EFFECTS OF POLYAMINES ON DIFFERENTIAL EXPRESSION OF PROTEINS DURING THE MATURATION OF SUGARCANE SOMATIC EMBRYOS

RICARDO DE SOUZA DOS REIS

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE
DARCY RIBEIRO – UENF

CAMPOS DOS GOYTACAZES – RJ
ABRIL – 2015

EFFECTS OF POLYAMINES ON DIFFERENTIAL EXPRESSION OF PROTEINS DURING THE MATURATION OF SUGARCANE SOMATIC EMBRYOS

RICARDO DE SOUZA DOS REIS

"Dissertação apresentada ao Centro de Ciências e Tecnologias Agropecuárias da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Mestre em Genética e Melhoramento de Plantas"

Orientador: Prof. Vanildo Silveira

CAMPOS DOS GOYTACAZES – RJ
ABRIL – 2015

EFFECTS OF POLYAMINES ON DIFFERENTIAL EXPRESSION OF PROTEINS DURING THE MATURATION OF SUGARCANE SOMATIC EMBRYOS

RICARDO DE SOUZA DOS REIS

"Dissertação apresentada ao Centro de Ciências e Tecnologias Agropecuárias da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Mestre em Genética e Melhoramento de Plantas"

Aprovado em 09 de Abril de 2015

Comissão Examinadora:

_____
Prof. Fábio César Sousa Nogueira (D.Sc., Bioquímica) - UFRJ

_____
Prof. Virginia Silva Carvalho (D.Sc., Fitotecnia) – UENF

_____
Prof. Gonçalo Apolinário de Souza Filho (D.Sc., Biociências e Biotecnologia) UENF

_____
Prof. Vanildo Silveira (D.Sc., Biotecnologia) - UENF
(Orientador)

ACKNOWLEDGMENTS

The research and scholarships were supported by the Carlos Chagas Filho Foundation for Research Support in the State of Rio de Janeiro (FAPERJ), the Coordination for the Improvement of Higher Education Personnel (CAPES), and the National Council for Scientific and Technological Development (CNPq).

I would like to thank the Universidade Estadual do Norte Fluminense Darcy Ribeiro and the Genetic and Plant Breeding Graduate Program for the quality of education.

SUMMARY

RESUMO

REIS, Ricardo de Souza dos; M.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Abril de 2015; EFEITO DAS POLIAMINAS NA EXPRESSÃO DE PROTEÍNAS TOTAIS DURANTE A MATURAÇÃO DE EMBRIÕES SOMÁTICOS DE CANA-DE-AÇÚCAR; Orientador: Prof. Vanildo Silveira; Conselheiros: Profª. Virginia Silva Carvalho e Prof. Gonçalo Apolinário de Souza Filho.

A cana-de-açúcar tem ganhado importância nos últimos anos principalmente por sua utilização como fonte renovável de biocombustível. O potencial de aplicação de ferramentas biotecnológicas para o melhoramento da safra de cana-de-açúcar é bastante promissor. Dessa forma, estudos sobre a embriogênese somática têm sido realizados objetivando diferentes aplicações, tais como, micropropagação, melhoramento e engenharia genética. O objetivo deste trabalho foi estudar os efeitos das poliaminas na indução da embriogênese somática e expressão diferencial de proteínas durante a embriogênese somática de cana-de-açúcar. Como material vegetal, utilizamos plantas de cana-de-açúcar da cultivar SP80-3280. Estas plantas foram utilizadas para indução de calos em meio de cultura MS. Os calos embriogênicos induzidos foram inoculados em meio de cultura MS contendo diferentes concentrações das poliaminas putrescina, espermidina e

espermina (0,10,100 e 500 µM), utilizadas separadamente. As análises de poliaminas livres foram realizadas por cromatografia líquida de alta performance, utilizando-se coluna de fase reversa. Para as análises proteômicas, utilizamos o sistema de LC-MS/MS para as análises de amostras complexas. Foram observadas diferenças no perfil de expressão de proteínas sob o efeito das poliaminas na maturação de embriões somáticos de cana-de-açúcar. Entre as poliaminas, putrescina na concentração de 500 µM resultou em um maior número de embriões formados. As análises de poliaminas livres demonstraram, como esperado, que a putrescina se encontrava em maior concentração nas culturas tratadas com putrescina. Para espermidina, não houve diferenças significativas entre o tratamento e o controle. Espermina foi identificada em baixa concentração em ambos o tratamento e controle. As análises proteômicas do controle e tratamento com putrescina mostraram proteínas diferencialmente expressas que estão relacionadas à embriogênese somática, sendo elas: proteínas arabinogalactanas, peroxidases, proteínas de choque térmico, glutationa s-transferase, proteínas LEA e proteínas 14-3-3. Todas elas desempenham funções essenciais na detoxificação e resposta a estresses nas plantas, reforçando a ideia de que a embriogênese somática possui um caráter induzido por estresse. Estes resultados consideraram a putrescina e as proteínas identificadas como desempenhando papéis importantes na proteção das células contra o estresse do ambiente *in vitro* e, assim, preparando as células para o desenvolvimento da embriogênese somática durante a maturação.

ABSTRACT

REIS, Ricardo de Souza dos; M.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; April 2015; EFFECTS OF POLYAMINES ON DIFFERENTIAL EXPRESSION OF PROTEINS DURING THE MATURATION OF SUGARCANE SOMATIC EMBRYOS; Adviser: Prof. Vanildo Silveira; Counselors: Profª. Virginia Silva Carvalho e Prof. Gonçalo Apolinário de Souza Filho.

Sugarcane crops have gained importance in recent years, mainly because of sugarcane's use as a renewable biofuel source. The potential for application of biotechnological tools for improvement of sugarcane crop is rather promising. Thus, somatic embryogenesis studies have been carried out aiming different applications such as micropropagation, plant breeding, and genetic engineering. The objective of this work was to study the effects of polyamines on somatic embryo induction and differential abundance of proteins during the somatic embryogenesis of sugarcane. As plant material, we utilized the cultivated variety SP80-3280 for callus induction. Embryogenic cultures were treated with different concentrations of putrescine, spermidine, and spermine (0, 10, 100 e 500 µM). Free polyamines analysis was performed by high performance liquid chromatography using a reversed phase column. Proteomics consisted of LC-MS/MS analysis using the method of shotgun. Among polyamines, putrescine at

500 µM gave rise to the highest number of somatic embryos in the embryogenic callus; however, no differences in the fresh matter were observed between polyamines and control. During polyamine analysis, putrescine was identified in higher concentration in putrescine-treated cultures, as expected. No significant differences were observed in spermidine content between treatment and control. Spermine was identified in small content in both treatment and control. Differences in protein abundance profiles resulting from the effect of putrescine at 500 µM on sugarcane somatic embryo maturation were observed. Proteomic analyses of putrescine and control treatment showed differently abundant proteins related to somatic embryogenesis, such as arabinogalactan proteins, peroxidases, heat shock proteins, glutathione s-transferases, late embryogenesis abundant proteins, and 14-3-3 proteins. These proteins play essential roles during stress responses in plant cells. These results showed that putrescine and the identified proteins play important roles in protecting the cells against an *in vitro* stress environment, preparing cells to undergo somatic embryogenesis during the maturation period.

1. INTRODUCTION

Currently, all of the cultivated sugarcane worldwide is derived from the crossing of two main species. *Saccharum officinarum* is a domesticated species that accumulates high sugar content and presents a basic number of chromosomes equal to 10 and a chromosome constitution of 2n=80. *Saccharum spontaneum* is a wild species presenting a basic number of chromosomes equal to 8 and a chromosome constitution of 2n=40-128. This later has high resistance to biotic and abiotic stresses (Casu et al., 2005; Cheavegatti-Gianotto et al., 2011; Arruda, 2012). As a result of the crossings, the hybrids show a genomic constitution even more complex than the parental genomes, featuring a chromosome constitution of 2n=100-130, where 60-70% of the chromosomes have been inherited from *S. officinarum* (Arruda, 2012). Sugarcane cultures allow several means of economic exploitation, such as sugar, ethanol, and biopolymers, as well as electricity generation and cellulosic ethanol from the bagasse and straw. The species has been cultivated on an industrial scale for sugar production in more than 90 countries worldwide for over 100 years, and the interest in this culture has increased due to the production of ethanol as a renewable energy source (Arruda, 2011).

The potential for the application of biotechnological tools to improve sugar production and agronomic performance of sugarcane crops is rather promising because the yield gains using conventional breeding may be reaching their limit due to the difficulties imposed by the complex genome of sugarcane (Arruda,

2012). Furthermore, the selection of superior genotypes within a population obtained by crossing two individuals is a long-term project that takes at least ten years to generate results (Dal-Bianco et al., 2012). Biotechnology has been considered important for sugarcane crops due to the insertion of new genes conferring advantageous agronomic characteristics (Matsuoka et al., 2009). Tissue culture plays a crucial role in the conservation, creation, and utilization of genetic variability of sugarcane, including cryopreservation, *in vitro* selection, genetic engineering, and commercial large-scale micropropagation of disease-free sugarcane (Altpeter e Oraby, 2010).

Under optimal culture conditions, *in vitro* plants may undergo a series of genetic and morphological alterations by reprogramming their genome, epigenome and, consequently, proteome, thus acquiring a new state of totipotency. This new state results from stimuli, often hormone dependent, producing effects that may culminate in a differentiation process resulting in a whole plant. One possible morphogenetic pathway for this process is through somatic embryogenesis, an *in vitro* culture technique in which a single somatic cell or small group of cells give rise to somatic embryos (Tautorus et al., 1991). In sugarcane, the first evidence of somatic embryos in callus cultures occurred in the 1980s (Ahloowalia e Maretzki, 1983; Ho e Vasil, 1983a; Ho e Vasil, 1983b). From these studies, several other research efforts using *in vitro* cultures of sugarcane have been carried out involving different applications, such as micropropagation, breeding, germplasm conservation, and genetic engineering (Lakshmanan et al., 2005). Although many breakthroughs have been reported regarding the discovery of genes involved in the development of somatic embryogenesis, the complete route that triggers the de-differentiation, re-differentiation, and development of somatic cells into embryos has not been completely elucidated.

In the study of somatic embryogenesis, various molecules have been described as inducers of the process. This includes the polyamines, mainly putrescine, spermidine, and spermine, which are considered a class of plant growth regulators. They are small polycationic aliphatic molecules bearing amino groups that are capable of electrostatically interacting with macromolecules, such as nucleic acids, phospholipids, cell wall components, and proteins (Baron e Stasolla, 2008; Tiburcio et al., 2014). They may be found in fungi, animals, bacteria, and plants, which implicates a common biological function to these

molecules in the modulation of cellular, physiological, and developmental processes (Baron e Stasolla, 2008). In plants, they have been associated with the regulation of physiological processes, such as organogenesis, embryogenesis, flower development, senescence, fruit maturation and development, and responses to biotic and abiotic stresses (Ahmad et al., 2012).

To gain a better understanding of the biochemical, physiological, and morphological changes that these molecules may cause in plant development, genomic studies may be insufficient for a complete comprehension of the factors involved in such alterations. Therefore, in addition to the genome studies, it is useful to apply proteomic tools to study gene expression products through the identification of proteins and, potentially, their interactions.

2. OBJECTIVES

2.1. General Objectives

The main objective of this work was to study the effects of polyamines on somatic embryo induction and differential abundance of proteins during the somatic embryogenesis of sugarcane cv. SP80-3280 to acquire a comprehensive understanding of the mechanisms underlying this complex process.

2.2. Specific Objectives

- Effects of exogenous polyamines on somatic embryo induction and callus fresh weight during the maturation of sugarcane embryogenic callus;

- Analysis of endogenous polyamines of sugarcane somatic embryos;

- Effects of exogenous polyamines on differential abundance of proteins during somatic embryogenesis of sugarcane.

3. REVIEW

3.1. Sugarcane

Sugarcane (*Saccharum sp*.) is a member of Poacea family and an interspecific hybrid between two main species, *S. officinarum* and *S. spontaneum*. Due to theses crossings, it is a species with a complex genome, being polyploidy and frequently aneuploid (Altpeter e Oraby, 2010). The interspecific hybrid has been backcrossed with *S. officinarum* to generate the sugarcane germplasm that retain both characteristics of high sugar content and the high stress resistance (Arruda, 2012). The *Saccharum* genus has probably originated before the continents might have assumed the current locations and shapes, and it consists of about 40 species, presenting two diversity centers: the Old World (Asia and Africa) and the New World (South, Central, and North America) (Cheavegatti-Gianotto et al., 2011).

Sugarcane crop has been important to the economy of Brazil since the beginning of the 16th century, after the arrival of the first sugarcane plants around 1515 and with the establishment of the first mill in 1532 (Cheavegatti-Gianotto et al., 2011). The crop has several means of economic exploitation such as sugar and ethanol as well as electricity generation and cellulosic ethanol production using the bagasse and straw. And regarding the economic values, in 2012 the annual revenue of the sector was greater than US$ 36 billion (1.6% of the Gross National Product) (Sugarcane, 2014; UNICA, 2014).

The second generation of biofuels depends on the development of efficient processes to breakdown cellulose into fermentable sugars, and current research has aimed the development of plants with improved formation of cell wall to produce more cellulose and less lignin (Scortecci et al., 2012). Given the scale of ethanol production in Brazil, the energy stored in the straw and bagasse of sugarcane would be enough to generate up to 10 gigawatts (GW) of energy. This would be close to the 14 GW generated by the Itaipu hydroelectric power plant, which responds by 1/5 of the energy demand in Brazil and 90% of Paraguay (Scortecci et al., 2012).

According to the latest survey by the National Supply Company (CONAB, 2015), 43,7% of sugarcane crop will be used for sugar production, while the remaining 56,3% will go to ethanol production. However, with the use of bagasse and straw for second-generation ethanol, there could be an increase of up to 40% in ethanol production (De Souza et al., 2013). Current sugar production makes of Brazil the world largest producer and exporter for this commodity, representing 25% of world production and 50% of exportation (USDA, 2013; Sugarcane, 2014), and the second largest producer of ethanol, behind only the United States, which produces ethanol from other energy source, the maize (FAO, 2013).

Worldwide, due to the increasing demand, there were progressive increases in the productivity of sugarcane. This was attributed to the development and widespread use of improved cultivars with resistance to diseases and pests, better management of resources (water, nutrients, etc.) and the availability of chemical fertilizers and pesticides with relatively lower costs (Lakshmanan et al., 2005).

3.2. Sugarcane Breeding and Biotechnology

The importance of sugarcane as raw material for the production of energy has increased the interest in generating new cultivars suitable for this function. However, the introduction of new cultivars not necessarily lead to major changes in the production system as a whole, thus there is always an expectation in the search for gains in productivity (Dal-Bianco et al., 2012). Furthermore, the selection of genotypes within a population obtained by crossing two individuals is a

long-term work, which takes not less than ten years to generate results (Dal-Bianco et al., 2012).

The potential of application of biotechnological tools for increasing sugar production and agronomic performance of sugarcane is rather promising, since the production gains using conventional breeding may be close to its limit, because of difficulties imposed by the complexity of sugarcane genome (Arruda, 2012). Furthermore, the adoption of new cultivars is impaired by the limitations of conventional vegetative propagation (planting internodes), for example, low multiplication rates (one bud per internode) and possible spread of diseases (Snyman et al., 2011). For this reason, micropropagation, the large-scale multiplication of selected explants originated from *in vitro* embryogenesis and organogenesis protocols, would help to address these problems (Snyman et al., 2011). In addition to the *in vitro* propagation techniques, another important technique is the genetic engineering, which is critical for the development of new cultivars with various agronomic characteristics.

Early studies with genetic transformation of sugarcane occurred in the 1980s and aimed to obtain plants resistant to kanamycin (Chen et al., 1987). Currently, several studies address the genetic transformation of sugarcane, demonstrating transgenic plants for various characteristics such as resistance to biotic (Arvinth et al., 2010; Weng et al., 2011; Zhu et al., 2011) and abiotic stresses (Wang et al., 2005; Zhang et al., 2006; Belintani et al., 2012), modification of sugar content (Wu e Birch, 2007; Groenewald e Botha, 2008), expression of recombinant enzymes (Harrison et al., 2011) for cellulosic ethanol production, and production of various compounds of economic interest, e.g., biopolymers such as polyhydroxybutyrate (PHB) (Petrasovits et al., 2007; Petrasovits et al., 2012). However, despite many studies in the area, there was no commercial planting of transgenic sugarcane in the world. Overall, it had only researches in experimental stages. Nonetheless, recently, Indonesia has approved the use of transgenic sugarcane with drought tolerance for commercial planting planned to have occurred in 2014 (Cheavegatti-Gianotto et al., 2011; ISAAA, 2014). In Brazil, the National Biosafety Technical Commission (CTNBio) has approved more than 40 requests for field testing of sugarcane containing genetically modified genes conferring different characteristics such as higher

sucrose content, tolerance to herbicides, drought, and resistance to insects (Cheavegatti-Gianotto et al., 2011).

3.3. Tissue Culture and Somatic Embryogenesis

Tissue culture is the aseptic culture of cells, tissues or organs isolated from an organism and maintained in an *in vitro* environment under physical and chemical controlled conditions (Thorpe, 2007). It is a science based on the principle of totipotency, established by Haberlandt in 1902, according to which a plant cell is able to divide and differentiate, forming a whole organism. It is an essential tool for transgenic plants, as well as it allows the production of disease-free plants, germplasm conservation, and mass propagation of plants of economic or ecological interest.

Tissue culture has existed for over 100 years and ever since it has undergone several breakthroughs, from the cell theory of Schleiden-Schwann to the present days with large-scale production by using the most diverse and modern bioreactor systems (Vasil, 2008). In the beginning, one of the most limiting factors for the *in vitro* culture was the lack of culture media suitable for the survival and growth of the cells for a long-term and thus many researchers gave focus to the development of more suitable culture media for plant cells (Vasil, 2008). Another factor that promoted a major breakthrough in the field of tissue culture was the discovery of plant growth regulators, which allowed researchers to have more control over the development of *in vitro* plants (Vasil, 2008).

In sugarcane, early studies in tissue culture occurred in the 1960s with the callus induction and also root induction from callus (Nickell, 1964). Sometime later, sugarcane plants were regenerated from callus-induced shoots (Barba e Nickell, 1969; Heinz e Mee, 1969). The first evidences of somatic embryos in sugarcane callus occurred in the 1980s (Ahloowalia e Maretzki, 1983; Ho e Vasil, 1983a; Ho e Vasil, 1983b). From these studies, several other studies with *in vitro* culture of sugarcane were performed aiming different applications such as micropropagation and production of healthy plants, plant breeding, germplasm conservation, and genetic engineering (Lakshmanan et al., 2005).

Micropropagation, the main application of tissue culture, has the advantage of controlling the environmental conditions during the process of propagation, thus ensuring the proper development of propagules, aside from allowing the large scale production of disease-free plantlets in a short time, using a relatively small area when compared to the conventional system (Cruz et al., 2009). The commercial sugarcane is propagated by planting of internodes containing the axillary bud. This is a slow process of multiplication, due to limitations imposed by the number of buds per internode, which may also cause the spread and perpetuation of pathogens in crops, since the replanting occurs in every 3-8 years (Snyman et al., 2011). For this reason, micropropagation provides a simple and rapid method for mass production of healthy clones of sugarcane (Lakshmanan et al., 2005; Snyman et al., 2011).

Tissue culture also allows to carry out the conservation of germplasm, which is an integral part of any plant breeding program and whose current methods include conservation stands and collections in greenhouses, which requires specific lands and facilities that generate high costs of maintenance and is laborious (Snyman et al., 2011). In addition, there is always the risk of loss of germplasm by disease, pests, and natural disasters (Snyman et al., 2011).

Somatic embryogenesis, the formation of embryos from somatic cells, is a process analogous to the zygotic embryogenesis. Somatic cell after perceiving some stimulus, usually provided by plant growth regulators or some kind of stress, undergo a process of gene reprogramming, acquiring the status of totipotent cells. After this de-differentiation process, the cell carries out a series of cell divisions and gene expressions, initiating the formation of a bipolar structure (somatic embryo), which if placed in appropriate condition will culminate in the development of a whole plant. However, the establishment of a whole new cell state is not governed only at the level of gene expression, but also requires the modification and/or removal of unnecessary proteins and the proper folding of newly synthesized proteins and protein complexes (Feher et al., 2003).

Somatic embryogenesis was first described by three independent researchers, Waris (1957), working with *Oenanthe aquatica* seedlings and Steward et al. (1958) and Reinert (1958), working with *Daucus carota* (carrot) (Krikorian e Simola, 1999; Vasil, 2008). Since then, the process has been observed in several other species and has been studied for different research

groups trying to elucidate at a molecular level how this process occurs. Thus, numerous studies have demonstrated the relationship of certain genes in the development of somatic embryos. However, the molecular basis of somatic embryogenesis, particularly the transition from somatic cells to embryogenic cells still requires further studies (Sun et al., 2012).

Somatic embryos may be originated either from an intermediary callus phase, and therefore termed as indirect somatic embryogenesis, or from the surface of the explants in a direct process. When callus are formed, two types may usually be distinguished morphologically: an embryogenic, which has the morphogenetic competence to generate embryos, and the non-embryogenic. For callus formation, several parts of the plant may be used, but the young leaves and immature inflorescences are more likely to form embryogenic callus, and therefore the preferred target tissues (Lakshmanan, 2006). As in many species of the Poaceae family, auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is the most effective for the callus induction in sugarcane (Lakshmanan, 2006).

The plant growth regulators are the primarily responsible for triggering the *in vitro* morphogenetic response, including somatic embryogenesis (Gaj et al., 2006). Auxin is considered the most important growth regulator in the modulation of somatic embryogenesis and its continuous presence induces protein synthesis necessary to initiate the formation of somatic embryos up to the globular stage and to inhibit their differentiation to other embryonic stages (Cooke et al., 1993; Zimmerman, 1993). In general, the maturation phase occurs with the removal of auxin from culture medium and the addition of maturation promoters, thus inducing the formation of mature somatic embryos capable of *in vitro* or *ex vitro* germination, which may also be used for the production of synthetic seeds (Steiner et al., 2008).

Compared to other techniques of tissue culture, somatic embryogenesis has several advantages. It allows the production of a large number of propagules (somatic embryos). The system enables a high degree of automation, allowing lower costs per unit produced using bioreactors. Somatic embryos may be produced with a high degree of uniformity and genetic purity. May be used as an integrated tool in plant breeding programs, especially when combined with cryopreservation techniques and genetic engineering (Guerra et al., 1999). However, the main limitation in somatic embryogenesis systems is the somaclonal

variation that may occur after successive subcultures, which in sugarcane and other species may affect plant development (Lakshmanan et al., 2005).

Somaclonal variation is usually considered as a major problem in tissue culture, because it may cause genetic and epigenetic changes that affect plant development. However, despite the possibilities of these changes to add undesirable characteristics, somaclonal variation may also be used as a tool for crop improvement, aiming to increase the quality and productivity of crops (Snyman et al., 2011; Suprasanna et al., 2011). The use of this approach becomes quite interesting, since the use of transgenic plants faces difficulties due to various bureaucratic and biosecurity issues that may prevent or delay the researches with breeding goals. Some agronomic characteristics for sugarcane have been explored using this approach, such as resistance to the herbicide glyphosate (Zambrano et al., 2003), diseases (Sengar et al., 2009), insects (White e Irvine, 1987), salt stresses (Patade et al., 2006), and characteristics related to productivity and growth (Khan et al., 2002; Doule et al., 2008; Rajeswari et al., 2009).

3.4. Polyamines

Polyamines demonstrate to be essential molecules to all organisms, participating in several important cellular activities and each polyamine has a specific relationship with certain cell functions and may have different effects when added to the culture medium (Bais e Ravishankar, 2002; Takahashi e Kakehi, 2010). They are considered a class of plant growth regulator and contain various properties that facilitate electrostatic interactions to other molecules such as nucleic acids, phospholipids, cell wall components, and proteins (Baron e Stasolla, 2008).

Advances in metabolism studies indicate that intracellular levels of polyamines in plants are mostly regulated by anabolic and catabolic processes and also by the conjugation of them with other molecules (Alcazar et al., 2010). The biosynthetic pathway of polyamines in plants is more complex than in other organisms, since it has two paths that lead to the synthesis of putrescine (Put); Put may be formed directly by decarboxylation of ornithine by ornithine decarboxylase

(ODC), or indirectly through a series of intermediates by decarboxylation of arginine by arginine decarboxylase (ADC). The diamine Put derived from ODC or ADC is subsequently converted to the triamine spermidine (Spd) by spermidine synthase (SPDS). This enzyme adds an aminopropyl group donated by a decarboxylated S-adenosylmethionine molecule (common precursor of ethylene biosynthesis); The subsequent addition of any other aminopropyl group to Spd molecule is mediated by spermine synthase (SPMS) and results in the formation of the tetramine spermine (Spm) (Kakkar et al., 2000; Bais e Ravishankar, 2002; Baron e Stasolla, 2008; Alcazar et al., 2010).

In works with *Araucaria angustifolia*, a species of gymnosperm considered recalcitrant to produce somatic embryos, the addition of polyamines to the culture medium had influences in both the morphogenetic evolution during the development of pro-embryogenic masses as in nitric oxide biosynthesis and action of proton pumps in embryogenic cell suspension cultures (Silveira et al., 2006; Dutra et al., 2013). Analysis of endogenous polyamine metabolism in embryogenic cultures of *Pinus sylvestris* also demonstrated the relationship between these molecules with the proliferative capacity of the cultures and the ability of cells to cope with stress caused during the growing season (Vuosku et al., 2012).

In sugarcane callus with different morphogenetic characteristics (embryogenic and non-embryogenic) and treated for maturation, the observed differences in endogenous levels of polyamines would be more related to the embryogenic capacity of callus than to the type of treatment (control and maturation) (Silveira et al., 2013). This demonstrates the importance of these molecules to the acquisition of embryogenic competence and further development of somatic embryo.

The metabolic pathway of polyamines is actually interconnected with other important metabolic pathways involved in the formation of several signaling molecules and metabolites that are relevant to different cellular responses (Alcazar et al., 2010). The intracellular concentration of polyamines is in the range of several hundred micromolars to few millimolars and is tightly regulated, since high concentrations are toxic and lead to cell death. Therefore, the levels of polyamines are perfectly regulated in several steps, including *de novo* synthesis, degradation, and transport (Kusano et al., 2008). In a review, Takahashi e Kakehi (2010) address some functions to which polyamines may be related. They

describe Put as responsible for specific responses to abiotic stresses; Spd would be related to plant development; and Spm to the control of ionic channels and to protect DNA from oxidative stress.

Despite the many functions already ascribed to polyamines, there is still the need for many molecular studies for a better understanding about the influences that these molecules have on cellular activities, especially during somatic embryogenesis. However, it is known that polyamines are essential to life, since the inhibition of biosynthesis blocks the growth and may lead to cell death (Kusano et al., 2008).

3.5. Proteomics

The proteome term refers to the complete set of proteins expressed by an organism, cell or tissue at a given time, under a specific condition, and therefore, it is the dynamic complement of proteins expressed by a genome (Wasinger et al., 1995; Wilkins et al., 1996a; Wilkins et al., 1996b). Proteomics, which is the study of the proteome, addresses a systematic investigation on the distribution, quantity, types of modifications, interactions, and functions of a protein or set of proteins, thus it exists a wide interest in the study of such molecules aiming the development of analytical strategies for the analysis of the different proteomes (Di Palma et al., 2012).

Proteins are molecules formed by amino acids composing polypeptide chains by covalent bonds involving the carboxyl group of an amino acid and the amino group of another. However, in order to properly perform their functions, proteins need to acquire a correct folding and frequently they also need to be bonded to other molecules, e.g., carbohydrates (glycoproteins), lipids (lipoproteins), and phosphate groups (phosphoproteins). Thus, these post-translational modifications may generate conformational and charge alterations in the proteins, which cause them to differently interact with other molecules. Amino acids have various physico-chemical properties that may be used as a basis for methods of separation, thus peptide separations may involve differences in polarity or charge (Di Palma et al., 2012). Furthermore, there are separations that employ more than one type of property for protein selectivity, therefore a mixed

separation mode may be achieved when different interaction mechanisms are combined (Di Palma et al., 2012).

Proteomic analysis allows qualitative and quantitative measurements of a large number of proteins that directly influence cellular metabolic processes and thereby provides an accurate analysis of changes during the growth, development, and responses to several environmental factors, and therefore, it is a key technology for the study of the most complex and dynamic biological systems (Chen e Harmon, 2006). The proteomic characterization is a complementary tool to genomics, since protein dynamics is influenced by a variety of external and internal factors that determine conformational and structural changes of proteins (Balbuena et al., 2011a). Therefore, the study of the protein dynamics is more complex than the genome, since the latter is generally static, i.e., equal in all cells of an organism.

One of the main methods used for protein separation is the two-dimensional electrophoresis (2-DE). The technique began to be used in the 1970s (Macgillivray e Wood, 1974; O'Farrell, 1975) and a breakthrough has occurred after the use of immobilized pH gradients (IPG) in the 1980s (Bjellqvist et al., 1982), which substantially improved the quality and reproducibility of electrophoresis. However, the technique has some limitations, such as difficulty of automation, it is quite laborious, it has limitations in the separations of proteins with extreme characteristics of pH or molecular weight and of low abundance proteins (Chen e Harmon, 2006; Rogowska-Wrzesinska et al., 2013). Because of this, the technique has been replaced by modern technologies that offer faster and accurate analysis, such as Multidimensional Protein Identification Technology (MudPIT) (Rogowska-Wrzesinska et al., 2013).

Proteins from biological samples are highly complex molecules, and require sophisticated analytical tools to provide reliable analysis of their components and therefore, proteomics needs a robust, automated, and high-throughput technology (Wolters et al.). The MudPIT technology has emerged to directly relate the separation of peptides and proteins with mass spectrometry (Wolters et al., 2001). The technique consists primarily in digesting a complex sample to generate a mixture of peptide fragments, which are then loaded onto one or two columns with different properties of separation (e.g., Strong Cation Exchange, Reversed Phase). Then, these peptides are analyzed by the mass

spectrometer, where they will be identified by generating a spectrum of these fragments. Spectra, in turn, are correlated by using specific algorithms (e.g., SEQUEST, MASCOT) with the sequences of protein databases (Link et al., 1999).

According to Schluter et al. (2009), for proteomic analysis there are three approaches that may be used. The classic strategy is the 2-DE-MS/MS, which consists in separating the proteins by two-dimensional electrophoresis (described above), followed by analysis in tandem mass spectrometry. The second approach starts directly with the digestion of complex samples. The digestion generates a large number of peptides, which are then separated by MudPIT. This approach is termed "Bottom up", because it begins at the level of peptide separation. The peptides eluted from the column are subsequently identified by mass spectrometry. These analyses generate amino acid sequences, by which it is possible to identify the original proteins. The third strategy is defined as "Top-down" and initiates the separation of entire proteins by liquid chromatography followed by identification of the protein by mass spectrometry. It also allows the analysis of post-translational modifications. The strategy of 2-DE-MS/MS is a middle ground between the other two approaches, therefore consisting of a “Top-down” separation and ”Bottom up" identification.

Several studies have reported proteins expressed during the development of embryogenesis (Bian et al., 2010; Li et al., 2010; Rode et al., 2012), in comparative studies between non-embryogenic and embryogenic callus or in callus development (Sharifi et al., 2012; Tan et al., 2013; Varhaníková et al., 2014), and between zygotic and somatic embryogenesis (Sghaier-Hammami et al., 2009; Noah et al., 2013). Besides these studies, there are others that aimed to understand the differences in the expression of proteins related to the morphogenetic process between normal and off-type plants obtained from somatic embryos (Fraga et al., 2013). Along with these studies to characterize the processes associated with somatic embryogenesis, there are other works that focus on the detection of potential markers (biomarkers) related to the different stages of embryogenesis development (Takac et al., 2011).

These biomarkers might be used for identification and differentiation of cultures with embryogenic potential, facilitating the choice of the cultures that would be used in genetic transformation studies (Stirn et al., 1995; Fellers et al., 1997). Arabinogalactan proteins (AGP), which are a family of plant glycoproteins,

have been identified as being important in several aspects of growth and development of cells, including somatic embryogenesis, being associated with a promoter activity of this process after addition to culture medium (Poon et al., 2012; Steinmacher et al., 2012; Mallón et al., 2013).

In this sense, to a better understanding of the aspects related to the acquisition of embryogenic competence and to somatic embryogenesis development, proteomic analysis appears as a valuable tool in the studies of the relations and interactions between different proteins that have been described as associated with the embryogenic process.

4. MATERIALS AND METHODS

4.1. Plant Material and Callus Induction

Sugarcane plants cv. SP80-3280 were obtained from the Universidade Federal Rural do Rio de Janeiro (UFRRJ), campus Leonel Brizola, localized in Campos dos Goytacazes, Rio de Janeiro, Brazil (21° 48'S and 41° 17'W). This variety was chosen based on a search using The Sugarcane EST Project (SUCEST) protein databank (http://sucest-fun.org/), which helped to acquire more reliable MS^E data.

Callus induction was performed as previously described by Silveira et al. (2013). Internodes with axillary buds were planted into plastic trays containing plant substrate for a period of two months. Subsequently, plants were processed by removing the outer mature leaves. The resulting leaf rolls were surface sterilized in 70% ethanol for 1 min, then in 30% commercial bleach (2-2.5% sodium hypochlorite) for 15 min, and subsequently washed three times in autoclaved distilled water. As explants, leaf rolls were transversely sectioned into 2-4 mm-thick slices and cultured in test tubes (150 x 25 mm) containing 10 mL of MS culture medium (Murashige e Skoog, 1962) (Phytotechnology Lab, Overland Park, KS, USA), supplemented with 20 g/L sucrose, 2 g/L Phytagel® (Sigma-Aldrich, St. Louis, MO, USA) and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich), pH 5.8. The cultures were kept in the dark at 25°C ± 1 for approximately 45 days.

Induced callus was transferred to Petri dishes (90 x 15 mm) containing 20 mL of the same culture medium and kept in the dark at 25 °C ± 1 and were subcultured every 21 days. During this multiplication period, callus was separated into embryogenic and non-embryogenic parts according to morphological characteristics as previously described by Silveira et al. (2013). Only embryogenic callus was used for this study.

4.2. Exogenous Polyamines in the Culture Medium

For analysis of polyamine effects on somatic embryo induction, we used three colonies of 200 mg of embryogenic callus per Petri dish containing 20 mL of MS medium supplemented with 30 g/L sucrose, 2 g/L Phytagel. Different concentrations (0, 10, 100 and 500 µM) of the polyamines putrescine, spermidine, and spermine were used; these were referred to as the control, Put10, Put100, Put500; Spd10, Spd100, Spd500; and Spm10, Spm100, and Spm500, respectively. Polyamines were filter-sterilized and added to the autoclaved MS medium with the pH adjusted to 5.8. The cultures were kept at 25°C ± 1 in the dark for seven days and transferred to light for an additional 21 days with a photoperiod of 16 h using LED lamps (90 µmol/m^2/s). After 0, 7, 14, 21, and 28 days of culture, calluses were evaluated for an increase in fresh matter and the number of somatic embryos formed. The best treatment regarding the production of somatic embryos was utilized for polyamines and proteomic analyses, in addition to the control treatment. Thereby, callus colonies were homogenized and 300 mg of fresh matter was stored at -20°C. For polyamine analysis, samples were collected from all maturation periods, whereas for proteomics, only samples from days 14 and 28 were utilized.

The embryos were regenerated on MS culture medium supplemented with 2 g/L Phytagel and 30 g/L sucrose. The pH of the culture medium was adjusted to 5.8 before Phytagel was added. The culture medium was sterilized by autoclaving at 121 ºC for 15 min. The *in vitro* cultures were incubated at 25ºC ± 1 and were exposed to a photoperiod of 16 h for 30 days. For acclimatization, sugarcane plants were transferred to 50 mL plastic cups containing plant substrate and vermiculite (1:1) and kept at 25°C ± 1 under a photoperiod of 16 h. Cups were

placed in plastic trays covered with PVC film for seven days to maintain high humidity and after 30 days of cultivation, they were transferred to larger trays and kept in green house.

4.3. Free Polyamine Analysis

The analysis of free polyamines was carried out using high-performance liquid chromatography (Shimadzu, Japan) as previously described by Silveira et al. (2004). Three-hundred-milligram samples of fresh matter were pulverized in liquid nitrogen and 1 mL of 5% perchloric acid (Merck, Darmstadt, Germany) was added, agitated, and incubated on ice for 60 min. Next, samples were centrifuged at 16,000 g for 20 min at 4°C and supernatants were collected. Extracted polyamine samples were derivatized with dansyl chloride (Sigma-Aldrich), vacuum dried in CentriVap® (Labconco, Kansas, MO, USA) and resuspended in pure acetonitrile (Merck) prior to analysis by HPLC using a reversed phase Shin-pack CLC ODS 5 µm column (Shimadzu). The mobile phase consisted of pure acetonitrile (solvent B) and acetonitrile 10% (pH 3.5). The solvent gradient was as follows: 65% solvent B for 10 min, 65% to 100% from 10 to 13 min, and 100% from 13 to 21 min, with a constant flow rate of 1 mL/min at 40°C. Free polyamine detection was made with a fluorescence detector (Shimadzu) using 340 nm excitation and 510 nm emission, and the concentrations of putrescine, spermidine, and spermine (Sigma-Aldrich) were determined by a standard curve.

4.4. Proteomic Analysis

4.4.1. Protein Extraction

For total protein extraction, the methodology used was that as previously described by Balbuena et al. (2011b). The extraction buffer consisted of 7 M Urea, 2 M Thiourea, 2% Triton X-100, 1% Dithiothreitol (DTT), 1 mM Phenylmethanesulfonyl fluoride (PMSF), and 5 µM Pepstatine. Three-hundred-milligram samples were pulverized using a mortar and pestle in liquid nitrogen.

Then, in microtubes, 1 mL of extraction buffer was added to the sample powder. Samples were vortexed and incubated on ice for 30 min, followed by a centrifugation step at 16,000 g for 20 min at 4°C. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA). Then, the five biological replicates were used to yield one pooled sample (Luge et al., 2014; Heringer et al., 2015) of 100 µg of proteins.

4.4.2. Protein Digestion

Before the trypsin digestion step, pooled samples were desalted on 5000 MWCO Vivaspin 500 membranes (GE Healthcare, UK) using 50 mM ammonium bicarbonate (Sigma-Aldrich) pH 8.5, as buffer. Membranes were fulfilled until maximum capacity with ammonium bicarbonate and centrifuged at 15,000 g for 20 min at 8°C. This procedure was repeated at least three times, resulting in approximately 50 µL per sample.

The methodology used for protein digestion was as previously described by Calderan-Rodrigues et al. (2014). Thus, 25 µL of 0.2% (v/v) RapiGest® (Waters, Milford, CT, USA) was added, and samples were briefly vortexed and incubated in an Eppendorf Thermomixer® at 80°C for 15 min. Then, 2.5 µL of 100 mM DTT (Bio-Rad Laboratories, Hercules, CA, USA) was added, and the tubes were vortexed and incubated at 60°C for 30 min under agitation. Next, 2.5 µL of 300 mM iodoacetamide (GE Healthcare) was added, and the samples were vortexed and then incubated in the dark for 30 min at room temperature. The digestion was performed by adding 20 µL of trypsin solution (50 ng/µL; V5111, Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate, and samples were incubated at 37°C overnight. For RapiGest® precipitation, 10 µL of 5% (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich) was added and incubated at 37°C for 90 min, followed by a centrifugation step of 30 min at 16,000 g. Samples were transferred to Total Recovery Vials (Waters).

4.4.3. Mass Spectrometry Analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, UK) was used for ESI-LC-MS/MS analysis. The chromatography step was performed by injecting 1 µL of digested samples to normalize them before the relative quantification of proteins. Normalization among samples was based on stoichiometric measurements of total ion counts of scouting runs prior to analyses, in order to ensure standardized molar values for all conditions. Subsequently, runs consisted of three replicates per pooled sample. During separation, samples were loaded onto the nanoAcquity UPLC 5 µm C18 trap column (180 µm x 20 mm) at 5 µL/min during 3 min and then onto the nanoAcquity HSS T3 1.8 µm analytical reversed phase column (100 µm x 100 mm) at 600 nL/min, with a column temperature of 60 °C. For peptide elution, a binary gradient was used and consisted of mobile phase A as water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich), and mobile phase B was acetonitrile (Sigma-Aldrich) and 0.1% formic acid. Gradient elution started at 7% B and was held for 3 min, then ramped from 7% B to 40% B up to 90.09 min, and from 40% B to 85% B until 94.09 min, being maintained at 85% until 98.09 min, then decreasing to 7% B until 100.09 min and kept at 7% B up to the end at 108.09 min. Mass spectrometry was carried out in positive and resolution mode (V mode), 35,000 FWMH, with ion mobility, and in data-independent acquisition (DIA) mode; IMS wave velocity was set to 600 m/s; the transfer collision energy ramped from 19v to 45v in high-energy mode; cone and capillary voltages of 30v and 2,800v, respectively; and a source temperature of 70°C. In TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50 to 2,000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol/µL was used as an external calibrant and lock mass acquisition was performed every 30 s.

4.4.4. Bioinformatics

Spectra processing and database searching conditions were performed by Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis used the following parameters: one missed cleavage, minimum fragment ion per peptide equal to 1, minimum fragment ion per protein equal to three, minimum peptide per protein equal to 1, fixed modifications of

carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY), and a default false discovery rate (FDR) value at a 4% maximum, score greater than five, and maximum mass errors of 10 ppm. The analysis used the SUCEST database (http://sucest-fun.org), which is an EST databank. Label-free relative quantitative analyses were performed by the ratio of protein ion counts among contrasting samples. After data processing and to ensure the quality of results, the following protein refinement parameters were used: only proteins present in 3 of 3 runs and with a coefficient of variation lesser than 0.5. For unique proteins, only those present in 2 of 3 runs were considered regardless of whether the coefficient of variation was greater than 0.5. Furthermore, differentially abundant proteins were selected based on a max fold change of at least 2. Functional annotation was performed using Blast2Go software v3.0 PRO (Conesa et al., 2005) and UniProtKB (http://uniprot.org).

4.5. Statistical Analysis

The experiment was conducted in a completely randomized factorial design with five biological replicates represented by five Petri dishes and three colonies of 200 mg fresh matter per Petri dish. Resulting data were submitted to analysis of variance (ANOVA), and the means were compared by the Student-Newman-Keuls (SNK) test (P-value < 0.01) using the statistical analysis software R (R Core Team, 2014) with the easyanova packet (Arnhold, 2013).

5. RESULTS

5.1. Effects of Polyamines on Somatic Embryo Induction

Putrescine at a concentration of 500 µM showed the best results among treatments, presenting an average of 55 embryos per callus after 28 days of culture (Table 1). The addition of different polyamines to the culture medium showed no significant effect on the increase of callus fresh matter between the polyamine treatments and controls (data not shown). Both the Put500 and control treatments enabled the conversion of somatic embryos into plantlets, and 100% of acclimatized plants survived (Figure 1F).

Table 1. Average number of embryos per callus after 28 days of culture.

Control	**Putrescine**			**Spermidine**			**Spermine**		
0µM	10µM	100 µM	500 µM	10µM	100 µM	500 µM	10µM	100 µM	500 µM
19 bc	35 bc	37 bc	55 a	30 bc	16 c	24 bc	39 b	34 bc	25 bc

Means followed by different letters are significantly different ($P < 0.01$) according to the SNK test. n=5 and coefficient of variation equal to 23.92%.

Based on the results described above, shown in Table 1, further analyses of endogenous polyamines and proteomics were limited to samples treated with 500 µM putrescine and the control treatment.

During the maturation period, morphological observations showed that all callus cultures presented anthocyanin pigments, which can be explained as due to light exposure because no pigments were observed for the first seven days in the

dark (Figure 1). However, somatic embryos developed from areas close to these anthocyanin pigments, which suggest that somatic embryogenesis is stress-induced.

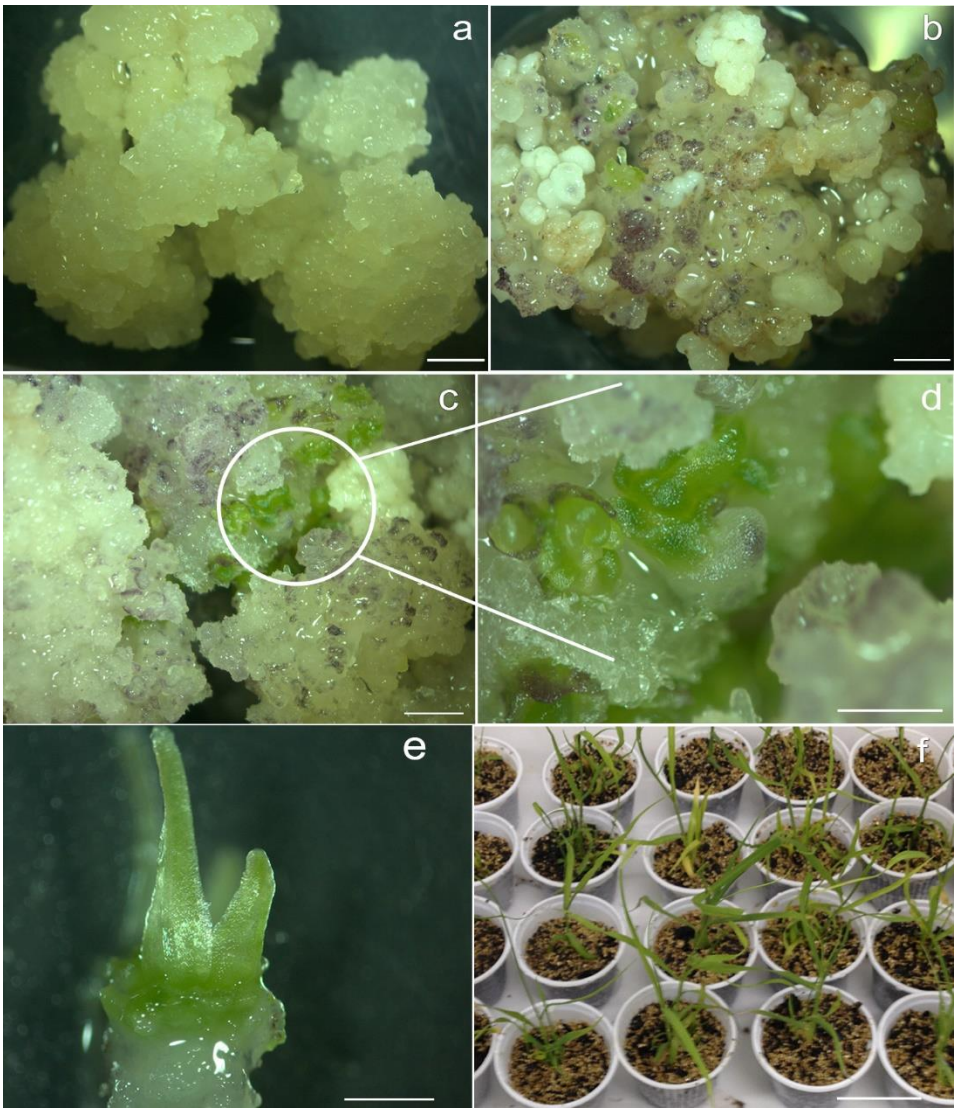

Figure 1. Morphological characteristics of embryogenic cultures of sugarcane var. SP80-3280 in time 0 (A) and subjected to maturation treatments (control) (B); (Put500) (C) and (D). Morphological characteristics of somatic embryos (E); and regenerated plantlets (F). Put: putrescine; Bars: (A-D): 0.5 mm; (E): 0.2 mm and (F): 15 mm.

5.2. Endogenous Polyamine Content

The analyses of free endogenous polyamines were performed in 7-day intervals until the end of the 28-day period of culture during maturation of callus in the control and Put500 treatments (Figure 2).

The results of free endogenous polyamines demonstrated that sugarcane callus cultures treated with 500 µM putrescine presented considerably higher levels of this free polyamine, which peaked on day 14 (Figure 2A). Spermidine levels demonstrated no differences among treatments but changed during the culture period, presenting the highest content at the seventh day and decreasing up to the end of the maturation period (Figure 2B). Spermine had the lowest levels among the three analyzed polyamines, and the control treatment showed higher levels of endogenous spermine on days 7, 14, and 21 compared to the Put500 treatment (Figure 2C).

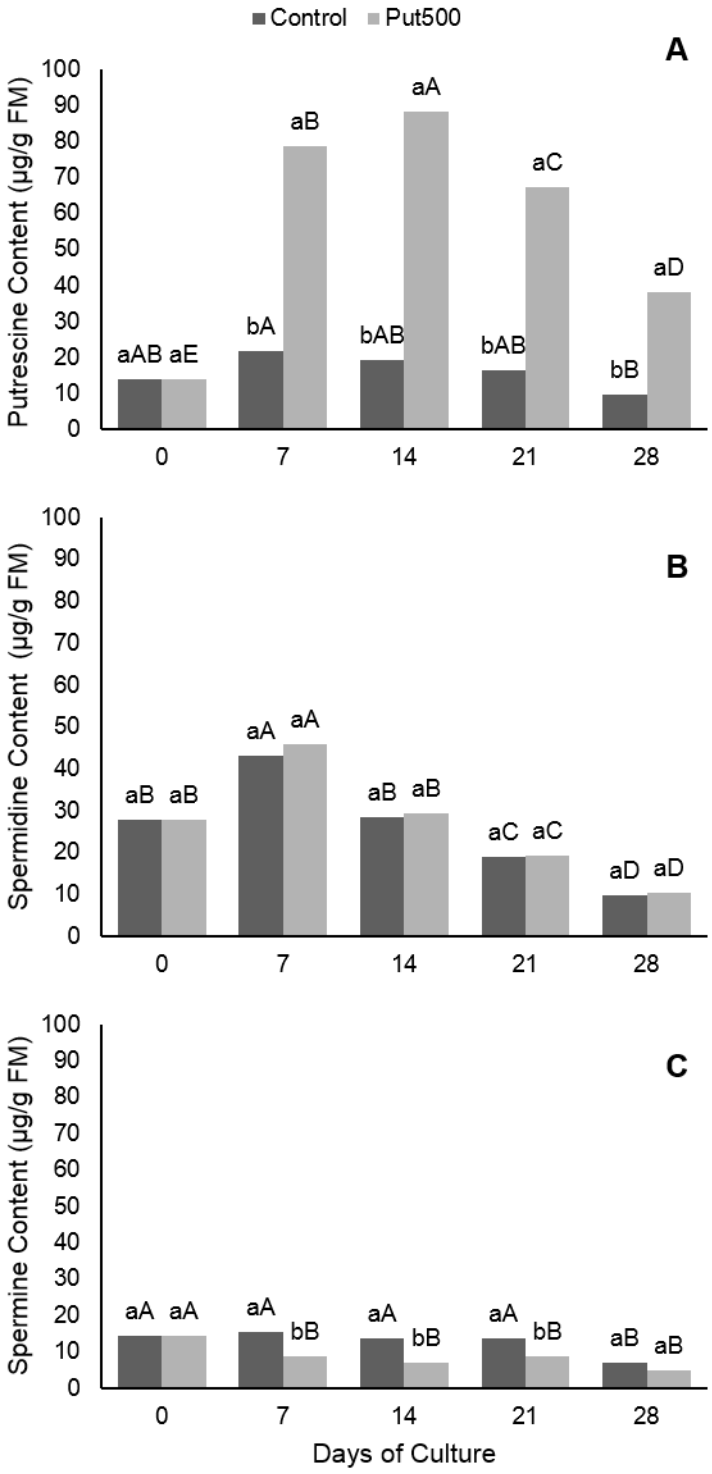

Figure 2. Content of polyamines along 28 days of culture in control and Put500. A, putrescine, CV=13.62%; B, spermidine, CV=13.31%; and C, spermine, CV=22.56%. Lowercase letters denote significant differences among treatments in the same period. Capital letters denote significant differences among days of culture within same treatment. Means followed by different letters are significantly different ($P < 0.01$) according to the SNK test (n=5). FM (Fresh Matter)

5.3. Protein Identification by LC-MS/MS

Proteomic analysis was performed for samples from days 14 and 28 (for both control and Put500). Day 14 was used because it was the first week of culture in a light exposure period, in contrast to the first seven days, in which the cultures were still in the dark. Day 28 was the end of the maturation period and was thought to be critical for a better comprehension of the somatic embryogenesis process.

As a relative quantification analysis, this proteomics study was based on comparative analyses among samples, where each comparison resulted in a list of proteins identified in both samples and proteins unique to one sample. In each list, protein refinement was performed to ensure the quality of the results, which consisted of establishing a cutoff for proteins with a coefficient of variation greater than 0.5, thus automatically excluding proteins present in only 1 or 2 replicates, aside from the exclusion of false positives. The only exceptions to this refinement were the unique proteins because these were considered, according to their presence or absence in the samples, as an important characteristic to study somatic embryogenesis without considering their quantification.

During the analysis, MS^E data were contrasted between the putrescine and control treatments within the same day (days 14 and 28). The differentially abundant protein raw list for the control and putrescine treatments on days 14 and 28 presented 2,611 identified proteins and, after refinement, remained at 1,291 and 1,161 proteins, respectively, on days 14 and 28. Another cutoff was established to show only proteins with differences in abundance levels of at least 2-fold. For Blast2Go analysis, in each period, functional annotation was divided between the two groups of up- and down-regulated proteins for putrescine treatment in relation to the control. Thus, on day 14, putrescine treatment had 61 up-regulated and 97 down-regulated proteins when compared to the control, whereas on day 28, putrescine treatment had 85 up-regulated and 56 down-regulated proteins; 12 and 10 unique proteins were identified on days 14 and 28, respectively. Of these 12 unique proteins, 11 were observed in the putrescine treatment group and only 1 in the control group. Of the 10 unique proteins, seven belonged to the putrescine treatment group and three to the control group. See

supplementary Appendix Tables S1, S2, S3, and S4 for a complete list of all differentially abundant proteins.

Proteins in Table 2 were grouped according to several biological processes that were mainly metabolic processes or a response to stress. Because a protein may be classified in more than one group, functional classification results are presented separately for each protein, emphasizing the main functional groups for that protein.

After processing the data, several stress and detoxification-related proteins were identified, such as eight peroxidases, three heat shock proteins (HSP), three 14-3-3 proteins, and nine glutathione s-transferases (GST) as well as five late embryogenesis abundant (LEA) proteins and two arabinogalactan proteins (AGP), which were differentially abundant on days 14 and 28 (Table 2) with putrescine treatment in relation to the control. Choosing these proteins was based on the relation they had to some type of stress and to having been described previously in other works as being important during somatic embryogenesis development.

On day 14, all the identified arabinogalactan proteins and HSPs showed increased abundance levels with putrescine treatment compared to the control, whereas only one GST of nine was up-regulated, aside from the increased abundance of three peroxidases (Table 2). The most up-regulated protein was an ubiquitin-like protein, which presented an abundance level that was changed by 61.3-fold. In addition, another ubiquitin-conjugating enzyme unique to the putrescine treatment was present, indicating that these proteins might play an important role during putrescine-induced somatic embryogenesis development (Table S2). Two 14-3-3 and three LEA proteins and peroxidases were down-regulated (Table 2).

On day 28, one of the two arabinogalactan proteins increased its abundance level with putrescine treatment in relation to the control. Furthermore, three LEA proteins, including a dehydrin, were also up-regulated in the putrescine treatment group (Table 2). One peroxidase was considered unique, another one showed increased abundance, and two showed decreased expression with putrescine treatment (Table 2). The most up-regulated protein during this stage was an indole-3-acetic acid-amido synthetase, which showed a 55.8-fold change in putrescine-treated cultures (Table S4). In contrast, the 40S ribosomal protein

s15 was the most down-regulated protein. Moreover, an auxin-induced protein pcnt115 was found to be unique to the control treatment during this developmental stage (Table S3).

Interestingly, although it would be logical to believe that somatic embryogenesis development would demand the increased synthesis of new proteins, our results showed that most of the ribosomal proteins were down-regulated with putrescine treatment (Tables S1 and S3).

Table 2. Max fold changes in somatic embryogenesis-related proteins with putrescine treatment compared to controls on days 14 and 28.

Accession	Peptide count	Unique peptides	Score	Description	Biological process	Down on day 14	Up on day 14	Down on day 28	Up on day 28
SCCCCL3001E12.b	8	6	50.68	Arabinogalactan protein	Unknown		7.0		
SCEZRT3069B05	1	1	5.81	Arabinogalactan protein precursor	Unknown		3.7		7.0
SCCCRZ3004A06	4	3	29.85	Heat shock 70 kDa protein	Protein metabolic process; response to stress		7.6		
SCRFST1041E06	3	1	11.77	Stromal 70 kDa heat shock-related chloroplastic-like	Protein metabolic process; response to stress		2.3		
SCCCST3C11C04	6	1	33.31	20 kDa heat shock mitochondrial-like	Protein metabolic process; response to stress		2.1		
SCJFLR1035D05	2	1	16.8	Peroxidase	Carbohydrate metabolic process; response to abiotic stimulus; metabolic process; biosynthetic process; transport; response to stress				Unique
SCJLRT1014B03	9	1	63.22	Loc100286338 (Peroxidase 16-like)	Response to stress; iron ion transport	13.0			
SCCCAD1001C08	1	1	6.60	Peroxidase 42 precursor	Catabolic process; metabolic process; response to stress				5.6
SCEQRT1025E05	11	1	116.36	Cytosolic ascorbate peroxidase	Metabolic process; response to stress		5.1		
SCRLAD1099B04	6	5	39.82	Class III peroxidase 66	Metabolic process; response to stress		2.4		
SCCCLB1002D05	8	1	61.36	Loc100286338 (Peroxidase 16-like)	Response to stress; iron ion transport	2.3			
SCEPRZ1011A06	3	2	18.95	Peroxidase 72 precursor	Response to stress; lignin biosynthetic process	2.1		2.9	
SCCCCL7C05F08	1	1	7.98	Class III peroxidase 66	Metabolic process; response to stress		2.0		
SCCCCL5003C11	7	2	57.51	Glutathione s-transferase 4	Metabolic process; response to stress			4.0	
SCJLRT1020A09	13	4	90.65	Glutathione s-transferase 31	Metabolic process; response to toxic substances; toxin catabolic process	2.8			
SCCCCL4003D01	7	5	39.93	Glutathione s-transferase 30	Response to stress, toxic substances, and growth hormones; regulation of growth; amino acid transport	2.5			
SCSFCL6068E03	10	3	127.14	Glutathione s-transferase parA	Metabolic process; auxin-activated signaling pathway			2.6	
SCJFRT1008A09	11	5	73.55	Glutathione s-transferase gstu6	Metabolic process; response to stress and toxic substances; toxin catabolic process			2.5	
SCCCCL4014B12	5	2	35.40	Glutathione s-transferase 31	Metabolic process; response to toxic substance; toxin catabolic process		2.5		
SCCCLR1048D04	20	8	253.48	Glutathione s-transferase parA	Metabolic process; auxin-activated signaling pathway	2.0		2.1	
SCCCCL4015B02	3	2	16.94	Glutathione s-transferase	Response to stress and toxic substances	2.0			
SCCCCL4007F05	12	6	85.82	Glutathione s-transferase gstu6	Metabolic process; response to stress and toxic substances; toxin catabolic process	2.0			
SCVPCL6061E12	5	5	33.73	Late embryogenesis abundant protein 14-a	Response to abiotic stimulus; response to stress	4.8			2.7
SCACLR1126F12	3	2	22.00	Late embryogenesis abundant protein 1	Response to stress		2.2		
SCCCCL4006B06	4	1	46.65	Late embryogenesis abundant protein group 3 variant 1	Response to stress				2.4
SCCCCL4007G11	10	4	92.35	Late embryogenesis abundant protein group 3 variant 2	Response to stress	2.7			
SCCCCL6001A04	5	2	53.12	Dehydrin 11	Response to abiotic stimulus; response to stress	2.8			2.5
SCEQRT2094B01	13	1	129.15	14-3-3-like protein A	Unknown	2.3			
SCEQRT1031D02	16	3	190.60	14-3-3-like protein	Unknown	2.2			
SCMCRT2102A01	14	1	136.60	14-3-3-like protein A	Unknown			3.9	

6. DISCUSSION

In our study, no significant effect on callus fresh matter was observed for all polyamine concentrations during the maturation period, which is different from that described for other species, such as *Ocotea catharinensis* (Santa-Catarina et al., 2007) and *Araucaria angustifoli* (Silveira et al., 2006). In these two species, exogenous putrescine also showed no effect on culture growth, but spermidine and spermine demonstrated an inhibitory effect. Dutra et al. (2013) studied an *A. angustifolia* cell suspension culture and proposed that this negative effect of spermidine and spermine might be explained by the inhibition of proton pumps, which has been described as part of the acid growth mechanism in plant cells. Paul et al. (2009) reported a positive effect of all polyamines on the fresh matter of *Momordica charantia* embryogenic callus, in which putrescine presented the best results in both fresh matter and somatic embryo development.

Exogenous polyamines have been studied as growth regulators in tissue culture as well as stress-mitigating compounds in plants or seeds of many species. In *in vitro* cultures, they have been tested on somatic embryos (Chiancone et al., 2006; Paul et al., 2009) and organogenesis induction (Viu et al., 2009; Arun et al., 2014), whereas some studies demonstrated the alleviating effects of polyamines during plant development under a variety of stresses, such as draught (Yin et al., 2014) and salinity (Hu et al., 2012).

Putrescine treatment at a concentration of 500 µM showed the best results regarding the production of somatic embryos (Table 1), and a high intracellular

level of putrescine may be related to best performance during the maturation of somatic embryos, as described in studies in which endogenous levels of putrescine were higher. Endogenous polyamines appear to have a similar pattern among some species under somatic embryogenesis induction, as demonstrated by having the highest abundance of putrescine, followed by an intermediate level of spermidine, and low level of spermine found in studies with *Vitis vinifera* (Bertoldi et al., 2004), *Coffea canephora* (De-La-Pena et al., 2008), *Pinus sylvestris* (Vuosku et al., 2012), and sugarcane (Silveira et al., 2013). In contrast, other studies have reported spermidine as the most abundant among polyamines in somatic and immature zygotic embryos of *Quercus ilex* (Mauri e Manzanera, 2011), in zygotic embryos of *P. sylvestris* (Vuosku et al., 2006), and in zygotic and somatic embryos of *Pinus radiata* (Minocha et al., 1999). Farias-Soares et al. (2014) reported high conversion rates of pro-embryogenic masses to somatic embryos and higher levels of polyamines in cultures of *A. angustifolia* under prematuration treatment in culture medium containing polyethylene glycol and maltose as osmotic agents. Among the polyamines, putrescine was found to be at higher levels.

These results demonstrated that polyamine metabolism may be quite different and show distinct responses depending on the species or culture condition. In our study, the control treatment presented spermidine with a higher content compared to putrescine and spermine (Figure 2), and no significant differences were observed for spermidine content between the control and putrescine treatments (Figure 2B). Despite these findings, the control was still able to produce somatic embryos but in a smaller number compared with the putrescine treatment group (Table 1), which suggests that putrescine alone might not be the main factor that contributed to the best induction rates that were found with the putrescine-treated culture. The best somatic embryogenesis induction rates might be due to the indirect action of putrescine in high concentrations, which plays a dual role of contributing as continuous supply for spermidine synthesis while helping cells cope with oxidative stress caused by an excessive production of reactive oxygen species (ROS) by modulating the expression of peroxidases and other related proteins. This scenario would be different in the control treatment due to the low concentration of endogenous putrescine, thus not

being able to cope with oxidative stress and produce embryos at the same efficiency as with the putrescine treatment.

Polyamine catabolism and back-conversion participate in nitrogen flow and generate hydrogen peroxide as part of these reactions (Moschou et al., 2012). Catabolism may occur either in apoplasts or in peroxisomes and hydrogen peroxide acts as a signaling compound to which some biological functions, such as programmed cell death and senescence, are ascribed (Moschou et al., 2012). Jung e Kim (2003) working with polyamine-deficient mutants of *Escherichia coli*, showed that spermidine and putrescine were directly responsible for the regulation of two genes that code for a transcription regulator and an alternative sigma factor subunit that in turn regulate the expression of other three genes that code for three peroxidases. Although plants and bacteria are not directly related organisms, polyamine pathways are present in all groups of organisms, from bacteria to animals, thus demonstrating a conserved characteristic among them.

Studies have already reported the involvement of polyamines in several cellular processes, e.g., cell growth, embryogenesis, and stress in both whole plants and *in vitro* cultures (Kusano et al., 2008). However, the precise way in which polyamines are able to induce somatic embryogenesis has not been elucidated. For that reason, the use of proteomic analysis as a tool for studying the effects of polyamines as inducers of somatic embryogenesis may be an important step toward unraveling the mechanisms that control this complex process.

Differentially abundant proteins between control and putrescine-treated cultures, which were both capable of producing embryos, might suggest that somatic embryogenesis has either more than one induction pathway, depending on the stimulus received by the cells, or has an enhanced effect promoted by polyamines. This complex process has already been shown to be influenced by a variety of molecules, such as growth regulators and carbohydrates, as well as different culture conditions and type of explant, thus not exhibiting a perfect induction protocol for all species, which makes it a species-dependent process (Prakash e Gurumurthi, 2010; Rodríguez-Sahagún et al., 2011; Businge et al., 2013). A promising possibility is searching for the molecular mechanisms that trigger somatic embryogenesis on a proteome level, thus tracking the pathways cells follow to become embryogenically competent and form somatic embryos.

Among the differentially abundant proteins, the six classes of proteins shown in Table 2 have been reported previously in cultures that have been submitted to somatic embryogenesis induction or in embryogenic and non-embryogenic callus. Exogenous putrescine at a concentration of 500 µM, which promoted the best results on somatic embryo induction, was able to change the protein abundance profile of the treated culture compared to the control, thereby modulating the expression of several proteins related to somatic embryogenesis. Thus, a signaling pathway induced by the addition of putrescine at a concentration of 500 µM is proposed for playing a role in the maturation of sugarcane embryogenic callus (Figure 3).

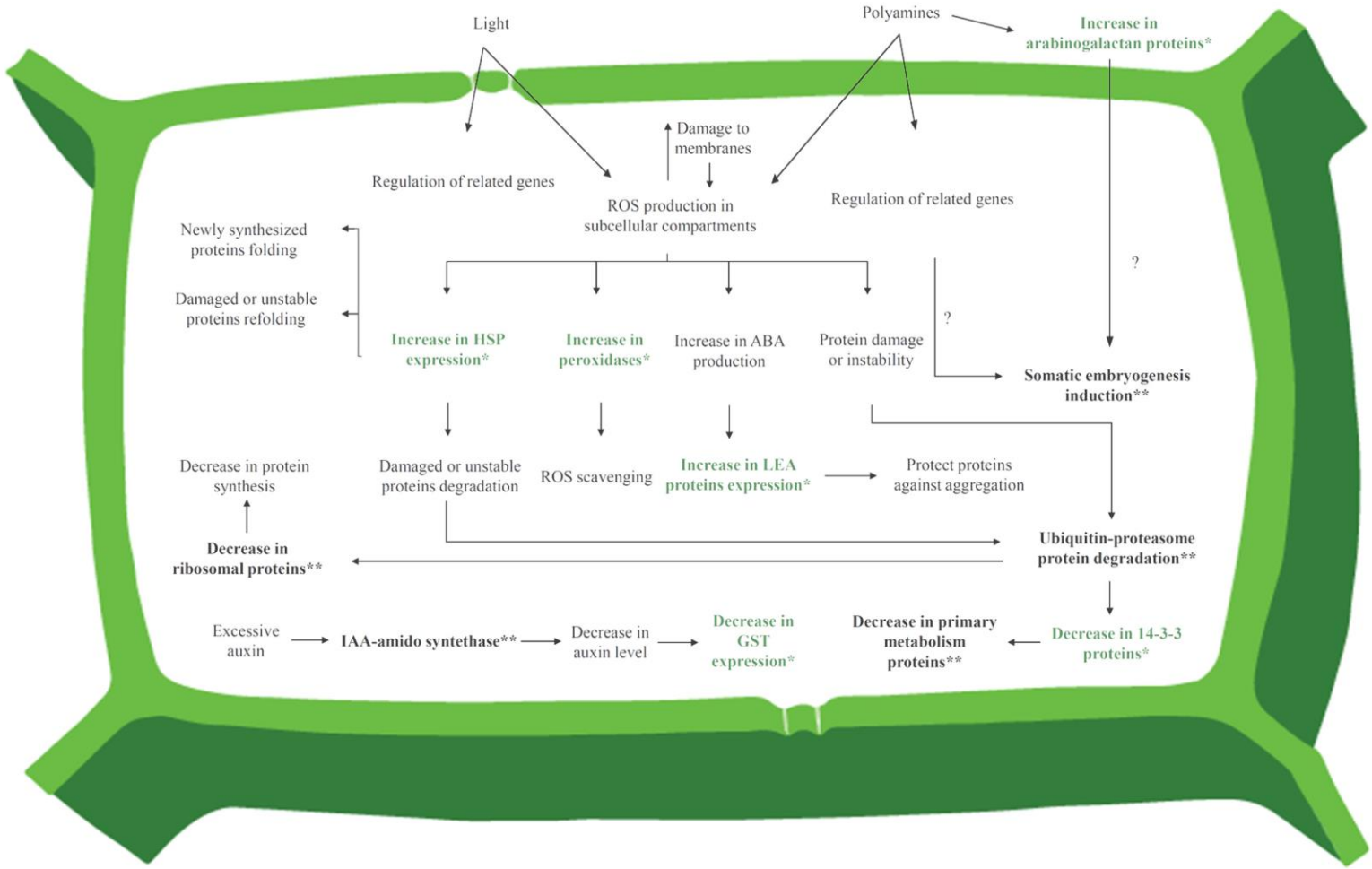

Figure 3. Model for the reactions occurring in somatic embryogenesis development under the effect of putrescine. *Somatic embryogenesis-related proteins up- and down-regulated; **Proteins possibly affected by putrescine action; and somatic embryogenesis induction.

AGPs

AGPs constitute an abundant class of plant glycoproteins consisting of a core protein of highly variable length and domain complexity, one or more arabinogalactan side chains, and often a glycosylphosphatidylinositol (GPI) lipid anchor (Seifert e Roberts, 2007). AGPs may be located in the cell walls, the outer side of the plasma membrane, in vacuoles, in intercellular spaces, and in different secretions and mucilages (Rumyantseva, 2005). They may be implicated in a variety of biological processes, such as cell division, programmed cell death, embryo development, growth, abscission, signaling, and stress responses, and may interact with plant growth regulators (Showalter, 2001; Seifert e Roberts, 2007). The addition of AGPs to embryogenic callus cultures has been reported to stimulate somatic embryogenesis development in cotton (Poon et al., 2012) and *Quercus bicolor* (Mallón et al., 2013) as well as shoot organogenesis in wheat cultures (Zhang et al., 2015). The use of Yariv reagent (β-glucosyl), a synthesized chemical antibody, has been described to interfere with AGP action, causing loss-of-function and thus affecting somatic embryogenesis and callus formation rates in peach palm (Steinmacher et al., 2012). The authors reported a decreasing rate of somatic embryos in the presence of β-glucosyl in a dose-dependent manner. They also found that the presence of β-glucosyl stimulated callus formation, whereas no effect was observed concerning the callus fresh matter. These results demonstrated the possible direct action of AGPs on somatic embryogenesis development, which would be consistent with our results, where two AGPs were up-regulated in putrescine treatment on day 14 (3.7- and 7.0-fold), and one of them further increased its abundance from 3.7- to 7.0-fold on day 28 (Table 2).

Peroxidases

The exposure of plants to stress conditions may induce the production of ROS; therefore, plants must employ essential mechanisms to cope with these harmful molecules and protect plant cells and their organelles against the toxic effect of these species (Caverzan et al., 2012). The ROS detoxification systems include enzymatic (e.g., peroxidases and catalases) and non-enzymatic (e.g., glutathione, anthocyanin, and ascorbate) antioxidant components (Caverzan et al.,

2012). Peroxidases may be found in nearly all subcellular compartments and participate in a wide variety of pathways, including the synthesis of the cell wall components lignin and suberin, the metabolism of hormones, such as indole-3-acetic acid, stress response mechanisms, and fatty acid metabolism (Schuller et al., 1996).

In our study, a wide variety of peroxidases were found to be up- and down-regulated on days 14 and 28 (Table 2), which demonstrated that these enzymes participate in a broad array of cellular processes, most of which are related to responses to stress, as well as in the biosynthesis of lignin and iron ion transport, as shown by functional classification. These results showed that the requirement of peroxidases is more important during the early stages of culture development because a wider variety of enzymes were expressed on day 14 (Table 2), which might be to protect and prepare cells for further development. Differences in peroxidase activity between embryogenic and non-embryogenic callus have been reported in some species as possible somatic embryogenesis markers. In date palm (*Phoenix dactylifera*) and lettuce (*Lactuca sativa*), peroxidase was found to be more active in embryogenic callus (Zhou et al., 1992; El Hadrami e Baaziz, 1995). In contrast, Gallego et al. (2014) showed lower peroxidase activity in embryogenic callus when compared to non-embryogenic callus. However, the authors noted that embryogenic callus presented a unique peroxidase isoform and that peroxidase activity varied with the culture period and an increase from the third month coincided with greater embryogenic frequency. Increased abundance of peroxidases was also found in the *Medicago truncatula* embryogenic line in the early stages of development when compared to the non-embryogenic line (Almeida et al., 2012).

Hydrogen peroxide has been described previously to be a somatic embryogenesis inducer in some cultures (Kairong et al., 1999; Zhang et al., 2015). Kairong et al. (1999) showed that the intracellular level of hydrogen peroxide peaked during the fifth day of culture in *Lycium barbarum* somatic embryogenesis. In strawberry, high levels of peroxide and low levels of hydrogen peroxide were correlated with low levels of callus organogenesis; however, the addition of hydrogen peroxide to the culture medium slightly promoted the percentage of callus showing shoot bud (Tian et al., 2003). The presence of peroxidases, mainly in early stages of development, might not be the key for increased somatic embryo

induction but rather the hydrogen peroxide levels inside cells, which might trigger a signaling cascade and promote the expression of various stress-related genes and thus, via a still unknown route, may stimulate somatic embryogenesis in a growth regulator-manner.

GSTs

GSTs are a family of stress-induced enzymes responsible for detoxifying xenobiotic compounds and ROS by conjugating these molecules to the tripeptide glutathione (GSH), thus tagging them for vacuolar import by specific ATP binding transporters (Edwards et al., 2000). Aside from these functions, GSTs also play roles in normal cellular metabolism, in response to auxin, in the metabolism of plant secondary products, such as anthocyanin, and in the stress caused by pathogen attack (Marrs, 1996). Regarding the induction of GSTs, some may be induced by lipid peroxidation, hydrogen peroxide, and either natural or synthetic auxins. They may also be inactivated by the latter as a result of binding to the enzyme catalytic site (Marrs, 1996). Galland et al. (2007) reported GST expression during the early stages of direct somatic embryogenesis in an interspecific chicory hybrid (*Cichorium intybus* var. *sativum* × *C. endivia* var. *latifolia*). They previously described the presence of GST transcripts extracted from leaf tissues of the embryogenic responsive genotype, but no detection was found in the non-embryogenic responsive genotype (Galland et al., 2001). In mustard (*Brassica juncea*), GST expression differed in young and old leaves (temporal expression), in stems, root, and in *in vitro* shoot regeneration. Furthermore, GST expression was up-regulated by many factors, such as high temperature, heavy metal (mercuric chloride), herbicide, and hydrogen peroxide, and it was down-regulated by spermidine, likely due to the stress-alleviating action of polyamines (Gong et al., 2005).

In our study, only one GST was found to be up-regulated with putrescine treatment, whereas the other eight were down-regulated (Table 2). This increased abundance of GSTs in control might be due to the lack of alternative mechanisms to cope with the excessive production of ROS, differently of putrescine treatment because polyamines are stress-related compounds that help plants to cope with a great diversity of stresses. Therefore, putrescine might facilitate the cellular

mechanisms used for ROS scavenging, avoiding the damages caused by the stress. Both treatments showed the presence of anthocyanin pigments, which help to protect cells from oxidative DNA damage caused mainly by light-induced ROS (Takahashi et al., 1991; Winkel-Shirley, 2002). However, excessive production of anthocyanin may also be harmful to cells. Thus, GSTs act by conjugating them with GSH and sending this GSH-conjugate to be stored in vacuoles (Marrs et al., 1995; Marrs, 1996; Alfenito et al., 1998).

LEA proteins

LEA proteins, as the name suggests, were first observed in the late stages of embryogenesis in cotton seeds during desiccation (Dure et al., 1981). Although they were first observed in plants, these proteins are also present in almost all organisms and are related to stress conditions, such as desiccation, low temperature, light, and osmotic stress (Harrison et al., 2011; Amara et al., 2014). Despite the suggestion that they play the role of molecular chaperone, LEA proteins alone were not capable of preventing heat-induced protein aggregation in citrate synthase; thus, they cannot be classified as HSPs. However, they prevented freezing and desiccation-induced aggregation (Goyal et al., 2005). On day 14, three LEA proteins were found to be down-regulated with putrescine treatment in relation to the control, whereas one was up-regulated. Two of these down-regulated LEA proteins were then up-regulated on day 28 together with a new protein (Table 2). Among them, a dehydrin was observed; dehydrins have been described to be localized mainly in chromatin in the nuclei of embryogenic cultures during somatic embryogenesis development and were not identified in non-embryogenic callus in sugarcane of the same variety (Burrieza et al., 2012).

The expression of LEA proteins may be induced either by the stress itself or by ABA, and this growth regulator has also been related to increase its concentration in response to stress conditions (Curry et al., 1991; Rinne et al., 1998; Hong-Bo et al., 2005; Dalal et al., 2009; Shinde et al., 2012). Dong e Dunstan (1996) reported the expression of abundant genes during somatic embryogenesis of white spruce (*Picea glauca*), including a specific LEA protein that was only expressed in the presence of ABA. The *in vitro* culture itself is a stressful condition to the cells in tissue culture, which means that some of the

identified LEA proteins in our study might be a direct effect of ABA, which in turn would be a response to the culture environment of sucrose (an osmotic agent), light (excessive light may generate ROS), and polyamine catabolism (hydrogen peroxide production).

14-3-3 proteins

The 14-3-3 proteins are a family of phosphoserine-binding proteins that are present in all eukaryotes. They are capable of regulating, via protein-protein interactions, several target proteins related to metabolism, signal transduction, chromatin function, ion transport, and vesicle trafficking and are also involved in stress responses (Roberts, 2003). Swatek et al. (2011) reported the interaction of 14-3-3 isoforms with many proteins extracted from *Arabidopsis thaliana* seeds. These proteins are related to many cellular processes, such as ribosomal proteins, glycolysis, HSPs, and ATPases. Protein-protein interactions may depend on the type of 14-3-3 protein isoform.

14-3-3 proteins may act either as positive or negative regulators of proteins. Swatek et al. (2011) also reported that a specific 14-3-3 isoform interacts with indole-3-acetic acid-amido synthetase, which, in our study, was the most up-regulated protein (55.8-fold) on day 28 with putrescine treatment (Table S4). Because 14-3-3 proteins were down-regulated during this same time period with the same treatment and were up-regulated in control, 14-3-3 proteins may have a negative regulation action on indole-3-acetic acid-amido synthetase, thus decreasing its abundance in the control treatment.

Indole-3-acetic acid-amido synthetase is involved in auxin homeostasis by conjugating the excess of auxins to amino acids, thus inactivating their functional role, although some authors have suggested a possible function for these conjugates (Staswick et al., 2005). High auxin concentration is important for the acquisition of embryogenic capacity and somatic embryogenesis initial stimulus; however, eliminating or reducing auxin concentration is also important for the further development of somatic embryos (Jimenez, 2005). Silveira et al. (2013) reported a negative effect of 2,4-D on somatic embryo maturation in sugarcane callus culture when compared to cultures grown on plant growth regulator-free medium supplemented with activated charcoal. Furthermore, the identification of

an auxin-induced protein pcnt115 unique to the control treatment (Table S3) might indicate a high intracellular auxin level. Thus, the auxin-conjugating enzyme indole-3-acetic acid-amido synthetase may play an important role during somatic embryogenesis development in putrescine-treated cultures.

Possible reactions occurring in somatic embryogenesis under putrescine effect

Based on the results of this study and previous literature reports, it was possible to propose a model for the reactions occurring inside cells undergoing somatic embryogenesis development after putrescine stimulus (Figure 3). In the model, both light and polyamine catabolism may generate ROS, which may cause damage to proteins and membranes. To cope with this stress condition, cells express proteins and antioxidant compounds (anthocyanin) to protect themselves and remain alive. Peroxidases are expressed to scavenge ROS and avoid major damage to the cellular components. HSPs must fold newly synthesized proteins and refold other damaged proteins. When not possible, they direct these proteins for degradation by the ubiquitin-proteasome system. 14-3-3 proteins may also be degraded by the ubiquitin-proteasome system, thus altering the abundance of its target proteins, such as primary metabolism proteins (Appendices). IAA-amido synthetase conjugates auxin with amino acids to cope with excessive concentration of auxins. A decrease in auxin levels may cause a decrease in the abundance of some auxin-regulated GSTs. The stress caused by ROS may increase the production of ABA and therefore increase the abundance of LEA proteins. These proteins act by protecting other proteins against aggregation promoted by a desiccation condition. Due to this capacity to cope with this unfavorable condition, cells decrease the excessive synthesis of new proteins by regulating the abundance of ribosomal proteins. These factors, together with an increased abundance of AGPs, make cells competent to develop into somatic embryos.

7. CONCLUDING REMARKS

The culture medium supplemented with 500 µM putrescine gave rise to the highest number of somatic embryos, when compared to the control. During the analysis, it was possible to identify six classes of proteins that have already been reported in the somatic embryogenesis process. These proteins were related to responses to stress conditions, which demonstrated the importance of stress to trigger the somatic embryo development in different species. AGPs, peroxidases, HSPs, GSTs, LEA proteins, and 14-3-3 proteins may play essential roles during detoxification, stress responses, and development in plant cells. According to the literature, hydrogen peroxide plays an important role during the induction of the process, acting as a growth regulator-like molecule. Thus, in addition to these protein levels being the key to somatic embryo induction, intracellular hydrogen peroxide levels may also be capable of triggering many important reactions.

It might also be possible that somatic embryogenesis acts as a survival mechanism in response to a stress condition faced by cells in tissue culture, which would explain the diversity in stress response proteins found in several studies. Thus, to continue the lineage of offspring, mother cells trigger signaling pathways that culminate in the formation of somatic embryos and subsequently a new plant.

REFERENCES

Ahloowalia, B. , Maretzki, A. (1983) Plant regeneration via somatic embryogenesis in sugarcane. *Plant Cell Reports* 2(1):21-25.

Ahmad, P., Kumar, A., Gupta, A., Hu, X., Hakeem, K.u.R., Azooz, M. , Sharma, S. (2012) Polyamines: Role in Plants Under Abiotic Stress. *In*: Ashraf, M., Öztürk, M., Ahmad, M.S.A. , Aksoy, A. (eds) Crop Production for Agricultural Improvement. Springer, p. 491-512.

Alcazar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P. , Tiburcio, A.F. (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 231(6):1237-1249.

Alfenito, M.R., Souer, E., Goodman, C.D., Buell, R., Mol, J., Koes, R. , Walbot, V. (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. *The Plant Cell Online* 10(7):1135-1149.

Almeida, A.M., Parreira, J.R., Santos, R., Duque, A.S., Francisco, R., Tomé, D.F.A., Ricardo, C.P., Coelho, A.V. , Fevereiro, P. (2012) A proteomics study of the induction of somatic embryogenesis in *Medicago truncatula* using 2DE and MALDI-TOF/TOF. *Physiologia Plantarum* 146(2):236-249.

Altpeter, F. , Oraby, H. (2010) Sugarcane Genetic Modification of Plants. Springer, p. 453-472.

Amara, I., Zaidi, I., Masmoudi, K., Ludevid, M.D., Pagès, M., Goday, A. , Brini, F. (2014) Insights into Late Embryogenesis Abundant (LEA) Proteins in Plants: From Structure to the Functions. *American Journal of Plant Sciences* 5(22):3440.

Arnhold, E. (2013) Easyanova: Analysis of variance and other important complementary analyzes.,

Arruda, P. (2011) Perspective of the sugarcane industry in Brazil. *Tropical Plant Biology* 4(1):3-8.

Arruda, P. (2012) Genetically modified sugarcane for bioenergy generation. *Current Opinion in Biotechnology* 23(3):315-322.

Arun, M., Subramanyam, K., Theboral, J., Ganapathi, A. , Manickavasagam, M. (2014) Optimized shoot regeneration for Indian soybean: the influence of exogenous polyamines. *Plant Cell, Tissue and Organ Culture* 117(2):1-5.

Arvinth, S., Arun, S., Selvakesavan, R., Srikanth, J., Mukunthan, N., Kumar, P.A., Premachandran, M. , Subramonian, N. (2010) Genetic transformation and pyramiding of aprotinin-expressing sugarcane with cry1Ab for shoot borer (*Chilo infuscatellus*) resistance. *Plant Cell Reports* 29(4):383-395.

Bais, H.P. , Ravishankar, G. (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell, Tissue and Organ Culture* 69(1):1-34.

Balbuena, T.S., Dias, L.L.C., Martins, M.L.B., Chiquieri, T.B., Santa-Catarina, C., Floh, E.I.S. , Silveira, V. (2011a) Challenges in proteome analyses of tropical plants. *Brazilian Journal of Plant Physiology* 23:91-104.

Balbuena, T.S., Jo, L., Pieruzzi, F.P., Dias, L.L.C., Silveira, V., Santa-Catarina, C., Junqueira, M., Thelen, J.J., Shevchenko, A. , Floh, E.I.S. (2011b) Differential proteome analysis of mature and germinated embryos of *Araucaria angustifolia*. *Phytochemistry* 72(4-5):302-311.

Barba, R. , Nickell, L. (1969) Nutrition and organ differentiation in tissue cultures of sugarcane, a monocotyledon. *Planta* 89(3):299-302.

Baron, K. , Stasolla, C. (2008) The role of polyamines during in vivo and in vitro development. *In Vitro Cellular & Developmental Biology-Plant* 44(5):384-395.

Belintani, N.G., Guerzoni, J.T.S., Moreira, R.M.P. , Vieira, L.G.E. (2012) Improving low-temperature tolerance in sugarcane by expressing the ipt gene under a cold inducible promoter. *Biologia Plantarum* 56(1):71-77.

Bertoldi, D., Tassoni, A., Martinelli, L. , Bagni, N. (2004) Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. *Physiologia Plantarum* 120(4):657-666.

Bian, F., Zheng, C., Qu, F., Gong, X. , You, C. (2010) Proteomic analysis of somatic embryogenesis in *Cyclamen persicum* Mill. *Plant Molecular Biology Reporter* 28(1):22-31.

Bjellqvist, B., Ek, K., Giorgio Righetti, P., Gianazza, E., Görg, A., Westermeier, R. , Postel, W. (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods* 6(4):317-339.

Burrieza, H.P., Lopez-Fernandez, M.P., Chiquieri, T.B., Silveira, V. , Maldonado, S. (2012) Accumulation pattern of dehydrins during sugarcane (var. SP80.3280) somatic embryogenesis. *Plant Cell Reports* 31(12):2139-2149.

Businge, E., Bygdell, J., Wingsle, G., Moritz, T. , Egertsdotter, U. (2013) The effect of carbohydrates and osmoticum on storage reserve accumulation and

germination of Norway spruce somatic embryos. *Physiologia Plantarum* 149(2):273-285.

Calderan-Rodrigues, M.J., Jamet, E., Bonassi, M.B.C.R., Guidetti-Gonzalez, S., Begossi, A.C., Setem, L.V., Franceschini, L.M., Fonseca, J.G. , Labate, C.A. (2014) Cell wall proteomics of sugarcane cell suspension cultures. *Proteomics* 14(6):738-749.

Casu, R.E., Manners, J.M., Bonnett, G.D., Jackson, P.A., McIntyre, C.L., Dunne, R., Chapman, S.C., Rae, A.L. , Grof, C.P. (2005) Genomics approaches for the identification of genes determining important traits in sugarcane. *Field Crops Research* 92(2):137-147.

Caverzan, A., Passaia, G., Rosa, S.B., Ribeiro, C.W., Lazzarotto, F. , Margis-Pinheiro, M. (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* 35(4):1011-1019.

Cheavegatti-Gianotto, A., de Abreu, H.M.C., Arruda, P., Bespalhok Filho, J.C., Burnquist, W.L., Creste, S., di Ciero, L., Ferro, J.A., de Oliveira Figueira, A.V. , de Sousa Filgueiras, T. (2011) Sugarcane (*Saccharum* X *officinarum*): a reference study for the regulation of genetically modified cultivars in Brazil. *Tropical Plant Biology* 4(1):62-89.

Chen, S. , Harmon, A.C. (2006) Advances in plant proteomics. *Proteomics* 6(20):5504-5516.

Chen, W., Gartland, K., Davey, M., Sotak, R., Gartland, J., Mulligan, B., Power, J. , Cocking, E. (1987) Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. *Plant Cell Reports* 6(4):297-301.

Chiancone, B., Tassoni, A., Bagni, N. , Germanà, M.A. (2006) Effect of polyamines on in vitro anther culture of *Citrus clementina* Hort. ex Tan. *Plant Cell, Tissue and Organ Culture* 87(2):145-153.

CONAB (2015) Terceiro Levantamento Cana-de-açucar Safra 2014/2015 2015. Disponível em: < http://www.conab.gov.br/ >. Acesso em: 28/03/2015.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M. , Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21(18):3674-3676.

Cooke, T.J., Racusen, R.H. , Cohen, J.D. (1993) The role of auxin in plant embryogenesis. *The Plant Cell* 5(11):1494.

Cruz, M.A.L., Da Silva, A.D.C., Veiga, C.F.d.M. , Silveira, V. (2009) Biofábricas para produção de mudas por micropropagação: estratégia para o aumento da produtividade de cana-de-açúcar no Rio de Janeiro. *InterSciencePlace*(5)

Curry, J., Morris, C. , Walker-Simmons, M.K. (1991) Sequence analysis of a cDNA encoding a Group 3 LEA mRNA inducible by ABA or dehydration stress in wheat. *Plant Molecular Biology* 16(6):1073-1076.

Dal-Bianco, M., Carneiro, M.S., Hotta, C.T., Chapola, R.G., Hoffmann, H.P., Garcia, A.A.F. , Souza, G.M. (2012) Sugarcane improvement: how far can we go? *Current Opinion in Biotechnology* 23(2):265-270.

Dalal, M., Tayal, D., Chinnusamy, V. , Bansal, K.C. (2009) Abiotic stress and ABA-inducible Group 4 LEA from *Brassica napus* plays a key role in salt and drought tolerance. *Journal of Biotechnology* 139(2):137-145.

De-La-Pena, C., Galaz-Avalos, R.M. , Loyola-Vargas, V. (2008) Possible role of light and polyamines in the onset of somatic embryogenesis of *Coffea canephora*. *Molecular Biotechnology* 39(3):215-224.

De Souza, A.P., Grandis, A., Leite, D.C. , Buckeridge, M.S. (2013) Sugarcane as a Bioenergy Source: History, Performance, and Perspectives for Second-Generation Bioethanol. *BioEnergy Research*:1-12.

Di Palma, S., Hennrich, M.L., Heck, A.J. , Mohammed, S. (2012) Recent advances in peptide separation by multidimensional liquid chromatography for proteome analysis. *Journal of Proteomics* 75(13):3791-3813.

Dong, J.-Z. , Dunstan, D. (1996) Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. *Planta* 199(3):459-466.

Doule, R., Kawar, P., Devarumath, R. , Nerkar, Y. (2008) Field performance and RAPD analysis for assessment of genetic variation in sugarcane somaclones. *The Indian Journal of Genetics and Plant Breeding* 68(3):301-306.

Dure, L., Greenway, S.C. , Galau, G.A. (1981) Developmental biochemistry of cottonseed embryogenesis and germination: Changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. *Biochemistry* 20(14):4162-4168.

Dutra, N.T., Silveira, V., de Azevedo, I.G., Gomes-Neto, L.R., Facanha, A.R., Steiner, N., Guerra, M.P., Floh, E.I.S. , Santa-Catarina, C. (2013) Polyamines affect the cellular growth and structure of pro-embryogenic masses in *Araucaria angustifolia* embryogenic cultures through the modulation of proton pump activities and endogenous levels of polyamines. *Physiologia Plantarum* 148(1):121-132.

Edwards, R., Dixon, D.P. , Walbot, V. (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends in Plant Science* 5(5):193-198.

El Hadrami, I. , Baaziz, M. (1995) Somatic embryogenesis and analysis of peroxidases in *Phoenix dactylifera* L. *Biologia Plantarum* 37(2):197-203.

FAO (2013) FAOSTAT - Crop production. 2013. Disponível em: < http://faostat.fao.org/ >. Acesso em: 10-06-2013.

Farias-Soares, F.L., Steiner, N., Schmidt, É.C., Pereira, M.L.T., Rogge-Renner, G.D., Bouzon, Z.L., Floh, E.S.I. , Guerra, M.P. (2014) The transition of proembryogenic masses to somatic embryos in *Araucaria angustifolia* (Bertol.) Kuntze is related to the endogenous contents of IAA, ABA and polyamines. *Acta Physiologiae Plantarum* 36(7):1853-1865.

Feher, A., Pasternak, T.P. , Dudits, D. (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell, Tissue and Organ Culture* 74(3):201-228.

Fellers, J.P., Guenzi, A.C. , Porter, D.R. (1997) Marker proteins associated with somatic embryogenesis of wheat callus cultures. *Journal of Plant Physiology* 151(2):201-208.

Fraga, H.P.d.F., Agapito-Tenfen, S.Z., Caprestano, C.A., Nodari, R.O. , Guerra, M.P. (2013) Comparative proteomic analysis of off-type and normal phenotype somatic plantlets derived from somatic embryos of Feijoa (*Acca sellowiana* (O. Berg) Burret). *Plant Science* 210:224-231.

Gaj, M.D., Trojanowska, A., Ujczak, A., Mędrek, M., Kozioł, A. , Garbaciak, B. (2006) Hormone-response mutants of *Arabidopsis thaliana* (L.) Heynh. impaired in somatic embryogenesis. *Plant Growth Regulation* 49(2-3):183-197.

Galland, R., Blervacq, A.-S., Blassiau, C., Smagghe, B., Decottignies, J.-P. , Hilbert, J.-L. (2007) Glutathione-S-transferase is detected during somatic embryogenesis in chicory. *Plant Signaling & Behavior* 2(5):343-348.

Galland, R., Randoux, B., Vasseur, J. , Hilbert, J.-L. (2001) A glutathione S-transferase cDNA identified by mRNA differential display is upregulated during somatic embryogenesis in *Cichorium*. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1522(3):212-216.

Gallego, P., Martin, L., Blazquez, A., Guerra, H. , Villalobos, N. (2014) Involvement of peroxidase activity in developing somatic embryos of *Medicago arborea* L.

Identification of an isozyme peroxidase as biochemical marker of somatic embryogenesis. *Journal of Plant Physiology* 171(2):78-84.

Gong, H., Jiao, Y., Hu, W.-w. , Pua, E.-C. (2005) Expression of glutathione-S-transferase and its role in plant growth and development in vivo and shoot morphogenesis in vitro. *Plant Molecular Biology* 57(1):53-66.

Goyal, K., Walton, L. , Tunnacliffe, A. (2005) LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal* 388:151-157.

Groenewald, J.-H. , Botha, F.C. (2008) Down-regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. *Transgenic Research* 17(1):85-92.

Guerra, M.P., Torres, A.C. , Teixeira, J.B. (1999) Embriogênese somática e sementes sintéticas. *In*: Torres, A.C., Caldas, L.S. , Buso, J.A. (eds) Cultura de tecidos e transformação genética de plantas. 1 ed., Brasília: Embrapa, vol 2, p. 533-586.

Harrison, M.D., Geijskes, J., Coleman, H.D., Shand, K., Kinkema, M., Palupe, A., Hassall, R., Sainz, M., Lloyd, R. , Miles, S. (2011) Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. *Plant Biotechnology Journal* 9(8):884-896.

Heinz, D.J. , Mee, G.W. (1969) Plant differentiation from callus tissue of *Saccharum* species. *Crop Science* 9(3):346-348.

Heringer, A.S., Barroso, T., Macedo, A.F., Santa-Catarina, C., Souza, G.H.M.F., Floh, E.I.S., de Souza-Filho, G.A. , Silveira, V. (2015) Label-Free Quantitative Proteomics of Embryogenic and Non-Embryogenic Callus during Sugarcane Somatic Embryogenesis. *PLoS One* 10(6):e0127803.

Ho, W.J. , Vasil, I.K. (1983a) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L) - Growth and Plant Regeneration from Embryogenic Cell Suspension Cultures. *Annals of Botany* 51(6):719-726.

Ho, W.J. , Vasil, I.K. (1983b) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L). I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma* 118(3):169-180.

Hong-Bo, S., Zong-Suo, L. , Ming-An, S. (2005) LEA proteins in higher plants: Structure, function, gene expression and regulation. *Colloids and Surfaces B: Biointerfaces* 45(3–4):131-135.

Hu, X.H., Zhang, Y., Shi, Y., Zhang, Z., Zou, Z.R., Zhang, H. , Zhao, J.Z. (2012) Effect of exogenous spermidine on polyamine content and metabolism in tomato exposed to salinity-alkalinity mixed stress. *Plant Physiology and Biochemistry* 57:200-209.

ISAAA (2014) Global Status of Commercialized Biotech/GM Crops: 2013. 2014. Disponível em: < www.isaaa.org >. Acesso em: 22/03/2014.

Jimenez, V.M. (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. *Plant Growth Regulation* 47(2-3):91-110.

Jung, I. , Kim, I. (2003) Transcription of ahpC, katG, and katE genes in *Escherichia coli* is regulated by polyamines: polyamine-deficient mutant sensitive to H2O2-induced oxidative damage. *Biochemical and Biophysical Research Communications* 301(4):915-922.

Kairong, C., Gengsheng, X., Xinmin, L., Gengmei, X. , Yafu, W. (1999) Effect of hydrogen peroxide on somatic embryogenesis of *Lycium barbarum* L. *Plant Science* 146(1):9-16.

Kakkar, R., Nagar, P., Ahuja, P. , Rai, V. (2000) Polyamines and plant morphogenesis. *Biologia Plantarum* 43(1):1-11.

Khan, I., Khatri, A., Javed, M., Siddiqui, M., Khanzada, M., Dahar, N. , Khan, R. (2002) Performance of sugarcane somaclones under field conditions at NIA, Tando Jam. *Pakistan Journal Of Botany* 34(1):65-71.

Krikorian, A.D. , Simola, L.K. (1999) Totipotency, somatic embryogenesis, and Harry Waris (1893–1973). *Physiologia Plantarum* 105(2):347-354.

Kusano, T., Berberich, T., Tateda, C. , Takahashi, Y. (2008) Polyamines: essential factors for growth and survival. *Planta* 228(3):367-381.

Lakshmanan, P. (2006) Somatic embryogenesis in sugarcane - an addendum to the invited review 'Sugarcane biotechnology: the challenges and opportunities,' in vitro cell. Dev. Biol. Plant 41(4): 345-363; 2005. *In Vitro Cellular & Developmental Biology-Plant* 42(3):201-205.

Lakshmanan, P., Geijskes, R.J., Aitken, K.S., Grof, C.L.P., Bonnett, G.D. , Smith, G.R. (2005) Sugarcane biotechnology: the challenges and opportunities. *In Vitro Cellular & Developmental Biology-Plant* 41(4):345-363.

Li, K.M., Zhu, W.L., Zeng, K., Zhang, Z.W., Ye, J.Q., Ou, W.J., Rehman, S., Heuer, B. , Chen, S.B. (2010) Proteome characterization of cassava (*Manihot esculenta* Crantz) somatic embryos, plantlets and tuberous roots. *Proteome Science* 8

Link, A.J., Eng, J., Schieltz, D.M., Carmack, E., Mize, G.J., Morris, D.R., Garvik, B.M. , Yates, J.R. (1999) Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnology* 17(7):676-682.

Luge, T., Kube, M., Freiwald, A., Meierhofer, D., Seemüller, E. , Sauer, S. (2014) Transcriptomics assisted proteomic analysis of *Nicotiana occidentalis* infected by *Candidatus* Phytoplasma mali strain AT. *Proteomics* 14(16):1882-1889.

Macgillivray, A.J. , Wood, D.R. (1974) The Heterogeneity of Mouse-Chromatin Nonhistone Proteins as Evidenced by Two-Dimensional Polyacrylamide-Gel Electrophoresis and Ion-Exchange Chromatography. *European Journal of Biochemistry* 41(1):181-190.

Mallón, R., Martínez, T., Corredoira, E. , Vieitez, A.M. (2013) The positive effect of arabinogalactan on induction of somatic embryogenesis in *Quercus bicolor* followed by embryo maturation and plant regeneration. *Trees* 27(5):1285-1296.

Marrs, K.A. (1996) The Functions and Regulation of Glutathione S-Transferases In Plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 47(1):127-158.

Marrs, K.A., Alfenito, M.R., Lloyd, A.M. , Walbot, V. (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2. *Nature* 375(6530):397-400.

Matsuoka, S., Ferro, J. , Arruda, P. (2009) The Brazilian experience of sugarcane ethanol industry. *In Vitro Cellular & Developmental Biology-Plant* 45(3):372-381.

Mauri, P.V. , Manzanera, J.A. (2011) Somatic embryogenesis of holm oak (*Quercus ilex* L.): ethylene production and polyamine content. *Acta Physiologiae Plantarum* 33(3):717-723.

Minocha, R., Smith, D.R., Reeves, C., Steele, K.D. , Minocha, S.C. (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. *Physiologia Plantarum* 105(1):155-164.

Moschou, P.N., Roubelakis-Angelakis, J., Wu, A., Cona, P., Tavladoraki, R., Angelini, K.A. , Moschou, K.A. (2012) The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. *Journal of Experimental Botany* 63(14):5003-5015.

Murashige, T. , Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15(3):473-497.

Nickell, L. (1964) Tissue and cell cultures of sugarcane: another research tool. *Hawaii Plant Rec* 57:223-229.

Noah, A.M., Niemenak, N., Sunderhaus, S., Haase, C., Omokolo, D.N., Winkelmann, T. , Braun, H.-P. (2013) Comparative proteomic analysis of early somatic and zygotic embryogenesis in *Theobroma cacao* L. *Journal of Proteomics* 78:123-133.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* 250(10):4007-4021.

Patade, V., Suprasanna, P., Bapat, V. , Kulkarni, U. (2006) Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. *Barc Newsletter* 273:244.

Paul, A., Mitter, K. , Raychaudhuri, S.S. (2009) Effect of polyamines on in vitro somatic embryogenesis in *Momordica charantia* L. *Plant Cell, Tissue and Organ Culture* 97(3):303-311.

Petrasovits, L.A., Purnell, M.P., Nielsen, L.K. , Brumbley, S.M. (2007) Production of polyhydroxybutyrate in sugarcane. *Plant Biotechnology Journal* 5(1):162-172.

Petrasovits, L.A., Zhao, L., McQualter, R.B., Snell, K.D., Somleva, M.N., Patterson, N.A., Nielsen, L.K. , Brumbley, S.M. (2012) Enhanced polyhydroxybutyrate production in transgenic sugarcane. *Plant Biotechnology Journal* 10(5):569-578.

Poon, S., Heath, R.L. , Clarke, A.E. (2012) A chimeric arabinogalactan protein promotes somatic embryogenesis in cotton cell culture. *Plant Physiology* 160(2):684-695.

Prakash, M.G. , Gurumurthi, K. (2010) Effects of type of explant and age, plant growth regulators and medium strength on somatic embryogenesis and plant regeneration in *Eucalyptus camaldulensis*. *Plant Cell, Tissue and Organ Culture* 100(1):13-20.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Rajeswari, S., Thirugnanakumar, S., Anandan, A. , Krishnamurthi, M. (2009) Somaclonal variation in sugarcane through tissue culture and evaluation for quantitative and quality traits. *Euphytica* 168(1):71-80.

Reinert, J. (1958) Untersuchungen uber die Morphogenese an Gewebekulturen. *Berichte der Deutschen botanischen Gesellschaft* 71:15.

Rinne, P., Welling, A. , Kaikuranta, P. (1998) Onset of freezing tolerance in birch (*Betula pubescens* Ehrh.) involves LEA proteins and osmoregulation and is impaired in an ABA-deficient genotype. *Plant, Cell & Environment* 21(6):601-611.

Roberts, M.R. (2003) 14-3-3 Proteins find new partners in plant cell signalling. *Trends in Plant Science* 8(5):218-223.

Rode, C., Lindhorst, K., Braun, H.-P. , Winkelmann, T. (2012) From callus to embryo: a proteomic view on the development and maturation of somatic embryos in Cyclamen persicum. *Planta* 235(5):995-1011.

Rodríguez-Sahagún, A., Castellanos-Hernández, O.A., Rodríguez-Domínguez, J.M., Rodríguez-Garay, B., Cervantes-Martínez, J. , Acevedo-Hernández, G. (2011) Effect of light quality and culture medium on somatic embryogenesis of *Agave tequilana* Weber var. Azul. *Plant Cell, Tissue and Organ Culture* 104(2):271-275.

Rogowska-Wrzesinska, A., Le Bihan, M.-C., Thaysen-Andersen, M. , Roepstorff, P. (2013) 2D gels still have a niche in proteomics. *Journal of Proteomics* 88:4-13.

Rumyantseva, N. (2005) Arabinogalactan proteins: involvement in plant growth and morphogenesis. *Biochemistry (Moscow)* 70(10):1073-1085.

Santa-Catarina, C., Silveira, V., Scherer, G.F.E. , Floh, E.I.S. (2007) Polyamine and nitric oxide levels relate with morphogenetic evolution in somatic embryogenesis of *Ocotea catharinensis*. *Plant Cell, Tissue and Organ Culture* 90(1):93-101.

Schluter, H., Apweiler, R., Holzhutter, H. , Jungblut, P.R. (2009) Finding one's way in proteomics: a protein species nomenclature. *Chemistry Central Journal* 3(11):11.

Schuller, D.J., Ban, N., van Huystee, R.B., McPherson, A. , Poulos, T.L. (1996) The crystal structure of peanut peroxidase. *Structure* 4(3):311-321.

Scortecci, K.C., Creste, S., Calsa Jr, T., Xavier, M.A., Landell, M.G., Figueira, A. , Benedito, V.A. (2012) Challenges, opportunities and recent advances in sugarcane breeding. *Plant Breeding*

Seifert, G.J. , Roberts, K. (2007) The Biology of Arabinogalactan Proteins. *Annual Review of Plant Biology* 58(1):137-161.

Sengar, A., Thind, K., Kumar, B., Pallavi, M. , Gosal, S. (2009) In vitro selection at cellular level for red rot resistance in sugarcane (Saccharum sp.). *Plant Growth Regulation* 58(2):201-209.

Sghaier-Hammami, B., Drira, N. , Jorrin-Novo, J.V. (2009) Comparative 2-DE proteomic analysis of date palm (*Phoenix dactylifera* L.) somatic and zygotic embryos. *Journal of Proteomics* 73(1):161-177.

Sharifi, G., Ebrahimzadeh, H., Ghareyazie, B., Gharechahi, J. , Vatankhah, E. (2012) Identification of differentially accumulated proteins associated with embryogenic and non-embryogenic calli in saffron (*Crocus sativus* L.). *Proteome Science* 10

Shinde, S., Nurul Islam, M. , Ng, C.K.Y. (2012) Dehydration stress-induced oscillations in LEA protein transcripts involves abscisic acid in the moss, *Physcomitrella patens*. *New Phytologist* 195(2):321-328.

Showalter, A. (2001) Arabinogalactan-proteins: structure, expression and function. *Cellular and Molecular Life Sciences* 58(10):1399-1417.

Silveira, V., Balbuena, T.S., Santa-Catarina, C., Floh, E.I.S., Guerra, M.P. , Handro, W. (2004) Biochemical changes during seed development in *Pinus taeda* L. *Plant Growth Regulation* 44(2):147-156.

Silveira, V., Santa-Catarina, C., Tun, N.N., Scherer, G.F.E., Handro, W., Guerra, M.P. , Floh, E.I.S. (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. *Plant Science* 171(1):91-98.

Silveira, V., Vita, A.M., Macedo, A.F., Dias, M.F.R., Floh, E.I.S. , Santa-Catarina, C. (2013) Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. *Plant Cell, Tissue and Organ Culture* 114(3):351-364.

Snyman, S.J., Meyer, G.M., Koch, A.C., Banasiak, M. , Watt, M.P. (2011) Applications of in vitro culture systems for commercial sugarcane production and improvement. *In Vitro Cellular & Developmental Biology-Plant* 47(2):234-249.

Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C. , Suza, W. (2005) Characterization of an Arabidopsis Enzyme Family

That Conjugates Amino Acids to Indole-3-Acetic Acid. *The Plant Cell* 17(2):616-627.

Steiner, N., Santa-Catarina, C., Andrade, J.B., Balbuena, T.S., Guerra, M.P., Handro, W., Floh, E.I. , Silveira, V. (2008) Araucaria angustifolia Biotechnology. *Functional Plant Science and Biotechnology* 2(1):20-28.

Steinmacher, D.A., Saare-Surminski, K. , Lieberei, R. (2012) Arabinogalactan proteins and the extracellular matrix surface network during peach palm somatic embryogenesis. *Physiologia Plantarum* 146(3):336-349.

Steward, F., Mapes, M.O. , Smith, J. (1958) Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. *American Journal of Botany*:693-703.

Stirn, S., Mordhorst, A.P., Fuchs, S. , Lörz, H. (1995) Molecular and biochemical markers for embryogenic potential and regenerative capacity of barley (*Hordeum vulgare* L.) cell cultures. *Plant Science* 106(2):195-206.

Sugarcane (2014) Sugarcane Industry in Brazil. 2014. Disponível em: < www.sugarcane.org >. Acesso em: 21/03/2014.

Sun, L., Wu, Y., Su, S., Liu, H., Yang, G., Li, S., Shan, X. , Yuan, Y. (2012) Differential gene expression during somatic embryogenesis in the maize (*Zea mays* L.) inbred line H99. *Plant Cell, Tissue and Organ Culture (PCTOC)* 109(2):271-286.

Suprasanna, P., Patade, V., Desai, N., Devarumath, R., Kawar, P., Pagariya, M., Ganapathi, A., Manickavasagam, M. , Babu, K. (2011) Biotechnological developments in sugarcane improvement: an overview. *Sugar Tech* 13(4):322-335.

Swatek, K.N., Graham, K., Agrawal, G.K. , Thelen, J.J. (2011) The 14-3-3 Isoforms Chi and Epsilon Differentially Bind Client Proteins from Developing Arabidopsis Seed. *Journal of Proteome Research* 10(9):4076-4087.

Takac, T., Pechan, T. , Samaj, J. (2011) Differential proteomics of plant development. *Journal of Proteomics* 74(5):577-588.

Takahashi, A., Takeda, K. , Ohnishi, T. (1991) Light-Induced Anthocyanin Reduces the Extent of Damage to DNA in UV-Irradiated *Centaurea cyanus* Cells in Culture. *Plant and Cell Physiology* 32(4):541-547.

Takahashi, T. , Kakehi, J. (2010) Polyamines: ubiquitous polycations with unique roles in growth and stress responses. *Annals of Botany* 105(1):1-6.

Tan, B.C., Chin, C.F., Liddell, S. , Alderson, P. (2013) Proteomic Analysis of Callus Development in *Vanilla planifolia* Andrews. *Plant Molecular Biology Reporter* 31(6):1220-1229.

Tautorus, T.E., Fowke, L.C. , Dunstan, D.I. (1991) Somatic embryogenesis in conifers. *Canadian Journal of Botany* 69(9):1873-1899.

Thorpe, T.A. (2007) History of plant tissue culture. *Molecular Biotechnology* 37(2):169-180.

Tian, M., Gu, Q. , Zhu, M. (2003) The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. *Plant Science* 165(4):701-707.

Tiburcio, A., Altabella, T., Bitrián, M. , Alcázar, R. (2014) The roles of polyamines during the lifespan of plants: from development to stress. *Planta* 240(1):1-18.

UNICA (2014) Detalhamento das exportações mensais de açúcar pelo Brasil. 2014. Disponível em: < www.unicadata.com.br >. Acesso em: 21/03/2014.

USDA (2013) Sugar: World Markets and Trade. Foreign Agricultural Service, 2013. Disponível em: < http://apps.fas.usda.gov/psdonline/circulars/sugar.pdf >. Acesso em: 30/01/2014.

Varhaníková, M., Uvackova, L., Skultety, L., Pretova, A., Obert, B. , Hajduch, M. (2014) Comparative quantitative proteomic analysis of embryogenic and non-embryogenic calli in maize suggests the role of oxylipins in plant totipotency. *Journal of Proteomics*

Vasil, I.K. (2008) A history of plant biotechnology: from the Cell Theory of Schleiden and Schwann to biotech crops. *Plant Cell Reports* 27(9):1423-1440.

Viu, A.F.M., Viu, M.A.O., Tavares, A.R., Vianello, F. , Lima, G.P.P. (2009) Endogenous and exogenous polyamines in the organogenesis in *Curcuma longa* L. *Scientia Horticulturae* 121(4):501-504.

Vuosku, J., Jokela, A., Laara, E., Saaskilahti, M., Muilu, R., Sutela, S., Altabella, T., Sarjala, T. , Haggman, H. (2006) Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. *Plant Physiology* 142(3):1027-1038.

Vuosku, J., Suorsa, M., Ruottinen, M., Sutela, S., Muilu-Makela, R., Julkunen-Tiitto, R., Sarjala, T., Neubauer, P. , Haggman, H. (2012) Polyamine metabolism during exponential growth transition in Scots pine embryogenic cell culture. *Tree physiology* 32(10):1274-1287.

Wang, Z.-Z., Zhang, S.-Z., Yang, B.-P. , Li, Y.-R. (2005) Trehalose synthase gene transfer mediated by *Agrobacterium tumefaciens* enhances resistance to osmotic stress in sugarcane. *Sugar Tech* 7(1):49-54.

Waris, H. (1957) A striking morphogenetic effect of amino acid in seed plant. *Suom Kemistil B* 30:121.

Wasinger, V.C., Cordwell, S.J., Cerpapoljak, A., Yan, J.X., Gooley, A.A., Wilkins, M.R., Duncan, M.W., Harris, R., Williams, K.L. , Humpherysmith, I. (1995) Progress with gene-product mapping of the mollicutes - *Mycoplasma genitalium*. *Electrophoresis* 16(7):1090-1094.

Weng, L.-X., Deng, H.-H., Xu, J.-L., Li, Q., Zhang, Y.-Q., Jiang, Z.-D., Li, Q.-W., Chen, J.-W. , Zhang, L.-H. (2011) Transgenic sugarcane plants expressing high levels of modified cry1Ac provide effective control against stem borers in field trials. *Transgenic Research* 20(4):759-772.

White, W. , Irvine, J. (1987) Evaluation of variation in resistance to sugarcane borer (Lepidoptera: Pyralidae) in a population of sugarcane derived from tissue culture. *Journal of Economic Entomology* 80(1):182-184.

Wilkins, M.R., Pasquali, C., Appel, R.D., Ou, K., Golaz, O., Sanchez, J.C., Yan, J.X., Gooley, A.A., Hughes, G., HumpherySmith, I., Williams, K.L. , Hochstrasser, D.F. (1996a) From proteins to proteomes: Large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Bio-Technology* 14(1):61-65.

Wilkins, M.R., Sanchez, J.C., Gooley, A.A., Appel, R.D., HumpherySmith, I., Hochstrasser, D.F. , Williams, K.L. (1996b) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnology and Genetic Engineering Reviews, Vol 13* 13:19-50.

Winkel-Shirley, B. (2002) Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology* 5(3):218-223.

Wolters, D.A., Washburn, M.P. , Yates, J.R. (2001) An automated multidimensional protein identification technology for shotgun proteomics. *Analytical Chemistry* 73(23):5683-5690.

Wu, L. , Birch, R.G. (2007) Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnology Journal* 5(1):109-117.

Yin, Z., Li, S., Ren, J. , Song, X. (2014) Role of spermidine and spermine in alleviation of drought-induced oxidative stress and photosynthetic inhibition in Chinese dwarf cherry (*Cerasus humilis*) seedlings. *Plant Growth Regulation* 74(3):209-218.

Zambrano, A., Demey, J. , González, V. (2003) In vitro selection of a glyphosate-tolerant sugarcane cellular line. *Plant Molecular Biology Reporter* 21(4):365-373.

Zhang, S.Z., Yang, B.P., Feng, C.L., Chen, R.K., Luo, J.P., Cai, W.W. , Liu, F.H. (2006) Expression of the Grifola frondosa Trehalose Synthase Gene and Improvement of Drought-Tolerance in Sugarcane (*Saccharum officinarum* L.). *Journal of Integrative Plant Biology* 48(4):453-459.

Zhang, W., Wang, X., Fan, R., Yin, G., Wang, K., Du, L., Xiao, L. , Ye, X. (2015) Effects of inter-culture, arabinogalactan proteins, and hydrogen peroxide on the plant regeneration of wheat immature embryos. *Journal of Integrative Agriculture* 14(1):11-19.

Zhou, X., Han, Y., Yang, W. , Xi, T. (1992) Somatic Embryogenesis and Analysis of Peroxidase in Cultured Lettuce (*Lactuca sativa* L.) Cotyledons. *Annals of Botany* 69(2):97-100.

Zhu, Y.J., McCafferty, H., Osterman, G., Lim, S., Agbayani, R., Lehrer, A., Schenck, S. , Komor, E. (2011) Genetic transformation with untranslatable coat protein gene of sugarcane yellow leaf virus reduces virus titers in sugarcane. *Transgenic Research* 20(3):503-512.

Zimmerman, J.L. (1993) Somatic embryogenesis - a model for early development in higher-plants. *Plant Cell* 5(10):1411-1423.

APPENDICES

Table S1- Down-regulated proteins on day 14

Accession	Max fold change	Peptide count	Unique peptides	score	Anova (p)	Description
SCCCLR1080G12	2.0	4	3	23.09	0.0053433145	Pre-mRNA-splicing factor 19
SCAGAD1073F08	2.0	1	1	5.19	0.0459856589	---NA---
SCSFRT2069F01	2.0	1	1	6.85	0.0001725279	Mpi
SCJFRT1005D01	2.0	6	4	53.71	0.0000187146	D-3-phosphoglycerate dehydrogenase
SCRURT2005F01	2.0	13	2	149.89	0.0001776092	Chloride intracellular channel 6
SCRLRZ3116A05	2.0	2	1	17.37	0.0295991934	NADH-ubiquinone oxidoreductase 23 kda subunit
SCEZRT2015E08	2.0	2	1	10.82	0.0321205981	Hypothetical protein SORBIDRAFT_06g006100 [Sorghum bicolor]
SCAGLR2011C02	2.0	9	1	92.98	0.0011438304	Fructokinase-2
SCCCLR1C08F03	2.0	1	1	5.40	0.0134238812	Hypothetical protein SORBIDRAFT_10g020170 [Sorghum bicolor]
SCCCCL4015B02	2.0	3	2	16.94	0.0002092389	Glutathione s-transferase
SCJFRT2053C06	2.0	2	1	20.63	0.0914700937	Bola-like protein
SCVPRT2080H05	2.0	4	2	29.92	0.0001560597	Bowman-birk serine protease inhibitor precursor
SCCCLR1C01D02	2.0	8	2	59.50	0.0751681877	Transcription factor expressed
SCCCLR1048D04	2.0	20	8	253.48	0.0000565666	Glutathione s-transferase para
SCCCCL4007F05	2.0	12	6	85.82	0.0034187801	Glutathione s-transferase gstu6
SCEQLB1063E01	2.1	7	1	43.18	0.0013183063	60s ribosomal protein l6
SCEQRZ3021A06	2.1	1	1	5.50	0.0128189636	Vacuolar-type h(+)-atpase
SCJLLR1011B02	2.1	6	4	35.93	0.0026228582	Hypothetical protein SORBIDRAFT_03g043970 [Sorghum bicolor]
SCEQLR1093H04	2.1	4	1	24.90	0.0064552559	Polyadenylate-binding protein 2
SCVPRT2076C02	2.1	3	2	17.54	0.0655386379	Isovaleryl- dehydrogenase
SCQSLR1089F07	2.1	5	4	57.68	0.0000768988	Acidic ribosomal protein p2a-2
SCEPRZ1011A06	2.1	3	2	18.95	0.0002211632	Peroxidase 72 precursor
SCSBSD2030G12	2.1	1	1	6.32	0.1387166593	Probable non-specific lipid-transfer protein 2
SCCCLB1001B05	2.1	2	2	11.86	0.0003676956	Chitin elicitor-binding
SCBFSB1048B04	2.1	1	1	6.21	0.0143142943	---NA---
SCCCLR1072E07	2.1	5	1	27.29	0.0003851477	Unknown [Zea mays]
SCCCLR1078C05	2.1	2	1	16.74	0.0011825256	Glycogenin-like protein

Table S1 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	score	Anova (p)	Description
SCCCLR1048A10	2.1	3	2	17.09	0.0089470229	60 ribosomal protein l14
SCEZLB1007F07	2.2	10	6	58.60	0.0093835742	Arginyl-trna synthetase
SCEPLR1051F02	2.2	4	3	29.78	0.0751355544	Hypothetical protein SORBIDRAFT_03g002080 [Sorghum bicolor]
SCUTLR2023H05	2.2	13	3	100.75	0.0277918625	R40g2 protein
SCEQLR1091B11	2.2	6	1	83.01	0.0016675128	40s ribosomal protein s12
SCCCCL3001B07.b	2.2	27	9	228.95	0.0002770124	Pyrophosphate-dependent phosphofructokinase alpha subunit
SCJLRZ1023B07	2.2	8	3	49.77	0.0013143127	Minichromosomal maintenance factor
SCCCRZ2001D09	2.2	9	2	59.31	0.0143189514	Substrate binding domain containing expressed
SCCCRZ1002D03	2.2	3	2	16.61	0.0169788274	Pinin sdk mema protein conserved region containing protein
SCVPLR1049H03	2.2	10	7	77.77	0.0000566329	60s acidic ribosomal protein p2b
SCCCLR1C02B02	2.2	4	1	31.64	0.1226422974	Aconitate cytoplasmic
SCACSB1037G08	2.2	8	2	87.88	0.0000269219	Fructose-bisphosphate aldolase cytoplasmic isozyme
SCEQRT1031D02	2.2	16	3	190.60	0.0125919190	14-3-3-like protein
SCCCRZ2002F09	2.3	12	2	71.27	0.0234694428	Alpha-soluble nsf attachment protein
SCSBFL1046B03	2.3	3	3	23.42	0.0000771300	Methionyl-tRNA synthetase
SCCCLR1075G03	2.3	3	1	26.96	0.0000301840	---NA---
SCJFRZ3C07D06a	2.3	1	1	5.17	0.0058139801	Transcription factor apfi
SCJLLR1108F04	2.3	5	3	36.20	0.0029443802	Mitochondrial import receptor subunit tom20
SCJLFL3017H10	2.3	2	1	11.83	0.0127165253	Hypothetical protein SORBIDRAFT_10g022230 [Sorghum bicolor]
SCCCLB1002D05	2.3	8	1	61.36	0.0005093358	Loc100286338 precursor
SCEZLR1052C03	2.3	11	1	97.24	0.0002000489	Sucrose synthase
SCEQRT2094B01	2.3	13	1	129.15	0.0008861754	14-3-3-like protein a
SCMCRT2107G02	2.3	3	1	17.70	0.0307619641	Aspartate-semialdehyde dehydrogenase
SCSGFL4C03A12	2.4	1	1	5.80	0.0000970617	Aaa family expressed
SCSFAD1108C05	2.4	2	2	11.06	0.0374893415	Ankyrin protein
SCCCLR2004H07	2.4	6	5	59.72	0.0004428675	Uncharacterized protein LOC100383393 [Zea mays]
SCUTCL6034D10	2.4	7	6	76.71	0.0000009286	Hypothetical protein SORBIDRAFT_05g022640 [Sorghum bicolor]
SCVPAM2068D11	2.4	1	1	5.88	0.0002170925	Uridylate kinase

Table S1 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	score	Anova (p)	Description
SCCCLB1023G10	2.5	4	2	33.82	0.0032466780	Armadillo beta-catenin-like repeat family protein
SCJLRZ1024A01	2.5	15	3	98.71	0.0002646829	Phosphoenolpyruvate partial
SCAGLR2026C06	2.5	9	6	55.12	0.0011559062	4-methyl-5(b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme
SCRLRZ3039G08	2.5	1	1	5.31	0.0006181117	F-box family expressed
SCCCCL4003D01	2.5	7	5	39.93	0.0029318253	Glutathione transferase 30
SCMCCL6054D04	2.5	2	2	13.86	0.0040250596	Mitochondrial glycoprotein
SCQSLB1052H09	2.5	8	1	91.05	0.0218711439	Ubiquitin 11
SCEPAM2013B01	2.6	2	1	11.83	0.0010349541	Growth regulator like protein
SCCCCL3002A02.b	2.6	13	1	157.18	0.0000694543	Chloride intracellular channel 6
SCCCCL5002C02	2.6	6	3	34.06	0.0419227006	Isopenicillin n epimerase
SCVPRZ2040E07	2.7	2	2	11.90	0.0551592425	GDP-l-fucose synthase 1
SCCCLR2002C01	2.7	6	3	34.78	0.0521578988	40s ribosomal protein s9
SCCCCL4007G11	2.7	10	4	92.35	0.0000391447	Late embryogenesis abundant protein group 3 variant 2
SCCCCL6001A04	2.8	5	2	53.12	0.0001418949	Dehydrin 11
SCJLRT1020A09	2.8	13	4	90.65	0.0015195415	Glutathione transferase 31
SCCCLR2003E04	3.0	2	2	11.37	0.0000063666	Transposon protein
SCJFLR1013G09	3.0	1	1	6.24	0.0000073811	Loc100281836 precursor
SCJLAM1064D11	3.0	1	1	6.70	0.0013721699	NADP-dependent malic enzyme
SCVPLR2012H02	3.0	4	3	23.15	0.0000410839	RNA binding protein 45
SCEQLR1092H10	3.1	4	2	23.23	0.0414052787	Snf1-related protein kinase regulatory subunit gamma-1-like
SCAGAD1073D06	3.1	1	1	5.73	0.0109057797	Hypothetical protein SORBIDRAFT_06g032580 [Sorghum bicolor]
SCEPCL6018B07	3.2	1	1	5.08	0.0192948204	Hypothetical protein SORBIDRAFT_09g002530 [Sorghum bicolor]
SCCCLR2001E08	3.2	14	9	126.50	0.0000512754	Hemoglobin 2
SCVPLR2027C10	3.2	16	7	118.13	0.0016156608	Tcp-1 cpn60 chaperonin family protein
SCCCLR1C05G04	3.2	1	1	5.44	0.0006012682	Receptor ser thr protein kinase-like
SCEQRT1028C03	3.3	13	1	91.55	0.0000211343	Pathogenesis-related protein 1
SCCCCL4014C12	3.3	3	2	17.54	0.0013094913	Hypothetical protein SORBIDRAFT_02g031470 [Sorghum bicolor]
SCCCHR1004F09	3.4	6	4	55.09	0.0000009414	Copper transport protein atox1-like

Table S1 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	score	Anova (p)	Description
SCEZLB1014H01	3.4	1	1	6.53	0.0002908673	Bowman-birk type wound-induced proteinase inhibitor
SCEQSD2074C06	3.7	2	1	21.92	0.0002966300	60s acidic ribosomal protein p3
SCQSRT1034F02	3.8	1	1	6.01	0.0001290981	Adenosine 5 -phosphosulfate reductase-like 1
SCCCHR1001A01	3.9	2	1	13.84	0.0000315020	Glycine-rich cell wall structural protein precursor
SCCCLR1065C12	4.1	1	1	11.71	0.0007470888	Probable cytokinin riboside 5 -monophosphate phosphoribohydrolase logl9-like
SCJLLR1104B01	4.2	1	1	5.95	0.0007059271	P8mtcp1
SCJLFL3014G01	4.3	1	1	8.36	0.0105288376	ATP synthase epsilon mitochondrial
SCJLLR1102F09	4.3	5	4	31.38	0.0004298808	Hypothetical protein SORBIDRAFT_01g015010 [Sorghum bicolor]
SCCCCL2001B11.b	4.6	3	1	17.48	0.0004797396	xylanase inhibitor protein 1 - class iii chitinase homolog flags: precursor
SCVPCL6061E12	4.8	5	5	33.73	0.0000817142	Late embryogenesis abundant protein lea14-a
SCUTFL1060A02.b	5.1	4	1	28.39	0.0008534473	Multidomain cystatin
SCCCLR1065D06	6.3	2	2	10.73	0.0000043138	ATP-dependent zinc metalloprotease chloroplastic
SCRFLR1012F02	10.2	4	4	22.58	0.0000007559	Alpha- -glucan-protein synthase
SCJLRT1014B03	13.0	9	1	63.22	0.0001916507	Loc100286338 precursor
SCJLLR1104B12	Unique to control	1	1	6.8777	0.1311576222	Erwinia induced protein 1

Table S2 - Up-regulated proteins on day 14

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCEZRT2019C05	2.0	8	1	51.75	0.0512329682	Soluble inorganic pyrophosphatase
SCBGLR1023G12	2.0	4	4	23.53	0.0050200113	Probable proteasome inhibitor-like isoform 1
SCCCCL7C05F08	2.0	1	1	7.98	0.0001546039	Tpa: class iii peroxidase 66
SCEQLB1064D12	2.1	6	3	40.23	0.0174647748	Protochlorophyllide reductase b
SCUTAM2088D10	2.1	4	1	27.39	0.0084629558	Substrate binding domain containing expressed
SCRUAD1061C12	2.1	5	2	34.55	0.0539948194	Hypersensitive-induced response protein
SCEPLR1030H06	2.1	6	2	36.71	0.0100105339	RNA binding protein
SCJFRT2057A10	2.1	1	1	5.68	0.0005884291	Uncharacterized protein LOC100272791 [Zea mays]
SCSFST1066G10	2.1	3	3	22.13	0.0000197130	Hypothetical protein SORBIDRAFT_08g018710 [Sorghum bicolor]
SCCCST3C11C04	2.1	6	1	33.31	0.0256140328	Hypothetical protein SORBIDRAFT_10g007590 [Sorghum bicolor]
SCMCRT2103A12	2.1	4	4	36.17	0.0003698382	Lipid transfer protein
SCMCST1054E03	2.1	1	1	11.61	0.0310829554	---Na---
SCCCLR1066B02	2.1	7	1	55.24	0.0072917177	40s ribosomal protein s6
SCMCST1055F02	2.1	5	2	30.65	0.0361126466	Lysyl-trna synthetase
SCBGRT1046B04	2.1	7	1	46.78	0.0209672357	GTP-binding nuclear protein gsp1 ran
SCAGCL6014G06	2.2	9	2	66.25	0.0000116737	Pdi-like protein
SCACLR1126F12	2.2	3	2	22.00	0.0444526817	Lea1_orysj ame: full=late embryogenesis abundant protein 1
SCSGFL5C03H04	2.2	3	2	18.51	0.0005617651	Chromatin complex subunit a101
SCCCCL4009G04	2.2	7	2	64.23	0.0070590064	Hypothetical protein SORBIDRAFT_01g004270 [Sorghum bicolor]
SCJFRZ2013G10	2.2	3	1	19.63	0.0001681455	Structural maintenance of chromosomes protein 6-like
SCJFRT1060F02	2.2	9	1	79.04	0.0191091482	Histone h3
SCRFAM1028B07	2.2	3	2	17.10	0.0602791062	Jmjc domain containing expressed
SCEZLB1010A03	2.2	4	3	25.51	0.0201565238	Spermidine synthase 1
SCEQRT2029H09	2.2	2	1	12.20	0.0139034203	Ef hand family expressed
SCAGFL3029B06	2.3	1	1	5.58	0.0095097880	---Na---
SCQGFL4080D04	2.3	10	1	61.99	0.0079011488	Clh2_orysj ame: full=clathrin heavy chain 2

Table S2 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCJFRT2054A08	2.3	2	1	10.90	0.0597580485	Hypothetical protein SORBIDRAFT_09g002290 [Sorghum bicolor]
SCRFST1041E06	2.3	3	1	11.77	0.0002972858	Stromal 70 kda heat shock-related chloroplastic-like
SCCCCL4006C07	2.3	15	3	102.86	0.0019744925	26s proteasome regulatory particle triple-a atpase subunit6
SCVPRT2080B07	2.3	3	3	23.20	0.0135870211	Thaumatin-like protein precursor
SCCCCL3001E04.b	2.4	7	3	47.89	0.0127541542	Non-photosynthetic nadp-malic enzyme
SCJLRT1014F08	2.4	1	1	5.28	0.0430103695	Progesterone 5-beta-
SCRLAD1099B04	2.4	6	5	39.82	0.0082639199	Tpa: class iii peroxidase 66
SCCCRZ2001C11	2.4	4	4	22.07	0.0005350224	Usp family protein
SCCCRZ2C03B06	2.4	3	1	18.68	0.0007822110	Hypothetical protein SORBIDRAFT_02g037540 [Sorghum bicolor]
SCCCCL4014B12	2.5	5	2	35.40	0.0010062597	Glutathione transferase 31
SCCCLR1069D09	2.5	2	2	12.42	0.0071263437	Swib domain-containing protein
SCJFRZ2031F05	2.5	2	2	10.81	0.0058746877	Ribonucleotide reductase
SCJLLR1011D01	2.7	2	2	10.48	0.0280315503	Cytochrome b-c1 complex subunit mitochondrial precursor
SCCCLR1076G12	2.7	10	4	74.24	0.0102353826	Act7_orysi ame: full=actin-7
SCCCLR1024C05	2.9	8	1	60.95	0.0000322592	Nonspecific lipid-transfer protein 3 precursor
SCCCLR1024A12	3.1	2	2	11.75	0.0119346460	Serine carboxypeptidase precursor
SCJFLR1013D04	3.2	3	2	16.26	0.0008245716	Cinnamoyl- reductase
SCJFRZ2015B06	3.2	3	2	26.56	0.0030192823	Peptidyl-prolyl isomerase fkbp12
SCRURT2012C09	3.3	3	2	18.69	0.0001356722	Zeamatin-like protein
SCCCLR1048C08	3.5	1	1	5.02	0.0000032059	Zinc finger c-x8-c-x5-c-x3-h type family protein
SCEQLB1067D01	3.6	1	1	5.91	0.0014819004	Dtdp-4-dehydrorhamnose reductase
SCEZRT3069B05	3.7	1	1	5.81	0.0070997372	Arabinogalactan protein precursor
SCJFRZ2026C07	3.7	2	2	16.96	0.0027248336	Hypothetical protein SORBIDRAFT_07g025350 [Sorghum bicolor]
SCCCLR2002E05	3.8	9	2	61.04	0.0001239816	60s ribosomal protein l7-2
SCCCRZ1C01D11	4.1	3	1	19.24	0.0005018291	Cytochrome c
SCBFAD1046B06	4.7	1	1	5.31	0.0011909222	Pyruvate kinase isozyme chloroplast precursor
SCBFST3133F03	4.8	1	1	11.60	0.0045271095	Rab1 small gtp-binding protein
SCCCLR1079G05	4.8	2	2	13.94	0.0138126107	40s ribosomal protein s15

Table S2 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCCCLR1C03C09	5.0	5	2	30.21	0.0010686904	Ras-related protein rab-18
SCEQRT1025E05	5.1	11	1	116.36	0.0028704811	Apx2 - cytosolic ascorbate peroxidase
SCCCCL3001E12.b	7.0	8	6	50.68	0.0015964810	Arabinogalactan protein
SCCCRZ3004A06	7.6	4	3	29.85	0.0000131118	Heat shock 70 kda protein
SCSGLV1008H10	8.2	1	1	5.30	0.0011013239	Hypothetical protein SORBIDRAFT_07g023700 [Sorghum bicolor]
SCAGHR1018G09	9.0	9	1	86.92	0.0003831598	Alpha- -glucan protein synthase
SCJFRT2055G07	61.3	9	1	92.25	0.0002541649	Ubiquitin-like protein
SCCCCL3002A09.b	Unique to putrescine	5	1	29.4324	0.0002023728	Alanine aminotransferase
SCCCRZ1004B03	Unique to putrescine	1	1	5.5611	0.0058273077	hypothetical protein SORBIDRAFT_10g026795 [Sorghum bicolor]
SCEZRZ1013F11	Unique to putrescine	1	1	5.4508	0.1919162912	RNA recognition motif -containing protein
SCEZRZ1015A07	Unique to putrescine	1	1	5.8313	0.0000157769	---NA---
SCRURT3062A10.b	Unique to putrescine	1	1	5.1101	0.0740628495	mitochondrial 2-oxoglutarate malate carrier protein
SCSBFL1043D08	Unique to putrescine	1	1	5.2005	0.0053498084	hypothetical protein SORBIDRAFT_02g010740 [Sorghum bicolor]
SCSBSD2056C12	Unique to putrescine	1	1	5.9304	0.0060156978	hypothetical protein SORBIDRAFT_08g016260 [Sorghum bicolor]
SCSGAM2075E07.b	Unique to putrescine	1	1	5.7005	0.1348881680	hypothetical protein SORBIDRAFT_05g001370 [Sorghum bicolor]
SCSGRT2066E06	Unique to putrescine	1	1	5.507	0.0413549123	Basic helix-loop-helix family protein
SCVPLR2027B12	Unique to putrescine	6	1	40.6889	0.0000017843	Pyrophosphate-dependent phosphofructo-1-kinase
SCVPRT2076C01	Unique to putrescine	1	1	5.8651	0.1361853747	Ubiquitin-conjugating enzyme e2

Table S3 - Down-regulated proteins on day 28

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCCCFL5061H09	2.0	6	2	47.15	5.72E-06	Dihydrolipoyl dehydrogenase mitochondrial-like
SCCCRZ1002E10	2.0	4	1	22.58	0.005607414	Mitochondrial rho gtpase 1-like
SCUTLR1058E09	2.1	1	1	6.77	0.001405416	40s ribosomal protein s13
SCMCSB1116D02	2.1	2	1	12.07	0.072252481	Phosphoenolpyruvate carboxylase
SCJLLR1054A06	2.1	12	11	87.65	0.009928565	1-aminocyclopropane-1-carboxylate oxidase 1
SCCCLR1048D04	2.1	20	8	253.48	0.000426962	Glutathione s-transferase para
SCMCRT2108A04	2.1	3	1	22.32	0.038143195	Hypothetical protein SORBIDRAFT_05g023700 [Sorghum bicolor]
SCRUFL1015F05	2.1	2	1	11.10	0.000787785	Hypothetical protein SORBIDRAFT_02g037270 [Sorghum bicolor]
SCQGLR1019G02	2.1	16	6	111.50	0.000995422	UDP-glucose 6- expressed
SCCCCL4006H08	2.1	19	6	169.88	5.78E-05	Alcohol dehydrogenase 2
SCUTLR1037G10	2.1	4	1	23.25	0.000696398	Glucose-6-phosphate 1- cytoplasmic isoform
SCQGHR1013F09	2.1	2	1	17.18	0.059499675	Rust resistance protein rp3-1
SCCCCL4017A09	2.1	21	1	166.55	0.000171976	NADP-specific isocitrate dehydrogenase
SCCCLR1C07G11	2.1	26	1	219.98	0.000484906	Eukaryotic initiation factor 4a
SCCCRT1002C05	2.1	24	19	189.43	0.000101703	Alanine aminotransferase
SCQSRT1035D12	2.1	3	2	18.84	0.000256316	Permatin precursor
SCACLR2007G05	2.1	3	2	21.65	0.009429698	Remorin-like isoform 1
SCRUAD1132H03	2.2	1	1	5.53	0.025601829	Phd-finger family homeodomain protein
SCJLRZ1024A01	2.2	15	3	98.71	9.62E-05	Phosphoenolpyruvate partial
SCJLAM1064D11	2.2	1	1	6.70	0.08721476	NADP-dependent malic enzyme
SCCCCL4002C10	2.2	1	1	6.47	0.027088811	Phi-1-like phosphate-induced protein
SCCCCL4011H08	2.2	33	16	310.19	0.000172415	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase beta subunit
SCEQLR1093H04	2.2	4	1	24.90	0.085570281	Polyadenylate-binding protein 2
SCCCLR1001E06	2.3	25	11	332.61	4.22E-06	Glyceraldehyde-3-phosphate cytosolic 3
SCCCLR1022C06	2.3	3	1	18.88	0.031565719	Aci-reductone dioxygenase-like protein
SCCCCL3001B07.b	2.4	27	9	228.95	0.000575917	Pyrophosphate-dependent phosphofructokinase alpha subunit
SCCCAM2002D07	2.4	1	1	5.87	0.006892055	---NA---
SCCCLR1048A10	2.4	3	2	17.09	0.016431681	60 ribosomal protein l14

Table S3 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCJLRZ1024A09	2.4	1	1	6.26	0.019322207	Succinate dehydrogenase iron-protein subunit
SCEQRT1025F12	2.4	2	1	11.89	0.084442005	Hypothetical protein SORBIDRAFT_06g033890 [Sorghum bicolor]
SCEZLR1052C03	2.5	11	1	97.24	0.006895342	Sucrose synthase
SCJFRT1008A09	2.5	11	5	73.55	0.000620549	Glutathione s-transferase gstu6
SCEPRZ1011H03	2.5	4	3	23.30	0.005015294	Pyrroline 5-carboxylate reductase
SCEZRT2023E09	2.5	3	1	18.24	0.000761129	Atpase family aaa domain-containing protein 1
SCCCCL4011D12	2.5	23	6	188.67	0.005067709	Pyruvate decarboxylase
SCSFCL6068E03	2.6	10	3	127.14	0.000791371	Glutathione s-transferase para
SCCCCL4003F09	2.6	10	4	70.70	5.65E-06	Xylanase inhibitor protein 1 precursor
SCJLLR1033G09	2.7	13	1	90.08	0.017223534	Eukaryotic translation initiation factor 3 subunit a
SCRLAM1006H09	2.7	4	1	23.56	0.047991546	Ribose-5-phosphate isomerase
SCEPRZ1011A06	2.9	3	2	18.95	0.001366187	Peroxidase 72 precursor
SCCCLR2001E08	2.9	14	9	126.50	4.85E-05	Hemoglobin 2
SCMCLR1053H06	3.0	3	3	16.74	0.021508868	Lysyl-tRNA synthetase
SCBGRT3014H03	3.1	1	1	5.23	7.56E-05	Hypothetical protein SORBIDRAFT_06g021280 [Sorghum bicolor]
SCVPLR1028E03	3.1	9	7	66.05	0.002611507	Pci domain containing protein
SCCCCL3001B04.b	3.2	13	11	78.18	0.005616706	Prolyl-tRNA synthetase -like
SCEQLR1091F02	3.3	1	1	5.70	0.00152623	Purple acid phosphatase precursor
SCJLRT1020A09	3.5	13	4	90.65	0.001619214	Glutathione transferase 31
SCSFRT2069F01	3.6	1	1	6.85	0.001998415	Mpi
SCSFRT2072E08	3.6	2	1	10.92	0.015552091	Tbc1 domain family member 13-like
SCQGST3123C08	3.8	1	1	5.78	0.007762619	Pectin methylesterase
SCCCCL4007F05	3.9	12	6	85.82	0.000287773	Glutathione s-transferase gstu6
SCMCRT2102A01	3.9	14	1	136.60	0.015881734	14-3-3-like protein a
SCCCCL5003C11	4.0	7	2	57.51	0.002531037	Glutathione s-transferase 4
SCCCCL4015B02	4.3	3	2	16.94	0.011768471	Glutathione s-transferase
SCRLSD2009C01	7.2	2	2	11.80	2.15E-05	Aldose reductase

Table S3 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCCCLR1079G05	7.4	2	2	13.94	0.00732895	40s ribosomal protein s15
SCCCCL3002B06.b	Unique to control	9	1	77.1302	0.124439952	isochorismate synthase 1
SCJLLR1107G01	Unique to control	8	1	51.9936	0.174152235	auxin-induced protein pcnt115
SCVPRZ3025F08	Unique to control	1	1	6.3046	0.116813698	protein kinase

Table S4 - Up-regulated proteins on day 28

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCRLLV1051E09	2.0	1	1	6.00	8.99E-05	Ubiquitin-protein ligase zinc ion binding protein
SCJLRT2049E06	2.0	1	1	5.41	0.000554175	Elongation factor mitochondrial
SCJLLR1033A07	2.0	5	2	35.56	0.001454689	Hydroxyacylglutathione hydrolase
SCJFRT2053G02	2.0	4	1	26.03	0.001457454	Tubulin alpha-3 alpha-5 chain-like partial
SCJLRT1023A07	2.1	8	1	54.21	0.001647579	Pyruvate dehydrogenase e1 component subunit beta
SCEZFL4044H06	2.1	2	1	11.05	0.000129913	Cytochrome p450
SCBGLR1120G03	2.1	3	2	28.61	1.54E-06	Grf-interacting factor 2
SCCCRT2003E01	2.1	2	1	12.63	0.001005493	Predicted protein [Hordeum vulgare subsp. Vulgare]
SCCCRZ2002E03	2.1	11	7	74.02	0.000744293	Tpr domain containing protein
SCRUAD1132B09	2.1	1	1	6.23	0.001001411	---NA---
SCSBFL1046B03	2.1	3	3	23.42	0.007549929	Methionyl-tRNA synthetase
SCVPRT2080G03	2.1	2	1	12.67	0.000137029	Spotted leaf protein 11
SCCCCL3002B12.b	2.1	7	6	42.41	0.007947454	Hypothetical protein SORBIDRAFT_01g015200 [Sorghum bicolor]
SCJFRT1060F02	2.1	9	1	79.04	0.004220062	Histone h3
SCRFRT3059D09	2.2	3	1	16.74	0.000909179	---NA---
SCAGLR1043D06	2.2	17	3	166.55	0.003659834	Methyl binding domain106
SCJFLR1073B11	2.2	7	5	50.69	0.003009622	Transcription factor
SCQGAM2026D09	2.2	4	1	24.18	0.002346442	Nuf2 family protein
SCQGHR1013C08	2.2	5	1	31.03	0.003357083	26s protease regulatory subunit 4
SCVPCL6062A06	2.3	4	2	22.80	0.019054299	Abi3-interacting protein 2 2
SCSFFL4085H10	2.3	1	1	6.19	0.010000877	Protein furry homolog-like
SCJLST1027H12	2.3	6	4	35.62	0.002084002	PREDICTED: uncharacterized protein LOC100840292 [Brachypodium distachyon]
SCJFHR1033B10	2.3	2	2	11.92	0.00538216	Cellular retinaldehyde-binding triple function
SCCCLR1048E11	2.4	1	1	5.98	0.0045428	GTP-binding protein chloroplastic-like
SCCCRZ1C01D11	2.4	3	1	19.24	0.005256076	Cytochrome c
SCVPLR2012E08	2.4	2	1	17.80	0.015048684	Seed maturation protein pm41
SCCCCL4006B06	2.4	4	1	46.65	0.001959636	Late embryogenesis abundant protein group 3 variant 1
SCSBSD2030G12	2.4	1	1	6.32	0.00929318	Probable non-specific lipid-transfer protein 2

Table S4 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCVPLR2012C11	2.4	1	1	6.50	0.008377305	Mfp1 attachment factor 1
SCJLLR1011D01	2.5	2	2	10.48	0.004905936	Cytochrome b-c1 complex subunit mitochondrial precursor
SCCCCL3002C07.b	2.5	9	5	70.63	0.000657761	Alpha-amylase isozyme 3c (alpha-d-glucan glucanohydrolase)
SCCCCL6001A04	2.5	5	2	53.12	0.042692542	Dehydrin 11
SCJFLR1013D11	2.5	2	1	11.35	0.001789154	Remorin-like isoform 1
SCJFRZ2031F05	2.6	2	2	10.81	0.005077039	Ribonucleotide reductase
SCCCLR2C01H05	2.6	1	1	5.56	0.003009118	Eukaryotic translation initiation factor 1a
SCCCLR1C02F11	2.6	2	1	11.02	0.014515799	Pyruvate kinase isozyme chloroplastic-like
SCEPAM1019D01	2.6	3	1	21.42	0.000363443	Nap16kda protein
SCJFLR1013D04	2.6	3	2	16.26	0.011444784	Cinnamoyl- reductase
SCBFRT1064B08	2.6	5	1	28.69	0.001988687	Plasma membrane h+ atpase
SCEZRT2019C05	2.6	8	1	51.75	0.001575158	Soluble inorganic pyrophosphatase
SCCCFL3003D04	2.6	1	1	5.81	0.012756205	Hmgc1 protein
SCVPRZ2043A12	2.6	6	3	37.44	0.010997599	Usp family protein
SCEQRT1030C08	2.7	2	2	13.38	0.017082325	Translin [Zea mays]
SCSBSD1032D07	2.7	1	1	5.45	0.055413282	Hypothetical protein FOXB_01634 [Fusarium oxysporum Fo5176]
SCCCLR1048A08	2.7	4	1	23.86	0.001270353	NADP-dependent malic enzyme
SCVPCL6061E12	2.7	5	5	33.73	0.014921141	Late embryogenesis abundant protein lea14-a
SCBGLR1082B02	2.7	2	2	11.63	0.019806762	Splicing factor 3b subunit expressed
SCCCRT1003G09	2.8	1	1	5.83	0.0237736	Isoform 1
SCEQLR1092H02	2.8	5	2	36.89	0.006923474	Oleosin 18 kda
SCCCCL3002C12.b	2.8	1	1	5.37	0.015053878	Reticulon-like protein b2-like isoform 1
SCACLR1127E11	2.8	1	1	5.74	0.00086288	26s proteasome non-atpase regulatory subunit 3
SCCCRZ2002G06	2.9	5	3	41.74	0.01320538	Hypothetical protein SORBIDRAFT_03g036890 [Sorghum bicolor]
SCQSFL3032H06	2.9	2	1	11.84	0.001264331	Pleckstrin homology domain family a
SCEPRZ1009D06	2.9	3	2	19.32	0.000144806	Unknown [Zea mays]
SCCCLB1025B01	3.0	3	2	23.60	0.000172853	Plasminogen activator inhibitor 1 rna-binding protein
SCRFLR2037A10	3.0	1	1	5.67	0.012849796	Type 1 membrane

Table S4 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCJFFL3C04H10.b	3.0	1	1	5.65	0.003724649	Splicing factor 3a subunit 3-like
SCUTLR1037A05	3.0	3	2	17.75	0.000888088	---NA---
SCRLSD2012G09	3.1	4	3	45.97	1.31E-05	Flower-specific gamma-thionin precursor
SCCCLR1C08E09	3.1	10	4	59.53	0.001380497	Ras-related protein rab11a
SCCCCL3004A09.b	3.2	2	2	11.95	0.004647455	Protein kinase
SCUTLR1037D04	3.5	16	1	159.58	0.058131406	Methyl binding domain106
SCJFLR1073B01	3.5	3	1	22.83	0.000194133	Uncharacterized protein LOC100276829 [Zea mays]
SCCCCL3003E02.b	3.6	3	2	18.25	0.007403397	Ubiquitin thioesterase otubain-like protein
SCJLRT3078H06	3.6	1	1	5.68	0.001134343	Disease resistance response protein 206-like
SCEZAM2032C01	3.6	3	2	16.74	0.057792696	Adhesion regulating molecule conserved region family protein
SCCCFL6002G10	3.7	2	1	11.76	0.00167844	Tmv-mp30 binding protein 2c
SCCCLR1024C05	3.7	8	1	60.95	0.019274987	Nonspecific lipid-transfer protein 3 precursor
SCQSRT2032F09	4.1	6	1	37.44	0.010554726	Uncharacterized wd repeat-containing protein alr3466-like
SCQGLR1041H09	4.1	2	2	10.49	0.011324197	Hypothetical protein SORBIDRAFT_01g041750 [Sorghum bicolor]
SCSBFL1108H06	4.5	1	1	5.68	0.001164142	Af466203_1 rire2 orf3
SCACCL6007B02	4.6	2	1	12.13	0.00077761	Hypothetical protein SORBIDRAFT_06g014936 [Sorghum bicolor]
SCEQLR1092H10	5.1	4	2	23.23	0.002081321	Snf1-related protein kinase regulatory subunit gamma-1-like
SCCCLR1078C05	5.3	2	1	16.74	0.015176058	Glycogenin-like protein
SCSFCL6068E04	5.3	3	2	18.06	0.000609425	Hypothetical protein SORBIDRAFT_04g010023 [Sorghum bicolor]
SCCCAD1001C08	5.6	1	1	6.60	0.001364968	Peroxidase 42 precursor
SCCCLR1072D04	6.1	2	1	10.60	0.017885796	Replication factor c subunit 2
SCCCLR1048C08	6.5	1	1	5.02	0.00130761	Zinc finger c-x8-c-x5-c-x3-h type family protein
SCVPCL6064B05	6.7	3	2	33.71	0.020884706	Oleosin 16 kda
SCEZRT3069B05	7.0	1	1	5.81	0.042375046	Arabinogalactan protein precursor
SCJFLR2036B04	7.4	8	1	73.70	0.007493408	Peptidyl-prolyl cis-trans isomerase
SCCCRZ1004C10	8.9	3	2	21.72	0.017561976	RNA recognition motif -containing protein
SCCCLB1004D05	12.1	3	1	16.18	0.005737443	NADH-cytochrome b5 reductase-like protein
SCCCCL4005F01	22.8	4	1	37.34	0.029517041	Oleosin 18 kda

Table S4 – Cont.

Accession	Max fold change	Peptide count	Unique peptides	Score	Anova (p)	Description
SCCCCL6002A08	55.8	1	1	5.49	0.017957436	Indole-3-acetic acid-amido synthetase
SCEPCL6018B07	Unique to putrescine	1	1	5.084	1.24E-07	hypothetical protein SORBIDRAFT_09g002530 [Sorghum bicolor]
SCEPLR1008F05	Unique to putrescine	4	2	44.3927	1.59E-05	deoxyuridine 5 -triphosphate nucleotidohydrolase
SCEQRT1030G05	Unique to putrescine	4	1	22.4274	0.117463672	ribose-phosphate pyrophosphokinase 4
SCJFLR1035D05	Unique to putrescine	2	1	16.8026	0.002783211	peroxidase
SCQSLR1040A11	Unique to putrescine	2	1	12.5619	0.11615097	transposon protein
SCRLFL1006D11	Unique to putrescine	1	1	5.1579	0.000318276	---NA---
SCSBSB1057C04	Unique to putrescine	1	1	5.2056	3.69E-05	hypothetical protein SORBIDRAFT_04g030120 [Sorghum bicolor]