

Protoplast Culture and Plant Regeneration in Wild *Oryza* Species with Promotion by Nurse Cell Liquid and Suitable Proliferation Media

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野生稻原生质体培养与植株再生

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摘 要:三个野生稻(*O. rufipogon*, *O. glumaepatula* 和 *O. latifolia*)成熟胚,经愈伤组织诱导和悬浮细胞培养,分离原生质体,利用简化原生质体培养基(SPCM),结合琼脂糖包埋,并附加看护细胞培养液,这三个野生稻原生质体均得到了成功培养,其中两个(*O. rufipogon* 和 *O. glumaepatula*)分化了绿色植株。看护细胞液在野生稻原生质体培养中,可促进细胞分裂,提早细胞分裂始期,细胞分裂频率(33.2%)和植板率(19.0%)分别比未加看护细胞液的培养方法高出13.3%和6.2%。看护细胞液可大大减少野生稻愈伤组织在液体中培养始期的死亡率,提高建立其悬浮细胞的成功率。原生质体克隆在适当的后培养基上进行培养,可以显著改良其结构,明显促进绿苗分化。尤其是L3培养基诱导原生质体克隆形成胚性愈伤组织或类似胚状体结构的频率达32.1%,显著高于MS和N6培养基,从而导致较高绿苗分化频率。

关键词:野生稻;原生质体培养;植株分化;看护细胞液;后培养基

Abstract: The protoplasts of three *Oryza* species (*O. rufipogon*, *O. glumaepatula* and *O. latifolia*) were successfully cultured by using agrose-bead method in simplified protoplast culture medium (SPCM) with or without the nurse cell liquid through cell suspensions from mature embryos. Two of them (*O. rufipogon*, *O. glumaepatula*) regenerated green plantlets from protoplast-derived calli. The nurse cell liquid covering agrose-bead protoplasts could promote cell division and significantly increase the frequency of cell division (33.2%) and colony formation (19.0%). The hardening for protoplast-derived calli on the suitable proliferation media greatly benefited green plantlet regeneration. In the course of callus hardening, L3 medium showed the highest efficiency (32.1%) in the formation of embryogenic calli and embryo-like structure, thus resulting in producing more green plants.

Key words: nurse cell liquid; plant regeneration; proliferation medium; protoplast culture; wild *Oryza* species

Wild *Oryza* species are widely distributed in the humid tropic area throughout the world. They have been used as genetic resources for resistance to diseases and insects, for tolerance to saline soil and environmental stresses in rice improvement^[5], as well as the donor of CMS cytoplasm for male sterile lines

in hybrid rice^[12-15].

In the utilization of some sophisticated charac-

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ters of wild rice, breeders often encounter the problem of interspecific hybrid embryo abortion^[8,9], and have to make backcross of wild rice with recurrent cultivars for 5-8 generations. Although a few of scientists had successfully used embryo rescue technique to overcome embryo abortion, and anther culture of F_1 progenies to shorten breeding period in cross breeding between cultivated rice and wild rice, asymmetric protoplast fusion is even much more effective and time saving in transferring the cytoplasmic traits, such as cytoplasmic male sterility in mitochondrion, from wild species to cultivated ones, alleviating the introgression of undesired characters controlled by nuclear of the donor. However, protoplast fusion only can be carried out when protoplast isolation, culture and plant regeneration are established. Several research workers have reported the successful culture of wild rice. Wang^[13] obtained the regenerated plants through somatic embryogenesis from mature seeds and young inflorescences of wild rice, *Oryza perennis* Moench. Hayashi^[2] established cell suspension from four *Oryza* species (*O. officinalis*, *O. eichingeri*, *O. brachyantha*, *O. perrieri*) for cell fusion. However, no report presented successful culture of protoplasts and plant regeneration in other wild *Oryza* species. In order to establish protoplast culture system in wild rice for asymmetric cell fusion, we collected 28 tested genetic resources of wild *Oryza* species which have potential characteristics of cytoplasmic male sterility^[11] to carry out mature embryo tissue culture. After tissue culture selection, we chose three wild rice resources, i. e. *O. rufipogon*, *O. glumaepatula* and *O. latifolia*, which showed a good culturability to continue cell suspension and protoplast culture. In this paper, we describe protoplast culture and plant regeneration through cell suspensions in these species of wild rice and promoting effects of nurse cell liquid and suitable proliferation medium on protoplast culturability in wild *Oryza* species.

1 Materials and Methods

1.1 Rice seeds

Seeds of *O. rufipogon* (IRRI Acc. No.

104866), *O. glumaepatula* (IRRI Acc. No. 100969) and *O. latifolia* (IRRI Acc. No. 101443) and other 25 wild rice accessaries were kindly provided by Dr. D. A. Vaughan at the International Rice Research Institute. Three wild *Oryza* species tested have good culturability (data not shown) and potential cytoplasmic male sterility^[11].

1.2 Induction of callus and establishment of cell suspensions

Dehulled seeds were surface-sterilized in 20% NaClO solution for 40 minutes and washed 3 times in sterilized water. The sterilized seeds were inoculated on MS medium supplemented with 2.0 mg/L 2,4-D, 0.2 mg/L BAP, 5.0 mg/L proline and 500 mg/L casein hydrate. When callus was induced, the embryogenic ones were selected during subculture on N6 medium with 2.0 mg/L 2,4-D, 5.0 mg/L proline, 5.0 mg/L tryptone, 0.2 mg/L BAP and 0.05 mg/L ABA for 1-2 times every 4 weeks.

After subculture and improvement of callus, the embryogenic calli with globular and loose structure were transferred to flask containing AA liquid medium supplemented with 2.0-4.0 mg/L 2,4-D, 0.2 mg/L KT, 0.1 mg/L GA₃, 10 mg/L proline, 5.0 mg/L tryptone and 10 mg/L AgNO₃ with shaking at 26°C. The sucrose in AA medium was substituted by 30 g/L maltose. At the beginning of liquid culture, calli were subcultured every 4 days by replacing all the original medium with two-thirds of the same fresh medium and one-third of the nurse cell liquid. The nurse cell liquid was referred to as the filtered and pH adjusted culture liquid from cell suspension of rice variety 02428. When aggregates grew well, cultures were subcultured every 6 days by replacing two-thirds of the original liquid medium with the fresh one.

1.3 Protoplast isolation and culture

Four days after subculture, 1-2 grams of cell suspensions were incubated in 10 mL enzyme solution^[14] shaking at 50 rpm in darkness. After 3-4 hours shaking, the digested cell were filtered through two layers of 3 μ m nylon mesh cell filtration apparatus. The protoplasts were collected in tubes and washed with CPW13 for at least two times by

being centrifuged for 5 minutes at 500 rpm.

After that, protoplasts were resuspended in simplified protoplast culture medium (SCPM)^[14], then cultured by using the agrose-bead method with a final cell density of $1-3 \times 10^5/\text{mL}$. After being solidified, 0.5 mL agrose-bead protoplasts in petri dish were covered by 1.0 mL osmoticum adjusted liquid composed of one half of SPCM and another of the nurse cell liquid. The medium was renewed by the fresh SPCM medium supplemented with the nurse cell liquid every 5 days with gradual osmotic reduction to a final composition of 0.175 mol/L.

1.4 Proliferation of protoplast-derived calli and plant regeneration

When protoplast-derived calli reached 1-2 mm in diameter, half of them were transferred onto the regeneration medium and the other half onto three solid proliferation media for callus hardening. The three media were MS, N6, L3^[3], of which L3 medium combined inorganic nitrogen sources with organic nitrogen, and contained maltose as carbon source. All supplemented with 20% potato extracts, 2.0 mg/L NAA and 0.5 mg/L ABA for 2-3 weeks.

Plant regeneration medium was MS medium with 4.0 mg/L BAP, 0.5 mg/L NAA and 0.5 mg/L IAA under 16 hour photoperiod. After 5 weeks of culture, green plantlets were counted. When plantlets fully developed, they were transferred to hormone free 1/2 MS medium for plant growth.

2 Results

2.1 Establishment of cell suspension cultures

At the beginning of the experiment, twenty wild *Oryza* species were tried to induce calli. There existed a significant genotype effect on culture response with a wide range of frequency of calli induction at 0-69% and embryogenic cell initiation at 0-43%, respectively. Three species, *i. e.*, *O. rufipogon*, *O. glumaepatula*, *O. latifolia*, with a higher frequency of embryogenic calli, were chosen to continue our studies.

For initiation of embryogenic cell suspension cultures, it was important to collect the calli with globular and compact but loose shattering structure

at first two subcultures on induction medium. After the calli were inoculated in modified AA liquid medium for about two weeks, some of them grew and some became brown in color. After the brown calli were discarded, 2,4-D concentration adjusted to 4.0 mg/L and the content of sugar reduced, the growth rate of cultures significantly sped up. Afterwards, cultures with fine aggregate were collected. During this period, it was essential to remove the old medium completely and add fresh medium containing one-third of the filtered culture liquid from nurse cell (c. v. 02428). After 3 weeks, no brown calli were seen and the growth of aggregates was greatly promoted. Subsequently, the well-grown cultures released many miniclusters into liquid medium. After separated from the cultures, the mini-clusters proliferated and increased rapidly. For about 3 months of culture, the cell aggregates each consisting of 20-80 cells with fresh yellow color and dense cytoplasm (Fig. 1A) constituted more than 90%, which were suitable for isolating protoplasts.

2.2 Protoplast isolation and culture

Protoplasts (Fig. 1 B) were isolated 4-5 days after subculture of cell suspensions. The yield of protoplast isolated from the cell suspensions could reach 2.4×10^7 prot./gram cells. When protoplasts were cultured in agrose-bead SPCM medium, protoplasts could be seen expanding and shaping ellipse on the second day after embedded in the medium. The first cell division (Fig. 1 C) could be observed on the third day when the agrose-bead protoplasts were covered in the nurse cell liquid, which was two days earlier than that without nurse cell liquid (conventional agrose-bead method, Table 1). The frequency of cell division (Fig. 1 C, D) and plating (Fig. 1 E) with the nurse cell liquid were much higher than the conventional agrose-bead method (Table 1).

2.3 Subculture of calli derived from protoplasts

When protoplast-derived calli were 1-2 mm in diameter, they were transferred to proliferation media for callus hardening. In the course of proliferation, the calli (Fig. 1 F) propagated quickly and developed into embryogenic calli. Compact, smooth surfaced embryo-like structures formed in about 3

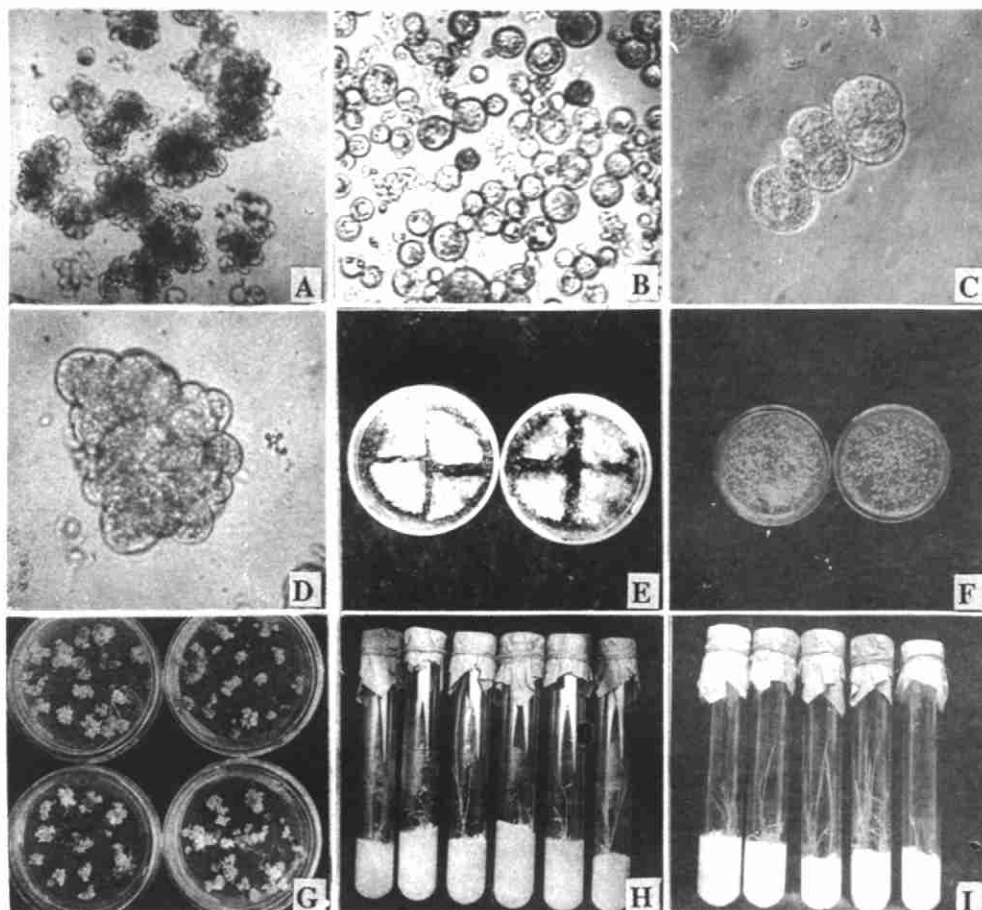


Fig. 1. Embryogenic cell suspension, protoplast culture and plant regeneration in wild *Oryza* species. A. Embryogenic cell suspensions initiated from callus cultures. B. Isolated protoplasts from cell suspensions. C. Initial cell division. D. Cell clone with several cell divisions. E. Protoplast-derived mini-clusters. F. Protoplast-derived calli hardening on L3 basal proliferation medium. G. Protoplast-derived calli regenerating. H. Green plantlets regenerated from *O. rufipogon*. I. Green plantlet from *O. glumaepatula*.

weeks after being transferred to the media. The embryogenic calli, somatic embryo-like structures and unembryogenic calli occurred simultaneously in each plate. A comparison of three protoplast proliferation media suggested that L3 was the best medium which led to a higher frequency of embryogenic callus and embryo-like structure formation (Table 2) and a faster growth of protoplast-derived callus.

2.4 Plant regeneration

In the case of being directly transferred to regeneration medium, protoplast-derived calli from 10^6 protoplasts per species, only produced 2 plantlets

from *O. rufipogon*, most of the calli failed to occur organogenesis or stopped growing or grew slowly and gradually became browning. When the calli from the same amount of protoplast per species were sub-cultured for callus hardening, 37 green plantlets (Fig. 1 G, H, I) were regenerated from two species, *O. rufipogon* (21 plants) and *O. glumaepatula* (16 plants). However, *O. latifolia* produced no green plantlets except several albino ones. Of three species, the calli capable of regenerating plant still proliferated (Fig. 1 G), but most of the calli only increased in size, some of them merely produced

Table 1. Comparison of different culture methods in protoplast culture of the three wild *Oryza* species

Species	CAM			NAM		
	1st cell division ¹⁾	Freq. of cell division (%)	Freq. of plating (%)	1st cell division	Freq. of cell division (%)	Freq. of plating (%)
<i>O. rufipogon</i>	4	23.7	14.5	3	30.1	19.6
<i>O. glumaepatula</i>	6	19.8	11.8	3	35.6	22.4
<i>O. latifolia</i>	5	18.5	11.8	4	33.9	15.0
Average	5	20.5	12.7	3.3	33.2	19.0

CAM, Conventional Agrose-bead method; NAM, Nurse cell liquid modified agrose-bead method.

¹⁾ The day from protoplast cultured in agrose-bead to 1st cell division.

Table 2. Effect of different basal proliferation media on the growth of wild *Oryza* species protoplast-derived calli

Genotype	No. of visible calli for proliferation (per treatment)	Calli proliferated						Embryogenic calli & embryo-like structures					
		MS		N6		L3		MS		N6		L3	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>O. rufipogon</i>	470	221	47.0	308	65.5	316	62.7	79	16.8	104	22.1	189	40.2
<i>O. glumaepatula</i>	470	192	40.9	231	49.1	275	58.5	50	10.6	124	26.4	166	35.3
<i>O. latifolia</i>	320	126	39.4	130	40.6	119	37.2	32	10.0	58	18.1	49	15.3
Total	1260	539	42.8	669	53.1	710	56.3	161	12.8	286	22.7	404	32.1

roots and others displayed green color on the callus surface without shooting and rooting.

3 Discussion

Green plants were successfully regenerated from protoplasts of wild *Oryza* species, *O. rufipogon* and *O. glumaepatula*. This study indicated that simple medium (SPCM) was also suitable to protoplast culture of wild rice in addition to cultivated rice^[14]. The results demonstrated that medium is not crucial to protoplast culture. Suspension cell line with a good growth status and dense cytoplasm, and embryogenic character played a decisive role in successful culture of protoplasts. In this experiment, the nurse cell liquid contributed much to cell suspension culture and protoplast culture. Many reports claimed that cell suspensions could liberate various biochemical factors to the medium^[1,10], which presumably belong to organic acids or oligosaccharides^[1,6] or proteins^[4]. Growth of the cultures in liquid medium and the colony formation were considered to be improved

by the factors liberated from nurse cell suspensions^[4]. On the other hand, in using the factors released from nurse cell suspensions for protoplast culture, most researchers directly placed nurse cells in the culture plate^[7]. In our experiment, the nurse cell liquid filtered from cell suspensions was put on the agrose-bead protoplasts, which had the equal effect on culture and greatly simplified the technique.

The culture system described above have been employed for asymmetric cell fusion between wild *Oryza* species and cultivated rice to develop a new-type of CMS line. Progress in the formation of cybrid and regeneration have being achieved.

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更正 Erratum

- 1)第9卷第1期,第3页图1左边的说明自上而下应为:W262、W178、W174、W165、W160、W157、W124、W121、W103、W101、W87、W85、W55、W54、W37、W36、W35、W34、W32、W29、W28、W25、W23、W8、W3 (*O. rufipogon*);福保、科情3号(japonica);桂朝2号、双桂36(indica)。
- 2)第9卷第2期,第125页Table 2倒2行,数据0.582应为6.582。