International Journal of Microbiology and Application
2016; 3(1): 1-5
Published online February 10, 2016 (http://www.openscienceonline.com/journal/ijma)
ISSN: 2381-442X (Print); ISSN: 2381-4438 (Online)

Open Science

In Vitro Studies on Callus Induction of Kenaf (*Hibiscus cannabinus* L.)

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To cite this article

R. Sultana, A. K. M. L. Quader, Maksudul Haque, S. Mazumder, S. K. Paul. In Vitro Studies on Callus Induction of Kenaf (*Hibiscus cannabinus* L.). *International Journal of Microbiology and Application.* Vol. 3, No. 1, 2016, pp. 1-5.

Abstract

The present study was undertaken to investigate the comparative regeneration potential of seedling explants of kenaf (*Hibiscus cannabinus* L.) at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, during the period from January, 2014 to June, 2014. An efficient regeneration protocol was developed for kenaf 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 hormones for callus induction formation NAA (0.0, 1.0, 1.0, 1.5, 2.0, 2.0 and 2.0 mg/L) and BAP (0.0, 1.0, 2.5, 5.0, 7.5, 10.0 and 15.0 mg/L) were used while, 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 callus induction (59.14) HC-95 genotype had the lowest value 37.70% for callus induction. The callus induction (71.41%) occurred the best on 2.0 mg/L NAA + 7.5 mg/L BAP combination. On the other hand, hypocotyl explant showed the highest callus induction (60.52%) followed by the other two explants (Root tip and cotyledon) which had lower callus induction (41.27%, 53.49%). The explant from the hypocotyl of genotype HC-2 showed the best performance on callus induction (91.33%) in 2.0 mg/L NAA +7.5 mg/L BAP combination within 7-10 days compared with genotype HC-95 and HC-3. 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.

Keywords

Hormonal Concentrations, Callus Induction, Explants, Kenaf (*Hibiscus cannabinus* L.)

1. 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 (Li, 1980). 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 (Naz*et al.*, 2007; Ozyigit & Gozukirmizi, 2008). 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.* (1992) reported organogenesis of kenaf via callus culture but failed or were irreproducible. Banks *et al.* (1993) demonstrated foreign gene expression in kenafcallus, however, they were not able to regenerate plants. Plant regeneration from the shoot apex of kenaf (*Hibiscus cannabinus*) was reported by Zapata *et al.* (1999) and Srivatanakul *et al*. (2000), from nodal segments Reichert and Baldwin (1996) and from cotyledons with plumes attached (Purwati and Sudarmadji, 1998). 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 experiment was under taken.

2. Materials and Method

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 are; 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 seedling production was found to be one of the major criteria for the plant regeneration from kenaf explants. MS (Murashige and Skoog, 1962) 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 (Murashige and Skoog, 1962) medium without 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 sterilized seeds 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.

Number of explants formed callus was recorded and the percentage of callus induction was calculated as,

$$\text{Percentage callus induction} = \frac{Numberofexplantshowingcallus}{Numberofexplantsinoculated} \times 100$$

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) (Gomez and Gomez, 1984).

3. Results and Discussion

The present piece of research work was conducted with three kenaf genotypes and investigation on comparative regeneration potential of seedling explants of kenaf (*Hibiscus cannabinus*L.) were accomplished by using root tip, cotyledon and hypocotyl as explants, which were cultured on MS medium supplemented with different concentrations and combinations of NAA and BAP. The calli were transferred to shooting medium for shoot induction. The shoots initiated were transferred to rooting medium. Here the results of the study are presented and discussed.

3.1. Effect of Genotypes of Kenaf on Callus Induction

Three kenaf genotypes were subjected to study their comparative callus induction potential. The highest callus induction percentage (59.14%) was found in HC-2 kenaf genotype within 10.47 days while the callus induction percentages of HC-3 and HC-95 were 58.44% (11.58 days) and 37.70% (13.48 days) respectively (Table 1). The variation among the genotypes for callus initiation for shoot development from different explants was highly significant. The result indicated that kenaf genotypes have influenced the callus induction from root tip, cotyledon and hypocotyl explants. McLean *et al.* (1992) also reported genotypic variation in the callus induction while working with different kenaf genotypes.

3.2. Effect of Different Explants of Kenaf on Callus Induction

To induce callus, three explants (root tip, cotyledon and hypocotyl) of each genotype of kenaf were cultured in MS medium with different combinations of NAA and BAP. The result had shown highly significant variation for percentage of callus induction. The hypocotyl explants showed the highest callus induction (60.52%) and lowest induction (41.27%) was achieved from root tip (Table 2). Tanusree (2011) also found hypocotyl explants had highest callus induction (78.06%) of kenaf.

Table 1. *Effect of genotypes of kenaf on callus induction.*

Genotypes	Days to callus induction	Percentage of callus induction
HC-2	10.47 c	59.14 a
HC-95	13.48 a	37.70 c
HC-3	11.58 b	58.44 b
$LSD_{0.05}$	0.131	0.308

Table 2. *Effect of explants of kenaf on callus induction.*

Explants	Days to callus induction	Percentage of callus induction
Root tip	15.88 a	41.27 c
Cotyledon	10.68 b	53.49 b
Hypocotyl	8.962 c	60.52 a
$LSD_{0.05}$	0.131	0.308

3.3. Effect of Hormonal Concentrations (NAA and BAP) on Callus Induction

In this experiment, hormonal combination (NAA and BAP) exhibited significant influence on the percentage of callus induction. The highest percentage of callus induction (71.41%) was observed in 2.0 mg/L NAA combined with 7.5 mg/L BAP and lowest percentage was found with the combination of 0.0 mg/L NAA combined with 0.0 mg/L BAP (Table 3). McLean *et al.* (1992) studied with kenaf genotype and showed that the most abundant callus production was observed at 1.0 mg/L NAA and 1.0 mg/L BAP.

Figure 1. *Callus initiation from hypocotyl of the genotype HC-95 on MS + 2.0 mg/L NAA + 7.5 mg/L BAP.*

Figure 2. *Callus initiation from hypocotyl of the genotypeHC-3 on MS + 2.0 mg/L NAA + 7.5 mg/L BAP.*

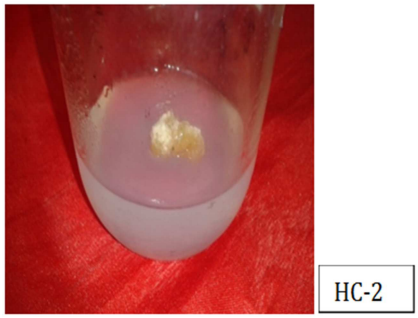

Figure 3. *Callus initiation from hypocotyl of the genotype HC-2 on MS + 2.0 mg/L NAA + 7.5 mg/L BAP.*

Table 3. *Effect of hormonal concentrations (NAA and BAP) on callus induction.*

Hormone concentration (mg/L) NAA + BAP	Days to callus induction	Percentage of callus induction
0.0 + 0.0	0.000 g	0.000 g
1.0 + 1.0	18.69 a	44.15 f
1.0 + 2.5	16.52 b	53.71 e
1.5 + 5.0	14.43 c	63.74 c
2.0 + 7.5	12.24 d	71.41 a
2.0 + 10.0	10.88 e	66.37 b
2.0 + 15.0	10.11 f	62.95 d
$LSD_{0.05}$	0.2001	0.4705

Table 4. *Combined effect of kenaf genotypes and hormonal concentrations (NAA and BAP) on callus induction.*

Genotypes	Hormonal concentration (mg/L) NAA + BAP	Days to callus induction	Percentage of callus induction
	0.0 + 0.0	0.000 l	0.000 o
	1.0 + 1.0	16.87 c	52.22 i
	1.0 + 2.5	14.87 e	64.22 f
HC-2	1.5 + 5.0	12.53 g	72.78 c
	2.0 + 7.5	10.47 i	79.22 a
	2.0 + 10.0	9.600 j	74.11 b
	2.0 + 15.0	8.933 k	71.44 d
	0.0 + 0.0	0.000 l	0.000 o
	1.0 + 1.0	20.37 a	27.78 n
	1.0 + 2.5	18.57 b	34.22 m
HC-95	1.5 + 5.0	16.53 c	47.00 l
	2.0 + 7.5	14.37 f	55.78 h
	2.0 + 10.0	12.83 g	51.11 j
	2.0 + 15.0	11.67 h	48.00 k
	0.0 + 0.0	0.000 l	0.000 o
	1.0 + 1.0	18.83 b	52.44 i
	1.0 + 2.5	16.13 d	62.67 g
HC-3	1.5 + 5.0	14.23 f	71.45 d
	2.0 + 7.5	11.90 h	79.22 a
	2.0 + 10.0	10.20 i	73.89 b
	2.0 + 15.0	9.733 j	69.41 e
$LSD_{0.05}$		0.3466	0.8149

3.4. Combined Effect of Kenaf Genotypes and Hormonal Concentrations (NAA and BAP) on Callus Induction

In this experiment, among the combination between genotypes and hormonal concentration (NAA and BAP), the highest percentage of callus induction (79.22%) was observed from HC-2 genotype of kenaf with 2.0 mg/L NAA + 7.5 mg/L BAP and HC-3 genotype of kenaf with 2.0 mg/L NAA + 7.5 mg/L BAP (79.22%) (Table 4).But there was significant difference between HC-2 (10.47) and HC-3 (11.90) genotypes for the days to callus induction on same hormonal concentrations. McLean *et al.* (1992) in his study with kenaf genotypes showed that the most abundant callus production was observed at 1.0 mg/L NAA and 1.0 mg/L BAP.

3.5. Combined Effect of Kenaf Explants and Hormonal Concentrations (NAA and BAP) on Callus Induction

The combination of explants and hormonal concentration (NAA and BAP) showed significantly variation on percentage of callus induction. Maximum percentage of callus induction (80.22%) of kenaf was found from the combination of hypocotyl with 2.0 mg/L NAA + 7.5 mg/L BAP (Table 5).

Table 5. *Combined effect of kenaf explants and hormonal concentrations (NAA and BAP) on callus induction.*

Explants	Hormonal concentration (mg/L) NAA + BAP	Days to callus induction	Percentage of callus induction
	0.0 + 0.0	0.000 n	0.000 o
	1.0 + 1.0	24.37 a	35.11 n
	1.0 + 2.5	22.00 b	43.34 m
Root tip	1.5 + 5.0	20.30 c	49.11 l
	2.0 + 7.5	17.13 d	58.00 i
	2.0 + 10.0	14.20 f	53.44 k
	2.0 + 15.0	13.13 g	49.89 l
	0.0 + 0.0	0.000 n	0.000 o
	1.0 + 1.0	17.23 d	42.56 m
	1.0 + 2.5	15.43 e	54.56 j
Cotyledon	1.5 + 5.0	12.83 g	64.34 g
	2.0 + 7.5	10.43 i	76.00 c
	2.0 + 10.0	9.767 j	70.11 e
	2.0 + 15.0	9.067 k	66.86 f
	0.0 + 0.0	0.000 n	0.000 o
	1.0 + 1.0	14.47 f	54.78 j
	1.0 + 2.5	12.13 h	63.22 h
Hypocotyl	1.5 + 5.0	10.17 i	77.78 b
	2.0 + 7.5	9.167 k	80.22 a
	2.0 + 10.0	8.667 l	75.56 c
	2.0 + 15.0	8.133 m	72.11 d
$LSD_{0.05}$		0.3466	0.8149

4. Conclusions

Considering the findings of present study, MS media supplemented with 2.0 mg/L NAA and 7.5 mg/L BAP was found to be the best for rapid callus initiation. The hypocotyl explant of kenaf genotypes was found to be the best for callus initiation.

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