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Full Length Research Paper

Direct shoot organogenesis and plant regeneration from cotyledonary node of kenaf (*Hibiscus cannabinus* L.)

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Using the whole cotyledonary node as explants, a rapid and efficient regeneration protocol was established for kenaf (*Hibiscus cannabinus* L.) on Murashige and Skoog (MS) basal medium. The effects of the plant growth regulators: N6-benzyladenine (BA), indole-3-aceticacid (IAA) and the non-ironic surfactant pluronic F-68 (F-68) on shoot regeneration were evaluated by the orthogonal design L16 (4^5). The best combination for shoot regeneration was MS medium supplemented with 5.0 mg l^{-1} BA, 0.3 mg l^{-1} IAA and 0.2% (m/v) F-68. The maximum number of shoots per explant and the frequency of shoot regeneration were 12.97 and 100%, respectively, after cultivation for 21 days. The results indicated that BA had the greatest contribution on shoot inducing, followed by F-68, and IAA showed no significant effect on shoot formation. Furthermore, 0.5 ~ 2.0 mgl^{-1} of IAA and 1-naphthyleneacetic acid (NAA) were used respectively for root induction. More than 13 roots were obtained on MS medium supplemented with 2.0 mgl^{-1} of IAA after culturing for 14 days. IAA was more effective than NAA on root induction. According to this method, the plantlets could be obtained within 2 months and showed no morphological difference from those naturally grown plants. This protocol could be used for kenaf genetic improvement *by Agrobacterium* mediated transformation.

Key words: Kenaf, *Hibiscus cannabinus* L., cotyledonary node, shoot regeneration, rooting.

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) belongs to the family of Malvaceae and is used widely in textiles and paper pulp industries (Zapata et al., 1999; Chin and Yousif, 2009). Kenaf could grow well in lean soil and saline-alkali land but susceptive to pests and pathogenic microbes.

Recently, genetic engineering opened a new avenue for plant improvement. The development of insect and/or disease-resistant transgenic kenafs would greatly enhance conventional breeding efforts. Up till now, reports on genetic manipulation of kenaf have been limited mainly due to the fact that there have been no efficient *in vitro* regeneration protocols. Mclean et al. (1992) reported organogenesis of kenaf via callus culture but failed or was unreproducible. Banks et al. (1993) demonstrated foreign gene expression in kenaf callus, however, they were not able to regenerate plants.

Rapid regeneration of plants directly from explants was much more time-saving and presented an effective strategy to avoid somaclonal variation as it decreased callus formation in culture (Zapata et al., 1999; Lakshmanan et al., 2006). Previous studies on kenaf organogenesis were achieved mainly from shoot apex (Zapata et al., 1999; Srivatanakul et al., 2000; Samanthi et al., 2004) and cotyledon (Khatun et al., 2003), and these protocols were found to be genotype dependent. To

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Abbreviations: MS, Murashige and Skoog; **BA,** N6-benzyladenine; **IAA,** indole-3-aceticacid; **NAA,** 1-naphthyleneacetic acid; **F-68,** pluronic F-68.

Table 1. The shoot regeneration on 16 different media of the orthogonal table [L16 (45)] from whole cotyledonary node of kenaf.

Treatment	Concentration			Percentage of shoot induction	Average number of shoots/explant
	BA (mg l^{-1})	IAA (mg l^{-1})	F-68 (% m/v)		
1	1(Level 1)	0.1 (Level 1)	0 (Level 1)	59.13±2.93 h	1.97±0.20 j
2	1	0.3 (Level 2)	0.05(Level 2)	65.07±2.54 g	3.07±0.20 i
3	1	0.5 (Level 3)	0.1(Level 3)	66.80±1.50 fg	3.67±0.30 h
4	1	1(Level 4)	0.2 (Level 4)	68.38±1.18 f	3.87±0.06 h
5	3 (Level 2)	0.1	0.05	94.64±1.89 c	7.97±0.50 e
6	3	0.3	0	94.39±1.57 c	7.57±0.40 e
7	3	0.5	0.2	97.70±0.44 ab	10.37±0.70 c
8	3	1	0.1	96.47±0.28 b	9.40±0.20 d
9	5(Level 3)	0.1	0.1	100.00±0.00 a	12.00±0.15 b
10	5	0.3	0.2	98.33±0.15 ab	12.97±0.20 a
11	5	0.5	0	96.31±0.10 b	10.97±0.20 bc
12	5	1	0.05	97.02±1.28 ab	11.60±0.47 b
13	7(Level 4)	0.1	0.2	78.20±2.55 d	6.07±0.20 f
14	7	0.3	0.1	74.93±2.98 e	6.07±0.15 f
15	7	0.5	0.05	71.16±2.13 f	5.13±0.20 g
16	7	1	0	63.86±1.58 g	3.83±0.25 h

Levels 1 – 4 were selected for each factor; the data reported correspond to the mean values which were determined from triplicate experiments. The differences within each column were obtained using a students t-test. Means (±standard deviation) with different small letters are significantly different at $P < 0.05$ level.

the best of our knowledge, there is no protocol for direct shoot regeneration from cotyledonary node of kenaf so far. At the present study, we described an efficient and reproducible protocol for direct shoot organogenesis and plant regeneration from cotyledonary node of kenaf.

MATERIALS AND METHODS

Plant material and explants preparation

Kenaf cultivar P_3B was conserved in the Laboratory of Bastfibre Crops of Guangxi University. Seeds were rinsed in tap water, subsequently surface sterilized in 70% (v/v) ethanol for 30 s, then in 0.1% $HgCl_2$ for 2 × 7 min with agitation, washed thoroughly with sterile double distilled water. The sterile seeds were germinated on the surface of 50 ml aliquots of agar-solidified (0.8% w/v) hormone free Murashige-Skoog (MS, 1962) medium contained in 250 ml capacity cotton-plugged conical flask. Seedlings were grown at 25 - 28°C 16-h photoperiod (60 – 80 µmol m^{-2} s^{-1}) for 3 – 4 days.

Whole cotyledonary nodes were excised from aseptic seedlings. Primary leaves and the epicotyls were detached from the seedling using sterile surgical blades. Then the seedling radicle was excised, leaving approximately 0.7 – 1.0 cm long hypocotyls intact.

Shoots initiation

Cotyledonary nodes were induced on 16 different MS media containing 100 mgl^{-1} myoinositol and 3% sucrose. This medium was further supplemented with 1.0 – 7.0 mgl^{-1} 6-benzyladenine (BA) and 0.1 – 1.0 mg l^{-1} indole-3-acetic acid (IAA) as regulators. Furthermore, the non-ionic surfactant, pluronic F-68 (F-68) was added to the final concentration of 0 – 0.2% (w/v). The pH of the medium was adjusted to 5.8 before gelling with 0.8% (w/v) agar. Molten medium was dispensed into 250 ml capacity cotton-plugged conical flask with a 50 ml aliquot, then autoclaved at 121°C for 20 min before been used. Shoots were counted after 21 days of cultivation. The shoots were elongated on hormone free MS medium for 7 days. Each treatment was replicated three times and each replicate consisted of 12 explants.

Roots induction and acclimatization

Individual elongated shoots roughly 2 – 3 cm in length were detached and transferred to MS medium supplemented with either IAA or 1-naphthyleneacetic acid (NAA) at 0 – 2.0 mgl^{-1}. Each treatment was replicated three times and each replicate consisted of at least 50 explants. After 14 days cultivation, conical flasks were uncovered to harden the plantlets for 2 days. Plantlets were moved out from the conical flasks and planted in mixture of soil, vermiculite and sand (1:1:1) in greenhouse.

Experimental design

The orthogonal test L16 (4^5) was used for shoots regeneration. Three factors including BA, IAA and F-68 were studied, and each of the three factors was selected at four levels (Table 1). Sixteen treatments were arranged according to the design and the experiments were repeated three times. For each treatment, the numbers of shoots of eight randomly chosen explants per repetition were recorded.

Cytological analysis

Excised root tips (2 cm long) from regenerated plantlets were examined to determine their chromosome number. The root tips were taken from the rooted shoots and treated with 0.2%

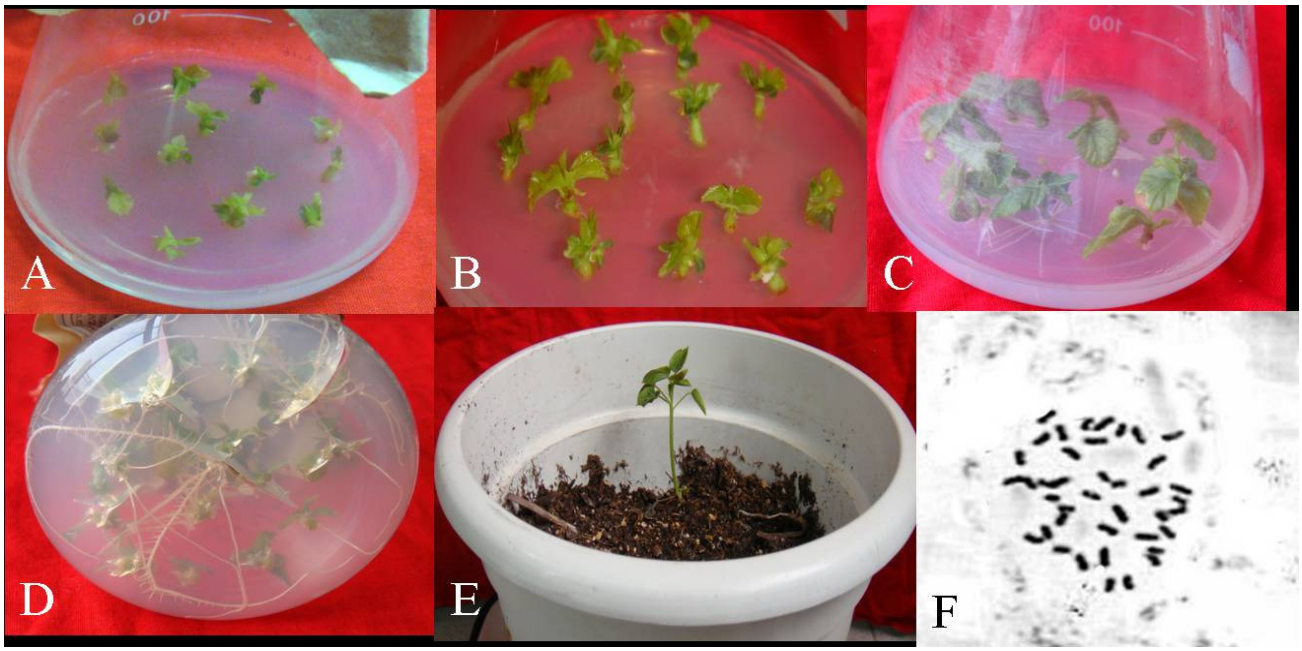

Figure 1. Plant regeneration of kenaf via direct adventitious shoot formation. (A) Adventitious shoot formation after 1 week of culture; (B) adventitious shoot formation after 2 weeks of culture; (C) excised shoots for rooting; (D) well rooted plantlet; (E) plants transferred to pots; (F) metaphase chromosomes of plantlet root.

colchicines for 3 ~ 4 h, then washed thoroughly with distilled water and immersed in Farmer's fixative (3 ethanol : 1 acetic acid). After 24 h, the tips were rinsed in distilled water and hydrolyzed for 10 ~ 15 min in 1 M HCl at 60°C. They were stained with phenol-fuchsin and squashed. The metaphase stages of the root tips and the different stages of morphological development were photographed with a camera.

Statistical analysis

The statistics test was carried out using the Statistical Package for the Social Sciences (SPSS) 13.0 for Windows (SPSS Inc.). The general linear model (GLM) was performed on the data obtained after treatment.

RESULTS

Shoot regeneration

New shoots first appeared at the axillary meristem regions of the nodes without any callus formation after 1 week (Figure 1A). The frequency of shoot induction (designated as "frequency" in this paper) and average number of shoots per explant (designated as "number" in this paper) were calculated. The results showed significant difference on both the frequency and the number among these 16 media (Table 1). The frequency ranged from 59.13 to 100%, and the treatment 9, 10 and 12 showed the most efficient (Table 1). The number ranged from 1.97 to 12.97, and the treatment 10 was the best among the 16 combinations (Table 1).

All three factors had significantly effects on the number (Table 2). BA is the most widely used cytokinin for multiple shoot formation. In this study, shoots formation were affected dramatically by BA concentration. By increasing BA (up to 5.0 mgl^{-1}), the number increased dramatically, highest number of shoots (11.88/explant) was induced at 5.0 mgl^{-1} (Table 2). The number decreased when the concentration of BA was more than 5.0 mgl^{-1} (Table 2). High concentration of BA (7.0 mgl^{-1}) led to lower number of shoots (5.27/explants) (Table 2) with calluses. Furthermore, too high concentrations of BA (7.0mg l^{-1}) brought about shoots fasciation and vitrification. The most suitable concentration of IAA was 0.5 mgl^{-1} (Table 2). Adding F-68, the number was increased significantly in each concentration when compared to the control ($P < 0.01$), and no harmful effect on morphology was observed. The most favorable F-68 concentration for shoots induction was 0.2% (m/v) (Table 2). Their R values were 8.74, 0.91 and 3.09, respectively (Table 2), indicating that the BA had the largest effect on the number then followed by F-68 and IAA. Therefore, the best combination of these three factors for the number would be $A_3B_3C_4$ (5.0 mgl^{-1} BA, 0.2%m/v F-68 and 0.5 mgl^{-1} IAA).

The frequency increased obviously when BA concentration in the range of 1.0 - 5.0 mgl^{-1}, and then decreased at 7.0 mgl^{-1} (Table 2). The F-68 also had notable effect on frequency when it was added to BA (Table 2). Adding IAA showed no significant effect on the frequency (Table 2). The R (extreme deviation) values were 33.07, 7.23 and 1.75, respectively (Table 2). BA showed the highest

Table 2. The percentage of shoot induction and the mean number of shoots per explant for each factor at four levels from whole cotyledonary node of kenaf.

Level	Percentage of shoot induction			Average number of shoots/explant		
	BA (A)	IAA (B)	F-68 (C)	BA (A)	IAA (B)	F-68 (C)
1	64.85 ±3.38 C	82.99±1.03	78.42±1.34 c	3.14±0.05 D	7.00±0.18 b	5.23±0.28 D
2	95.80±1.95 A	83.18±3.56	81.97±1.17 b	8.82±0.17 B	6.77±0.18 c	6.94±0.12 C
3	97.92±2.30 A	82.99±3.84	84.55±0.91 a	11.88±0.55 A	7.53±0.23 a	7.13±0.28 B
4	72.04±3.58 B	81.43±4.77	85.65±1.01 a	5.27±0.21 C	6.62±0.18 c	8.32±0.23 A
R	33.07	1.75	7.23	8.74	0.91	3.09

R, Extreme deviation; the data reported correspond to the mean values which were determined from triplicate experiments. The differences within each column were obtained using a students t-test. Means (±standard deviation) with different capital letters are significantly different at $P < 0.01$ level and small letters are significantly different at $P < 0.05$.

Table 3. Rooting response to different concentration of IAA and NAA.

Concentration (mgl^{-1})		Percentage of root induction	Average number of root/shoot
IAA	NAA		
0	0	53.60±1.31 g	5.2±0.51 e
0.5	0	75.90±0.82 e	8.0±0.73 cd
1.0	0	88.61±1.56 c	10.5±1.02 b
2.0	0	100.00 a	13.0±0.97 a
0	0.5	69.53±1.28 f	7.2±1.11 d
0	1.0	80.44±2.11d	8.9±0.53 c
0	2.0	93.80±1.15 b	11.1±0.66 b

The data reported correspond to the mean values which were determined from triplicate experiments. The differences within each column were obtained using a students t-test. Means (±standard deviation) with different small letters are significantly different at $P < 0.05$ level.

effect on the frequency, followed by F-68. So the best combination of these three factors for frequency would be $A_{2/3}B_{1-4}C_{3/4}$.

Rooting

Roots started to emerge from the cut end of the shoots within 7 days after transfer to rooting medium (Figures 1C - D). The greatest response with 100% root induction and an average of 13.0 roots per shoot after 14 days cultivation was achieved on MS medium supplemented with 2.0 mgl^{-1} IAA. Whereas, 2.0 mgl^{-1} NAA also yielded a significant number of roots, but was found to be not as efficient as IAA (Table 3). A high survival rate of plants, approaching 95%, was obtained by transferring rooted shoots to a soil, sand and vermiculite (1:1:1, v/v/v) mixture (Figure 1E).

The regenerated plantlets did not show any morphological difference from those naturally grown plants. Furthermore, they showed the same chromosome number (2n = 36) based on a 35 metaphase spreads (Figure 1F). Neither mixoploidy nor aneuploidy was observed in the regenerated plantlets of kenaf. The diploid chromosome numbers in all regenerated plantlets clearly indicated that there was no numerical variation in the complements of the regenerated plantlets.

DISCUSSION

There were some reports on the direct shoots induction from cotyledon (Khatun et al., 2003) and shoot apex (Srivatanakul et al., 2000) of kenaf in previous studies. Cotyledonary node was proved to be a good candidate explant for shoot regeneration in many species (Donaldson and Simmonds, 2000; Shyamkumar et al., 2003; Jha et al., 2004; Aslam et al., 2009; Mallikarjuna and Rajendrudu, 2009). But to the best of our knowledge, this is the first report of rapid shoots induction from the whole cotyledonary node of kenaf. The present regeneration system may have some advantages over cotyledon and shoot apex systems. Firstly, this system takes less time than other explants used. The whole process shortened to 2 months. Secondly, using cotyledonary node as explants may lead fewer calluses on the medium supplemented with high concentrations of BA (5.0 – 7.0 mgl^{-1}). While cotyledons are easily dedifferentiate to callus on the medium in high concentrations of BA (data not shown), the callus can compete with axillary meristems on nutrition absorption. The over-proliferating callus can also have negative impact on meristematic nutrition transmission and supply, thereby compromising shoot regeneration ability (Ma and Wu, 2008). Furthermore, highest number of shoots (11.88/explant) could be induced at 5.0 mgl^{-1} (Table 2). This was higher than ever reported (Zapata et al., 1999; Srivatanakul et al., 2000; Samanthi et al., 2004; Khatun et al., 2003). Finally, because there was barely any callus formation, the regenerated plantlets were very stable in heredity.

In this study, surfactant, pluronic F-68 was used for the enhancement of shoot regeneration from kenaf cotyledonary node explants. Number of shoot production/ explant was increased in each concentration when

compared to the control, and no harmful effect on morphology was observed. It will give full scope on shoot formation when the concentration of F-68 is at 0.2% (m/v). The results are similar with the previous studies on jute (Khatun et al., 1993), kenaf (Khatun et al., 2003) and *Arabidopsis thaliana* (Lowe et al., 1993). The mechanism that F-68 enhances shoots formation significantly may be that it can inhibit the accumulation of polyphenolic compounds in culture and increase the membrane permeability. So F-68 is a potential useful supplement for kenaf shoot regeneration combined with other regulators.

Elongated shoots (>2 cm long) were detached and rooted on a full strength MS supplemented with various concentrations of IAA or NAA (0.5 - 2.0mgl^{-1}) with 3% (w/v) sucrose, within 14 days cultivation. The highest percentage of rooting (100%) occurred on a medium containing a MS medium supplemented with 2 mgl^{-1} IAA (Table 3). The results indicated that IAA was more effective than NAA on rooting of kenaf.

In conclusion, a simple, efficient, high frequency and reproducible direct regeneration protocol from cotyledonary node of kenaf was developed. The highest number of shoot could be obtained on MS supplemented with 5.0 mgl^{-1} BA, 0.5 mgl^{-1} IAA and 0.2% (m/v) pluronic F-68, and the medium for rooting had MS added with 2.0 mgl^{-1} IAA. This protocol would facilitate genetic improvement of kenaf through genetic transformation.

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