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Regeneration of Plants by Pollen Culture in Rice (*Oryza sativa* L.)

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Pollen calluses and plants were obtained in rice (*Oryza sativa* L.) through pollen culture without preculture of anthers. The spikes were pretreated at 10℃ for 10-30 days. Isolated pollen grains at the late uninucleate stage were cultured in a medium consisting of one eighth to three eighths of the nutrient constituents of N_6[1] supplemented with 1% sucrose, 1% glucose, 0.1% yeast extract, 0.1% casein hydrolysate, and 10^{-5}M 2,4-dichlorophenoxyacetic acid. After 11 days of culture, small calluses were induced from the pollen grains. To regenerate plants, viable calluses were transferred from the liquid medium onto the agar media for differentiation[2]. All the regenerated plantlets, including haploid ones, were planted in paddy fields to obtain mature plants.

A culture of isolated pollen grains is a suitable approach for the analysis of haploid cell physiology and genetics, and for obtaining haploid plants. However, this method has been associated with only limited success so far. The regeneration of plants from the pollen culture is restricted to few plant species such as tobacco (*Nicotiana tabacum*),[3] barley (*Hordeum vulgare*),[4] etc. The technique for the culture of isolated pollen grains is difficult compared to that of anthers. This difference is largely due to a auxiliary function of the anther wall tissue as in the case of tobacco anther culture.[3] The successful pollen culture of barley reported by Wei et al[4]. may be ascribed to be regulation of the sucrose concentration of the culture medium rather than to the function of anther tissue. In rice, Chen et al.[5] reported that calluses could only be derived from the pollen from anthers precultured for a period of 5 days, which floated on the liquid medium.

In the current experiment, we report that calluses could be induced from isolated rice pollen without 'preculture' of anthers and that plants could be regenerated from the calluses.

Materials and Methods

Oryza sativa L. var. 'Nipponbare' was used as the material in this experiment. Spikes of rice with pollen at the late uninucleate stage were collected and pretreated at about 10℃ for 10-30 days. Pollen grains were isolated by gently crushing the anthers with a glass stick on a petri dish in distilled water. After filtration with a 100 μm pore stainless steel sieve, the pollen grains were washed twice in distilled water by centrifugation at $100 \times g$ for 2 min. Then the pollen grains were resuspended into the liquid medium, and the pollen density was adjusted to $2\text{-}5 \times 10^5$ pollen/ml. The culture was performed in ϕ 35 mm petri dishes containing 0.5 ml of pollen suspension. The culture medium consisted of the nutrient constituents of the N_6 medium[1] supplemented with 1% sucrose and glucose, 0.1% yeast extracted and casein hydrolysate, and 10^{-5}M 2,4-dichlorophenoxyacetic acid (pH 5.8). When the medium was used, it was diluted at six strengths; zero, one eighth, one fourth, three eighths, half

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and normal strength. For plant regeneration, vigorously growing calluses in the liquid medium were transferred onto two kinds of differentiation media, Murashige and Skoog medium containing 10^{-6}M NAA, 70 g/*l* sucrose and either 3 g/*l* yeast extract and 2 g/*l* casein hydrolysate or 10 mg/*l* benzyladenine[2].

Results and Discussion

No callus was obtained when the pollen grains were cultured in normal strength N_6 media. Under these conditions, the pollen viability was completely lost at the early stage of the culture, and shrivelled protoplasts were observed inside the grains. This phenomenon which was particularly conspicuous for small pollen grains was not observed when the microscopic observation was performed before isolation or culture in distilled water. Therefore, the medium was diluted (**Table 1**).

After an 11-day period, small calluses were observed in one eighth to three eights strength media (**Figs. 1, 2**) while no calluses were observed in the half strength medium even after 24 days of culture. The formation of the calluses was most remarkable in the one eighth strength the most diluted-medium. In addition, the higher the degree of dilution of the medium, the larger the number of calluses within the limit of established strength of the medium. It appears that proportion of dead pollen increased with an increase in the concentration of the culture medium. When the isolated pollen was cultured in distilled water only, it became gradually darker and the grains lost their viability.

Table 1. Effect of strength of the culture medium on the viability of pollen and callus formation in *Oryza sativa* L. var. 'Nipponbare.'

Strength of medium[a]	0[b]	1/8	1/4	3/8	1/2	1
Pollen viability[c]	±	++	++	+	−	−
Number of calluses[d]	0	11	7	3	0	0

[a] Normal strength: N_6[1] basic nutrient constituents supplemented with 1% sucrose and 1% glucose, 0.1% yeast extract, 0.1% casein hydrolysate, and 10^{-5}M 2,4-D.

[b] Cultured in distilled water only.

[c] Microscopic observation after 11 days of culture. Pollen grains with shrivelled protoplasts were counted as dead pollen.

[d] Number of calluses per petri dish after 24 days of culture.

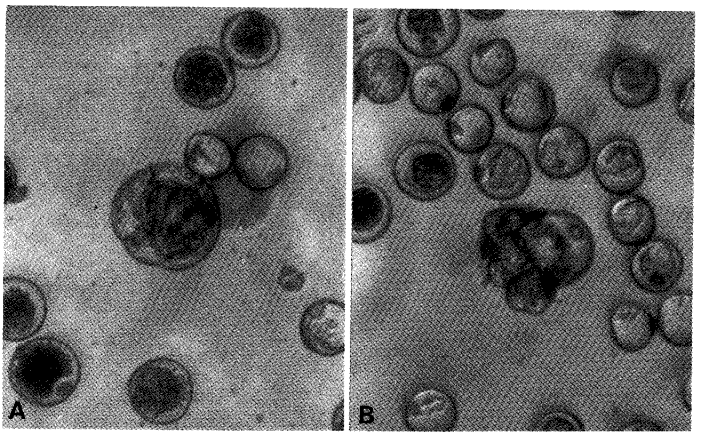

Fig. 1. Division of the pollen grain cells (A), and multicellular mass breaking out of pollen wall (B) in diluted N_6 pollen culture medium (one eighth strength) after 11 days of culture.

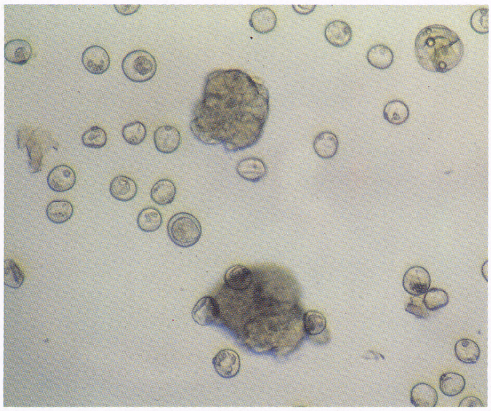

Fig. 2. Callus formation from pollen grains in diluted N_6 pollen culture medium (one eighth strength) after 24 days of culture.

Fig. 4. Plant regeneration from pollen callus on the differentiation agar medium 30 days after transfer from the callus induction medium. Diameter of petri dish is 90 mm.

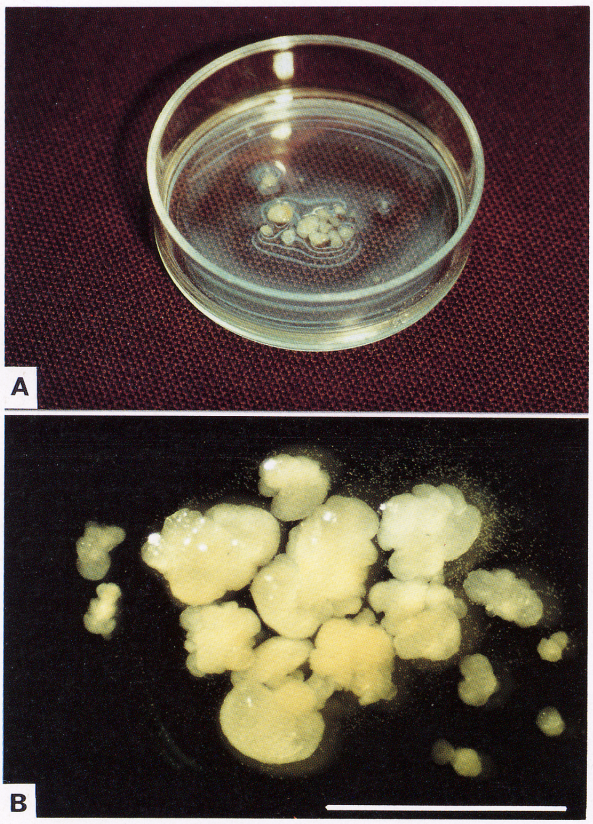

Fig. 3. Calluses growing vigorously in liquid medium ϕ 35 mm petri dish (A) after 40 days of culture; B magnification. Bar indicates 5 mm.

Fig. 5. Haploid plant obtained from pollen callus.

For plant regeneration, calluses growing vigorously in the liquid medium as indicated in **Fig. 3** were transferred onto two types of differentiation media. Several plants were regenerated on the both media. **Fig. 4** shows regenerated plantlets from pollen calluses on an agar plate, and some of them were matured in pots as illustrated in **Fig. 5**. All these plants were transplanted in a paddy field to examine agronomic characters. From the morphological traits, such as small sizes of panicle, glume and pollen with complete sterility[2] these plants were recognized as haploid.

In this experiment it was revealed that calluses could be obtained from isolated pollen grains in rice without preculture of anthers in diluted N_6 medium, and that it was possible to regenerate plants from these calluses on differentiation agar media. On the basis of these results it appears that the method should be further modified and improved to enhance the pollen viability, especially during the period from pollen isolation to early culture. In conclusion, the results obtained suggest that this simple culture technique for isolated pollen grains may enable to obtain haploid plants for the use of haploid breeding and genetical studies in rice.

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≪和文要約≫

イネの花粉培養による植物体復原

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前培養を行わない葯から単離したイネの花粉から，半数体植物を復原することに成功した．低温処理を施した葯から単離した，1核期後期の花粉を，ショ糖1%，ブドウ糖1%，イースト抽出物 0.1%，カゼイン加水分解物 0.1%，および 2,4-D 10^{-5}M を含む N_6 培地を基本の培地濃度として，それらを 1/8～3/8 に希釈した培地中で培養を行ったところ，11 日後に小カルスの形成をみた．花粉の生存，カルスの発生は 1/8 濃度の培地で最もよかった．液体培地中で形成したカルスを，植物体再分化用の寒天培地に移植することにより，数個体の植物体を復原できた．得られた植物体を順化・育成し，形態および花粉を調査したところいずれも半数体であると認められた．