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CALLUS FORMATION AND PROTOPLAST ISOLATION IN RICE (*Oryza sativa* L.) AND ITS RELATED SPECIES

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Introduction

Rice is one of the most important staple foods in world and various kinds of traditional breeding methods have played an important role in rice improvement. In addition, some new techniques such as tissue cultures and gene engineering are essential to raise the efficiency of rice breeding. In culture techniques, the success of callus formation, protoplast isolation and plant regeneration from the various tissues or organs are required for the universal genotypes.

Especially protoplast isolation and plant regeneration from the calli using a wide range of materials are indispensable for the use of somaclonal selection and production of somatic hybrids and cybrids.

In this paper the authors examined the effects of various genotypes involving 12 species of the genus *Oryza* for callus formation and protoplast isolation.

Materials and Methods

The genome constitution of species and the number of strains used are shown in Table 1. Dehulled seeds were surface sterilized using 70% alcohol for 1 minute, then by 10% commercial bleach for 2-3 minutes, followed by washing 2-3 times with sterilized distilled water. 10 seeds per strain were planted on the N6 medium supplemented with 4.4mg/l 2,4-D, 50g/l sucrose and 10g/l agar, and incubated in the darkness at 27°C. Calli from the seeds were subcultured every 10 days. Callus growth was assessed by measuring the diameter of callus 50 days after seed planting. The interspecific variation of callus growth was evaluated by Duncan's multiple range test. In this calculation, intraspecific variance were estimated by means of strains within the same species except for *O. nivara* which was estimated from variance among individuals.

Seed calli (0.5g) at 90 days after plating for the 40 species were added to 10ml

Table 1. 12 species of the genus, *Oryza* used for the experiments

Species	Genome	No. of strains
O. sativa	AA	63
O. glaberrima	A^gA^g	8
O. rufipogon	AA	38
O. punctata	BBCC, BB	5
O. minuta	BBCC	5
O. officinalis	CC	8
O. australiensis	EE	5
O. latifolia	CCDD	5
O. gradiglumis	CCDD	5
O. brachyantha	FF	5
O. barthii	A^gA^g	5
O. nivara	AA	1
Total		153

Table 2. Enzyme solution for protoplast isolation

Driselase	1%
Cellulase "Onozuka" RS	2%
Cellulase "Onozuka" R-10	2%
Macerozyme R-10	2%
Hemicellulase	1%
Pectoryase Y-23	0.1%
Potassium dextran sulfate	0.2%
Albumin	0.05%
Sorbitol	0.35M
Mannitol	0.35M
MES	20mM
$MgCl_2$	5mM
pH	5.6

enzyme solution (Table 2) and incubated in the darkness on a reciprocal shaker at 50 rpm for 6 hours at 28°C. The protoplast enzyme mixture was filtered through a 42μm nylon filter. Filtrated protoplasts were used for microscopic observation for counting.

Yields of protoplast were calculated by hemacytometer. The experiments were repeated nine times, and the average number of nine replications was transformed to a number of protoplasts per one gram of fresh weight of calli.

Results

1. Callus induction and growth

Callus induction was achieved in the strains of 12 species except for a strain of *O. rufipogon* and a strain of *O. latifolia* as shown in Table 3. In the cultivated species, *Oryza sativa* L., a considerable range of variation was observed in the callus growth both in Japonica and Indica type. It is noted that the mean diameter of calli in Japonica was significantly larger than that in Indica after 50 days of culture. In addition, the callus growth of African rice (*Oryza glaberrima*) was nearly equivalent to that of Indica. The wild species indicated an inferior growth as compared with the cultivated species. According to Duncan's multiple range test (Table 4), the interspecific variation of callus growth was classified into 4 or 5 groups.

2. Protoplast isolation

An isolation of intact protoplasts was achieved in 15 strains of Japonica, 12 strains of Indica in *Oryza sativa*, 2 strains of *O. officinalis* and *O. barthii* and 16 strains of *O. rufipogon*. Different protoplast yields in Japonica strains were obtained (Table 5). It is noted that the some superior cultivars such as Koshi-

Table 3. Comparison of callus growth among 12 species

Species	No. of strains	No callus formation	Diameter of callus(mm)											Mean
			0	1	2	3	4	5	6	7	8	9	10	
O. Sativa	63		1		3	10	5	14	15	4	6	3	2	5.4
Japonica	30					1	1	7	8	2	6	3	2	6.7
Indica	33		1		3	9	4	7	7	2				4.3
O. glaberrima	8					2	1	2	2	1				4.9
O. rufipogon	38	1		1	7	19	8		2					3.0
O. punctata	5				3	2								2.4
O. minuta	5			3	2									1.3
O. officinalis	8			5	2	1								1.3
O. australiensis	5			1	1	3								2.5
O. latiforia	5	1	1		3									1.7
O. gradiglumis	5				1	2	2							3.0
O. brachyantha	5		2		3									1.1
O. barthii	5				1	2	1		1					3.6
O. nivara	1					1								3.0
Total	153	2	4	10	26	42	17	16	20	5	6	3	2	2.7

Note; Diameter after 50 days from plating.

Table 4. Dancan's multiple range test for the interspecific variation

Species	Mean diameter of callus (mm)	Dancan's test (p=0.05)
O. sativa (Japonica)	6.7	a*
O. glaberrima	4.9	b
O. sativa (Indica)	4.3	bc
O. barthii	3.6	cd
O. rufipogon	3.0	de
O. nivara	3.0	de
O. gradiglumis	3.0	de
O. australiensis	2.5	def
O. punctata	2.4	efg
O. latifolia	1.7	fgh
O. officinalis	1.3	gh
O. minuta	1.3	gh
O. brachyantha	1.1	h

Note *; Means designated by the same letter show no significance.

hikari, Shiokari and Kinmaze showed a high yield of protoplasts. In Indica type, only one glutinous strain (Thaiglu. rice) from Thailand indicated the highest yield than the other strains (Table 6). In the strains from wild species, the protoplast yields varied between 0 and 4.0×10^9 protoplasts per g fresh weight of calli (Fig. 1).

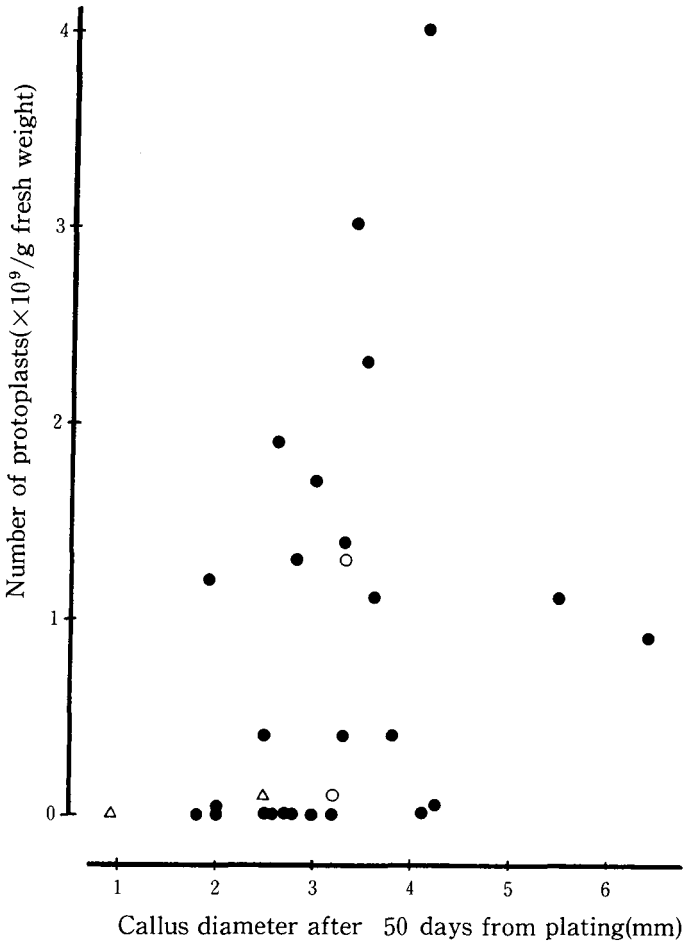

Fig. 1. Correlation between callus growth and protoplast yield in three wild species

● : *O. rufipogon*
○ : *O. officinalis*
△ : *O. barthii*

3. Relation between callus growth and protoplast yield

Fifteen strains of Japonica type showed no correlation ($r=-0.456$) between callus growth and protoplast yield (Table 5). The strains that produced prolific calli indicated rather inferior yields of protoplasts in Japonica strains. In Indica strains, the strain that occupied the second rank in callus growth resulted in the highest yield of protoplasts. Although the correlation coefficient was not significant ($r=0.538$), there was a tendency for protoplast yields to increase proportionally when callus growth is promoted in a restricted range as shown in Table 6. In protoplast yields of three wild species, there were no correlation ($r=0.305$)

Table 5. Relation between callus growth and protoplast yield in Japonica strains of *O. sativa* L.

Name of strains	Diameter of callus (mm)	No. of protoplasts ($x10^9$/gfw)
Norin 10	9.2	1.1
Kochihibiki	8.4	0.1
Kochikaze	8.2	0.2
Todoroki-wase	7.6	0.6
Reimei	6.6	0.4
Jukkoku	6.3	0.3
Reihou	6.1	0.4
Shiokari	5.8	3.1
Akibare	5.4	0.4
Houyoku	5.4	0.6
Kinmaze	4.9	2.0
Tangin-bouzu	4.9	3.2
Akihikari	4.5	1.3
Koshihikari	3.8	4.0
Hokuriku 100	3.4	0.4
Mean	6.0	1.2

Table 6. Relation between callus growth and protoplast yield in Indica strains of *O. sativa* L.

Name of strains	Diameter of callus (mm)	No. of protoplasts ($x10^9$/gfw)
Calrose 76	7.0	1.4
Thai-glu. rice	6.7	6.1
Assam III	6.3	0.1
Calrose	5.9	0.4
Dalashaita	4.5	0.0
Surjumkhi	4.5	0.0
Bhutmuri 36	4.2	0.04
Dular	3.5	0.1
Hu-nan-zao	3.1	0.2
Mitsutari	3.0	0.2
Charnock	2.4	0.03
Mushakdanti	2.2	0.02
Mean	4.4	0.7

between callus growth and protoplast yield (Fig. 1). But the same tendency was seen in Indica strains. For *O. officinalis* and *O. barthii*, the number of strains used for protoplast isolation were only two, however the tendency of relation was almost similar with that of *O. rufipogon*.

Discussion

Intervarietal variation in callus induction from rice seeds was examined using Japonica, Indica and their hybrids. According to MAEDA[9], there was no difference between Japonica and Indica for the nature and growth rate of callus. However, the present results indicated a superiority in Japonica type, supporting the findings by ABE and SASAHARA[1]. In addition, there was a wide range of variation in the both Japonica and Indica type. Therefore, it is possible to screen the strain showing a high callus formation in their respective types. In the anther culture, MIAH *et al.*[11] elucidated the inheritance mode of callus formation and suggested the possibility of improvement in callus induceability using the Indica genotypes.

The callus formation and plant regeneration in African rice (*Oryza glaberrima*) were also reported (FATOKUN and YAMADA[6]). In this experiment, there was no significant difference in callus development between Indica and African rice. Most of the strains in wild species succeeded in callus formation though the callus growth was rather inferior. Therefore, it is necessary to improve certain media suitable for these species.

Protoplast isolation in rice succeeded by using seed calli or suspension cells (YAMADA *et al.*[19], COULIBAR *et al.*[4], FUJIMURA *et al.*[7] and TORIYAMA and HINATA[15]), by anther calli (CAL *et al.*[3] , WAKASA *et al.*[17], TORIYAMA and HINATA[15]) and young leaves or mesophyll cells (DEKA and SEN[5], SHIH *et al.*[13]). The authors isolated protoplasts from the seed calli induced from both cultivated and wild species. It is noted that Japanese familiar cultivars, such as Koshihikari, Kinmaze and Shiokari showed a high yielding ability of protoplasts. In Indica, a glutinous strain from Thailand produced the highest yield of protoplast in this experiment. In the wild species, *Oryza rufipogon,* there were a small number of strains possessing a high yielding ability. In general, there is no intimate relation between the callus growth and protoplast yield.

The plant regeneration from isolated rice protoplasts were reported by many researchers (FUJIMURA *et al.*[7], YAMADA *et al.*[19], TORIYAMA *et al.*[16], ABDULAH *et al.*[2] and COULIBALY *et al.*[4]). But there were quite a few reports for protoplast culture from wild species in *Oryza* (HAYASHI *et al.*[8]). Now, we have been attempting to culture protoplasts from wild species and a detailed examination is now progressing.

Since the formation of somatic hybrids through protoplast fusion was demonstrated between rice and soybean (NIIZEKI *et al.*[12]), and between cultivated rice and four wild *Oryza* species (HAYASHI *et al.*[8]), this technique will be effectively used for rice breeding for the transfer of male sterile cytoplasm and favorable characters from related species through somatic hybrids or cybrids.

Summary

In vitro culture methods is quite important techniques for rice breeding. Especially callus induction and protoplast isolation are essentially basic techniques for the use of somatic hybrids or cybrids.

The induction and growth of seed calli was investigated examining 153 strains of *Oryza sativa* L. and 11 species of the genus *Oryza*. A basal N6 medium containing 4.4 mg/l 2,4-D, 50 g/l sucrose and 10 g/l agar was used for callus induction from seeds. Most of the materials except a strain of *O. rufipogon* and a strain of *O. latiforia*, showed successful callus induction. A remarkable difference in callus growth was observed among the strains of wild species as well as among those of cultivated species. Seed calli from the two cultivated species, *O. sativa* and *O. glaberrima* showed better growth than the other species in *Oryza*.

Protoplast isolation from seed calli was conducted by using an enzyme solution under conditions of 6 hours at 28°C. The protoplast isolation from the callus was achieved in 15 Japonica strains, 10 Indica strains, one strain of *O. officinalis*, two strains of *O. barthii* and 16 strains of *O. rufipogon*. In the relation between callus growth and protoplast yield, the strain showing prolific callus was not always superior in the yield of protoplasts.

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