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In-ovule Embryo Culture: A Novel Method of Cotton Transformation

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Abstract: The study was undertaken to develop a culture technique permitting to grow cotton embryos from fertilization to germination with minimum of manipulation and maximum yield of viable plants. During the study, conditions were optimized for transfer of zygotic ovules after post anthesis and selection of media for the growth of ovules. Forty-eight hours post anthesis was selected as the most appropriate time since >50% of ovules were found alive, floating and developing. Also, ST medium was found suitable as it supported the growth very well and ovules continued to grow till the end of 10 weeks. This in-ovule technique has several advantages over culture of isolated embryos. The aseptic removal of 48 h old ovules from the ovaries was rapid and efficient and minimum damage was observed in this technique. No change of medium was needed during the entire culture period. This technique was used for the first time as a means of cotton transformation using Sonication assisted *Agrobacterium* mediated transformation (SAAT) method while a marker gene was used to transform cotton ovules.

Key words: Embryo culture, cotton ovules, GUS assay, cotton improvement, ST medium

INTRODUCTION

Cotton is the major nonfood cash crop of Pakistan, playing a key role in strengthening national economy. Cotton earns a lion share of foreign exchange from its export to other countries, as Pakistan is the 5th largest producer in the world. It provides 90% of the raw material for textile industry. It is also the source of more than 60% foreign exchange earnings while nearly 66% of the domestic oil is produced from cottonseed oil^[1]. The total area under cotton cultivation in Pakistan is 2997 thousand hectares producing about 11240 thousand bales with an average yield of 641 kg ha⁻¹^[2]. Cotton being referred as “Silver fibre” of Pakistan is susceptible to attack by more than 15 economically important insects, the major Lepidopteran being, American bollworm (*Heliothus armigera*), Pink bollworm (*Pectinophora gossypiella*), Spotted bollworm (*Earias insulana/vitella*), Cotton leaf roller (*Sylepta derogata*), Cotton semi looper (*Tarache notabilis*), Bud moth (*Phycita infusella*) and Corn ear worm (*Heliothus zea*). At present, crop protection in agricultural system relies almost exclusively on the use of broad-spectrum highly toxic agrochemicals.

This has resulted in serious environmental problems and human health concerns, leading to make efforts towards developing biological control measures. The total spray of insecticides exceeds about 41576 tons every year. In order to protect cotton against 40% damage without spray, the government is spending as much as 23 million rupees per annum on the cost of insecticides^[3].

Cotton breeders have continuously sought to improve the cotton crop through conventional plant breeding which has introduced numerous improvements in crop yields during the past centuries. However, resistance to insects and diseases does not exist in the available germplasm which led to limited new genetic information into plants and create plant varieties with novel characters through plant breeding techniques.

Current approaches of cotton improvement include the use of genetic engineering that is gaining momentum in developed as well as developing countries to improve the agricultural crops. However, progress in this area is limited because of the notoriously recalcitrant tissue culture nature of most elite cotton cultivars. In the absence of a well-characterized regeneration procedure for cotton, the present study was an attempt to develop a

culture technique to grow cotton embryos from fertilization to germination with minimum of manipulation and maximum yield of viable transformed plants.

MATERIALS AND METHODS

Plants of cotton were grown under green house conditions at National Centre of Excellence in Molecular Biology (NCEMB), University of the Punjab, Lahore. Flowers were self pollinated and the ovaries harvested at 24, 48 and 72 h post anthesis. These were surface sterilized by dipping in 70% ethanol for 2-3 min with slow shaking followed by flaming. After dissection^[4], about 20 zygotic ovules were aseptically removed and floated on the Beasley's medium^[5] containing no hormones in the flasks and were incubated at 32±2°C in darkness. Counts of floating ovules were made during the entire culture period. At the end of 10 weeks, ovule development and maturation was found at different stages.

Agrobacterium strain LBA 4404 was used as host for the plasmid pBinGUSINT used in this study. It was constructed by introducing the GUSINTRON gene from p35SGUSINT^[6] into pBinHyg vector at *Hind*III site and used for transformation by *Agrobacterium* with the advantage that there was no expression in bacteria. *Agrobacterium* was grown overnight in a modified Luria-Bertani^[7] containing 5 g L⁻¹ NaCl and supplemented with 5 g L⁻¹ sucrose and 50 mg L⁻¹ kanamycin. Bacteria were centrifuged at 6 Krpm for 10 min, washed twice with MS^[8] broth and finally resuspended in MS broth (MS media without gelling agent).

SAAT treatment: Approximately 50 zygotic ovules were placed in 10 mL polystyrene ultra clear centrifuge tube (Beckman) containing 2 mL MS broth with and without *Agrobacterium*. Tubes were placed in ice bucket during sonication treatment. An electronic timer (W-375 model, heat systems controlled the sonicator, Ultrasonic Inc., 1938, New Highway Farming Dale New York, 11735) was attached with the sonicator. After SAAT treatment for different timings, zygotic ovules were removed from the tubes and immediately co-cultivated with *Agrobacterium* suspension. Similarly, these were co-cultivated at different timings. Each experiment was repeated in triplicate.

Pre and post SAAT culture conditions: To optimize the effects of sonication duration, continuous and pulse sonication, zygotic ovules were sonicated for 1, 2, 3, 4, 5 and 6 sec using *Agrobacterium* at 0.1 OD600 nm and transient expression levels were recorded. In another attempt, different co-cultivation periods (1, 2, 3, 4 h or overnight) were evaluated using 3 sec sonication

treatment for zygotic ovules. After co-cultivation in all cases, ovules were rinsed in sterile water, blotted dry on sterile filter paper and suspended in medium supplemented with 500 mg L⁻¹ cefataxime to prevent *Agrobacterium* growth.

Histochemical GUS assay: The histochemical GUS assay was performed after 48 h of co-cultivation of zygotic ovules. These were washed for 30 min with 50 mM phosphate buffer (pH 7.0) and immersed for 10 min in fixation solution (0.3% formaldehyde, 10 mM MES, 0.3 M mannitol). The samples were put in 1 mM X-gluc solution and incubated at 37°C overnight for blue color development^[9]. No GUS activity was detected with overnight cultures of *Agrobacterium* harboring pGus-Int by histochemical assays. The GUS mixture was removed and the tissues were rinsed twice with 70% ethanol. GUS activity was then determined by placing the explants on a grid and estimating the percentage of the explant surface that showed blue sectors under a dissecting microscope.

RESULTS AND DISCUSSION

Figure 1 confirm the optimization of time of transfer of zygotic ovule to media after post anthesis, as 48 h after post anthesis was selected when more than 50% of ovules were found floating and developing. The relative ability of BT medium^[5] to support ovule growth during the first 2 weeks of culture was reported which agree with results of the present investigation. After 2 weeks, growth of ovules slowed down while growth on ST^[10] continued very well till the end of 10 weeks (Fig. 2) and a few ovules showed some germination at this point. Similarly, ST medium^[10] was selected for future use (Fig. 3). The discovery of multicellular fibres during cotton ovule culture^[4] suggested a unique means for evaluating gene

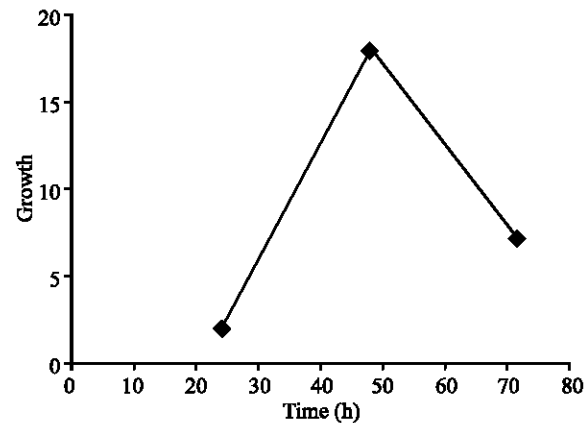


Fig. 1: Optimization of post anthesis time for zygotic ovule

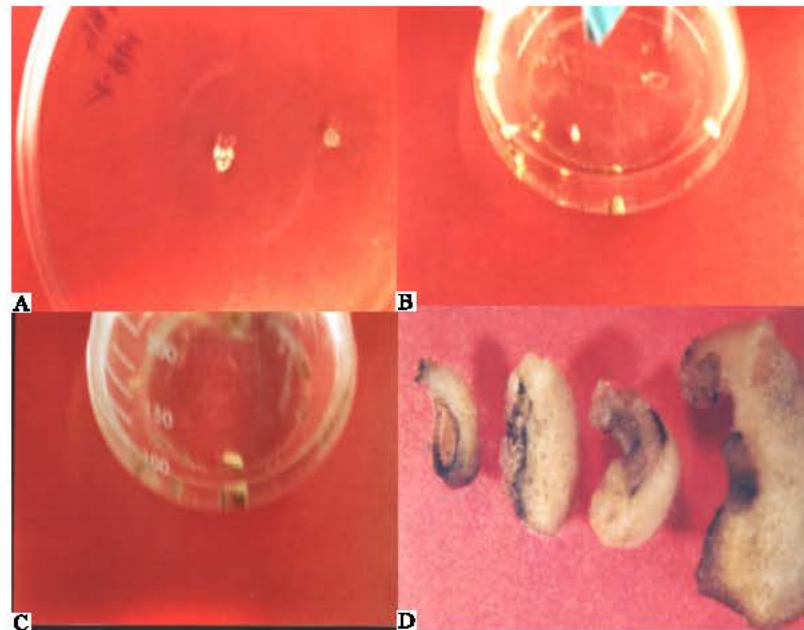


Fig. 2: Optimization of in-ovule embryo culture

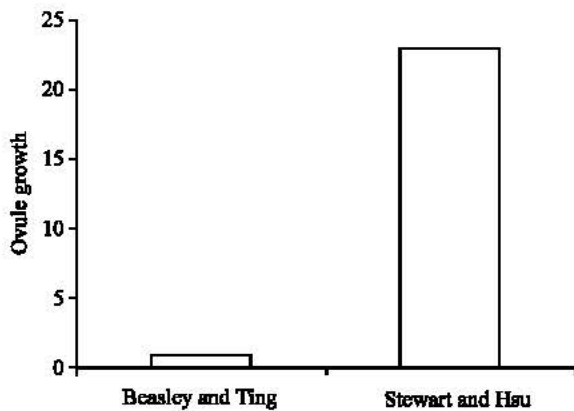


Fig. 3: Selection of media for growth of zygotic ovules

expression and incorporation in cotton fibre cells. Multicellular fibres are multiple single cells arranged linearly which have developed from homogenous mass of unicellular fibre initials and accordingly, transgenic expression can easily be detected through the different cycles of cell division.

Normally, an upland cotton ovule has about 32,000 fibre cells with about 30% of them dividing within the first 72 h of culture^[4]. Thus, time of transfer of ovule after post anthesis to culture is most important. Similarly, new cells in a multicellular fibre will express the transgene if transformation is successful.

A reliable method for isolation and culturing self fertilized embryos of *Gossypium hirsutum* (Fig. 4) is necessary before an embryo culture technique can be

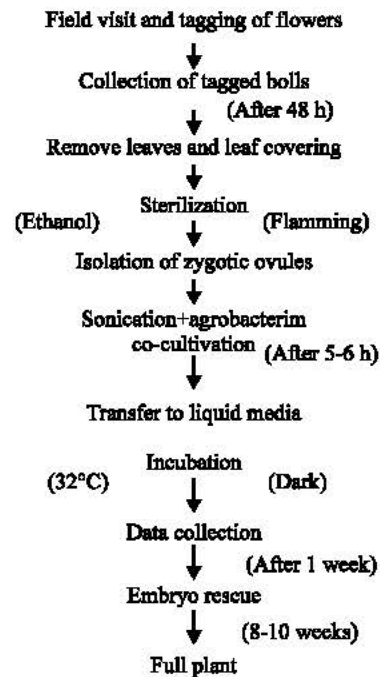


Fig. 4: In-ovule embryo culture protocol

used confidently for transformation and hybridization work. This in-ovule technique has several advantages over culture of isolated embryos^[11] and made significant progress towards developing the technique of in-ovule culture using MS medium supplemented with hormones. The mineral and hormonal requirements for early cotton ovule growth were determined indicating the potential of

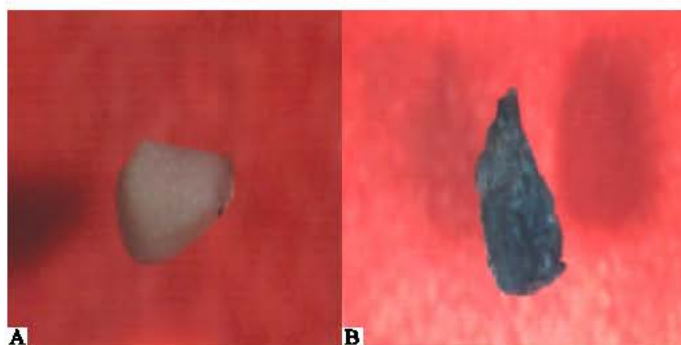


Fig. 5: GUS expression in zygotic ovules; A: Control, B: Experimental

their system for embryo culture^[5]. Present results demonstrated that cotton embryos can be cultured in-ovule from zygote to maturity with reasonable success. One can anticipate that many embryos will germinate precociously and that additional embryos can be obtained by dissection of the germinated ovules^[6]. However, both germinated and ungerminated embryos *in vitro* have less vigor than *in situ* embryos. The aseptic removal of 48 h post anthesis ovules from the ovaries is rapid, efficient and with minimum of damage. Similarly, no dissection of minute proembryos is necessary. Also no change of medium is needed during 8-10 weeks of culture period^[7]. Adequate replications may be achieved both within and between ovaries. Flowers can be easily tagged on the day of anthesis and ovules of known age that are fertilized or non-fertilized may be collected^[5]. It also appears that, with the use of certain growth regulators, cultured ovules afford fibre development similar to that obtained from ovules left attached to the parent plant.

In another series of experiment, SAAT conditions for cotton ovule transformation were optimized as 3 sec pulse sonication, 1 h co-cultivation and *Agrobacterium* at 0.1 OD600 nm. These conditions were used to transform cotton ovule in rest of the experiments. Here lengthy sonication result in breaking of ovules and lengthy culture period made it difficult to control *Agrobacterium* growth during culture period. Similarly, pulse sonication was found to be better than continuous sonication.

Histochemical GUS assay was performed by incubation of sonicated ovules with X-gluc overnight. Blue color formation occurred in the expression sites where X-gluc was catabolized by GUS gene product (Fig. 5). As expected, the intron-containing GUS reporter gene construct showed no GUS activity in pGus- Int-containing cultures of *Agrobacterium* where the intron-splicing process normally does not occur.

Considering the economic importance of cotton, the use of organ and tissue culture in *gossypium* is new and relatively limited^[2]. In contrast, cotton ovule culture has

received the most attention. This organ is particularly attractive as an explant for physiological research, for it bears both the fibres and the embryo^[3]. Current study also presents a system for evaluating gene introduction and incorporation utilizing sonication assisted *Agrobacterium* mediated transformation (SAAT) of multicellular fibres, which developed during ovule culture in media without hormones within a short period of time.

REFERENCES

1. Hashmi, L.A., M.H. Qazi and P. Khaliq, 1995. Food production in Pakistan. Progressive farming, World Food Day Issue, 15: 7-1.
2. Economic survey of Pakistan, 1999-2000. Ministry of Food, Agriculture and Livestock, Govt. of Pakistan, Finance Division, Economic Wing, Islamabad, Pakistan.
3. Food and Agriculture Organization of United Nations, 1995. FAO Quarterly Bullet. Stat.
4. Van't Hof, J. and S. Saha, 1996. Cotton fibres can undergo cell division. Proc. Beltwide Cotton Conf., pp: 590-591.
5. Beasley, C.A. and I.P. Ting, 1974. Effects of plant growth substances on *in vitro* fibre development for unfertilized cotton ovules. Am. J. Bot., 61: 130-139.
6. Vancanneyt, G., A. Schmidt, R.O' Connor-Sanchez, L. Willmitzer and M. Rocha-Sosa, 1990. Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated plant transformation. Mol. Gen. Genet., 220: 245-250.
7. Sambrook, J., E.F. Fritch and T. Maniatis, 1989. Molecular Cloning. A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, New York.
8. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultivars. Plant Physiol., 150: 473-497.

9. Jefferson, R.A., 1987. Assaying chimeric gene expression in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.*, 5: 387-405
10. Stewart, J.M. and C.L. Hsu, 1977. In-ovule embryo culture and seedling developments of cotton (*Gossypium hirsutum* L.). *Plant*, 137: 113-117.
11. Eid, A.A.H., E. DeLange and L. Waterkeyn, 1973. *In vitro* culture of fertilized cotton ovules. I. The growth of cotton embryos. *Cellule*, 69: 361-371.
12. Davidonis, G.H. and R.H Hamilton, 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. *Plant Sci. Lett.*, 32: 89-93.
13. Stewart, J. McD., 1986. Organ and Tissue Culture of Cotton. In: Mauney, J.R. and Stewart, J. McD. (Eds.) *Cotton Physiology*. Cotton Foundation, Memphis, Tennessee, pp: 623-631.