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In vitro Studies on Shoot Proliferation of Kenaf (*Hibiscus cannabinus* L.)

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Abstract: An efficient regeneration protocol was developed for kenaf (*Hibiscus cannabinus L.*) genotypes HC-2, HC-95 and HC-3 using root tip, cotyledon and hypocotyl as explants. For regeneration, Murashige and Skoog (MS) medium was used as culture medium and supplemented with different concentrations and combinations of NAA (α-Napthaleneacetic acid) and BAP (6-Benzylaminopurine) as growth regulators. Different concentrations and combinations of Growth regulators for shoot initiation BAP (0.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/L) were used. The HC-2 genotype had the highest shoot initiation and HC-95 genotype had the lowest value 18.83% shoot initiation. The shoot initiation resulted the best on 8.0 mg/L BAP. On the other hand, hypocotyl explant showed the highest shoot initiation (49.60%) followed by the other two explants (Root tip and cotyledon) which had lower shoot initiation (0.0%, 33.75%). Shoot initiation was observed the best on MS medium supplemented with 8.0 mg/L BAP in case of hypocotyl of genotype HC-2 (87.67%) within 7-8 days. The frequency of variation was found to be genotype dependent. Certain changes were found in regeneration of all the three genotypes, suggesting the existence of a mutation-sensitive part of the kenaf genome and possibility of improvement through somaclonal variation.

Key words: Hormonal concentrations · Shoot initiation · Explants · Kenaf (*Hibiscus cannabinus* L.)

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is a short-day, fast growing annual, herbaceous plant cultivated for its stem fiber. It is native to tropical regions of Asia and Africa. It belongs to the Malvaceae family. Kenaf is closely related to cotton, roselle and hollyhocks. The kenaf plant has a wider range of adaptation to climate than any other fiber plant grown for commercial use. It can grow well and produce high fiber yield on an enormously wide range of soils. It has been widely planted due to its multiple uses ranging from basic animal feed to a variety of bio-composite products focuses on fibre production, such as making ropes, sacks, canvases and carpets [1]. Advanced biotechnology provides both an innovation method for kenaf breeding and germplasm multiplication and accelerates the process of kenaf breeding. The plant breeding methods can be combined with tissue culture methods in order to form genetic variability for desired traits [2-3]. The application of biotechnology in combination with the traditional breeding methods will cause the gigantic task of increasing food production. The development of insect and/or disease-resistant transgenic kenafs would greatly enhance conventional breeding efforts. Mclean *et al.* [4] reported organogenesis of kenaf *via* callus culture but failed or were irreproducible. Banks *et al.* [5] demonstrated foreign gene expression in kenaf callus, however, they were not able to regenerate plants. Plant regeneration from the shoot apex of kenaf (*Hibiscus cannabinus*) was reported by Zapata *et al.* [6] and Srivatanakul *et al*. [7], from nodal segments Reichert and Baldwin [8] and from cotyledons

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with plumes attached [9]. Plant regeneration *via* organogenesis form diverse explants, using different concentrations and combinations of auxins and cytokinins have been described in kenaf. A successful regeneration of plant by tissue culture critically depends upon potentiality of explants, application of suitable hormones and as well as favorable environmental condition. Application of both tissue culture and genetic transformation techniques could lead to the development of kenaf plants more resistant to different disease. Cell and tissue cultures have been applied successfully to the selection of variant cells exhibiting increased resistance to abiotic stress but no plants exhibiting the selected traits have been regenerated. Plant regeneration in kenaf is severely limited due to the formation of ill structures either resisting elongation of producing rosettes of distorted leaves, which generally do not produce normal shoot. Those limitations must be overcome to exploit the potentials of modern biotechnologies for kenaf improvement. Considering the above facts, the experiments were undertaken.

MATERIALS AND METHODS

The experiment was conducted at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, during the period from January, 2014 to June, 2014. Three different varieties of kenaf (*Hibiscus cannabinus*) were used in the present investigation to study different parameters. The varieties were HC-2, HC-3 and HC-95. The seeds used for seedling production in the experiment were collected from Bangladesh Jute Research Institute (BJRI), Dhaka. Experiment was conducted in growth room and arranged in Completely Randomized Design (CRD) with 3 replications. Healthy seedlings productions were found to be one of the major criteria for the plant regeneration from kenaf explants. MS [10] medium was used for seed germination and seedling development. Each culture bottles contained in 10-12 seeds and placed in growth room with 25±1°C under 2000 lux fluorescent illumination with 16 hours photo period. The following culture media used in the present investigation depending on specific purposes. (1)For seed germination: MS [10] medium free growth regulators. Separate stock solution for macronutrients, micronutrients, irons, vitamins, growth regulators etc. were prepared and used. The stock solution of micro-nutrients (except $FeSO_4$ and Na_2-EDTA) was made to 100 times the final strength of necessary constituents of the medium in 1000 ml of distilled water. The stock solution was filtered, labeled and stored in a refrigerator at 4°C.It was made up to 100 folds the final strength of the medium in 1000 ml of distilled water. Here two constitutes, $FeSO_4$ and Na-EDTA were dissolved in 750 ml of distilled water in a beaker by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml further addition of distilled water. Finally the stock solution was filtered and stored by wrapping with aluminum foils in a refrigerator at 4°C for further use. In addition to the nutrients, it is generally necessary to add growth regulators (hormones) such as auxin and cytokinin to the medium to support good growth of plants. Separate stock solution of growth regulators were prepared by dissolving the desired quantities of ingredients to the appropriate solvent and the final volume was made with distilled water. The following growth regulators were used in the present investigation. Auxin: α-Naptheleneacetic acid (NAA). Cytokinin: 6- benzyleaminopurine (BAP). Culture vessels, beakers, pipettes, measuring cylinder, metal instruments such as forceps, scalpel, needles, spatula and aluminium foils were sterilized in a pressure cooker or in a autoclave at a temperature of 121°C for 20 minutes at 15 psi. Required amount of were germinated aseptically on a seed germination medium (half strength MS medium) in vials. In each vials, 10-12 seeds were inoculated and then incubated in the inocubation room till the germination of seeds. The age of seedling used as explants were 7-10 days. The seedlings raised in axenic culture were used as the source of different kinds of explants. Cotyledons, hypocotyls and root tips were used as explants. Attempt has been taken for the induction of organogenesis using different explants in MS medium supplemented with different phytohormones. The aseptically grown 7-10 days old seedling was rescued placed on a sterile petridish. The cotyledons were then excised and cut into small pieces with the help of scalpel and forceps and inoculated on MS medium augmented with different concentrations of NAA and BAP for callus induction. In each vial, 4-5 cotyledon segments were placed. Hypocotyl from each germinated seedlings was cut into 2-3 mm in length using sterilized surgical blades. In each vials, 4-5 pieces of hypocotyls segments were inoculated on MS medium with various concentrations of NAA and BAp for callus induction. Root tips from each germinated seedlings were cut into 2-3 mm with the help of a sterile scalpel and forceps. In each vial, 4-5 pieces of root tip segments were placed on the culture medium supplemented with various concentrations of NAA and BAp. The culture vessels or culture vials containing

inoculated explants were placed in incubation room with controlled temperature of 25±2 °C and 16 h photoperiod (2000-3000 lux illumination) the vessels were checked daily to note the response and the development of unwanted organisms, if any.

The number of days required for shoot initiation was recorded.

The number of explants formed shoot was recorded and the percentage of shoot regeneration was calculated as;

$$\text{Percentage of shoot regeneration} = \frac{Number\ of\ \text{explants showing shoots}}{Number\ of\ \text{explant sinoculated}} \times 100$$

Length of the shoot was measured in centimeter (cm) from the base to the top of the plantlet by measuring scale. It was recorded at 10 days interval.

Number of shoots per explant was recorded at 10 days interval up to one month of culture and mean number of shoots per explant was calculated by using the following formula:

$$X = \frac{\sum xi}{n}$$

where,

X = Mean number of shoots per explant.
Xi = Number of shoot per explant.
N = Number of observation.
□ = Summation

Data recorded for different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The significance of difference between the pair of means was calculated at 5% level of significance by Duncan's Multiple Range Test (DMRT) [11].

RESULTS AND DISCUSSION

Effect of Genotypes of Kenaf on Shoot Initiation: Significant interaction effect of genotypes was observed on percentage of shoot regeneration. The highest percentage of shoot regeneration (34.14%) was obtained from the genotypes HC-2 of kenaf and took the minimum days (4.71 days). Shoot length (1.34 cm) also higher in HC-2 genotype of kenaf. On the other hand, the lowest percentage of shoot regeneration (18.83%) was obtained from the genotypes HC-95 of kenaf within 7.41 days. The lowest shoot length (0.89 cm) was observed in genotype HC-95 of kenaf (Table 1). Parvin *et al.* [12] observed that four varieties of *Corcho rusolitorius* had significant interaction effect of genotypes for days to shoot initiation, shoot regeneration (%), days required for shooting. The highest percentage of shoot regenerates were found from O-9897 (88.94%) followed by O-72 (85.83%) and OM-1 (72.61%).

Effect of Explants of Kenaf on Shoot Initiation: The result had shown highly significant variation for percentage of shoot regeneration. The hypocotyl explants showed the highest percentage (49.60%) of shoot regeneration and no shoot regeneration was achieved from root tip (Table 2).

Effect of BAP on Shoot Initiation: In the experiment to determine the effect of BAP on the days for shoot initiation, shoot regeneration (%), length of shoot (cm). The highest percentage of shoot regeneration (42.67%) was produced when explants were cultured on the medium containing 8.0 mg/L BAp (Table 3). On the other hand, the maximum length of shoot (1.584 cm) was in 8.0 mg/L BAP. This indicated that 8.0 mg/L BAp produced significantly higher percentage of shoot initiation per explants within a short duration (5.800 days). Similar findings were also obtained by Khatun [13] and Paul [14] who reported that, low concentration of IAA enhanced shoot regeneration in *C. capsularis.*

Combined Effect of Kenaf Genotypes and BAP on Shoot Initiation: In the experiment, the genotypic response to different concentrations of BAP was observed. HC-2 kenaf genotype produced higher percentage of shoot initiation (51.45 %) on media containing 8.0 mg/L BAp within a short duration (4.800 days) (Table 4). Shoot length (1.780 cm) were also higher in HC-2 genotype in the same concentration. 0.0 mg/L BAP showed no shoot initiation for different genotypes of kenaf. Shamsunnaher *et al.* [15] reported the highest frequency of shoot regeneration was observed in case of 3mg/l BAP concentration in two kenaf varieties (HC-2 and HC-95). No shoot regeneration was observed in case of 0 and 1 mg/l BAp concentration in both cases. Due to combined effect of BAp concentration and variety, days to shoot regeneration were more or less similar in both varieties.

Combined Effect of Kenaf Explants and BAP on Percentage of Shoot Initiation: Different BAP concentrations had significantly different response on days to shoot initiation, shoot regeneration (%) and length of shoot (cm). The higher shoot initiation (%) (73.34 %) was observed in hypocotyl explants cultured on MS medium supplemented with 8.0 mg/L BAp (2.450 cm) (Table 5). Purwati and Sudarmnadji [16] studied

Table 1: Effect of genotypes of kenaf on shoot initiation

Genotypes	Days to shoot initiation	Shoot regeneration (%)	Length of shoot (cm)
HC-2	4.71 c	34.14 a	1.34 a
HC-95	7.41 a	18.83 c	0.89 c
HC-3	4.92 b	30.38 b	1.29 b
$LSD_{0.05}$	0.06963	0.2927	0.01115

Table 2: Effect of explants of kenaf on shoot initiation

Explants	Days to shoot initiation	Shoot regeneration (%)	Length of shoot (cm)
Root tip	0.00 c	0.000 c	0.000 c
Cotyledon	9.25 a	33.75 b	1.711 b
Hypocotyl	7.79 b	49.60 a	1.800 a
$LSD_{0.05}$	0.06963	0.2927	0.01115

Table 3: Effect of BAp on shoot initiation

BAP concentration (mg/L)	Days to shoot initiation	Shoot regeneration (%)	Length of shoot (cm)
0.0	0.000 g	0.000 g	0.000 g
2.0	8.756 a	18.08 f	1.099 f
4.0	7.722 b	25.85 e	1.180 e
6.0	6.833 c	32.85 d	1.363 d
8.0	5.800 d	42.67 a	1.584 a
10.0	5.411 e	38.52 b	1.521 b
12.0	5.233 f	36.52 c	1.443 c
$LSD_{0.05}$	0.1064	0.4471	0.01703

Table 4: Combined effect of kenaf genotypes and BAp concentrations on shoot initiation

Genotypes	BAp concentration (mg/L)	Days to shoot initiation	Shoot regeneration (%)	Length of shoot (cm)
HC-2	0.0	0.000 n	0.000 o	0.000 o
	2.0	7.233 e	23.67 k	1.268 h
	4.0	6.233 h	33.00 f	1.361 f
	6.0	5.500 j	43.00 c	1.580 d
	8.0	4.800 l	51.45 a	1.780 a
	10.0	4.667 lm	44.89 b	1.707 b
	12.0	4.500 m	43.00 c	1.653 c
HC-95	0.0	0.000 n	0.000 o	0.000 o
	2.0	11.33 a	10.78 n	0.800 n
	4.0	10.43 b	15.00 m	0.867 m
	6.0	9.267 c	20.33 l	0.970 l
	8.0	7.533 d	31.11 g	1.260 h
	10.0	6.767 f	28.22 i	1.196 j
	12.0	6.567 g	26.33 j	1.100 k
HC-3	0.0	0.000 n	0.000 o	0.000 o
	2.0	7.700 d	19.78 l	1.230 i
	4.0	6.500 g	29.55 h	1.313 g
	6.0	5.733 i	35.22 e	1.540 e
	8.0	5.067 k	45.45 b	1.713 b
	10.0	4.800 l	42.44 c	1.660 c
	12.0	4.633 lm	40.22 d	1.577 d
$LSD_{0.05}$		0.3466	0.1842	0.7744

Table 5: Combined effect of kenaf explants and BAp concentrations on shoot initiation

Explants	BAp concentration (mg/L)	Days to shoot initiation	Shoot regeneration (%)	Length of shoot(cm)
Root tip	0.0	0.000 l	0.000 m	0.000 k
	2.0	0.000 l	0.000 m	0.000 k
	4.0	0.000 l	0.000 m	0.000 k
	6.0	0.000 l	0.000 m	0.000 k
	8.0	0.000 l	0.000 m	0.000 k
	10.0	0.000 l	0.000 m	0.000 k
	12.0	0.000 l	0.000 m	0.000 k
Cotyledon	0.0	0.000 l	0.000 m	0.000 k
	2.0	14.00 a	19.34 l	1.617 j
	4.0	12.50 b	26.00 k	1.710 i
	6.0	11.07 d	39.11 i	1.990 g
	8.0	9.567 f	54.67 e	2.303 c
	10.0	8.933 g	50.00 g	2.223 d
	12.0	8.700 h	47.11 h	2.133 e
Hypocotyl	0.0	0.000 l	0.000 m	0.000 k
	2.0	12.27 c	34.89 j	1.681 i
	4.0	10.67 e	51.56 f	1.831 h
	6.0	9.433 f	59.44 d	2.100 f
	8.0	7.833 i	73.34 a	2.450 a
	10.0	7.300 j	65.55 b	2.339 b
	12.0	7.000 k	62.44 c	2.197 d
$LSD_{0.05}$		0.3466	0.1842	0.7744

the response of five kenaf accessions for shoot regeneration and established regeneration protocol for kenaf from cotyledons with attached plumules. Purwati and Sudarmnadji [16] used MS based medium containing BAp (2 mg/L) and GA_3 (0.5 mg/L) for shoot initiation.

CONCLUSIONS

Considering the findings of present study, MS media supplemented with 8.0 mg/L BAp performed the best for regeneration of shoots of kenaf genotypes. The hypocotyl explant of kenaf genotypes was found to be the best for shoot regeneration.

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