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Full Length Research Paper

In vitro production of double haploid plants from two rice species (*Oryza sativa* L. and *Oryza glaberrima* Steudt.) for the rapid development of new breeding material

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Eight genotypes of two rice species (*Oryza sativa* and *Oryza glaberrima*) were studied for their response to anther culture in terms of callus induction and frequency of plant regeneration. N6 medium (Chu et al., 1975) was used for callus induction, and MS medium (Murashige and Skoog, 1962) with 1 mg/l BAP and 0.5 mg/l NAA for plant regeneration. Generally, *O. glaberrima* genotypes produced more callus than *O. sativa* genotypes. All *O. glaberrima* genotypes regenerated plants. Among *O. sativa* genotypes only japonica variety IKP produced plants. Other *O. sativa* genotypes showed low frequency of callus induction without plant regeneration. Many albino plants were obtained from the culture. Only one *O. glaberrima* genotype (6202 Tog) produced green plants. In this experiment, a total of 93 plants were regenerated with 14 green plants and 79 albino plants. Anther culture response is largely species and genotype dependent.

Key words: Rice species, *Oryza sativa* (L.), *Oryza glaberrima* (Steudt.), anther culture, medium, callus induction, plant regeneration.

INTRODUCTION

Varietals improvement to increase rice production in West Africa is a recent trend. In Asia, it was associated at the beginning with varietals testing for yield, plant size (semi-dwarf), photoperiod-insensitiveness, and pest resistance (Miezan and Sie, 1997; Gosal et al., 1997). Success in varietals improvement was minimal because of limited resistance of *Oryza sativa* to many of the stresses typical for West Africa.

Less attention has been paid to exploitation of wide genetic variability available in indigenous African cultivated rice, *Oryza glaberrima* (2*n* = 24, AA). This species is known to have been selected and cultivated in many parts of West Africa for more than 3500 years (Jacquot, 1977). *O. glaberrima* has many useful traits such as weed competitiveness, drought tolerance and ability to grow under low input conditions (Sarla and Mallikarjuna, 2005). Moreover, *O. glaberrima* has many other characteristics valuable to breeders and to farmers. It is a potential source of useful genes for a wide range of economically important characters (Jones et al., 1996) and it is resistant to many of the prevailing stresses. Many new rice cultivars have been developed through biotechnological techniques like anther culture, embryo rescue and somaclonal variation (Zapata-Arias et al., 2004; Brown and Thorpe, 1995).

In China, anther culture has contributed to more than 100 new rice cultivars (Meifang, 1992; Zhang and Chu, 1986). The production of doubled haploids through anther or isolate microspores culture *in vitro* is a rapid approach to homozygosity that shortens the time required for development of new rice cultivars through conventional methods, which require at least 6 - 7 generations. Haploids are also valuable to detect and fix desirable recessive traits introduced through mutation (Chen et al., 2001) or hybridisation (He et al., 2006). Double haploid techniques which accelerate the breeding cycle and allow

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Table 1. Species, origin and genotypes of rice used as donor plant in the experiment.

Genotype	Origin	Species	Type
Tog 7178	Warda	*O. glaberrima*	
Tog 7205	Warda	*O. glaberrima*	
Tog 6202	Warda	*O. glaberrima*	
IKP	Taiwan	*O. sativa*	Japonica
Sahel 108	Irri	*O. sativa*	Indica
IR 31851	Irri	*O. sativa*	Indica
Farox 304	Nigeria	*O. sativa*	Indica
Farox 239	Nigeria	*O. sativa*	Indica

better discrimination between genotype within any generation became very attractive (Maria et al., 2006). In anther and / or isolated microspores culture technique, the immature pollen grain (haploid) are induced to divide and double their chromosome number so that the plant regenerated from them have two set of chromosomes (double haploid). Anthers are cultivated in a specialized solid medium while isolated microspores are generally cultivated in a liquid medium.

This technique has been routinely used for many crop plants worldwide. For rice, both anther and isolated microspore culture methods have been successful in generating double - haploid plants. However, the use of anther culture as a routine technique for breeding of indica rice is extremely limited by the poor induction of androgenic calli and subsequent plant regeneration (Sripichitt et al., 2000).

Combining the best traits of *O. sativa* and *O. glaberrima* by interspecific hybridisation could be an interesting method to develop new breeding material suitable for low input conditions. High sterility of F1 populations is the major problem of interspecific hybrids between *O. sativa* and *O. glaberrima* (Heuer and Miezan, 2004). Several backcrosses with *O. sativa* are necessary to obtain fertile plants (Heuer and Miezan, 2004). Production of doubled haploids through anther culture of F1 hybrids would facilitate the use of interspecific crosses.

A first step is the development of a reproducible protocol for anther culture of parental genotypes. Anthers with microspores at the uninucleate stage are more efficient for callus induction or direct embryo regeneration (Chen, 1976; Huang et al., 1986). The objective of this study was to establish a method for *in vitro* production of doubled haploid plants from eight genotypes of *O. sativa* and *O. glaberrima*.

MATERIALS AND METHODS

An experiment was set up in the greenhouse using five genotypes of *O. sativa* and three genotypes of *O. glaberrima* (Table 1). Pre-germinated seeds from each genotype were sown in pots containing sterilised soil under greenhouse conditions.

The temperature was maintained at 35ºC with a 14/10 h photoperiod. Plants were watered twice a day. Nitrogen as urea, phosphorus and potassium were used to fertilize plants. Anthers were collected at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Selection was based on a maximum distance between the auricle and the next subtending leaf of 5 - 10 cm.

This coincides with the mid-uninucleate stage which is most responsive to anther culture. Panicles were collected from plants between 9:00 - 10:00 h in the morning and were washed with tap water. After clipping the flag leaves, panicles were sprayed with 70% ethyl alcohol. They were kept in polyethylene bags and incubated in the dark at 4ºC for 5 - 7 days. After cold pre-treatment, panicles were put in a sterile Erlenmeyer and sterilised with 5% $Ca(OCl)_2$ for 5 min. $Ca(OCl)_2$ was then drained off, followed by three washes with sterile water. Immature anthers were cultivated in Petri dishes containing 25 ml of Chu et al., (1975) N6 medium supplemented with 3 mg/l 2, 4-D, 1 mg/l NAA and 1 mg/l Kin. Medium was solidified with 0.8% w/v agar. Petri dishes containing anther and callus medium were then incubated at 27ºC in the dark under 60% humidity to develop organogenic callus.

Anthers were subcultured onto fresh medium every week. Induced calli from anthers were transferred in fresh medium for growing during 30 - 45 days. After this period, white calli were transferred onto regeneration medium MS (1962) + 1 mg/l BAP + 0.5 mg/l NAA and incubated in a culture room at 27ºC with a 14/10 h photoperiod. Plantlets of 2 - 3 cm height obtained after 4 - 6 weeks culture were cultivated in MS medium without hormones for root development. After 7 - 14 days, well-developed young plants with vigorous roots were transferred to the greenhouse in pots containing sterilized soil. Plants reaching 8 - 10 cm in height were transferred to larger pots.

RESULTS

Callus induction and plant regeneration, cytological investigation was experimented using 4,6-Diamidino-2-phenylindole (DAPI) and Aceto-carmine. Tetrad and uni-nucleate microspores obtained after meiosis were observed (Figures 1a and b). Callus production from anthers cultivated in N6 medium and resulting haploid plants regenerated are summarized in Table 2.

The overall frequency of induced calli and regenerated plants are markedly low with regard to the potential of inducible microspores per anther. In fact, between 2 - 30 calli were obtained from cultures initiated with anther numbers of 231 - 1023 per genotype. Callus production

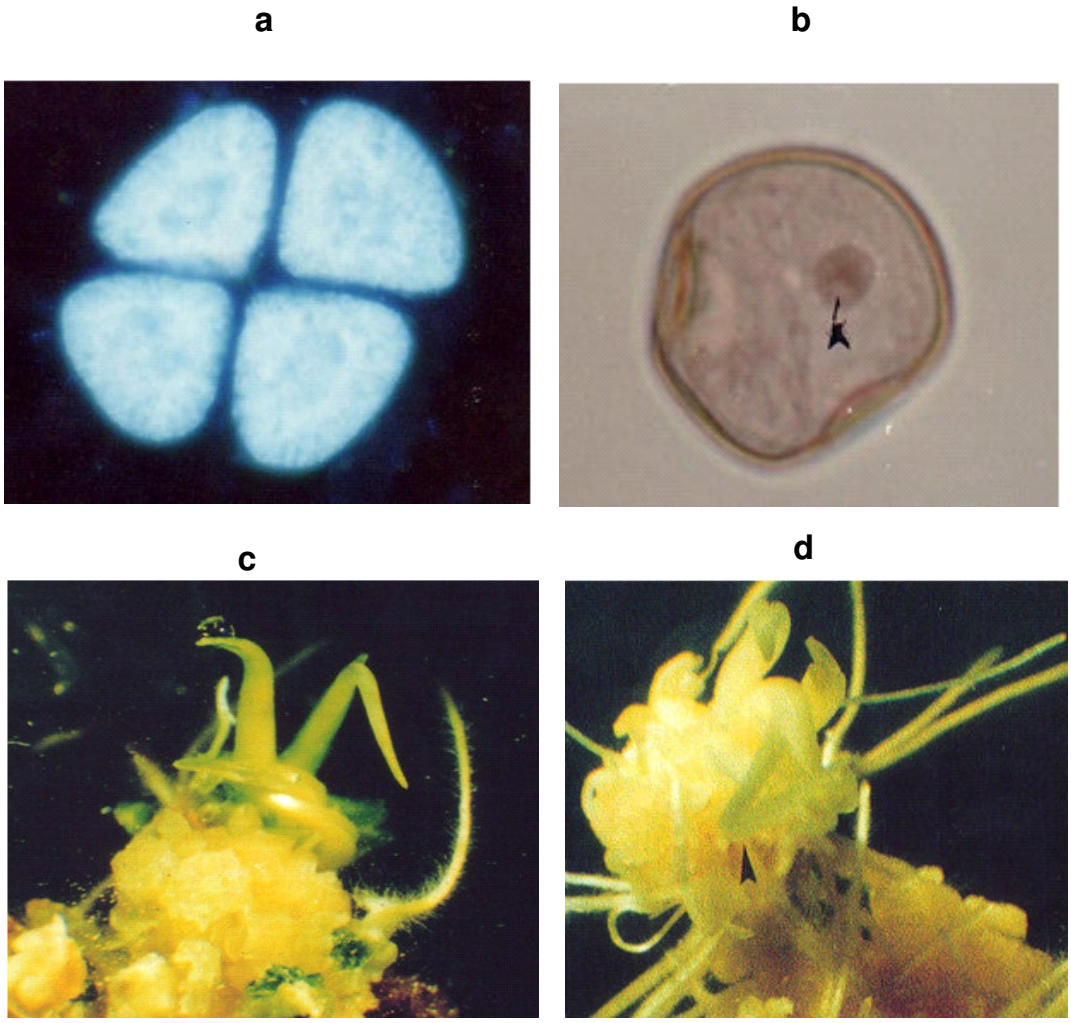

Figure 1. (a) Formation of tetrad (n) after meiosis during microsporogenesis stained with Diamidino phenylindole (DAPI), (b) uninucleate microspore (n) stained with Aceto carmine, (c) Regeneration of haploid green plant from callus in MS medium and (d) regenerated albino plants in MS medium.

Table 2. Callus induction and plant regeneration of *O. sativa* and *O. glaberrima* genotype.

	A	B	[a][B/A*100]	C	D	E	[b][D+E/A*100]
Variety	**Number of cultivated anthers**	**Number of calli**	**Callus frequency %**	**Number of calli that regenerated green or albino plants**	**Number of green plants**	**Number of albino plants**	**Androgenic plant frequency**
7178 Tog	528	30	5.68	6	0	26	4.92
6202 Tog	528	13	2.46	2	14	0	2.65
7205 Tog	231	2	0.86	1	0	15	6.49
IKP	660	28	4.24	7	0	38	5.75
Sahel 108	1023	7	0.68	0	0	0	0
Farox 304	924	5	0.54	0	0	0	0
Farox 239	660	2	0.03	0	0	0	0
IR 31851	792	2	0.02	0	0	0	0
Total	5346	89	1.66[a]	16	14	79	1.74[b]

[a] Frequency of callus induction (number of calli divided by the total number of anthers cultured).
[b] Frequency of plant regenerated (number of regenerated plants divided by the total number of anthers cultured).

Low rate of callus induction and or plant regeneration is generally observed in anther culture with indica rice. Tran and Vuong (2002) obtained frequency of 3.53 of callus induction in N6 medium and 1.12% in plant regeneration.

Best reponse of japonica rice compared to indica has been earlier demonstrated by other authors (Mandal and Bonyopadhyay, 1997; Gosal et al., 1997; Raina and Zapata, 1997; Chen et al., 1991). In rice, it has been demonstrated that japonica genotypes produce more androgenic callus and plants than indica genotypes (Zapata et al., 1990; Yamagishi et al., 1998). Species dependence has been found by Tang et al. (1999) in wild rice species, in callus production and plant regeneration from microspore-derived calli. A diallel analysis has revealed that anther culturability in rice is a quantitative trait controlled by the nuclear genome. Miah et al. (1985) shows that, callus induction ability is inherited as a recessive character conditioned by a single block of genes and japonica appear to be a good combiner for callus induction.

Albino plants are often produced in anther (Niizeki and Oono, 1968), tissue (Tang and Sun, 1979), ovary (Liu and Zhou, 1984), cell (Zou et al., 1986) and protoplast-culture (Lei et al., 1986) which limited the application of anther culture technique in breeding. There are not many studies with *O. glaberrima* varieties. Only some experiment was done with hybrids *O. sativa* x *O. glaberrima* to produce DH line (Jones et al., 1996). Many authors have shown that, anther culture depends on diverse factors, including the developmental stage of microspores (Chen, 1976), period of cold pre-treatment of anthers (Zapata et al., 1982), growing conditions of donor plants (Lee et al., 1988), culture medium (Mandal and Gupta, 1997), orientation of plated anthers (Mercy and Zapata, 1987), and plant genotype (Shen et al., 1983). Abundance of albinos (Gosal et al., 1997; Raina and Zapata, 1997) and low percentage of green regenerated plants are the major constraints which need to be improved through manipulation of chemical and physical environments together with other innovative methods (Alejar et al., 1995). Much more attention should be paid to the physiological state of donor plants and their growing conditions. Low ratio green/albino by anther culture of recalcitrant indica rice can be improved with (i) early transplanting of young calli onto regeneration medium, (ii) a low temperature incubation (< 26°C), and (iii) improving media for callus induction and plant regeneration (Bishnoi et al., 1999). A diallel analysis of anther culturability and green plant regeneration of Quimo and Zapata (1990) shows significant effects of genotype and genotype x medium. A predominance of additive effects controlling both characters was suggested by a combining ability analysis, with japonica cultivars having higher combining ability for green plant regeneration. Many protocols of rice anther culture are now established with different degree of success. Growing conditions of donors plants should be take into account. Collecting panicles with plant under field conditions (Lee et al., 1988) will contribute to increase callus induction and plant regeneration from anther-derived calli. *O. glaberrima* genotypes seem to be interesting for callus induction and plant regeneration from anther-derived calli. More investigation is needed with this species including interspecific hybrid. Concerted efforts are also required to enhance the frequency of responding microspores to improve callus growth and subsequent green plant regeneration particularly with recalcitrant indica rice (Gosal et al., 1997). Pre-treatment stress of anthers and use of maltose as the carbohydrate source (Raina and Irfan, 1998) can help to produce a high frequency of callus and plants. On the other hand, enhanced efforts should be done on genetic engineering and molecular genetic studies in order to really understand the genetic mechanism that controls the anther culturability, particularly in the relatively recalcitrant indica rice.

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