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In vitro direct organogenesis of kenaf (*Hibiscus cannabinus* L.) using different types of explants

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Abstract

Kenaf (*Hisbiscus cannabinus* L.) is grown mainly for its fiber. In Malaysia, this species is relatively known as a new plant and has a potential to be cultivated and improved to produce high quality of fibers. Direct organogenesis of explants is one of the ways to provide high quality of seedling for the purpose. This study aimed to develop protocol for producing kenaf plantlets through direct organogenesis of explants. Different types of explants (shoot tips, nodes, leaves, and petioles) of kenaf were surface sterilized and cultured on the MS media supplemented with different combination of hormones. The results showed that shoot could be induced using different combination of hormones in both shoot tip and node cultures, but not in leaf and petiole cultures. On the other hand, root could form in all cultures easily even on MS basal media without hormones added.

Keywords: Kenaf, *Hibiscus cannabinus*, Java jute, Direct organogenesis, Explant

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Introduction

Kenaf (*Hibiscus cannabinus* L.,) or also known as java jute, is closely related to cotton (*Gossypium hirsutum* L.) and okra (*Abelmoschus esculentus* L.) that belong to the Malvaceae family and section Furcaria. Kenaf is an annual fiber crop cultivated for several uses, such as paper pulp, fabrics, textile, building materials, bio-composites, bedding material, oil absorbents (Monti and Alexopoulou, 2013; Mahmood et al., 2018). The production volume of paper and cardboard worldwide has increased significantly during the last years from 399.2 million metric tons in 2001 to 410.9 million metric tons in 2016 (Statista, 2018).

Nowadays, it is almost impossible to produce fibers to meet domestic demands from forests. Together with other non-wood fiber plants, kenaf is one of the potential annual species to meet the need (Favero et al., 2017). In Malaysia, however, this plant is considered new and cultivated especially in the eastern part of Peninsula to replace tobacco plantations that are no longer supported by the government. The Malaysian Government has allocated RM5.6 million to strengthen the kenaf industry by targeting the expansion area of 2,200 hectares in 2017 (Mohamad, 2017). Samanthi et al. (2013) stated that the establishment of *in vitro* plant regeneration through callus can be used for the development of gene transformation of kenaf. Gest (2004) showed that shoot organogenesis and regeneration of plant from callus by somatic

embryogenesis or by organogenesis revealed the highest rate of variation. On the contrary, direct shoot regeneration from explants without a callus phase would maintain the genotype fidelity that could be lost with the shoot arising from callus (Srivatanakul et al., 2000). Furthermore, the axillary bud culture can be implemented for conserving and restoring of plant biodiversity as this technique demonstrates the minimum variation among the propagated plants (Ngezahayo and Liu, 2014). Direct somatic embryogenesis and recurring embryogenesis express the best balance of high propagation percentage with fairly few off-types (Gamborg, 2002). Ayadi et al. (2011) isolated shoot tips and nodes from 15 days old seedlings cultivated on Murashige and Skoog (MS) medium and those explants were tested on different combination and concentration of auxin or cytokinin. Samanthi et al. (2013) tested the response of two varieties of kenaf (V36 and G4) on *in vitro* shoot regeneration using leaf explants treated by three different combinations of hormones. There was no significant difference between two varieties in both induction of callus and plant regeneration, but a negative correlation between the hormone concentration and callus induction was detected.

The objective of the study was to develop a protocol for kenaf *(Hibiscus cannabinus* L.) var KB6 (one of the varieties grown and developed in Malaysia) *in vitro* regeneration through direct organogenesis using different explants (shoot tips, nodes, leaves and petioles) as a first step towards the production of haploid and dihaploid kenaf for plant improvement.

Material and Methods

Plant material and treatments

The kenaf seeds of KB6 variety were collected from Mardi, Telung Kelantan and grown in AgroPark Universiti Malaysia Kelantan. Different types of explants (shoot tips, nodes, leaves, and petioles) were collected from mature mother plants. All the explants were initially washed with running tap water for 10 minutes, and soaked in detergent for 5 minutes. Then the explants were gently brushed with water color brush for another 15 minutes followed by repeated washing in running tap water until all traces of detergent were removed. Furthermore, the explants were rinsed 3-5 times with distilled water.

Surface sterilization of these explants was made by immersing them in 70% alcohol for 20 seconds followed by rinsing for 3 times in sterile distilled water. Finally the explants were treated with 0.1% HgCl2 (w/v) for 40 seconds. The surface sterilization was followed by 4-5 rinses in sterile distilled water. The cut ends of the explants were structurally trimmed with sharp edge sterile surgical blades and blotted on sterile filter paper discs to absorb the excess of water. For shoot induction, the explants were inoculated in MS medium supplemented with different combinations of 6-Benzylaminopurine (BAP) and Indole-3-acetic acid (IAA) as treatments. For root induction, the treatments were either 1-Naphthylacetic acid (NAA) or IAA with different concentrations.

The cultures were maintained in a growth room with 25ºC – 27ºC under daylight fluorescent illumination of 16 hours photoperiod. Sub culturing was performed once in every 2 weeks to fresh medium (same type). Explants observation was performed every day. When the shoots achieved 1-2 cm in length, they were rescued aseptically from cultured jars and separated from each other and again cultured individually with freshly prepared root induction medium. In this study, the multiplication rate was considered after five subcultures. Data were collected every week for percentage of shoot induction (%), average number of shoot per explants, average of shoot length (cm) and average number of leaves per explants.

Experimental design and statistical analysis

The experiment was designed as a Completely Randomized Design (CRD) with one factor. For shoot organogenesis, 20 levels of treatments were tested to select the best combination of BAP (0.0, 0.05, 0.10, 0.50, 1.00 mg/l) and IAA (0.0, 0.01, 0.03, 0.05 mg/l); and for root induction, the treatments were either NAA or IAA with the concentrations of 0.00, 0.10, 0.20, 0.30, 0.04 and 0.50 mg/l. All treatments were replicated three times.

The dependent variables observed included the percentage of shoot and root induction, number of shoots and roots, length of shoot and root, and number of leaves. Data were analyzed using One-way Analysis of Variance (ANOVA) with the help of using SPSS version 17.0. The means and the differences within the treatments were compared based on the Tukey Test at $p \leq 0.05$.

Results and Discussion

Shoot and root direct organogenesis from shoot tip cultures

New shoots appeared without any callus formation after 2-3 weeks depending on the treatment media and the multiple shoot started to appear after 5 weeks. Treatment TS6 (0.05 mg/l BAP+ 0.01 mg/l IAA) gave the highest values for all variables observed: 75% of shoot induction, 10 shoots per explant, 6.7 cm in length, and about 8.0 leaves per explant (Table 1, Figure 1d). The number shoot induction ranged from 3.7-10.0 shoot per explant. The number of shoots tended to decrease when the combination concentration of treatment BAP + IAA was more than 1.0 mg/l (BAP) + 0.01 mg/l (IAA) (Table 1). The kenaf shoot tips could regenerate and give rise to several plants within approximately 8 weeks. Zapata et al. (1999) failed to stimulate multiple shoots from the kenaf shoot apex by using 6-benzyladenine (BA). Meanwhile, Ayadi et al. (2011) could generate shoot within 5 weeks using the sources of explants from the sterile seedling germinated *in vitro*. Herath et al. (2004) found that the highest number of shoot induction of kenaf could be achieved by 8.8 µM of BAP and stated that the higher concentration of BAP could not increase the number of shoot but increased the callus growth.

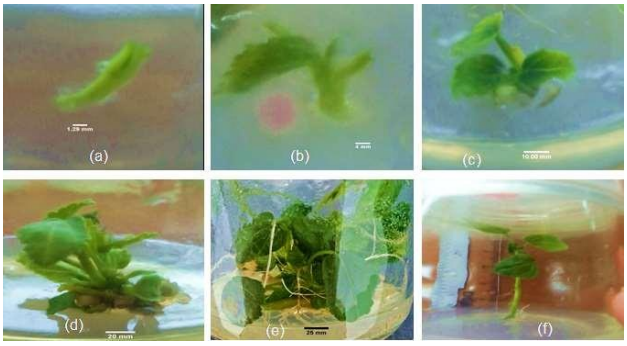

Figure 1: Development of shoot organogenesis on MS media supplemented with 0.05 mg/L BAP +0.01 mg/L IAA (TS6) in shoot tip culture:
(a) Single initiation of shoot tip explant on the 1st day of inoculation; (b) Single shoot initiation of shoot tip explants after 2 weeks. (c) Shoot proliferation after 3 weeks. (d) Multiple shoot production after 5 weeks. (e) Induction and elongation of multiple shoot after 6 weeks. (f) Elongation of single plantlet and rooting of *in vitro* raised shoot on MS basal medium on 8 weeks.

Roots started to emerge from the cut end of the shoots within 4 days after transferred to rooting medium (Table 2). The highest percentage of root induction (75%) and the highest number of roots were achieved by TR0, TR1, and TR2, while the longest root length (4 cm) was achieved by treatment TR9 (0.40 mg/L IAA). Khatun et al. (2003) stated that root formation was found quite easy on MS medium without hormone, and it was related to its high endogenous auxin content.

Direct organogenesis from node cultures

Nodes of kenaf with the size of 1.0 cm were put in MS medium containing combination of hormones (BAP+IAA) according to the treatments. The explants typically showed some swelling after 5-10 days of culture and new shoots started to appear without any callus formation in all treatment media (Figure 2a).

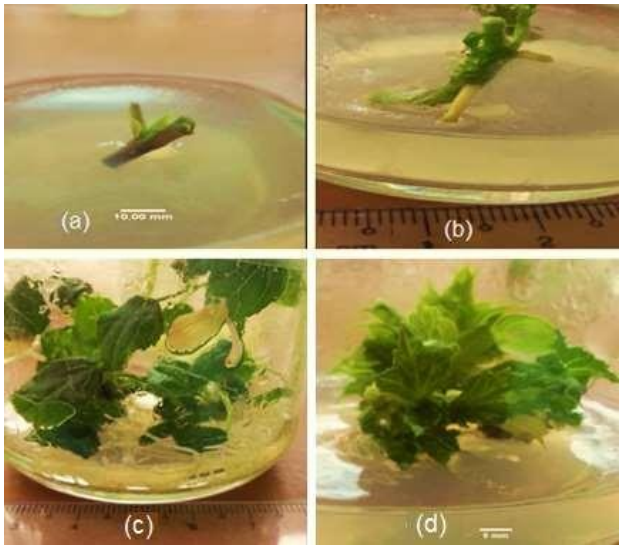

Figure 2: Direct organogenesis of node explants:
(a) Shoot initiation after 6 days in TS9. (b) Shoot proliferation and petiole fall down from node explant after 14 days in TS16. (c) Multiplication of shoot after 5 weeks in TS16. (d) Elongation of single plantlet and rooting of in vitro raised shoot on MS medium basal on 8 weeks in TS0.

The highest percentage of shoots induction (75%) were achieved by five different treatments: TS6, TS7, TS11, TS13, and TS16 (Data not shown). The highest number of shoot per explant (16.3) was achieved by TS16 (0.5 mg/l BAP + 0.05 mg/l

IAA), while the longest of shoot and highest number of leaves were achieved by TS9 (8.00) and TS5 (8.67) both were without IAA. This means that different treatment induced different variables on kenaf explant node.

The roots emerged from all explants as early as 8 days of inoculation and all explants responded positively to the root medium (Figure 2d), even in TR0 (no hormone), TR3 and TR4, all explants (100%) could produce roots. The number of roots per explant ranged from 1.7 to 4.0 and the root length ranged from 2.67 to 6.67 cm. From these data, it seemed that MS medium basal was enough to induce roots without any plant growth regulator added (Table 3).

In some plants, cotyledonary nodes were showed to be good candidate explants for shoot regeneration (Aslam et al., 2009) but there is no report for kenaf explants from nodes. Chen et al. (2010) was the first person who managed rapid shoot regeneration from the whole cotyledonary nodes of kenaf from *in vitro* seedling. That study was the first rapid shoot induction from nodes of mother plants. MS medium is frequently used for micropropagation of a large number of plants (Feyissa and Nagesh, 2005), and many types of plants were successfully induced to the shoot through node culture (Mungole et al., 2009).

Direct organogenesis from leaf cultures

One month old leaves along with the petioles were cultured in MS medium containing the combination of BAP and IAA according to the treatments. Leaf explants were inoculated about 8 weeks in the shoot induction media. The leaves were cut at the edge. After the first 2 weeks of culture the leaf explants distorted shapes and sparse. The light yellow line formed around the cut edge with the light green crystalline callus.

After fourth weeks of inoculation, some roots formed but there was no shoot appeared from the leaf explants (Figure 3b, Table 4). Trigiano et al. (1994) conducted an experiment of the leaf explants culture for direct organogenesis in chrysanthemum and also resulted some roots at their leaf cultures without any shoots. In this case, the rhizogenesis (the formation of roots) came directly from mesophyll cell without intervening callus phase. About 33.3 – 75.0% of explants formed very vigorous and healthy roots, about 6.33 – 12.33 cm of length (Figure 3c).

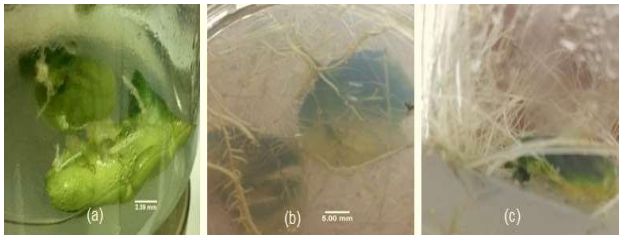

Figure 3: Direct organogenesis from leaf cultures: (a) root organogenesis in first week. (b) Root direct organogenesis on MS medium + 0.05 mg/1 BAP + 0.01 mg/1 IAA after 9 days. (c) Abundant of roots from leaves culture on MS medium 0.1 mg/l BAP + 0.05 mg/l IAA after 4th weeks.

Different species has different response in the cultures. In this study, no shoot was formed from leaf cultures. Studying of *Aerva lanata*, Varutharaju et al. (2014) showed that low concentrations (0.25–1.0mg L^{-1}) of TDZ had a significant effect on the percentage of shoot bud regeneration from leaf segments, but the higher concentration exhibited inhibitory effect. Direct somatic embryogenesis was also observed in cultures of the all four genotypes of *Nicotiana* (*N tabacum*, *N. benthamiyana*, *N. xanthi*, *N. tobacum* cv *petihavana*). The best medium for direct somatic embryogenesis was MS supplemented with 2.5 mg/l, 0.2 mg/l IAA and 2% sucrose (Pathi et al., 2013). Kanna and Jayabalan (2015) observed high frequency shoot organogenesis (79-81%) from leaf explant of Indian variety of *Solanum melongena* L. by culturing leaf eggplant on MS medium supplemented with (2iP) 2.0 mg L^{-1} and Naphthalene acetic acid (NAA) 1.0 mg L^{-1}. Dhaliwal et al. (2003) used transferred treatments between shoot induction medium and root induction medium, clearly showed that meristematic centers that initiated at these two sites in tobacco leaf disc could not be interconverted from a shoot to root meristem or vice versa. In contrast, field bindweed (*Convulvulus*) root explants initiated both roots or shoots internally from cells associated with the protoxylem (Ramzy et al., 2008); and in petunia (*Petunia inflate*), leaf explants produced both organs along the edge (Talona et al., 2012).

Table-1: The effects of hormone combinations on the percentage of shoot induction, number of shoots, shoot length and number of leaves in shoot tip cultures

Treatment	Hormone combination (mg/l)		Percentage of shoot induction (%)	Number of shoot per explant	Shoot length (cm)	Number of leaves per explant
	BAP	IAA				
TS_0	0.00	0.00	50.00^{b}	5.00^{d}	3.67^{b}	4.33^{c}
TS_1	0.00	0.01	41.67^{bc}	7.33^{bc}	3.83^{b}	5.67^{bc}
TS_3	0.00	0.03	25.00^{c}	7.67^{b}	5.17^{ab}	5.67^{bc}
TS_4	0.00	0.05	58.33^{ab}	8.67^{ab}	4.67^{ab}	6.33^{bc}
TS_5	0.05	0.00	66.67^{a}	10.00^{a}	5.33^{ab}	6.67^{bc}
TS_6	0.05	0.01	75.00^{a}	10.00^{a}	6.67^{a}	8.00^{a}
TS_7	0.05	0.03	41.67^{bc}	9.00^{ab}	5.33^{ab}	7.67^{a}
TS_8	0.05	0.05	58.33^{ab}	7.00^{bc}	3.67^{b}	7.00^{a}
TS_9	0.10	0.00	58.33^{ab}	5.67^{cd}	6.67^{a}	5.67^{bc}
TS_{10}	0.10	0.01	41.67^{bc}	6.00^{c}	6.67^{a}	4.67^{c}
TS_{11}	0.10	0.03	58.33^{ab}	6.00^{c}	4.67^{ab}	4.00^{c}
TS_{12}	0.10	0.05	41.67^{bc}	6.00^{c}	3.00^{b}	6.00^{bc}
TS_{13}	0.50	0.00	41.67^{bc}	6.33^{bc}	4.00^{b}	6.33^{bc}
TS_{14}	0.50	0.01	58.33^{ab}	7.00^{bc}	6.00^{a}	6.67^{bc}
TS_{15}	0.50	0.03	50.00^{b}	6.67^{bc}	6.67^{a}	6.67^{bc}
TS_{16}	0.50	0.05	50.00^{b}	6.67^{bc}	4.67^{ab}	6.33^{bc}
TS_{17}	1.00	0.00	58.33^{ab}	5.00^{d}	4.67^{ab}	5.67^{bc}
TS_{18}	1.00	0.01	50.00^{b}	5.67^{cd}	5.33^{ab}	5.33^{bc}
TS_{19}	1.00	0.03	41.67^{bc}	3.67^{d}	5.67^{ab}	5.33^{bc}
TS_{20}	1.00	0.05	41.67^{bc}	3.67^{d}	4.67^{ab}	5.33^{bc}

Note: Means values having the same letter in each column do not differ significantly at P≤0.05 (Tukey Test)

Table-2: Effects of NAA and IAA on the percentage of root induction, root length, and root number per explant in shoot tip cultures

Treatment	Concentration (mg/l)		Percentage of root induction (%)	Root length per explant (cm)	Number of root per explant
	NAA	IAA			
TR0	0.00	0.00	75.0^{a}	2.3^{ab}	6.0^{a}
TR1	0.10	0.00	75.0^{a}	3.3^{a}	6.0^{a}
TR2	0.20	0.00	75.0^{a}	3.3^{a}	6.0^{a}
TR3	0.30	0.00	41.7^{bc}	2.0^{ab}	4.0^{b}
TR4	0.40	0.00	66.7^{a}	3.0^{a}	3.3^{b}
TR5	0.50	0.00	50.0^{b}	3.0^{a}	3.3^{b}
TR6	0.00	0.10	58.3^{ab}	3.0^{a}	4.0^{b}
TR7	0.00	0.20	41.7^{bc}	2.3^{ab}	5.0^{ab}
TR8	0.00	0.30	50.0^{b}	2.0^{ab}	5.7^{a}
TR9	0.00	0.40	41.7^{bc}	4.0^{a}	5.7^{a}
TR10	0.00	0.50	66.7^{a}	2.7^{ab}	3.3^{c}

Note: Means values having the same letter in each column do not differ significantly at P≤0.05 (Tukey Test).

Table-3: Percentage of root induction, number of root, and root length in node cultures with different concentration of NAA (mg/l) of IAA (mg/l) in node cultures

Treatment	Concentration		Percentage of root induction (%)	Number of roots per explant	Root length per explant (cm)
	NAA	IAA			
TR0	0.00	0.00	100.00^{a}	4.00^{a}	5.33^{a}
TR1	0.10	0.00	83.33^{a}	3.67^{a}	6.67^{a}
TR2	0.20	0.00	75.00^{ab}	3.33^{a}	4.67^{ab}
TR3	0.30	0.00	100.00^{a}	3.67^{a}	4.00^{b}
TR4	0.40	0.00	100.00^{a}	4.00^{a}	4.33^{ab}
TR5	0.50	0.00	83.33^{a}	3.67^{a}	4.67^{ab}
TR6	0.00	0.10	83.33^{a}	3.33^{a}	4.67^{ab}
TR7	0.00	0.20	75.00^{ab}	3.00^{b}	4.00^{b}
TR8	0.00	0.30	50.00^{b}	3.00^{b}	4.00^{b}
TR9	0.00	0.40	50.00^{b}	1.67^{c}	4.33^{ab}
TR10	0.00	0.50	58.33^{ab}	2.33^{bc}	2.67^{a}

Note: Means values having the same letter in each column do not differ significantly at P≤0.05 (Tukey Test),

This differential response seen between tissues sources may be due to tissue competency, which in turn could be due to different levels of the inductive factors (morphogens) pre-existing in the tissue.

Tissue maturity of explants donor is also critical to the explant's competency in response to morphogenetic signals to form adventitious organs (Ana et al., 2012). A plant's life cycle from seedling through vegetative into reproductive phases has distinct characteristic (Hartmann et al., 2002). Juvenile cutting tend to be easy-to- root and form root primordial directly from existing phloem parenchyma cells. As plants are mature, these cells lose their competency to directly form adventitious root. Basto et al. (2012) studied the effects of donor plant age (adult or juvenile) and type of explant on the organogenic potential of *Cedrela montana* and showed that the explants from the juvenile plants produced the better results.

Direct organogenesis from petiole cultures

Petioles of the kenaf were put in the MS medium supplemented with the combination of BAP and IAA as treatments. After several observations and subculture, explants remained same and did not react to the medium positively. There were no shoot or callus appearing from the explants. The explants only showed some swollen tissues at both end cut edges that might be due to the absorption of nutrient into the explants. After 8 weeks, the explants turned into a light brown color at the both ends, showing the exposed tissue dead. Kumar and Kanwar (2006) reported that petiole explant of *Gerbera jamesonii* failed not only in organogenesis but also in producing callus as response to any treatments. Some researchers have successfully induced organogenesis from petiole cultures in various species of plants including African violet (Sunpui et al., 2002), *Withania somnifera* (Ghimire et al., 2010), *Begonia tuberhybrida* (Nada et al., 2011); *Jatropha curcas* (Ying et al., 2015); and *Fragaria vesca* L. (Ling and Wetten, 2017), and *Solanum nigrum* L. (Li-Juan, 2017).

There was no research that has been done using petioles as explants for direct organogenesis in kenaf. According to George et al. (2008), the induction of direct shoot regeneration depends on the nature of the plant organ from which the explants are derived, and highly depends on the plant genotype. Direct morphogenesis is rarely observed or is unknown in many plant genera yet.

Table-4: Frequency of shoot induction, number of roots per explant, root length per explant in leaf cultures using MS media with different concentration of BAP and IAA.

Treatment	Treatment (mg/l)		Percentage of shoot induction (%)	Number of root per explant	Root length per explant (cm)
	BAP	IAA			
TS0	0.00	0.00	0.00	41.67^{b}	6.67^{bc}
TS1	0.00	0.01	0.00	33.33^{c}	7.67^{bc}
TS3	0.00	0.03	0.00	58.33^{ab}	9.00^{b}
TS4	0.00	0.05	0.00	50.00^{ab}	10.67^{ab}
TS5	0.05	0.00	0.00	58.33^{ab}	11.33^{a}
TS6	0.05	0.01	0.00	75.00^{a}	12.33^{a}
TS7	0.05	0.03	0.00	41.67^{b}	10.67^{ab}
TS8	0.05	0.05	0.00	58.33^{ab}	12.00^{a}
TS9	0.10	0.00	0.00	75.00^{a}	7.67^{bc}
TS10	0.10	0.01	0.00	75.00^{a}	6.33^{c}
TS11	0.10	0.03	0.00	50.00^{ab}	6.67^{bc}
TS12	0.10	0.05	0.00	50.00^{ab}	10.00^{ab}
TS13	0.50	0.00	0.00	50.00^{ab}	9.67^{ab}
TS14	0.50	0.01	0.00	58.33^{ab}	9.00^{b}
TS15	0.50	0.03	0.00	50.00^{ab}	7.67^{bc}
TS16	0.50	0.05	0.00	58.33^{ab}	8.00^{bc}
TS17	1.00	0.00	0.00	66.67^{a}	9.67^{ab}
TS18	1.00	0.01	0.00	75.00^{a}	7.00^{bc}
TS19	1.00	0.03	0.00	58.33^{ab}	6.67^{c}
TS20	1.00	0.05	0.00	41.67^{b}	7.00^{bc}

Note: Means values having the same letter in each column do not differ significantly at P≤0.05 (Tukey Test)

Conclusion

A high frequency of multiple shoot induction and proliferation to complete propagation were achieved using nodes as the source of explants. Node explant was the best part that produced more shoot via direct organogenesis compared to the other parts (shoot, leaf and petiole). In term of rooting, MS basal was the best rooting induction medium compared to MS medium supplemented with PGRs.

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Contribution of Authors

Susanto D: Conceived idea, analyzed data and wrote up of article
Mat Husen ZES: conducted experiment and analyzed data and wrote of article

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