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RESEARCH ARTICLE

Enhanced *in vitro* shoot multiplication and elongation in sugarcane using cefotaxime

Ajinder Kaur · M. S. Gill · D. Ruma · S. S. Gosal

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Abstract Antibiotic cefotaxime has been found to be a growth promoting substance in sugarcane tissue culture. Young leaf segments (1-2 cm long) of three commercially important Indian sugarcane varieties viz., CoJ 83, CoJ 85 and Co 89003, regenerated into shoots and multiplied on semi-solid MS medium supplemented with 6-benzylaminopurine (BA, 0.2 mg/l) and kinetin (0.2 mg/l) for 2 cycles of 2 weeks each. Shoots (2 cm long) were separated and cultured on semi-solid half-strength MS medium supplemented with NAA (2 mg/l) and cefotaxime at various concentrations viz., 0, 250, 500 and 750 mg/l. Maximum shoot multiplication and elongation with respect to number of microtillers, shoot length and plantlet fresh weight in all the genotypes was obtained with cefotaxime used at the rate of 250 and 500 mg/l in the medium. Among the different varieties, on the basis of mean of 50 shoot cultures, number of microtillers per culture was highest in Co 89003 (7.50, 145.90% increase over control), whereas, shoot length and plantlet fresh weight were highest in CoJ 83, i.e. 10.30 cm (60.94% increase over control) and 360.45 mg (50.75% increase over control), respectively after 2 weeks of culturing with cephotaxime used at the rate of 500 mg/l in the medium. Statistical analysis of data revealed significant differences among varieties and media for different shoot multiplication and elongation parameters. Therefore, use of cefotaxime during tissue culture and genetic transformation of sugarcane can improve frequency of shoot multiplication and transformation, respectively.

Keywords Young leaf segments, tissue culture, microtillers, shoot length, fresh weight, antibiotic

Ajinder Kaur · M. S. Gill · S. S. Gosal (✉)
Department of Plant Breeding,
Genetics and Biotechnology,
Punjab Agricultural University,
Ludhiana - 141 004, Punjab, India
E-mail: ssgosal@rediffmail.com

D. Ruma
Department of Vegetable Crops,
Punjab Agricultural University,
Ludhiana - 141 004, Punjab, India

Introduction

In sugarcane (*Saccharum* spp. hybrid), shoots can be easily regenerated from various cultured tissues, such as nodal buds (Cheema *et al.,* 1992), shoot apices (Gosal *et al.,* 1998), embryogenic calli (Gill *et al.,* 2004) and young leaf segments (Gill *et al.,* 2006). However, many times due to excessive microtillering *in vitro*, there is a problem of crowding, which inhibits subsequent normal shoot elongation. This lowers the rate of multiplication through micropropagation in sugarcane. Besides this, during plant regeneration from long-term maintained callus cultures, there is a problem of albinism in the regenerated shoots. Further, during *Agrobacterium*-mediated genetic transformation, use of antibiotics such as carbenicillin, hygromycin and kanamycin also results into regeneration of weak shoots and sometimes albino shoots, ultimately ending into a very low frequency of transformation (Arencibia *et al.,* 1998, Bower and Birch, 1992). In this regard, use of cefotaxime ($C_{16}H_{16}N_5O_7S_2Na$), a semi-synthetic, broad-spectrum and cephalosporin antibiotic, instead of carbenicillin can improve genetic transformation frequency by killing the adhering *Agrobacteria* after co-cultivation and improving the growth of regenerated shoots. Cefotaxime is a β-lactam antibiotic that inhibits cell wall synthesis in dividing bacterial

cells and results in cell lysis (Selwyn, 1980). Cefotaxime can also be added into the culture medium to overcome bacterial contamination, which is a major hurdle in *in vitro* success of plant cultures, as microbial contamination in cultured plantlets can reduce multiplication or rooting rates or even induce plant death (Cassells, 1991; Leifert *et al.,* 1991).

After explant establishment and shoot regeneration, cultures are expected to be free from contamination. But, even after these stages, contamination due to endogenous microorganisms in tissue culture system has been reported (Reed *et al.,* 1997, Tanprasert and Reed, 1997). Plant tissue cultures harbor latent bacteria (Leifert and Waites, 1990), which affect multiplication rate of plant cultures when these are transferred to a medium containing reduced concentration of salt and sucrose. The antibacterial property of cefotaxime has been harnessed to enhance the frequency of plant regeneration in finger millet (Eapen and George, 1990), wheat (Borrelli *et al.,* 1992), pearl millet (Pius *et al.,* 1993), sorghum (Rao *et al.,* 1995) and rice (Grewal *et al.,* 2006). However, in sugarcane, as such there is no report regarding the influence of cefotaxime on shoot multiplication and elongation. Therefore, the present study was carried out with the objective to investigate the effect of cefotaxime on shoot multiplication and elongation in sugarcane tissue culture.

Materials and Methods

Tops of three commercially important Indian sugarcane varieties namely, CoJ 83, CoJ 85 and Co 89003 were excised from field-grown (6-month old) healthy plants. Spindle tissues (5-6 cm long and 1 cm thick) were taken out of the tops, surface-sterilized with 0.1% $HgCl_2$ for 8-10 min. and rinsed thrice with sterile distilled water, and reduced to 1-2 cm long segments under aseptic conditions. Young spindle leaf segments were cultured on agar-solidified MS (Murashige and Skoog, 1962) + naphthaleneacetic acid, NAA (5 mg/l) + kinetin (0.5 mg/l) medium for shoot regeneration. Regenerated shoots were multiplied and maintained on agar-solidified MS medium supplemented with 6-benzylaminopurine, BA (0.2 mg/l) and kinetin (0.2 mg/l) for 2 cycles of 2 weeks each. Thereafter, fifty shoot cultures of each variety from a culture jar were inoculated on each of the following media: (i) agar-solidified half-strength (½) MS medium (containing half the concentration of MS salts) supplemented with 2 mg/l NAA (control), and (ii) agar-solidified ½ MS + 2 mg/l NAA + 250, 500 and 750 mg/l cefotaxime medium (treatment). The experiment was replicated twice. Shoot cultures were kept in an incubation room with 16/8 h light/dark regime having a light intensity of 68 µ mol $m^{-2}s^{-1}$ and temperature of 25 ± 1 °C. Cefotaxime salt (1 g, omnatax™) was dissolved in 4 ml sterile water and sterilized by passing through membrane filter assembly having a pore size of 0.2 mm. Cefotaxime solution was added into the molten medium before solidification under aseptic conditions. Then 15 ml of this medium was dispensed in pre-autoclaved culture tubes (150 x 25 mm, Borosil) and closed with cotton plugs wrapped in muslin cloth. The medium without cefotaxime served as control. Data were recorded on different parameters of shoot multiplication and elongation viz., number of microtillers formed per shoot culture, shoot length and fresh weight (FW) of plantlets, after 2 weeks of culturing. Data were analyzed using factorial experiment in Completely Randomized Block (CRD) Design to see level of significance and critical difference for variety, media and variety x media interactions.

Results and Discussion

Young leaf segments of sugarcane varieties viz., CoJ 83, CoJ 85 and Co 89003, during first week of culturing, followed by differentiation of shoot buds from the cut ends without any callus interphase. Shoot buds developed into shoots within 2 weeks of culturing. Regenerated shoots were multiplied on agar-solidified MS + BA (0.2 mg/l) + kinetin (0.2 mg/l) medium for 2 cycles of 2 weeks each. Thus obtained shoots (2.0 cm) were separated and transferred on control and treatment media. Data were recorded on three traits viz., number of microtillers formed per shoot culture, shoot length and FW of plantlets after 2 weeks of culturing.

Number of Microtillers

Inclusion of antibiotic cefotaxime in the culture medium fostered the development of microtillers in tissue culture regenerated shoots of all the three sugarcane varieties at most of the concentrations. Number of microtillers per culture in control and treatment media, after 2 weeks of culturing is presented in Table 1.

As is evident from Table 1, maximum number of microtillers per culture were formed in variety Co 89003 (7.50, 145.90 % increase over control), followed by 6.65 (107.81% increase) in CoJ 83 on ½ MS + 2 mg/l NAA medium supplemented with 500 mg/l cefotaxime (Fig. 1). However, highest number of microtillers per culture in variety CoJ 85 (5.40 - 120.41 % increase over control) was obtained with the use of 250 mg/l cefotaxime in the medium. Previously, in apple Yepes and Aldwinckle (1994) observed higher number of

Table 1. Effect of antibiotic cefotaxime on average number of microtillers per shoot culture in sugarcane varieties after 2 weeks of culturing

Cefotaxime (mg/l)	Average no. of microtillers								
	CoJ 83			CoJ 85			Co 89003		
	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean
Control	3.10*	3.30	3.20	2.80	2.10	2.45	3.10	3.00	3.05
250	3.90	4.10	4.00	5.30	5.50	5.40	3.80	4.00	3.90
500	6.60	6.70	6.65	3.10	3.30	3.20	7.60	7.40	7.50
750	2.70	2.90	2.80	2.60	3.00	2.80	4.00	4.20	4.10

*Each figure is a mean of 50 shoot cultures

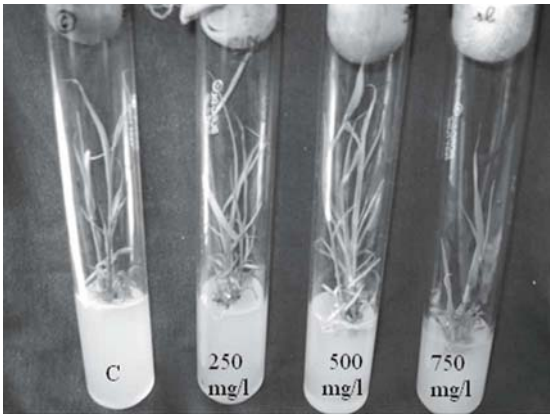

Fig. 1. Microtillering in sugarcane variety CoJ 83 on ½ MS + 2 mg/l NAA (control), ½ MS + 2 mg/l NAA + 250 mg/l cefotaxime, ½ MS + 2 mg/l NAA + 500 mg/l cefotaxime and ½ MS + 2 mg/l NAA + 750 mg/l cefotaxime, after 2 weeks of culturing

shoots per culture with 200 mg/l cefotaxime as compared to control, and at higher cefotaxime doses, the rate of shoot multiplication and elongation was reduced. Increase in number of microtillers may be associated with inhibition of ethylene production in the cultures by cefotaxime, which is positively correlated with plantlet differentiation (Pius *et al.,* 1993). Number of microtillers followed a downward trend in all the varieties with the highest dose of cefotaxime (750 mg/l) (Table 1). Thus, cefotaxime application in the medium beyond an optimum concentration may be toxic.

Results in Table 2 showed significant interaction between variety and cefotaxime or media, indicating that the varietal differences with respect to number of microtillers were significantly affected by the cefotaxime level applied and the cefotaxime effect differed significantly with the varieties tested. The effects both of variety and cefotaxime were significant.

Shoot Length

Unlike most of the cereals and legumes, different sugarcane varieties respond almost equally well to callus induction, shoot regeneration and subsequent shoot proliferation (Kaur *et al.,* 2001; Gill *et al.,* 2004). Under several instances, excessive shoot proliferation results in crowding, that leads to the formation of abnormal weak shoot clumps. Such clumps normally do not exhibit shoot elongation and rooting upon their transfer to respective shoot elongation and rooting media. In the present study, it was found that cefotaxime (up to a level of 500 mg/l) promoted shoot length in all the sugarcane genotypes tested. The maximum shoot length of 10.30 cm was achieved with the use of cefotaxime at the dose of 500 mg/l in variety CoJ 83 (Fig. 2, Table 3). It was found to be higher over control (6.40 cm) by 60.94 %, and all other combinations of genotype x cefotaxime concentrations. However, maximum increase in shoot length in varieties CoJ 85 and Co 89003 over control, i.e. 51.48 % and 52.69 % respectively, was obtained when culture medium in the respective variety was supplemented with 250 mg/l cefotaxime. Likewise, Kumar *et al.* (personal communication) observed significant increase in shoot length reported in *Vitis* species through the use of cefotaxime (250-500 mg/l) in the culture medium. Increase in cefotaxime concentration beyond the optimum limits reduced the shoot length progressively in all the genotypes. Minimum shoot length in all the varieties was noted with 750 mg/l cefotaxime (Table 3).

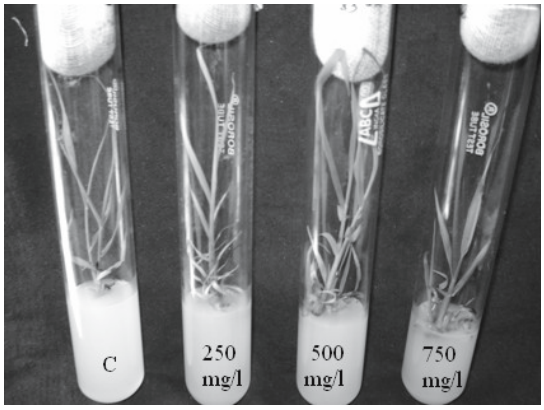

Fig. 2. Effect of different cefotaxime concentrations (0, 250, 500 and 750 mg/l) on shoot length in sugarcane variety CoJ 83, after 2 weeks of culturing

Table 2. Analysis of variance of data in Table 1 from a 3 x 4 factorial experiment in a CRD design

Source	df	M.S.	F-ratio	CD (5%)	C.V.
Reps.	1	0.04	0.81		
Variety (A)	2	2.79	66.67**	0.22	
Cefotaxime (B)	3	10.27	244.98**	0.26	
A x B	6	3.47	82.89**	0.45	
Error	11	0.04			5.01

**Significant at 1% level

Table 3. Effect of antibiotic cefotaxime on average shoot length in sugarcane varieties after 2 weeks of culturing

Cefotaxime (mg/l)	Average shoot length (cm)								
	CoJ 83			CoJ 85			Co 89003		
	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean
Control	6.30*	6.50	6.40	5.10	5.00	5.05	4.80	4.50	4.65
250	7.80	7.10	7.90	7.60	7.70	7.65	7.00	7.20	7.10
500	9.90	10.70	10.30	5.80	5.90	5.85	6.90	7.20	7.05
750	6.20	6.00	6.10	4.10	4.50	4.30	3.70	3.90	3.80

*Each figure is a mean of 50 shoot cultures

Analysis of average shoot length data in a factorial experiment in CRD design shows that variety MS (9.44), antibiotic or media MS (13.18) and variety x antibiotic interactions were significant at 1% level with CDs being 0.29, 0.34 and 0.59 respectively (Table 4). Therefore, there was a significant difference for shoot length among varieties and among media containing different cefotaxime doses. Significance of variety x antibiotic interactions envisages that the varieties responded differently with respect to shoot length in the four media. Thus, it is concluded that addition of cefotaxime in the culture medium (250-500 mg/l) enhanced the process of shoot elongation in different sugarcane varieties. In this regard, use of cefotaxime in the culture medium can promote shoot multiplication and elongation even from vitrified shoot clumps, and thus improve rate of shoot multiplication through micropropagation in sugarcane.

Table 4. Analysis of variance of data in Table 3 from a 3 x 4 factorial experiment in a CRD

Source	df	M.S.	F-ratio	CD (5%)	C.V.
Reps.	1	0.42	0.58		
Variety (A)	2	9.44	131.81**	0.29	
Cefotaxime (B)	3	13.18	183.92**	0.34	
A x B	6	1.97	27.54**	0.59	
Error	11	0.72			4.24

**Significant at 1% level

Fresh weight (FW) of plantlets

Perusal of data in Table 5 reveals stimulatory effect of cefotaxime on FW of plantlets in all the three varieties tested. Maximum FW in all the genotypes viz.,

360.45 mg in CoJ 83, 289.70 mg in CoJ 85 and 344.45 mg in Co 89003 was achieved when the medium was supplemented with 500 mg/l cefotaxime. This was significantly higher over control in the respective genotype. In an earlier study, cefotaxime (250 and 500 mg/l) has been reported to markedly enhance FW of callus in eggplant (Picoli *et al.,* 2000). However, no mention has been made in literature regarding its effect on FW of plantlets in any crop. The stimulatory impact of cefotaxime on FW may be associated with the inhibition of endogenous microbial growth (Yepes and Aldwinckle, 1994). There was a decrease in FW of plantlets in all the genotypes with 750 mg/l cefotaxime (Table 5). Thus, cefotaxime addition in the medium beyond an optimum concentration is undesirable.

Results in Table 6 show that variety x media interactions were non-significant at 5% level, whereas, variety MS and media MS were significant at 1% level with CDs being 20.32 and 23.47, respectively. This implied that there was a significant difference in the varieties with respect to average fresh weight of pantlets using different levels of cefotaxime in the media. Comparing the efficiency of different doses of the antibiotic thus used, 500 mg/l of cefotaxime was found to be the best for improving fresh weight of pantlets.

Table 6. Analysis of variance of data in Table 5 from a 3 x 4 factorial experiment in a CRD

Source	df	M.S.	F-ratio	CD (5%)	C.V.
Reps.	1	319.25	0.94		
Variety (A)	2	3732.00	10.93**	20.32	
Cefotaxime (B)	3	9162.03	26.84**	23.47	
A x B	6	670.72	1.97	NS	
Error	11	341.32			6.57

**Significant at 1% level

The addition of cefotaxime in the culture medium also boosted up the photosynthetic machinery of sugarcane plants, as they were greener than plants of the control group after 2 weeks of transfer. Enough evidence for this is provided by Zaghmout and Torello (1992), who obtained more number of green plants when ryegrass calli were treated with 60 mg/l cefotaxime for 6 weeks prior to placement on regeneration medium. Without cefotaxime pretreatment, only albino plants were produced. Therefore, during regeneration of plants from relatively older calli for induction of somaclonal variation in sugarcane, use of cefotaxime in the shoot regeneration medium can give a high frequency of green and healthy shoots or somaclones. So, cefotaxime in somatic tissue culture may be acting at the level of chlorophyll synthesis. Further studies are required to know its actual mechanism of action. Cefotaxime did not inhibit *in vitro* root induction, multiplication and elongation in the regenerated sugarcane shoots as equally good rooting was observed in shoots on both

Table 5. Effect of antibiotic cefotaxime on average fresh weight of pantlets of sugarcane varieties after 2 weeks of culturing

Cefotaxime (mg/l)	Average fresh weight of pantlets (mg)								
	CoJ 83			CoJ 85			Co 89003		
	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean
Control	240.70*	237.50	239.10	229.50	232.00	230.75	242.40	235.00	238.70
250	300.30	300.10	300.20	241.00	300.10	270.55	252.50	288.20	270.35
500	380.00	340.90	360.45	278.90	300.50	289.70	330.90	358.00	344.45
750	300.00	289.40	294.70	244.20	230.00	237.10	290.10	306.40	298.25

*Each figure is a mean of 50 shoot cultures

the media. However, in tobacco, cefotaxime has been reported to inhibit rooting of shoots regenerated from leaf discs, but had no effect on rooting of shoots regenerated from cotyledons (Nauerby *et al.,* 1997).

Conclusions

In conclusion, cefotaxime (250-500 mg/l) has a growth promoting activity in sugarcane tissue culture as demonstrated by enhanced growth in terms of number of microtillers per shoot culture, shoot length and FW of plantlets in the present investigation. Thus, during micropropagation, induction of somaclonal variation in callus cultures and genetic transformation of sugarcane, cefotaxime can augment shoot multiplication and elongation and regeneration of green shoots to a significant extent. Owing to positive effect of antibiotic cefotaxime on shoot multiplication and elongation, it can be routinely used in sugarcane tissue culture.

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