Sugar cane buds as an efficient explant for plantlet regeneration

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Abstract

An efficient and reproducible protocol for regeneration of plantlets at a high frequency was developed by using sugar cane buds. Disinfected buds were firstly submerged in ethanol sodium hypochlorite solution with 0.1 % polyvinyl-pyrrolidone, 1.5 % ascorbic acid and 1.75 % citric acid as antioxidants and subsequently treated with solution of agrimicin:captan (1:1). The upper stalk segment was better to obtain bud *in vitro* culture compared to lower segments. The medium for induction of multiple shoots consisted of Murashige and Skoog basal medium (MS) supplemented with 2 mg dm^{-3} thidiazuron and 1 mg dm^{-3} naphthalene acetic acid. An average of 24 shoots per bud was obtained for cv. Mex 68-P23 within four weeks and 29 shoots for cv. MY 55-14 within six weeks. Indole-3-butyric acid induced more roots in both cultivars compared to the untreated plantlets. Plantlets transferred to soil showed normal growth with up to four axilliary buds in each node. It was concluded that the germplasm obtained through the above mentioned technique generated stalks with more buds in each node which would give farmers more vegetative material for plantations in field with 100 % germination.

Additional key words: bud culture, indole butyric acid, naphthalene acetic acid, *Saccharum* spp., thidiazuron.

Introduction

Farmers obtain seed-cane from clonal propagation of sugar cane using bud culture. The main problem, however, related to this technique is bud disinfection (Moutla and Dookum 1999), explants oxidation and the limited number of plants obtained per bud (Jaime-Gomez 1994). Bud position in the sugar cane stalk is a main factor to obtain *in vitro* cultures that produce less phenolic and oxidant compounds as reported for *Aesculus hippocastanum* (Ćalić *et al.* 2003/04). Jaime-Gomez (1994) tried different concentrations of naphthalene acetic acid (NAA) to increase plants per buds, but this treatment was not successful. Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) (TDZ) has been used to increase the number of plants obtained from *in vitro* cultures of *Hordeum vulgare* (Ganeshan *et al.* 2003), *Lilium* spp. (Bacchetta *et al.* 2003), *Zantedeschia albomaculata* (Chang 2003), *Epipremnum aureum* (Qu 2002), *Stachys sieboldii* (Li 2002), *Vigna radiata* (Amutha *et al.* 2003), *Cajanus cajan* (Singh *et al.* 2003), *Cimicifuga racemosa* (Lata *et al.* 2002) and *Saintpaulia ionantha* (Mithila *et al.* 2003). However, TDZ sometimes shows negative effects. Zhao *et al.* (2003/04) found difficulties in subculturing *Sophora flavescens* and the rooting of regenerated shoots. The remnant TDZ in the regenerated shoots probably inhibited root organogenesis. In some plants, larger shoot proliferation was obtained when TDZ was supplemented with NAA at different concentrations (Bedir *et al.* 2003, Cui and Ezura 2003), but no information exist about shoot induction of sugar cane from buds culture with TDZ + NAA. Indole-3-butyric acid (IBA) was used successively in different plantlets to promote shoot rooting (Shyamkumar *et al.*

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Abbreviations: BA - 6-benzyladenine; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; NAA - naphthalene acetic acid; HS - humic substances; TDZ - thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea).
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2003/04), while humic substance (HS) has been used to stimulate plant germination and root growth (Masciandaro *et al*. 2002).

The objective of the present work was to develop an efficient protocol to obtain plantlets from buds of two commercial available sugar cane cultivars using TDZ + NAA for shoot induction and IBA and HS for shoot rooting. Plantlets were acclimatized and their growth monitored in the field.

Materials and methods

Two sugar cane (*Saccharum* spp.) cultivars (Mex 68-P23 and MY 55-14) were obtained from the La Fe sugar factory in Venustiano Carranza (Chiapas, México). Stems were collected from 10 - 12 months old plants. Axilliary buds were obtained with a scoop and washed with commercial soap. Three different treatments were applied to disinfect buds. In a first treatment, the buds were submerged in 70 % ethanol for 30 s, in $HgCl_2$ (0.1 %) for 10 min and washed four times with sterile distilled water. The buds were then immersed in 3 % (m/v) aqueous calcium hypochlorite suspension for 25 min and washed four times with sterile distilled water. In a second treatment, the buds were treated in the same way, but 0.1 % polyvinylpyrrolidone, 1.5 % ascorbic acid plus 1.75 % citric acid were added as antioxidants to the sterile distilled water for the last washes. In the third treatment buds were first immersed in agrimicin 500 (0.5 % m/v) - captan (0.5 % m/v) solution (1:1), placed on an orbital shaker (120 rpm) for 10 min and then treatment two was repeated. Ten buds were incubated in 150 cm^3 bottles containing 30 cm^3 Murashige and Skoog (1962; MS) medium. Eight bottles were used per treatment and incubated under cool white fluorescent light (irradiance of 45.8 $\mu mol\ m^{-2}\ s^{-1}$), 12-h photoperiod, and temperature of 26 - 28 ºC for one month. Percentage of aseptic buds was defined as the number of buds that showed no contamination after four weeks of culture divided by the total buds placed in each bottle. Blacking of the white explants in the culture medium indicated phenolization and was also expressed on a percentage basis. The percentage of oxidation was calculated as the number of buds with necrotic tissue divided by the total amount of buds placed in each bottle.

Sugar cane stalks of cultivars Mex 68-P23 and MY 55-14 were divided in three segments: upper, middle and lower and buds were obtained of each segment. Buds were disinfected as described earlier and ten buds were placed on 30 cm^3 MS medium in 150 cm^3 bottles under aseptic conditions. Eight bottles were used per treatment, incubated under above mentioned conditions for two weeks and percentage of aseptic buds, phenolization and oxidation was determined.

The effect of different combinations of NAA+TDZ was investigated in a completely randomised design (eight bottles per treatment) (Table 1). Buds were kept in culture until shoots formation and shoots formed per bud and height of shoots were determined.

Different combinations of IBA+HS, known to improve rooting efficiency (Martin *et al*. 2003), were also tested (Table 2). Number of roots formed per plantlet and length of roots were determined. Plantlets were transferred to disinfected soil in *Unicel* pots and covered with plastic cover, irrigated every three days for one month. The plants were planted in the field and normal management practices for water and fertilizer were applied (Gutiérrez-Miceli *et al*. 2004). Buds in each node were counted after four months. The vegetative capacity of each bud was then tested by cultivating them in soil.

All statistical analysis were performed using the statistical package *SAS* (version 6, SAS Institute 1989).

Results

In the first treatment, only 30 % aseptic buds were obtained after two weeks, and after four weeks, all buds died due to oxidation and phenolization (Table 3). In the second treatment with antioxidants added to the media culture, the amount of aseptic buds had increased to 70 %. However, phenolization and oxidation was high. The third treatment with a captan-agrimicin pre-treatment gave the best results.

The place in the sugar cane stalk where the buds were derived from was a factor that affected the percentage of aseptic buds, phenolization and oxidation (Table 4). Buds derived from the upper segment were 100 % aseptic with lowest values for phenolization and oxidation, while those of the lower stalk segment were 100 % infected with 100 % phenolization and oxidation.

Table 1. The number of shoots per bud and shoot height of two sugar cane cultivars cultivated on MS medium with different concentrations of NAA and TDZ [mg dm^{-3}] for four weeks. Means ± SE, n = 8.

NAA	TDZ	Mex 68-P23		MY 55-14	
		shoot number [bud^{-1}]	height [cm]	shoot number [bud^{-1}]	height [cm]
1	1	4 ± 2	4.0 ± 0.5	6 ± 1	4.2 ± 0.4
1	2	24 ± 4	4.2 ± 0.6	29 ± 5	4.4 ± 0.5
1	3	11 ± 1	4.2 ± 0.4	15 ± 3	4.3 ± 0.3
0	1	12 ± 2	3.9 ± 0.4	14 ± 2	4.5 ± 0.4
1	0	2 ± 1	4.1 ± 0.5	3 ± 1	4.4 ± 0.4

After four weeks, buds from cv. Mex 68-P23 gave 24 shoots per bud for the 1:2 NAA:TDZ ratio, but < 12 shoots per bud for the other NAA:TDZ ratios tested (Table 1). The mean height of the shoots was 4.2 cm for the 1:2 NAA:TDZ ratio. After six weeks, buds of cv. MY 55-14 gave 29 shoots per bud for the 1:2 NAA:TDZ ratio, but < 15 shoots per bud for the other NAA:TDZ ratios. The average height of the shoots was 4.2 cm for the 1:2 NAA:TDZ ratio.

Table 2. The number of roots per plantlets and root length of two sugar cane cultivars cultivated on MS medium with different ratios of IBA and HS for four weeks. Means ± SE, $n = 16$.

IBA	HS	Mex 68-P23 root number [plantlet^{-1}]	length [cm]	MY 55-14 root number [plantlet^{-1}]	length [cm]
0	0	6 ± 2	3.0 ± 0.4	6 ± 1	2.7 ± 0.2
0	1	12 ± 4	4.7 ± 0.5	4 ± 1	1.5 ± 0.1
0	3	6 ± 1	4.0 ± 0.4	8 ± 2	2.3 ± 0.2
0	9	6 ± 2	5.3 ± 0.5	10 ± 3	5.7 ± 0.4
1	0	17 ± 5	3.0 ± 0.6	13 ± 3	3.8 ± 0.2
3	0	11 ± 3	3.7 ± 0.4	14 ± 2	4.0 ± 0.2
9	0	12 ± 4	5.0 ± 0.5	17 ± 4	4.5 ± 0.3
$LSD_{0.05}$		6	2.1	6	1.9

IBA induced more roots per shoot for both cultivars than humic substances (HS) (Table 2). Application of 9 mg HS dm^{-3} significantly increased root length compared to the untreated plantlets in both cultivars, but not IBA. Survival of rooted plants *in vivo* was > 80 % when plantlets were transferred first to the culture room for 4 weeks, and then transferred to disinfected soil. In the nursery, the plantlets developed more shoots and four plantlets were obtained after one month. Plantlets then transferred to the field, grew normally and large amount of stalks had two, three or four axillary buds in each node (Table 5). The vegetative capacity of each bud was 100 % (no data shown).

Table 3. Percentage of aseptic buds, phenolization and oxidation [%] obtained after three disinfection treatments. Mean for cultivars Mex 68-P23 and MY 55-14 after two weeks of incubation.

Treatment	Aseptic buds	Phenolization	Oxidation
1	30	100	100
2	70	50	60
3	97	4	1

Table 4. Influence of bud position in the sugar cane stalk on the percentage of aseptic buds, phenolization and oxidation [%] obtained after the treatment 3. Mean for cultivars Mex 68-P23 and MY 55-14 after two weeks of incubation.

Bud position	Aseptic buds	Phenolization	Oxidation
Upper	100	4	3
Middle	100	50	55
Lower	0	100	100

Table 5. Bud number of sugar cane micropropagated plants obtained for bud culture (MP) and plants obtained from vegetative cuttings grown for four months in the field (VC). Means ± SE, $n = 20$.

		Nodes with 2 buds	3 buds	4 buds
Mex 68-P23	VC	0	0	0
Mex 68-P23	MP	50 ± 6	20 ± 3	7 ± 1
MY 55-14	VC	0	0	0
MY 55-14	MP	52 ± 7	19 ± 5	10 ± 3

Discussion

Micropropagation from axillary buds *in vitro* is difficult as pathogen contamination often occurs (Moutla and Dookum 1999). Several methods have been described to overcome this problem. Mercury chloride and sodium hypochloride were used to sterilize olive buds and antibiotics were added to the culture media (Zacchini and De Agazio 2004). The procedure in which sugar cane buds were immersed in captan-agrimicin solution gave the least contamination, oxidation and phenolization. Bud disinfection is a very important step not only to obtain aseptic cultures, but also to avoid production of phenolic compounds. Moutla and Dookum (1999) used hot water treatments to obtain aseptic buds. However, bud tissues were damaged and produced phenolic and oxidant compounds. Immersing buds in captan-agrimicin solution was presumably less drastic than submerging them in hot water and consequently the buds did not produce phenolic and oxidant compounds. The use of 0.1 % polyvinylpyrrolidone, 1.5 % ascorbic acid and 1.75 % citric acid was very effective. Similar results were obtained with young cashew trees (Thimmappaiah and Sadhana 2002).

Buds from the upper segment are younger and grow faster so produce less phenolic and oxidant compounds (Ćalić *et al*. 2003/04) as found in the experiment reported here. Similar results were also reported for the medicinal plant *Hypericum brasiliense* (Abreu *et al*. 2003/04). They used apical buds from plants at different development stages and apical buds from juvenile plants grew faster compared to buds from adult plants.

All NAA:TDZ ratios induced shoots proliferation. However the 1:2 ratio induced more shoots per bud than the other ratios. None of TDZ concentrations tested did induce other changes. Buds are more organized than other explants used, so the morphogenic action of TDZ was limited (Mok *et al*. 1982, Thomas and Katterman 1986). Maybe TDZ induced accumulation of purines stimulating cell division (Capelle *et al*. 1983, Malik and Saxena 1992). The effects of TDZ were very specific as has been reported for leaf and petiole explants of African violet (Mithila *et al*. 2003). At concentrations below 2.5 µM, TDZ induced shoot organogenesis, whereas at higher doses (5 - 10 µM) somatic embryos were formed (Saxena and Malik 1992, Lantcheva *et al*. 1999). These responses were presumably due to changes in cytokinin metabolism (Mok *et al*. 1982) as TDZ stimulates the conversion of cytokinin nucleotides to nucleosides (Thomas and Katterman 1986) and phenylurea inhibits cytokinin oxidase (Laloue and Fox 1989). These biochemical changes should stimulated macroscopic changes in the buds, for example promoted bud opening and increased fresh and dry masses (Kapchina-Toteva and Stoyanova 2003).

The protocol presented here was very efficient as more shoots were formed per bud compared to values reported in literature for sugar cane and other plants. Jaime-Gomez (1994) reported an average of only 2.4 shoots per bud for sugar cane, while in this study 24 shoots per but were formed with Mex 68-P23 and 29 with MY 55-14. Martin *et al.* (2003) reported 8.3 shoots per bud for *Wedelia chinensis*, a medicinal plant. Li (2002) reported 9.1 shoots per explant for *Stachys sieboldii*, a traditional Chinese medicinal plant and vegetable, when cultured on MS medium containing 1.0 mg dm^{-3} TDZ after four weeks of incubation. Hiregoudar *et al*. (2003) induced axillary shoot proliferation of *Feronia limonia* on MS medium supplemented with BAP, kinetine and TDZ and obtained a maximum number of 11.3 shoots per explant. A shoot regeneration of 12.3 shoots per explant was achieved by Cui and Ezura (2003) with stem explants of *Nemesia strumosa* using MS medium containing 0.75 mg dm^{-3} TDZ and 0.1 mg dm^{-3} NAA. Over 20 shoots per 0.3 g (f.m.) of cell clusters was obtained with the liliaceous ornamental plant *Hosta sieboldiana* on media containing 0.1 mg dm^{-3} NAA and either 1 or 5 mg dm^{-3} TDZ (Saito and Nakano 2002).

The time for shoot development of sugar cane cv. Mex 68-P23 was similar as for *Epipremnum aureum*. Qu *et al*. (2002) reported shoot regeneration from leaf and petiole explants after four weeks. The response time was different for the two cultivars. Mex 68-P23 developed shoots after 4 weeks whereas MY 55-14 needed two weeks more. Differences might be attributed to genotype. In the field, Mex 68-P23 also has a more rapid stem growth than MY 55-14. Differences in shoots formation of different cultivars have been reported for other plants, such as for lily (Bacchetta *et al*. 2003). The rooting rate of sugar cane plantlets treated with 1.0 mg IBA dm^{-3}, *i.e*. 11 roots per shoot, was larger than that of *Terminalia chebula* plantlets (5.5 roots per shoots), treated with the same amount of IBA (Shyamkumar *et al*. 2003/04).

It was concluded that the germplasm obtained through the above mentioned technique generated stalks with more buds in each node which would give the farmers more vegetative material to plant in the field with 100 % germination.

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