RAPID PROCEDURE FOR PRODUCTION OF ANTI-*AZOSPIRILLUM* SERUM

K. ANNAPURNA and Y. D. GAUR
Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110 012, India.

ABSTRACT

Antiserum of high titre and specificity is essential for any serological study. Prolonged immunization increases antibody titre; however, specificity decreases due to the development of antibodies against minor non-specific antigens. An immunization schedule involving 4 intramuscular and 4 intravenous injections within 49 days is suggested producing high quality anti-*Azospirillum* serum. This is roughly half the period earlier reported for producing similar antibodies.

INTRODUCTION

THE potential importance of *Azospirillum* as an associative symbiont in the roots of cereals and grasses[1–3] has attracted worldwide interest in the ecology of this diazotroph. Serological techniques (being most successful for understanding the ecology of many soil bacteria[4–6]) have also been used to study the ecology of strains of *Azospirillum*[7–11], in which anti-*Azospirillum* sera were produced by immunizing either goats or rabbits for 90–120 days (table 1). This period is quite long not only for the experiment but also for keeping the small animal alive because continuous immunization sometimes proves fatal to the animal due to anaphylactic shock or painful granuloma. It is also known that during the long period of immunization, antibodies against minor antigens may develop which may not be strain-specific[12]. An attempt was therefore made to produce anti-*Azospirillum* sera within a shorter period by following three immunization schedules (table 2).

A strain of *Azospirillum* Azs 8, isolated by Dr K. V. B. R. Tilak of this Department from the roots of sorghum (*Sorghum bicolor*) cv. B-3, grown at IARI Farm, New Delhi, was incubated at 28 ± 1°C in Okon's modified broth (table 3). The pH of the broth was adjusted to 6.8. The phosphates were sterilized separately and mixed after cooling. The cells were harvested by centrifuging the 5-day growth at 7,000 *g* and washed with phosphate buffer saline (PBS)[13]. They were then stored on a thick paste at −10°C.

A suspension containing 1×10^{10} cells/ml PBS was injected into the rabbit either through intramuscular (im) or intravenous (iv) routes or both according to the immunization protocols (table 2). Suspension for im injections contained cell suspension, well homogenized with equal volume of Freund's complete bacto-adjuvant. Titre and potency of antisera were tested following tube agglutination[12] and Ouchterlony's gel immunodiffusion (ID)[14] techniques. Cells for ID tests were subjected to ultrasonication and heat treatment. Specificity of the antisera was tested by examining the immunodiffusion reactions of the antisera with 5-day-old cells of 9 other strains of *Azospirillum* (table 4), grown and harvested as described for strain Azs 8.

The results (table 4) show that the anti-*Azospirillum* Azs 8 serum with good agglutination titre and strong and specific immunodiffusion reactions could be produced by following immunization schedule III which involved 4 im and 4 iv injections in 49 days.

Table 1 *Immunization schedules followed for producing anti-Azospirillum sera in earlier studies*

Studies*	Antigen	Adjuvant	Injection	Animal	Days	Titre
a*	Whole cell	yes	sc, im & iv	goat	104	640 Aggl 1280 Imf
b*	Whole cell	yes	id	rabbit	90–120	1000 Imf
c*, d*	Whole cell	no	id	rabbit	nd	nd

sc, subcutaneous; im, intramuscular; iv, intravenous; id, intradermal; Aggl, agglutination; Imf, immunofluroscence; nd, not described.
* see reference 7, 9, 10 & 11 for a, b, c & d respectively.

Table 2 *Immunization and bleeding schedules used for producing antiserum against Azospirillum brasilense strain Azs 8 in rabbit*

	Schedule I		Schedule II[a]		Schedule III	
Day	ml Ag/blood	im/iv	ml Ag/blood	im/iv	ml Ag/blood	im/iv
0	5.0	Ns	5.0	Ns	5.0	Ns
1	0.5	iv	2.0	im	1.0	im
2	0.5	iv				
3	0.5	iv				
8	1.0	iv	0.5	iv	1.0	im
9	1.0	iv				
10	1.0	iv				
12			1.0	iv		
15	1.0	iv			1.0	im
16	1.0	iv	2.0	iv		
17	1.0	iv				
22					1.0	im
23			20 0	As		
24	20.0	As				
29					0.5	iv
35					1.0	iv
39					1.5	iv
42					2.0	iv
49					20.0	As

im, Intramuscular injection of an emulsion of bacterial suspension 1×10^{10} cell/ml in saline and Freund's bacto-adjuvant (1:1); iv, Intravenous injection of antigen suspension without adjuvant; Ag, Antigen suspension; As, Bled for antiserum; Ns, Bled for normal serum; [a] Procedure found suitable for producing antisera against rhizobia by Sharma and Sen[15].

This period is half of that reported earlier for producing anti-*Azospirillum* sera (table 1). The specificity of antiserum was high as it did not show ID reaction with the other 9 strains of *Azospirillum* except Azs 9, with which cross-reaction of partially identical nature was seen for three of the seven antigens.

Antiserum, produced by following schedule I involving 9 (iv) injections in 24 days, (when tested undiluted) showed ID reaction equally strong and specific as to the antiserum produced by immunization schedule III. However, its agglutination and ID titre were lower than the antiserum obtained by immunization schedule III. Nevertheless, it demonstrated that anti-*Azospirillum* serum having strong and specific ID reactions could be produced in shorter time (i.e. one-fourth the time reported earlier, table 1), though the suitability of such antiserum would depend on the technique employed for antigen-antibody reactions (antisera with low ID titre are not always successful in producing flurochrome labelled conjugates for immunofluorescence studies). Anti-*Azospirillum* serum produced by following immunization schedule II, in 23 days had lower agglutination titre and was also poor in ID reactions as it reacted only for 4 of the 7 antigens.

Table 3 *Composition of Okon's modified broth*

K_2HPO_4	6.0	g
KH_2PO_4	4.0	g
Malic acid	5.0	g
NaOH	3.0	g
NH_4Cl	1.0	g
$MgSO_4.7H_2O$	0.2	g
$CaCl_2$	0.02	g
Yeast extract (Difco)	0.05	g
$FeCl_3$	2000	μg
Na_2MoO_4	2000	μg
H_3BO_3	1400	μg
$MnSO_4$	1000	μg
$ZnSO_4$	210	μg
$Cu(NO_3)_2$	40	μg
Bromothymol blue (0.5% alcoholic)	2	ml
Distilled water	1000	ml

Table 4 *Potency of antisera prepared against 5-day-old whole cells of Azospirillum-strain Azs 8 by three immunization schedules*

Antigen	Antiserum dilution in fold	U/H	Immunization schedules I (400)	II (200)	III (1600)
Azs 8	0	U	abc_1c_2de	-bcd-	$ab_1b_2c_1c_2de$
		H	abc_1c_2-e	-bcd-	$ab_1b_2c_1c_2$-e
	2	U	-bc_1c_2--	-bc--	$ab_1b_2c_1c_2$d-
		H	-bc_1c_2--	-bc--	$ab_1b_2c_1c_2$--
	4	U	------	-----	-b_1-c_1---
		H	-bc_1---	-----	-b_1-c_1---
Azs 9	0	U	-ss---	--ss-	-sss---
		H	--s---	---s-	--ss---
Azs 1 to 7 & 10	0	U	------	-----	-------
		H	------	-----	-------

U, Unheated 5-day-old whole cells; H, Five-day-old cells steamed for 30 min; Identical alphabets denote completely identical precipitin line, '–' denotes lack of reaction; 's' denotes precipitin line forming spur at the junction of two precipitin lines showing partial identical reaction; line 'a' is nearest to the antigen well and line 'f' is farthest. Azs 1 from *Digitaria decumbens* cv *transvala* (original No. Sp 7); Azs 2 to 7 from *S. bicolor* cv CHS 5,6,7R,J3,J7 & J15 respectively; Azs 9 from *Zea mays* and Azs 10 from *Hordeum vulgare*; Figure in brackets denote agglutination titre.

Thus, it can be concluded that highly specific anti-*Azospirillum* sera with strong ID reactions could be produced in less than 50 days by following combined immunization protocol involving im and iv injections of schedule III.

ACKNOWLEDGEMENTS

The authors thank Dr K. V. B. R. Tilak for providing strains of *Azospirillum*, and Dr N. S. Subba Rao and Dr A. C. Gaur for their keen interest.

30 October 1987; Revised 24 February 1988

1. Neyera, C. A. and Dobereiner, J., *Adv. Agron.*, 1977, **29,** 1.
2. Barber, L. E., Russell, S. A. and Evans, H. J., *Plant Soil*, 1979, **52,** 49.
3. Van Berkum, P. and Bohlool, B. B., *Microbiol. Rev.*, 1980, **44,** 491.
4. Gray, T. R., In: *The soil ecosystem*, (ed.) J. Sheals, The systematics Associative Publication, London, 1969, p. 73.
5. Schmidt, E. L., *Soil Sci.*, 1974, **118,** 141.
6. Dudman, W. F., *Appl. Microbiol.*, 1971, **21,** 973.
7. Dazzo, F. B. and Milam, J. R., *Soil Crop Sci. Soc. Florida*, 1975, **35,** 121.
8. Sampaio, M. J. A., Vasconcellos, L. de and Dobereiner, J., *Granhall. Ecol. Bull.*, 1978, **26,** 364.
9. Schank, S. C. *et al.*, *Soil Biol. Biochem.*, 1979, **11,** 287.
10. De-polli, H., Bohlool, B. B. and Dobereiner, J., *Arch. Microbiol.*, 1980, **126,** 217.
11. Kosslak, R. M. and Bohlool, B. B., *Can. J. Microbiol.*, 1983, **29,** 649.
12. Vincent, J. M., In: *A manual for the practical study of the root nodule bacteria*, Blackwell Scientific Publications, Oxford, 1970, p. 164.
13. Nairn, R. C., In: *Fluorescent protein tracing*, Livingstone, Edinburgh and London, III, 1969.
14. Gaur, Y. D. and Sen, A. N., *Indian J. Exp. Biol.*, 1984, **22,** 302.
15. Sharma, C. R. and Sen, A. N., *Curr. Sci.*, 1979, **48,** 173.