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# *In Vitro* Shoot Regeneration from Leaf Explants of Kenaf (*Hibiscus cannabinus* L.)

(Penjanaan Semula Pucuk Kenaf (*Hibiscus cannabinus* L.) Secara *In Vitro* Menggunakan Eksplan Daun)

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ABSTRACT

*Kenaf (*Hibiscus cannabinus *L.) is a versatile plant with multiuse ranging from animal feed to a wide variety of biocomposite products such as pulp and paper and fibre reinforce plastic. Therefore genetically improved planting materials are needed to tailor made requirement of the industry. Thus, development of plant regeneration through callus is important for* in vitro *genetic manipulation of kenaf. Currently development of successful genetic transformation of kenaf is through* in planta *transformation means.* In vitro *shoot regeneration was conducted using leaf explants from varieties V36 and G4 treated to three different combinations of* $N^6$ *Benzyl adenine (BA) and Indole-3-butyric acid (IBA). High percentage of healthy callus induction was produced in MS medium supplemented with combination of 1.5 mgL*$^{-1}$ *BA and 0.5 mgL*$^{-1}$ *IBA. In addition 68.7% plant regeneration was obtained in MS medium supplemented with 0.3 mgL*$^{-1}$ $GA_3$*. All plantlets produced roots in hormone free medium. There was no significant difference among varieties in terms of callus induction (number of callus) and plant regeneration (number of plantlets). This protocol is useful to be used for the development of gene transformation protocol of kenaf through callus.*

*Keywords: Callus;* in vitro*; kenaf (*Hibiscus cannabinus *L.); leaf explants; plant regeneration*

ABSTRAK

*Kenaf (*Hibiscus cannabinus *L.) merupakan sejenis tumbuhan berdaya saing yang mempunyai pelbagai guna. Kegunaannya berjulat daripada pemakanan haiwan kepada pembangunan pelbagai produk biokomposit seperti kertas dan pulpa serta plastik diperkuat gentian. Bahan tanaman yang diperbaiki secara genetik adalah diperlukan untuk memenuhi keperluan tertentu industri. Pembangunan kaedah regenerasi tumbuhan melalui kalus amat penting bagi manipulasi genetik secara* in vitro *untuk tumbuhan kenaf. Buat masa kini, pembangunan kaedah transformasi genetik pada kenaf hanya dapat dijayakan melalui kaedah transformasi* in planta*. Regenerasi melalui pembentukan pucuk* in vitro *telah berjaya dihasilkan dengan mengunakan eksplan daun daripada varieti V36 dan G4 yang dikulturkan dalam tiga kombinasi* $N^6$ *Benzil adenin (BA) dan asid Indol-3-butirik (IBA). Penghasilan kalus segar dalam peratusan yang tinggi terbentuk dalam media MS yang mengandungi campuran 1.5 mgL* $^{-1}$ *BA and 0.5 mgL*$^{-1}$ *IBA. Manakala 68.7% pembentukan anak pokok telah berjaya dihasilkan dalam media MS yang dicampurkan dengan 0.3 mgL*$^{-1}$ $GA_3$*. Plantlet membentuk akar dalam media tanpa hormon. Pembentukan kalus (bilangan kalus) dan regenerasi tumbuhan (bilangan anak pokok) tidak menunjukkan perbezaan yang bererti antara varieti. Protokol ini boleh digunakan untuk membangunkan transformasi kenaf melalui pembentukan kalus.*

*Kata kunci: Eksplan daun;* in vitro*; kalus; kenaf (*Hibiscus cannabinus *L.); penjanaan semula tumbuhan*

## Introduction

Kenaf (*Hibiscus canabinus* L.) is native to tropical regions of Asia and Africa. It is a short-day fast-growing annual crop belonging to the family Malvaceae (Ayadi et al. 2011; Reichert et al 1995). It has been widely planted due to its multiple uses ranging from basic animal feed to a variety of biocomposite products focuses on fibre production, such as making ropes, sacks, canvases and carpets (Li 1980). The importance of *H. cannabinus* as a fibre crop with many commercial uses has increased tremendously in recent years. It has the potential to be alternative resource for all the papers consumed worldwide. New applications of kenaf have been developed in the pulp and paper industry, oil absorption and potting media, board making, filtration media and animal feed (Ayadi et al. 2011; Keshk et al. 2006; Sellers & Reichert 1999). But the production of genetically improved planting material has been achieved quite slow. Therefore the production of high yielding quality planting material through genetic engineering was started with *H. cannabinus*. The development of successful *in vitro* manipulation techniques including an efficient plant regeneration system is important.

There are several reports on the establishment of *in vitro* regeneration techniques of *Hibiscus* genus (Dar et al. 2012; Gomez-Leyva et al. 2008; Govinden-Soulange

et al. 2009; Jenderek & Olney 2001; Ma'arup et al. 2012; Sakhanokho & Kelley 2009) including some reports on organogenesis regeneration of *H. cannabinus* (Ayadi et al. 2011). Banks et al. (1992) demonstrated the possibility of gene transformation to the callus of *H. cannabinus*, but they were unable to produce transformed plants. Mclean et al. (1992) reported that organogenesis of kenaf via callus culture was not reproducible because it is very low in frequency. A direct and simple regeneration procedure using the Kenaf shoot apex has been reported by Zepata et al. (1999). The shoot apex includes the shoot apical meristem and several leaf primordia. Plant regeneration from apex of kenaf (Srivatanakul et al. 2000, 2001; Zepatal et al. 1990) and nodal explants (Reichert & Baldwin 1996) has also been reported. These works have used shoot apices from 3-5 days old seedling for co-cultivation with *Agrobacterium tumefaciens* containing genes resistant to Phosphinothricin and Hygromycin and obtained the highest percentage of rooted plants in selection media (Srivatanakul et al. 2000). In all these methods they tried for genetic transformation through callus but no success story has been reported. Khatun et al. (2003) have reported plant regeneration from cotyledonary petioles. They used surfactant pluronic-F-68 for the enhancement of shoot regeneration from kenaf cotyledon explants, whereas Kojima et al. (2004) have reported on *in planta* transformation of kenaf. In this method they directly inoculated into either apical meristem or lateral bud meristem in the bio-hazard controlled rooms and only the upper part of the $T_0$ plants showed transformation while the lower parts remained untransformed. Further, Samanthi et al. (2005) optimized the factors for *in vitro* gene transformation through shoot apex (without callus phase) of kenaf. In the most recent study on *in vitro* shoot regeneration from shoot tips and nodes, Ayadi et al. (2011) found that the highest shoot regeneration frequency (90.5%) was obtained on MS control medium where addition of growth regulators to the MS medium didn't affect the shoot regeneration but only lead to a decrease in shoot induction and length and root induction rates. All these studies report direct shoot regeneration but so far no successful indirect shoot regeneration method through callus has been developed for kenaf.

Here, we explore the possibility to produce an efficient reproducible indirect plant regeneration procedure which can be used to develop transgenic plants of Kenaf.

## Materials and Methods

### Explants used

Seeds of two kenaf varieties viz variety 36 (V36) and Guatemala 4 (G4) were used for this purpose. The former from an intermediate flowering which is currently Malaysia's production variety while the latter from a late flowering variety which is a promising production variety. The seeds were initially surface sterilized in 70% ethanol for 3 min, soaked for 60 min in commercial Clorox (sodium hypochlorite 5.25%) containing 0.2% Tween 20 and washed 4 to 5 times with sterile distilled water. These seeds were germinated on Murashige and Skoog medium (Murashige & Skoog 1962) and 2% (w/v) sucrose without any growth regulators. pH of the media was adjusted to 5.8 before the addition of 0.4% gelrite. These cultures were kept in a controlled room, maintained at a temperature of 25°C ± 2°C under 24 h light for 7 to 10 days.

### *In vitro* callus formation, shoot regeneration and rooting

Cotyledonary leaf explants from aseptically grown 7 to 10 days old plantlets were cut into small squares of 0.5 × 0.5 $cm^2$ and placed on MS medium by dipping the cut surfaces on medium supplemented with three different combinations of benzyl adenine (BA) and indole-3-butyric acid (IBA) i.e. 1.5 $mgL^{-1}$ BA + 0.05 $mgL^{-1}$ IBA, 2 $mgL^{-1}$ BA + 0.1 $mgL^{-1}$ IBA and 2.5 $mgL^{-1}$ BA + 1.00 $mgL^{-1}$ IBA for callus induction. The cultures were maintained for 24 days under dark condition at 25±2°C. All treatment combinations were repeated two times. Each treatment consisted of 170 to 200 leaf explants per replicate.

Three to five mm diameter calli masses were excised from the leaf and transferred into similar growth medium with same composition of BA and IBA. In addition, 0.3 $mgL^{-1}$ of Gibberellic acid was added into the medium for shoot regeneration. Cultures were maintained under continuous light (200 μmol $mL^{-2}$ $S^{-1}$ flourescent lamp) at 25±2°C for nine weeks with regular sub culturing (every 12 to 14 days intervals) on to fresh medium.

The regenerated plantlets (0.5 - 1.0 cm height with one or two new leaves) were excised from calli and transferred on similar medium to ensure good growth of the *in vitro* plantlets for 10 days. The plantlets were then transferred to the MS free medium for rooting and maintained under similar growth condition as mentioned above. Rooted plantlets were maintained for 3 to 4 months on MS free medium with sub culturing to fresh medium at every two weeks for further acclimatization.

### Statistical analysis

Data on the percentage of calli induced and percentage of shoots regenerated were statistically tested by analysis of variance (ANOVA) ($p<0.05$).

## Results and Discussion

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic viability and adventitious shoot formation (Dodds & Roberts 1982). It has been discovered that the plant hormone combination and the concentrations are the deciding factors influencing embryogenic callus and plant regeneration. Generally cytokinin and auxin are known to promote callus formation (Akiyoshi et al. 1983). 1-naphthaleneacetic acid (NAA),

Indole acetic acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), (IBA) and BA are the most commonly used growth regulators for callus induction and plant regeneration from leaf petiole and shoot explants (Khalafalla et al. 2007). An addition of 2, 4-D to the medium did not change multiple shoot formation. In addition, increasing 2, 4-D concentration in the medium resulted in a reduction in multiple shoot regeneration. With higher 2, 4-D concentration, the number of explants; that responded; was reduced since more explants produce callus. (Srivatanakul et al. 2000). The effectiveness of 2, 4-D and NAA in combination with cytokinins in inducing callus might be due to their role in DNA synthesis and mitosis. The difference in some nitrogen bases maybe the cause of difference in the morphology of Kenaf plant from non-branching to branching phenotype. Either NAA/IAA/2, 4-D with BA induced profuse light green to dark green colour or fragile to nodular callus formation from leaf explants. At higher concentration of auxins occasional root formation directly from the leaf explants was also observed. Indirect shoot organogenesis was achieved from the callus using BA where the highest number of shoots with maximum frequency was regenerates (Arumingtyas et al. 2010).

There was no significant difference between V36 and G4 in both induction of callus and plant regeneration. This result was different from Zouzou et al. (2008) who observed that callus induction was highly genotype dependent in *Gossypium hirsutum*. Gowher et al. (2007) also observed callus initiation in all hormone combinations tested irrespective of varietal different but shoot regeneration was markedly affected by the genotype of tobacco. But hormone combination showed a highly significant effect on both callus induction and plant regeneration (Figure 1). There was no callus induction but roots were produced on cut surface, as observed in the medium without any hormone (Figure 2(a)) depicting that there is a need of hormones for callus induction in Kenaf. Vegetative plant parts especially leaves are desirable explants for *in vitro* improvement because regeneration from these explants would preserve the genetic homozygosity of the parent genotype. When applying *in vitro* techniques, it is a must to surface sterilize plant parts which are going to be cultured on artificial nutrient media. Since plants and plants parts are in contact with a wide range of fungi and bacteria, it is necessary to disinfect tissues with a minimum amount of cellular damage to the host tissue (Conger 1987).

The medium supplied with 1.5 $mgL^{-1}$ BA and 0.05 $mgL^{-1}$ produced significantly high number of calli masses (3 - 5 mm diameter) (Figure 2(b)). Two to five number of nodular calli masses were produced on the cutting edge of the explants. It was also observed that callus induction was affected by IBA and BA concentration i.e. with increase in the IBA and BA, a reduction in the callus induction took place (Table 1). Similar trend was reported in *Azadirachta indica* by Murthy and Saxena (1998) and Khalafalla et al. (2007) and in *Broussonetia papyrifera* by Meru et al. (2008). Khalafalla et al. (2007) also reported that IBA with BAP produced higher callusing percentage and best callusing when compared to NAA and 2, 4 D. They also further observed IBA at higher concentration induced the lowest callusing percentage. Embryogenic calli developed at the end of cut surfaces and most of them showed compact yellow colour structure. Upon transfer to the plant regeneration medium these calli turned into greenish colour.

After 9 weeks in all hormone combinations, most of calli masses showed differentiation into plants (Figure 2(c)) except the medium without hormone. But medium with 1.5 $mgL^{-1}$ BA and 0.05 $mgL^{-1}$ IBA and 0.3 $mgL^{-1}$ $GA_3$ showed significantly high number of plant regeneration compared with other two combinations. It shows 68.7% plant regeneration. Similar results were reported in *Azadirachta indica* by Akiyoshi et al. (1993) and Khalafalla et al. (2007). They observed MS basal

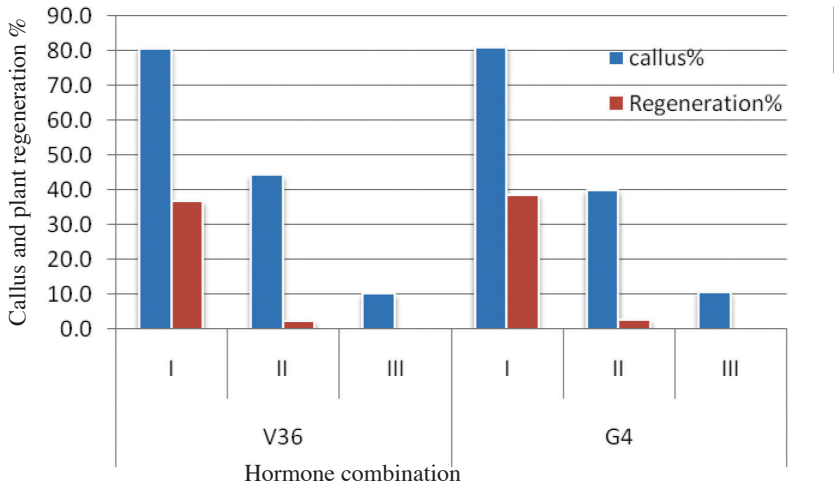


FIGURE 1. The callus induction and plant regeneration (as a %) in different hormonal combinations

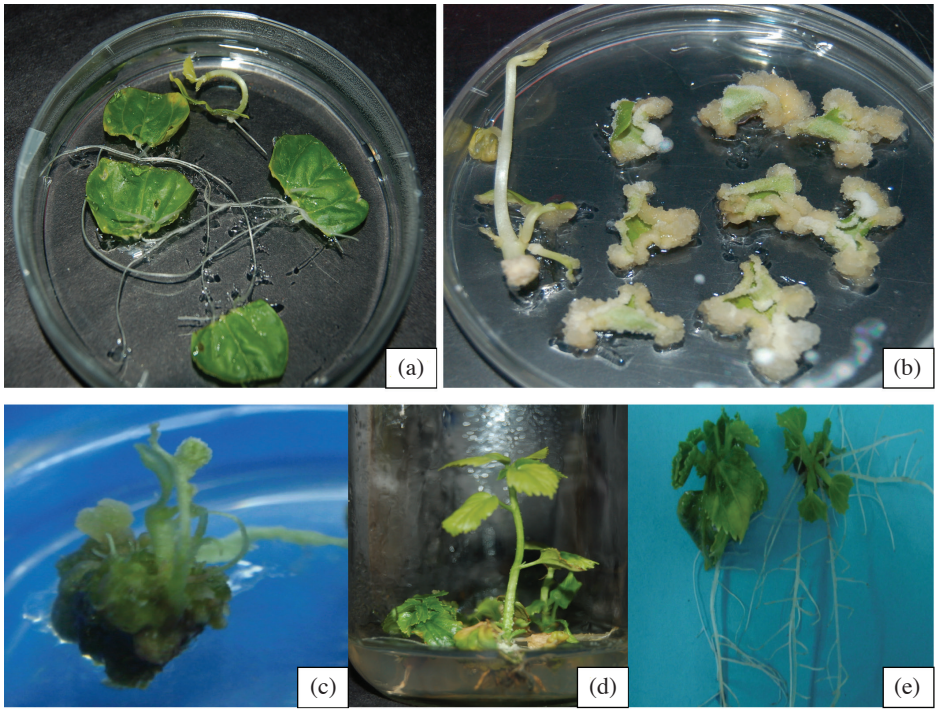


FIGURE 2. The plant regeneration from *in vitro* grown leaf explants of kenaf (a) explants were producing roots, without callus in hormone free medium; (b) induction of callus on medium containing 1.5 $mgL^{-1}$ BA, 0.05 $mgL^{-1}$ IBA (130 mm size Petri dishes); (c) shoots appeared on the MS medium supplemented with 1.5 $mgL^{-1}$ BA, 0.05 $mgL^{-1}$ IBA and 0.3 $mgL^{-1}$ GA 3; (d) regenerated plantlets after 4 months (6 × 11 cm glass bottles) and (e) rooted plantlets

TABLE 1. Mean and Duncan's multiple range tests for callus induction and plant regeneration

| Hormone combinations | Mean | |
|---|---|---|
| | No. of calli masses | No. of plants regenerated |
| 1.5 $mgL^{-1}$ BA+ 0.05 $mgL^{-1}$ IBA | 165.75[a] | 68.7[a] |
| 2 $mgL^{-1}$ BA+ 0.1 $mgL^{-1}$ IBA | 75.5 [ab] | 5.5[b] |
| 2.5 $mgL^{-1}$ BA+ 1 $mgL^{-1}$ IBA | 20.75 [b] | 0.00[b] |
| Control without hormone | 0.00[b] | 0.00[b] |

[a] Means with the same letters are not significantly different from each other at 5% level by Duncan's multiple range test. These mean values are from two independent replicates

medium containing 1.0 $mgL^{-1}$ of IBA in combination with 0.5 $mgL^{-1}$ of BAP promoted rapid growth, produced the highest callusing percentage (95.0%) and the best callusing degree. *Broussonetia papyrifera* also showed 100% callus induction in a combination of BA and IBA (Meru et al. 2008). It agrees with our results that a good callus can be obtained in a combination of IBA and BA. Regenerated plants were excised from callus (Figure 2(d)) and allowed to grow 7 to 10 days in the same hormone combination before transfer to the rooting medium. Almost all plants produced roots in 4 to 6 weeks (Figure 2(e)). Around 2 to 4 cm tall plantlets were obtained after maintaining in the MS media without hormone which took around 3 to 4 months. Plants were acclimatized in plastic pots containing autoclaved peat, sand and clay soil (3:1:1) for one month and then transferred to the field.

## CONCLUSION

In summary, an adventitious regeneration protocol developed from *H. cannabinus* was attempted on various ornamental *Hibiscus* species. Previous studies with kenaf showed that the adventitious regeneration protocol could induce mutations (somaclonal variation in the regenerants.), variation in Kenaf stem colour and flower shapes was noted. Since many ornamental *Hibiscus* species are asexually propagated, once a desired mutant is identified, it could be maintained and propagated without loss of the unique trait(s). Some types of mutations are transition, transversion, insertion and deletion. Insertion and deletion of N base are capable of inducing frame shift mutation. This causes large changes in the protein translated from the codon. (Preethi et al. 2011). The

results of this study showed that there is a possibility of indirect plant regeneration from Kenaf callus. But further work is needed to develop a gene transformation system (especially *Agrobacterium* mediated) to produced transgenic plants.

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