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/1 2,4-D, 50g/1 sucrose and 10g/1 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
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 42 μm 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

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

Note: Diameter after 50 days from plating.

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

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

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 (Thai-glu. 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 X 10^9 protoplasts per g fresh weight of calli (Fig. 1).
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 \( (r = 0.305) \)
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, 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. 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. 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). 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 8, 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 Shiokiari 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. latifolia, 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.
310  K. MORI, T. KINOSHITA AND Y. YAMADA

Literature Cited