Chapter 5

Transcriptome, Genetic Transformation and Micropropagation: Some Biotechnology Strategies to Diminish Water Stress Caused by Climate Change in Sugarcane

Evelyn Arlette Carrillo Bermejo,
Miguel Angel Herrera Alamillo,
Samuel David Gamboa Tuz,
Miguel Angel Keb Llanes,
Enrique Castaño de la Serna, Manuel L. Robert and
Luis Carlos Rodríguez Zapata

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72438

Abstract

Global climate change caused by natural processes results in major environmental issues that affect the world. Climate variability results in changes that cause water stress in plants. Sugarcane is a tropical grass C4, perennial and a multi-purpose industrial cash crop which serves as the main source of raw material for the production of sugar and biofuel. Farmers face the challenge to provide biotech alternatives with potential benefits and minimize potential adverse impacts on sugarcane's production. In order to find biotechnology strategies to diminish the impact of climate change, our laboratory teamworks with micropropagation, transcriptome and genetic transformation of sugarcane using the var. MEX69290. In the transcriptome of sugarcane, a total of 536 and 750 genes were differentially regulated under normal and water stress treatment respectively, of which key genes were selected to be inserted into sugarcane for tolerance to abiotic stress. Regarding results of micropropagation, it was concluded that the continuous immersion propagation system was the best culture strategy. This may be as result of the elimination of gelling agent, which additionally helps reduce production costs.

Keywords: biotechnology, micropropagation systems, transcriptome, genetic transformation, sugarcane, abiotic stress

1. Introduction

Climate change alters environmental conditions and therefore has direct and biophysical effects on agricultural production. The biophysical and direct effects of climate change induce alterations on the prices and production of agriculture. Such changes are reflected on the economic system as farmers and other market participants make adjustments autonomously. They are both compelled to modify their crop combinations, use of supplies, level of production, and food demand, consumption and trade. Climate change causes a changes in rainfall regimes which have direct effects on crop yields as well as indirect effects through changes in the availability of water irrigation [1].

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous perennial plant belonging to the gramineous family *Saccharum officinarum* L. [2]. Sugarcane is a commercial crop in tropical and subtropical regions. According to FAOSTAT [3], sugarcane is cultivated in 26.1 million hectares producing 1.83 trillion canes. Sugarcane is a multi-purpose industrial cash crop and the main source of raw material for sugar production. It is responsible for almost 70% of world-produced centrifugal sugar [4]. Some mitigation and adaptation strategies for climate change in sugarcane production are the use of biotechnological techniques such as transcriptome, genetic transformation and *in vitro* micropropagation. In this chapter, we will talk about water stress in sugarcane caused by the climatic change and the biotechnological alternatives such as transcriptome, genetic transformation and micropropagation which are currently being carried out in our laboratory to counteract this problem.

2. Climate change, water stress and its effect in sugarcane

A large scale of plant production grown under different agricultural production systems is lost under the effects of abiotic stresses, which may result in a 70% reduction of the potential yields of crop plants [5]. During growth and developmental periods, crops suffer seasonal floods and droughts, extreme temperatures or salinity all year round. Globally, about 22% of global agricultural land is saline, and the increased damage caused by drought has been reported to limit plant growth and development followed by a loss of productivity, especially in crop species [6, 7]. Thus, drought stresses are one of the most serious kind of abiotic stresses that implies a threat on crop productivity worldwide.

Sugarcane, an important source of sugar and ethanol, is a relatively high water-demanding crop and its growth is highly sensitive to water deficit [8]. It is estimated that sugarcane produces 8–12 ton cane per ML of water irrigation [9], and water deficit can lead to productivity losses of up to 60% [10–13]. For this reason, production areas are concentrated in regions with favorable rain regime to sugarcane growth and development [14], while in other areas crop production requires supplemental or full irrigation [15].

According to various studies, water stress triggers many physiological, biochemical, and molecular responses that influence various cellular processes in plants and this impacts on its productivity [16, 17].

Severe water stress such as drought affects the entire plant. Morphological and physiological responses in sugarcane plants vary according to its genotype, duration (rapid or gradual)

and intensity (severe or mild) of stress and also the type of affected tissue [18–21]. Water stress also affects both cane and sugar yield substantially. The most common water stress responses in sugarcane are leaf rolling, stomatal closure, inhibition of stalk and leaf growth, leaf senescence and reduced leaf area [12, 22]. Moreover, under water stress, both cell division and cell elongation are interrupted [23] and stem and leaf elongation are the most severely affected growth processes [24, 25]. Root development is also influenced by water deficit [19, 26] but its overall biomass is relatively less than the above-ground biomass. Sugarcane is a tropical crop with C4 photosynthetic metabolism. A moderate water stress causes a stomatal limitation, which triggers a decrease in stomatal conductance (gs), transpiration rate (E), internal CO_2 concentration (Ci), and photosynthetic rate [26–30]. Under water stress, a decline in photosynthetic rate is mainly caused by a decrease in phosphoenolpyruvate carboxylase (PEPcase) and ribulose-1,5-biphosphate carboxylase (Rubisco) activity [26, 27, 31]. It is worth to note that photosynthesis rate is also impacted by sugar accumulation in leaves [32]. Under non-stressed condition low leaf sugar content is conducive to photosynthesis, while high sugar content moderates carbon fixation [33]. Interestingly, increased levels of some sugars, such as trehalose, can help plants to cope with water deficit, reducing the damage on cell membrane [34]. The capacity to accumulate trehalose was demonstrated in sugarcane roots under drought conditions. Sales et al. [35] reported an increase in starch hydrolysis, leading to higher levels of soluble sugars that helped sustain carbon supply even in a reduced CO_2 fixation condition, facilitating growth recovery after stress.

3. Sugarcane and biotechnology

Sugarcane crop productivity has progressively increased to remarkable levels worldwide in the last century [36]. This increase in productivity has been ascribed to the development and widespread use of improved cultivars with increased resistance to diseases and pests, better management of water, nutrients and other resources, and the availability of relatively cheap chemical fertilizers and pesticides. Sustaining this pace of improvement in crop productivity by innovative and intensive agriculture, whilst ensuring minimal environmental impact, will be one of the major challenges to maintain a profitable sugar industry in the future.

Biotechnology offers excellent opportunities for sugarcane crop improvement. Commercial sugarcane, mainly the interspecific hybrids of *S. officinarum* and *S. spontaneum* [37], would greatly benefit from biotechnological improvements due to its complex polyploid-aneuploid genome, narrow genetic base, poor fertility, susceptibility to various diseases and pests, and the long duration (12–15 years) required to breed elite cultivars. More importantly, there is an ongoing need to provide durable disease and pest resistance commercial clones in combination with superior agronomic performance. This led to considerable research in different areas of biotechnology pertinent to sugarcane breeding and disease control. Despite the availability of molecular tools and strategies and advancements in our understanding of stress responses, engineering crops for drought tolerance remains a major challenge. This is not only due to the complexity of the plant responses to water deficit but also due to the difficulty of identifying and exploiting of large effect genes and alleles and the associated selection traits for developing drought tolerant varieties suitable for commercial crop production conditions [38].

4. Micropropagation an alternative to develop plants tolerant to water stress "hyperhydricity"

Various micropropagation systems such as liquid cultures and automation have proven the potential to resolve manual handling of *in vitro* cultures at various stages and decrease production cost. However, hyperhydricity is a major problem during *in vitro* culture of many crops in liquid culture systems. Hyperhydricity (also known as "vitrification") is a physiological disorder occurring in plant material of tissue culture, which causes a reduction of propagation and death of tissues when transferred to *ex vitro* conditions [39–41]. The environment inside culture vessels normally used for plant micropropagation is characterized by high humidity, limited gaseous exchange between the internal atmosphere of the culture vessel and its surrounding environment, and the accumulation of ethylene; conditions that may induce physiological disorders [42]. The development of hyperhydric deformities represents a disadvantage for plant micropropagation and a barrier for the exploitation of bioreactor technologies to scale-up its production [41]. The concept of stress in relation to hyperhydricity is not completely established. Therefore, it remains difficult to assume when hyperhydric tissues are stressed. Previous studies argued that abnormal morphology observed in hyperhydricity could be attributed to changes occurring at cellular level due to the modifications of membrane composition or DNA content [42]. However, Rojas-Martínez and coworkers [41] considered this disorder as the result of the stressful conditions brought out by waterlogging of the apoplast. This causes hypoxia and thereby leads to severe oxidative stress. They concluded that hyperhydric features like vitreous appearance and wrinkled leaves are secondary events resulting from waterlogging of the apoplast.

The temporary immersion system (TIS) consists on the use of bioreactors with automated devices that control features such as gas exchange, liquid medium culture and lighting, required for the growth, development and survival of plants. TIS mainly consist of three phases: multiplication, elongation and rooting phase. Plantlets propagated in TIS have better performance than those propagated by conventional methods of micropropagation. TIS provides a rapid and efficient plant propagation system for many agricultural and forestry species, it utilizes liquid media avoiding intensive manual handling [43].

With the objective of evaluating the stress caused by hyperhydricity in the *in vitro* culture of sugarcane var. MEX69290, three types of culture were analyzed: Semisolid (Magenta) was used as control; Continuous immersion (250 ml Flask); and Temporary Immersion (BioMINT II Bioreactor). Multiplication, maturation, and *ex vitro* adaptation phases of sugarcane under these three types of culture were evaluated.

The obtained results in the adaptation of *in vitro* plants of *S. officinarum* at three different types of culture in the multiplication phase were surprising, as it is observed in **Figure 1**, where a notorious formation of shoots occurs in continuous immersion medium. Plants of var. MEX69290 obtained a much higher average shoot formation at the temporary immersion bioreactors than those observed in semi-solid medium. It was observed that invariable of the inoculum density applied (5, 10, 15 plants per bottle) was higher in continuous immersion. Similarly, growth index factor was higher in this culture system than that obtained in semi-solid medium or temporary immersion bioreactors (**Figure 1**). We can observe comparing our results with other

http://dx.doi.org/10.5772/intechopen.72438

works that the treatment response depends on the type of explant and variety of sugarcane. Several studies have reported that the rate of shoot formation is higher in temporary immersion bioreactors than in semi-solid cultures. It is important to mention that none of the previous works reported any problem with the hyperhydricity in the obtained *in vitro* plants. Only, Snyman [44] reports this condition on the induction and germination of somatic sugarcane embryos. Tesfa and coworkers [45], didn't report problems of hyperhydricity or a decrease in field survival rate out of *in vitro* plants after using a liquid culture medium with agitation

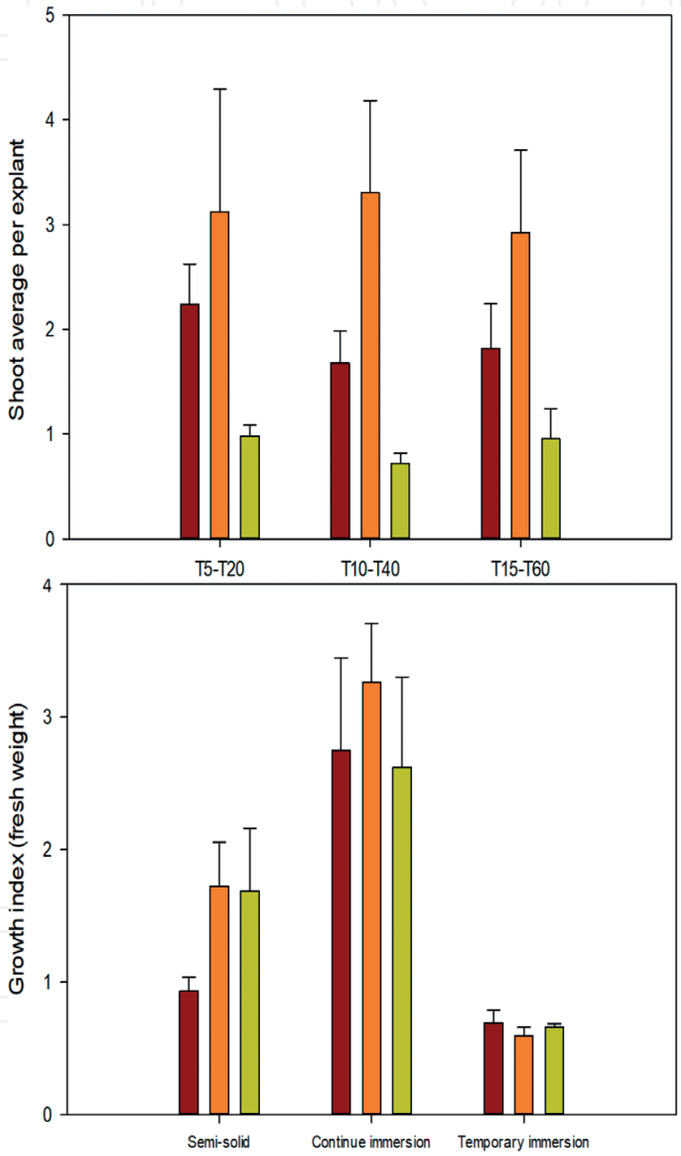

Figure 1. Average of shoots at different densities of inoculum. T5-T20: 5 inoculum plants were used in semi-solid and continuous immersion medium, 20 plants in temporary immersion; T10-T40: 10 inoculum plants were used in semi-solid and continuous immersion medium, and 40 plants in temporary immersion; T15-T60: 15 inoculum plants were used in semi-solid and continuous immersion medium, and 60 plants in temporary immersion; semisolid (red rectangle), continue immersion (orange rectangle) and temporary immersion (green rectangle). At the bottom of the figure, the calculated growth index factor is reported using the obtained fresh weight under the same inoculum density conditions; T5-T20 (red rectangle), T10-T40 (orange rectangle) and T15-T60 (green rectangle). Five replicates were carried out for each treatment.

(80 rpm) in which they obtained an average shoot emission of 6.95 and 6.30 in the two cultivars used. The shoot emissions and growing index of the sugarcane variety MEX69290 was not affected when cultivated in a stationary liquid medium for 28 days (**Figure 1**).

The variety MEX69290 clones' response at the maturation phase showed the same behavior as that observed at the multiplication phase, with the average shoot emission and the growth index being higher in the liquid culture than the one obtained in half semi-solid or in the temporary immersion bioreactor culture (**Figure 2**).

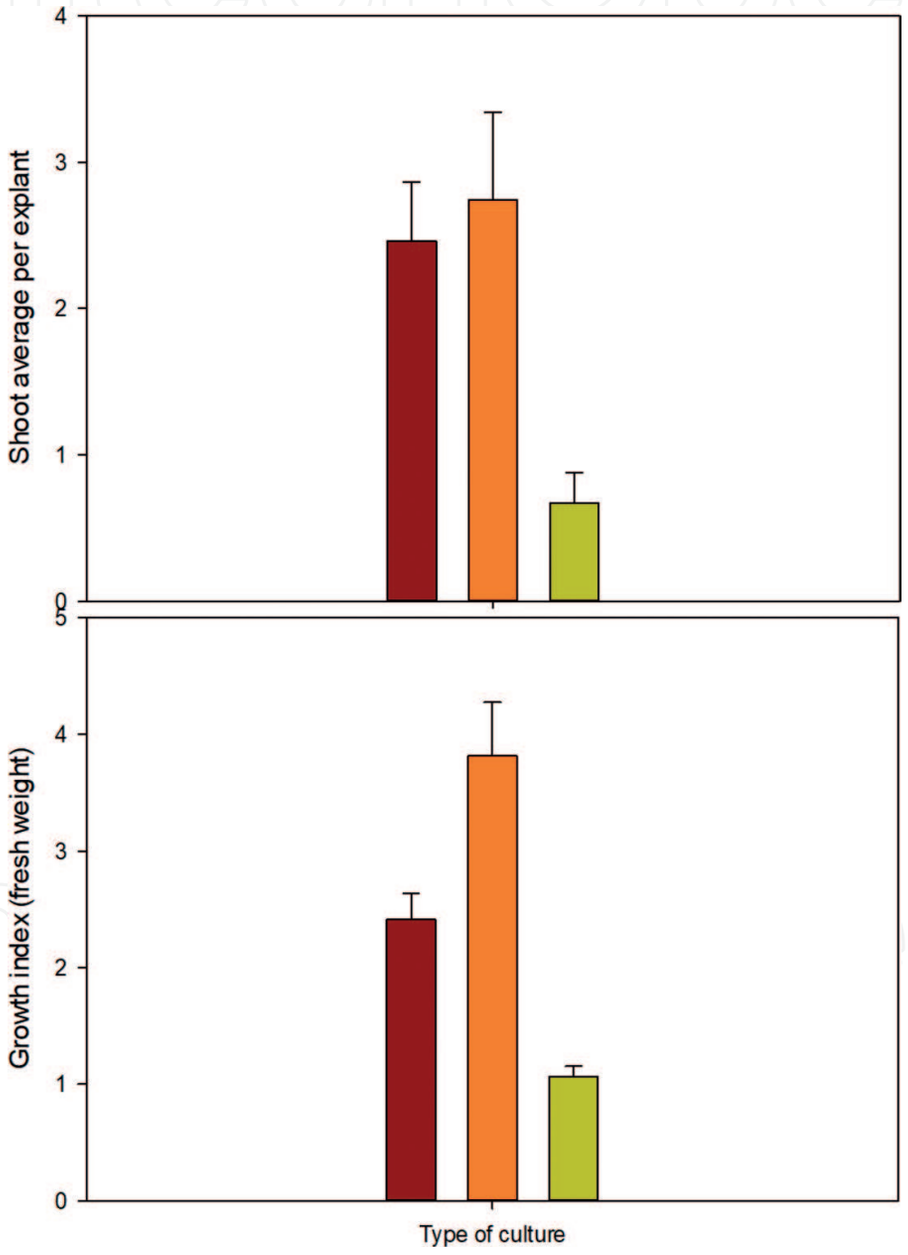

Figure 2. Mean of shoots using 10 in vitro plants in semi-solid and continuous immersion cultures and 60 plants in temporary immersion bioreactors. At the bottom of the figure the calculated growth index factor is reported using the obtained fresh weight under the same inoculum density conditions. Five replicates were carried out for each treatment. Semisolid (red rectangle), continue immersion (orange rectangle) and temporary immersion (green rectangle).

http://dx.doi.org/10.5772/intechopen.72438

After 28 days in maturation phase, 120 plants from semi-solid culture, 120 plants under continuous immersion, and 75 from BIOMINT were adapted. In **Figure 3**, we can observe the quality of the plants from the same clone at the three different cultivation systems.

Plants underwent a 28 days preadaptation period, and afterward were planted and placed in greenhouse conditions. Once plants where transferred into the greenhouse, their survival rate was evaluated, being 100% in all cases (**Figure 4**). Plants from the temporary immersion bioreactors were taller and with longer leaves, but those from semi-solid medium and continuous immersion continued to emit shoots during the following 4 months evaluation at the greenhouse. The results obtained in this phase are very similar to those reported by Arencibia et al. [46], Bernal et al. [47], and Silva et al. [48], who reported survival rates higher than 96% in the different cultivars using a temporary immersion bioreactor, and our result is much higher than the studies reported by Snyman et al. [44], with only 34% of survival rate from sugarcane grown in the RITA system.

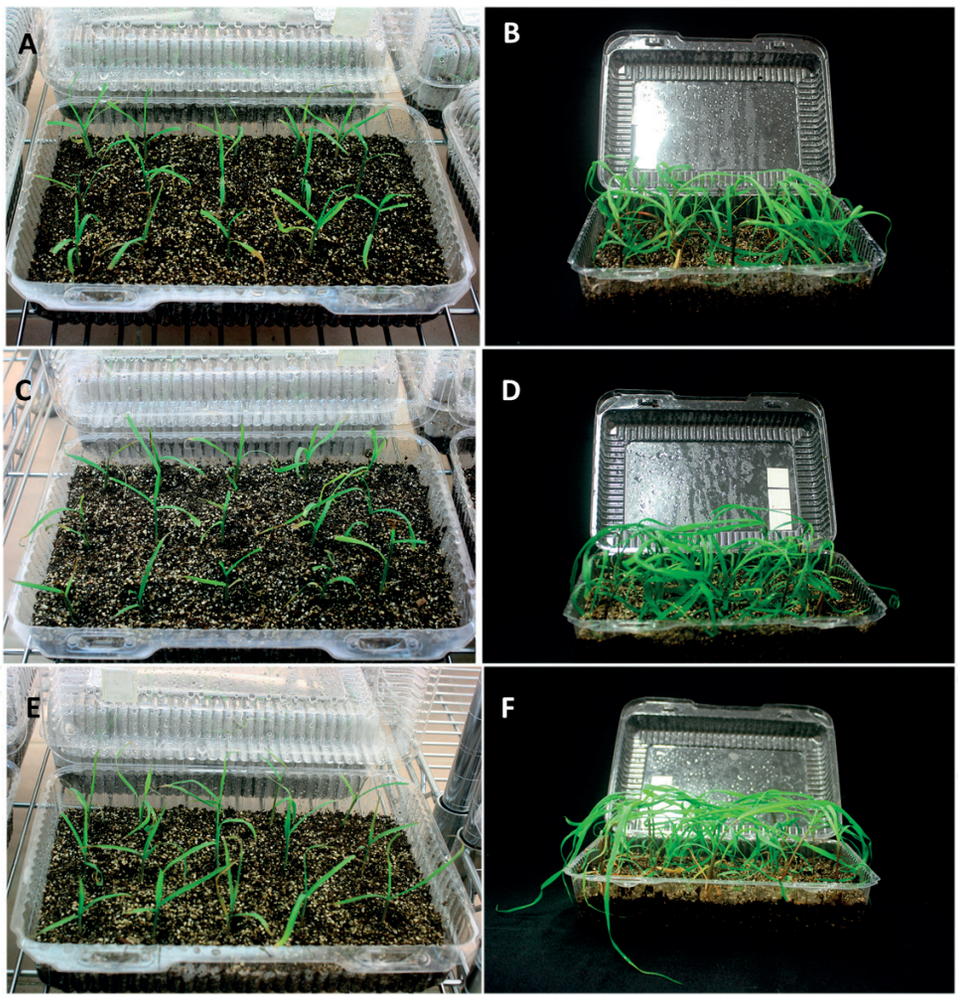

Figure 3. Phase adaptation of *in vitro* plants of *S. officinarum* var. MEX69290, seeded in a germination mixture BM2, previously autoclaved. 15 plants per container were adapted in growth culture room at 25°C with 16/8 hours photoperiod light/dark. (A) and (B) day zero and twenty-eight, of plants coming from semi-solid culture; (C) and (D) day zero and twenty-eight, of plants coming from liquid culture; (E) and (F) day zero and twenty-eight, of plants coming from temporary immersion system (BioMINT).

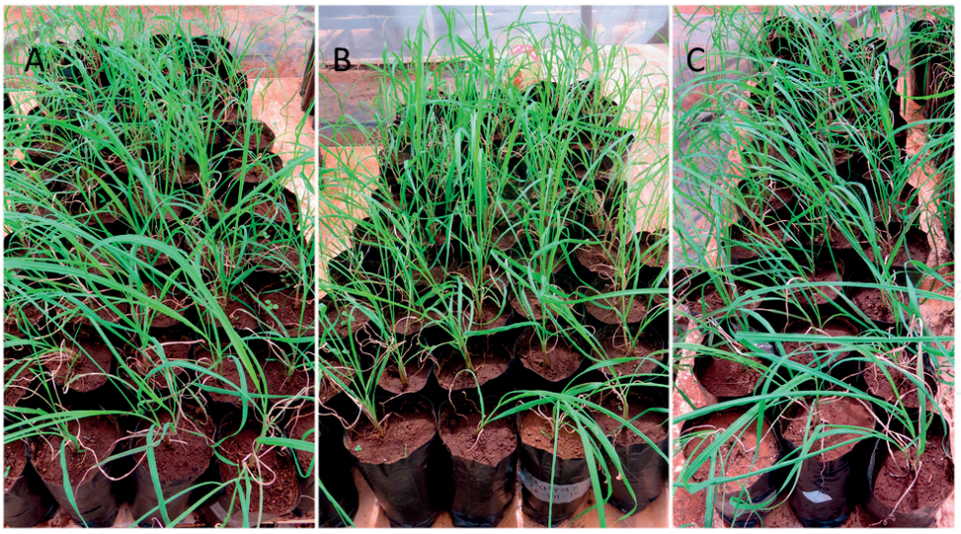

Figure 4. Greenhouse adaptation of *in vitro* plants of *S. officinarum* var. MEX69290, from culture: (A) semi-solid; (B) continuous immersion; (C) temporary immersion. Substrate consisted on a 3: 1 mixture of sunshine: soil. All plantlets survived 100% after 30 days in the greenhouse.

The best results out of the measured parameters were obtained from the continuous immersion propagation system. It was concluded the reason for this may reside in the elimination of gelling agent, which additionally lowers production costs in the process of delivering this sugarcane's variety to the field. Plants obtained under this system achieved normal development, they developed shoots and roots cyclically and no vitrification was detected in any of the evaluated micropropagation phases. This suggests that the clone obtained from the MEX69290 variety is tolerant to liquid culture conditions. Apparently this system does not generate an abiotic stress, stationing it as a prospective medium to perform genetic transformation processes and to study its gene expression pattern that could further make enhanced tolerant clones.

5. Transcriptomic analysis of an elite Mexican sugarcane cultivar ('Mex 69-290') in response to osmotic stress. Identification of genes with biotechnological potential

Modern sugarcane cultivars have been obtained by inter-specific hybridizations between the high-sucrose-yielding of *S. officinarum* (2n = 8x = 80) and the stress-tolerant *S. spontaneum* (2n = 40–128). As a consequence, sugarcane cultivars present large (10 Gb) and poly-aneuploid genomes with numerous gene alleles and repetitive sequences. Such genome complexity has made it difficult to obtain a complete sequenced reference genome that could aid in the identification of novel genes with biotechnological potential for the improvement of this important C4 crop. Alternatively, de novo transcriptome assembly of reads produced by high-throughput sequencing technologies (also referred to as Next Generation Sequencing (NGS)) offers a mean to unravel global gene expression changes in response to various conditions in

sugarcane. For example, some recent works have employed High-throughput sequencing to identify sugarcane genes involved in leaf abscission [49], biomass content and composition [50], and abiotic stress [51]. Li and cols. [49] performed a transcriptome analysis to identify genes associated with leaf abscission in sugarcane. They employed the Illumina HiSeq 2000 platform (2x90pb) to analyze six cDNA libraries from parents and their F1 offspring, which present different leaf abscission behaviors. After a total assembly, they found 275,018 transcripts corresponding to 164,803 genes. Then, to identify genes related to leaf abscission in sugarcane [49], analyzed a core set of 1, 202 transcripts which were up-regulated in leaf abscission sugarcane plants (LASP) in comparison to leaf packaging sugarcane plants (LPSP). They found that some of these genes were associated with plant-pathogen interaction, response to stress, and ABA-associated pathways. On the other hand [50], performed an extensive transcriptome analysis to identify genes associated with biomass content. They employed the Illumina HiSeq 4000 platform to analyze cDNA libraries from 20 internodal samples of 10 different sugarcane genotypes, which were divided in low and high fiber containing groups. They found 5601 and 4659 unique expressed transcripts in High and Low fiber containing genotypes; and 83,421 shared expressed transcripts between both groups. Furthermore, they found 555 differentially expressed transcripts between low and high fiber containing genotypes. Of these, 151 and 23 transcripts corresponded to sugar and fiber accumulation, respectively. Some of these genes were involved in Carbohydrate metabolism, Photosynthesis, Cell-wall metabolism and Lignin Pathway; DIR proteins were also represented [50].

Regarding abiotic stress, Belesini and cols. [51] analyzed the transcriptomic profile of the drought-tolerant 'SP81-3250' and the drought-sensitive 'RB855453' sugarcane cultivars under drought stress conditions for 30, 60, and 90 days. They analyzed a total of 54 cDNA libraries by Illumina HiScanSQ System and HiSeq 2500 platforms. Among the genes that were induced in the drought-tolerant cultivar, they found an ascorbate peroxidase, a MYB TF, an E3 SUMO-protein ligase SIZ2, a coenzyme A ligase (a key enzyme for the biosynthesis of flavonoids), and an aquaporin, among others. These types of genes are well known to play a role in abiotic stress tolerance. In the drought-sensitive cultivar they found several kinases that were induced upon stress like Receptor like protein kinases (RLK), which might play a role in stress stimulus perception; bHLH transcription factors; ACC oxidase from the ethylene biosynthetic pathway; and many undescribed genes. More recently (2017), in our laboratory Pereira-Santana and cols. [52] analyzed the transcriptomic profile of the 2nd most important sugarcane cultivar in Mexico, 'Mex 69-290', in response to osmotic stress. In such study, authors employed the High-throughput sequencing system HiSeq-Illumina (2x100bp) to analyze 16 cDNA libraries representing leaves and roots of *in vitro*-grown plantlets exposed to PEG-8000 during 0, 24, 48, and 72 hours. After assembly of a total of 140, 339 unigenes, Pereira-Santana and cols. Found core sets of 536 and 750 up-regulated genes in response to osmotic stress in roots and leaves, respectively; and core sets of 1093 and 531 down-regulated genes in roots and leaves, respectively. After gene annotation, the authors found that sugarcane 'MEX69290' responds to osmotic stress by increasing the expression of genes involved in transcription regulation, oxide-reduction, carbohydrate catabolism, and flavonoid and other secondary metabolites biosynthesis. Genes responsive to ABA, water deprivation, and heat stress were also up-regulated. On the other hand, this sugarcane cultivar responds to osmotic stress by

decreasing the expression of genes involved in sucrose and starch metabolic processes, cell wall biogenesis, cellulose biosynthesis, anion transport, and light response. A handful of the genes found by Pereria-Santana and cols. Are presented along with their expression profiles in the heat map of **Figure 5A**. Because of the well-defined expression pattern of some of these genes, they could prove to be useful as expression markers in the response of 'MEX69290' to osmotic stress. For example, ABA 8-hydroxylase 3, Isoflavone 2-hydroxylase, LEA 14A, and NAC TF 25 showed clear patterns of up-regulation. In fact, in our laboratory further expression and functional analyses are currently being carried out regarding this NAC TF25 gene. Conversely, Bidirectional sugar transporter SWEET11, Cellulose synthase E6, and Sugar transporter ERD6 16 showed clear patterns of down-regulation. These down-regulated genes are also interesting, not just because of their responsiveness to osmotic stress but also due to their involvement in sucrose metabolism. The engineering of these genes might increase biomass production in sugarcane and tolerance to osmotic stress simultaneously. Furthermore, many TFs known to play important roles in the stress responses of plants, i.e. HSF, ZN, bZIP, WRKY, NAC, and MYB, were found in abundance in the total assembly of the 'MEX69290' transcriptome (**Figure 5B**). Even when some of these TF families seemed underrepresented (like NAC and MYC), they still provide a useful benchmark to conduct phylogenetic, expression, and functional analysis.

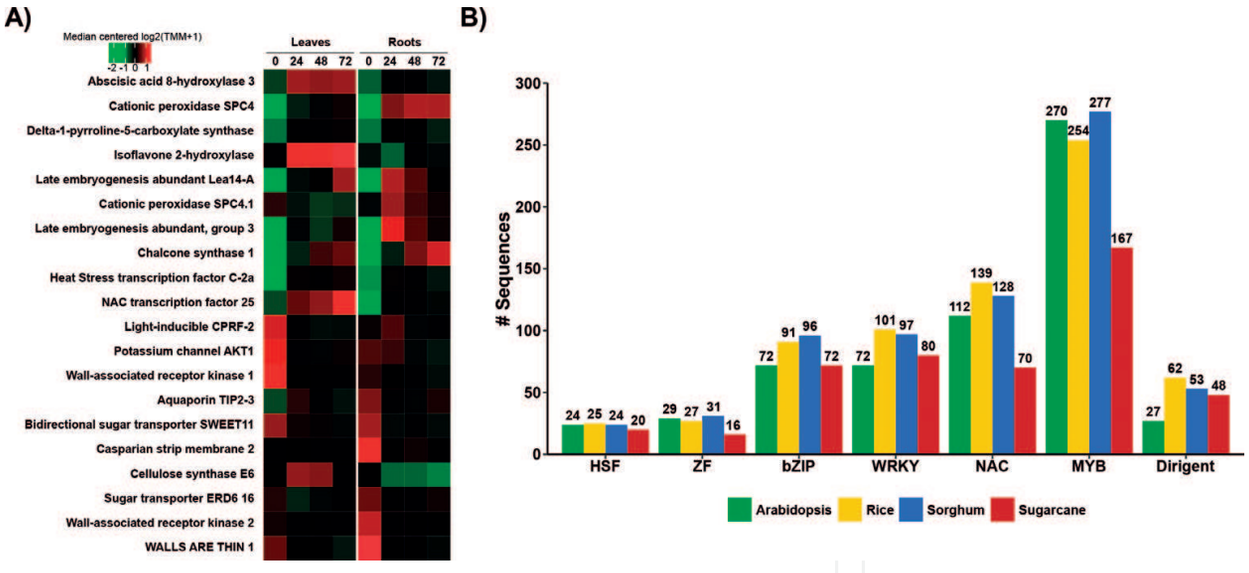

Figure 5. Selected DEGs in response to osmotic stress and abundance of major TF families and Dirigent protein family in sugarcane 'MEX69290' transcriptome. (A) Expression profile of 20 selected DEGs in leaves and roots of sugarcane 'MEX69290' plantlets submitted to PEG-8000 treatment during 0, 24, 48, and 72 hours. Data was obtained from the work of Pereira-Santana and cols [52]. The heat map was generated with the ComplexHeatmappackage v1.14.0 [52] in R v3.4.1 [53]. (B) Abundance of major stress-related TF families and Dirigent protein family in arabidopsis, rice, sorghum, and sugarcane. The results were obtained by means of HMM searches using the profiles of the HSF (PF00447), ZF (PF00096), bZIP (PF00170), WRKY (PF03106), NAC (PF02365), MYB (PF00249), and Dirigent (PF03018) proteins obtained from the Pfam database (http://pfam.xfam.org) [54]. For this analysis the complete predicted proteomes (primary transcripts only) of arabidopsis, rice, and sorghum were obtained from Phytozome v. 12 [55]. Sugarcane predicted protein dataset was obtained from the transcriptome assembly of Pereira-Santana and cols [52] HMM searches were performed using HMMER3 v3.1b2 (http://hmmer.org/) and set to a cut-off e-value of 1e-05 and a score above the inclusion threshold of each HMM profile.

In addition to the insights about the global gene expression dynamics of 'Mex 69-290' in response to osmotic stress and the identification of novel TFs, the work of Pereira-Santana and cols. Provides a useful benchmark for the study of other specific gene families of biotechnological significance for sugarcane engineering, for example the DIR protein family. Plant DIR proteins are believed to be involved in lignin biosynthesis, defense [56, 57], and abiotic stress responses such as dehydration [58], and salinity and oxidative stress [59]. In a recent study, 5 available sequence databases for sugarcane were surveyed, a total of 120 DIR proteins were identified [60]. Phylogenetic analysis showed that these DIR proteins are divided in 64 groups and 7 major clades: Dir-a, Dir-b/d, Dir-c, Dir-e, Dir-g, Dir-h, and Dir-i [60]. In the sugarcane transcriptome assembly of 'sugarcane Mex 69-290' performed in our laboratory by Pereira-Santana and cols, a total of 48 predicted proteins with DIR-like domains were identified. These DIR proteins were clustered in 7 groups according to their expression patterns (**Figure 6**). DIR42 protein from cluster 1 was significantly up-regulated in all time points of osmotic stress in root tissues. Conversely, DIR40 protein from cluster 7 was significantly down-regulated in all time points of osmotic stress in leaf tissues. In general, DIR genes from cluster 4 seem to possess a relative high expression in roots under control conditions, and those from cluster 7 seem to possess a relative high expression in leaves under control conditions. DIR genes from both clusters are down-regulated in response to osmotic stress. On the other hand, we also recovered a homolog of the ScDir gene (GenBank: JQ622282.1) from the sugarcane variety FN39 (DIR38 in cluster 5). The expression of ScDir from FN39 has been

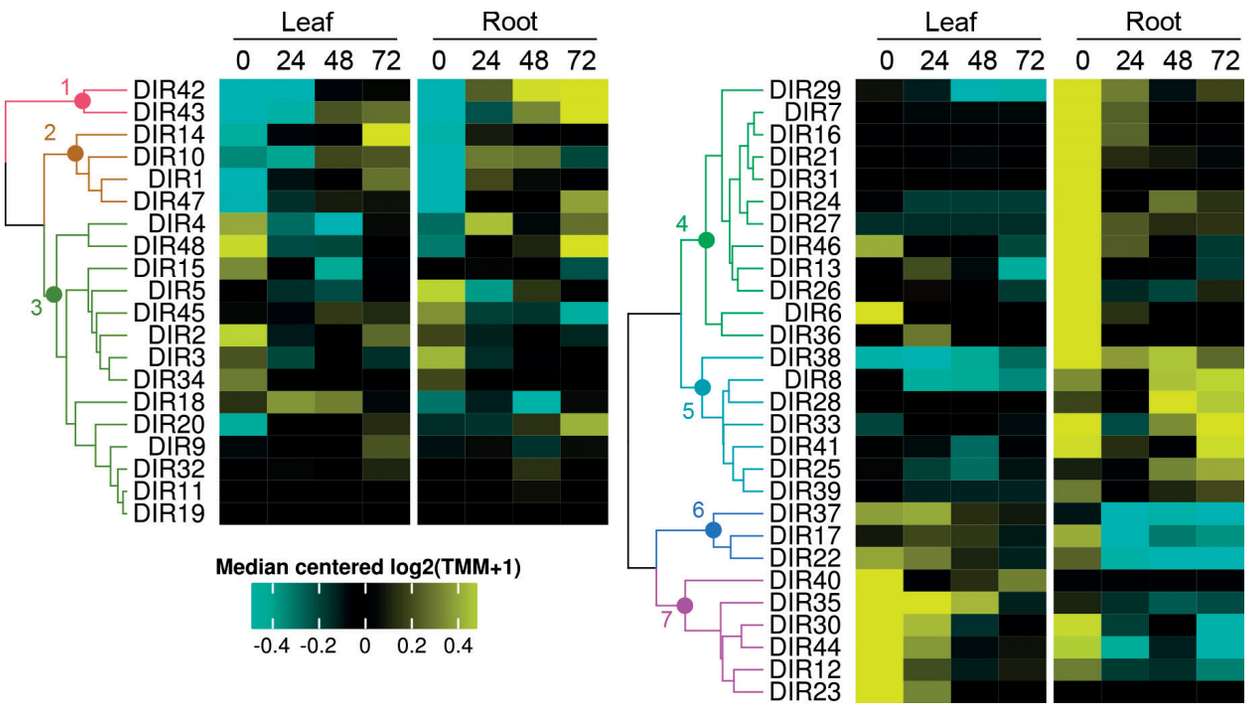

Figure 6. Differential expression in response to osmotic stress of 48 Dirigent proteins found in sugarcane 'MEX69290' transcriptome. The 48 Dirigent sequences from sugarcane were grouped according to their expression profiles in 7 clusters (1–7). Data was obtained from Pereira-Santana and cols [52]. Heat map and sequence clustering were generated with ComplexHeatmap v1.14.0 [53] in R v3.4.1 [54] using the "euclidean" distance method and "complete" clustering method.

reported to be up-regulated in response to H_2O_2, NaCl, and PEG treatment [59]. Furthermore, its heterologous expression in *Escherichia coli* increases the bacterial host's tolerance to NaCl and PEG [59]. The homolog of this gene in 'Mex 69-290' was slightly up-regulated in leaves, but down-regulated in roots (**Figure 6**, cluster 5). All of these mentioned DIR genes from sugarcane 'MEX69290' are interesting because they show differential expression patterns in leaves and roots in response to osmotic stress. However, their functional roles in osmotic stress tolerance and biomass accumulation still need to be experimentally analyzed. In summary, in the absence of a complete sequenced genome for sugarcane, high-throughput sequencing technologies applied to the elucidation of elite cultivars' transcriptome profile are one of the most valuable resources for the identification of genes involved in both stress tolerance and biomass accumulation, which are important agronomic traits to face global climate change.

6. Genetic transformation of cane, a very powerful biotechnological tool to generate tolerant plants to water stress

According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the worldwide distribution of genetically modified crops involves a total of 26 developing countries and 7 industrialized countries, headed by USA, Brazil, Argentina, Canada, India, China and South Africa. There is a current approval on the use of two commercial varieties of genetically modified cane in Brazil and Indonesia. On the former, plants containing the Cry1Ab gene, which produces an insecticidal toxin capable of killing the *Diatraea* caterpillar, are being cultivated. In Indonesia plants transformed with the EcBetA gene are resistant to drought.

Scientific research in genetic transformation have focused on resistance to biotic and abiotic factors such as weed control, production of renewable primary products, energy crops and production of pharmaceutically active substances.

Some of the methods in genetic transformation of plants are by Agrobacterium or biolistic which are time consuming, laborious and have low transformation efficiency. Thus we have attempted different options to optimize genetic transformation in sugar cane. An option for efficient transformation is by using different types of vectors, for example Anderson & Birch [61] used Binary super vectors in addition of different types of promoters (constitutive and inducible). Niu et al. [62] is other case who used the SoCINI inducible promoters and the ScMybRI constitutive promoters respectively [62, 63].

On the other hand, different *in vitro* culture protocols have been tried for decades to optimize the efficiency (time and management of the explant) as well as the number of transgenic plants. Yogesh and collaborators transformed cane leaves by Biolistic [64], regenerating seedlings via direct (ED) and indirect (EI) embryogenesis [65]. Arencibia and Carmona [66] reported genetic transformation by *Agrobacterium tumefaciens* and via indirect morphogenesis resulting in regenerated seedlings. Manickavasagam et al. reported regenerated seedlings

after *A. tumefaciens* transformation via axillary shoots [67]. These latter two protocols require a time lapse between 3 and 6 months to generate seedlings.

In contrast, a genetic transformation protocol using *A. tumefaciens* has been developed in our laboratory (in the process of obtaining patent) where *in vitro* basal micro-shoots of MEX69290 cultivars underwent the insertion of the CpRap2.4b gene from the AP2/ERF transcription factor family, and out of cDNA of papaya stressed at 40°C. This genetic transformation protocol requires only 20 minutes and has a contamination rate of 0%, as well as a 21-day seedling regeneration rate. Our results showed a 70% survival in the first subculture and 100% in the second subculture with Kanamycin; similar results were reported by Manickavasagam regenerating transgenic seedlings using micro axillary outbreaks out of field plants [67], with a very laborious genetic transformation system and with 50% survival in the first crop. In addition, this work would be the second in sugarcane to report a gene of the AP2/ERF family of transcription factors inserted in sugar cane, the other work is the one reported by Reis et al. where they over expressed AtDREB2A CA (constitutive activity) in sugar cane [68]. In the transformed sugarcane seedlings generated by the genetic transformation protocol that was developed in our laboratory, the presence of the GFP was observed at the fluorescent emission of 395–475 nm, which indicates that the seedlings are transformed (**Figure 7**).

It should be clarified that the functionality of the CpRap2.4b gene belonging to the (AP2/ERF) transcription factors family was tested in tobacco plants, which were segregated to obtain F2 plants and were then subjected to water stress (drought) conditions to evaluate their function.

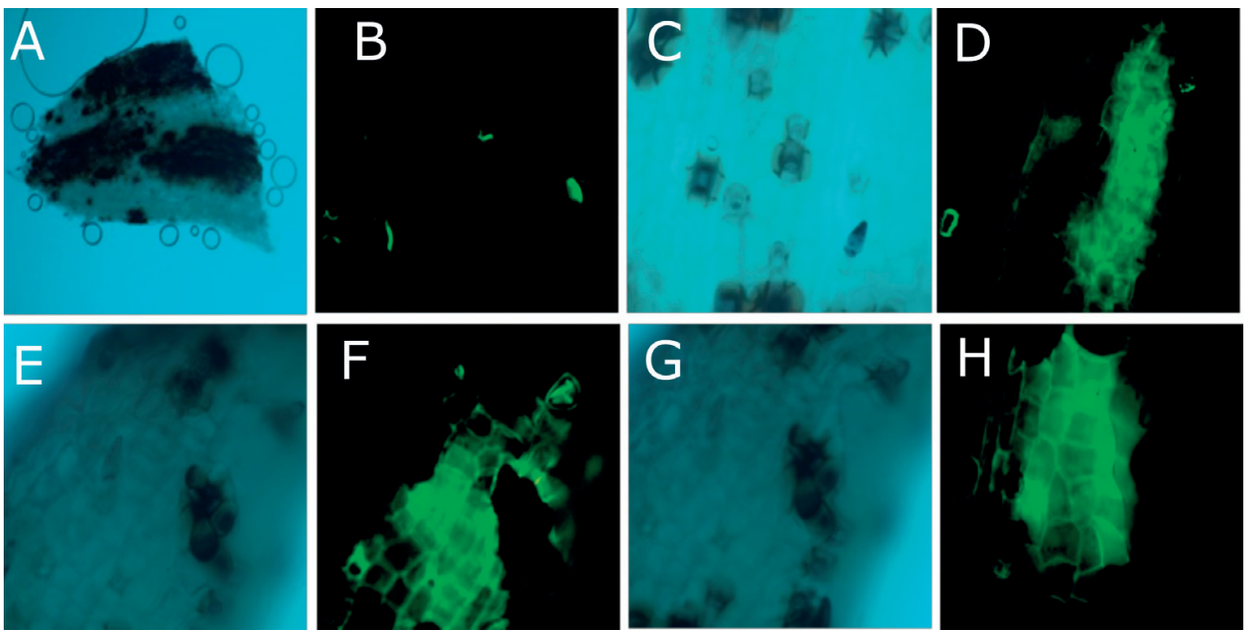

Figure 7. GFP fluorescence of different plant leaves of sugar cane var. MEX69290. (A) Segment of wild leaf in visible light. (B) Wild leaf segment with emission at 509 nm. (C, E and G) Transgenic plants 1, 2 and 3 in visible light. (D, F and H) Transgenic plants 1, 2 and 3 with emission at 509 nm.

7. Conclusions

Climate change affects farmers economically, causing drought floods, which affect the productivity of the plant. Biotechnology is an alternative to reduce the impact of climate change on plants. In recent years there has been a continuing need to provide commercial clones of resistance to pests and long-lasting diseases in combination with superior agronomic performance. This led to considerable research in different areas of biotechnology including: micropropagation, transcriptomics and genetic transformation.

These areas of biotechnology together are a key tool in the pursuit of genetically enhanced plants that resist climate change.

Abbreviations

BAC	Bacterial artificial chromosome
EST	Expressed sequence tag
NGS	Next generation sequencing
GO	Gene Ontology
ABA	Abscisic acid
LEA	Late embryogenesis abundant
NAC	NAM, ATAF, and CUC
MYB	Myeloblastosis
HSF	Heat shock factor
ZF	Zinc Finger
TF	Transcription factor
ORF	Open reading frame
aa	Amino acids
DEG	Differentially expressed genes
HMM	Hidden Markov Model
TMM	Trimmed mean of M values
DIR	Dirigent
nt	Nucleotide
H_2O_2	Hydrogen peroxide
NaCl	Sodium chloride

PEG Polyethylene glycol

CA Constitutive activity

GFP Green fluorescent protein

Author details

Evelyn Arlette Carrillo Bermejo[1], Miguel Angel Herrera Alamillo[1], Samuel David Gamboa Tuz[1], Miguel Angel Keb Llanes[1], Enrique Castaño de la Serna[2], Manuel L. Robert[1] and Luis Carlos Rodríguez Zapata[1]*

*Address all correspondence to: lcrz@cicy.mx

1 Biotechnology Unit, Yucatan Scientific Research Center, Yucatan, Mexico

2 Unit of Biochemistry and Molecular Biology of Plants, Center for Scientific Research of Yucatan, Yucatan, Mexico

References

[1] Nelsona G, Valinb H, Sandsc R, Havlíkb P, Ahammadd H, Derynge D, et al. Climate change effects on agriculture: Economic responses to biophysical shocks. PNAS. 2014;**111**:3274-3279. DOI: 10.1073/pnas.1222465110

[2] Jahangir GZ, Nasir IA. Various hormonal supplementations activate sugarcane regeneration in vitro. Journal of Agricultural Science. 2010;**2**:231-237. DOI: 10.5539/jas.v2n4p231

[3] FAOSTAT. Sugarcane Production. Rome, Italy: Food and Agriculture Organization; 2014 [Internet]. Available from: http://faostat.fao.org/site/567/DesktopDefault.asp [Accessed: 21-02-2017]

[4] Sengar K. Developing an efficient protocol through tissue culture techniques for sugarcane micropropagation. BioInfoBank Library Acta. 2010;**18**:56

[5] Agarwal PK, Agarwal P, Reddy MK, Sopory SK. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Reports. 2006;**25**:1263-1274. DOI: 10.1007/s00299-006-0204-8

[6] Burke EJ, Brown SJ, Christidis N. Modelling the recent evolution of global drought and projections for the twenty-first century with the Hadley Centre climate model. Journal of Hydrometeorology. 2006;**7**:1113-1125

[7] Shao HB, Chu LY, Jaleel CA, Zhao CX. Water-deficit stress-induced anatomical changes in higher plants. Comptes Rendus Biologies. 2008;**33**:215-225

[8] Moore PH, Paterson AH, Tew T. Sugarcane: The crop, the plant, and domestication. In: Moore PH, Botha FC, editors. Sugarcane: Physiology, Biochemistry, and Functional Biology. Chichester: John Wiley & Sons, Inc.; 2013. pp. 411-435. DOI: 10.1002/ 9781118771280.ch1

[9] Kingston G. Benchmarking yield of sugarcane from estimates of crop water use. Proceedings of the Australian Society for Sugar Cane Technologists. 1994;**16**:201-209

[10] Robertson MJ, Inmam-Bamber NG, Muchow RC, Wood AW. Physiology and productivity of sugarcane with early and mid-season water deficit. Field Crops Research. 1999;**64**:211-227. DOI: 10.1016/S0378-4290(99)00042-8

[11] Ramesh P. Effect of different levels of drought during the formative phase on growth parameters and its relationship with dry matter accumulation in sugarcane. Journal of Agricultural Science. 2000;**185**:83-89. DOI: 10.1046/j.1439-037x.2000.00404.x

[12] Basnayake J, Jackson PAN, Inman-Bamber G, Lakshmanan P. Sugarcane for water-limited environments. Genetic variation in cane yield and sugar content in response to water stress. Journal of Experimental Botany. 2012;**63**:6023-6033. DOI: 10.1093/jxb/ers251

[13] Gentile A, Dias LI, Mattos RS, Ferreira TH, Menossi M. MicroRNAs and drought responses in sugarcane. Frontiers in Plant Science. 2015;**6**:58. DOI: 10.3389/fpls.2015.00058

[14] Moreira J, Goswami D, Zhao Y, editors. Solar Energy and Human Settlement. Bioenergy-successes and barriers. In: Proceedings of ISES Solar World Congress. Vols. I–V; 2017. pp. 38-45

[15] Walter A, Galdos M, Scarpare F, Seabra J, Leal M, Cunha M. Brazilian sugarcane ethanol: Developments so far and challenges for the future. Wiley Interdisciplinary Reviews: Energy and Environment. 2013;**3**:70-92. DOI: 10.1002/wene.87

[16] Wang WX, Vinocur B, Shoseyov O, Altman A. Biotechnology of plant osmotic stress tolerance and physiological and molecular considerations. Acta Horticulturae. 2001;**560**:285-292. DOI: 10.17660/ActaHortic.2001.560.54

[17] Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. International Journal of Molecular Sciences. 2013;**14**:9643-9684. DOI: 10.3390/ijms14059643

[18] Bartels D, Sunkar R. Drought and salt tolerance in plants. Critical Reviews in Plant Sciences. 2005;**24**:23-58. DOI: 10.1080/07352680590910410

[19] Smit M, Singels A. The response of sugarcane canopy development to water stress. Field Crops Research. 2006;**98**:91-97. DOI: 10.1016/j.fcr.2005.12.009

[20] Da Graça J, Rodrigues F, Farias J, Oliveira MD, Hoffmann-Campo C, Zingaretti S. Physiological parameters in sugarcane cultivars submitted to water deficit. Brazilian Journal of Plant Physiology. 2010;**22**:189-197. DOI: 10.1590/S1677-04202010000300006

[21] Inman-Bamber N, Lakshmanan P, Park S. Sugarcane for water limited environments: Theoretical assessment of suitable traits. Field Crops Research. 2012;**134**:95-104. DOI: 10.1016/j.fcr.2012.05.004

[22] Hemaprabha G, Nagarajan R, Alarmelu S. Response of sugarcane genotypes to water deficit stress. Sugar Tech. 2004;**6**:165-168. DOI: 10.1007/BF02942718

[23] Machado R, Ribeiro R, Marchiori P, Machado D, Machado E, Landell M. Biometric and physiological responses to water deficit in sugarcane at different phenological stages. Pesquisa Agropecuária Brasileira. 2009;**44**:1575-1582

[24] Inman-Bamber N. Sugarcane water stress criteria for irrigation and drying off. Field Crops Research. 2004;**89**:107-122. DOI: 10.1016/j.fcr.2004.01.018

[25] Inman-Bamber N, Bonnett G, Spillman M, Hewitt M, Jackson J. Increasing sucrose accumulation in sugarcane by manipulating leaf extension and photosynthesis with irrigation. Australian Journal of Agricultural Research. 2008;**59**:13-26. DOI: 10.1071/AR07167

[26] Inman-Bamber N, Smith D. Water relations in sugarcane and response to water deficits. Field Crops Research. 2005;**92**:185-202. DOI: 10.1016/j.fcr.2005.01.023

[27] Du Y, Kawamitsu Y, Nose A, Hiyane S, Murayama S, Wasano K, et al. Effects of water stress on carbon exchange rate and activities of photosynthetic enzymes in leaves of sugarcane (*Saccharum* sp.). Australian Journal of Plant Physiology. 1996;**23**:719-726. DOI: 10.1071/PP9960719

[28] Endres L, Silva JV, Ferreira VM, Barbosa GVS. Photosynthesis and water relations in Brazilian sugarcane. The Open Agriculture Journal. 2010;**4**:31-37. DOI: 10.2174/1874331501004010031

[29] Medeiros DB, da Silva EC, Mansur Custodio Nogueira RJ, Teixeira MM, Buckeridge MS. Physiological limitations in two sugarcane varieties under water suppression and after recovering. Theoretical and Experimental Plant Physiology. 2013;**25**:213-222. DOI: 10.1590/S2197-00252013000300006

[30] Basnayake J, Jackson PA, Inman-Bamber NG, Lakshmanan P. Sugarcane for water-limited environments. Variation in stomatal conductance and its genetic correlation with crop productivity. Journal of Experimental Botany. 2015;**66**:3945-3958. DOI: 10.1093/jxb/erv194

[31] Lakshmanan P, Robinson N. Stress physiology: Abiotic stresses. In: Moore PH, Botha FC, editors. Sugarcane: Physiology, Biochemistry, and Functional Biology. Chichester: John Wiley & Sons, Inc.; 2014. pp. 411-434

[32] McCormick AJ, Cramer MD, Watt DA. Regulation of photosynthesis by sugars in sugarcane leaves. Journal of Plant Physiology. 2008;**165**:1817-1829. DOI: 10.1016/j.jplph.2008.01.008

[33] Goldschmidt EE, Huber SC. Regulation of photosynthesis by endproduct accumulation in leaves of plants storing starch, sucrose, and hexose sugars. Plant Physiology. 1992;**99**:1443-1448. DOI: 10.1104/pp.99.4.1443

[34] Delorge I, Janiak M, Carpentier S, Van Dijck P. Fine tuning of trehalose biosynthesis and hydrolysis as novel tools for the generation of abiotic stress tolerant plants. Frontiers in Plant Science. 2014;**5**:147. DOI: 10.3389/fpls.2014.00147

[35] Sales CRG, Ribeiro RV, Machado DFSP, Machado RS, Dovis VL, Lagôa AMMA. Gas exchange and carbohydrate balance in sugarcane plants under root stressful conditions. Bragantia. 2012;**71**:319-327. DOI: 10.1590/S0006-87052012000300001

[36] Keating BA, Wilson JR. Intensive Sugarcane Production: Meeting the Challenge beyond 2000. Wallingford: CAB International; 1997

[37] Bakker H. Sugar Cane Cultivation and Management. New York: Kluwer Academic/ Plenum Publishers; 1999

[38] Ferreira THS, Tsunada MS, Bassi D, Araújo P, Mattiello L, Guidelli GV, Righetto GL, Goncalves VR, Lakshmanan P, Menossi M. Sugarcane water stress tolerance mechanisms and its implications on developing biotechnology solutions. Frontiers in Plant Science. 2017;**8**:1077. DOI: 10.3389/fpls.2017.01077

[39] Gomathi R, Gururaja Rao PN, Chandran K, Selvi A. Adaptive responses of sugarcane to waterlogging stress: An over view. Sugar Tech [Internet]. Springer India. 2015;**17**:325-338. Available from: http://dx.doi.org/10.1007/s12355-014-0319-0 [Accessed: 23-06-2017]

[40] Avivi S, Soeparjono S, Arieza Ramadhan R. Physiological characters of sugarcane after flooding stress. Agriculture and Agricultural Science Procedia [Internet]. Elsevier Srl. 2016;**9**:31-39. Available from: http://dx.doi.org/10.1016/j.aaspro.2016.02.119 [Accessed: 07-03-2017]

[41] Rojas-Martínez L, Visser RGF, de Klerk GJ. The hyperhydricity syndrome: Waterlogging of plant tissues as a major cause. Propagation of Ornamental Plants. 2010;**10**:169-175

[42] Dewir YH, Insoliya Y, Chakrabarty D, Paek KY. Biochemical and physiological aspects of hyperhydricity in liquid culture system. In: Paek KY, editor. Prod. Biomass Bioact. Compd. Using Bioreact. Technol. Netherlands: Springer; 2014. pp. 693-709

[43] Lyam PT, Musa ML, Jamaleddine ZO, Okere UA, Odofin WT. The potential of temporary immersion bioreactors (TIBs) in meeting crop production demand in Nigeria. Journal of Biology and Life Science. 2012;**3**:66-86

[44] Snyman SJ, Nkwanyana PD, Watt MP. Alleviation of hyperhydricity of sugarcane plantlets produced in RITA vessels and genotypic and phenotypic characterization of acclimated plants. South African Journal of Botany. [Internet]. SAAB. 2011;**77**:685-692. Available from: http://dx.doi.org/10.1016/j.sajb.2011.03.004 [Accessed: 09-05-2017]

[45] Tesfa M. In vitro shoot multiplication of elite sugarcane (*Saccharum officinarum* L.) genotypes using liquid shake culture system. Journal of Biology, Agriculture and Healthcare. 2016;**6**:35-40. DOI: 10.1590/S0100-204X2009001200003

[46] Arencibia AD, Bernal A, Yang L, Cortegaza L, Carmona ER, Pérez A, et al. New role of phenylpropanoid compounds during sugarcane micropropagation in Temporary Immersion Bioreactors (TIBs). Plant Science. 2008;**175**:487-496

[47] Bernal A, MacHado P, Cortegaza L, Carmona ER, Rivero O, Zayas CM, et al. Priming and biopriming integrated into the sugarcane micropropagation technology by Temporary Immersion Bioreactors (TIBS). Sugar Tech. 2008;**10**:42-47

[48] Silva M, Cabral E, Mota G, Willadino L, Camara T. In vitro propagation in Temporary Immersion System of sugarcane plants variety "RB 872552" derived from somatic embryos. Biotecnología Vegetal. 2015;**15**:187-191

[49] Li M, Liang Z, Zeng Y, Jing Y, Wu K, Liang J, Wang L. De novo analysis of transcriptome reveals genes associated with leaf abscission in sugarcane (*Saccharum officinarum* L.). BMC Genomics. 2016;**17**(1):195. DOI: 10.1186/s12864-016-2552-2

[50] Hoang NV, Furtado A, O'Keeffe AJ, Botha FC, Henry RJ. Association of gene expression with biomass content and composition in sugarcane. PLoS One. 2017;**12**(8):1-31. DOI: 10.1371/journal.pone.0183417

[51] Belesini AA, Carvalho FMS, Telles BR, Castro GM, Giachetto PF, Vantini JS, Carlin SD, Cazetta JO, Pinheiro DG, Ferro MIT. De novo transcriptome assembly of sugarcane leaves submitted to prolonged water-deficit stress. Genetics and Molecular Research: GMR. 2017;**16**(2):1-20. DOI: 10.4238/gmr16028845

[52] Pereira-Santana A, Alvarado-Robledo EJ, Zamora-Briseño JA, Ayala-Sumuano JT, Gonzalez Mendoza VM, Espadas-Gil F, et al. Transcriptional profiling of sugarcane leaves and roots under progressive osmotic stress reveals a regulated coordination of gene expression in a spatiotemporal manner. PLoS One. 2017;**12**(12):1-25. DOI: 10.1371/journal.pone.0189271

[53] Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;**32**(18):2847-2849. DOI: 10.1093/bioinformatics/btw313

[54] R Development Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria [Internet]; 2017. Available from: https://www.r-project.org/ [Accessed: 10-08-2017]

[55] Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Bateman A. The Pfam protein families database: Towards a more sustainable future. Nucleic Acids Research. 2016;**44**(D1):D279-D285. DOI: 10.1093/nar/gkv1344

[56] Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Rokhsar DS. Phytozome: A comparative platform for green plant genomics. Nucleic Acids Research. 2012;**40**(D1):D1178. DOI: 10.1093/nar/gkr944

[57] Ralph S, Park JY, Bohlmann J, Mansfield SD. Dirigent proteins in conifer defense: Gene discovery, phylogeny, and differential wound- and insect-induced expression of a family of DIR and DIR-like genes in spruce (*Picea* spp.). Plant Molecular Biology. 2006;**60**(1):21. DOI: 10.1007/s11103-005-2226-y

[58] Zhu L, Zhang X, Tu L, Zeng F, Nie Y, Guo X. Isolation and characterization of two novel dirigent-like genes highly induced in cotton (*Gossypium barbadense* and *G. hirsutum*) after infection by *Verticillium dahliae*. Journal of Plant Pathology. 2007;**89**:41-45

[59] Wu R, Wang L, Wang Z, Shang H, Liu X, Zhu Y, Deng X. Cloning and expression analysis of a dirigent protein gene from the resurrection plant *Boea hygrometrica*. Progress in Natural Science. 2009;**19**(3):347-352. DOI: 10.1016/j.pnsc.2008.07.010

[60] Jin-long G, Li-ping X, Jing-ping F, Ya-chun S, Hua-ying F, You-xiong Q, Jing-sheng X. A novel dirigent protein gene with highly stem-specific expression from sugarcane, response to drought, salt and oxidative stresses. Plant Cell Reports. 2012;**31**(10):1801-1812. DOI: 10.1007/s00299-012-1293-1

[61] Nobile PM, Bottcher A, Mayer JLS, Brito MS, dos Anjos IA, de Andrade Landell MG, Mazzafera P. Identification, classification and transcriptional profiles of dirigent domain-containing proteins in sugarcane. Molecular Genetics and Genomics. 2017;**292**(6):1323-1340. DOI: 10.1007/s00438-017-1349-6

[62] Anderson DJ, Birch RG. Minimal handling and super-binary vectors facilitate efficient, Agrobacterium-mediated, transformation of sugarcane (*Saccharum* spp. hybrid). Tropical Plant Biology. 2012;**5**:183-192. DOI: 10.1007/s12042-012-9101-1

[63] Niu J-Q, Wang A-Q, Huang J-L, Yang L-T, Li Y-R. Isolation, characterization and promoter analysis of cell wall invertase gene SoCIN1 from sugarcane (*Saccharum* spp.). Sugar Tech. 2014;**17**(1):65-76. DOI: 10.1007/s12355-014-0348-8

[64] Yogesh T, Maria G, Fredy A. Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. Plant Cell, Tissue and Organ Culture (PCTOC). 2012;**111**:131-141. DOI: 10.1007/s11240-012-0177-y

[65] Zale J, Jung JH, Kim JY, Pathak B, Karan R, Liu H, Chen X, Wu H, Candreva J, Zhai Z, Shanklin J, Altpeter F. Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass. Plant Biotechnology Journal. 2016;**14**:661-669

[66] Arencibia AD, Carmona ER. Sugarcane (*Saccharum* spp.). In: Wang K, editor. Agrobacterium Protocols. 2nd ed. Totowa, NJ: Humana Press; 2006. pp. 227-235. DOI: 10.1385/1-59745-131-2:227

[67] Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S. Agrobacterium-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Reports. 2004;**23**:134-143. DOI: 10.1007/s00299-004-0794-y

[68] Reis RR, da Cunha BADB, Martins PK, Martins MTB, Alekcevetch JC, Chalfun-Júnior A, et al. Induced over-expression of AtDREB2A CA improves drought tolerance in sugarcane. Plant Science. 2014;**221**:59-68. DOI: 10.1016/j.plantsci.2014.02.003