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An approach towards induction of double haploids in okra (*Abelmoschus esculentus* L. Moench)

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Abstract

Okra (*Abelmoschus esculentus* L. Moench), also known as "lady's finger", belonging to the Malvaceae family, is an alloploid. Availability of haploids and doubled haploid lines are essential for the development of improved okra hybrid varieties. Anthers were excised from flower buds at different stages. The ability to produce haploid callus or somatic embryogenesis and thereby, regenerate into haploid plants was investigated. Several factors, such as flower bud initiation time, type of media and plant growth regulator combinations have been evaluated. The flower buds of different sizes were dissected to determine stages of development before subjecting to various pre-treatments and then the anthers. These were cultured on different PGR combinations (NAA, IAA, 2,4-D, KIN, BAP, IBA, ZTN, 2iP, GA₃ and TDZ) and various concentrations. The cultures were incubated in both dark and light conditions. The suitable developmental stage of microspore for callus induction was obtained from 12 mm length of flower buds in okra for calli and root development. The effect of flower bud initiation time was an important factor in anther cultures. The media, MSNB, gave highest percentage (95 %) of callus induction. Incubation for 28 days in dark gave highest percentage (92.5 %) of callus induction. The ultimate aim of this study was to investigate the potential of okra anther culture. The study will ultimately help in double haploid development for faster crop improvement.

Key words: Okra anther culture, microspores, callus, primary shoots.

Abbreviations: NAA: 1-Napthalene acetic acid, IAA: Indole-3-acetic acid, 2,4-D: 2,4- Dichlorophenoxyacetic acid, KIN: Kinetin, BAP: 6-Benzylaminopurine, IBA: Indole 3-butyric acid, ZTN: Zeatin, 2iP: N6 (Isopentenyl) adenine, GA₃:Gibberellic Acid/Gibberellin 3 and TDZ: Thidizuron, PGR: Plant growth regulators. CPPU: N-(2-Chloro-4-pyridyl)-N'-phenylurea

Introduction

Okra (*Abelmoschus esculentus* (L.) Moench.) is one of the most widely grown vegetables in tropics and subtropics. Yield and fruit quality play important role in okra productivity and marketability (Diaz *et al.*, 1997, Choudhury, 1979). Production of new hybrid varieties with shorter life cycle and higher yield could increase the fruit production; fetch higher profits, to the growers.

It has also considerable medicinal and industrial value (Kirtiker and Basu, 1984). Abelmoschus was originally included in the genus Hibiscus (Linnaeus, C. 1753). The chromosome number of okra is 2n = 130, although Dutta and Naug (1968) suggested that the numbers 2n=72, 108, 120, 132 and 144 are in regular series of polyploids with n=12. Okra is allotetraploid. The chromosome number of wild species is 2n = 58. Haploids are produced through the process of gynogenesis (ovule, ovary culture - maternal haploid) and androgenesis (immature anther or microspore culture - paternal haploids), which are extensively studied in various crops like maize, wheat, rice, barley and brassica species and is now well documented worldwide. Still there is a need and further scope for improvement of double haploid (DH) line development through improved tissue culture practices, such as growth of donor materials and media combinations, while in okra no successful studies are reported. Therefore, this study is timely and makes advances in our understanding of double haploid in okra.

DH technology has various applications in commercial cultivar development (Klima et al., 2004 and Babbar et al., 2004) which includes inbred line production in the shortest period of time, genetic studies, gene mapping, genomics, mutation, cytological studies etc. Guha and Maheshwari reported pollen plant through androgenesis in Datura annoxia in the year 1964 and 1966. In some species, it is possible to produce haploids by microspores (Keller et al., 1987). To improve crop productivity, it is important to speed up breeding technology with recent developments in biotechnology assisted tools. No double haploid studies were conducted in okra, only few tissue culture assisted plant regeneration reports have been described (Kabir et al., 2008, 2016 and Muhammed et al., 2017). Suitably, in-vitro tissue culture techniques like androgenesis (an anther culture technique) can be used for homozygous line/ double haploid line development, in the shortest period of time. Breeder needs best inbred line (homozygous) for better cultivar development. As okra is very tedious crop, it takes long duration for homozygous line development by conventional breeding method which can be reduced by generation of double haploid through anther culture. Therefore, efforts were made to initiate anther culture studies in okra with a view to decrease varietal developmental timelines. Essentially, double haploid technique acts as a time machine to compress lengthy conventional breeding timelines. Also, okra being polyploid is a difficult crop for double haploid production and is extremely challenging.

Materials and methods

Plant material and determination of microspore development stage: Seeds of okra were used as anther donor after eight weeks from seeds sowing. Internal breeding material was chosen for this study as these would have been more appropriate for further proprietary germplasm development. Flower buds of different lengths *viz.*, 6, 8, 12, 16, and 20 mm after the date of the flower bud initiation were collected for anther culture, having the different stages of microspore and pollen grains. Five buds of each respective bud size were collected and carried into the laboratory immediately for processing. Prior to anther culture,

the stage of microspores was checked under a microscope. The staining process was performed by removing anthers from buds in a random manner and were subsequently stained with 4 % acetocarmine (W/V) in 50 % (v/v) acetic acid. The length of flower buds, anther length, and anther bunch diameters were measured by the digital microscope (Fig.1).

Pre-treatment and sterilization: First, the buds were washed with sterile distilled water for 5 min to remove the dust and then disinfected for 45 sec in 70 % alcohol and 8-10 min in 3 % sodium hypochlorite with 2-3 drops of Tween 20 or by using $HgCl_2$. The buds were rinsed four times with double sterile distilled water



Fig.1. Different sizes of flower bud and development stages of microspore in okra. (A) <10 mm long flower bud, (B) 12 -15 mm flower bud, (C-D) >15 mm length flower bud, (E-H) microspore stage under light microscope. (H) Mature stages (including pollen grains) under light microscope.



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Table 1. Details of culture media cused for callus induction

S No.	Media	Total number of anthers cultured
1	MSOC	750
2	MSO	750
3	MSB	750
4	MS2T	750
5	MST-3	750
6	MSAg	500
7	MSNB	500
8	MAgC	400
9	MS+C	400
10	MSIK	400
8 9 10	MAgC MS+C MSIK	400 400 400

MSOC: MS salts + vitamin + 3 % sucrose + 0.8 % agar + 1 mg/L CPPU MSO: MS salts + vitamin + 3 % sucrose + 0.8 % agar.

MSB: MS salts + vitamin + 3 % sucrose + 0.8 % agar + 1 mg/L BAP

MS2T: MS salts + vitamin + 3 % sucrose + 0.8 % agar + 0.044 uM/L TDZ [Kabir *et al.*(2016) with modifications]

MST-3: MS salts + vitamin + 3 % sucrose + 0.8 % agar + 1.0 mg/L TDZ

MSAg: MS salts + vitamin + 3 % sucrose + 0.8 % agar + 20 mg/L AgNO₃ +1.0 mg/L Kinetin [Mangat & Roy (1989) with modifications]

MSNB: Mangat and Roy (1989) with modifications

- MAgC: MS salts + vitamin + 3 % sucrose + 0.8 % agar+200 mg/L Charcoal+20 mg/LAgNO₃+1.0 mg/L KIN [Mangat & Roy (1989) with modifications]
- MS+C: MS salts + vitamin + 3 % sucrose + 0.8 % agar +200 mg/L Charcoal
- MSIK: MS salts + vitamin + 3 % sucrose +0.5 mg/L IBA+0.1 mg/L KIN+0.3 % phytagel +0.3 % glucose

for three minutes each time and then dried on sterile filter paper. The buds were carefully dissected and anthers were removed. The filaments were cut from anthers to avoid *in vitro* callusing from cut places.

The culture medium: Suitable anthers having the majority of microspores in the late uni-nucleate stage were inoculated on the solid medium that contained different types of plant growth regulator (PGR) *i.e.* a combination of auxins and cytokinins in MS media (Table 1). Each type of media consisted of six Petri dishes (25 mL of MS medium), where 10 anthers were inoculated. The pH was adjusted to 5.8 and incubated at 25 °C in darkness. After six weeks, the induced calli were transferred to new media at 24-28 °C and were exposed to light for 16 hours per day.

Results and discussion

An overview of anther culture of okra is depicted in Fig. 3.

Determination of developmental stage of microspore: Flower buds of different sizes were collected, evaluated, and the suitable size of the flower buds was found to be about 12 mm long. The anthers in such buds contained the majority of mid-or late uninucleate microspores and were most suitable for culture (Fig.1). Specifically, Fig.1 shows stages of microspore development corresponding to the flower bud. Whereas, Fig. 2 shows callus formation from individual anther.



Fig. 3. An over-view of anther culture of okra

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Flower bud collection: Essentially, a flower bud transition into a full-fledged flower also entails microspore transition from uninucleate to mature pollen grain. For double haploid formation microspore at a particular stage can transform into the cluster of cells. The flower buds of different sizes were dissected to determine their stage of development before subjecting to various pre-treatments and then the anthers were cultured on different PGRs (Table 1). Of all the PGR combinations used only MSNB showed callus initiation. The frequency of callus induction was maximum after 1-2 weeks and decreased with time. The higher percentage (90 %) of callus formation was from anthers collected from plants which started flowering after 1-2 weeks. After 4 weeks, the callus formation decreased to 60 % and after 7 weeks, it was reduced to 35 % (Table 2).

Table 2. Frequency of callus formation of flower buds collected at a different time interval after bud initiation on MSNB media

Days after bud initiation	Frequency of callus formation
1-2 weeks	92.5-95 %
4-6 weeks	50-60 %
After 7 weeks	35 %

Callus induction: Callus started to appear after 2-4 weeks of incubation in the dark place. A high frequency of callus formation was observed from MSNB medium. PGR combinations of auxin and cytokinin have been widely used for indirect organogenesis in various protocols developed for the member of Malvaceae family. After two weeks of incubation in dark, the callus were sub-cultured to the fresh media and incubated in light conditions. In okra, the morphogenic callus induction was observed in the highest frequency from anther explants by culturing in MSNB medium. The results of the study revealed that the effect of flower bud initiation time was an important factor in anther cultures. MSNB media gave the highest percentage (95 %) of callus induction. Incubation for 28 days in dark gave highest percentage (92.5 %) of callus induction, thus facilitating the interaction of multiple factors in cell multiplication in okra.

The present study concludes that the suitable microspore stage of the okra flower buds was about 12 mm long, and the anther collected from plants at the beginning of flower initiation showed higher frequency of callus formation. The best hormone combination for callus induction was found to be MSNB medium. The protocols developed under this study are valuable to induce callogenesis from anthers, which may help in the production of haploid plants in the future. Further, attempts are being made to induce shooting and rooting from okra callus from a shoot like structures. Therefore, it is expected that results provided in this study will hasten further research for haploid/double haploid production in okra. Being a polyploid crop, any success obtained in DH production will pave the path for double haploid production in other economically important crops like cotton.

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