Proteomic and Physiological Indices-Based Selection of Broadly Diverse Sugarcane (*Saccharum* Spp) New Genotypes

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ABSTRACT

Distantly-related 42 (S1-S42) genotypes of sugarcane (*Saccharum* spp) were selected phenotypically from new genotypes generated from seeds of an open-pollinated parent, EH 26-2 (pedigree, LCP 81-10 X cp76-331) unfortunately, at the age of 100-days-old, two of the selected genotypes, namely S29 and S36, could not adapt to environmental conditions and were excluded from consideration. Proteomic analysis was carried out involving quantitative analysis of total soluble protein of leaf tissue extracts at five month-old plants and electrophoresis separation of proteins. The analysis showed significant differences among genotypes, in the total soluble protein content which ranged from 4.42 ± 0.22 to 2.73 ± 0.06 mg/g tissues, which were observed in genotype S37 and S28, respectively. Despite the results of total protein analysis of sugarcane leaf tissues appeared to vary among genotypes, it did not suggest evidence that protein content alone was a reliable marker for identifying genotypes. The analysis of protein by SDS-PAGE revealed clear differences in intensity and number of bands amongst genotypes. Furthermore, phylogenetic analysis of the 40 genotypes elucidated by electrophoresis, showed a wide range of total protein polymorphic variants. At least four (4) distinguishable clusters of genotypes were identified, amongst which was a cluster containing genotypes S17, S18, S25, S26 and S27; which were most distantly-related to the sub-cluster containing S35, S37, S42, S38, S39 and S40. Moreover, the physiological profiles of these genotypes were assessed at the age of 180 days, the leaf physiological characters of the remaining eleven potential sugarcane genotypes, relevant to plant response of drought, were evaluated. The studied parameters; included stomata length, width and number, epicuticular wax, relative leaf water content, chlorophyll content, peroxidase activity and proline content revealed that the distantly-related genotypes S25 and S40 which could be potential as future varieties in addition, the eleven genotypes may be useful parents in future sugarcane breeding programs in crosses to identify hybrids with high specific combining ability.

Keywords: clonal identification, drought, electrophoresis, germplasm maintenance, isozymes, *Saccharum* spp, sugarcane.

INTRODUCTION

In Egypt, the Sugar Crops Research Institute has invested much effort over the past three decades to generate new varieties of sugarcane with high sugar accumulation and resistance to pests and diseases. This effort began with Allam's work (Allam et al., 1977) and is being achieved through breeding, selection and evaluation of hybrids, based mainly on broadening the genetic base through interspecific hybridization of cultivars. However, the progress has been affected by the limited genetic diversity among cultivars currently available. As a result, efforts of generating variability through open pollination would achieve genetic gains in breeding programs. This approach has allowed sugarcane breeders worldwide for accomplishing a great success (Srivastava, 1993).

Molecular markers based on protein and enzyme polymorphism among genotypes are of the most popular selection tools in discriminating among genotypes and hold potentials utility in sugarcane breeding (Ramagopal, 1990). Synthesis of proteins is coded for by genes in genotypes; hence proteins are valuable source of genetic markers in sugarcane and could be useful to identify specific genotype and stages of its development (Menéndez *et al*., 1994 and Glaszmann, 1989).

Many recent proteomic studies have been performed on various species under different abiotic stresses, but there have been only a few proteomic studies in sugarcane (Sugiharto *et al*., 2002 and Amalraj *et al.*, 2010). Disc-polyacrylamide gel electrophoresis (Disc-PAGE) was used to separate native soluble proteins by Davis (1964) and Wagih *et al*. (2005). The general characterization of the proteome to identify the largest possible number of proteins or the differentially expressed proteins between genotypes is a commonly used procedure in proteomics (Park, 2004).

In addition, certain sugarcane leaf physiological characters are closely related to the characterization of genotypes. Low density of stomata, thick cuticle, short and narrow leaves would be selection criteria in screening genotypes for drought tolerance (Yadav and Prasad, 1988). Routine methodologies were described

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for epicuticular wax, stomata characters (Rai, 1999) leaf water content (Karakas *et al*., 1997), chlorophyll content and proline content (Jagtap *et al*., 1988).

Therefore, the investigation aimed at using some leaf proteomic and physiological analysis of some new genotypes of sugarcane for identification and selection of the distantly-related genotypes as potential genotypes and for use in future breeding programs.

MATERIALS AND METHODS

Plant material

Seeds were planted at 24/8/2014 in greenhouse of Sabahia Research Station, Alexandria, Sugar Crops Research Institute (SCRI). Leaf tissue samples were obtained from 150-days-old sugarcane plants of 40 new genotypes (S1-S42), where at the age of 100-days-old, two of the selected genotypes, namely S29 and S36, could not adapt to environmental conditions and were excluded from consideration, generated from seeds of open-pollination of the parent EH 26-2(pedigree, LCP 81-10 X cp76-331) known to be vigorous and drought tolerant (Badawy et al., 2006). Samples were collected from sets in grown greenhouse, grown in the absence of water shortage, salinity, or other intended stresses and stored in -20^{o}C freezer prior using for protein profiling within days from collection. After one month fresh leaves of the eleven selected genotypes were collected for studying leaf physiological characteristics to assess plant response to drought.

Extraction of Whole Protein from Sugarcane Leaves:

Total soluble proteins of sugarcane frozen leaves were extracted as described by Shewry *et al*. (1996). Firstly, 0.5 g of fresh sugarcane leaves was grinded into powder in liquid nitrogen. Grounded samples were mixed in 200 µl lyses buffer (84 mM citric acid, 32 mM Na_2HPO_4, 15 mM β-mercaptoethanol and pH was set to 8.2). Secondly, the mixed liquid was precipitated on ice for 30 min and centrifugation at 10,000 g for 10 min at 4 °C and then the supernatant was discarded and the pellet was re-suspended with cold acetone containing 0.07% β-mercaptoethanol and kept overnight at −20 °C. Thirdly, after centrifugation for 30 min with 10,000 g, the pellet was resuspended with 80% acetone and kept for 1h at −20 °C, and then it was centrifuged again. Finally, the pellet was dried into powder by vacuum drying at low temperature and stored at−80 °C until use. Pellets were suspended in rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2 mM DTT, 0.8% (w/v) IPG buffer and 0.2% bromophenol blue.

The supernatant used for 1-DE analysis. Total protein content was measured according to the method developed by Bradford (1976) using bovine serum albumin as the standard, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) was carried out using the discontinuous buffer system as described by Sambrook *et al*. (1989), with the gel concentration of 12.0% samples were then denatured by heating at 100 °C for 3 min followed by immediate cooling on ice. Then, samples were loaded into the gel and electrophoresis was carried out for 3-h at 80 V across the polyacrylamide gel using consort power supply (Biometra, USA) and mini protein cell (Bio-Rad, USA) containing running buffer. When, SDS-PAGE finished, the gel was taken out and stained in 50 ml of staining solution consisting of 0.1% coomassie blue R-250 as described by Hames and Rickwood (1990). Protein pattern bands were measured used Hyper PAGE II pre-stained protein marker (BIOLINE, United Kingdom).

Quantification of soluble protein contents: Total soluble protein extracts concentration was determined by a colorimetric method using a commercially available reagent (Bio-Rad protein assay dye reagent), as described by Bradford (1976). Tubes containing 100 µl aliquots of a standard known concentrations of Bovine Serum Albumin (BSA; 0.156 mg l-1 to 10 mg l-1 in 0.15 M NaCl), were prepared. Blank tubes containing 100 µl of 0.15 M NaCl were also prepared. One ml Coomassie Brilliant Blue solution was added to each tube and vortexed. The reactions were left at room temperature for 2 min. The absorbance at wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted. The proteins concentration of the extracts was determined from the standard curve, using a Unicam 8620 UV/VIS (USA) Spectrophotometer.

Assessment of leaf physiological parameters

Leaf physiological parameters including stomata length, width and number, epicuticular wax, relative leaf water content (RWC), chlorophyll content, peroxidase activity and proline content, were measured in the eleven selected genotypes, including no., S17, S18, S25,S26, S27, S35, S37, S38, S39, S40 and S42 at the age of 180-days.

Stomata characteristics were measured following nail paint peeling technique as described by Capellades et al. (1990).

Epicuticular wax was measured using a micrometer as described by Rai (1999).

Leaf relative water content (RWC) was determined by excising 100 mm^2 of leaf, weighed, and then re-weighted after floated on distilled water for 24 hours, oven drying at 80^{o}C to get the dry weight. RWC = (FW –DW)./(TW – DW) where FW = leaf fresh weight, DW

= dry weight and TW = turgid weight (Mu-Qing and Ru-Kai, 1998).

Chlorophyll content was essentially carried out using a modified method used by Wagih *et al.* (2005). Under deem light, 0.25 g of leaf tissue were grinded with 20 ml of 80% acetone, 0.1 g (approx.) of CaCO3 to neutralize acid in the cell and to prevent loss of Mg from the chlorophyll centre in a pre-cooled mortar, in the presence of small quantity of washed sand. The homogenate was centrifuge at 10,000 g for 10 minutes. The supernatants were adjusted to 50 ml with acetone. Optical density was determined at 663 nm (absorption peak of Chlorophyll a) and 645 nm (absorption peak of Chlorophyll b) in the spectrophotometer, using acetone 80 % as a blank. The concentrations of chlorophyll were calculated by the following formula: [(Chlorophyll a = 12.7 x A663 – 2.69 x A 645)], [(Chlorophyll b = 25.8 x A645 – 4.68 x A 663)]; and [(Chlorophyll a + b = 20.21 x A645 + 8.02 x A 663)]. Content of chlorophyll, mg/g fresh weight = [(C-x mgL-1 x total amount of extract (ml) x dilution) / (gram fresh weight of sample x 1000)], where C-x represents the concentration of Ca, Cb and Ca+b, respectively.

Peroxidase activity was determined by the Zymoblot technique essentially as described by Wagih and Wagih (1997). One gram of leaf sample was ground in a mortar with Tris-buffered saline pH 7.4 (TBS; 25 mM Tris-HCl, consists of 8 g L-1 NaCl; 0.2 g L KCl; Tris 3 g/L and Phenol-red 0.015 g/L) at a ratio of 1:5 w/v. The crude tissue extract was centrifuge at 10,000 g for 10 min and the supernatants were recovered and tested for enzyme activity. Aliquots of 2µl of clarified extracts were blotted onto nitrocellulose membrane. Blots were air-dried, then marinated in a suitable enzyme-reaction mixture. Instead of applying 1-5 µl clarified extracts to nitrocellulose membrane, 20 µl clarified extracts were applied to the membrane; which was air-dried, then marinated in a reaction mixture contained 5 ml TBS buffer plus 1 ml of substrate mix (3 mg of 4-chloro-1-napthol in 1 ml of methanol) plus 20 µl of 3% hydrogen peroxide, shacked gently under light at room temperature until a color reaction was developed. Result was code visually as a percentage of color density develops on the blot after incubation. The color density was scored as percentage, compared to the control dense reaction.

Proline content was determined as mg/g fresh weight of leaf, according to Bates *et al.* (1973).

Statistical analysis

The quantitative amounts of total protein were statistically analyzed using Duncan's Multiple Range Test with three replications. Phylogenetic trees were constructed using PAST, Version 3.08, 2015. The physiological traits were analyzed by ANOVA, LSD, simple multiple linear correlation using Statistix 1.0 for Windows software (Analytical Software, Tallahassee FL).

RESULTS AND DISCUSSION

Assessment of soluble protein content

Assessment of soluble protein content of leaf tissues of 40 new genotypes grown in the absence of any intended stress is presented in Figure 1. The results showed significant differences ($p<0.05$) amongst genotypes, labeled (a), (b) and (c). The values of total soluble protein content ranged from 4.42 ± 0.22 to 2.73 ± 0.06 mg/g tissues. The maximum value was observed in genotype S37, which did not differ significantly ($p>0.05$) from 24 other genotypes labeled (a). The minimum value was observed in genotype S28, that did not differ significantly ($p>0.05$) from three (3) other genotypes labeled (c). Between them, a group of eleven (11) genotypes labeled (b) did not differ significantly ($p>0.05$) from one another. The major disadvantages of proteomic markers are that they may be liited in number and are influenced by environmental stages of the plant.

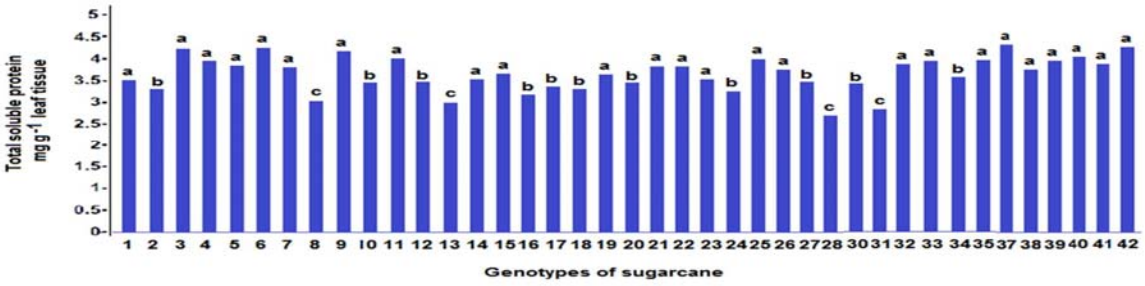

Figure 1. Differential accumulation of total protein content (mg/g) in leaf tissues of 40 new genotypes of sugarcane during formative phase of growth (150-days-old sugarcane). The letters a, b and c represent the significance of means (three replications) difference according to Duncan's Multiple Range Test.

Analysis of soluble proteins by electrophoresis

Analysis of soluble proteins of sugarcane leaf tissues extracted at pH (8.3), separated by SDS-PAGE gels following coomassie blue staining shown in Figure 2, revealed at least four (4) protein bands, and a maximum of thirteen (13) bands. Comparison of the protein profiles revealed clear differences in intensity and number of bands amongst genotypes. These results suggest a mean of differentiating among sugarcane genotypes. Separation of proteins by electrophoresis is advantageous as it gives overview of tissue by molecular mass.

Differential protein expression of genotypes appeared, not only in the expression capacity of the same protein, but also in totally absent proteins. Park (2004) stated that the general characterization of the proteome to identify the largest possible number of proteins or the differentially expressed proteins between genotypes is a commonly used procedure in proteomics. The protein expression pattern was contrasting, suggesting that the differentially expressed proteins may be related to different performance of genotypes under the growing environment.

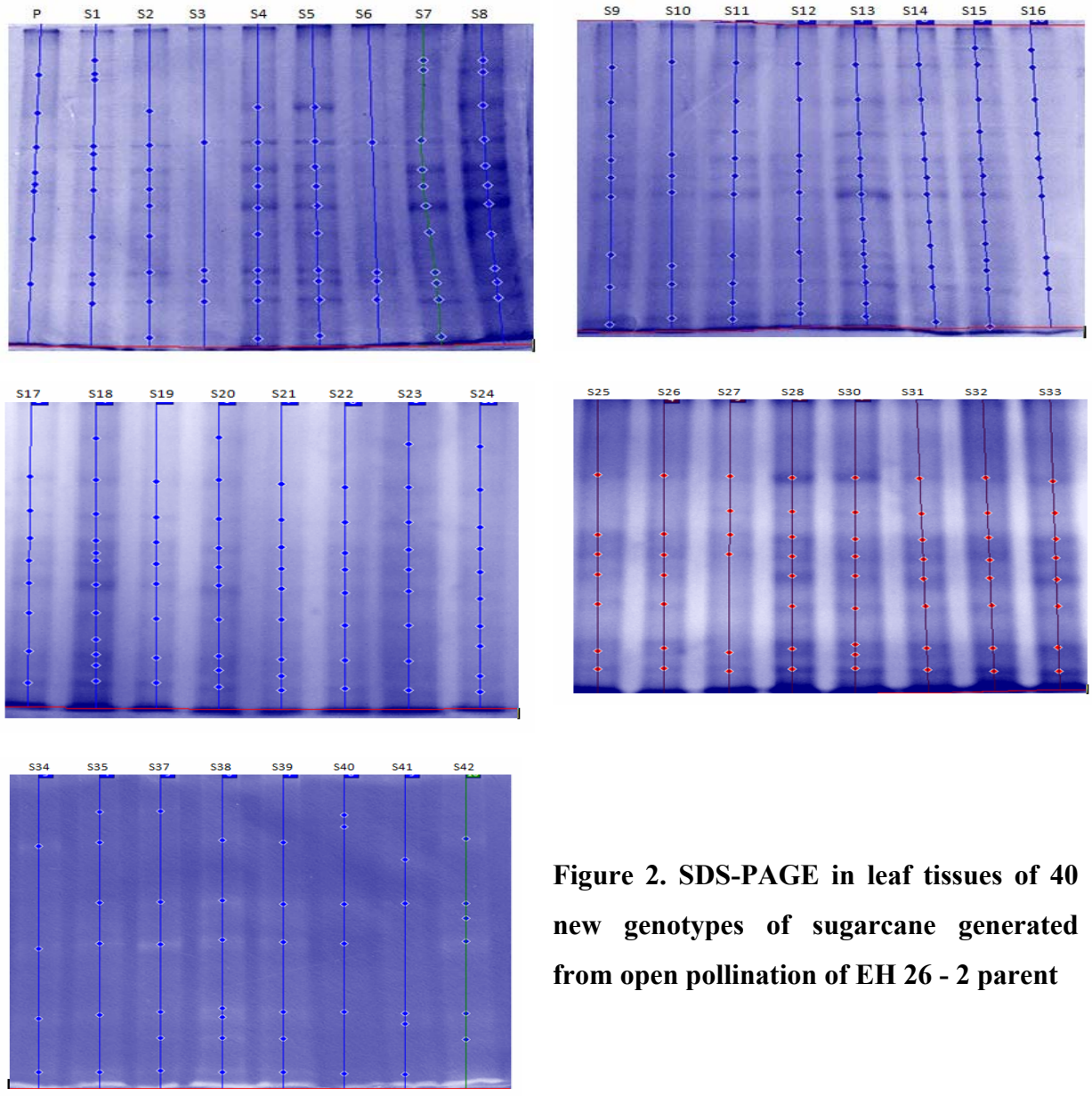

Figure 2. SDS-PAGE in leaf tissues of 40 new genotypes of sugarcane generated from open pollination of EH 26 - 2 parent

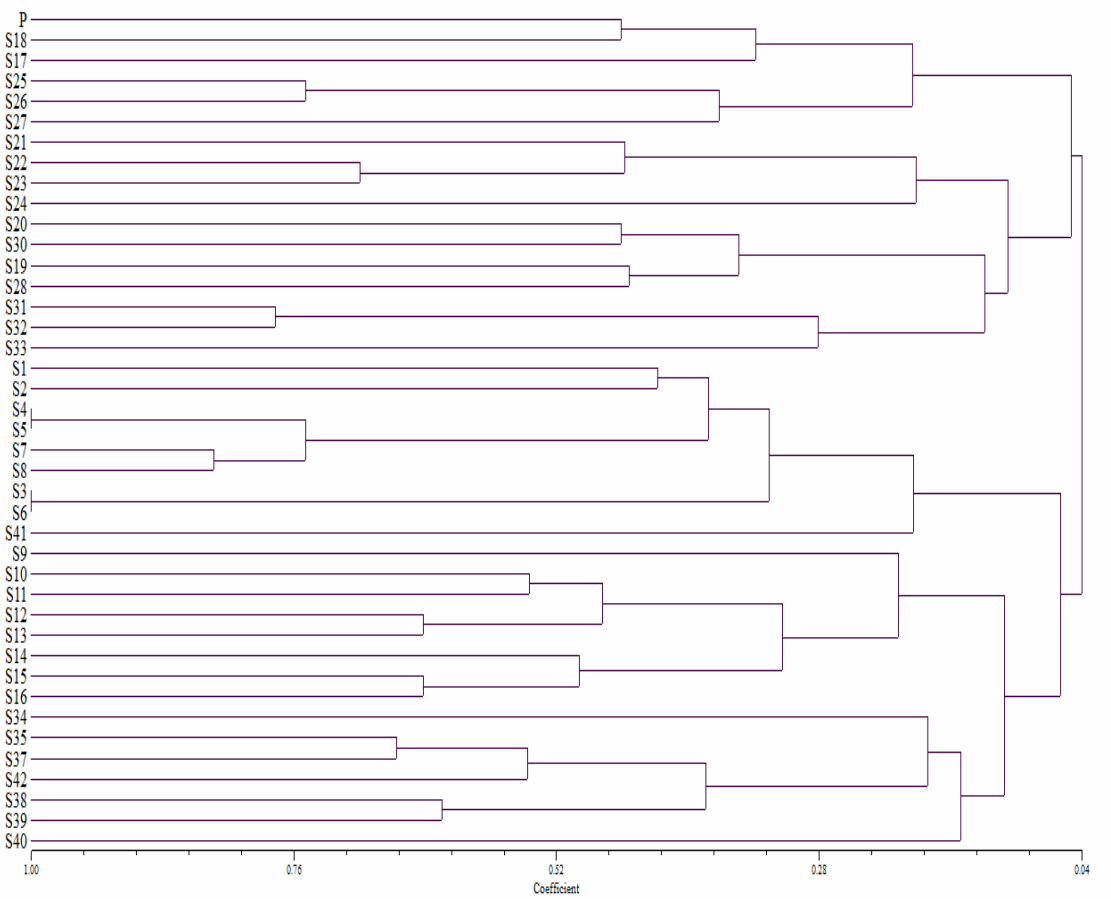

Figure 3. Dendrogram of cluster analysis based on total soluble protein electrophoretic fractions of leaf tissues of 40 new genotypes of sugarcane generated from open pollination of EH 26-2 parent

Phylogenetic analysis

The analyses of phylogenetic relationships among total soluble protein patterns of 40 sugarcane genotypes were presented in Figure 3. Cluster patterns of the 40 genotypes, elucidated by protein electrophoresis showed a wide range of total protein variants. The bands deduced by protein staining and matching genotypes of similar banding patterns in different genotypes. Pattern differences between band frequencies were a reflection of genetic distance between pairs of genotypes.

Stomata features play an important role in transpiration of water and gas exchange. The size and number of stomata determined the ability of the plant to lose or retain moisture. Reduced stomata length and width, and number per unit area are characteristics of plant tolerance to drought (Rai, 1999). Despite the differences of stomata measurements shown in Table 1, which showed that the tested genotypes were not significantly differ from the mother plant (M) that known as drought tolerant (Badawy et al., 2006).

Assessment of leaf physiological parameters

At the age of 180-days-old, the leaves were collected for physiological characteristics of the eleven selected genotypes and were evaluated for plant response to drought. Table 1, showed the differences of physiological profiles of the genotypes, reflecting their response to drought.

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Table 1. Physiological profiles of two distantly-related groups of genotypes at the age of 150 days

Parameter	Genotype											
	M*	Group I						Group II				
		S17	18	S25	S26	S27	S35	S37	S38	S39	S40	S42
Stomata Length, (µm)	30.5	28.3	24.7	28.2	29.6	31.2	29.8	27.9	28.9	32.3	28.6	29.8
Stomata Width, (µm)	14.1	12.5	12.7	13.8	12.9	13.3	14.2	14.1	12.8	12.9	12.5	13.9
Stomata Number (no. mm^{-2})	192	202	202	178	188	198	194	204	198	203	187	190
Epicuticular Wax Thickness (µm)	1.9	1.9	2.0	2.2	2.1	1.9	2.2	2.3	2.1	1.9	2.1	2.0
Leaf Relative Water Content (%)	90.4	89.5	91.2	87.5	88.9	93.3	88.2	89.3	91.4	92.0	87.8	89.7
Chl. a (mg g^{-1} of leaf)	3.4	3.3	3.2	4.2	3.9	3.2	4.3	3.8	3.7	4.8	4.5	3.6
Chl. b (mg g^{-1} of leaf)	0.9	0.9	0.9	1.2	1.1	0.9	1.1	1.0	0.9	1.2	1.0	1.0
Chl. a + b (mg g^{-1} of leaf)	4.3	4.2	4.1	5.4	4.6	4.4	5.4	4.8	4.6	6.0	5.5	4.7
Peroxidase Activity	80	80	80	75	77	75	80	80	80	75	70	76
Proline (mg g^{-1} leaf fresh weight)	35.1	37.4	42.2	45.0	37.6	34.6	47.2	36.1	35.0	38.7	49.7	36.2

*M=mother plant

Epicuticular Wax Thickness (EWT) of all tested genotypes were thicker than that on the leaves of the mother plant; except genotypes S17, S27 and S39, which were equal to that of the mother plant. Thicker leaf epicuticular reduces water loss (Rai, 1999). Leaf relative water content (RWC) varied among genotypes, where RWC was lower in all genotypes compared with the mother genotype; except genotypes S18, S27, S38 and S39. Higher total leaf chlorophyll content (CC) is an indicator of a more efficient photosynthetic capacity of a plant genotype. It was higher in all genotypes compared with the mother genotype; except genotypes S17 and S18. Low Peroxidase activity (PA) is a characteristic of tolerance to drought (Eksomtramage *et al*., 1992). It was lower in all genotypes compared with the mother genotype; except genotypes S17, S18, S35, S37 and S38. There is a strong association between Proline content (PC) and tolerance to drought in sugarcane, where high leaf PC occurs in genotypes more tolerant to drought (Jagtap *et al*., 1988). It was shown that accumulation of free proline was greater in drought tolerant than sensitive genotypes, while greater accumulation was found in the leaves of 30-day than in older plant (Mu-Qing and R-Kai, 1998 and Jagtap *et al*., 1988). It was higher in all genotypes compared with the mother genotype; except S27 genotype.

CONCLUSION

Proteomic analysis of total soluble protein in leaf tissue extracts involving quantitative analysis, electrophoresis separation of proteins, dendrogram analysis and physiological profiling of genotypes allowed for identifying eleven diverse and distantly-related potential sugarcane genotypes, among them two distantly-related genotypes S25 and S40 would be of potential future varieties, and the eleven genotypes may be useful parents in future sugarcane breeding programs in crosses to identify hybrids with high specific combining ability.

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الملخص العربي

الانتخاب المعتمد علي دلالات بروتينية وفسيولوجية لتراكيب وراثية جديدة متسعة التباين من قصب السكر

نجاة عبدالقادر موسى، العربي سالم رمضان سالم، محمد زهدي عطا الله

تم انتخاب عدد ٤٢ سلالة جديدة من قصب السكر ناتجة من بذرة من التلقيح المفتوح للصنف EH26-2 على اساس الشكل المظهرى ولكن عند عمر ١٠٠ يوم لم تتمكن الـسلالتين S29,S36من التاقلم للظروف البيئية وتعرضتا للموت وتـم استبعادهما. التحليل البروتيني تم اجرائه متـضمنا التقـدير الكمي للبروتين الكلي في مستخلص أنـسجة الاوراق عنـد عمر ١٥٠ يوم(خمسة أشهر) وكـذلك الهجـرة الكهربيـة للبروتينات. وقد أظهر التحليل فروق معنوية بين التراكيـب الوراثية في محتوى البروتين الكلي الذائب والتي تراوحـت بين ٤,٤٢ ±٠,٢٢ الـى ٢,٧٣ ± ٠,٠٦ مجـم/جـم مـن الانسجة و التي تم ملاحظتها في الـسلالة S37 وS28علـى الترتيب.على الرغم من أن تحليل البروتين الكلي قد أظهـر اختلافات بين التراكيب الوراثية إلا أنه لا يمكـن الاعتمـاد عليه منفردا كدليل حقيقي لتقييم التراكيب الوراثية.

تحليل الهجرة الكهربية للبروتين باستخدام SDS-PAGE أظهرت فروق واضحة بين التراكيب الوراثية مـن حيـث عدد الحزم وكثافة الحزم. تحليل التنوع الوراثي لعـدد ٤٠ تركيب وراثي بواسطة الهجرة الكهربية أظهرت مدى واسع من الاختلافات المظهرية في البروتين. وقد تم تمييز عـدد (٤) تجمعات Clusters على الأقل واحدة تضم التراكيـب الوراثية التالية S17, S18, S25, S26, S27 والتي كانت أبعد الأقارب عن ال Sub-cluster الذي يضم التراكيب S35, S37, S38, S39, S40, S42 .

وتم تقييم الصفات الفسيولوجية عند عمر ١٨٠ يوم للأوراق والتي تشير الى الاستجابة للجفاف في عـدد ١١ تركيـب وراثي هي أبعد الأقارب من خلال التحاليل الـسابقة. وتـم تقدير عدد من الخصائص هي طول وعرض وعدد الثغور التنفسية وسمك الطبقة الشمعية والمحتوى النسبي للرطوبـة في الورقة ومحتوى الكلوروفيـل. ونـشاط البيروكـسيديز والمحتوى النسبي من البروتين والتي أظهرت أن السلالتين S25 وS40 يمكن اعتمادهما كأصناف محتملة في المستقبل. بالاضافة الى أن الاحدى عشر(١١) سلالة يمكن استخدامها مستقبليا كأباء في برامج التربية لانتاج هجـن ذات قـدرة خاصة عالية على الخلط.

كلمات البحث: تحديد التراكيب الوراثية والتحمـل للجفـاف والفصل الكهربائي، وحفـظ الأصـول الوراثيـة، نظـائر الانزيمات، قصب السكر، النشاط الأنزيمي.