# ESTABLISHMENT OF AN EFFICIENT MICROPROPAGATION PROTOCOLS FOR THREE KENAF (*HIBISCUS CANNABINUS* L.) CULTIVARS

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**ABSTRACT**

**This study was aimed to establish an efficient micropropagation protocol for three popular kenaf (*Hibiscus cannabinus* L.) cultivars (V36, HF992 and KB6) using different types of plant growth regulators (PGR's) on Murashige and Skoog medium (MS). The shoot tip, cotyledon and hypocotyl isolated from 2 weeks old seedlings were used as explants. MS medium fortified with different types and concentrations of cytokinins and auxin were evaluated for establishment callus formation, shoot multiplication and rooting. Among different PGR's used, 5.0 mg/l 2iP + 0.1 mg/l NAA was the best concentration for direct shoot regeneration from cotyledon with shooting percentage of 55% in HF992 cultivar. In multiple shoot induction experiment, 1.0 mg/l TDZ + 0.1 mg/l NAA mg/l concentration has induced the highest (10.4) shoots/explant. Moreover, indirect shoot regeneration was established from callus which was induced from cotyledon, maximum callus induction percentage (88.75%) was recorded on 0.5 mg/l TDZ and 1.0 mg/l NAA in V36 variety. While, for shoot elongation and rooting, MS media half strength supplemented with 1.0 mg/l kinetin + 1.0 mg/l IBA was found to be an optimum concentration.**

**Keywords: direct shoot; *in vitro* culture; callus; plant growth regulators**

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## تثبيت طرائق فعالة للاكثار الدقيق لثلاثة اصناف من نبات التيل (*Hibiscus cannabinus* L.)

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**المستخلص**

**الهدف من هذه الدراسة لتثبيت طرق للاكثار الدقيق لثلاثة أصناف من التيل (*Hibiscus cannabinus* L.) (V36 و HF992 و KB6) باستخدام أنواع مختلفة من منظمات النمو النباتية على الوسط الغذائي موراشك وسكوك (MS) . تم استعمال القمه النامية والاوراق الفلقيه والسويقات تحت الفلقية المعزولة من شتلات عمرها أسبوعين كمصدر للزراعة. اختيرالوسط MS مدعم بأنواع وتراكيز مختلفة من السيتوكينينات والأوكسينات من أجل الاكثار الدقيق ومرحلة التضاعف والتحفيز على انتاج الكالس وكذلك تحفيز الجذور. من بين مختلف أنواع منظمات النمو النباتية المستعملة كان الوسط الغذائي المدعم بـ 5.0 ملغ / لتر 2iP + 0.1 ملغ / لتر NAA أفضل تركيز في انتاج النبيتات بصورة مباشره من الاوراق الفلقية بنسبة 55٪ في صنف , HF992, وفي تجربة التضاعف المتعدد للنبيتات ، اعطى 1.0 ملغ / لتر من TDZ مع 0.1 ملغ / لتر NAA أعلى نسبة (10.4) براعم / نبات. علاوة على ذلك ، الاستحثاث غير المباشر للبراعم من الكالس الناتج من النبيتات تم تسجيل اعلى نسبة لاستحثاث الكالس (88.75٪) على 0.5 ملغ / لتر TDZ و 1.0 ملغ / لتر NAA في صنف V36. بينما ، من أجل الاستطالة والتجذير ، وجد أن نصف قوة وسط MS المضاف إليها 1.0 ملغ / لتر KIN + 1.0 ملغ / لتر IBA هو التركيز الأمثل.**

**الكلمات المفتاحية: الانبات المباشر, الزراعة المختبرية, الكالس, منظمات النمو النباتية.**

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## INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is an herbaceous plant belong to the family of Malvaceae, and it was believed to be originated from Africa, and being distributed in the 20th century in Asia and America (11, 27, 29). Currently, China and India are the world's largest Kenaf producers which representing approximately 70% of world total Kenaf production (12, 19). Kenaf (*Hibiscus cannabinus* L.) is one of the most important industrial fiber crops with various applications from paper production to furniture and from biofuel to textile. These attributes have caught the attention of several industries and investors, and enables plentiful product types to be obtained (9, 13). In Malaysia, Kenaf has become the country's seventh industrial crop after first being announced in 2010 as an alternative crop to replace tobacco in line with its commitments under the WHO agenda on tobacco control (22). Furthermore, the "Malaysia Ministry for Plantation Industries and Commodities" allocated financial support for Kenaf research and development programs. In addition, several agencies have provided agricultural lands and technical support for smallholders to cultivate Kenaf and transform the industry (5). Owing to its multipurpose applications, many farmers were encouraged and started to cultivate Kenaf as a potential industrial crop of economic importance. The Kenaf oil market value is projected to be about USD 180 million in 2023, and its fibers are continued to grow in the world market rapidly (1).Kenaf plant is characterized by its fast growth and reaches its maturity within 4 to 6 months. Traditionally, Kenaf plant propagated by seed. However, seed availability is limited (4, 10, 29). Seed production strategies are affected by several factors such as cultivar, location-especially latitude, and cultural practices (29). Heterogeneous nature of the seedlings owing to its cross pollination. These will eventually decrease in fiber yields and quality. The application of plant biotechnology i.e., tissue culture techniques could be an ideal approach for mass propagation of healthy and genetically identical plant material regardless of the season in a relatively short time (29). Plant biotechnology has played and continues to play an important role in agricultural crop improvement (14, 25, 33). Over the past few years, the use of these technologies has increased in order to improve crops and develop novel varieties. The most important traits and characteristics that many breading programs are focused on are mainly on efficiency of crop production, diseases-free, high yielding and better quality in terms of nutritional values and increased shelf life (2, 6, 18). As shown in the earlier reports, micropropagation methods have been used in Kenaf, especially for shoot regeneration and propagation (28). Although some earlier researches have been conducted for *in vitro* culture of *Hibiscus cannabinus* L. (3, 31). Researches are still required to development the micropropagation protocols for Kenaf to make them more efficient, inexpensive and convenient for practice. This study was carried out to establish an efficient micropropagation system for three Kenaf cultivars (V36, HF992 and KB6) through multiple explants using different types of plant growth regulators (PGR's) on Murashige and Skoog medium (MS).

## MATERIAIS AND METHODS

**Plant material and surface sterilization**

Cotyledon and shoot tip of three Kenaf cultivars (V36, HF992 and KB6) were used as explants. The seeds of Kenaf were provided by the "National Kenaf and Tobacco Board, Kelantan (NKTB)". Surface sterilization was done under aseptic conditions following the protocol (8). Briefly, the seeds were cleaned thoroughly under running water for 15-minute to remove dust and other contaminants, then disinfected with 70% ethanol for 45 seconds to reduce the populations of bacteria and fungi on the seed surface. Then chemically sterilized with 5% sodium hypochlorite (NaClO) for 10-12 minutes with addition of 2-3 drops of Tween 20 to improve the effectiveness of the sterilization process, before being rinsed 4 times with sterile water to remove any residual chemicals. The surface sterilized seeds were cultured in jars contained sterilized soaked peat moss (already autoclaved for 30 minutes with 121°C) for germination. Two or three seeds were placed in each jar, and then covered by plastic cap and incubation in growth room with controlled temperature in light and dark place (16/8h) under 25 ±2°C,

after 2-3 days seeds have begun to germinate (3).

**Collection of explants**

The explants were obtained from aseptically germinated seeds after 10-12 days. To maintain the sterilization of the tissue culture system, the cotyledons and shoot tips were carefully excised using sterilized surgical blades. The cotyledons were then cut into small pieces, approximately 1 cm in length, while the shoot tips were trimmed to a length of 3-4mm before being cultured.

**Direct shoot regeneration**

The explants taken from aseptically germinated seeds were inoculated on MS growth medium media, which consisted mineral salts, four hormones combination of TDZ, BA, 2iP, KN and NAA to promote cell division and shoot development. Cotyledon leaf and shoot tip were used for direct shoot regeneration. Cotyledon were cut into small pieces one cm and shoot tips at 3-4mm length before were cultured. The cultures were then incubated in a growth room with 16-hour light photoperiod at 25 °C.

**Indirect shoot regeneration**

Indirect shoot regeneration could be achieved by two steps, callus induction then shoot regeneration. Cotyledons obtained from the in vitro seedling cultures were subjected to callus induction. Each cotyledon explants were cut into small sections before being cultured into MS media fortified with four types of hormones combination of Thidiazuron (TDZ), 6-benzylaminopurine (BA), 6-(γ,γ-Dimethylallylamino)purine (2iP), Kinetin (KN) and α-naphthalene acetic acid (NAA). Four pieces of cotyledons were used as explants and inoculated into per Petri dish with four replications. Cultures were placed in the growth room under dark place condition for callus induction. Callus produced after 5-6 weeks later were removed and transferred into fresh MS media supplemented with the same hormone combination but at different concentration. All cultures were kept in the growth room under light and dark place(16/8h). Number of shoots formed per callus was scored after 6-week culture under the 16 hours photoperiod in growth room at 25°C.

**In vitro root induction and acclimatization**

For root formation, an individual shoots were excised from the callus clumps and then sub-cultured onto 1/2 strength MS growth medium fortified with 1 mg/l IBA combined with 1mg/l KIN and shoot growth. Once the roots have developed, the new plantlets were transferred into tubes which contains a small amount of tap water and covered with a piece of cotton wool to allow the root development and growth of the plantlets in a semi-controlled environment, prior to acclimatization. Plantlets were kept in the growth room for 1-2 weeks, then transferred to a small plastic pot with peatmoss media, covered by a plastic cap with a small hole for gas exchange. The cap was removed for an hour daily, and exposure time was gradually increased to 2-4 hours over 2 weeks. Finally, the plants were moved to a soil-based growing medium in the greenhouse nursery for further growth.

**RESULTS AND DISCUSSION**

**Shoot regeneration from shoot tip**

Direct shoot is a widely used technique for generating new plant directly from plant tissues without the need for intermediate stages such as callus formation or induction of somatic embryos. This is often achieved through tissue culture techniques where plant tissues such as shoot tips are placed in a suitable growth medium that promotes cell division and shoot development, and then the formation of a new plant. In the present study, the shoot tip explants of three Kenaf cultivars (i.e., V36, HF992 and KB6) were cultivated in MS media fortified with different types and concentration of PGRs. The PGRs types and concentrations were selected based on this preliminary studies. Explants in MS basal medium (without PGRs) served as control treatment. The shoot proliferation was evaluated two-week after the inoculation in shoot regeneration medium. All the Kenaf cultivars except HF992, regenerated significantly ($P \leq 0.05$) highest number of shoots per explant on MS medium fortified with 5 mg/L BAP + 0.1 mg/L NAA (Table 1). Whereas, in cultivar HF992, the maximum number of shoots per explant (10.40) was recorded in MS medium fortified with 1.0 mg/l TDZ + 0.1 mg/l NAA. The regenerated shoots

developed after two-week of culture (Figure 1 a-d). However, in medium supplemented 0.1 mg/l IBA + 3.0 mg/l KIN, lower number of shoots/explant was observe

**Table 1. Effect of PGRs on multiple shoot formation from shoot tips explant of Kenaf**

| PGRs (mg/L) | Number of shoots/shoot tip | | |
|---|---|---|---|
| | HF992 | V36 | KB6 |
| Control | 1.20 ± 0.04$^{d}$ | 1.10 ± 0.03$^{d}$ | 1.15 ± 0.02$^{d}$ |
| TDZ (1.0) + NAA (0.1) | 10.40 ± 1.10$^{a}$ | 9.55 ± 0.91$^{b}$ | 8.05 ± 0.04$^{b}$ |
| BAP (5.0) + NAA (0.1) | 8.60 ± 1.21$^{a}$ | 10.40 ± 1.01$^{a}$ | 9.20 ± 1.40$^{a}$ |
| 2iP (5.0) + NAA (0.1) | 5.10 ± 0.45$^{b}$ | 4.45 ± 0.89$^{c}$ | 5.00 ± 0.06$^{c}$ |
| KIN (3.0) + NAA (0.1) | 1.05 ± 0.03$^{c}$ | 1.15 ± 0.34$^{d}$ | 1.20 ± 0.03$^{d}$ |

**Letter (s) within a column are not statistically significant difference ($P \leq 0.05$, DMRT).**

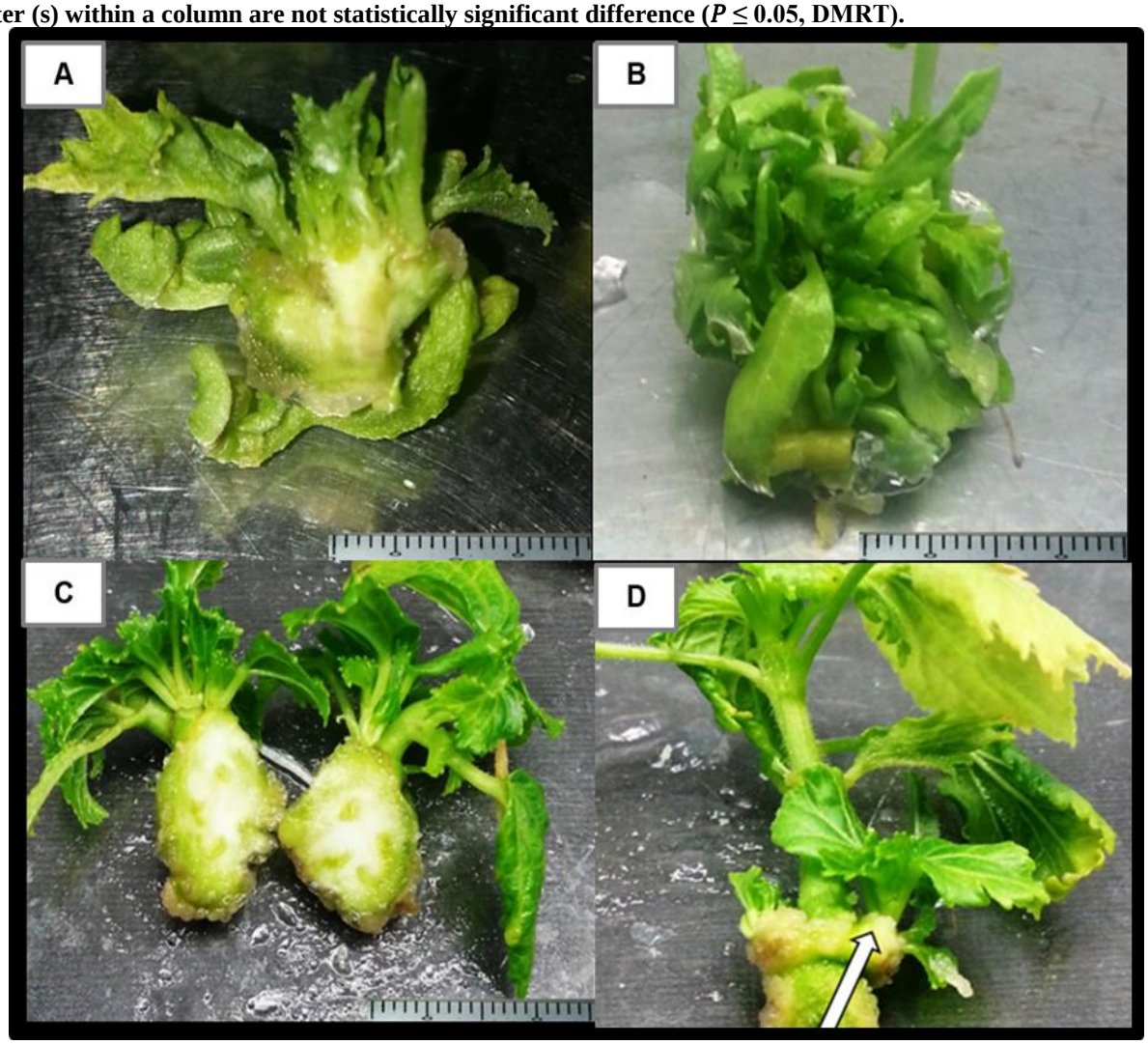


**Figure 1. Multiple shooting generated from the shoot tip of different Kenaf cultivars (a) Kenaf KB6 from MS media supplemented 0.1 mg/l NAA + 5.0 mg/l BAP, (b) Kenaf V36 from MS media fortified with 0.1 mg/l NAA + 5.0 mg/l 2iP. (c) Kenaf KB6 from MS media supplemented 0.1 mg/l NAA + 5.0 mg/l BAP, (d) Kenaf V36 from MS media supplemented 0.1 mg/l NAA + 5.0 mg/l 2Ip**

In direct shoot regeneration, the shoot proliferation response was evaluated through the number of shoots per explant two weeks after the explant was placed on the shoot regeneration medium. In this study, significantly ($P \leq 0.05$) highest number of shoots per explant on MS medium fortified with 5 mg/L BAP + 0.1 mg/L NAA. However, in medium supplemented 0.1 mg/l IBA + 3.0 mg/l KIN, lower number of shoots/explant was observed, and the shoot tips had started to produce root and induced shoot elongation after one week. This could be the fact that the explant might contain high auxin levels that

are favorable for rooting and shoot elongation. It has been shown to stimulates the development of axillary shoots and concurrently prevents root formation. Furthermore, compared to other cytokinins, BAP is more stable, and exert a prolonged effect (20). This result is consistent with (29) in Kenaf's study of three genotypes, demonstrated shoot regeneration and at the molecular level characterized regenerative plants. They demonstrated that all three genotypes had the ability to regenerate buds in vitro, and in the presence of BAP, the bud regeneration frequency was higher. The regenerated plants are genetically stable for commercial propagation of kenaf and their potential use in the production of fibers and other industrial products. They also indicated that explanted plants with shoot tips are a suitable source for laboratory regeneration of kenaf genotypes. Zain Al-Din et al. (34) They discovered that a high response to bud regeneration was obtained with media supplemented with IBA and BAP. Studying the in vitro micropropagation of kenaf from the tips of shoots.

**Shoot regeneration from cotyledon**

During this research, the most effective shoot regeneration was attained by culture cotyledon explants on MS media complemented with 0.1 mg/l NAA and 5.0 mg/l 2iP PGRs with the percentage of 55.34% (HF992) and 45.21.92% (V36) in 6 weeks of culture (Table 2, Figure 2). However, no shoot regeneration was observed for the cultivar KB6 in all PGRs treatments. Both of the Kenaf cultivars (HF992 and V36) responded well on shoot regeneration medium, whereas KB6 didn't respond to all treatments. Shoot regeneration from cotyledons is an extensively used technique in tissue culture for the production of genetically uniform and true-to-type plants. It could be used in plant breeding programs to produce plants with improved traits such as diseases resistance, improved yield, and better growth characteristics. This could be achieved through tissue culture techniques, where the cotyledons are excised and cultured in a suitable growth medium to promote cell division and shoot development. Cotyledons could be a valuable source of plant material for shoot regeneration as they are rich in stored nutrients and could provide the energy needed for shoot development. According to (20) reported that the appearance of new shoot from the meristematic area at the shoot tip is directly linked to the occurrence of cytokines and auxins. However, (4) observed decreases in shoot and root induction rates in plant growth regulators fortified culture medium. These could be due to the difference in genotypes of the Kenaf plant. Kenaf cultivars that are suitable for the Malaysian climate condition are reported to be V36, HC2, HC78, HF992, KB6, and V133. These genotypes have been also recommended as commercially suitable by the Malaysian Agriculture Research Center (MARDI) (4, 5, 10, 22).

**Table 2. Effect of plant growth regulator on direct shoot regeneration from cotyledon Kenaf cultivars**

| Plant growth regulators (mg/L) | Direct shoot (%) | | |
|---|---|---|---|
| | HF992 | V36 | KB6 |
| Control | NR | NR | NR |
| TDZ (1.0) + NAA (0.1) | NR | NR | NR |
| BAP (5.0) + NAA (0.1) | NR | NR | NR |
| 2iP (5.0) + NAA (0.1) | 55.34 ± 2.23 | 45.21±1.92 | NR |
| KIN (3.0) + NAA (0.1) | NR | NR | NR |

*NR- No response

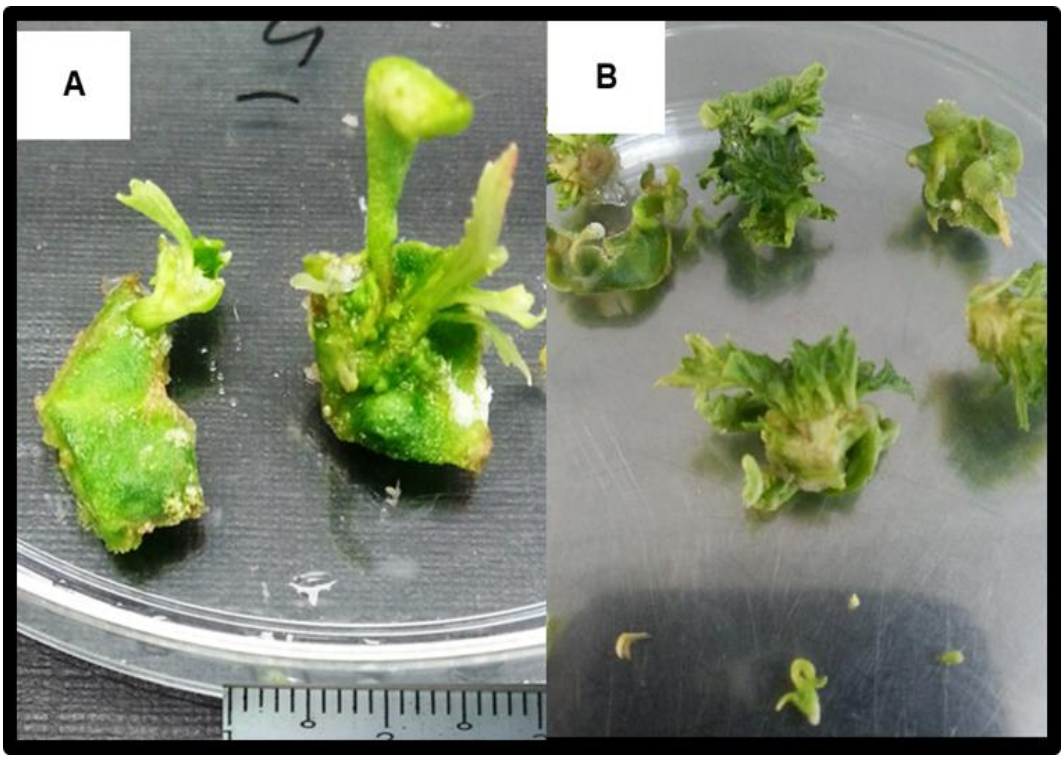


**Figure 2. Direct shoot regeneration observed from cotyledon culture. (a) Shoot of Kenaf HF992 cultivar induced using 5.0 mg/l 2iP + 0.1 mg/l NAA MS media after 6 weeks of culture. (b) Shoot of Kenaf V36 cultivar induced using 5.0 mg/l 2iP + 0.1 mg/l NAA on MS media after 6 weeks of culture.**

**Callus initiation from cotyledon**

Callus was induced from cotyledon explants of three Kenaf cultivars (HF992, V36 and KB6) on MS media, containing different combinations and concentrations of growth regulators. Callus induction was observed in all cultures that contained different PGRs within 5 and 6-week of incubation of cotyledon explants depending upon the concentration and combination of PGRs (Table 3, Figure 3). The percentage of callus formed was calculated after 6-week. In HF992 cultivar, the highest frequency of callus induction (83.75%) was recorded on 1 mg/L NAA + 0.5 mg/L TDZ, while mini-mum callus percent (20.00%) was observed in MS containing 0.1 mg/L IBA + 3.0 mg/L KIN (Table 3). The percentage of callus in the V36 Kenaf cultivar exhibited significant variability. Maximum callus induction percentage (88.75%) was recorded on 0.5 mg/l TDZ and 1.0 mg/l NAA and the lower callus induction percentage (11.25%) was observed in MS containing 0.1 mg/L IBA + 3.0 mg/L KIN. Similarly, in KB6 cultivar, the highest callus induction percentage was achieved on 0.5 mg/l TDZ and 1.0 mg/l NAA and the minimum callus percent was observed in 0.1 mg/L IBA + 3.0 mg/L KIN (Table 3).

**Table 3. Effect of PGRs on callus induction from cotyledon explant of Kenaf**

| Plant growth regulators (mg/L) | Callus (%) | | |
|---|---|---|---|
| | HF992 | V36 | KB6 |
| Control | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ |
| NAA (1.0) + TDZ (0.5) | 83.75 ± 2.34$^{a}$ | 88.75 ± 4.6$^{a}$ | 80.00 ± 5.10$^{a}$ |
| NAA (1.0) + BAP (5.0) | 60.00 ± 3.0$^{b}$ | 68.75 ± 6.10$^{b}$ | 58.75 ± 4.3$^{b}$ |
| NAA (1.0) + 2iP (5.0) | 57.50 ± 4.1$^{b}$ | 65.00 ± 2.9$^{b}$ | 55.00 ± 3.5$^{b}$ |
| IBA (0.1) + KIN (3.0) | 20.00 ± 2.9$^{c}$ | 11.25 ± 3.7$^{c}$ | 11.25 ± 1.23$^{c}$ |

**Letter (s) within a column are not statistically significant difference ($P \leq 0.05$, DMRT).**

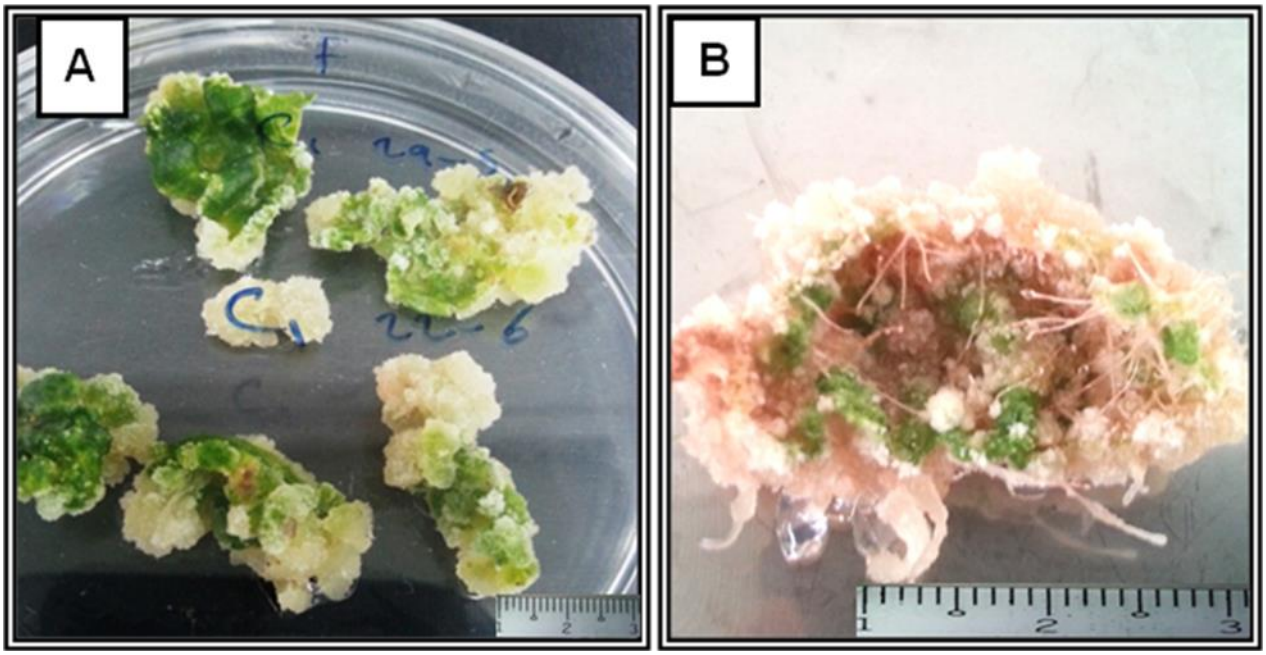


**Figure 3. Callus induction from cotyledon. (a) Compact callus induction observed after 6 weeks (b) Shoot induction after six weeks**

Callus induction from cotyledons refers to the process of inducing callus tissue from cotyledon explants. Cotyledons are embryonic leaves found in the seeds of most plants, and they could be used as starting material for callus induction in plant tissue culture. The process involves cutting a piece of the cotyledon and inoculating a nutrient-rich medium that supports cell division and growth. The cotyledon piece will start to form a callus, which is a mass of undifferentiated cells. By manipulating the culture conditions and hormones, the callus could be directed to differentiate into various plant tissues, such as roots and shoots. In this study, indirect shoot regeneration was established from callus, which was induced from cotyledon. Callus induction was observed in all cultures that contained different PGRs within 5 and 6-week of incubation of cotyledon explant depending upon the concentrations and combination of PGRs. In HF992 cultivar, the highest rate of callus induction (83.75%) recorded on 1 mg/L NAA + 0.5 mg/L TDZ, while minimum callus percent (20.00%) was observed in MS containing 0.1 mg/L IBA + 3.0 mg/L KIN. Among all cultivars, maximum callus induction percentage (88.75%) was recorded on 0.5 mg/l TDZ and 1.0 mg/l NAA in cultivar V36. (25), optimized callus induction conditions from cotyledons of Hibiscus cannabinus using IBA and BA as supplements to MS medium. Their result shows that a hormonal combination of 1.5 mg/L BA + 0.01 mg/L IBA yielded the highest callus percentage, while the lowest callus induction was observed in single hormone concentration (1.5 mg/L BA). In comparison, highest callus percentage was observed in MS medium supplemented with 1 mg/L NAA + 0.5 mg/L TDZ in all cultivars of Kenaf, suggesting that NAA and TDZ is the most suitable hormonal combination than others and are important for Kenaf callus induction. Auxins are extensively used for initiation of callus and their ideal concentrations have been observed to vary significantly from species to species of Kenaf. Several studies have reported to produce callus from Kenaf explants (7, 16, 21, 28, 30). (26) obtained higher callus from cotyledon. However, in this study, the callus induction percentage from cotyledon was lower than that obtained by (26). In this study, observation of callus induction in all cultures that contained different plant growth regulators (PGRs) within 5-6 weeks of incubating cotyledon explants suggests that the PGRs were effective in promoting the formation of callus from the explants.

**Shoot organogenesis from callus**

Calli of three Kenaf cultivars (HF992, V36 and KB6), taken from previous experiments were transferred to MS media enhanced with various levels and combinations of PGRs for indirect shoot organogenesis. In cultivar HF992, among the different types and

combinations of PGRs, the highest number of shoots/explant (8.40) was observed on MS fortified with 1.0 mg/l TDZ + 0.1 mg/l NAA, while the lowest number of shoots/explant (1.40) was observed in 0.1 mg/L IBA + 3.0 mg/L KIN. In V36 cultivar, the maximum number of shoots/explant (9.00) was recorded by 0.1 mg/L NAA + 5 mg/L 2iP (Table 4, Figure 4). In KB6 Kenaf cultivar, the highest number of shoots/explants was achieved with 0.1 mg/L NAA + 1.0 mg/L TDZ. In this study, didn't found shoot regeneration was observed when using MS medium without any added PGRs. in all Kenaf cultivars. Calli of three Kenaf cultivars (HF992, V36 and KB6), taken from previous experiments were transferred to MS medium fortified with various concentrations and combinations of PGRs for indirect shoot organogenesis. The highest number of shoots per explant (8.40) was observed on MS supplemented with 1.0 mg/l TDZ + 0.1 mg/l NAA, while the lowest number of shoots per explant (1.40) was observed in 0.1 mg/L IBA + 3.0 150 mg/L KIN. In this research, the high numbers of shoots per explant indicate that this culture condition was able to respond well to the treatment and very supportive of the regeneration process and that the explants resulted in an optimal bud proliferation response to achieve the highest number of shoots. The shoots of each plant are in a specific plant species. The success of organ formation in buds depends on many factors, such as the stage of callus development, the type of callus and the type of plant. To produce new plants, this process is commonly used in plant tissue culture, especially in plants that are difficult to propagate using traditional methods. These results demonstrated the importance of plant growth regulators on bud formation from explants and their effect in plant tissue culture. Different concentrations and combinations of PGRs can greatly affect the outcome of shoot formation, and it is crucial to optimize these conditions for maximum shoot production.

**Table 4. Effect of PGRs on indirect shoot regeneration from cotyledon of Kenaf**

| Plant growth regulators (mg/L) | Number of shoots/explant | | |
|---|---|---|---|
| | HF992 | V36 | KB6 |
| Control | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{d}$ |
| TDZ (1.0) + NAA (0.1) | 8.40 ± 0.45$^{a}$ | 6.35 ± 0.67$^{c}$ | 7.15 ± 0.19$^{a}$ |
| BAP (5.0) + NAA (0.1) | 6.50 ± 0.23$^{b}$ | 7.50 ± 0.87$^{b}$ | 6.15 ± 0.29$^{b}$ |
| 2iP (5.0) + NAA (0.1) | 8.15 ± 0.65$^{a}$ | 9.00 ± 1.23$^{a}$ | 6.65 ± 0.02$^{ab}$ |
| KIN (3.0) + NAA (0.1) | 1.40 ± 0.026$^{c}$ | 2.30 ± 0.03$^{d}$ | 1.00 ± 0.00$^{c}$ |

**Letter (s) within a column are not statistically significant difference ($P \leq 0.05$, DMRT).**

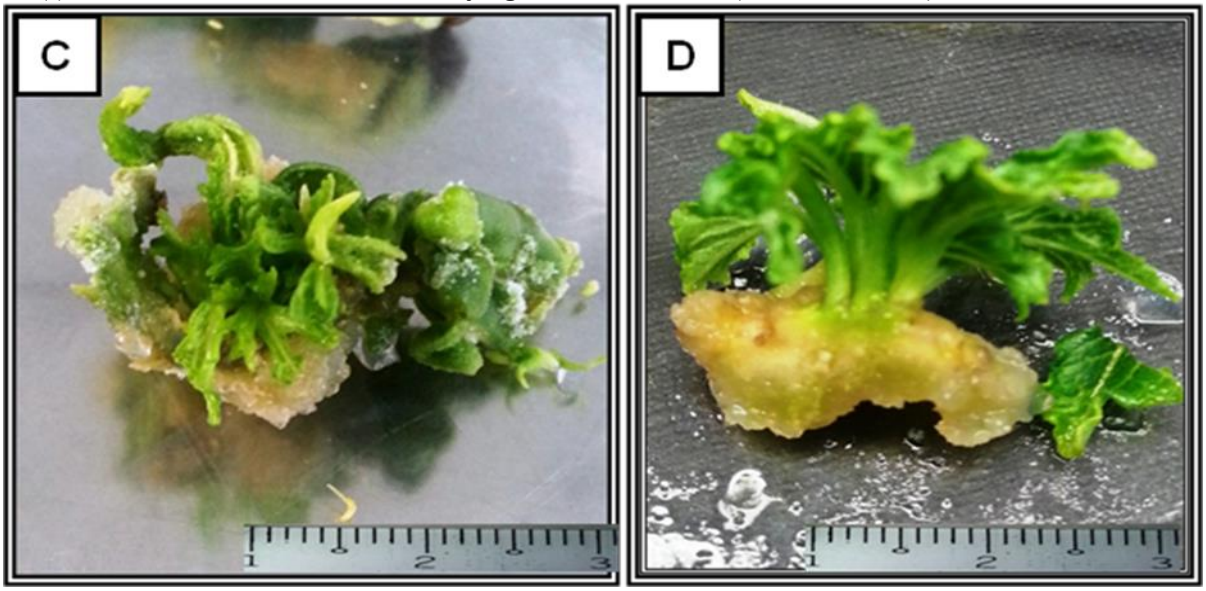


**Figure 4. Different stages of indirect shoot regeneration observed in the callus cultures of HF992 cultivar into MS media supplemented 0.1 mg/l NAA + 1.0 mg/l TDZ, (a) shoot regeneration after 12-week; (b) plantlets after 16-week**

**In vitro rooting and acclimatization**

Well-developed shoots (Figure 5 b & c) were cultured in rooting medium for root induction. High percentage of root induction with shoot development was observed after two weeks of sub-cultured in media supplemented MS media half strength, enhanced with 1 mg/l IBA with 1mg/l KIN (Figure 5b). The well-rooted plantlets were acclimated in vermiculite, perlite and peat moss at 1:1:1 (w/w) growing media under greenhouse conditions, with a 100% survival rate. In plant tissue culture, growth regulators play crucial roles in controlling the growth and development of in vitro plants. Therefore, supplementation of tissue culture growth media with some plant growth regulators (PGRs) is important (15, 20). PGRs are natural compounds synthesized in plants and effect growth and development (17). Auxins, cytokinins, ethylene, gibberellins, and abscisic acid considered the most popular of plant growth regulator. Auxins and cytokinins are typically used together in a balanced ratio to promote shoot and root development. In the present study, 1 mg/l IBA with 1mg/l KIN was effective to induce strong plantlets with well rooting. Optimization acclimatization is essential for plants that was propagated in a controlled (in vitro conditions). This process involves gradually exposing the rooted plantlets to environmental conditions that are similar to those growing in soil in a greenhouse or outdoor environment. This process is often used to propagate plants with desirable traits or to produce a large number of plants in a short amount of time. above all, the current study effectively induced root growth in mature shoots over sub-culturing in a rooting media complemented with KIN and IBA. The root initiation was notice to be high-rise after 14 days and the good-rooted with 100% being under greenhouse environments were acclimated in a growing medium. The mixture of peat moss, perlite and vermiculite give drain well and well-adjusted growing conditions of the plantlets, lead to well growing. Peat moss, perlite and vermiculite are frequently used growing medium in greenhouse. Perlite is perfect for plants that need moral oxygen and drainage around their roots, whereas peat moss is improved suited for those which need a moist and a little acidic environment. These mediums can encourage strong root development and growth, serving plants to acclimatize in the nursery of their new environments. Vermiculite offers a stable condition for root development and growth due to its excellent water-holding volume and drying. Ultimately, the optimal of media depends on the specific needs of the plant species being grown. (25) reported successful method for acclimatizing grapevine (*Vitis vinifera* L.) plantlets using a mixture of sand, perlite, and peatmoss in a 1:1:1 (v:v) ratio. Based on their results, this method gave the best rate of vegetative development, success rate, and 100% survival rate. Our results demonstrate the potential for successful rooting induction and plantlet acclimation through the use of this exact rooting media. The *in vitro* rooting and adaptation process is offered in Figure 5.

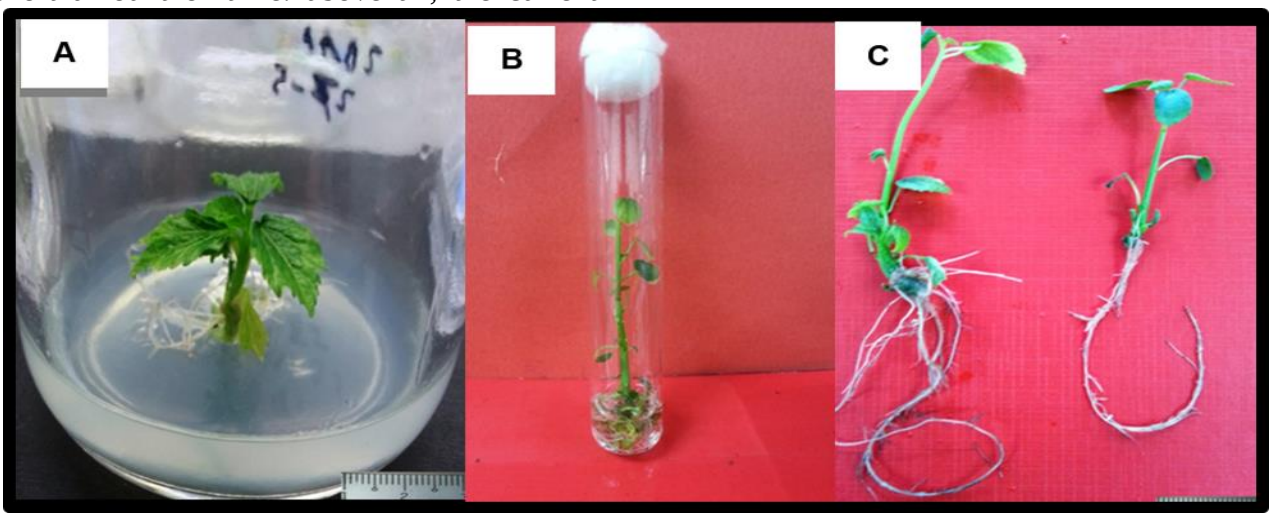


**Figure 5. In vitro rooting and acclimatization process (A) shoot cultured in rooting medium for root induction (B, C) well rooted plantlets.**

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