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Culture of Unfertilized Embryo Sacs of Pearl Millet - *Pennisetum glaucum* (L.) R. Br.

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Abstract

The morphology and structure of isolated unfertilized embryo sacs of pearl millet, *Pennisetum glaucum* (L.) R.Br. is similar to those *in situ.* In all cultivars a minimum of five intact embryo sacs could be isolated from 30 ovaries per hr. Isolated embryo sacs (30-50%) were viable for four days on MS medium. An increase in the number of polar nuclei was osberved in 15% of the embryo sacs cultured for two - four days on MS + 3.0 mg/l BAP. These results hold promise in generating gynogenetic haploids from some pearl millet cultivars.

Introduction

Isolation of viable gametes is a prerequisite for generation of haploids and for *in vitro* fertilization. Isolation of gametes *per se* is a difficult task compared to the isolation of gametophytes. Different methods have been developed for the isolation of gametes/gametophytes. These methods are micromani-pulation, enzymatic maceration or a combination of both enzymatic maceration and micromanipulation. The enzymatic maceration technique was used to isolate viable egg cells from maize (Kranz et al. 1991), perennial rye grass (Van der Mass et al. 1993), tobacco (Hu et al. 1992) and *Torennia* (Mol 1986). In case of wheat egg cells were isolated by means of non-enzymatic micromanipulation (Kovacs et al. 1994) and barley (Holm et al. 1994). In maize enzymatic isolation has been shown to have a deleterious effect on embryo sacs and egg cells (Leduc et al. 1995); the egg protoplasts isolated by treatment with enzymes do

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not fuse with sperms as efficiently as their counterparts isolated mechanically (Kranz et al. 1995). Because of this limitation, manual isolation of gametes/ gametophytes is gaining more attention. Mechanical isolation of fertilized (Lakshmi et al. 1999) and unfertilized (Aruna Lakshmi and Lakshmi, unpublished) embryo sacs from pearl millet has been achieved in our laboratory. We report here an increase in the number of haploid nuclei in 15% of embryo sacs cultured for four days as well as the viability of unfertilized embryo sacs from two different cultivars of pearl millet, IP 8166 and Vg 272.

Materials and Methods

Two varieties of pearl millet viz. Vg 272 and IP 8166 (a dwarf with purple pigmentation) were investigated in the present study. The ear heads were bagged before the emergence of styles from the ovaries. Embryo sacs were isolated from unpollinated ovaries as described by Lakshmi et al. (1999b). The isolated embryo sacs were surface-sterilized and quickly transferred on to the culture medium with a needle or a brush with only a few bristles.

The culture media used were : (i) MS + 2.5 mg/l 2,4-D, (ii) MS + 3.0 mg/l BAP, (iii) MS + 3.0 mg/l Kn, (iv) MS + 1.0 mg/l IAA + 2.0 mg/l GA + 3.0 mg/l 2,4-D and (v) N_6 + 3.0 mg/l 2,4-D.

These culture media were chosen because they were earlier demonstrated to be best suited for the culture of fertilized embryo sacs (Lakshmi et al. 1999b), mature and immature embryos (Lakshmi et al. 1999a) of the cultivar Vg 272. The osmolality of the medium was not adjusted, as the isolated embryo sacs of these cultivars were stable in a wide range of osmolality (Aruna Lakshmi and Lakshmi 2001). The cultures were examined intermittently.

A few intact ovaries were also cultured under similar culture conditions and embryo sacs from these ovaries served as control. Liquid media were used since embryo sac viability rate was higher in liquid media compared to their solid counterparts (Lakshmi 1991). Embryo sacs immediately after isolation and thereafter on different days of culture were checked for their viability by fluorescein diacetate (FDA) test (Heslop-Harrison and Heslop-Harrison 1970). The reactions were observed under Leitz Laborlux S epifluorescent microscope using appropriate filters. The detailed morphology of the nuclei was studied using fixed (1 : 3 acetic : alcohol) material stained with 0.8% aceto-carmine.

Results and Discussion

Isolation of embryo sacs from unfertilized ovaries was found to be more difficult than those from the fertilized ovaries, possibly due to their small size and fragile nature. We found that the isolation of embryo sacs from the variety IP 8166 was easier (20 embryo sacs from 30 ovaries per hr) compared to the

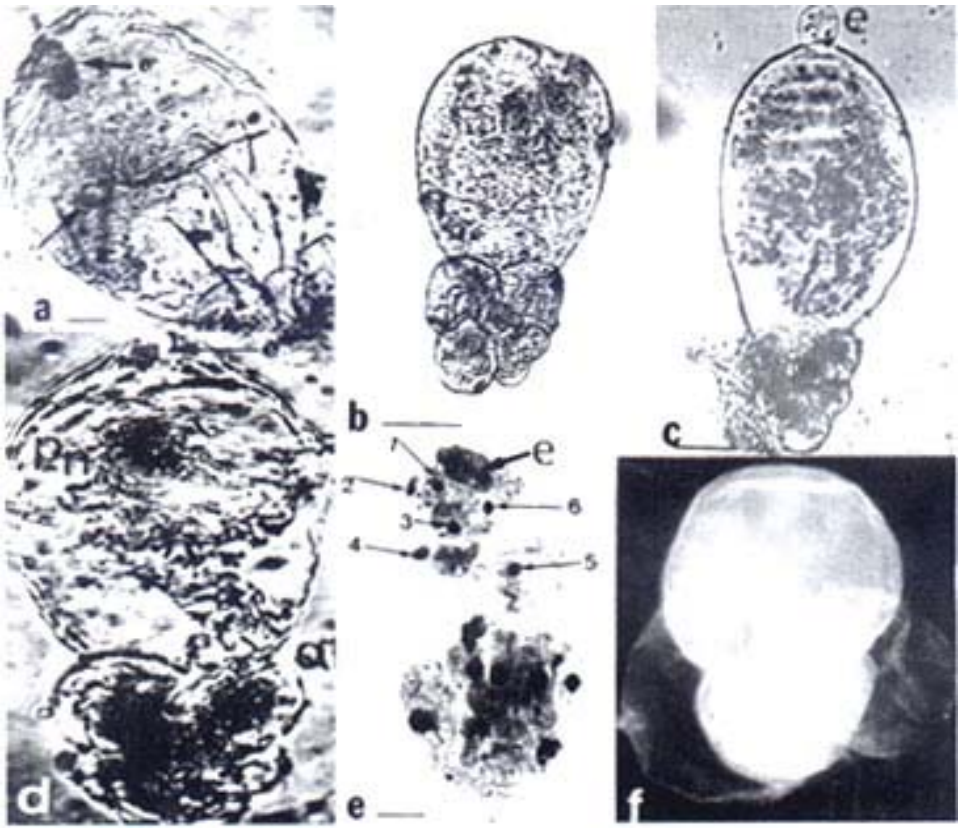

Fig. 1. (a) Embryo sac of IP 8166. (b) Embryo sacs with broad micropylar end, isolated from purple bristled ear heads of Vg 272 (light microscope). (c and d) Oval shaped embryo sac, egg (e) protruding out, isolated from green bristled Vg 272. (c - light microscope; d - phase contrast microscope). (e) An acetocarmine stained embryo sac (Vg 272) showing six degenerated nuclei in the central cell. (f) FDA positive and negative embryo sacs of VG 272. Bar = 10 μm.

variety Vg 272 (ten embryo sacs from 30 ovaries for the purple bristled strain and only five embryo sacs from 30 ovaries in the green bristled strain). This observation may have relevance to the shape of the embryo sac. The variety IP 8166 has an elliptical emrbyo sac (Fig. 1a) while the variety Vg 272 has an oval/oblong embryo sac with a blunt micropylar end).

The microscopic observation (phase contrast microscope) of isolated embryo sacs revealed a richly granular cytoplasm with small starch grains. The egg apparatus were identified by its oval to pear shape or nearly that of a spherical egg flanked by synergids (Figs. 1b, c). The egg is always nearly oval in the embryo sacs of IP 8166 (Fig. 1a). The egg in either cultivar is filled with glistening starch grains and is therefore, readily identified. The egg of IP 8166 is larger and denser than that of Vg 272. The polar nuclei were rarely identified, because of their large nucleoli appearing darker than the rest of the contents; they were found in the vicinity of the egg apparatus (Fig. 1d). It should be noted that the embryo sacs float in the liquid medium and a continuous focusing is required to get a clear picture of polar nuclei in the live specimens. The antipodals, the components of the embryo sac were identified first following dissection of an ovule filled with starch grains. The structure and morphology of isolated embryo sacs were comparable to those *in situ*.

The viability test showed that 90% embryo sacs were viable immediately after their isolation (Fig. 1f), about 50% four days after culture (Table 1) and only 2% after 15 days. The egg apparatus of the damaged embryo sacs remained viable for 1 hr. However, the exposed egg apparatus of intact embryo sacs did not loose its shape and remained viable up to four days in the medium.

Table 1. Culture of unfertilized embryo sacs of Vg 272 and IP 8166 from pollinated ear heads.

Line	Composition of the medium (mg/l)	No. of cultured embryo sac	Remarks
Vg 272	MS + 2.5 2,4-D	53	39 - 50% viable up to four days
Vg 272	MS + 3.0 BAP	60	Increased number (six) of free polar nuclei in a degenerate state after four days. No visible changes in polar nucleus or egg. Degenerated embryo sacs
Vg 272	MS + 3.0 Kn	55	30 - 35% viable up to four days with no visible change in the contents
Vg 272	MS + 1.0 IAA + 2.0 GA + 3.0 2,4-D	71	32 - 48% viable up to four days (100% degeneration by sixth day)
Vg 272	N_6 + 3.0 2,4-D	51	48 - 60% viable
IP 8166	MS + 3.0 BAP	37	44 - 56% viable up to four days

An increase in the number of polar nuclei to six (Fig. 1e) was observed in 15% of embryo sacs cultured on MS + 3.0 mg/l BAP for four days (Table 1). Each nucleus contained a prominent nucleolus and a very deeply stained clumped chromatin. Clear mitotic stages were not detected.

Nearly 60% of the cultured ovaries were swollen while the rest shrivelled after a week. Embryo sacs isolated from the swollen ovaries were found to be viable while those isolated from the shrivelled ones degenerated (Table 2). There were no signs of division in the egg/synergids/polar nuclei in any of the cultivars in any of the culture media.

Table 2. Unfertilized embryo sacs isolated from cultured ovaries of Vg 272.

Composition of the medium (mg/l)	No. of cultured ovaries	Ovaries		% viable embryo sacs
		Swollen	Shrivelled	
N_6 + 3.0 2,4-D	96	61	15	80
MS + 3.0 BAP	43	17	26	40
MS + 2.5 2,4-D	66	60	6	90

It has ben demonstrated that intact, viable unfertilized embryo sacs with no visible mechanical injury can be dissected out with the help of a fine-tipped steel needle from unfixed ovules of pearl millet. Isolation of egg cells has been reported in maize (Kranz et al. 1995), wheat (Kovacs et al. 1994), barley (Holm et al. 1997), rye grass (Van der Maas et al. 1993), *Brassica* (Katoh et al. 1997), *Plumbago* (Huang and Russell 1989) and tobacco (Tian and Russell 1997a). Isolation of gametophytes rather than gametes for induction of division is not only easy, but also logical. As the gametes are enclosed within the gametophyte they are not in direct contact with the medium, hence not subjected to shock. Besides, long time viability of gametes is possible since they are enclosed in protective gametophytes. Clear visibility of the egg apparatus gives access to micromanipulate the egg.

Because of difficulty to isolate the embryo sacs manually the yield is low. With the available materials it is possible to dissect out 5 - 20 embryo sacs from 30 ovaries per hr depending upon the cultivar. In the present study it was observed that the "shape of embryo sacs" varies among the inbred lines of pearl millet and that the shape is an important character to facilitate dissection. A broad micropylar end is a desireble feature. Identification or more features conducive to easy dissection will enhance the speed of manual isolation.

In this experiment we used low concentrations of 2,4-D (2.5 and 3.0 mg/l), which were most suitable for the growth of zygotes (Lakshmi et al. 1999b) and mature embryos (Lakshmi et al. 1999a) of cultivar Vg 272. High concentrations

of 2,4-D up to 50 mg/l for a short duration induced divisions in the isolated egg protoplasts of maize (Kranz et al. 1995). We intend to subject the egg containing embryo sacs to high pulse of 2,4-D, which might trigger divisions in the egg cell. We report here for the first time an increase in the number of polar nuclei in the cultured unfertilized embryo sacs. In maize, divisions were induced in the *in vitro* zygotes without feeder cells, but for their sustained growth co-culture with feeder cells was mandatory (Kranz et al. 1995). In wheat, isolated zygotes developed into embryos and plantlets when co-cultured with ovular tissues/feeder cells (Kumlehn et al. 1997, 1998b). A modification of the medium through a suitable nurse culture probably is needed to sustain the viability and growth of unfertilized embryo sacs in pearl millet too. Besides haploid induction, the *in vitro* embryo sacs have a great potential for use in a variety of investigations viz, in the study of the structure, physiology and transformation of the egg cell into an viable embryo.

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