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Improved rate of callus induction from rice anther culture following microscopic staging of microspores in iron alum-haematoxylin

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Summary. High frequencies of callusing were obtained in indica rice from the microspores which were staged in acetic acid iron alum-haematoxylin stain prior to culture on G5 medium. Two local varieties, 'Khonorullo' and 'Namyi', and two advance pre-release cultures, 'PK 1-1-3' and 'PK 12-22', were used in this investigation. All the cultures exhibited a wide adaptation to varying medium; however, the frequency of callusing was highest (45.5%) in 'PK 1-1-3' followed by 'PK 12-22' (32.4%) and 'Khonorullo' (31.6%). Cold shock (10 °C) for 11 days enhanced the frequency of callusing by 200% in 'Khonorullo'.

Key words: Microspore – Anther culture – Haploid production – *Oryza sativa* L. ssp. *indica*

Introduction

The demonstration by Guha and Maheshwari (1964, 1966) that culturing immature anthers of *Datura* in artificial nutrient medium can directly give rise to embryoids with haploid numbers of chromosomes, generated worldwide interest in developing the technique of haploid production through androgenesis for a variety of crop plants. Niizeki and Oono in 1968 obtained seven haploid rice plants through anther culture. Breeding through haploids reduces the time needed to reach homozygosity since spontaneous or induced doubling of haploid chromosome number results in homozygous diploid plants. In addition, the screening of haploid cells against cold tolerance, salinity, pathotoxins and other biotic and abiotic factors before plant regeneration also becomes possible.

Several studies indicate that anthers containing immature pollen are more likely to produce callus than those having pollen in advanced stages (Guha et al. 1970; Niizeki and Oono 1971; Guha-Mukherjee 1973). However, Chen's (1977) work in particular has demonstrated that maximum callus and green plant regeneration is obtained by culturing anthers with microspores at the miduninucleate stage of development. Most rice researchers have used japonica types for such a study and the highest frequency reported so far is 68% in 'Norin 21', a japonica type (Genovesi and Magill 1979). The indica rice is recalcitrant to callusing in vitro (Chaleff 1979; Raina 1983; Miah et al. 1985); however, 52.5% callusing has recently been reported by Reddy et al. (1985) in indica rice.

The present study reports high frequencies of callus induction in indica rice obtained through the use of microscopic staging of microspores followed by culturing on G5 medium. This investigation also confirms the role of cold pre-treatment in enhancing the frequency of callusing.

Materials and methods

Two varieties, 'Khonorullo' and 'Namyi', and two advance cultures, 'PK 1-1-3' and 'PK 12-22' (progeny of 'Pusa-33' × 'Khonorullo'), were raised in the field at the ICAR Research Complex for N.E.H. Region, Shillong, with normal fertilization and crop management. Panicles were excised while still enclosed within the sheath and brought to the laboratory. They were wrapped in polythene bags and stored in a BOD incubator maintained at 10 °C.

Preparation of iron alum-haematoxylin stain

A stock solution of 4% haematoxylin in 45% acetic acid was prepared and 1 g of iron alum was added to this. The solution was allowed to stand for 24 h. To 10 ml of this stock solution

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4 g of chloral hydrate was added and after complete dissolution it was used (Chang et al. 1978).

Staging of microspores

Anthers were squashed in a drop of staining solution under a coverglass. After 2–5 min the slide was observed under a research microscope.

Anther culture

Panicles of the 4 varieties named above were surface-sterilized. Since individual panicles contain microspores at different stages of development, the sheath was opened at five places and one spikelet was taken out from each opening. Anthers were squashed and the portion containing microspores at the mid-uninucleate stage were taken out, passed through 70% ethanol for a few seconds, and then sterilized in 10% w/v calcium hypochlorite solution. Spikelets were rinsed in autoclaved double distilled water and placed in one of the following culture media: 1) Minimal medium: medium no. 12 of Song et al. (1978) fortified with 2,4-D (2 mg/l), nicotinic acid (0.5 mg/l) pyridoxin-HCl (0.1 mg/l), thymine-HCl (0.1 mg/l) and glycine (2 mg/l); 2) MS medium: fortified with the supplements used in (1); 3) Blaydes medium: fortified with IAA (1.5 mg/l), kinetin (1.5 mg/l) and 2,4-D (1 mg/l); 4) B5 medium: fortified with 2,4-D (2 mg/l), nicotinic acid (0.5 mg/l), pyridoxin-HCl (0.5 mg/l), thymine-HCl (1 mg/l) and glycine (2 mg/l). Here designated as G5 medium; 5) N6 medium: fortified with the supplements used in (4); 6) Genovesi-Magill: N6 supplemented with thymine-HCl (0.5 mg/l), and NAA (2 mg/l); 7) E24 medium: B5 basic salts fortified with 2,4-D (1 mg/l), IAA (0.5 mg/l), BAP (0.5 mg/l) and inositol (160 mg/l) (Zapata et al. 1983); 8) G4 medium: B5 salts and vitamins fortified with 2,4-D (1 mg/l), BAP and IAA (0.5 mg/l each).

Small amounts of calli (10 mg) were transferred to J-19 (B5 salts fortified with NAA (1 mg/l), kinetin (1 mg/l) and inositol (160 mg/l) or N-19 (MS supplemented with NAA and kinetin (1 mg/l each) and inositol (100 mg/l) liquid medium for proliferation. After 10 days they were transferred to a regenerating medium (MS supplemented with NAA and kinetin (2 mg/l each) and BAP (1 mg/l) and kept under white fluorescent light (3,000–3,500 lux) with a 14/10 h photoperiod.

Cytology of calli

Calli were pretreated with a saturated solution of p-dichlorobenzene for 3 h. After thorough washings with distilled water they were fixed in acetic-alcohol (1 : 3) for 24–72 h and stained in 2% acetoorcein at 90° for 2 min. They were squashed in 1% acetoorcein.

Results

Staging of microspores

The microspore nucleus when stained in iron-alum-haematoxylin, assumed a deep grey colour against the colourless cytoplasm. Microspores at uninucleate (Fig. 1 a) and binucleate stages were distinctly visible.

Callusing of anthers

The microspores of 'Khonorullo', plated in six culture media-minimal medium, MS, Blaydes, B5, Genovesi-Magill and N6 – showed callusing in MS, Genovesi – Magill, B5 and minimal medium. Since callusing was better in B5 and minimal medium, further experiments were conducted using B5 basic and minimal medium.

Anthers of 'Khonorullo', 'Namyi', 'PK 1-1-3' and 'PK 12-22' were inoculated in G5, E24, G4 (all contain B5 basic salts) and minimal medium. During the first 3–4 weeks uninucleate microspores enlarged (Fig. 1 b), and later became binucleate through mitosis. Subsequently, both cells divided and the callus mass bursted out of the anthers which had already turned brown after 40–50 days of culture in the dark. The frequency of callusing in different media has been presented in Table 1.

In 'Khonorullo' an average of 31.6% callusing was recorded on minimal medium (Fig. 1 c), 3.9% in MS fortified with supplements of medium no. 1, 6.1% in MS supplemented with the supplements of E24 medium; 15.5% in G4, 12.2% in G5 and 11.7% in E24. 'PK 12-22' showed 32.4% calusing in E24 medium. The average callusing frequency was 45.5% for 'PK 1-1-3' and 9.5% for 'Namyi' when cultured in the G5 medium. Although the average callusing was 45.5% for 'PK 1-1-3', in some of the tubes the frequency was up to 95% (Fig. 1 d).

Effect of cold pretreatment

A mild temperature shock (10 °C) of up to 11 days given to the anthers, enhanced the frequency of cal-

Table 1. Frequency of callus production in different media

Media / Variety	G5		E24		G4		Minimal medium	
	Anthers plated	Callus induction (%)	Anthers plated	Callus induction (%)	Anthers plated	Callus induction (%)	Anthers plated	Callus induction (%)
'Khonorullo'	844	12.2	811	11.7	653	15.5	566	31.6
'PK 1-1-3'	266	45.5	141	35.4	–	–	199	30.6
'PK 12-22'	142	20.4	342	32.4	–	–	–	–
'Namyi'	126	9.5	–	–	153	3.9	145	4.8

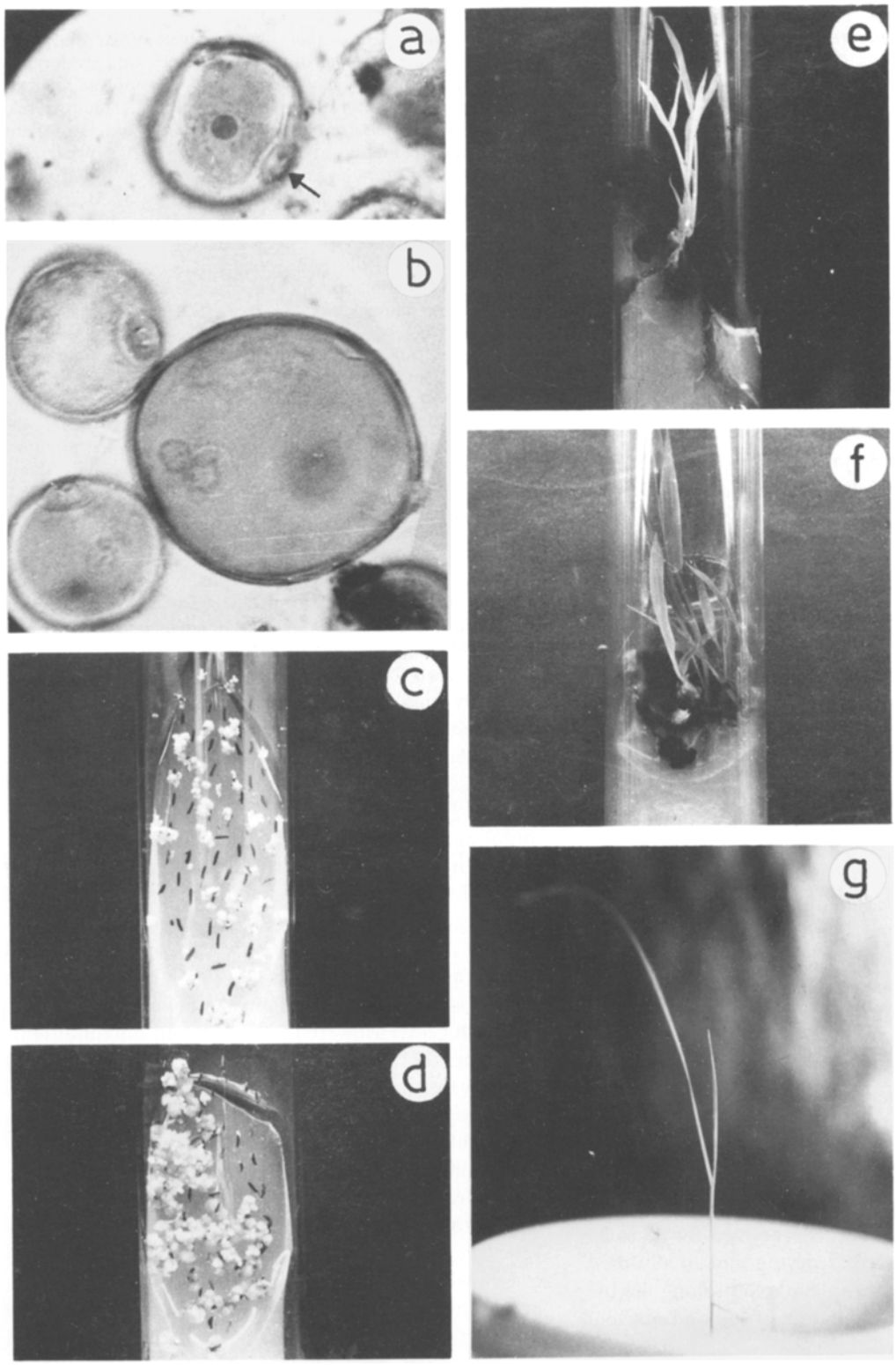

Fig. 1. a, b Photomicrographs of microspores of *Oryza sativa* L. ssp. *indica* (X900): **a** microspore, stained in acetic acid-iron alum-haematoxylin, showing distinct nucleus and germ pore (arrow); **b** Enlarged microspores after 30 days of culture on G5 medium (B5 basic salts + 2,4-D and glycine (2 mg/l each) + nicotinic acid and pyridoxin-HCl (0.5 mg/l each) + thymine-HCl (1 mg/l); (note the degree of enlargement in **b**). **c** Anthers of cv. 'Khonorullo' showing an average of 32% callusing on minimal medium; **d** Anthers of 'PK 1-1-3' showing profuse callusing up to 95% on G5 medium; **e–f** Calli-derived haploid and diploid plantlets of 'PK 1-1-3' and 'Khonorullo' redifferentiated on MS + NAA and Kin (2 mg/l each) + BAP (1 mg/l), when kept under white fluorescent light (3,500 lux) 14/10 h photoperiod; **g** Haploid plantlet of cv. 'Khonorullo' in pot, 30 days after transfer to soil

Table 2. Effect of cold pretreatment on the anthers of cv. 'Khonorullo'

Duration of cold pretreatment	Anthers		
	Plated (no.)	Producing (no.)	Callus (%)
0	461	58	12.5
6	469	80	17.0
11	421	138	32.8
18	665	98	14.7
23	318	54	16.9
25	315	150	47.6

lusing from 12.5% in the control to 32.8% (Table 2). Although a cold shock for 25 days also enhanced the frequency of callusing (47.6%) the plantlets regenerated from these microspores mostly gave rise albinos.

Proliferation of callus and their ploidy status

Ten to 15 mg of calli were transferred to N-19 and J-19 liquid medium for further proliferation. Both media were found to support the proliferation of callus equally well; however, the proliferation took place only in the dark or under low intensity, diffused light.

The cytology of calli revealed that the callus cells were mixoploids, haploid cells were, however, in abundance and their frequency ranged from 30 to 40%.

Regeneration of plantlets

Six media compositions were tried for redifferentiation of the androgenic calli. MS medium supplemented with NAA 2 mg/l, kinetin 2 mg/l, BAP 1 mg/l and sucrose 3%, supported regeneration in the presence of fluorescent tube light (3,600 lux intensity, 14/10 h photoperiod). The frequency of redifferentiation was, however, low (6–12.5%). Frequency of albinos was 20–25% of the regenerated plantlets and it went up to 50% when the calli were taken after 25 days of cold shock. The cytological examination of plantlet root-tips revealed their haploid (Fig. 1 e) and diploid (Fig. 1 f) nature. After regeneration, the plantlets were removed to the sterilized soil (Fig. 1 g) which was kept wet with Hoagland's nutrient medium. Plantlets survived for 25 to 30 days, after which they started drying and finally died after 45 days. Efforts are being made to prolong the life of the plants through manipulation of soil and nutrient medium.

Discussion

One of the important factors for haploid production through androgenesis is the culturing of anthers at the mid-uninucleate stage (Chen 1977). When cultured at this stage there is shifting in the normal pathway of pollen development and after repeated mitotic divisions of the microspores, calli are formed. Although microscopic identification of the mid-uninucleate stage is necessary before the plating of anthers, some researchers have used the external morphological characters of the panicle to select anthers at this stage (Chu 1980). However, the use of such morphological features has been found to be erroneous both in our laboratory as well as by Mercy et al. 1984. Microscopic staging is an answer to this problem. The stain generally used in microscopic staging is 2% acetocarmine (Mercy et al. 1984) which in our experience does not stain the nucleus and cytoplasm differentially. The use of modified acetocarmine staining was advocated by Genovesi and Magill 1979. Nevertheless, some researchers have not found even this method very satisfactory. Chang et al. 1978 suggested a staining technique using haematoxylin and iron-alum.

In our laboratory, the microspore nuclei of rice could not be observed by simple acetocarmine staining. However, the visibility of the nuclei becomes slightly better when modified acetocarmine staining is employed (Genovesi and Magill 1979). Using the Feulgen stain (Gupta et al. 1986) the nucleus can be seen distinctly but the time required is 2–5 h and hence this technique may have only a limited application, especially when a large number of anthers are to be plated every day. Acetic acid-ironalum-haematoxylin stains microspore nuclei very quickly (2–5 min) and the nucleus becomes very distinctive. The clarity of nuclear staining using the haematoxylin method may be due to the use of iron-alum which acts as a mordant.

Induction of callusing in japonica rice has been generally high. However, it has not been so in indica, consequently work on haploid production in indica rice has been slow. In addition to hormonal and other growth supplements (Guha et al. 1970; Niizeki and Oono 1971), the concentration of nitrogen plays an important role in callusing (Chu 1975; Song et al. 1978). In the present investigation, when B5, MS and N6 basic media were used, the highest frequency of callusing was recorded from the B5 medium, which contains 26.72 mM of total nitrogen as against 60 mM in MS and 35 mM in N6. In the minimal medium where callusing was also high, the concentration of N, P, K and Ca was lowered to a third and micronutrients like zinc, boron, iodine, copper, molybdenum and cobalt were eliminated as suggested by Song et al. 1978. It appears that a low concentration of nitrogen is favourable to callus induction. In the present investigation, callusing was generally high in the G5 medium (which contains B5 basic salts): 'PK 1-1-3' and 'PK 12-22' recorded 45.5% and 20.4% callusing, respectively. The response of 'PK 12-22' was still better (32.4%) in E24 which again contains B5 salts. It may, therefore, be concluded that B5 basic medium is the most suitable for the callusing of indica rice. However, varietal preference has been observed by the present authors. Genotypic differences in anther response to callusing have already been reported (Guha-Mukherjee 1973; Maheshwari et al. 1982). Our results on the suitability of media is in conformity with that of Zapata et al. (1983) where the B5 basic medium was reported to be the best.

Recently Reddy et al. (1985) have reported anther callusing ranging from 2.0% to 52.6% (In cv. 'Radhunipagal' and Small fruit, respectively) after extensive changes in the composition of the culture medium, including growth supplements. However, they have used external morphological features of the panicle for obtaining the microspore at the mid-uninucleate stage. In the present investigation although an average of 45% callusing was recorded in one of the cultivars it was as high as 95% in some of the tubes. This phenomenal response could be atributed to the precise determination of the uninucleate stage of microspores through microscopic staging.

Cold shock has been extensively used by several researchers (Hu et al. 1978; Chaleff and Stolarz 1981) for enhancing the frequency of callusing in rice. In the present investigation, 11 and 25 days of cold shock resulted in an increase of callusing by 200% and 400%, respectively. However, calli from 11 days of cold shock were found to be better for plantlet regeneration as the frequency of albinos was high in the latter. This indicates that the ability to produce green pigment is lost with time during a cold pretreatment. The result of the cold pretreatment is in agreement with that of Genovesi and Magill (1979) who have reported a 100% increase in callusing over the control in anthers treated at 10 °C for 10 days and that of Zapata et al. (1983) where a 50% increase was recorded after 8 days of pretreatment at 8 °C.

The frequency of regeneration in the present investigation was rather low and the regenerated plantlets survived only up to 30 days in pots. An effort is being made to enhance the frequency of redifferentiation and to prolong the life of plantlets in pots through the manipulation of regeneration media and environmental conditions.

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