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EFFECTS OF PLANT GROWTH REGULATORS ON INDIRECT SOMATIC EMBRYOGENESIS IN KENAF (*Hibiscus cannabinus*)

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ABSTRACT

To develop protocols for regeneration of kenaf via indirect somatic embryogenesis, hypocotyl and cotyledon explants of two genotypes were cultured at different 2,4- dichlorophenoxyacetic acid and kinetin concentrations. The highest callus yields were obtained in medium containing both 0.5mg of kinetin and 0.1mg of 2, 4-dichlorophenoxyacetic acid per litre of medium. The calli were further cultured in twelve different media containing different plant growth regulators to induce somatic embryogenesis. Friable calli were mostly produced in one genotype (Ifeken100) and non-friable in the other (Ifeken400). Among the plant growth regulator regimes, media containing 3.0mg/l of kinetin and that containing 1.5mg/l each of kinetin and NAA did not support production of globular embryos in both varieties. The friable and non-friable calli were most responsive to kinetin and NAA respectively in terms of formation of torpedo embryos. The most advanced walking stick embryos were produced only in Ifeken400 in 1.5mg/l NAA. The importance of genotype and callus type in successful regeneration of kenaf by indirect somatic embryogenesis is discussed. Research is ongoing to achieve whole plantlet regeneration.

Keywords: Kenaf, *Hibiscus cannabinus*, embryogenesis, plant regeneration, plant growth regulators.
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INTRODUCTION

Kenaf is a fibre crop that has been rated as the most viable replacement for trees in paper-making (Rymsza, 1999). This is because it is an environment-friendly, low-input, short-duration crop (Rymsza, 1999) which yields five times as much fibre as southern pine (LeMahieu *et al.*, 2003). In addition, the ban on the use of synthetic packaging materials in international trade has necessitated the use of natural fibres in the industry. This is especially relevant in Nigeria where the use of kenaf in the manufacture of bags for importation and exportation is yet to be fully adopted.

Kenaf production is seriously constrained by its susceptibility to pests and diseases, especially root-knot nematodes (Ogunlola and Adeoti, 1990) sensitivity to photoperiod (Scott, 1982; Webber *et al.*, 2002) and high level of out-crossing which causes high intra-cultivar variation (Siepe *et al.*, 1997), especially in morphological and agronomical characteristics. Using Tissue culture techniques, crop improvement is possible via somaclonal variation, wherein desirable changes are manifested by plantlets regenerated from different explants via a callus phase (Ogunbodede and Novak, 1998). Also, vegetative propagation *in vitro* (micropropagation) ensures true-to-type

production of plantlets with no fear of out-crossing (Ng and Ng, 1997; Srivatanakul *et al.,* 2000; Khatun *et al.,* 2003). Although there have been few reports on protocols for somatic embryogenesis in kenaf, inherent genotypic differences and specificity of laboratory conditions have made it necessary to develop protocols for our laboratory. In addition, roles of various chemicals in somatic embryogenesis still lack a comprehensive biochemical framework (Ganguli, 2002). The development of improved varieties of kenaf from somaclonal variants will require protocols for plantlet regeneration by indirect somatic embryogenesis through the callus phase. This study was conducted to investigate the effects of plant growth regulators (PGRs) on callus induction and somatic embryogenesis in kenaf.

MATERIALS AND METHODS

Seeds of two varieties of kenaf (Cuba 108 and Ifeken400) were cultured in Murashige and Skoog (MS, 1962) medium containing (per litre) 30g sucrose, 0.1g myo-inositol and 7g agar set at a pH of 5.7. When the embryos had germinated (four days after culturing), the hypocotyls and cotyledons were excised, cut into 1cm^2 sizes and cultured in modified MS medium containing seven different combinations of 2,4-dichlorophenoxyacetic acid and kinetin regimes for callus induction (Table 1). Four weeks later, data were taken on degree of callus formation (on a scale of 0: no callus, 1: low, 2: medium, 3: high callus formation) and weight of callus (g) per explant. There were three explants per petri plate and 4 petri plates (replicates) per medium. Analysis of variance was performed based on a completely randomized design and means were separated at p=0.05 to identify the optimum medium for callus induction.

More calli were then induced in the medium found to be optimum using two varieties of kenaf (Ifeken100 and Ifeken400). Four weeks later, the calli were transferred into 12 media, each containing (per litre) 0.44g MS medium, 30g sucrose, 0.1g myo-inositol, 7g agar and different regimes of benzylaminopurine (BAP), indoleacetic acid (IAA), naphthalene acetic acid (NAA) and kinetin for somatic embryogenesis. The media were set to a pH of 5.7. The types and concentrations of plant growth regulators in each medium are shown in Table 2.

After 5 months, data were taken on percentage of cultures of each treatment that had friable callus, globular, torpedo and walking stick stages of embryogenesis by observation under dissecting microscope. There were three units per treatment, and the experiment was replicated three times. Arcsine transformation was done on the data and analysis of variance was carried out using the Statistical Analysis Systems (SAS, 2002). Means were separated at p=0.05.

RESULTS AND DISCUSSION

The main effect of variety was not significant for all the parameters, while among explants, the cotyledons had higher degree of callus formation and callus weight than hypocotyls (Table 1). Treatment effect was significantly different ($p<0.01$), with as high as 2.13 degree of callusing in media containing 0.1mg/l 2,4-D and 0.5mg/l kinetin, and as low as 0.00 in medium containing only 0.1mg/l kinetin. The same trend was observed for callus weight produced per explant. 2,4-D has been reported to enhance callus formation in the presence of Isopentenyl adenine (2-iP) and adenosine sulphate in kenaf, although it took 6-8 weeks after culturing (Ogunbodede and Novak, 1998). In this study, cotyledon explants formed callus 4 weeks after culturing. Although

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Table 1. Callus formation by cotyledon and hypocotyl explants of two varieties of kenaf at different plant growth regulator regimes.

Cultural factor	Degree* of callus formation	Weight of callus (g)
PGR regime (per litre)		
0.1mg/l 2,4-D	0.88c	0.32de
0.1mg 2,4-D; 0.1mg Kinetin	1.38bc	0.88bc
0.1mg 2,4-D; 0.5mg Kinetin	2.13a	1.50a
0.1mg 2,4-D; 1.0mg Kinetin	1.63ab	1.44ab
0.1mg Kinetin	0.00d	0.09e
0.5mg 2,4-D; 0.1mg Kinetin	1.25bc	0.96abc
1.0mg 2,4-D; 0.1mg Kinetin	1.50abc	0.65de
Genotype		
Cuba 108	1.44a	1.00a
Ifeken400	1.22a	0.82a
Explant		
Cotyledon	1.63a	1.12a
Hypocotyl	1.03b	0.70b
Lsd	0.32	1.18

** : on a scale of 0: no callus, 1: low, 2: medium, 3: high callus formation.*

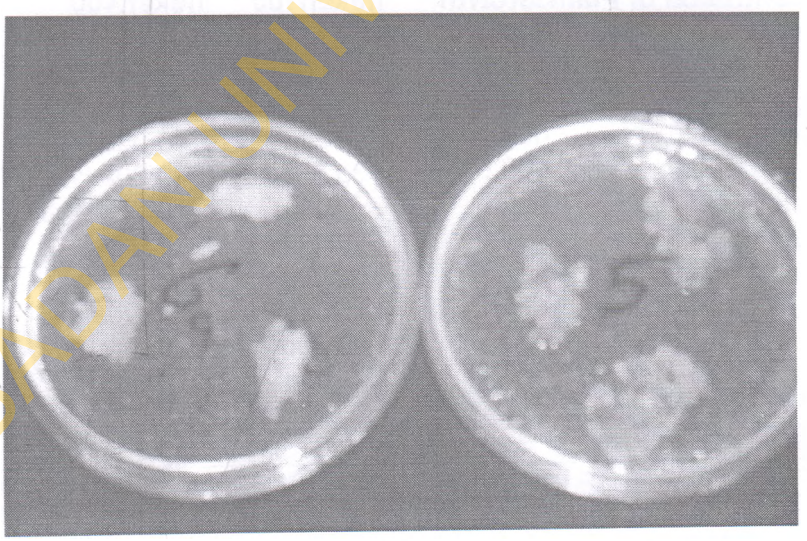

Figure 1. Types A and B calli in two varieties of Kenaf.

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the non-significance of varietal differences suggests that this callus inducing medium may be genotype-independent, more genotypes may have to be tested to confirm this.

Two types of calli were produced (Figure 1). The friable, white to cream, soft textured Type A callus and the non-friable, yellow to brown hard-textured Type B callus, which were mostly characteristic of the varieties Ifeken100 and Ifeken400 respectively (Table 2). The type B callus produced in Ifeken400 was irrespective of the culture medium composition, while in Ifeken 100, the degree of friability differed among the media. In Ifeken100, type B calli were produced in 3.0mg/l KIN, 3.0mg/l KIN plus 1.5mg/l NAA, 1.5mg/l KIN plus 3.0mg/l NAA and the control without plant growth regulators (Table 2). Different forms of calli were reported from different explants of kenaf (Ogunbodede and Novak, 1998). In maize, two types of callus were also reported (Finer, 1995), and each type differed in the rate of development of advanced stages of somatic embryos. The type of callus can therefore have implications on the ease of regeneration of kenaf, which could also be an indication of the genetic closeness of kenaf varieties.

Table 3 shows that in Ifeken100, media containing 1.5mg/l NAA, 3.0mg/l KIN plus 1.5mg/l NAA and 1.5mg/l KIN plus 3.0mg/l NAA had 66.67, 100 and 100% of the cultures at the globular stage of embryogenesis while, in other media, the callus phase persisted. This suggests that NAA is supportive of this stage of embryogenesis as it is common to the three media. In Ifeken400 however, globular embryos were produced in all the media except 3.0mg/

Table 2. Percentage formation of friable (Type A) callus in two varieties of Kenaf at different plant growth regulator regimes.

Type and concentration of Plant Growth regulator (l^{-1})	Ifeken100	Ifeken400	Mean
Control	0.00d	0.00a	0.00d
3mgBAP, 1.5mgIAA	100a	0.00a	50.00b
1.5mgBAP, 3.0mgIAA	100a	0.00a	50.00b
1.5mg NAA	33.33cd	0.00a	16.67cd
1.5mgNAA, 1.5mgBAP	100a	0.00a	50.00b
3mgBAP, 1.5mgNAA	80.00b	0.00a	40.00bc
1.5mgBAP, 3.0mgIAA	50.00bc	0.00a	25.00c
1.5mg kinetin	100a	25.00a	62.50a
3.0mg Kinetin	0.00d	0.00a	0.00d
1.5mg kinetin, 1.5mgNAA	100a	0.00a	50.00b
3.0mg Kinetin, 1.5mgNAA	0.00d	0.00a	0.00d
1.5mg Kinetin, 3.0mg NAA	0.00d	0.00a	0.00d
Mean (Variety)	55.28a	2.08b	28.68 ± 5.21

Values in each column followed by the same letters are not significantly different at p=0.05 by Duncan multiple range test. Mean values in each row followed by the same upper case letters are not significantly different at p=0.05.

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Table 3. Percentage formation of globular embryos in two varieties of Kenaf at different plant growth regulator regimes.

Plant Growth regulator regime (l^{-1})	Ifeken100	Ifeken400	Mean
Control	0.00b	100a	50.00b
3mgBAP, 1.5mgIAA	0.00b	100a	50.00b
1.5mgBAP, 3.0mgIAA	0.00b	50.00b	25.00c
1.5mg NAA	66.67a	0.00c	33.33bcd
1.5mgNAA, 1.5mgBAP	0.00b	100a	50.00b
3mgBAP, 1.5mgNAA	0.00b	100a	50.00b
1.5mgBAP, 3.0mgIAA	0.00b	16.67c	8.33de
1.5mg kinetin	0.00b	75.00ab	37.50bc
3.0mg Kinetin	0.00b	0.00c	0.00e
1.5mg kinetin, 1.5mgNAA	0.00b	0.00c	0.00e
3.0mg Kinetin, 1.5mgNAA	100a	0.00c	50.00b
1.5mg Kinetin, 3.0mg NAA	100a	100a	100a
Mean (Variety)	22.22^{b}	53.47^{a}	37.84 ± 5.00

Values in each column followed by the same letters are not significantly different at p=0.05 by Duncan multiple range test. Mean values in each row followed by the same upper case letters are not significantly different at p=0.05.

Table 4. Percentage formation of torpedo embryos in two varieties of Kenaf at different plant growth regulator regimes.

Plant Growth regulator regime (l^{-1})	Ifeken100	Ifeken400	Mean
Control	0.00b	0.00b	0.00b
3mgBAP, 1.5mgIAA	0.00b	0.00b	0.00b
1.5mgBAP, 3.0mgIAA	0.00b	0.00b	0.00b
1.5mg NAA	0.00b	50.00a	25.00a
1.5mgNAA, 1.5mgBAP	0.00b	0.00b	0.00b
3mgBAP, 1.5mgNAA	0.00b	0.00b	0.00b
1.5mgBAP, 3.0mgIAA	0.00b	0.00b	0.00b
1.5mg kinetin	0.00b	0.00b	0.00b
3.0mg Kinetin	33.33a	0.00b	16.67a
1.5mg kinetin, 1.5mgNAA	0.00b	0.00b	0.00b
3.0mg Kinetin, 1.5mgNAA	0.00b	0.00b	0.00b
1.5mg Kinetin, 3.0mg NAA	0.00b	0.00b	0.00b
Mean (Variety)	2.78^{b}	4.17^{a}	3.48 ± 1.58

Values in each column followed by the same letters are not significantly different at p=0.05 by Duncan multiple range test. Mean values in each row followed by the same upper case letters are not significantly different at p=0.05.

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Table 5. Percentage formation of walking stick embryos in two varieties of Kenaf at different plant growth regulator regimes.

Plant Growth regulator regime (l^{-1})	Ifeken100	Ifeken400	Mean
Control	0.00	0.00b	0.00b
3mgBAP, 1.5mgIAA	0.00	0.00b	0.00b
1.5mgBAP, 3.0mgIAA	0.00	0.00b	0.00b
1.5mg NAA	0.00	50.00a	25.00a
1.5mgNAA, 1.5mgBAP	0.00	0.00b	0.00b
3mgBAP, 1.5mgNAA	0.00	0.00b	0.00b
1.5mgBAP, 3.0mgIAA	0.00	0.00b	0.00b
1.5mg kinetin	0.00	0.00b	0.00b
3.0mg Kinetin	0.00	0.00b	16.67a
1.5mg kinetin, 1.5mgNAA	0.00	0.00b	0.00b
3.0mg Kinetin, 1.5mgNAA	0.00	0.00b	0.00b
1.5mg Kinetin, 3.0mg NAA	0.00	0.00b	0.00b
Mean (Variety)	0.00^{b}	4.17^{a}	2.08 + 1.12

Values in each column followed by the same letters are not significantly different at p=0.05 by Duncan multiple range test. Mean values in each row followed by the same upper case letters are not significantly different at p=0.05.

l KIN, 1.5mg/l KIN plus 1.5mg/l NAA and 3.0mg/l KIN plus 1.5mg/l NAA in which the callus phase persisted. This may be an indication that kinetin is inhibitory to this phase of embryogenesis in this variety.

Table 4 shows that the more advanced stage of embryogenesis, the torpedo stage, was formed only in medium containing 1.5mg/l NAA in Ifeken400 (50% torpedo embryos) and that containing 3.0mg/l kinetin in Ifeken 100 (33.33%). The latter two media contain only NAA and kinetin respectively. It seems therefore, that types A and B calli are more responsive to kinetin and NAA respectively for successful somatic embryogenesis.

The walking stick phase, which is the final precursor of plantlet regeneration (Phillips *et al.*, 1995), was produced only in the presence of 1.5mg/l NAA in Ifeken400 (50%) while this phase of embryogenesis was not produced in any of the media in Ifeken100 (Table 5). Shoot formation by direct organogenesis from internodal and leaf sections of kenaf was achieved in medium containing NAA and thidiazuron (Reichert and Liu, 1994; Reichert *et al.*, 1999)

No plantlets were regenerated in any of the media throughout the experimental period in both varieties. Although a genotype-independent regeneration system is most desirable, it may be difficult to remove the influence of genotype in kenaf somatic embryogenesis through the callus phase. We hope to achieve regeneration by culturing in more plant growth regulator regimes based on the type of callus formed in each genotype

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