only emerged as adults. Working on the biology of nepids Rao[3] showed the variation in the egg number in different months of the year. However, he has not observed repeated egglaying in any of the hemipterans. We have clearly recorded repeated egglaying by *L. griseus* and perhaps it may be the first report in this aspect.

Financial support from the CSIR, New Delhi, is gratefully acknowledged.

29 September 1984

1. Hoffman, W. E., *Lingnam Agric. Rev.*, 1927, **4**, 73.
2. Rao, T. K. R., *Proc. R. Entomol. Soc. London*, 1962, **37**, 61.
3. Rao, T. K. R., *Entomology*, 1976, **1**, 123.
4. Jansson, A. and Scudder, G. G. E., *J. Entomol. Soc. Brit. Columb.*, 1972, **69**, 44.
5. Panicker, K. N. and Rajagopalan, P. K., *Indian J. Med. Res.*, 1977, **66**, 772.
6. Gopal, R., M.Phil. Thesis, Madurai Kamaraj University, Madurai, T.N., India, 1980.
7. Cannings, S. G., *Can. Entomol.*, 1978, **110**, 599.
8. Mathavan, S., Ph.D. Thesis, Madurai Kamaraj University, Madurai, T.N., India, 1975.

PROTOPLAST FORMATION AND REGENERATION IN *RHIZOBIUM* AND *AZOSPIRILLUM*

S. S. SINDHU and K. R. DADARWAL
Department of Microbiology, Haryana Agricultural University, Hisar 125 004, India.

PROTOPLAST of gram-positive bacteria have successfully been used for cell fusion between strains of same species[1-3] as well as of distinct species for transferring genetic factors[4-7]. The cell walls of gram-negative bacteria being complex, protoplast formation and their regeneration have been reported only in a few genera like *Escherichia*, *Providentia* and *Pseudomonas*[8-10]. *Rhizobium* and *Azospirillum* are the two major groups of gram-negative soil bacteria which have acquired significance, since the former fixes nitrogen in symbiosis with legumes and the latter in association with cereals and grass roots. Genetics of both the bacterial groups is poorly understood with regard to symbiotic association, host specificity and nitrogen fixation potential and because of that strain selection from native soil population is the only accepted method of getting better isolates. Since protoplast fusion provides a method to transfer genetic factors among distinct species and strains where normal methods of gene transfer is not possible, it was proposed first to standardize methods for protoplast formation and their regeneration in *Rhizobium* and *Azospirillum* so that it could be used subsequently for genetic recombinations. In this communication we report conditions for preparation of protoplasts and regeneration in *Rhizobium* strains Ca42 and Ca141 nodulating chick pea (*Cicer arietinum L.*), S24 nodulating green gram (*Vigna radiata Var. aureus*) and an associative nitrogen fixing bacterium of sorghum *A. brasilense* strain 12S. It was observed that protoplasting and regeneration frequencies varied with the strains as well as with the protoplasting method. However, by slight modification in plating technique 12 to 30% of the protoplasts formed showed regeneration.

Rhizobium strains Ca42, Ca141 and S24[11] were maintained on yeast extract mannitol agar slopes[12] whereas *A. brasilense* strain 12S was maintained on Dobereiner's malate medium[13]. For preparation of protoplasts, the basal medium used for the growth of *Rhizobium* was BMR medium containing: $g\,l^{-1}$ yeast extract, 0.5; mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; sodium glutamate 0.32 and $mg\,l^{-1}$: $FeSO_4.2H_20$, 27; $CaCl_2.2H_2O$, 78; EDTA disodium salt, 32; H_3BO_3, 3; $MnSO_4.H_2O$, 4; $NaMoO_4.2H_2O$ 5; $ZnSO_4.7H_2O$, 0.21; KI, 0.78 and $CoCl_2.6H_2O$ 0.025. For *A. brasilense* mannitol in the BMR medium was replaced with $3.2\,g\,l^{-1}$ of sodium malate (BMA medium). Hypertonic sucrose BMR or BMA broth contained 0.5 M sucrose in the basal medium. The broth was always boiled and filtered before autoclaving. In solid medium 1.5% agar was added. For preparation of protoplasts, *Rhizobium* was grown for 96 hr and *A. brasilense* for 48 hr in BMR and BMA broth, respectively. All incubations were done at $28 \pm 1°C$ and centrifugation at 4000 g for 30 min at 4°C. All solutions and media used, were sterilized by autoclaving.

Both, tris-sucrose-EDTA-lysozyme[8] as well as sucrose-cation-lysozyme[10] methods were tried for protoplast preparation. In the former method, cells harvested by centrifugation from BMR or BMA broth were resuspended in 100 mM tris-HCl buffer (pH 8.0) containing 0.5 M sucrose, washed twice and finally taken in the same buffer. Cell density in the buffer was then adjusted to have viable cell counts between 10^8 to 10^9 cells ml^{-1}. To 9 ml of the cell suspension, 1 ml of 100 mM EDTA (disodium salt) was added drop by drop

and incubated for 1 hr. Crystalline lysozyme was then added to the suspension (2 mg ml^{-1}), mixed gently and further incubated for 2 hr. The protoplasts formed were centrifuged twice by suspending into hypertonic sucrose-BMR or BMA broth. The pellet was finally collected in 9 ml of the hypertonic sucrose medium broth. Dilutions of the suspension were then made in the respective hypertonic sucrose broth for counting protoplasts as well as in distilled water for counting cells after osmotic cell lysis.

By sucrose-cation lysozyme method, the cell pellet from BMR or BMA broth was similarly washed twice by suspending in sucrose cation solution (sucrose, 0.5 M; Na_2HPO_4, 0.125 M; KH_2PO_4, 0.05 M; NH_4Cl, 0.05 M; $CaCl_2 . 2H_2O$, 0.02 M) followed by centrifugation and finally suspended in 10 ml of the above hypertonic solution. Viable cell count in the suspension were determined by dilution plating. For protoplasting, the cell suspension in the sucrose cation solution was incubated for 90 min followed by treatment with lysozyme (2 mg ml^{-1}) for 2 hr. The protoplasts formed were centrifuged and the pellet was diluted in hypertonic sucrose broth as well as in distilled water for plating.

Dilutions prepared for total viable cell counts in the cell suspensions used initially for preparation of protoplasts and protoplast dilutions prepared in sterile distilled water, were plated on respective BMR or BMA agar medium plates. For plating hypertonic sucrose broth dilutions, double layered medium plates were prepared. For this sterilized 20 ml BMR or BMA agar medium was poured per plate in 10 cm diam. plates followed by a second layer of 10 ml of respective hypertonic sucrose medium. Hypertonic broth dilutions were then plated in the plates to determine total revertible cells after protoplasting.

Protoplasts were formed in *Rhizobium* strains as well as in *A. brasilense* by both the treatments, however, regeneration frequencies showed variations by the two methods (table 1). In *A. brasilense* more than 99% cells were converted into protoplasts by both the methods but proportion of original cells regenerated to colony forming units on hypertonic medium was 1.4% by sucrose-cation and 14.9% by tris-sucrose-EDTA treatment. Similarly when *Rhizobium* strain Ca141 was treated with sucrose-cation only 0.06% of the original cell population regenerated to colonies as compared to 32.0% regeneration by tris-sucrose-EDTA method. It appears, therefore, that sucrose-cation treatment probably results into rapid lysis of the cells in these strains during protoplast preparation. In *Rhizobium* strain S24, protoplasts formed with a low frequency by both the treatments. *Rhizobium* strain Ca42 showed a high rate of protoplasting and maximum cells regenerated into colony forming units by both the methods.

Normally 0.5 M sucrose hypertonic agar medium plates have been reported to be used for regeneration of protoplasts[2,3,10,14]. In our studies we could get maximum regeneration only on double layer plates of normal and hypertonic medium. In these plates colonies from protoplasts formed after 15 days and

Table 1 *Frequency of protoplast formation and regeneration in Rhizobium and Azospirillum.*

Bacterial strain	Treatment	Initial cell count (ml^{-1}) (A)	Viable cells after osmotic shock (ml^{-1}) (B)	Viable cells after regeneration (ml^{-1}) (C)	Protoplast frequency (%) [(A – B/A)]	Regeneration frequency (%) [C/(A – B)]
Rhizobium Ca42	TSE	$56.3 \pm 4.3 \times 10^8$	$56.3 \pm 8.8 \times 10^6$	$10.8 \pm 0.8 \times 10^8$	99.0	19.38
	SC	$83.7 \pm 8.2 \times 10^8$	$45.3 \pm 5.2 \times 10^6$	$28.3 \pm 3.5 \times 10^8$	99.46	34.00
Rhizobium Ca 141	TSF	$80.3 \pm 9.5 \times 10^8$	$32.3 \pm 5.7 \times 10^7$	$24.7 \pm 4.2 \times 10^8$	95.98	32.05
	SC	$53.0 \pm 7.1 \times 10^8$	$52.7 \pm 5.0 \times 10^3$	$34.7 \pm 5.1 \times 10^5$	99.99	0.06
Rhizobium S24	TSE	$30.0 \pm 3.6 \times 10^9$	$13.0 \pm 1.1 \times 10^8$	$36.0 \pm 4.6 \times 10^8$	95.67	12.54
	SC	$31.7 \pm 2.7 \times 10^9$	$46.7 \pm 3.9 \times 10^7$	$39.0 \pm 5.9 \times 10^8$	98.53	12.49
A. brasilense	TSE	$30.0 \pm 4.3 \times 10^8$	$72.0 \pm 5.3 \times 10^4$	$44.7 \pm 6.8 \times 10^7$	99.98	14.90
	SC	$33.3 \pm 8.1 \times 10^8$	$32.3 \pm 5.2 \times 10^4$	$47.0 \pm 6.7 \times 10^6$	99.99	1.41

TSF: *tris-sucrose-EDTA: lysozyme treatment and* SC: *sucrose cation-lysozyme treatment.*

Initial cell counts are the viable counts in the cell suspension used for protoplasting. Viable cells after osmotic shock are the colonies formed on BMR or BMA medium plated from water dilutions of protoplasts. Viable cells after regeneration are the colonies formed on double layer agar plates, plated from protoplast dilutions, prepared in hypertonic sucrose broth. Protoplast frequency is calculated as per cent of cells converted to protoplasts (A – B/A × 100) and regeneration frequency is the per cent of protoplasts regenerated into colony forming units (C/A – B × 100).

8 days of incubation in the case of *Rhizobium* and *Azospirillum*, respectively. The regeneration frequencies observed by this method were relatively much higher than what has been reported in other bacteria[8, 10, 14]. On plates of pure hypertonic medium no colony appeared from protoplasts even after 20 days of incubation. With regard to the method of protoplasting, the sucrose cation lysozyme method used for protoplast preparation in *Pseudomonas putida*[10] was found to be less suitable as compared to the tris-sucrose-EDTA lysozyme method used in *E. Coli* and *providentia*[8, 9] although there were variations from strain to strain.

This article is a part of the research project sponsored by the Indian council of Agricultural Research, New Delhi.

7 August 1984; Revised 1 November 1984.

1. Fodor, K. and Alfoldi, L., *Proc. Natl. Acad. Sci.*, USA, 1976, **73**, 2151.
2. Hopwood, D. A., Wright, H. M., Bibb, M. J. and Cohen, S. N., *Nature (London)*, 1977, **268**, 171.
3. Hotchkiss, R. D. and Gabor, M. H., *Proc. Natl. Acad. Sci.*, USA, 1980, **77**, 3553.
4. Dancer, B. N., *J. Gen. Microbiol.*, 1980, **121**, 263.
5. Gryczan, T. J., Contente, S. and Dubnau, D., *J. Bacteriol.*, 1978, **134**, 318.
6. Reid, S. J., Allcock, E. R., Jones, D. T. and Woods, D. R., *Appl. Environ. Microbiol.*, 1983, **45**, 305.
7. Vorobjeva, I. P., Khmel, I. A. and Alfoldi, L., FEMS *Microbiol. Lett.*, 1980, **1**, 261.
8. Weiss, R. L., *J. Bacteriol.*, 1976, **128**, 668.
9. Coetzee, J. N., Sirgel, F. A. and Lecatsas, G., *J. Gen. Microbiol.*, 1979, **114**, 313.
10. Maksimova, N. P., Zheldakova, R. A. and Fomichev, Yu. k., *Microbiol.*, 1983, **51**, 521.
11. Dadarwal, K. R., Batra, R. and Tauro, P., *Arch. Microbiol.*, 1982, **133**, 303.
12. Vincent, J. M., *A mannual for the practical study of root nodule bacteria*. Blackwells, Oxford, 1970, p. 3.
13. Dobereiner, J., Marrel, I. E. and Nery, M., *Can. J. Microbiol.*, 1976, **22**, 1464.
14. Minton, N. P. and Morris, J. G., *J. Bacteriol.*, 1983, **155**, 432.

MORPHOLOGICAL VARIATION AND INHERITANCE IN A PIGEONPEA INTERGENERIC HYBRID

P. SATEESH KUMAR, N. C. SUBRAHMANYAM and D. G. FARIS*

School of Life Sciences, University of Hyderabad, Hyderabad 500 134, India.
** ICRISAT, Patancheru 502 324, India.*

AN intergeneric cross between *Cajanus cajan* ($2n = 22$) and *Atylosia albicans* ($2n = 22$) yielded hybrid progeny in 7% of the pollinations. These hybrids were very luxuriant with profuse branching and thick leaf canopy. The *Cajanus* parent has an erect habit while *A. albicans* is a twiner. The F_1 had a twining (semi-sreading) habit. The leaflets in *C. cajan* are lanceolate with an acute tip while those in *A. albicans* are obovate with an obtuse tip. The hybrids were intermediate for leaf shape and leaf texture in their initial stages of growth, which continued for about 100 days. During this period the leaf tip was obtuse (figure 1b). The leaves in some of the hybrid branches (figure 1a–2) which appeared after 100 days resembled the *Cajanus* parent, having an acute tip (figures 1c: 4–6), while leaves developed on other branches were obtuse (figures 1a–1, 3, 4, 5, 6). However, as the plant grew further, the remaining branches also developed leaves similar to those of the *Cajanus* parent. Though the change in leaf shape was observed in all the three leaflets it was more pronounced in the terminal ones which enabled an easy classification. The texture of the leaves produced after 100 days also resembled the *Cajanus* parent. Scanning electron microscopic studies of the upper surface of the terminal leaflets revealed similarities between the leaf surfaces of the *Cajanus* parent (figure 1d) and the *Cajanus*-like leaves produced on the hybrid (figure 1g). Both the leaf surfaces exhibited long trichomes which were uniformly spread over the surface. Leaves of the *A. albicans* parent had a very dense population of trichomes (figure 1e) whereas the initial intermediate type leaves had sparse population of short trichomes (figure 1f) when compared to the leaves of the *Cajanus* parent and the *Cajanus*-like leaves in the hybrid. Floral initiation took place only on those branches which had developed leaves similar in shape and texture to the *Cajanus* parent.

The development of leaves similar to the *Cajanus* parent initially led us to look for the possibility of selective chromosome elimination similar to that of *Nicotiana*[2] or *Hordeum* interspecific[5] and intergeneric[1] hybrids. But our cytological investigations[3]