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AN EFFICIENT METHOD FOR ANTHER AND OVARY CULTURE IN *ABELMOSCHUS ESCULENTUS* (L.) MOENCH. *IN-VITRO*

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ABSTRACT: Okra (*Abelmoschus esculentus* (L.) Moench.) is an important nutrient-rich vegetable crop also known as "lady's finger", widely grown in the tropics and sub-tropics mainly for its edible pods and is an alloploid. The haploid technique has been used in plant breeding for the improvements of plants and to develop new varieties in a relatively short time. Hence, we have optimized several factors such as plant growth regulators (PGR), sucrose concentration, type of media and culture conditions for callus induction from the anther and ovary of okra (Parbhani Kranti Hybrid). In this research, the effects of the microspore developmental stage, time of bud collection, different combinations of hormones, and culture condition on anther culture were studied. The explants were cultured on various combinations of PGRs i.e., (IAA, 2,4-D, KIN, BAP, IBA and TDZ) and various concentrations. The optimum developmental stage of microspore for callus initiation was achieved from flower buds of okra and its size was about 12 mm long, and showed significantly higher percentage of callus induction. The optimum stage for ovary culture was one or two days prior to anthesis and the flower buds stage was 25±1mm. In conclusion, our study investigated the effect of several factors that affect callus induction in okra and optimized culture conditions. The ultimate aim of this study was to investigate the potential of okra anther culture and ovary culture for obtaining haploid plants. The study will ultimately help in double haploid production that can aid in faster okra improvement.

INTRODUCTION: Okra *Abelmoschus esculentus* L. Moench. often called as lady's finger in England, belongs to the family Malvaceae, as an important vegetable crop throughout the tropical and warm temperate regions. It is valued for its immature green fruits and leaves. Okra seeds are a good source of vitamins, minerals and medicinally important compounds (Uda *et al*., 1997). One of the mucilaginous preparations from pod can be used as a plasma replacement of blood volume expander [4].

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Protein content of the seeds is 20% and oil content 14% or more. Therefore, okra is considered as a potential protein and oil crop (Martin *et al.*, 1981). Considering tremendous importance in world's vegetable supply, conventional breeding and agronomic practises have been adapted to improve yield and quality of this crop (Martin *et al.,* 1981). However, use of modern tissue culture techniques to improve this crop is limited.

Recognizing its economic importance, still there is a vast scope to utilize modern biotechnology for further improvement of this valuable crop. Success of utilizing plant tissue culture largely depends on an efficient *in-vitro* culture of okra. Limited number of protocols were reported for another culture in okra. Recognizing its economic importance, there is a massive scope of utilizing modern biotechnological methods for further

improvement of okra, because conventional plant breeding methods are cumbersome and time consuming. Very few regeneration protocols have been reported to date [5, 7-8], probably due to low percentage of regeneration, excretion of mucilage and phenolic compounds from explants into the medium and browning of callus. The present investigation aims to develop anther and ovary culture protocols for haploid plant production. As okra is very tedious crop, it takes a long duration for the development of homozygous lines by conventional breeding methods. Such prolonged breeding cycles can be reduced by generating double haploid (DH) through another culture. Therefore, efforts were made to initiate anther culture studies in okra with a view to decrease varietal developmental timelines. Successful initiation of callus culture is crucial for the production of haploid plants. In order to establish a callus culture, it is important to select the optimal stage of development, use of appropriate pre-treatments and the right combination of plant growth regulators [17]. Callus induction is one of the pathways required for haploid plant regeneration. The findings of our research are important for future research in this area to produce fully homozygous line in one generation which can be used in breeding programs. Therefore, this study aimed to investigate the effect of growth regulators, sucrose concentration, type of media and size of flower bud, on okra callus induction.

MATERIALS AND METHOD:

Plant Material and Establishment of Experimental Nursery: Mature seeds of okra (*Abelmoschus esculentus* L. *Monech.*) variety Parbhani Krantiwere collected from the VNMKV, Parbhani. The seeds were grown in the field **Fig. 1A** for further growth and development. After 4-5 weeks, flower buds of various length (10,12,14,16,18,20,22 mm) were collected **Fig. B** to determine the appropriate anther and ovary development stages at defined intervals of 2,3 and 4 weeks after bud initiation. The length of flower buds was measured by scale **Fig. B**. Three bud sizes were collected and carried into the laboratory immediately for processing.

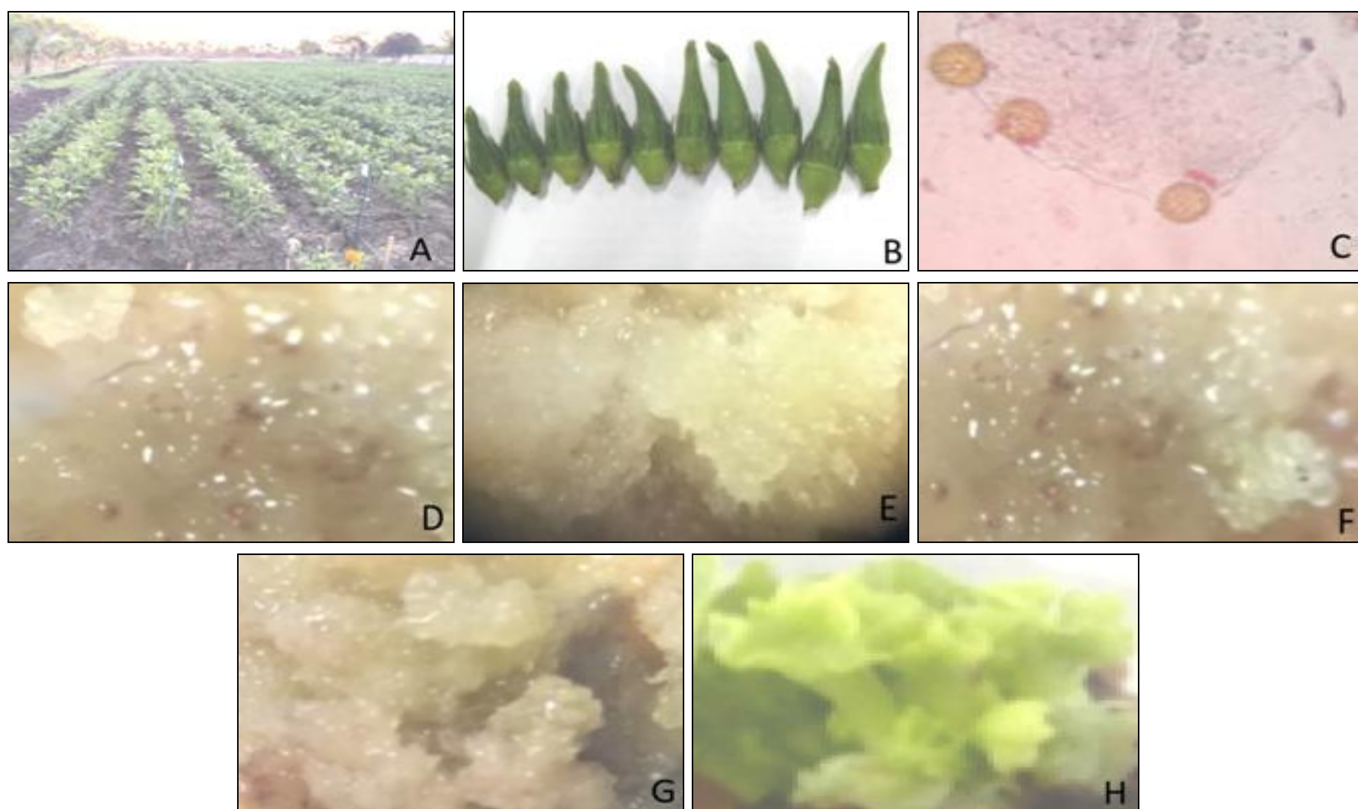

FIG. 1: A. OKRA FIELD FIG. B. DIFFERENT SIZES OF FLOWER BUD FIG. C. MICROSPORE STAGE UNDER LIGHT MICROSCOPE. FIG D AND E. STAGE WISE CALLUS INDUCTION IN OKRA FROM ANTHER CULTURE FIG. F AND G. CALLUS DERIVED FROM OVULE CULTURE FIG. H. DIRECT SHOOT INDUCTION IN OVULE CALLUS

Cytological Examination of Microspore: The stage of the microspore was examined under light microscope after the cold pre-treatment and before culturing. This was carried by taking 2-3 anthers out of an anther bunch randomly and were subsequently stained with 4% acetocarmine (w/v) in 50% (v/v) acetic acid **Fig. C.**

Explant Sterilization, Chilling Pretreatment and Culture Conditions: Selected flower buds were washed 2-3 times with tap water and also sterile water for removing dust. Then disinfected with 70% ethanol for 2 min and subsequently three times with sterile water for 5 min. After surface sterilization, chilling pretreatment was given for 0, 2, and 4 days by placing them in a container with sterilized wet filter paper and storing them in the refrigerator (4±2°C). The callus induction rates were then recorded and analyzed to determine the effect of pretreatment and plant growth regulators on callus induction from anther and ovary explants of okra and then buds were placed on a sterile paper for drying.

After surface sterilization, the buds were carefully dissected and anthers removed from buds and inoculated on to MS media [11]. The filaments were removed from anthers to avoid any *in-vitro* callusing from the cut places. Ovaries were separated from each flower bud. All 10 anthers, 10 ovaries, were inoculated into the test tube containing 20 ml of three types of growth media MS1, MS2 and MS3 supplemented with 7 g/l agar, 3% sucrose with different PGR combinations **Table 1**. The pH of all media was adjusted to 5.7 using 1M NaOH or 1N HCl, before being autoclaved for 10 min at 121°C. For four weeks, the cultures were incubated under regulated temperature (25±2°C) in the dark (24 hours), light and dark conditions (16/8 hours). Then, all the explants were subjected to light and dark for 16/8 hours.

Callus Induction: After sterilization of flower buds, the isolated anthers and ovaries were inoculated onto MS media containing different concentrations of PGRs i.e. BAP and kinetin (KIN) (0.5,1,1.5,2.2, 5,3µM/L). Callus started to appear after 3-weeks of incubation in the dark. High frequency callus formation was observed on MS medium **Fig. D & E.**

Callus Subculture: Different types of calli produced from anthers and ovaries were sub-cultured for shoot initiation ability in fresh medium containing various combinations of PGRs and incubated in light and dark for 8/16 hours under 25 ± 2°C for four-weeks. The hormonal combinations for culturing were MS-2 media (BAP 2.8 µM + KIN 0.7µM + IAA 0.14µM). Then the calli showed proliferation **Fig. F & G**. After subculturing of callus, shoots were initiated **Fig. H.**

SEM Sample Preparation: Samples (anther, ovary and callus morphogenic structures at different stages of development) were fixed in FAA mixture (70% ethanol (100): formalin (7): glacial acetic acid (7) as described by Berlin and Miksche (1976) [3] and stored in the fixing solution until further studies. A common fixing preparation for plant material analysed by the SEM method is a glutaraldehyde solution [10].

Data Analysis: Experiments were planned using randomized block design (RBD) and data were analyzed using ANOVA and DUNCAN multiple range test to compare significant differences.

RESULTS AND DISCUSSION:

Stage of Microspore and Ovary Development: The present study found that flower buds of a specific size were ideal for callus induction **Table 1**. The flower bud size with suitable microspore stage of the okra was about 12mm long. Anthers with mid or late-uninucleate microspore-stage were the best for anther culture.

At a bud length of 9 mm, the pollen mother cells were observed and the anthers appeared white with no visible stigma. Flower buds measuring 20mm, 25mmand 35mm have developed mature pollen grains, with anthers and ovaries of different colors and lengths. Our findings are in agreement with Varandani *et al*. (2021) [16] who reported that the best developmental stage of microspores for callus initiation was the 12 mm length in okra. In most cases, the optimum stage for ovary and ovary culture is nearly mature embryo sac, one or two days prior to anthesis and flower bud size was 35±2 mm long and the ovary had a nearly or fully mature embryo sac. For various species, an optimum gynogenesis was obtained with embryo sacs that were close to its maturity [9].

Specific stage of microspore development at the time of culture establishment plays a significant role for successful microspore embryogenesis [14].

The best hormonal combination for callus induction of anther and ovary was found to be MS medium. The results of the study revealed that the effect of flower bud initiation time was an important factor in anther and ovary cultures. MS media gave the highest percentage (92%) of callus induction.

TABLE 1: BUD SIZE AND DIFFERENT STAGES OF MICROSPORE

Size of buds (mm)	Stage of microspore
10-12	Uninucleate
12-14	Mid uninucleate
14-16	Mid uninucleate
16-18	Late uninucleate
18-20	Late uninucleate
20-22	Late uninucleate

Influence of Flower Initiation Time and Bud Collection on Callus Growth: Okra exhibited a significant increase of callus formation in early collected flower buds in comparison to flower buds collected at later stages. Flower buds collected 1-week after flowering showed significantly high percentages of callus induction compared to those collected 5-week after flowering induction **Table 2**. 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. This study found that early collected flower buds (1-week) had 95% callus formation in anthers and 85% in ovaries. However, the percentage of callus formation **Table 2** decreased in flower buds collected later (3-weeks), with 80% in anthers and 85% in ovaries. The study also found that after 5-weeks, the percentages of callus formation decreased to 70 in anthers. In ovaries **Fig. 2**, collecting plant explants at the start of flowering generally results in a significant response in most plant species.

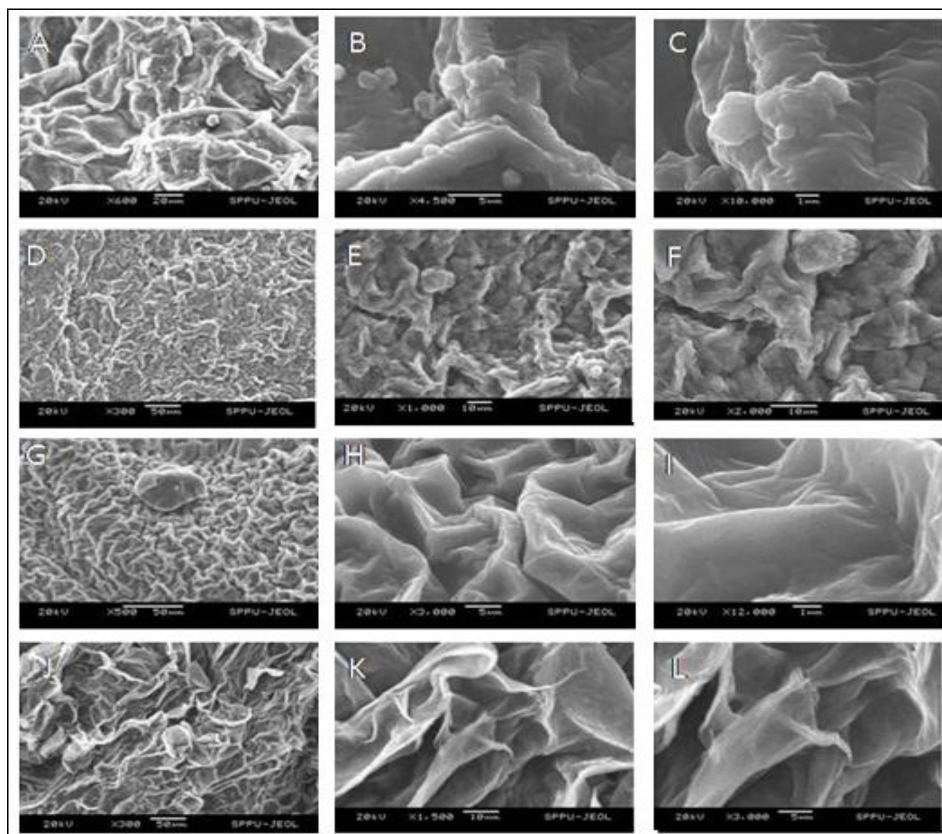

FIG. 2: A. SCANNINIG ELECTRON MICROGRAPH OF ANTHER SURFACE AND B AND C POLLEN GRAINS D, E AND F DIRECT EMBRYO FORMATION FROM CULTURED ANTHER G, H AND I SCANNING ELECTRON MICROGRAPH OVULE SURFACE AND EMBRYO INDUCTION, J, K AND L. DIRECT SHOOT INDUCTION FORM OVULE OF OKRA

Hence, it is recommended to collect anthers from buds as early as possible during the flowering period for better results Incubation for four-week period in light/dark gave the highest percentage (92%) of callus induction, under optimum hormone concentration, suitable stage of microspore and culture conditions facilitating the interaction of multiple factors in cell multiplication in okra.

TABLE 2: FREQUENCY OF CALLUS FORMATION FROM FLOWER BUDS COLLECTED AT DIFFERENT INTERVALS OF TIME AFTER THE INITIATION OF BUDS IN MS MEDIA

Days after bud initiation	Frequency of callus (%)
1-2weeks	92-95
2-3 weeks	75-80
After 5 weeks	less than 70

Effect of Chilling Pre-treatment on Callus Formation: The pre-treatment of explants is a crucial step in the callus initiation process for anther and ovary culture. As reported by Galan-Avila *et al*., (2021) [6], the chilling pre-treatment reduces degradation processes in anther tissues thus protecting microspores from the toxic chemicals when the anther is decayed. In the present study, the anther explants that underwent no pre-treatment (0-day), induced high callus induction percentage 95%. However, the results showed that a chilling pre-treatment for 4 days increased callus induction percentages in ovaries, resulting in 85% callus induction. These results suggest that while pre-treatment was not essential for callus induction in anthers, chilling pre-treatment was beneficial for ovaries [2].

Also, the percentage of callus initiation differed considerably depending on hormonal concentration, with the optimal callus formation of 95%) when 2 μM/l BAP + 2 μM/l KIN were supplemented. Chilling pre-treatment was shown to be effective in transitioning microspores from the gametophytic pathway to the sporophytic pathway in rice anther cultures [12].

Effect of PGRs on Callus Induction: Anthers containing the suitable stage of microspores when cultured on MS medium fortified with PGRs, the colour changed from light yellow to dark-yellow and swelled in size after 5-8 day. Thereafter, the wall of anthers cracked with a small globular protuberance which gradually turned into a mass of cells after 3 weeks. Differential responses in the induction of calli were observed among different media used both in anther and ovaries. Variability in size, number, and colour of calli were observed for both the anther and ovaries. PGRs play an important role in the formation and differentiation of calli in tissue culture [9]. PGRs particularly auxins such as 2,4-D, IAA and NAA have been reported to promote direct embryogenesis and their ideal concentrations have been reported to differ relatively from species to species [18]. In the present study, the two explants (anthers and ovaries) of okra were cultured on MS media fortified with different PGR combinations. Callus formation was observed after 19 days of culture. The most effective frequency of callus induction (95%) was recorded when anthers were cultured on 2 μM/L BAP and 2 μM/L KIN. This hormonal combination is effective when compared with other hormonal combinations such as BAP 2.88μM + KIN 0.7 μM +IAA 0.14 μM for ovary.

Whereas, the optimum callus induction rate (35%) was observed at 3 μM/l BAP + 3 μM/l KIN. It can be concluded that the percentage of callus induction was also affected by the types of auxins and their concentrations used. These results highlight the importance of using diverse PGRs and concentrations for effective callus induction. In general, induction of high frequency callus from various explants is a crucial step for obtaining haploid plants. To achieve this, optimization of PGR concentrations is a key strategy [15]. Observed 89.66% callus induction when ovaries of marigold (*Tagetes sp.*) were cultured on MS media fortified with 4.44 μM BAP+2.26 μM 2,4-D. Similarly, Yarali and Yanmaz (2017) induced callus efficiently from onion *(Allium cepa* L.) using a combination of 2,4-D and BAP inferring that different PGR concentrations are key for the successful induction of callus.

Callus Subculture: Periodic subculturing onto a fresh medium is crucial to maintain callus growth, proliferation, differentiation and also to provide new inoculums for increasing the amount of callus. Therefore, in this study, the calli induced from anthers and ovaries of okra were subcultured in MS media fortified with various combinations and concentrations. Greenish yellowish calli obtained from anther and ovaries were selected for plantlet regeneration.

Calli were cut into 3-4 pieces and subcultured. After subculture on regeneration medium shoot buds initiated after 21 days in ovary culture while in anther culture only embryogenic callus was observed. The combination of BAP, KIN and IAA (BAP 2.8 µmol + KIN 0.7µM +IAA 0.14 µM) induced friable callus and found to be most effective for sub culturing of callus [1]. Callus cultured on MS medium containing various types of PGRs and sub-culture for more than five times, has resulted callus proliferation with different textures and morphology with shoot induction and embryogenesis. Shoots were maintained on the same medium for 28 days to allow shoot differentiation (subculture medium).

SEM Analysis: Morphogenic structures such as embryoids, calli with embryoids, and calli with buds and non-embryogenic callus were noticed in okra anther **Fig. 2A** and ovary cultures **Fig. G**. Direct shoot induction **Fig. 2 J, K & L** was observed from the cultured ovaries and formation of embryoids that is embryo-like structures from the anther cultures **Fig. 2 G, H & I**. SEM structures clearly exhibit the presence of embryo-like structures as shown in **Fig. 2D, E** & **F**. Our study revealed a specific morphogenetic pathway as characterized by microspore development into structures with multiple organs **Fig. F**. Similar structures were obtained in wheat cultivars [13]. SEM analysis also revealed the presence of embryoids. The initial stages of development of these embryoids are similar to those of typical somatic embryos **Fig. I.** During the transition to organogenesis, their apical part increased and formed multiple scutella and shoot meristems, with a common root pole. SEM and light microscopy analysis also revealed indirect embryo formation.

CONCLUSION: The present study concludes that the optimum microspore stage of the okra flower buds was about 12 mm in lengths and the explants obtained from plants at the mid or late-uninucleate displayed higher percentages of callus induction. The optimum media type for callus induction from anthers, and ovary was found to be MS media. KIN or BAP in combination with IAA or 2,4-D was found as the best plant growth regulator combination for callus induction. Dark conditions were suitable for efficient callus induction. The protocols developed in this study are invaluable to produce callogenesis from anthers and ovary which might help in the production and improvements of haploid plants in the future breeding programs.

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CONFLICTS OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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