Effect of Growing Conditions of Rice Donor Plants on Anther Culture *in Vitro*

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Abstract: An experiment was conducted to find the effect of three types of donor plants growing conditions (growth chamber, open space + growth chambers and open space) on callus induction and subsequent plant regeneration and productive shoot regeneration from the anthers culture *in vitro* of four rice hybrid (1-2, 2-1, 7-1, 13-3) developed by Primorsky Scientific Research Institute of Agriculture. Five variants of N₆ medium (N₆-1, N₆-2, N₆-3, Mix-1, N₆-4) were used as basal medium. Mean value of callus induction frequency on three types of conditions ranged from 5.68% to 9.44% and the difference was non-significantly. In general, callus derived from donor plants grown on condition of open space + growth chambers showed significantly better performances for plant regeneration (0.23 green regenerants on anther and 3.77 green regenerants on callus) and productive shoot regeneration (0.06 productive regenerants on anther and 0.56 productive regenerants on callus). Favourable conditions for donor plant growth in open space positively affect on callus induction and regeneration. It is possible to get assured results on many hybrids, but not the highest. In growth chamber, frequency of callus induction can be the maximal only on some samples, few hybrids are resulted in deficiency of callus induction.

Key words: *Oryza sativa* L., anther culture *in vitro*, growth conditions of rice donor plants, regenerant.

1. Introduction

Double haploid is an important resource for breeding and genetic research. It allows shortening of the breeding cycle and production of homozygous lines from a segregating population in the immediate generation [1-4]. Anther culture *in vitro* is the routine technique for haploid induction in rice improvement [1, 3, 5, 6].

Most of the donor plants were grown in the field, since anthers removed from field-grown plants gave a better response than those picked from greenhouse-grown plants [7]. Several other researchers raised donor plants under the optimized conditions of climatic chambers or phytotrons, thereby enabling plants to maintain a healthy growth. Rice plants yield in the controlled conditions gave a high degree of success of anther culture response with reproducible results [1]. Moreover, it is possible to fix

a relatively low temperature in climatic chambers or phytotrons which extend time of forming panicles and anthers. In this case, there is possibility to prolong a period of inoculation of anthers *in vitro* and to execute the bigger volume of works. The controlled conditions reduce the intensity of disinfection of panicles by any surface-sterilized agents before inoculation of anthers [8]. On the other hand, unfavourable condition for donor plants raises the frequency of response of the anthers to *in vitro* culture [1, 3, 4, 7]. There are no unique recommendations for growing of donor plants.

This study was undertaken to choose preferable conditions for growing of donor plants intended for anther culture *in vitro* of Far Eastern rice hybrids.

2. Materials and Methods

2.1 Plant Material

Four hybrids of F₂ rice *Oryza sativa* L. subspecies *japonica* Kato were used for experiment from three to seven plants: 1-2 ((Russvet × Novator) × Novator);

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2-1 {Novator × [Priozerny × (Dalnevostichny × Hayakaze)]}; 7-1 (Husar × Dary 23); 13-3 (Lugovoy × Virag). The parents of the hybrids were derived from Russian Far Eastern and foreign varieties.

Donor rice plants were cultivated during summer of 2013 under three types of conditions:

(1) In growth chamber at 20 °C, 16 h photoperiod and light of 4,000 lux in pot (growth chamber);

(2) At the beginning, plants grew in open space for vegetation in pot, then from middle of tillering, it was in growth chambers at 20 °C, 16 h photoperiod and light of 4,000 lux (open space + growth chambers);

(3) In open space for vegetation in pot (open space).

The panicles were sampled in the morning when distance between flag leaves and next leaves were 5-7 cm. It is according to florets with microspores at the mid- to late-uninucleate stage as described by Goncharova [3]. Acetocarmine stain was used for determining the developmental stage of the microspore [9]. Panicles with short stem and leaves were located in glass cylinders with water and stored in the refrigerator at 5 °C for 7 d. Panicles containing florets were not disinfected by any surface-sterilized agents. Anthers were extracted in aseptic conditions and inoculated into tuber of 14 mm in diameter by pairs.

The number of cultured explants varying between hybrids ranged from 38 to 116. In summary, 3,542 anthers were inoculated on several medium.

2.2 Culture

The anthers were inoculated on five types of induction medium based on N₆ medium [6]. Their full compositions were given as Ilyushko [10]. The cultures were incubated in darkness at 25-28 °C. Calluses (after 4-5 weeks of culture with diameter 2-5 mm) were transplanted for differentiation on tubes (21 mm diameter) to the shoot regeneration medium N₆ containing 6% sucrose, 1.0 mg/L 6-benzylaminopurine (BAP) and 1.0 mg/L kinetine. The shoots were transferred on 1/2 MS [11] medium supplemented

with naphthalene acetic acid (NA) 0.25 mg/L and sucrose 20 g/L. Plants with roots and leaf were transplanted into autoclaved soil before harvested. Plants with mature seeds were regarded as productive shoots. Calluses, shoots and plants were subjected to a temperature of 25 °C, 16 h photoperiod and light of 4,000 lux.

2.3 Statistical Analysis

The Chi-square " χ^2 " of mean was used to determine the significant difference in callus induction frequency. A probability of difference on the indexes was calculated between mediums and hybrids. The student "*t*" pairwise comparison of means was used to determine the significant difference in types of donor plants growing conditions. The significant differences at $\alpha = 0.05$ are marked with *.

3. Results

3.1 Callus Induction

Data on callus induction were recorded from three to six weeks. No hybrids were totally non-responsive. The callusing ability of the hybrid anthers on various mediums was significantly different (Table 1). Callus induction frequencies were variable and ranged from 0 to 55.0%. Hybrid 13-3 showed very poor performance in induction callus on conditions of growth chamber and open space + growth chambers, but in open space, the callus induction was observed on five mediums. Hybrid 7-1 had anthers response on all conditions and mediums, except N₆-4 medium in growth chamber. The average of the interaction between the types of donor plants and growing conditions ranged from 5.68 to 9.44 and were non-significant (Table 1, Fig. 1).

3.2 Plant Regeneration

Results of plant regeneration from callus are presented in Table 2. The results showed that number of green shoots regenerated per anther was higher on open space + growth chambers condition and open space

Fig. 1 Frequency of callus induction from rice anthers, observed on three types of donor plants growing conditions.

Table 1 Frequency of callus induction (%) from rice anthers, observed on five types of callusing medium and three types of donor plants growing conditions.

Hybrid	Frequency of callus induction (%)					χ^2
	N ₆ -1	N ₆ -2	N ₆ -3	Mix-1	N ₆ -4	
Growth chamber						
1-2	25.0	10.0	5.0	2.5	-	12.84*
2-1	1.5	0.0	5.1	4.9	0.0	7.71
7-1	5.0	12.5	7.5	55.0	0.0	59.00*
13-3	0.0	0.0	0.0	0.0	0.0	0.00
χ^2	10.69*	12.11*	3.38	74.34*	0.00	
Mean	7.05					
Open space + growth chambers						
1-2	0.0	0.0	0.0	0.0	2.5	40.20*
2-1	0.0	15.0	7.5	0.0	0.0	16.50*
7-1	22.5	10.0	12.5	31.7	10.3	17.07*
13-3	0.0	0.0	1.5	0.0	0.0	3.03
χ^2	32.03*	16.58*	14.11*	48.34*	10.52*	
Mean	5.68					
Open space						
1-2	16.2	3.9	6.4	7.8	2.6	14.90*
2-1	3.7	0.0	0.0	5.2	1.7	5.58
7-1	3.4	11.5	13.2	38.8	7.1	5.28
13-3	14.5	11.6	9.5	22.1	7.7	9.15
χ^2	12.18*	14.55*	6.28	41.54*	4.25	
Mean	9.44					

*Significant at $\alpha = 0.05$. Between growth chamber and open space + growth chambers $t = 0.38$; between growth chamber and open space $t = 0.66$; between open space + growth chambers and open space $t = 1.33$.

than that in growth chamber, but they are statistically insignificant (Table 2, Fig. 2). Number of green shoots regenerated per callus were found 3.77 on condition open space + growth chambers, this mean value was significantly higher than that in another conditions (Fig. 3). In open space, three hybrids formed green regenerants on four mediums, except hybrid 2-1 which showed plant regeneration only on medium Mix-1. Very few regenerants were obtained on medium N₆-4 in all the growth conditions.

3.3 Productive Shoots Regeneration

The number of productive shoots regenerated on callus was the highest on condition of open space + growth chambers (0.56), followed by open space (0.22) (Table 3, Fig. 4). The same results were obtained on the number of productive shoots regenerated on anther. The value was significantly higher on condition of open space + growth chambers. These results demonstrate

that the frequency of productive shoots regenerated depends on conditions for growing of donor rice plants.

4. Discussion

It is widely known that growth conditions influence the androgenic response by affecting the vigor and quality of donor plants [2, 12, 13]. The main factors include light intensity and quality, nutrition, photoperiod and temperature. Environmental stress including diseases and insects should be avoided [12]. Raising donor plants for anthers culture in growth chamber reduces the risks of diseases and insects. Plants from open space have some diseases and insects, and anther inoculations without surface sterilization are infected until 15% [8]. However, several hybrids are clear in full measure, but other ones are infected until 100%. Probably there is internal infection in plants without visible affection on

Table 2 Number of green shoots regenerated per anther and per callus on three types of donor plants growing conditions and different medium.

Hybrid	Number of regenerated green shoots				
	N ₆ -1	N ₆ -2	N ₆ -3	Mix-1	N ₆ -4
Growth chamber					
1-2	0.55 ^a /2.20 ^b	0/0	0/0	0/0	-
2-1	0/0	0/0	0/0	0.35/7.30	0/0
7-1	0/0	0.10/0.80	0.80/10.70	0.43/0.77	0/0
13-3	0/0	0/0	0/0	0/0	0/0
Mean	0.12/1.98				
Open space + growth chambers					
1-2	0/0	0/0	0/0	0/0	0.10/4.00
2-1	0/0	0.65/4.33	0.68/9.00	0/0	0/0
7-1	0.60/2.67	0/0	0.60/4.80	1.53/4.84	0.44/4.29
13-3	0/0	0/0	0/0	0/0	0/0
Mean	0.23/3.77				
Open space					
1-2	0.24/1.50	0.12/3.00	0.14/2.20	0.44/5.60	0/0
2-1	0/0	0/0	0/0	0.19/3.60	0/0
7-1	0.01/0.33	0.31/2.70	0.22/1.70	1.14/3.00	0/0
13-3	0.61/4.19	0.47/2.85	0.34/3.55	0.09/0.39	0/0
Mean	0.22/1.92				

^aNumber of green shoots regenerated per anther; ^bNumber of green shoots regenerated per callus.

^aBetween growth chamber and open space + growth chambers $t = 1.06$; between growth chamber and open space $t = 1.17$; between open space + growth chambers and open space $t = 0.13$. ^bBetween growth chamber and open space + growth chambers $t = 1.23$; between growth chamber and open space $t = 0.06$; between open space + growth chambers and open space $t = 2.14^*$.

Fig. 2 Number of green shoots regenerated per anther from three types of donor plants growing conditions.

Fig. 3 Number of green shoots regenerated per callus from three types of donor plants growing conditions.

Table 3 Number of productive shoots regenerated per callus and per anther from three types of donor plants growing conditions grown different medium.

Hybrid	Number of regenerated productive shoots per callus				
	N ₆ -1	N ₆ -2	N ₆ -3	Mix-1	N ₆ -4
Growth chamber					
1-2	0.40	0.00	0.00	0.00	-
2-1	0.00	0.00	0.00	0.25	0.00
7-1	0.00	0.20	0.00	0.27	0.00
13-3	0.00	0.00	0.00	0.00	0.00
Mean	0.06				
Number of regenerated productive shoots per anther 0.01					
Open space + growth chambers					
1-2	0.00	0.00	0.00	0.00	3.00
2-1	0.00	2.33	3.67	0.00	0.00
7-1	0.89	0.00	0.40	0.63	0.29
13-3	0.00	0.00	0.00	0.00	0.00
Mean	0.56				
Number of regenerated productive shoots per anther 0.06					
Open space					
1-2	0.75	0.25	0.00	0.60	0.00
2-1	0.00	0.00	0.00	0.00	0.00
7-1	0.00	0.11	0.20	0.68	0.00
13-3	0.75	0.15	0.82	0.00	0.00
Mean	0.22				

Between growth chamber and open space + growth chambers $t = 0.06$; between growth chamber and open space $t = 2.05^*$; between open space + growth chambers and open space $t = 1.35$.

Fig. 4 Number of productive shoots regenerated from callus from three types of donor plants growing condition.

phase of sampling of panicles. On the author's observation, the plants were weak at the end of period of vegetation. Consequently, for raising donor plants in open space, the authors selected off loose donor hybrids plants intended for anthers culture *in vitro* without surface sterilization at the beginning.

The growth conditions of the donor plants used for pollen culture have a profound effect on the embryogenetic response [13]. Some other authors were noted on significant influence of donor plants growth conditions on anthers response and plant regeneration [1, 3]. Other authors were detected large differences only on callus production frequency among wheat donor plant from greenhouse and environments of growth chamber and sandy field, but regeneration frequency did not differ significantly among environments [14]. In this experiment, differences in frequency of callus induction were absent among types of donor plants growing conditions. In previous attempt, the frequency of callus induction in open space exceeds this index from growth chamber and open space + growth chambers [8]. Large differences on the rice regeneration among donor plants growing conditions in this test were good conformed by opinion that donor plant conditions play a role in regeneration of the embryos to plants [2]. Moreover, for rice callus induction, open space is preferable [8], while for wheat callus production, greenhouse is preferred [14]. High plant regeneration requires other conditions. It has been reported that donor plants of barley grown under growth chamber conditions produced more green plants than donor plants grown under greenhouse conditions [2]. Type of conditions open space + growth chambers was the best for rice regeneration in this experiment. However, the maximal indexes of frequency of callus induction, number of green shoots regenerated per anther and number of green shoots regenerated per callus were fixed on growth chamber. So, it is not possible to make general recommendations about the optimal growth conditions, as they seem to vary between

hybrids.

The temperature at which the donor plants are grown plays a critical role in microspore culture response in several agricultural species [2, 7, 13, 15]. The lower limit of the optimum temperature gave the maximal embryogenetic potential for *Brassica* species, winter barley [1, 2], rice [3], tobacco [15] and wheat [1]. This slow of the growth of the donor plants allows for a longer time period, during which buds at the appropriate developmental stage can be selected [2]. It was supposed that the cold temperature for the donor plants resulted in absent callus induction on 13-3 hybrid in growth chamber and only 1.5% on N₆-3 medium on condition open space + growth chambers. Temperature of 20 °C is critical for rice plant growth and unfavourable for fluorescence. It was too low for 13-3 hybrid. Reducing of the temperature until 20 °C was lower limit for 7-1 hybrid and gained excellent result on callus induction and regeneration. In whole, it seems that favourable conditions for donor plant growth in open space positively affect on callus induction regeneration. It is possible to get assured results on many hybrids, but not the highest. In growth chamber, frequency of callus induction can be the maximal only on some samples, few hybrids are resulted in deficiency of callus induction. Low temperature switches the effect on plant regeneration and leads to spontaneous doubling of chromosomes particularly if it is abruptly like stress. Preculture treatment of the rice anthers before culture by low temperature influences on the release of microspores from the anther and their subsequent divisions leading to plant regeneration similarly. At the temperature 10 °C, frequency of callus induction is higher than that at 5 °C. The treatment of anthers by 5 °C temperature is significant effective for plant regeneration in comparison with 10 °C [16].

The ability to undergo androgenesis is controlled by more than one gene [7, 13, 15]. Foroughi-Wehr et al. 1982, cited in Ref. [7], distinguished four independently and differently inherited traits, namely,

callus induction, callus stabilization, plant regeneration and albino versus green plantlet formation. Difference in preferable temperature indicates that callus induction and shoot regeneration processes may be under independent genetic control. It is good in accord with data in indica $\times$ Basmati rice hybrids [17].

5. Conclusions

For breeding programmes, haploid technology *in vitro* was recognized as a valuable tool to help plant improvement. In every seasons, plant breeders cross new hybrids combinations and obtain a new sets of heterozygous genotypes which are required for transfer to homozygous lines by haploid technology for accelerating plant breeding program. Some part of them responses to anther or other tissue culture, while some other part of them can not form callus and/or regenerants, since it is impossible to unify the conditions for cultivation *in vitro* for all genotypes. In this case, valuable hybrids are selected in field in 5-7 years. But, it is necessary to grow donor plants in different conditions and inoculate anthers on several medium for increasing callusing ability and productive shoot regeneration for each hybrid. In open space, donor plants ensure results on callus induction and regeneration on many hybrids, but not the highest. In growth chamber, frequency of callus induction can be the maximal only on some samples, and few hybrids are resulted in deficiency of callus induction. Low temperature switches the effect on plant regeneration and leads to spontaneous doubling of chromosomes particularly if it is abruptly like stress.

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