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Factors Affecting Callus Induction from Anther and Ovary of Okra (*Abelmoschus esculentus* L.)

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ABSTRACT

Background: Okra [*Abelmoschus esculentus* (L.) Moench.] is a nutrient-rich vegetable crop widely grown in the tropics and sub-tropics mainly for its edible pods. The haploid technique has been used in plant breeding for the improvements of plants and to develop new varieties in relatively a short time. Hence, we have optimized several factors such as plant growth regulators (PGR), sucrose concentration, cold treatment, type of media and culture conditions for callus induction from the anther and ovary of okra (557 F1 hybrid).

Methods: The flower buds of different sizes were collected to determine various stages of development and then subjected to cold pre-treatments. The explants were then cultured on various combinations of PGRs *i.e*., naphthyloxy acetic (NOA), Indole acetic acid (IAA), 2, 4-Dichlorophenoxy acetic acid (2, 4-D), Benzyl amino purine (BAP), isopentenyl adenine 2iP, Kinetin (KIN) and Thidiazuron (TDZ).

Result: The optimum developmental stage of microspore for callus initiation was achieved from flower buds of okra and its size was about 11 mm long. Flower buds that emerged one week after the flowering showed significantly higher percentage of callus induction. The optimum stage for ovary and ovule culture were one or two days prior to anthesis and the flower buds stage was 35±1mm. In conclusion, our study investigated the effect of several factors that affects callus induction in okra and optimized cultured conditions for future haploid development for okra.

Key words: *Abelmoschus esculentus,* Anther culture, Callogenesis, Haploid, Okra, Ovule culture.

INTRODUCTION

Okra [*Abelmoschus esculentus* (L.) Moench.], also known as “lady’s finger” or “gumbo”, is a member of the Malvaceae family and its native to African countries. It is an economically important nutritive vegetable and widely cultivated in the tropics, sub-tropics and warmer areas mainly for its edible pods (Durazzo *et al*., 2018; Mkhabela *et al*., 2022). The pods have several nutritional values and is rich in vitamins, minerals and other bioactive substances. The pods of okra plant have been also reported to have several medicinal values (Badrie *et al*., 2016). However, improvements of okra plant using conventional breeding approaches has been a difficult task and time consuming (Bortey and Dzomeku, 2016).

Scientists have made an effort to improve okra plant and circumvent the recurring problems (Krishnakumar *et al*., 2018). In this regard, modern biotechnological tools such as haploid through anther/ovule culture, embryo culture and molecular breeding have been used for further improvement of okra crop. Haploid culture techniques are effective for the rapid production of homozygous lines, which can be used for the production of F1 hybrids. The 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 (Venkadeswaran and Sundaram, 2016).

Callus induction is one of the pathways required for haploid plant regeneration and a number of callus induction protocol have been reported to date. Sadhu *et al*. (2021), established callus induction and plant regeneration protocols through unpollinated ovary culture *Callistephus chinensis* L. Nees. Another study by Silva *et al*. (2021) successfully induced callus for haploid plant regeneration through anther culture. To the best of our knowledge, this is the first study on the induction of callus from okra gametophyte and we believe that the findings of our research is 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, illumination condition, sucrose concentration, type of media and cool pre-treatment on okra callus induction.

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MATERIALS AND METHODS

Plant material and establishment of experimental nursery

The seeds of Okra hybrid cultivar F1 557 were provided by Leckat Corporation Sdn. Bhd, Kuala Lumpur, Malaysia and it was used as the source of anthers and ovaries explants. The seeds were grown in 50 Jefy-7 Peat pellets for one week in a greenhouse before being transferred to the field at University Malaysia Kelantan research field for further growth and development. After 4-5 weeks, flower buds of various lengths (10, 12, 20, 25, 35 and 40 mm) were collected to determine the appropriate anther, ovary and ovule development stages at defined intervals of 1, 3 and 5 weeks after bud initiation. Different stages of explants were extracted from the anthers, ovary and ovule.

Cool pretreatment and sterilization

The flower buds were sterilized using a two-step process involving 70% alcohol for one minute and 15 minutes in 3% sodium hypochlorite with a few drops of Tween-20. They were then rinsed few times in sterile water and given 0, 2, or 4 days of cool pre-treatment 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 pre-treatment and plant growth regulators on callus induction from anther, ovary and ovule explants of okra.

Cytological examination of microspore

The stage of the microspore was examined under light microscopy after the cold pre-treatment and before culturing; this was done by taking two to three anthers out of an anther bunch. Afterwards, they were dyed with 4% acetocarmine (w/v) in 50% acetic acid (v/v). The bulk of pollen grains were evaluated to be at the tetrad microspore stage for anthers and 1-2 days before anthesis for ovules when flower buds were chosen.

Callus induction with different factors

After surface sterilization, anthers, ovaries and ovules were separated from each flower bud and 20 anthers, 10 ovaries, or 20 ovules were inoculated in Petri dishes containing 20 ml of three types of growth medium MS, N6 and MN6 (Thuzar *et al.,* 2011) supplemented 7 gm/l agar with different PGR combinations (Table 1). The pH value of all media was adjusted to pH 5.7 using 1M NaOH or 1N HCl, before being autoclaved for 15 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 area (16/8 hours). Then,

Table 1: Type of PGR concentrations with different influential callus induction factors.

Treat.	Cool treat			Type of media		Dark and light			Type of PGR				
	Concentration mg/l												
	BAP	IAA	NOA	KIN	2iP	IAA	KIN	2,4-D	NOA	TDZ	BAP	IAA	NOA
T1	2.0	2.0		2.0		2.0	0.5		2.0	0.1		2.0	
T2	2.0		2.0	2.0		0.5	0.5	2.0		0.1		0.1	
T3	0.5	2.0			0.5	2.0	3.0		2.0	0.1			2.0
T4	0.5		2.0		0.5	0.5	3.0	2.0		0.1			0.1
T5	2.0	2.0		2.0		2.0	0.5		2.0		0.2	2.0	
T6	2.0		2.0	2.0		0.5	0.5	2.0			0.2	0.1	
T7	0.5	2.0			0.5	2.0	3.0		2.0		0.2		2.0
T8	0.5		2.0		0.5	0.5	3.0	2.0			0.2		0.1
T9	2.0	2.0		2.0		2.0	2.0						
T10	2.0		2.0	2.0		0.1	0.1						
T11	0.5	2.0			0.5	2.0	2.0						
T12	0.5		2.0		0.5	0.1	0.1						

Table 2: Influence of various PGR concentration on subculturing of callus.

Treatment level (S)	Hormone combination	Concentration (mg/l)	Callus texture observation	Callus color
1	BAP+2,4-D	3.0+0.1	Friable	Yellow
2	2iP+NAA	3.0+2.0	Compact	Yellow
3	BAP+NAA	3.0+2.0	Friable	White
4	2iP+2,4-D	3.0+2.0	Friable	White
5	TDZ+NAA	0.5+3.0	Compact	Greenish yellow
6	TDZ+NAA	0.5+1.0	Compact	Greenish yellow
7	TDZ+NAA	0.5+2.0	Compact	Greenish yellow
8	KIN+2,4-D	2.0+2.0	Friable	White
9	TDZ+2,4-D	0.5+2.0	Friable	Green
10	KIN+NAA	2.0+2.0	Compact	Yellow

all the explants were subjected to light and darkness for 16/8 hours each day.

Callus subculture

The different types of calli produced from anthers, ovaries and ovules were sub-cultured for shoot induction ability in fresh medium contain various combinations of PGR and incubated in light and dark for 16/8 h per day under 25±2°C for 6 weeks. The hormonal combination and concentrations for subculturing were 3.0 + 0.1 mg/l (BAP+2,4-D), 3.0+2.0 mg/l (2iP+NAA, BAP+NAA, 2iP+2,4-D), 0.5+1.0 mg/l (TDZ+NAA), 2.0, 3.0 mg/l (TDZ+NAA), 2.0+2.0 mg/l (KIN+2,4-D), 0.5+2.0 mg/l (TDZ+2,4-D), 2.0+2.0 mg/l (KIN+NAA).

Data analysis

Experiments were created using completely random design (CRD) and data was analysed using ANOVA and Duncan multiple range test to compare significant differences.

RESULTS AND DISCUSSION

Stage of microspore and ovule development

This study found that flower buds of a specific size were ideal for callus induction. The flower buds size 11 mm long were found to be the best stage and anthers with mid or late-uninucleate microspores 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. Meanwhile, buds of lengths 20 mm, 25 mm and 35mm had developed mature pollen grains, with anthers and ovules of different colors and lengths (Fig 1). Our study is in agreement with Varandani *et al*. (2021) 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 ovule culture is the nearly mature embryo sac, one or two days prior to anthesis and flower buds stage was 40±2 mm long and the ovule had a nearly or fully mature embryo sac. For most species, an optimum gynogenesis were obtained with embryo sacs that were close to its maturity (Ibrahim *et al*. 2015). The specific stage of microspore development at the time of culture establishment plays a significant role for successful microspore embryogenesis (Shen *et al*. 2023).

Influence of flower initiation time and buds' 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 stage. Flower buds collected 1 week after flowering showed a significantly high percentages of callus induction compared to those collected 3-5-week after flowering induction (Fig 2).

This study found that early collected flower buds (1 week) had the highest rate of callus formation, with 95% observed in anthers, 85% in ovaries and 85% in ovules. However, the percentage of callus formation decreased in later collected explants (3 weeks), with 80% in anthers, 82% in ovaries and 85% in ovules. The study also found that after 5 weeks, the percentages of callus formation decreased to 75% in anthers, 70% in ovaries and 60.0% in ovules (Fig 2). Collecting plant explants at the start of flowering generally results in a significant response in most plant species. Hence, its recommended to collect anthers from buds as early as possible during the flowering period for better results (Dissanayake *et al*., 2020).

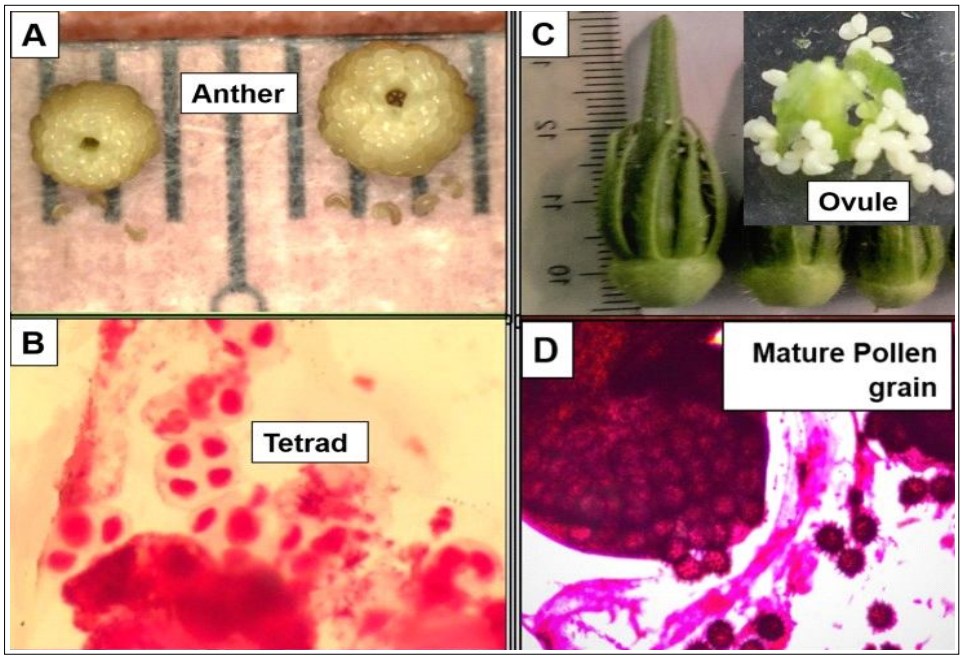


Fig 1: (A and B); Anther at microspore stage (Tetrad), (C); Flower 1-2 days before anthesis (immature ovule); (D): Mature pollen grain stage.

The effect of cool 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 Galán-Ávila *et al*. (2021), the cold pretreatment reduces degradation processes in anther tissues thus protect microspores from release of toxic chemicals when the anther is decayed. In the present study, the anthers explants that underwent no pre-treatment (0 day), induced high callus induction percentage 95%. However, the results showed that a cool pre-treatment for 4 days increased callus induction percentages in ovules, resulting in 80% callus induction. These results suggest that while no pre-treatment was sufficient for callus induction in anthers, a cool pre-treatment was beneficial for ovaries and ovules (Fig 3). Also, the percentage of callus development differed considerably depending on hormone concentration, with the optimal callus formation (95.0%) observed in T1 (2.0 mg/l BAP + 2.0 mg/l IAA) (Fig 3).

Cold pre-treatment was shown to be effective in transitioning microspores from the gametophytic pathway to the sporophytic pathway in several crops, including wheat. This result suggests that cold pre-treatment can play a significant role in promoting the efficient transition of microspores, leading to improved tissue culture success.

The effect of PGRs on callus induction

Plant growth regulators play an important role in the formation and differentiation of calli in tissue culture (Zur *et al*., 2015). Growth regulators, particularly auxins such as 2, 4-D, IAA, NOA and NAA have been reported to promote direct embryogenesis and their ideal concentrations have been reported to differ relatively from species to species (Xu *et al.,* 2019). In the present study, the three explants (anthers, ovaries and ovule) of okra were cultured on MS media fortified with different plant growth regulator combination. Callus formation was observed after 14 days of culture.

In anther culture, highest callus induction rate (92.0%) was recorded when anthers were cultured on T1 (0.1 mg/L TDZ and 2.0 mg/L IAA) treatment. This result is significantly highest when compared with other hormone combinations. Whereas, the lowest callus induction rate (15%) was observed in T4 (0.1 mg/l TDZ + 0.1 mg/l NOA) and T8 (0.2 mg/l BAP + 0.1 mg/l NOA) (Fig 4). It can be concluded that the percentage of callus induction was also affected by the types of auxins and their concentrations used (Fig 4).

The highest callus formation in ovary and ovule were (70% and 75%) observed in T1 (0.1 mg/l TDZ + 2.0 mg/l IAA), which used a combination of TDZ and IAA. On the other hand, the lowest callus induction in both ovary and ovule was observed in T8, (15% and 12%) which used different

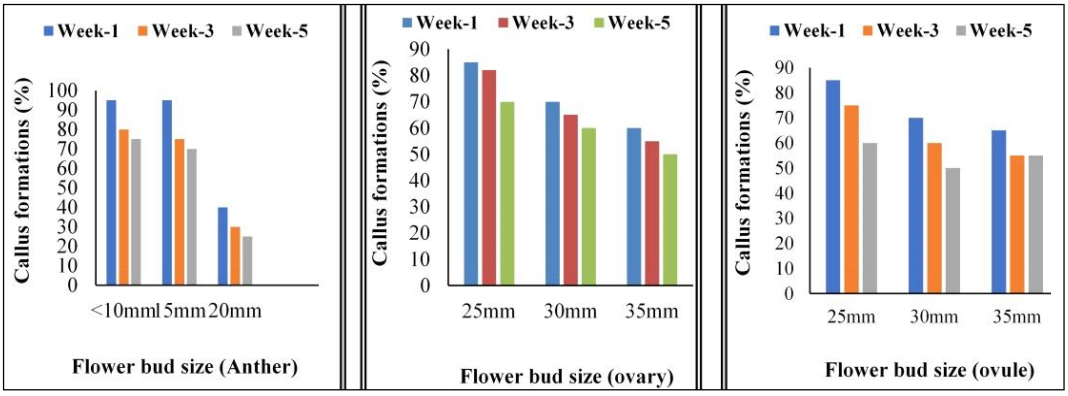


Fig 2: Callus induction rate of flower buds collected at different times after bud formation.

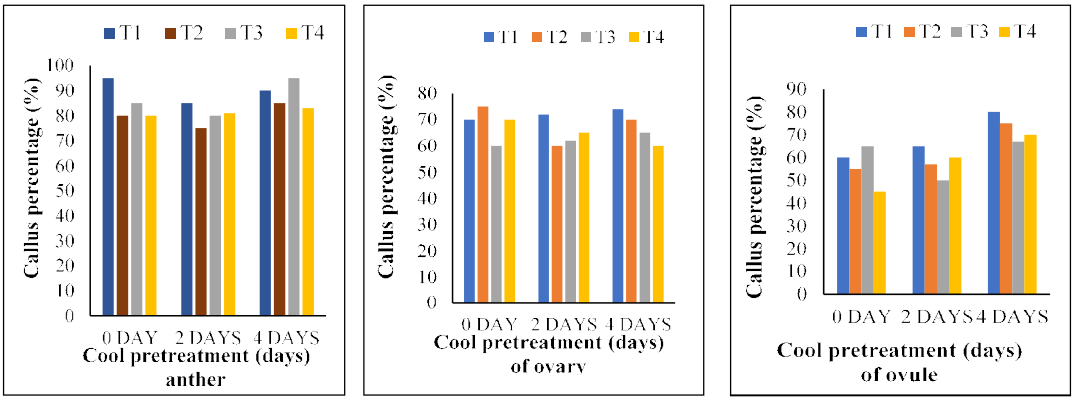


Fig 3: Effect of cool pretreatment and different types of PGR on callus induction.

combinations of (BAP 0.2 mg/l + 0.1 mg/l NOA). These results highlight the importance of using the high Auxin concentration of growth regulators for optimal callus induction in different plant tissue explants (Fig 5). In general, induction of high-quality callus from various explants is a crucial step for obtaining haploid plants. To achieve this, optimization of plant growth hormone concentrations is a key strategy. Thaneshwari, (2018), observed high callus induction (89.66%) when ovaries of marigold (*Tagetes spp*) 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.

The effect of media type on callus induction

In this study, the three types of media (MS, N6 and MN6) were used to identify suitable medium for callus initiation. In this study, MN6 media was the best media for callus induction for all explants (Fig 5). Higher rate of callus induction was observed from the anther culture with the percentages of 85%, 92% and 85%, were observed from M6, MN6 and MS media respectively. Ovary culture, the rate of callus induction was 77%, 77% and 76% obtained for M6, MN6 and MS media respectively. The callus induction efficiency on different culture mediums might be due to the quantity and proportion of NO_3^-/NH_4^+, which is an important factor in nitrogen uptake (Pavelek *et al*. 2020).

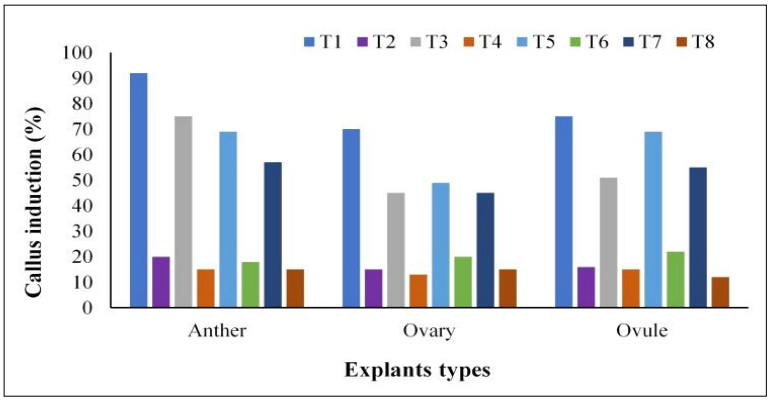
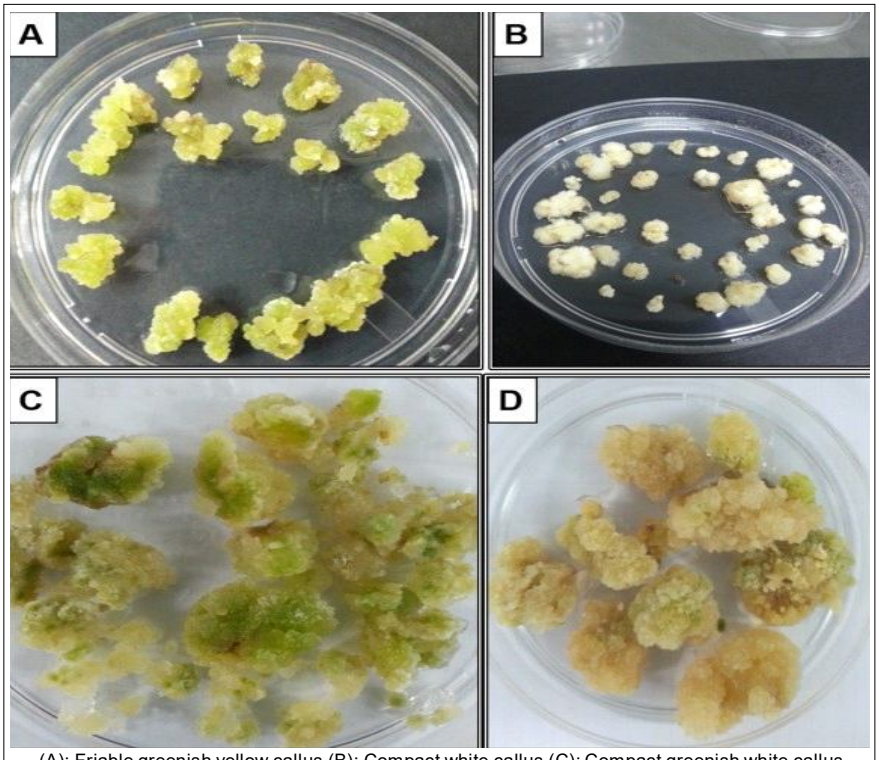


Fig 4: Effect of PGR on percentage of callus induction.



(A); Friable greenish yellow callus (B); Compact white callus (C); Compact greenish white callus (D); Compact white callus.

Fig 5: Effect of PGR on percentage of callus induction.

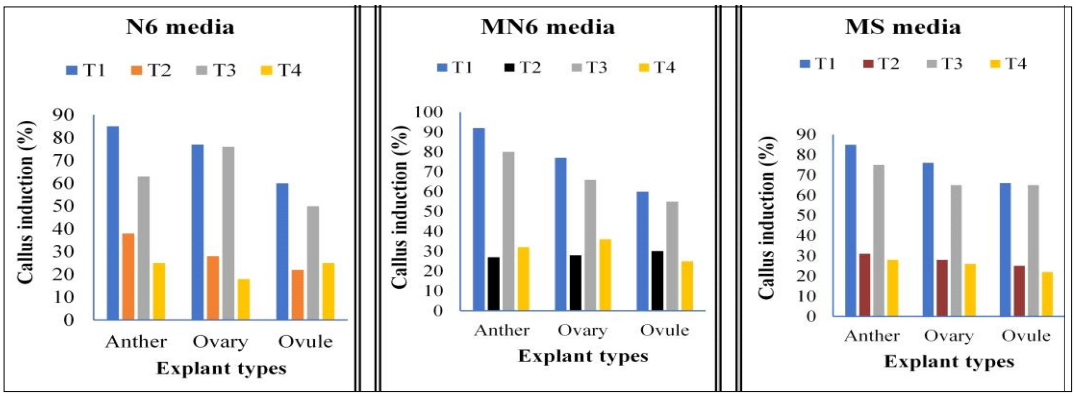


Fig 6: Effect type of mediums with various types of PGR on callus formation.

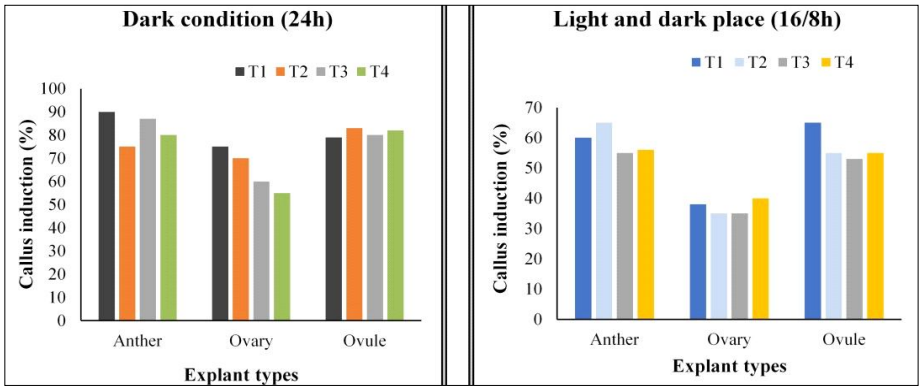


Fig 7: Effect of dark and light place with various types of PGRs on callus formation.

The effect of light condition on callus induction

Light intensity of *in vitro* culture has a great influence on callus induction. Callus induction from all explants varied significantly depending on culture conditions. The effects of different culture conditions (complete darkness and light conditions) with different hormone combinations on callus induction are presented in Fig 6. The highest callus inductions in full darkness were 90% of anther culture in T1. While the highest callus induction in ovary (75.0%) in T1 and ovule (83%) in T2. While the lowest callus percentage observed on ovary culture was 35.0% in treatment T2 and T3 under light and dark conditions (16/8h).

In this study, media combination and culture conditions have been observed to be a substantial influence on callus induction. We observed that callus induction was greatly affected by the light intensity. Strikingly, continuous incubation of anthers in the dark have been considered as important. *Citrus clementina* Hort. ex Tann. Has also been demonstrated to benefit from a light and dark cycle following the induction period (Chiancone *et al*., 2015).

Callus subculture

Periodic subculturing on a fresh media is crucial to maintain callus growth and also to provide new inoculums for increasing the amount calli. Therefore, in this study, the calli induced from anther, ovary and ovules of okra were sub-cultured using MS media fortified with various combinations and concentrations. The callus cultured on MS medium containing various types of PGR and sub-culture for more than ten times, has resulted more callus proliferation with different textures and morphology but without shooting or embryogenesis (Table 2, Fig 7). The combination of BAP and NAA induced friable callus and found to be effective for subculturing of callus.

CONCLUSION

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

Conflict of interest: None.

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