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A New One-step Anther Culture Method which Allows Short Duration of Culture for Regeneration of Rice Plant through Somatic Embryogenesis

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With the aim of making anther culture more applicable to rice breeding, a new method of anther culture has been developed. The method consists of the pretreatment of anthers with phytohormone before inoculation on a hormone-free medium and regeneration of plants without transfer of the calli to a regeneration medium. The best results were obtained when anthers were pretreated with NAA or NAA plus BA. This method (one-step culture) has the following features compared with the conventional two-step culture method; 1) a higher frequency of callus formation and plant regeneration, 2) a much shorter duration of culture for plant regeneration (a half of the two-step culture method), 3) a higher ratio of haploid to diploid regenerants, 4) no segregation within A2 lines. The histological observation showed that plants in the one-step culture method were regenerated through somatic embryogenesis.

Introduction

Anther culture technique has proved useful for rice breeding. It shortens the breeding term and increases selection efficiency by producing doubled haploid plants from hybrids. In fact, a number of new cultivars have already been bred using this technique[1-3]. In spite of the practical examples, however, there are still some problems needed to be solved to make the anther culture technique more applicable to rice breeding. These are as follows:

1. A tremendous number of anthers have to be inoculated on a callus formation medium because of the low frequency of callus formation as well as plant regeneration.
2. Callus formed is usually transferred to a plant regeneration medium, which is labor intensive and time consuming.
3. A relatively long duration of culture is normally required before plant regeneration.
4. Segregants are often found in the progeny population of spontaneously doubled haploid plants, which reduces the superior selection efficiency of anther culture for rice breeding.

The first and the second points were partially solved by modifying anther culture methods. It was reported that skillful combination of the stages of pollen, cold pretreatment of anther and optimal culture conditions gave a high frequency of callus formation and plant regeneration[4-8]. It was also observed that inoculation of anthers on the medium containing a low concentration (10^{-8} -10^{-6}M) of auxin allowed direct plant regeneration on the same medium[9-11]. However, the rest of the problems have remained unsolved. We have developed a new method of rice anther culture, aiming at solving the above mentioned problems. The method is characterized by pretreatment of

anthers with phytohormone before inoculation on a hormone-free medium and regeneration of plants without the transfer of calli to a regeneration medium, thus called a new "one-step culture method[9-11]". We report here interesting features of the method on the frequency of plant regeneration, duration of culture and the ratio of haploid to diploid in the regenerated plants, using a rice cultivar 'Koganebare' as an example. Histological study was also carried out on the regeneration process in the method.

Materials and Methods

1. *Culture Conditions and Plant Growth*

Plants of rice (*Oryza sativa* L. cv. 'Koganebare' and 'Milyang 23') used in this study were grown in an experimental field of Hyogo Prefecture.

Spikes with flag leaf sheath were harvested 3 to 4 days before heading. They were wrapped in plastic film and kept at 5°C for 7 days. Young inflorescences containing pollen at the mid- to late-uninucleate stages were collected and then surface sterilized in 80% ethanol for 1 min., followed by three rinses with sterile distilled water. The developmental stage of the pollen grains was determined according to Nishiyama's method[12]. The sterilized anthers were soaked for 24 hours in a phytohormone solution of naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), indoleacetic acid (IAA), indolebutilic acid (IBA) or NAA plus benzyladenine (BA), for which concentrations were shown in **Table 1**. The anthers pretreated with the phytohormone were inoculated on a hormone-free N6 medium[13] with MS[14] vitamins, 3% sucrose and 0.9% agar at pH 5.8 and

Table 1. Effect of phytohormone pretreatment for 'Koganebare' on callus formation and plant regeneration in the one-step anther culture method.

phytohormone pretreated*1	No. of anthers inoculated	anthers forming callus		callus formed		plants regenerated		No. of albino plants
		No.	%	No.	%	No.	%	
0.33 mg/*l* NAA	371	5	1.4	19	5.1	0	0	0
1.0 mg/*l* NAA	373	6	1.6	9	2.4	2	0.5	1
3.3 mg/*l* NAA	369	7	1.9	10	2.7	2	0.5	1
10 mg/*l* NAA	349	50	14	84	24	8	2.3	2
100 mg/*l* NAA	362	0	0	0	0	0	0	0
10 mg/*l* NAA, 1.0 mg/*l* BA	330	48	15	106	32	37	11	2
10 mg/*l* NAA, 5.0 mg/*l* BA	343	53	16	114	33	16	4.7	3
10 mg/*l* NAA, 25 mg/*l* BA	320	43	13	100	31	13	4.1	3
0.2 mg/*l* 2, 4-D	317	7	2.2	17	5.4	0	0	0
1.0 mg/*l* 2, 4-D	303	11	3.6	13	4.3	0	0	0
5.0 mg/*l* 2, 4-D	341	4	1.2	8	2.4	0	0	0
5.0 mg/*l* IAA	929	1	0.1	1	0.1	0	0	0
15 mg/*l* IAA	780	8	1.0	11	1.4	2	0.3	0
5.0 mg/*l* IBA	866	4	0.5	5	0.6	0	0	0
15 mg/*l* IBA	846	11	1.3	23	2.7	0	0	0
10 mg/*l* NAA*2	380	35	9.2	76	20	10	2.6	3
two-step method*3	347	19	5.5	46	13	4	1.2	1

*1 Anthers were inoculated on the N6 hormone-free medium described as in the text after treatment with hormone except for the case of *2 and *3.

*2 Anthers were inoculated on the N6 medium supplemented with 0.5 mg/*l* BA for callus formation and plant regeneration.

*3 Anthers were inoculated on the N6 medium supplemented with 2.0 mg/*l* 2, 4-D and 0.5 mg/*l* BA for callus formation and calli formed were transferred to the N6 medium with 0.1 mg/*l* NAA and 5.0 mg/*l* BA for plant regeneration.

25°C under continuous dim light of 3000 lux intensity. In a special case, the above N6 medium supplemented with 0.5 mg/*l* BA was used for inoculation instead of the hormone-free medium.

Regenerated plants of 1 to 3 cm in height were transferred to test tubes containing the same medium for further developement. Plants of 10 to 15 cm in height were then planted in potted soils and grown to maturity in a greenhouse.

In the conventional two-step culture method, the sterilized anthers were inoculated on the N6 medium supplemented with 2.0 mg/*l* 2, 4-D and 0.5 mg/*l* BA at 25°C under the continuous dim light until calli were formed. Calli of 2 to 3 mm in diameter were cultured in test tubes containing the N6 medium with 0.1 mg/*l* NAA and 5.0 mg/*l* BA for plant regeneration. These conditions on phytohormone were chosen by trial and error so as to give the highest efficiency in the two-step anther culture of 'Koganebare'. Regenerated plants, about 10 to 15 cm in height, were planted in potted soils and grown in a greenhouse as described above.

2. *Observation of Regeneration Process*

Calli formed in the one-step culture method were fixed in Craf 3 solution (chromic acid : formalin : acetic acid : water = 0.3 : 10 : 2 : 87.7). The fixed calli were washed, dehydrated, embedded in Paraffin and sectioned to 5 μm in thickness, followed by three stainings with hematoxylin, safranin and fast green FCF. The regeneration process was thus observed microscopically.

3. *Ploidy Level of Regenerant and Segregation in A2*

Ploidy level of each regenerant was determined based on seed fertility, glume shape and plant size. Chromosome number was counted using root tips of several regenerants stained with Giemsa method to confirm the ploidy level determined by the phenotypic observation. In the case that ploidy level of regenerant was not certified from the morphological observation, chromosome number was counted using A2 seeds to determine the level (here 'A' represents regenerant. A2 means the 2nd generation of regenerant).

Twenty four individuals in each line of A2 generation of diploid regenerants were grown in a paddy field. Segregation was investigated by observation on each line in A2.

Results and Discussion

1. *Callus Formation and Plant Regeneration*

Table 1 shows results for 'Koganebare' on callus formation and plant regeneration by the one-step culture method.

It is apparent that the frequency of callus formation and plant regeneration strongly depended upon phytohormone and its concentration. Through 24-hour-treatment, NAA and 2, 4-D were shown to be effective in inducing callus. For plant regeneration, however, only NAA is effective at relatively low concentration. Since NAA is known to be more unstable than 2, 4-D in plant tissues or cells, the results on these two phytohormones may suggest that the existence of auxin in pollen is necessary for callus formation and that the removal of auxin from pollen is crucial for plant regeneration. The results of pretreatment with the same concentration range of the four auxins indicates that IAA and IBA are less effective than NAA in inducing callus as well as plant regeneration. The most distinct effect is observed at 10 mg/*l* of NAA, where the one step method gives twice the frequency of callus formation and plant regeneration of the two-step method. Furthermore, the frequency of plant regeneration was enhanced up to five times by the addition of BA. It should be noted that all of the frequencies shown in **Table 1** were obtained under the same conditions of stage of pollen, cold pretreatment, culture temperature, light strength and so on for both the one-step and two-step culture methods, although the values for the two-step method in

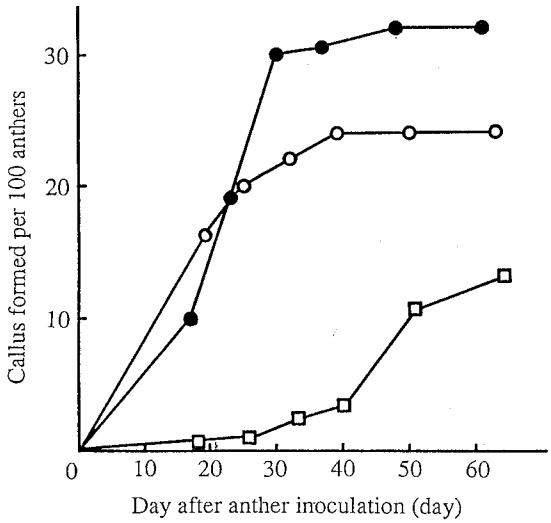

Fig. 1 Time course of callus formation in the one-step and the two-step anther culture methods.
○: one-step method where anthers were pretreated with 10 mg/*l* of NAA, ●: one-step method where anthers were pretreated with 10 mg/*l* of NAA and 1.0 mg/*l* of BA, □: two-step method of which culture conditions are given in the text.

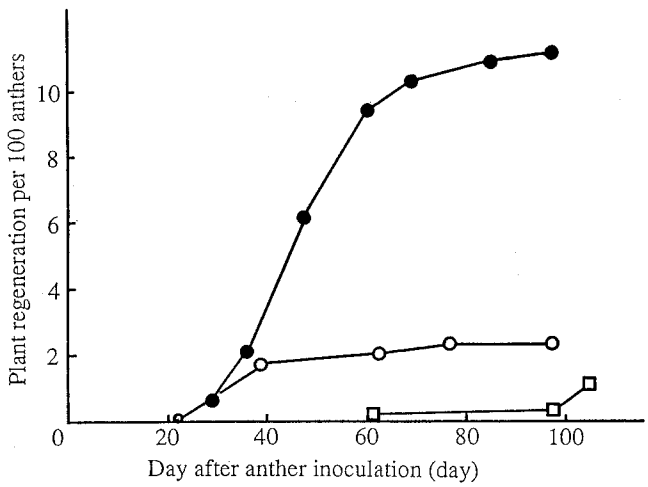

Fig. 2 Time course of plant regeneration in the one-step and two-step anther culture methods. For treatments and indications, refer to **Fig. 1**.

Table 1 were smaller than the large values recently reported[4-8].

Another remarkable feature is that the duration of culture for both callus formation and plant regeneration in the one-step method was half that of the two-step method (**Figs. 1** and **2**). A similar tendency was observed in another one-step culture method[10]. In practical application of anther culture to rice breeding, this is a strong advantage of the one-step culture method for it not only shortens the breeding period but also reduces the emergence of undesirable variants[15], in addition to being labor-saving due to no transfer of callus.

It is known that the genotype of plants used for anther culture can influence the frequency of both callus formation and plant regeneration and that indica varieties of rice usually offer much lower efficiency of anther culture than japonica varieties[16,17]. For indica rice cultivar Milyang 23, the one-step culture method did actually give regenerants (one out of 376 anthers). However, the frequency

was similarly low(2 regenerants out of 454 anthers) in the conventional two-step method. From these results, it can be said that the one-step method can be useful in plant regeneration not only for japonica but also indica varieties of rice but that it is not always able to enhance the regeneration frequency.

2. *Regeneration Process*

The regeneration process was carefully observed for the one-step culture method. Crucial points

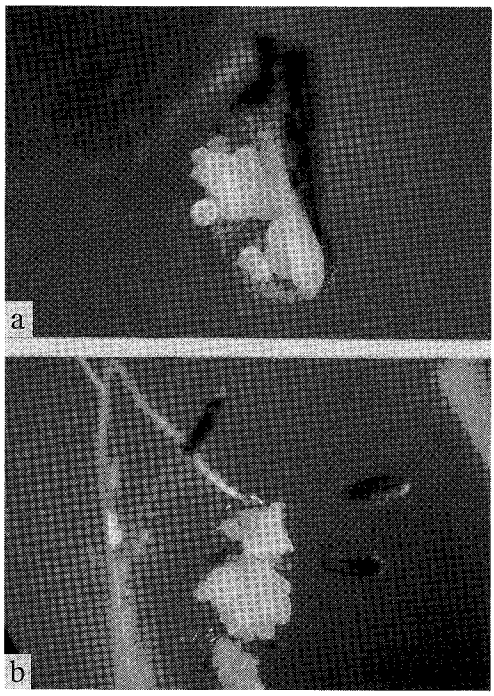

Fig. 3 Callus formation and plant regeneration in the one-step anther culture method.
a: globular embryo-like callus emerged from anther, b: plant regenerated from embryo-like callus.

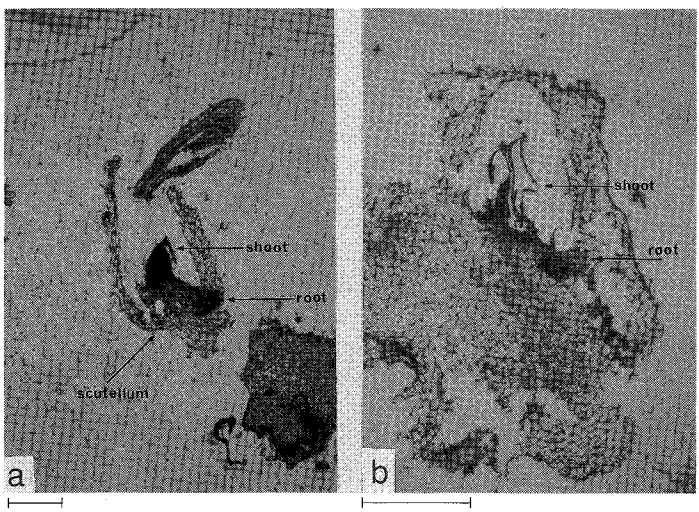

Fig. 4 Anatomical observation of sectioned embryo-like callus.
a: One has shoot, root and scutellum, b: the other has shoot and root, but no scutellum was observed. bar=500 μm.

Table 2. Ploidy of regenerants for 'Koganebare'.

culture method	No. of plants regenerated	haploid plants No.	haploid plants %	diploid plants No.	diploid plants %
one-step*1	62	44	71	18	29
two-step*2	146	52	36	94	64

*1 Anthers were inoculated on the N6 hormone-free medium after treatment with hormone.

*2 Culture conditions are described in **Table 1**. A small number of tetraploid plants were observed in the two-step culture method. However, these were excluded from this table.

in the process are shown photographically (**Fig. 3**). Globular embryoid or embryo-like callus derived from pollen came out through the cleavage of the anthers inoculated on the hormone-free medium (**Fig. 3-a**). Then soon, both a shoot and a root directly emerged from the embryoid in most cases (**Fig. 3-b**). Regeneration from ordinary callus has not been observed in the one-step culture mothod. Anatomical observation revealed that all of the embryoids had not only a shoot but also a root and occasionally a scutellum as well (**Fig. 4-a** and **b**). From these histological observations, it is concluded that the regeneration of the plants occured through somatic embryogenesis[18,19] in the one-step culture method. This could be responsible for the shortened duration of culture for plant regeneration in the one-step method.

However, the total process of regeneration seems to be more complicated in the two-step culture method. An embryoid derived from pollen turned to be callus in a callus formation medium, and then the regeneration took place through adventitious shoot bud[20] or secondary embryogenesis[21,22] in the regeneration medium.

3. *Ploidy Level of Regenerant*

Ploidy level of plants regenerated in the one-step and the two-step culture methods are compared in **Table 2**. It is clear that the former method produced more haploid but fewer diploid plants than the latter method. This may be attributed to a culture duration of about half as long in the former, as seen above (**Fig. 2**). From the viewpoint of practical application of anther culture to rice breeding, it would be advantageous to obtain such a high frequency of haploid plants in the culture if chemical doubling could be effectively achieved. Indeed, it is reported that segregation in the A2 progeny of doubled haploid produced by colchicine treatment is less than that of spontaneously doubled haploid[23].

4. *Segregation in A2*

Segregation of as many as 30% (24 out of 85) was found in the A2 line for the two-step method, which certainly decreases selection efficiency for rice improvement through anther culture. On the other hand, no segregation (0 out of 13) was observed in the A2 population of diploid (spontaneously doubled haploid) plants for the one-step method. This is an advantage of the one-step method over the two-step method.

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《和文要約》

体細胞不定胚を経由して迅速に植物体を再分化するイネ(*Oryza sativa* L.)の新しい一段階葯培養法

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イネ(*Oryza sativa* L.)の葯を植物ホルモンで前処理した後ホルモンを含まない培地で培養し，形成したカルスを再分化培地に移すことなく再分化植物を得る，新しい葯培養一段階法を開発した．植物ホルモンとして，ナフタレン酢酸(NAA)または，NAAとベンジルアデニンを用いたときに，最も良い結果が得られた．常法の二段階法と比較して本一段階法では，①高いカルス形成率・再分化率が得られること，②植物体再分化が速く，二段階法の約1/2の時間であること，③再分化植物のうち半数体が70%と高いこと，④次世代(A2世代)で分離がみられないこと，等の優れた特徴を持つ．組織学的観察から，その理由は本一段階法においては体細胞不定胚を経由して再分化が起こるためと結論された．