ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology, Volume 2, Number 2: 86-93, 2003
ISSN 1682-2978
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An Efficient Protocol for Plant Regeneration from the Cotyledons of Kenaf (*Hibiscus cannabinus* L.)

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Abstract: Plant regeneration was obtained from the cotyledonary petioles of kenaf (*Hibiscus cannabinus* L. var. HC-2) on MS medium supplemented by different levels of IAA or NAA and BAP. The effects of non-ionic surfactant, Pluronic F-68 have been studied on shoot regeneration of kenaf cotyledons with attached petioles ***in vitro***. The effect was most marked in 0.1% and 0.5% level of Pluronic F-68 in addition to usual plant regeneration medium. These results were found significantly much higher than the control. The difference between control and Surfactant-treated cotyledons was marked. Plants regenerated from the Pluronic-treated cotyledons were found morphologically normal. Pluronic F-68 has been proved to be a growth stimulating agent for increasing the shoot regeneration efficiency of kenaf explants and this system can used for other crop plants.

Key words: 6-benzylaminopurine, indole-3-acetic acid, α-napthalene acetic acid, Murashige and Skoog (1962), Thidiazuron

Introduction

Kenaf (*H. cannabinus*) is a potential bast fibre crop mostly used as a jute substitute. Next to cotton, jute and kenaf are the important natural fibres. Kenaf has two main centres of distribution, the major one being in tropical Africa and the lesser one in tropical America. It yields higher biomass in poor lowland with less care. In Bangladesh, kenaf is grown in those marginal areas where jute does not grow well. Kenaf is important to the economics of many countries of the world e.g. Bangladesh, India, China, Thailand, Burma and Nepal. Kenaf is also adapted to the Southern United States. Its uses are manifold and of all the recent uses, paper pulp appears to have much potential.

Several diseases hamper production of kenaf cultivars. For example, dieback is one of the most important diseases of kenaf causing severe damage to the crop. Mealy bug, a major pest of kenaf in Bangladesh causes severe damage to the crop both in yield and quality of fibre. Spiral borer is another serious pest of this crop. Not much variability is available for this crop. Genetic transformation is one possible way to introduce resistant genes against insects and fungal diseases into kenaf cultivars. The pre-requisite for the genetic transformation in kenaf was to establish an efficient plant regeneration system from explants to matured fertile plants. Plant regeneration from the shoot apex of kenaf (*Hibiscus cannabinus*) was reported by Zapata ***et al.*** (1990) and Srivatanakul ***et al.*** (2000), from nodal segments Reichert ***et al.*** (1996) and from

cotyledons with plumes attached (Purwati ***et al.***, 1998). However, an efficient plant regeneration system from kenaf explant was still remaining the prerequisite to be used for genetic transformation. Plant regeneration from the cotyledonary petioles of jute (*C. **capsularis***) was reported earlier (Khatun ***et al.***, 1993a) and this system is being used for genetic transformation (Khatun, 1993). Plant regeneration from the cotyledonary petioles of kenaf was reported earlier (Khatun, 1999). However, the rate of shoot production was found to be not adequate to be used for developing a transformation protocol.

Previous work has shown that the non-ionic, block of co-polymer surfactants, Pluronic F-68, Triton and Tween are valuable growth-promoting supplement in transformed roots and jute cotyledons (Khatun ***et al.***, 1993b). Pluronic F-68 promoted the growth of callus and protoplast cultures of ***Solanum dulcamara*** (Kumar ***et al.***, 1991, 1992) and jute (Khatun ***et al.***, 1993a; Lowe ***et al.***, 1994). As in the previous experiments with kenaf cotyledons it was found that number of shoot regeneration from the explants were quite low compared to jute explants. This system was not adequate for further exploitation. Therefore, in the present experiments, the objective was to see whether Pluronic F-68 could increase the efficiency of plant regeneration from the cotyledons of kenaf. The aim of this experiment was to establish a plant regeneration protocol from the explants of kenaf and to be used for gene insertion in future.

Materials and Methods

The present experiment was conducted in the Biotechnological Laboratory of Bangladesh Jute Research Institute (BJRI) during the period of 2000-2002.

Development of seedlings from kenaf cultivars

Seeds of ***Hibiscus cannabinus*** (var. HC-2) were surface sterilized with 0.1% $HgCl_2$ (w/v) solution for 20 min and thoroughly washed 5-6 times with sterilized tap water. Sterilized seeds were then germinated on the surface of 80 ml aliquots of agar-solidified (0.8%, w/v; Sigma, Poole, U.K.) hormone-free MS-based medium (Murashige and Skoog, 1962) contained in 500 ml capacity of conical flasks. Each flask contained 12-15 seeds and was placed in a growth room with 27-28°C under 1.0 Wm^{-2} of daylight fluorescent illumination with 12 h photoperiod. Seedlings were used for cotyledon culture after the emergence of the shoots between the cotyledons. It was made sure that the emerging shoots were not remained attached with the petioles. Cotyledons with attached petioles were then cultured in 250 ml capacity of conical flasks on the surface of MS agar solidified medium with 0.5 mg l^{-1} of NAA or IAA and various concentrations of BAP (1.0, 3.0,5.0, 7.0 mg l^{-1}). In all cases, the cut ends of the petioles were inserted into the medium to a depth of 2-3 mm. The cultures were maintained in a growth room with 27-28°C under 1.0 Wm^{-2} of daylight fluorescent illumination of 12 h photoperiod. Number of shoots regenerated from the cotyledons of kenaf in the culture medium were recorded 49 days after of culture. Hundred cotyledons were used for each treatment.

In another set of experiment, 10 day old cotyledons (with attached petioles) of kenaf (var. HC-2) were cultured in 250 ml conical flasks on the surface of 50 ml of MS agar solidified medium containing growth regulators IAA (0.5 mg l^{-1}) and BAP (5.0 mg l^{-1}). This hormone combination was

found to be the best for shoot regeneration from kenaf cotyledons (with attached petioles) in the previous experiment. This experiment was used as control. Additionally, for another experiment, the growth medium was supplemented with 0.001, 0.01, 0.1 or 0.5% (v/v) commercial grade Pluronic F-68 (Sigma, UK) in addition to the established medium for plant regeneration. Excised cotyledons were also cultured on hormone free MS medium containing Pluronic F-68 at the same concentrations. The cultures were maintained at 27-28^{0}C with a day length of 12 h (1.0 Wm^{-2} of daylight fluorescent illumination). The regenerated cotyledons were transferred to hormone free MS medium after 21 days of culture, for shoot elongation. After a further 21 days, isolated shoots were transferred to hormone free agar solidified medium for rooting. Hundred cotyledons were used for each treatment. Number of cotyledons responded for shoot regeneration and number of shoots regenerated from the cotyledonary petioles of kenaf cotyledons of both control and surfactant-treated medium was recorded. Each experiment was repeated three times with three replications.

Transfer system for regenerated kenaf plantlets into soil

The ***in vitro*** regenerated and rooted kenaf plantlets were then transferred to pots containing mixed soil. As peat soil was not available, 70% dairy soil (Savar Dairy, 70%) was mixed with 30% commercial sand. The mixture was sterilized before use. The idea of mixture was to make soil pours for good aeration. Plastic pots (6 cm dia and 7 cm height) with a small whole at the bottom were used for transfer purpose. Pots were placed on a 9 cm Petri dishes each containing 20 ml of water. The plantlets were washed with sterilized tap water to remove agar and then transferred into the pots. The plantlets were then covered with a cellophane paper bag and placed in a well-ventilated place. After one week, two wholes were made in each bag to allow some fresh air. In the second week, more wholes were made in the bags and during the third week the bags were removed. During the fourth week the plants were finally transferred to 30 cm pots (two plants in each pot) containing dairy soil mixed with chemical fertilizers. Survival rate of the plants was recorded.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) method of Minitab Statistical Package, Version- 13. Test of differences between means was made at the 5% probability level when a significant F value was obtained for varietal effect. Calculating a Least Significant Difference (LSD) as follows compared different varietal means:

$$LSD = v(2EMS)/n \times t(0.05)\ df$$, where

EMS= error mean square

n = number of replication (3)

t = 0.05

df = values from the t distribution tables at 5% probability level and appropriate error degrees of freedom

Results

Plant regeneration from kenaf explants

Seeds of kenaf (***Hibiscus cannabinus***; var. HC-2) were readily germinated on the surface of 80 ml of agar solidified MS medium in 500 ml flasks. The seedlings were found to be normal and healthy. Vigorous root production was observed from the cotyledonary petioles of kenaf (var. HC-2) which was found to be a common phenomenon with most of the hormone concentrations used for these species. Cotyledons cultured with low concentration of BAP (1.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) or NAA (0.5 mg l^{-1}) directly produced roots from the cut ends of the petioles. Callus production was observed from the cut ends of the petioles on rest of the hormone combinations used. Shoot regeneration was obtained from the petioles of the cotyledons of kenaf via callus production on MS medium containing IAA (0.5 mg l^{-1}) or NAA (0.5 mg l^{-1}) and all combinations of BAP except BAP (1.0 mg l^{-1}). The highest percentage (59.50±6.25) of cotyledons produced shoots was found in a medium supplemented by IAA (0.5 mg l^{-1}) and BAP (5.0 mg l^{-1}). This was found to be significantly higher than the other combinations. However, in the case of NAA (0.5 mg l^{-1}) + BAP (5.0 mg l^{-1}), the difference was not significant. The over all differences among the treatments were found to be highly significant ($P<0.001$). On the other hand, the average highest number of shoots produced cotyledon^{-1} was recorded 4.50±1.0 with MS medium supplemented by IAA (0.5 mg l^{-1}) and BAP (5.0 mg l^{-1}). The over all differences among the treatments were found significant ($P<0.05$).

The plantlets of kenaf produced roots in rooting medium and were transferred to pots containing soil. These plants produced flower and fruits after maturity. Morphological variation was not found.

Effect of Pluronic F 68 on shoot regeneration

Multiple shoots started to emerge from the cut ends of the petioles of ***H. cannabinus*** (var. HC-2) after two weeks of culture via callus production from the medium containing Pluronic F-68 and without Pluronic-68. However, no morphogenic response or callus induction was observed from the excised cotyledons cultured on hormone free MS medium containing Pluronic F-68. Differences in number of shoot production cotyledon^{-1} and percentage of explants producing shoots have been observed in the presence of various concentrations of surfactants are shown in Table 2. The percentage of cotyledons produced shoots was recorded 59.00±3.37 on surfactant free medium (control). In 0.5% Pluronic F-68, the highest number of cotyledons regenerated shoots was 81.25±4.27. All cotyledons produced shoots in the presence different levels of surfactants were significantly higher than the control. As concentration of surfactant was reduced, the percentage of cotyledons produced shoot was also reduced. All the concentrations were found significantly higher than the control. Among the treatments, 0.5% Pluronic F-68 was found significantly higher 0.01% and 0.001%. However, the difference was not significant with 0.1% Pluronic F-68.

For average number of shoot production from the cotyledons, the over all differences were found significantly higher ($P< 0.001$) than the control. Pluronic F-68 at 0.5% level produced the highest number of shoot (11.00±1.83) cotyledon^{-1} and at 0.001% level the cotyledons produced

Table 1: Percentage of cotyledons (with attached petioles) produced shoots and average number of shoot production cotyledon^{-1} of kenaf varieties using MS basal medium supplemented by NAA or IAA and BAP

Verities	Medium used (mg l^{-1})	Number of cotyledons producing shoots	Percentage of cotyledons producing shoots
	NAA 0.5+BAP 1.0	No regeneration	No regeneration
	IAA 0.5+BAP 1.0	No regeneration	No regeneration
	NAA 0.5+BAP 3.0	No regeneration	No regeneration
HC-2	IAA 0.5+BAP 3.0	25.00±4.40	2.00±0.82
	NAA 0.5+BAP 5.0	47.25±3.59	3.00±0.82
	IAA 0.5+BAP 5.0	59.50±6.25	4.50±1.0
	NAA 0.5+BAP 7.0	43.25±4.99	3.00±0.82
	IAA 0.5+BAP 7.0	57.50±5.07	3.25±0.96

LSD for% shoot production = 7.44***

LSD for number of shoot production = 1.33*

The over all differences among the treatments were found significant (P<0.05)

Table 2: Percentage of cotyledons producing shoots and number of shoots produced per cotyledon after 50 day of growth on MS-based medium containing IAA with BAP and supplemented with surfactants Pluronic F-68

Surfactant used (%) Pluronic F-68	Number of cotyledons cultured	Percentage of cotyledons producing shoots	Number of cotyledons producing shoots
Control	50	59.00±3.37	4.25±0.96
0.001	50	68.00±4.40	4.50±1.0
0.01	50	71.25±7.46	7.25±1.71
0.1	50	76.50±5.92	10.75±1.50
0.5	50	81.25±4.27	11.00±1.83

LSD for% shoot production = 7.96***

LSD for no. of shoot production = 2.17***

the lowest number of shoots (4.50±1.0). Average number of shoot production cotyledon^{-1} was increased with the increase of the surfactant level. The differences among the treatments were found highly significant (P< 0.001).

Root production was found quite easy on hormone free MS medium. The 95% kenaf plantlets survived after transfer to soil and grew into maturity. The matured plants produced flower and fruits without showing any morphological abnormality.

Discussion

Vigorous root production was observed from the cotyledonary petioles of kenaf (***Hibiscus cannabinus*** var. HC-2) at various concentrations of auxins and cytokinins used. In this study, root production was found to be a very common phenomenon with most of the hormone concentrations used for this species. The regenerated shoots of kenaf produced roots readily after transfer to rooting media. This result also shows similarity with the findings of Srivatanakul ***et al.*** (2000). They reported that rooting of regenerated kenaf shoots was not found to be difficult to achieve, even, when high levels of TDZ was used. Though Huetteman and Preece, 1993 reported TDZ as a potent cytokinin. They have reported that TDZ inhibited root production in woody plant tissue culture but not inhibited for kenaf culture. According to Srivatanakul ***et al.***

(2000) kenaf might contain high auxin levels that are favorable for rooting and overcome a "carry over" effect of TDZ from the multiple soot induction medium.

Shoot regeneration was observed from the cotyledons of kenaf in the presence of high level of BAP (3.0 mg l^{-1} to 5.0 mg l^{-1}). This result is comparable with shoot regeneration from cotyledons of the results of jute (Khatun ***et al.***, 1993a) where plant regeneration was obtained with BAP (1.0 mg l^{-1}). Like jute, the best response for shoot regeneration from each cotyledon and percentage of cotyledons producing shoots was observed from kenaf when the media contained the hormone combination of IAA and BAP in comparison to medium contained NAA and BAP.

The present finding also indicate that the cotyledons of kenaf will respond to shoot regeneration provided the petioles remained attached to the cotyledons. This finding is comparable to jute, ***Corchorus capsularis*** (Khatun ***et al.***, 1993a), ***Brassica spp.*** (Sharma ***et al.***, 1991) and apple (Kouider ***et al.***, 1984). Like kenaf, these species similarly require an attached petiole to undergo morphogenesis.

In this study, surfactant, Pluronic F-68 was used for the enhancement of shoot regeneration from kenaf cotyledon explants. Number of shoot production cotyledon^{-1} was increased in each concentration compared to the control. This has a similarity with the report of Khatun ***et al.*** (1993a) on jute (*C.* ***capsularis***). On the other hand, disadvantages of using TDZ in culture medium include: a decrease in shoot elongation, difficulty of rooting of regenerated shoots, vitrification, shoot fascination, which has been reported in many plant species (Huetteman and Preece 1993; Lu, 1993). Srivatanakul ***et al.*** (2000) reported that TDZ treated kenaf plants exhibited some of the problems associated with TDZ. Multiple shoots regenerated from media containing high levels of TDZ exhibited vitrification and formed fasciated shoots. In this study, number of shoot production per explants could be increased by using Pluronic F-68, which is comparable to the study of Srivatanakul ***et al.*** (2000) using TDZ. However, all these negative affects of TDZ on regenerated shoots could be avoided using Pluronic F-68. Unlike TDZ (Srivatanakul ***et al.***, 2000), no harmful effect of the Pluronic F-68 on regenerated shoot was observed.

The stimulatory effect of Pluronic F-68 on cultured cotyledons of ***Hibiscus cannabinus*** is comparable to that reported previously with jute *C.* ***capsularis*** (Khatun ***et al.***, 1993a). In the present study, culture of kenaf cotyledons with attached petioles in the presence of Pluronic F-68 increased shoot multiplication up to 0.5% with no further stimulation at higher concentrations which shows similarity with jute regeneration (Khatun ***et al.***, 1993a). In contrast, maximum growth of *S.* ***dulcamara***-transformed roots occurred in the presence of 0.01% commercial grade Pluronic F-68 and that of leaf derived callus at 0.1% (Kumar ***et al.***, 1991, 1992). Furthermore, cell suspension cultures of *S.* ***dulcamara*** will grow normally in the presence of Pluronic upto 1.0% (w/v), although growth is inhibited at higher concentrations (King ***et al.***, 1990).

In this study, the experiments reported in this paper demonstrate a growth promoting effect of Pluronic F-68 on cultured ***Hibiscus*** tissues and this compound is a valuable supplement in plant tissue culture media. This work also suggests that Pluronic F-68 may act, at least in part through plasma membrane-associated changes and thus could influence permeability (Lowe ***et al.***, 1993). Related studies with animal cells have shown that Pluronic F-68 stimulates 2-deoxyglucose uptake

and amino acid incorporation into protein (Cawrse ***et al.***, 1991), perhaps increasing cytoplasm membrane permeability. This supported by patch clamp experiments using artificial lipid bilayers in which Pluronic F-68 caused the formation of short-lived, transmembrane pores (King ***et al.***, 1991).

It may be concluded that, supplementation of culture medium with 0.001-0.5% (w/v) commercial grade Pluronic F-68 showed a marked stimulation of shoot production from kenaf explants. Both differences in number of shoot production cotyledon^{-1} and percentage of explants producing shoot has been increased in the presence of surfactant without showing any morphological abnormality. On the other hand, root production was found to be a very common phenomenon for ***in vitro*** kenaf culture. The biotechnological implications of these results are important in relation to the potential value of non-ionic surfactant as growth stimulating additives to plant culture media.

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