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Antioxidants and Phenolic Secretion in Sugarcane Genotypes Shoots Culture

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Abstract

Secretion of phenolic compounds is a major limitation for sugarcane *in vitro* shoot culture, causing a loss of regenerative capacity and subsequent cell death. In this study, micropropagation and phenolic secretion of four Saccharum genotypes were evaluated in presence of different antioxidants. Aseptic cultures of *S. officinarum* (PI 184794 and PI 88652), *S. sinense* (PI 29109) and *S. robustum* (UNK R65P35) were propagated on medium containing antioxidants, citric acid (100 mg/L), L-cysteine (100 mg/L), polyvynylpirrolidone (300 mg/L) and L-glutathione (50 mg/L) in two consecutive subculture cycles. Interaction between genotypes and antioxidants was significant in both cycles. All genotypes showed good shoot formation, shoot vigor and color, except in PI 88652 which had less shoot development in both the presence and absence of the antioxidants tested. PI 184794 displayed the highest shoot proliferation in the presence of citric acid, and UNK R65P35 produced more shoots per explant in the 2nd subculture. For *S. sinense* (PI 29109), in both subcultures, most shoots were observed in the presence of polyvynylpirrolidone. Medium discoloration due to phenolic secretion was reduced in the presence of citric acid and polyvynylpirrolidone. The type of secreted phenolic compounds differed with genotype as the Principal Component Analysis of cultivation media separated PI 88652 from PI 29109 and UKN R65P35. Phenolic compounds varied in composition and were secreted at various levels as a function of genotype and antioxidant type. Loadings plots indicated the genotype and antioxidant separations were broadly driven by flavonoid compounds.

Keywords: flavonoids, genotypic response, micropropagation, *Saccharum*

1. Introduction

The *Saccharum* genus belongs to the Andropogoneae tribe of the Poaceae (Gramineae) family (Daniels & Roach, 1987). It is a perennial grass cultivated in tropical and subtropical regions of the world and the global crop production in 2014 was 1900 million metric tons (The Statistics Portal, 2017); in 2013, sugarcane ranked first in commodities (FAO, 2013).

Sugarcane is asexually propagated by stem cuttings or by plants derived by micropropagation (Lal et al., 2015; Michael, 2007). Usually, the cane stems are infected by various pathogens without exhibiting any symptoms (Parmessur et al., 2002), and the propagation rate from field-grown stems is low. In contrast, micropropagation provides a rapid multiplication of healthy sugarcane shoots once aseptic cultures are established. Besides multiplication, *in vitro* cultures are also used in detection and elimination of sugarcane virus diseases (Snyman et al., 2012) and are a preferred form for conservation of genetic resources, either by preserving shoots in slow growth conditions or by cryopreservation.

During *in vitro* organogenesis or somatic embryogenesis, browning of sugarcane culture caused by phenolic secretion can be a major limitation causing a loss of regenerative capacity and subsequent cell death. According to Ndakidemi et al. (2014), the relationship between medium chemical compounds and phenolic exudation influences substantially the intensity of medium discoloration, rooting, explant browning and tissue deterioration. In a culture establishing phase, the phenolic secretion and its oxidation can affect the culture initiation and development (Kerns & Meyer, 1986; Kumari & Verma, 2001). Thus the prevention of culture browning is essential in micropropagation of *Saccharum* plants. Studies conducted by Qin et al. (1997) indicated that sugarcane has a high content of phenolics, and their oxidation is affected by genotypes, the sources of explants and exogenous growth regulators. Similar observations were recorded for *Gossypium* spp., cotton (Ozyigit et al., 2007) and *Strelitzia reginae*, bird of paradise (North et al., 2012). Lux-Endrich et al. (2000) suggested that the composition and synthesis of phenolic compounds in plant tissue may be determined by genetic and environmental conditions.

The possibility of controlling phenolic secretion from sugarcane explants by pretreatment with solutions of ascorbic or citric acid, polyvynylpirrolidone (PVP) and cysteine or culturing the explants on medium with these substances, or with activated charcoal has been suggested (Kumari & Verma, 2001; Lorenzo et al., 2001; Huang et al., 2003; Khan et al., 2007; Lal et al., 2015; Shimelis et al., 2015). However, there is little information on tissue culture responses of various sugarcane species to the type of antioxidants used during culturing and the type of phenolic compounds secreted. Adding from 0.5 to 1 g/L PVP to medium of callus derived sugarcane culture controlled tissue browning but was variety specific (Michael, 2007). Callus derived culture might exhibit somaclonal variation and compromise genetic integrity of propagated material (Nehra et al., 1992; Skrivin et al., 1993; Sahijram et al., 2003; Bairu et al., 2006, 2008, 2011).

The National Plant Germplasm System of the United States Department of Agriculture (USDA) preserves over 400 sugarcane accessions in a clonal form as field plantings. The security backup of the field collection is done by cryopreservation of 0.6-1 mm shoot tips derived from aseptic shoot culture. The shoot cultures are established from apical fragments of field grown canes and it takes usually from 2 to 4 months (genotype dependent; 30-day subculture intervals), after contamination free shoots are obtained, to produce a sufficient number of shoots for cryopreservation. Rooting of the shoot culture is not necessary because the shoots will not be planted before cryopreservation; however, multiplication of a large number of shoots in a short time with a low benzyl aminopurine concentration is desired. Secretion of phenolic compounds and high genotypic variation in the cultivation of tissue culture affects the growth and multiplication of the shoots, and impedes the preservation of the *Saccharum* genetic resources. Limiting the phenolic occurrence in culture medium might improve shoot multiplication, and limit the number of subcultures needed to produce a large number of shoots, and also it might increase the shoot vigor that supports successful preservation of sugarcane.

The objective of this study was to evaluate in vitro sugarcane shoot performance in the presence of phenolic compounds secreted into medium with selected antioxidants and to characterize the phenolic intensity and composition in the culture medium of three sugarcane species (*S. officinarum*, *S. robustum* and *S. sinense*).

2. Material and Methods

2.1 Plant material and Shoot Cultures

Apical cane segments of four *Saccharum* genotypes were obtained from the USDA-ARS, National Germplasm Repository, Miami, FL and established aseptically *in vitro* at the USDA-ARS PAGRP Unit, Fort Collins, CO (*S. officinarum* PI 184794 and PI 88652, *S. sinense* PI 29109 and *S. robustum* UNK R65P35). After a 10 min sterilization in 70% isopropyl alcohol (1000033127, Cumberland Swan®) followed by 20% commercial bleach (6.0% NaOCl; 20 min), and three rinses in sterile water, the segments were cultured in GA-7 magenta™ vessels [WxLxH (77 ×77 ×97 mm); V8505, Sigma-Aldrich®] with 60 mL of Murashige & Skoog (1962) propagation medium (MS-519, Sigma-Aldrich®) with 20 g/L sucrose, 0.1 mg/L kinetin, 0.2 mg/L use µM for PGR n^6-benzyl aminopurine and 8.0 g/L of agar (Sigma-Aldrich® A7002) with the pH adjusted to 5.8, autoclaved for 27 min at 121 °C/22 psi. The cultures were grown in a growth chamber at 25±2 °C with a 16-h photoperiod with 50 mol/m^2/s light intensity provided by cool daylight fluorescent lamps (Osram Sylvania, Wilmington, MA). Regenerated shoots were excised and subcultured after 30 days.

2.2 Micropropagation and Antioxidants

Excised shoots (from 15 to 20 mm long), 30±10 days old, 9 shoots per box, were transferred to fresh MS propagation medium (as described before) with the following antioxidants individually or in combination: 100 mg/L of citric acid (C277, PhytoTechnology Laboratories), 100 mg/L L-cysteine (168149, Sigma-Aldrich®), 300 mg/L PVP 40,000 (PX 1300, EM Science) and 50 mg/L L-glutathione (G4251, Sigma-Aldrich®). The selection

of the antioxidant type and concentration was based on in-house observations made during micropropagation of other plant species (data not published). After 30 days, cultures were transferred to fresh MS propagation medium with the same antioxidant treatment for another 30 days under same growing conditions, 9 shoots per box and 6 boxes per treatment.

2.3 Assessment of Micropropagation and Phenolic Secretion

Genotypic and antioxidant effects were evaluated by observing the number of shoots per explant, their vigor and color, and by assessing the intensity of medium color caused by phenolic secretion during two micropropagation cycles. The two cycles were selected due to previous observations in which the most intense medium discoloration appeared during this time. The shoot vigor was ranked: 1-poor vigor; 2-average good vigor; 3-fully healthy with excellent shoot vigor (adapted from Debnath, 2005). The shoot color was ranked as 1-brown; 2-yellow; 3-light green; 4-dark green and the phenolic secretion intensity (color of medium) was ranked as 1-no phenolic secretion; 2-some; 3-moderate secretion; 4-heavy secretion.

Analysis of secreted phenolic compounds was carried out in propagation media containing antioxidants (1) 100 mg/L citric acid; (2) 100 mg/L L-cysteine; (3) 300 mg/L PVP; or (4) 50 mg/L L-glutathione) separately for the three genotypes (PI 88652, PI 29109 and UNK R65P35) at the end of the 1st subculture cycle (30 days after inoculation). The assay of phenolic compounds was performed according to Heuberger et al. (2014), with modifications as noted below.

2.4 Preparation of Extract

For metabolite profiling, an approximate 200 mg of culture medium pooled from the culture boxes in each treatment (four media combined with three accessions) was collected by a micropipette and kept in Eppendorf® tubes. The samples were freeze dried and homogenized in a bullet blender. A sequential extraction from 7.5 mg of culture medium was performed, first extracting with 1.0 mL methanol and secondarily with 1.0 mL of MTBE/MeOH/Water (6/3/1 v/v/v). The supernatant from each step was collected and pooled. The solvent was evaporated under nitrogen, and the metabolites re-suspended in 100 µL methanol and stored at -80 °C until further analysis.

2.5 UPLC-MS Analysis (CSH Phenyl-Hexyl Method)

The amount of each phenolic compound in each extract was determined by an ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis (CSH Phenyl-Hexyl method); 5 µL of the methanol extract was injected twice (n = 2 replicates) onto a Acquity UPLC system (Waters Corporation, Milford, MA) in discrete, randomized blocks, and separated using a Acquity UPLC CSH Phenyl-Hexyl column, 1.7 µM, 1.0 x 100 mm (186005402, Waters©), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). Calibration was conducted using sodium iodide with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temp at 150 °C, and nitrogen de-solvation temperature at 350 °C with a flow rate of 800 L/hr.

2.6 Experimental Design and Statistical Evaluation

Experimental design to evaluate the effect of the antioxidants and genotypes on the growth and phenolic secretion was fully randomized in a 2 × 4 × 5 factorial scheme (2 subculture cycles × 4 genotypes × 5 treatments with or without antioxidants) with six replications per treatment and nine shoots per replication (n = 54). The means were compared by Scott-Knott test at 5% probability. Pearson's correlation coefficient was calculated to denote the relationship between phenolic secretion and number of shoot/explant, their vigor and color, during the 1st and 2nd subculture cycles. Pearson correlation coefficient for each dependent variable between actual and predicted values was maximized, which is an indicator of the predictive performance of the algorithm. The hypothesis of the correlation coefficient was tested with a two-sided t test. Statistical analyses were performed with the SAS-9.4 program (SAS Institute, 2013).

To explore the metabolome of the culture medium with emphasis on phenolic compound determination, samples from the first experiment were separated for another experiment and a fully randomized 3 × 4 factorial scheme (3 genotypes × 4 antioxidants) with three replications per treatment was considered. Analysis of variance was carried out for each compound using the Analysis of Variance (AOV) function in R, and p-values were adjusted for false positives using the Bonferroni-Hochberg method in the p adjust function in R (Benjamini & Hochberg, 1995). Principal component analysis (PCA) was carried out on mean-centered and Pareto variance-scaled data using the PCA Methods package in R (Stacklies et al., 2007). The results were plotted as a function of retention time and the -log of the P-value. For all experiments, the differences between the data were considered significant at 5%.

3. Results

3.1 Effect of Genotypes and Antioxidants on Growth Variables (Shoot Number, Color and Vigor)

The subculture cycle factor (S) was significant only for the number of shoots/explant and in combination with the genotype (S×G) for the shoot vigor (Table 1). The genotype and antioxidants were significant for all the factors and presented very high values. A significant interaction was observed between the evaluated genotypes (G) and the applied antioxidants (A).

The evaluated cultures showed good shoot formation, vigor and color, except the PI 88652 (*S. officinarum*), which displayed low proliferative capacity and shoot vigor in the presence and the absence of the four antioxidants (Table 2).

S. officinarum (PI 184794) showed a significantly higher number of proliferated shoots in the presence of 100 mg/L citric acid (12.53 shoots/explant) and a significantly lower number with 300 mg/L PVP (8.28 shoots/explant). S. *officinarum* (PI 88652), *S. robustum* (UNK R65P35) and *S. sinense* (PI 29109) showed no significant improvement in shoot number with antioxidants in the medium compared to the control. The antioxidants had no effect on the shoot vigor S. *officinarum* (PI 88652), *S. sinense* (PI 29109) and *S. robustum* (UNK R65P35). However, *S. officinarum* (PI 184794) had significantly higher vigor with three of the antioxidants compared to PVP and the control.

Table 1. Analysis of variance for number of shoots/explant (NS), shoot color (SC) and shoot vigor (SV) for four *Saccharum* genotypes

Source of variation	df	MS shoot number	Fr shoot number	MS shoot color	Fr shoot color	MS shoot vigor	Fr shoot vigor
Subculture cycle-S	1	6.564	5.126*	0.204	1.541^{ns}	0.017	0.111^{ns}
Genotype-G	3	706.570	551.805**	3.548	26.782**	11.678	77.852**
Antioxidant-A	4	5.036	3.933**	1.7645	13.318**	0.267	1.778**
S×G	3	0.284	0.222^{ns}	0.1819	1.373^{ns}	0.428	2.852*
S×A	4	0.108	0.085^{ns}	0.0479	0.362^{ns}	0.0167	0.111^{ns}
G×A	12	12.503	9.765**	1.114	8.412**	0.622	4.148**
S×G×A	12	0.140	0.109^{ns}	0.081	0.613^{ns}	0.067	0.444^{ns}
Error	200	1.280		0.132		0.150	
VC (%)		12.91		10.54		15.70	

Note. ns: not significant; * significant at 5% by F test; ** significant at 1% by F test; df: degree freedom; ms: mean square; Fr: F ratio; VC: variation coefficient.

Shoot color was significantly better for *S. officinarum* (PI 184794) and *S. robustum* (UNK R65P35) with some of the antioxidant treatments while the others were not significantly different. All of the genotypes tested were light green to dark green regardless of the antioxidant added to the culture medium. In general an intensive green color was observed on shoots of all genotypes grown on medium with L-cysteine.

Table 2. Effect of genotypes and antioxidants (GxA) on the number of shoots/explant, shoot vigor and color of four *Saccharum* genotypes (means of 1st and 2nd subculture cycles)

Antioxidants	*S. officinarum* PI 184794	*S. officinarum* PI 88652	*S. sinense* PI 29109	*S. robustum* UNK R65P35	Means
Number of shoots/explant					
Control	11.23 aB	3.71 cA	10.13 bA	11.67 aA	9.18
Citric acid	12.53 aA	2.42 dB	9.49 cB	10.77 bB	8.80
L-cysteine	10.35 bB	4.46 cA	9.73 bB	11.28 aA	8.96
PVP	8.32 bC	3.68 cA	10.45aA	11.29 aA	8.44
L-glutathione	10.37 aB	4.12 cA	9.04 bB	10.26 aB	8.46
Shoot vigor[1]					
Control	2.08 bB	1.92 bA	2.92 aA	2.83 aA	2.44
Citric acid	2.58 bA	1.92 cA	2.58 bA	3.00 aA	2.52
L-cysteine	2.75 aA	1.75 bA	2.75 aA	2.92 aA	2.54
PVP	1.92 bB	1.83 bA	2.92 aA	2.75 aA	2.35
L-glutathione	2.50 aA	2.00 bA	2.83 aA	2.58 aA	2.48
Shoot color[2]					
Control	3.08 cB	4.00 aA	3.58 bA	3.08 cC	3.44
Citric acid	3.00 bB	3.00 bB	3.25 bB	3.92 aA	3.29
L-cysteine	3.58 bA	3.92 aA	3.58 bA	4.00 aA	3.77
PVP	3.00 bB	3.75 aA	3.50 aA	3.58 aB	3.46
L-glutathione	3.00 bB	3.83 aA	3.17 bB	3.25 bC	3.31

Note. Means followed by the same lowercase letter (rows) and the same uppercase letter (columns) are not different from each other according to Scott-Knott test at 5% probability.

[1]Shoot vigor rank: 1-poor vigor; 2-average good vigor; 3-fully healthy with excellent shoot vigor; 4-heavy secretion; [2]Shoot color rank: 1-brown; 2-yellow; 3-light-green; 4-dark green.

In all genotypes, the most shoots/explant was observed in the second subculture cycle (Table 3). The GxV interaction was indicated by the high shoot vigor maintained in *S. sinense* (PI 29109) and *S. robustum* (UNK R65P35) in the second subculture.

Table 3. Effect of subculture cycle on the number of shoots/explant and the shoot vigor of four *Saccharum* genotypes

Subculture Cycle	*S. officinarum* PI 184794	*S. officinarum* PI 88652	*S. sinense* PI 29109	*S. robustum* UNK R65P35	Means
Number of shoots/explant					
1st subculture	10.41	3.51	9.51	10.96	8.60B
2nd subculture	10.71	3.84	10.03	11.15	8.93A
Shoot vigor[1]					
1st subculture	2.30 bA	2.00 cA	2.77 aA	2.77 aA	2.46
2nd subculture	2.43 bA	1.77 cB	2.83 aA	2.87 aA	2.48

Note. Means followed by the same lowercase letter (rows) and the same uppercase letter (columns) are not different from each other according to Scott-Knott test at 5 % probability.

[1] Shoot vigor rank: 1-poor vigor; 2-average good vigor; 3-fully healthy with excellent shoot vigor.

3.2 Phenolic Secretion and Correlation With Growth Variables

All the factors and interactions were significant for phenolic secretion (Table 4). Phenolic secretion into the medium and its intensity (measured by the medium discoloration) was significant for the genotype × antioxidant × subculture interaction.

Table 4. Analysis of variance for phenolic color rank in medium with shoot cultures of four *Saccharum* species

Source of variation	df	ms	Fr
Subculture cycle-S	1	0.600	3.529^{ns}
Genotype-G	3	9.6778	56.928**
Antioxidant-A	4	2.598	15.282**
S×G	3	1.167	6.863**
S×A	4	1.402	8.248**
G×A	12	1.125	6.622**
S×G×A	12	0.580	3.411**
Error	200	0.170	
VC			

Note. ns: not significant; * significant at 5% by F test; ** significant at 1% by F test; df: degree freedom; ms: mean square ; Fr: F ratio; VC: variation coefficient.

Variations in the medium color intensity due to phenolic secretion were observed in both cycles for all genotypes (Table 5). In the 1st subculture cycle the genotypes showed different phenolic intensity. *S. officinarum* (PI 184794) and *S. robustum* (UNK R65P35) presented lowest phenolic secretion in the presence of PVP (2.50; 2.50, respectively). The activated charcoal (AC) induced the lowest discoloration of medium for *S. robustum* (UNK R65P35). No difference in medium discoloration between the applied antioxidants was observed in *S. sinense* (PI 29109) culture media.

Table 5. Effect of antioxidants (A) and subculture cycles (S) on the phenolic secretion[1] in cultures of four *Saccharum s*pp. genotypes

Antioxidants	*S. officinarum* PI 184794		*S. officinarum* PI 88652		*S. sinense* PI 29109		*S. robustum* UNK R65P35		Means
	1st SC	2nd SC	1st SC	2nd SC	1st SC	2nd SC	1st SC	2nd SC	
Control	2.67a	3.00a	4.00a	4.00a	2.83b	3.67a	4.00a	4.00a	3.52A
AC	2.83a	3.00a	4.00a	3.67a	3.17b	3.67a	3.00b	3.50a	3.35B
L-CYS	3.33a	2.00b	4.00a	4.00a	4.00a	4.00a	4.00a	4.00a	3.67A
PVP	2.50b	3.00a	3.00a	3.33a	3.17a	3.50a	2.50b	3.33a	3.04C
L-GLUT	3.50a	2.50b	3.67a	3.83a	3.17a	3.00a	3.67a	4.00a	3.42B
S Means	2.97a	2.70b	3.73a	3.77a	3.27b	3.57a	3.43b	3.77a	3.42B
G Means	2.83c		3.75a		3.42b		3.60a		

Note. Means followed by the same lowercase letter (rows) and the same uppercase letter (columns) are not different from each other according to Scott-Knott test at 5 % probability.

[1]Phenolic secretion rank: 1-no phenolic secretion; 2-some; 3-moderate secretion. AC: ascorbic acid; L-CYS: L-cysteine; PVP: polyvinylpyrrolidone; L-GLUT: L-glutathione.

Except in PI 184794, all other genotypes in the 2nd subculture cycle did not show any effect of the antioxidants on the medium discoloration. PI 184794 showed the lowest phenolic rank in the presence of L-cysteine (2.00) and L-glutathione (2.50). Antioxidants did not present effect on phenolic rank in both subcultures for PI 88652.

For *S. sinense* (PI 29109) and *S. robustum* (UNK R65P35), the average ranking for culture medium discoloration due to phenolic secretion seemed to be slightly higher in the 2nd subculture than in the 1st one. The expectation was to observe less phenolic secretion with each subsequent subculture, as it was observed for *S. officinarum* (PI 184794). However, an increase in phenolic secretion in *in vitro* cultivation of other sugarcane genotypes was noticed, even in the 4th and 5th subculture (data not shown).

In both subculture cycles, the intensity of medium color caused by phenolic secretion was negatively correlated with the number of shoots/explant (-0.2520; -0.2391, respectively, $p < 0.01$) (Table 6). Negative correlation indicated an inhibitory effect of phenolic compounds on *in vitro* shoot proliferation. Correlation between phenolic secretion and shoot vigor was not significant in either cycle ($p > 0.05$); however, in the 2nd subculture, a

positive correlation between phenolic secretion and shoot color (0.3148, $p < 0.01$), and shoot vigor with the number of shoots/explant (0.6121, $p < 0.01$) was observed.

Table 6. Bivariate Pearson's correlations among medium color due to phenolic secretion, shoot number, shoot color and shoot vigor, during the 1st and 2nd subculture cycles of *Saccharum* genotypes

	Phenolic secretion	Shoot color	Shoot vigor
1st subculture			
Number of shoots/explant	-0.2520**	-0.1467^{ns}	0.4557**
Phenolic secretion		0.1527^{ns}	0.0179^{ns}
Shoot color			0.1187^{ns}
2nd subculture			
Number of shoots/explant	-0.2391**	-0.2089*	0.6121**
Phenolic secretion		0.3148**	-0.0797^{ns}
Shoot color			-0.0427^{ns}

Note. ns: not significant; * significant at 5% by t test; ** significant at 1% by t test.

3.3 Effect of Antioxidants and Genotypes on the Secreted Phenolic Compounds

Based on the UPLC-MS data analysis, the Principal Component (PC) 2 and PC3 collectivity offered the best separation of the three genotypes based on the phenolics secreted into the culture medium (Figure 1A). PC3, in particular, offered separation of PI 88652 from the other two genotypes. The PC loadings plot (Figure 1B) indicated that this separation was largely driven by flavonoid compounds, as dihydroxyflavone, dihydroxy-methoxyflavone and 5,6-dihydroxy-7-methoxyflavone which accumulated in this genotype.

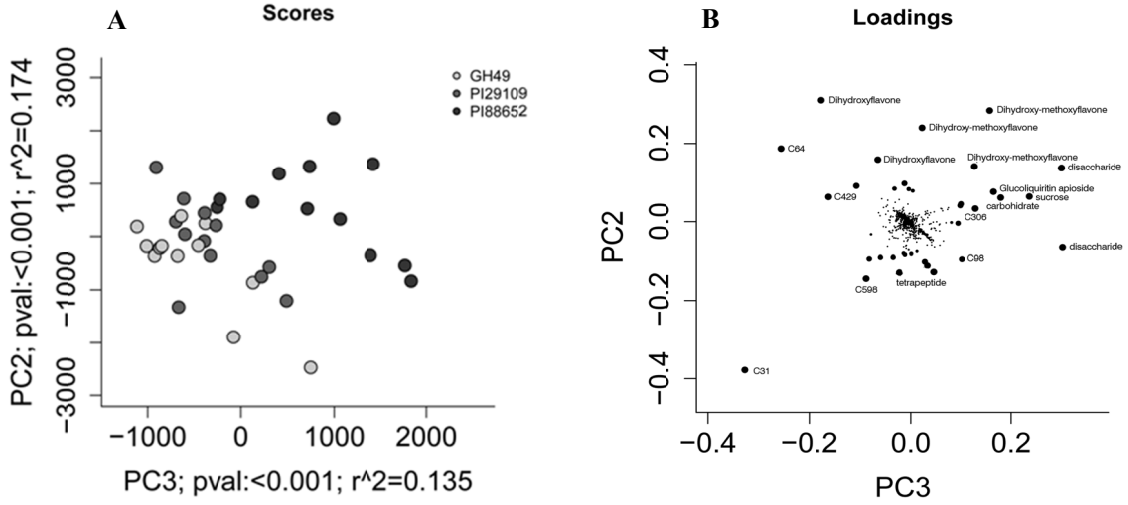


Figure 1. A-Principal Component (PC) variation plots of phenolic compounds secreted into culture media cultivated with three *Saccharum* genotypes (PI 88652, PI 29109 and UNK R65P35) by UPLC-MS; B-PC loading plots of phenolic compounds show correlations between different *Saccharum* genotypes

The same two PCAs groups (PC2 and PC3) separated phenolic compounds present in the medium with the antioxidants; in the separation, treatment with PVP (300 mg/L) was distinguishable (Figure 2A, group 3) from the three other antioxidants (citric acid, L-cysteine, and L-glutathione) that were largely driven by flavonoid compounds (Figure 2B).

According to ANOVA, the flavonoid compounds had different abundance responses as a function of the genotypes and antioxidants (Figure 3). The genotype and antioxidant type had a significant effect at the dihydroxy-methoxyflavone, dihydroxyflavone-rhamnoside and hydroxyl-methoxyflavone abundances. PI 88652 in the presence of all antioxidants, except 300 mg/L PVP, presented higher dihydroxy-methoxyflavone

abundance than UNK R65P35 and PI 29109. UNK R65P35, in the presence of 50 mg/L L-glutathione, showed a higher dihydroxyflavone-rhamnoside and hydroxyl-methoxyflavone presence than PI 29109 and PI 88652.

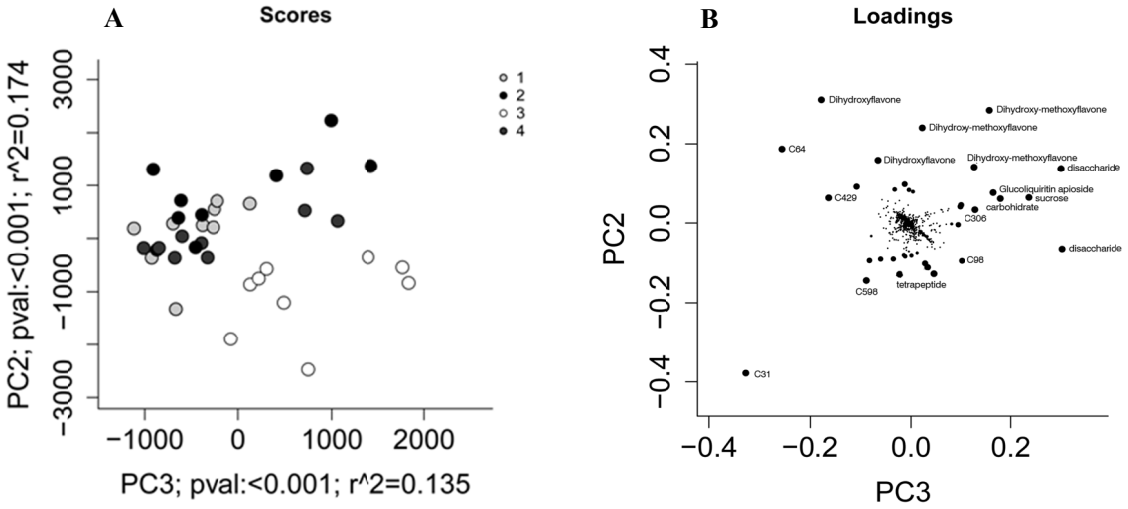
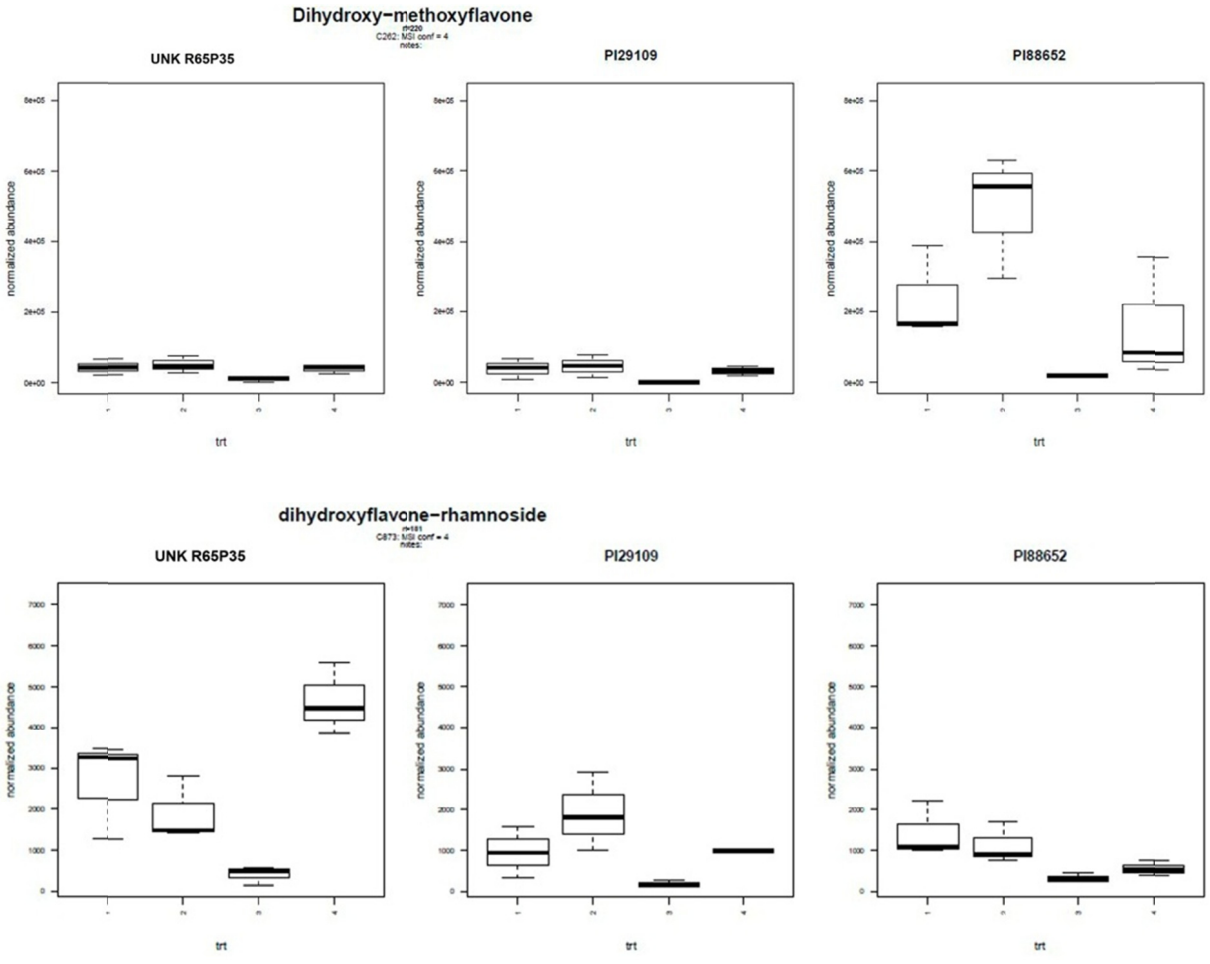


Figure 2. A-Principal Component (PC) variation plots of phenolic compounds secreted into culture media supplemented with four antioxidants (1-100 mg/L citric acid; 2-100 mg/L L-cysteine; 3-300 mg/L PVP and 4-50 mg/L L-glutathione) by UPLC-MS; B-PC loading plots of phenolic compounds show correlations between different antioxidants



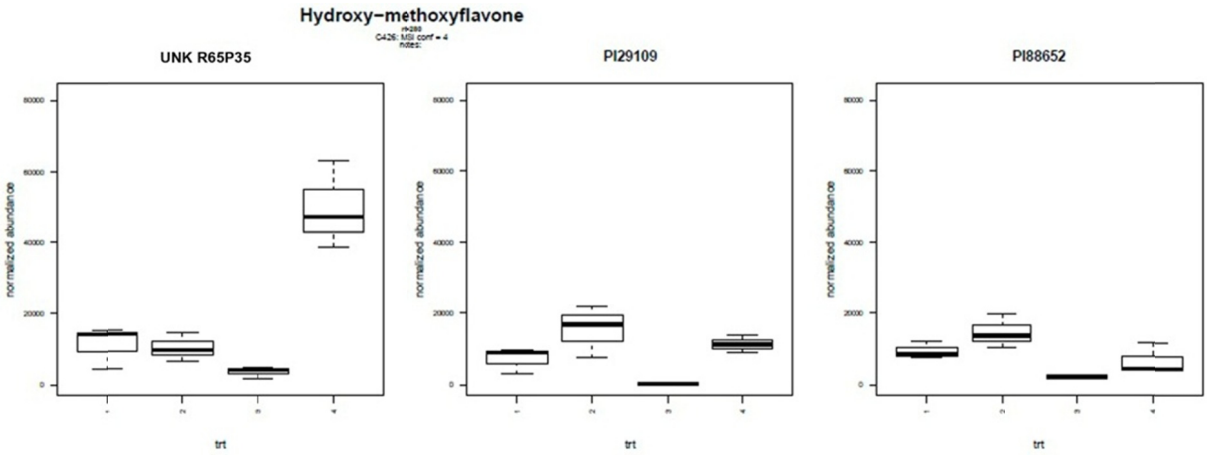


Figure 3. Relative quantification of some flavonoids (normalized abundance) as a function of genotypes and antioxidants (1-100 mg/L citric acid; 2-100 mg/L L-cysteine; 3-300 mg/L PVP and 4-50 mg/L L-glutathione)

4. Discussion

Pretreatment of sugarcane explants using solutions of ascorbic acid, citric acid, PVP and cysteine or culturing the explants on medium with the substances or activated charcoal was reported by several authors (Kumari & Verma, 2001; Lorenzo et al., 2001; Huang et al., 2003; Khan et al., 2007; Michael, 2007; Lal et al., 2015; Shimelis et al., 2015). Studies on *in vitro* shoot proliferation in the presence of antioxidants and their control of phenolic secretion are sparse and predominantly pertain to *S. officinarum*. In the present research, beyond the *S. officinarum* accessions, we studied *S. robustum* and *S. sinense* shoot cultures and genotype specific responses to the antioxidant types were observed.

The results of this study underlined the significant interaction between genotypes and antioxidants during *in vitro* cultivation of sugarcane. The higher shoot proliferation of the PI 184794 (*S. officinarum*) in the presence of 100 mg/L citric acid and lower at 300 mg/L PVP than without the antioxidants, contrasts with the results reported by Huang et al. (2003); Michael (2007) and Shimelis et al. (2015). Huang et al. (2003) observed that PVP reduced shoot browning in GT11 and ROC16 sugarcane varieties (*S. officinarum*) and Shimelis et al. (2015) in C86-12 (*S. officinarum*) at 300 mg/L PVP. According Michael (2007), an addition from 500 to 1000 mg/L of PVP in medium for cultivation of callus derived culture in constant darkness lessened tissue browning. In the present experiment, discoloration (due to phenolic secretion) of medium with 300 mg/L PVP showed the lowest average color intensity. However, both *S. officinarum* accessions produced a significantly fewer shoots on medium with PVP. The unexpected behavior in regard to shoot number and vigor of *S. officinarum* in the presence of PVP might be due to the genotypic makeup of the accessions and the *in vitro* regeneration path applied. In this study, the quantity of citric acid incorporated in the medium probably moderately reduced the leaching of the phenolic compounds and supported a healthy morphogenetic response in *S. officinarum* (PI 184794). On the other hand, the *S. robustum* (UNK R65P35) and *S. sinense* (PI 29109) showed a larger number of shoots per explant in the presence of PVP but a similar number of shoots was observed in the absence of antioxidant (control).

Significant differences between the three sugarcane species in the abundance of flavonoids secreted into cultivation medium were observed. Fewer shoots per explant induced in *S. officinarum* (PI 88652), in the presence and absence of all antioxidants in both subculture cycles, was most likely due to the moderate and heavy phenolic secretion observed for the genotype, in combination with the high abundance of flavonoids present in the medium, as dihydroxy-methoxyflavone, and a low genetic capacity of the accession to produce shoots. An inhibitory effect of flavonoids, such as tephroleocarpin, glabranin, and methylglabranin isolated from the *Tephrosia* species, on some photosynthesis phases, was described by Céspedes et al. (2001). Recently, this repressive effect was reported in photosynthetic electron transport chain and *in vivo* during germination and growth of *Physalis ixocarpa*, *Trifolium alexandrinum* and *Lolium perenne* plants (Morales-Flores et al., 2015). Genes involved in isoflavone biosynthesis were identified in sugarcane (Franca et al., 2001). Colombo et al. (2006) described a high content of flavonoids found in sugarcane juice and its by-products (bagasse and leaves)

in comparison to other flavonoid sources. Despite the high levels of dihydroxyflavone-rhamnoside and hydroxyl-methoxyflavone, *S. robustum* (UNK R65P35) showed good shoot proliferation.

Another observation pertains to the variation in genotypic responses between the two subculture cycles in shoot vigor and phenolic secretion. In PI 184794, during the 1st cycle, phenolic secretion was higher than in the 2nd cycle in the presence of L-cysteine and L-glutathione, disagreeing with Lorenzo et al. (2001) that observed an increase of phenol compounds in sugarcane cultivar C-1051-73 when the culture medium was changed. This might be a genotype specific reaction of *S. officinarum* (PI 184794) considering that *S. sinense* (PI 29109) and *S. robustum* (UNK R65P35) had the lowest phenolic ranks on the 2nd subculture cycle. The make-up and synthesis of phenolics in plant tissue may be determined by genetics and environmental conditions (Lux-Endrich et al., 2000). The results of this study agree with Rodrigues et al. (2011) and Kala et al. (2012), who observed that *Saccharum* sp. and *Psidium* sp. genotypes showed variation in secretion of phenolic compounds. Genotype dependence of the *in vitro* organogenesis responses was also reported by Gandonou et al. (2005) for nine sugarcane (*Saccharum* sp.) commercial cultivars. Another aspect to consider is the relation between auxin metabolism and phenolic secretion (Lorenzo et al., 2001). In studies on the effect of phenolic acids and their derivatives upon the growth of *Avena sativa* L. coleoptiles, Wolf et al. (1976) reported different effects of phenolic compounds on the plant growth and inhibition in the presence of indole acetic acid oxidation.

Based on Pearson's correlation coefficient, in both subculture cycles the number of shoots/explant was negatively correlated with phenolic secretion. Kerns and Myer Jr. (1986) emphasized the phenolic secretion and other exudate discharge observed during explant initiation into tissue culture systems lessened with growth and development. However, controversial opinions have been published on the relation between cell and tissue proliferation and low intensity of phenolics found in different species as *Medicago sativa* L. (Cvikrová et al., 1996), *Nicotiana tabacum* L. (Chirek, 1990) and *Pinus* sp. (Herman, 1991). Lorenzo et al. (2001) showed that the increase of phenolic content during the first three days of sugarcane culture is due to the most intensive cell division period, but later the secretion decreased.

The separation by principal components suggests that phenolic compounds, as flavonoids, are secreted at different levels as a function of the genotype and antioxidant. These aspects were observed by other authors for cotton (Ozyigit et al., 2007) and Hawk tea (Tan et al., 2016). These results suggest that additional antioxidant types, not included in this research, might be effective in controlling phenolic secretion in diverse *Saccharum* genotypes.

5. Conclusions

The research reinforced the strong interaction between *Saccharum* genotypes and phenolic secretion into *in vitro* shoot culture medium. The studies demonstrated that including citric acid (100 mg/L *S. officinarum* PI 184794) or PVP (300 mg/L *S. robustum* UNK R65P35 and *S. sinense* PI 29109) in culture medium reduced the medium discoloration caused by the phenolic secretion. The number of shoots was negatively correlated with the phenolic secretion; hence, antioxidants might promote shoot development. The high levels of flavonoids secretion by *S. officinarum* PI 88652 had an adverse effect on *in vitro* shoot development and the culture vigor. The ability of *S. officinarum* and *S. robustum in vitro* cultures to produce flavonoid compounds in the presence of antioxidants might be a desired characteristic in producing flavonoids on a larger scale throughout the year under controlled environmental conditions.

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