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Protoplast Culture and Plant Regeneration of Indica Rice by Nurse Culture and Encapsulation Technique

应用滋养和包埋技术进行籼稻原生质体培养和植株再生

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Abstract Protoplasts with densities of 2.2×10^5/mL to 4.6×10^5/mL on average were isolated from different developing stages of young panicles of indica rice. The technique in which the isolated protoplasts encapsulated in sodium alginate beads and nursed on calli of japonica rice showed most efficiency. Another method of protoplasts encapsulated in sodium alginate beads without nurse feeder was also successful. Protoplasts were stable and their division and colony establishment in sodium alginate beads with nurse feeder were shown up to 48.4% and 5.3%, respectively, and without nurse feeder up to 38.8% and 4.7%, respectively. The both methods were superior to agarose entranpment and agarose bead methods with the latter being a standard method. The two successfully improved methods would be useful for gene transfer and other biotechnological studies in indica rice.

Key words indica rice, protoplast culture, plating efficiency, plant regeneration

摘要 从几个籼稻品种不同发育阶段的幼穗分离出密度平均为 2.2×10^5 个/mL~4.6×10^5 个/mL 的原生质体。将这些原生质体包埋在藻酸钠颗粒里并分别在粳稻愈伤组织滋养下培养和没有愈伤组织滋养。在这两种培养条件下原生质体稳定，分裂频率分别达到48.4%和38.8%，群体存活率达到5.3%和4.7%，愈伤组织绿苗分化率达到71.6%和62.3%。这两种方法优于常用的琼脂糖包埋法和琼脂糖颗粒法。

关键词 籼稻 原生质体培养 植板率 植株再生
中图法分类号 S 511.210.353

Protoplast culture and plant regeneration in indica rice are still in lower efficiency compared with that in japonica rice[9~11]. Identification of responsive genotypes, development of suitable methods for protoplast isolation and nurse culture are necessary to japonica and indica rice[7,13,14,16]. Efficient and reproducible protocols for protoplast culture, high frequency colony formation and subsequent regeneration are prerequisites for gene transfer studies.

In this study the young panicles of indica rice were dealt by encapsulating isolated protoplasts in sodium alginate beads and nurse culture. High plating efficiency in protoplast division, colony formation and plant regeneration were obtained. The protocol can be extended and utilized in developing transgenic rice with more efficiency.

1 Materials and methods

1.1 Materials preparation

About 1.5 g of young panicles of each sample in the stages with < 5 mm, 6 mm ~ 10 mm and 11 mm ~ 20 mm in developing length was collected from Indica rice varieties, PD 12, PMS 2A, PR 106, UPR 92-26 and IR62871, and chipped into thin pieces before digested with 15 mL of an enzyme mixture containing 6% (w/v) cellulase RS and 1.0% (w/v) macerozyme R10, 0.5% (w/v) driselase, 0.4 mol mannitol, 5

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mol 2N MES (N－morpholinoethane sulphonic acid) with salts of CPW medium[5], at pH 5.6. Protoplasts were purified from the cell debid by passing the protoplast enzyme suspension through 35 μ to 45 μ nylon mesh, then pelleted and washed twice in CPW medium with 0.4 mol mannitol by centrifugation at 70 g for 7 min. Protoplast yield was determined using a haemocytometer. Protoplast viability was estimated using fluoresce in diacetate[8].

1.2 Protoplast culture

Fresh protoplasts isolated were cultured through the following techniques:

1.2.1 Protoplast encapsulated in alginate beads

Equal volumes of double strength N_6[2] protoplast culture medium containing protoplasts and 2.4% (w/v) sodium alginate solution with 0.27 mol sucrose, 0.08 mol maltose were mixed properly. Alginate beads were prepared in a solution of 100 mol $CaCl_2$ and 0.4 mol mannitol in a petridish, in which the formed beads were allowed to immersed in $CaCl_2$ solution for 30 min to 35 min, and then the $CaCl_2$－mannitol solution was replaced by protoplast culture medium.

1.2.2 Protoplasts encapsulated in alginate beads and nursed on feeder calli

Young embryos of japonica genotype Taichung 65 were cultured in sodium alginate containing N_6 medium supplemented with 2.0 mg/L 2, 4－D. Globular calli were induced. These calli in size of about 2 mm were picked up and laid in a petridish, and embedded in the condition medium for callus induction. A filter paper was placed on the feeder calli. The alginate beads containing protoplasts which were prepared as the former method (1.2.1) were formed on the filter paper.

1.2.3 Protoplasts encapsulated in agarose beads

Equal volumes of protoplast suspensions in double strength N_6 protoplast culture medium and 2.4 % agarose were mixed. The mixture as small beads was immediately placed in a plastic petridish. Subsequently, the beads were encapsulated by spreading of single strength N_6 protoplast culture medium onto the petridish.

1.2.4 Protoplasts embedded in agarose

In this agarose entrapment technique, equal volumes of double strength N_6 protoplast culture medium containing protoplasts and 2.4% agarose were mixed. The mixture of suspension containing protoplasts was plated in petridishes. This is a standard technique used for inducing calli from protoplasts of indica and japonica genotypes.

Observation from first division of protoplasts to callus formation was recorded in all the treatments. Plating efficiency in terms of microcallus formation was estimated by the total number of protoplasts encapsulated accounting for the number of colonies obtained.

1.3 Plant regeneration

The 1/2 strength MS[11] supplemented with various combinations of 1.0, 2.0 or 3.0 mg/L IAA (indoleacetic acid), 0.5, 1.0 or 2.0 mg/L BA (6－benzylaminopurine) and 0.1, 0.2, 0.4, 0.6, or 1.0 mg/L kinetin were used for plant regeneration. The calli (＞1.5 mm) induced in agarose media were picked up from the agarose surface and transferred to regeneration medium. The microcalli induced in alginate media were freed by dissolving the beads in a depolymerizing solution containing 0.1 mol sodium citrate, 0.175 mol sucrose and transferred to regeneration medium.

The frequency of plant regeneration was recorded. Plantlets with well－developed roots were cleaned up and transferred to opened containers in which the roots were submerged in the distilled water for 5 days of hardening, then to soil in pots.

2 Results

2.1 Protoplast yields obtained from different varieties and in different panicle developing stages

Protoplasts were isolated from the five varieties, PD 12, PMS 2A, PR 106, UPR 92－6 and IR 62871 in three developing stages with young panicle lengths, ＜5 mm, 6 mm～10 mm and 11 mm～20 mm. The protoplast yields in terms of final density are showed in Table 1. The difference of protoplast density between the varieties is less as compared with their panicle developing stages. The data clearly shows that the shorter the length of young panicles, the higher the final protoplast density obtained. However, even though the panicles has developed up to 20 mm in length, they are still suitable for protoplast isolation. But in the case of rice, the young panicles is not easily collected.

Table 1 Final protoplast density isolated from different developing stages of young panicles

Variety	Densities in different developing stages ($\times 10^{-5}$, no. /mL)			Mean ($\times 10^{-5}$, no. /mL)
	< 5 mm	6 mm ~ 10 mm	11 mm ~ 20 mm	
PD 12	4.5	4.2	2.6	3.8
PMS 2A	4.2	3.8	1.7	3.3
PR 106	3.8	3.6	2.0	3.1
UPR 92-6	5.6	3.8	2.3	3.9
IR 62871	4.9	4.0	4.2	3.7
Mean	4.6	3.9	2.2	

2.2 Relation of protoplast encapsulation methods and plating efficiency

The method for protoplast encapsulation in sodium alginate beads and subsequent culture was found to be superior to both agarose entrapment and agarose bead methods, while the nurse culture of alginate encapsulated protoplasts was the best method (Table 2). No significant difference was found within varieties. Protoplasts encapsulated in sodium alginate beads were more stable than encapsulated in agarose. Highest division speed of protoplasts was obtained on N_6 protoplast culture medium supplemented with 1 g/L L-proline, 2 mg/L 2, 4-D, 0.27 mol sucrose and 0.08 mol maltose.

The plating efficiency was analyzed by estimating the number of microcalli obtained out of the total number of protoplasts encapsulated after dissolving the alginate beads using a depolymerizing solution and subsequent counting in a haemocytometer. Nurse culture of alginate encapsulated protoplasts gave rise to earliest (2 days) and highest frequency (48.4%) of protoplast division, microcallus formation and plating efficiency (5.3%). Alginate bead method gave rise to 38.8% plating efficiency in terms of protoplast division and 4.7% microcallus formation and was significantly greater as compared with agarose bead and agarose entrapment methods (Table 2).

The Ca^{++} ions perform an important function during cell wall synthesis[6]. The higher plating efficiency was obtained in alginate beads than in agarose beads, and agarose entrapments may be due to the positive response of protoplast membranes to high Ca^{++} concentration during the calcium alginate bead formation.

2.3 Plant regeneration related to different concentrations of hormone supplements and encapsulation methods

Maximum efficiency of plant regeneration was obtained on 1/2 strength MS medium with 1 mg/L IAA, 2 mg/L 6-BA, 0.6 mg/L kinetin and 3% sucrose (Table 3). This hormone combination was applied to the plant regeneration of all calli obtained by the four protoplast culture methods in the Table 2.

The calli grew most healthy in nurse culture with alginate encapsulation and further led to the highest plant regeneration frequency, 71.6% of the 680 calli being regenerated. In the case of alginate encapsulation method without nurse feeder, out of the 705 calli plated on regeneration medium, the 439 calli (62.3%)

Table 2 Mean division, microcallus and callus formation and plant regeneration from young panicle derived from protoplasts of five indica rice varieties[a]

Method	Protoplast culture					Plant regeneration		
	Stability $x \pm$%	Callus formation		Plating efficiency[b] $x \pm$%		Total no. of calli plated	No. of calli regenerating green plants[c]	Plant regenerating (%)
		2~4[d]	15~18[d]	PD	CF			
Alginate beads	93±3	First division	Microcallus visible	38.8±2.2	4.7±0.46	705	439	62.3
Nurse culture with alginate beads	95±4	First division	Microcallus visible	48.4±3.5	5.3±0.34	680	487	71.6
Agarose	70±2	—	Cell colony	20.2±1.5	0.8±0.17	380	170	44.7
Agarose entrapment	68±1	—	Cell colony	18.3±2.8	0.7±0.15	452	256	56.6

a) Varieties: PD 12, PMS 2A, Pr 106, VPR 92-6 and IR 62871; b) PD = Protoplast division; CF = colony formation; c) Plant regeneration medium: 1/2 strength MS + 1 mg/L IAA + 2.0 mg/L 6-BA + 0.6 mg/L KT + 6 g/L agar; d) Days after culture.

Table 3 Mean plant regeneration frequency in different concentration of supplements *

IAA (mg/L)	6-BA (mg/L)	Regeneration frequency (%) 0.1	0.2	0.4	0.6	1.0
1.0	0.5	36.4	45.2	59.3	58.6	52.3
1.0	1.0	40.3	47.7	60.2	64.3	58.1
1.0	2.0	52.0	50.2	69.3	70.0	68.2
2.0	0.5	38.7	44.4	50.6	62.9	62.4
2.0	1.0	47.5	52.2	58.2	63.2	60.3
2.0	2.0	56.9	60.9	62.5	64.3	62.8
3.0	0.5	42.2	42.9	44.3	48.2	60.3
3.0	1.0	46.6	47.2	47.4	50.1	52.6
3.0	2.0	45.3	46.6	48.1	50.6	51.3

*1) Basic medium: 1/2 strength MS+1 g/L L-proline+3% sucrose+6 g/L agar; 2) Varieties: PD 12 and UPR 92-6; 3) Protoplasts cultured by alginate bead method

showed regeneration response to green plantlets. In agarose bead and agarose entrapment methods both 380 and 452 calli were transferred to regeneration media, and 170 (44.7%) and 256 (56.6%) showed regeneration responses, respectively (Table 2).

Duration from protoplast isolation to plant regeneration by using the alginate bead methods (60 days) was reduced by 15 days in comparition with agarose bead and agarose embedding methods (75 days), because the microcalli were induced earlier and grew faster and healthier in the alginate beads.

The rooting was done by the plantlets being transferred to 1/2 strength MS supplemented with 0.5 mg/L NAA, and 75% of the plantlets with well developed root systems survived in soil.

3 Discussion

Plant regeneration from protoplasts through encapsulation in alginate beads has been reported in dicots[4]. In indica rice, using encapsulation method for the plant regeneration from protoplasts derived from mature and immature embryo or anther suspension cultures has been reported[3,9]. Protoplast culture from immature inflorescences of rice was also reported by X. Y. Cheng, but the protoplasts were not stable and most of them collapsed within 48 h in either liquid or agarose solidified medium. No remarkable result of plant regeneration was obtained[1]. Method of protoplasts capsulated in agarose beads has also been used but only a few plants were regenerated[15]. In the present study with more varieties involved. young panicles were directly used for protoplasts isolation, a large number of protoplasts being cultured and plantlets being regenerated. The method of nurse culture with alginate encapsulation was most successful, and the method of alginate encapsulation without nurse feeder also showed good results in application to the culture of young panicles derived from protoplasts of indica rice. It is considered using the young panicles for producing protoplasts could save the time of in vitro culture, compared with the protoplasts derived from cell suspension culture and further lead to high frequency of plant regeneration. The protocol of protoplast culture and plant regeneration are useful for the gene transfer or other biotechnology studies in indica rice.

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