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DEVELOPMENT OF A PROTOCOL FOR EMBRYO RESCUE IN WIDE HYBRIDIZATION OF RICE

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ABSTRACT

In rice improvement programs, the lack of rice varieties tolerant to biotic and abiotic stresses is always a problem. Rice Research and Development Institute at Batalagoda has already begun to use wild relatives of rice to draw genes needed to construct varieties that are resistant to various biotic and abiotic stresses. However, hybridization between cultivated and some of the wild rice varieties belonging to different genomic groups is incompatible due to genomic distance. To overcome this problem, embryo rescue can be carried out. At present, the major limiting factor for its use is the lack of suitable media. Therefore, the objective of this study was to develop an effective medium for rice embryo rescue. Premature embryos (5-12 days after pollination) derived from different wide cross combinations between the AA (*Oryza sativa*) and CC (*Oryza eichingeri and Oryza rhizomatis*) genomic groups were cultured in five embryo rescue media. Based on the germination percentage, out of five media used in the experiment, ¼ MS medium modified with 2.5 mg/l GA_3, 1 g/l Casein hydrolysate, 0.002 mg/L NAA, 1 mg/L Kinetin and 3% Sucrose was found to be the best medium. Embryos at the age of 8-12 days after pollination showed 100% germination and produced complete plants within 6 weeks in culture. Thus the present medium could be used for embryo rescue in wide hybridization programs in rice.

KEYWORDS: Culture medium, Embryo rescue, Rice, Wide hybridization.

INTRODUCTION

The wild relatives of *Oryza* species are rich sources of useful traits such as tolerance to biotic and abiotic stresses which can be used in rice improvement programs. A number of important traits such as grassy stunt virus resistance from *O. nivara* (AA genome), BPH, WBPH and bacterial blight resistance from *O. officinalis* (CC genome), blast and bacterial blight resistance from *O. minuta* (BBCC genome) and cytoplasmic male sterility from *O. rufipogon* (AA genome) have been transferred from wild species into cultivated species by inter-specific hybridization. Hybridization between cultivated rice (AA genome) and some of the wild species sharing AA genome such as *O.nivara and O.rufipogon* has been successful (Vaughan and Merishima, 2003). However, more remote hybridization between *O.sativa* and the wild species such as *O.eichingeri* (CC genome) and *O.rhizomatis* (CC genome) is difficult because of failure in embryo development due to embryo abortion or endosperm degeneration owing to genomic distance (Sitch *et al.*, 1989). This inability of remote hybridization to undergo normal sexual reproduction might be overcome by embryo rescue.

In Sri Lanka, there are five different wild rice species, namely *O. nivara* (AA genome), *O. rufipogon* (AA genome), *O. eichingeri* (CC genome), *O. rhizomatis* (CC genome) and *O. granulata* (GG genome). These species serve as a very important gene pool for the breeders. Rice Research and Development Institute (RRDI) at Batalagoda has begun to utilize such genetic resources to draw important genes for rice improvement.

During the last decade, several successful attempts have been made in overcoming reproductive barriers in rice using biotechnological tools such as embryo rescue, *invitro* pollination and protoplast fusion. A number of hybrids have been produced between cultivated and wild species, but only a few protocols on emasculation, pollination and embryo rescue are available (Jena and Khuss, 1990). For example, no protocol is available to rescue embryos that degenerate at very early stages of development. Thus, the goal of the present investigation was to find an effective and efficient medium for the rescue of the embryos of *O.sativa* x *O.eichingeri* and *O.sativa* x *O.rhizomatis* hybrid combinations.

MATERIALS AND METHODS

This study was conducted at RRDI Batalagoda, as a three factor factorial experiment, the factors being, 3 hybrid combinations *O. sativa* x *O. sativa*, *O.sativa* x *O. eichingeri* and *O. sativa* x *O. rhizomatis*, 2 different times of embryo excision (measured as 5-7 and 8-12 days after pollination) and 5 different types of culture media.

Since the experiment had no replications, the highest order interaction (the three way interaction) was used as the error term in the analysis of variance.

Five different media were prepared using 1/4 Murashige and Skoog (1962) medium modified by supplementing different concentrations of Gibberelic acid, Casein hydrolysate, Naphthalene acetic acid, Kinetin and Sucrose as shown in Table 1. The pH of the media was adjusted to 5.8 ± 1 and the cultures were maintained at 25 °C.

Cross combinations of cultivated varieties and wild rice varieties in different genomic groups (*O. sativa* x *O. sativa*, *O. sativa* x *O. eichingeri*, *O. sativa* x *O. rhizomatis*,) were prepared. The cultivated varieties were always used as the female parent. Pollination was performed with fresh pollen of the wild species collected during anthesis. Hybrid embryos were detached from spikelets 5, 6, 7, 8, 9, 10, 11 and 12 days after pollination (DAP). The embryos detached at 5, 6 and 7 DAP were grouped as early embryos while the embryos detached at 8, 9, 10, 11 and 12 DAP were grouped as late embryos. Dehusked, premature seeds were washed in tap water and Teepol before being

used in the culture room. Further sterilization events were carried out in the clean air bench. Premature seeds were sterilized by using 20% sodium hypochloride for 15-20 min. and washed three times with sterile distilled water and then transferred to 70% alcohol for 2 min. and rinsed two times in sterile distilled water. That part of the seed containing the embryo was cut and cultured on different modified MS agar media as shown in Table 1 and kept in the dark at 25° C until germination. The culture tubes containing germinated embryos were then kept for 16 hrs in the light and 8 hrs in the dark and incubated at 25° C. The seedlings were kept in the incubation room until the development of shoots and roots and then transferred to soil. Premature embryos collected at the same time intervals from self pollinated cultivated plants were used as the controls.

Table 1. Constituents of media used for embryo rescue and regeneration in inter-specific hybrids.

Medium	*Constituents*
1	1/4 MS+ 0.002mg/L NAA +1 mg/L kinetin
2	1/4 MS + 1.5 mg/GA_3 + 0.002 mg/L NAA+ 1 mg/L kinetin
3	1/4 MS + 2.5 mg/L GA_3 +0.002 mg/L NAA + 1 mg/L kinetin
4	1/4 MS + 1.5 mg/L GA_3 + 0.002mg/L NAA+ 1 mg/L kinetin + 1g/L casein hydrolysate
5	1/4 MS + 2.5 mg/L GA_3 + 0.002 mg/L NAA+ 1 mg/L kinetin + 1g/L casein hydrolysate

RESULTS AND DISCUSSION

Embryo survival at different DAPs in inter-specific hybrid combinations was studied before examining the efficiency of embryo rescue in inter-specific hybrids. Number of pollinated florets and embryo survival percentages in inter and intra-specific hybrid combinations up to 5 and 12 DAP are presented in Table 2. Embryo survival was observed on a visual basis. Degenerated seeds were dark brown in colour and dead cells in embryos were observed under the light microscope. When the developments in inter-specific combinations were compared with the self-pollinated *O sativa* (intra-specific combination), a slower embryo development and late regeneration were observed in wide crosses.

Embryo survival percentage decreased with the increase in the number of days after pollination in both inter-specific cross combinations, whereas embryo survival of the intra- specific cross combination increased up to 100% at 12 DAP. Embryo survival percentage showed a tremendous decrease (more than 6%) in the first period (5 to 7 DAP) in both inter-specific crosses, but *O.*

sativa x *O. rhizomatis* showed a higher embryo survival percentage than that of *O. sativa* x *O, eichingeri.* However, both inter-specific crosses showed almost the same (about 11%) embryo survival percentage in the second period (8-12 DAP) as indicated in Table 2.

The embryo survival percentage and embryo rescue on plant regeneration efficiency are dependent on the appropriate time of embryo detachment from the mother parent. Thus, comparatively higher embryo survival was observed in all the combinations at early stages of embryo growth (at 5-7 DAP), which does not necessarily mean that early embryo detachment is comparatively better, without knowing the actual efficiency of embryo rescue or plant regeneration.

Table 2. Number of pollinated florets and embryo survival percentage in inter and intra-specific hybrid combinations of pre-mature embryos from 5 to 12 day age groups.

Intra/Inter specific hybrid combination	*Number of pollinated florets*	*Embryo survival %*	
		5 to 7 DAP	*8 to 12 DAP*
O. sativa x *O. eichingeri*	1310	16.9	11.1
O. sativa x *O. rhizomatis*	945	28.0	11.4
O. sativa x *O.sativa*	913	100%	100%

Plant regeneration and embryo rescue efficiency from embryos of inter and intra specific cross combinations when embryos were excised at 5-7 and 8-12 DAP and cultured in different culture media are presented in Table 3. In the analysis of variance, the highest order interaction effect of hybrid combination (C) x Time of embryo excised (T) x culture medium (M) was used as the error term. The two way interaction effects of C x T ($p=0.02$) and T x M ($p=0.02$) were found to be significant when tested against the error term so that they were further analyzed using response curves (Figure 1).

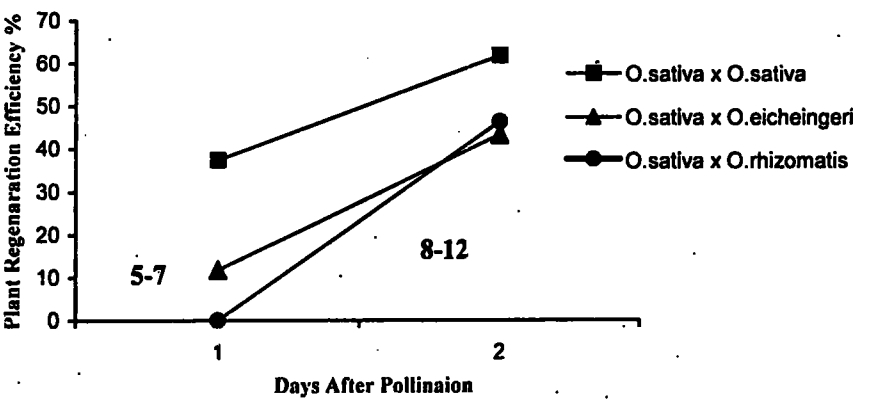

Figure 1. Plant regeneration efficiency of different cross combinations in response to age of the embryo as measured by days after pollination.

Table 3. Influence of time of embryo excision and culture medium on efficiency of embryo rescue in inter and intra- specific cross combinations.

Cross combination	*Time of embryo excised (Days after pollination)*	*Medium*	*Inoculated embryos (No.)*	*Regenerated plants (No.)*	*Regeneration efficiency %*
Intra-specific					
1. *O. sativa x O. sativa*	5-7 DAP	1	20	5	25
		2	22	5	20
		3	25	8	32
		4	20	7	35
		5	20	15	75
	8-12 DAP	1	23	14	56
		2	22	16	56
		3	25	20	80
		4	20	14	75
		5	25	25	100
Inter-specific					
2. *O sativa X O. eichingeri*	5-7 DAP	1	20	0	0
		2	24	0	0
		3	24	2	8.3
		4	20	2	10
		5	24	4	17
	8-12 DAP	1	24	4	17
		2	24	4	17
		3	20	8	40
		4	24	10	42
		5	24	24	100
3. *O. sativa x O. rhizomatis*	5- 7 DAP	1	15	0	0
		2	12	0	0
		3	14	0	0
		4	11	0	0
		5	15	0	0
	8-12 DAP	1	12	2	20
		2	10	4	40
		3	14	9	64
		4	14	7	50
		5	14	12	86

In all the hybrid combinations, embryo rescue efficiency improved significantly with increased DAP. Different cross combinations showed different rates of increase. *O. sativa* x *O. rhizomatis* showed zero % and *O. sativa* x *O. eichingeri* showed only 11.8% embryo rescue efficiency when embryos were excised at 5-7 DAP. Thus, irrespective of the type of inter specific cross combination, 8-12 DAP appeared to be a better time than 5-7 DAP to excise embryos for embryo rescue and plant regeneration.

The culture media have been improved by testing the efficiency and effectiveness of the constituents of the media, and the embryo rescue efficiency also depends on the type of cross combination. However, the rate of improvement was higher when the embryos were excised 8-12 DAP than those excised 5-7 DAP.

In a cytological study, Randrangboon *et al.* (2002) revealed that the growth rate of inter-specific hybrid embryos was slower than that of intra-specific embryos and the growth mostly terminated at the zygote and pro-embryo stages. Therefore, embryo abortion occurred at the very early stages in wide crosses and the rate of success for inter-genomic crosses was very low. The present study showed that irrespective of the cross combination, the embryo rescue efficiency of inter-specific cross combinations could be improved by the use of culture medium 5 (1/4 MS+2.5 mg/L GA_3+0.002 mg/L NAA+1 mg/L kinetin +1g/L casein hydrolysate) and in embryos that are excised at 8-12 DAP.

Survival ability until maturity rate was lower in wide cross combinations than *O. sativa x O. sativa* combination. Twenty plants were produced from *O. sativa x O. eichingeri cross* and two plants were obtained from *O sativa x O. rhizomatics* cross combination (Plate 1).

CONCLUSIONS

Time of embryo rescue and nutrients in the media have a significant effect on embryo survival. Excising embryos 8-12 days after pollination is much better than 5-7 DAP for regeneration. The medium consisting of 1/4 MS + 2.5 mg/L GA_3 + 0.002 mg/L NAA+ 1 mg/L kinetin + 1g/L casein hydrolysate was the best for embryo rescue.

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Plate 1. Plants of *O.eichingery* (1), *rescued* F1 hybrid (2) and *O. sativa* (3).