Detection of bacteriocinogenic strains of *Cicer–Rhizobium* by modified simultaneous antagonism method

Many strains of bacteria, including rhizobia, are known to produce bacteriocins. Of the various methods described for detecting freely produced bacteriocins[1], the most commonly used methods are those developed by Gratia and Fredricq. In all these methods, the antagonism is performed on solid media and involves detection of inhibition of growth of an indicator (sensitive) strain by a test (producer) strain. These methods are based either on simultaneous (direct) or deferred antagonistic activity[2]. Excepting few studies[3], mostly the deferred antagonism procedures have been employed for studying bacteriocinogeny among strains of rhizobia[4,5]. There are two procedures of studying deferred antagonism. One is to grow both the producer and indicator strains on the same surface of a gel, where the growth of the producer strain is removed and the surface is sterilized by fumigation with chloroform, before cross-streaking the indicator strains. The other procedure is based on the fact that diffusion of bacteriocin is tridimensional[6]. The producer strain is strip-inoculated at the centre of the surface of agar medium. After the full growth, the gel is inverted onto the lid of the petri dish and the indicator strain is cross-streaked over the sterile reverse surface of the gel. In this laboratory, while working with *Cajanus*-rhizobia, chloroform was found to inactivate the bacteriocinogenic property of the cultures, while the method of Kekessy and Piguet[6] was successfully used to screen out bacteriocin-producing strains[7–9]. We further successfully identified bacteriocin-producing strains of *Cicer*-rhizobia using this deferred antagonism procedure on a medium containing D-xylose. However, the method was found highly prodigious in terms of time, particularly to screen out bacteriocin negative mutants from a collection of several thousand transconjugants generated after Tn5 mutagenesis[10]. Therefore we used the simultaneous procedure involving double layers, and modified it to further improve the clear visibility of the inhibition zone.

The indicator (sensitive) strain was grown in yeast extract D-xylose (YEDX) broth for 3 days, and 0.5 ml of it was mixed with 9.5 ml of molten YEDXA containing 1.0% agar. The inoculated medium was over-layered on a 3 mm gel of YEDXA containing 2% agar in a plate. After solidification of the soft agar, the test strains were gently spotted. Observation for antagonism was taken from 3 to 7 day of inoculation.

It was interesting to observe that there was no inhibition of the growth of the sensitive culture around the growth spot of the producer strain. However, a very weak halo zone was visible under the growth. This was more sharply visible as a well, when the growth was gently swabbed off. Though we have no clear explanation for the lack of inhibition in the horizontal direction, probably the entrapment of antagonistic substance in diffusing exopolysaccharides over the surface of the gel limits the horizontal diffusion, while under the cell growth most of the antagonistic substance diffuse down into the soft agar rapidly. The results by this simultaneous antagonism method and those obtained with deferred antagonism were totally identical.

In order to observe inhibition around the growth spot, we made further attempts by modifying the composition of the media, particularly the solidifying agent, the agar. Four different media containing agar from three different sources were used (Table 1). Again the inhibition as a well-like halo zone under the growth was

Table 1. Detection of antagonism on different media varying in the source by simultaneous antagonism (double layer) method

Medium	Source of agar		Halo zone produced by test strains (PR2042c, PR2015b, PR2109a, PR2303)	
	Bottom layer	Top layer	Indicator strain PR2005b	Indicator strain 2007a
YEDXA	Qualigens 2%	Qualigens 1%	+	+
	Qualigens 0.5% + Difco 1.5%	Qualigens 0.5% + Difco 0.5%	++	++
	Sisco 1.5%	Sisco 1%	±	±
YEMA	Qualigens 2%	Qualigens 1%	+	+
	Qualigens 0.5% + Difco 1.5%	Qualigens 0.5% + Difco 0.5%	++	++
	Sisco 1.5%	Sisco 1%	±	±
Defined medium	Qualigens 2%	Qualigens 1%	+	+
	Qualigens 0.5% + Difco 1.5%	Qualigens 0.5% + Difco 0.5%	++	++
	Sisco 1.5%	Sisco 1%	±	±
SEYA	Qualigens 2%	Qualigens 1%	+	+
	Qualigens 0.5% + Difco 1.5%	Qualigens 0.5% + Difco 0.5%	++	++
	Sisco 1.5%	Sisco 1%	±	±

++, Very clear; +, clear; ±, weakly clear.
Qualigens, Glaxo India Ltd; Difco, Detroit, Michigan; Sisco, Sisco Research Laboratory, Mumbai, India.
Defined medium: According to Bergersen[11] but D-xylose as the carbon source.
YEMA, Yeast extract mannitol agar[12]; YEDXA, Yeast extract D-xylose agar (YEMA-mannitol + 0.5% D-xylose); SEYA, Soil yeast extract agar[13] except that no glucose but 0.4% yeast extract was incorporated.

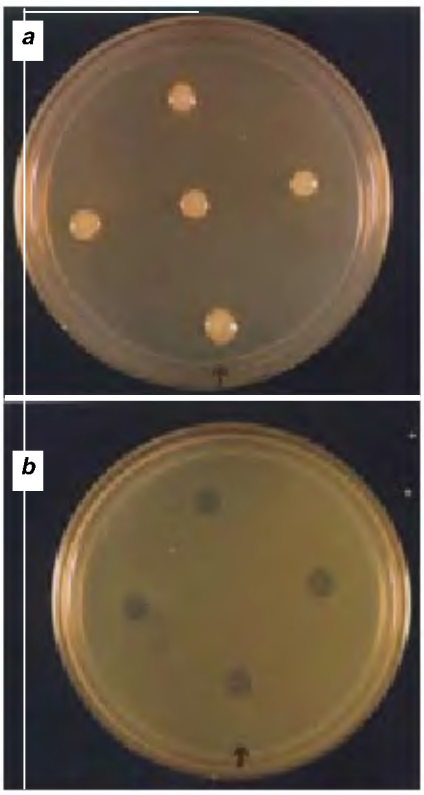

Figure 1. Production of inhibition zone by the antagonistic strains of *Cicer–Rhizobium* against the sensitive strain PR2005b (*center*: 2005b, from ↑ clockwise: PR2303, PR2042c, PR2015b and PR2109a). ***a***, Growth; ***b***, Halo zone seen after removal of growth.

observed. It was found that when the bottom layer of YEDXA contained 0.5% Qualigens and 1.5% Difco agar, and the top layer contained 0.5% of each of these two agars, the halo zone was most sharp (Table 1, Figure 1). Both the layers of gel prepared in Sisco agar showed poorly visible halo zones, while with Qualigens, the halo zone was of moderate intensity. Similar type of results were observed when different media varying in composition, particularly in carbon sources were used. A well-like halo zone under the spot, which was clearer after removing the growth with cotton swab, was considered as positive antagonism.

The examination of 38 strains as test strains against 15 non-bacteriocin producing sensitive strains on YEDXA, made with a mixture of Qualigens and Difco agars, showed 23 strains producing clearly visible inhibitory halo zones. These 23 strains showed an inhibitory spectrum identical to that obtained with deferred antagonism procedure.

Based on this modified simple method, 8648 transconjugants could successfully be screened to identify three bacteriocin-defective mutants[10].

1. Mayr-Harting, A., Hedges, A. J. and Berkelay, R. C. W., in *Methods in Microbiology* (eds Norris, J. R. and Ribbons, D. W.), Academic Press Inc, New York, 1972, pp. 315–472.
2. Tagg, J. R., Dajani, A. and Wannamaker, L. W., *Bacteriol. Rev.*, 1976, **40**, 722–756.
3. Schwinghamer, E. A. and Brockwell, J., *Soil Biol. Biochem.*, 1978, **10**, 383–387.
4. Lotz, W. and Mayer, F., *J. Virol.*, 1972, **9**, 160–173.
5. Gissman, L. and Lotz, W., *J. Gen. Virol.*, 1975, **27**, 379–383.
6. Kekessy, D. A. and Piguet, J. D., *Appl. Microbiol.*, 1970, **20**, 282–283.
7. Chauhan, S., M Sc thesis, Indian Agricultural Research Institute, New Delhi, 1984, p. 148.
8. Chauhan, S. and Gaur, Y. D., in *Current Status of Biological Nitrogen Fixation* (eds Singh, R., Nanawatee H. S. and Sawhney S. K.), HAU, Hissar, 1986, pp. 136–137.
9. Gaur, Y. D., in *Nitrogen: Soil, Physiology, Biochemistry, Microbiology, Genetics* (eds Abrol, Y. P., Tilak, K. V. B. R., Kumar, S. and Katyal, J. C.), INSA, New Delhi,1993, pp. 173–198.
10. Nirmala, J., Ph D thesis, Indian Agricultural Research Institute, New Delhi, 1999, p. 262.
11. Bergersen, F. J., *Aust. J. Biol. Sci.*, 1961, **14**, 349–360.
12. Vincent, J. M., in *A Manual for the Practical Study of Root Nodule Bacteria*, Blackwell Scientific Publications, Oxford, 1970, p. 164.
13. Subba Rao, N. S., in *Interaction of Nitrogen-Fixing Microorganism* (ed. Subba Rao, N. S.), Oxford & IBH, New Delhi, 1984, pp. 37–64.

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