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Callus Induction through Anther and Ovary of Kenaf (*Hibiscus cannabinus L.*)

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Abstract

In this research new protocol has been developed that would induce callus from anther and ovary of Kenaf. The haploid technique is a fast and efficient tool for developing new varieties in a comparatively short time. The present study was carried out to investigate the effect of cold treatment, plant growth regulators (PGR) and culture condition on callus induction from Kenaf anther and ovary culture. Kenaf HF992 cultivar was chosen as an explants material, several trails were carried out to investigate the Androgenesis and gynogenesis ability before we succeed to get high percentage of callus. Flower buds at the appropriate stage of anther and ovary development were sterilized and the anthers and ovary were carefully excised from the flowers and underwent to various pretreatments and inoculated into media contained different combinations of 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 best callus induction frequency from anther was 90.00 % in the optimized MS medium fortified with 3.0 mg/l 2iP + 3.0 mg/l NAA at the stage the microspores were at the uninucleate stage and the anther was about 7±1 mm long collected. And the best callus induction frequency from ovary was 91.25% in the optimized semi solid MS medium fortified with 3.0 mg/l 2iP + 3.0 mg/l NAA, and the flower buds was about 24±1 mm length which was collected 3-5 weeks after flower initiation. The effect of culture condition was highly significant, the root induction was highest (83.75% & 87.50%) of anther and ovary respectively when it kept in dark place for 28 days.

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1. Introduction

Kenaf (*Hibiscus cannabinus L.*) is native to tropical regions of Asia and Africa. In Malaysia considered new and cultivated especially in the eastern part of Peninsula Malaysia to replace tobacco plantations that have no longer supported by the government. This plant has potential breeding methods to improve the plant is by the production of hybrid varieties. It is a short-day, fast growing annual. It 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 will achieve 5 to 6m in height and 2.5 to 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 fertilizer. When kenaf is grown in home gardens for fiber, the more tender upper leaves and shoots are sometimes eaten either raw or cooked [1].

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The ability to produce haploid plants is a tremendous asset in genetic and plant breeding studies. Heritability studies are simplified, because with only one set of chromosomes, recessive mutations are easily identified. In addition, doubling the chromosome number of a haploid to produce a doubled haploid result in a completely homozygous plant [2].

The first time haploid plants were discovered in Datura stramonium by A.D Bergner in 1921. After the initial reports of successful production of haploids from another culture in Datura [3], haploids have been obtained in more than 150 species belonging to 23 families of angiosperms [4] while Production of haploid plants through gynogenesis by culturing unfertilized ovaries was first described in barley [5]. In vitro gynogenesis involves the culture of unfertilized ovules or ovaries while Androgenesis is the process by which haploid plants develop from the male gametophyte. As in androgenesis, gynonegic haploids may develop directly or indirectly via regeneration from the callus. Ovary and flower bud cultures have generally been found to be more efficient than ovule culture because of the less intrusive manipulation. Plant breeding can produce lines that are more resistant and have higher yield; however standard plant breeding takes many years. Doubled haploid lines can be produced in one step by tissue culture of flower parts.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 [6].

As with androgenesis, gynogenic haploids may develop directly or indirectly via regeneration from callus. The first cell divisions of gynogenesis are generally similar to those of zygotic embryogenesis. Direct gynogenesis usually involves the egg cell, synergids or antipodals with organized cell divisions leading first to the formation of proembryos and then to well-differentiated embryos. In indirect gynogenesis, callus may be formed directly from the egg cell, synergids, polar nuclei or antipodals, or may develop from proembryos. Plants regenerated from callus maybe haploid, diploid or mixoploid.

Gynogenesis experiments are usually conducted using unfertilized ovules or ovaries, although entire immature flower buds have been cultured in a few species. It is easier to dissect ovaries than ovules without damaging the female gametophyte. However, in polyovulate ovaries, it may be advantageous to excise the ovules so that they can be in direct contact with the culture medium.

Five factors affected on haploid production, (a) Genotype: genotype plays a major role in determining the success or failure of an experiment. Haploid plant production via androgenesis has been very limited or nonexistent in many plant species. Furthermore, within a species, differences exist in the ability to produce haploid plants. (b) Condition of donor plants, The age and physiological condition of donor plants often affect the outcome of androgenesis experiments. In most species, the best response usually comes from the first set of flowers produced. (c) Stage of microspore development, the most critical factor affecting haploid production from anther and microspore culture is the stage of microspore development; for many species, success is achieved only when anthers are collected during the uninucleate stage of pollen development. (d) Pretreatment, for some species, a pretreatment following collection of buds before surface disinfestations and excision of anthers, has been found to be beneficial. In general, lower temperatures require shorter durations, whereas a longer pretreatment time is indicated for temperatures at the upper end of the cold pretreatment. (e) Media, For most other species, the commonly used media for [2].

Anther culture include MS (Murashige and Skoog, 1962), N6 [7], or variations on these media. In some cases, complex organic compounds, such as potato extract, coconut milk, and casein hydrolysate, have been added to the media. (f) Temperature and light, various cultural conditions, such as temperature and light, may also affect androgenic response. Anther cultures are usually incubated at 24 to 25° C. In some species, an initial incubation at a higher or lower temperature has been beneficial. Some species respond best when exposed to alternating periods of light and dark, whereas continuous light or dark cultural conditions have proven beneficial in other species. [2].

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2. Materials and methods

This study was carried out in the tissue culture laboratory of Faculty of Base Industry (FIAT), University Malaysia Kelantan.

2.1 Plant material and growth

The seeds of Kenaf HF992 were collected through (National Kenaf and Tobacco Board). Seeds were germinated in the tissue culture laboratory of Faculty of Base Industry (FIAT), University Malaysia Kelantan. Flower buds of different microspores and ovary stage 6, 8 and 12mm for anther culture and 16, 20 and 24 mm for ovary culture (Table 1 & Figure 1). In different time after the date of start flowering were collected. Anthers and ovary were excised as explants material having the different microspore and ovary stages. Five buds from each length were collected and anthers were removed to determined stage of microspore, this was performed by removing one or two anthers then stained with 4% acetocarmine (W/V) in 50% (v/v) acetic acid [8]. The lengths of flower buds, ovary length, ovule diameter, anther length and anther bunch diameters were measured by the digital microscope.

2.2 Pre-treatment & Sterilization

The flower buds were surface sterilized with 70% alcohol for 45 sec and 10-12 min in 3% sodium hypochlorite (NaClO) with few drops tween 20, followed by three washings in sterile-distilled-water for three minutes/time, the anthers and ovarys were dissected out with the aid of a stereo-microspore and underwent to The cool pretreatment for 0, 2 and 4 days under 4-6^0 celsius [18,21]. Twenty anthers were cultured in disposable Petri dish contain 15 ml [9]

2.3 Culture Medium & condition

Anthers and Ovaries were inoculated on the solid medium (7% agar) that contained four types of plant growth regulator (PGR): one type of Auxins NAA and three types of cytokinins, BAP, 2iP and TDZ. Each type of media consisted of four Petri dish where 20 anthers or 10 ovaries have been inculcated (contained 15ml of MS medium) (Figure 3a). The pH was adjusted to 5.7, and incubated at 25° C in darkness place for 0, 7, 14 and 28 days, afterward the cultures were exposed to light at an intensity of about 2000 Lux for 16 hours per day [10].

2.4 Data analysis

All the experiment was designed according to CRD (completely Random design) and the data was analyzed using ANOVA significant differentiations were compared by DMRT (Duncan Multiple Range Test).

Table 1: Relation between flower bud length, anther, ovary, ovule size, and stigma color.

Bud length (mm)	Diameter of Anther bunch (mm)	Ovary length (mm)	Anther length (mm)	Ovule diameter (mm)	Anther color	Ovule color	Stigma color
<6	<2.0	0.6<	<0.2	<0.1	white	white	white
7.0±1	2.0±0.2	0.8±0.2	2.0±0.1	0.1±0.05	white	white	yellow
10.0±1	3.7±0.2	1.0±0.2	0.6±0.2	0.2±0.1	white	white	pink
15.0±1	4.4±0.2	1.6±0.2	0.9±0.2	0.6±0.2	yellow	white	Red
20.0±1	5.2±0.2	2.4±0.2	1.1±0.2	1.0±0.2	brown	white	Red
24.0±1	>5.4	3.2±0.2	1.4±0.2	1.2±0.2	carmine	white	Red

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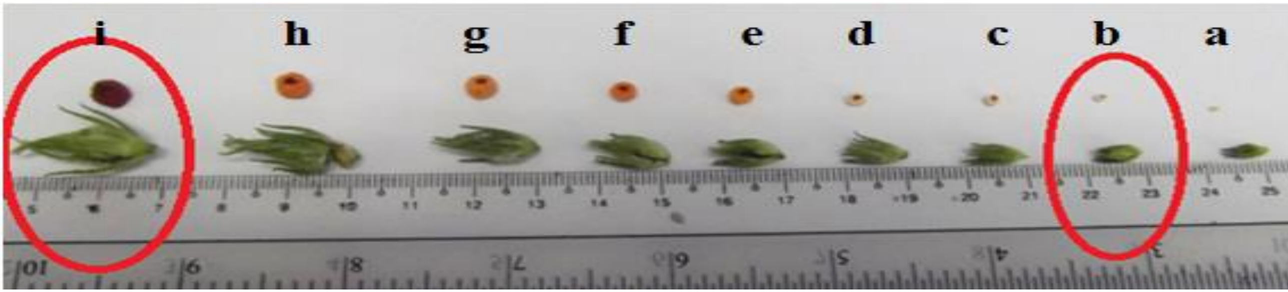

Figure 1: Different size of flower buds (a) pollen mother cell, (b) Tetrad, microspore stage, (c)-(h) pollen grain stage (i) flower 1-2 days before anthesis.

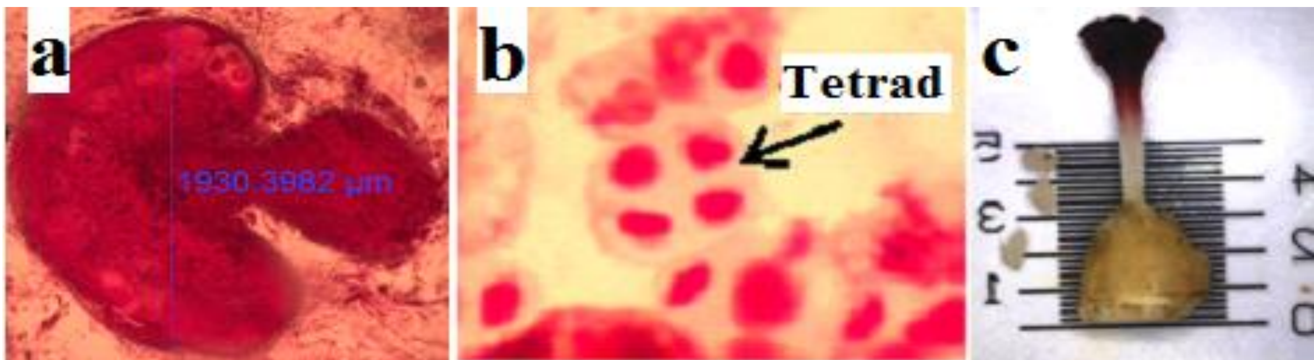

Figure 2: (a) anther during tetrad stage, (b) Tetrad, microspore stage, (c) ovary before anthesis flower.

3. Result & Discussion

Plant tissue culture has emerged as the rapidly developing science because of its proven success in agricultural improvements that is elimination of disease enhance multiplication rates of selected clones of importance, evolution of new varieties in a reduced span of time and a shortened breeding cycle.

3.1 Stage of anther and ovary

In this research, flower buds about 7±1 mm long were best size for callus induction (Table 1 & Figure1b). The anthers containing mid or late-uninucleate microspores were most suitable for anther culture while the best ovary stage was 24±1mm one or two days before anthesis. The buds of less than 6 mm long before starting of meiosis stage for pollen mother cell (PMC), had white color for anther and stigma (Table 1 & Figure1a)., and buds of length 10, 15, 20, and 24mm(Table 1 & Figure1) have mature pollen grain (after pollen mitoisis1) with different color and length anthers. After the pollen grains begin to accumulate storage reserves, they usually lose their embryogenic capacity and follow the gametophytic developmental pathway [11]. Sunderland [12] reported that anthers from the first flush of flowers in the season were found to be more responsive. In developing a protocol for anther culture, one anther from each bud is usually set aside and later cytologically observed to determine the stage of microspore development. In many cases, anthers within a bud are sufficiently synchronized to allow this one anther to represent the remaining cultured anthers. Measurements of physical characteristics of the flower, such as calyx and corolla length and anther color, shape, and size, are also recorded [2].

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Table 2: Effect of days to flowering on androgenesis and gynogenesis

Days after flower beginning	Frequency of Callus formations from anther (%)	Frequency of Callus formations from ovary (%)
2-4 week	90.6[a]	90.0[a]
6 weeks	74.0[b]	81.6[b]
8 weeks	48.3[c]	74.3[b]

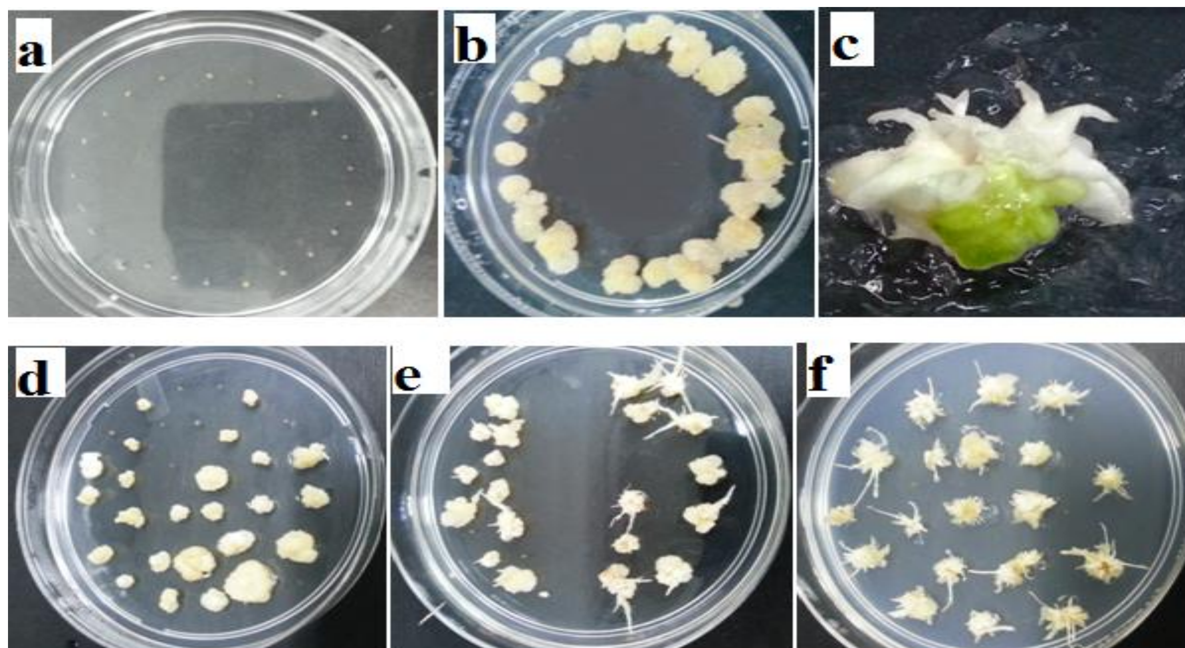

Figure 3: (a) anther inoculation, (b) callus induction from anthers for T1, (c) callus and root induction from ovary, (d) callus and root induction from anthers for T5, (e) callus and root induction from anther for T3, (f) callus and root induction from anther for T1.

3.2 Cool pretreatment

Temperature shock is considered to be the most effective treatment to induce pollen embryogenesis development. The optimum temperature and duration of pretreatment vary with the genotype. With regarding cool pretreatment in this research, there were no significant differences between chilling treatment 2 and 4 days with control treatment on callus induction (Table 3 & 4), the best callus induction from anthers and ovaries were 90% and 91.25 respectively with 4 days cool pretreatment. Bohanec [13] reported that in vitro gynogenesis is generally not stimulated by shock treatment such as low or high temperatures or pregrowth on starvation medium. Also Ali [14] reported that some of crops were not affected by cool pretreatment.

3.3 Culture media

Callus formation was observed in 12 days after inoculation from the anther and ovary. Among the different combinations of plant growth regulator, the concentration of T1, 3.0 mg/L 2iP and 3.0 mg/L NAA was the best to induce callus production after 4 weeks with 90% for anther culture respectively and it was significant differences with another hormone combination (Table 3 & 4), and less callus induction

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was observed in combination T2, 3.0 mg/L 2iP and 0.1 mg/L NAA . Results of callus induction from anther explants are shown in Tables 3 and 4, the effect of auxin NAA was important on callus induction.

And with regarding callus induction from ovary, the combination T1 3.0 mg/L 2iP and 3.0 mg/L NAA was the best with 91.25% of callus induction, and there were no significant differences with T3, 0.5 mg/l TDZ and 2.0 mg/l NAA, but it was significant differences with another combinations, whereas the minimum was noticed in T2, 3.0 mg/L 2iP and 0.1 mg/L NAA. The most commonly used basal media for anther culture are N6 medium [7], (modified) MS medium (Murashige and Skoog), Nitsch and Nitsch (1969) medium and B5 medium [15], but there are many others. Generally, half strength MS salt mixtures are suggested for the Solanaceae, and N6 medium for the cereals [7].

3.4 Culture condition

The effects of various culture conditions with different hormone combination of the roots induction are given in Table 5 &6 and Figure 3. The effect of culture condition was highly significant, the root induction was highest (80%) on media T1 with 28 days dark place for anther culture and (85%) on media T1 with 28 days dark place for ovary culture, and the lowest was (5%) in media T6 with control dark place for anther culture and (4%) in media T6 with control dark place for ovary culture, on this basis of revised medium and culture condition the different auxin concentration and different dark place days has been observed to be an important determinate for the success of callus and root induction, the main factor on root induction was dark place, the explants has been kept in dark place for 28 days butter from 14, 7 and 0 days of root induction, in other word the root induction affected significantly by dark place factor.

Anther cultures are usually incubated at 24–27°C and exposed to light at an intensity of about 2,000 lux for 14 h per 24-h day [16], The incubation of anthers continuously in the dark has, on occasion, been found to be essential. An alternating light and dark period has also been shown to be beneficial after the induction period in several species [17].

4.0 Conclusion

This study concludes that anther collected from plants at the beginning of flower initiation (7±1mm length) and ovary collected 1 or 2 days before anthesis (24±1mm length) was induced higher frequency of callus formation, The best hormone combination for callus induction was found to be MS medium containing NAA 3.0 mg/l and 2iP 3.0 mg/l, The protocols developed under this study are valuable to induce callogenesis from kenaf anthers, which may help to the production of haploid plant in kenaf.

Table 3: Effect of cool pretreatment with different types of PGR on callus induction of anther.

Growth regulator mg/l					Cool pretreatment (days)		
					0	2	4
No.of treat	2iP	TDZ	BAP	NAA	Callus %		
T1	3.0			3.0	83.75^{a}	81.25^{a}	90.00^{a}
T2	3.0			0.1	12.50^{d}	13.75^{cd}	5.00^{d}
T3		0.5		2.0	80.00^{ab}	72.50^{ab}	75.00^{b}
T4		0.5		0.1	25.00^{c}	22.50^{c}	22.50^{c}
T5			3.0	3.0	72.50^{b}	70.00^{b}	75.00^{b}
T6			3.0	0.1	8.75^{d}	6.25^{d}	8.7d^{c}

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Table 4: Effect of cool pretreatment with different types of PGR on callus induction of ovary.

Growth regulator mg/l					Cool pretreatment (days)		
					0	2	4
No.of treat	2iP	TDZ	BAP	NAA	Callus %		
T1	3.0			3.0	78.75[a]	78.75[a]	91.25[a]
T2	3.0			0.1	10.00[bc]	15.00[b]	17.50[cd]
T3		0.5		2.0	75.00[a]	82.50[a]	85.00[a]
T4		0.5		0.1	17.50[b]	20.00[b]	26.25[c]
T5			3.0	3.0	72.50[a]	77.50[a]	72.50[b]
T6			3.0	0.1	3.75[c]	5.00[c]	7.50[d]

Values followed by the same letters within a column are not significantly different ($P \leq 0.05$); Duncan's Multiple Range Test (DMRT)

Table 5: Effect of dark condition and different types of PGR on Root induction of anther.

Growth regulator mg/l					Dark place (days)							
					0		7		14		28	
No.of treat	2iP	TDZ	BAP	NAA	Response %	Root%	Response%	Root%	Response%	Root%	Response%	Root%
T1	3.0			3.0	83.75[a]	8.75[a]	81.25[a]	21.25[a]	86.25[a]	40.00[a]	92.50[a]	83.75[a]
T2	3.0			0.1	12.50[d]	2.50[a]	8.75[c]	5.00[b]	7.50[d]	2.50[c]	13.75[d]	8.75[e]
T3		0.5		2.0	80.00[ab]	6.25[a]	78.75[a]	18.75[a]	82.50[ab]	33.75[b]	90.00[a]	76.25[b]
T4		0.5		0.1	25.00[c]	3.75[a]	20.00[b]	8.75[b]	17.50[c]	7.50[c]	27.50[c]	17.50[d]
T5			3.0	3.0	72.50[b]	3.75[a]	76.25[a]	21.25[a]	76.25[b]	32.50[b]	80.00[b]	66.25[c]
T6			3.0	0.1	8.75[d]	1.25[a]	8.75[c]	2.50[b]	3.75[d]	1.25[c]	6.25[d]	2.50[e]

Values followed by the same letters within a column are not significantly different ($P \leq 0.05$); Duncan's Multiple Range Test (DMRT).

Table 6: Effect of dark condition and different types of PGR on Root induction from ovary.

Growth regulator mg/l					Dark place (days)							
					0	7	14	28				
No.of treat	2iP	TDZ	BAP	NAA	Response %	Root%	Response%	Root%	Response%	Root%	Response%	Root%
T1	3.0			3.0	78.75[a]	8.75[ab]	85.00[a]	31.25[a]	80.00[a]	36.25[a]	93.75[a]	87.50[a]
T2	3.0			0.1	10.00[bc]	2.50[c]	6.25[b]	2.50[b]	17.50[bc]	7.50[b]	20.00[c]	17.50[c]
T3		0.5		2.0	75.00[a]	12.50[a]	81.25[a]	26.25[a]	86.25[a]	31.25[a]	86.25[ab]	77.50[b]
T4		0.5		0.1	17.50[b]	5.00[bc]	15.00[b]	5.00[b]	22.50[b]	7.50[b]	26.25[c]	20.00[c]
T5			3.0	3.0	72.50[a]	11.25[a]	77.50[a]	27.50[a]	80.00[a]	36.25[a]	78.75[b]	73.75[b]
T6			3.0	0.1	3.75[c]	1.25[c]	6.25[b]	1.25[b]	10.00[c]	3.75[b]	10.00[d]	6.25[d]

Values followed by the same letters within a column are not significantly different ($P \leq 0.05$); Duncan's Multiple Range Test (DMRT)

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