ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 09, pp.37778-37781, September, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

A PRELUDE TO *IN VITRO* FERTILIZATION IN PEARL MILLET- *Pennisetum glaucum* (L.) R.BR.

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ARTICLE INFO

Article History:
Received 17th June, 2016
Received in revised form
16th July, 2016
Accepted 29th August, 2016
Published online 20th September, 2016

Key words:

Pennisetum glaucum, In vitro fertilization, Pollen grains, Egg cells and synergids.

ABSTRACT

This research is part of an attempt to establish *In vitro* fertilization in pearl millet which aids an understanding the mechanisms of fertilization. We demonstrate here co-culture of isolated embryo sacs with pollen grains and/or male gametes of pearl millet in two culture media. The pollen grains in the first medium pseudo germinated, while in the second medium germinated as in planta. The pollen tubes of some pollen grains were guided to the egg apparatus, while those of others were not. Precise guidance of pollen tubes to the eggs is crucial for successful fertilization. The probable reasons for the variation in the guidance of pollen tube growth to the egg apparatus are discussed.

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Citation: Lakshmi, T. V. R., Shahid Nazir, M., Raja Rao, K. G. and Aruna Lakshmi, 2016. "A prelude to *in vitro* fertilization in pearl millet- *Pennisetum glaucum* (L.) R.BR", *International Journal of Current Research*, 8, (09), 37778-37781.

INTRODUCTION

Fertilization plays a key role in higher plants. To gain insights into cellular and molecular events of fertilization and its application to create new hybrids prelude of IVF is mandatory (Wang *et al*, 2006). *In vitro* pollination/fertilization if successful becomes a valuable tool for plant breeders (Zenkteler, 1990; Kranz and Scholten, 2008). Hence an emergent need for investigations in the field of experimental embryology was felt, a couple of decades back. During the last two decades, techniques have been standardised for the isolation of embryo sacs and egg cells in monocots viz., pearl millet (Aruna Lakshmi and Lakshmi 2001; Lakshmi *et al*., 2002), rice (Okamoto, 2011), maize (Kranz and Lorz, 1994), barley, wheat (Kovacs *et al*., 1995) rye(Van Der Mass *et al*., 1993) and *Alstroemeria* (Hoshino *et al*., 2006), *Crinum asiaticum* (Ohshika and Ikeda, 1994) and in dicotyledons viz., rape seed and tobacco (Hu *et al* 1985; Tian and Russell, 1997b), *Nicotiana tabacum* and *Impatiens glandulifera* (Hussein, 2013), *Plumbago zeylanica* (Huang and Russell, 1989), *Brassica* (Katoh *et al*., 1997), *Dianthus* species (Hoshino *et al*., 2000), sunflower (Popielarska and Przywara, 2003). Up to the present time *in vitro* fertilization (IVF) is accomplished only in maize (Kranz *et al*., 1991), wheat (Kovacs *et al*., 1995) and rice (Uchiumi *et al*., 2007). Although IVF has been successful in maize, rice and wheat, fusion of male and female gametes is induced and the information on gamete recognition, adhesion and fusion is meagre. The use of gametophytes rather than isolated gametes seems to be more rational approach towards the study of IVF, as *in vivo* germinated pollen grains when co-cultured with the ovules, the pollen tubes specifically grew towards the embryo sacs (Higashima *et al*., 1998). So far, there are no systems involved in the culture of isolated embryo sacs with pollen grains. The present paper focuses on the co-culture of isolated embryo sacs with pollen grains and also with germinating pollen grains in pearl millet.

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MATERIALS AND METHODS

The cultivar Vg 272 (a local variety) of *P. glaucum* (L.) R.Br. was grown in the experimental field (day temperature 25-27 ^{0}C, night temperature 15-19^{0}C). Ear heads of field grown plants were bagged 2-3 days before the styles emerged. After the style emergence unpollinated ear heads together with 4-5 inch long peduncles were cut under water and kept in 3% sucrose solution for 4-6 hr until further use.

Isolation of embryo sacs

The procedure for isolation of embryo sacs is essentially the same as described by Aruna Lakshmi and Lakshmi (2001).

Viability

The viability of isolated embryo sacs was determined with 0.01% of FDA (Heslop- Harrison and Heslop- Harrison, 1970). The reactions were observed under a Leitz Laborlux S epifluorescent microscope system using appropriate excitation filters.

Co-culture of embryo sacs with pollen grains

The isolated embryo sacs were carefully transferred to liquid culture media taken in the well of a cavity slide. A freshly dehisced anther was tapped over the medium depositing the pollen grains.

(a) MS + 2.5 mg/l 2,4-D pH 7.0 (Murashige and Skoog, 1962) and
(b) 25% sucrose + 0.3% $CaNo_3$ + 0.01% Boron pH 7.0.

The media were so chosen as the former yielded up to 50% embryo sacs of the same cultivar viable up to 4 days (Lakshmi *et al*., 2002) while the latter was the standardized optimum medium for germination of pollen grains and the tube growth of the same inbred line (Raju, 1983). The culture conditions were essentially the same as those described for culture of fertilized embryo sacs of Vg 272 of pearl millet (Lakshmi *et al*., 1999). The slides were examined intermittently up to 6h with an Eliza inverted binocular microscope (Model TCM and TCMP (Toyo Optical Co., Ltd., Tokyo, Japan) fit with phase contrast objectives (10X, 40X with 10X eye piece).

RESULTS

The maceration yielded 30-40% of embryo sacs which are intact and filled with contents. About 25% of the isolated embryo sacs were FDA positive (Fig.1a).

Co-culture of embryo sacs with pollen grains in MS +2.5 mg/L 2,4-D medium

Freshly collected pollen grains are added to the liquid medium MS+ 2.5 mg/ L 2,4-D in which embryo sacs are freshly inoculated. A total of 57 embryo sacs were cultured with the pollen grains in 5 experiments. A few pollen grains burst at the germ pore through which the contents oozed out in the form of a thread. The contents were full of shining, small starch grains which hindered the visibility of male gametes or vegetative nuclei. A few pollen grains adhered at micropylar end of embryo sacs (Fig 1b), some at the antipodals and some at other regions (Fig. 1C); their distribution appeared to be random. The pollen grains did not show any signs of germination including those at micropylar end of the embryo sac revealing this medium to be unsuitable for germination of pollen grains and also attraction of male gametes to the egg apparatus.

Co-culture of embryo sacs with pollen grains in 25% sucrose + 0.3% $CaNo_3$ + 0.01% Boron medium

Alternately the medium reported as optimum for pollen germination and tube growth is attempted. A total of 100 embryo sacs were co-cultured in this medium in 6 experiments. A few pollen tubes were seen growing towards the embryo sac/egg apparatus (Fig.1d, arrows). Some of the pollen grains at the micropylar end of the embryo sac did not germinate but

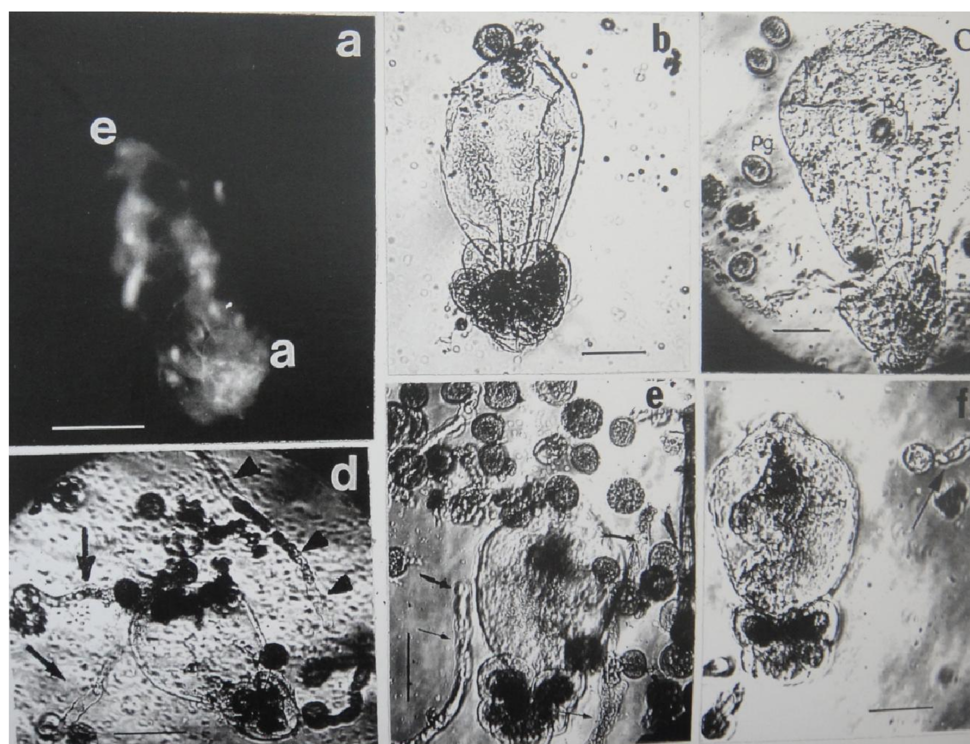

Figure 1. Isolated embryo sacs of *Pennisetum glaucum* (L.)R.Br.

a :FDA positive embryo sac, b& c : Co-culture of embryo sacs with pollen gains in the MS medium, b: light microscopic view of an adhered pollen grain at the egg apparatus of an embryo sac, c: Pollen grain adhered on surface of the embryo sac corresponding to the location of polar nuclei, d-f : Co-culture of embryo sacs with pollen gains in the Sucrose medium, d: Pollen tubes are guided towards the egg apparatus (arrows), e: Pollen tube growing along the embryo sac (arrow) to reach the egg apparatus, f: pollen tube growing away from the embryo sac, Figs a: Viewed under Fluorescent microscope, b: Light microscope, c-e: viewed under phase contrast microscope. Figs. A-E Bar= 100 μ

those at other regions (near antipodals) germinated, had their pollen tubes growing the entire length of the embryo sac and finally towards the egg apparatus region (Fig.1e). There was no firm contact between the embryo sac and the pollen tube as the pollen tubes dissociated easily from the embryo sac when the medium was gently agitated. There were no signs of distortion of shape or degeneration of synergids observed in any of the embryo sacs. Some of the tubes have grown past the micropylar end which appeared as not to identify the egg apparatus/ embryo sac (Figs. 1f arrow, 1d arrow heads).

DISCUSSION

In the present study, evidence of the pollen tube growth towards the egg apparatus/ micropylar end of embryo sac *in vitro* is achieved. The growth of some pollen tubes seems to be preferential as they grew towards the egg apparatus. The medium was adequate to allow pollen grains germinate, and pollen tubes grow towards the egg apparatus. Among the germinated pollen grains, some grew away from the egg apparatus /embryo sac. It has been stated that pollination builds communication pathway from pollen tube to ovule, demonstrating that a pollination-related signal elicits changes in the ovule and embryo sac in tobacco (Tian and Russel, 1997a). In *Arabidopsis* it is proposed that there are two types of control for guidance of pollen tubes towards the embryo sacs: 1. Embryo sac secretes signaling molecules and 2. The signal of the embryo sac causes changes in the surface properties of the sporophytic cells (Ray *et al*., 1997). It has been shown in *Arabidopsis* and tobacco that the signalling molecules, D-Serine (D-Ser) are secreted by the female sexual organ that open the Glutamate receptors-like channels (GLRs) to facilitate calcium ion concentrations for pollen tube growth (Michard *et al*., 2011). In the present study and also in *Torenia* the pollen grains germinated in the medium grew around the ovules but were not guided towards the embryo sacs (Higashiyama *et al* 1998). This failure of directed growth of some pollen tubes towards the egg could be 1. Due to the absence/dissipation of signal (D-Ser) from ovule; 2. Due to inadequate amount of the signal to direct the large number of pollen tubes present in the medium. In the current study, more than one pollen tube is attracted to a single embryo sac. In *Torenia* the embryo sac after receiving the pollen tube doesnot attract any more pollen tubes (Higashiyama *et al* 1998). The difference in response of pollen tube growth towards the embryo sac between the two genera is 1. In *Torenia* the pollen grains were germinated *in vivo* while in *Pennisetum* they germinated *in vitro* and 2. the embryo sacs in *Torenia* were not devoid of ovular tissues; while in pearl millet the pollen germinated in culture and the embryo sacs were isolated from ovules.

In the present experimental set up fertilization was not affected in embryo sacs that received one or more pollen tubes. In tobacco, it has been stated that pollination builds communication pathway from pollen tube to the ovule indicating that a pollination-related signal (D-Ser?) elicits changes in the ovule, and the embryo sac and intense calcium gradients are set up in the synergids and filiform apparatus before fertilization (Tian and Russell, 1997a). In pearl millet, in natural conditions "Pollination triggered synergid degeneration is required for the entry of pollen tube into the embryo sac (Chaubal and Reger 1990, 1992). In the present investigation, the synergids did not show any signs of degeneration or distortion and if any ultra-structural changes could not be detected under light microscopic study. Since the embryo sacs have been isolated from the ear heads prior to pollination, the synergids without receiving any signal to facilitate fertilization, failed to mimic their *in vivo* behaviour (fertilization). In wheat, activation of egg is by calcium concentration which is initiated/triggered by the pollen tubes (Ponya *et al*, 2014). It would be interesting to vary calcium levels in the media and study the effect on egg activation and subsequently *in vitro* fertilization.

IVF is possible in maize, wheat, rice and tobacco at present. The fusion of gametes is mediated either through electric pulse as in maize, wheat and rice (Kranz *et al*., 1991; Kovacs *et al*., 1995; Toda *et al*., 2016; Uchiumi *et al*., 2007) or through Calcium (Faure *et al*., 1994) or through Calcium and pH (Kranz and Lorz, 1994) or through PEG- polyethylene glycol as in tobacco (Tian & Russell 1997b). Although IVF is possible in these plants, only in maize and rice the *in vitro* zygotes grew into mature plants through a callus phase. From these experiments, 2 points of interest emerge- 1. The gametes which are fusigenic, lost their natural high tendency to fuse *in vitro*. 2. The *In vitro* zygotes undergo repeated divisions to form a callus initially, indicating that the information for a differentiated growth be erased during their formation.

The present approach is novel though did not lead to successful IVF, further efforts in this direction may enable us to identify the factors responsible for fertilization creating *in vitro* zygotes that develop like the ones in planta.

Acknowledgement

Part of the work was done in UMS, Dubai; and part of the work in AU, India. Authors [1]&[2] thank UMS, [4] thanks AU, India.

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