EMBRYOGENIC COMPETENCE IN SUGARCANE: STUDY OF FACTORS AFFECTING THE LOSS AND ACQUISITION OF COMPETENCE IN EMBRYOGENIC AND NON-EMBRYOGENIC CALLUS

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"Thesis presented to Centro de Ciências e Tecnologias Agropecuárias of Universidade Estadual do Norte Fluminense Darcy Ribeiro, as part of the requirements for the degree of Doctor in Genetics and Plant Breeding"

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RESUMO

PASSAMANI, Lucas Zanchetta; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Março, 2019; Competência embriogênica em cana-de-açúcar: estudo dos fatores que afetam a perda e aquisição de competência embriogênica em calos embriogênicos e não-embriogênicos; Orientador: Prof. Dr. Vanildo Silveira; Conselheiros: Prof. Dr. Gonçalo Apolinário de Souza Filho and Prof. Drª Clícia Grativol Gaspar.

A embriogênese somática é uma ferramenta biotecnológica com alto potencial para propagação *in vitro* e regeneração de espécies que possuem interesse econômico, como a cana-de-açúcar. Neste contexto, a análise dos processos de aquisição e perda da competência embriogênica em calos de cana-de-açúcar é importante para a otimização do processo e maior integração com programas de melhoramento genético da espécie. Portanto, o objetivo deste trabalho foi analisar os processos de aquisição e perda da competência embriogênica durante a multiplicação e maturação de calos embriogênicos de cana-de-açúcar. No primeiro capítulo desta tese, os calos embriogênicos foram multiplicados, no primeiro momento, em intervalos de 21 dias, por 8 subcultivos, na presença de 10 µM de 2,4-D, e, ao final de cada ciclo, foram submetidos ao processo de maturação. Em um segundo momento, os calos embriogênicos foram multiplicados, em intervalos de 21 dias, por 6 subcultivos, na presença (S6) e ausência de 2,4-D [S6(-)], e os calos no primeiro (S1) e sexto [S6 e S6(-)] ciclos de multiplicação foram submetidos ao processo de maturação. Foram analisados a histomorfologia, histoquímica, conteúdo endógeno de poliaminas (PAs) e o perfil proteômico dos calos nos

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ABSTRACT

PASSAMANI, Lucas Zanchetta; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; March, 2019; Embryogenic competence in sugarcane: study of factors affecting the loss and acquisition of competence in embryogenic and non-embryogenic callus; Advisor: Prof. Dr. Vanildo Silveira; Committee members: Prof. Dr. Gonçalo Apolinário de Souza Filho and Prof. Drª Clícia Grativol Gaspar.

Somatic embryogenesis is a biotechnological tool with high potential for in vitro propagation and regeneration of economically interesting plants, such as sugarcane. In this context, the analysis of the processes of acquisition and loss of embryogenic competence in sugarcane callus is an important tool for the optimization of the process and integration in breeding programs of the species. Thus, the objective of this work was to develop an analysis of the acquisition and loss processes of embryogenic competence during multiplication and maturation of embryogenic callus of sugarcane. In the first chapter of this thesis, embryogenic callus were multiplied, in the first moment, at 21 days intervals, by eight subcultures, with 10 µM 2,4D, and at each cycle, they were submitted to the maturation process. In the second moment, embryogenic callus were subcultured every 21 days, by six subcultres, with (S6) and without 2,4-D [S6(-)], and callus in first (S1) and sixth [S6 and S6(-)] multiplication cycle were submitted to the maturation process. We analysed the histomorphology, histochemistry, endogenous content of polyamines (PAs) and proteomic profile of all callus in S1, S6 and S6(-) subculture. Callus cultivated for long-term with 2,4-D (S6) showed reduced embryogenic competence, while callus cultivated in the absence of 2,4-D [S6(-)] maintained high embryogenic

competence. All callus presented organized structures, however, different from that observed in S6, callus in first subculture (S1) and S6(-) presented prominent nuclei, a high nucleus/cytoplasm ratio and high content of proteins and polysaccharides. The long-term culture with 2,4-D affected the synthesis and regulation of proteins, especially of proteins important for the process of somatic embryogenesis such as, late embryogenesis abundant, chitinase, oleosins and heat shock proteins. Therefore, we demonstrate that long-term culture with 2,4-D induces a decline of embryogenic competence in sugarcane embryogenic callus through morphological and protein abundance changes. In the second chapter of this thesis, we performed a microsomal proteomics analysis and analyzed changes in extracellular H^+ flux and H^+ pump (P-H^+-ATPase, V-H^+-ATPase and H^+-PPase) activity in embryogenic and non-embryogenic callus. A total of 657 proteins were identified, 16 of which were H^+ pumps. We observed a high abundance of P-H^+-ATPase and H^+-PPase in embryogenic callus and a high H^+ influx, especially at maturation day 14 as well as higher H^+ pump activity, mainly P-H^+-ATPase and H^+-PPase activity, when compared to non-embryogenic callus. H^+-PPase appears to be the major H^+ pump in embryogenic callus during somatic embryo formation, functioning in both vacuole acidification and PPi homeostasis. These results provide evidence for an association between higher H^+ pump protein abundance and, consequently, higher H^+ flux and embryogenic competence acquisition in the callus of sugarcane, thus allowing the optimization of the somatic embryo formation process through modulation of H^+ pumps activity. Finally, results presented in this document showed news insights about loss and acquisition embryogenic competence in sugarcane callus, highlighting the possible relationship between constant exposure to 2,4-D with loss of embryogenic competence, and high H^+ influx with this competence acquisition. These results are essential for higher elucidation to somatic embryogenic process, as well to the optimization this process in sugarcane.

1. INTRODUCTION

Sugarcane is one of the main agricultural crops of the country, being economically important for Brazilian producing states, among them the state of Rio de Janeiro. In view of its economic importance, the obtainment of agronomically superior cultivars will have a direct impact on sugarcane industries and consequently on the national economy (Arruda, 2011). For this reason, sugarcane is constantly inserted in breeding programs, and in the last decades, it has been observed the achievement of cultivars from breeding programs in species that present higher productivity and resistance to biotic and abiotic stresses (de Morais et al., 2015). However, sugarcane presents high genomic complexity and lack of diversification in the genotypes that hinder the use of conventional methods of plant breeding (Singh et al., 2008). Thus, the integration of molecular techniques with conventional methods is crucial for the constant improvement of cultivars in sugarcane.

Among molecular techniques used for genetic improvement, genetic engineering has become an important alternative for the obtainment of the superior cultivars for this specie (Ming et al., 2006). In genetic engineering protocols, associated with efficient transformation protocols, it is necessary to develop efficient protocols for the regeneration of transformed cells, making tissue culture an important tool in modern breeding programs. Somatic embryogenesis is the most studied tissue culture technique in sugarcane (Lakshmanan et al., 2005), and is considered an important part of processing systems in the species (Bower e Birch,

1992). In this technique, the embryos to be produced directly or indirectly from the explant. The indirect production of the somatic embryos requires the step of dedifferentiation of the explant resulting in a little organized cell mass, called callus. In many species, the formation of two distinct types of callus (embryogenic and non-embryogenic callus), differentiated in relation to embryogenic competence, is observed, in which only embryogenic callus are able to form embryos (Namasivayam, 2007). In addition, embryogenic callus can also be used as explants for transformation, because it is a material with high transformation rate in sugarcane (Arencibia et al., 1998).

The analysis of different types of callus, allows an in-depth analysis of the main molecular mechanisms, associated with the process of acquisition of embryogenic competence (Jimenez, 2001). The identification of the main factors associated with the process of acquisition of embryogenic competence is important for the optimization through the modulation of these metabolic pathways in order to obtain a larger number of somatic embryos. Increased synthesis of sugar compounds in the gluconeogenesis, such as starch and sucrose as well as increased proteolytic activity are, among others, factors that are being associated as important for the process of somatic embryogenesis (Neves et al., 2003). However, several metabolic pathways associated with the process of acquisition of embryogenic competence remain unknown.

Embryogenic callus can be maintained at constant multiplication in the in vitro culture system, but maintenance for long periods in the multiplication phase may accumulate genetic and epigenetic variations, known as somaclonal variations (Bairu et al., 2011). Dubrovina e Kiselev (2016), have shown that long-term maintenance in in vitro culture may induce numerous molecular modifications, which promotes changes in gene expression profile. Such variations are known to cause undesirable effects, such as loss of morphogenetic competence in embryogenic callus (Fitch e Moore, 1993) and obtaintion of *off-types* clones (Landey et al., 2015). On the other hand, these variations may also be desirable, since it can be associated with increased genetic variability in plant breeding programs (Rastogi et al., 2015). Thus, it is observed that somaclonal variation presents both negative and positive effects, and both should be treated in parallel, in light of the importance of both for the development of the culture.

In recent years, studies have demonstrated the main metabolic pathways regulated in embryogenic callus during somatic embryo formation (Heringer et al., 2015; Reis et al., 2016; Heringer et al., 2017) and the main effects caused by genetic and epigenetic variations during in vitro culture (Rodriguez-Enriquez et al., 2011; Nwauzoma e Jaja, 2013). Despite the great advances in the mechanisms associated with the loss and acquisition of morphogenetic competence, the biochemical and molecular bases associated with these events remain unknown. In this context, studies in genomics, proteomics and metabolomics can provide important information regarding the molecular bases involved in such events (Al-Khayri et al., 2016). The greater understanding of these factors will contribute to elucidate how a single somatic cell has the capacity to transform into a complete organism, as well as serve as a basis for the optimization of the process of somatic embryo formation in sugarcane, aiming the application of this technique in modern programs of genetic improvement.

2. OBJECTIVES

2.1 General objective

Aiming at a better understanding of the molecular mechanisms associated with processes of acquisition and loss of embryogenic competence and allowing the optimization of the somatic embryogenesis in sugarcane, we develop an analysis in theses processes during multiplication and maturation of embryogenic callus of sugarcane,

2.2 Specific objectives

- To investigate the effect of long-term culture of sugarcane embryogenic callus, with or without 2,4-D, on the decrease in embryogenic competence.
- To analysis the effect of long-term culture of sugarcane embryogenic callus, with or without 2,4-D, on the morphological modifications and changes in PAs and proteins profile.
- To investigate the association between H^+ flux and embryogenic competence acquisition during somatic embryogenesis in sugarcane.
- To analysis a microsomal proteomics and changes in extracellular H^+ flux and H^+ pump (P-H+-ATPase, V-H+-ATPase and H+-PPase) activity during the maturation of embryogenic and non-embryogenic sugarcane callus.

3. CHAPTERS

3.1. LONG-TERM CULTURE WITH 2,4-DICHLOROPHENOXYACETIC ACID AFFECTS EMBRYOGENIC COMPETENCE IN SUGARCANE CALLUS VIA CHANGES IN STARCH, POLYAMINE AND PROTEIN PROFILES

3.1.1. INTRODUCTION

Sugarcane is an economically important crop in several countries, mainly due to the commercialization of its main products: ethanol and sugar (ISO, 2017). The high economic potential of sugarcane cultivation makes this species an important crop in breeding programs that use classic strategies, such as recurrent selection (Lingle et al., 2009), and modern strategies, such as genetic engineering (Kumar et al., 2014b), to improve agronomic traits. Although superior cultivars can be efficiently obtained by classic strategies, several limitations, such as a long durations of breeding programs and high genomic complexity, prevent these programs from achieving greater success (Singh et al., 2008).

Therefore, advances in genetic engineering have made this method an efficient tool for the breeding of different species (Wang et al., 2009; Arruda, 2012). In addition to efficient transformation protocols, the regeneration of transformed

plants is crucial to the success of the technique; therefore, advances in tissue and plant cell culture are directly associated with the success of modern breeding programs (Ijaz et al., 2012).

Among the in vitro propagation techniques for sugarcane, direct and indirect regeneration pathways are well established and are extremely important for species improvement via genetic engineering (Snyman et al., 2011). Somatic embryogenesis is one of the most well studied processes in the cultivation of sugarcane; in this process, by means of an in vitro cultivation technique, isolated cells or a small group of somatic cells give rise to embryos via a morphogenetic process that is similar to the sequence of events that occurs during zygotic embryogenesis (Tautorus et al., 1991). Somatic embryogenesis has been considered to be the best biotechnological technique for regeneration of plants derived from both particle bombardment and Agrobacterium-mediated transformation (Ming et al., 2006; Arruda, 2012).

Despite its importance, in some species, somatic embryogenesis could give rise to genetic instability of cultures (Geijskes et al., 2003), which can have several undesirable effects, such as the decreasing embryogenic competence in embryogenic callus (Fitch e Moore, 1993) and formation of off-type clones (Pandey et al., 2012).

One of the factors that may cause alterations in these cultures is the type and concentration of auxin used. 2,4-dichlorophenoxyacetic acid (2,4-D) is associated with somaclonal variations, especially when cultures are cultivated for long durations with high concentrations of auxin (Brisibe et al., 1994). High concentration or long exposure to 2,4-D can result in formation of abnormal somatic embryos due to genetic and epigenetic disorders (Garcia et al., 2019). In addition, the use of callus during propagation as well as long-term culture is among the main factors associated with this genetic instability (Pontaroli e Camadro, 2005; Bairu et al., 2011). Considering the potential of somatic embryogenesis as a model for plant development studies and a tool for the regeneration of transformed cells, an improved understanding of the molecular mechanisms associated with these variations is crucial to enable the use of these techniques in modern genetic breeding programs.

In recent years, several studies have investigated the molecular aspects associated with the process of somatic embryogenesis in sugarcane (Mahmud et

al., 2015; Kaur e Kapoor, 2016; Reis et al., 2016; Ferreira et al., 2017; Heringer et al., 2017). In particular, a comparison between embryogenic and non-embryogenic callus showed the possible important regulatory pathways for embryogenic competence acquisition. Morphological modification, changes in the endogenous content of free polyamines (PAs) and protein profiles have been highlighted as being important for the acquisition of competence in embryogenic callus of sugarcane (Silveira et al., 2013; Heringer et al., 2015; Reis et al., 2016; Passamani et al., 2018), although the association of these modifications with long-term culture with 2,4-D and the decrease in embryogenic competence in embryogenic callus of sugarcane remains unclear.

Thus, the aim of this study was to investigate the effect of long-term culture of sugarcane embryogenic callus, with or without 2,4-D, on the decrease in embryogenic competence by analysis of somatic embryo formation, morphological modifications, and changes in the PA content and proteomic profile.

3.1.2. REVIEW

3.1.2.1 Sugarcane

Sugarcane belongs to the Poacea family, from the genus Saccharum. Evidence supports the theory that the origin of sugarcane occurred in New Guinea, in which noble cultivars were domesticated and dispesed in the Pacif and South and Southeast Asia by human migrations. In addition, it is belivied that complex introgressions between *S. spontaneum* and other genera, particularly *Erianthus* and *Miscanthus* have been essential for formation of the other species of the Saccharum genre (Grivet et al., 2004). Currently, the cultivation of sugarcane extends acroos all continents, being predominant in the tropical zone where the climate is considerated ideal for the cultivation of the species.

The genus Saccharum comprises hundreds species, among them are cultivable sugarcanes: *S. barberi*, *S. edule*, *S. officinarum*, *S. robustum*, *S. sinense* and *S. spontaneum*. The main cultivars currently used are interspecific hybrids, especially from the cross between *S. spontaneum* (a vigorous wild species with

many aneuploidy forms) and *S. officinarum* (the domesticated sugar-producing species). Sugarcane presents high ploidy levels. In general, modern hybrids exhibiting a n+n constitution and are composed of 10–20% of S. spontaneum chromosomes, 5-17% of recombinant chromosomes and the remainder composed of S. officinarum chromosomes (Cheavegatti-Gianotto et al., 2011). The sugarcane plants are perennial grasses that form stools of stalks or culms. The stalk or culm consists of alternating nodes and internodes and leaves are alternate and attached to the stalk. The root system of sugarcane is composed of adventitious and permanent roots, and the ratio between root types is species-dependent. *S. spontaneum* is characterized by more supporting roots than *S. officinarum*, which may explain the greater tolerance of this species to environmental variations (Cheavegatti-Gianotto et al., 2011).

Similar to other grass family, such as *Sorghum bicolor* L. and *Zea mays* (that presents high genomic similarity with sugarcane) the cultivation of sugarcane have high economic value, mainly through the commercialization of its main products (ethanol and sugar) (ISO, 2012). Most of the sugar produced and marketed worldwide comes from the sugarcane crop, with Brazil being the largest producer, followed by China and India (FAO, 2017). In Brazil, in the 2017/2018 harvest, there was a production of 633.3 million tons, which moved more than 8 billion dollars in exports. The high production of sugarcane in Brazil is a result of a high production in the center-south region, which represents more than 92% of the total produced in the country. Within this region, the southeast presents the highest production rate, mainly due to the participation of the states of São Paulo and Minas Gerais, which represent two of the three states with the highest production in Brazil (CONAB, 2017). Rio de Janeiro, although with a less expressive participation, also contributes to the total sugarcane produced in the southeast region, being well represented by the north and northwestern region of Rio de Janeiro, specifically Campos dos Goytacazes city, which has the highest production rates of State (IBGE, 2013).

Although it has obtained high production values, the current harvest presents a production of 3.6% less when compared to the 2016/2017 harvest (CONAB, 2018). According to the Companhia Nacional do Abastecimento (CONAB), the reduction in the sugarcane production rate in the current harvest is directly associated with smallest area for sugarcane planting, which reduced 3.5% in relation to the 2016/2017 harvest (CONAB, 2018). In addition, another factor that

negatively influenced sugarcane production in the current harvest was the intense climate changes, especially variations in the levels of precipitation in the different producing states (CONAB, 2018). Abiotic stresses are among the main causes for a deficit in the sugarcane productivity, especially drought and salt stress (Andrade et al., 2015). Thus, the elucidation tolerance mechanisms would allow the development of cultivars more tolerant to drought and salinity, capable of being cultivated in several areas and consequently increasing productivity. In this context, breeding programs and tissue culture techniques are important to obtain superior cultivars to maintain a high productivity even in conditions adverse to the cultivation of the species (Jackson, 2005; Rai et al., 2011).

3.1.2.2 Genetic improvement in sugarcane

Early genetic improvement programs in sugarcane were developed in 1888 in two independent studies conducted in Indonesia and Barbados (Kennedy, 2004), and the following years were important for other countries to also initiate their programs for genetic improvement in sugarcane (Ming et al., 2006). Since the first breeding programs created, the main objectives in genetic improvement in sugarcane are associated with productivity, sugar content, fiber level and resistance to diseases (Jackson, 2005). The obtainment of superior cultivars from the breeding programs represented a 40% increase in production in the last 50 years (de Morais et al., 2015), and these cultivars were obtained from classical strategies with recurrent selection (Lingle et al., 2009), as well as modern strategies such as genetic engineering (Kumar et al., 2014b).

In the last 50 years, the acquisition of hybrids through the cross between the five principals sugarcane species (*Saccharum officinarum, Saccharum barberi, Saccharum sinense, Saccharum spontaneum* and *Saccharum robustum*) has resulted in a significant increase in both productivity and resistance to diseases in sugarcane (de Morais et al., 2015), in which they demonstrate the efficiency in the use of genetic improvement programs in sugarcane. Lingle et al. (2010), demonstrated the increase in productivity and juicy quality in sugarcane by means of the recurrent selection technique and this technique was also used successfully to increase the biomass production (Barbosa et al., 2004), sugar content (Lingle et al., 2009) and disease resistance (Zhou e Mokwele, 2016), evidencing the efficiency

of the technique for the genetic improvement of sugarcane. However, intrinsic characteristics of the species such as long period in obtaining superior cultivars, high genomic complexity and low fertility make it difficult to obtain superior cultivars by means of traditional strategies of genetic improvement (Singh et al., 2008).

Analysis carried out in classic breeding programs in sugarcane pointed to a less pronounced genetic gain in advanced cycles, due especially to low genetic diversity, and emphasizes the importance of biotechnology to the continuous productive advance of the species (Dal-Bianco et al., 2012). In this context, in the last 30 years, part of the studies aimed at achieving advances in the area of biotechnology and these advances allowed the use of these tools in modern programs of genetic improvement, minimizing the difficulties of the programs of classic genetic improvement (de Morais et al., 2015). One of the most used molecular strategies in sugarcane is genetic engineering and has been shown to be an efficient strategy for the species (Wang et al., 2009; Arruda, 2012). According to Ming et al. (2006) sugarcane is an ideal candidate for the genetic engineering strategy, since there are efficient transformation systems for the species and the superior cultivars obtained through transformation can be maintained indefinitely through vegetative propagation. The efficiency of the technique for sugarcane was demonstrated by the development, by the Centro de Tecnologia Canavieira (CTC), of the first genetically modified sugarcane approved for commercial use (Unica, 2017). This transgenic sugarcane was developed aiming at greater resistance to biotic stress, however, genetic engineering is used in the species with several objectives such as herbicide resistance, disease and insect resistance, drought tolerance, increased sucrose accumulation, and delayed flowering time (Hotta et al., 2010).

The development of genetically modified sugarcane is entirely dependent on advances in the areas of physiology and molecular biology for the elucidation of important new functions of genes. However, one of the main limitations in the generation of new transgenic is the silencing and/or inconsistency of expression of the genes inserted in the new cultivars (Mudge et al., 2009), therefore, advances in the techniques of transformation are crucial for a greater success of genetic engineering in sugarcane. Although microparticle bombardment is the most commonly used technique in sugarcane (Birch, 1997), the development of other techniques is important for the continued success in the efficiency of genetic

transformation. Compared with other techniques, the transformation by *Agrobacterium tumefaciens* presents several advantages such as appropriate manipulation, selection of the best age of the callus, type and stage of embryogenic culture besides presenting lower cost (Hotta et al., 2010). In recent years, many studies have sought to optimize the main methods of transformation in sugarcane, especially via *A. tumefaciens* (Kumar et al., 2014a; Mayavan et al., 2015). In addition, advances in more modern genome-editing techniques such as zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs) and especially CRISPR/Cas9, concomitantly with advances in bioinformatics will be crucial for the improvement of culture in the next years (Mohan, 2016). Kandel et al. (2018), report that the use of the CRISPR / Cas9 technique in sugarcane is valuable in the production of superior cultivars due to the high complexity of the genome of the species. Associated with to efficient transformation protocols, the regeneration of transformed plants is crucial to the success of the technique, so advancement in tissue culture and plant cells is directly associated with the success of modern breeding programs (Ijaz et al., 2012). Arencibia et al. (1999) revealed that somaclonal variation induced in in vitro culture is a bottleneck in the development of genetically modified sugarcane. Thus, greater knowledge and optimization of plant cell and tissue culture techniques is fundamental to increase the efficiency of genetic engineering in the species.

3.1.2.3 Somatic Embryogenesis

Plant cell and tissue culture is the science in which it is intended to isolate organ, tissues or cells from the parent plant and cultivate them in artificial medium under controlled and pathogen free conditions (George et al., 2008). The techniques of plant cell and tissue culture are based on works conducted in the early century XX, especially the work conducted by Haberlandt in 1902 which coined the term totipotentiality (Vasil, 2008). The totipotentiality refers to that each plant cell presents the genetic competence to reproduce an entire organism, being this the basic principle associated with the current techniques of plant cell culture (Krikorian e Berquam, 1969). Based on these theories, the main techniques of plant cell and tissue culture were only developed in the middle of the 60's and presented a great scientific advance in the years 70 and 80 (Smith, 2013). Similar to that presented

for other species, the importance of plant cell culture for sugarcane also began in the mid-1960s through in studies development by Nickell (1964), Barba e Nickell (1969) and Heinz e Mee (1969). The recent advances in methods of in vitro culture of sugarcane had a great impact in both basic research and commercial interest. In particular the applications in micropropagation of superior clones, production of pathogen-free plants, generation of somaclones with superior agronomic characteristics, screening for tolerance to biotic and abiotic stresses, and conservation of germplasm (Suprasanna et al., 2011).

Techniques for the in vitro propagation of sugarcane both through direct regeneration pathways and indirect regeneration pathways are well established and are important in various efforts to improve species through genetic engineering (Snyman et al., 2011). According to Lakshmanan et al. (2005), among the techniques of in vitro cultivation, somatic embryogenesis is the most studied and important in the cultivation of sugarcane, being used in methods of genetic transformation and obtaining a large number of commercial cultivars.

Somatic embryogenesis is a process in which, by means of the in vitro culture technique, isolated cells or a small group of somatic cells give origin to embryos, in a morphogenetic process that approaches the sequence of events representative of zygotic embryogenesis (Tautorus et al., 1991). Somatic embryos can be obtained either directly or indirectly. In the direct embryogenesis pathway, the somatic embryo is formed directly on the surface of the explant used, while of indirect embryogenesis, the explant is subjected to a dedifferentiation process in which it will give rise to a little organized cellular mass, called callus, and subsequently, the somatic embryos can be obtained from the maturation of the embryogenic callus produced (Suprasanna et al., 2011). Recent studies have shown that both ways of obtaining somatic embryos are used successfully in species such as *Gossypium hirsutum* L. (Yang et al., 2014), *Musa accuminata* (Jimtha et al., 2014) and *Carica papaya* L. (Vale et al., 2014). In sugarcane, the first protocols of embryogenesis were developed between 1974 and 1979 (Liu e Chen, 1974; Nadar e Heinz, 1977; Zeng, 1979) and currently the process of somatic embryogenesis in the species presents satisfactory results (Lakshmanan, 2006).

Steiner et al. (2008) demonstrated that the process of somatic embryogenesis consists of two main cycles. Initially, the explant from the parent plant is introduced in vitro to form the embryogenic cultures. These cultures can be

maintained for long periods in the multiplication phase, or they can be directed to the stage of maturation, in which the somatic embryos are produced. The transition between phases requires physical and chemical signals such as the removal of auxin from the culture medium, presence of light and addition of maturation promoters in the culture medium. After obtaining the somatic embryos, these can be germinated and established ex vitro. In particular for sugarcane, somatic embryogenesis protocols include four main step: Induction, multiplication, maturation and conversion phases (Heringer et al., 2018).

Associated with different transformation methods, such as biobalistics and electroporation, indirect somatic embryogenesis was widely used as a method of regeneration of transformed cells (Ming et al., 2006). Taparia et al. (2012), demonstrated that a higher transformation efficiency is obtained when indirect somatic embryogenesis is used in comparison with direct somatic embryogenesis. However, due to the genetic instability of cultures during indirect somatic embryogenesis, the technique was replaced by other techniques, such as the direct production of adventitious shoots (Geijskes et al., 2003) which reduces the incidence of genetic and epigenetic variations observed during indirect somatic embryogenesis. The genetic instability in plants obtained through indirect somatic embryogenesis was demonstrated in a study developed by Basnayake et al. (2011), in which it was associated with the long time to obtain the seedlings as the main factor for the accumulation of somaclonal variations in the cultures. Considering the efficiency of somatic embryogenesis as a tool for the regeneration of transformed cells, a better understanding of the molecular mechanisms associated with these variations is crucial to make the use of techniques viable in modern breeding programs viable.

3.1.2.4 Changes in embryogenic competence

The in vitro culture system may induce genetic and epigenetic variations between plant cells or tissues that may result in loss of embryogenic competence. Genetic variations commonly found in tissue culture, such as polyploidy, aneuploidy, (point) mutations, and new insertions of transposons can occur randomly. In contrast, epigenetic changes are caused by changes in the expression of the information in the DNA brought about by alterations in DNA methylation, in histones,

or in both, in which can influence gene transcription (Smulders e Klerk, 2011). These variations may be spontaneous or induced and may be termed somaclonal variations (Larkin e Scowcroft, 1981; Yang et al., 2010), in which are common to many vegetatively propagated crops, generating the production of *off-types* and in some cases genetic variability in these species (Bairu et al., 2011).

Among the types of in vitro culture, the use of callus culture is known to increase the incidence of somaclonal variations in comparison to direct sprouting of the shoots and embryos (Bairu et al., 2011). Studies conducted at *Rosa hybrida* L. (Arene et al., 1993), *Titanotrichum oldhamii* (Takagi et al., 2011) and *Clivia miniata* (Wang et al., 2012), have shown that seedlings obtained by indirect organogenesis show more variations than plantless obtained by direct organogenesis. Lee e Phillips (1988), report that the processes of dedifferentiation and redifferentiation that are conducted in indirect organogenesis can induce quantitative and qualitative changes in the genome and consequently increase the frequency of somaclonal variations. In addition, the poor organization of the cells observed in the callus is also seen as an important point for increasing variations in these tissues (Skirvin, 1978). Along with these factors, the type and concentration of growth regulators and number of subcultures are constantly associated with the emergence of somaclonal variations in cultures (Bairu et al., 2011).

Constant exposure to the chemicals present in the culture medium is primarily responsible for the high rate of somaclonal variations in the in vitro culture system (Skirvin et al., 1994). Several studies have shown that the application of high concentration of cytokinin and auxin during in vitro development may provide an increase in the number of somaclonal variations (LoSchiavo et al., 1989; Giménez et al., 2001; Roels et al., 2005; Siragusa et al., 2007; Mohanty et al., 2008). Especially with the use of synthetic auxin 2,4-D, commonly used to induce callus, the appearance of somaclonal variations is frequent (Mohanty et al., 2008). Ahmed et al. (2004) revealed that the use of 2,4-D promotes a higher number of somaclonal variations in cultures than when using other auxins such as NAA and IBA, suggesting that exposure to high concentration or long-term exposure to 2,4-D may induce the emergence of crop variations. Although the presence of plant growth regulators has been consistently associated with the emergence of somaclonal variations, this effect is species-dependent and some species do not exhibit somaclonal variations in the presence of these compounds (Bairu et al., 2011).

In addition, studies have shown that the maintenance of in vitro culture for long periods also stimulates a greater accumulation of somaclonal variations in different species, and may lead to the reduction of embryogenic competence (Fitch e Moore, 1993; Pontaroli e Camadro, 2005). During in vitro multiplication, the high multiplication rate can induce somaclonal variations (Bairu et al., 2011), providing the genetic instability of these cultures. Rodrigues et al. (1997), showed that somaclonal variants appeared from the fifth subculture, and similar results was found with progressive subculture of micropropagated bananas (Bairu et al., 2006). Based on the results found in different species, a model was proposed in which two main conclusions were discussed: that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and secondly, after a given number of multiplication cycles, variable off-type percentages can be expected (Côte et al., 2001). However, similar to that observed for the effect of growth regulators, the effect of long-term of culture is also a species-dependent and some species such as *Pisum sativum* L (Smýkal et al., 2007) and *Foeniculum vulgare* Mill (Bennici et al., 2004) do not show somaclonal variations when maintained for long periods in in vitro culture.

The presence of somaclonal variations during in vitro culture has been described in several species including *Arabidopsis thaliana* (Polanco e Ruiz, 2002), *Asparagus officinalis* (Pontaroli e Camadro, 2005), *Saccharum* ssp. (Singh et al., 2008) and *Coffea arabica* (Landey et al., 2015). Different from that observed in other species (Wei et al., 2016), in sugarcane, somaclonal variations may cause several effects such as the loss of morphogenetic competence in embryogenic callus (Fitch e Moore, 1993) and also in the formation of *off-types* somaclones (Pandey et al., 2012). These effects make it difficult to use in vitro culture techniques, such as somatic embryogenesis, in the methods of genetic transformation, since in these methods the formation and multiplication of the transformed plants is objective, without undesirable variations occurring.

Based on all the effects generated by somaclonal variations, it is observed that these variations are directly associated with breeding programs. Recent studies have shown that the main effects associated with somaclonal variations are related to morphological alterations, alterations in chromosome structure and number and in the change in the overall methylation profile (Rodriguez-Enriquez et al., 2011; Landey et al., 2015). However, the biochemical and molecular bases remain

unknown and are important to make this strategy viable. In this sense, a better understanding of the biochemical and molecular bases associated with these events becomes crucial for the best use of this tool in breeding programs.

3.1.2.5 Morphological and biochemical analysis

Molecular breeding and plant biotechonology are potential alternatives for used in association with classical breeding techniques aimed to obtain agronomically superior cultivars (Al-Khayri et al., 2016). The progress in these different areas of studies, allow a breakthrough in knowledge about molecular genetics, which can be applied directly to different breeding programs. Among the approaches carried out, morphological analysis, proteomic analysis and endogenous polyamines contents may be important to increase the understanding of the events related to tissue culture and plant cell techniques, and consequently to help in the development of new plant breeding programs (Silveira et al., 2013; Reis et al., 2016; Heringer et al., 2018).

Morphological aspects are known to be directly associated with the acquisition of embryogenic competence in sugarcane callus (Rodriguez et al., 1996). Silveira et al. (2013), demonstrated that embryogenic cells are characterized by being small and isodiametric containing prominent nuclei and dense cytoplasm, while non-embryogenic cells are highly vacuolated and elongated. In addition, long-term of culture induces morphological changes in which affects the somatic embryo formation (Breton et al., 2006; Fu et al., 2012) suggesting that morphological changes in embryogenic cells may be associated with reduced embryogenic competence.

The proteomics analysis appear as important tools to elucidate changes in the protein profile resulting from the process of loss and gain of morphogenetic competence. These alterations can be directly associated to morphological changes and be an important tool in the obtaining of superior cultivars within the programs of genetic improvement of plants (Bairu et al., 2011). In addition, Al-Khayri et al. (2016) reveal that proteomic analysis can contribute to the understanding of molecular mechanisms that are essential for plant growth and development as well as interactions with the environment and has been rapidly adopted in the last decade by genetic breeding programs. Especially associated with the technique of somatic

embryogenesis in sugarcane, recent studies reveal the main groups of proteins regulated during the process of formation of the somatic embryo (Heringer et al., 2015; Reis et al., 2016; Heringer et al., 2017) suggesting possible important proteins in the regulation of somatic embryogenesis and emphasizing the importance of the use of this strategy to elucidate the biochemical and molecular bases associated to the process.

Finally, the analysis of the endogenous content of PAs can also be an important tool used to determine embryogenic competence in callus during in vitro culture. These molecules are known to regulate various physiological processes in plants such as cell division and differentiation, embryogenesis, seed germination, responses to abiotic and biotic stresses, among others (Santa-Catarina et al., 2007; Dias et al., 2009; Vuosku et al., 2012; Grzesiak et al., 2013). In particular, a recent study suggests PAs as molecules modulating somatic embryogenesis in sugarcane (Reis et al., 2016). Based on this, the alteration in the metabolism of PAs can be an important effect associated with somaclonal variations during somatic embryogenesis in sugarcane, which alteration in metabolism may contribute to the loss of morphogenetic competence in embryogenic callus.

3.1.3. MATERIALS AND METHODS

3.1.3.1. Plant material and growth conditions

Sugarcane variety SP803280 was used for callus induction as described by Silveira et al. (2013), with modifications. Briefly, immature nodal cane segments were grown in the commercial substrate PlantMax (DDL Agroindustria Paulínia, São Paulo, Brazil) for 60 days under environmental conditions. Then, plants that were approximately 45 cm high were selected for callus induction. After removal of the mature leaves, the resulting leaf rolls were surface sterilized in 70 % ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 1 min, immersed in 30 % commercial bleach (2.25 % sodium hypochlorite) for 30 min and subsequently washed 3 times with sterilized water. Leaf rolls were transversely sectioned into approximately 3-mm-thick slices and cultured in test tubes (150 × 25 mm) containing 10 mL of MS culture medium

(Phytotechnology Lab, Overland Park, KS, USA) (Murashige e Skoog, 1962) supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel® (Sigma-Aldrich) and 10 µM 2,4-D (Sigma-Aldrich). The pH was adjusted to 5.8, and the culture medium was sterilized by autoclaving at 121 °C for 15 min. The tubes with explants were maintained in the dark at 25 ± 1 °C for 45 days. Embryogenic callus were separated from induced callus based on morphological characteristics (Silveira et al., 2013) and transferred to Petri dishes (90 × 15 mm) containing 20 mL of the MS culture medium supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel®, and 10 µM 2,4-D. Embryogenic callus were kept in the dark at 25 ± 1 °C and subcultured every 21 days in a total of eight subcultures. To evaluate the embryogenic competence, a piece of callus was submitted to maturation in each subculture up to the eighth subculture (S8) (Figure 1).

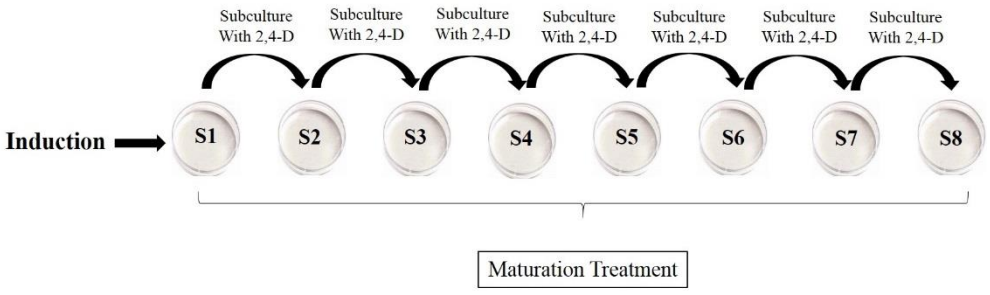

Figure 3. Experimental workflow used to evaluate the effect of long-term culture on the presence of 2,4-D in embryogenic callus of sugarcane.

Second, the induced embryogenic callus (S1) was cultivated in 20 mL of MS culture medium supplemented with 20 g L^{-1} sucrose and 2 g L^{-1} Phytagel® with (S6) and without [S6(-)] 2,4-D for six subcultures under the same conditions as those previously described. The embryogenic callus S1, S6 and S6(-) were subjected to maturation to evaluate embryogenic competence, and a piece of each callus was collected for morphological and biochemical analysis and stored at -80 °C (Figure 2).

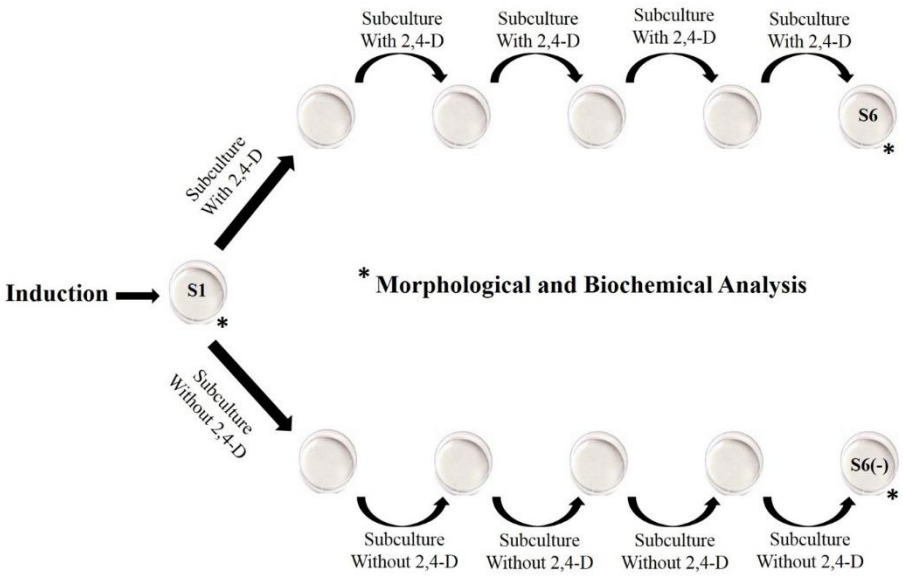

Figure 4. Experimental workflow used to evaluate the effect of long-term culture on the presence and absence of 2,4-D in embryogenic callus of sugarcane.

3.1.3.2. Callus maturation in different subcultures

Maturation of embryogenic callus was carried out as described by Silveira et al. (2013). Briefly, three embryogenic callus colonies containing 250 mg of fresh mass (FM) were inoculated into Petri dishes (90 × 15 mm) containing MS culture medium supplemented with 30 g L^{-1} sucrose, 2 g L^{-1} Phytagel® and 1.5 g L^{-1} activated charcoal (Sigma-Aldrich). For each treatment, three Petri dishes were used, each containing three embryogenic callus, totaling nine callus per treatment. The pH was adjusted to 5.8, and the culture medium was sterilized by autoclaving at 121 °C for 15 min. After inoculation, the cultures were maintained in a growth chamber at 25 ± 1 °C in the dark for the first 7 days. Thereafter, photoperiods of 16 h with a GreenPower TLED 20W light (W_mB) (Koninklijke Philips Electronics NV, Netherlands) (55 µmol m^2 s^{-1}), emitting white and medium blue light (12-14 % blue), were used for 21 days (Heringer et al., 2017). On the 28th day, the number of somatic embryos formed in each subculture was evaluated.

3.1.3.3. Histomorphological and histochemical analysis

Histomorphological analysis was performed according to the methodology described by Silveira et al. (2013). The callus samples from the different subcultures

were fixed in aqueous solution containing 2.5 % glutaraldehyde (Merck, Darmstadt, Germany) and 4.0 % formaldehyde (Merck) diluted in 0.1 M sodium cacodylate buffer (pH 7.2) (Merck) at room temperature for 24 h. Subsequently, the samples were washed for 45 min in 0.1 M sodium cacodylate buffer (pH 7.2) and subjected to an increasing ethanol series (30, 50, 70, 90 and 100 %), with 1 h for each step. The samples were then infiltrated with Historesin (Leica Biosystems, Wetzlar, Germany) and 100 % ethanol (1:1; v/v) for 12 h, followed by 100 % resin for 24 h. Then, 5-µm-thick slices were obtained on a microtome (Leica Biosystems).

For histomorphological analysis, samples were stained with 1.0 % aqueous toluidine blue solution (Sigma-Aldrich). For histochemical analysis, samples were stained with Coomassie brilliant blue R-250 (CBB; GE Healthcare, Freiburg, Germany) for proteins and with periodic acid-Schiff (PAS) for polysaccharides (Gahan, 1984). Samples were analyzed using an Axioplan ZEISS (Carl Zeiss, Jena, Germany) optical microscope equipped with an AxioCam MRc5 digital camera for image capture and AxioVisionLE software version 4.8 (Carl Zeiss) for image analysis.

3.1.3.4. Free polyamine (PA) determination

Free PA determination was performed using embryogenic callus in the first subculture (S1) and after six subcultures with 2,4-D (S6) and without 2,4-D [S6(-)]. Extraction of the PAs putrescine (Put), spermidine (Spm) and spermine (Spd) was performed as described by Silveira et al. (2004). For extraction, four samples (300 mg FM each) were homogenized with 1.2 mL of 5 % (v/v) perchloric acid (Merck Millipore, Tullagreen, Co. Cork, Ireland) at 4 °C. After 1 h of incubation at 4 °C, samples were centrifuged at 16,000 × g for 20 min, and the supernatants were collected. The remaining pellets were re-extracted in 0.1 mL of 5 % (v/v) perchloric acid and centrifuged used the same conditions as those previously described. The supernatants were collected, and both were mixed and stored at -80 °C.

Free PA levels were determined by supernatant derivation with dansyl chloride (Merck) and HPLC analysis. For identification and quantification, a fluorescence detector was used at 340 nm (excitation) and 510 nm (emission). For the stationary phase, a 5-µm reverse-phase column (Shimadzu Shim-pack CLC ODS) was used. The column temperature was maintained at 40 °C.

The mobile phase consisted of 10 % acetonitrile (Merck) in water (pH 3.5) (solvent A) and absolute acetonitrile (solvent B). The programmed gradient for absolute acetonitrile was as follows: 60 % for the first 13 min, from 60 to 100 % between min 13 and 16, 100 % between min 16 and 24, and 60 % till min 30. The flow rate was 1 mL min^{-1}, and the injected volume was 10 µL. The areas and retention times of Put, Spd and Spm (Sigma-Aldrich) were evaluated by comparison with the respective PA standards.

3.1.3.5. Proteomic analysis

3.1.3.5.1 Extraction and quantification of proteins

Proteomic analysis was performed on the embryogenic callus from the first subculture (S1) and after six subcultures with 2,4-D (S6) and without 2,4-D [S6(-)]. Total proteins were extracted following the methodology described by Balbuena et al. (2009). Samples (300 mg FM each, in triplicate) were homogenized in liquid N_2 and placed in a 2 mL microtube, and then, 1 mL of urea/thiourea extraction buffer (7 M urea and 2 M thiourea) (GE Healthcare) containing 1 % dithiothreitol (GE Healthcare), 2 % Triton X-100 (GE Healthcare), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) was added, and the samples were vortexed for 5 min at room temperature. Samples were centrifuged at 12,000 × *g* for 10 min. The supernatant was then collected and stored at -20 °C. Protein concentrations were determined using the 2D-Quant Kit (GE Healthcare) according to the manufacturer's instructions.

3.1.3.5.2 Protein digestion

Total protein samples were prepared as described by Reis et al. (2016), with modifications. Briefly, 100 µg of total protein from each sample was desalted on Amicon Ultra 0.5−3 kD centrifugal filters (Merck Millipore, Darmstadt, Germany) using 50 mM ammonium bicarbonate (pH 8.5; Sigma-Aldrich) as the buffer. For protein digestion, 25 µL of 0.2 % (v/v) RapiGest® (Waters, Milford, CT, USA) was added, and the samples were briefly vortexed and incubated in an Eppendorf Thermomixer® at 80 °C for 15 min. Then, 2.5 µL of 100 mM DTT (GE Healthcare, Piscataway, NJ, USA) was added, and the tubes were briefly vortexed and

incubated at 60 °C for 30 min with stirring. Next, 2.5 µL of 300 mM iodoacetamide (GE Healthcare) was added, and the samples were incubated in the dark for 30 min at room temperature. The iodoacetamide was quenched by adding 2.5 µL of 100 mM DTT (GE Healthcare) to each sample, followed by incubation at 37 °C for 30 min. Digestion was performed by addition of 20 µL of trypsin solution (50 ng mL^{-1}) (V5111, Promega, Madison, WI, USA) followed by overnight incubation at 37 °C with agitation. Subsequently, for RapiGest® precipitation, 10 µL of 5 % (v/v) trifluoroacetic acid was added, and the samples were incubated at 37 °C for 90 min, followed by centrifugation for 30 min at 15,000 × *g*. The samples were then transferred to total recovery vials (Waters).

3.1.3.5.3 Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for ESI-LC-MS/MS analysis. During separation, samples were loaded onto the nanoAcquity UPLC 5-µm C18 trap column (180 µm × 20 mm) at 5 µL min^{-1} for 3 min and then onto a nanoAcquity HSS T3 1.8-µm analytical reverse-phase column (75 µm × 150 mm) at 400 nL min^{-1}. The column temperature was 40 °C. For peptide elution, a binary gradient was used: mobile phase A consisted of water (Tedia, Fairfield, OH, USA) and 0.1 % formic acid (Sigma-Aldrich), and mobile phase B consisted of acetonitrile (Sigma-Aldrich) and 0.1 % formic acid. Gradient elution started at 5 % B and was then ramped up from 5 % B to 40 % B over 91.12 min and from 40 % B to 99 % B until min 95.12, maintained at 99 % until min 99.12, decreased to 5 % B until min 101.12 and maintained at 5 % B until the end of experiment at 117.00 min. Mass spectrometry was performed in the positive and resolution mode (V mode) with a resolution of 35000 FWHM with ion mobility and in the data-independent acquisition mode. The IMS wave velocity was set to 600 m s^{-1}; the transfer collision energy was ramped from 19 to 45 V in high-energy mode; the cone and capillary voltages were 30 and 2,800 V, respectively; and the source temperature was 70 °C. The nanoflow gas was set to 0.50 Bar, and the purge gas flow ranged from 145 to 150 L h^{-1}. The TOF parameters included a scan time of 0.5 s in the continuum mode and a mass range of 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol $µL^{-1}$

was used as an external calibrant, and lock mass acquisition was performed every 30 s.

3.1.3.5.4 Proteomics data analysis

Spectral processing and database searching were performed using the ProteinLynx global server (PLGS; version 3.0.2) (Waters) and ISOQuant workflow software (Distler et al., 2014; Distler et al., 2016). The PLGS data were processed using a low-energy threshold of 150 (counts), an elevated energy threshold of 50, and an intensity threshold of 750. In addition, the analysis was performed using the following parameters: two missed cleavages, 3 minimum fragment ions per peptide, 7 minimum fragment ions per protein, 2 minimum peptides per protein, fixed modifications of carbamidomethyl and variable modifications of oxidation and phosphoryl. The false discovery rate (FDR) for peptide and protein identification was set to a maximum of 1 %, with a minimum peptide length of six amino acids. The proteomic data were processed against the *Saccharum spontaneum* database (Zhang et al., 2018). Comparative label-free quantification analysis was performed using ISOQuant software with previously described settings and algorithms (Distler et al., 2014; Distler et al., 2016). Briefly, the analysis included retention time alignment, exact mass retention time (EMRT) and IMS clustering, as well as data normalization and protein homology filtering. The FDR within ISOQuant was set to 1 %, with a peptide score greater than six. Only proteins identified by at least two peptides with a minimum length of six amino acids were considered for label-free quantitation using the TOP3 approach. For comparative proteomics, after ISOQuant data analyses, only the proteins that were present or absent (for uniques) in all 3 biological replicates were considered for differential accumulation analysis. Data were analyzed using Student's t-test (two tailed). Proteins with $P < 0.05$ were considered up-accumulated if the $\log_2$ fold change (FC) was greater than 0.6 and down-accumulated if the $\log_2$ FC was less than -0.6.

3.1.3.6. Statistical analysis

The experiment was arranged in a completely randomized design with embeyogenic callus, 3 subcultures [S1, S6 and S6(-)] for analysis. For somatic embryo formation analysis nine callus was used for subculture, in which each callus

was considered experimental unit. For biochemical analysis, 3 biological repetitions (3 Petri dishes of each callus type per subculture) was used. The biochemical analyses were statistically analyzed by analysis of variance (ANOVA) followed by the SNK test at ($P < 0.05$) using the Assistat program (e Silva e de Azevedo, 2016).

3.1.4. RESULTS

3.1.4.1. Maturation of callus in different subcultures

Every 21 days, subcultured callus were subjected to a maturation process, and the number of somatic embryos formed was determined. For callus maintained with 2,4-D from the first (S1) to eighth subculture (S8) during multiplication, embryogenic competence did not change until subculture 4 (S4), and more than 20 somatic embryos were observed per callus (Figure 1). However, from subculture 5 (S5), the embryogenic competence decreased significantly, especially from subculture 6 (S6) to S8, which exhibited less than 5 somatic embryos per callus (Figure 1).

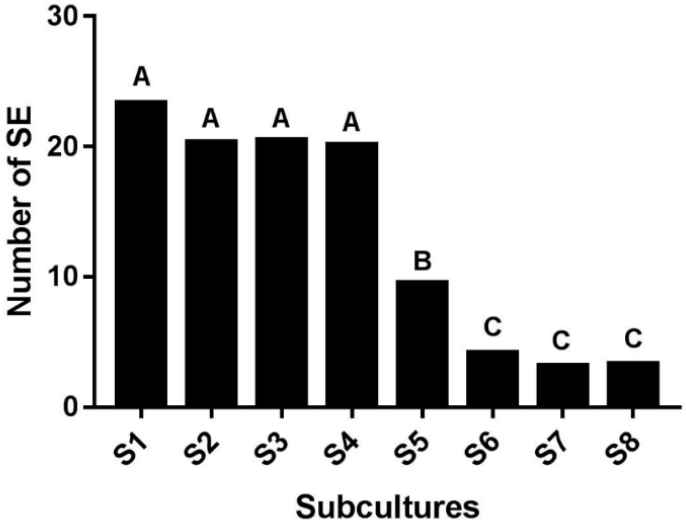

Figure 3. Number of somatic embryos (SE) formed by callus in the different subcultures in the presence of 2,4-D after 28 days of maturation. Means followed by different letters are significantly different (P<0.05) according to the Student-Newman-Keuls test. S1-S8 are callus from the first to eighth subculture with 2,4-D. (n=9, coefficient of variation = 19.35 %).

In contrast, the callus at the sixth subculture without 2,4-D [S6(-)] presented the same number of somatic embryos per callus as callus S1 but presented a higher number of somatic embryos per callus than callus S6 with 2,4-D (Figure 4). After 28 days of maturation, somatic embryo formation could be observed by the emergence of green points in the callus, which were more evident in callus S1 and S6(-) than in S6 (Figure 5).

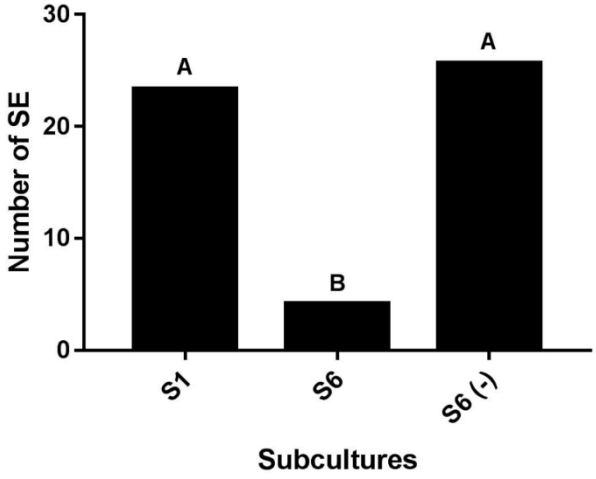

Figure 4. Number of somatic embryos (SE) formed by callus in the different subcultures in the presence of 2,4-D (S1 and S6) or absence of 2,4-D [S6(-)] after 28 days of maturation. Means followed by different letters are significantly different ($P<0.05$) according to the Student-Newman-Keuls test. S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D (n=9, coefficient of variation = 15.10 %)

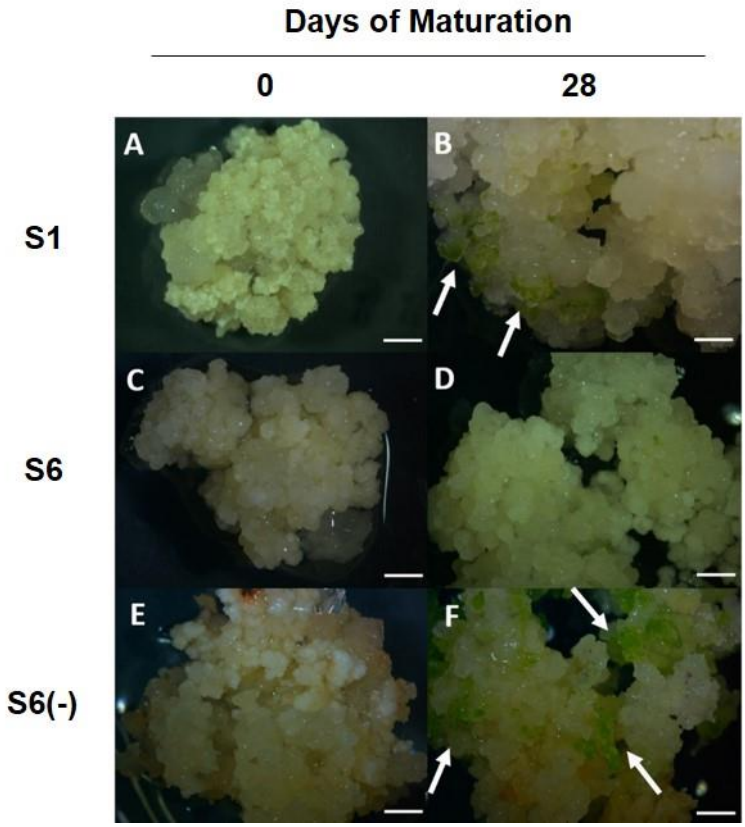

Figure 5. Morphological features of callus during the process of somatic embryo formation. Callus from S1 on days 0 (A) and 28 (B) of maturation. Callus from S6 on days 0 (C) and 28 (D) of maturation. Callus S6(-) on days 0 (E) and 28 (F) of maturation. White arrows indicate somatic embryos (SE). S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D. Bars A and C = 10 mm. Bars B, D, E, F = 20 mm.

3.1.4.2. Histomorphological and histochemical changes

Histomorphological analysis showed that all callus presented organized structures on the surface, in which rounded and compact cells were observed (Figure 6). Callus S1 had small and isodiametric clumps of cells containing prominent nuclei and a dense cytoplasm (Figure 6A). However, the callus from the 6th subculture (S6) presented smaller nuclei, a less dense cytoplasm, and more vacuolated cells than the callus from the first subculture (S1), demonstrating differences in cell characteristics with successive subcultivation. These differences could be associated with the loss of embryogenic competence in the callus from S6 (Figure 6B). In contrast, callus S6(-) showed a morphological pattern similar to that observed for callus S1, presenting cells with prominent nuclei and a dense

cytoplasm, without or with small vacuoles, which is characteristic of meristematic cells (Figure 6C).

Histochemical analysis indicated the presence of reserve compounds in callus of different subcultures. Protein bodies were detected by positive reaction to CBB (Figures 4D-F). There were relatively few protein bodies in callus S6 (Figure 6E). In contrast, callus S6(-) presented the highest number of protein bodies, which were larger than those observed in the callus of other subcultures (Figure 6F). Polysaccharides were detected by positive reaction to PAS (Figures 4G-I). The pattern of polysaccharide formation in callus of different subcultures was similar to that observed for proteins. Callus S1 presents the formation of small starch grains in the cells (Figure 6G). No starch grains were identified in cells from callus S6 (Figure 6H). However, callus S6(-) presented a positive reaction to PAS, showing the highest number of starch grains, which were larger than those observed in the callus of other subcultures (Figure 6I). In addition, decreasing trends for protein and starch concentrations were observed in callus with successive subcultivation in the presence of 2,4-D, while callus that were subjected to long-term culture without 2,4-D [S6(-)] showed high accumulation of proteins and starch (Figure 7).

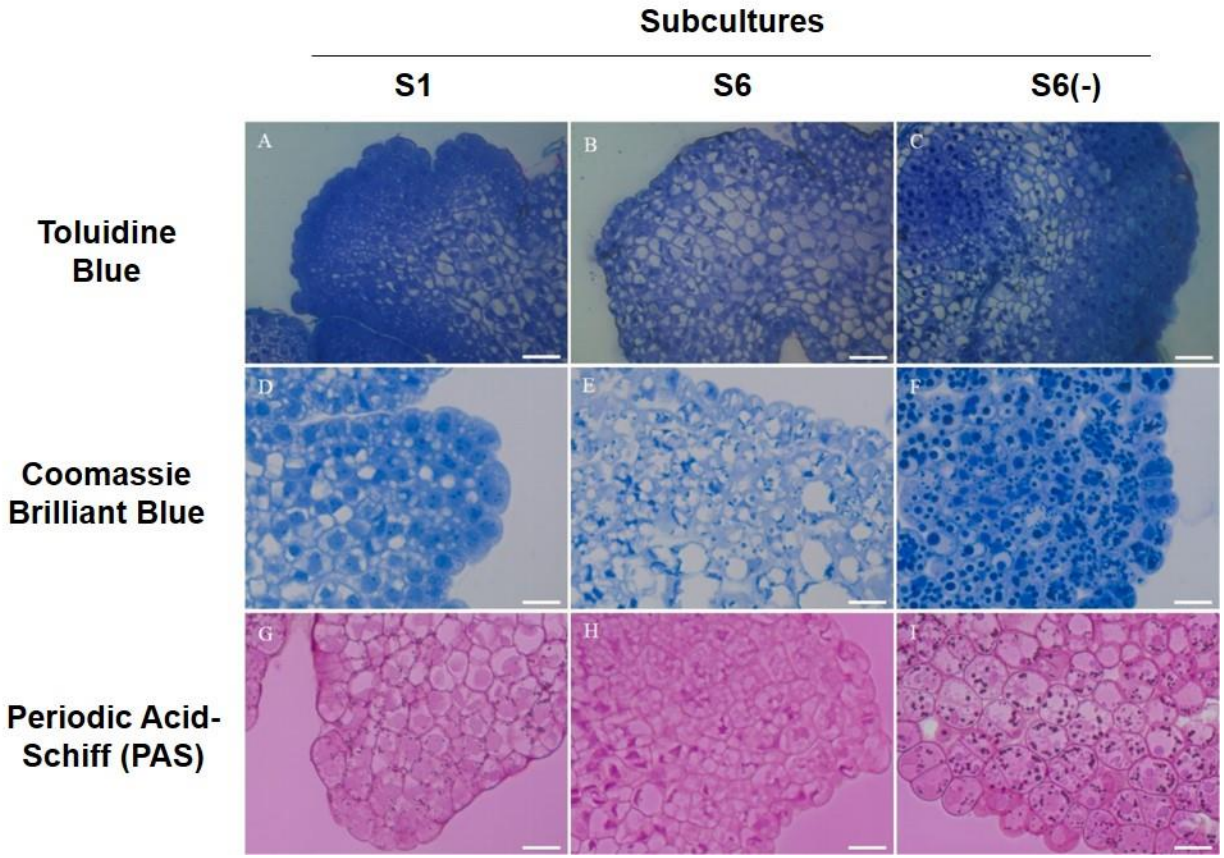

Figura 6. Histomorphological and histochemical aspects of cells from callus in subcultures S1, S6 and S6(-). Histological sections stained with toluidine blue (A-C), Coomassie brilliant blue (CBB) (D-F) and periodic acid-Schiff (PAS) (G-I). S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D. Bars A-D = 50 µm; Bars D-I = 20 µm.

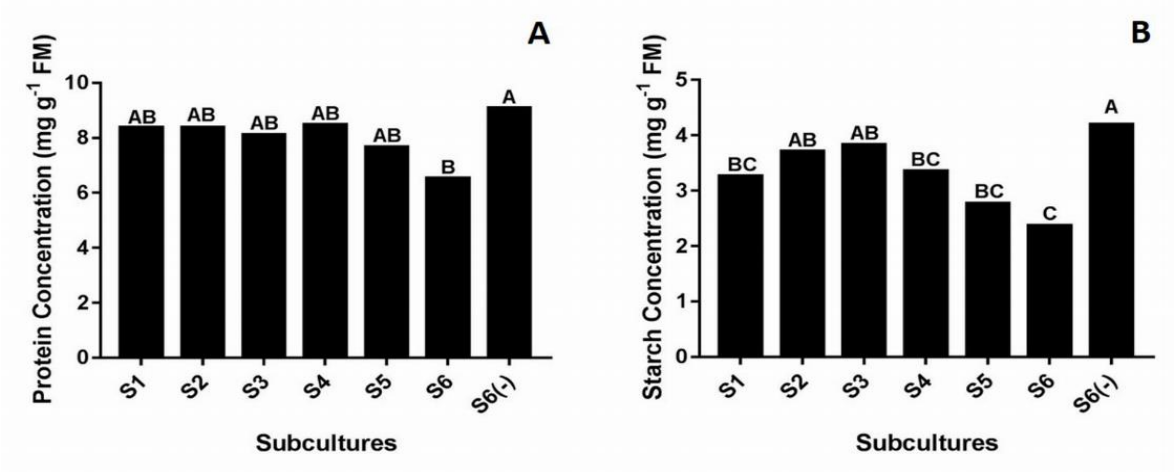

Figure 7. Protein (A) and starch (B) concentrations in callus in the different subcultures with or without 2,4-D. Means followed by different letters are significantly different (P<0.05) according to the SNK test. S1-S6 are callus from the first to sixth subculture with 2,4-D, and S6(-) is the callus from the sixth subculture without 2,4-D. Coefficient of variation = CV (n = 3; $CV_{Protein}$= 10.54 %, CV_{starch}= 10.19 %).

3.1.4.3. Alterations on PAs contents

The endogenous PA content was analyzed in callus from the S1, S6 and S6(-) treatments. The total free PA levels were affected by long-term culture; the callus from S1 showed higher total free PA levels that those from S6 with and without [S6(-)] 2,4-D (Figure 8A). In addition, the callus from the sixth subculture without 2,4-D [S6(-)] showed lower total free PA levels than the callus cultivated with 2,4-D (S6). The levels of Put, Spd and Spm were also altered in different subcultures (Figure 8). Long-term culture significantly affected the levels of Put and Spd, in which the callus from the S1 treatment showed high levels of Put and Spd, while the callus from S6 and S6(-) showed low levels of both PAs (Figures 6B and 6C). In contrast to the other PAs, the level of Spm was not significantly different in the callus from both S1 and S6 (Figure 8D). Callus S6(-) showed lower levels of all the PAs than the callus from other subcultures (Figure 8).

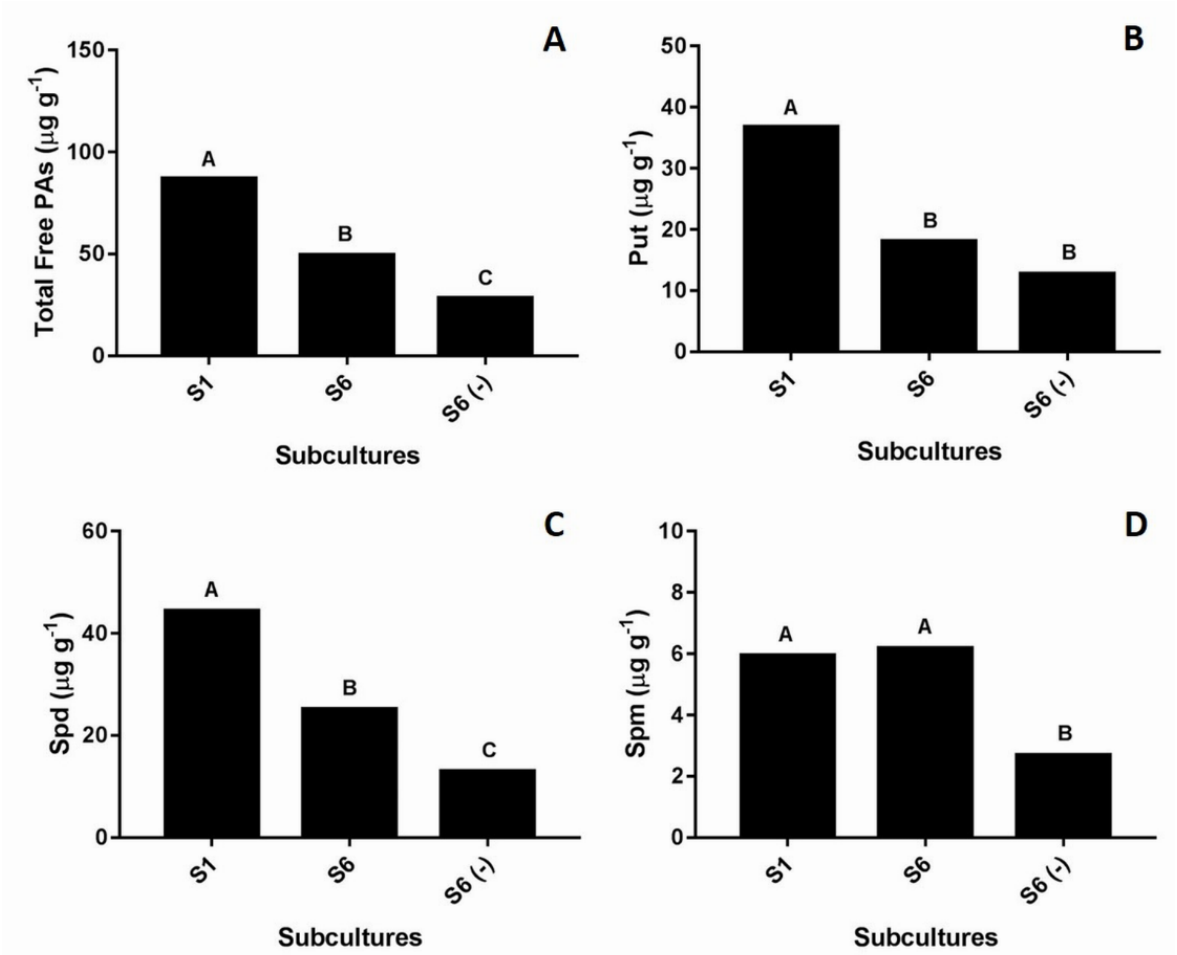

Figure 8. Total free PAs (A), Put (B), Spd (C) and Spm (D) in callus from subcultures S1, S6 and S6(-). Means followed by different letters are significantly different ($P<0.05$) according to the Student-Newman-Keuls test. S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D. CV = coefficient of variation (n=3; CV_{Total}=14.34 % CV_{Put}= 29.38 %, CV_{Spd}= 12.93 %, CV_{Spm}= 24.02 %).

3.1.4.4. Changes in proteomic profile

Proteomic analysis was performed by comparing callus with embryogenic competence (S1 and S6(-)) and the callus with reduced embryogenic competence (S6), for which three differential comparisons were made: S1/S6, S6(-)/S6 and S1/S6(-). A total of 694 proteins were identified; 618 were common in all callus, 13 were shared among only S1 and S6(-), 15 were shared among only S6(-) and S6, 12 were shared among only S1 and S6, four were unique to S1, 19 were unique to S6 and 13 were unique to S6(-) (Figure 9A).

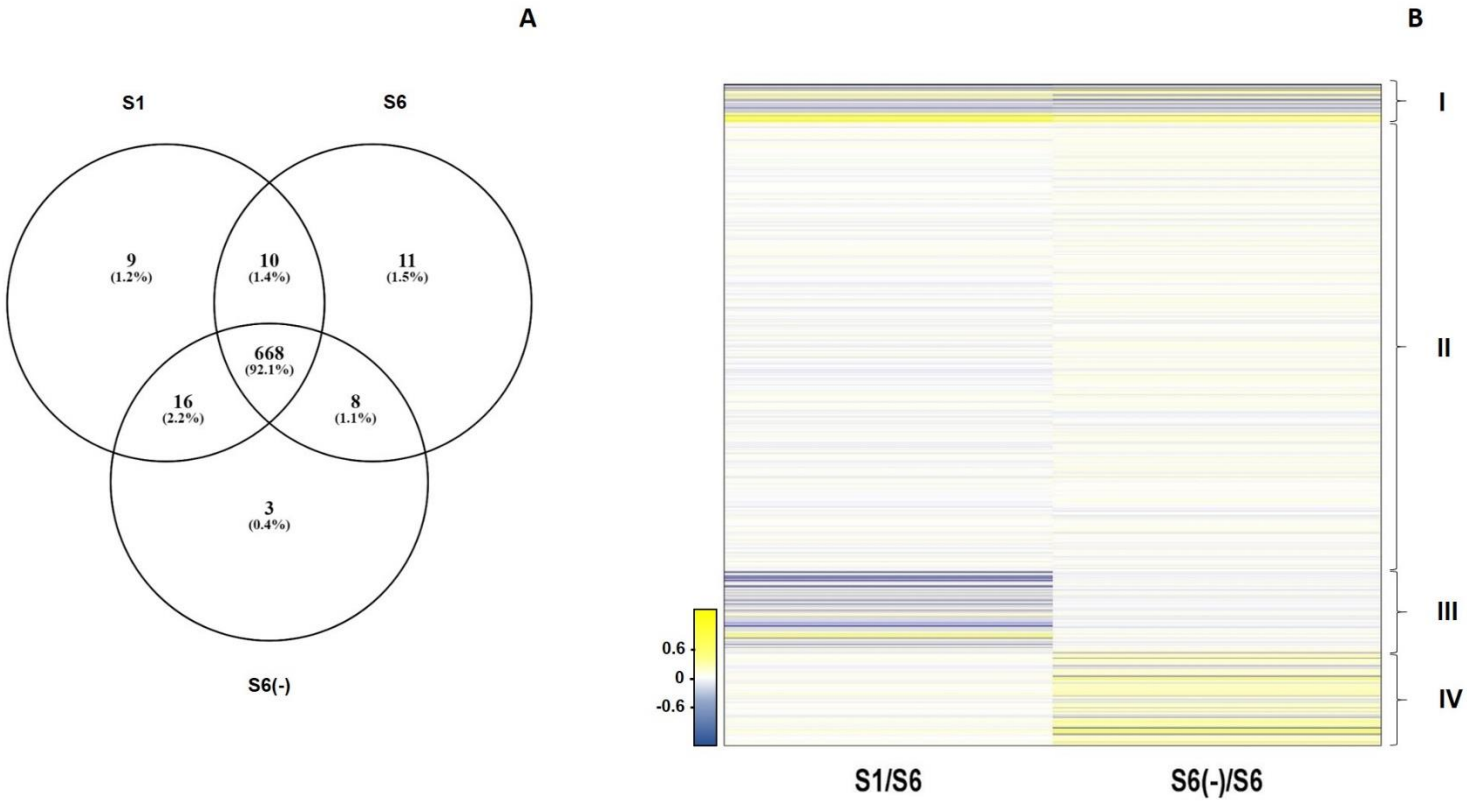

Figure 9. (A) Venn diagram illustrating the number of proteins identified in all callus. The diagram shows unique proteins and proteins presenting differential abundances in callus S1, S6 and S6(-). (B) Heatmap of 618 proteins identified in all callus that shows regulation of abundance profiles for the comparisons S1/S6 and S6(-)/S6. Blue lines represent up-accumulated proteins. Yellow lines represent down-accumulates proteins. White lines represent unchanged proteins. Proteins that showed log2 fold change (log2_FC) values higher than 0.6 and significance in Student's t-test ($P<0.05$) were considered up-accumulated, and proteins that showed log2_FC values lower than -0.6 and significance in Student's t-test ($P<0.05$) were considered down-accumulated. S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D.

Among the identified proteins, 13 that were shared among only S1 and S6(-) could be directly associated with embryogenic competence (Table 1). These proteins were identified in only callus S1 and S6(-), but only 23 % of these proteins exhibited significant differential abundances. Here, we highlight the late embryogenesis abundant protein (LEA) (Sspon.01G0003390-2B; Sspon.07G0003050-3D), heat shock protein Hsp90 (Sspon.02G0011980-1A) and oil body-associated protein 1A (Sspon.04G0006260-1A).

The 618 proteins identified in all callus were used to perform an analysis of differential abundance in the different callus using a heatmap graph (Figure 9B). Proteins were divided into four main groups according to differential abundance between the S1/S6 and S6(-)/S6 comparisons. Group I is composed proteins that showed similar abundance variations for callus S1 and S6(-) compared to callus S6. Group II is composed of proteins that did not show abundance variations in any of the callus. Group III is composed of proteins that exhibited abundance variations only compared to callus S1 and S6. Group IV is composed of proteins that varied only compared to callus S6(-) and S6 (Figure 9B). Among the groups generated by the heatmap, group I, composed of 35 proteins, indicates the possible proteins that have direct associations with the processes of acquisition and loss of embryogenic competence (Table 2). We highlight heat shock 70 kDa (HSP-70KDa) (Sspon.01G0052010-1C), oleosin 18 kDa (Sspon.01G0056570-2D), chitinase (Sspon.08G0024680-1P; Sspon.08G0024680-2P), methyl-CpG-binding domain (MBD) proteins (Sspon.02G0031680-2D) and eukaryotic initiation factor 4A (Sspon.04G0018470-1P; Sspon.04G0018470-3C), all of which except chitinase were more abundant in S1 and S6(-) than in S6.

Table 1. Proteins identified only on sugarcane callus from S1 and S6(-). S1 is the callus from first subculture with 2,4-D; S6(-) is the callus from the sixth subculture without 2,4-D.

Accession	Reported Peptide	Score	Relative abundance regulation			Description
			S1/ S6	S6(-)/S6	S1/S6(-)	
Sspon.01G0003390-2B	7	2135.2	Unique S1	Unchanged	Unique S6(-)	Late embryogenesis abundant protein D-34
Sspon.01G0006100-1A	2	776.3	Unique S1	Unchanged	Unique S6(-)	UDP-glucose 6-dehydrogenase 4
Sspon.01G0042790-1B	23	2534.6	Unique S1	Unchanged	Unique S6(-)	Alanine aminotransferase 2
Sspon.01G0048710-1B	12	6219.8	Unique S1	Unchanged	Unique S6(-)	Globulin-1 S allele-like
Sspon.02G0004530-1A	8	1753.7	Unique S1	Up	Unique S6(-)	Succinate--coa ligase [ADP-forming] subunit alpha, mitochondrial
Sspon.02G0011980-1A	13	7449.1	Unique S1	Up	Unique S6(-)	Heat shock protein Hsp90
Sspon.03G0009670-3D	4	2474.8	Unique S1	Unchanged	Unique S6(-)	Aspartic proteinase oryzasin-1
Sspon.04G0006260-1A	2	1269.6	Unique S1	Unchanged	Unique S6(-)	Oil body-associated protein 1A
Sspon.05G0032150-2D	6	3102.3	Unique S1	Unchanged	Unique S6(-)	Peroxygenase 2
Sspon.06G0001460-1A	4	5514.8	Unique S1	Up	Unique S6(-)	Uncharacterized protein
Sspon.06G0023030-3D	3	1367.8	Unique S1	Unchanged	Unique S6(-)	Flower-specific gamma-thionin precursor
Sspon.07G0003050-3D	9	18970.4	Unique S1	Unchanged	Unique S6(-)	Late embryogenesis abundant protein 3
Sspon.08G0015870-1A	11	3704.4	Unique S1	Unchanged	Unique S6(-)	Proteasome subunit beta type-6

Table 2. Proteins that present similar regulation of abundance profiles in sugarcane callus from S1 and S6(-) compared to S6. S1 and S6 are callus from the first and sixth subcultures with 2,4-D, respectively; S6(-) is the callus from the sixth subculture without 2,4-D.

Accession	Reported Peptide	Score	Differential abundance regulation*			Description
			S1/S6	S6(-)/S6	S1/S6(-)	
Sspon.01G0008860-4D	22	5557.9	Up	Unchanged	Up	Hydroxyproline-rich glycoprotein family protein
Sspon.01G0024930-2B	19	8921.3	Up	Unchanged	Up	Globulin-1 S allele
Sspon.01G0040770-1B	3	8793.3	Down	Unchanged	Down	Cysteine proteinase inhibitor
Sspon.01G0048710-2D	15	5965.5	Up	Unchanged	Up	Globulin-1 S allele-like
Sspon.01G0052010-1C	29	9189.2	Up	Unchanged	Up	Heat shock cognate 70 kda protein
Sspon.01G0056570-2D	3	6127.9	Up	Unchanged	Up	Oleosin 18 kda
Sspon.01G0058110-1D	6	4600.3	Down	Up	Down	Pathogenesis-related protein 1
Sspon.02G0004530-4D	8	2074.2	Down	Unchanged	Down	Succinate--coa ligase [ADP-forming] subunit alpha, mitochondrial
Sspon.02G0004830-1A	8	1172.1	Down	Down	Down	Polygalacturonase inhibitor 1
Sspon.02G0013220-3P	29	5378.6	Up	Unchanged	Up	Alpha-amylase isozyme 3C
Sspon.02G0029080-1A	7	3516.4	Up	Down	Up	Osmotin-like protein
Sspon.02G0031680-2D	15	5705.6	Up	Unchanged	Up	Methyl-cpg-binding domain-containing protein 11
Sspon.02G0034650-2D	7	8727.4	Down	Unchanged	Down	Zeamatin-like protein

Table 2. Cont.

Accession	Reported Peptide	Score	Differential abundance regulation			Description
			S1/S6	S6(-)/S6	S1/S6(-)	
Sspon.02G0041800-2C	9	3205.4	Down	Unchanged	Down	Xylanase inhibitor protein 1
Sspon.03G0003060-1A	12	4910.8	Up	Down	Up	Osmotin-like protein
Sspon.03G0006750-2B	4	761.0	Up	Unchanged	Up	NADP-dependent malic enzyme
Sspon.03G0009670-1P	14	4147.9	Up	Unchanged	Up	Aspartic proteinase oryzasin-1-like
Sspon.03G0010210-3C	11	7625.7	Down	Up	Down	Malate dehydrogenase, mitochondrial
Sspon.04G0010770-4D	5	4099.2	Up	Unchanged	Up	Uncharacterized protein
Sspon.04G0017360-1A	12	3587.2	Down	Up	Down	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
Sspon.04G0018470-1P	23	6150.7	Up	Unchanged	Up	Eukaryotic initiation factor 4A
Sspon.04G0018470-3C	28	6917.5	Up	Unchanged	Up	Eukaryotic initiation factor 4A-3
Sspon.04G0019100-2B	10	1678.6	Up	Unchanged	Up	Retrovirus-related Pol polyprotein LINE-1
Sspon.05G0003520-1A	8	11405.1	Down	Unchanged	Down	Glycine-rich protein 2
Sspon.05G0008290-1A	21	1815.2	Up	Unchanged	Up	Malate synthase, glyoxysomal
Sspon.06G0001460-2B	5	6418.4	Up	Unchanged	Up	Uncharacterized protein
Sspon.06G0017150-2C	11	3718.8	Up	Unchanged	Up	Proteasome subunit alpha type-5

Table 2. Cont.

Accession	Reported Peptide	Score	Differential abundance regulation			Description
			S1/S6	S6(-)/S6	S1/S6(-)	
Sspon.06G0027590-1B	18	10251.0	Down	Unchanged	Down	Basic 7S globulin-like
Sspon.06G0027590-1P	13	11710.5	Down	Down	Down	Basic 7S globulin-like
Sspon.07G0006270-3C	19	4265.7	Up	Unchanged	Up	Heat shock 70 kda protein
Sspon.07G0012310-1A	6	4779.1	Down	Down	Down	Cysteine-rich repeat secretory protein 55-like
Sspon.07G0012310-2P	3	4159.7	Down	Down	Down	Cysteine-rich repeat secretory protein 55-like
Sspon.08G0011360-4D	21	4690.2	Down	Unchanged	Down	Annexin D7
Sspon.08G0024680-1P	5	3648.3	Down	Down	Down	Chitinase
Sspon.08G0024680-2P	5	4609.3	Down	Down	Down	Chitinase

* Proteins that showed significantly changes ($P < 0.05$) between callus and showed Log2 of the fold change (Log2_FC) higher than 0.6 were considered up-accumulated (Up) or Log2_FC smaller than -0.6 were considered down-accumulated (Down).

3.1.5. DISCUSSION

In sugarcane somatic embryogenesis, we observed a progressive decrease in embryogenic competence in successive subcultures in the presence of 2,4-D. The decrease in embryogenic competence occurred from S5 and was maintained in subsequent subcultures until S8 (Figure 3). Similar results have been observed in different species, such as *Pinus pinaster* (Breton et al., 2006), *Coffea arabica* (Landey et al., 2015) and *Olea europaea* (Bradaï et al., 2016). For sugarcane, the decreased in callus embryogenic competence was first studied by Fitch e Moore (1993). The authors showed loss of totipotency in cell suspensions of different genotypes after the eighth month of culture and the appearance of abnormal plants after the fifth month of culture. In addition, these authors also showed the influence of the composition of the culture medium on the production and maintenance of sugarcane callus.

Several studies have shown that the type and concentration of auxin used in the culture medium can influence the maintenance of embryogenic competence (Brisibe et al., 1994). Among auxin types, 2,4-D is one of the most commonly used auxins in somatic embryogenesis process. For sugarcane, a relatively high concentration of 2,4-D may be required for callus induction (Fitch e Moore, 1993); however, a high concentration of this auxin during the multiplication phase can induce a decrease in embryogenic competence (Brisibe et al., 1994). Although the effects of prolonged exposure to 2,4-D are known, the major molecular factors associated with these effects remain unknown, and elucidation of these factors is essential for an improved understanding of the process of loss of embryogenic competence in sugarcane callus. The loss of embryogenic competence associated with prolonged exposure to 2,4-D is demonstrated by the results obtained in our study (Figure 4). Delbarre et al. (1996) revealed that 2,4-D is rapidly transported into cells by carriers and is not metabolized at the same rate, resulting in cytosolic accumulation of this compound. Cytosolic accumulation of 2,4-D can negatively regulate auxin transport by regulating the activity of polar auxin transport inhibitor sensitive 1 (PIS1) (Fujita e Syōno, 1997; Bennett, 1998), and correct auxin transport is essential for success of the somatic embryogenesis process (Su et al., 2009; Abrahamsson et al., 2012). In a review published by Garcia et al. (2019), it is

suggested that the accumulation of intracellular 2,4-D by long exposure to this hormone and consequent imbalance of auxin metabolism is one of the main causes for the appearance of abnormalities in somatic embryos. Thus, long-term culture in the presence of 2,4-D can influence auxin metabolism and can consequently affect the production of somatic embryos in sugarcane callus. In addition to the regulation of auxin metabolism, long-term culture can modulate morphological changes in cells of embryogenic callus (Fu et al., 2012), interfering with the production of well-developed somatic embryos (Breton et al., 2006).

We observed that long-term culture with 2,4-D (S6) induced the growth of highly vacuolated cells with low nucleus/cytoplasm ratios (Figure 6), which are characteristic of non-embryogenic cultures. Silveira et al. (2013) demonstrated that embryogenic cells are characterized by small isodiametric cells containing prominent nuclei and a dense cytoplasm, while non-embryogenic cells are highly vacuolated and elongated. Thus, sugarcane cells from long-term cultures with 2,4-D presented similar characteristics to non-embryogenic cells, directly influencing the process of somatic embryo formation.

Histochemical analysis showed that long-term culture of callus in the presence of 2,4-D resulted in decreased amounts of protein bodies and polysaccharides, especially starch grains (Figure 6), which are essential for in vitro morphogenesis, including for somatic embryo development (Branca et al., 1994; Martin et al., 2000). According to Pinto et al. (2010), starch and proteins are important sources of reserves for the early stages of the somatic embryo formation process. Protein bodies accumulate during callus formation (Rocha et al., 2016) and are rapidly degraded by proteolytic enzymes in the early stages of embryo development as sources of carbon and nitrogen (Cangahuala-Inocente et al., 2004). The degradation of protein bodies in the early stages of somatic embryogenesis is considered crucial for the acquisition of embryogenic competence (Cangahuala-Inocente et al., 2014), suggesting that high concentrations of protein bodies in embryogenic callus can be fundamental for the success of somatic embryo formation.

Similar to protein bodies, starch grains also accumulate during callus formation (de Carvalho Silva et al., 2014). Studies carried out with *Elaeis guineensis* have shown that starch accumulation occurs during callogenesis and that degradation of these starch grains occurs during the late stages of somatic embryo

formation (Gomes et al., 2014). Similar results were obtained in *Feijoa sellowiana* (Cangahuala-Inocente et al., 2004), *Acrocomia aculeate* (Moura et al., 2008), *Eucalyptus globulus* (Pinto et al., 2010) and *Passiflora edulis* (Rocha et al., 2016), suggesting that the accumulation of starch grains in embryogenic callus is an important step for the success of somatic embryogenesis. According to de Carvalho Silva et al. (2014), the accumulation of starch grains in embryogenic callus may be associated with the process of embryogenic competence acquisition. Therefore, the decreasing concentrations of these reserve compounds in callus subjected to long-term culture with 2,4-D could be associated with the decrease in embryogenic competence in these callus, due to the low synthesis and mobilization of protein and starch for the process of embryo formation. However, callus from S1 and S6(-) showed high accumulation of these compounds, suggesting that these callus provide adequate conditions for the formation and development of somatic embryos.

In addition to morphological changes, it is known that physiological changes are also associated with the transition from somatic cells to the embryogenic state (Karami e Saidi, 2010). Among the physiological factors, PAs appear to be important modulatory molecules of the somatic embryogenesis process in different species, such as *Gossypium hirsutum* (Sakhanokho et al., 2005), *Ocotea catharinensis* (Santa-Catarina et al., 2007), *Picea abies* (Vondráková et al., 2015) and *Eleusine coracana* (Satish et al., 2016). Sugarcane was shown to have embryogenic and non-embryogenic callus with different concentrations of PAs during somatic embryo formation (Silveira et al., 2013). Additionally, Reis et al. (2016) reported that addition of putrescine to the culture medium increased the number of somatic embryos formed in embryogenic callus of sugarcane. These results suggest that PAs act directly in the regulation of somatic embryogenesis in sugarcane. The low free PA content observed in callus S6(-) (Figure 7) can be explained by the close association between PA and auxin metabolisms. Cona et al. (2003) revealed that exogenous addition of auxin inhibits the activity of the polyamine oxidase (PAO), preventing the catabolism of these molecules and consequently increasing the levels of free PAs. Therefore, low exposure to 2,4-D in callus S6(-) may alleviate the inhibition of PAO and consequent catabolism of PAs. The low level of free PAs, especially Put and Spd, is associated with the acquisition of embryogenic competence in *Pinus nigra* (Noceda et al., 2009), suggesting that the low PA content observed in callus S6(-) (Figure 8) may be associated with maintenance of embryogenic competence in

these callus. The high free PA content observed in callus S1 can be explained by the association of PA metabolism with DNA methylation (Noceda et al., 2009), since the callus induction process is associated with the regulation of the DNA methylation profiles that may be related to the levels of free PAs in these tissues. Thus, although PAs have been implicated as important modulators in somatic embryo formation in sugarcane, our results suggest that maintenance of embryogenic competence in callus for prolonged periods in culture is associated with low levels of PAs, especially Put and Spd (Figure 8).

Long-term in vitro culture leads to an increased frequency of genetic and epigenetic changes that can regulate the expression of important genes and cause physiological changes (Dubrovina e Kiselev, 2016). Zeng et al. (2010) demonstrated that long-term culture can lead to silencing and regulation of the expression of different genes. In addition, long-term culture with 2,4-D may result in the mutation or inactivation of genes important for embryo development (Garcia et al., 2019). In our study, we highlight proteins that act directly in the process of embryogenesis in plants, such as MBD proteins, LEA, chitinase, HSP-70KDa, eukaryotic initiation factor 4A and oleosins.

MDB proteins are known to be involved in DNA methylation and chromatin remodeling, consequently participating in the regulation of transcription processes and gene silencing (Grafi et al., 2007). Chromatin remodeling is an important process during the transition from somatic to embryogenic cells and is therefore important for the acquisition of embryogenic competence (Feher et al., 2003). Berg et al. (2003), reported that the MDB11 protein is crucial for normal plant development, and silencing of this protein generated several phenotypic effects in Arabidopsis. Based on the importance of this protein in plant development, the high abundance of MDB11 in the S1 and S6(-) callus (Table 2) could be directly associated with the maintenance of embryogenic competence.

LEA proteins are associated with desiccation tolerance during late stages of embryo development (Chugh e Khurana, 2002), being described for the first time in embryos of *Triticum aestivum* (Cuming e Lane, 1979). A recent study has shown that LEA proteins are highly abundant in the final stage of somatic embryo production in sugarcane (Reis et al., 2016); however, during somatic embryogenesis, LEA proteins are involved in both early and late stages of embryo formation (Chugh e Khurana, 2002). Lara-Chavez et al. (2012) reported that, unlike

zygotic embryogenesis, LEA proteins are identified during all stages of somatic embryo development, suggesting the importance of these proteins throughout the somatic embryogenesis process. Thus, the absence of LEA proteins in callus S6 (Table 1) might influence the decrease in embryogenic competence of these callus.

Chitinases are proteins that are known to be involved in plant defense against biotic stress, especially against attacks by fungi and insects (Sharma et al., 2011). In addition, chitinases are also involved in embryogenesis, but few studies have examined the precise metabolic pathways regulated by these proteins (von Arnold et al., 2002). Studies suggest that chitinases may be important for normal development of the somatic embryo, especially under stress conditions, and a lack of secretion of these proteins can result in abnormal development of the embryos (De Jong et al., 1992; de Jong et al., 1995). Thus, the high abundance of chitinases in callus S6 can represent a callus response to stress during long-term culture with 2,4-D. However, in contrast to studies showing that chitinases act directly in the normal development of somatic embryos, our results suggest that chitinases are associated with the loss of embryogenic competence in callus maintained for a prolonged period with 2,4-D, because these proteins were more abundant in callus S6 than in callus S1 and S6(-).

Protein synthesis and degradation promote changes in protein metabolism during somatic embryogenesis in different species (Zi et al., 2013). HSPs are associated with protein metabolism and play an important role during the process of somatic embryogenesis (Rode et al., 2012), as demonstrated for the species *Vitis vinifera* (Marsoni et al., 2008), *Cyphomandra betacea* (Correia et al., 2012), and *Saccharum* ssp. (Reis et al., 2016). According to Zi et al. (2013), HSPs may perform several functions during somatic embryogenesis, such as stabilization of protein conformation, prevention of aggregation, and refolding under stress. Recent studies have demonstrated that HSPs are more abundant in embryogenic callus than in non-embryogenic callus (Zhao et al., 2015; Kumaravel et al., 2017), which indicates that HSP accumulation may play a fundamental role in the acquisition and maintenance of embryogenic competence in callus. In the present study, we observed that three HSPs were more abundant in callus S1 and S6(-) than in callus S6 (Table 2), suggesting that the low abundance of these proteins could be associated with the decrease in competence observed in callus cultivated for a prolonged period with 2,4-D. In addition, eukaryotic initiation factor 4E proteins are

known to act on the binding of mRNA to the ribosome and are therefore directly associated with the translation process (Sonenberg e Hinnebusch, 2009). These proteins were identified as being important in embryogenesis and morphogenesis in *Drosophila* (Gong et al., 2004; Hernández et al., 2004). Based on our results (Table 2), these proteins can act in a similar manner in plants, being important for the maintenance of metabolic activity for the developmental process and for the synthesis of proteins that will act directly in the development of the somatic embryo.

Protein degradation is important for most diverse biological events that occur during the plant life cycle. In our study, a higher abundance of proteasomal proteins was observed in callus S1 and S6(-) than in callus S6 (Table 1 and Table 2). These results indicate the occurrence of active protein metabolism in the callus, leading to maintenance of embryogenic competence in a culture medium without 2,4-D [S6(-)]. During the dedifferentiation process, an increase in the proteasomal synthesis is observed, and these proteins act directly in the process (Jamet et al., 1990). Feher et al. (2003) suggested that the increased activity of these proteins is important for genetic reorganization during the transition from cell to embryogenic cell. Studies in different species have demonstrated the regulation of proteasomes during the development of the somatic embryo (Lippert et al., 2005; Bian et al., 2010; Lara-Chavez et al., 2012), reinforcing the hypothesis regarding the importance of these proteins for the process, and based on our results, these proteins are important for the maintenance of embryogenic competence. The callus cultivated with 2,4-D (S6) exhibited reduced levels of this protein, which could be associated with the loss of competence observed.

Finally, we also observed that proteins associated with lipid metabolism, such as oleosins and oil body-associated protein 1A (Table 1 and Table 2), were more abundant in callus S1 and S6(-) than in callus S6. These proteins have been characterized as important sources of energy reserves in plants and are closely associated with the embryogenic process (Frandsen et al., 2001). In somatic embryogenesis, proteins associated with lipid metabolism are highly accumulated during the maturation of somatic embryos (Stone et al., 2001; Che et al., 2006), due to a strong interaction with leafy cotyledon (LEC) genes (Guo et al., 2013). Our results suggest that oleosins play an important role in the early process of somatic embryogenesis, because accumulation of these proteins was observed in only the callus in the first subculture (S1) and after six subcultures without 2,4-D [S6(-)],

which showed the competence of somatic embryo development. Similar results were obtained in *Arabidopsis thaliana* (Gliwicka et al., 2012), reinforcing the hypothesis that oleosin accumulation may be associated with the acquisition of embryogenic competence. Our results suggest that long-term culture with 2,4-D affects the abundance of this protein, as callus cultivated without 2,4-D [S6(-)] showed higher abundances than those subcultured with 2,4-D (S6) and maintained competence for somatic embryo development.

3.1.6. CONCLUSION

Based on the results presents in this chapter, the following conclusion:

- Long-term culture with 2,4-D affects the embryogenic competence of sugarcane callus;
- Cells were highly vacuolated and exhibited low levels of proteins and polysaccharides in callus cultivated with 2,4-D for a prolonged period, which could be associated with the reduced ability to develop somatic embryos;
- Lower content of PAs in successive subcultures is associated with maintence of embryogenic competence in sugarcane callus;
- The regulation of proteins associated with protein and lipid metabolism, such as HSPs and oleosins, is associated with the maintenance of embryogenic competence in callus from S1 and S6(-);

3.2. EMBRYOGENIC COMPETENCE ACQUISITION IN SUGARCANE CALLUS IS ASSOCIATED WITH DIFFERENTIAL H^+-PUMP ABUNDANCE AND ACTIVITY

3.2.1. INTRODUCTION

Sugarcane (*Saccharum* ssp.) is sown on more than 27 million hectares of land worldwide, resulting in more than 18 billion tons of harvested cane annually (FAO, 2017). Sugarcane is the most important source of sugar production, representing 80 % of the total sugar produced in the world (ISO, 2017). Additionally, ethanol biofuel production from sugarcane has garnered international acclaim (Arruda, 2011), especially due to the production of second-generation bioethanol (Bezerra e Ragauskas, 2016).

Due to its economic importance, sugarcane is involved in several breeding programs; however, classic breeding programs take 12 to 14 years to develop cultivars (Butterfield et al., 1996). Molecular approaches comprise an important alternative for continuous improvement of sugarcane (Pan, 2010; Arruda, 2012). For instance, in 2017, the Brazilian Sugarcane Breeding and Technology Company (Centro de Tecnologia Canavieira - CTC) developed the first genetically modified sugarcane (Bt Sugarcane) variety (CTC 20 BT) approved for commercial use (Unica, 2017).

Genetic transformation has been successfully used for several purposes, such as to promote resistance to herbivory, drought and herbicides and to increase

sugar content (Arruda, 2012); however, low transformation rates and inefficient regeneration protocols prevent high-throughput transformation of sugarcane (Hotta et al., 2010). Optimization of the regeneration protocol is dependent on advances in in vitro culture techniques, and somatic embryogenesis demonstrates great potential as a regeneration technique for transformed plants (Ithape Dinesh et al., 2017). Therefore, somatic embryogenesis is more effective for the regeneration of plants derived from both particle bombardment and *Agrobacterium*-mediated transformation techniques (Arruda, 2012).

In general, somatic embryogenesis protocols include induction, multiplication, maturation and conversion phases (Heringer et al., 2018), and recent studies have sought to elucidate the main molecular mechanisms associated with the process of somatic embryo formation in different species (Wu et al., 2015; Campos et al., 2017; Kumar e Van Staden, 2017; Long et al., 2018). During sugarcane somatic embryogenesis, two types of callus are induced, embryogenic and non-embryogenic (Silveira et al., 2013); embryogenic callus is able to differentiate into somatic embryos. Recently, studies have aimed to identify the main molecular aspects associated with embryogenic competence (Oropeza et al., 2001; Nieves et al., 2003; Silveira et al., 2013; Heringer et al., 2015; Mahmud et al., 2015; Reis et al., 2016); however, the basis of morphogenetic acquisition remains unclear.

The evolution of biotechnology and the integration of -omics technologies show tremendous promise for improving our understanding of the physiological and molecular bases for morphogenetic cell competence during somatic embryogenesis (Heringer et al., 2018). In a recent study using shotgun proteomics during somatic embryogenesis in sugarcane, ATPase-related proteins were observed to be more abundant during somatic embryo maturation in embryogenic versus non-embryogenic callus (Heringer et al., 2015). Additionally, enhanced somatic embryo maturation in sugarcane, using putrescine as a maturation agent, revealed the differential accumulation of proteins associated with H^+ flux (Reis et al., 2016). These results suggest that H^+ flux regulation is an important factor during somatic embryogenesis. Thus, microsomal proteomics analysis can reveal new and important insights into the acquisition of embryogenic competence, in which the greater understanding of this relationship may be important for the optimization of the process of the somatic embryogenesis process, allowing the production of a higher number of somatic embryos through the modulation of the H^+ flux.

The ion-selective probe system is a noninvasive technique that has been used to measure H^+ flux in plants, especially to study the physiology of plant development (Feijó et al., 1999; Certal et al., 2008; Ramos et al., 2008). H^+ flux is primarily regulated through the action of P-H^+-ATPase, V-H^+-ATPase and vacuolar H^+-PPase (Gaxiola et al., 2007) and can cause changes in intra- and extracellular pH, altering several biological processes such as embryogenesis (Feher et al., 2003). Although the relationship between pH changes and somatic embryogenesis has been described, few studies have demonstrated the regulation of H^+ flux in callus (Gorst et al., 1987; Rathore et al., 1988; Pasternak et al., 2002a; Dutra et al., 2013). In addition, the relationship between H^+ flux and somatic embryo formation remains unknown, and detailed investigation may provide important information regarding the acquisition of embryogenic competence in sugarcane.

Thus, the purpose of this work was to investigate the association between H^+ flux and embryogenic competence acquisition during somatic embryogenesis in sugarcane. We performed a microsomal proteomics analysis and analyzed changes in extracellular H^+ flux and H^+ pump (P-H^+-ATPase, V-H^+-ATPase and H^+-PPase) activity during the maturation of embryogenic and non-embryogenic sugarcane callus.

3.2.2. REVIEW

3.2.2.1 Acquisition of competence embryogenic

Somatic embryogenesis is a phenomenon wherein a somatic cell can regenerate into a whole plant and this process is observed in plants because they have cellular totipotency (Namasivayam, 2007). However, somatic cells are not naturally embryogenic capacity, so is necessary the process for induction of competence embryogenic in these cells (Dodeman et al., 1997). Jimenez (2001) report that somatic embryogenesis consist in two main phases: induction phase, in which somatic cells are dedifferentiated and they acquire embryogenic competence; expression phase, in which somatic cell are differentiate to form somatic embryo.

The success in induction phase is dependent of many factors like culture conditions, genotype, tissue and stage of development of the explant (Carman, 1990).

In induction phase, not all somatic cells acquire embryogenic competence. Thus, two group of cell are formed, embryogenic cells are cells that presents embryogenic competence while non-embryogenic cells not present embryogenic competence (Namasivayam, 2007). Analysis of the differences between embryogenic and non-embryogenic cells is important to understand the main factors associated with the process of acquisition of embryogenic competence, in which reveal that the main factors that influencing this process are morphological aspects, and gene expression regulation (Jimenez, 2001).

Embryogenic and non-embryogenic cells are characterized by differences in morphological changes, in which embryogenic cells are characterized by being small and isodiametric containing prominent nuclei and dense cytoplasm, while non-embryogenic cells are highly vacuolated and elongated (Silveira et al., 2013). These morphological aspects are directly associated with the acquisition of embryogenic competence, since the alteration in this morphological profile in embryogenic cells interferes with the formation of the somatic embryo (Breton et al., 2006; Fu et al., 2012).

Although it presents two types of morphologically distinct cells, the process of acquisition of differential embryogenic competence for both cell types remains not elucidated. Dedifferentiation and acquisition of embryogenic competence is strongly influenced by many factors such as nutritional components of culture medium, illumination, explant type and growth regulators (Karami e Saidi, 2010). One of the main factors associated with these processes are plant hormones homeostasis and/or stress conditions. The plants promoted a strong control of the gene expression when submitted to different abiotic stresses to maintain their survival under these conditions (Hasegawa et al., 2000), and specifically in somatic embryogenesis, stress condition promotes gene expression regulation, which can induces the dedifferentiation of the somatic cells (Grafi e Barak, 2015). In addition, Fehér (2015) reported that stress conditions are important for the dedifferentiation process due to the modulation of the endogenous contents of plant hormones, especially auxin. Auxins, in special 2,4-D, are important for induction process in several species (Gaj, 2004), mainly due to the control of expression of genes important for the process. The formation of embryogenic cells is related to high DNA

methylation rate promoted by the presence of high concentration of 2,4-D (Leljak-Levanić et al., 2004), and the changes in DNA methylation results in genetic reprogramming and acquisition of competence embryogenic (Karami e Saidi, 2010).

Somatic embryogenesis is a process characterized by high gene regulation, especially in the process of somatic cell dedifferentiation for transition in the embryogenic cell (Feher et al., 2003). Lee e Seo (2018) report that the process of dedifferentiation of somatic cells is characterized by an intense modification in the global pattern of DNA methylation. The intense epigenetic modifications result in the control of gene expression, and genetic reprogramming of the cell. In recent years, several works have discovered the major genes expressed during cell reprogramming that are important for the acquisition of embryogenic competence in these cells. Genes like somatic embryogenesis receptor kinases (SERK), calcium dependent protein kinases (CDPK), leafy cotyledon (LEC) and clavata (CLV) are known to act in the process of initiation of somatic embryogenesis in different species (Chugh e Khurana, 2002). In addition to these genes, Salvo et al. (2014) have shown that genes associated with stress response are directly related to the process of genetic reprogramming in maize cells. Similar results were observed in *Gossypium hirsutum* (Jin et al., 2014) and *Cinnamomum camphora* (Shi et al., 2016) suggesting the participation of these stress response genes in the induction process of embryogenic cells. Fehér (2015) revealed that the induction of embryogenic cells can be regulated by several molecular pathways, the regulation of the genes associated with the stress response being one of these pathways.

In addition to genomic and transcriptomic analysis, proteomic analysis are being used as an important tool to elucidate the main molecular factors associated with the process of acquisition of embryogenic competence. The use of proteomics analysis in studies in the process of acquisition of embryogenic competence is reported in different species, including *Araucaria angustifolia* (dos Santos et al., 2016), *Cyathea delgadii* (Domżalska et al., 2017), *Elaeis guineensis* (Silva et al., 2014), *Saccharum ssp* (Heringer et al., 2015; Reis et al., 2016). In these studies, proteins related to stress and detoxification, protein metabolism, hormone synthesis and signal transduction, carbohydrate and energy metabolism, cell transport, lipid metabolism and cell wall formation are related to directly associated with somatic embryogenesis process in different species (Heringer et al., 2018). In sugarcane, proteins associated with stress response and H^+ flux are also strongly regulated

(Heringer et al., 2015; Reis et al., 2016) and may be associated with the process of somatic embryogenesis in this specie. Similar results were observed in other species (Gomez-Garay et al., 2013; Zhao et al., 2015; Quinga et al., 2018; Zhu et al., 2018), reinforcing the hypothesis of importance of these proteins in the processes of acquisition of embryogenic competence and somatic embryo formation.

3.2.2.2 Plant proton pumps

In plants, the most known and studied H^+ pumps are those of plasma membrane (P-H^+-ATPase), and of vacuoles (V-H^+-ATPase and H^+-PPase) (Gaxiola et al., 2007). P-H^+-ATPase has the main function of generating an electrochemical gradient by means of H^+ transport in which it functions as a driving force for the activity of secondary transporters located in the plasma membrane, internalizing nutrients into the cytoplasm (Sze et al., 1999). In addition, P-H^+-ATPase also play an important role in cell expansion, abiotic stress tolerance, stomatal opening regulation, and embryonic development (Rober-Kleber et al., 2003; Merlot et al., 2007; Janicka-Russak, 2011; Krajinski et al., 2014). The activity of these proteins can be regulated by binding of the 14-3-3 protein to the C-terminal region (Fuglsang et al., 1999). However, P-H^+-ATPase activity is more effectively regulated at post-translational level, especially by the increase in H^+ transport coupling and ATP hydrolysis (Gaxiola et al., 2007).

Among the vacuolar pumps, V-H^+-ATPase has the function of hydrolyzing ATP while H^+-PPase has the function of hydrolyzing inorganic pyrophosphate (PPi). V-H^+-ATPase are complex proteins and have as main function to maintain the electrochemical gradient of H^+ by the endomembrane compartments, including the vacuole, which maintains an optimum pH due to the regulation of H^+ transport performed by V-H^+-ATPase (Sze et al., 1999). Due to the fact that ATP is the main source of energy in cells, V-H^+-ATPase is known to be the main H^+ pump in endomembranes, except for young tissues where H^+-PPase activity occurs (Martinoia et al., 2006). In the plants, during the process of embryogenesis, the accumulation of PPi generated by the high metabolic activity results in a greater activity of H^+-PPase (Ferjani et al., 2011), being that in some species only the activity of H^+-PPase is sufficient for the initial processes of the development of the embryo

(Krebs et al., 2010). The H^+-PPase act in synchronism with P-H^+-ATPase and V-H^+-ATPase in the energization of the secondary transport system and in the control of the cytoplasmic homeostasis (Gaxiola et al., 2007). However, in situations such as abiotic stress, PPi can assume the role of ATP acting as a donor of metabolic energy of the cell (Ramos et al., 2005), in which result in H^+-PPase assuming the role of the main H^+ pump in the cell under these conditions.

The transport of H^+ is also important in the regulation of intra and extracellular pH, and Feher et al. (2003), report that pH regulation is closely related to the embryogenic potential during somatic embryogenesis. These authors suggest that the regulation of H^+ pump activity may be an important factor in relation to the process of acquisition of embryogenic competence. The activity of the H^+ pumps is important for the growth and development of the zygote embryo (Rober-Kleber et al., 2003), however the knowledge about the relation of the H^+ pumps to the development of the somatic embryo remains unknown. Recent studies have shown that during the development of the somatic embryo it is observed a regulation in the synthesis of the H^+ pumps, increasing the abundance of these proteins (Gomez-Garay et al., 2013; Reis et al., 2016). In addition, the higher abundance of these proteins is found in cells that have the embryogenic competence to form the somatic embryo when compared to cells that do not have the competence (Heringer et al., 2015; dos Santos et al., 2016; Quinga et al., 2018; Zhu et al., 2018). Thus, it is suggested that the regulation of the synthesis and activity of H^+ pumps is an important factor in the process of acquisition of competence and formation of the somatic embryo.

3.2.2.3 Subcellular proteomics

The term "Proteomics" was first used in the mid-1990s, referring to the protein content of an organism at a given time and in a given condition, and could present information on the quantification of total proteins, post-translational modifications and protein interaction (Wilkins et al., 1996; Baginsky, 2009; Vanderschuren et al., 2013). Most studies in proteomics used the methodology of two-dimensional electrophoresis (2-DE) for separation for subsequent identification of proteins. Most studies in proteomics used the methodology of two-dimensional electrophoresis for separation for subsequent identification of proteins. However, in the last decades

other methodologies, such as gel-free techniques in which they have several advantages over 2-DE, have been used concomitantly the 2-DE methodologies in proteomics studies (Heringer et al., 2018). One of the main advantages presented by the gel-free methodologies regarding 2-DE is the greater capacity of identification of proteins, considering that the gel-free methodologies present a greater capacity of identification of proteins with low level of abundance due to the greater amplitude of identification (Washburn et al., 2001). In addition, advances in mass spectrometry techniques as well as the development and improvement of the algorithms used in bioinformatics also allowed an advance in obtaining a greater number of proteins identified (Heringer et al., 2018).

In view of the advances in proteomics techniques in recent years, there is a constant increase in the number of studies carried out using the proteomics technique, in which they fit into the most diverse physiological events of the plants, such as germination and seed development, somatic embryogenesis, programmed cell death, among others (Jorrin-Novo, 2009; Jorrin-Novo et al., 2009). Especially for somatic embryogenesis, proteomics has been used for several species to identify the main groups of proteins regulated during the process (Lippert et al., 2005; Marsoni et al., 2008; Pan et al., 2009; Vale et al., 2014; Heringer et al., 2015; Reis et al., 2016; Heringer et al., 2017). Most studies using proteomics techniques during somatic embryogenesis use an approach for total protein identification. However, new approaches such as subcellular proteomics may provide important new information regarding the molecular aspects associated with the embryogenesis process (Heringer et al., 2018). One of the main advantages of performing subcellular proteomics is in relation to sample enrichment, where it makes possible the identification and analysis of previously unidentified proteins in a crude sample (Dreger, 2003). In species such as *Arabidopsis thaliana* and *Panicum virgatum*, the subcellular proteomics technique has been successfully used to identify the main plasma membrane and endomembranes-regulated proteins (Lao et al., 2015; de Michele et al., 2016). Valot et al. (2004) carried out a study with *Medicago truncatula* and observed that the enrichment of the microsomal fraction allowed the identification of proteins located in the plasma and vacuolar membranes previously unidentified in the total protein sample, evidencing the effectiveness of this methodology for the study of this specific group of proteins. Considering that membrane proteins have important functions such as intercellular

communication, ion transport and signaling cascade (Mbeunkui e Goshe, 2011), the proteomics of the microsomal fraction appears as an alternative for in-depth analysis of the abundance and activity of membrane proteins during the process of somatic embryogenesis.

3.2.3. MATERIALS AND METHODS

3.2.3.1. Plant material and growth conditions

Sugarcane variety SP803280 was used for the induction of embryogenic and non-embryogenic callus according to Silveira et al. (2013), with modifications. Briefly, immature nodal cane segments were planted in the commercial substrate PlantMax (DDL Agroindustria, Paulínia, SP, Brazil) and were grown for 60 days in a greenhouse. Then, plants of approximately 45 cm were selected for callus induction. Mature leaves were removed, and the resulting leaf rolls were surface disinfected in 70 % ethanol (Sigma-Aldrich, St. Louis, USA) for 1 min, immersed in 30 % commercial bleach (sodium hypochlorite from 2 to 2.5 %) Qboa® (Anhembi SA, Osasco, Brazil) for 30 min and, subsequently, washed 3 times with autoclaved water in a laminar flow hood. Subsequently, leaf rolls were transversely sectioned into 2–4 mm-thick slices and cultured in glass test tubes (150 × 25 mm) containing 10 mL of MS culture medium (Murashige e Skoog, 1962) (Phytotechnology Lab, Overland Park, USA), supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel® (Sigma-Aldrich) and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.8. The culture medium was sterilized by autoclaving at 121 °C for 15 min. The tubes containing explants were maintained in the dark at 25 ± 1 ºC for 45 days. Induced callus were transferred to Petri dishes (90 × 15 mm) containing 20 mL of the same culture medium, kept in the dark at 25 ± 1 °C, and then subcultured three times, every 21 days. During this multiplication period, embryogenic and non-embryogenic callus were separated according to morphological characteristics, as previously described by Silveira et al. (2013).

3.2.3.2. Maturation of embryogenic and non-embryogenic callus

Maturation experiments were performed according to (Heringer et al., 2015). Embryogenic and non-embryogenic callus were maintained for three subculture cycles. Then, three cultures (250 mg fresh mass [FM] each) of embryogenic and non-embryogenic callus were placed in Petri dishes (90 × 15 mm) containing 20 mL of MS culture medium supplemented with 30 g L^{-1} sucrose, 2 g L^{-1} Phytagel and 1.5 g L^{-1} activated charcoal (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.8, and the medium was sterilized by autoclaving at 121 ºC for 15 min. After inoculation, the cultures were maintained in a growth chamber at 25 ± 1 ºC and grown in the dark for the first 7 days. Thereafter, the cultures were grown under a 16 h photoperiod using GreenPower TLED 20W WmB (Koninklijke Philips Electronics NV, Netherlands) at 55 µmol m^2 s^{-1} for 21 days. Samples were collected before (time 0) and after 7, 14, 21 and 28 days for further analyses. At 28 days of incubation, the FM, morphology and number of formed somatic embryos were determined.

3.2.3.3. Measurements of H^+ fluxes using the ion-selective vibrating probe system

H^+ flux measurements were performed with samples of embryogenic and non-embryogenic callus before incubation (at the beginning of the experiment, time 0) and after 7, 14, 21 and 28 days of culture in the maturation cycle. At each time of analysis, collected callus were placed on Petri dishes (90 × 15 mm) filled with 30 mL of lowsalt adapted nutrient medium (without MES) composed of 0.037 mM NaH_2PO_4, 0.1 mM $MgSO_4$, 0.5 mM $Ca(NO_3)_2$, 0.1 mM KCl, 0.1 mL L^{-1} Clark micronutrients (Clark, 1975) and 0.025 mL L^{-1} Fe-EDTA at pH 5.5 and were used for H^+ flux measurement in the ion-selective vibrating probe system (Applicable Electronics, New Haven, CT, USA) (Feijó et al., 1999; Ramos et al., 2008). Micropipettes were pulled from 1.5 mm borosilicate glass capillaries and treated with dimethyl dichlorosilane (Sigma-Aldrich). After silanization, pipettes were backfilled with a 15–20 mm column of electrolyte (15 mM KCl and 40 mM KH_2PO_4, pH 6.0) and then frontloaded with a 20–25 µm column of the corresponding ion-selective liquid exchange cocktail (Fluka, Milwaukee, WI, USA). A Ag/AgCl wire electrode

holder (World Precision Instruments, Sarasota, FL, USA) was inserted into the back of the microelectrode, establishing an electrical contact with the bathing solution, and the ground electrode dry reference (DRIREF-2; World Precision Instruments) was inserted into the sample bath. The microelectrodes were calibrated at the beginning and end of each experiment using standard solutions covering the experimental range of each ion, to obtain a calibration line. Both the slope and intercept of the calibration line were used to calculate the corresponding ion concentration from the mV values measured during the experiments, and the vibration distance was 15 µm.

To confirm the differential extracellular acidification promoted by embryogenic and non-embryogenic callus, cultures were placed in MS medium supplemented with Phytagel (2.0 g L^{-1}) and bromocresol purple (0.03%) (w/v) at pH 5.8. After 72 h, the change in the coloration of the culture medium, from purple to yellow, which indicates medium acidification, was analyzed (Zandonadi et al., 2010).

3.2.3.4 Preparation of membrane vesicles

For the proteomics analysis as well as for enzymatic and proton pump assays, samples of embryogenic (E) and non-embryogenic (NE) callus at time 0, i.e., before incubation (NE-0 and E-0), and after 14 days of culture in maturation treatment (NE-14 and E-14) were used.

Membrane vesicles were isolated using the differential centrifugation method, essentially as described by Giannini e Briskin (1987), with modifications. Approximately 8 g FM of each type of callus (in triplicate) was homogenized using a mortar and pestle in 2 mL g^{-1} (FM) of ice-cold buffer containing 250 mM sucrose (Sigma-Aldrich), 10 % (v/v) glycerol (Sigma-Aldrich), 0.5 % (v/v) polyvinylpyrrolidone (PVP-40, 40 kD; Sigma-Aldrich), 5 mM EDTA (Sigma-Aldrich), 0.3 % (w/v) bovine serum albumin (BSA; Sigma-Aldrich), 0.1 M Tris-HCl buffer pH 7.6 (Sigma-Aldrich), 150 mM KCl (Sigma-Aldrich), 3.3 mM dithiothreitol (DTT; Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 1 mM benzamidine (Sigma-Aldrich). The homogenate was strained through four layers of cheesecloth and centrifuged at 1,500 g for 15 min. The supernatant was centrifuged once more at 10,000 g for 20 min and then at 100,000 g for 45 min. The pellet was resuspended in 2 mL of ice-cold buffer containing 10 mM Tris-HCl, pH 7.6, 10 % (v/v) glycerol, 1

mM EDTA, 1 mM DTT, 1 mM PMSF and 1 mM benzamidine. All preparative steps were performed at 4 °C. The vesicles were frozen under liquid N_2 and stored at -80 °C. Protein concentrations were determined by the Bradford method (Bradford, 1976).

3.2.3.5 Microsomal proteomics analysis

Three biological replicates of the microsomal fractions of embryogenic and non-embryogenic callus before (NE-0 and E-0) and after 14 days (NE-14 and E-14) of culture in maturation treatment were used for proteomics analysis. Briefly, 100 µg of total protein from each sample was desalted on Amicon Ultra 0.5-3 KD centrifugal filters (Merck Millipore, Darmstadt, Germany) using 50 mM ammonium bicarbonate (pH 8.5; Sigma-Aldrich) as the buffer. For protein digestion, 25 µL of 0.2 % (v/v) RapiGest® surfactant (Waters, Milford, CT, USA) was added to each sample. The resulting mixtures were briefly vortexed and incubated in a Thermomixer® for 15 min at 80 °C, and 2.5 µL of 100 mM DTT (Bio-Rad Laboratories, Hercules, CA, USA) was added. Afterward, the samples were vortexed and incubated at 60 °C for 30 min under agitation at 350 rpm, 2.5 µL of 300 mM iodoacetamide (GE Healthcare, Piscataway, NJ, USA) was added, and the samples were vortexed and incubated in the dark for 30 min at room temperature. The iodoacetamide was quenched by adding 2.5 µL of 100 mM DTT (Bio-Rad Laboratories) to each sample, followed by incubation at 37 ºC for 30 min. Digestion was performed by adding 20 µL of trypsin solution (50 ng μL^{-1}; V5111; Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate to each sample, followed by incubation overnight at 37 °C. For RapiGest® precipitation, 10 µL of 5 % (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich) was added, and samples were incubated at 37 °C for 90 min and then centrifuged at 16,000 g for 30 min. The samples were subsequently transferred to Total Recovery Vials (Waters) for mass spectrometry analysis.

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for ESI-LC-MS/MS analysis. During separation, the samples (1 µg of digested protein) were loaded onto the nanoAcquity UPLC 5-µm C18 trap column (180 µm x 20 mm) at 5 µL min^{-1} for 3 min and then onto the nanoAcquity HSS T3 1.8-µm analytical reverse-phase column (75 µm x 150 mm) at 400 nL min^{-1}. The column temperature was 45 °C. For peptide elution, a binary

gradient was used: mobile phase A consisted of water (Tedia, Fairfield, USA) and 0.1 % formic acid (Sigma-Aldrich), and mobile phase B consisted of acetonitrile (Sigma-Aldrich) and 0.1 % formic acid. Gradient elution was performed as follows: 7 % B for 3 min, ramping from 7 to 40 % B until 90.09 min; ramping from 40 to 85 % B until 94.09 min; holding constant at 85 % until 98.09 min; decreasing to 7 % B until 100.09 min; and holding constant at 7 % B until the end of the run at 108.09 min. Mass spectrometry was performed in positive and resolution mode (V mode), with a resolution of 35000 FWHM with ion mobility and in the data-independent acquisition mode. The IMS wave velocity was set to 600 m s^{-1}; transfer collision energy was ramped from 19 to 45 V in high-energy mode; the cone and capillary voltages were 30 and 2,800 V, respectively; and the source temperature was 70 °C. The nano flow gas was set to 0.50 Bar, and the purge gas flow ranged from 145 to 150 L h^{-1}. The TOF parameters included a scan time of 0.5 s in the continuum mode and a mass range of 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol μL^{-1} was used as an external calibrant, and lock mass acquisition was performed every 30 s.

Spectral processing and database searching were performed using ProteinLynx Global Server (PLGS; version 3.0.2) (Waters) and software ISOQuant workflow (Distler et al., 2014; Distler et al., 2016). The PLGS was processed using a low-energy threshold of 150 (counts), an elevated energy threshold of 50, and an intensity threshold of 750. In addition, the analysis was performed using the following parameters: two missed cleavage, minimum fragment ion per peptide equal to 3, minimum fragment ion per protein equal to 7, minimum peptide per protein equal to 2, fixed modifications of carbamidomethyl and variable modifications of oxidation and phosphoryl. The false discovery rate (FDR) for peptide and protein identification was set to a maximum of 1 %, with a minimum peptide length of six amino acids. The proteomics data were processed against the SUCEST sugarcane database (www.sucest-fun.org) (Vettore et al., 2003).

The comparative label-free quantification analysis was performed using ISOQuant software using previously described settings and algorithms (Distler et al., 2014; Distler et al., 2016). Briefly, the analysis included retention time alignment, exact mass retention time (EMRT) and IMS clustering, as well as data normalization and protein homology filtering. ISOQuant annotates the resulting feature clusters by evaluating consensus peptide identifications and identification probabilities. Protein

identification parameters in ISOQuant were set to a false discovery rate (FDR) of 1%, a peptide score greater than 6, a minimum peptide length of 6 amino acids, and at least 2 peptides per protein. Label-free quantification were estimated using the TOP3 quantification approach (Silva et al., 2006) followed by the multidimensional normalization process implemented within ISOQuant (Distler et al., 2016).

After ISOQuant data analyses, only the proteins that were present or absent (for uniques) in all 3 biological replicates were considered for differential abundance analysis. Data were analyzed using Student's t-test (two-tailed). Proteins with *P*-values of $P < 0.05$ were considered upregulated if the $\log_2$ of fold change (FC) was greater than 1.0 and downregulated if the $\log_2$ of FC was less than -1.0. Finally, proteins were blasted against the Nonredundant (nr) Plants/Viridiplantae_Protein_Sequences database using the Blast2GO software (www.blast2go.com) (Conesa et al., 2005) for protein description and cellular component term annotation. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al., 2014) via the PRIDE (Vizcaíno et al., 2015) partner repository with the dataset identifier PXD009858.

3.2.3.6 Enzyme assay

The hydrolytic activities of P-H^+-ATPase, V-H^+-ATPase and H^+-PPase were determined colorimetrically by measuring the release of Pi (Fiske e Subbarow, 1925). The reaction media contained 50 mM Tris-HCl pH 6.5, 3 mM $MgSO_4$ (Sigma-Aldrich), 100 mM KCl, 0.2 mM $NaMoO_4$ (Sigma-Aldrich) and 1 mM ATP or PPi (Sigma-Aldrich). Reactions were started by the addition of protein (60 µg mL^{-1}) and stopped with ice-cold 5 % (w/v) trichloroacetic acid (Sigma-Aldrich) after 30 min of incubation at 25 °C. Specific inhibitors, including 0.2 mM vanadate (Na_3VO_4; Sigma-Aldrich) and 5 nM concanamycin a (Sigma-Aldrich), were used to determine the hydrolytic activities of P-H^+-ATPase and V-H^+-ATPase, respectively. PPi hydrolysis, performed by H^+-PPase, was determined through its dependence on K^+ (Venancio et al., 2014).

3.2.3.7 Proton pumping assay

ATP- and PPi-dependent H^+ transport across membranes was measured as the initial rate of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA; Sigma-Aldrich) at 25 °C in a spectrofluorimeter (F-3010; Hitachi, Tokyo) using a protocol adapted by Façanha e de Meis (1998). The excitation and emission wavelengths were set at 415 nm and 485 nm, respectively. The reaction medium contained 10 mM Tris-HCl pH 6.5, 2 µM ACMA, 5 mM $MgCl_2$ (Sigma-Aldrich), 100 mM KCl and 1 mM ATP or PPi. The reaction was initiated by the addition of 60 µg mL^{-1} of membrane vesicles. The addition of 20 mM NH_4Cl (Sigma-Aldrich) was used to show a recovery of fluorescence that indicated a collapse of the preliminarily H^+ gradient. Specific inhibitors, including 0.2 mM vanadate and 5 nM concanamycin a, were used to determine the H^+ pumping activities of P-H^+-ATPase and V-H^+-ATPase, respectively. H^+-PPase pumping activity was determined through its dependence on K^+. Based on the H^+ pumping analysis, was calculated the initial velocity (V_0) and delta maximum fluorescence (ΔMF). V_0 was calculated based on the values of maximum possible fluorescence and initial decrease of ACMA fluorescence in which it is represented by the curve decline at the initial moments after substrate insertion (ATP or PPi). The ΔMF was determined according to formule: ΔMF=Feq/Fmax(x100), in which Feq represents maximum fluorescence obtained and Fmax represents maximum possible fluorescence. The coupling rate was calculated as the ratio of the V_0 of H^+ pumping and ATP or PPi hydrolysis.

3.2.3.8 Statistical analysis

The experiment was arranged in a completely randomized design with two callus types (embryogenic and nonembryogenic), 5 analysis time points (0, 7, 14, 21, and 28 days) and 3 biological repetitions (3 Petri dishes of each callus type per time point). The H+ fluxes were analyzed by ANOVA, and the means were compared using the Tukey test ($P < 0.05$) in GraphPad Prism 7.0. The enzyme assay and coupling rate were analyzed by ANOVA ($P < 0.05$). When a factor or any interaction between factors was deemed statistically significant ($P < 0.05$), differences between averages were examined using Tukey's test in GraphPad Prism 7.0.

3.2.4. RESULTS

3.2.4.1. Maturation of embryogenic and non-embryogenic callus

The formation of two types of callus, embryogenic and non-embryogenic, was observed during induction, as previously characterized by Silveira et al. (2013). Both callus were submitted to maturation treatment to form somatic embryos. After 28 days of culture in maturation treatment, the embryogenic callus presented a high rate of somatic embryos. As expected, the non-embryogenic callus was unable to form somatic embryos, even in the maturation treatment (Figure 10, Table 3). Embryogenic callus showed no changes in morphogenesis before incubation (time 0) or after 7 days of culture in maturation treatment. On the 14th day of maturation, treatment embryos formed particularly well at the base of the callus in contact with the culture medium. On subsequent days, the continuous development of somatic embryos was observed, resulting in a high degree of embryo formation after 28 days of culture (Figure 10). Non-embryogenic callus showed few morphological changes during maturation treatment showing a mucilaginous aspect, in contrast to the granular appearance of embryogenic callus. At 28 days of culturing, no somatic embryos were observed, and some callus samples presented oxidation, as observed by the development of a brownish color, most likely resulting from exposure to light (Figure 10). In addition, embryogenic callus showed a higher increase in FM in comparison to non-embryogenic callus during maturation (Table 3).

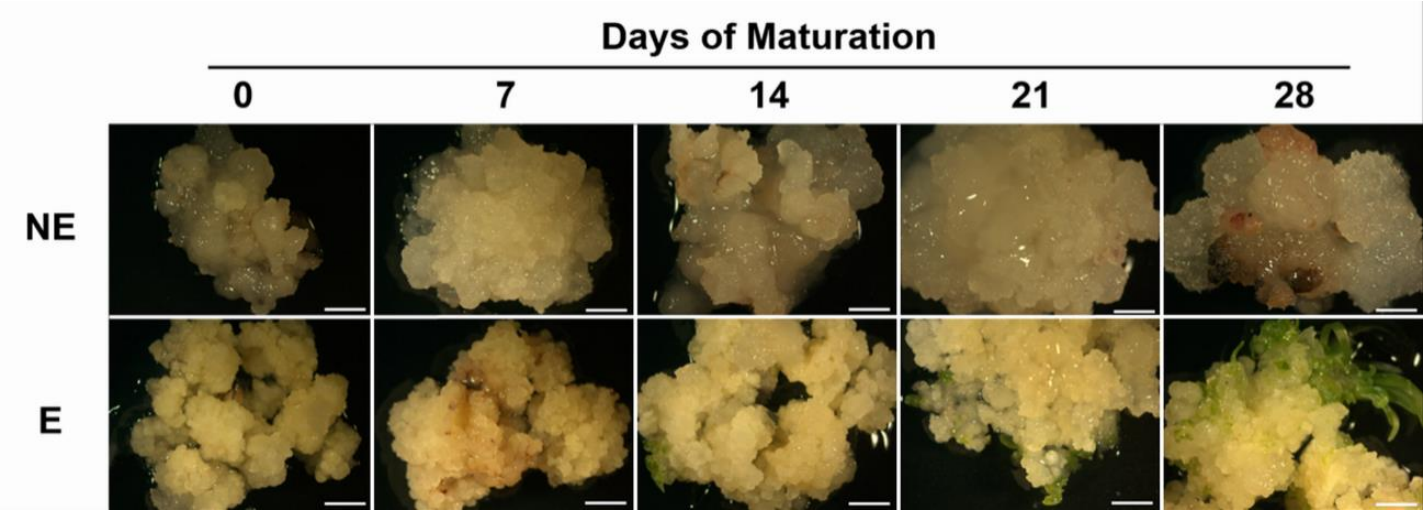

Figure 10. Morphological aspects of embryogenic (E) and non-embryogenic (NE) sugarcane callus under maturation treatment; bars = 20 mm

Table 3. Number of somatic embryos formed and amount of fresh matter under maturation treatment in embryogenic and non-embryogenic sugarcane callus at 28 days of culture.

Type of Callus	Fresh Matter (mg/callus)	Somatic Embryos (SE/callus)
Non-Embryogenic	355.8 B*	0.0 B
Embryogenic	503.9 A	20.53 A

*Means followed by different letters indicate significant differences ($P < 0.05$) according to Tukey's test (n = 9).

3.2.4.2 Changes in H^+ flux, extracellular pH and H^+ extracellular voltage difference during maturation

Embryogenic and non-embryogenic callus were analyzed on an ion-selective vibrating probe system to measure H^+ flux, extracellular pH, and extracellular voltage differences during maturation. H^+ fluxes were altered during maturation in both callus types (Figures 9A and 9B). During maturation, a reduction in H^+ flux was observed in non-embryogenic callus, especially on days 7, 14, and 21 of culturing (Figure 11A). The highest value of H^+ flux (−1.08 pmol cm^{-2} min^{-1}) in non-embryogenic callus was detected at time 0 of the maturation treatment. In embryogenic callus, the lowest H^+ influx was observed at time 0 and after 7 days of culture, followed by a significant 460% increase in H^+ influx at 14 days of culture during somatic embryo formation, which was the highest value of H^+ influx measured (−11.59 pmol cm^{-2} min^{-1}). H^+ influx subsequently decreased, reaching values similar to those observed for embryogenic callus at time 0 (Figure 11B).

Surface extracellular pH values in non-embryogenic callus samples were significantly altered during maturation treatment, ranging from 5.4 to 6.2 (Figure 11C), while embryogenic callus samples showed less variation, with the highest (5.9) and lowest (5.4) values observed at 14 and 28 days of culture in maturation treatment, respectively (Figure 11D). To verify whether changes in pH also occur in culture medium, we performed additional analysis using bromocresol purple. This analysis demonstrated that embryogenic callus present a small acidification on their surface (indicated by the yellow coloration), while the culture medium remains more alkaline (indicated by the purple coloration) (Figure 12). In contrast, non-embryogenic callus causes a high acidification of the culture medium, especially on day 14 of the maturation treatment (Figure 12).

The analysis of H^+ extracellular voltage differences showed that non-embryogenic callus presented a low microvolt difference, with averages varying from −0.7 to −2.2 µV on all days, except at 21 days of culture, exhibiting an inward voltage (Figure 11E). Embryogenic callus showed only inward voltage, with an especially large negative current (−49 µV) at 14 days of culture, when somatic embryos were developed (Figure 11F).

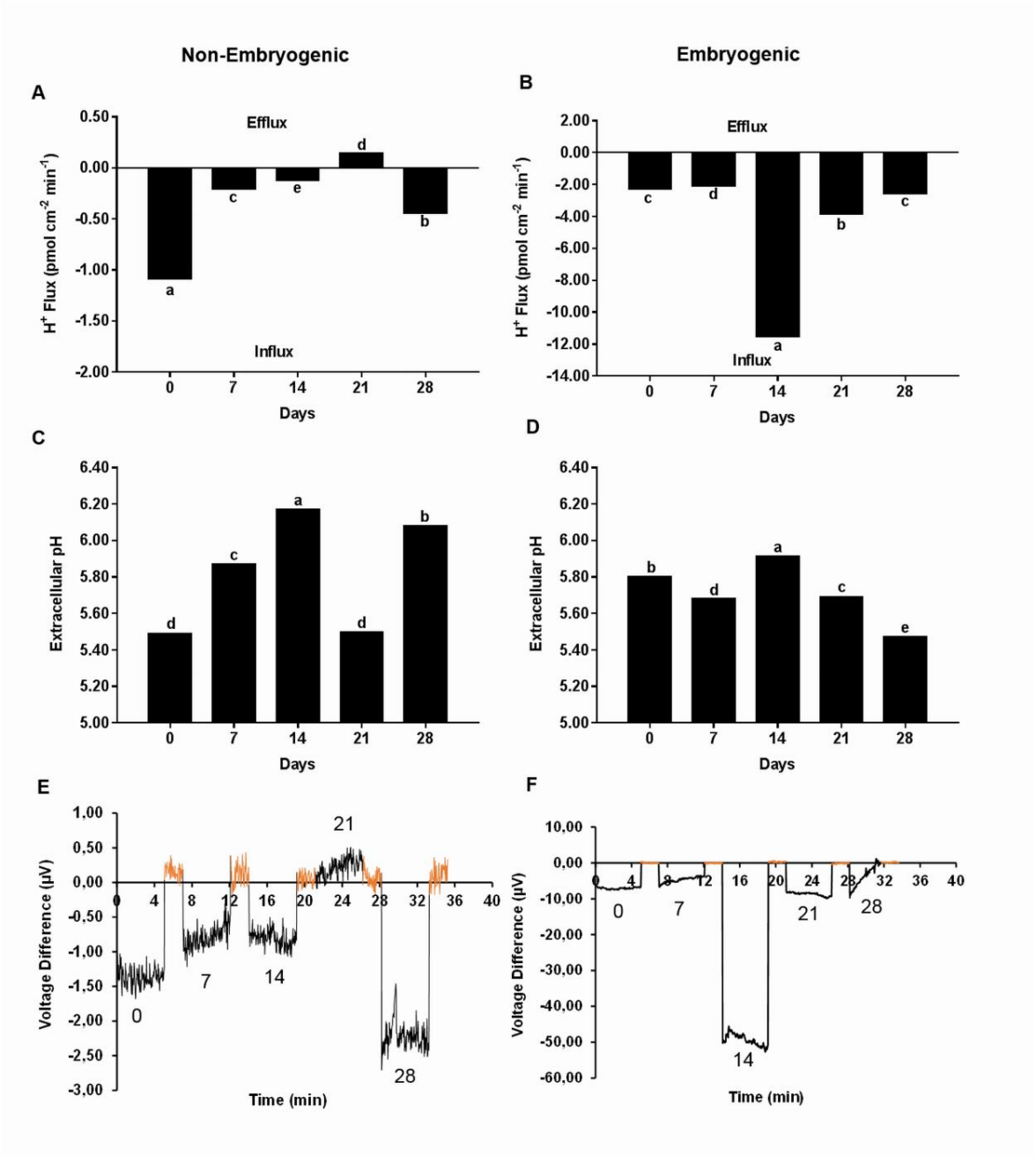

Figure 11. Measurements of H^+ flux, pH and microvolt in embryogenic and non-embryogenic callus during maturation treatment using an H^+-specific vibrating probe. Graphical representation of H^+ flux (A,B), extracellular pH (C,D) and H^+ microvolt difference (E,F) in non-embryogenic (A,C,E) and embryogenic (B,D,F) callus under maturation treatment. Orange lines represent reference values. Means followed by different letters indicate significant differences according to Tukey´s test ($P < 0.05$) ($n = 3$).

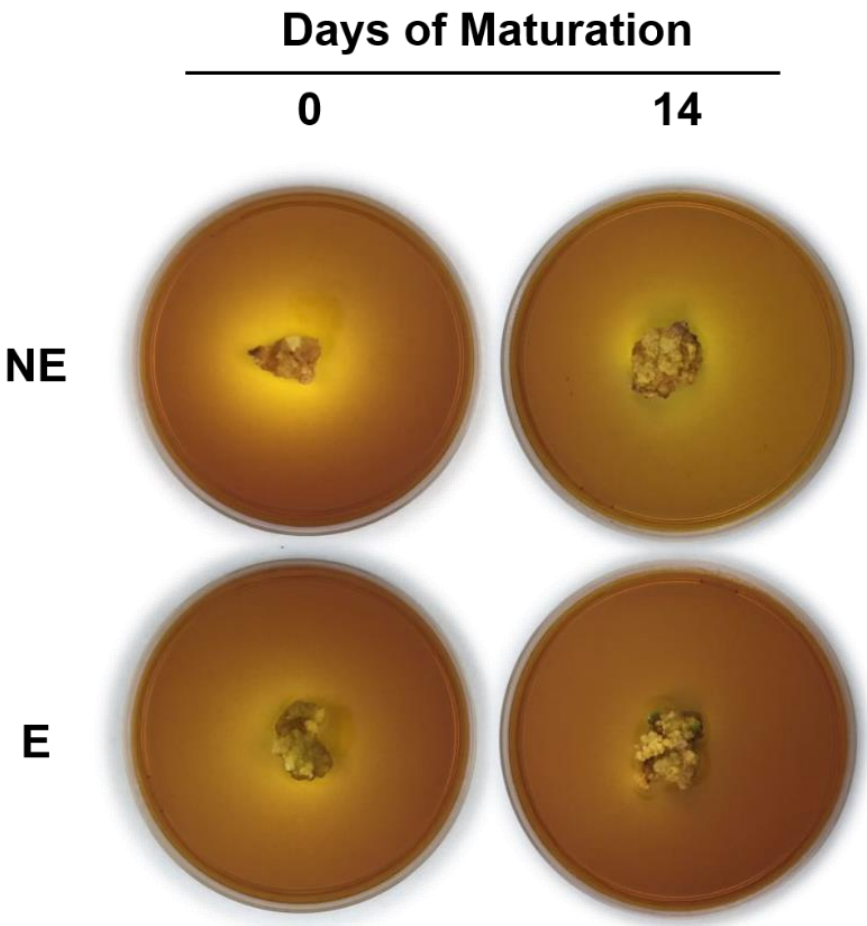

Figure 12. pH changes in the culture medium on days 0 and 14 during maturation treatment in embryogenic and non-embryogenic callus of sugarcane. pH changes from acid to alkaline are indicated by changes in color from yellow (acid) to purple (alkaline).

3.2.4.3 Changes in the proteomic profiles of microsomal fractions

Shotgun proteomics analysis allowed for the identification and quantitation of a total of 657 proteins. The cellular component classification indicated the efficiency of the microsomal proteomic analysis with 65.6% of total identified proteins (431) belonging to the microsomal fraction.

Among the total identified proteins, 527 were identified in all conditions, 17 were identified in only embryogenic callus, 8 proteins were identified in only embryogenic callus on day 14, and 1 protein was identified only non-embryogenic callus on day 14 of maturation (Figure 13). Among the 527 proteins identified in both

callus types, a total of 16 H+ pumps (P-H^+-ATPase, V-H^+-ATPase, or H^+-PPase) were identified (Table 4).

When we compared the differential abundances of H^+ pumps between embryogenic and non-embryogenic callus samples at time 0, we observed four H^+ pumps that were upregulated, 4 that were unchanged, 1 that was down-regulated, and 6 that were identified in only embryogenic callus (Table 4). Additionally, a total of 9 proteins were upregulated, 5 were unchanged, and 2 were identified only in embryogenic callus when we compared embryogenic and non-embryogenic callus at 14 days under the maturation treatment (Table 4).

In both callus types, a large variation in the abundance of V-H^+-ATPase during the maturation of somatic embryos was not detected, while P-H^+-ATPase and H^+-PPase were more abundant in embryogenic callus than those in non-embryogenic callus (Table 4). In particular, we observed the regulation of H^+-PPase during somatic embryo development, wherein the protein vacuolar H^+-pyrophosphatase (SCCCRZ2C03E07) and proton translocating pyrophosphatase (SCEQRT1024C07) were strongly upregulated in embryogenic callus at 14 days under maturation treatment in comparison with those in non-embryogenic callus at the same time point (Table 4).

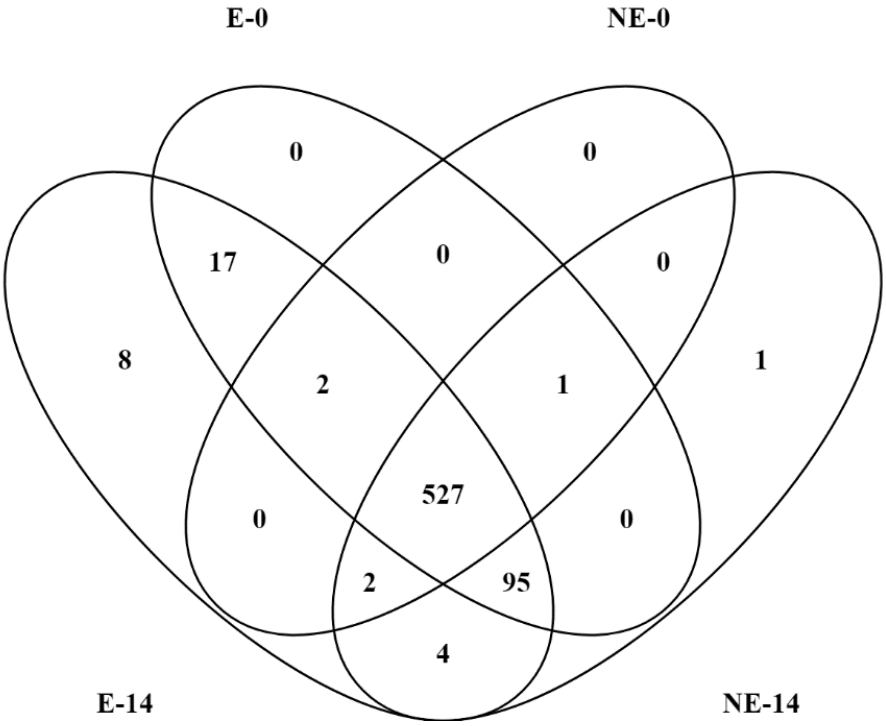

Figure 13. Venn diagram illustrating the numbers of proteins identified in embryogenic (E) and non-embryogenic (NE) callus types before (time 0; NE-0 and E-0) and at 14 days (NE-14 and N-14) of culture in the maturation treatment. The diagram shows unique proteins and proteins with differential abundances in both callus types.

Table 4. Proton pumps identified in embryogenic (E) and non-embryogenic (NE) sugarcane callus at 0 and at 14 days of maturation treatment.

Accession	Reported Peptides	Score	Differential abundance regulation*				Description
			E-0/NE-0	E-14/NE-14	E-14/E-0	NE-14/NE-0	
SCACLR2007H10	15	6803.8	Up	Up	Unchanged	Unchanged	V-type proton ATPase subunit d2
SCAGLR1043D04	44	19083.5	Up	Unchanged	Unchanged	Up	V-type proton ATPase catalytic subunit A
SCBFLR1026E05	31	14522.9	Unique E-0	Up	Unchanged	Unique NE-14	V-type proton ATPase catalytic subunit A
SCBFRT1064B08	14	4552.7	Unique E-0	Up	Up	Unique NE-14	Plasma membrane ATPase 1
SCCCCL1001C10.b	31	19558.2	Unchanged	Unchanged	Unchanged	Unchanged	V-type proton ATPase subunit B 1
SCCCLB1004B02	8	10138.5	Unchanged	Unchanged	Unchanged	Unchanged	ATPase subunit 1 (mitochondrion)
SCCCLR1022D02	9	3529.2	Up	Unchanged	Unchanged	Up	Probable V-type proton ATPase subunit H
SCCCRT1002F07	37	10912.8	Unchanged	Up	Unchanged	Unchanged	Plasma membrane ATPase
SCCCRZ2C03E07	17	29485.8	Unique E-0	Up	Up	Unique NE-14	Pyrophosphate-energized vacuolar membrane proton pump
SCEQRT1024C07	14	27944.4	Down	Up	Up	Down	Pyrophosphatase

Table 4. Cont.

Accession	Reported Peptides	Score	Differential abundance regulation				Description
			E-0/NE-0	E-14/NE-14	E-14/E-0	NE-14/NE-0	
SCEZRT2019G09	8	5393.4	Unique E-0	Unique E-14	Unchanged	-	Vacuolar proton pump3
SCEZRZ1012B07	12	3447.0	Unchanged	Up	Up	Up	Plasma membrane ATPase 1
SCJFLR1074G03	18	11814.6	Up	Unchanged	Unchanged	Unchanged	V-type proton ATPase catalytic subunit A
SCSFSD1065B09	5	21235.9	Unique E-0	Unique E-14	Unchanged	-	Pyrophosphatase
SCSGAD1007A08	7	5805.6	-	Up	Unique E-14	Unique NE-14	Plasma membrane ATPase 3
SCVPLR2019H02	9	23252.3	Unique E-0	Up	Unchanged	Unique NE-14	Pyrophosphate-energized vacuolar membrane proton pump 1

*E-0 = Embryogenic callus at time 0; NE-0 = Non-embryogenic callus at time 0; E-14 = Embryogenic callus at 14 days of culture in maturation treatment; NE-14 = Non-embryogenic callus at 14 days of culture in maturation treatment.

In addition to H^+ pumps, another 76 identified proteins also had transport functions; we were especially interested in plasma membrane proteins associated with ion, protein, and carbohydrate transport that may be associated with H^+ influx (Figure 14). Proteins associated with ion and protein transport, such as mechanosensitive ion channel protein 6 (SCCCFL5062H06), copper transport protein ATX1-like isoform X1 (SCCCHR1004F09), and importin subunit α-1b (SCCCCL3003D06.b), showed no variation in their abundance during somatic embryo formation or were less abundant in embryogenic callus during this process. In contrast, calcium and carbohydrate transporters, such as plastidic glucose transporter 4 (SCEZLR1009H06) and calcium load-activated calcium channel-like (SCJFLR1074C12), were more abundant in embryogenic callus during somatic embryo formation (Figure 14).

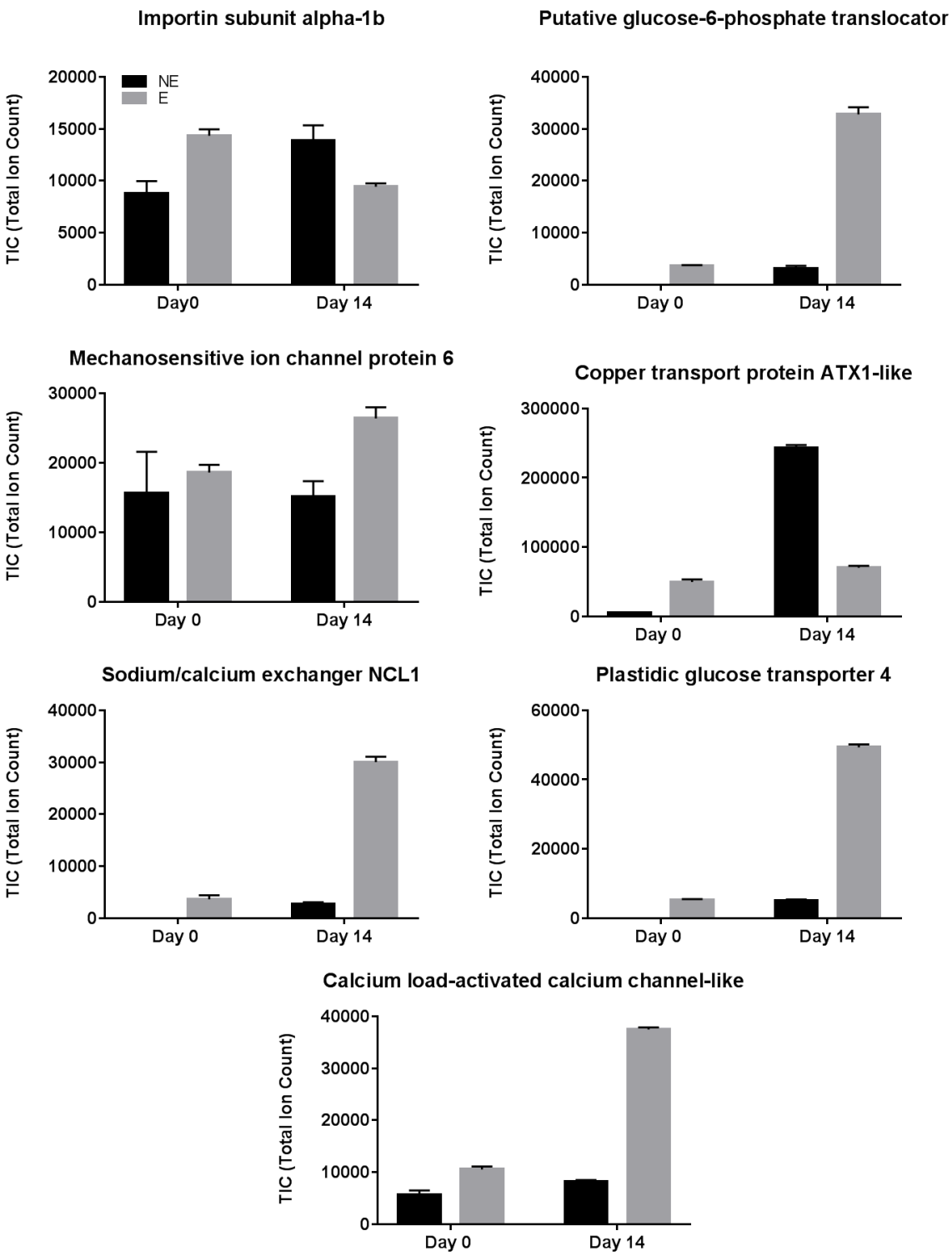

Figure 14. Differential abundance of proteins associated with ions, protein and carbohydrate transport. The abundances of importin subunit alpha-1b, putative glucose-6-phosphate translocator, mechanosensitive ion channel protein 6, copper transport protein ATX1-like isoform X1, sodium/calcium exchanger NCL1, plastidic glucose transporter 4 and calcium load-activated calcium channel-like are shown for both callus types on days 0 and 14 of the maturation treatment.

3.2.4.4. ATP and PPi specific hydrolysis activities (SA)

To evaluate the activity of these H^+ pumps during somatic embryo formation, we analyzed the hydrolytic activity of these pumps during maturation treatment in both types of callus. We observed that the ATP and PPi hydrolytic activity profiles changed during the maturation of embryogenic and non-embryogenic callus (Figure 15). There were no significant changes in P-H^+-ATPase hydrolytic activity in non-embryogenic callus during maturation treatment, while in embryogenic callus, ATP hydrolysis was stimulated at 14 days under maturation compared to time 0. For both time points, ATP hydrolysis by the P-H^+-ATPase was higher in embryogenic callus than in non-embryogenic callus (Figure 15A). V-H^+-ATPase hydrolysis was altered in both callus types, being reduced in embryogenic callus from time 0 to 14 days of culture and stimulated in non-embryogenic callus at 14 days of culture compared to time 0. V-H^+-ATPase hydrolytic activity was also higher at time 0 in embryogenic callus, while V-H^+-ATPase hydrolytic activity was higher at 14 days under maturation in non-embryogenic callus (Figure 15B). PPi hydrolysis was enhanced in both embryogenic and non-embryogenic callus at 14 days under maturation compared with time 0, especially in embryogenic callus that showed a significant increase in H^+-PPase hydrolytic activity. Embryogenic callus at time 0 showed lower PPi hydrolysis compared to non-embryogenic callus; however, at 14 days of culture, PPi hydrolysis was 1.5 times higher in embryogenic callus than that in non-embryogenic callus (Figure 15C).

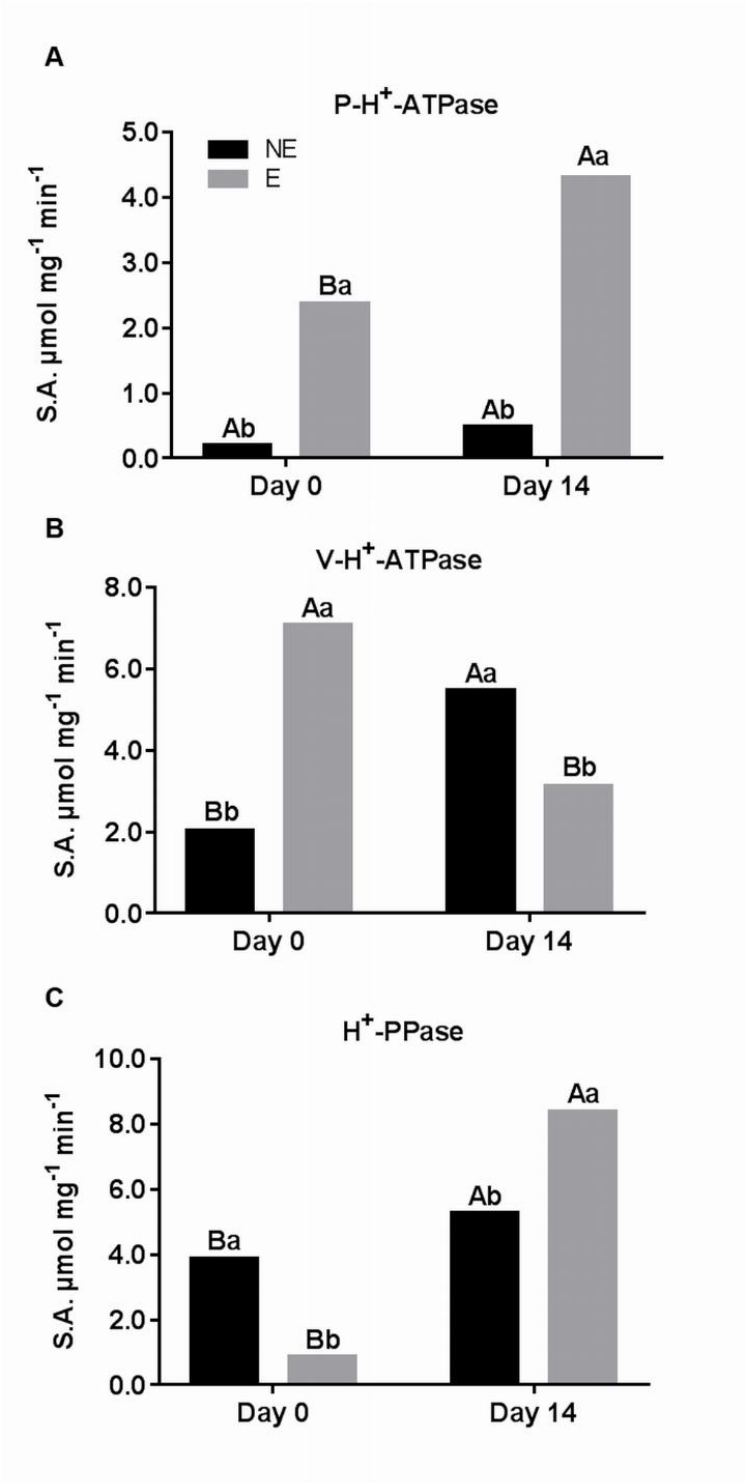

Figure 15. ATP or PPi specific hydrolytic activities (SA, µmol mg^{-1} min^{-1}) of H^+-ATPases or H^+-PPases in the microsomal fraction of embryogenic (E) and non-embryogenic (NE) callus at time 0 and at 14 days of culture in maturation treatment. Specific hydrolytic activities of plasma membrane H^+-ATPases (P-H^+-ATPase) (A), vacuolar H^+-ATPases (V-H^+-ATPase) (B), and vacuolar H+-pyrophosphatases (H^+-PPase) (C). Means followed by different letters indicate significant differences ($P < 0.05$) according to Tukey's test. Different capital letters indicate significant differences between the times of maturation (0 and 14 days) in each callus type (E or NE). Different lowercase letters indicate significant differences between callus type (E and NE) at each time (0 or 14 days) of culture in maturation treatment (n = 3).

3.2.4.5 Regulation of H^+ pumping activity

In addition to hydrolytic activity, we also analyzed H^+-pumping activity during maturation treatment. We observed enhanced H^+ transport by all 3 types of pumps at 14 days of culture compared to time 0 for both callus types except for P-H^+-ATPase in non-embryogenic callus, which showed no H^+ transport at 14 days of culture (Figure 16 and Table 5). Additionally, for all three pumps, H^+ transport was higher in embryogenic callus than in non-embryogenic at both time points, especially for the V0 of H^+-PPase in embryogenic callus, which showed 53% higher activity in embryogenic callus than in non-embryogenic callus at 14 days of culture (Figure 16C and Table 5).

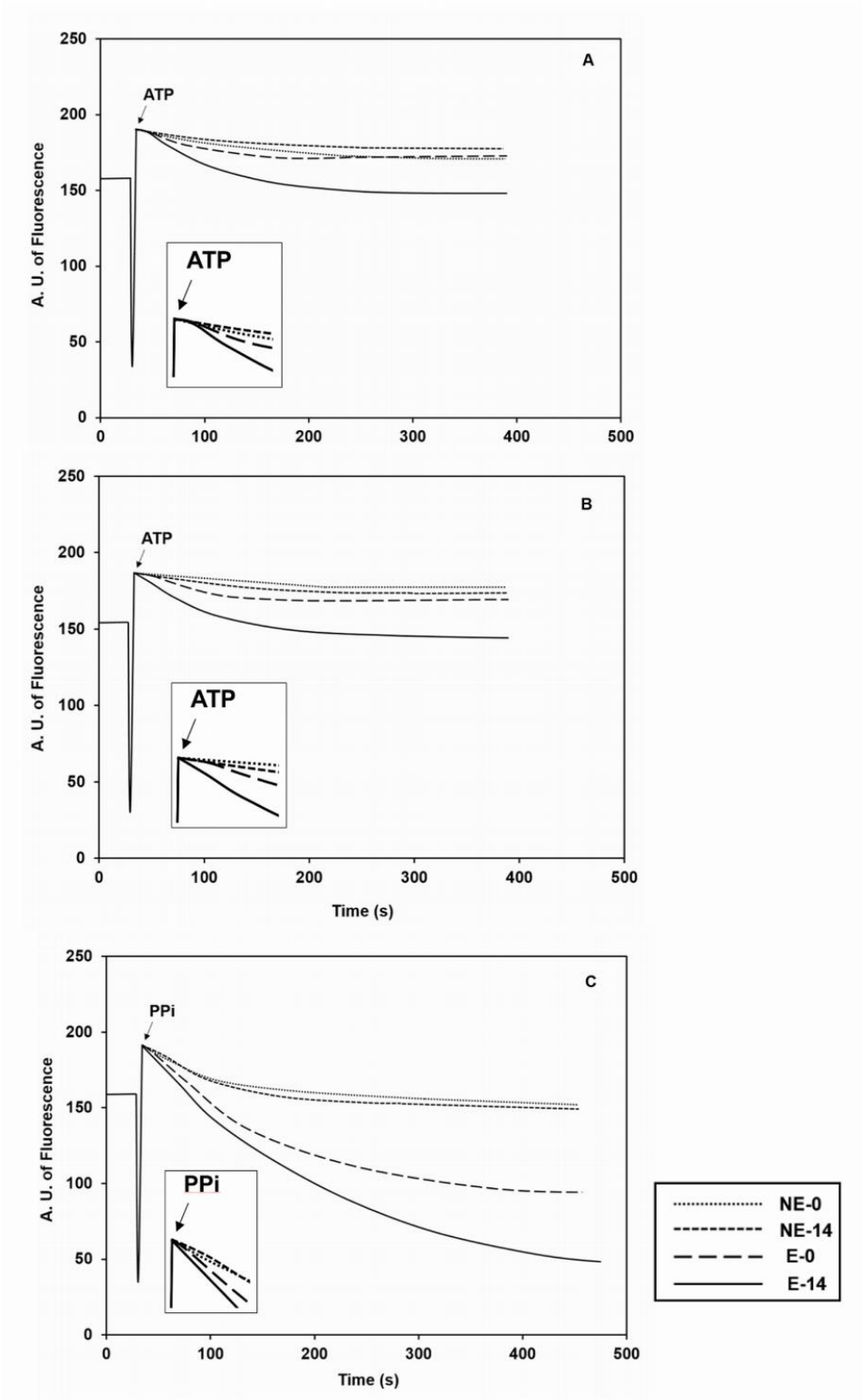

Figure 16. H^+ pumping activities of H^+-ATPases or H^+-PPases in the microsomal fraction of embryogenic (E) and non-embryogenic (NE) callus at time 0 and at 14 days of culture in maturation treatment. H^+-pumping activities of P-H^+-ATPase (A), V-H^+-ATPase (B) and H^+-PPase (C). H^+ translocation across membrane vesicles was monitored by fluorescence quenching of ACMA in the presence of 60 µg protein of membrane vesicles. The reaction was started by the addition of 1 mM ATP for P-H^+-ATPase and V-H^+-ATPase and 1 mM PPi for H^+-PPase and was stopped with 20 mM NH_4Cl (n = 3).

Table 5. Comparison of the initial velocity (V_0) of H^+ pumping activities and the difference between maximum fluorescence (ΔMF) of P-H^+-ATPase, V-H^+-ATPase and H^+-PPase in embryogenic and non-embryogenic sugarcane callus at time 0 and at 14 days of culture in maturation treatment.

	P-H^+-ATPase		**V-H^+-ATPase**		**H^+-PPase**	
	V_0 (%)	ΔMF (%)	V_0 (%)	ΔMF (%)	V_0 (%)	ΔMF (%)
NE-0	2.16 Ab*	6.32 Ab	1.31 Bb	4.21 Bb	9.92 Ab	16.85 Bb
E-0	4.78 Ba	7.32 Ba	4.07 Ba	7.69 Ba	14.45 Aa	50.0 Ba
NE-14	0 Bb	0 Bb	2.95 Ab	7.69 Ab	8.93 Ab	18.75 Ab
E-14	7.82 Aa	17.0 Aa	8.23 Aa	18.0 Aa	13.65 Aa	54.0 Aa

* Means followed by different letters indicate significant differences ($P < 0.05$) according to Tukey's test. Different capital letters indicate significant differences between the times of maturation (0 and 14 days) in each callus type (E or NE). Different lowercase letters indicate significant differences between the callus type (E and NE) at each time (0 or 14 days) of culture in maturation treatment (n = 3).

The regulation of H^+-pumping activity and hydrolytic activity results in alterations in the efficiency of H^+ pumps (Figure 17). The coupling rate of P-H^+-ATPase was higher in non-embryogenic callus at time 0, while no significant changes were observed in embryogenic callus (Figure 17A). The coupling rate of V-H^+-ATPase was higher in embryogenic callus at 14 days of culture, while no significant changes were observed in non-embryogenic callus, which showed a similar coupling rate to embryogenic callus at time 0 (Figure 17B). The higher H^+-PPase coupling rate appeared in embryogenic callus before incubation (14.45%) and was 5.2 times higher compared to that in non-embryogenic callus at the same time point. However, for both callus types, the coupling rate of H^+-PPase was lower at 14 days of culture, especially in embryogenic callus, which was 89.27% less efficient at 14 days than at time 0 (Figure 17C).

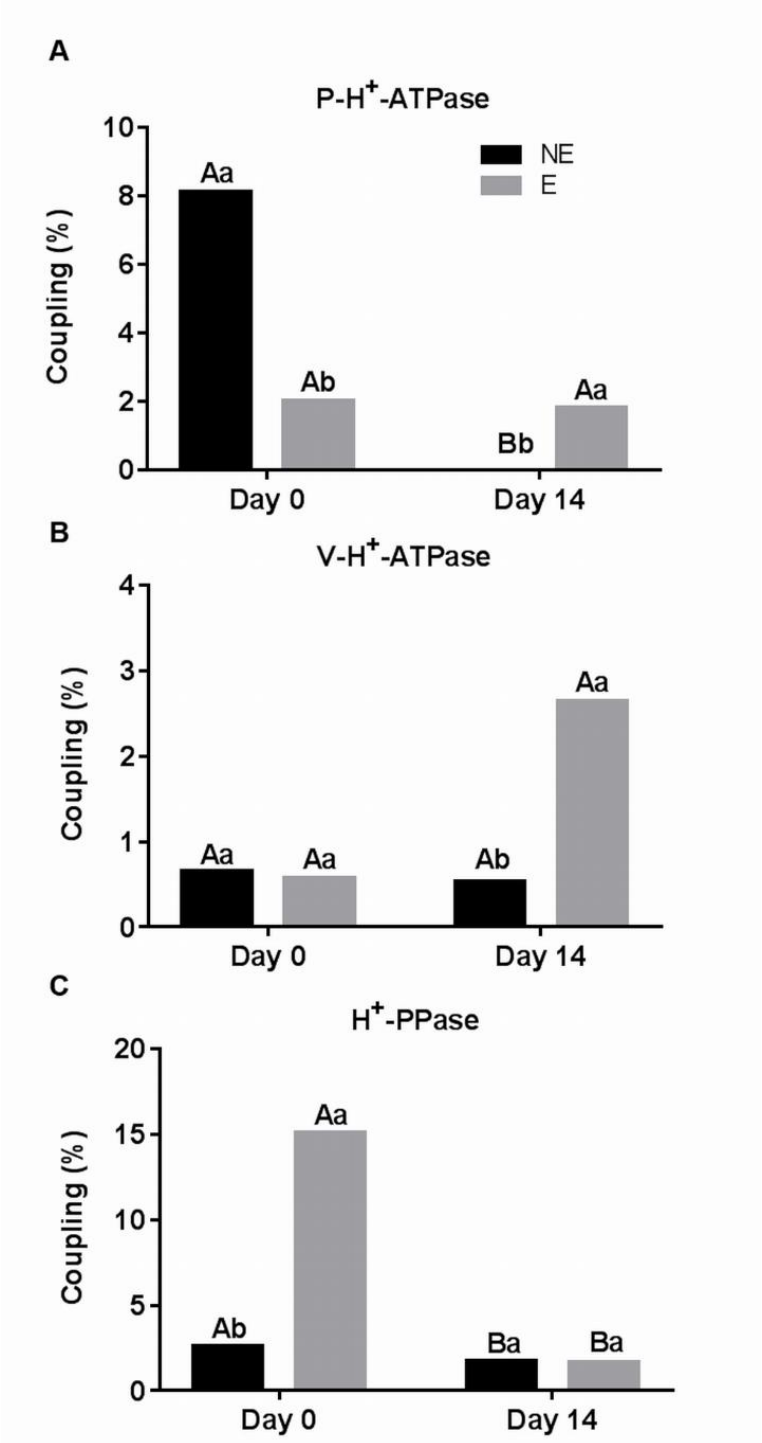

Figure 17. Coupling ratio (V_0 of H^+ pumping/ATP or PPi hydrolysis) of H^+-ATPases or H^+-PPases in microsomal fractions of embryogenic (E) and non-embryogenic (NE) callus at time 0 and at 14 days of culture in maturation treatment. Coupling ratio of P-H^+-ATPase (A), V-H^+-ATPase (B) and H^+-PPase (C). Means followed by different letters indicate significant differences ($P < 0.05$) according to Tukey's test. Different capital letters indicate significant differences between the times of maturation (0 and 14 days) in each callus type (E or NE). Different lowercase letters indicate significant differences between the callus type (E and NE) at each time (0 or 14 days) of culture in maturation treatment (n = 3).

3.2.5. DISCUSSION

Embryogenic and non-embryogenic callus types have been reported in somatic embryogenesis of sugarcane and are distinguished by their characteristic morphologies (Silveira et al., 2013). As expected, these two types of callus performed differently under maturation treatment, with significant competence for somatic embryo development (Figure 10, Table 3), and our results suggest that regulation of H^+ flux is directly associated with the difference in embryogenic competence in sugarcane callus.

We showed that H^+ flux was altered during maturation in both types of callus, but the most important change was observed in embryogenic callus, which presented a high H^+ influx during somatic embryo formation (Figure 11). Thus, our results suggest that the reduction of H^+ flux during maturation treatment in non-embryogenic callus (Figure 11A) can be associated with the observed absence of embryogenic competence. On the other hand, the high H^+ influx observed in embryogenic callus at 14 days of culture (Figure 11B) suggests that H^+ influx is associated with the production of the first somatic embryos. The alterations in P- and V-H^+-ATPase gene expression regulate H^+ flux across the plasmatic and vacuolar membranes (Sze et al., 1999), which is important for several physiological processes, including root development (Fan e Neumann, 2004; Ramos et al., 2015), photosynthesis (Sacksteder et al., 2000; Avenson et al., 2005) and zygotic embryogenesis (Thavarungkul, 1997). Particularly in regard to embryogenesis, H^+ flux (both influx and efflux) is essential for embryo, somatic and zygotic polarized growth (Rathore et al., 1988). H^+ influx is also thought to be associated with the maintenance of embryogenic competence in *Daucus carota* callus (Gorst et al., 1987) via metabolite transport and/or polar development of callus during embryo somatic formation. Additionally, the observed high H^+ influx may be associated with high carrier transporter activity, especially calcium and carbohydrate transporters that are more abundant in embryogenic callus (Figure 14).

The regulation of H^+ flux (both influx and efflux) also affects intra- and extracellular pH. Changes in intracellular pH can be associated with a transition from a somatic to an embryogenic cell state (Feher et al., 2003). The alkalinization of cytosolic pH has been suggested to influence cell division (Pichon e Desbiez, 1994),

while differentiation is related to the acidification of cytosolic pH and alkalinization of extracellular pH. Alkaline medium pH is associated with high somatic embryo production in several species (Chung et al., 2006; Ma et al., 2010; Yuan et al., 2012; Chung et al., 2016). It has been shown that somatic embryo formation is interrupted by highly acidic medium pH (<4) (Chung et al., 2006). In addition, small variations in pH are commonly required for the successful production of somatic embryos, in which the highest variation observed is 0.5 pH units in species such as *Primulina tabacum* (Ma et al., 2010), *Brassica oleracea* (Yuan et al., 2012), and Musa spp. (Chung et al., 2016) Maintaining pH with minimal variations is a result of cellular ionic homeostasis (Steinacher et al., 2012), which regulates several physiological processes (Pittman, 2012). We observed that, compared to embryogenic callus, non-embryogenic callus showed higher variations in extracellular pH (Figures 9C and 9D), thus demonstrating that embryogenic callus more effectively maintains ionic homeostasis, with a direct effect on somatic embryo formation in sugarcane.

The difference in ionic homeostasis between embryogenic and non-embryogenic sugarcane callus can be associated with the differential activity of H^+ pumps, especially P-H^+-ATPase, which proved to be incapable of H^+ transport in non-embryogenic callus at 14 days of culture (Figure 16 and Table 5). P-H^+-ATPase activity generates a potential difference across the plasma membrane that is crucial for maintaining ion homeostasis (Morsomme e Boutry, 2000) and alleviates the stress caused by several abiotic conditions (Bose et al., 2013; Morgan et al., 2014; Zhang et al., 2017). Additionally, the knocking out P-H+-ATPase genes reduced the development of Arabidopsis seedlings grown under stress conditions, highlighting the importance of P-H^+-ATPase activity for seedling development in response to abiotic stress (Zhang et al., 2017). Given that in vitro culturing induces moderate stress, the absence of P-H^+-ATPase H^+ transport in nonembryogenic callus (Figure 16 and Table 5) may be responsible for the lack of embryo development under maturation treatment in this callus type. In contrast, embryogenic callus showed high P-H^+-ATPase H^+ transport, especially at 14 days of culture (Figure 16 and Table 5), suggesting that this pump regulates ionic balance and allows somatic embryo production. We also observed that the 14-3-3 protein, which binds the C-terminal region of P-H^+-ATPase and activates this enzyme (Jahn et al., 1997), was not differentially accumulated during maturation, suggesting that the regulation of this

pump indeed occurs at the protein level, as observed by the increased abundance of P-H^+-ATPase in embryogenic callus on day 14.

V-H^+-ATPase activity and coupling rate are regulated by lumen and cytosolic pH (Davies et al., 1994; Dietz et al., 2001). As observed in the present work, the high H^+ influx observed in the embryogenic callus at 14 days of culture (Figure 11B) leads to an accumulation of H^+ in the cytoplasm, which increases cytosolic pH and results in increased V-H^+-ATPase transport of H^+ into the vacuole (Figure 17) to maintain ionic homeostasis.

Although increased V-H^+-ATPase efficiency was observed in embryogenic callus, only low hydrolytic activity was observed in both types of callus under maturation treatment (Figures 14 and 15). Similar results were observed in proembryogenic masses of *Araucaria angustifolia*, suggesting that callus development is not dependent on V-H^+-ATPase activity (Dutra et al., 2013). Additionally, low V-H^+-ATPase activity can be compensated by high H^+-PPase activity, especially in young tissues, which have a higher H^+-PPase activity compared with mature tissues (Martinoia et al., 2006). H^+-PPase activity has been reported to be necessary for embryo development (Krebs et al., 2010), which agrees with the high H^+ transport activity of this pump in embryogenic callus compared to non-embryogenic callus (Figure 16 and Table 5) observed in the present work. In addition, the high abundance of H^+-PPase (Table 4) suggests the regulation of this H^+ pump at the protein level. The preference of H^+-PPase accumulation in relation to V-H^+-ATPase in embryogenic callus may be associated with energy economy because H^+-PPase is composed of a single polypeptide, whereas V-H^+-ATPase is more complex, being formed by various subunits (Martinoia et al., 2006).

Differences in vacuolar pH between embryogenic and non-embryogenic cells have been reported previously in *Medicago sativa*, in which more-acidic vacuoles are associated with lytic function, whereas less-acidic vacuoles have a storage function (Pasternak et al., 2002b). Thus, the high H^+-PPase activity observed in sugarcane embryogenic callus suggests that this callus contains a more-acidic vacuole, which is a characteristic of callus with embryogenic competence.

PPi is generated through the metabolism of important molecules such as DNA, RNA, and proteins (Martinoia et al., 2006), and young tissues are known to have high metabolic activity, resulting in the high production of these molecules and, consequently, high PPi production (Nakanishi e Maeshima, 1998). Post-germinative

growth in *Arabidopsis thaliana* generates elevated PPi concentrations, inducing high H^+-PPase hydrolytic activity (Ferjani et al., 2011). Like post-germinative events, somatic embryo formation may induce high PPi production in embryogenic callus due to its high metabolism, thereby increasing cytosolic PPi concentrations and inducing a higher H^+-PPase hydrolytic activity, as observed here (Figure 15), suggesting that PPi is more-efficiently converted to Pi is in embryogenic callus than that in non-embryogenic callus.

Efficient PPi metabolism is important for maintaining PPi homeostasis and plant development, as suggested by the H^+-PPase loss-of-function *A. thaliana* mutants that exhibit growth inhibition due to excessive PPi accumulation (Fukuda et al., 2016), which has been associated with the inhibition of several metabolic processes, especially sucrose synthesis (Ferjani et al., 2012) and auxin metabolism (Li et al., 2005). In our study, we observed a high H^+-PPase hydrolytic activity in embryogenic callus during somatic embryo production (Figure 15), which may regulate sucrose and auxin metabolism in this callus. Both compounds have been associated with somatic embryogenesis in several species (Iraqi e Tremblay, 2001; Karami et al., 2006; Su et al., 2009; Xu et al., 2013), suggesting that the correct production of these metabolites is essential for somatic embryo formation. In particular for sucrose metabolism, high H^+ influx (Figure 11) coupled with increased levels of carbohydrate transporters (Figure 14) suggests an influx of carbohydrates into embryogenic cells and, consequently, greater activation of these metabolic pathways during somatic embryo formation.

Finally, based on our results, we suggest that the relationship between proton flux and somatic embryo formation in sugarcane occurs through the regulation of six main processes: (1) cytosolic PPi accumulation due to the high metabolic activity of embryogenic cells; (2) PPi hydrolysis and release of Pi through elevated H^+-PPase activity; (3) high H^+ influx through increased abundance and activity of plasma membrane transporters; (4) cytosolic acidification due to an increase in H^+ influx, resulting in increased H^+-pumping activity by H^+-PPase and acidification of the vacuole; (5) similar to H^+-PPase, P-H^+-ATPase also increases its H^+-pumping activity, generating an electrochemical gradient that drives the operation of secondary transporters; and (6) secondary transporters internalize important compounds such as ions and carbohydrates to maintain the high metabolic activity as a result of embryo development (Figure 18).

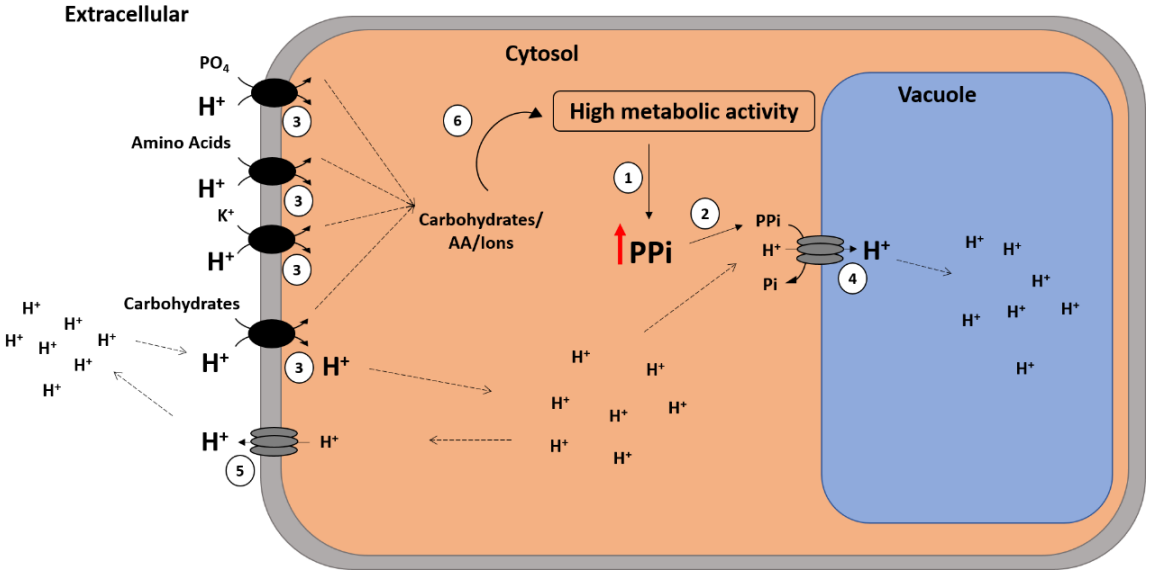

Figure 18. Proposed scheme of the relationship between proton flux and somatic embryo formation in sugarcane. 1) High formation and accumulation of PPi in the cytosol; 2) H^+-PPase increases hydrolytic activity; 3) high H^+ influx; 4) increased H^+ pumping activity of H^+-PPase; 5) increased H^+ pumping activity of P-H^+-ATPase; and 6) secondary transport of important compounds such as ions, amino acids and carbohydrates maintains high metabolic activity.

3.2.6. CONCLUSION

Based on the results presents in this chapter, the following conclusion:

- high H^+ influx was observed in embryogenic callus during maturation treatment and can be associated with embryogenic competence;
- Microsomal proteomics showed that this high H^+ influx is due to the accumulation of H^+ pumps, especially P-H^+-ATPase and H^+-PPase;
- H^+-pump transport and hydrolytic activity, especially P-H^+-ATPase and H^+-PPase, is essential for maintaining ionic homeostasis;
- H^+-PPase is the major plant H^+ pump during somatic embryo formation, functioning both to transport H^+ into the vacuole and to hydrolyze PPi;
- The regulation of H^+-PPase occurs at the protein level, with embryogenic callus showing higher H^+-PPase levels than non-embryogenic callus at 14 days of culture in maturation treatment;

4. FINAL COMMENTS

In this work we advance the understanding of maintence of embryogenic competence in embryogenic callus of sugarcane, and we highlight the possible relationship between the long-term with 2,4-D and the loss of embryogenic competence. The next steps related to this work will be the evaluation of the viability of the somatic embryos formed as well as the analysis of the seedlings obtained through somatic embryogenesis in order to identify possible morphological and molecular alterations resulting from somaclonal variations. Finally, new studies will be fundamental for a better understanding of the effects caused by long-term with 2,4-D and these results may be used to optimize the process of somatic embryogenesis in sugarcane.

In addition, we also advance the understanding of the process of somatic embryo formation in sugarcane, obtaining unpublished results that demonstrate a possible relationship between the flow of H^+ and the formation of the somatic embryo. The next steps related to this work will be the analysis of the participation of these proteins in the process of somatic embryogenesis through molecular or biochemical inhibition of the H^+ pumps, to prove the importance of these in the different stages of embryo development. New studies focusing on this relationship between H^+ flow and somatic embryo formation will be important both to increase understanding of important molecular pathways in somatic embryo development as well as to optimize somatic embryogenesis process in sugarcane.

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