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Callus Induction from Ovules of Kenaf (*Hibiscus cannabinus* L.)

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ABSTRACT

Kenaf (*Hibiscus cannabinus* L.) is an important crop cultivated for fiber production. The haploid production using tissue culture technique is a fast and efficient tool for developing new varieties in comparatively short-time. Kenaf HF992 cultivar was chosen as an explants material, several trials were carried out to investigate the gynogenesis ability before we succeed to get high percentage of callus induction from ovule. Flower buds at the appropriate stage of ovule development were sterilized and the ovules were carefully excised from the flowers and underwent to various pretreatments and inoculated onto MS media supplemented with different combinations of Plant Growth Regulators (PGR) like NAA (a-naphthaleneacetic acid), BAP (N6-benzyladenine), 2iP (N6-(2-isopentenyl) adenine) and TDZ (Thidiazuron) and kept in the dark place for different periods before transferred to light conditions. The high frequency of callus induction (98%) was observed when the ovules collected from the flower bud size of 22-24 mm length after 2-4 weeks of the flower initiation and inculcated onto the optimized semi solid MS medium (3:1 gelrite:agar by weight) fortified with 3.0 mg L^{-1} NAA+3.0 mg L^{-1} 2iP.

Key words: Haploid, ovule culture, gynogenesis, kenaf HF992

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is native to tropical regions of Asia and Africa. It is a short-day, fast growing annual crop belongs to the Malvaceae family and has been cultivated for its stem fiber which is used for ropes, textiles and paper. Kenaf grows quickly and can achieve 5-6 m in height and 2.5-3.5 cm in diameter within 5 to 6 months. Fifty five percent of dried kenaf stalks are used to make paper. Waste products from the process can be made into organic fertilizer and feed binder. The tender upper leaves and shoots are sometimes eaten either raw or cooked when kenaf is grown in the kitchen gardens (Fisher, 1994).

The first step in hybrid seed development inbred parent lines by repeated self-pollination. This can be a very slow process which requires 6-7 generation (Trigiano and Gray, 2010). However, haploid plants that containing only one copy of each chromosome can be produced by culturing anther or ovule on the appropriate plant tissue culture media in laboratory. These plants then can be induced to double their chromosome number by a chemical treatment to quickly result in plants that have two identical sets of chromosomes, or completely homozygous. This procedure can dramatically reduce the time required to develop inbred parents for the production of F1 hybrid varieties.

The main advantage of double haploid lines is their complete homozygosis. This makes phenotypic selection for qualitative and quantitative characters much easier. Thus, DHs can improve the efficiency and speed of the usually cumbersome, time-consuming and laborious and sometimes rather inefficient conventional breeding methods. DH systems are widely applied in breeding (Thomas *et al*., 2003) and genetic mapping (Forster and Thomas, 2005).

The first recognition of haploids in plants was exercised by A.D. Bergner in 1921 in *Datura stramonium* L. (Blakeslee *et al*., 1922).

After the initial reports of successful production of haploids from anther culture in *D. stramonium* (Guha and Maheshwari, 1967), haploids have been obtained in more than 150 species belonging to 23 families of angiosperms (Maheshwari *et al*., 1980). Haploids are sporophytic plants that contain the gametic chromosome number. When they arise from diploid species they contain a single genome and are described as monoploids in contrast to haploids derived from polyploid species, containing two or more genomes and are called polyhaploids. Following chromosome doubling, haploid plants become Doubled Haploids (DHs). The process of haploid regeneration though unpollinated female gametophyte is usually described as gynogenesis. Gynogenesis has been shown to be a possible alternative source for haploid production in plants, particularly in species where androgenesis is recalcitrance or where the level of albino regenerated plants is high (reaching in most cases 100%), or due to male sterility and dioecious nature of plants (Thomas *et al*., 2000). Gamete cells may be manipulated to produce embryos, in contrast to normal fertilization of ovules by pollen grains. Induced or spontaneous chromosome doubling can generate completely homozygous doubled haploid plants. Compared with androgenesis approach, the gynogenic approach has the following advantages (Chang and Hong-Yuan, 1981).

The gynogenic approach increases the frequency of green plant regeneration and has low frequency of aneuploidy and character variation.

In this study, we explored the possibility of haploid induction through gynogenesis. Apparently haploid and double haploid shoot production occurs as a result of organogenesis from an initial proliferation of callus and not via androgenesis (Perera *et al*., 2008).

Generally, gynogenesis has two or many stages and each stage may have distinct nutritional requirements. During induction, ovaries require low levels of growth regulators and to be kept in the dark or light while for regeneration they are transferred to medium with higher growth regulator concentration and incubated in light. Culture medium is a principal factor controlling induction and development of intact plants. However, it is still difficult to draw a conclusion as to the most suitable composition for different plant species. No general recommendation can be given with regards to culture medium for *in vitro* gynogenesis due to differential nutritional requirements at intra- and inter specific levels (Mukhambetzhanov, 1997).

This investigation aimed to study the effects of flower bud length, time of f collection, cold treatment, Plant Growth Regulators (PGR) and culture condition on induction from Kenaf HF992 ovule culture. To our knowledge, this is the first report regarding Kenaf ovule culture.

MATERIALS AND METHODS

Plant material and growth: The seeds of Kenaf HF992 were collected from National Kenaf and Tobacco Board, Kelantan, Malaysia and grown at the tissue culture laboratory Faculty of Base Industry (FIAT), University Malaysia Kelantan. After 10 weeks of transplantation, flower buds of different length 6, 8, 12, 16, 20 and 24 mm (Table 1, Fig. 1) were collected at time intervals of 2 weeks (Table 2). Ovules were excised as explants material having the suitable ovule stages (22-24 mm) for callus induction. Buds were emasculated to avoid damage to the inflorescence meristem from emasculation; fine forceps and needle were used to excise the ovule under dissection microscope, the lengths of flower buds, diameter of anther bunch, ovary and ovule were measured by the digital microscope (Fig. 1).

Pre treatment and explant sterilization: The explants were washed thoroughly under tap water for 15 min to remove dust and disinfected for 45 sec in 70% ethanol and 10-12 min in 5% sodium hypochlorite (NaClO) with 2-3 drops of Tween 20 (Cistue *et al*., 2003), the explants were rinsed 4 times with sterile double distilled water and dried on sterile filter paper and stored in sterile bottles. For the cold pretreatment flower buds exposed for 0, 2 and 4 days at 4-6°C (Osolnik *et al*., 1993; Powell, 1988).

Culture medium: Sterilized ovule explants were transferred on the semi solid MS medium (3 Gel:1 Agar) that contained six types of combination Plant Growth Regulator (PGR): One type of Auxins NAA and three

Table 1: Relation between flower bud length, diameter of anther, ovary and ovule, stigma and ovule color

Bud length (mm)	Diameter of anther bunch (mm)	Ovary diameter (mm)	Ovule diameter (mm)	Ovule color	Stigma color
<6.0	<2.0	<0.6	<0.1	White	Yellow
8.0±2	2.0±0.2	0.8±0.2	0.1±0.05	White	Yellow
12.0±2	3.7±0.2	1.0±0.2	0.2±0.10	White	Pink
16.0±2	4.4±0.6	1.6±0.2	0.6±0.20	White	Red
20.0±2	5.2±0.2	2.4±0.2	0.8±0.20	White	Red
24±2	>5.4	3.2±0.2	1.0±0.20	White	Red

Table 2: Effect of days to flowering on callus induction of gynogenesis.

Days after flower beginning (week)	Frequency of callus formations from ovule (%)
2-4	98[a]
6	60[bc]
8	40[c]

Values followed by the same letters within a column are not significantly different ($p \leq 0.05$)

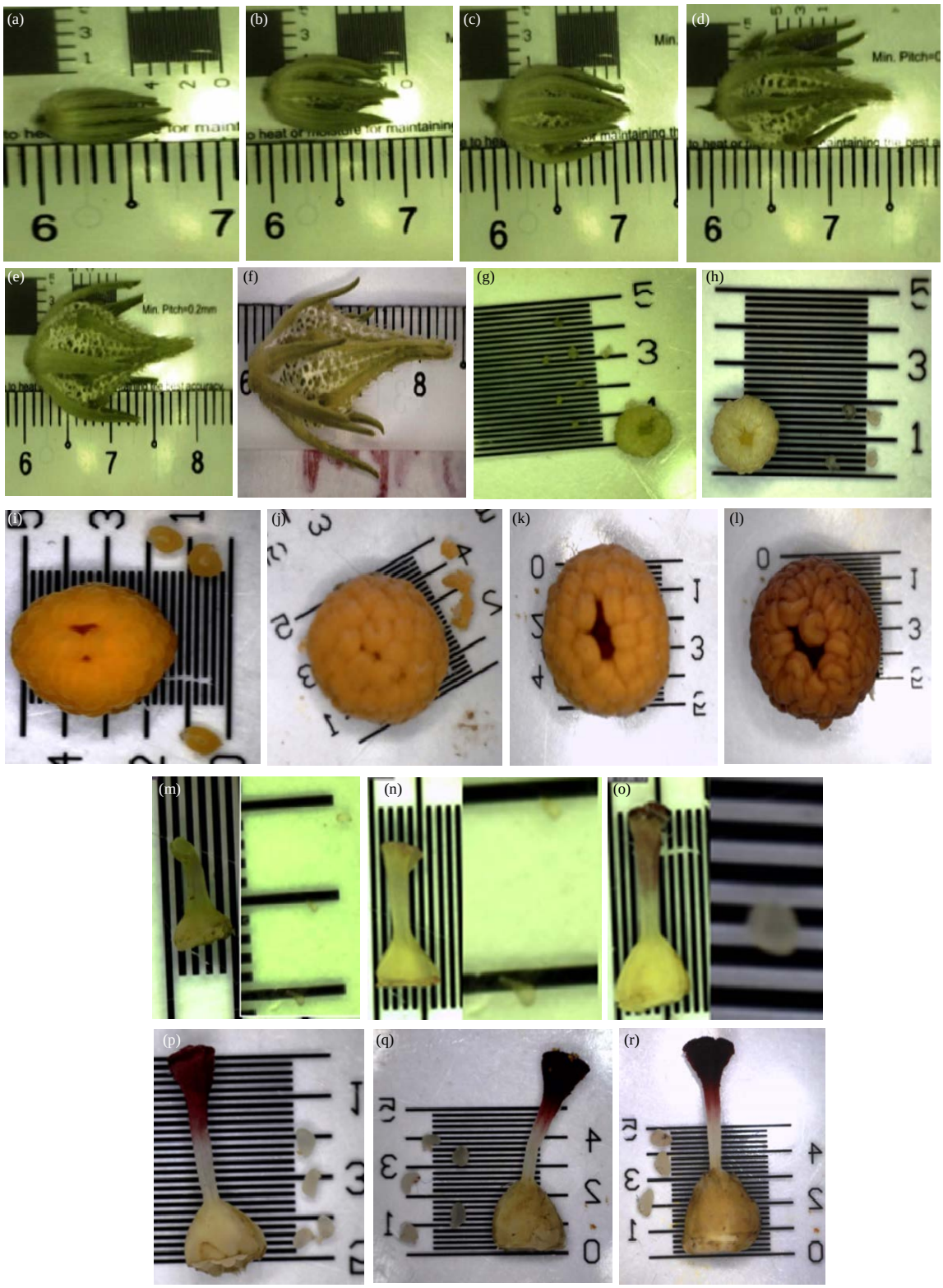

Fig. 1(a-r): (a-f) Different size of flower buds, (g-l) Anther length and diameter of anther bunch and (m-r) Ovary and ovule diameter

types of cytokinins, BAP, 2iP and TDZ supplemented with 3% sucrose. Twenty ovule explants were transferred to each petri dish containing 25 mL of MS media supplemented with different combination of hormones (Fig. 2-4), pH 5.7 and incubated at 25°C in dark for 0, 7 and 14 days. Afterward the cultures were exposed to light at an intensity of about 2000 Lux for 16 h per day (Reinert and Bajaj, 1977) for 4 weeks.

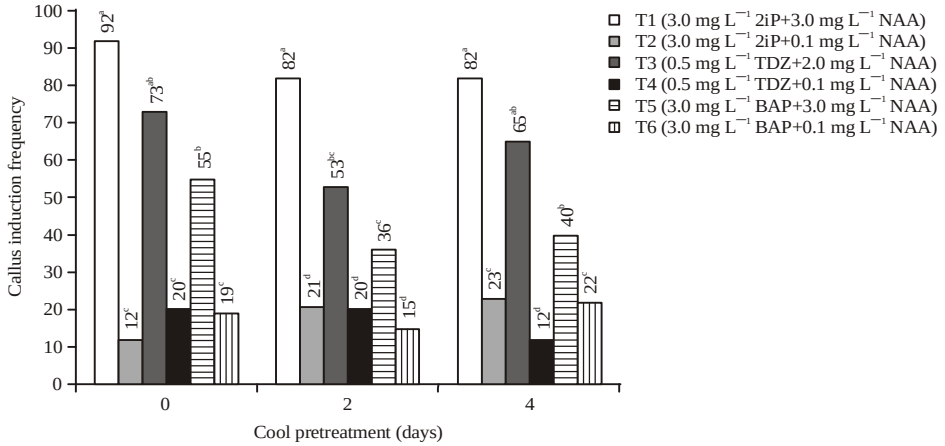

Fig. 2: Effect of cold pretreatment and different plant growth hormones on callus induction from kenaf ovule, values followed by the same letters within a column are not significantly different ($p \leq 0.05$)

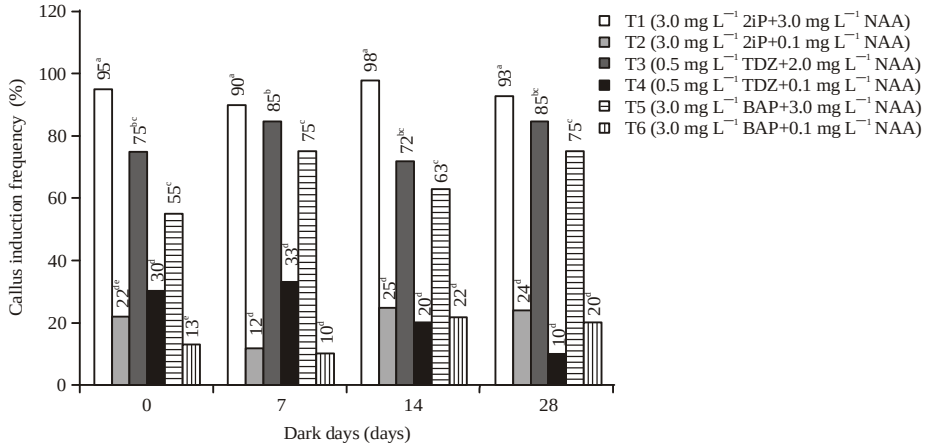

Fig. 3: Effect of dark condition and different types of PGR on callus induction from kenaf ovules, values followed by the same letters within a column are not significantly different ($p \leq 0.05$), Duncan's Multiple Range Test (DMRT)

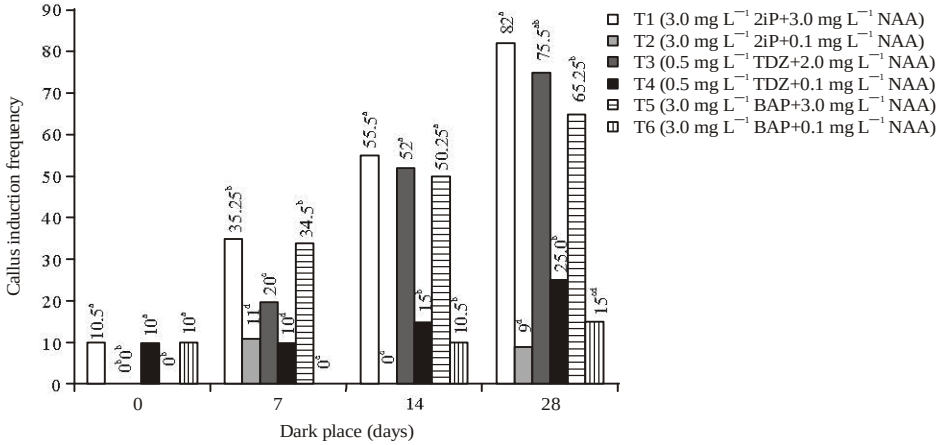

Fig. 4: Effect of dark condition and different types of PGR on root induction from kenaf ovules

Data analysis: All experiments were designed according to CRD (Completely Random Design) and data were analyzed using ANOVA and significant differentiations were compared by DMRT (Duncan Multiple Range Test).

RESULTS

The most critical factor affecting haploid production from ovule culture is the stage of ovule development. In this study, the best flower sizes for ovule culture was about 22-24 mm long for callus induction and the ovary diameter was about 3.2±0.2 mm while ovule length was near 1.0±0.2 mm (Table 1, Fig. 1).

The ovules which were collected from flower buds after 2-4 week from the date of flowering induced higher percentage (98%) of callus formation and after 6 weeks the callus formation was decreased to 60% and after 8 weeks reduced to 40% (Table 2).

Significant differences observed between chilling treatments and control (0 day, no chilling treatment) in callus formation (Fig. 2). However insignificant differences were observed in callus formation when ovule explants were incubated at 4-6°C for 2 and 4 days before transferring onto hormone supplemented MS media (Fig. 2). Use of NAA (3.0 mg L^{-1}) in combination with 2iP (3.0 mg L^{-1}) has supported high callus formation (92%) as compared to NAA (0.1 mg L^{-1}). Callus started to appear after 2-3 weeks of incubation in the light and dark place, less frequency of callus formation was observed in media T2, T4 and T6 containing NAA 0.1 mg L^{-1} (Fig. 2, 5).

No significant differences were observed in callus formation incubated continuously in dark, however, darkness did support root formation in time dependent manner (Fig. 2).

DISCUSSION

For many plant species, success is achieved only when ovule is used before flower anthesis 1 or 2 days. Several studies found that optimal gynogenesis was obtained with nearly mature embryo sacs (Hosemans and Bossoutrot, 1983; Noeum and Gelebart, 1986; Lux *et al*., 1990). Wu and Cheng (1982) reported that ovaries with young uninuclear to mature embryo sacs were responsive to gynogenesis in tobacco ovule cultures. The most responsive ovaries (ovules) had nearly mature or fully mature embryo sacs (Gemes-Juhasz *et al*., 2002).

The physiological state of the mother plants is also a factor. Wu and Cheng (1982) reported that anthers harvested from old and senescent citrus tree had a lowered ability to undergo embryogenesis than those collected from young and vigorous trees. Also anther from spring flowers produced embryos while those from the summer flowering season failed to produce embryos (Segui-Simarro and Nuez, 2007).

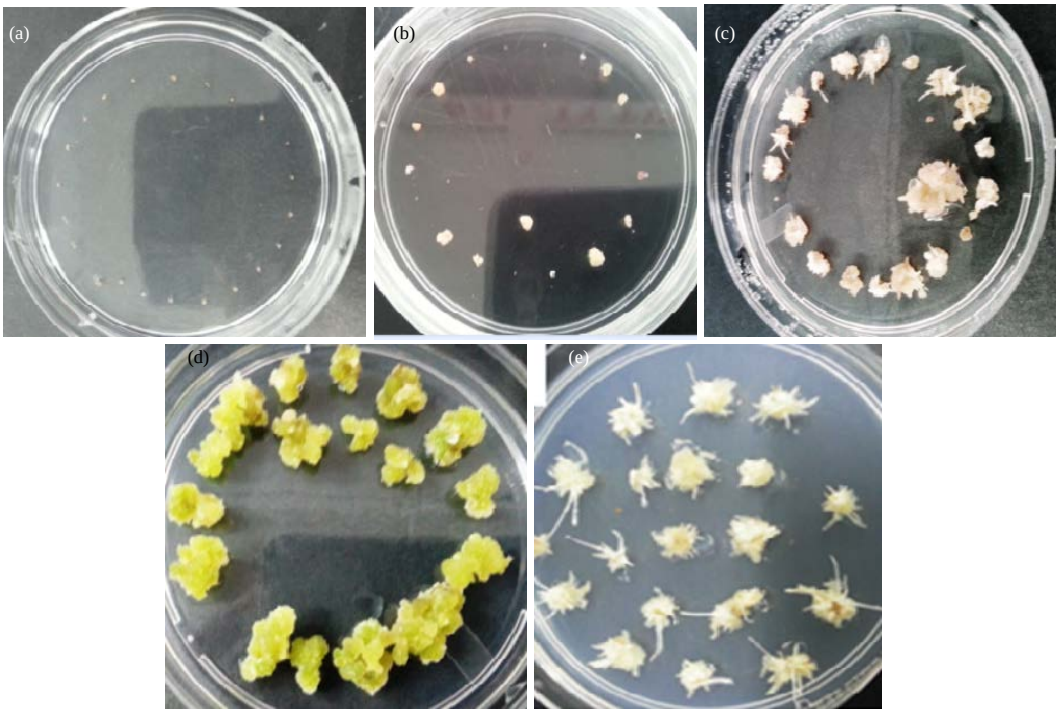

Fig. 5(a-e): Ovule culture, (a) First day of inoculation, (b) Callus induction after 2 weeks, (c) Callus induction after 6 weeks, (d) Green callus from ovule and (e) Root induction from callus

Ali (2008) reported that some of crops were not affected by cool pretreatment. Bohanec (1998) reported that *in vitro* gynogenesis is generally not stimulated by shock treatment such as low or high temperatures or pre growth on starvation medium, also Bhat and Murthy (2007) reported there were no beneficial effect of cold pretreatment (at 4°C) on gynogenesis was observed as compared with control. Yields of tobacco haploids are often increased by storing excised buds at 7-8°C for 12 days prior to anther excision and culture (Sunderland and Roberts, 1979). Stress treatments are the most common factor affecting embryogenesis, with cold/heat shock and starvation treatment being commonly used. Without stress, the change from gametophytic to sporophytic phase is very difficult (Puddephat *et al*., 1999).

Auxins are widely used for induction of gynogenesis and their optimum concentrations have been reported to vary considerably from species to species. Bhagyalakshmi (1999) found that neither NAA nor BA alone supported caulogenesis in saffron. Specific ratios of NAA and BA supported caulogenesis, where an increase in NAA (up to 26.9 mμ) progressively increased the percentage response and higher levels of BA enhanced callus formation and abnormal shoots. TDZ (Thidiazuron) is another widely used and active growth regulator in induction and regeneration media for improving gynogenic response. In cucumber, TDZ is successfully used for gynogenic induction and regeneration of embryos (Diao *et al*., 2009).

Afshari *et al.* (2011) reported in all cultivars and explants light stimulated callus growth, inhibits root growth and increased browning in cotyledon derived calli.

CONCLUSION

This study concludes that the suitable ovules stage of the kenaf were obtained from flower buds after 2-4 weeks of initial flowering with 22-24 mm in length. Ovules collected from plants at the beginning of flower initiation were induced higher frequency of callus formation. The optimum hormone combination for callus induction was found to be NAA 3.0 mg L^{-1} with 2iP 3.0 mg L^{-1}. The experimental protocol developed in this study can be valuable to induce callogenesis from kenaf ovules helping the production of haploid kenaf plants.

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