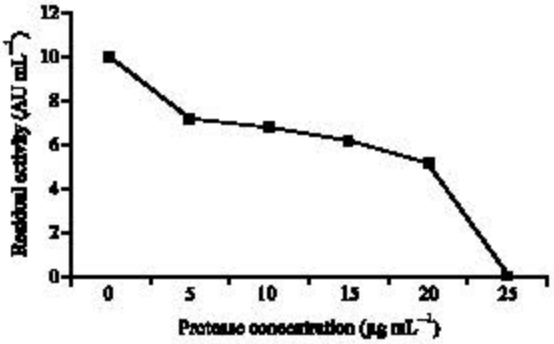

Fig. 1: Effect of protease concentration on bacteriocin activity

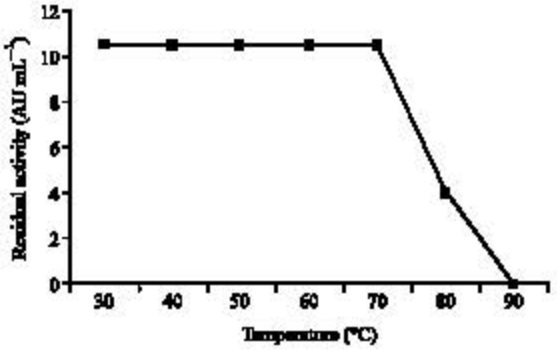

Fig. 2: Effect of temperature on bacteriocin activity

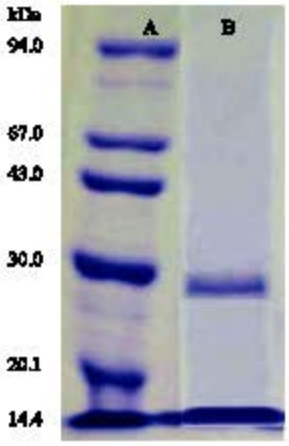

Fig. 3: SDS-PAGE of purified bacteriocin of *Rhizobium* sp. from *C. alata*, Lanes; (A) Molecular weight markers; (B) Purified bacteriocin of 29 kDa

remained at 80°C for 15 min and at 90°C, the activity diminished. That the bacteriocin from *Cicer Rhizobium* was found to be heat stable even after 5 min at 80°C was reported earlier (Nirmala *et al*., 2001).

SDS-PAGE analysis of protein isolated from the *Rhizobium* sp. showed the presence of 29 kDa protein band (Fig. 3). This band was visible only in samples isolated from 30-75% ammonium sulphate

saturation of CFS. The molecular mass showed that this bacteriocin to be much smaller than those reported in *R. leguminosarum* bv. *trifolii* (Joseph *et al*., 1983).

CONCLUSION

The results of this study have shown that bacteriocin production may play an important role in interspecific competition. In this study the broad spectrum activity of *Rhizobium* sp. from *C. alata* may help in the improvement of legume inoculants.

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