Differentiation of Sugarcane Cultivars for Green Energy Using Microscopy and Tissue Culture

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ABSTRACT

As mankind has become increasingly dependent on fossil fuel, a finite resource, significant progress has been made in developing favorable alternative methods of energy production. Sugarcane *(Saccharum* sp.) is, because of its competitiveness, a potential natural resource. By applying biotechnology to quality sugarcane varieties it is possible to develop a new array of plants with high added value including sugarcane by-products, such as fiber and wood. This research may serve as an aid in exploring novel approaches, based on structural (cell wall, epidermic, subepidermic and vascular bundles constitution) and physiological (plantlet growth rate) criteria for practical methodologies in order to select new sugarcane varieties that are not attainable by conventional tissue culture methods. The histologic analysis performed on Mex 70-485, Mex 69-290 and B 43-337 sugarcane cultivars which have been classified as having low, medium and high fiber content, respectively, allowed us to demonstrate that their fiber content is directly related to: 1) the mean number of rows of thick-wall cells that constituted the vascular bundles: 4.68, 5.68 and 7.92 in the varieties Mex 70-485, Mex 69-290 and B 43-337 respectively. The mean diameter of these vascular bundles were, in the same order: 299, 355 and 333 mm. 2) the number of epidermal and sub-epidermal layers of thick-wall cells of these varieties: Mex 70-485 showed one, Mex 69-290 two and B 43-337 had up to four layers. We did not find differences among these varieties regarding: 1) the thickness of the walls of cells forming the vascular bundles, which varied between 1.90 and 2.11 mm, and 2) the number of vascular bundles per unit area observed under light microscopy. Callus cultures established from the leaf tissue of these varieties did not show morphological differences. Plantlets regenerated from calluses from the Mex 69-290 variety grew twice as fast as the other two in the same incubation period. This finding is in agreement with the precocity of Mex 69-290 in the field.

Keywords: biomass, cell wall, epidermal and subepidermal cells, fiber, vascular bundles

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RESUMEN

Diferenciación de cultivares de caña de azúcar para energía verde usando microscopía y cultivo de tejidos. El ser humano ha venido incrementando su dependencia en el petróleo, que es una fuente finita. Afortunadamente, ciertos avances en el desarrollo de métodos alternos para la producción de energía parecen viables. Con la aplicación de la biotecnología para mejorar la caña de azúcar se pueden obtener plantas de alto valor agregado, bioproductos como fibra y madera. La caña de azúcar *(Saccharum* sp.) por su competitividad es un recurso natural potencial. Un bioensayo fue diseñado para identificar variedades o individuos de rápido desarrollo a partir de variedades que difieren en otro importante carácter como contenido de fibra. Este pudiera estar relacionado con diferencias histológicas. El análisis histológico practicado a los cultivares de caña de azúcar Mex 70-485, Mex 69-290 y B 43-337 que han sido clasificados como de bajo, medio y alto contenido de fibra respectivamente, nos ha permitido demostrar que el contenido de fibra está directamente relacionado con: 1) el número promedio de hileras de células con pared gruesa que constituyen los haces vasculares: 4,68; 5,68 y 7,92 en las variedades Mex 70-485, Mex 69-290 y B 43-337, respectivamente. El diámetro promedio de estos haces vasculares, en el mismo orden son: 299, 355 y 333 mm. 2) El número de capas de células epidérmicas y subepidérmicas con pared celular engrosada que presentan estas variedades: Mex 70-485 mostró una, Mex 69-290 dos y B 43-337 hasta cuatro capas. No se encontraron diferencias respecto a: 1) el grosor de la pared celular de las células que constituyen los haces vasculares, que osciló en estas variedades entre 1,90 y 2,11 mm, y 2) el número promedio de haces vasculares por campo visual en el microscopio de luz. Los cultivos de callos establecidos a partir de tejido foliar de estas variedades no mostraron diferencias morfológicas. Las plántulas regeneradas de callos de la variedad Mex 69-290 tuvieron desarrollo más rápido que las otras dos variedades, lo que concuerda con el carácter de precocidad de esta variedad en el campo.

Palabras claves: biomasa, células epidérmicas y subepidérmicas, fibra, haces vasculares, pared celular

*I*ntroduction

Mankind for millennia has been burning biomass as fuel. Thermal conversion procedures to transform biomass into fuel are currently under development so as to reduce both our reliance on fossil fuels [1–3], that are finite resources, and the discharge of carbon dioxide in the atmosphere. Biomass is formed during plant photosynthesis through the combination of carbon dioxide and water to produce carbohydrates which constitute the building blocks of biomass. Roughly, the chemical composition of biomass is 75% carbohydrates and 25% lignin. Sugarcane has been commercially grown for sugar production and varieties have been selected specifically for high yields. The production of sugar and electricity from bagasse will likely increase in some countries due to the fact that it represents a massive amount of a renewable energy resource [1, 4]. Bioconversion and thermal conversion techniques for transforming biomass into fuel have been successfully applied by several countries [3–6] for which reason sugarcane bagasse will be economi-

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cally viable in the near future. The new sugar industry must consider a wider array of by-products and improve the stages of the process, starting with land productivity and ending with high sugar yields and a wide range of co-products: oxygenated fuels, molasses for the production of yeast, citric acid, aconitic acid, the production of livestock feeds, the cogeneration of energy and xylitol based on bagasse, the production of charcoal and activated carbon from boiler ash pit and other products giving added values. According to this vision, parallel efforts must come from sugarcane breeders and biotechnologists in order to obtain new varieties to enable the revival of the industry. Plant tissue culture may help plant breeders obtain genotypes in different ways, which would be more difficult to attain by conventional methods. Liu and Chen [7] performed histological studies to determine the origin and process of plantlet redifferentiation from calluses, while Nadar *et al.* [8] studied the fine structure of the sugarcane embryogenic callus. Bonnel *et al.* [9] demonstrated that in sugarcane calluses the first anatomical modifications observed along the redifferentiation process was the appearance of non-lignified fibers. The involvement of cortical microtubules in wood formation has been demonstrated in Angiosperm plants [10]. Preliminary results on the use of histological differentiation of sugarcane varieties according fiber content and the novel use of plant tissue culture in sugarcane to differentiate precocious sugarcane and non precocious varieties are presented in this study. Here we show the results of a bioassay designed to identify fast growing sugarcane varieties or individuals with properly balanced high sugar recovery and fiber yield as well as the precocious ones, based on their *in vitro* growth rate potential and histological differences. The success of these selection procedures in the coming years will be crucial to determine the extent of the adoption of biomass energy technologies.

Materials and Methods

Three varieties of *Saccharum* sp. used in this preliminary study were selected according to their fiber content which has been classified as: low, Mex 70-485 (12.0%); medium, Mex 69-290 (12.5%) and high, B 43-337 (15.5%) [11].

Plant tissue cultures

Sterile young 3 mm thick leaf rolls from the region above the apical meristem were used to establish plant tissue cultures. The explants were placed on the MS3 solid medium which contained the Murashigue and Skoog [12] inorganic compounds and (in amounts per litre): inositol, 100 mg; thiamine-HCl, 1 mg; 2,4-dichlorophenoxiacetic acid (2,4-D), 3 mg; sucrose, 30 g; coconut milk, 180 mL; agar, 7.5 g and the pH was adjusted to 5.8. Calluses were subcultured every 30 days on the MS3 medium. Shoot induction and plant micropropagation was attained when the calluses were transferred onto the MS0 medium, the MS3 medium without 2,4-D. All cultures were incubated at 26–29 ºC and 16 h of light daily. In order to determine the morphogenetic activity of the calluses, three independent experiments were performed using 80 day-old calluses. After a month of culturing on MS0, the number of points where shoots emerge were determined in 10 cultures per variety per experiment, each week.

Histological studies

For histological studies, tissue samples were obtained from the middle of a basal internode (the samples were obtained from the same internode position) of the stem from 8 to 10 month old field grown plants.

Fixation, inclusion and staining

Transverse 3-mm thick segments of internodes from the basal part of the stalk and 2 x 4 x 4 mm pieces of callus tissue were fixed in a CRAF III solution for 24 h. The fixed samples were dehydrated, using a device that automatically changed the samples and embedded them in paraffin. They were then sectioned into 8-μm segments and stained with safranin-fast green. At least 50 visual fields were analyzed per variety, using a Carl Zeiss model 67791 light microscope, to determine: 1) the epidermic and subepidermic region, 2) the number of vascular bundles per visual area, 3) the diameter of the vascular bundle (mm), 4) the number of layers of thickened wall cells and 5) thickness (mm) of the walls of cells forming the vascular bundles.

The data were analyzed through PROC GML of SAS [13] in order to determine statistical significance ($\mu = 0.05$) and the correlation between treatments.

Results and Discussion

These are preliminary results on a procedure that may be used to identify fast growing sugarcane varieties or individuals, based on their *in vitro* growth potential, through a difference that may be related to histological differences in their fiber content.

Histological analysis

The morphological differences existing in differentiated stem tissues of field grown sugarcane plants from varieties that differ in fiber content were determined. One of them, Mex 69-290 covered 23.8% of the sugarcane area in México in 1999. Light microscopy examination of the transversely sectioned internodes from the basal part of the stem revealed the following characteristics.

Epidermic and subepidermic region. The epidermic cells of the three varieties presented thick cell walls. The Mex 70-485 variety showed, in addition to the epidermic cell layer, a row of subepidermic cells with thick walls and the B 43-337 variety had up to four rows of subepidermic cells with thick walls (Figures 1A–C).

Vascular bundles. Under light microscopy, the number of vascular bundles per visual area, the number of rows of thickened cell walls forming them, their diameter as well as their cell wall thickness (Table) were determined. According to these results, no differences were observed in either the number of vascular bundles per visual field, which varied between 1.6 to 1.9, and the wall thickness of cells forming the vascular bundles. The mean thickness estimated in mm were: 1.90 (B 43-337), 2.00 (Mex 70-485) and 2.11 (Mex 69-290).

Significant positive correlations ($P \geq 0.05$) were found between: the number of rows of cells forming the vascular bundles as well as their diameter, with the fiber content of the varieties. Among the parameters stud-

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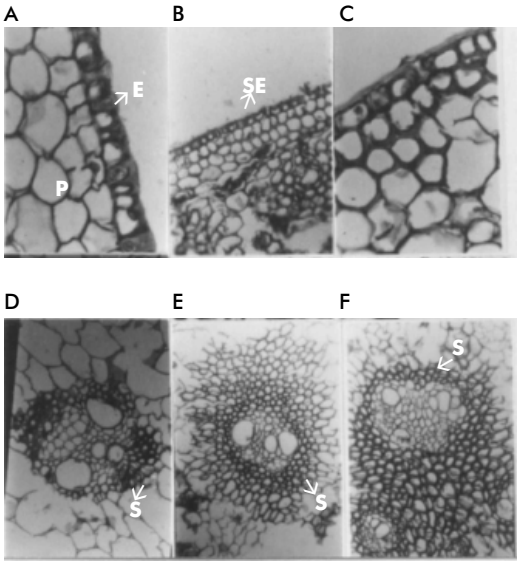

Figure 1. Comparison of epidermal tissues and vascular bundles of sugarcane stems from varieties Mex 70-485 (A, D), Mex 69-290 (B, E) and B 43-337 (C, F) respectively. E, epidermis; P, parenchyma; SE, subepidermis; S, sclerenchyma.

ied, the number of rows of cells forming the vascular bundles and their diameter showed positive correlations with values of 0.70, 0.72 and 0.80 for Mex 69-290, Mex 70-485 and B 43-337 respectively. When the pooled data of the three varieties were considered, the same situation was observed: the diameter of the vascular bundle and the number of rows of cells forming them are highly correlated (r = 0.69). The number of vascular bundles per visual field showed the highest variation (C. V. = 45.86%) which contrasted with the number of rows of cells forming the vascular bundles (C. V. = 21.97%). In Figures 1D–F we show the hystological aspects indicated. These results allow us to demonstrate that the parameters analyzed in the three varieties are related to the fiber content of the plants. Therefore, the histological criteria may be an alternative procedure to determine the fiber content in field-grown plants.

Micropropagation

Callus induction. During the first three months of *in vitro* culture on the MS3 medium, the leaf tissue explants from these varieties suffered a drastic transformation into calluses. Under light microscopy, two kinds of cytodifferentiation zones were detected in all microscopic fields of the callus samples analyzed: 1) areas with densely stained small cells, that are characteristic in actively dividing meristematic cells and 2) areas with bigger and less stained cells, that resembled parenchymatous cells. The *in vitro* cultured cells from the three varieties, at this stage, showed no differential morphologic characteristics (Figures 2A–C). In Angiosperm plants, all cell types derive from fusiform cambial cells. At early stages of redifferentiation, cortical microtubules of fibres and axial parenchyma cells increase in number as secondary wall thickening proceeds. Vessel elements have been detected by marking the sites of bordered pits [10]. The analysis performed to redifferentiate plantlets from cloned calluses, may

Table. Mean values (Tukey) obtained in the sugarcane varieties indicated for the traits: 1) number of vascular bundles per visual area observed under light microscopy; 2) vascular bundle diameter (μm); 3) number of layers of thickened cell walls forming the vascular bundles and 4) wall thickness (μm) of cells forming the vascular bundles. The groups designated with different letters are statistically different.

Variety	Trait	N	Mean	Group	DMS	C. V.
Mex 70-485	1	50	1.90	A	0.384	45.86
Mex 69-290			1.80	A		
B 43-337			1.60	A		
Mex 69-290	2	50	355.67	A	39.67	25.44
B 43-337			333.06	A		
Mex 70-485			299.21	B		
B 43-337	3	50	7.92	A	0.634	21.97
Mex 69-290			5.68	B		
Mex 70-485			4.68	C		
Mex 69-290	4	50	2.11	A	0.229	24.09
Mex 70-485			2.00	A		
B 43-337			1.90	A		

allow us determine their fiber content. Zeier and Schreiber [14] have shown the relationship between the chemical composition of the primary and tertiary endodermal cell walls and the fine structure of five monocotyledoneous species. This information is indeed valuable in order to develop suitable selection systems.

Shoot induction. Callus cultures were transferred to the MS0 culture medium to determine their organogenic potential. After four weeks of culture, areas of shoot induction appeared in 45, 72 and 32% of the calluses from Mex 70-485, Mex 69-290, and B 43-337 respectively and the mean number of shoot induction sites per calluses were, in the same order: 2.6, 5.1 and 2.1. The Mex 69-290 variety showed both the highest percentage of calluses with shoot induction and the highest number of shoots regenerated per explant. These facts benefit this variety by expressing these highly useful traits for biotechnologists. Furthermore, a very important result was that after 60 days of culture, the regenerated plantlets from this variety had grown twice as fast as the other two sugarcane varieties (Figure 3). In this Figure we only show the redifferentiated Mex 69-290 (A) and B 43-337 (B) plantlets. Performing *in vitro* selection, we have successfully detected clear growth potential differences in early stages of

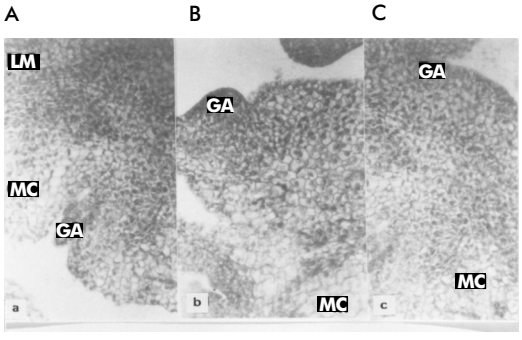

Figure 2. Aspect of callus sections under de light microscope from callus cultures established from Mex 70-485 (A), Mex 69-290 (B) and B 43-337 (C). LM, light microscopy fields; GA, growth areas; MC, mature cells.

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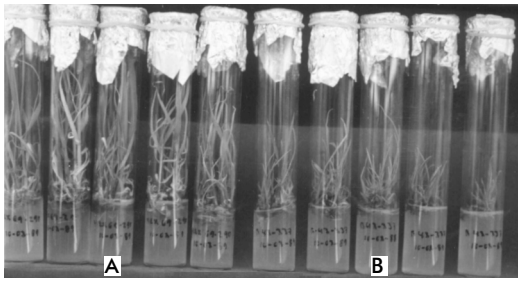

Figure 3. Aspect of *in vitro* sugarcane plantlet height of Mex 69-290 (A) and B 43-337 (B) varieties. Shoot induction was performed on the MS0 culture medium and the plantlets had been incubated for 60 days.

plantlet development; dwarf and a slow growing chrysanthemum *(Dendrathema grandiflora)* plantlets that have been identified from irradiated and ethylmethane sulphonate treated calluses (paper in preparation). In the Eucalyptus genus that includes fast-growing tree species, the influence of *in vitro* conditions on the cellular organization and the associated structural changes during plant regeneration has been investigated. The thickness of the cell wall, a dynamic structure that plays key roles in cell development and differentiation, was affected in the region of the plasmodesmata by the culture treatments [15]. On the other hand, in the development of cotton fiber, a four-stage process (initiation, elongation, secondary wall thickening, and maturation), *in vitro* culture techniques did not effect the number of fibers produced, although the *in vitro* cultured cells take longer to reach the maximum number of fibers and this experimental condition induced the formation of multicellular fibers as well [16].

In vitro selection primarily depends on spontaneous cellular mutation. In corn plants, for instance, spontaneous mutation rates at certain specific loci have been determined to be of between 0.12 and 49.2 mutations per 100 000 gametes [17]. Biotechnologists are used to manipulating *in vitro* suspension cultures that may contain up to 100 000 plant cells per milliliter. In a previous paper based on the analysis of 15 agroindustrial phenotypic traits [18] we have demonstrated the high degree of performance of 4-month-old callus regenerated plants. Histological studies showed the basic differences between highly specialized and less specialized sugarcane cells [19], and based on isozyme analysis it was demonstrated that the succession of isozymes expressed by *in vitro* sugarcane calluses are related to stages of the redifferentiation process and shoot induction potential. The presence of abnormalities was detected in the long term subcultured calluses [20]. Shoot induction was performed in the absence of exogenous regulatory compounds avoiding the problem of levels of cell sensitivity to auxins [21, 22].

Field trails. Growth rate screening trials must be performed in the field to confirm the *in vitro* results in order to select varieties for biomass energy [15, 23]. In this respect, Mex 69-290 has been recognized by farmers as a fast field-growing and a precocious variety. In a different direction, a practical and important application of plant tissue culture has been the development of a procedure for precocious flowering. Under green house conditions, at least 3 years are required after sowing *Phalaenopsis* seeds to flower development; however, under certain *in vitro* conditions, plants reach floral induction in 9-month-old shoots [24]. The delay in flowering is a major problem in the breeding and commercial production of orchids.

Conclusions

The results presented here allowed us to demonstrate that: the mean number of rows of thickened cell walls forming the vascular bundles, the mean diameter of these vascular bundles and the number of epidermal and sub-epidermal layers of thickened cell walls are related to fiber content in these varieties. The histological results offer an alternative procedure to determine the fiber content of field-grown plants.

We were able to identify fast-growing plantlets. The micropropagated plantlets from the Mex 69-290 variety grew twice as fast as the other two sugarcane varieties. In fact, Mex 69-290 has been recognized as a fast field-growing plant and a precocious variety. Growth rate screening trials must be performed in the field to confirm the *in vitro* results [16, 17].

Novel procedures using hystological data will significantly help in our understanding of cellular processes and provide clues for improving protocols to select sugarcane varieties with properly balanced high sugar recovery and fiber yield, as well as plants with enhanced photosynthetic capacity, reproductive capacity and growth rate.

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