Development of a system for rapid plant regeneration from in vitro *sugarcane* (Saccharum officinarum *L.) meristematic tissue*

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RESEARCH

ABSTRACT

In this report we describe a methodology for rapid sugarcane shoot regeneration using meristematic tissue from *in vitro*-grown plantlets. Shoot regeneration from previously induced meristematic tissue, in the presence of up to 22.62 μM of 2,4-D was evaluated. The explants were transferred, after a week induction, to a propagation medium supplemented with activated charcoal and then transferred to a regeneration medium for shoot development. The highest regeneration efficiency, averaging 9.34 shoots/explant, was obtained from cut meristematic tissue previously induced in the presence of 4.52 μM 2,4-D. We evaluated the first five stem segments from the basal stem to the leaves in two Cuban cultivars to determine their capacity for plant regeneration. The largest number of regenerated plantlets was obtained from the first two segments of the basal stem. For the first segment the regeneration frequency was 16.95 and 8.0 shoots/explant for C1051-73 and C86-12 varieties respectively. We concluded that this procedure is useful for the regeneration of large amounts of sugarcane plants thus minimizing production time.

Key Words: auxin, callus, monocot species, phytohormone, somaclonal variation, tissue culture

Biotecnología Aplicada 2006;23:22-24

RESUMEN

Desarrollo de un sistema para la regeneración rápida de plantas a partir de tejido meristemático de caña de azúcar (*Saccharum officinarum* L.) cultivada *in vitro*. En este reporte describimos una metodología para la regeneración rápida de plantas de caña de azúcar utilizando el tejido meristemático de plántulas cultivadas *in vitro*. Fue evaluada la regeneración de brotes a partir del tejido meristemático inducido previamente en presencia de 2,4-D hasta 22.62 μM. Los explantes fueron transferidos, después de una semana a medio de propagación que contenía carbón activado, y después se transfirieron a medio de regeneración para el desarrollo de los brotes. La mayor eficiencia de regeneración como promedio fue de 9.34 brotes/explante, a partir del meristemo segmentado, previamente inducido en medio con 4.52 μM de 2,4-D. Nosotros evaluamos los primeros cinco segmentos del tallo desde la base del tallo hacia las hojas en dos variedades cubanas con el objetivo de determinar su capacidad de regenerar plantas. El mayor número de plántulas regeneradas fue obtenido de los dos primeros segmentos de la base del tallo. Para el primer segmento la frecuencia de regeneración alcanzó 16.95 y 8.0 brotes/explante en las variedades C1051-73 y C86-12, respectivamente. Concluimos que este procedimiento es útil para la regeneración de gran cantidad de plantas de caña de azúcar minimizando el tiempo de producción.

Palabras claves: auxinas, callos, cultivo de tejido, especies monocotiledóneas, fitohormonas, variación somaclonal

*I*ntroduction

Sugarcane *(Saccharum officinarum* L.) is an important crop for many tropical and subtropical countries. Properties such as an efficient photosynthesis and efficient biomass production make this graminae, an excellent target for industrial processing and a valuable alternative for animal feeding and the production of by-products.

The application of biotechnology for the genetic improvement of traits in sugarcane could have a significant impact on agricultural yields and industrial production. However, the development of genetic engineering methodologies based on the appropriate tissue culture techniques to obtain genetically improved varieties is not as yet a routine process. Early reports demonstrated that sugarcane regeneration from callus cultures take place either by organogenesis [1, 2] or somatic embryogenesis [3, 4] upon prolonged callus culture. This long procedure is not particularly desirable during the genetic transformation. Currently, several new approaches to regenerate sugarcane plants without passing through the development of a callus mass have been reported [5, 6]. These new strategies lead to the formation of large amounts of plants from immature inflorescences [5] and buds [6], thus minimizing the probability of somaclonal variation. Although the regeneration from spindle segments have been exploited widely because of the high frequency of callus formation and the efficient plant regeneration obtained from them, the potential of these explants to rapidly regenerate minimizing callus formation has not yet been tested. These explants are excellent in producing prolific callus masses which are the basal segment of young leaves (spindle), although calluses can be obtained from almost any sugarcane tissue [3]. Spindle segment cultures on a Murashige and Skoog (MS) [7] medium supplemented with high auxin concentrations can generate three types of calluses. Only Type II is good for an efficient conversion to plants [8].

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In the present paper, we report a simple and efficient system for shoot regeneration from sugarcane meristematic tissue carried out in a short period of time. Here we showed that no callus development-propagation is needed to generate large amounts of plants from single segments of *in vitro* propagated plants.

Materials and methods

Plant material

Conditions for the aseptic culture of sugarcane cultivars C1051-73 and C86-12 on the MS basal medium [7] were established as previously described [9, 10]. The plant material was sterilized in a 1.5% NaClO solution for 20 min. Spindle sections from 6-month-old field-grown plants were used to obtain *in vitro* plants from the above mentioned commercial cultivars. The aseptic culture of meristematic tissues until the formation of whole plants followed a standard procedure [10].

The composition of the culture medium used here is described in table 1. The components were purchased from SIGMA.

Table 1. Composition of the media for callus induction and plant regeneration of sugarcane cv. C1051-73 and C86-12

Medium	Composition
PM [3]	MS salts, 1mg l-1 nicotinic acid, 0.8 mg l-1 vitamin thiamine, 0.5 mg l-1 vitamin pyridoxine,100 mg l-1 myo-inositol, 20 g l-1 sucrose, 7 g l-1 Phytoagar
P+N	MS salts, 1 mg l-1 nicotinic acid, 0.8 mg l-1 vitamin thiamine, 0.5 mg l-1 vitamin pyridoxine, 100 mg l-1 myo-inositol, 20 g l-1 sucrose, 500 mg l-1 casein hydrolysate, 8 g l-1 Phytoagar, supplemented with 2,4-D
PM- AC	MS salts, 1 mg l-1 nicotinic acid, 0.8 mg l-1 vitamin thiamine, 0.5 mg l-1 vitamin pyridoxine, 100 mg l-1 myo-inositol, 20 g l-1 sucrose, 7 g l-1 Phytoagar, 0.5% activated charcoal
RM	MS salts and vitamins, 30 g l-1 sucrose, 2 mg l-1 Kinetin, 1.3 mg l-1 IAA, 8 g l-1 Phytoagar
RM-AC	MS salts and vitamins, 30 g l-1 sucrose, 2 mg l-1 Kinetin, 1.3 mg l-1 IAA, 8 g l-1 Phytoagar, 0.5% activated charcoal

PM: propagation medium; RM: regeneration medium; P^{+N} basal medium (N= 4.5, 9.0, 13.5, 18.0 or 22.6 μM 2,4 -D); AC: activated charcoal

Callus formation after a short induction by 2,4-D

Five concentrations of 2,4-D (4.5, 9.0, 13.5, 18.0 and 22.6 μM) were tested for callus initiation over a one-week culture period. The stems from *in vitro* plantlets were cut into five pieces (about 2-3 mm each) and the explants were classified from 1 to 5, according to their position on the stem from the radicle to the leaves (Figure 1). They were separately transferred to Petri dishes containing 25 ml of the basal medium P^{+N} [7] with different 2,4-D concentrations, and cultivated in the dark (Table 1).

Regeneration

After a week of callus induction in the presence of 2,4-D, the explants were transferred to a propagation medium supplemented with activated charcoal (PM-AC) or a regeneration medium with activated charcoal (RM-AC) (see Table 1), and incubated for another week at 25 °C under a 16 h photoperiod of 2000 lux day intensity. Then, the explants were transferred to regeneration medium (RM) until shoot formation. After 15 days on RM, the number of shoots per explant was counted and the efficiency of the different combinations was analyzed. All shoots were classified into three size categories: 2, 5 and 7 mm or more. The regeneration ability from the different stem positions was also evaluated.

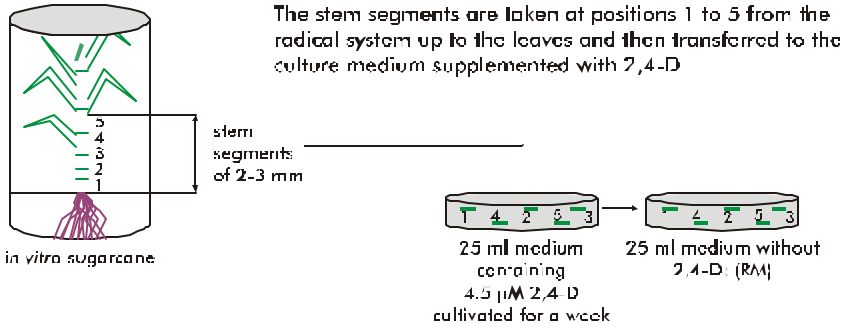

Figure 1. Diagram representing the general procedure for testing the influence of the position of the sugarcane stem on tissue culture response.

Transformation into plants, rooting and propagation

After two weeks in RM, the regenerated plants were transferred to the propagation medium (PM) for growth and rooting.

Results and discussion

Callus formation

Specific genetic variations have been observed in sugarcane after prolonged *in vitro* tissue cultures [11]. To minimize this variation, which could be caused by a long culture in the presence of 2,4-D, and to improve the regeneration methodologies, different concentrations of 2,4-D were evaluated using a short exposure time of the sugarcane segments to this auxin. As a result, the explants cultured without 2,4-D turned brown within a few days, while the incubation of explants on a low-auxin-content medium resulted in callus formation in both varieties. During this stage, the small calluses were mucilaginous, soft and gummy, and composed of associated elongated cells (data not shown). In this case, the calluses were similar to non-morphogenic calluses (Type III) [3]. The frequency of callus formation from the stem segments varied in relation to the 2,4-D concentrations. The frequency of callus formation in the $P^{+4.5}$ medium (61.9 %) was greater than in the other 2,4-D levels (Table 2).

Plant regeneration

The presence of activated charcoal in the regeneration medium has a significant influence on the development of shoots *in vitro* [12,13]. Here we observed that sugarcane plantlets developed faster and some differences were observed in the regeneration efficiency on both PM and RM when activated charcoal was used. The addition of charcoal could

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Table 2. Regeneration efficiency for different 2,4-D levels on sugarcane variety C1051-73

Treatments (μM of 2,4-D)	Number of explants	Explants producing small calluses (%)	Callus producing shoots (%)	Average number of shoots per explant
0	60	0	0	0
4.5	63	61.9	79.48	9.34 [a] ± 5.3
9.0	62	45.16	57.14	4.20 [b] ±1.8
13.5	57	57.89	57.57	3.90 [b] ± 2.5
18.0	56	53.57	63.33	4.95 [b] ± 2.5
22.6	51	58.82	40.00	3.78 [bc] ± 2.2

The values represent the mean ± S.E. of 3 separate experiments. Means followed by different letters are significantly different at p=0.05 level using Duncan´s multiple range test

decrease the content of phenolic compounds and prevent auxin accumulation. PM yielded a larger number of well-developed shoots (data not shown).

Increasing the 2,4-D concentration in the callus induction medium had a negative effect on shoot formation and/or elongation. A higher 2,4-D concentration caused an increase in the mass of calluses, many of which failed to develop shoots in the regeneration medium.

The formation of very small calluses and a greater shoot production occurred in the presence of 4.5 μM 2,4-D, as described in figure 2. The percentage of calluses producing shoots and the average number of shoots per explant were 79.48 and 9.34, respectively (Table 2). For the other concentrations of 2,4-D these parameters were lower. Nonetheless, vigor and growth rate of the developed shoots were pronounced.

Influence of the position of the stem

The response of the stem segment in relation to its position was evaluated by callus induction and shoot regeneration in the RM medium with 4.5 μM 2,4-D.

The two basal positions of the stem are most appropriate for obtaining a large number of shoots, especially the one proximal to the roots. As shown in table 3 for C1051-73, the segments of positions 1 and 2 regenerate 16.95 and 6.61 shoots/explant respectively and the C86-12 segment of position 1 originated 8.0 shoots/explant. In general, the C1051-73 variety showed a greater response demonstrating the genotype dependent behavior of these varieties.

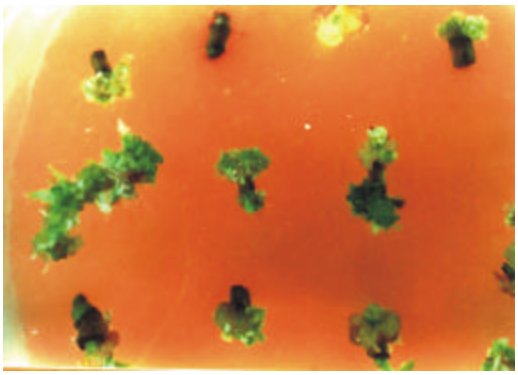

Figure 2. *In vitro* sugarcane explants produced from 4.5 mM 2,4-D. The shoots regenerated on the RM medium ten days later. (photo magnified 1x).

Table 3. Influence of stem position on callus and shoot regeneration in two sugarcane varieties

Stem Position1	Explants producing small calluses (%)		Callus producing shoots (%)		Average number of shoots per explant	
	C1051-73	C86-12	n=21 (C1051-73)	n=11 (C86-12)	C1051-73	C86-12
1	100.00	100.00	100.00	100.00	16.95 ±1.1	8.00 ±7.4
2	71.42	90.00	52.38	9.09	6.61 ±4.4	0
3	14.28	18.18	4.76	0*	0	0
4	9.52	0	0	0*	0	0
5	0	0	0	0*	0	0

The values represent the mean ± S.E. of 2 separate experiments
1 Stem position from the base to the leaves (1-5) (see Figure 1)
* Presence of roots

Transformation into plants:propagation

Rooting and leaf development in all regenerated plants were normal. We obtained 100% adaptation to the soil and the plants emerged from our procedure had a normal morphology. No differences in the adaptation between these two varieties were observed.

After this study, we routinely use this procedure to regenerate sugarcane plants. Small pieces (2-3 mm) of the basal stem from *in vitro* plantlets are kept on a $P^{+4.5}$ medium in the dark for a week. After that, the explants are transferred to PM containing 0.5% activated charcoal and incubated in the light for another week. Finally, shoot regeneration proceeds in the RM medium under photoperiod conditions.

Conclusions

All procedures for shoot regeneration and transformation previously described for sugarcane include prolonged culture periods on a medium containing 2,4-D [3, 9-10, 14-18]. In this paper we demonstrate a reliable method for shoot regeneration from explants derived from *in vitro* cultured sugarcane plantlets that involves a very short exposure period to 2,4-D and subsequently, a rapid regeneration of plants from stem segments.

Acknowledgements

We would like to thank Mrs. Milagro Ponce for her technical assistance and Mr. Jesús Seobany for the photographic service. This work was supported by the Cuban Council of State (Project number 3031-248).

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Received in october, 2005. Accepted for publication in december, 2005.