DÉBORA DE OLIVEIRA PRUDENTE

SOMATIC EMBRYOGENESIS, ANTIOXIDANT METABOLISM AND RESERVE MOBILIZATION DURING CRYOPRESERVATION OF *Passiflora ligularis* Juss. ZYGOTIC EMBRYOS

LAVRAS - MG

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Thesis presented to the Federal University of Lavras, as part of the requirements of the Plant Physiology Graduate Program to obtain the Doctor degree.

Advisor:

Renato Paiva, Ph.D.

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APPROVED on July 17, 2017

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BIOGRAPHY

DÉBORA DE OLIVEIRA PRUDENTE, daughter of Sebastião Prudente and Maria Lúcia de Oliveira Prudente, was born on november 7, 1990 in Barbacena - MG. She attended elementary school at the Abgar Renault Municipal School and the high school at the Instituto Tenente Ferreira (ITF) in Barbacena - MG, concluding her high school in 2008. In march 2009, she began her undergraduate course in biological sciences, at the Federal University of São João Del Rei (UFSJ), São João Del Rei-MG, finishing in april, 2013. During this period, she supervised the disciplines of plant physiology and plant anatomy, developing research projects under the supervisor of profª Fernanda Carlota Nery, as a scientific initiation fellow of the National Council for Scientific and Technological Development (CNPq) for three consecutive years. In May 2013, she began a master's degree in the Plant Physiology Graduate Program (PPGFV) at Federal University of Lavras (UFLA), Lavras-MG, where she carried out research in the area of micropropagation and cryopreservation of native Brasilian Cerrado species under the supervisor of prof. Renato Paiva, finishing it in august 2014. In september of the same year, she joined the doctorate in the PPGFV at UFLA, under the supervisor of Prof. Renato Paiva, where she participated in the following year of the Doctorate Sandwich Program-PDSE of Coordination of Improvement of Higher Level Personnel (CAPES), in Katholieke Universiteit Leuven (KULeuven), Belgium, under supervisor of the researchers: Bart Panis and Sebastien Carpentier. In april 2017, she received a student award at the "7th International Symposium on Production and Establishment of Micropropagated Plants", promoted by International Society for Horticultural Science (ISHS) for the best poster presentation. She completed her doctorate on July 17, 2017.

"Without dreams, life has no brightness.
Without goals, dreams have no objective.
Without priorities, dreams don't come true"

- Augusto Cury

GENERAL ABSTRACT

Passiflora ligularis Juss. is an endemic species from South America with important medicinal properties. The sexual propagation of *P. ligularis* present non-uniformity, extended dormancy and your conservation in conventional storage banks is limited by the loss of germination potential. The objectives of this work are obtain plants via somatic embryogenesis from zygotic embryos, characterizing the origin of somatic embryos, and evaluate the influence of exposure time to the cryoprotectant PVS2 on the mobilization of reserves and on antioxidant metabolism during the germination of cryopreserved *P. ligularis* zygotic embryos. The results demonstrate that the highest frequency of somatic embryo formation was observed on a culture medium supplemented with 27.2 µM 2,4-diclorophenoxyacetic acid + 4.5 µM 6-benzyladenine. The complete independence of somatic embryos from the adjacent tissues was histologically confirmed by the absence of vascular continuity, after 60 days. It was verified an increase in the activity of key enzymes for the glyoxylate cycle, malate synthase (Msy) and isocitrate lyase (ICL), and changes in antioxidant metabolism after exposure of zygotic embryos to 60 min of PVS2 before cryopreservation, accelerating the germination in *P. ligularis*. This study describes a complete micropropagation route for *P. ligularis* and moreover shows the precise origin of embryogenic cells. The PVS2 vitrification technique was successfully used to cryopreserve *P. ligularis* zygotic embryos.

Keywords: Sweet granadilla, Histological analysis, Plant regeneration, Biotechnology, Long-term storage, Cryopreservation

RESUMO GERAL

Passiflora ligularis Juss. é uma espécie endêmica da América do Sul com importantes propriedades medicinais. A propagação sexuada de *P. ligularis* apresenta não-uniformidade, dormência prolongada e sua conservação em bancos convencionais de armazenamento é limitada pela perda do potencial germinativo. Objetivou-se com este trabalho obter plantas via embriogênese somática a partir de embriões zigóticos, caracterizando a origem dos embriões somáticos, e avaliar a influência do tempo de exposição ao crioprotetor PVS2 na mobilização de reservas e no metabolismo antioxidante durante a germinação de embriões zigóticos criopreservados de *P. ligularis*. Os resultados demonstram que a maior freqüência de formação de embriões somáticos foi observada no meio de cultura suplementado com 27,2 μM de ácido 2,4-diclorofenoxiacético + 4,5 uM 6-benziladenina. A independência completa dos embriões somáticos dos tecidos adjacentes foi confirmada histologicamente pela ausência de continuidade vascular, após 60 dias. Verificou-se um aumento na atividade das principais enzimas para o ciclo do glioxilato, malate sintase (Msy) e isocitrato liase (ICL) juntamente com alterações no metabolismo antioxidante após exposição de embriões zigóticos a 60 min de PVS2 antes da criopreservação, acelerando a germinação em *P. ligularis*. Este estudo descreve uma rota de micropropagação completa para *P. ligularis* e, além disso, mostra a origem precisa de células embriogênicas. A técnica de vitrificação PVS2 foi utilizada com sucesso para criopreservar embriões zigóticos *P. ligularis.*

Palavras-chave: Sweet granadilla, Análises histológicas, Regeneração de plantas, Biotecnologia, Armazenamento em longo prazo, Criopreservação

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FIRST PART

1 INTRODUCTION

The family Passifloraceae Juss. EX Roussel, comprises 18 genera and about 630 species (SACCO, 1980; ROSA; BELLO; DORNELAS, 2015). The genus *Passiflora* L. stands out for its more than 400 species, with more than 90% distributed mainly in South America (VANDERPLANK, 2000; TIWARI et al., 2015). The economic representativeness of this genus has been increasing, and the countries responsible for about 80% of the production are: Brazil, Colombia, Peru, Ecuador and Venezuela (ARIAS SUÁREZ; OCAMPO PÉREZ; GÓMEZ, 2016). Its commercialization is based on the export of fresh fruit, pulp or concentrated juice, and the main importers are European countries, such as the Netherlands, Belgium and Germany, the United States, Japan and Argentina (ARIAS SUÁREZ; OCAMPO PÉREZ; GÓMEZ, 2016). There is also a great interest in the medicinal properties attributed by literature to the genus, as anxiolytic, sedative, anti-inflammatory, antihypertensive and diuretic (DHAWAN; DHAWAN; SHARMA, 2004; RAMOS et al., 2007; RUDNICKI et al., 2007; LIMA et al., 2012).

In Brazil, cash crops are mainly based on two *Passiflora* L. species: *Passiflora edulis* Sims and *P. alata* Curtis (SALOMÃO et al., 2002). Studies on Passifloraceae-based phytotherapeutic compounds are concentrated on *P. incarnata* L. and *P. alata*, official species of the Brazilian and European Pharmacopoeia, respectively (SOARES et al., 2012). *Passiflora ligularis* Juss., commonly known as “Sweet Granadilla”, native from Indonesia, New Guinea, Jamaica, Sri Lanka, India and other tropical regions, including Brazil, is one of the species from genus *Passiflora* L. which has been outstanding in South America in recent decades (OCAMPO; ARIAS; URREA, 2015). It has large participation in the international market of exotic fruits, and it is widely cultivated and commercialized in Colombia and Peru (BRACK EGG et al., 1985, SUÁREZ et al., 2016). In Brazil, there are few technified plantations of this species, with a high cost to the consumer (SOUZA; AOYAMA; FURLAN, 2015). This can be justified by the fact that their propagation occurs

mainly through sexual reproduction, and the main problems are non-uniformity in germination, extended dormancy and diseases, such as *Cowpea aphid-borne mosaic virus* - CABMV, leading to low quality of seedlings and fruits (MANICOM et al., 2003; HERNÁNDEZ et al., 2012; SOUZA et al., 2015).

In this context, the application of plant tissue culture through micropropagation techniques has become an important tool to propagate elite genotypes, in reduced physical space and time, seeking high yields and/or plants resistant to diseases and pests (STROSSE et al., 2003; SHAHZAD et al., 2017. Among micropropagation techniques, somatic embryogenesis is based on isolated cells or a small group of somatic cells that give rise to embryos. Somatic embryogenesis offers a number of advantages over other *in vitro* propagation methods, including, high multiplication rate; elimination of the dependence on specific availability periods of the propagating material, allowing to set the desired period for the obtention of embryos; production of synthetic seeds and conservation of genetic resources in long-term, through cryopreservation techniques (STROSSE et al., 2003; MATSUMOTO, 2017).

Long-term conservation has become an important tool to maintain species diversity as an efficient way to reduce costs and losses common to conventional seed banks (DULLOO et al., 2009). Given the growing concern with the *in vitro* conservation of plant material, cryopreservation techniques, which conserve the living biological material over a long period at ultra-low temperature (-196 °C) (ENGELMANN, 2011), can ensure survival and subsequent growth of these species after exposure to liquid nitrogen (LN) (DULLOO et al., 2009). The storage of *P. ligularis* zygotic embryos in seed banks is limited by losses in germination potential (SALAZAR, 2000). In order to preserve *P. ligularis* zygotic embryos, every effort is made to know the physiological and biochemical factors that may influence their development after the use of cryoprotectants, such as *Plant Vitrification Solution* – PVS, and the sequential cryopreservation steps. In addition, studies

on the *in vitro* propagation of *P. ligularis* are insufficient, contributing to the lack of an adequate protocol for long-term *in vitro* conservation of the species.

The work aims were to obtain plants via somatic embryogenesis from zygotic embryos, characterizing the origin of somatic embryos; besides evaluate the influence of exposure time to the cryoprotectant PVS2 on the reserve mobilization and also on antioxidant metabolism during germination of cryopreserved *P. ligularis* zygotic embryos.

2 LITERATURE REVIEW

2.1 *Passiflora ligularis* Juss.

Passiflora ligularis Juss. belongs to the family Passifloraceae Juss. EX Roussel and the genus *Passiflora* L. (MORTON, 1987), and is a highlight in the agricultural sector of South America (Figure 1), due to its medicinal and food properties, exhibiting a considerable amount of vitamin C and crude fiber (SARAVANAN; PARIMELAZHAGAN, 2014).

Figure 1 Immature and mature fruit of *Passiflora ligularis* Juss. with flower details. Image source: John Ocampo.
UFLA/ Lavras, MG. 2017

Fruit peels have high molecular weight polysaccharides such as xylose, glucose, galactose, galactosamine and fructose, which ensure the fruit is not as acidic as those of commercial species, such as *P. edulis* (TOMMONARO et al., 2007). In addition to their popular use, as leaf infusions or direct pulp ingestion, acting in the control of fever, renal calculi, tranquilizer, antioxidant potential and antimicrobial agent (CERDAS ARAYA; CASTRO RETANA, 2003; SARAVANAN; PARIMELAZHAGAN, 2014). Studies have proved that the isolated compound of *P. ligularis* pulp was significantly effective against Gram-Positive *Staphylococcus aureus* and Gram-Negative *Proteus vulgaris*, when compared to the methanolic extract of its leaves and the standard drug Ciprofloxacin, under similar conditions (KANNAN et al., 2011).

Even though it is a crop with a high economic and medicinal potential to be exploited, its large-scale production has been carried out through traditional agricultural techniques, leading to the formation of heterogeneous crops, requiring the excessive use of pesticides, which have caused reduction in fruit production and quality (ARIAS SUÁREZ; OCAMPO PÉREZ; GÓMEZ, 2016). Another difficulty for technified production is the species requirement for colder climates, with a minimum germination temperature of 13 ºC to 22 ºC, demanding a treatment with low temperatures to break dormancy (HERNÁNDEZ et al., 2012), like stratification for 200 hours at 5 ºC (SANTOS ALVORADO et al., 1994), or maintenance in a cold chamber for a period longer than 101 days (SOUZA et al., 2015), which results in a wide variation in germination percentage, from 20% to 52% (SALAZAR 2000; GUTIÉRREZ et al., 2011).

In order to maximize the production of healthy seedlings in a short period of time, studying alternative methods for *P. ligularis* propagation becomes necessary taking into account there are no reports in literature.

2.2 Somatic embryogenesis

Among the main plant tissue culture techniques, micropropagation using somatic embryogenesis occurs when somatic embryos originate from matrix tissues, in a morphogenetic sequence that approximates the events representative of zygotic embryogenesis (TAUTORUS; FOWKE; DUNSTAN, 1991). Somatic embryogenesis can occur through the direct model, in which somatic embryo differentiation occurs without intermediate stages, which are cell mass clusters, called callus; or by indirect model, in which embryos differ from callus (ZIMMERMAN, 1993; SHAHZAD et al., 2017). The processes involved in somatic embryogenesis induction can be divided into four phases: (i) induction in culture medium containing growth regulators; (ii) multiplication in culture media containing auxins at low concentration; (iii) maturation in presence of ABA and/or osmotic agents; and (iv) germination in culture media free from phytoregulators (TAUTORUS et al., 1991; PULLMAN; BUCALO, 2014).

This process has advantages in comparison to other techniques used in plant micropropagation, such as production of a large number of embryos from a single explant; uniformity with the formation elite genotype clones; use as an integrated tool for *in vitro* conservation programs of plant material, especially when associated with cryopreservation techniques (MERKLE; DEAN, 2000). It is known that the induction and control of somatic embryogenesis protocol is linked to different questions, like, source and physiological stage of explants, genotype of the mother plant, the culture medium used and time in which the growth regulators are maintained in the culture medium (SHAHZAD et al., 2017). Among the difficulties presented in the literature for application of somatic embryogenesis for *Passiflora* species are the lack of synchronization in the processes of embryo maturation and low germination and acclimatization rates of somatic embryos. Recently, protocols for somatic embryogenesis from zygotic embryos of *Passiflora* species have been reported (SILVA et al., 2009; PINTO et al., 2010; ROCHA

et al., 2012; FERREIRA et al., 2015; SILVA, 2015). The development of a protocol for somatic embryogenesis in *P. ligularis* will also significantly contribute to *in vitro* propagation of the species and for future protocols of cryopreservation and genetic transformation, since seedling regeneration by this morphogenetic pathway has not yet been described.

2.2.1 Histological analysis

From the formation of embryogenically competent cells, it is possible to observe the proliferation of Pro-Embryogenic Masses (PEM), defined as cell clusters capable of producing somatic embryos (CANGAHUALA-INOCENTE et al., 2004). These cells have ability to divide continuously, forming Embryogenic Masses (EM) (CANGAHUALA-INOCENTE et al., 2004). EM resemble meristematic cells, since they have isodiametric cells of reduced size. They are rich in amyloplasts where a dense cytoplasm predominates with an organized cellular system, microvacuoles and large nucleus stained together with the nucleolus (DODEMAN; DUCREUX; KREIS, 1997).

The analyses that allow to follow the sequence of events that culminate with the differentiation of PEM in EM, are mainly those histologically conventional, with the use of dyes, such as Toluidine Blue, that acts by identifying isodiametric cells with evident nuclei and nucleoli through the blue and violet coloration (O'BRIEN; FEDER; MCCULLY, 1964). In order to determine the origin and location of embryogenically competent cells, these analyses may support molecular studies that will investigate into the initial somatic cell differentiation events for embryogenesis (GUZZO et al., 1994; SCHMIDT et al., 1997). It is the key to increasing the methodological efficiency of this technique for more species, and to unravel even more about this important path for large-scale propagation.

2.3 Cryopreservation techniques

In vitro conservation consists in the maintenance of living biological material under aseptic conditions and can be carried out in the medium or long term (PINHAL et al., 2011; SHAHZAD et al., 2017). In medium-term conservation, it is possible to highlight the slow-growing technique, in which propagules are kept at reduced temperatures (4 °C - 15 °C) with low luminous intensity and culture medium containing osmotic agents (LÉDO et al., 2014). Cryopreservation, a method that guarantees the viability and genetic integrity of plant material for a long period of time, through its ultra-low temperature (-196 ºC) stands out for long-term conservation (MATSUMOTO, 2017).

Cryopreservation can be used for the conservation of different types of explants: seeds, cell suspensions, embryogenic callus, stem apices, lateral and axillary buds, zygotic and somatic embryos (BENSON, 2008). Cryopreserved material remains protected from contamination in small physical spaces and requires minimal maintenance in comparison to conventional storage methods, such as cold storage, germplasm banks and slow growth *in vitro* conservation (SANTOS, 2004; DULLOO et al., 2009).

For most *Passiflora* species, however, the lack of protocols for *in vitro* cultivation has not yet allowed the extensive use of this germplasm conservation technique. Most of the species of *Passiflora* L. genus have intermediate or orthodox seeds, which tolerate dehydration at relatively low levels, but are damaged by temperatures below zero when dry (HONGS; ELLIS, 1996; PACHECO et al., 2016). The species *P. edulis* and *P. ligularis* were described with intermediate and orthodox behavior, respectively (LIU et al., 2008; GONZÁLEZ-BENITO et al., 2009), both of which had their germination decreased after dried (OSPINA et al., 2000). In particular, the establishment of protocols for cryopreservation and subsequent explant survival is dependent on application of the most appropriate technique used for size and water content in plant material (VEIGA-BARBOSA et al., 2013). Thus, the choice of a method that allows a rapid cooling and, at the same time,

leads to the direct transition of water from the liquid state to an amorphous or vitreous solid state, called vitrification, can ensure the reduction in crystallization (MAZUR, 1984; BENSON, 2008).

Among the main cryopreservation techniques are slow cooling, characterized by the use of low concentrations of cryoprotectants and gradual temperature reduction, controlled by a programmable freezer (NAIK et al., 2005). The classical vitrification technique – PVS2 vitrification, which is based on the increase of solution viscosity, using highly concentrated cryoprotectant solutions (such as PVS2), followed by ultrafast cooling of explants, wherein solidification occurs without crystallization (PANIS; PIETTE; SWENNEN, 2005; PANIS; SAKAI; ENGELMANN, 2007; KAVIANI, 2011; FONSECA et al., 2012). Droplet vitrification method (SCHÄFER-MENUHR; SCHUMACHER; MIX-WAGNER, 1994) – which differs from PVS2 vitrification, since the samples are placed on a small sterilized aluminum foil. One PVS2 drop is placed on samples and all aluminum foil is dipped in LN (WESLEY-SMITH et al., 2001).

Encapsulation-dehydration is a technique in which explants are encapsulated in alginate matrix, pre-cultured in a medium containing high concentrations of sucrose, dehydrated by exposure to air in a laminar flow chamber or drying in a container with silica gel, reducing the water content to 20% to 30%, followed by rapid immersion in LN (PANIS; PIETTE; SWENNEN, 2005). Encapsulation-vitrification is a combination of encapsulation-dehydration and vitrification methods, in which the explants are encapsulated in an alginate matrix, transferred to cryotubes, exposed to cryoprotectant solutions and rapidly frozen in LN (SAKAI; ENGELMANN, 2007).

Such techniques seek to minimize cellular damage, and consequently increase the regeneration rate of explants (SKRLEP et al., 2008; CHEN et al., 2011; LAMBARDI; OZUDOGRU; BENELLI, 2008). Thus, cryopreservation becomes a promising tool for the successful creation of germplasm banks, in

which cryopreserved plant material will be readily available for regeneration (MATSUMOTO, 2017).

2.4 Biochemical cryopreservation aspects

Cryopreservation techniques have been used to store live biological material, as zygotic embryos, for long periods (CEJAS et al., 2012). However, during the sequential stages of cryopreservation, the plant material is exposed to some cryoprotectant solutions such as Loading Solution - LS; Plant vitrification solution - PVS; and Recovery Solution - RS, which basically consist of distilled water, high concentrations of sucrose, MS basal salts, ethylene glycol, glycine and dymethylsulfoxide- DMSO (SAKAI; KOBAYASHI; OIYAMA, 1990). All these compounds in contact with the intracellular space for a certain period can lead cells to osmotic and oxidative stress and thus damage the tissues, making it impossible to regenerate the explants. Nevertheless, all the factors affecting the success of cryopreservation, especially biochemicals with important implications for plant material survival, are not yet known (BENSON, 2008).

Stress factors such as dehydration from vitrification solutions, as well as freezing and thawing, can induce the production of *Reactive Oxygen Species* (ROS) (SHOHAEL et al., 2006; SUN et al., 2010). As a consequence of aerobic life, ROS are formed by the partial reduction of molecular oxygen (CASSELLS; CURRY, 2001). Oxidative stress conditions are characterized by the accumulation of different ROS: superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Under normal physiological conditions, there is a balance between the formation of ROS and the protective antioxidant mechanisms of cells (ARORA et al., 2002; VINOCUR; ALTMAN, 2005). In plant cells, ROS can be formed preferentially in mitochondria, chloroplasts and peroxisomes (SHOHAEL et al., 2006). On the other hand, these organelles act in an integrated way to overcome the situation of oxidative stress and to maintain cellular redox

balance (AHMAD et al., 2008). When this balance is disrupted, the increase in ROS can induce cell damage and even leads to cell death (CASSELLS; CURRY, 2001; SCANDALIOS, 2005). It is known that increased levels of ROS can cause damage to biomolecules such as DNA and proteins (GILL; TUTEJA, 2010) and, by reacting with membrane lipids, a process called lipid peroxidation can occur, which may trigger programmed cell death (SINGH et al., 2011), shown in figure 2.

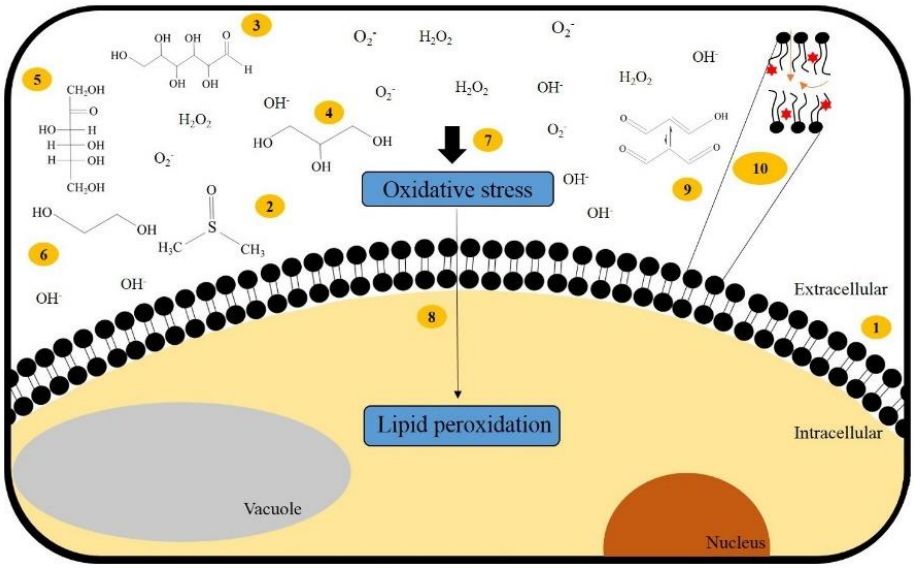

Figure 2 Schematic of ROS overproduction in the extracellular space (1) and their interaction with cryoprotectant substances such as dimethylsulfoxide (DMSO) (2), glucose (3), glycerol (4), fructose (5) and ethylene (6), resulting in oxidative stress (7) and lipid peroxidation (8) through the oxidative deterioration of polyunsaturated lipids present at cell membrane, leading to reduced malondialdehyde (9). These factors lead to changes in membrane permeability disorders, changing the flow of ions and other substances, causing the loss of selectivity for incoming and/or outgoing water and toxic substances to the cell, such as cryoprotectants (10).

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In general, plant cells have two mechanisms to maintain stable levels of ROS: (i) enzymatic: represented by the action of enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) and; (ii) non-enzymatic: includes the ascorbate (ASC) / glutathione (GSH) cycle (APEL; HIRT, 2004; AHMAD et al., 2008). The activity of the antioxidant system is a defense mechanism triggered when the cells are confronted with some type of stress (EL-BELTAGI et al., 2011). However, proper ROS concentrations can stimulate the transcription of defense genes and trigger adaptive responses in the plant (VAN BREUSEGEM, 2006).

Recent studies support the hypothesis that oxidative stress may be an implicit component in somatic embryogenesis and seed germination (EL-BELTAGI et al., 2011). However, at the same time, proteins were identified, expressed after the explant underwent cryoprotectant, freezing treatments, and an influence of cryoprotectant excess was verified at the intracellular space (UCHENDU et al., 2010). Together with these observations, it was found that lipid peroxidation shows a negative correlation with the cryopreserved explants survival, an important factor to evaluate cell survival during the cryogenic treatment, thus modulating the effects of excessive ROS production (UCHENDU et al., 2010).

Different factors can trigger stress signals that end up being responsible for the convergence of cell signaling pathways (HUANG et al., 2016). Therefore, studies on biochemical aspects of cryopreservation may increase the understanding of stress associated with cryopreservation and provide clear solutions for the oxidative stress control and elimination of free radicals, essential for the technique's success for a greater number of species.

2.5 Germination and reserve mobilization of oilseeds

Germination is a key process in plant metabolism responsible for embryo growth and development into a complete plant (BEEWLEY et al., 2013). From the physiological point of view, germination comprises four

phases: (i) water imbibition; (ii) cell stretching; (iii) cell division; and (iv) cell differentiation into tissues (POPINIGIS, 1985). Knowledge about seed biology and germination process of each species is fundamental to understanding the establishment of a plant community, as well as its survival and natural regeneration (IZQUIERDO, 2017).

In oilseeds, as the case of some *Passiflora* sp. (NYANZI; CARSTENSEN, SCHWACK, 2005), the main endosperm reserve is lipid, which is in form of Triacylglycerol (TAG) stored in organelles called of lipid bodies or oleosomes (GRAHAM, 2008). The TAG present in the lipid bodies are initially cleaved by lipases, release fatty acids into glyoxysomes and are subsequently degraded by the ß-oxidation enzymes, producing acetyl-CoA (DIETZ et al., 2016). Acetyl-CoA is converted into sucrose through the glyoxylate cycle and gluconeogenesis, where they have two key enzymes: malate synthase (Msy) and isocitrate lyase (ICL), both act on the lipid metabolism stored in oilseeds. Activity these enzymes increases during germination, obtaining maximum values when the highest proportion of degraded lipids occurs and in the sucrose synthesis, which is transported to the embryonic axis and serves as energy and carbon support for root growth (EASTMOND; GRAHAM, 2001; IZQUIERDO, 2017).

Despite the importance of lipid catabolism in glyoxysomes during germination, this process is responsible for the potential production of ROS (JASPER; BIAGGIONI; SILVA, 2013). The amount of ROS is closely regulated by the balance between production and elimination, playing a dual role in the seed physiology: by one side, they behave as cellular signals and may even act as a break in the dormancy of orthodox seeds (BAILLY et al. 2008) On the other hand, they can accumulate as toxic products under stress conditions interfering with cellular homeostasis (DIETZ et al., 2016).

The main ROS formed inside glyoxysomes is the hydrogen peroxide (H2O2) produced during β-oxidation (GRAHAM, 2008). H_2O_2 is a moderately reactive ROS, whose small size allows it to cross cell membranes and migrate in different compartments (BARBOSA et al., 2014). However,

the antioxidant defense system contributes to the maintenance of redox balance (FARHOUDI; HUSSAIN; LEE, 2012). Under stress conditions, as in cryopreservation process, there is an intensification of other formed ROS during the cellular respiration, for example, the superoxide radical (O_2^-). O_2^- is a potentially harmful ROS, which can be rapidly disrupted to H_2O_2 in a reaction catalyzed by the antioxidant enzyme SOD (VAN BREUSEGEM et al., 2001). Therefore, SOD activity may increase the cellular content of H_2O_2, evidencing the importance in keeping together the enzyme operation of the antioxidant system. This unbalance may result in a cascade of events beginning with the peroxidation of structural lipids, advancing towards membrane degradation and cell death (GREGGAINS et al., 2000).

In this sense, the study of metabolic characteristics involved in *P. ligularis* germination, such as the reserve mobilization and the antioxidant metabolism in the initial stages of germination process, especially after stress conditions, is a determining factor to maximize and to standardize the plant productivity.

3 GENERAL CONCLUSIONS

Passiflora ligularis is an economically important species with a medicinal potential to be exploited. Delays in germination due to prolonged dormancy, result in non-uniformity of plants and give it priority in its *in vitro* establishment, through micropropagation techniques. In addition, this *Passiflora* species does not tolerate excessive dehydration, which interferes with its germination potential, and does not have protocols for storage in germplasm banks for long periods by cryopreservation techniques, which may be a viable alternative for its use.

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SECOND PART - PAPERS

PAPER 1 - Characterization of the formation of somatic embryos from mature zygotic embryos of *Passiflora ligularis* Juss.

The following chapter is entitled "Characterization of the formation of somatic embryos from mature zygotic embryos of *Passiflora ligularis* Juss." and contains data from the published paper:

Prudente D O, Paiva R, Carpentier S, Swennen R, Nery F C, Silva L C and Panis B (2017) Characterization of the formation of somatic embryos from mature zygotic embryos of *Passiflora ligularis* Juss. Plant Cell, Tissue and Organ Culture (PCTOC), 131: 1-9. doi: https://doi.org/10.1007/s11240-017-1266-8

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ABSTRACT

Passiflora ligularis Juss. is an endemic species from South America with important medicinal and economical properties. The development of improved micropropagation techniques is necessary to provide rapid and efficient clonal propagation of elite genotypes with high resistance and uniform production, as well as a system that can be used for genetic transformation. For this reason, we focused on establishing a protocol for somatic embryogenesis in *P. ligularis* from mature zygotic embryos. Our results demonstrate that the highest frequencies of somatic embryo formation was observed on a culture medium supplemented with 27.2 µM 2,4-diclorophenoxyacetic acid plus 4.5 µM 6-benzyladenine. Histological analyses of somatic embryogenesis were performed every 7 days after induction over 60 days of exposure to the medium. We present clear evidence for the precise origin of de-differentiation. Initial cell divisions occurred from a group of cells (multicellular origin) on the abaxial surface of the cotyledon in the periphery of the epidermal tissues of mature zygotic embryos after 14 days of incubation. After 21 days, internal segmenting divisions resulted in the embryogenic character of the tissue. Globular embryos contain a protoderm surrounding a mass of vacuolated parenchymatous cells and meristematic regions with an observable procambium zone after 45 days. The complete independence of somatic embryos from the adjacent tissues was histologically confirmed by the absence of vascular continuity between them, after 60 days. We describe for the first time the development of somatic embryos in *P. ligularis* and demonstrate that somatic embryos develop into plants that can later on be acclimatized.

Keywords: Sweet granadilla, Histological analysis, Plant regeneration, Biotechnology

1 INTRODUCTION

Species of the genus *Passiflora* L. are important for their ornamental, alimentary and pharmacological properties (SARAVANAN; PARIMELAZHAGAN, 2014). Among them, *Passiflora ligularis* Juss. is highly relevant for agribusiness in South America (SUÁREZ et al., 2016) since its fruits are not as acidic as other commercial Passifloras (MEDINA; LOBO 2000; TOMMONARO et al., 2007). *P. ligularis* differs from others species of *Passiflora* in being exigent in places with altitudes between 1500 and 2300 m and having a preference for moderate temperatures for germination and development throughout the year, between 13 ℃ and 22 ℃ (VANDERPLANK, 1996). The current methods for sexual propagation of *P. ligularis* have some drawbacks, such as delayed and non-uniform germination due to prolonged dormancy (HERNÁNDEZ et al., 2012). Dormancy can be overcome using long-term treatments like stratification for 200 hours at 5 °C (SANTOS ALVARADO et al., 1994) or maintaining the seeds in a cold chamber (7 °C to 10 °C) for a period exceeding 101 days (SOUZA et al., 2015). Even with these treatments, *ex vitro* germination percentage is highly variable, from less than 20% up to 52% (SALAZAR, 2000; GUTIÉRREZ et al., 2011). Propagating by cuttings is the other common method for commercial propagation of *P. ligularis*, however it has presented serious restrictions through difficulty of rooting (MORA et al., 2005; LOBO; MEDINA, 2009).

In Colombia, *P. ligularis* is often grown on a commercial scale as a monoculture, but it is also commonly grown in mixed agroforestry systems, for example, in Peru (BRACK EGG et al., 1985). However, significant production losses are caused by the incidence of viral diseases like the *Cowpea aphid-borne mosaic virus*-CABMV that is disseminated by aphids (MANICOM et al., 2003), resulting in rugosity, distortion of the leaves and deformed fruits. CABMV affects normal plant development and commercial production (MCKERN et al., 1994; FREITAS et al., 2015), requiring annual

replanting. One of the most viable alternatives to control CABMV is clonal propagation of elite genotypes with high resistance and uniform production, but to accomplish this, it is necessary to develop an efficient protocol for *in vitro* plant regeneration (SANTOS et al., 2015).

Micropropagation techniques were originally developed to meet three main goals: clonal propagation, storage of plant germplasm and the development of cellular tools for genetic improvement (STROSSE et al., 2003). Micropropagation studies through organogenesis (direct and indirect) in the genus *Passiflora* L. were initiated as early as 1966 (VIEIRA; CARNEIRO, 2004; NHUT et al., 2007; DIAS et al., 2009; PINTO et al., 2010; SILVA et al., 2011; PACHECO et al., 2012; OTONI et al., 2013; VIEIRA et al., 2014). However, somatic embryogenesis (SE) is considered the most important technique for mass-propagation and establishment of biotechnological strategies including genetic transformation, somatic hybridization, and production of artificial seeds, among others (KUMAR; THOMAS, 2012). Recently, protocols for SE from mature zygotic embryos (MZE) of wild species of *Passiflora* were reported for *Passiflora miniata* Vanderpl. (FERREIRA et al., 2015), *Passiflora speciosa* Gardner (FERREIRA et al., 2015), *Passiflora edulis* Sims (ROSA et al., 2015), *Passiflora cincinnata* Mast. (ROCHA et al., 2012; SILVA et al., 2009; PINTO et al., 2010), *Passiflora alata* Curtis (ROSA et al., 2015), *Passiflora crenata* Feuillet & Cremers (ROSA et al., 2015) and *Passiflora Gibertii* N.E. Br. (ROSA et al., 2015). However, most of these protocols failed to regenerate normal plants from zygotic embryos.

In our study, we show that cultures of *P. ligularis* can acquire embryogenic competence when MZE were exposed for 60 days to a high concentration of 2,4-diclorophenoxyacetic acid - 2,4-D (27.2 µM). Somatic embryos started to develop exclusively from the cotyledonary regions, resulting in the suppression of zygotic embryo germination. This method will enable clonal propagation in order to maximize production, avoiding

problems of dormancy and non-uniformity of germination, and allow for the possibility of genetic transformation protocols in *P. ligularis* in the future.

Despite the economical interest in *P. ligularis*, successful studies on this topic are scarce. The present investigation was undertaken with the aim of developing an efficient *in vitro* somatic embryogenesis protocol for *P. ligularis* and examining the origin of cell differentiation during the development of SE.

2 MATERIAL AND METHODS

2.1 Plant material and culture media

Seeds of *P. ligularis* were supplied by Tabutins sementes Brasil Ltda. (Gramado-RS, Brazil; http://www.tabutinssementes.com.br) collected separately from elite productive plants with different genotypes. Prior to surface sterilization, the seeds were decoated under a laminar flow hood with a mini-vise (Hever Front 100) (REIS et al., 2007). The total of 500 decoated seeds were surface-sterilized for 30 sec in 200-mL of ethanol 70% (v/v) following immersion for 30 min in 200-mL commercial sodium hypochlorite 2.5% active chlorine (v/v) with two drops of Tween-20 per 100-mL of solution. Decontaminated embryos were washed three times in autoclaved distilled water. Zygotic embryos were excised from decoated seeds with tweezers and a scalpel and inoculated on an induction medium: MS (MURASHIGE; SKOOG, 1962) basal salts, B5 vitamins (GAMBORG; MILLER; OJIMA, 1968), myo-inositol 0.01% (w/v), sucrose 3% (w/v), and Phytagel® (Sigma-Aldrich®, Steinheim, Germany) 0.3% (w/v). SE was induced using different concentrations of 2,4-D: 0.0; 18.1; 27.2; 36.2 and 72.4 µM, combined or not combined with 4.5 µM 6- benzyladenine (BA) (PINTO et al., 2011; ROCHA et al., 2012; FERREIRA et al., 2015). The pH was adjusted to 5.7 with 2 N NaOH and HCl prior to autoclaving at 121 °C, 1.1 atm for 20 min, and cultures were maintained at 25 °C ± 2 °C in the dark.

Throughout the experiments, MZE were cultured in 90 x 15 mm sterile polystyrene Petri dishes (Greiner®, Frickenhausen Germany) containing 25-mL aliquots of medium, and were sealed with vinyl polycloret film. The percentage of explants with calli was evaluated for each treatment after 30 days, and the percentage of explants with somatic embryos were evaluated after 60 days.

2.2 Somatic embryo maturation and germination media

After 60 days on induction medium, 200 MZE showing the highest somatic embryogenic frequency were transferred to the embryo maturation media: MS basal salts, B5 vitamins, sucrose 3% (w/v), and Phytagel® (Sigma-Aldrich®, Steinheim, Germany) 0.3% (w/v). This medium was supplemented with (i) no growth regulators (GR) (Control); (ii) 1 $g.L^{-1}$ of activated charcoal (AC) without GR; (iii) 27.2 µM 2,4-D + 4.5 µM BA; and (iv) 1 g of AC with 27.2 µM 2,4-D + 4.5 µM BA. After 30 days, mature somatic embryos were transferred to the germination medium: MS basal salts, B5 vitamins, sucrose 3% (w/v) and Phytagel® (Sigma-Aldrich®, Steinheim, Germany) 0.3% (w/v) (FERREIRA et al., 2015). During this phase, cultures were maintained under culture room conditions at 25 °C ± 2 °C, with a 16 h photoperiod with 36 µmol m^{-2} s^{-1} lighting provided by two fluorescent tubes (20 W, Osram, Munich, Germany). The number of mature somatic embryos was evaluated in each treatment after 30 days, and the number of normal plantlets was evaluated after 60 days.

2.3 Plantlet acclimatization

Regenerated plantlets were separated and roots treated with Rootone NA 98% TC (20 mg L^{-1}), according to onkha and Dzyazko (2014), Subsequently plantlets were transferred to polyethylene pots (500-mL) filled with vermiculite substrate (500 g) (Nº 3) with a plastic bag to maintain relative

humidity. The plantlets were grown under a controlled temperature of 27 °C ± 2 °C in a greenhouse. After 21 days of acclimatization, the plastic bag was gradually removed and the percentage of plantlet survival was evaluated.

2.4 Histological preparation

Explants incubated in culture were collected during the induction phase (7; 14; 21 and 28 days after inoculation) and during the maturation phase (35; 45 and 60 days after inoculation), then preserved in 70% ethanol (v/v) and infiltrated with a mix of Technovit Kulzer 7100 solution and Hardener I (100-mL: 1 g) (Heraeus Kulzer GmbH & Co. KG, Germany), stored for 48 hours in the fridge (4 °C). Samples were transferred into embedding forms, which were filled with a mix of infiltration solution and Hardener II (1-mL: 15 mL) and incubated for 2 min at room temperature (15 °C ± 2 °C). The samples were placed in plastic caps and allowed to rest for 30 min so that the plastic caps could be easily removed with a scalpel. The blocks were wetted before cutting, and 5 µm thin sections were produced with a Reichert-Jung 11300- Biocut microtome. The sections were mounted on microscope slides and stained with toluidine blue (O'BRIEN; MCCULLY 1981), pH 7.0, for 5 min. Observations and photographs were taken using an Nikon TPL-210 stereo microscope and an Olympus UC30 U-Photo camera system.

2.5 Statistical analysis

The experimental design was completely randomized for all experiments. Each experimental unit consisted of a Petri dish with five MZE, and there were ten repeats per treatment. Data were submitted to analysis of variance (ANOVA) using the statistical software SISVAR 4.3 (System Analysis of Variance for Balanced Data, Lavras, Brazil) (FERREIRA, 2014). According to the results of the ANOVA, data from the qualitative factors were

compared by Tukey's test ($p<0.05$), and data from quantitative factors were analyzed by polynomial regression ($p<0.05$).

3 RESULTS

After 30 days on induction media, 80% of the MZE (Fig. 1C) growth of compact, white calli could be observed but no significant difference was observed among all tested combinations of 2,4-D (Fig. 2). Somatic embryo formation was observed after 60 days of culture on different 2.4 D containing media with or without 4.5 µM BA (Fig. 3), with a concentration of 27.2 µM 2,4-D + 4.5 µM of BA resulting in the highest percentage of visible somatic embryos (Fig. 1D).

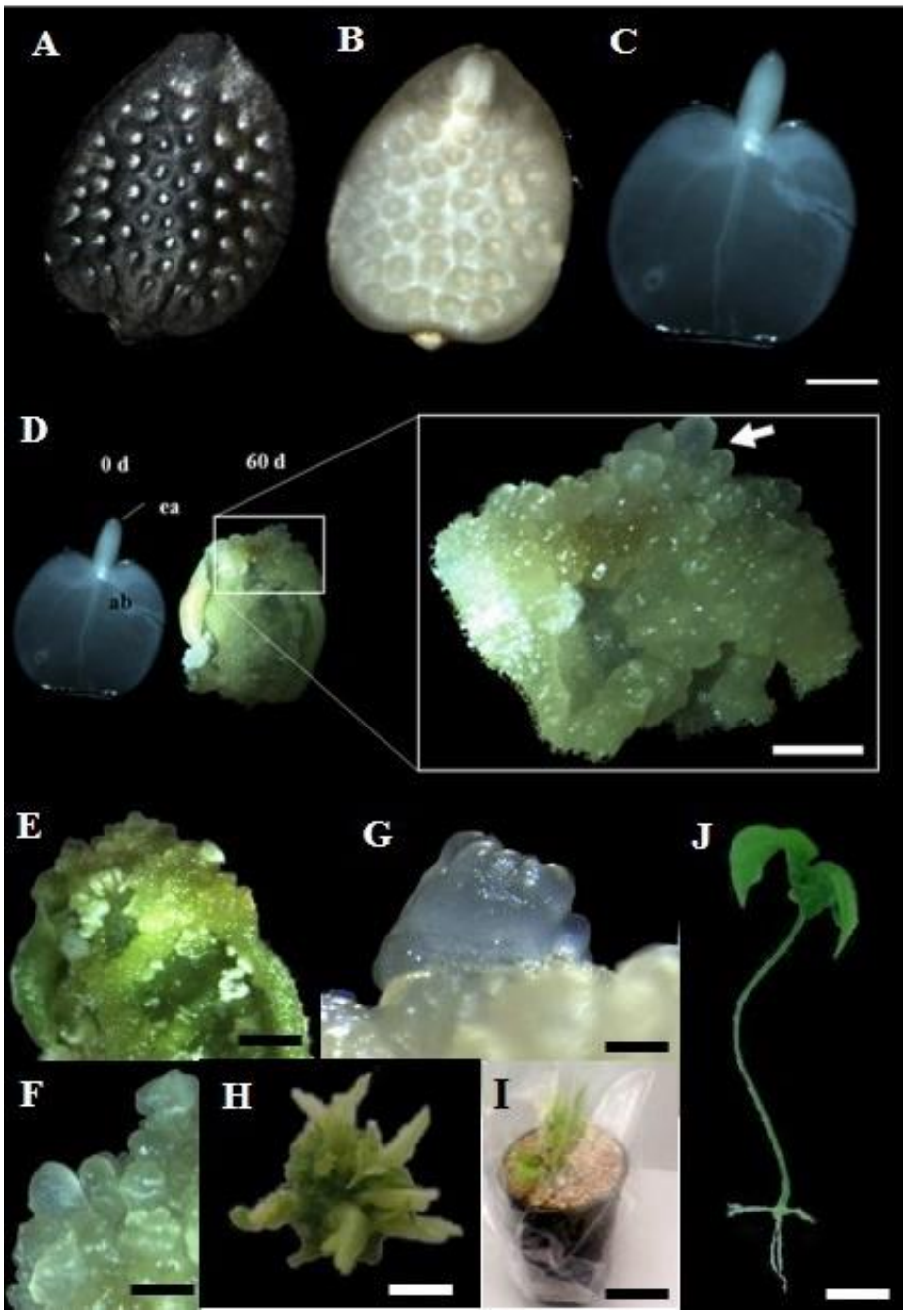

Figure 1 General aspects of somatic embryogenesis formed on MZE of *P. ligularis*. Representative mature seeds (A), seeds without tegument (B) and MZE (C). Embryo initiation on the abaxial surface of a

cotyledon (arrow) after 60 days in medium (D). Explants with embryos after transfer to a maturation media and light conditions (E). Embryos at different developmental stages (globular and cotyledonary) in maturation media after 30 days (F-G). Growing individual plantlets after 30 days in germination medium (H). A completely regenerated plant from an isolated somatic embryo after 21 days of acclimatization (I-J). ab: abaxial surface of the cotyledon; ea: embryonic axis. Bars = 5 mm (A, B, C, D and E); 2 mm (F and G); 1 cm (H, and J); 5 cm (I).

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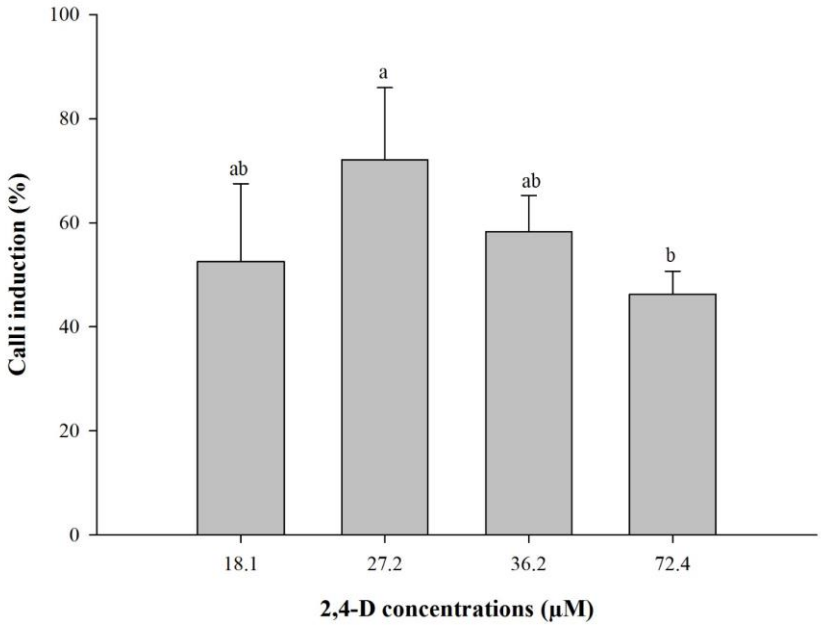

Figure 2 induction on MZE after 30 days on induction media supplemented with different concentrations of 2,4-D. Median values followed by the same letters within the same morphogenetic response are not significantly different according to Tukey's test (p<0.05). Vertical bars indicate standard error of the median.

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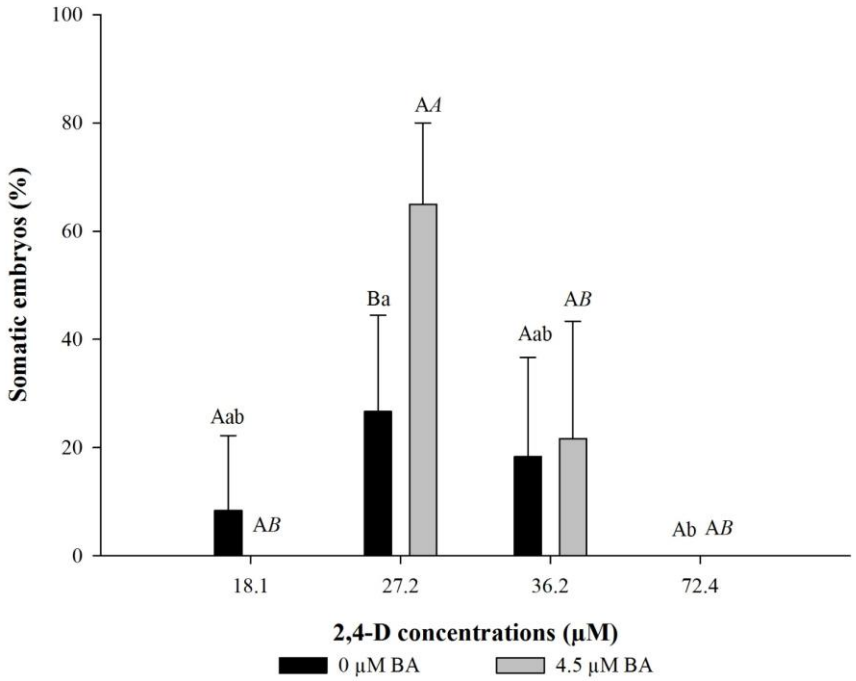

Figure 3 Somatic embryos after 60 days on induction media supplemented with different concentrations of 2,4-D and BA. Normal upper-case letters compare BA concentrations within each 2,4-D concentration; lowercase letters compare the concentrations of 2.4 on the black

columns (0.0 μM BA); Capitalized italics letters compare concentrations of 2.4 on ash columns (4.5 uM BA). Vertical bars indicate standard error of the median.

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Differentiation of somatic embryos occurred mainly on the abaxial surface of the cotyledon when they were placed face-up on the medium, and they could be easily distinguished by their granular appearance and lighter and more translucent color (Fig. 1D). After transfer to maturation media, MS control medium (without AC and GR) promoted significant development and maturation of somatic embryos (81%) (Fig. 4), with 44.2 ± 1.2 mature embryos per explant (Fig. 1F and 1G). Embryos turned green, with subsequent conversion into plantlets (12.7 ± 1.6 per explant) after 60 days of culture in a medium without GR under light conditions (Fig. 1E). After this period, 52% ± 0.8 of individualized plants that were transferred to greenhouse conditions for an acclimatization period presented leaves and roots (Fig. 1I and 1J).

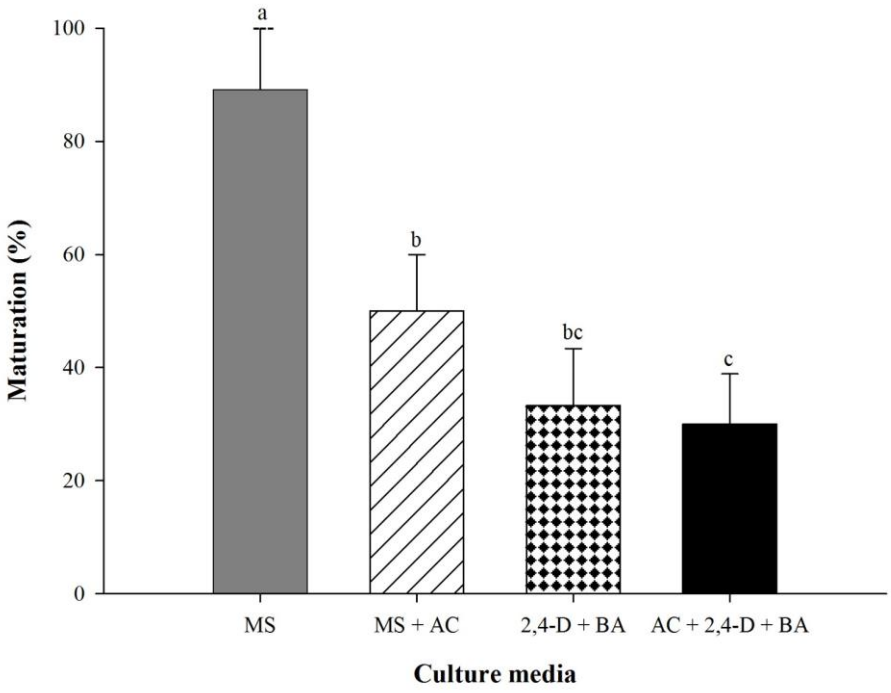

Figure 4 Maturation of embryos after 30 days of culture from somatic embryogenesis of *P. ligularis*. Median values followed by the same letters within the same morphogenetic response for different combinations of AC and GR are not significantly different according to Tukey's test (p<0.05). Vertical bars indicate standard error of the median.

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Histological analysis showed that cellular proliferation started mainly at the epidermal tissues, with start of cell differentiation within 14 days of culture directly from MZE (Fig. 5A and 5B). After 21 days, due to internal

segmenting divisions the callus acquired embryogenic characteristics (Fig. 5C and 5D). Globular embryos show a protoderm surrounding a mass of vacuolated parenchymatous cells and contain meristematic regions with an observable procambial zone after 45 days in culture (Fig. 5E). The complete independence of somatic embryos from the adjacent tissues was histologically confirmed by the absence of vascular continuity between them (Fig. 5F and 5G).

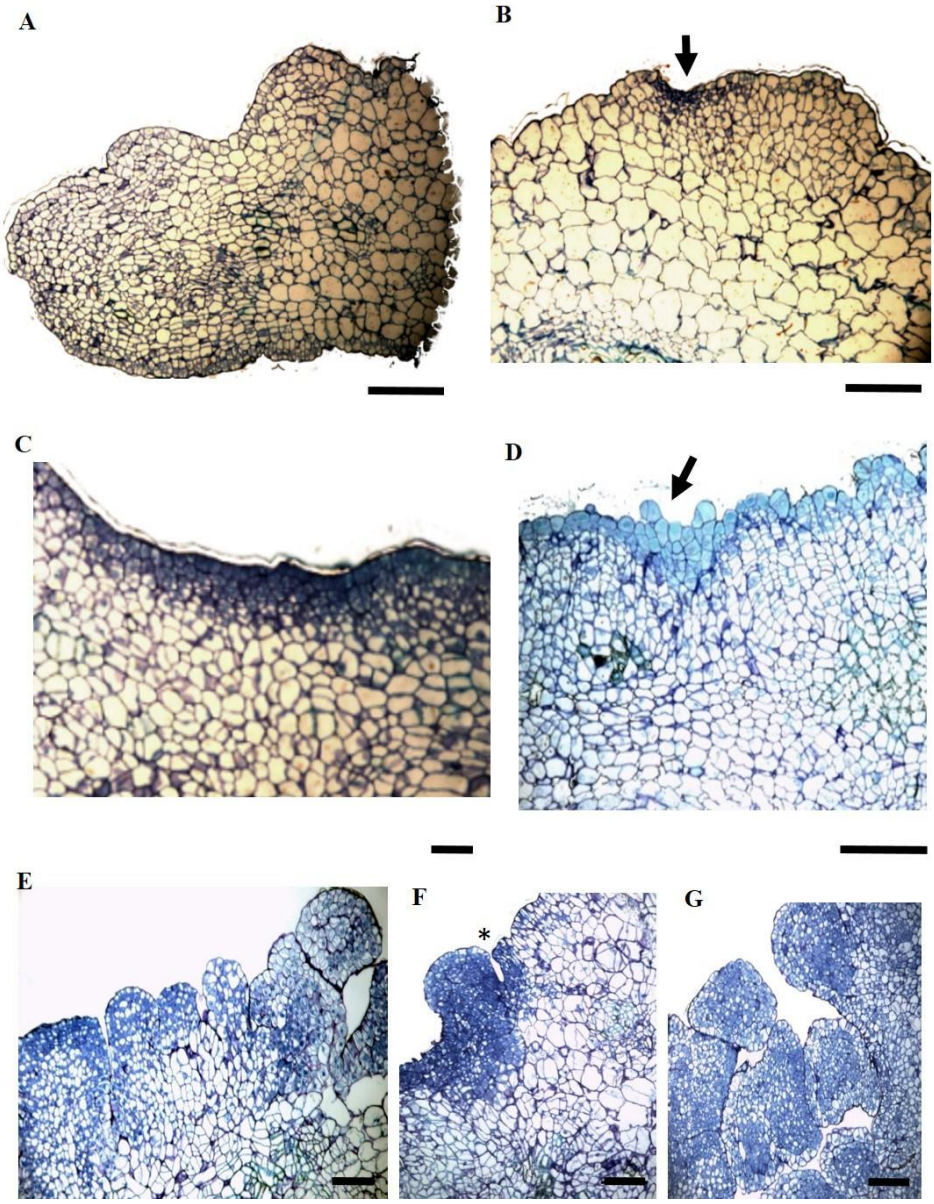

Figure 5 Longitudinal sections showing histological organization of embryogenic culture from MZE of *P. ligularis*. MZE after seven days in induction media (bar= 50 μM) (A). MZE after 14 days in induction media, with start sites of differentiation indicated by an arrowhead (bar= 25 μM) (B). MZE after 21 days in induction media, with

numerous divisions of epidermal cells (bar= 50 μM; C). MZE after 28 days in induction media, showed cells with prominent nuclei and nucleoli (arrowhead; bar= 50 μM; D). After 35 days in induction media, the MZE developed protuberances from the epidermis. Small cells with dense cytoplasm in the outer layers of the protuberance cells were visible (bar= 100 μM; E). After 45 days in maturation media, embryos were forming (asterisk) on the external surface of the explant (bar= 100 μM; F). After 60 days in maturation media, the MZE showed an absence of vascular connection with the parental tissue (bar= 100 μM; G).

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4 DISCUSSION

Endogenous hormone levels may be a major factor involved in determining the embryogenic competence of an explant (THOMPSON et al., 2000). In general, “young” cells, like those of zygotic embryos, are the most competent and sensitive to exogenous auxin application (FEHÉR, 2015). Among auxins applied exogenously, 2,4-D is the most commonly used for the induction of SE in recent protocols for *Passiflora* spp. (OZAROWSKIA; THIEMA, 2013), however, the amount of 2,4-D used in other species of passiflora in the literature is very variable. Therefore the choice of a large spectrum of 2,4-D concentration for the species under study (PINTO et al.,

2010; ROSA et al., 2015; FERREIRA et al., 2015). This strong synthetic auxin operates by increasing the endogenous concentration of indoleacetic acid (IAA) (PASTERNAK et al., 2002), which is associated with fundamental processes such as cell elongation and division (KARAMI; SAIDI, 2010), cell signaling (FEHÉR et al., 2003), histone methylation (SCHMIDT et al., 2013) and nuclear DNA (hyper)methylation (KARAMI; SAIDI, 2010). Our results indicated a high protein content around epidermal cells of MZE (stained darker purple with toluidine blue). Studies mention many differences concerning types of proteins in somatic tissues and 2,4-D may facilitate the transcription of many regulatory genes (NOWAK; GAJ, 2016) resulting in structural and storage proteins required for induction of somatic embryos (ALTAMURA et al., 2016). 2,4-D may also elevate the endogenous levels of the abscisic acid (ABA) biosynthetic gene 9-cis-epoxycarotenoid dioxygenase (NCED) (XIONG et al., 2003). The carotenoid cleavage reaction catalyzed by NCED is the key regulatory step in ABA biosynthesis (SEO et al., 2002; FEHÉR et al., 2005). ABA is a critical chemical messenger for stress responses (FINKELSTEIN, 2013; ZHAO et al., 2013; DANQUAH et al., 2014) and altered cellular energy status, resulting in cascades of calcium (KNIGHT, 1999; HEPLER, 2005; BATISTIČ; KUDLA, 2012), indicating that there is an important role for stress signaling in SE induction (FEHÉR, 2015).

In the maturation phase, the embryos continued their development after withdrawal of auxin from the culture medium, with 44.2 maturated

embryos per explant. At this stage, the continuous presence of auxin inhibits differentiation to other stages of embryonic development (ALTAMURA et al., 2016). Furthermore, after establishing the polarity of the embryo (pole root and stem), which is maintained with elimination and/or reduction of 2,4-D in the medium, auxin efflux start from pole root occurs due to the presence of the auxin transporter PIN1 (GELDNER et al., 2001; ROSE et al., 2010). After the acclimatization phase, we obtained 52% of regenerated plants surviving in the greenhouse under *ex vitro* conditions. The low survival rate after acclimatization in this study may be due to the exposure of the plants to many stresses during the transfer to photoautotrophic conditions (HAZARICA, 2003) and the sensitivity of leaf tissue of *P. ligularis*. Adjustments to the acclimatization methodology are therefore still necessary in order to ensure a higher percentage of plant survival after the SE protocol. In other passiflora species, similar results were found with respect to the number of embryos formed and regenerated plants, such as *P. miniata*, with 36 embryos per callus and 60% regenerated plants (FERREIRA et al., 2015) and *P. cincinnata*, with 19 embryos per callus and 49% regenerated plants (PINTO et al., 2010). On the other hand, many species did not obtain satisfactory results for the regeneration and acclimatization of plants (not significant) after the formation of the embryos, such as *P. alata* (26 embryos per callus), *P. crenata* (28 embryos per callus), *P. edulis* (32 embryos per callus) and *P. gibertii* (35 embryos per callus).

In this study, embryogenic competence occurred directly from cotyledonary tissues, which was confirmed through histological analysis. The ability of cells to change division and cellular polarity has different origins. In general, embryos can begin differentiation through reorganization from clusters of cells that are at a similar stage of embryogenic induction, so a multicellular origin (MAHESWARAN; WILLIAMS, 1986; SU et al., 2009; SU et al., 2015; FEHÉR, 2015). In contrast, embryos can arise from a single predetermined cell, which is called unicellular origin (MAHESWARAN; WILLIAMS, 1985; FEHÉR, 2015; SAEED; SHAHZAD, 2015). In both cases, it is necessary to change the *in vitro* conditions to divert the cell from standard development pathways, from a vegetative to embryogenic state. This means that these cells are not inherently embryogenic but become embryogenic in response to external and subsequently internal stimulation (SU; ZHANG, 2009; SU et al., 2015).

During the course of SE in *P. ligularis*, we observed clear evidence for the exact origin of de-differentiation (Fig. 2B). This event occurred during embryogenic responses obtained from a group of cells (multicellular origin) in the periphery of cotyledonary tissue in *P. ligularis* with subsequent formation of protuberances (Fig. 2D), with independent somatic embryos confirmed by the absence of vascular continuity between the two (Fig. 2G). The origin of somatic cells via a multicellular pattern in small groups of individualized cells were observed also to Rocha et al. (2012) for *P. cincinnata*.

This study has shown that plants contain a variety of tissues that may be more amenable to express totipotency than most other, like the mature zygotic embryos (JAYARAJ et al., 2015). These explant could present advantages in your variations in the plane of zygotic division regulated by its proximity to an auxin secreting meristematic region (MIKULA et al., 2015). The basal medium containing both auxin and cytokinin was able to induce somatic embryogenesis but differences were observed caused by the type and concentration of plant growth regulators. The histological analysis confirmed that the somatic embryo formation initiated directly from zygotic embryos, contributing evidence for the multicellular origin of embryogenic cells. As such somatic embryogenesis could represent an important way for *in vitro* clonal propagation in *P. ligularis.*

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PAPER 2 - Reserve mobilization and antioxidant metabolism on the germination of *Passiflora ligularis* zygotic embryos after cryopreservation

ABSTRACT

Cryopreservation is a process where live biological structures are preserved by cooling to very low temperatures (−196 °C using liquid nitrogen). Among cryopreservation techniques, PVS2 vitrification consists in the use of cryoprotectants such as *Plant Vitrification Solution 2*-PVS2 garanting the vitrification process before imeersion on liquid nitrogen. In this work we evaluate the influence of different exposure times to PVS2 (0, 30, 60 and 120 min) on the germination of *Passiflora ligularis* zygotic embryos. Our analyzes showed that the 60 min of exposure to PVS2 solution increase the activity of exclusive enzymes of the glyoxylate cycle: Malate synthase and isocitrate lyase, accelerating the reserve mobilization and consequently the germination process. Besides, increase the proline content, antioxidant enzymes (SOD, CAT, APX) and decrease the lipidic peroxidation what was able to optimize the long-term conservation of this species.

Keywords: Sweet granadilla, Long-term storage, Cryopreservation, Glyoxylate cycle, Antioxidant metabolism

1 INTRODUCTION

Passiflora L. is the most representative genus of the Passifloraceae Juss. family, which comprises 18 genera containing approximately 630 species widely distributed throughout South America (MARTIN; NAKASONE, 1970; TIWARI et al., 2015). Among all these species, *Passiflora ligularis* Juss., commonly known as sweet granadilla, grows in tropical regions at high altitude such as those found in Brazil (OCAMPO; ARIAS; URREA, 2015). The pulp of *P. ligularis* has anti-diabetic and antimicrobial properties and is used for consumption *in natura* or as industrialized fruit (SARAVANAN; PARIMELAZHAGAN, 2014).

Despite their economic and medicinal potential, many species of *Passiflora* L. are threatened by destruction of their natural habitat (CERQUEIRA-SILVA et al., 2014). Studies based on the long-term storage of seeds such as cryopreservation have demonstrated success in preserving the viability of 22 *Passiflora* species belonging to intermediate and orthodox classes (VEIGA-BARBOSA et al., 2013; POSADA et al., 2014). Cryopreservation has been suggested for live biological material conservation at ultralow temperature (-196 °C) (ENGELMANN, 2011). Cryopreserved collections are maintained in small physical spaces, protected from contamination, and require minimal upkeep and little financial support during preservation compared to other available systems of plant material storage (DULLOO et al., 2009). One of the benefits of seed cryopreservation include 175 times greater longevity than that achieved at temperatures used in conventional seed banks (PRITCHARD, 1995; WALTERS et al., 2004, 2005).

A study about the *in vitro* conservation of *P. ligularis* has proven that germination decreases as seeds became desiccated for 2, 5 and 8 h under laboratory conditions, in a drying room and over silica gel, respectively (OSPINA et al., 2000). Nevertheless, the method used was direct immersion in liquid nitrogen without cryoprotection. The protocols used to reduce water

content with concentrated solutions (cryoprotectors) that osmotically remove water from cells can lead to better results (VOLK; WALTERS, 2006). The use of cryoprotectants compounds such as glycerol, ethylene glycol, and dimethylsulfoxide (DMSO), as well as sucrose, which are all present in *Plant vitrification solution* (PVS2), proved to be very effective for a wide range of explants (SAKAI et al., 1990; MATSUMOTO, 2017). Moreover, the exposure time to PVS2 is crucial to the success of cryoprotection. In addition, the PVS2 compounds may also act as antioxidants, helping to regulate the balance between the production and detoxification of *Reactive Oxygen Species* (ROS), which maintain cellular protection (REED, 2014).

The reduction in lipid peroxidation may favor the accumulation of low-molecular-weight compounds such as proline that act to protect membranes (HOSSAIN et al., 2014). There are many reports showing that exposure of embryos and seeds to ultralow temperatures following thawing may result in the modification of essential metabolic functions. Those modifications interfere in both speed and the final percentage of germination (SALOMÃO, 2002; JOHNSON et al., 2012; KHOLINA; VORONKOVA, 2012; GANTAIT et al., 2016).

For a better understanding of signaling pathways that influence germination triggering, we should understand the main type of endosperm reserve of each species (RAJJOU et al., 2012). Seed reserves consist of complex carbohydrates, protein and lipid molecules that can vary in quantity and proportion (BEWLEY; BLACK, 1994). Oilseeds (lipid-rich endosperm), which occur in some *Passiflora* L. species (NYANZI; CARSTENSEN; SCHWACK, 2005; TOZZI; TAKAKI, 2011), differ from other types of seeds that have dominant carbohydrate or protein reserves because oilseeds have specific metabolic characteristics (THEODOULOU; EASTMOND, 2012).

A positive effect on germination of oilseeds is related to the fast and efficient use of lipid reserves at the beginning of seed germination through coordinated induction of a number of biochemical pathways with different subcellular locations (GRAHAM, 2008). The first step in lipid breakdown is

catalysis by lipases, which provides free fatty acids (FFAs) and glycerol from triacylglycerol (TAG) molecules (XU; SHANKLIN, 2016). The FFAs are then introduced into membrane-bound organelles of glyoxysomes, where β-oxidation and the glyoxylate cycle occur (LI-BEISSON et al., 2013). Glyoxysomes are structurally similar but metabolically distinct from peroxisomes and contain two enzymes unique to the glyoxylate cycle: malate synthase (MSy) and isocitrate lyase (ICL) (GRAHAM, 2008); the products of these two enzymes are used respectively for cycle regeneration and for sucrose synthesis, of which sucrose will be transported to developing tissues to promote seedling growth (GEIGENBERGER; FERNIE, 2014).

Cryoprotection by the use of PVS2 for long periods can interfere in cellular homeostasis (DIETZ; MITTLER; NOCTOR, 2016) and, consequently, in the performance of key enzymes, such as MSy and ICL, which are both involved in the mobilization of lipids in oilseeds (UPCHURCH, 2008). Therefore, this study aims to evaluate the influence of exposure time to the cryoprotectant PVS2 on the mobilization of reserves and on antioxidant metabolism during the germination of cryopreserved *P. ligularis* zygotic embryos.

2 MATERIALS AND METHODS

2.1 Moisture content, centesimal composition and histological analysis of seeds

Passiflora ligularis seeds were obtained from Tabutins sementes Brasil Ltda. (Gramado, RS, Brazil; http://www.tabutinssementes.com.br). The moisture content was determined using the drying oven method at 105 °C ± 3 °C for 24 h, with five replications of 25 seeds, in accordance with the rules for seed testing (BRASIL, 2009). The centesimal composition of fixed mineral residues, lipids, proteins, crude fiber and total carbohydrates was analytically determined using 100 g of freshly harvested *P. ligularis* seeds

according to methods of Horwitz (2007). For histological analysis, zygotic embryos were subjected to transverse sections using Sudan IV for the identification of lipid bodies, according to the methods of Kraus and Arduin (1997). The preparation of semipermanent slides was performed according to techniques described by Johansen (1940). Observations were made using an Axiophot microscope equipped with DIC optics (Zeiss®, Oberkochen, Germany), and photographic documentation was carried out using a Powershot A640 digital camera (Canon®, Tokyo, Japan).

2.2 Germination index

Prior to surface sterilization, the seed tegument was removed using a mini-vise (REIS et al., 2007). This removal was followed by surface sterilization in 200 mL of 70% (v/v) ethanol (30 sec), 200 mL of a commercial sodium hypochlorite solution 2.5% (v/v), and active chlorine (30 min) with two drops of Tween-20 per 100 mL of solution. Afterward, the material was washed three times in autoclaved distilled water. Different culture media were tested to enhance the *in vitro* germination of zygotic embryos: (1) MS (MURASHIGE; SKOOG, 1962) basal salts + B5 vitamins (GAMBORG; MILLER; OJIMA, 1968); (2) MS basal salts + MS vitamins; (3) half-strength MS basal salts + half-strength B5 vitamins; and (4) half-strength MS basal salts + half-strength MS vitamins. All media were supplemented with 0.01% (w/v) myo-inositol and gelified with 0.3% (w/v) Phytagel® (Sigma-Aldrich®, St. Louis, MO, USA). The pH was adjusted to 5.8 with 2 N NaOH and HCl prior to autoclaving at 121 °C for 20 min. Culture rooms were maintained at 25 °C ± 2 °C with a 16 h photoperiod and a photosynthetic photon flux of 36 µmol m^{-2} s^{-1} irradiance at the top of the culture vessels provided by Phillips cool-white 18 W fluorescent lamps. After 21 days of *in vitro* culture, the percentage of germination, leaf number and shoot length were evaluated. The germination was evaluated for 21 days, at 2-day intervals. The percentage of germinated seeds with 2-mm rootlets was recorded in each treatment, and the

germination speed index (GSI) was calculated according to the methods of Maguire (1962).

2.3 Cryopreservation procedures

Five zygotic embryos were placed into cryotubes (TruCool®, Sigma-Aldrich®, St. Louis, MO, USA) containing 2 mL of loading solution (LS; 2 M glycerol and 0.4 M sucrose) for 20 min at 25 °C. Afterward, the LS was removed and replaced with 2 mL of PVS2 [30% (w/v) glycerol; 15% (w/v) ethylene glycol; 15% (w/v) DMSO and 0.4 M sucrose] (SAKAI et al., 1990) at 0 °C for different exposure times (0, 30, 60 and 120 min) and then immersed into liquid nitrogen (LN) for 90 min. The rewarming of cryopreserved zygotic embryos was performed in a water bath at 40 °C for 2 min. Afterward, the PVS2 was removed, and 2 mL of recovery solution (RS; 1.2 M sucrose) was added; the solution then sat for 15 min at room temperature (~25 °C). The control treatment (without LN plunge) consisted of rewarming in a water bath at 40 °C for 2 min followed by direct transfer to the RS. All cryoprotectant solutions (LS, PVS2 and RS) were dissolved in MS liquid basal medium with the pH adjusted to 5.8 prior to filter sterilization, accomplished using a 0.22-µm-pore-size micropore filter (Millipore Filter Corp., Bedford, Mass.). Afterward, the cryopreserved (+LN) or control (-LN) embryos were transferred to germination media. Culture rooms were maintained at 25 °C ± 2 °C with a 16 h photoperiod and a photosynthetic photon flux of 36 µmol m^{-2} s^{-1} irradiance at the top of the culture vessels provided by Phillips cool-white 18 W fluorescent lamps. After 30 days of *in vitro* culture, the percentage of germination and GSI were evaluated.

2.4 Protein content

Protein concentration was quantified in accordance with the standard Bradford protocol (BRADFORD, 1976) using commercially available

reagents (Sigma-Aldrich®, St. Louis, MO, USA). Protein was extracted from tissue powder using 0.1 M HEPES (pH 7.0) containing 0.5% CHAPS and 0.1% SDS on ice. Bovine serum albumin was used as a standard (all extracts measured separately).

2.5 Proline content

To determine free proline content, 400 mg of zygotic embryos was sampled at 7, 14 and 21 days after cryopreservation process. The samples were homogenized in 3% (w/v) sulfosalicylic acid, after which the homogenate was filtered through a Whatman® (No. 4) filter paper. Two milliliters of filtrate was reacted with 2 mL of ninhydrin and 2 mL of glacial acetic acid in a microtube (Brand®, Sigma-Aldrich®, St. Louis, MO, USA) for 1 h at 100 °C, after which the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL of toluene mixed vigorously with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, and this fraction was set aside for absorbance readings using a UV–vis spectrophotometer (Shimadzu UVmini-1240, Kyoto, Japan) at a 520-nm wavelength (BATES; WALDREN; TEARE, 1973). Proline concentrated using a calibration curve and was expressed in nanomoles per milligram of fresh weight (FW).

2.6 Hydrogen peroxide content

To determine hydrogen peroxide (H_2O_2) content, 200 mg of zygotic embryos was sampled at 7, 14 and 21 days after cryopreservation process. Samples were ground in LN, homogenized in 1.5 mL of 0.1% trichloroacetic acid (TCA) (w/v) and centrifuged at 12,000 × *g* at 4 °C for 15 min. The H_2O_2 content was assessed by measuring the absorbance at 390 nm of the reaction containing 45 µL of 10 mM potassium phosphate (pH 7.0) and 90 µL of potassium iodide (1 M) (VELIKOVA; YORDANOV; EDREVA, 2000).

2.7 Glyoxylate cycle enzymatic assays

To determine the activity of isocitrate lyase (ICL) and malate synthase (MSy), 200 mg of zygotic embryos was sampled at 7, 14 and 21 days after cryopreservation process. Samples were ground in LN, and ICL was extracted on ice with 50 Mm Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EDTA and 2 mM cysteine. ICL samples were centrifuged at 15,000 x *g* for 10 min at 4 °C, and the supernatant was collected. ICL activity was measured in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 2 mM L-cysteine, 10 mM L-isocitrate and 4 mM phenylhydrazine hydrochloride at 324 nm using the extinction coefficient of glyoxylic acid phenylhydrazone (14.6 mM cm^{-1}) according to the methods of Eprintsev et al. (2014). MSy was extracted on ice using 0.1 M HEPES (pH 7.8) containing 5 mM $MgCl_2$, 1 mM EDTA and 2 mM DTT. After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatant was collected. MSy activity was determined in 0.1 M HEPES (pH 7.8) containing 6 mM $MgCl_2$, 5 mM sodium glyoxylate, 2.5 mM acetyl-CoA and 2 mM DTNB at 412 nm using the extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB; 13.6 mM^{-1} cm^{-1}) according to the methods of Ma et al. (2016).

2.8 Lipidic peroxidation and antioxidant enzyme activity assays

For these analyses, 200 mg of zygotic embryos was sampled at 7, 14 and 21 days after cryopreservation process. Lipidic peroxidation was evaluated through the quantification of species that are reactive to thiobarbituric acid (TBA), as described by Buege and Aust (1978). Samples were ground in LN, and 0.5% (w/v) TBA and 10% (w/v) TCA were added to the reaction medium followed by incubation at 95 °C for 30 min. The reaction was stopped by fast cooling on ice, and readings were taken using a spectrophotometer at 535 nm and at 600 nm. TBA forms reddish complexes consisting of low-molecular-mass aldehydes, such as malondialdehyde (MDA), a peroxidation process by-

product. The MDA/TBA complex concentration was calculated using the following equation: [MDA] = (A^{535} –A^{600}) / (ξ.b), where ξ is the extinction coefficient equal to 1.56 x 10^{-5} cm^{-1} and b is the optical length equal to 1.

Antioxidant enzyme extracts were prepared in 2-mL microtubes (Brand®, Sigma-Aldrich®, St. Louis, MO, USA) by adding 1,500 µL of extraction buffer containing the following reagents: 375 µL of 0.1 M potassium phosphate buffer (pH 7.8), 15 µL of 0.1 mM EDTA, 75 µL of 10 mM ascorbic acid and 1,035 µL of distilled water. Next, the homogenous solution was centrifuged at 13,000 × *g* at 4 °C for 10 min. The supernatant was collected and used to measure the enzymatic activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (BIEMELT; KEETMAN; ALBRECHT, 1998).

The SOD activity was determined based on the ability of the enzyme to inhibit nitroblue tetrazolium chloride (NBT) photoreduction, as described by Giannopolitis and Ries (1977), with modifications. Ten microliters of enzymatic extract was added to 190 µL of incubation medium, which was composed of 100 µL of potassium phosphate buffer (100 mM; pH 7.8), 40 µL of methionine (70 mM), 3 µL of EDTA (10 µM), 31 µL of distilled water, 15 µL of NBT (1 mM) and 2 µL of riboflavin (2 µM). Tubes containing the incubation buffer and the samples were illuminated (~ 250 µmol m^{-2} s^{-1}) with a fluorescent lamp (20 W, Osram, Barueri, Brazil) for 10 min. The control treatment was performed using the same reaction medium without the enzymatic extract and maintained for 10 min in a dark room at 25 °C ± 1 °C. Readings were performed at 560 nm, and the SOD activity was assessed using the following equation: % inhibition = (A_{560} sample with enzymatic extract – A_{560} sample without enzymatic extract) / (A_{560} sample without enzymatic extract). One unit of SOD can inhibit 50% of the photoreduction of NBT under the assay conditions.

The CAT activity was quantitatively assessed every 15 sec for 3 min as a decrease in absorbance at 240 nm, which was monitored by H_2O_2

consumption. A applied molar extinction coefficient was 36 M^{-1} L^{-1} cm^{-1} (HAVIR; MCHALE, 1987).

The APX activity was quantified every 15 sec for 3 min by monitoring the ascorbate oxidation rate at 290 nm. One aliquot of 9 µL of enzymatic extract was added to 162 µL of incubation buffer containing 90 µL of potassium phosphate (200 mM; pH 7.0), 9 µL of ascorbic acid (10 mM) and 63 µL of distilled water. Immediately prior to measurements, 9 µL of H_2O_2 (0.1 mM) was added to the incubation medium. A molar extinction coefficient of 36 M^{-1} L^{-1} cm^{-1} was used (NAKANO; ASADA, 1981).

2.9 Experimental design and statistical analysis

The experiments were carried out in a completely randomized design with six replicates per treatment. A replicated unit consisted of a sterile polystyrene Petri dish (90 x 15 mm) (J. Prolab®, Paraná, Brazil) containing 25 mL of culture medium, with five zygotic embryos each. The Petri dishes were sealed with polyvinyl chloride film (Rolopac®, Santana do Parnaíba, Brazil). Data were subjected to analysis of variance (ANOVA) using the statistical software SISVAR 4.3 (System Analysis of Variance for Balanced Data, Lavras, Brazil) (FERREIRA, 2014). Data were compared using Tukey's test (p<0.05).

3 RESULTS

3.1 Moisture content, centesimal composition and histological analysis of seeds

The initial moisture content of *P. ligularis* mature seeds was 11%. According to the results obtained from the centesimal composition, after being dried in an oven at 70 °C ± 3 °C for 24 h, the seeds presented 4.06% moisture content. Our results indicate *P. ligularis* seeds present a large percentage of

lipids (31%) in their reserves, in which they are stored in the form of Triacylglycerol, and can be characterized as oilseeds (Table 1).

Table 1 Centesimal composition of *Passiflora ligularis* seeds (g 100 g^{-1}).

Compounds	Seeds*
Moisture content	4.06 ± 0.12
Dry weight	95.94
Fixed mineral residue	3.05 ± 0.05
Lipids	31.15 ± 0.01
Proteins	13.17 ± 0.09
Crude fiber	22.99 ± 0.07
Total carbohydrates	25.58

* Mean and standard deviation (±).

Mature seeds of *P. ligularis* consist of a brown seed coat and a relatively thick layer of white endosperm, which surround the embryo. The predominant seed reserve content is stored in the endosperm in the form of lipid bodies (Figure 1A).

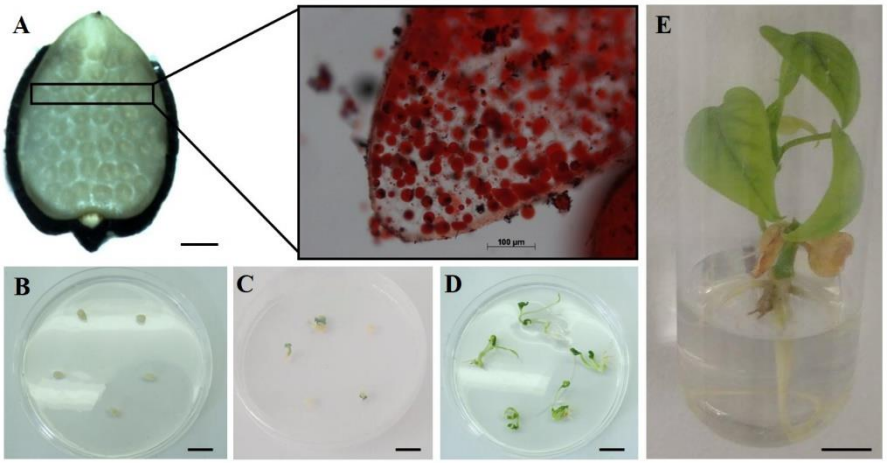

Figure 1 Representative mature seeds with teguments removed and transverse section showing lipidic bodies with toluidine blue dye (bar= 0.5 cm) (A). Visual appearance of the following: zygotic embryos of the

control, 14 days after inoculation in germination medium (bar= 2 cm) (B); zygotic embryos after 0 min of exposure to PVS2 before cryopreservation and 14 days after inoculation in germination medium (bar= 2 cm) (C); and zygotic embryos after 60 min of exposure to PVS2 before cryopreservation, 14 days after inoculation in germination medium (bar= 2 cm) (D). Completely regenerated plant 60 days after cryopreservation (60 min in PVS2) (bar= 2 cm) (E).

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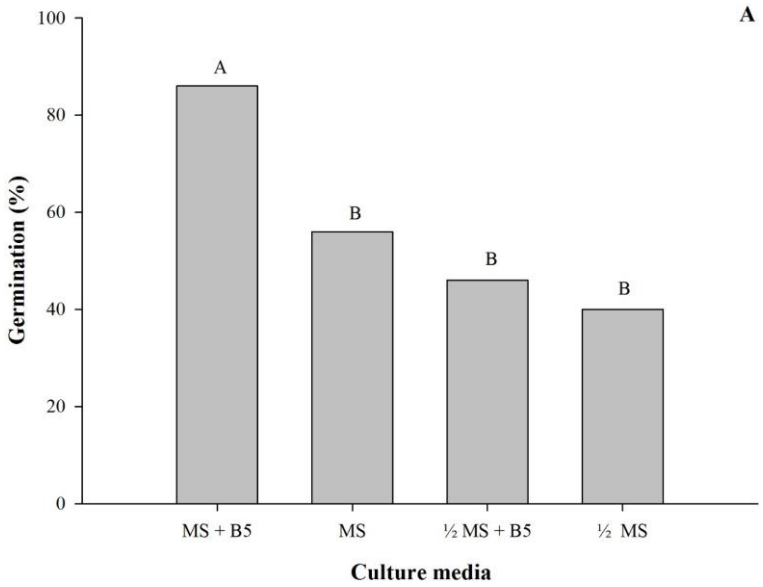
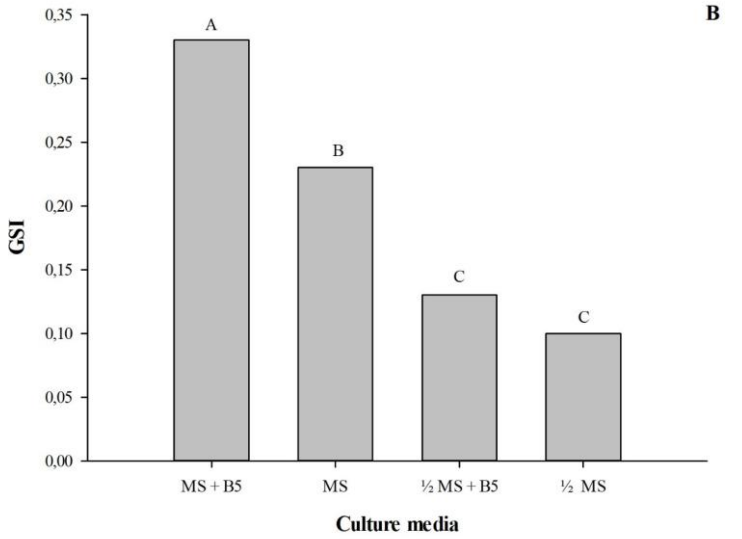
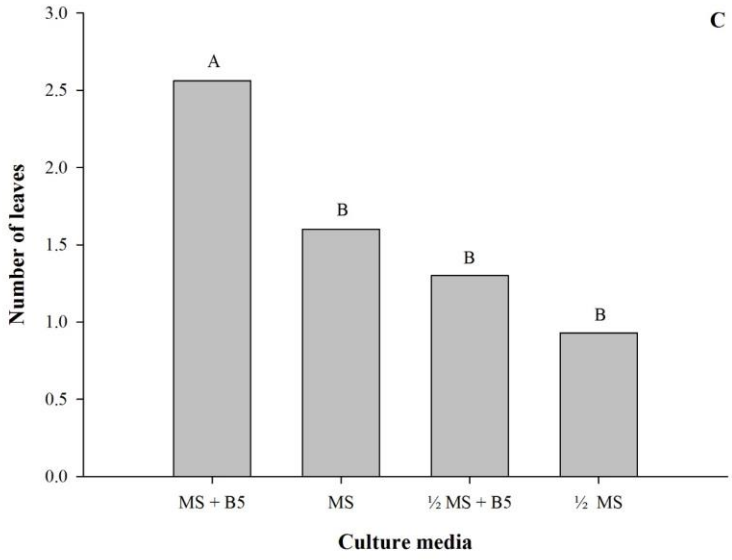
3.2 Germination index

There was significant difference regarding germination in MS basal medium supplemented with B5 vitamins (Figure 2A). This combination resulted in higher GSI, number of leaves and shoot length (Figure 2B-D).




B
GSI
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
A
B
C
C
MS + B5
MS
½ MS + B5
½ MS
Culture media


C
Number of leaves
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
B
B
MS + B5
MS
½ MS + B5
½ MS
Culture media

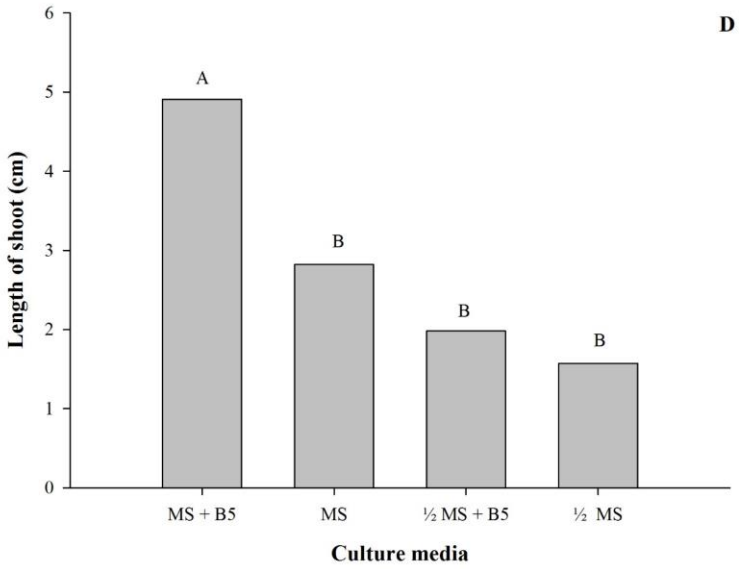


Figure 2. Influence of different concentrations of MS media and types of vitamins (MS vitamins or B5 vitamins) on germination (A), the average number of leaves (B), average root number (C), and the length of shoots (D) of *P. ligularis* cultivated *in vitro* for 30 days. Means followed by the same letter are not significantly different (p>0.05) according to Tukey's multiple range test. MS= MS media; B5 = Gamborg vitamins; ½ MS = half-strength MS; ½ B5 = half-strength Gamborg vitamins.

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3.3 Cryopreservation of seeds

The percentage of germination from zygotic embryos treated with PVS2 and without LN immersion gradually decreased with time of exposure to increasing PVS2 (Figure 3A). Nevertheless, the germination from cryopreserved zygotic embryos increased significantly (Figure 1D) and reached the maximum (86%) after 60 min of PVS2 exposure (Figure 3A). The

highest values for GSI (0.6) were recorded after cryopreservation, representing a considerable advance in the germination process, mainly for the treatment of 60 min of exposure to PVS2 (Figure 3B).

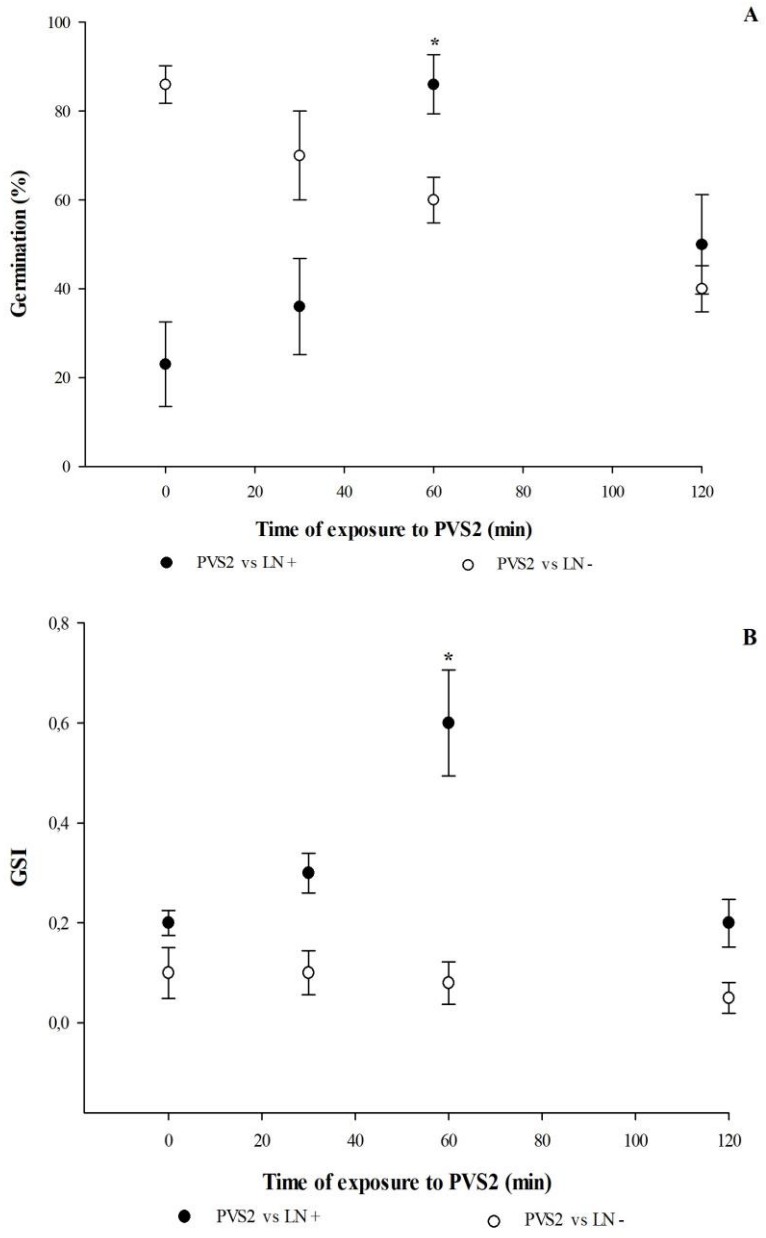

Figure 3 Effect of time exposure to PVS2 (0, 30, 60 and 120 min) in zygotic embryos of *P. ligularis* before immersion in liquid nitrogen (LN +) or without immersion in liquid nitrogen (LN -) on germination percentage (A) and GSI (B) of *P. ligularis*.

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3.4 Proline content

Proline showed a significant increase until each period of time that preceded a higher percentage of germination. Significant changes in the content of proline were observed after 60 min of PVS2, with the highest average after 7 days (Figure 4).

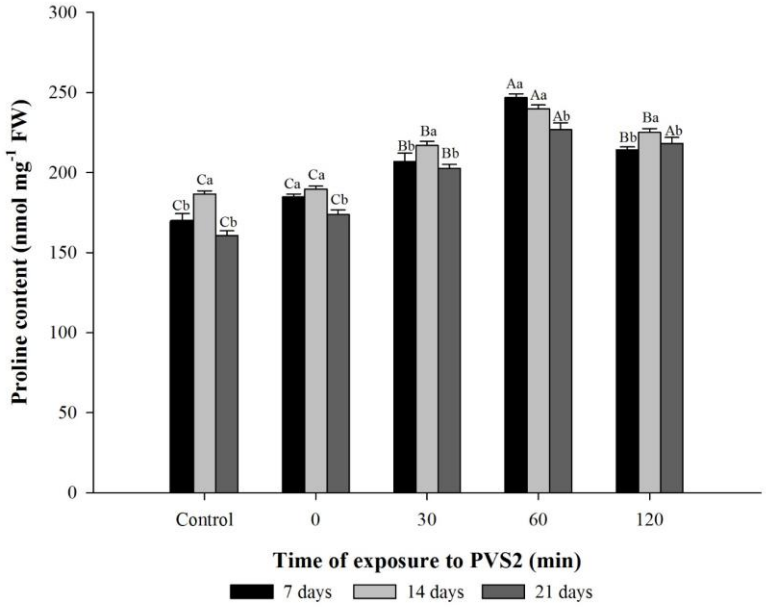

Figure 4 Influence of PVS2 exposure time (0, 30, 60 and 120 min) on proline content of cryopreserved zygotic embryos after 7, 14 and 21 days in germination medium; the control is represented by zygotic embryos

that were not cryopreserved. Means followed by the same uppercase letter (for a given day) and lowercase latter (for a given PVS2 time) are not significantly different using Tukey's test at $p<0.05$.

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3.5 Content of hydrogen peroxide

It is possible to see significant differences in the content of hydrogen peroxide (H_2O_2) due to PVS2 exposure time before cryopreservation compared to that in the embryos without cryopreservation (control) and between the germination days for each treatment (Figure 5).

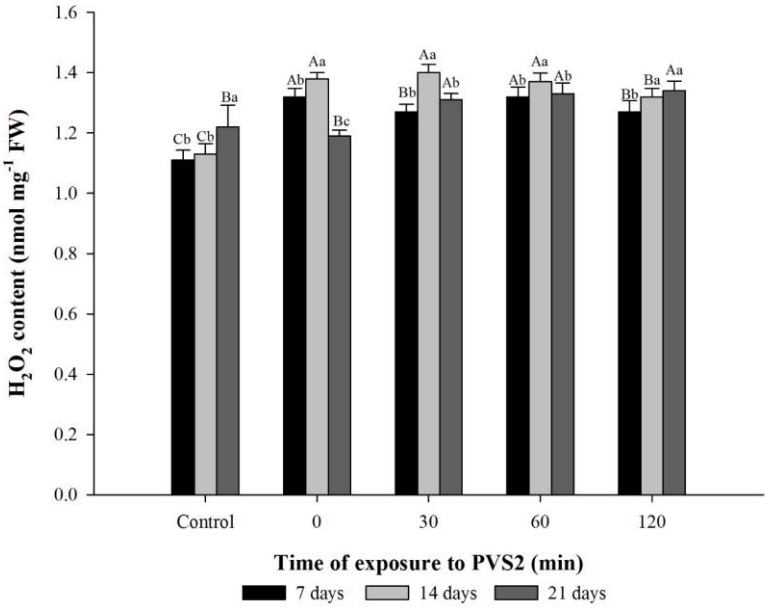

Figure 5 Influence of PVS2 exposure time (0, 30, 60 and 120 min) on the hydrogen peroxide content of cryopreserved zygotic embryos after 7, 14 and 21 days in germination medium; the control is represented by zygotic embryos that were not cryopreserved. Means followed by the same uppercase letter (for a given day) and lowercase latter (for a

given PVS2 time) are not significantly different using Tukey's test at $p<0.05$.

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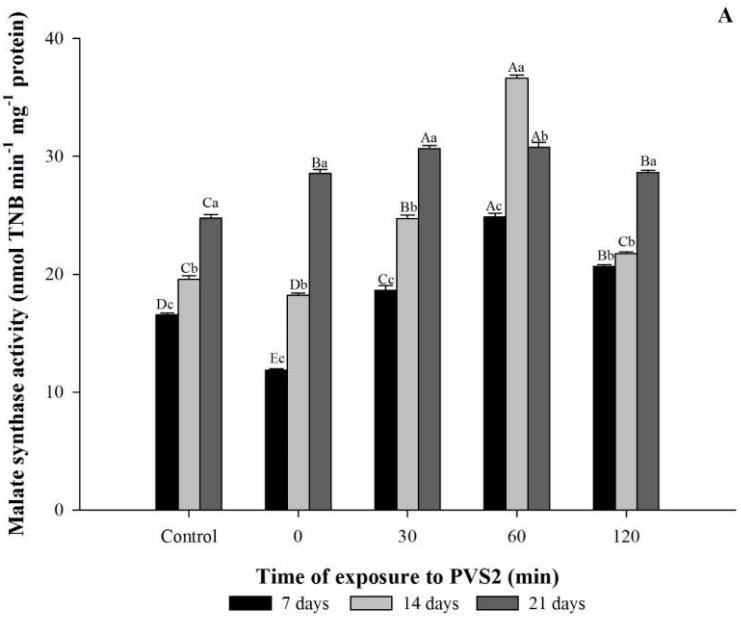
3.6 Measurement of malate synthase and isocitrate lyase

The enzymatic activity showed significant differences between the treatment of 60 min of PVS2 before cryopreservation compared to that of the embryos without cryopreservation (control).



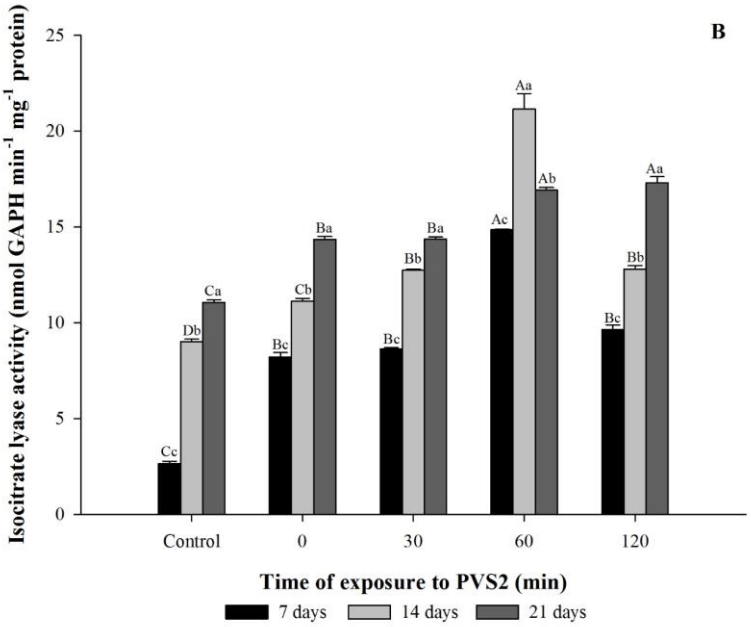


Figure 6 Influence of PVS2 exposure time (0, 30, 60 and 120 min) on isocitrate lyase activity (A) and malate synthase activity (B) of cryopreserved zygotic embryos after 7, 14 and 21 days in germination medium; the control is represented by zygotic embryos that were not cryopreserved. Means followed by the same uppercase letter (for a given day) and lowercase latter (for a given PVS2 time) are not significantly different using Tukey's test at $p<0.05$.

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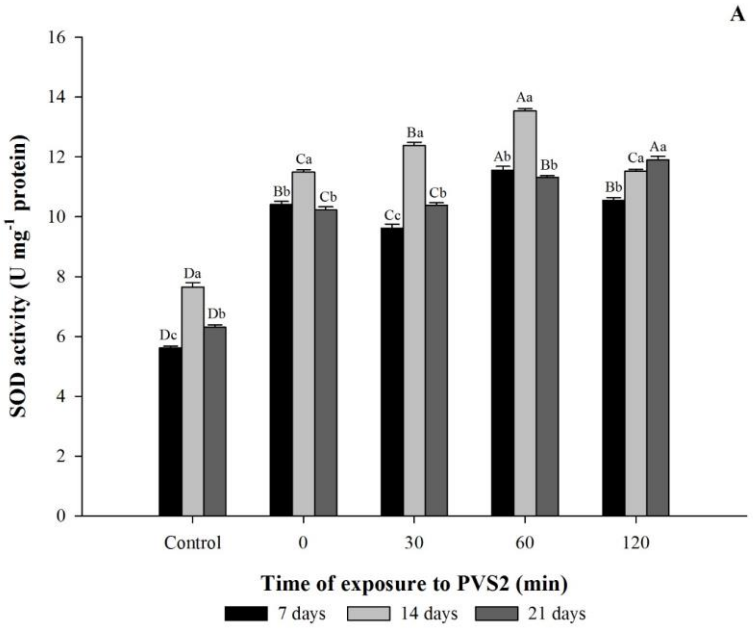
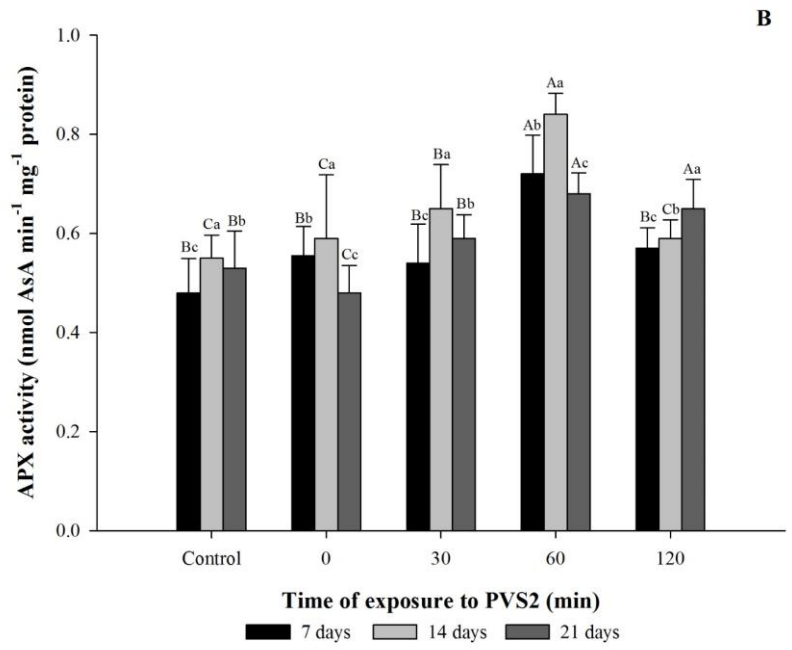
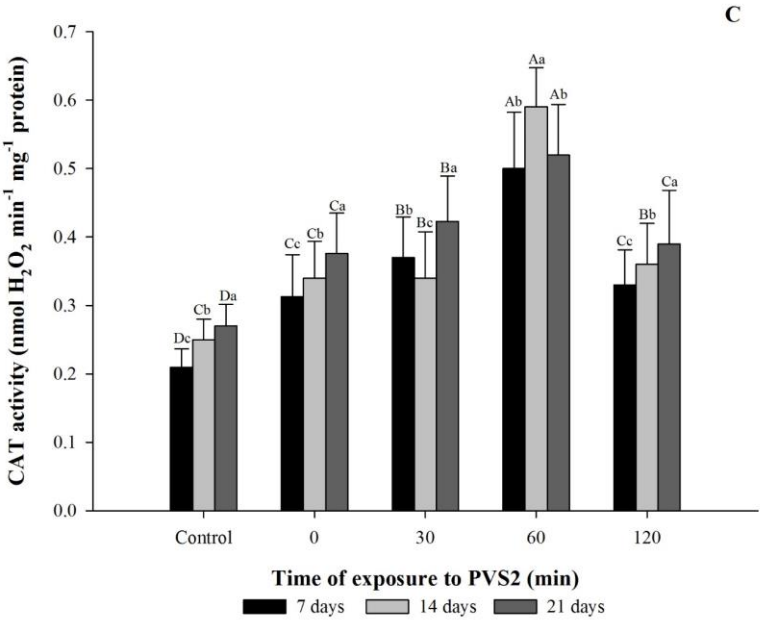
3.7 Lipidic peroxidation and antioxidant enzyme activity assays

The differences in antioxidant enzyme (SOD, CAT and APX) activities are given in Figure 7A-C. Within the treatments, 60 min of PVS2 at 14 days resulted in the highest activity means of both enzymes. For lipid peroxidation, the control had the highest mean values after 21 days of germination (Figure 7D).


A
SOD activity (U mg-1 protein)
16
14
12
10
8
6
4
2
0
Dc
Da
Db
Bb
Ca
Cb
Cc
Ba
Cb
Ab
Aa
Bb
Bb
Ca
Aa
Control
0
30
60
120
Time of exposure to PVS2 (min)
7 days
14 days
21 days


B
APX activity (nmol AsA min-1 mg-1 protein)
1.0
0.8
0.6
0.4
0.2
0.0
Bc
Ca
Bb
Bb
Ca
Cc
Bc
Ba
Bb
Ab
Aa
Ac
Bc
Cb
Aa
Control
0
30
60
120
Time of exposure to PVS2 (min)
7 days
14 days
21 days


C
CAT activity (nmol H_2O_2 min^{-1} mg^{-1} protein)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Dc
Cb
Da
Cc
Cb
Ca
Bb
Bc
Ba
Ab
Aa
Ab
Cc
Bb
Ca
Control
0
30
60
120
Time of exposure to PVS2 (min)
7 days
14 days
21 days

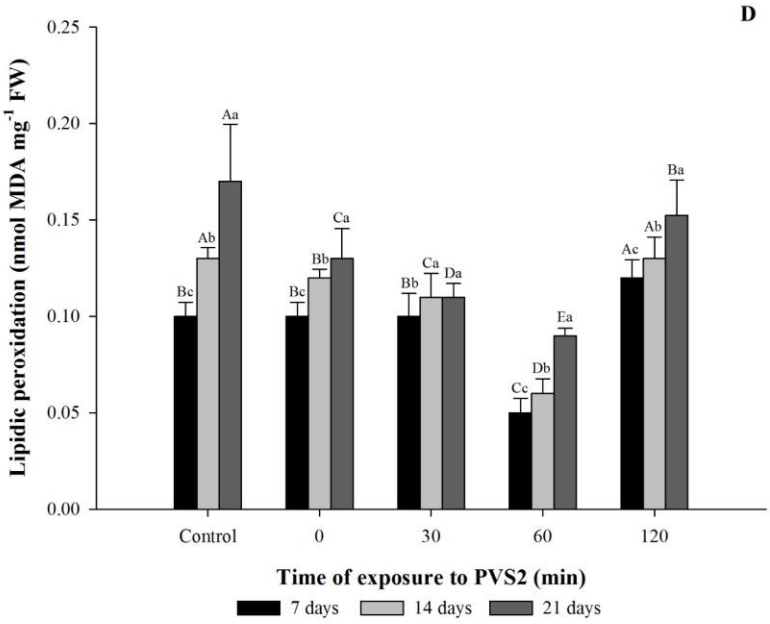

D
Lipidic peroxidation (nmol MDA mg^{-1} FW)
0.00
0.05
0.10
0.15
0.20
0.25
Bc
Ab
Aa
Bc
Bb
Ca
Bb
Ca
Da
Cc
Db
Ea
Ac
Ab
Ba
Control
0
30
60
120
Time of exposure to PVS2 (min)
7 days
14 days
21 days

Figure 7 Influence of PVS2 exposure time (0, 30, 60 and 120 min) on SOD activity (A), APX activity (B), CAT activity (C), and lipidic peroxidation (D) of cryopreserved zygotic embryos after 7, 14 and 21 days in germination medium; the control is represented by zygotic embryos that were not cryopreserved. Means followed by the same uppercase letter (for a given day) and lowercase latter (for a given PVS2 time) are not significantly different using Tukey's test at $p<0.05$.

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4 DISCUSSION

For the majority of wild *Passiflora* species, the absence of *in vitro* regeneration and conservation protocols has not allowed the establishment of gene banks using cryopreservation techniques, even with their advantages and efficiency reported for some species (VEIGA-BARBOSA et al., 2013). In this study, *in vitro* germination of *P. ligularis* was optimized for MS culture medium basal salts supplemented with B5 vitamin complex. However, higher efficiency for different combinations of basal salts and vitamins was observed for *in vitro* germination of other *Passiflora* species: *Passiflora edulis*, ½ MS medium supplemented with B5 vitamins (SILVA et al., 2011); *P. alata*, MS medium with MS vitamins (PINTO et al., 2010); *P. cincinnata*, MS medium with B5 vitamins (SILVA et al., 2009; ROCHA et al., 2012); and *P. suberosa*, ½ MS medium with ½ MS vitamins (GARCIA et al., 2011). When comparing the MS and B5 vitamin formulations, the main difference is their concentrations. For example, the vitamin thiamine is 10 times more concentrated in the B5 formulation (GAMBORG; MILLER; OJIMA, 1968). This fact may be linked to better performance of MS culture medium supplemented with this vitamin complex because thiamine is an essential

cofactor for aerobic respiration reactions in plants, acting as a greater stimulus for growth and germination (GOYER, 2010).

After establishing the culture medium, which maximizes *in vitro* germination, the second step for *in vitro* conservation is determining a technique for the cryopreservation of zygotic embryos without affecting their vigor. The increase of intracellular solute viscosity by highly concentrated solutions is easily achieved with the cryoprotector PVS2 (GONZALEZ-ARNAO et al., 2007). Therefore, the success of cryopreservation using the PVS2 vitrification protocol involves determining the optimal time of exposure of samples to the cryoprotector and fast cooling for improved tolerance to cryopreservation (TSAI et al., 2009).

It is known that the addition of sucrose, ethylene glycol (the simplest diol), glycerol (sugar alcohol with three hydroxyl groups) and DMSO (organosulfur compound) offers excellent protection against a number of different cellular systems from the damaging effects associated with freezing (ASAHINA; TAKAHASHI, 1978; HARVEY et al., 1983; WILLHITE; KATZ, 1984; ROBERTSON et al., 1988; TOWEY et al., 2013). Sucrose is frequently used for the dilution of LS, RS and PVS2 solutions to prevent excessive swelling that can occur when the dilution is directly exposed to the physiological medium (LEUNUFNA; KELLER, 2005). Ethylene glycol and glycerol further share a hydroxyl functionality and the ability to both accept and donate hydrogen atoms (BOUTRON; KAUFMANN, 1979). Both ethylene glycol and glycerol further have low viscosity, resulting in a difficult crystallizations, and in small quantities, they can be rapidly reduced and can be incorporated into metabolic pathways (HUANG; CAUGHEY; DONG, 1995). Moreover, glycerol containing only single bonds is highly flexible for readily adapting to the hydrogen bond structure of surrounding water (STUMPF, 1955; FAHY; WOWK, 2015).

The zygotic embryos treatment for 60 min in PVS2 accelerated the germination process, significantly increasing GSI (Figure 3B). The hypothesis is that during different times of exposure to PVS2 the biochemical reactions

were modified by PVS2 compounds, because after rewarming these compounds may be not entirely removed from explants (ROWLEY et al., 1999).

In this study, was verified that the activity of the enzyme MSy is much higher than that of ICL, which means that the glyoxilate cycle is supplied with glyoxylate from other reactions. The possibilities to increase substrate for the MSy and metabolic pathways for each compound to PVS2 is showed in figure 8. Ethylene glycol for exemple, is primarily produced by the metabolite glycol, which is formed from glycolaldehyde by the action of alcohol dehydrogenase (WALDER; TYLER, 1994). In the glyoxysome, through the glycolate oxidase (OG) enzyme, glycolate can be converted to glyoxylate (YUE et al., 2012). In addition, glycine can be converted into glyoxylate through transamination in the glyoxysome (DELLERO et al., 2016).

During ß-oxidation, excessive production of H_2O_2 can occur, which is broken down by the action of CAT inside the glyoxysome. APX acts only when excess H_2O_2 escapes from the glyoxysome to the cytosol (CORPAS; GUPTA; PALMA, 2015). Glycerol, originating from the breakdown of TAG or as residuals from PVS2, can be phosphorylated and dephosphorylated by glycerol kinase and general phosphatase (HAMMERSTEDT et al., 1990), converted to pyruvate in the cytosol, which enters mitochondria by feeding the tricarboxylic acid (TCA) cycle. The oxidation of dicarboxylic acids by TCA cycle generates reducing power, which is directed to the electron transport chain (CTE) to allow ATP to be formed by reduction of superoxide anion ($\bullet O_2^-$). However, under stressful conditions, such as low temperatures and dehydration, partial reduction of O_2 and formation of $\bullet O_2^-$ can occur (OGAWA; IWABUCHI, 2001). In addition, in mitochondria, there is an enzyme (SOD) capable of converting singlet oxygen ($^1O_2$) to H_2O_2, and when the stressful conditions worsen, the amount of H_2O_2 produced may be greater than the ability of the antioxidant system to contain it (CAT and APX) (CORPAS; GUPTA; PALMA, 2015). In that case, H_2O_2 may be transported to the mitochondria, and this process may function as a signaling mechanism

(Figure 8). Also, in mitochondria, malate formed in the TCA cycle can be easily transported between organelles (FERNIE et al., 2004), exported to the cytosol, where the enzyme that converts malate to OAA (PEP carboxykinase) is present, to form sucrose via gluconeogenesis. The sucrose may protect membranes during cryopreservation or be broken down and assist in embryo growth.

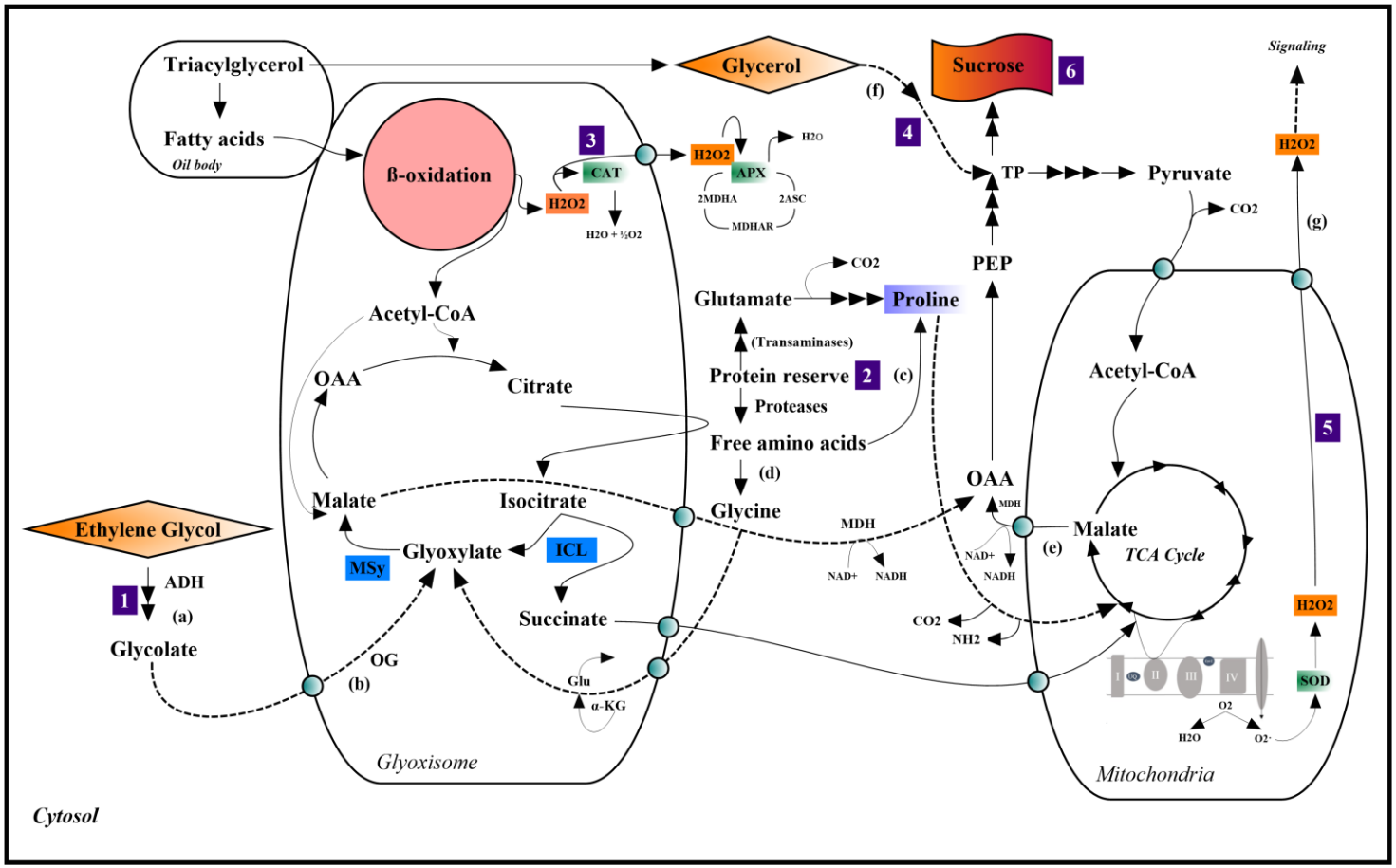
Triacylglycerol
Fatty acids
Oil body
ß-oxidation
Glycerol
Sucrose
6
Signaling
(f)
4
3
CAT
H2O2
H2O + ½O2
H2O2
APX
H2O
2MDHA
2ASC
MDHAR
TP
Pyruvate
CO2
H2O2
(g)
PEP
CO2
Glutamate
Proline
(Transaminases)
Protein reserve
2
(c)
Proteases
Free amino acids
(d)
Acetyl-CoA
OAA
Citrate
Malate
Isocitrate
Glycine
MDH
NAD+
NADH
OAA
MDH
Malate
(e)
TCA Cycle
5
Ethylene Glycol
1
ADH
(a)
Glycolate
MSy
Glyoxylate
ICL
Succinate
CO2
NH2
OG
(b)
Glu
α-KG
H2O2
SOD
O2
H2O
O2·
Glyoxisome
Mitochondria
Cytosol

Figure 8 Proposed pathways of fatty acid catabolism and PVS2 residual compounds during embryo development in *P. ligularis*. The entry of ethylene glycol (1) and passage to the glyoxysome to be broken down into glyoxylate is shown by dashed lines together with the breakdoown of reserve proteins (2) and their possible pathways for the formation of proline and glycine, which can be converted to glyoxylate. In ß-oxidation, increased peroxide produced is broken down by CAT in the glyoxysome or by APX in the cytosol (3). Glycerol can be incorporated into gluconeogenesis and converted to sucrose or from triose phosphate converted to pyruvate, which enters the TCA cycle (4). During the TCA cycle, excessive production of ROS can occur, generating H_2O_2 that can act on the cytosol as signaling (5). Sucrose can be derived from gluconeogenesis and can be a residual compound from the LS, PVS2 or RS (6). *Dashed lines* = Possibilities to occur. *Complete lines* = Pathways previously studied. (a) Walder and Tyler, 1994; (b) Yue et al., 2012; (c) Bewley and Black, 1994; (d) Murray, 2013; (e) Eastmond et al., 2001 and Neill et al., 2002 (f) Penfield et al., 2006; (g) Bailly et al., 2008.

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The DMSO cryoprotective effect is associated with loosening membranes, resulting in electrostatic interactions between the polar sulfoxide moiety of DMSO and phospholipid membranes, favoring the entrance of other cryoprotectants and quick exit of water from cell membranes (YU; QUINN, 1994). However, DMSO is moderately toxic to plant cells, depending on temperature and concentration (FAHY; WOWK, 2015). This is because after rewarming, the excess DMSO can results in an increase of cytosolic calcium which, in turn, activates phospholipases and proteases, affecting the spatial organization of biological membranes (FRANZ; BRUGGEN, 1967; HAMMERSTEDT et al., 1990; SPINDLER et al., 2011; GALVAO et al.,

2014). Thereby, the optimal osmoprotection is directly related to the ability of explants to equilibrate the quantity of DMSO that favors vitrification and not toxicity. The percentage of germination in *P. ligularis* was affected by time of exposure to PVS2, as the ideal internal concentration of DMSO was related to the prevention of oxidative damage resulting from the production of an abundance of H_2O_2 (Figure 5) and the consequent increase in lipid peroxidation (Figure 7D).

Reactive oxygen species, such as H_2O_2, O_2• and hydroxyl radical (•OH), are produced within cells as a consequence of normal metabolic processes, but the production of ROS often increases under stress (SMIRNOFF, 1993; GUTTERIDGE; HALLIWELL, 2006). When ROS are produced at levels high enough to overcome the antioxidant defenses that normally control cellular ROS levels, the oxidation of DNA, proteins and membrane fatty acids occurs. The last can result in lipid peroxidation and loss of membrane function (GUTTERIDGE; HALLIWELL, 2006). Detoxification of ROS by antioxidants usually occurs at the site of production. However, under stress-inducing conditions, the local ROS detoxification capacity is not able to manage the levels of ROS produced, and H_2O_2 can spread to other cellular compartments, e.g., the cytosol and can affect β-oxidation of fatty acids (Figure 8).

H_2O_2 may be also modulates the expression of various genes involved with transcript proteins such as protein kinases and calmodulin, the latter suggesting longer-term effects in the cytoplasm (BAILLY et al., 2008). A recent study on oxidative stress and antioxidant metabolism during the cryopreservation of olive (*Olea europaea* L.) somatic embryos demonstrated the importance of antioxidant metabolism for successful cryopreservation (LYNCH et al., 2011).

The above findings clearly demonstrate that the endogenous accumulation of proline (Figure 4) can play important roles in embryo and seed development induced by various abiotic stresses, including low temperature. Proline is an organic osmoprotectant synthesized from glutamate

in the cell cytosol (Figure 8), especially when the plant is exposed to osmotic stress (SZABADOS; SAVOURE, 2010). Several studies show that proline can function as a molecular chaperone capable of protecting protein integrity against denaturation during severe water stress and modulating antioxidant enzymes activities by increasing nonenzymatic antioxidant contents in addition to its potential role in osmotic adjustment (MATTIOLI et al., 2008). Proline can be easily disseminated when the conditions normalize to serve as a carbon skeleton, providing energy for embryo growth after adverse conditions (ZADEHBAGHRI et al., 2014).

Corroborating this study, significant positive correlations between cellular proline accumulation in the cytosol have been demonstrated in *Arabidopsis thaliana* in which the proline contributes to cytoplasmic osmotic adjustment in response to water loss without interfering with normal cellular processes and biochemical reactions, improving cold tolerance (XIN; BROWSE, 2000). In regenerated *H. rumeliacum*, the concentration of free proline increased twice compared to that of embryos that were not preserved by cryopreservation processes, which could be a clear indication of both the presence of oxidative stress and the low tolerance of that species to freezing (GEORGIEVA et al., 2014).

The present study revealed new aspects of the organization of metabolic processes during *P. ligularis* germination after PVS2 vitrification cryopreservation. The possibilities listed in this study suggest that the components of PVS2 may have a residual effect after cryopreservation, interfering in the speed and germination potential of seeds through antioxidant metabolism and incorporation compunds into metabolic pathways. The high activity of the MSy enzyme demonstrates that operation of the glyoxylate cycle in oilseeds may be important for the conversion of fatty acids to carbohydrates. In conclusion, the application of the cryopreservation technique, in addition to being a tool for conservation, can be applied to accelerate and standardize germination and also may have long-term practical importance for oilseeds.

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