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Influence of polyamines on shoot regeneration of sugarcane *(Saccharum officinalis. L)*

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Abstract

We studied the effect of polyamines (spermidine, putrescine and spermine) along with benzyladenine, kinetin and napthaleneacetic acid on multiple shoot regeneration of sugarcane. Murashige & Skoog medium containing a combination of benzyladenine (8.3 µM), kinetin (4.6 µM), napthaleneacetic acid (2.6 µM) and spermidine (68 µM) induced the maximum number of shoots (42 shoots/explant) compared to benzyladenine (8.3 µM), kinetin (4.6 µM) or napthaleneacetic acid (2.6 µM) alone or with putrecine (68 µM), spermine (32 µM) or combinations of polyamines. Plantlets raised were successfully transplanted in soil with a 90% survival rate.

Keywords:

Introduction

Polyamines are a class of low-molecular-weight aliphatic cations prevalent in all living organisms. They are essential for growth and development in prokaryotes, and eukaryote polyamines, specifically spermidine, spermine and putrescine, are present in all plant cells (Galston 1983; Bais & Ravishankar 2002). Polyamines act as intracellular growth factors by increasing the rate of cell growth. They play a major role in cell division and differentiation, and have distinct physiological and developmental effects on plants.

Polyamines are involved in shoot morphogenesis, growth, organ development, leaf senescence, stress responses, flower inducement, improving fruit quality and as biomarkers for plant regeneration (Zhu & Chen 2005; Alcazar et al. 2006; Kusano et al. 2007). They induce shoot regeneration from *Passiflora* leaves, cotyledonary explants of *Brassica campestris* and shoot tip explants of *Cucumis sativus*.

Polyamines have stimulatory effects on phytohormonal signalling, endogenous growth and synergism (Jang et al. 2002). They are essential factors for cell viability, protein phosphorylation, post-transcriptional modification and for the formation of secondary metabolites, acting as promotors of growth and survival (Kusano et al. 2008). It is evident from the literature that polyamines play a major role in morphogenic processes in plant tissue culture.

Sugarcane is a global agro-economic crop, grown vegetatively in most parts of the world. This natural process requires large areas for cultivation and a time-consuming methodology. An effective regeneration protocol is needed for multiple shoot regeneration. Hence, we investigated the role of polyamines on *in vitro* shoot regeneration from the axillary bud explants of sugarcane.

Materials & Methods

Axillary bud explants of sugarcane (Variety Co671 from Tamilnadu Agricultural University, Sirugamani, Tiruchirapalli, India) were isolated using a sterile blade and soaked with tap water for 15 min. They were then surface-sterilized with 70% alcohol for 1 min and 2.5% (*v/v*) Teepol (Commercial detergent, Reckitt & Benckiser, Bangalore, India) for 15 min, followed by

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three rinses with sterile distilled water. The isolated buds were treated with the fungicide Carbendazim (Bavistin, India) for 20 min to eradicate fungal contamination before they were further surface-sterilized by soaking in a 0.1% (*w/v*) aqueous mercuric chloride ($HgCl_2$) solution for 10 min and then rinsing four times with sterile distilled water.

Axillary bud explants (one per tube) from 6-mon-old healthy stalks of sugarcane (Co671) were excised and inoculated in 25x150-mm test tubes plugged with non-absorbent cotton (Borosil, New Delhi, India) containing 20 ml MS liquid medium (Murashige & Skoog 1962). Multiplication was doubled in temporary immersion medium (Lorenzo et al. 1998; Murashige & Skoog 1962) consisting of sucrose 0.086 M (3%) and 0.8% (w/v) agar (Himedia, India) with different concentrations of benzyladenine (2.1, 4.1, 6.2, 8.3, 10.4, 12.5, 14.6 and 16.7 µM) for primary shoot induction.

Multiplication medium containing MS liquid medium supplemented with different concentrations of benzyladenine (4.1, 8.3 and 12.5 µM), kinetin (2.3, 4.6 and 9.2 µM) and napthaleneacetic acid (1.3, 2.6 and 4.0 µM) was used for secondary shoot regeneration. Primary shoots excised from the axillary bud explants were trimmed of leaves and further subcultured in the multiplication medium for secondary shoot regeneration for three cycles each of three-weeks duration (Fig. 1). Whatman no. 3 filter papers were used for physical support.

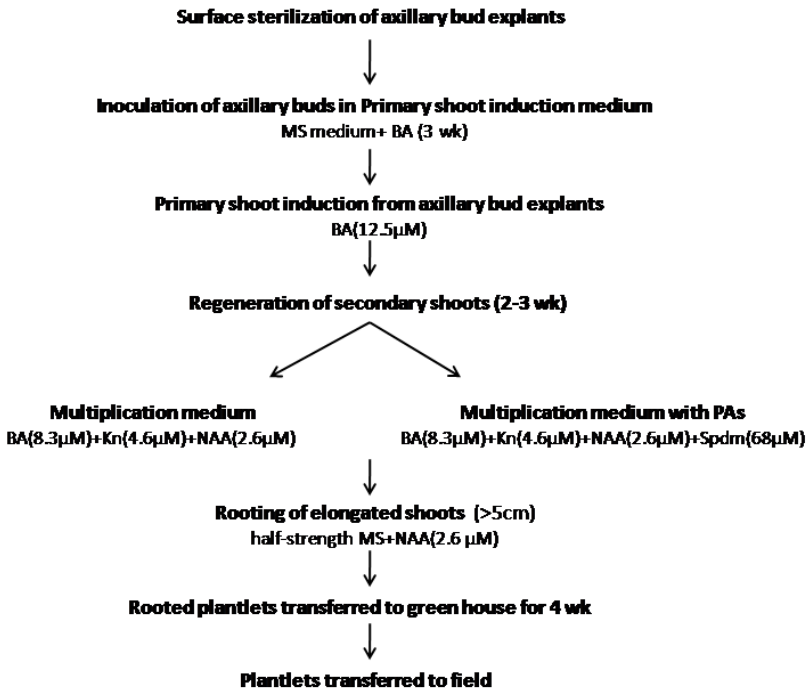

Fig. 1 Experimental design

Separate experiments were carried out using polyamines. Primary shoots produced after 3 wks were excised from axillary bud explants and trimmed of leaves. The primary shoots excised were subcultured in multiplication medium containing the determined optimal concentrations of benzyladenine (8.3 µM), kinetin (4.6 µM) and napthaleneacetic acid (2.6µM) supplemented with polyamines (spermidine [10, 32, 68, 102 and 500 µM], putrescine [10, 32, 68, 102 and 500 µM], combinations of polyamines [spermidine (50 µM) + putrescine (50 µM), spermidine (100 µM) + putrescine (100 µM), spermidine (50 µM) + spermine (50 µM), spermidine (100 µM) + spermine (100 µM), putrescine (50 µM) + spermine (50 µM),

putrescine (100 µM) + spermine (100 µM)] (Sigma Research labs) for secondary shoot regeneration, repeated for three cycles each of three-weeks duration. Appropriate controls were maintained for the experiment (without polyamines).

The pH of the medium was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} (121 ˚C) for 20 min. Elongated secondary shoots (>5cm) were selected and transferred to half-strength MS medium containing napthaleneacetic acid (2.6 µM) [rooting medium] for root induction. All the cultures were maintained at 25 ± 2˚C under a 16 h photoperiod with a light intensity of 15 µmol m^{-2} s^{-1}.

Rooted plantlets (>5cm) were washed thoroughly with sterile water to remove agar and transplanted to plastic pots containing a mixture of autoclaved sand, soil and vermiculite (1:1:1 *v/v/v*). Potted plants were grown in a growth chamber (Sanyo, Japan) at 80% relative humidity for 3-4 weeks and then moved to a greenhouse for a period of 4 weeks before being transferred to the field. At early stages plants were covered with polyethylene bags to maintain high humidity (80%) and supplied with Hoagland nutrient solution (Hoagland & Arnon 1950). The polyethylene bags were removed when the plants had shown acclimatization. After 4 weeks of acclimatization, plants were transplanted to terracotta and grown in the garden.

Each experiment (three cycles) consisted of 20 explants and was repeated twice. The data from the two separate experiments were pooled and the mean and standard error derived from these data. The mean number of shoots was calculated per responding explant. Comparisons between the mean values of treatments were carried out using Duncan's multiple-range test with significance determined at 5% level (Gomez & Gomez, 1976).

Results

The number of axillary bud explants responding to primary shoot induction increased with increasing concentrations of benzyladenine (Table 1). However, at higher concentrations of benzyladenine (14.6 µM and 16.7 µM), primary shoot formation was reduced. The highest percentage response (91%) was noted in MS liquid medium supplemented with 12.5 µM benzyladenine (Fig. 2). The addition of kinetin and napthaleneacetic acid to MS liquid medium did not enhance primary shoot production, but induced callus around the cut end of axillary buds and suppressed shoot formation. After three weeks of inoculation, secondary shoots emerged from the basal region of primary shoots.

benzyladenine (µM)	Number of buds cultured	Number of buds responded	Response of buds (%)
2.1	20	5.8 ± 0.06^{g}	24
4.1	20	7.5 ± 0.06^{e}	32
6.2	20	$8.1 \pm 0.06^{d,e}$	42
8.3	20	12.0 ± 0.12^{c}	61
10.4	20	14.0 ± 0.12^{b}	71
12.5	20	16.0 ± 0.12^{a}	87
14.6	20	11.0 ± 0.12^{c}	60
16.7	20	$6.1 \pm 0.06^{f,g}$	29

Table 1: Effect of growth regulators on primary shoot induction from axillary buds. Means ± s.e. The same superscript letter indicates no significant difference (at the 5% level) between the mean values, according to Duncan's multiple range test.

The rate of secondary shoot multiplication rate was low using benzyladenine alone. The addition of kinetin and napthaleneacetic acid with benzyladenine increased the number of secondary shoots. The number of shoots (30.1) produced in the combination of 8.3 µM

benzyladenine, 4.6 µM kinetin and 2.6 µM napthaleneacetic acid (Table 2) was higher compared to other combinations (cf. Manickavasagam 2002). Higher concentrations of napthaleneacetic acid suppressed multiplication of secondary shoots.

benzyladenine (µM)	kinetin (µM)	napthaleneacetic acid (µM)	Mean number of shoots	Regeneration of shoots (%)
4.1	2.3	1.3	10.1 ± 0.12^{h}	63
8.3	4.6	2.6	25.5 ± 0.21^{b}	88
12.5	9.2	4.0	$10.3 \pm 0.12^{g,h}$	65
4.1	2.3	1.3	13.9 ± 0.14^{e}	74
8.3	4.6	2.6	30.1 ± 0.28^{a}	91
12.5	9.2	4.0	9.2 ± 0.11^{i}	60
4.1	2.3	1.3	$14.4 \pm 0.14^{d,e}$	75
8.3	4.6	2.6	19.3 ± 0.19^{c}	85
12.5	9.2	4.0	11.5 ± 0.13^{f}	68

Table 2: Effect of plant growth regulators on secondary shoot regeneration. Means ± s.e. The same superscript letter indicates no significant difference (at the 5% level) between the mean values, according to Duncan's multiple range test.

Our aim was to asses the involvement of polyamines in shoot regeneration. At the optimum level of spermidine (68 µM), 90% of primary shoots from axillary bud explants produced an average of (42.8 ± 0.23) secondary shoots per explants (Table 3). Although the addition of putrescine (68 µM), spermine (32 µM) (Table 3) and combinations of polyamines (Table 4) produced more shoots per explant, the differences were not statistically significant.

Polyamines (µM)	Regeneration of shoots (%)	Mean number of shoots	Mean shoot length(cm)
0	78	29.8 ± 0.11^{h}	5.3 ± 0.18
Spermidine			
10	82	34.5 ± 0.14^{d}	5.9 ± 0.21
32	86	37.3 ± 0.16^{b}	6.5 ± 0.24
68	91	42.8 ± 0.23^{a}	6.8 ± 0.26
102	79	32.4 ± 0.12	5.3 ± 0.18
500	74	29.6 ± 0.11^{h}	4.9 ± 0.16
Putrescine			
10	76	30.5 ± 0.11^{h}	5.2 ± 0.18
32	80	$33.2 \pm 0.14^{e,f}$	5.4 ± 0.19
68	84	$35.1 \pm 0.15^{c,d}$	6.2 ± 0.22
102	72	28.5 ± 0.11^{i}	4.9 ± 0.16
500	69	26.7 ± 0.10^{k}	4.7 ± 0.14
Spermine			
10	70	$27.7 \pm 0.10^{j,k}$	4.7 ± 0.14
32	78	32.7 ± 0.12^{f}	5.7 ± 0.20
68	79	$31.8 \pm 0.12^{g,h}$	5.1 ± 0.18
102	68	26.2 ± 0.10^{k}	4.5 ± 0.12
500	64	24.5 ± 0.10l	4.3 ± 0.12

Table 3: Effect of PAs on secondary shoot regeneration from primary shoots produced from the axillary bud explants. Means ± s.e. The same superscript letter indicates no significant difference (at the 5% level) between the mean values, according to Duncan's multiple range test.

Polyamine combination (μM)	Regeneration of shoots (%)	Mean number of shoots	Mean shoot length (cm)
0	78	$29.6 \pm 0.19^{c,d}$	5.3 ± 0.11
Spermidine+Putrescine			
50 + 50	80	33 ± 0.21^{a}	5.4 ± 0.22
100 + 100	77	29 ± 0.17^{c}	4.9 ± 0.12
Spermidine+Spermine			
50 + 50	78	31 ± 0.19^{b}	5.2 ± 0.22
100 +100	72	$26 \pm 0.16^{e,f}$	4.5 ± 0.12
Putrescine+Spermine			
50+ 50	72	28 ± 0.17^{d}	4.8 ± 0.12
100 + 100	64	25 ± 0.15^{f}	4.4 ± 0.12

Table 4: Effect of combination of PAs on secondary shoot regeneration from primary shoots produced from axillary bud explants. Means ± s.e. The same superscript letter indicates no significant difference (at the 5% level) between the mean values, according to Duncan's multiple range test.

Promotive hardening and acclimatization was obtained when plantlets of at least 5 cm long were used. More than 90% of hardened plants survived. A similar correlation between plant height, acclimatization and subsequent survival has been reported for watermelon, *Cucumis hystrix*, *Cucumis sativus* (Compton et al. 1993; Selvaraj et al. 2002; Vasudevan et al. 2008).

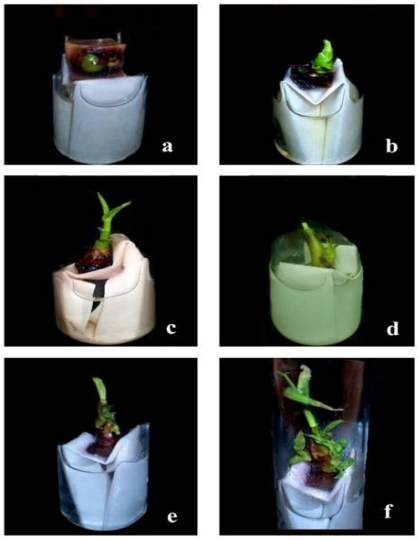

Fig 2: Regeneration stages of sugarcane from axillary bud explants. a-c: primary shoot initiation; d-f: secondary shoot initiation.

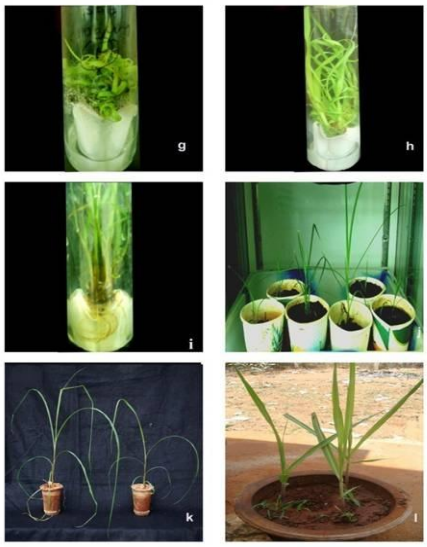

Fig 3: Regeneration of sugarcane from axillary bud explants with polyamines. g-h: regenerated multiple shoots; i: rooted plantlets; j-k: individual plantlets; l: plantlets raised in field.

Discussion

Several research findings have been reported on the vital role of polyamines in shoot regeneration and somatic embryogenesis. Putrescine stimulates cell division in *Pinus oocarpa* and *Pinus patuta* embryonic cultures, and spermine was indispensable for adventitious shoot formation in cucumber (Zhu & Chen 2005). Spermidine was found to affect shoot development

in carnation cultures of *Arabidopsis thaliana* (Jang et al. 2006). Conclusively in the present study, spermidine rather than putrescine or spermine was found most effective in multiple shoot regeneration.

Spermidine rather than putresine or spermine was involved in enhancing multiple shoot differentiation from the shoot tips of cucumber (Vasudevan et al. 2008). These lines of evidences show that spermidine, putrescine and spermine may play dissimilar roles in different species or in different explants as reported by Zhu & Chen (2005). Exogenously added polyamines have been widely used for studying the function of polyamines in cell growth and differentiation (Martin-Tanguy 2001).

With the use of these protocols, approximately 42 plants per axillary bud explants were obtained within a short period. From the present study, it is clear that the exogenous application of benzyladenine (8.3 µM), kinetin (4.6 µM), napthaleneacetic acid (2.6 µM) and spermidine (68 µM) in the MS medium promoted the highest shoot induction frequency along with shoot elongation (Fig. 3).

Our investigation suggests that exogenous application of spermidine along with benzyladenine, kinetin, napthaleneacetic acid plays a synergistic role in enhancing the multiple shoot formation from axillary bud explants of sugarcane *in vitro.* It is presumed that spermidine, which provides a nitrogen source (Altman et al. 1993), along with the growth regulators triggered the enhancement of shoot regeneration from the axillary bud explants of sugarcane. In our study sugarcane variety Co671 was selected mainly due to its high sucrose content and regeneration efficiency. The protocol developed in the study offers a simple and improved method to mass-propagate sugarcane, which ultimately resulted in better acclimatization and survival of regenerated shoots.

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الملخص العربى

تأثير الأمينات على تجدد براعم قصب السكر (*Saccharum officinalis. L*)

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الملخص العربي

تناول البحث دراسة تأثير الأمينات (سبيرمدين وبوتريسين وسبيرمين) مع مادة البنزيل أدينين والكينتين وحامض النفثالين أسيتيك على تجدد العديد من براعم قصب السكر. لقد تسبب استخدام بيئة موراشيج وسكوج (Murashige & Skoog) المحتوية على مزيج من مواد البنزيل أدينين (8.3 ميكرومتر) والكينتين (4.6 ميكرومتر) وحامض النفثالين أسيتيك (2.6 ميكرومتر) وسبيرمدين (68 ميكرومتر) في الوصول إلى العدد الأقصى من البراعم (42 برعم / النبات) مقارنة بمواد البنزيل أدينين (8.3 ميكرومتر) أو الكينتين (4.6 ميكرومتر) أو حامض النفثالين أسيتيك (2.6 ميكرومتر) وحدهم أو مع بوتريسين (68 ميكرومتر) أو السبيرمين (32 ميكرومتر) أو مزيج من الأمينات. وقد تم زرع الشتلات بنجاح في التربة وكان معدل البقاء 90 ٪.