Original Article

Bacteriocin production by rhizobia isolated from root nodules of Horse gram

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Abstract

Thirty two Rhizobium strains were isolated from the root nodules of Horse gram plants grown in 32 soil samples collected from various regions in Andhra Pradesh, India. They were identified as rhizobia by nodulation test. These isolates were used to study the production of bacteriocins. All the isolates produced bacteriocins against the remaining isolates. The isolates HGR-4 and 9 showed maximum activity/inhibition zone among all the isolates, and also found to be inhibiting more number of isolates than that of other bacteriocin producing isolates. The bacteriocin protein substance produced by the isolates HGR-4 and 9 showed a molecular weight of ~ 50 kDa by SDS-AGE and bacteriocin plasmids molecular weight is > 22 kb and > 24 kb.

Key words: bacteriocin, rhizobia, competition.

Introduction

The Rhizobium legume symbiosis is the most promising plant bacterium association so far known. Inoculated Rhizobium spp. strains often fail to compete with the indigenous soil rhizobia and do not increase nodulation [1,2]. 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 [3,4,5]. Bacteriocins are proteins or protein complexes with bacteriocidal activity directed against species that are usually closely related to producer bacterium [6]. Bacteriocins are ribosomally encoded peptide antibodies. Both Gram negative and Gram positive bacteria produce them. The Gram negative bacteriocins are well studied. Rhizobium leguminosarum strains have been shown to produce bacteriocins which have been characterized small, medium or large based on their assumed size characteristics [7,8]. Small bacteriocins were found to be chloroform soluble and have molecular mass less than 2000 Da [7,9]. Oresnik et al.[5] found that the bacteriocins appear to play a major role in determining competitiveness for nodulation when assayed against some strains.

Genetic evidence suggests that some of the genes involved in Rhizobium-legume symbiosis may be located on plasmids [10,11,12]. Many bacteriocins of enterobacteria are plasmid determined 13 and this may be a feature common to other bacterial genera. Bacteriocin production by Rhizobium spp. has been described [4,14,15,16] but there are no data to suggest that it may be plasmid determined.

So the successful preparationof mixed inoculum requires the knowledge of bacteriocin producing ability of the inocula strains as well as their effect on the related rhizobia. In the present study 32 rhizobia isolated from various regions in Andhra Pradesh, India has been investigated for their bacteriocin production.

Materials and Methods

Isolation of root nodulating bacteria from horse gram Root nodules were collected from horse gram plants grown in thirty two soil samples collected from various regions in Andhra Pradesh, India. Rhizobia were isolated from fresh nodules by the standard method on Yeast Extract Mannitol Agar (YEMA) medium [17]. They were designated as HGR-

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1 (Horse Gram Rhizobia) to HGR-32. Root nodulating ability of these isolates was determined by nodulation test [18]. Morphological, cultural and biochemical activities of the isolates were studied using standard methods. Four strains were selected for 16S rRNA sequence and were submitted to the NCBI GenBank under the accession numbers GQ483457, GQ483458, GQ483459 and GQ483460.

Bacteriocin Activity Assay

The bacteriocin producing ability of the strains was bioassayed by simultaneous antagonism method 6. 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 of the medium). Activity was quantified by two fold serial dilution [19] and is expressed in arbitrary units mL-1 (AU mL-1).

Bacteriocin protein purification

Purification of proteins was carried out using the procedure of Yang et al. [20]. Cell Free Supernatant (CFS) was used to carry out protein extractions. Twenty percent chloroform was added to the CFS in a separatory funnel. The aqueous phase formed was separated and used for precipitating out the proteins. Protein precipitation was carried out on ice or at 4oC by the addition of analytical grade ammonium sulphate. The aqueous phase was saturated with cold ammonium sulphate from 20 to 80% (w/v) saturations and gradually stirred with a glass stirrer for 10-15 min. The aqueous phase was kept overnight at 4oC.

The precipitate was collected by centrifugation at 15000xg for 20 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 dialysates obtained were lyophilized. Lyophilized material of 0.01 g was added in 100 ml Tris-HCl (pH 6.5) buffer and tested for inhibitory activity. The quantification of protein concentration was done by standard Bradford method [21]. Bovine serum albumin (BSA) was used to construct the standard curve.

SDS-PAGE

Analysis of total cellular proteins of rhizobia was carried out by SDS-PAGE described by Laemmli [22] with slight modifications. 3 ml of YEM broth was inoculated and incubated for 12 h at room temperature. It was centrifuged at 10000xg for 4 min at 4°C. To the pellet sample buffer was added at 1:1 ratio. The samples were lysed by heating at 95°C for 2 min and immediately transferred to the cold water. Lysed samples and marker protein samples were loaded into the different gel slots. Electrophoresis was carried out at 100 V for 4 h at RT. The gel was stained with Coomassie blue (Sigma). The image was photographed using Gel documentation unit (Alpha Innotech, USA).

Plasmid analysis

An overnight culture of rhizobia in YEM broth was centrifuged at 14000xg, for 4 min at 4°C and the plasmid analysis was carried out according to Sambrook and Russell [23].

Results and Discussion

The cells are Gram negative rods, colonies are small, white on YEMA medium at room temperature. The optimum pH was in the range of 7-7.5. These strains grow at a temperature between 10 to 40oC. Lower concentrations of NaCl favoured growth of these rhizobia. All the isolates were resistant to ampicillin and rifampicin. The isolates HGR-[11, 22] and [23] showed resistance to most of the antibiotics tested, where as the isolate HGR-4 showed susceptibility towards most of the antibiotics.

The production of bacteriocins is an advantageous character for strain competition [24]. Bacteriocin production was indicated by the formation of clear zones around the colonies. All the thirty two isolates produced clear zones on YEMA medium (0.6% w/v) inoculated with the isolates from the same host. No test strains inhibited its own growth, which is characteristic of bacteriocin producers [13,25].

The bacteriocin production started after 48 h and reached maximum after 72 h of incubation. Further incubation does not affect the zone size, therefore, 72 h of growth of the producer colonies at 30oC 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

Table.1. Purification of isolated bacteriocin protein from HGR 4 and HGR-9

Sample material	**Potency**		**Diameter of inhibition zone (mm)**		**Arbitrary Units (AU mL^{-1})**		**Protein concentration (mg mL^{-1})**	
	HGR-4	HGR-9	HGR-4	HGR-9	HGR-4	HGR-9	HGR-4	HGR-9
Chloroform extract	+++	+++	22.0	15.0	190	160	6.0	7.25
Ammonium sulphate concentration (%)								
20	++	++	18.0	15.2	100	98	7.60	8.10
30	++	++	22.6	18.6	104	106	8.85	8.00
40	++	++	24.0	20.2	110	109	9.00	8.65
50	+++	+++	33.0	32.0	240	220	10.25	9.25
60	++	++	28.0	25.2	114	112	8.50	7.20
70	++	++	28.0	14.0	86	82	7.00	5.00
80	--	--	--	--	--	--	--	--

-: Ineffective, +: less effective, ++: Moderately effective, +++: Highly effective. All the results are means of triplicates.

growth phase as a result of degradation by proteases was reported earlier in Micrococcus sp. [26.] All the strains of Rhizobium were found to produce antimicrobial activity, which inhibited the growth of the related strains on the agar medium.

In the present study maximum activity/inhibition zone was observed with the isolate HGR-9 and the isolate HGR-4 was also found to be inhibiting more number of isolates than that of other bacteriocin producing isolates, further studies were carried out with these two strains (Table. I). Thus the activity spectrum varied from strain to strain was reported earlier in Rhizobium leguminosarum bv. viciae. This is consistent with the earlier report that the strains of Rhizobium leguminosarum bv. viciae differ in their activity spectrum 27 .

Present results showed that when the sample is successfully 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 50% ammonium sulphate saturated pellet. The activity does not depend on the quantity of the protein produced.

SDS-PAGE analysis of protein isolated from the strain HGR-4 and 9 showed the presence of ~ 50 kDa protein band (Fig. I). Plasmid analysis of this strains showed that it contains two plasmids similar to > 22 kb and 24 kb (Fig. II).

Thus, these studies indicate that bacteriocin production is strain specific character. It is clear that bacteriocin production may play an important role in interspecific competition. These strains may help in the improvement of legume inoculants.

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