Research Article

Intrinsic Antibiotic Resistance (IAR) of Different Rhizobial Strains Isolated From Root Nodules of Clusterbean (*Cyamopsis tetragonoloba* (L.) Taub.)

Subha Dhull*, Kuldeep Singh and Rajesh Gera

Department of Microbiology, CCS Haryana Agricultural University, Hisar-125004, India

Abstract

The present study was carried out at Department of Microbiology, CCS HAU, Hisar with the objective to identify intrinsic antibiotic resistance in rhizobia isolated from root nodules of Clusterbean (*Cyamopsis tetragonoloba* (L.) Taub.). A total of fourteen rhizobia isolated from clusterbean growing in Bhiwani, Hisar and Mahendergarh districts of Haryana were used to investigate their intrinsic resistance to different concentrations of eight antibiotics *viz*., Ampicillin, chloramphenicol, gentamicin, kanamycin, neomycin, nalidixic acid, tetracycline and streptomycin. The results of this study revealed that most of rhizobial strains tested on yeast extract mannitol agar (YEMA) medium having antibiotics had good growth on ampicillin, chloramphenicol, nalidixic acid and streptomycin upto concentrations of 100 µg ml^{-1} while using gentamicin showed medium growth upto concentrations of 20 µg ml^{-1}. None of rhizobial isolates showed growth on YEMA medium having antibiotics kanamycin, neomycin and tetracycline even at concentration of 10 µg ml^{-1}. Rhizobial isolate GB-5c showed growth on the entire antibiotic tested except kanamycin and neomycin.

Keywords: *Rhizobium*, Antibiotics, Clusterbean, YEMA medium, Intrinsic antibiotic resistance

***Correspondence**

Author: Subha Dhull
Email: subhachoudhary45@gmail.com

Introduction

Use of high attributes rhizobial inoculants on agricultural legumes has share significantly to the N market of agriculture system due to profit from biological nitrogen fixation (BNF). Significant community of symbiotically efficient rhizobia ought to be existing in the rhizosphere for symbiotic BNF with host plants. The act of *Rhizobium* inoculation in farmland and its effectiveness are decisive because of contest taking place among the introduced rhizobia and the native rhizobia existing at elevated densities. Contest for nodulation is commonly calculated by measuring the capability of introduced *Rhizobium* isolates to form large digit of nodules on the selected host. In spite of this, the rhizosphere constitutes huge populations of diverse microorganisms. A number of these microorganisms typically generate antibiotics which are fatal to sensitive rhizobial populations in the soil [1]. Resistance to antibiotics increases the *Rhizobium* probability of continued existence in the rhizosphere. Antibiotic resistance has been regularly applied in differentiating the applied inoculant strain from native rhizobia and observes their continuity and habitation of legume nodules [2, 3]. Rhizobial strains ought to resistant to concentrations of antibiotics that obstruct the development of other soil bacteria and they must be adept to keep their affectivity and symbiotic efficacy. The resistance may be grown up for single or manifold antibiotic groups [4]. Although bulks of rhizobial isolate in the soil are sensitive to antibiotics, remains have advanced resistance in reaction to commonly produced antibiotics [5, 6, 7]. Hence, intrinsic resistance to antibiotics is an advantageous feature for the rhizobial population. It boosts the rhizobial probability of development, reproduction and determination in the soil. The logic for making of antibiotics by a few soil habiting microbes is not yet understandable. Several informations has implied a lot of hypotheses for the construction of antibiotics. The antibiotics lyse living cells of sensitive bacterial group, thus given that food for the organism [8]. Different group of antibiotics show diverse modes of response on the bacteria like interference with cell wall synthesis, inhibition of protein, nucleic acid and metabolic pathway synthesis, disruption of bacterial membrane structure. Benefits of this process of detection enclosed its loyalty in that resistance to low levels of antibiotics came to be a durable assets of the *Rhizobium* strains investigated, as outcome were reproducible over lengthy stage of period. In inclusion, this process needed no cellular variations, which may occur through mutant selection or mutagenesis and may interfere with strain performance. The resistance of rhizobial strains to antibiotics is being broadly applied in study of the stability in soil or other environment and their competitiveness as a marker for nodulation of the host plant and success of nitrogen fixation [9, 10, 11, 12, 13].

Clusterbean is an important kharif legume of arid and semi-arid regions of India. It is primarily grown for seed, green fodder, vegetable and green manuring. Like other legumes, clusterbean is nodulated by *Rhizobium* or

Bradyrhizobium strains belonging to cowpea miscellany group. The productivity of clusterbean is low [14] and the inoculation through effective rhizobial isolates does not essentially result in crop development. Cause for inoculation failing is the little symbiotic specificity among clusterbean and rhizobia in adverse conditions such as drought, high temperature and also competition with the indigenous soil bacteria. This examination shows the necessity for advance information of biological criterion, for example the dynamics of native rhizobial populations, continuity and competitiveness. For identification of strains high concentration of antibiotic resistance markers have been used [15]. It has advantage of ease of isolation and recognition of inoculum strains from nodules and also from the soil. Antibiotic resistance pattern has traditionally been used as a marker in competition studies because this method is simple and requires no specialized equipment [16]. Therefore, aim of this study was to characterize 14 strains of rhizobia isolated from root nodules of clusterbean plant based on their intrinsic resistance to different concentrations of eight antibiotics.

Materials and Methods

Isolation of root nodule bacteria (Rhizobia)

Evaluation of root nodule bacteria (rhizobia) for intrinsic antibiotic resistance

Intrinsic antibiotic resistance of different rhizobia was evaluated according to Eaglesham's technique on YEMA medium plates supplemented with different concentrations of antibiotics [18]. Stocks of different antibiotics (ampicillin, chloramphenicol, gentamicin, kanamycin, neomycin, nalidixic acid, tetracycline and streptomycin) were prepared by dissolving them in appropriate sterilized water or acid/alkaline solution. These stocks were diluted as per the requirement of different concentration of each antibiotic and were further filter sterilized. Filter sterilized aliquots of each antibiotic were added aseptically to YEMA medium having the temperature 45-50^{0}C. Five µl of log phase grown cells of different rhizobia was spotted on YEMA medium plates supplemented with different antibiotics. YEMA plates without antibiotic (control) and with antibiotics were incubated at 28±2^{o}C for 7 days and growth of each rhizobial isolates on different antibiotic supplemented plates was compared with growth on plates without antibiotic. The maximum concentration where colonies diameter was equivalent to growth on control plate was recorded as resistance level of different rhizobia.

Results and Discussion

A total of fourteen rhizobia were isolated from root nodules of clusterbean (*Cyamopsis tetragonoloba* (L.) Taub.) (Table 1). Among the 14 rhizobial strains, nine rhizobia from Bhiwani, one from Hisar and four rhizobial strains from Mahendergarh district. These regions have a usual temperature vary from l0°C (in winters) to 47°C (in summers). All the rhizobia were Gram negative, rod shaped and motile. Similarly Singh *et al.* [19] also isolated forty-nine isolates from root nodules of Pigeon pea [*Cajanus cajan* (L.) Millspaugh] growing in Haryana.

Table 1 Nomenclature of rhizobial isolates and description of samples collection sites

S. No.	Rhizobia isolates	Village	Districts
1	GB-1a	Bhagi	Bhiwani
2	GB-1b	Bhagi	Bhiwani
3	GB-3a	Neemali	Bhiwani
4	GB-5c	Sanspur	Bhiwani
5	GB-7a	Achina	Bhiwani
6	GB-10b	Achina	Bhiwani
7	GB-10c	Achina	Bhiwani
8	GB-14a	Morwala	Bhiwani
9	GB-16a	Bilawal	Bhiwani
10	GH-6d	Kirtan	Hisar
11	GM-10a	Sighara	Mahendergarh
12	GM-11b	Paldi	Mahendergarh
13	GM-11c	Paldi	Mahendergarh
14	GM-13a	Basai	Mahendergarh

In present study, the development of rhizobial strains tested to various antibiotic concentrations was visibly decided depend on improvement on colony diameter. The outcome of our study (Table 2) revealed that majority of the rhizobial strains had fine expansion on YEMA medium augmented by ampicillin, chloramphenicol, nalidixic acid and streptomycin upto concentrations of 100 µg ml^{-1}. Especially elevated intrinsic resistance was found in strains GB-1a, GB-5c, GB-10c, GB-14a, GM-11b and GM-13a. The moderate level of intrinsic resistance of largely strains certfied was to the gentamycin in concentrations up to 20 µg ml^{-1}. Only one rhizobial strain GB-5c showed growth on tetracycline up to concentration 50 µg ml^{-1} but growth was poor. None of the rhizobial isolates showed growth on YEMA medium supplemented with antibiotics *viz*., kanamycin and neomycin even at concentration of 10 µg ml^{-1} (Fig.1 and Table 2). The results of this study were in conformity with the results of earlier researchers [20] because they found a different antibiotic resistance pattern in *Rhizobium*, *Bradyrhizobium* and *Rhizobium radiobacter*. Raza *et al.* [21] observed that rhizobial strains were inherently susceptible to clindamycin (2 µg ml^{-1}) and alienated into four sets on the foundation of their intrinsic resistance pattern. Set A, counting ARC 400 and ARC 402, exhibit resistance to all eleven antibiotics whereas set C, constitute two blue lupin (ARC 413 and ARC 414) strains and one Egyptian white lupin (ARC 404) strains were inherently susceptible to entirely antibiotics studied. Young and Chao [22] described that both the quickly and leisurely developing isolates of rhizobia display extensive fluctuation in resistance to antibiotics. Intrinsic antibiotic resistance property have been applied to recognize isolates of *Rhizobium leguminosarum* bv. *viceae* [23].

Table 2 Characterization of rhizobia for intrinsic antibiotic resistance pattern

Sr. No.	Rhizobial isolates	Ampicillin			Chloramphenicol			Gentamycin		Kanamycin	
		20	50	100	20	50	100	20	50	10	20
1	GB-1a	+++	+++	++	+++	+++	+	++	+	-	-
2	GB-1b	+++	+++	+++	+++	+++	+++	++	-	-	-
3	GB-3a	+++	+++	+	+++	+++	++	+++	-	-	-
4	GB-5c	+++	+++	+++	+++	+++	+++	+++	+++	-	-
5	GB-7a	+++	+++	+++	+++	+++	+	+	-	-	-
6	GB-10b	+++	+++	+++	+++	+++	+	++	+	-	-
7	GB-10c	+++	+++	+++	+++	+++	+++	+++	++	-	-
8	GB-14a	+++	+++	+++	+++	+++	+++	+++	+	-	-
9	GB-16a	+++	+++	+++	+++	+++	++	+++	-	-	-
10	GH-6d	+++	+++	++	+++	+++	++	+++	-	-	-
11	GM-10a	+++	+++	+++	+++	+++	+++	++	-	-	-
12	GM-11b	+++	+++	+++	+++	+++	+++	+++	+	-	-
13	GM-11c	+++	+++	+	+++	+++	+++	++	-	-	-
14	GM-13a	+++	+++	+++	+++	+++	++	+++	-	-	-

Sr. No.	Rhizobial isolates	Neomycin		Nalidixic acid			Tetracycline		Streptomycin		
		10	50	20	50	100	10	50	20	50	100
1	GB-1a	-	-	+++	+++	+++	-	-	+++	+++	+++
2	GB-1b	-	-	+++	+++	+++	-	-	+++	+++	++
3	GB-3a	-	-	+++	+++	+++	-	-	+++	+	+
4	GB-5c	-	-	+++	+++	+++	++	+	+++	++	+
5	GB-7a	-	-	+++	+++	+++	-	-	+++	++	-
6	GB-10b	-	-	+++	+++	+++	-	-	+++	++	+
7	GB-10c	-	-	+++	+++	+++	-	-	+++	++	++
8	GB-14a	-	-	+++	+++	+++	-	-	+++	+++	++
9	GB-16a	-	-	+++	+++	+++	+	-	+++	++	+
10	GH-6d	-	-	+++	+++	++	-	-	+++	+++	+
11	GM-10a	-	-	+++	+++	+++	-	-	+++	+++	+++
12	GM-11b	-	-	+++	+++	+++	-	-	+++	+++	++
13	GM-11c	-	-	+++	+++	+	-	-	+++	++	++
14	GM-13a	-	-	+++	+++	+++	-	-	+++	+++	++

(+++= good growth, ++= moderate growth, += poor growth and -= no growth)

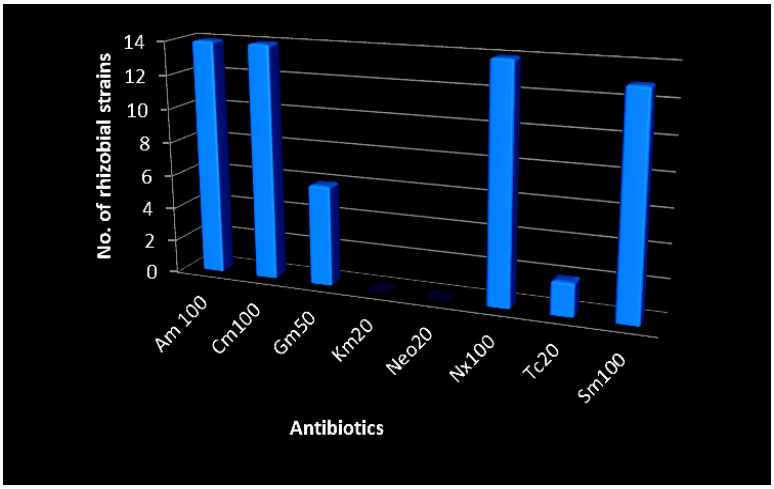

Figure 1 Intrinsic antibiotic resistance pattern of Clusterbean rhizobial strains

Similar type of antibiotic resistance pattern was also observed by Berrada *et al*. [24]. Hartman and Amarger [25] and Tas *et al*. [26] point out the intrinsic resistance to antibiotics of distinct rhizobial isolates associated with the similar group as a important phenotypic traits. Kaur and Sharma [27] tested ten rhizobacteria of *Pseudomonas* spp. isolated from rhizosphere of chickpea for their reactivity to antibiotics along with reference strain LK884. All the isolates showed resistance against ampicillin, whereas LK884 was sensitive to this antibiotic. Ansari *et al*. [28] reported that out of 20 leisurely developing bradyrhizobia, 15 were resistant to trimethoprim, 16 to nalidixic acid, 13 to polymyxin B, 14 to vancomycin and 9 to novobiocin. While out of 15 quick developing rhizobia, 10 were resistant to trimethoprim, 9 to nalidixic acid and 8 to novobiocin. This outcome point out that the leisurely developing were extra resistant to different antibiotics than quick growers. Different *Rhizobium* strains of fababean and soybean were found to be resistant to different antibiotics *viz.*, amoxicillin, ampicillin and cloxacilline while very few strains were found to be resistant to ampicillin and cloxacilline only [29].

Conclusion

It was observed that most rhizobial strains tested showed good growth on ampicillin, chloramphenicol, nalidixic acid and streptomycin at concentrations upto 100 µg ml^{-1}. An elevated amount of intrinsic resistance was found in strain GB-5c to most of the antibiotics studied except kanamycin and neomycin. It was concluded that different rhizobial strains have different antibiotic resistance pattern.

Acknowledgement

We thank the Department of Microbiology, CCS Haryana Agricultural University, Hisar, India for providing necessary facilities for this work.

References

[1] Naamala, J., Jaiswal, S.K. & Dakora, F.D. (2016) Antibiotics Resistance in *Rhizobium*: Type, Process, Mechanism and Benefit for Agriculture. Current Microbiology, 72, 804–816.

[2] Bromfield, E., Lewis, D. & Barran, L. (1985) Cryptic plasmid and rifampin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. Journal of Bacteriology, 164, 410–413.

[3] Simon, T. & Kalalova, S. (1996) Preparation of antibiotic resistant mutants of rhizobia and their use. Rostlinna Vyroba-UZPI (Czech Republic).

[4] Anand, A., Jaiswal, S.K., Dhar, B. & Vaishampayan, A. (2012) Surviving and thriving in terms of symbiotic performance of antibiotic and phage-resistant mutants of *Bradyrhizobium* of soybean [*Glycine max* (L.) Merrill]. Current Microbiology, 65, 390–397.

[5] Beringer, J. (1974) R factor transfer in *Rhizobium leguminosarum*. Journal of general microbiology, 84, 188–198.

[6] Wiener, P. (1996) Experimental studies on the ecological role of antibiotic production in bacteria. Ecology and Evolution, 10, 405–421.

[7] Xavier, G., Martins, L., Neves, M. & Rumjanek, N. (1998) Edaphic factors as determinants for the distribution of intrinsic antibiotic resistance in a cowpea rhizobia population. Biology and Fertility of Soils, 27, 386–392.
[8] Kumbhar, C. & Watve, M. (2013) Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. Natural Science, 5(4A), 26-40.
[9] Levin, R.A. and Montgomery, M.P. (1974) Symbiotic effectiveness of antibiotic resistant mutants of *Rhizobium japonicum*. Plant and Soil, 41, 669-674.
[10] Schwinghamer, E.A. (1979) Effectiveness of *Rhizobium* as modified by mutation for resistance to antibiotics. Antonie van Leeuwenhoek, 33, 121-136.
[11] Yosey, D.P., Beyonon, F.L., Jonson, A.W. and Beringer, F.E. (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. Journal of Applied Microbiology, 46, 343-350.
[12] Pugashetti, B.K. and Wagner, G.H. (1980) Survival and multiplication of *Rhizibium japonicum* strains in silt loam. Plant and Soil, 56, 217-227.
[13] El Hasan, G.A., Hernandez, G.S. & Focht, D. (1986) Comparasion of hup trait and intrinsic antibiotic resistance for assessing rhizobial competitiveness axenically and in soil. Applied and Environmental Microbiology, 22, 546-551.
[14] Lal, S. (2001). Seed production. In: Guar in India (Eds. Kumar, D. and Singh, N. B.). Scientific publishers, Jodhpur, India.
[15] Schwinghamer, A. & Dudman, W. F. (1973) Evaluation of spectinomycin resistance as a marker for ecological studies with *Rhizobium* spp. Journal of Applied Bacteriology, 36, 263-272.
[16] Ramirez, M.E., Israel, D.W. & Wollum, A.G. (1998) Using spontaneous antibiotic-resistant mutants to assess competitiveness of bradyrhizobial inoculants for nodulation of soyabean. Canadian Journal of Microbiology, 44, 753-758.
[17] Vincent, J.M. (1974) Root-nodule symbiosis with *Rhizobium*. In D. Quispel (eds.), The biology of nitrogen fixation. Elsevier/North-Holland Publishing Co., Amsterdam, pp. 265-341.
[18] Hashem, F.D., Swelim, D.M., Kuykendell, L.D., Mohamed, A.I., Abdel-Wahab, S.M. & Hegazi, N.I. (1998) Identification and characterization of salt tolerant Leuceana- nodulation *Rhizobium* strains. Biology and Fertility of Soils, 27, 35-341.
[19] Singh K., Rani, A., Padder S.A., and Gera R. (2017). Plant Growth Promoting (PGP) Attributes of stress tolerant Rhizobial isolates from root nodules of Pigeon pea [*Cajanus cajan* (L.) Millspaugh] growing in Haryana, India. International Journal of Current Microbiology and Applied Sciences, 6(12), 461-473.
[20] Ansari, P.G. & Rao, D.L.N. (2014) Differentiating indigenous Soybean *Bradyrhizobium* and *Rhizobium* spp. of Indian Soils. Indian Journal of Microbiology, 54, 190-195.
[21] Raza, S., Jornsgard, B., Abou-Taleb, H. & Christiansen, J.L. (2001) Tolerance of *Bradyrhizobium* sp. (Lupin) strains to salinity, pH, $CaCO_3$ and antibiotics. Letters in Appled Microbiology, 32, 379-383.
[22] Young, C.C. & Chao, C.C. (1989) Intrinsic antibiotic resistance and competition in fast and slow-growing soybean rhizobia on a hybrid of Asian and US cultivars. Biology Fertile Soils, 8, 66–70.
[23] Josey, D.P., Beynon, J.L., Johnson, A.W.B. & Beringer, J.E. (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. Journal of Applied Bacteriology, 46, 343–350.
[24] Berrada, H., Nouioui, I., Houssaini, M.I., Ghachtouli, E.N., Gtari, M. and Benbrahim, K.F. (2012) Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of multiple legume species native of Fez, Morocco. African Journal of Microbiology Research, 6, 5314-5324.
[25] Hartman, A. & Amarger, N (1991) Genotipic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles DNA fingerprinting and insertion sequence typing. Canadian Journal of Microbiology, 37, 600-608.
[26] Tas, E., Leinonen, P., Saano, A., Rasanen, I.A., Kaijalainen, S., Piipola, S.,Hakola, S. and Lindstrom, K (1996) Assessment of competitiveness of Rhizobia infecting *Galega orientalis* on the basis of plant yield, nodulation and strain identification by antibiotics resistance and PCR. Environmental Microbiology, 62, 529-535.
[27] Kaur, N. & Sharma, P. (2013) Screening and characterization of native *Pseudomonas* spp. as plant growth promoting rhizobacteria in chick pea (*Cicer arietinum* L.) rhizosphere. African Journal of Microbiology Research, 7(16), 1465-1474.
[28] Ansari, P.G., Rao, D.L.N. & Pal, K.K. (2014) Diversity and phylogeny of soybean rhizobia in central India. Annals of Microbiology, 64, 1553-1565.

[29] Abera, T., Semu, E., Debele, T., Wegary, D. & Kim, H. (2015) Determination of soil *Rhizobium* populations, intrinsic antibiotic resistance, nodulation and seed yield of faba bean and soybean in western Ethiopia . World Journal of Agricultural Sciences, 11 (5), 311-324.

Publication History
Received 10th May 2018
Revised 30th May 2018
Accepted 10th June 2018
Online 30th June 2018