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RESEARCH ARTICLE

ESTABLISHMENT OF IN VITRO PROPAGATION OF *Hibiscus cannabinus* (KENAF)

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ABSTRACT

Hibiscus cannabinus or commonly known as kenaf is a versatile plant that serves as resources for numerous manufacturing and livestock industries. Originally planted in West Africa, kenaf is now distributed in many countries including Malaysia as its fibres were proved to be an ultimate alternative resource for major industries such as automotive, paper and bio-composite. In fact, in Malaysia, due to its adaptation to wide range of climatic conditions, kenaf has potentially be chosen as a new industrial crop replacing tobacco. There have been many interests on regenerating kenaf via micropropagation as the demand for this crop has been increasing tremendously since the past decades. Hence, this study is initiated with the objective to establish *in vitro* propagation system of *H. cannabinus*. The callus induction was achieved on Murashige and Skoog (MS) media supplemented with different concentrations of benzylaminopurine (BAP). It was observed that calli were successfully induced on all the BAP concentrations tested. The optimum concentration of BAP that induced the healthiest and biggest calli was 3.0 mg/l. Shoot and root induction from the calli were attempted using MS medium supplemented with different combinations and concentrations of IBA, BA and GA_3. From the seven treatments, three treatments successfully induced formation of shoot; treatment T3 (MS + 1.0 mg/l IBA + 2.5 mg/l BA), treatment T5 (MS + 0.1 mg/l IBA + 2.0 mg/l BA + 0.3 mg/l GA_3) and treatment T6 (MS + 1.0 mg/l IBA + 2.5 mg/l BA + 0.3 mg/l GA_3). The results obtained in this study can paved for more research on tissue culture of *H. cannabinus*.

KEYWORDS

callus, *Hibiscus cannabinus*, *in vitro*, root, shoot.

1. INTRODUCTION

Hibiscus cannabinus or locally known as kenaf has many hidden benefits and remarkable qualities to both economy and industries that very little had known. This annual and fast-grown plant belongs to the family Malvaceae (Ibrahim et al., 2014). Originated from Sub-Saharan Africa, kenaf is predominantly distributed in Asia and Latin America (Ayadi et al., 2017; Xia et al., 2017). This plant has been aggressively planted due to its various range of uses from basic animal feed to production of bio-composite products such as ropes, canvases and carpets. It was also stated that *H. cannabinus* has the potential to be an alternative resource for all the papers used worldwide (Samanthi et al., 2013). Thus, due to its undeniable potential contribution towards an improved country's economy and society's life, an effort to produce an efficient reproducible plant regeneration procedure for *H. cannabinus* plant has to be explored.

Kenaf consists of 65% core and 35% of bast fibres from which high-grade pulps are produced (Khalid et al., 2010). It was reported that fibre from *H. cannabinus* is an ideal alternative to manufacture biodegradable and much lower cost polylactic acid (PLA) that is widely used in biomedical field (Khalid et al., 2015). Kenaf was also nominated as one of the most promising non-wood alternative for paper and pulp production along with other 500 crop species by United States Department of Agriculture in 1960 (Alexopoulou et al., 2013). Moreover, the seed of *H. cannabinus* has multiple benefits especially its oil content. Edible oil extracted from kenaf seeds is utilized as top-grade cooking oil and margarine production (Ryu et al., 2013). By-product of kenaf seed extraction was also suggested to provide protein for feed and food (Mariod et al., 2010).

Due to the immense benefits and potentials from the fibre of *H. cannabinus*, a lot of countries have been paying a lot of attention in its cultivation including Malaysia. An additional land of 185 hectares worth RM 411,255 was allocated in Setiu, Terengganu to 109 kenaf farmers, in order to boost their income (David, 2017). It was also reported that kenaf industry in Malaysia is expected to bloom and generate a revenue of approximately RM 350 million and RM 3.5 billion domestically and globally by 2025 (Zakariah, 2018). With low capital investment and renewable source of plant fibres, it will be a huge advantage for a country to venture into *H. cannabinus* cultivation (Khalid et al., 2010). Propagation of *H. cannabinus* conventionally still cannot meet the current demand to fulfill its purpose of cultivation. Cultivation of *H. cannabinus* via tissue culture is very much needed to meet the current market demand. Developing *H. cannabinus* varieties with high quality and high yield is an effective guarantee to create a harmonious sufficient supply of natural fibre demands and elevate

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country's agricultural sector development (An et al., 2017). Hence, this study aims to establish an optimum *in vitro* propagation system of *H. cannabinus* for rapid and mass production of this beneficial plant.

2. Material and Methods

2.1 Mother plant preparation

The seedlings of *H. cannabinus* cultivar b27 were acquired from the University College of Yayasan Pahang (UCYP) and managed at the glasshouse and nursery of International Islamic University Malaysia, Kuantan Campus. The seedlings were maintained for three months and propagated by stem cuttings to get a multitude of mature leaves. The branches of the mother plants were cut into 10 to 15 cm long and dipped about an inch into the rooting hormone powder containing indole-3-butyric acid (IBA) before planting into the polybags consisted of 3:2:1 ratio of top soil, sand and peat moss. The mother plants were completely exposed to the sunlight in the open area, whereas the propagated cuttings were kept under the nursery shade. The plants were watered twice a day with a time-automated fertigation system. For maintenance of the samples, each of the polybags was fertilised and weeded weekly. The mother plants were fully grown and ready for the leaf samples collection after three months. The green, healthy and clean leaves were collected for further tissue culture process.

2.2 Surface sterilization of explants and culture initiation

Firstly, for surface sterilization, the collected leaf samples were washed with distilled waterto remove dirts and impurities on their surfaces. Then, the leaf samples were gently stirred in 0.2% carbendazim (fungicide) for 30 minutes. The leaf samples were made sure to be completely immersed in the fungicide to ensure the most efficient anti-fungal treatment. Once the treatment was done, the leaf samples were washed with distilled water and brought into the sterilised laminar airflow hood to be soaked and stirred constantly into 70% ethanol for 45 seconds. Then, the samples were soaked and stirred gently in 3% sodium hypochlorite (NaOCL) with a few drops of Tween 20 for 10 minutes. After that, the leaf explants were washed with sterile distilled water for 3 or more times until the foam was completely removed. The leaf explants were then blotted on filter papers.

2.3 Callus induction

To induce callus, the leaf explants were cut into approximately 1 cm x 1 cm using sterile scalpel. The kenaf leaf explants were treated on different concentrations of 6-benzylaminopurine (BAP): 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l in the MS medium. The treatment that gave the fastest callus induction time and that produced the largest size of callus were recorded. From there, the most optimum concentration of BAP to induce callus formation from kenaf leaf explants was determined.

2.4 Shoot and root initiation

To induce shoot and root from calli of kenaf, plant growth regulators (PGRs) which were IBA and benzyladenine (BA), together with gibberellic acid (GA_3) were used. Table 1 shows the different treatments tested for shoot and root initiation. The concentration and combination of IBA, BA and GA_3 that gave the fastest shoot and root induction time were recorded.

Table 1: Different concentrations and combinations of IBA, BA and GA_3 added to MS medium for shoot and root induction

Treatment	BA concentration (mg/l)	IBA concentration (mg/l)	GA_3 concentration (mg/l)
T0	0.00	0.0	0.0
T1	1.5	0.05	0.0
T2	2.0	0.10	0.0
T3	2.5	1.0	0.0
T4	1.5	0.05	0.3
T5	2.0	0.10	0.3
T6	2.5	1.0	0.3

2.5 Culture conditions

All cultures were incubated in a growth room with temperature of 25±2°C, the humidity of 60 – 79%, 24 hours light with the intensity of 2500 lux provided by white luminescence bulb. The samples were observed daily and any changes in its shape and size were recorded.

3. Results and Discussion

In this study, the formation of callus for *H. cannabinus* was induced by implementing a range of BAP concentrations in the MS media. Based on the results obtained, there was no indication of callus formation on the leaf explants cultured in the MS media without BAP, instead browning on the surface of the explants was noticed after 2 weeks of culture. In general, the leaf explants positively responded to the treatment of every BAP concentration supplemented to the MS media. In average, the calli formation was seen mostly on 14th day after the culture of explants on the media. Calli formation was initiated earliest in 3.0 mg/l of BAP treatment which was only 13 days after the leaf explants were cultured. Explants cultured in 2.0 mg/l of BAP media able to induce small callus formation on the 18th day which is later from other media.

The calli induced on the media of 1.0, 1.5, and 2.0 mg/l of BAP were observed to exhibit similar medium-sized, non-nodular growth pattern of the calli. The callus induction was observed to be highly responsive to the MS media supplemented with 2.5 mg/l (Figure 1A) and 3.0 mg/l of BAP (Figure 1B). Both concentrations formed large friable calli that are healthy and whitish-green in colour. However, 3.0 mg/l of BAP able to produce consistent size of compact-structured calli on all of the survived leaf explants compared to 2.5 mg/l of BAP. From the observation, 3.0 mg/l of BAP treatment has resulted in the extensively optimum callus induction and growth for *H. cannabinus* leaf explants. However, a higher concentration of BAP may regenerate shoot by where MS media supplemented with 8.0 mg/l BAP was the optimum media for shoot regeneration of *H. cannabinus* (Sultana et al., 2016). In terms of the morphology, calli formed from treatment with 0.5, 1.0 and 1.5 mg/l of BAP induced small and fresh green-coloured calli that accentuated at a corner of the leaf samples.

According to the study done on *Onobrychis sativa*, freshly developed calli were green-coloured while light green, white and then cream colours of calli were usually developed after 11th days until 22nd days of culturing (Mohajer et al., 2012). Meanwhile, compact-structured calli formed in 2.0 mg/l of BAP were in fresh green and light green colours. This showed that calli in 2.0 mg/l of BAP were more mature than in 0.5, 1.0 and 1.5 mg/l of BAP supplemented MS media. Furthermore, 2.5 and 3.0 mg/l of BAP produced the most mature calli compared to the other concentrations of BAP due to the large size of the compact calli with light green, white and cream colours. Since the healthy calli were induced earliest and consistently largest in the MS media supplemented with 3.0 mg/l of BAP, thus 3 mg/l is the most optimum concentration of BAP needed for kenaf leaf samples callus induction.

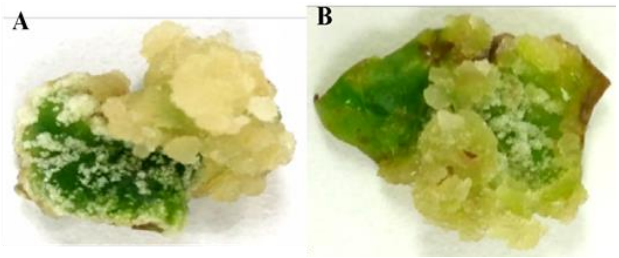

Figure 1: Callus induced on MS medium with A: 2.5 mg/l BAP and B: 3.0 mg/l BAP

Calli obtained were then excised from the explants and cultured on MS medium with different concentrations and combinations of BA, IBA and GA_3 in order to induce shoots and roots. Results obtained showed that there is no formation of shoot or root when calli were cultured on MS medium only (T0), MS + 0.05 mg/l IBA + 1.5 mg/l BA (T1), MS + 0.10 mg/l

IBA + 2.0 mg/l BA (T2) and MS + 0.05 mg/l IBA + 1.5 mg/l BA + 0.3 mg/l GA_3 (T4). After 9 weeks of culture calli turned brown and died for all these four treatments. For T3 (MS + 1.0 mg/l IBA + 2.5 mg/l BA) box, it was observed that a small shoot bud protruded from one of the calli after 2 weeks of culture (Figure 2A). Similar observations were shown when calli were cultured on treatments T5 (MS + 2.0 mg/l BA + 0.10 mg/l IBA + 0.3 mg/l GA_3) (Figure 2B) and T6 (MS + 2.5 mg/l BA + 1.00 mg/l IBA + 0.3 mg/l GA_3).

The usage of BA to induce shoot formation from callus of *H. cannabinus* was supported by a study conducted where indirect shoot organogenesis from the callus of *H. cannabinus* was accomplished using BA, producing the largest number of shoots with maximum frequency of generation (Arumingtyas et al., 2010). According to the number of shoots regenerated can be increased via the addition of BA and cytokinin-like PGR, thidiazuron (TDZ) into MS medium (Srivatanakul et al., 2000). This suggests that the higher the concentration of BA, the higher the rate of shoot regeneration. Similar results were obtained in this study where shoot bud formed from callus at the concentration of 2.5 mg/l BA. IBA was the most appropriate PGR for inducing root from leaf explants of *Labisia pumila*, which resulted in the highest rooting percentage and number of roots per explant formed (Ling et al., 2013). However in this study no root formation was observed when IBA was added to MS medium. Based on the study by [18] the effect of GA_3 is not significant in the number of shoots produced for tissue culture of tea Iran 100 (Gonbad et al., 2014). However, shoot elongation was observed when GA_3 was incorporated into the media resulting in prominent nodal segments which can be used for further multiplication during subculture.

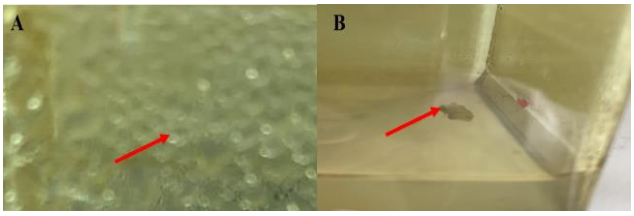

Figure 2: Shoot bud formation on A: T3 (MS + 1.0 mg/l IBA + 2.5 mg/l BA) and B: T5 (MS + 2.0 mg/l BA + 0.10 mg/l IBA + 0.3 mg/l GA_3) *Note: Red arrows show the shoot bud*

4. Conclusion

The micropropagation technique offers a good alternative to the rapid propagation of plants under *in vitro* conditions. In a short period, the desired plant parts such as callus, shoot and root can be obtained by maintaining the cultures under aseptic conditions and PGR-supplemented media. In many years, plant tissue culture has been an effective tool in regenerating plants that are genetically identical, free from diseases and uniform in growth without being restricted to any weather and climate conditions. Despite on the lack of researches on callus induction of *H. cannabinus*, a range of BAP concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) tested in this study were capable of producing healthy calli. Ultimately, 3.0 mg/l of BAP induced the most optimum growth of healthy calli. Significantly shoot was successfully induced from the calli using combination of PGR of BA, IBA and GA_3. To conclude, the objective of this study was successfully achieved.

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