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SUELLEN NUNES SARMENTO

DINÂMICA DE RESPOSTA DE CARBOIDRATOS E PERFIL PROTEÔMICO DURANTE A REGENERAÇÃO DE GEMAS ADVENTÍCIAS A PARTIR DO CULTIVO *IN VITRO* DE ENDOSPERMAS DE *Passiflora cincinnata* Mast.

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RESUMO

SARMENTO, Suellen, M.sc., Universidade Federal de Viçosa, março de 2024. **Dinâmica de resposta de carboidratos e perfil proteômico durante a regeneração de gemas adventícias a partir do cultivo *in vitro* de endospermas de *Passiflora cincinnata* Mast.** Orientador: Diego Ismael Rocha. Coorientadores: Claudete Santa-Catarina e Wagner Otoni.

Características como a totipotencialidade das células vegetais é base para as técnicas de cultivo *in vitro*. O endosperma é um tecido naturalmente triploide, resultado do processo de dupla fecundação. O cultivo *in vitro* de endospermas tem mostrado potencial como via direta para a regeneração de plantas triploides, oferecendo uma alternativa mais rápida para a obtenção de indivíduos triploides em larga escala. Este estudo apresenta dois capítulos que buscam reunir os avanços nos estudos sobre a regeneração *in vitro* de plantas triploides a partir do endosperma, e investigar mudanças estruturais e bioquímicas, identificando padrões na utilização de carboidratos e acúmulo diferencial de proteínas relacionados ao processo de regeneração dos explantes de *Passiflora cincinnata* Mast. Visamos aprimorar a compreensão dos mecanismos envolvidos na regeneração de plantas triploides e otimizar os protocolos de cultivo *in vitro* de endospermas para subsidiar o uso roteiro desta técnica e estudo de melhoramento genético.

Palavras-chave: endosperma. regeneração *in vitro*. triploidia.

ABSTRACT

SARMENTO, Suellen, M.Sc., Federal University of Viçosa, March 2024. Dynamic response of carbohydrates and proteomic profile during the regeneration of adventitious shoots from *in vitro* culture of *Passiflora cincinnata* Mast. endosperms. Advisor: Diego Ismael Rocha. Co-advisors: Claudete Santa-Catarina and Wagner Otoni.

Characteristics such as the totipotency of plant cells underpin *in vitro* culture techniques. Endosperm is a naturally triploid tissue resulting from the double fertilization process. *In vitro* culture of endosperms has shown potential as a direct method for the regeneration of triploid plants, offering a faster alternative for large-scale production of triploid plants. This study presents two chapters that aim to consolidate advancements in the field of *in vitro* triploid plant regeneration from endosperm and investigate structural and biochemical changes, identifying patterns in carbohydrate utilization and differential protein accumulation related to the regeneration process of *Passiflora cincinnata* Mast explants. Our goal is to enhance the understanding of the mechanisms involved in triploid plant regeneration and optimize *in vitro* endosperm culture protocols to support the use of this technique in genetic improvement studies.

Keywords: endosperm, *in vitro* regeneration, triploidy.

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SUMÁRIO

1 INTRODUÇÃO GERAL

A cultura de tecidos está baseada na totipotencialidade das células vegetais, capazes de assumir novas rotas morfogênicas e regenerar um indivíduo inteiro. Sendo o uso desta técnica, fundamental para a contribuição em estudos voltados para a conservação, propagação de espécies de interesse e melhoramento genético. Além disso, a cultura de tecidos, têm se mostrado indispensável para garantir pesquisas que buscam investigar processos bioquímicos, moleculares e fisiológicos ao longo de programas de desenvolvimento vegetal.

Através da regeneração *in vitro*, meristemas caulinares e/ou radiculares, podem ser formados a partir da organogênese *de novo*, enquanto embriões somáticos podem ser originados a partir da indução de embriogênese somática. Na organogênese *de novo* ocorre a formação de meristemas apicais ectópicos em um padrão monopolar, temporalmente e espacialmente separados. Na embriogênese somática, por sua vez, os embriões formados a partir de células somáticas consistem numa estrutura bipolar, semelhante a embriões zigóticos (Rocha et al. 2018), que culminarão na formação de indivíduos inteiros. Para isso, a utilização de balanços hormonais exógenos, principalmente de auxina (AUX) e citocinina (CK), são necessários para a modulação de respostas morfogênicas, possibilitando determinar o destino celular. Um balanço maior de AUX em relação a CK pode gerar a indução de respostas embriogênicas, enquanto maiores concentrações de CK, geralmente, induzem a formação de gemas adventícias (Rocha et al. 2018, Ikeuchi et al. 2019). Ambas as vias morfogênicas podem ocorrer diretamente a partir do explante usado para iniciar a cultura *in vitro* ou, indiretamente a partir de um calo. Esse estádio intermediário na regeneração *in vitro* consiste numa proliferação de células com certo grau de diferenciação geralmente induzida quando AUX e CKs apresentam proporções similares no explante (Atta et al. 2009, Kareem et al. 2016).

Os mecanismos moleculares relacionados à formação de calo a partir da proliferação de células perivasculares, ou especificamente do periciclo, são conservados e bem descritos pela literatura como um processo semelhante a formação de raízes laterais (Sugimoto et al. 2010, Shin et al. 2019). No entanto, sabe-se que a formação de calo pode ocorrer a partir da reprogramação de diferentes tipos celulares, como do parênquima vascular, e de células epidérmicas e/ou subepidérmicas (Yumbla-Orbes et al. 2017, Rocha et al. 2018, Popielarska-Konieczna et al. 2020). Em contrapartida, os mecanismos bioquímicos e fisiológicos relacionados à aquisição de competência morfogênica de tipos celulares não associados com o tecido vascular, ainda são preliminares, necessitando de maiores investigações (Rocha et al. 2018, Ikeuchi et al. 2019).

O endosperma é caracterizado por, na maioria das angiospermas, ser um tecido triploide formado ao longo de um processo de dupla fecundação, e desempenhar função de armazenamento nas sementes (Cailleau et al. 2010). Mas, diante da plasticidade vegetal e possibilidade de reprogramar o destino celular a partir das condições em que células vegetais são submetidas, protocolos de regeneração *in vitro*, a partir do cultivo de endospermas, tem sido estabelecido como via de produção de plantas triploides (Thomas et al. 2000, Thomas e Chaturvedi 2008, Sun et al. 2011, Razdan et al. 2014, Antoniazzi et al. 2018, Silva et al, 2020). Em estudos recentes, utilizando *Passiflora* spp. como modelo, protocolos de regeneração *in vitro* a partir de endosperma foram estabelecidos (Antoniazzi et al. 2018, Silva et al. 2020). Dentre eles, explantes de *P. cincinnata* foram considerados responsivos, apresentando intensa proliferação celular e transformações, com a formação de calo ao longo do tecido em estádios iniciais de indução (Silva et al. 2020), o que torna a espécie um modelo interessante para o entendimento dos aspectos bioquímicos e moleculares envolvidos na aquisição de competência organogênica. Neste sistema de regeneração, endospermas de *P. cincinnata* apresentaram gemas adventícias formadas após em 30 dias de cultivo, ocorrendo este processo a partir da reprogramação de células da face dorsal do endosperma e a triploidia das plantas regeneradas foi confirmada (Antoniazzi et al. 2018, Silva et al. 2020).

A compreensão das sinalizações que desencadeiam aquisição de competência morfogênica em explantes de endospermas pode ser interessante para a otimização de sistemas de regeneração de plantas triploides, tornando possível a produção em larga escala comercial. Do ponto de vista biológico, o endosperma se destaca por ser um potencial tecido modelo interessante para utilização em investigações acerca dos processos fisiológicos, que governam a regeneração celular em plantas. Na presente dissertação apresentamos informações quanto às mudanças bioquímicas e moleculares em endospermas de *P. cincinnata* ao longo do processo de regeneração *in vitro* de gemas adventícias. O presente trabalho está dividido em dois capítulos. No primeiro, intitulado "Cultivo *in vitro* de endosperma: ferramenta biotecnológica para a produção direta de plantas triploides" é apresentado uma revisão detalhando os recentes avanços nos estudos voltados para a produção de plantas triploides, e principalmente, a utilização do endosperma como alternativa direta. No segundo capítulo, intitulado "Dinâmica de resposta de carboidratos e perfil proteômico durante a regeneração de gemas adventícias a partir do cultivo *in vitro* de endospermas de *Passiflora cincinnata* Mast." é apresentado a análise proteômica e bioquímica ao longo da regeneração de gemas adventícias a partir de endospermas. A fim de identificar padrões na utilização de carboidratos e o acúmulo diferencial de proteínas, que podem estar relacionados com a formação de calo e indução da organogênese *de novo* no tecido.

1.2 REFERÊNCIAS BIBLIOGRÁFICAS

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2 CAPÍTULO I

IN VITRO CULTURE OF ENDOSPERM: BIOTECHNOLOGICAL TOOL FOR THE DIRECT PRODUCTION OF TRIPLOID PLANTS - AN OVERVIEW

2.1 ABSTRACT

Polyploidy in plants is generally associated with the expression of superior traits, such as larger lateral organs, seedlessness, better adaptation to climate changes, and increased resistance to biotic and abiotic factors. This arouses interest in plant breeding programs. Polyploidy can be achieved through sexual or somatic hybridization, chemical treatments with anti-mitotic agents, or in vitro cultivation of plant tissues with higher ploidy levels, such as the endosperm. The endosperm is a naturally triploid tissue in most angiosperms. Therefore, the in vitro cultivation of this plant tissue is an intriguing biotechnological method that allows the direct regeneration of triploid plants. The present study aims to summarize recent advancements in the use of endosperm for triploid plant regeneration, providing information to support further studies and efficient methodologies.

Keywords: Plant tissue culture, endosperm, micropropagation, *in vitro* regeneration, triploidy.

2.2 INTRODUCTION

Polyploidy is present in the evolutionary history of a significant part of angiosperms (Hieter and Griffiths 1999, Shakede et al. 2001, Xinge et al. 2010, Hoshino et al. 2011, Wang et al. 2016 Trojak-Goluch et al. 2021). Genome duplication in plants, in general, is associated with the expression of advantageous traits, such as larger flowers and fruits, better adaptation to climate changes, and increased resistance to biotic and abiotic factors (Levin 1983, Trojak-Goluch et al. 2021). These attributes are of interest to plant breeding programs aiming to develop cultivars with superior traits (Sattler et al. 2016, Touchell et al. 2020, Trojak-Goluch et al. 2021). In this context, triploidy is considered a favorable genomic condition for the development of plants with greater vigor and larger vegetative and reproductive organs (Sun et al. 2011).

Triploid organisms have three sets of chromosomes and are generally sterile. However, this characteristic can contribute to the increased longevity of flowers and the flowering period, avoiding the loss of vigor caused by fertilization and seed formation, as well as the production of seedless fruits (Sun et al. 2011, Nakano et al. 2021). Triploids can be found naturally in the environment or can be artificially obtained through somatic hybridization (Liu et al. 2002, 2010, Guo et al. 2006, Eeckhaut et al. 2013, Wang et al. 2016), using chemical treatments with anti-mitotic agents (Wang et al. 2016), or through *in vitro* cultivation of endosperm, which is naturally triploid in most angiosperms (Wang et al. 2016, Datta 2022).

The *in vitro* cultivation of endosperm is a direct method for obtaining triploid plants (Thomas and Chaturvedi 2008, Hoshino et al. 2011, Kumar and Gupta 2015). However, this method is still technically complex due to the influence of the species genotype, endosperm development stage, and the culture medium used (Thammina et al. 2011, Popielarska-Konieczna et al. 2013, Wang et al. 2016).

This study aimed to compile the main protocols described for *in vitro* endosperm regeneration, providing information to assist in the development of more efficient strategies for obtaining triploid plants from this plant tissue.

2.3 METHODS FOR OBTAINING TRIPLOID PLANTS

Triploid plants can be naturally obtained through sexual hybridization between diploid and tetraploid organisms, as well as through somatic hybridization, chemical treatments with anti-mitotic agents, and *in vitro* endosperm culture (Smith et al. 1993, Liu et al. 2002, 2010, Guo et al. 2006, Eeckhaut et al. 2013, Datta 2022, Wang et al. 2022). Natural triploids are widely present in nature, and their development can be favored by genetic factors, such as abnormalities in the meiotic process, resulting in unreduced gametes that can form polyploid zygotes (Wang et al. 2016).

This polyploidy occurs more frequently in plants subjected to stresses such as frost, injuries, high irradiance, herbivory attack, and water or nutrient scarcity (Ramsey et al. 1998, Mason et al. 2011, Sora et al. 2016, Trojak-Goluch et al. 2021, Wang et al. 2022). The availability of natural triploid lineages, however, is rare due to seed inviability (Datta 2022).

(i) *Sexual hybridization* - Sexual hybridization is the most commonly used technique for obtaining triploid organisms and involves generating hybrid populations by crossing diploid and tetraploid individuals, followed by selecting promising individuals (Oliveira et al. 2014). During controlled interploid crossing, embryo death is sometimes observed at an early stage of development, possibly resulting from endosperm degeneration during the early stages of embryogenesis (Guo et al. 2011, Trojak-Goluch et al. 2021). Thus, one way to overcome the barriers of seed inviability in triploid plants is embryo rescue. This technique allows overcoming issues such as cross incompatibility, dormancy, and low germination, ensuring cloning and plant development through micropropagation for genetic improvement studies (Sharma et al. 1996). The effectiveness of this method depends on genotypes, the number of days after pollination, and *in vitro* cultivation conditions (Trojak-Goluch et al. 2021). For species like *Hemerocallis* L., protocols for embryo rescue have been established from triploid genotypes kept in germplasm banks, with the aim of enabling commercial-scale propagation of these cultivars for ornamental use (Li et al. 2009).

(ii) *Somatic hybridization* - Somatic hybridization involves producing hybrids by fusing protoplasts (Gmitter et al. 1992, Liu et al. 2005). This process allows combining parents with distinct traits without significant loss of vigor, using sterile clones, and diversifying tetraploid genotypes (Carvalho and Sena 2007). Additionally, it allows overcoming cross incompatibility by obtaining hybrid forms and the direct synthesis of triploid hybrids through the fusion of haploid and diploid protoplasts (Carvalho and Sena 2007).

Protoplasts can be isolated from various plant tissues. However, cell suspensions and calli are more commonly used due to the ease and efficiency in manipulation and isolation (Carvalho and Sena 2007, Vieira 1997). After isolation, explants undergo disinfection, degradation of the cell wall through pectocellulolytic enzymes, and finally, they are cultivated in a culture medium rich in organic and inorganic components, supplemented with growth regulators (Carvalho and Sena 2007, Vieira 1997).

Fusion induction can be achieved through chemical treatments, such as the use of polyethylene glycol, or through physical methods, such as electroporation. Regardless of the method, the success of protoplast fusion depends on the genotype, physiological state of the plant, age of the explant, incubation time in the enzymatic solution, type of solution, agitation, concentration, and types of osmotic stabilizers, density of cultivation, cultivation methods, culture media, light, and temperature (Carvalho and Sena 2007).

(iii) *Polyploidizing agents* - Triploidy can also be induced through the use of a ‘spindle inhibitor, which aims to interrupt mitosis (Wittmann and Dall’agnol 2003, Trojak-Goluch et al. 2021). These inhibitory agents act by disrupting cellular microtubules and preventing the movement of sister chromatids of the chromosome during anaphase (Trojak-Goluch et al. 2021). Various commercial herbicides have been tested for use as polyploidizing or anti-mitotic agents, such as trifluralin, oryzalin, dithiopyr, thiazopyr, amiprofos-methyl (APM), among others (Emsweller and Ruttle 1941, Craig 2003).

Colchicine, oryzalin, and trifluralin are the most commonly used anti-mitotics to induce polyploidy in plants (Vainola 2000, Blakesley et al. 2002, Kadota and Niimi 2002, Shao et al. 2003). Among these, colchicine is the most well-known antimitotic, used in concentrations ranging from 0.25 to 38 mM (Chen et al. 2006, Stanys et al. 2006). The significant advantages of using this antimitotic are its ease of application, low toxicity, and efficiency in polyploid production. Moreover, application can be done on seeds, seedlings, or vegetative parts with active meristematic tissues (Wittmann and Dall’agnol 2003). However, colchicine has a low affinity for tubulin dimers compared to herbicides and causes side effects such as sterility, abnormal growth, and chromosomal alterations (Zeng et al. 2019). Consequently, colchicine has been progressively replaced by mitosis-inhibiting herbicides like oryzalin, trifluralin, and amiprofos-methyl (Zeng et al. 2019).

Oryzalin, in turn, can be used in lower concentrations compared to colchicine, which could minimize cytotoxic effects (Dhooghe et al. 2009). Additionally, oryzalin has been more effective than colchicine in inducing chromosomal duplication in Rhododendron (Vainola, 2000) and Solanum (Greplová et al. 2009) and can be used for inducing tetraploids in bananas (Van Duren et al. 1996, Ganga and Chezhiyan 2002, Kanchanapoom and Koarapatchaikul 2012).

Polyploidizing agents can be applied in both *in vivo* and *in vitro* conditions. The concentration and duration of treatment of these agents, as well as the species, cultivar, genotype, and type of tissue, affect the percentage of polyploidization success (Allum et al.

2007, Datta 2022, Trojak-Goluch et al. 2021). High concentrations of these polyploidization agents are toxic to plant cells, and usually cause abnormal plant growth or reduced viability, while low concentrations may be ineffective or induce many mixoploids or aneuploids (Manzoor et al. 2019, Trojak-Goluch et al. 2021).

(iv) In vitro *culture of endosperms* **-** Triploid plants can also be developed directly through *in vitro* culture of endosperm (Thomas and Chaturvedi 2008, Hoshino 2011). This technique involves cultivating the endosperm in a culture medium supplemented with growth regulators, in test tubes, Petri dishes, or similar containers, under aseptic conditions (Cid 2001). Protocols for endosperm culture have been established, but their routine application has not been feasible for most plant species (Datta 2022). However, it presents itself as an alternative approach in genetic improvement for the regeneration of triploid plants (Thomas and Chaturvedi 2008, Hoshino et al. 2011, Wang et al. 2016) and will be the main topic of discussion in this work.

2.4 THE ENDOSPERM

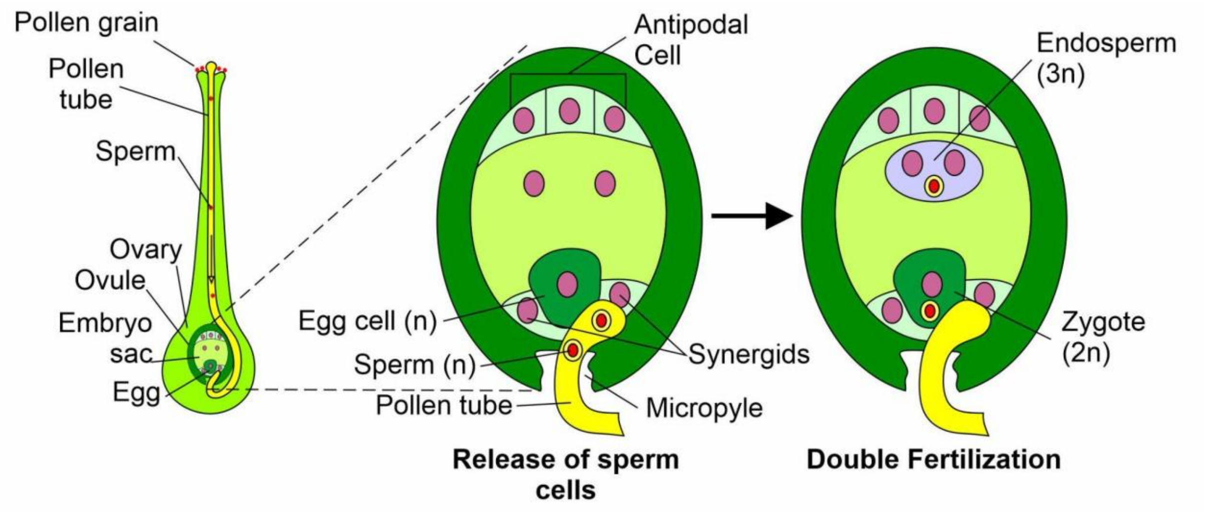
The endosperm is a triploid plant tissue, natural and unique in its origin, ploidy level, and growth nature (Kumar and Gupta 2015). Although, this tissue assumes various functions, its primary role is to provide essential nutrients to the zygotic embryo and, at times, to seedlings, regulating embryonic growth during germination and early seedling development (Brink and Cooper 1947, Raghavan 1966, Costa et al. 2011, Yan et al. 2014).

Endosperm can function as a mechanical barrier capable of detecting environmental signals and producing and secreting signals to regulate zygotic embryo growth, playing a critical role during the embryogenic process (Van Hengel et al. 1998, Okamoto et al. 2006, Costa et al. 2011, Yan et al. 2014). This tissue is also involved in maintaining the osmotic pressure gradient, preventing premature seed germination (Aqüila 2004).

The endosperm can be absorbed during embryonic development or persist partially or entirely after the physiological maturity of the seeds (Costa et al. 2011, Hoshino et al. 2011). It is found in all angiosperm families, except Orchidaceae, Trapaceae, and Podostemaceae, where endosperm development is interrupted early in its formation (Maheshwari 1950, Vijayaraghavan and Prabhakar 1984, Hoshino et al. 2011, Kumar and Gupta 2015).

This tissue results from double fertilization (Figure 1), a typical process in angiosperms (Chaudhury et al. 1998, Niedojadlo and Bednarska-Kozakiewicz 2022). During fertilization, one of the male gametes fuses with the ovule, forming a zygote. The other male gamete fuses with the central cell of the embryo sac, containing two haploid nuclei (Figure 1). This triple fusion, therefore, gives rise to a triploid structure known as the endosperm (Hoshino et al. 2011, Kumar and Gupta 2015, Hater 2020, Niedojadlo and Bednarska-Kozakiewicz 2022).

Figure 1 – Schematic representation of double fertilization.



Source: Adapted from sciencefacts.net.

Depending on the process that originated the embryo sac, this tissue can assume distinct ploidy levels (Costa et al. 2011). Furthermore, during its development, the endosperm can become a mixoploid tissue due to an increase in the size of the nuclei, which become hypertrophied and polyploid (Vijayaraghavan and Prabhakar 1984).

The evolutionary origin of this tissue is still a subject of debate (Friedman 1998, Berger 2003, Friedman and Williams 2004), but there are two widely accepted theories. The first theory, supported by Friedman (1994), argues that in ancestral species of current angiosperms, double fertilization produced two embryos, and one of these specialized in the accumulation of nutrient reserves, giving rise to the current endosperm later (Costa et al. 2011). The second, supported by several authors (Berger 2003, Friedman and Williams 2004), contends that there was a parallel and synchronized development of the embryo and cells of the embryo sac, which later specialized in storing nutrient reserves for the developing embryo (Costa et al. 2011).

The development of the endosperm can be divided into various phases, including the formation of the nuclear endosperm, cellularization, differentiation, maturation, and cell death (Berger 1999, Yan et al. 2014). This development involves repetitive nuclear divisions without cytokinesis (Berger 1999). Subsequently, cellularization occurs, forming a periclinal cell wall and separating the sister nuclei (Yan et al. 2014). This stage is followed by the differentiation of functional tissues, and eventually, most endosperm cells die during seed maturation (Berger 1999).

2.5 *IN VITRO* ENDOSPERM CULTURE

The first attempts at *in vitro* endosperm culture took place in the 1930s, using corn endosperm in a medium fortified with extracts from potatoes and young corn kernels (Lampe and Mills 1936). Subsequently, various research laboratories initiated their studies with this tissue (Straus and La Rue 1954, Tamaoki and Ullstrup 1958, Sehgal 1969, Felker and Goodwin 1988, Faranda et al. 1994).

Currently, endosperm culture has been tested for approximately 40 species (Table 1), including several studies established for *Actinidia* spp., *Passiflora* spp. and *Eucalyptus* sp. (Kin et al. 1990, Mohamed et al. 1996, Li et al. 2008, Popielarska-Konieczna et al. 2011, Antoniazzi et al. 2018, Mikovski et al. 2021, Machado et al. 2022).

Table 1 – Description of the culture medium conditions for plant regeneration from endosperm, the appropriate explant stages and the frequency of regeneration obtained.

Species	**Endosperm stage**	**Culture conditions**	**Responses**	**References**
Acacia nilotica (L.) Willd. ex Delile	Immature	MS + 3% Sucrose + 0.8% agar	Embryogenic callus induction: 7%	Garg *et al.* 1996
		Embryogenic callus induction: 2,4-D (10 µM) + BAP (25 µM) + casein (1000 mg/l)		
		Somatic embryo germination: MSm + B5 major salts, inositol, glutamine, CW, CH, e 0.2% phytagel		
Actinidia sp.	Mature	Callus induction: LS + 13.7 µM zeatin + 0.54 µM NAA + 0.4 g/l casein.	-	Kin *et al. 1990*
		Buds induction: LS+ 4.6 µM zeatin + 0.4 g/l CH + 3% sucrose		
		Induction of roots and seedlings: LS+ 490 µM IBA		
Actinidia arguta (Siebold & Zucc.) Planch. ex Miq.	Mature	MS + macro, microelements and vitamins (Duchefa) + sucrose (3%) + ágar (Duchefa) (0,6%) and PGRs	-	Abdullah *et al.* 2021
		Callus induction: TDZ (0.25, 0.5 or 1.0 mg/l) + 2,4-D (2 mg/l) + kinetin (5 mg/l).		

Actinidia chinensis Planch.	–	Callus induction: MS + Zeatin (3 ppm) + 2,4-D (0.5 ppm) + CH (400 ppm)	–	Gui *et al.* 1982
Actinidia deliciosa (A. Chev.) C.F. Liang & A.R. Ferguson	Mature	Callus induction: MS + 2 mg/l de 2,4-D + 5 mg/l kinetin	Callus formation: 80%	Góralski *et al.* 2005
		Shoot regeneration: MS + TDZ (0.5 mg/l)		
		Rooting: the shoots were extracted from the callus → soaked in a solution of IBA (100 mg/1) → 1/2 MS		
		Callus induction: MS + 0,5 mg/l TDZ + 8% agar	Callus formation: 80%	Popielarska-Konieczna *et al.* 2011
Actinidia kolomikta (Maxim. & Rupr.) Maxim.	Mature	Callus induction: MS + 0,1 mg/l 2,4-D + 1/2 or 5 mg/l kinetin	Callus formation: 60%	Asakura and Hoshino, 2017
Santalum album L.	Immature	Callus induction: MS + BAP (0,5–2 mg/l) + NAA (1 mg/l).	-	Lakshmi Sita *et al. 1980*
		Embryo differentiation: MS + GA (1–2 mg/l) or MS + BAP (0,3 mg/l) + IAA (1 mg/l) or MS + GA (1 mg/l) + kinetin (0,3 mg/l).		
		Shoot and root development: White culture medium + IAA (0,5 mg/l)		
Annona squamosa L.	Mature	White + 3% sucrose + 0.8% agar or Nitsch + 2% sucrose + 0.8% agar	*De novo* organogenesis: 25%	Nair *et al.* 1986
		Callus induction: White + kinetin (0.1 mg/l) + BAP (0.2 mg/l) + NAA (1 mg/l) + GA_3 (1 mg/l)		
		Shoot regenerations: Nitsch + BAP (2 mg/l) + NAA (0.5 mg/l)		
Azadirachta indica A. Juss.	Mature	MS + 3% sucrose + 0.8% agar	Callus formation:53% *De novo* organogenesis: 95%	Chaturvedi *et al.* 2003
		Callus induction: NAA (5 µM) + BAP (2 µM) + CH (500 mg/l)		
		Shoot regeneration: BAP (5 µM)		
		Stretching: BAP (0.5 µM)		
		Multiplication: BAP (1 µM) + CH (250 mg/l)		
		Rooting: 1/2 MS + IBA (0.5 µM)		
Barringtonia racemosa (L.) Spreng.	Mature	Callus induction: MS + 1,0 mg/l de 2,4-dichlorophenoxyacetic acid + 1,5 mg/l de kinetin	Callus formation: 56,7%	Osman *et al.* 2016

Carica papaya L.	Immature	Callus induction: MS + 6.0 μM 2,4-D + 2.5 μM NAA + 4.0 μM KT.	Callus formation: 67,8% *De novo* organogenesis: 93,8%	Sun *et al. 2011*
		Shoot induction: 3.0 μM TDZ + 1.5 μM NAA or 1.5 μM BA + 3.0 μM IAA		
		Shoot and root development: 1/2MS + 2.0 μM IBA		
Carthamus tinctorius L.	Immature	Callus induction: MS + 4.44 μM BAP or 2.32 μM kinetin.	-	Walia *et al. 2007*
		Shoot induction: MS + 4.5 μM 2,4-D, 2.27 μM TDZ		
Citrus sp.	Immature	Callus induction: MT + 22.2 μM BA + 9.04 μM kinetin + 23.2 μM or MT + 2,4-D + 1 g/l CH +0.5 g/l ME.	-	Gmitter *et al.* 1990
		Shoot and root development: 2 MT + 5.5 μM BA + 14.8 μM Ad + 5.77 μM GA3 + 0.5 g/l casein		
Codiaeum variegatum Blume	Mature	Callus induction: White culture medium + 4.5 μM 2,4-D + 4.65 μM kinetin + 0.5 g·/l CH + 10% CM.	-	Chikkannaiah and Gayatri 1974
		Shoot induction: 4.5 μM 2,4-D + 0.5 g/l CH + 10% CM		
Codiaeum variegatum Blume	Mature	Callus induction: White culture medium + 9.04 μM 2,4-D + 23.2 μM KT + 2.5 g/l YE.	-	Bhojwani and Johri 1971
		Shoot induction: 0.1 μM IBA		
Dendrophthoe falcata (L.f.) Ettingsh.	Mature	Callus induction: White culture medium + 24.6 μM IBA.	*De novo* organogenesis: 80%	Johri and Nag 1968
		Shoot induction: 23.2 μM KT or 148 μM Ad		
Diospyros kaki Thunb.	Mature	Callus induction: MS (½N) + 10 μM de zeatin + 10 μM IAA + 500 mg/L casein	Callus formation: 24% *De novo* organogenesis: 37%	Tao *et al.* 1997
		MSm (1/2 force for nitrats)		
		Callus induction: Zeatin (10 μM) + IAA (10 μM) + CH (500 mg/l)		
		Subculture: Zeatin (10 μM) + IAA (1 μM)		
		Adventitious shoot: Zeatin (10 μM) + IAA (0.1 μM)		
		Growth: Zeatin (5 μM) + IBA (1 μM)		
		Rooting: IBA (1.5 mM) → 1/2 MS		
Emblica officinalis Gaertn.	Mature	MS + 2% sucrose + 0.8% agar	*De novo* organogenesis: 50%	Sehgal and Khurana 1985
		Callus induction: IAA (1 mg/l) + BAP (1 mg/l) or 2,4-D (1 mg/l) + kinetin (1 mg/l)		
		Regeneration: IAA (0.1 mg/l) + BAP (0.2 mg/l)		
		Stretching and rooting- NAA (0.002 mg/l)		

Eucalyptus sp.	Immature	Callus induction: MS + 6.66 μM BA + 5.34 μM IAA. Shoots induction: MS + 4.44 μM BA + 5.37 μM NAA. Shoot and root development: 1/2 MS + 2.46 μM IBA.	-	Li *et al.* 2008
Euonymus alatus (Thunb.) Siebold	Mature and Immature	Callus induction: 2.22 μM BA + 2.7 μM NAA. Shoots induction: MS + 4.4 μM BA + 0.5 μM IBA Shoot and root development: WPM + 4.9 μM IBA	Callus formation: 51%. *De novo* organogenesis: 85%	Thammina *et al.* 2011
Exocarpos cupressiformis Labill.	Mature	Callus induction: White culture medium + 9.04 μM 2,4-D + 23.2 μM KT + 2.5g/l CH	-	Johri and Bhojwani 1965
Haemanthus albiflos Jacq.	Immature	Callus induction: MS + picloram (5 mg/l) + BAP (5 mg/l)	-	Nakano *et al.* 2021
Hordeum vulgare L.	Immature	Callus induction: MS + 4.5 μM 2,4-D +0.5 g/l CH Shoots induction: MS + 2.32 μM KT + 1.07 μM NAA	-	Sun and Zhu 1981
Jatropha curcas L.	Mature	*de novo* organogenesis induction: MS + 0.25 mg/l IAA + 0.5 mg/l kinetin + 1.0 mg/l de BAP + 0.25 mg/l GA$_3$	*De novo* organogenesis: 85,2%	Zhu *et al.* 2011
Lolium Multiflorum Lam.	Mature	Callus induction: White + 5.7 μM IAA + 117 mM Sucrose + 5 g/l YE	-	Smith and Stone 1973
Lonicera caerulea L.	Immature	MS + 3% sucrose + 0.2% gellan gum Callus induction: BA (2.22 μM) + IBA (0.49 μM) Shoot regeneration: BA (2.22 μM) + IBA (0.49 μM) Shoot proliferation: 1/2 MS + GA$_3$ (2.89 μM) stretching and rooting: 1/2 MS + BA (0.44 μM) + GA$_3$ (2.89 μM) shoot development: 1/2 MS	Callus formation: 63.3% *De novo* organogenesis: 10%	Miyashita *et al.* 2009
Lycopersicon esculentum Mill	Mature	Callus induction: 0.44 μM BA + 4.5 μM 2,4-D + 28.9 μM GA$_3$	-	Kagan-Zur *et al.* 1990
Melia azedarach L.	Immature	Callus induction: MS + 2.0 mg/l de NAA + 1.0 mg/l de BAP *de novo* organogenesis induction: MS + 1.5 mg/l BAP + 0.5 mg/l NAA	Callus formation: 55,9% *De novo* organogenesis: 98%	Van Thang *et al.* 2018

			16,7 media of shoot/explant	
Morus alba L.	Immature	MS + 3% sacarose + 0.8% ágar	Callus formation: 70–72% Callus *De novo* organogenesis: 62.5–75%	Thomas *et al.* 2000
		Callus induction: BAP (5 µM) + NAA (1 µM) + CM (15%) or YE (1000 mg/l)		
		Multiplication: 2,4-D (5 µM)		
		Shoot regeneration: TDZ (1 µM) or BAP (5 µM) + NAA (1 µM)		
		Shoot multiplication: 5 or 7 µM BAP		
		Rooting: 1/2 MS + IBA (7 µM)		
Oryza sativa L.	Mature and immature	Callus induction: MS + 2,4-D (2 mg/l)	–	Bajaj *et al.* 1980
		Subculture: MS + 2,4-D (1 mg/l)		
		Shoot regeneration: MS + IAA (4 mg/l) + kinetin (2 mg/l)		
Passiflora Cincinnata Mast.	Mature	Callus induction via *de novo* organogenesis: MS medium supplemented with 1.5, 2.0, and 3.0 mg L^{-1} of BA or TDZ		Silva et al. 2020
Passiflora cincinnata Mast.	Mature	Callus induction: MS + 18.1 µM 2,4-D + 4,5 µM BA	Somatic embryo number: 8,8 ± 1,3	Machado *et al.* 2022
		MS + mio-inositol (0,01%) + sucrose (3%) + agar (8 g/L)		
Passiflora edulis Sims	Mature	*de novo* organogenesis induction: MS + vitamins MS + 0,01% mio-inositol + 3% sucrose + 0.8% agar + 9 µM TDZ	*De novo* organogenesis: 32%	Antoniazzi *et al.* 2018
Passiflora foetida L.	Mature	*de novo* organogenesis induction: MS + TDZ 1.5 mg/l	*De novo* organogenesis: 27%	Mikovski *et al.* 2021
		de novo organogenesis induction: MS + TDZ 2mg/l		
		MS + 0.09 M sucrose		Mohamed *et al.* 1996
		Regeneration: BA (2 µM)		
		Growth and development: GA_3 (29 µM) + CH (1000 mg/l)		

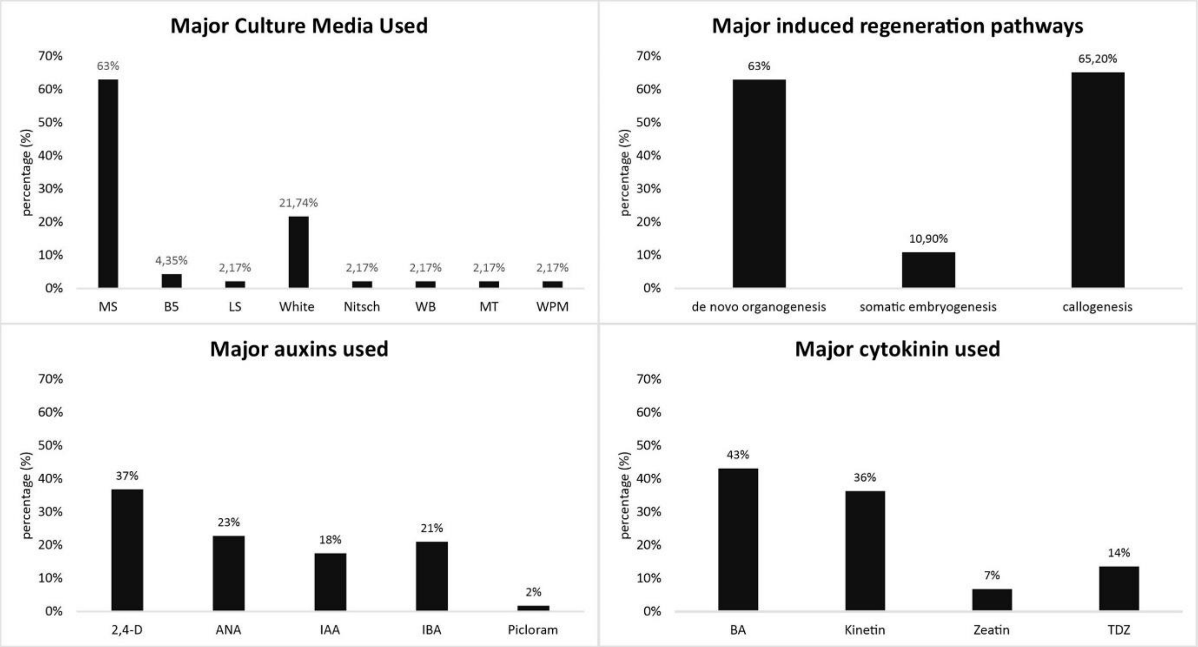
		Stretching: MS Rooting: NAA (5 µM)		
Petroselinum hortense L.H. Bailey	Mature	Embryogenic calli induction: MS + 3% sucrose + 0.6% agar	Callus formation: 24,5%	Masuda *et al.* 1977
Phlox Drummondii Hook.	Immature	Callus induction: MS + 5 µM BAP + 10 µM NAA. Shoot and seedling induction: MS + 10 µM BAP + 2.5 µM IAA	*De novo* organogenesis: 82%.	Tiku *et al.* 2014
Ricinus communis L.	Mature	Callus induction: WB + 9.04 µM 2,4-D + 23.2KT + 2.5 g/l YE	-	Srivastava 1971
Santalum álbum L.	Immature	Callus induction: MS + BAP (0.5–2 mg L^{-1}) + NAA (1 mg l^{-1}) Embryo differentiation: MS + GA (1–2 mg/l) or MS + BA (0.3 mg/l) + IAA (1 mg/l) or MS + GA (1 mg/l) + kinetin (0.3 mg/l) Shoot and root development: White + IAA (0.5 mg/l)	–	Lakshmi Sita *et al.* 1980
Sapium sebiferum (L.) Dum. Cours.	Mature	Callus induction: MS + 4.44 µM BA + 5.37 µM NAA. Shoot induction: 8.88 µM BA + 1.07 µM NAA Induction of roots and seedlings: MS + 9.84 µM IBA + activated carbon	Callus induction: 80% *De novo* organogenesis: 40–66.7% Root regeneration 80%	Tian *et al.* 2012
Taxillus vestitus (Wall.) Danser	Mature	Shoot induction: White + 50 µM kinetin	-	Johri and Nag 1970
Taxus chinensis (Pilg.) Rehder	Mature	Callus induction: Gamborg medium (B5) + sucrose (30 g /l) + 2,4-D (2.5 mg/l) + BA (0.5 mg/l) + agar (7 g/l)	-	Li *et al.* 2016
Vasconcellea pubescens A. DC.	Mature	Callus induction: MS + kinetin (0, 2, 4 mg/l) and 2,4-D (0, 1, 2, 3, 4, 5 mg/l)	-	Zuhro *et al.* 2021
Zea mays L.	Mature	White + YE	No calluses	Straus and La Rue 1954

MS: Murashige and Skoog culture medium (Murashige and Skoog 1962), BAP: 6-benzylaminopurine, Benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid, GA3: gibberellic acid, IAA: indole-3-acetic acid, IBA: indole-3-butyric acid, NAA: 1-naphthaleneacetic acid, YE: yeast extract. White culture medium (White 1963), Nitsch culture medium (Nitsch, 1969), MT: Murashige and Tucker culture medium (1969), WPM: Woody Plant Medium, LS: Linsmaier and Skoog (1965) culture meidum, CH: hydrolyzed casein, ZT: zeatin, KT: kinetin, TDZ: thidiazuron. Table based on Hoshino et al. (2011) and Wang et al. 2016 with updates.

According to the data collected in this study, the culture medium consisting of basic MS salts (Murashigue and Skoog 1962) is the most used for *in vitro* endosperm culture, being used in 69% of established protocols (Figure 2).

Regeneration from *in vitro* endosperm culture can occur via *de novo* organogenesis or via somatic embryogenesis. *De novo* organogenesis refers to the induction and formation of shoot apical meristem or adventitious root apical meristem, arising from the expression of plant cell pluripotentiality. *De novo* organogenesis constitutes the main regeneration pathway from *in vitro* endosperm culture (Table 1), reported in 64% of studies (Figure 2). This regeneration route is usually induced by supplementing the culture medium with auxins and cytokinins, or only with cytokinins (Hoshino et al. 2011, Silva et al. 2020). Among the auxins used, dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (ANA) stand out (Figure 2). Among the cytokinins, most studies reported the use of 6-benzylaminopurine (BAP), although kinetin (KT) and thidiazuron (TDZ) have also been used in smaller quantities (Figure 2).

Figure 2 – Key culture mediums and PGRs used in studies related to *in vitro* regeneration from endosperm culture.



MS= Murashige and Skoog; BS= bismuth sulfide; LS= Linsmaier and Skoog; MT= Murashige and Tucker; WPM= woody plant medium; 2,4-D= 2,4-dichlorophenoxyacetic acid; ANA= 1-naphthaleneacetic acid; IAA= indole-3-acetic acid; IBA= indole-3-butyric acid; BA= 6-benzylaminopurine, Benzyladenine; TDZ= thidiazuron. Source: Personal archive.

Somatic embryogenesis is the true expression of plant totipotency (Fehér 2019), in which, under suitable conditions, somatic cells differentiate into somatic embryos. These structures establish their apical-basal axis with the specification of shoot apical meristems and root apical meristems formed synchronously (Rocha and Dornelas 2013). However, few studies have reported somatic embryo formation from *in vitro* endosperm culture (Table 1), which includes *Acacia nilotica* H. Karst., *Petroselinum hortense* Hoffm., *Santalum album* L., and *Passiflora cincinnata* Mast. (Masuda et al. 1977, Lakishmi et al. 1980, Garg et al. 1996, Machado et al. 2022). In these studies, endosperm culture occurred in a medium rich in auxin, mainly 2,4-D (Hoshino et al. 2011), in the absence of light.

In this context, we observe that these regeneration processes, *de novo* organogenesis, and somatic embryogenesis, are not autonomous but depend on the signaling of growth regulators added to the culture medium to induce the reprogramming of gene expression patterns and the reorganization of cellular proliferation and differentiation mechanisms (Fehér

2019). Another aspect that also influences the success of the technique, is the developmental stage at which the endosperm is collected and cultivated, in which both mature and immature endosperms can be used.

In addition to the limitations arising from technical complexity, anomalies during cultivation are common, such as albinism, which is often observed in the progeny of interspecific crosses and in endosperm regenerants, and mixoploid chimeras (Tiku et al. 2014, Wang et al. 2016). Albinism reduces the survival rate of seedlings due to the absence of chlorophyll pigments (Eeckhaut et al. 2007, Kita et al. 2005, Kumari et al. 2009). All these obstacles decrease the likelihood of obtaining viable triploid plants from endosperm culture. However, compared to conventional methods, the culture of this tissue enables the development of a protocol for triploid plant production with a much faster response time (Thomas and Chaturvedi 2008, Hoshino et al. 2011).

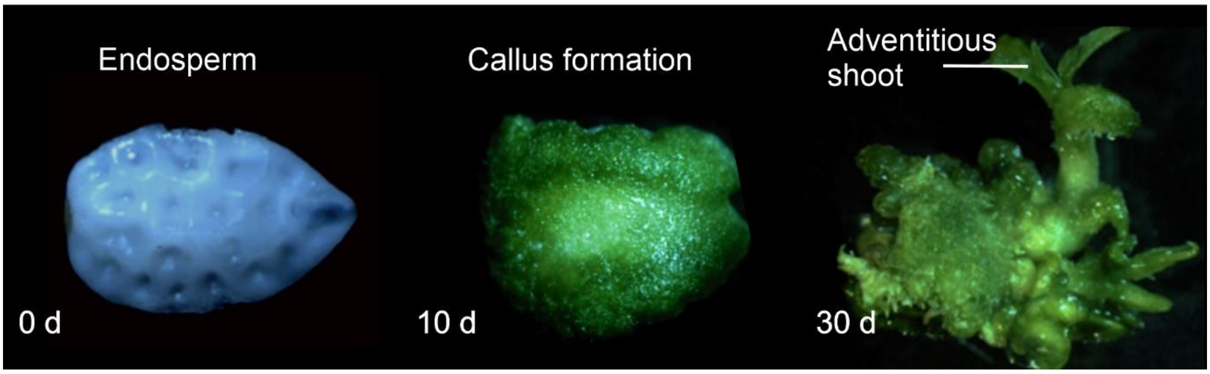
2.6 MORPHOPHYSIOLOGICAL CHANGES ASSOCIATED WITH PLANT REGENERATION IN *PASSIFLORA* FROM *IN VITRO* ENDOSPERM CULTURE

Regenerating plants from *in vitro* endosperm culture requires reserve parenchyma cells of this tissue to acquire competence to reprogram and take on a new developmental route. During the regeneration process, morphological changes can be observed on the surface of the endosperm as early as the first induction days.

Studies on *in vitro* endosperm culture of *Passiflora* spp. demonstrate that during *de novo* organogenesis, the initially white tissue composed of isodiametric cells, after inoculation in a cytokinin-rich medium in the presence of light, acquired typical green coloration and showed areas of cell proliferation that led to the formation of meristems and subsequently adventitious buds (Figure 3) (Antoniazzi et al. 2018, Silva et al. 2020, Mikovsky et al. 2021).

Meristems were observed only on the dorsal side of the explants and consisted of small cells with dense cytoplasm, a voluminous nucleus with heterochromatin regions, and one or more prominent nucleoli (Figure 4a). Interestingly, in the more internal regions of the explant, not directly involved in the organogenic process, zones of cell elongation were observed (Figure 4a), highlighting the polarity of the endosperm during the *de novo* organogenesis process (Silva et al. 2020).

Figure 3 – Morphological changes in the endosperm of *P. cincinnata* over 30 days of induction in a cytokinin-rich culture medium for the induction of *de novo* organogenesis.



Source: Personal archive.

In the process of somatic embryogenesis, the explants exhibit swelling on the dorsal side. Subsequently, the formation of embryogenic calli begins, which are yellowish, with a friable and granular texture, with areas containing smooth and shiny pro-embryogenic masses of light color (Figure 4b) (Silva 2021). The pro-embryos consisted of cells containing dense cytoplasm, voluminous nuclei with unique and prominent nucleoli (Figure 4b), differing from the regeneration process via *de novo* organogenesis (Silva 2021)

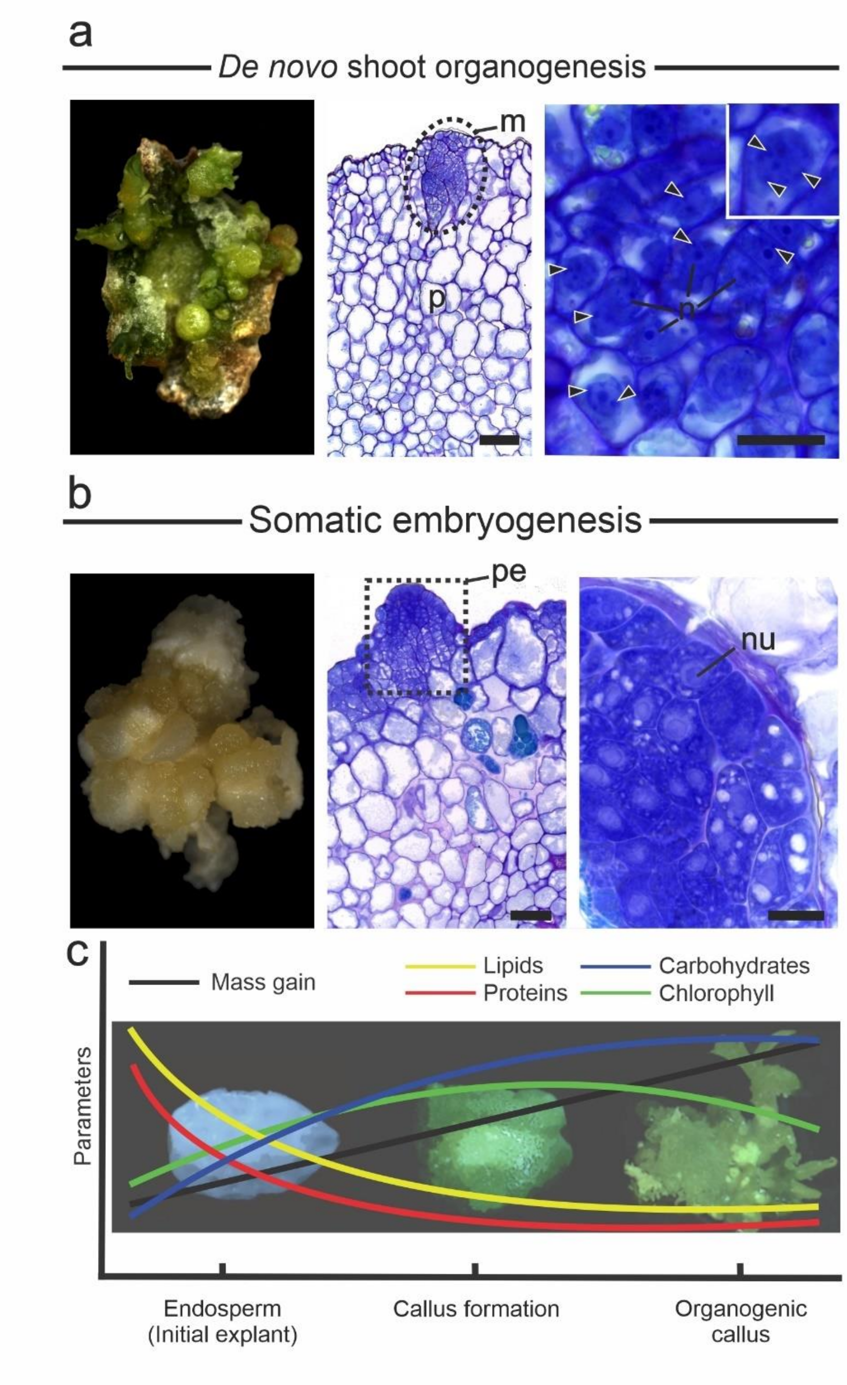
Regeneration processes and induction of morphogenic pathways, such as callus and lateral root formation originating from pericycle or pericycle-like cells, are widely discussed (Sugimoto et al. 2010, Shin et al. 2019). Pericycle cells are pluripotent and possess intrinsic organogenic potential to follow a developmental route after reactivation (Rocha et al. 2018, Ikeuchi et al. 2019). However, regeneration and morphogenesis processes have also been observed from epidermal and/or subepidermal cells, as reported in the endosperm (Yumbla-Orbes et al. 2017, Rocha et al. 2018, Popielarska-Konieczna et al. 2020). Nevertheless, the molecular and biochemical mechanisms related to the reprogramming of peripheral cells of explants for acquiring new cellular fates are still poorly discussed in the literature (Rocha et al. 2018, Ikeuchi et al. 2019).

Some studies have shown that morphogenic processes such as somatic embryogenesis and *de novo* organogenesis require different cellular mechanisms. Recently, Popielarska-Konieczna and collaborators (2020) demonstrated that chemical constituents of the cell wall can be markers of morphogenic events in *Actinidia arguta* (kiwi) endosperms, as tissues with morphogenic potential accumulate wall components in relation to endosperm regions not involved in regeneration. However, further analysis is needed to verify if this mechanism is conserved in other plant species.

Considering the reserve nature of the endosperm, studies have also shown that during the regeneration process, reserve compounds such as lipids and proteins are rapidly mobilized (Figure 4c) (Silva et al. 2020). The highest concentration of lipids is observed in the initial explant and gradually reduced over the cultivation days (Silva et al. 2020). On the other hand, soluble carbohydrates and photosynthetic pigments show the opposite behavior. Initially, these compounds are present in reduced amounts and increase in concentration over the cultivation days (Figure 4c) (Silva et al. 2020, Zhao et al. 2021). During endosperm development, the accumulation of reserves is accompanied by the degradation and death of tissue cells (Kobayashi et al. 2013, Li et al. 2018, Sabelli, Larkins, 2009, Wang et al. 2012). The initial mobilization of lipids and proteins observed in *in vitro* endosperm culture may be associated with intense metabolic activity throughout the processes of reorganization and cell proliferation for adventitious shoot regeneration (Silva et al. 2020).

Interestingly, the results obtained from organ regeneration processes using endosperm highlight significant potential for utilizing this tissue as a model for in vitro regeneration studies. This aims to comprehend the molecular mechanisms and signaling processes involved in acquiring competence for the reprogramming of differentiated parenchymal cells, such as reserve parenchyma. The importance of conducting biochemical, molecular, and physiological analyses is emphasized, aiming to enhance the understanding of the mechanisms related to the transformation of initially white tissue, primarily composed of reserve parenchyma, into the development of numerous adventitious shoots composed of complex tissues, including the formation of vasculature and photosynthetic tissue.

Figure 4 – Histological and biochemical changes during endosperm regeneration in *Passiflora edulis*.



(a) *De novo* organogenesis. Meristemoid cells (m) show nuclei with one or more nucleoli (nu) and various heterochromatin regions (arrowhead). (b) Somatic embryogenesis. Cells in the pro-embryogenic regions (pe) have cells with dense cytoplasm and a nucleus containing a single conspicuous nucleolus (nu). (c) Diagram based on the work of Silva et al. (2020) highlighting the dynamics of synthesis/mobilization of reserve compounds and photosynthetic pigments throughout the process of adventitious bud formation from *in vitro* endosperm culture. Source: Images from figures (a) and (b) were provided by Dr. Lázara Aline Simões Silva (Silva 2021).

2.7. CHARACTERISTICS OF ENDOSPERM-DERIVED PLANTS

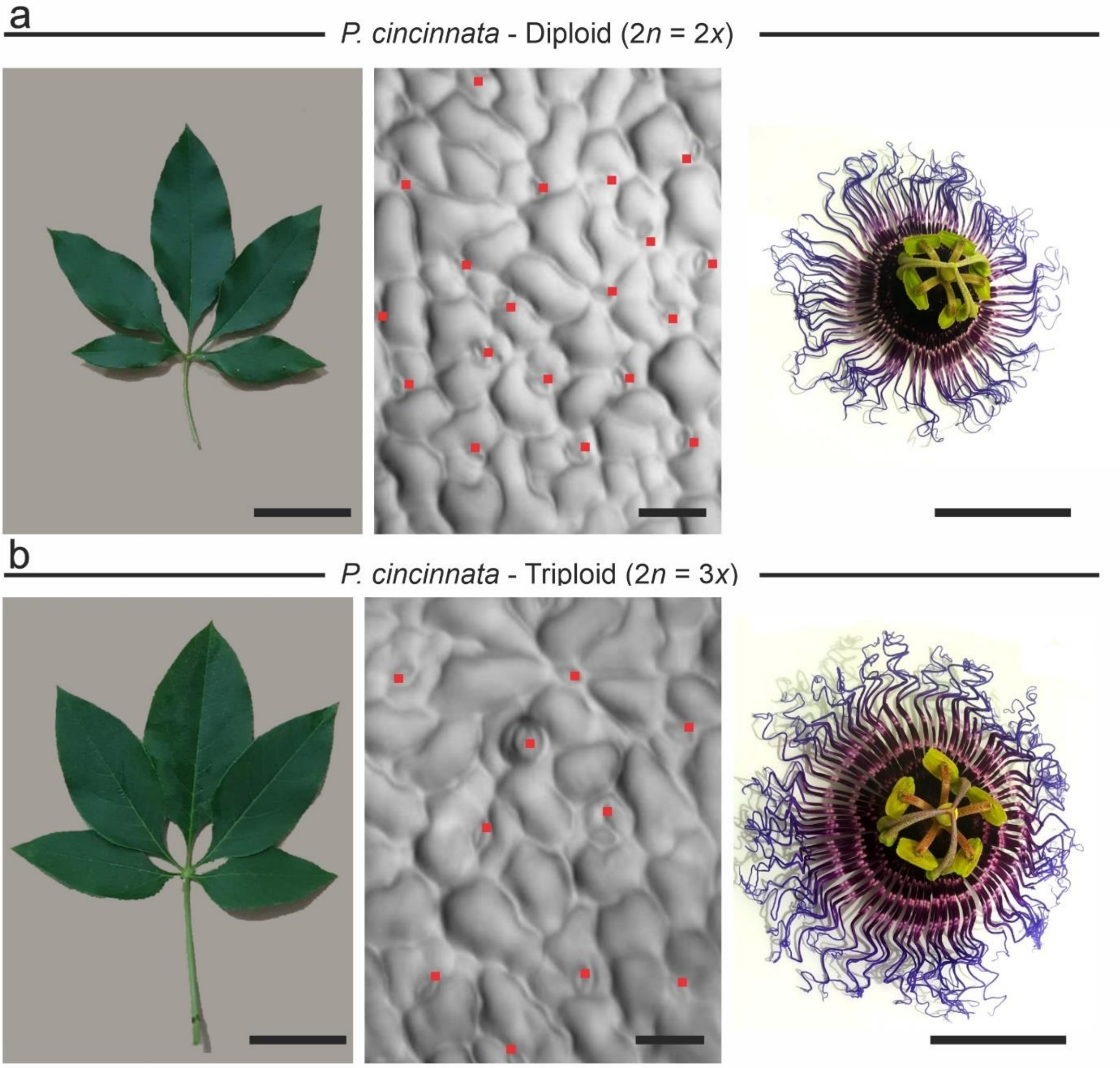
Changes in ploidy levels generally affect the morphological, physiological, and molecular characteristics of plants. The size and density of stomata, the quantity of leaves, shoots, roots, and flowers, the length-to-width ratio, the size of the flower, seed, and pollen, plant height, and habit are among the most frequently observed morphological changes (Figure 4) (Te Beest et al. 2012, Urwin 2014, Hannweg et al. 2016, Trojak-Goluch et al. 2021).

Furthermore, cell size (Figure 5), nucleus volume, and cell cycle duration, defined as phenotypic characteristics, are influenced by the substantial amount of DNA produced by polyploidization (Doyle and Coate 2019, Trojak-Goluch et al. 2021). These triploid cells, when compared to diploids, exhibit a higher surface-to-volume ratio (Figure 5) (Lavania 2013), leading to a slowdown in vital processes such as transpiration (Schwanitz 1953). They also have an increased stomatal size (Figure 5), although intercellular gas exchange is weaker due to reduced intercellular spaces (Trojak-Goluch et al. 2021). Triploids also show enhanced CO_2 assimilation and optimized biomass production (Urwin 2014, Dudits et al. 2016). However, they exhibit slower growth, possibly due to slower rates of cell division, reduced metabolic rate, or lower amounts of growth hormones (Imery and Cequea 2001).

Higher ploidy generally results in better plant adaptation to changes in environmental conditions and increased resistance to abiotic and biotic factors (Levin 1983, Trojak-Goluch et al. 2021), the latter especially due to sub or neofunctionalization in resistance genes and genes related to plant defense (Innes et al. 2008, King et al. 2012). Recently, Machado and colleagues (2022) reported an almost 30% increase in the activity of defense enzymes such as β-1,3-glucanase and polyphenoloxidase in triploid plants compared to their respective diploid counterparts.

Structural genome alterations may also occur, such as rearrangements, DNA deletion, meiotic or mitotic defects (Yang et al. 2011, Trojak-Goluch et al. 2021). Sterility, for example, results from the irregular distribution of chromosomes during meiotic division due to multiple associations formed by bivalents. Additionally, epigenetic modifications in gene expression, changes in chromatin density through methylation or demethylation, modifications in histones, or genetic alterations in terms of gene loss or subfunctionalization may occur (Yang et al. 2011, Trojak-Goluch et al. 2021).

Figure 5 – Comparison between the leaves, stomata, and flowers of diploid and triploid *P. cincinnata* plants.



Source: Leaf images - personal file, Stomata images - published by Silva et al. 2020, Flower images - published by Machado et al. 2022 (with modifications). Scale bar: Leaves and flowers: 5 cm, Stomata: 100 µm.

2.8 TRIPLOIDY IN AGRICULTURE

Polyploidy is a strategic tool in plant breeding programs (Sattler et al. 2016, Touchell et al. 2020). The increase in genome size can cause genetic and epigenetic changes, possibly leading to phenological, phenotypic and physiological alterations (Adams and Wendel 2005, Otto and Whitton 2007, Yang et al. 2011, Van de Peer et al. 2017).

Polyploids are used to generate varieties with higher productivity, improved quality, better morphological characteristics, increased tolerance to biotic and abiotic stresses and even to overcome genetic barriers (Sattler et al. 2016, Touchell et al. 2020, Trojak-Goluch et al. 2021). Moreover, they can be employed as a bridge for gene transfer between incompatible species or to restore fertility in newly synthesized sterile hybrids (Dewey 1980, Sattler et al. 2016).

Triploid plants exhibit larger somatic and guard cells, along with an increased number of chloroplasts, optimizing the photosynthesis process (Jones and Reed 2007, Padoan et al. 2013, Tapan 2014). These characteristics result in larger organs, short internodes, broad, thick, and dark green leaves (Sugiyama 2005, Hoshino et al. 2011, Wang et al. 2016). These attributes also favor higher biomass production and yield or harvest index, resistance to biotic and abiotic stresses, delayed flowering, or reduced fertility in some cases (Hoshino et al. 2011, Wang et al. 2016). Triploid plants are also characterized by their high economic value, justified by the absence of seeds in the fruits, an especially important feature in *Citrus* spp. (Hoshino et al. 2011). Sterility in triploid plants is undesirable when seeds are used commercially. However, seed sterility can enhance physiological quality in various fruits such as grapes, papaya, apples, watermelon, bananas, and citrus (Thomas and Chaturvedi 2008, Hoshino et al. 2011, Wang et al. 2016). Furthermore, triploid organisms are widely used in the improvement of various crops, attempting to overcome the inviability and infertility of interspecific hybrids, obtain seedless cultivars and increase resistance to abiotic and biotic factors (Wang et al. 2016, Trojak-Goluch et al. 2021).

The additional genome is often associated with increased gene expression, which, in turn, can lead to a higher quantity of proteins involved in plant immunity-related metabolic processes (Vleugels et al. 2013). Increased stress resistance is also linked to higher antioxidant enzyme activity, as well as elevated stress hormone levels (Dudits et al. 2016, Zhang et al. 2010). Additionally, triploidy can directly lead to genetic modifications through gene silencing and genetic or epigenetic interactions, capable of altering host-pathogen interactions (Vleugels et al. 2013). These characteristics make triploid genotypes unique organisms for genome studies (Wang et al. 2016, Yang et al. 2011).

Seedless watermelon (*Citrullus vulgaris*), seedless lemon (*Citrus limon*), neem (*Azadirachta indica*), banana (*Musa paradisiaca*), petunia (*Petunia violacea*), and sugar beet (*Beta vulgaris* L.) are some examples of triploid plants available in the market (Kumar and Gupta 2015). The advantageous characteristics of triploid plants are expected to be increasingly studied in the coming years.

2.9 CONCLUSIONS AND PERSPECTIVES

Triploidy is a promising strategy in plant breeding programs. Triploid plants are expected to be used for monitoring genomic and transcriptomic changes in unstable genomes, producing more adapted varieties with desirable traits. They may also play an interesting role as rootstocks for genotypes of interest.

Although in vitro endosperm culture for triploid plant production is not a routine practice, several successful protocols have been developed in recent years. It is anticipated that this study will contribute to the development of new protocols for various species. Studies prioritizing the understanding of physiological and molecular mechanisms governing the cellular reprogramming process in triploid regeneration from endosperm can aid in optimizing these protocols, thus enabling routine use of these techniques. Phytohormone analyses can contribute to understanding physiological processes related to tissue induction and responsiveness. Similarly, proteomic, metabolic, and structural analyses can help identify factors triggering gene activities involved in the cellular reprogramming process that leads to organ regeneration.

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3 CAPÍTULO 2

DYNAMIC RESPONSE OF CARBOHYDRATES AND PROTEOMIC PROFILE DURING THE REGENERATION OF ADVENTITIOUS SHOOTS FROM *IN VITRO* CULTURE OF *Passiflora cincinnata* Mast. ENDOSPERMS

3.1 ABSTRACT

The study aims to identify and characterize the dynamics of carbohydrates and the protein profile during the reprogramming and acquisition of organogenic competence from *Passiflora cincinnata* Mast. endosperms. The endosperms were cultured in Murashige and Skoog medium supplemented with 2.0 mg L^{-1} benzyladenine. Over 30 days of culture, explants were collected for histological, biochemical, and protein extraction analyses. Endosperm explants were initially white and composed of isodiametric cells, but under induction culture conditions, they started to exhibit typical green coloration and the initiation of callus formation at 10-15 days. By 30 days, well-developed adventitious buds with leaf primordia were observed throughout the tissue. During callus formation (10-15 days), there was an increase in hexose concentrations. A significant accumulation of starch occurred from 15 days, and malate concentrations increased during stages where tissue greening was observable (15-30 days), especially at 30 days. From proteomic analysis, 563 differentially accumulated proteins were identified, with the callus formation period standing out for having a higher concentration of up-acumulated proteins. In contrast, at 30 days, abundant proteins were observed, but when compared with the previous stage, a significant number of proteins were down-acumulated. Indeed, substantial mobilization of organic compounds is suggested during processes involving intense cell proliferation and reprogramming. It is noteworthy that proteins related to stress responses and energy metabolism are crucial for signaling dynamics, triggering cell proliferation, and developmental programs. These proteins serve as potential molecular markers for callus and adventitious bud formation from the endosperm.

Keywords: Callus formation, proteomics, shoot regeneration.

3.2 INTRODUCTION

The endosperm consists of a fundamental reserve tissue for the zygotic embryo in Angiosperms (Lopes and Larkins 1993, Hoshino et al. 2011). Serving as a source of energy and carbon throughout embryonic development, the endosperm results from the fusion of a haploid sperm nucleus with two nuclei from the central cell of the embryo sac, forming this reserve tissue containing three sets of chromosomes (Cailleau et al. 2009). Given its unique nature, *in vitro* cultivation of endosperms has emerged as a direct alternative for the production of triploid plants (Wang et al. 2016). These genotypes are recognized for their vigorous vegetative growth, increased resistance, and adaptive capacity compared to their diploid counterparts. Despite being generally sterile, they exhibit interesting characteristics for supporting genetic improvement studies in economically targeted plants (Thomas and Chaturvedi 2008, Chen et al. 2014).

In *Passiflora* L. (Passifloraceae), a neotropical genus known for the production of passion fruits and the ornamental potential of its species, *in vitro* regeneration protocols have been reported from various plant organs (Otoni et al. 2013, Rocha et al. 2020). In recent years, regeneration protocols via somatic embryogenesis and *de novo* organogenesis from the endosperm have been developed for *Passiflora cincinnata* Mast., confirming triploidy (Silva et al. 2020, Machado et al. 2022). Triploid plants of *P. cincinnata*, regenerated from *in vitro* endosperm cultivation, exhibited interesting characteristics such as the development of larger and longer vegetative and floral structures, higher nectar concentration, and increased activity of defense enzymes such as β-1,3-glucanase and polyphenol oxidase compared to diploid individuals of the same species (Machado et al. 2022). *P. cincinnata* is a native species in Brazil with widespread distribution, recognized in different regions as "maracujá-da-caatinga" and "maracujá-do-mato" (Bernacci et al. 2024). Besides being considered potentially ornamental, *P. cincinnata* has also stood out for its potential use of different plant organs for medicinal, cosmetic, and food purposes (Araújo et al. 2018). Notably, researchers have pointed out its increased tolerance to water stress and attacks by major passion fruit pathogens. Therefore, the species is considered a potential rootstock and an interesting model for commercial passion fruit breeding programs (Araújo et al. 2018).

During the cultivation of *P. cincinnata* endosperm explants, cells from the dorsal face of the endosperm underwent reprogramming followed by callus formation and subsequent development of adventitious buds (Antoniazzi et al. 2018, Silva et al. 2020). The initial explant, composed mainly of reserve parenchyma cells, underwent intense structural modification and, during *in vitro* cultivation, began to exhibit photosynthetic tissue and the formation of leaf primordia. In the literature, molecular mechanisms during organogenic callus formation are well-described, involving cell proliferation from the pericycle, resembling the process of lateral root formation (Sugimoto et al. 2010, Shin et al. 2019). However, the mechanisms involved in the reprogramming of epidermal and subepidermal cells during the acquisition of competence for callus and organogenic tissue formation are not well-understood yet (Rocha et al. 2018, Ikeuchi et al. 2019).

Several studies highlight the relevance of detailed investigations to understand the cellular events triggered during regeneration. Studies like those by Calamar and De Klerk (2002) emphasize the performance of various essential functions and the influence of carbohydrates on biological processes in plants. Carbohydrates can serve as substrates for respiration, control development processes, play essential roles in the synthesis of numerous compounds, and contribute to the construction of macromolecules (Calamar and De Klerk, 2002). Recently, in photosynthetic organisms, the fundamental role of sugars as signaling molecules has become more evident (Rolland et al. 2006). Additionally, growth and development responses suggest the involvement of dynamic interactions between sugars and hormonal regulation (Rolland et al. 2006).

In this context, the integration of "omics" analyses has been considered promising for advancing molecular and physiological knowledge about morphogenetic competence acquisition processes (Passamani et al. 2018). Proteomics is defined as the systematic analysis of a proteome, or the set of proteins expressed by a cell, tissue, or organism at a given moment or condition (Chen and Harmon, 2006). The advent of proteomics has allowed researchers to identify a broad spectrum of proteins in living systems (Chakraborty et al. 2015). This information is especially useful for agriculture, as these studies provide fundamental knowledge that can be used to characterize various properties of a wide variety of plants, as well as identify molecular markers for use in genetic improvement programs (Chakraborty et al. 2015). Many studies have utilized these analyses for a deeper understanding of the biochemical and molecular aspects involved in *in vitro* morphogenesis processes.

To enhance the understanding of the biochemical and physiological mechanisms involved in the acquisition of organogenic competence of endosperm explants during *in vitro* culture, the present study proposes to associate the results of global protein analysis and carbohydrate dynamics during the induction of the regeneration process in *P. cincinnata*, aiming to identify key factors in the modulation of organogenic responses in the tissue.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and *in vitro* culture

For the induction of *de novo* shoot organogenesis from mature endosperms of *P. cincinnata*, the regeneration system reported by Silva et al. (2020) was utilized.

Seeds of *P. cincinnata* had their seed coats removed using a mini-vise. Disinfection was performed in a laminar flow hood, using 70% (v/v) ethanol for 1 minute, followed by immersion in commercial sodium hypochlorite (2.5% v/v) with three drops of 0.1% (v/v) Tween-20 for 15 minutes. After this period, four rinses were conducted with deionized and autoclaved water. The seeds remained immersed in water for two hours, facilitating the extraction of the endosperms.

The endosperms were longitudinally sectioned, separated from zygotic embryos, and inoculated on induction medium consisting of MS (Murashige and Skoog 1962) culture medium basal salts, B5 vitamins (Gamborg et al. 1968), 0.01% (w/v) myo-inositol, 3% (w/v) sucrose, and 0.28% (w/v) Phytagel® (Sigma Chemical Company, USA), supplemented with 2 mg/L benzyladenine (BA) for adventitious shoot induction. The culture medium pH was adjusted to 5.7 ± 0.1 and autoclaved at 1.1 Pa, 120 °C for 20 minutes.

Endosperms were inoculated on 60 x 15 mm crystal polystyrene Petri dishes (J. Prolab, Brazil) containing 12-15 mL aliquots of culture medium. Cultures were maintained in an incubator (Diurnal Growth Chamber, Forma Scientific, USA) at 27 ± 2 ºC for up to 30 days with a 16-hour photoperiod. Initial explants were collected, and samples were collected after 10 and 30 days of induction. These samples will be appropriately stored according to the needs of the evaluations described below.

3.3.2 Optical and scanning electron microscopic analysis

The samples were initially recorded in a stereomicroscope, fixed in 70% FAA (Formaldeyde:acetic acid; alcohol) and subsequently dehydrated in an ethanol series (70, 80, 90, and 100%) for three hours each. After dehydration, critical point drying (CPD 030, Bal-Tec, Balzers, Germany) was performed, and the samples were fixed onto aluminum supports with conductive carbon tape and gold-coated (SCD 050, Bal-Tec, Balzers, Germany). Investigation and documentation were carried out using a scanning electron microscope (LEO 435VP, Cambridge, England).

3.3.3 Proteomic analysis

Samples of endosperm at the initial stage (before incubation) and after 10 and 30 days of *in vitro* induction were collected, frozen in liquid nitrogen, followed by maceration and lyophilization. TCA/Acetone extraction protocol was carried out according to Damerval et al. (1986), using 300 mg fresh mass (FM) macerated material for each sample, in biological triplicate. For each sample, 1 mL of extraction buffer composed of 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% dithiothreitol (DTT, GE Healthcare), 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), were added. The mixture was vortexed, centrifuged, and supernatants were collected. Protein concentration was measured using the 2-D Quant kit (GE Healthcare, Piscataway, NJ, USA). Protein samples were precipitated using the methanol/chloroform method (Nanjo et al. 2012). Samples were then resuspended in a solution of 7 M urea and 2 M thiourea, and protein digestion was performed using 30 kDa Microcon filter units (Millipore) with the Filter-Aided Sample Preparation (FASP) method according to Burrieza et al. (2019). After digestion, resulting peptides were quantified using the proteins and peptides A205 nm method with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), and then injected into a nanoAcquity UPLC mass spectrometer connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK). Runs consisted of five biological replicates of 1 μg of digested proteins. Injections were performed randomly to avoid bias. Spectra processing and database searching were carried out using ProteinLynx Global SERVER (PLGS) v.3.02 software (Waters), and quantification analyses were performed using ISOQuant v.1.7 software (Distler et al. 2014). To ensure the quality of results after data processing, only proteins present in at least four runs were accepted for differential abundance analysis. Strict parameters were employed, such as a False Discovery Rate (FDR) of <1%, identification parameters 3-7-2 (minimum fragment ion per peptide - minimum fragment ion per protein - minimum peptide per protein) for identification and quantification, ensuring high reliability of the obtained data. Proteins meeting two criteria, t-test ($P<0.05$) and significant Log2 fold change (FC) (if Log2 FC > 0.6 = UP-accumulated and if <-0.6 = Down-accumulated), were considered regulated. Functional annotations were performed using Blast2Go v.5.0 software (Conesa et al. 2005) and UniProtKB (www.uniprot.org).

3.3.4 Quantification of soluble sugars, malate and starch

Soluble sugars, and starch, were determined by hot methanol extraction, as described by Porra et al. (1989). Glucose, fructose, sucrose, and malate concentrations were determined as described by Fernie et al. (2001). Furthermore, starch was quantified in the insoluble fraction of the methanolic extract (Bradford 1976, Fernie et al. 2001).

3.4 RESULTS

3.4.1 Morphological transformations: from reserve tissue to the formation of adventitious shoot *in vitro* cultivation of endosperms

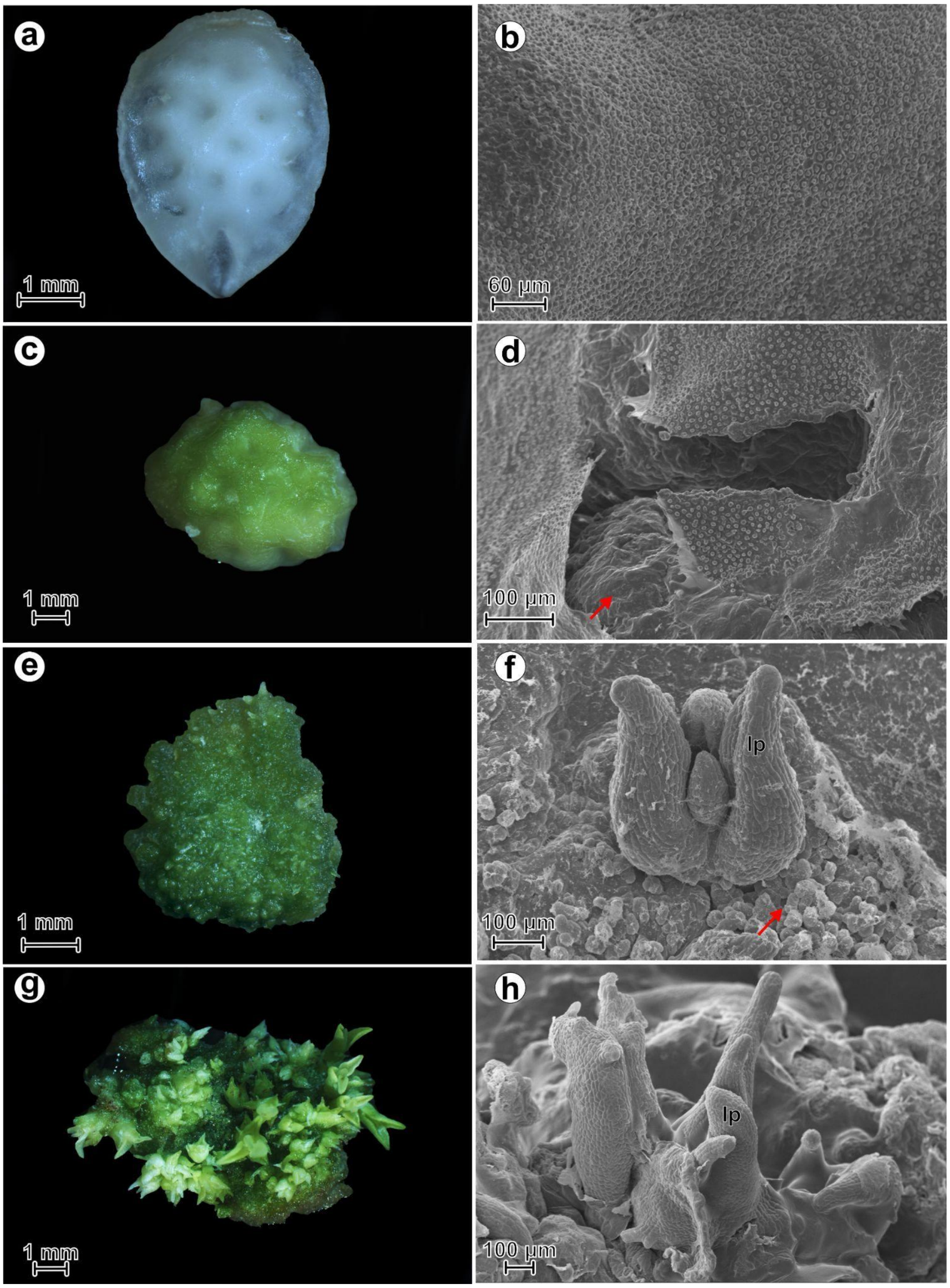
Morphological changes are noticeable throughout the induction process of *de novo* shoot organogenesis in passion fruit endosperms. During cultivation, the endosperms of *P. cincinnata*, initially whitish, ovoid with reticulated ornamentations, and primarily composed of parenchymatous reserve tissue (Fig. 1a), already show significant morphological modifications after 10 days of incubation. At this time, changes in tissue coloration can be observed in the explants, transitioning from whitish to yellowish or greenish, along with the formation of organogenic calli (Fig. 1c). Scanning results on the explant surface demonstrate that, within 10 days of cultivation, intense cell proliferation occurs during callus formation, leading to noticeable expansion in the explant's size, resulting in ruptures in the initial endosperm tissue (Fig. 1d). After 15 days of incubation, the tissue appears fully green, with adventitious buds and early-stage leaf primordia development (Fig. 1e-f). At 30 days of cultivation, well-developed adventitious buds undergoing elongation are notably present (Fig. 1g-h).

3.4.2 Global protein analysis

For understanding the mechanisms associated with *de novo* shoot organogenesis in the formation of adventitious shoot from *in vitro* endosperm culture, a global protein analysis was conducted on explants at the initial stage (i.e., before *in vitro* incubation) and with callus induced after 10 days and organogenic callus at 30 days of incubation. The proteomic analysis revealed a total of 563 proteins. Three comparisons were considered among the three collected treatments: callus formation (10 d)/initial explant, organogenic callus (30 d)/initial explant, and organogenic callus (30 d)/callus formation (10 d). In the comparison between 10 days of induction (callus formation) and the initial explant (Fig. 2a), 465 proteins were differentially accumulated (DAPs), with 190 up-acumulated and seven down-acumulated. Additionally, 208 proteins were uniquely found at the callus formation stage (10 d), while only three were unique to the initial explant (Fig. 2a).

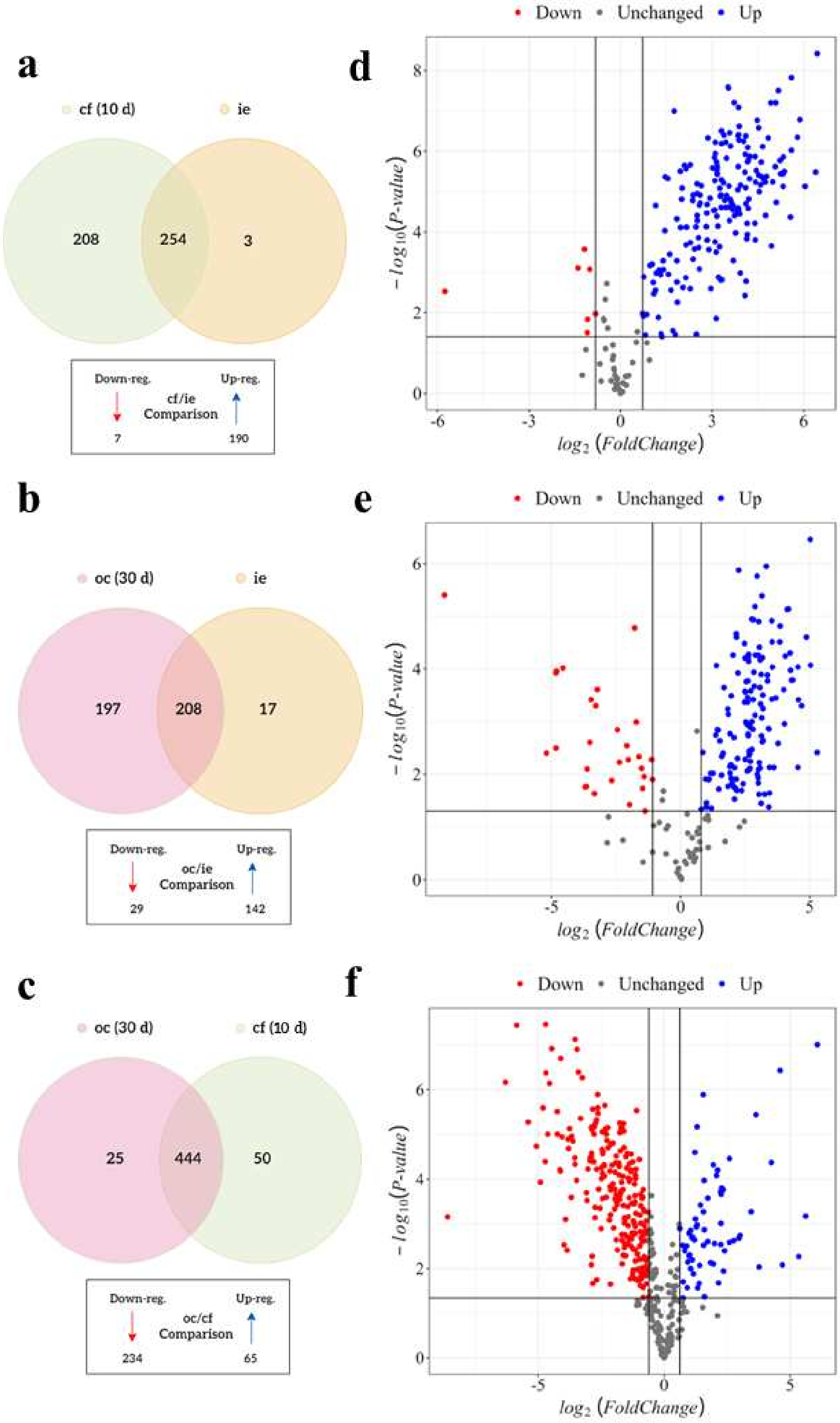
When comparing organogenic callus stages (30 d)/initial explant (Fig. 2b), 422 DAP were found, including 142 up-acumulated and 29 down-acumulated, 197 proteins were unique to organogenic callus stage at 30 days of induction (Fig. 2b). In the comparison of organogenic callus (30 d)/callus formation (10 d) (Fig. 2c), 519 proteins were DAPs, with 50 proteins unique to callus at 10 d and 25 unique to 30 d organogenic callus. Interestingly, in this comparison, 234 of these proteins were down-acumulated, and 65 were up-acumulated (Fig. 2c).

Figure 1 – Images under stereomicroscope and scanning electron microscopy during the *de novo* organogenesis from endosperm explants of *P. cincinnata*.



a-b. surface of the initial explant **c-d.** early stage of callus formation at 10 d of induction, **e-f.** initial formation of shoot meristems at 15 d **g-h.** Development of adventitious buds with well-developed leaf primordia (lf) at 30 d. Emphasis on regions of cell proliferation and tissue expansion (arrow). Source: Personal archives.

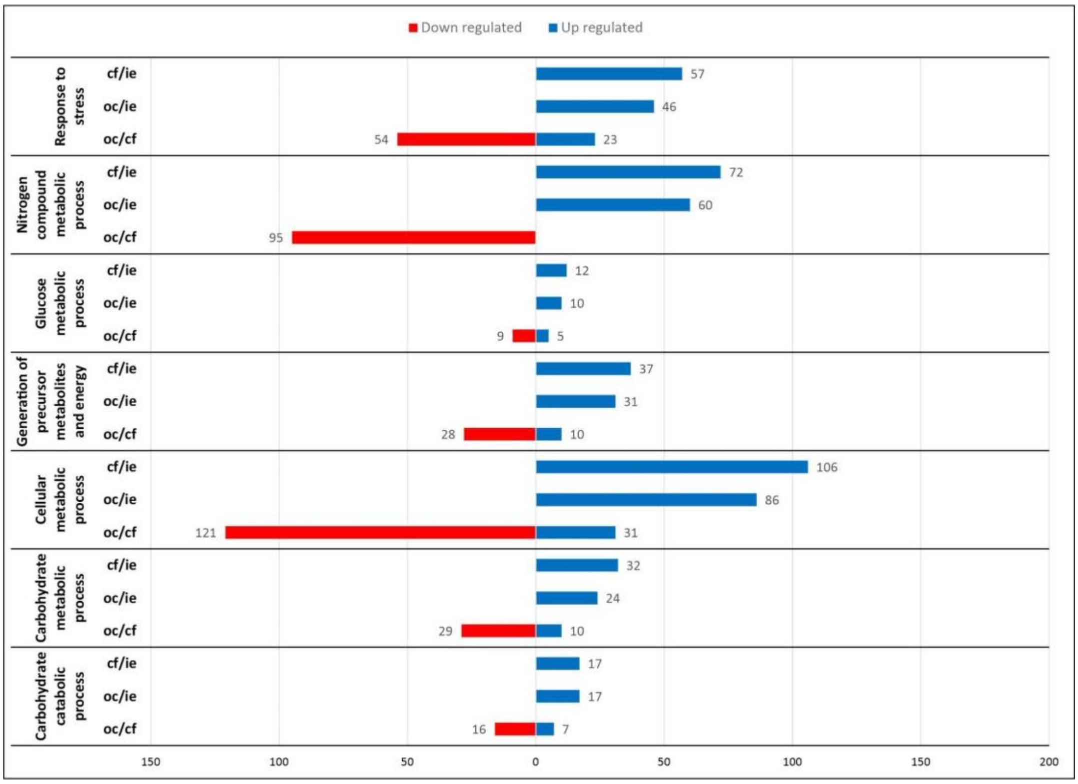
Figure 2 – Venn diagram of the proteins identified for *P. cincinnata* when comparing treatments



a. cf (10 d)/initial explant **b.** oc (30 d)/initial explant **c.** oc (30 d)/cf (10 d). Volcano plot of the DRPs identified from the comparison between **d.** cf (10 d)/initial explant **e.** oc (30 d)/initial explant **f.** oc (30 d)/cf (10 d). Proteins with a significant result in the Student's t-test (two-tailed, $P < 0.05$) were considered DAPs, up-acumulated if log2 of fold change (FC) was greater than 0.6 and down-acumulated if log2 FC was less than -0.6. Sources: Personal archives.

The DAPs were classified into seven groups according to their biological processes: carbohydrate metabolic process, carbohydrate catabolic process, cellular metabolic process, generation of precursor metabolites and energy, glucose metabolic process, nitrogen compound metabolic process and response to stress (Fig. 3).

Figure 3 – Venn diagram of the proteins identified for *P. cincinnata* when comparing treatments Number of proteins in biological processes considering differentially accumulated proteins during *in vitro* cultivation of *P. cincinnata* endosperm according to comparisons df/ie; oc/ie and oc/cf.



ie = initial explant before incubation, cf = callus formation – 10 d incubation, oc = organogenic callus 30 d incubation. Source: Personal archives.

3.4.3 Stress response during the induction of *de novo* organogenesis from endosperms

In this study, antioxidant enzymes related to stress response showed an increase at 10 and 30 d of induction compared to the initial explant (table 1), such as superoxide dismutase [Fe] 1, catalase-2, peroxidase 12, peroxiredoxin-2E, and peroxiredoxin-2B (*fold change*: 3, 5, 1.4, 1.9, and 2.5, respectively). For 2-Cys peroxiredoxin, an accumulation was observed in callus at 10 d of induction, followed by a substantial reduction in callus at 30 d. Interestingly, its increase coincided with structural changes during callus formation. Heat shock proteins

(Hsps) are associated with a large number of physiological processes (Kurashova et al. 2020). Among them, it has been demonstrated that the initiation of Hsps synthesis is closely related to cellular stress (Kurashova et al. 2020). In this work, we identified an accumulation in Hsps proteins in callus after 10 d of incubation, coinciding with the results obtained for oxidative enzymes. When comparing the stage of organogenic callus (30 d) with 10 d of induction (during callus formation), Hsps were mostly down-accumulated, with the exception of Heat shock protein 90-1 (TRINITY_DN12594_c0_g1_i1_p1_P51819). Cytosolic isocitrate dehydrogenase (NADP; TRINITY_DN1656_c0_g1_i3_p1_P50218), generally associated with the production of NADPH to promote redox and homeostasis signaling in response to oxidative stress, showed an increase in accumulation in the comparisons between 10 and 30 d of induction and the initial explant. However, in the comparison between callus of 30/10 d of induction, the enzyme was down-accumulated.

3.4.4 Energy and carbohydrate metabolism in the modulation of regenerative responses in endosperm explants

The dynamics of soluble carbohydrate and malate contents were significantly different during *in vitro* regeneration (Fig. 4). The concentrations of glucose and fructose increased by 75 and 60%, respectively, up to 15 days of induction, when considerable changes were already observed due to intense cell proliferation, tissue greening, and the initial formation of meristemoids along the explant. By 30 d of incubation, when adventitious buds were evidently differentiated, the levels of glucose and fructose decreased, compared to the earlier developmental stage (callus formation at 10 d). Unlike hexoses, a transient accumulation of sucrose was observed in the early stages of development. However, the concentration of this sugar decreased as hexose concentrations increased. The sucrose/hexose ratio significantly decreased throughout the regeneration process. During this process, a redirection of metabolic flux towards the accumulation of storage products, such as starch, was observed. Starch accumulation increased significantly during callus formation up to 30 days. Additionally, the malate concentration increased from day 10, particularly in the later stages of development when the explants were already green with well-defined leaf primordia.

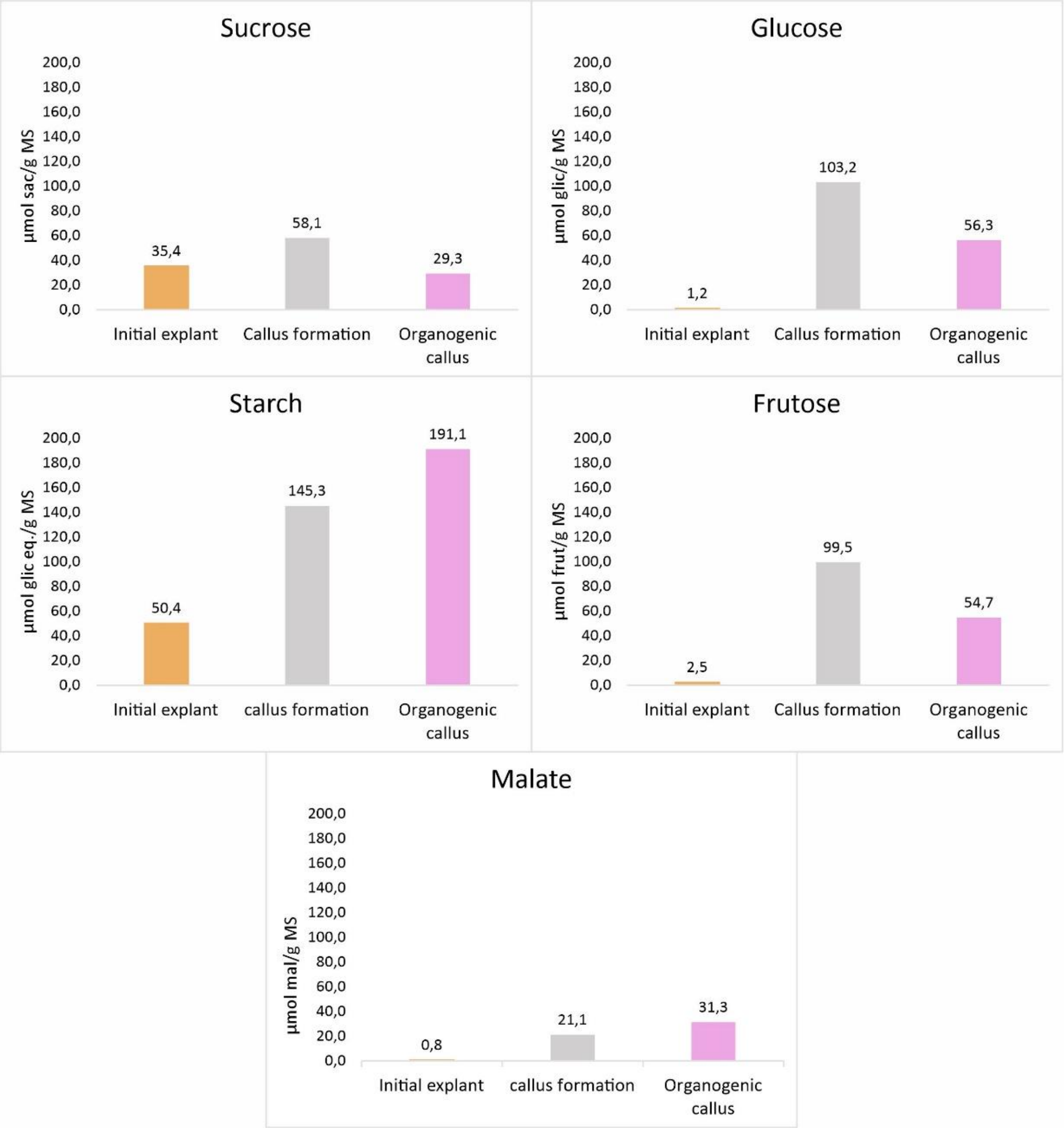
Table 1 – Proteins during *P. cincinnata* regeneration from endosperm *in vitro* culture.

			10 d/ie	**30 d/ie**	**30 d/10 d**
Accession	**Protein Description**	**Annotation**	**Log2Fold Change**		
TRINITY_DN5048_c0_g1_i39_p1_P22302	FSD1	Superoxide dismutase [Fe] 1	UP	DOWN	Unchanged
TRINITY_DN1674_c0_g1_i5_p1_P11796	MSD1	Superoxide dismutase [Mn] 2, mitochondria	Unchanged	DOWN	DOWN
TRINITY_DN2130_c0_g1_i2_p1_P17598	CAT2	Catalase-2	UP	UP	DOWN
TRINITY_DN12847_c0_g1_i1_p1_Q949U7	PRXIIE	Peroxiredoxin-2E	UP	UP	Unchanged
TRINITY_DN19335_c0_g1_i3_p1_A7NY33	PER52	Peroxidase 52	-	-	Unchanged
TRINITY_DN47386_c0_g1_i1_p1_Q9SZE7	PER51	Peroxidase 51	-	-	UP
TRINITY_DN9408_c0_g1_i5_p1_Q96520	PER12	Peroxidase 12	UP	UP	Unchanged
TRINITY_DN9931_c0_g1_i3_p1_Q9C5R8	2-Cys_Prx_B	2-Cys peroxiredoxin BAS1-like, chloroplastic	UP	UP	UP
TRINITY_DN2755_c0_g2_i1_p1_A9PCL4	PRXIIB	Peroxiredoxin-2B	UP	UP	Unchanged

TRINITY_DN2266_c0_g1_i10_p1_P53492	ACT7	Actin-7	UP	Unchanged	DOWN
TRINITY_DN2129_c0_g1_i17_p1_O23939	AOR	NADPH-dependent alkenal/one oxidoreductase, chloroplastic;	UP	UP	UP
TRINITY_DN12594_c0_g1_i1_p1_P51819	HSP90-1	Heat shock protein 90-1	UP	UP	UP
TRINITY_DN1997_c0_g1_i1_p1_Q07078	HSP90-2	Heat shock protein 90-2	UP	UP	DOWN
TRINITY_DN652_c0_g1_i1_p1_Q03685	BIP2	Heat shock 70 kDa protein BIP2	UP	UP	Unchanged
TRINITY_DN12442_c0_g1_i1_p1_Q01899	HSP70-10	Heat shock 70 kDa protein 10, mitochondrial	UP	UP	DOWN
TRINITY_DN105_c0_g1_i1_p1_P09189	HSP70-4	Heat shock 70 kDa protein 4	UP	Unchanged	DOWN
TRINITY_DN636_c0_g3_i1_p1_F4HQD4	HSP70-14	Heat shock 70 kDa protein 14	UP	UP	Unchanged
TRINITY_DN4197_c0_g1_i1_p1_Q02028	HSP70-6	Heat shock 70 kDa protein 6, chloroplastic	UP	UP	Unchanged
TRINITY_DN3195_c0_g1_i16_p1_P27880	HSP17.6B	17.6 kDa class I heat shock protein 2	Unchanged	DOWN	DOWN
TRINITY_DN1656_c0_g1_i3_p1_P50218	CICDH	Cytosolic isocitrate dehydrogenase [NADP]	UP	UP	DOWN

TRINITY_DN9148_c0_g1_i2_p1_P19446	PMDH1	Malate dehydrogenase 1, peroxisomal	UP	UP	DOWN
TRINITY_DN1740_c0_g2_i6_p1_Q39769	GAPC2	Glyceraldehyde-3-phosphate dehydrogenase GAPC2, cytosolic	UP	UP	DOWN
TRINITY_DN992_c0_g1_i13_p1_Q9SDX3	UGP1	UTP--glucose-1-phosphate uridylyltransferase 1	UP	Unchanged	DOWN
TRINITY_DN8260_c0_g1_i2_p1_P15479	ICL	Isocitrate lyase	UP	-	-
TRINITY_DN5168_c0_g1_i17_p1_P17815	MLS	Malate synthase	UP	DOWN	DOWN
TRINITY_DN220_c0_g2_i1_p1_P17614	T2K12.11	ATP synthase subunit beta-1, mitochondrial	UP	UP	Unchanged
TRINITY_DN1864_c0_g1_i11_p1_Q9SJQ9	FBA6	Fructose-bisphosphate aldolase 6, cytosolic	UP	UP	DOWN
TRINITY_DN4900_c0_g3_i1_p1_P52901	E1_ALPHA	Pyruvate dehydrogenase E1 component subunit alpha-1, mitochondrial	UP	UP	DOWN
TRINITY_DN11552_c0_g1_i1_p1_P48641	GR1-2	Glutathione reductase, cytosolic	UP	UP	Unchanged

Figure 4 – Hexoses, starch and malate contents during *in vitro* regeneration of *P. cincinnata* from the endosperm



Sources: Personal archives.

The results obtained from the protein analysis are consistent with the biochemical analyses that investigated the carbohydrate content during *de novo* shoot organogenesis in endosperm explants. The accumulation of proteins related to the carbohydrate catabolic process, carbohydrate metabolic process and glucose metabolic process, increased in callus with 10 days of induction compared to the initial explant. In comparisons between callus from 30 d of induction with the initial explant, the proteins remained up-accumulated with a slight reduction in abundance. However, when comparing callus at 30 d with those with 10 d, approximately 70% of the proteins related to these pathways were down-accumulated, reducing the contents at the end of incubation.

In biological processes related to cellular energy metabolism, there was a considerable increase in the accumulation of proteins in callus at 10 d induction compared to the initial explant and 30 d, and a decrease in callus at 30 d of incubation compared with the initial explant. When comparing callus at 30 d with the callus formation stage at 10 d of incubation, there was a decrease in the number of up-accumulated proteins, and on the other hand, a significant number of proteins were down-accumulated, unlike other treatment comparisons, which did not show down-accumulated proteins.

In the generation of precursor metabolites and energy pathway process, key proteins associated with cellular energy production through glycolysis, such as glyceraldehyde-3-phosphate dehydrogenase (GAPC2; TRINITY_DN1740_c0_g2_i6_p1_Q39769), fructose-bisphosphate aldolase 6 (FBA6; TRINITY_DN1864_c0_g1_i11_p1_Q9SJQ9), and pyruvate dehydrogenase E1 component subunit alpha-1 (PDHA1; TRINITY_DN4900_c0_g3_i1_p1_P52901), were up-accumulated in callus at 10 and 30 d of incubation both compared to initial explant, and down-accumulated in callus at final stage of development (30d) when compared to the callus formation stage (10 d). The abundance of UTP glucose-1-phosphate uridylyltransferase 1 (UGP1; TRINITY_DN992_c0_g1_i13_p1_Q9SDX3) protein, also observed in the nitrogen compound metabolic process pathway, a key enzyme in the sucrose production and glycogen synthesis pathway, increased at 10 d of incubation during callus formation when sucrose and glucose levels increased, while in callus 30 d, this protein decreased the abundance, both compared to the initial explants. The same regulation occurred for malate synthase and isocitrate lyase (up-accumulated at 10 d compared to initial explant).

3.5 DISCUSSION

According to previous studies, callus formation in endosperm explants of *Passiflora* sp. under *in vitro* culture occurs between 10 and 15 d of induction in a culture medium supplemented with plant growth regulators (PGRs). During this period, the initially white explants, consisting of isodiametric cells and composed solely of reserve parenchymatous tissue, undergo intense transformation when inoculated in a cytokinin-rich culture medium. This transformation includes cell proliferation, greening, and the formation of callus and meristems (Antoniazzi et al. 2018, Silva et al. 2020). By 30 d of induction, well-developed adventitious buds were observed along the explants, with the presence of leaf primordia.

Several studies have proposed a deeper understanding of the biochemical and physiological mechanisms that allow *in vitro* regeneration, especially from perivascular tissues. Therefore, endosperm serves as an interesting model for studying the processes involved in the reprogramming of parenchymal, epidermal, and/or subepidermal cells, allowing *in vitro* regeneration of non-vascular tissues.

To identify key factors influencing cell proliferation and callus formation, analyses were prioritized for the initial explant, callus formation (10 d), and organogenic callus (30 d). Quantitative results from the proteomic analysis show proteins more abundant during the initial callus formation stage compared to other treatments, especially when compared to the initial explant. Approximately 90% of DAPs during callus formation and the establishment of organogenic calli, compared to the initial explant, are up-accumulated. This up-accumulation may be associated with intense tissue transformations resulting from reprogramming induction, cell proliferation, and the acquisition of organogenic competence.

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the cell's antioxidant control capacity (Libik-Konieczny et al. 2012). Mechanisms regulating ROS levels are considered well-developed in plant cells, involving the activity of various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), peroxiredoxins (Prxs), and glutathione (GSH) (Mittler 2002, Mittler et al. 2011). In this study, antioxidant proteins were identified as widely expressed, especially during the stage where cell proliferation was most evident and during callus formation (10 d) and the stage of organ differentiation (30 d). The increase in SOD enzyme during callus formation may indicate its participation in both controlling superoxide radicals in the tissue and triggering cellular signaling processes that coordinate organogenic competence acquisition and differentiation. CAT and POX, in turn, catalyze the dismutation of H_2O_2 into H_2O and O_2. Besides neutralizing peroxides, POX may be involved in various biological functions, such as lignin biosynthesis and degradation, suberization, auxin catabolism, and defense response against pathogens. In this study, a significant the protein CAT was up-accumulated in callus at 10 d induction compared to initial explants, increase accumulated in CAT occurs, especially at 10 days, indicating increased control of H_2O_2 during tissue regeneration and promoting greater homeostasis during increased oxidative stress. Prxs are ubiquitously expressed thiol-dependent peroxidases that also modulate the levels of a broad spectrum of peroxides through reduction reactions (Gomes 2016). Their function is directly associated with maintaining cellular redox homeostasis under oxidative stress signals. CAT, Prxs, and POX can be considered interesting molecular markers due to their role in controlling oxidative stress, allowing controlled levels of H_2O_2 in cells, avoiding significant tissue damage and enabling greater action of agents in this pathway as signaling molecules. Furthermore, greater tissue homeostasis may allow higher energy expenditure directed toward other metabolic functions, such as triggering growth and development programs. The increase in antioxidant enzyme activity may be associated with stress imposed on cells when subjected to tissue culture conditions. However, it is noteworthy that the increase during the initial tissue transformations at 10 days indicates an important role of oxidative stress in modulating regenerative responses and callus formation.

Heat Shock Proteins (HSPs) act as molecular chaperones, assisting in the folding and unfolding, degradation, and transport of proteins under stress conditions (Zhang et al. 2009, Kurashova et al 2019). In addition to preventing the aggregation of denatured proteins, they can also control the cell cycle and signaling dynamics (Huang et al. 2008, Usman et al. 2014). HSPs are classified into various families based on molecular weight, hence considered large (Hsp90, Hsp70, Hsp60, Hsp40) and small (ranging between 12 and 43 Kda) (Huang et al 2008). Studies report a significant increase in the expression of HSPs in tissues subjected to oxidative stress conditions during morphogenetic events, highlighting them as essential components in adaptive responses under conditions where maintaining cellular homeostasis is crucial to ensuring development programs (Zhang et al. 2009, Marsoni et al. 2007, Corredor-Prado et al. 2019). In this study, identified primarily HSPs 70 KDa proteins with a predominant increase at 10 d of incubation comparing with initial explants following a reduction in callus from 30 d compared to 10 d of incubation coincide with the dynamics of oxidative stress response in the tissue during callus formation and adventitious bud differentiation. HSPs 70 kDa protein 6, chloroplastic showed an increase at 30 d, certainly due to the tissue condition, which exhibits photosynthetic activity, as typical greening and well-developed leaf primordia are observed.

During stages of cell growth and proliferation, plant cells require a significant availability of energy, ensuring metabolic activities, cell division, and synthesis of new cellular components (Calamar and De Klerk 2002). In this study, a significant increase in hexose content in callus after 10 d of cultivation is related to the stage at which we noted heightened cell proliferation activity in the tissue during callus formation. Hexoses are directly related to providing immediate energy to the cell, enabling a series of events and metabolic activities. In glycolysis, glucose molecules are converted into pyruvate, and throughout this reaction, energy is released in the form of ATP. The pyruvate molecules produced in the cytosol from glycolysis are transported to the mitochondria, where they will be fully oxidized, releasing more energy into the system. The abundance of enzymes involved in glycolysis and the Krebs cycle during callus formation (10 d) compared to initial explant, such as glyceraldehyde-3-phosphate dehydrogenase (GAPC2), fructose-bisphosphate aldolase 6 (FBA6), and pyruvate dehydrogenase E1 component subunit alpha-1, indicates an increase in cellular respiration and a positive correlation between these factors (including increased hexoses and enzymes associated with glycolysis) and programs of growth and organization of meristematic tissue. In addition to being a key factor in cellular respiration and energy production, sugar signaling dynamics are reported as modulators of gene expression involved in phytohormone metabolism, such as CK (Cosic et al. 2022, Guleria and Kumar 2023). Consequently, the interaction between glucose signaling and CK may be an important element influencing *de novo* organogenesis and may corroborate the increase in sugar and hexoses during stages where the acquisition of organogenic competence was observed, as seen at 10 days in this study (Guleria and Kumar 2023). When adventitious buds are well-developed, and we begin to observe photosynthetic tissue along the explant incubation (30 d), significant changes occur in the sugar utilization pattern. Part of the glucose and fructose may be redistributed and converted into other compounds, such as malate. Additionally, the establishment of adventitious buds may mark a shift, with photosynthetic activities serving as a means to generate energy. The progressive accumulation of malate may be related to an increase in its activity as a carbon transporter, essential for sustaining growth and resource distribution, in addition to its participation in the Calvin cycle.

In plants, the glyoxylate cycle is an anabolic pathway and a variation of the Krebs cycle (Beevers 1980). It involves the utilization of acetyl-CoA into succinate for carbohydrate synthesis and has been described as significant, particularly during seed development phases and growth under low nutrient availability conditions (Beevers 1980). In this study, two key enzymes involved in this cycle, Malate synthase (MS) and Isocitrate lyase (IL), showed an accumulation in callus at 10 d of incubation compared to initial explant and absence (IL) or a drastic reduction in the accumulation in the subsequent developmental stage (MS). The observed increase in these enzymes may result from specific adaptation mechanisms to in vitro culture conditions in early developmental stages when more complex carbon sources were not available. This increase on accumulation could also facilitate early developmental programs and callus formation. Their drastic reduction may be associated with a metabolic redirection as the tissue develops and becomes more specialized during the differentiation of adventitious buds.

The endosperm, being a reserve tissue, exhibits a significant starch content in the initial explant, and a substantial accumulation is recorded at 10 and 30 ds. Starch is an efficient source of energy. Studies such as those by Mangat and colleagues (1990) have demonstrated that starch accumulation in *Begonia* sp. explants may be related to the induction of adventitious bud regeneration. Interestingly, starch accumulation occurs during the initial organ formation process, and as leaf primordia develop, a constant decrease in starch in cells is observed. This suggests a crucial role of starch both in the initial stage and in the subsequent development of organs, as the organogenic process requires an intense energy supply (Mangat et al. 1990). In this study, we observed that the progressive accumulation of starch may be related to the glucose stock during physiological changes in the tissue to support subsequent developmental programs. Essentially, as the supply of carbon sources is obtained from photosynthetic activity and the sugar utilization pattern changes during the differentiation of adventitious buds.

Our results suggest that metabolic changes, such as carbohydrate dynamics, energy metabolism, and stress response, are evident during cellular reprogramming in the induction of callus and adventitious bud regeneration from *in vitro* culture of endosperms. The mechanisms associated with these pathways, the cells' ability to activate signaling cascades, and the production of organic compounds are essential to support developmental programs, enabling metabolic adaptations, and tissue homeostasis to take on new morphogenic routes. Furthermore, the identification of proteins and metabolic pathways involved in different stages of development contributes to understanding the biochemical and molecular processes involved in bud formation through the induction of *de novo* organogenesis in explants like the endosperm. These studies provide support for further investigations on the subject and for genetic improvement programs and micropropagation of commercially interesting *Passiflora* species.

3.6 FUTURE PROSPECTS

Among the protocols developed for *in vitro* cultivation of *Passiflora* spp. endosperms, *P. cincinnata* can be considered a responsive species, while *P. edulis*, the main commercial passion fruit plant, is considered a recalcitrant species. In other words, despite the success in establishing these regeneration protocols and confirming the triploidy of both *P. edulis* and *P. cincinnata*, the low percentage of responsive endosperms and the number of regenerated plants per explant, especially in *P. edulis*, hinder the routine use of these systems as methods for clonal propagation or regeneration of triploid passion fruit plants.

Considering that biochemical and proteomic analyses have been conducted for both species, the next step is to perform comparative analyses to identify significant differences in the modulation of organogenic responses. These comparisons may lead to interesting discussions to either support or strengthen hypotheses about the mechanisms involved in triggering regenerative responses, as well as to propose strategies for the genetic improvement programs of commercial passion fruit plants.

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