1 Kinashi, H., Shimaji, M. and Sakai, A., *Nature*, 1987, **320**, 454–456.
2 Kinashi, H. and Shimaji, M., *J. Antibiotics*, 1987, **40**, 913–916.
3 Hayakawa, T., Tanaka, T., Sakaguchi, K., Otake, N. and Yonehare, H., *J Gen. Appl Microbiol*, 1979, **25**, 255–260.
4 Keen, C. L., Mendelovitz, S., Cohen, G., Aharonowitz, Y. and Roy, K. L., *Mol Gen Genet*, 1988, **212**, 172–176.
5 Skiffman, D. and Cohen, S. N., *Proc. Natl Acad. Sci USA*, 1992, **89**, 6129–6133
6. Chen, C. W., Yu, T-N., Lin, Y-S., Kieser, H. M. and Hopwood, D. A., *Mol Microbiol*, 1993, **7**, 925–932.
7 Kirby, R. and Hopwood, D. A., *J. Gen Microbiol*, 1977, **98**, 239–252.
8. Vivian, A. and Hopwood, D. A., *J. Gen. Microbiol.*, 1973, **76**, 147–162.
9. Rathos, M. J., Verma, N. C. and Notani, N. K., *Curr Sci*, 1989, **58**, 1235–1239
10 Cundliffe, E., *Annu Rev. Microbiol*, 1989, **43**, 207–233
11. Martin, J. F. and Liras, P., *Annu Rev Microbiol*, 1989, **43**, 173–206
12. Dharamlingam, K., in *Experiments with M13 Gene Cloning and DNA Sequencing*, Macmillan, Madras, India, 1986.
13. Ferdows, M. S. and Barbour, A. G., *Proc Natl. Acad Sci. USA*, 1989, **86**, 5969–5973.
14. Taylor, K. A., Barbour, A. G. and Thomas, D. D., *Infect Immun*, 1991, **59**, 323–329.
15 Chen, C. W., Lin, Y-S., Kieser, H. M. and Hopwood, D. A., in Proceedings of the 17th International Congress of Genetics, Birmingham, UK, 1993, p. 194
16. Reynolds, R. J., *Mutat. Res.*, 1987, **184**, 197–207.

ACKNOWLEDGEMENT. We are grateful to Prof D. A. Hopwood, John Innes Institute, England, for critical comments and suggestions

Received 30 November 1993; revised accepted 25 May 1994

Alginate encapsulation technique for indica rice protoplast culture and plant regeneration

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Encapsulation and culture of indica rice protoplasts derived from anther suspension cultures of cv. Rasi in sodium alginate beads was standardized for the first time. Protoplast division (up to 40%) and colony formation (5–6%) were observed without nurse culture. This new technique was superior in terms of division and colony formation compared to agarose entrapment and agarose bead methods. The present protocol would be useful for gene transfer in rice.

PLANT regeneration from protoplasts of japonica rice has been standardized[1-4]. However, protoplast culture and efficient plant regeneration in indica rice are still a major problem and only a few reports are available[5-7]. Use of nurse culture, identification of responsive genotypes and development of suitable embryogenic suspensions for protoplast isolation have been the key to success. Efficient and reproducible protocol for protoplast culture, high frequency colony formation and subsequent regeneration are prerequisites for gene transfer studies. The present study deals with a technique of encapsulation and culture of isolated protoplasts of rice in sodium alginate beads. High plating efficiency in terms of protoplast division, colony formation and plant regeneration was obtained. Regenerated plants were successfully transferred to pots. The protocol can be extended to other cultivars and helps in developing transgenic rice with greater efficiency.

Suspension cultures were established from embryogenic calluses of anthers of indica rice cv. Rasi, a hybrid of T(N)1 × Co29, obtained from Directorate of Rice Research, Rajendranagar, Hyderabad, Andhra Pradesh. High frequency callusing from anthers was obtained on N_6 (ref. 8) medium supplemented with $AgNO_3$. Callus with globular stage embryos was used for establishment of suspension cultures. Suspension cultures were maintained in an amino acid (AA)[9] containing medium supplemented with proline prior to isolation of protoplasts. Four month old suspension culture derived from anther callus was used for protoplast isolation. Embryogenic suspension was obtained on AA medium with 1 g/l proline (AAP) supplemented with 2 mg/l 2,4-D and 0.08 M sucrose. Initial growth of suspensions in N6P medium and transfer of suspension cells to AAP medium prior to protoplast isolation was critical for high yield of stable protoplasts. About 1 g of suspension cells was incubated in 10 ml of an enzyme mixture containing 6% (w/v) cellulase RS, 1.0% (w/v) macerozyme R10 (Yakult Honsha, Japan), 0.5% (w/v) driselase (Sigma, USA), 0.4 M mannitol, 5 mM 2N, *N*-morpholinoethane sulphonic acid (MES) with CPW salts[10], at pH 5.7 (filter sterilized). Protoplasts were purified from the cell debris by passing the protoplast enzyme suspension through 30–45 μ nylon mesh. Protoplasts were pelleted and washed twice in CPW medium with 0.4 M mannitol by centrifugation at 70 *g* for 7 min. Protoplast yield was determined using an haemocytometer. Protoplast viability was estimated using fluorescein diacetate[11].

Freshly isolated protoplasts were cultured by the following three techniques – a) Protoplasts encapsulated in alginate beads; b) Protoplasts encapsulated in agarose beads; c) Protoplasts embedded in agarose.

a) In alginate bead technique, equal volumes of 2.4% (w/v) sodium alginate (BDH, UK) solution with 0.35 M sucrose or 0.4 M mannitol and double strength (DS) N6 PCM (protoplast culture medium) containing protoplasts were mixed properly. Alginate beads were prepared in a solution (50 to 100 mM $CaCl_2$ + 0.4 M mannitol) in a petri dish or a flask. Beads thus formed

were allowed to be immersed in $CaCl_2$ solution for 30–45 min and subsequently the $CaCl_2$ solution was replaced by PCM.

b) In agarose bead technique, protoplast suspensions in N6 PCM DS and agarose (Sea plaque, FMC Biochem, USA) 2.4% were mixed equally. The suspension was placed as small beads in a plastic petri dish and allowed to gel. Subsequently, N6 PCM single strength (SS) was spread onto the petri dish.

c) In agarose entrapment technique, equal volume of DS agarose 2.4% was mixed with N6 PCM DS containing protoplasts. Agarose suspension containing protoplasts was plated in sterile disposable petri dishes. This is a standard technique, in use for inducing callus from protoplasts of indica and japonica types.

Protoplast growth from first division to callus formation was monitored in all the three culture techniques. Plating efficiency (PE) in terms of colony formation was estimated by the total number of protoplasts encapsulated to the number of colonies obtained. For plant regeneration, the calluses (> 0.8 mm) were picked up from the agarose surface and transferred to regeneration medium. In the case of alginate technique, the microcalluses were freed by dissolving the beads in a depolymerizing solution containing 0.1 M sodium citrate, 0.175 M sucrose. Subsequently, these calluses were transferred to regeneration medium [half strength MS[12] medium with different concentrations of acetic acid, (IAA, 1, 2 and 3 mg/l), 6-benzylaminopurine (BAP, 0.5, 1 and 2 mg/l) and kinetin (kn, 0.1, 0.2, 0.4, 0.6 and 1 mg/l)]. The frequency of plant regeneration was recorded. Plantlets with well-developed roots were initially transferred to small plastic pots containing soil vermiculite mixture (1 : 1) and subsequently to soil in pots.

The technique of encapsulation of protoplasts in sodium alginate beads and subsequent culture was found to be superior to both agarose entrapment and agarose bead methods (Table 1). Protoplast stability was higher when protoplasts were encapsulated in sodium alginate compared to other methods. Optimum division of protoplasts was obtained on N6 PCM supplemented with 1 g/l L-proline, 2 mg/l 2,4-D and 0.35 M sucrose. Plating efficiency in terms of protoplast division (40%) and colony formation (5–6%) was significantly greater in alginate beads compared to agarose bead and agarose entrapment methods (Table 1). Stages of protoplast development in alginate beads and regeneration to whole plants are given in Figure 1.

An accurate analysis of number of colonies/microcalluses obtained with respect to the total number of protoplasts encapsulated can be calculated by dissolving the beads using a depolymerizing solution and subsequent counting in a haemocytometer. The Ca^{2+} ions play a major role during cell wall synthesis[13]. The high plating efficiency obtained using 'alginate beads method' compared to agarose beads and agarose entrapment procedures may be due to the exposure of protoplasts membrane to high Ca^{2+} concentration during the complexation reaction for calcium alginate bead formation.

Plant regeneration was obtained on half strength MS medium with 1 mg/l IAA, 2 mg/l BAP, 0.6 mg/l kinetin and 3% sucrose. In the case of alginate encapsulation technique, out of the 218 calluses plated on regeneration medium, 98 calluses showed regeneration response with both green (76) and albino (22) (Table 1). The overall regeneration frequency included green (35%) and albino (10%). In agarose bead and agarose entrapment methods 198 and 184 calluses were transferred to regeneration medium, respectively, and the regeneration response is depicted in Table 1. Following these techniques, overall regeneration frequency was decreased with increased frequency of albinos. Duration from protoplast isolation to plant regeneration was reduced by two weeks when compared to agarose bead and agarose embedding methods. (Table 1). Following these techniques, 172 independent green plantlets were obtained. Profuse rooting was obtained when the plantlets were transferred to 1/2 strength MS supplemented with 0.5 mg/l NAA.

Table 1. Role of culture techniques on division, colony, callus formation and plant regeneration from anther derived protoplasts of indica rice *Oryza sativa* cv. Rasi

Technique	Stability (%)	Days after culture		PE (%)		No. of calluses plated	No. of calluses regenerating		Plant regeneration time (weeks)
		2–4	14–21	PD	CF		Green	Albino	
Alginate beads	95 ± 2	1st division	35–40 micro callus visible	40 6 ± 1 9	5 2 ± 0 63	216	76	22	8
Agarose beads	65 ± 2	–	10–15 cell colony	17 2 ± 2 0	0 4 ± 0 1	198	63	24	10
Agarose entrapment	70 ± 1	–	15–20 cell colony	19 6 ± 3 1	0 8 ± 0 19	187	49	27	10

PE = Plating efficiency, PD = Protoplast division, CF = Colony formation.

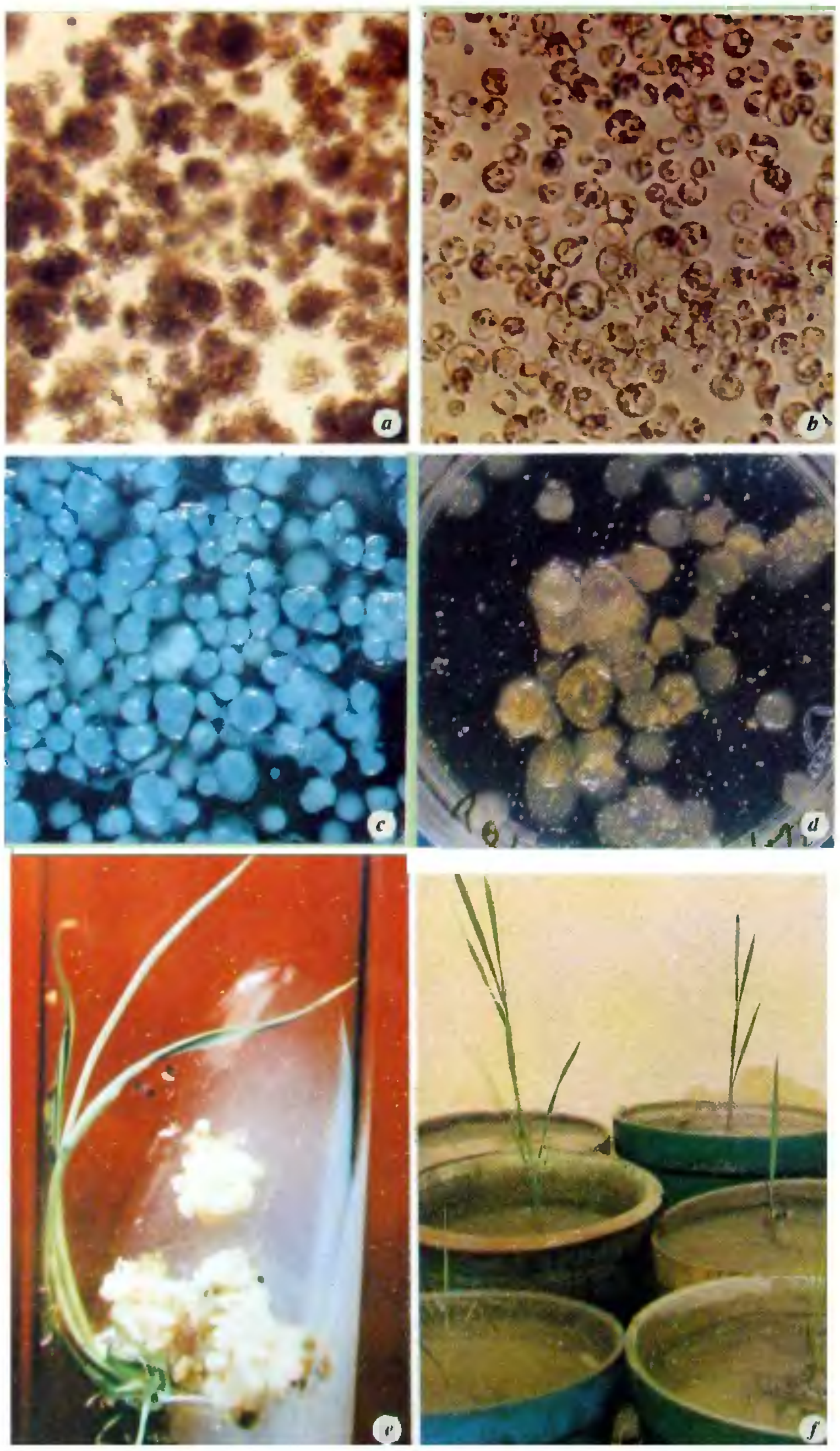

Figure 1. Alginate encapsulation technique for indica rice protoplast culture and regeneration. ***a***, Embryogenic suspension culture derived from anther culture; ***b***, Freshly isolated protoplasts; ***c***, Alginate beads after protoplast encapsulation; ***d***, Micro callus and colony formation in beads; ***e***, Plant regeneration from protoplast-derived callus; ***f***, Protoclones in pots (soil).

Regenerated plants (40) with developed roots were transferred to soil (pots) with 80% survival.

Regeneration of plants from protoplasts through encapsulation in alginate beads is reported in dicots[14, 15]. The present technique has been applied successfully to indica rice for the first time. This protocol of protoplast culture and plant regeneration can be successfully exploited for gene transfer in rice by direct DNA uptake.

1. Abdullah, R , Cocking, E C. and Thompson, J. A , *Bio/Technology*, 1986, **4**, 1087–1090.
2 Thompson, J A., Abdullah, R., Chen, W H and Gartland, K. M A , *J Plant Physiol* , 1987, **127**, 367–370.
3. Toriyama, K., Hinata, K and Sasaki, T , *Theor Appl Genet.*, 1986, **73**, 16–19.
4 Kyozuka, J , Hayashi, Y and Shimamoto, K , *Mol Gen. Genet* , 1987, **206**, 408–413.
5. Lee, L., Schrooll, R E., Grimes, H D. and Hodges, T. K , *Planta*, 1989, **178**, 325–333
6. Datta, K., Potrykus, I. and Datta, S K , *Plant Cell Rep* , 1992, **11**, 229–233.
7. Peng, J , Ononowicz, H and Hodges, T K., *Theor. Appl Genet.*, 1992, **83**, 855–863.
8. Chu, C C , Wang, C. C , Sun, C. S., Hsu, S. C , Yin, K. C., Chu, C. Y. and Bi, F. Y., *Sin. Sin* ., 1975, **18**, 659–668
9. Muller, A J. and Grafe, R , *Mol. Gen. Genet* , 1978, **161**, 67–76
10. Frearson, E. M., Power, J. B and Cocking, E. C., *Dev. Biol* , 1973, **33**, 130–137.
11 Larkin, P. J , *Planta*, 1976, **128**, 213–216.
12. Murashige, T and Skoog, F , *Physiol. Plant* , 1962, **15**, 473–497.
13. Kauss, *Annu Rev Plant Physiol.*, 1987, **38**, 147–172.
14 Damon, B. and Willmitzer, L., *Mol Gen Genet* , 1988, **213**, 15–20.
15. Daraget, K I , Myhre, S , Skjak-BRK G. and Ostgaard, K. J *Plant Physiol.*, 1988, **132**, 552–556.

ACKNOWLEDGEMENTS Financial support from DBT, New Delhi and USPL-480 Rice project, USA is gratefully acknowledged

Received 13 December 1993, revised accepted 2 June 1994

Modified procedure for bromide estimation with ion-selective electrode for predicting nitrate movement in soil

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Because of the similar behaviour, bromide (Br^-) could be used as a tracer for nitrate (NO_3^-) in soil-water systems. However, successful use of Br^- depends upon accurate recovery of added Br^- from soil and detection by instrument. In the present study efficiency of different extraction procedures for Br^- estimation was examined with ion-selective electrode. These procedures gave variable recoveries of added Br^-. The method which gave the highest recovery (90–93%) of the added Br^- was selected and modified to obtain precise and near 100% recovery in an Alfisol and a Vertisol of ICRISAT Centre, near Hyderabad.

LEACHING of fertilizer-derived NO_3^- in soils can be measured accurately with ^{15}N techniques but is cost prohibitive. A tracer bromide (Br^-), which is similar in charge and behaviour to NO_3^- in soil-water systems[1, 2] may provide the basis for an alternative technique of NO_3^- estimation. Br^- has several other characteristics to its advantages – it is in low background concentration, nonreactive with soil constituents[3], nontoxic to plants[4] biologically conserved[5], less likely to contaminate the environment[6], easy to analyse, and inexpensive.

Development of ion-selective electrodes has rendered measurement of Br^- more precise. However, successful use of Br^- as a tracer for NO_3^- movement in the soil depends upon accurate recovery of added Br^- from soils and accurate detection of it by Br^- ion-selective electrode. We therefore conducted experiments to evaluate several procedures used by past researchers for Br^- extraction from soils[6–12]. These procedures involved the use of different soil-to-water (or soil-to-electrolyte) ratios, shaking time, etc. Our objective was to identify and refine the most appropriate procedure to obtain accurate recovery of added Br^- from Alfisols and Vertisols at ICRISAT Centre, Patancheru, near Hyderabad.

These procedures gave variable recoveries of added Br^- (88–95%). The method which gave the highest recovery in both Alfisol (90%) and Vertisol (93%) involved shaking of 25 g soil samples with 49 ml distilled water and 1 ml of 5 M $NaNO_3$ as an ion strength adjuster for 30 min, followed by filtration[7]. This method was selected and further modified to improve the accuracy of Br^- recovery, using four alternative physico-chemical treatments applied to the filtrate[13]. The method which gave the most accurate Br^- recovery (98–100%) (Table 1) involved addition of 0.5 ml of H_2O_2 (30% w/v) into the filtrate and heating for 10 min on a water bath at 80–85°C. After cooling, Br^- was estimated by ion-selective electrode (PHM85 precision pH meter, Radiometer, Copenhagen). The excellent performance of this method may be due to the elimination of some interfering ions, particles of organic substances or dissolved gases, which