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SHEHERYAR

PROTEOME CHANGES ASSOCIATED WITH THE REMOTE GERMINATION IN CARNAÚBA (*Copernicia prunifera*) SEEDS

FORTALEZA
2023

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Thesis presented to the Doctorate Course in Biochemistry of the Department of Biochemistry and Molecular Biology of the Federal University of Ceará, as a partial requirement for obtaining the title of Doctor in Biochemistry. Area of concentration: Plant Biochemistry

Advisor: Prof. Francisco A.P. Campos.
Co-advisor: Prof. Fábio C.S. Nogueira.

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With heartfelt gratitude,

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ABSTRACT

The carnauba palm (*Copernicia prunifera*), a member of the Arecaceae family, acquires a characteristic way of protecting its embryonic tissues during its early organogenesis by adopting a tubular type of remote germination. This palm has great ecological and socioeconomic significance because every part of it can be used: the roots are therapeutic and have medicinal value; the leaves are used to make textiles and handicrafts; and the most crucial production of wax is by its younger leaves, which are used in cosmetics, electronics, pharmaceutical capsules, coatings, and polishing waxes and generate more than $55 million annually. Therefore, understanding its germination and mobilization of seed reserves is fundamental to establishing biotechnological strategies to increase the production and utilization range of this well-known "tree of life." The objective of the current study was to determine the spatiotemporal changes in the proteome of haustorium and cotyledonary petiole guided by morphoanatomical alterations during emergent organogenesis. The haustorium and cotyledonary petiole were evaluated morphoanatomically during germination, and four stages (the mature embryo, 2, 5, and 10 days after germination) were selected for proteomic analysis. The morphoanatomical analysis revealed that, after germination, the embryonic axis continues to divide, organogenesis occurs inside the emerging cotyledonary petiole, and the plant body ascends from the cotyledonary petiole with developed leaves and a complex root system. Samples from both tissues' stages were submitted to the bottom-up proteomics approach, where the peptides were analyzed in an nLC-MS/MS Orbitrap system. Proteome Discoverer v. 2.5 was used for the protein and peptide identifications, and Perseus v. 1.6.14 was used for the statistical analysis of the quantitative data. In proteomics analysis, 4776 and 4473 proteins were identified in the cotyledonary petiole and haustorium, respectively. In a total of 1673 up-regulated proteins in the haustorium, cellular catabolic processes, carbohydrate and lipid metabolic processes, and carbohydrate derivative biosynthesis activities were identified in GOBP, catalytic activity, peptidase activity, and hydrolase activity were identified in GOMF. Whereas, 318 differentially abundant proteins in the cotyledonary petiole, in particular, developmental growth, cell growth, and metabolic processes involving carbohydrate derivatives were found in GOBP, whereas catalytic activity, lyase activity, fructokinase activity, and cytoskeleton structural components were common in GOMF. Additionally, in the cotyledonary petiole, we quantified proteins involved in lipids, and protein mobilization, along with proteins involved in the biosynthesis of growth regulators such as salicylic acid (SA), jasmonic acid, ethylene, indole-3-acetic acid (IAA), cytokinin, and gibberellins (GA), which play a pivotal role in plant growth and

development. This work shows that the haustorium plays a pivotal role in the synthesis of hydrolases and transports the reserve to the seedling, whereas the embryonic axis continues its growth and development inside the cotyledonary petiole by utilizing these reserves and the action of other essential proteins responsible for growth and development.
Keywords: emergent organogenesis; cotyledonary petiole; reserve mobilization; carnauba; haustorium; embryonic axis; Arecaceae; remote germination.

RESUMO

A carnaúba (*Copernicia prunifera*), membro da família Arecaceae, adquire uma forma característica de proteger seus tecidos embrionários durante sua organogênese inicial, adotando um tipo tubular de germinação remota. Essa palmeira tem grande importância ecológica e socioeconômica porque todas as suas partes podem ser aproveitadas: as raízes são terapêuticas e têm valor medicinal; as folhas são usadas para fazer tecidos e artesanato; e a produção mais importante de cera é por suas folhas mais jovens, que são usadas em cosméticos, eletrônicos, cápsulas farmacêuticas, revestimentos e ceras de polimento e geram mais de $ 55 milhões anualmente. Portanto, entender sua germinação e mobilização de reservas de sementes é fundamental para estabelecer estratégias biotecnológicas para aumentar a produção e a faixa de utilização dessa conhecida "árvore da vida". O objetivo do presente estudo foi determinar as mudanças espaço-temporais no proteoma do haustório e pecíolo cotiledonar guiadas por alterações morfoanatômicas durante a organogênese emergente. O haustório e o pecíolo cotiledonar foram avaliados morfoanatomicamente durante a germinação, e quatro estádios (o embrião maduro, 2, 5 e 10 dias após a germinação) foram selecionados para análise proteômica. A análise morfoanatômica revelou que, após a germinação, o eixo embrionário continua a se dividir, a organogênese ocorre dentro do pecíolo cotiledonar emergente e o corpo da planta ascende do pecíolo cotiledonar com folhas desenvolvidas e um sistema radicular complexo. Amostras de ambos os estágios dos tecidos foram submetidas à abordagem proteômica bottom-up, onde os peptídeos foram analisados em um sistema nLC-MS/MS Orbitrap. Proteome Discoverer v. 2.5 foi usado para as identificações de proteínas e peptídeos, e Perseus v. 1.6.14 foi usado para a análise estatística dos dados quantitativos. Na análise proteômica, foram identificadas 4776 e 4473 proteínas no pecíolo cotiledonar e no haustório, respectivamente. Um total de 1673 proteínas reguladas positivamente no haustório, processos catabólicos celulares, processos metabólicos de carboidratos e lipídios e atividades de biossíntese de derivados de carboidratos foram identificados em GOBP, atividade catalítica, atividade de peptidase e atividade de hidrolase foram identificadas em GOMF. Considerando que, 318 proteínas diferencialmente abundantes no pecíolo cotiledonar em particular, crescimento de desenvolvimento, crescimento celular e processos metabólicos envolvendo derivados de carboidratos foram encontrados em GOBP, enquanto atividade catalítica, atividade de liase, atividade de frutoquinase e componentes estruturais do citoesqueleto foram comuns em GOMF. Além disso, no pecíolo cotiledonar, quantificamos proteínas envolvidas em lipídios e mobilização de proteínas, juntamente com proteínas envolvidas na biossíntese de reguladores

de crescimento, como ácido salicílico (SA), ácido jasmônico, etileno, ácido indol-3-acético (IAA). , citocinina e giberelinas (GA), que desempenham um papel fundamental no crescimento e desenvolvimento da planta. Este trabalho mostra que o haustório desempenha um papel fundamental na síntese de hidrolases e transporta a reserva para a muda, enquanto o eixo embrionário continua seu crescimento e desenvolvimento dentro do pecíolo cotiledonar utilizando essas reservas e a ação de outras proteínas essenciais responsáveis pelo crescimento e o desenvolvimento.

Palavras-Chave: organogênese emergente; pecíolo cotiledonar; mobilização de reservas; carnaúba; haustório; eixo embrionário; Arecaceae; germinação remota.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis Of variance
CP	Cotyledonary Petiole
CID	Collision-induced dissociation
CE	Collision Energy
DAG	Days After Germination
DDA	Data Dependent Acquisition
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EN	Endosperm
ESI	Electron Spray Ionization
FDR	False Discovery Rate
FWHM	Full Width at Half-Maximum
GO	Gene Ontology
HCD	Higher-energy Collision dissociation
HU	Haustorium
iTRAQ	Isobaric tag for relative and absolute quantitation
IAA	Indole-3-acetic acid
LTQ	Linear Trap Quadropole
MALDI	Matrix-Assisted Laser Desorption/Ionization
MBR	Match Between Runs
MS/MS	Tandem Mass Spectrometry
nLC	Nano Liquid Chromatography
PRM	Parallel Reaction Monitoring
PSM	Peptide Spectrum Match
TFA	Trifluoroacetic acid
TOF	Time-of-flight
Tris	2-Amino-2-(hydroxymethyl) propane-1,3-diol

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1 INTRODUCTION

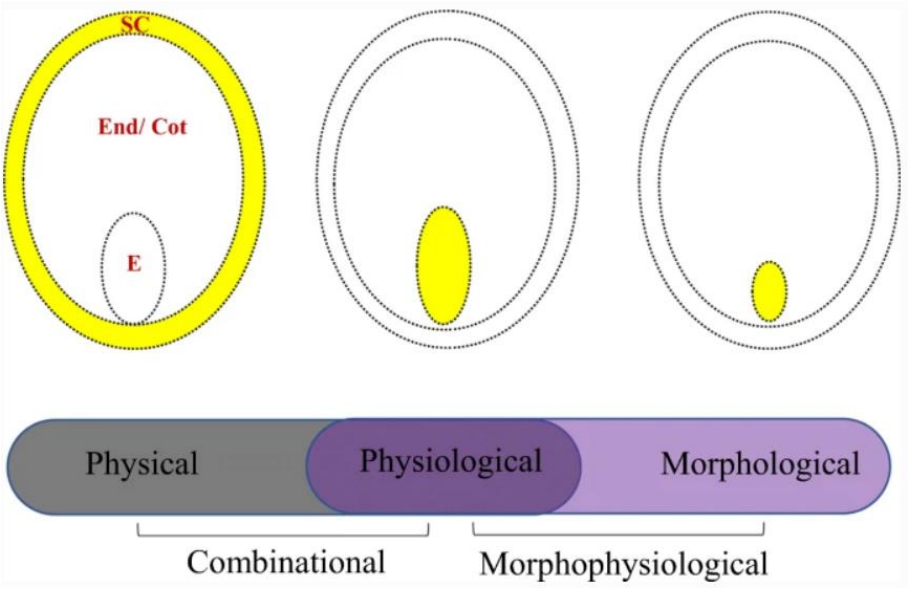
Arecaceae, or Palmae, is a diverse family of palms comprising about 202 genera and 2600 species (MUSCARELLA et al., 2020). This family is divided into five subfamilies: Nypoideae, Ceroxyloideae, Calamoideae, Coryphoideae, and Arecoideae (DRANSFIELD et al., 2008). The family shows different germination patterns and life forms, varying from climbers to shrubs and tall trees, demonstrating a significant morphological adaptation across a wide geographic range (PARMAR et al., 2023). Palms are economically important as they supply tradable commodities such as their nutritious fruits with phytochemicals and medicinal qualities. Good examples of these are the açaí (*Euterpe oleracea* Mart.), peach palm (*Bactris gasipaes* Kunth.), dates (*Phoenix dactylifera* L.), and coconut (*Cocos nucifera* L.) as well as vegetable fibers (Borassus, Chamaerops, Sabal, and Trachycarpus), sugar (*Borassus flabellifer* L.), oil (*Elaeis guineensis* Jacq.) wax (*Copernicia prunifera* (Mill.) H.E Moore), etc. (ANDRADE et al., 2020; DRANSFIELD et al., 2008; NASCIMENTO et al., 2020). Only 10% of the palms inhabit subtropical and seasonal tropical vegetation, while the rest, 90%, are found in tropical rainforests (COUVREUR; BAKER, 2013). Due to their ubiquity in the Amazon rainforest six of the top ten most common plants are palms (ONSTEIN et al., 2017; TER STEEGE et al., 2013). Due to the lack of secondary growth (i.e., woody development), about 40% of palm species are capable of growing stems that are ten cm in diameter and 1.3 m high (GOODMAN et al., 2013). Acaulescent to robust plant bodies, armed or unarmed leaves, inter-foliar or infra foliar inflorescences, staminate or pistillate flowers with discrete appearances, and fruits with apical or basal stigmatic and smooth epicarp are just a few of the traits that palms display (KADAM et al., 2023). Around 480 native palm species may be found in Brazil, which has a rich and varied palm flora. The palms like carnauba, macauba, tucuma, and babaçu are endemic to caatinga biome. As a result, the country was once known as "Pindorama" in the Tupi-Guarani language, which means "land of many palms" (BARRETO; PARISE; DE ALMEIDA, 2019; DE SOUZA et al., 2020). These palms are valuable for a variety of functions, including food, fiber production, fuel, folk medicine, traditional handicrafts, foraging shelter, landscape, and ornamental uses (BRANDÃO; DE CASTRO; FUTEMMA, 2019; CAMPOS et al., 2019).

The Arecaceae seeds vary greatly in size from millimetric seeds such as *Prestoea carderi* W.Bull Hook to seeds weighing more than 25kg in *Lodoicea maldivica* J.F.Gmel Pres. and are considered the largest seed of all plants. The shape and size of seeds and fruits reflect a range of dispersal methods, frequently via animals and water, with a majority of ellipsoid, oval,

or circular morphology (DEL POZO et al., 2020). In most of the palms, the endocarp and exocarp are single-layered while the mesocarp is multi-layered and thick; after pollination, these layers are differentiated into three topographic layers, and the hypodermis; the aerenchyma is formed from the outer mesocarp at late developmental stages; the second mesocarp layer constitutes the big stone; due to seed growth, the innermost parenchymatous multi-layered mesocarp is highly compressed and partially destroyed in mature fruit (BOBROV et al., 2012). When the palms' fruits mature, they fall by weight and are consumed by some rodent species (*Cuniculus paca* Linnaeus, 1758), which helps their dispersal (CEVALLOS et al., 2013). Berries and drupes are the natural dispersal units of palm fruits, which usually have only one seed inside, but sometimes this number may reach 10 depending on the species (DRANSFIELD et al., 2008). The thick and hard endocarp that holds the embryo and endosperm is called "pyrene." Pyrenes are rich in phenolic compounds and lignin, which lie beneath most of the species' fibrous mesocarp and exocarp. Upon the exocarp and mesocarp consumption by dispersers or decomposition along the way, the pyrene becomes the germination unit, and the fruit of carnauba is a drupe; because of its thickness and rigidness, it is considered pyrene (OLIVEIRA et al., 2013; RENCORET et al., 2018). Even in ideal germination conditions, most of the palm seeds germinate slowly and unevenly, which is frequently caused by the resistance and impermeability of the endocarp to water, therefore, a pre-germination treatment of the palm seed is required to accelerate the germination process (FERREIRA et al., 2021; RODRIGUES et al., 2014). For this purpose, seeds are imbibed to increase water content and humidity in the tissues, which ultimately initiate various biochemical processes and metabolic activities for the production of essential nutrients for the germination, growth, and development of the embryonic axis and, in addition, increase the germination percentage and speed index of the seeds up to 10 times (FERREIRA et al., 2021; SOUZA DIAS et al., 2018).

Most palm diaspores, possibly all of them, have embryos that roughly make up 10% of the diaspore size at the time of dispersal. The term morphological dormancy refers to this characteristic, that is, the embryo needs time to mature and grow inside the diaspores before germination. Morphophysiological dormancy is present in most trees, although only 10% of palm species have morphophysiological dormancy (Figure 1) (BASKIN; BASKIN, 2014; JAGANATHAN, 2020).

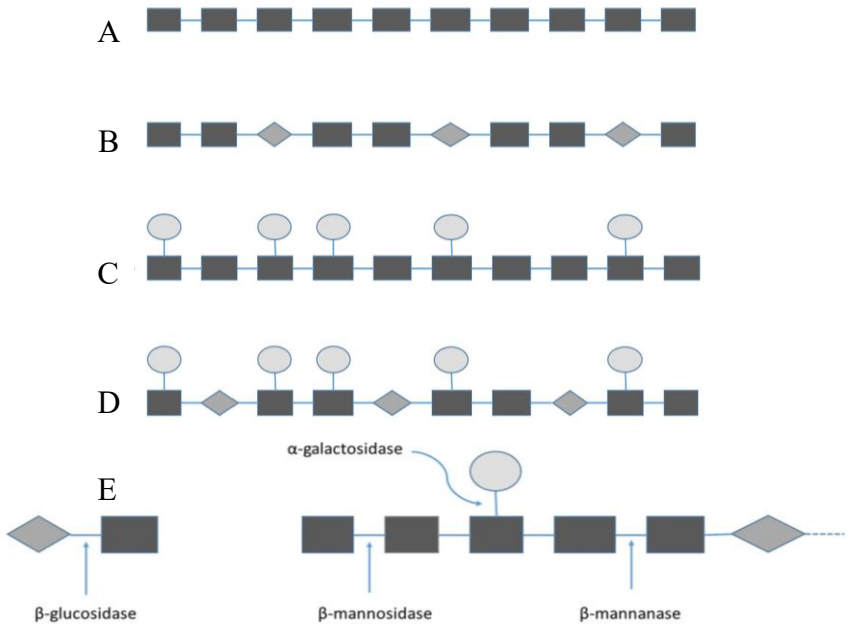
Figure 1- The illustration of seed dormancy. The three forms of dormancy physical, physiological, and morphological are depicted in the image. The yellow indicates the cause of dormancy. E, embryo; End/Cot, endosperm/cotyledon; SC, seed coat.



Source: First proposed by Nikolaeva in 1969 and improved by (BASKIN; BASKIN, 2014).

The primary reserve tissue which is utilized during germination and post-germination period by seedlings is the endosperm, which has a high amount of cell wall polysaccharides (HABIBI et al 2005), For the açaí, mannans make up ~80% of the total seed carbohydrates (MONTEIRO et al., 2019). Mannans are polysaccharides composed of connected mannose sugar units. Four primary varieties of mannans exist, each with distinct structures. Linear mannans are the first type which are homopolysaccharides containing mannopyranosyl residues joined by 1,4 links to create a linear chain. Unlike linear mannans, glucomannans have a 3:1 ratio of mannose to D-glucose units. A chain of 1,4-linked mannopyranosyl residues interspersed with irregular glucose residues is formed by the linear linking of these units. The 1,6-galactose residue is connected to the 1,4-linked mannose backbone at regular intervals in the third type of mannan known as galactomannans. Galactoglucomannans are more complex because they incorporate linear mannans with branches made of galactose and glucose residues that are variously linked to the mannose backbone (Figure 2) (VAN ZYL et al., 2010).

Figure 2- Shows an illustration of the general structure of mannans and hetero-mannans. (A) shows the structure of linear mannans (B) shows the structure of glucomannans (C) shows the structure of typical galactomannans (D) shows the structure of galactoglucomannan and (E) shows the enzyme site for the complete degradation of linear and heteromannans



Source: (MALGAS; VAN DYK; PLETSCHKE, 2015)

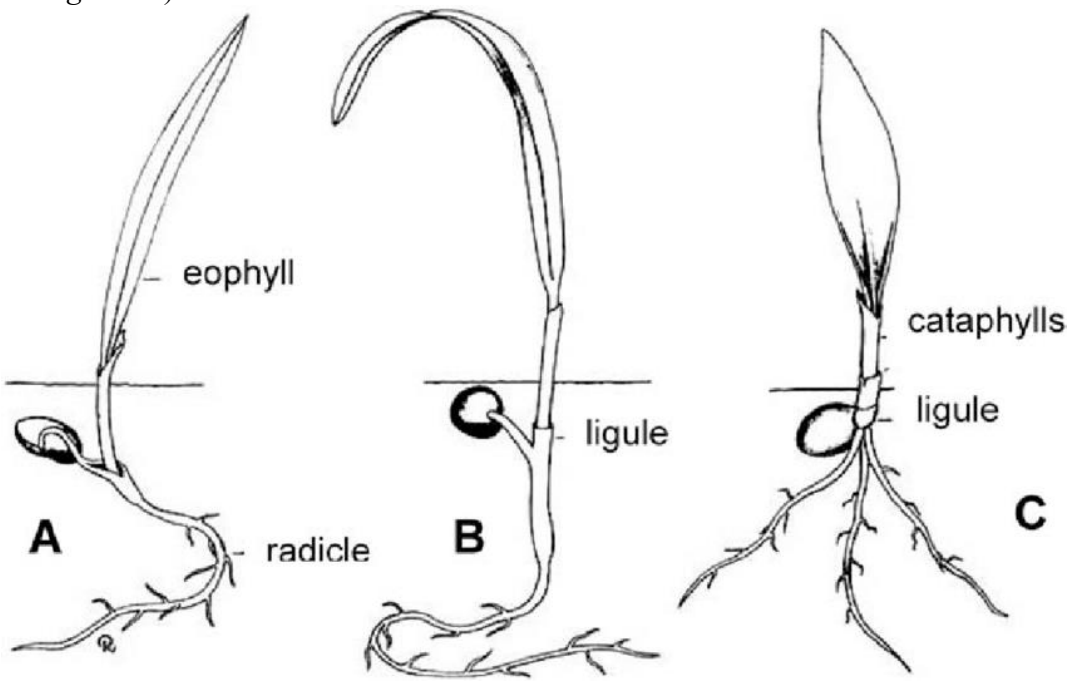
This high amount of mannose in the Arecaceae seed may be used as a residue for bioethanol production (RAMBO; SCHMIDT; FERREIRA, 2015). Additionally, these by-products and raw materials can be used in the production of cosmetics, pharmaceuticals, chemicals, and food products (DE SOUZA et al., 2020). The seed's integument contains phenolic compounds, which have stimulated research into the extracts' potential as medicines, especially given their proven anti-inflammatory and anti-cancer potential (MARTINS et al., 2020).

Along with mannoses, palm seeds also accumulate proteins, lipids, and other polysaccharides, which are utilized during seed germination and post-germination processes (NASCIMENTO et al., 2020). During the early developmental stages of seedlings, the haustorium (a highly specialized cotyledonary structure) plays a vital role in the mobilization of these reserves through a continuous increase in size within the seed (DIAS et al., 2020). It was believed that enzymes involved in the breakdown of these reserves are present but not active in the endosperm, and upon germination, they are activated, and the haustorium is just a transport structure (MAZZOTTINI-DOS-SANTOS et al., 2020); However, the role of the haustorium in the synthesis of hydrolyses during germination was observed in açai, It was also proposed that these hydrolases are synthesized during the digestion of the endosperm by the

endosperm and/or haustorium and transported to the digestion zone rather than accumulated in the mature seed and only activated with germination. The pattern of abundance of mannans hydrolyses suggests that such hydrolases are involved in the mobilization of seed reserves during germination (NASCIMENTO et al., 2020). The seed storage reserves are mobilized properly during germination; in the first step, protein bodies and storage proteins in the endosperm are mobilized, followed by carbohydrates and lipids (DIAS et al., 2020).

Desert palms have developed a unique adaptation to the desert environment to protect their meristematic tissue in early organogenesis. After germination, the developing embryo remains in the continuously growing cotyledonary petiole (XIAO, T. T. et al., 2019). Since (JU¨; TILLICH, 2007) disagrees with the term "petiole," he proposes the name “apocole” which would only be used for palm trees with remote germination. This author claims that the apocole is a segment of the cotyledonary hyperphyl that extends and separates the endosperm (seed) from the cotyledonary sheath (a portion of the cotyledon that encloses the cotyledon node and shields the plumule). According to the author (SEEDS; et al., 2015), the cotyledonary sheath, which houses the embryonic axis, is positioned in the proximal part of the zygotic embryo. However, according to several other authors (BASKIN; BASKIN, 2014; FERREIRA MOURA et al., 2019; MAZZOTTINI-DOS-SANTOS; RIBEIRO; OLIVEIRA, 2017; SOUZA DIAS et al., 2018), the proximal part of the embryo is equivalent to the cotyledonary petiole. The organogenesis occurs inside this tubular structure, and the complex root system and developed leaves arise from the shoot and root meristems of the embryonic axis inside the cotyledonary petiole, which increase the efficiency of water and nutrient uptake (XIAO, T. T. et al., 2019). Martius 1823–1850 studied for the first time the germination types in palms and explained that when the development of the seedling occurs near the seed it is germination admotiva, whereas remote germination (germination remotiva) is the development of the seedling at a distance from the seed, which can be a *germinatio remotiva tubulosa* (remote tubular) or *germinatio remotiva ocreata* (remote ligular) (Figure 3) (JU¨; TILLICH, 2007).

Figure 3- Illustration of the remote germination types in the family Arecaceae. (A) shows remote tubular (*P. dactylifera*). (B) shows remote ligular (*Sabal*) and (C) adjacent ligular (*P. borsigianum*)



Source: (Uhl et al., 1987).

The mobilization of reserves during seedling development in the palm family has received little attention (VISSCHER et al., 2020). Among the palm genera, the genus *Copernicia* Mart. ex Endl. stands out for its ecological and socioeconomic significance (CÁSSIA-SILVA et al., 2019; FREITAS et al., 2019). *Copernicia prunifera* Mart. ex Endl. is a xerophyte palm, a species of Brazilian origin commonly known as carnauba. This palm has opaque green leaves arranged on the plant's crown, 10-15 m in height, and almost 200 years of productive life. It is highly resistant to extreme drought and flood conditions (PEREIRA JUNIO et al., 2023). The population of carnauba is expected to grow by 23.88% until 2050–2070 (COSTA et al., 2022). Every part of the carnauba palm can be used; the roots of the palm are therapeutic and have medicinal value, the fruits are frequently utilized as animal food, and the trunk is used as building wood. Manufacturing lubricants and anti-corrosives makes considerable use of wax obtained from carnauba. The leaves are used to make textiles, handicrafts, and house covers (PEREIRA JUNIO et al., 2023). This economically significant palm is also known as the "tree of life" and is becoming more important because of the production of wax (the important Carnauba wax) by its younger leaves, which are used in cosmetics, electronics, pharmaceutical capsules, coatings, and polishing waxes (FERREIRA DE SOUSA et al., 2015). Carnauba's fiber and wax production generates more than $55 million annually (DOS SANTOS et al., 2021). Carnauba like other plants containing cellulose, makes

it appropriate for creating paper and wood for a variety of uses. After drying and being wax-free, the leaves are used by the industry to make mats, hats, and other items (ARRUDA; CALBO, 2004; LORENZI et al., 2004). Climate change can lead to a decrease as well as redistribution of the possible areas of the growth development and occurrence of carnauba. The high potential area is predicted to reduce from 25.3% in the present state to 19.6% in 2050 (VIEIRA, 2022). The germination of these palm seed reports is absent from the literature (PIVETTA et al., 2013). Carnauba is still not well explored despite its importance to both the economy and the environment (NOGUEIRA et al., 2017). The *C. prunifera* populations show signs of intense exploitation, such as fire, extraction and cutting of leaves, soil impacted by livestock, and low or absence of regeneration. Deforestation, agricultural expansion, and modernization of agriculture have led to a rapid decline in these populations (DOS SANTOS et al., 2021). Determining the germination morphoanatomy and proteomics study of *C. prunifera* is crucial because of the species' economic and social significance and because it is a factor that must be taken into account in the management, conservation, and genetic advancement of the species. This is important not only for understanding the early developmental stages of carnauba but also for identifying important factors influencing successful germination.

To open useful routes for managing, conserving, and developing this species by disclosing molecular insights and investigating the proteome changes associated with post-germination organogenesis and mobilization of reserves during germination in *C. prunifera*. This study was planned to investigate the effective mobilization of reserves inside the seed and to identify the location of hydrolase synthesis associated with mannans mobilization in the endosperm. To acquire important insights regarding emergent organogenesis, we simultaneously carried out a thorough quantitative proteomic analysis of the cotyledonary petiole. The accurate identification and quantification of proteins in the haustorium and entire cotyledonary petiole during germination were combined with the morphoanatomical study of cotyledonary petiole and haustorium development to accomplish these goals. The morphoanatomical analysis aimed to delimit which region of the embryo originates the haustorium and describe which changes occur in the anatomy of the haustorium that may be related to the mobilization of reserves in the endosperm. The quantitative proteomic analysis using, nLC-MS/MS techniques was used to identify and quantify proteins present in the haustorium and cotyledonary petiole during germination, to describe the spatiotemporal protein profiles between the two tissues and among the seedling stages.

2 THEORETICAL BACKGROUND

Copernicia prunifera (Carnauba) is a palm that belongs to the family Arecaceae. The species is endemic to north-eastern Brazil and is known for being the source of carnauba wax, an economically lucrative epicuticular wax (DA SILVA ANDRADE et al., 2018). It is a valuable commercial commodity that is widely utilized in lubricants, surface coatings, polishes, cosmetics, and other products (MEHYAR et al., 2012). Extracts from carnauba wax powder could also be useful sources of natural antifungal and antioxidant compounds (DA SILVA ANDRADE et al., 2018). The potential for medicinal applicability of carnauba (*C. prunifera*) wax extracts may be due to the presence of natural dammarane triterpenoid derivatives, which show antiprotozoal bioactivity (DE ALMEIDA et al., 2016). This wax has been the subject of numerous studies focusing on its usage in food processing and preservation, emphasizing its role in edible films, superhydrophobic packaging, and taste microencapsulation (RIBEIRO JUNIO, 2021). Long-chain fatty acids, aromatic acids, free alcohols, esters, hydroxy acids, cinnamic acids, aliphatic acids, triterpene diols, and proteins are among the numerous chemical components of carnauba wax (DE ALMEIDA et al., 2017). Aluminum, zinc, calcium, sodium, copper, magnesium, manganese, and iron are among the inorganic elements that are also present in carnauba wax (ALLAN et al., 2013).

The primary component of carnauba wax, p-methoxy cinnamic diester (PCO-C), was isolated by (FILHO et al., 2017) who noted a potential hypolipidemic and hypoglycaemic effect in the production of dyslipidemia in mice. Furthermore, (FREITAS et al., 2016) proved PCO-C's great thermal stability and high antioxidant potential in addition to its effective biological activity for the treatment of chronic diseases, indicating promise for the food and pharmaceutical industries. The p-hydroxycinnamic diesters (HCE) found in carnauba wax (CW) have been found to have good pharmacological properties, HCE was safe and was found to lower serum total cholesterol and LDL levels in mouse models, it may also lessen the harmful effects of dyslipidemia brought on by long-term consumption of western diets (SILVA et al., 2021). Furthermore, it also showed excellent *in-vitro* and *in-vivo* antioxidant activity, and it also decreased glycemia, reactive oxygen species in liver tissue, MDA, body mass, and water consumption in animals (RODRIGUES et al., 2014).

Mannans are one of the most prevalent polysaccharides in softwood hemicelluloses, as well as in palm seeds, endosperms, and fruits. The cell walls of some fungi, yeasts, and bacteria, legume seeds, coconut kernels, and palm kernels can all be used for the production of mannans because these plants contain mannans as a major component of their cell wall

(OLANIYI, 2013; SINGH; SINGH; ARYA, 2018; WANG et al., 2022). D-mannose has a wide range of uses in the food, pharmaceutical, and beverage industries due to its good physiological qualities and biological activities, including being low in calories and sweetness (HUANG et al., 2018; SHARMA et al., 2018). Mannose, for instance, can effectively slow tumor cell proliferation and is crucial in the chemical modification of anti-cancer medications and nano vaccines (DONG et al., 2019; GONZALEZ et al., 2018; YANG et al., 2018). Additionally, it positively impacts the management of type I diabetes, a lack of mannose phosphate isomerase (MPI), and can successfully help cure urinary tract infections (MILANDRI et al. 2019). On the other hand, mannose can also be a crucial raw material for the production of bioenergy. For example, mannan can be used as a substrate for the fermentation of ethanol, which is then converted into ethanol under the combined action of β-mannanase and β-mannosidase (DE LONLAY; SETA, 2009; ISHII et al., 2016; ZHANG et al., 2017). D-mannose production is a focus of study in the food and pharmaceutical industries since it is a high-value product. Numerous techniques, including biotransformation, chemical synthesis, microbial fermentation, etc., have been created thus far (HU et al., 2016), and each of these approaches has particular benefits. However, the reagents used and by-products created during the chemical synthesis of mannose may pollute the environment. The desire for environmental standards, sustainable growth, and a "zero waste economy" is growing in the context of the current social development, which also implies that prevention, assurance, and sustainability are important metrics for green chemistry (WANG et al., 2022).

The combination of acid and enzymatic hydrolysis with oil palm seeds (*Elaeis guineensis*), reached a yield of up to 92.1% recovery of seed mannose, and more for açaí (*E. oleracea*), with a yield of 96.8%, the highest ever recorded (FAN et al., 2014; MONTEIRO et al., 2019). As far as our knowledge there is no literature on the seed composition of *C. prunifera*, however, the presence of mannoses in *E. oleracea* and *Elaeis guineensis* and palm kernel cake is a strong indication of the presence of mannose in this species (DONG et al., 2022; MONTEIRO et al., 2019; NASCIMENTO et al., 2020). Besides numerous physiologic benefits for health, including the immune system, intestinal diseases, diabetes mellitus, and urinary tract infections. D-mannose is used as a preliminary material to synthesize vitamins, immunostimulatory agents, anti-tumor agents, and D-mannitol (WU; ZHANG; MU, 2019). Mannitol, which has applications in the pharmaceutical, chemical, and medical industries and is consumed in amounts of about 150,000 t annually, offers some intriguing advantages (DAI et al., 2017). This naturally occurring sugar alcohol is employed in medicine largely for its

osmotic diuretic effects, in the treatment of cerebral edema and elevated intracranial pressure (ICP) due to a variety of reasons, mannitol is frequently employed in cardiac and vascular surgery, during renal transplantation, and in the treatment of rhabdomyolysis, mannitol is used to protect the kidneys (SHAWKAT; WESTWOOD; MORTIMER, 2012). Therefore, understanding the physiological mechanisms involved in the mobilization of seed reserves is crucial for supporting biotechnological strategies that make use of the mannose reserves present in the polysaccharides of the cell wall of the carnauba seed.

2.1 Seed reserves in the family Arecaceae

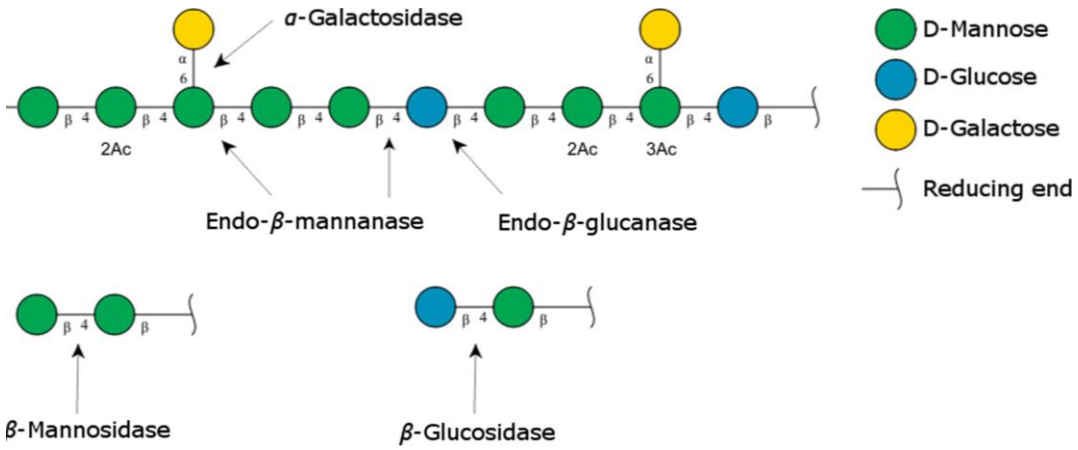
The quantity and complexity of reserves present in the endosperm of palm seeds, as well as the formation of the highly specialized haustorium, make such seeds interesting research models for study on seed reserve mobilization at the tissue level (SOUZA DIAS et al., 2018). In the endosperm of the mature seed, polysaccharides are accumulated in the cell walls, and lipid bodies and protein bodies are uniformly distributed in the cytoplasm of the endosperm, Whereas, in embryo, the proteins and lipid bodies are accumulated in the cotyledon (BICALHO et al., 2016; CORRÊA; ARAÚJO; MENDONÇA, 2019; DEMASON, 1988).

The haustorium, a highly specialized structure that emerges from the distal section of the cotyledonary leaf blad that eventually fills the whole interior of the seed previously occupied by the endosperm, is developed as a part of the mobilization of reserves (DIAS et al., 2020). During the seed germination a digestion zone forms around the endosperm closest to the haustorium. The events leading to the formation of such a digestion zone are well understood as regarded as the mobilization of protein bodies and cell wall reserves (BICALHO et al., 2016; MAZZOTTINI-DOS-SANTOS et al., 2020). In the endosperm near the haustorium, the protein bodies take on a granular and tiny appearance until most of them coalesce into a vacuole that fills the whole interior of the cell. The protein bodies have a uniform shape and take over a considerable amount of the cytoplasm (ALLAN et al., 2013; MAZZOTTINI-DOS-SANTOS et al., 2020). The cytoplasmic disappearance forms thick-walled empty cells, and finally the degradation of these walls also starts after the fading of protein bodies, and then cell wall digestion happens, leaving only the middle lamella near the haustorium, which results in the growth of the haustorium occupying the space of the endosperm (DIAS et al., 2020). The vacuoles formed as the result of protein bodies mobilization interact with the surface proteins commonly lipoxygenases or caleosin of lipid bodies which results in the formation of fatty acids that are either transported to the haustorium by the pressure exerted by haustorium on endosperm or utilized in the endosperm (POXLEITNER et al., 2006; ZIENKIEWICZ et al.,

2014). The lipid reserves are converted into carbohydrates via the glyoxylate cycle, which leads to the accumulation of starch in the haustorium (EASTMOND; GRAHAM, 2001).

Upon absorption by haustorium, the protein reserves are either transported to the developing seedling or utilized by haustorium itself for the synthesis of new enzymes, whereas, for haustorium expansion, the cell wall carbohydrates are utilized for the remodeling of the new cell wall (BARROS-GALVÃO et al., 2017; FERREIRA et al., 2020). Linear mannan, galactomannan, glucomannan, and galactoglucomannan are the different types of β-mannan, a key hemicellulose component. The combination and synergistic action of β-glucosidase, β-mannanase, α-galactosidase, and β-mannosidases are needed for the complete mobilization of mannans (Figure 4) (MALGAS; VAN DYK; PLETSCHKE, 2015; SINGH; SINGH; ARYA, 2018). The primary mannanolytic enzyme endo-mannanases (EC 3.2.1.78) hydrolyze internal β-1,4-glycosidic linkages in the mannans backbone to produce manno-oligosaccharides (MALGAS; VAN DYK; PLETSCHKE, 2015). The ends of mannans are acted on by β-mannosidases (EC 3.2.1.25), which are exo-type carbohydrases and act on non-reducing ends of mannans. They hydrolyze the β-(1→4) linkages, releasing manno-oligosaccharides and mannose (DOMINGUES et al., 2018). The side chains of manno-oligosaccharides in the case of galactomannan and galactoglucomannan are hydrolyzed by the action of α-galactosidase (EC. 3.2.1.22) and β-glucosidase (EC. 3.2.1.22) respectively. These carbohydrases are responsible for cleaving the β-(1→4) glycosidic bond between glucose and mannose residues in oligosaccharides (XIE et al., 2020).

Figure 4- The schematic diagram of the site of action of enzymes during mannan hydrolysis



Source: (VON FREIESLEBEN, et al. 2018).

There are different hypotheses regarding the hydrolases' origins; one of the studies on proteomics analysis of different developmental stages of açai (*E. oleracea* Mart.) seed identified different hydrolases as an indication of their presence in the seed before germination (FM NETO et al., 2023). There are three possibilities for the origin of these hydrolases in the seeds. Either these hydrolases are stored in the endosperm and activated during germination by a signal passed from the haustorium or they are stored in the haustorium and secreted to the endosperm during initiation of germination or they are synthesized de novo in the endosperm during germination initiation (DEMASON, 1988; MAZZOTTINI-DOS-SANTOS; RIBEIRO; OLIVEIRA, 2017; SOUZA DIAS et al., 2018). However, some findings revealed that the endosperm is only a living storage tissue, whereas the hydrolases are present in the digestion zone and activated during germination initiation (DIAS et al., 2020). Furthermore, it was shown that the presence of hydrolases in the haustorium, and their abundance was increased during germination and development, which indicates that the haustorium is the possible site of production of these hydrolases (NASCIMENTO et al., 2020). Since these hydrolyses have been found in both endosperm and haustorium, it is hard to pinpoint their precise origin utilizing studies at this level. It can be suggested that, while completely avoiding endosperm contamination, proteome analyses of the haustorium at different embryonic stages will provide the clearest picture possible of their origins.

2.2 Emergent ontogenesis

The diaspores of palms show a variety of dormancy, including physiological (MAGALHÃES et al., 2013), morphological, morphophysiological, and mechanical (PÉREZ et al.,2008). Most palms display morphophysiological and morphological dormancy (BASKIN; BASKIN, 2014). In morphological dormancy, embryo differentiation takes place inside the diaspores (BASKIN; BASKIN, 2004; JAGANATHAN, 2020). To cope with the environmental condition, palms may germinate in three different ways: adjacent ligular, remote ligular, or remote tubular. The cotyledonary petiole, a tubular root-like structure that develops in all these types from a germination seed, carries the embryo during the process of organogenesis as it grows (IOSSI; VITTIMORO; SADER, 2006; TILLICH, 2007).

The palm embryo may be divided into two regions: the cotyledonary petiole, which is close to the operculum, micropylar endosperm, and seed coat, and the cotyledon blade, which remains inside the seed and becomes the haustorium (BASKIN; BASKIN, 2014). The operculum dislodging and the cotyledonary petiole lengthening is a common definition of palm seed germination (NEVES et al., 2013). It was observed in a study on *Attalea vitrivir* Zona,

Butia Capitata Mart.*,* and *Mauritia flexuosa* L.F. that a small elongation in the embryo length is sufficient for the removal of the operculum to initiate germination (DIAS et al., 2017; FERREIRA MOURA et al., 2019). However, a study on *Euterpe precatoria* hypothesized that the utilization of embryonic protein reserves is associated with the energy supply required for germination bud emission (FERREIRA et al., 2020). Palm seed germination has two different stages: cotyledonary petiole protrusion and shoot and radicle emergence from cotyledonary petiole, In *Salanoia concolor* the cotyledonary petiole emerges from the seed plantation after 8-9 days, and after 80 days plumule and roots emerge from cotyledonary petiole (PINHEIRO, 2001). Anatomical characteristics of the *Corypha umbraculifera* L. cotyledonary sheath's growing tip mirror the usual root apex, which consists of an elongating region and a "root cap" (SEEDS et al 2015). Among angiosperms, the cotyledonary petiole of the palm has a distinctive and multifunctional role in seed germination and seedling establishment (FERREIRA MOURA et al., 2019). The downward extension (positive geotropism) pushes the embryonic axis to various depths where the seedling develops away from the seed into the soil within the cotyledonary petiole during remote germination in palms (BASKIN; BASKIN, 2014).

After germination, the date palm (*Phoenix. dactylifera*) protects its meristematic tissues inside the growing cotyledonary petiole. It was discovered that the developing tip of the cotyledonary petiole contains such underdeveloped embryo´s meristematic tissues. This developmental embryonic delay is accompanied by a decreased rate of cell division, a decrease in the expression of crucial developmental genes, an accumulation of hormones linked to dormancy, as well as reactions to biotic and abiotic stimuli. It was astonishingly discovered that organogenesis takes place inside the cotyledonary petiole (XIAO, T. T. et al., 2019). In macaw palm (*Acrocomia aculeata*) seedlings, the formation of the vegetative axis was correlated with increases in salicylic acid (SA) and jasmonic acid (JA) concentrations and concluded that these hormones play a vital tissue-specific role in regulating germination, dormancy, and initial development (RIBEIRO et al., 2015). Through organogenic activity, the shoot apical meristem (SAM) achieves post-embryonic development. Apart from self-maintenance, it also maintains the formation and initiation of lateral organs by its continuous cell division and generates leaves, stems, and meristems of the axillary shoot (JOUANNIC et al., 2011). In *Acrocomia aculeata,* the apical meristem activity and increase in cell volume were observed to cause the growth of the embryonic axis (RIBEIRO; OLIVEIRA; GARCIA, 2012).

The growing seedling maintains its encapsulation and develops root, shoot, and leaf primordia that express genes exclusive to those organs. The leaf pokes through the surrounding

tissue as it continues to expand. The date palm's aboveground organs can grow in diameter and height because the shoot meristem generates new primordia that multiply and lengthen (XIAO, T. T. et al., 2019). It was observed in *Phoenix. roebelenii* that the interior portion of the primary roots seems to produce the secondary roots (IOSSI; VITTIMORO; SADER, 2006). The primary root begins to protrude near the end of germination, and this indicates that the embryo has successfully overcome the resistance of the tissues surrounding it (BEWLEY; BRADFORD; HILHORST, 2012; FINCH-SAVAGE; LEUBNER-METZGER, 2006). In *Euterpe precatoria*, 20 days after germination, euphyll differentiation and complete protrusion of the root were observed, along with complete protein reserve depletion (FERREIRA et al., 2020). Compared to those documented for other angiosperms, the cotyledon petiole functions during germination and seedling development in this family are more diverse (FERREIRA MOURA et al., 2019). There is little information known regarding the functions of that particular type of cotyledonary petiole in germination and early seedling development in palms (D. DIAS et al., 2017; MAZZOTTINI-DOS-SANTOS et al., 2017; RIBEIRO et al., 2015). Therefore, more morphoanatomical and proteomics studies are needed to explore the emergent organogenesis and role of cotyledonary petiole in palm species that show remote germination.

2.3 Proteomic studies in the Arecaceae family

Literature on proteomics of the family Arecaceae is scarce. Besides some of the contributions of our group on the proteomics analysis of *Euterpe. oleracea* pericarp of the developing fruit that identified 4286 proteins using quantitative proteomics based on iTRAQ labeling. The authors were able to associate the changes in protein abundance during açai berry development with particular metabolic processes. Key enzymes and secondary metabolites were identified, opening up new directions for study and potential uses in the agriculture and food sectors (ANDRADE et al., 2020). A study of the haustorium and endosperm proteome during germination identified 1965 proteins. The haustorium actively mobilizes endosperm reserves during germination, according to this study on the açai seed (*Euterpe oleracea* Mart.). Hydrolases implicated in reserve mobilization were discovered by proteomic analysis, indicating that they are produced during endosperm digestion and transported to the digestion zone (NASCIMENTO et al., 2020) and a recent study on proteomic changes associated with seed development of açai *Euterpe oleracea* Mart. in which 2465 proteins with high confidence were identified using the shot-gun proteomics technique. The study reveals the conversion of linear mannans into galactomannans during seed development and identifies important enzymes involved in the synthesis of nucleotide sugars. The results also provide insight into the

main process of flavonoid biosynthesis that produces catechin and epicatechin, the two major procyanidin components in açai seeds (FM NETO et al., 2023). There are some other studies such as in a study the coconut meat proteome was analyzed using shot-gun proteomics and identified 1686 proteins using databases from NCBI and the *Cocos. nucifera* transcriptome repository. The study also revealed that the globulins were discovered to be connected to the Cupin and Oleosin sub-networks, whilst the antioxidant proteins were connected to the glutathione metabolism and peroxisome sub-networks (MA et al., 2022). In another study, the cold response of two varieties of coconut leaf was analyzed using iTRAQ tagging and identified 2975 proteins using a combined dataset of *Cocos nucifera* and *Elaeis guineensis* sequences. For 2 days and 5 days after treatment, the proteome response of the varieties under cold stress (8 °C) was examined. The findings revealed that the two species of coconut had varied protein expression, with Hainan Tall (BD) showing a stronger up-regulation of stress-responsive proteins than Aromatic coconut (XS) (YANG et al., 2020). In a study on coconut protein, nine proteins were identified using MALDI-TOF/TOF-MS with a combined dataset of *Elaeis guineensis* and *Phoenix dactylifera*. With the aid of dialysate membrane, HPLC-UV, and MALDI-TOF/TOF-MS, two different 63 kDa globulin types were identified and isolated from coconut protein. These proteins predicted tertiary structures were found to resemble vicilin (LIN et al., 2020). A differential proteomic analysis of date palm (*Phoenix dactylifera*) leaves infested with the red palm weevil using two-dimensional differential gel electrophoresis (2D-DIGE) followed by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) identified 32 proteins involved in stress, defense and other important biological pathways which were found affected in the infested plant (RASOOL et al., 2022). A study on coconut milk (*Coco nucifera*) that combined a mass spectrometer LTQ-XL with an nLC system and a search of the Viridiplantae-UniProt database found 307 proteins. According to the study, infested plants had altered levels of proteins related to plant stress, defense, photosynthesis, glucose utilization, and protein breakdown. As indicators for early detection of red palm weevil infestation in date palm trees, the differentially expressed proteins can be used (D'AMATO; FASOLI; RIGHETTI, 2012). In another study on *Phoenix. dactylifera* the protein expression changes in leaves were evaluated under variable growth temperature and soil water deprivation by LC-MS/MS where the authors used a dataset of the genome of this species combined with a SwissProt dataset with sequences from the green plants database. The study revealed that the plant can withstand the harsh circumstances of the Arabian Peninsula by adjusting protein abundances associated with photosynthesis, abiotic stress, and secondary metabolism. Under hot summer conditions and water restriction, heat shock proteins and the antioxidant system are

essential in preventing the development of reactive oxygen species. Notably, PdIspS, the gene encoding the isoprene synthase from *P. dactylifera*, has been upregulated, suggesting a potential role for it in surviving harsh conditions (GHIRARDO et al., 2021). In a study on the identification of differentially expressed proteins during the somatic embryogenesis of *Elaeis. guineensis*, 538 proteins were identified using 2-DE (two-dimensional electrophoresis) MALDI-TOF-MS/MS using the NCBI protein database and viridiplantae taxa. Certain proteins were found as possible candidates for future biomarker development by examining the proteome changes that occurred during the acquisition of somatic embryogenesis. Notably, proteins such as fructokinase, the type IIIa membrane protein cp-wap13, and PR proteins have been linked to the development of pro-embryogenic calluses (SILVA et al., 2014). The haustorium and cotyledonary petiole of germinating *C. prunifera* seeds have not yet been the subject of any proteomic and morphoanatomical research. Determining the involvement of the haustorium in endospermic mobilization and investigating proteome alterations based on the morphoanatomical development throughout the early development of the cotyledonary petiole and embryonic axis were the goals of the current investigation.

2.4 Objectives

The general and specific objectives of the work are as follows.

2.4.1 General objectives

The general objective of the work was to Understand the spatiotemporal changes at the cellular and subcellular levels during the organogenesis in the carnauba palm (Copernicia prunifera) as a model to explain the biological function of the haustorium and cotyledonary petiole in Arecaceae using proteome and morphonatomical analyses.

2.4.2 Specific objectives

- Describing the morphoanatomical analysis of the development of the haustorium during the germination of *Copernicia prunifera;*

- Describing the morphoanatomical analysis of the development of cotyledonary petiole containing embryonic axis during the germination of *Copernicia prunifera;*

- Studying the dynamics of haustorium and cotyledonary petiole proteomes during the germination of *Copernicia prunifera*.

3 MATERIALS AND METHODS

The materials and methods adopted for this study are mentioned below in detail.

3.1 Plant materials

The mature fruits of *Copernicia prunifera*, identified by their dark purple color, were collected from plants grown under natural conditions in Fortaleza, Ceará, Brazil, between February and April 2020. The fruits were transported to the Department of Biochemistry and Molecular Biology at the UFC and sun-dried in a greenhouse. Next, the fruits were peeled with the aid of a hammer, taking care not to damage the seed inside. The seeds were initially weighed and dried in the sun, with regular monitoring of their weight every day until it was constant. Seeds weighing 2 g or more were selected for the experiments described below.

3.2 Seeds germination

Seeds were sterilized with 1% of NaClO for 15 min followed by three washes with distilled water for the same duration. Then the seeds were left for imbibition overnight in distilled water and sowed in the washed autoclaved sand in the perforated plastic tray while watered every day without exceeding the field capacity. Given that the germination of carnauba seeds occurs unevenly, some seeds may begin to show signs of germination (the germination button, which is the extension of the cotyledonary petiole brought on by rupturing the operculum) after 10 days, whereas other seeds may take up to 20 days after sowing to show the germination button. In this way, germination was monitored daily, and when the seeds met the criteria mentioned above, they were transferred to other trays containing a mixture of organic compost and washed sand in a ratio of 1:3. Where they remained until the days defined for tissue collection were completed.

3.3 Sample preparation for anatomy

The morphoanatomical modifications that occurred during germination and initial development of carnauba seedlings were evaluated in the haustorium (HU) and cotyledonary petiole (CP). The sample groups comprised the embryo from mature seeds (non-germinated) and whole CP from seedlings with 1, 2, 3, 4, 5, 6, 7, 10, 14, and 16 days after germination (DAG). For the morphoanatomical study, 10 seeds were collected daily after germination (emission of germination button), until the seeds showed their first cataphyll which is usually after 17-18 DAG. Then washed carefully with distilled water, and were fixed in fixative 1% glutaraldehyde; and 4% formaldehyde in phosphate buffer (KARNOVSKY, 1965). For initial

anatomy, the selected samples were (Mature, 1, 2, 3, 4, 5, 7, 10, 14, and 16 DAG) (Figure 5). The samples were kept in fixative under vacuum for ~48h. For the embedding process, samples were dehydrated in a crescent ethanolic series (10-20-30-40-50-60-70-80-90 and 100%, for two hours each). After de-hydration the material was pre-infiltrated in a mixture of glycol-methacrylate resin (Historesin, Leica) and alcohol (1:1) for not less than 5 days under vacuum followed by infiltration in pure resin for 5 days then the resin was changed to new resin and left for more 10 days. After infiltration the material was embedded in glycol-methacrylate resin and 5 µm thick cross sections were made in an automatic rotating microtome (Leica® RM 2065). Then, sections were sequentially stained with 0.05% toluidine blue in 0.12% borax and fuchsin basic 0.5-1% for ~1 min each. The images were visualized and recorded with a digital camera (HP Photosmart R967) attached to the eyepiece of an optical microscope (Leica® DM4000).

3.4 Collection and preparation of the sample for proteomic analysis

Based on the results of the morphoanatomical study, four developmental stages were selected to proceed with the study of the carnauba proteome, which went: mature embryo and seedling with two, five, and ten DAG (Figure 6). For each stage of development, the HU and the whole CP containing the embryonic axis were isolated, represented by three biological replicas (with a minimum of 50 seeds each). The collected samples were washed in ice-cold Milli-Q water, then cut with the help of a sterilized scalpel blade kept under refrigeration (-20 °C) and later lyophilized (LABCONCO, model 195). All the lyophilized samples were macerated until a fine powder was used for protein extraction. The smaller samples were macerated with the help of a pestle and motel while an electrical grinder was used for the later stages.

3.5 Proteins extraction

Proteins from each sample were extracted according to the modified protocol (Fei et al., 2021), with some modifications. According to this protocol, four mg of the sample plus 10% of PVPP (w/w) was mixed with the washing solution (1.6 ml 10%TCA-acetone) vortexed for 1 min and centrifuged for 5 min at 12,000 ×g on 4°C removed the supernatant by pipetting carefully to remove the residual TCA, 80% of methanol containing 0.1M ammonium acetate was added to the sample and vortexed for 1 min followed by centrifugation at 12,000×g for 5 min at 4°C. The supernatant was discarded and 80% of methanol was added to the pellet vertex for 1 min and 12,000 ×g centrifugation for 5 min. supernatant was removed carefully, each washing step was repeated three times before moving to the next step, the pellet was left in the

speed vac for 5 min to remove the residual acetone. Upon drying 1.5 ml of extraction buffer i.e., 1:1 phenol (pH 7.9) and SDS buffer (30% sucrose, 2% SDS, 0.1M Tris-HCl, pH 8.0, 5% β-mercaptoethanol and 1mM PMSF) were added mixed well and incubated for 5 min at 4°C on a shaker incubator, then centrifuged at 12,000 ×g for 10min at 4°C. The upper phenol layer was collected and 4-8 volumes of methanol containing 0.1M ammonium acetate and left for precipitation overnight at -30°C. After centrifugation, the phenolic supernatant was removed, and the pellet was washed three times with pre-cooled methanol and acetone respectively, the pellet was dried in the speed vac concentrator (Thermo Fisher SRF 110). Protein solubilization was performed in 7M urea/2M thiourea and quantification was performed using the Bradford method (BRADFORD, 1976) and the QuibtTM kit (Invitrogen) according to the manufacturer's instructions.

3.6 SDS-PAGE analysis

Polyacrylamide mini gels were prepared using the reagents composition shown in (Table 1). 15 µg proteins from each Stage were subject to 1-D gel electrophoresis using a Mini-PROTEAN® Tetra Cell vertical electrophoresis system (Bio-Rad).

Table 1- shows the composition of polyacrylamide resolving gel (15%) and stacking gel (5%) for SDS-PAGE.

Reagents	**Volume**
Resolving gel (15%) composition	
Acrylamide/Bisacrylamide (29.2%/0.8%)	2.5ml
Tris-HCL (pH: 8.8) 1.5M	1.25 ml
Sodium dodecyl sulfate (SD S) 10%	100.0 µl
H2O (Milli-Q)	1.2 ml
Ammonium persulfate (AP) 10%	100.0 µl
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	5.0 µl
Stacking gel (5.0%) composition	
Acrylamide/Bisacrylamide (29.2%/0.8%)	1.33 ml
Tris-HCL (pH: 6.8) 0.5M	2.5 ml
SDS 10%	50.0 µl
H2O (Milli-Q)	6.0 µl
AP 10%	50 µl
TEMED	5.0 µl

Source: Prepared by the author

The EPS 3501 XL power supply (GE Healthcare) was used. For this, protein samples contained in the tubes were solubilized in 60 µl of 7 M urea / 2 M thiourea and quantified by the Qubit assay (Invitrogen). Appropriate volumes containing 15 µg of proteins, from each sample, were separately mixed with the sample diluting buffer [Tris-HCL (0.125 M) pH: 6.8, SDS (4%), Glycerol (20%), Bromophenol blue, DTT (0.02M)] to a final volume of 20 µl. This solution was kept in boiling water for 5-10 min, brought to room temperature, and loaded on the gels. Gels were placed in the gel tank, containing running buffer (Tris 25mM, Glycine 192mM, SDS 0.1%). Finally, the Mini-PROTEAN Tetra Cell (Bio-Rad) assembly was connected to EPS 3501 XL Power Supply (GE Healthcare), and appropriate current (15A for stacking gel and 20A for resolving gel) was applied to run the mini gels. Once completed, gels were stained with Coomassie Brilliant Blue R-250.

3.7 Samples preparation for LC-MS/MS analysis

A total of 100 µg of protein from each sample was used for enzymatic hydrolysis in solution as described by (NASCIMENTO et al., 2020) with slight modifications. The proteins were reduced with 10 mM DTT for 1 h at 30 °C and alkylated with 40 mM iodoacetamide (IAA) for 30 min at room temperature and protected from light. Then the urea concentration was diluted, and the pH of the solution was raised to 8, using 50 mM ammonium bicarbonate in a 1:9 ratio (sample: ammonium bicarbonate). Subsequently, the proteins were digested with trypsin (Promega, Madison, WI, USA) for 18 hours at 37 ºC, in a 1:50 ratio (1 µg of trypsin for each 50 µg of protein). The trypsin reaction was stopped by the addition of 10% TFA until the pH of the solution was around 2.

For the desalination of the peptides, cleaning was performed in stage tips columns manually made in C18 Poros R2 resin (Applied Biosystems). The column was washed in 100% acetonitrile three times, then equilibrated in 0.1% TFA, adding the peptides to the equilibrated column in 0.1% TFA. The peptides were passed through the column 3 times, which was washed with 200 µl of 0.1% TFA to remove the salts and detergents contaminations and finally, the peptides were eluted in acetonitrile 0.1% TFA, 50% ACN 50 µl followed by 50 µl of 0.1% TFA 70% ACN. The eluted peptides were concentrated in a vacuum concentrator (Thermo Fisher SRF 110). The peptides were solubilized in 15 µl of 0.1% formic acid and were quantified again by QuibtTM kit (Invitrogen) to check the recovery of the peptides.

3.8 Peptides analysis by nLC-MS/MS

Aliquots corresponding to 6 µg of peptides from each sample were diluted to a final concentration of 0.5 $\mu g.\mu L^{-1}$ using 0.1% FA and analyzed in a nano LC-EASY 1000 system (Thermo Fisher Scientific) coupled online to an nESI-Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). Each biological replica was analyzed in duplicates four µL of the sample was loaded initially onto a C18 trap column (~2 cm length, 100 µm internal diameter, 5 µm particle diameter, ReproSil-Pur C18-AQ resin, Dr. Maisch GmbH; Ammerbuch, Germany) and separated in homemade C18 column (20 cm length, 75 µm internal diameter, 3 µm particle diameter, ReproSil-Pur C18-AQ resin, Dr. Maisch GmbH; Ammerbuch, Germany). The chromatographic separation took place for 120 min, under a flow rate of 200 $nL.min^{-1}$, following the following gradient method: from 5% to 30% of B for 100 min, from 30% to 45% of B for 9 min, ending with isocratic 95% B for 5 min. Phase A was composed of H_2O 95%, ACN 5%, FA 0.1%, while phase B was H_2O 5%, ACN 95%, FA 0.1%. The peptides

were ionized in an nESI source at 2.30 KV with positive polarity. The ion transfer capillary was held at 200 °C. The MS1 spectra comprised a full scan in the m/z range of 350.0 – 1800.0, a resolution of 60,000 FWHM (for m/z 400), and a minimum intensity threshold of 2000. Data were acquired in DDA mode and the 20 most intense precursor ions were fragmented by HCD for MS2 acquisition, with a resolution of 7,500 FWHM (for m/z 400), normalized CE (collision energy) 30 V, isolation window 2.5 m/z, and dynamic exclusion of 45 s.

3.9 Data analysis

The spectra files (raw format) were analyzed by the software Xcalibur v.2.1 (Thermo Scientific) for signal strength and reproducibility between technical and biological replicates. The spectral correspondence of the tryptic peptides to identify the proteins was performed against a database of~345,000 protein sequences, which was elaborated from three different databases, namely: all sequences of plant proteins of the family Aceraceae deposited at the UniProt (114,326 sequences) (https://www.uniprot.org/uniprotkb?query=arecaceae accessed July 2022), all *A. thaliana* protein sequences deposited in the UniProt database (136,783 sequences) and the translated sequences from a transcriptomic study of the açaí mesocarp (67,458 sequences), available at https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=GFLX01, as described by (NASCIMENTO et al 2020).

The search for spectra for protein identification used the Proteome Discoverer 2.5 v software (Thermo Scientific), with the Sequest algorithm to obtain PSMs (Peptide Spectrum Match) against the aforementioned data bands and the validator of PSM Target Decoy with a restricted FDR (False Discovery Rate) of 0.1 and an FDR reliability of 1% at the peptide level and less than 5% at the protein level. The identification and quantification approach by MBR (Match Between Runs), where the identification of peptides is considered even though it is not selected for fragmentation in a given sample. The MBR parameters were a signal-to-noise ratio of 5 and a retention time variation limit of 3 min. As a way to reduce false identifications, the cotyledonary samples were analyzed independently in the haustorium samples.

Reproducibility was verified by the Personal correlation between technical and biological replicates and the distribution of abundance values in the histogram. Only proteins detected in a minimum of two biological replicates of a sample that has been identified with at least one unique peptide for peptide identification were considered as identified for that sample and used for the other analysis. The gene ontology analysis for identified proteins was done by

using string tools (https://string-db.org/) whereas, the BLASTp tool with a maximum target sequence of 1 and an E-value cutoff of 0.0000001 was used for the functional annotation analysis against CAZy (Carbohydrate-Active Enzymes), WallProt (Cell Wall Proteins), MEROPS (Peptidases), and TCDB (Transporter Classification Database) databases.

3.10 Quantitative analysis

Differentially abundant proteins were identified using permutation-based ANOVA employing 250 randomizations and Tukey tests with 0.05 FDR value for both tests. The abundance values obtained with the Precursor Ion Quantifier node by Proteome Discoverer 2.5 were loaded into the Perseus software (version 1.6.14), transformed to a logarithmic scale (log2(x)), and grouped according to biological replicate and developmental stage. First, the average between technical replicates was made so that each sample presents only three abundance values corresponding to each biological replicate, these values were filtered to a minimum of 87% of valid values considering all the groups, normalized by subtracting the median per sample and applying the tests of statistical significance. MetaboAnalyst, a potent tool for data visualization and analysis in proteomics research, was used to plot the figures.

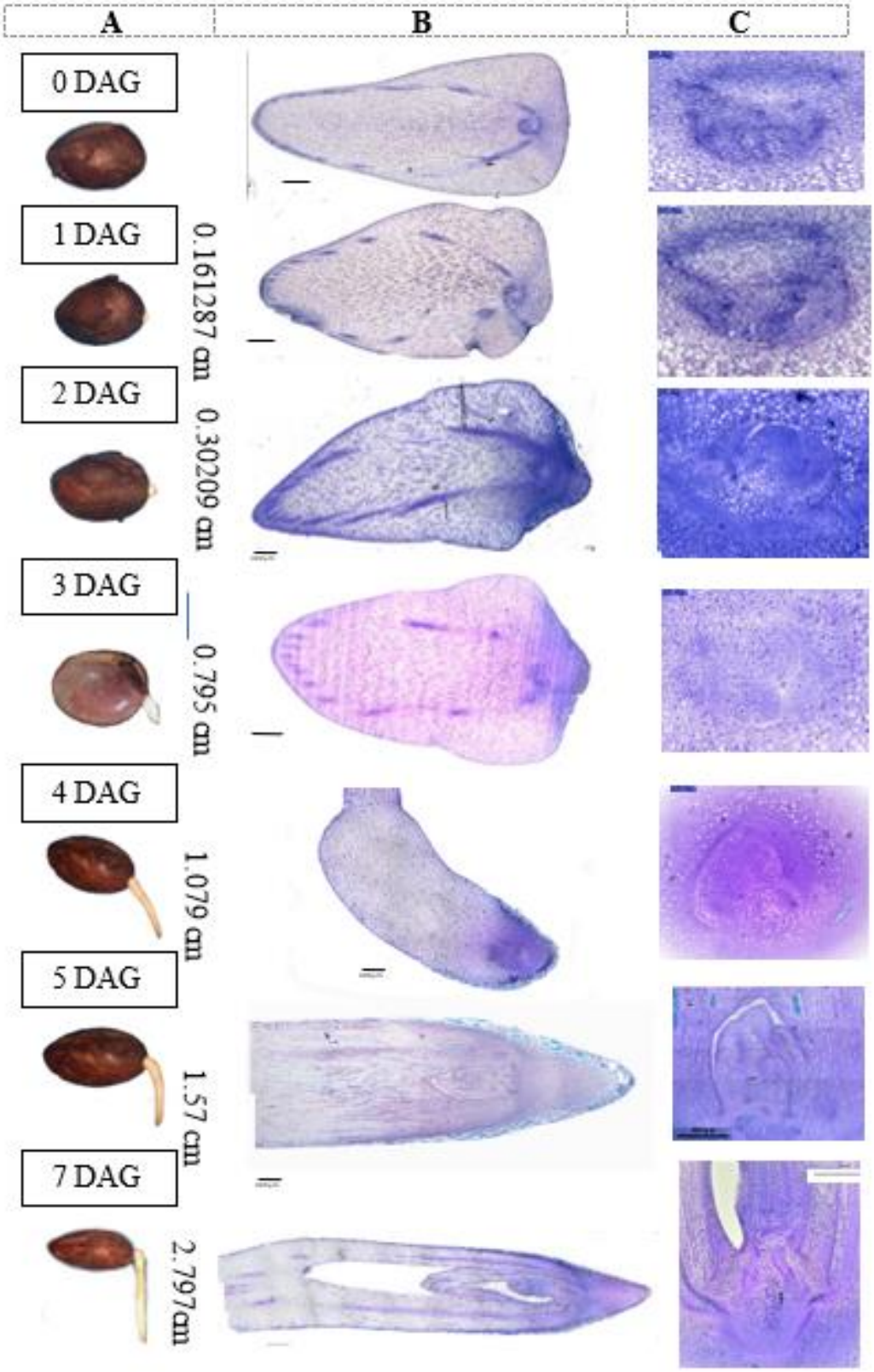
4 RESULTS AND DISCUSSION

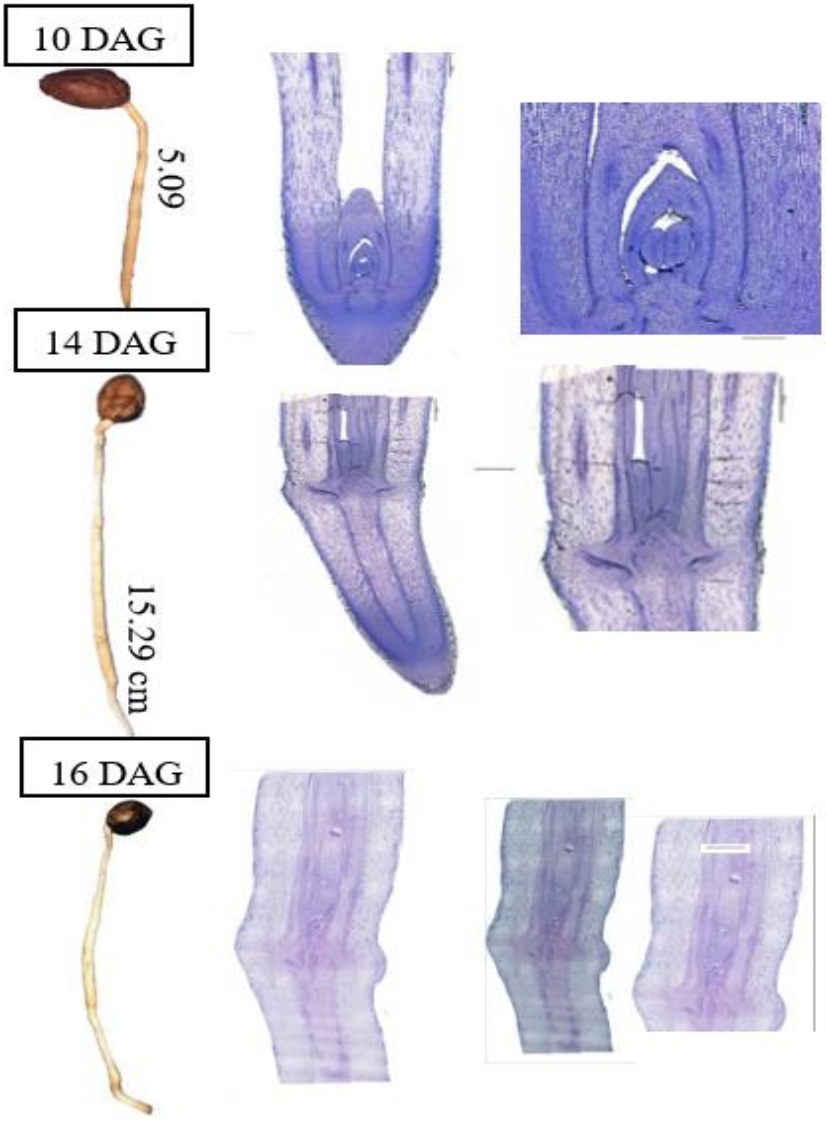
The morphoanatomical examination of the cotyledonary petiole and haustorium of the germinating *C. prunifera* seed constitutes the first section. To choose the stages that demonstrate significant structural alterations for subsequent proteomics analysis to discover the proteins involved in bringing about molecular changes, initially ten different stages were scrutinized for the morphoanatomical investigation.

4.1 Morphological and anatomical analysis of the seed development

The first part of this study consisted of the exploratory morphological and anatomical analysis of HU and CP of the mature, one, two, three, four, five, seven, ten, fourteen, and sixteen days after germination of *C. prunifera* seedlings (Figure 5). Four developmental stages were selected, which were representative of the main changes that occurred in these tissues, and the same stages were subjected to proteomics analysis (Figure 6). Germination started around 10 days after planting and reached 75% of seed germination at 20 days. The mature seed had a rigid consistency and a white, ruminated endosperm, surrounded by a dark brown integument. The embryo was held within the endosperm, in a compartment called the embryonic cavity, the embryo size is comparatively small concerning the seed size and in constant contact with the endosperm at the distal region and cotyledonary endosperm adjacent to the operculum at the proximal region. The embryo had a conical or cylindrical shape and could be divided into two regions, (i) cotyledonary haustorium (distal region), of light color and positioned more internally and in contact with the endosperm, and (ii) cotyledonary petiole, of darker color and positioned facing the operculum (proximal region) (Figure 7).

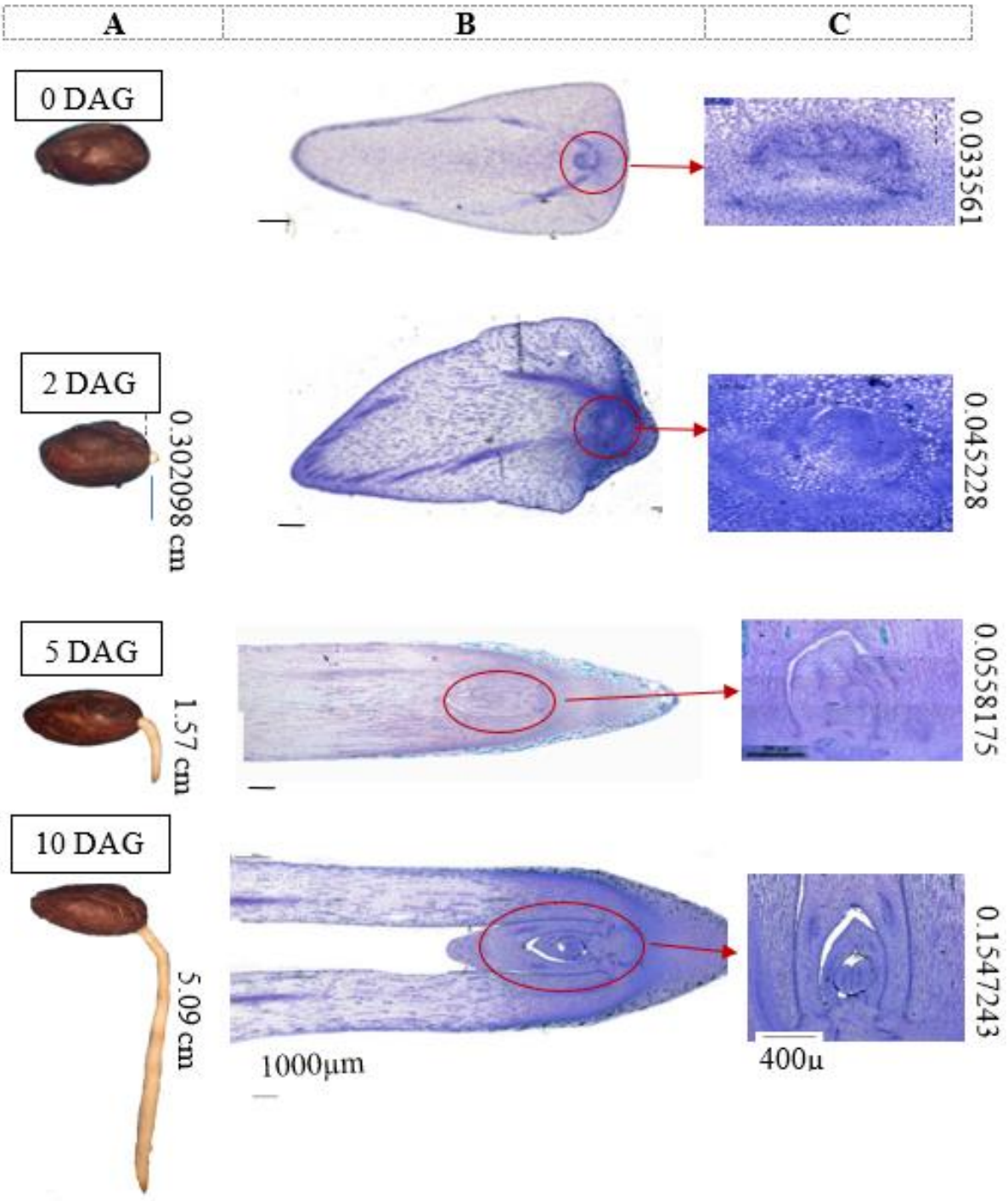
Figure 5- Morphoanatomical analysis of *C. prunifera* during seedling germination. Column (A) shows the seedling. Column (B) shows the morphoanatomical transverse sections of the complete seedling and the cotyledonary petiole 5 days after germination, and column (C) shows a magnification of the transverse section at the embryonic axis.





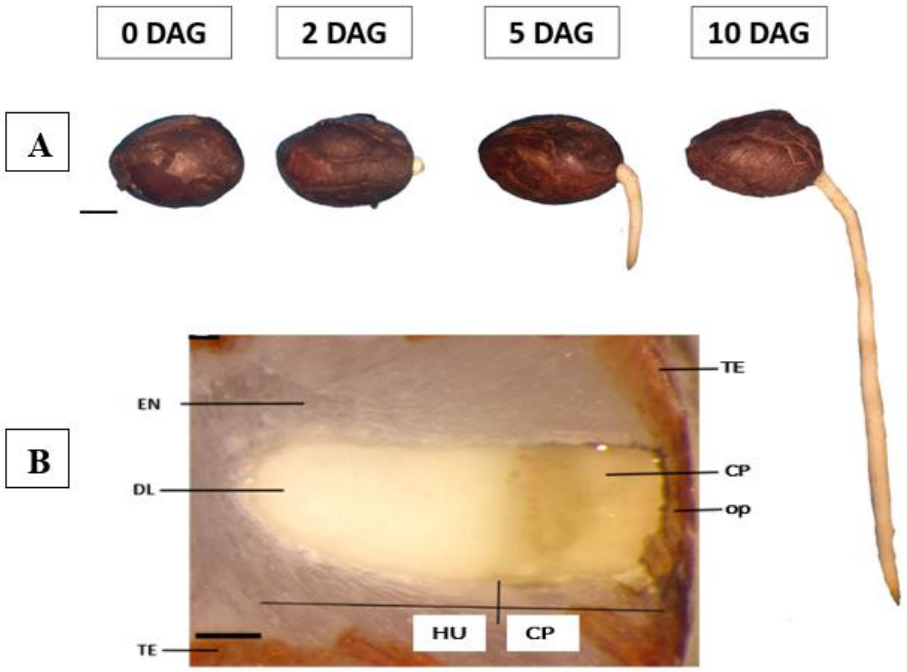
Source: Prepared by author.
DAG: Days after germination

Figure 6- Morphoanatomical results of the selected stages of *C. prunifera*. Selected based on major differences for further analysis, column (A) shows the seedling. Column (B) shows the morphoanatomical transverse sections of the complete seedling and the cotyledonary petiole after 5 days after germination and column (C) shows the zoomed transverse section of the embryonic axis. The numbers show the length in cm of the cotyledonary petiole and embryonic axis.



Source: Prepared by author
DAG: Days after germination

Figure 7- Developmental stages of germination of *C. prunifera* seed. (A) shows different developmental stages of germination of C. prunifera seed. (B) side view of the mature embryo inside the seed. CP, cotyledonary petiole; HU, cotyledonary haustorium; TE, integument, OP, operculum; EN, endosperm; DL, the distal region of the embryo. Scale, 0.5mm.



Source: Prepared by author.

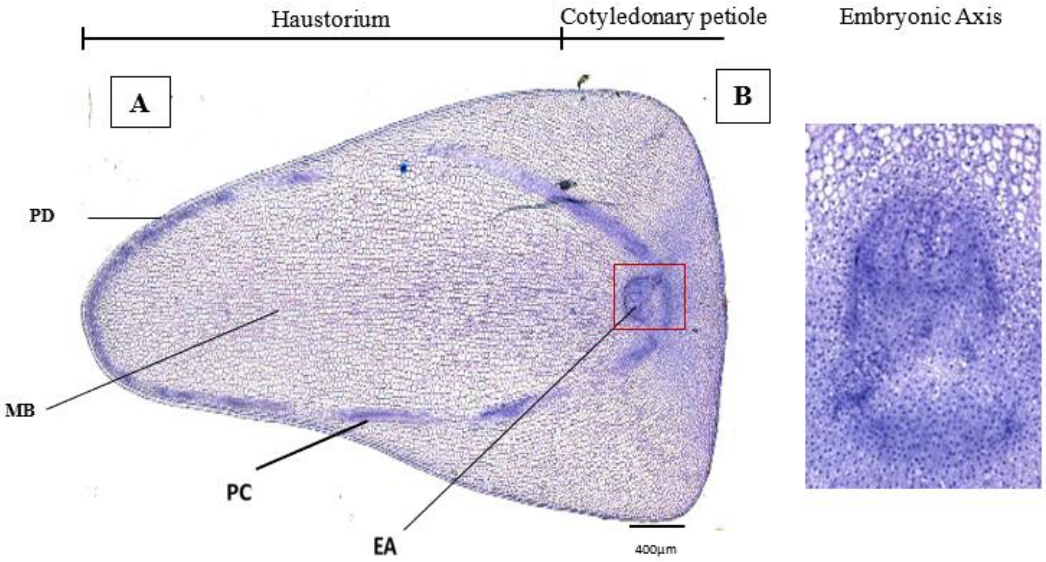
In summary, the morphological and anatomical examination of the *C. prunifera* seedlings at different developmental stages revealed important information on the structural alterations that take place during germination and the early stages of growth. To capture the main changes in these tissues, four typical stages were chosen (as per subtopic 4.1.1). Our understanding of the early stages of *C. prunifera* seedling growth and the spatial organization of various tissues within the seed is aided by these observations. The cotyledonary haustorium and cotyledonary petiole, two separate areas of the embryo, probably have a significant impact on how nutrients are distributed, how the embryo grows, and how it interacts with the surrounding endosperm.

4.1.1 Anatomical and morphological analysis of selected developmental stages

The cotyledonary petiole rose from the distal part of the mature embryo, whereas the haustorium derived from the proximal region as shown in the anatomical study. Procambium, ground meristem, and protoderm, each with unique cell morphologies, were all involved in the formation of the haustorium. The ground meristem cells were vacuolated and isodiametric, the protoderm cells were uniseriate and tabular, while the procambium cells were

thin, elongated, and densely stained. During haustorium development, the ground meristem cells in the core region significantly expanded (Figure 8). Near the cotyledonary node, the cotyledonary petiole portion also had densely packed cells. Germination began with the growth of the embryo against the operculum, which pressed the operculum until it opened and for the cotyledonary petiole protrusion. The cotyledonary petiole carried within itself the meristematic tissues of the embryonic axis while the cotyledonary lamina remained in contact with the endosperm. At 2 DAG it was possible to notice the development of the cotyledonary petiole and haustorium, the haustorium started taking up the space previously occupied by the endosperm, as well as the formation of a gelatinous layer in the portion of the endosperm adjacent to the haustorium (Figure 9). At 5 DAG the cotyledonary petiole continued to grow in length and haustorium continued to grow concomitantly with the consumption of the endosperm. At that stage, the establishment of the cataphyll leaf was more evident in the cotyledonary petiole (Figure 10). At 10 DAG there was a reasonable increase in the size of the haustorium, a good part of the endosperm had already been digested and the haustorium occupied more space in the seed (Figure 11). At that point, the cotyledonary petiole had the euphyll (first photosynthetic leaf) completely visible, indicating the initiation of the preparation for the photosynthetic independence of the seedling.

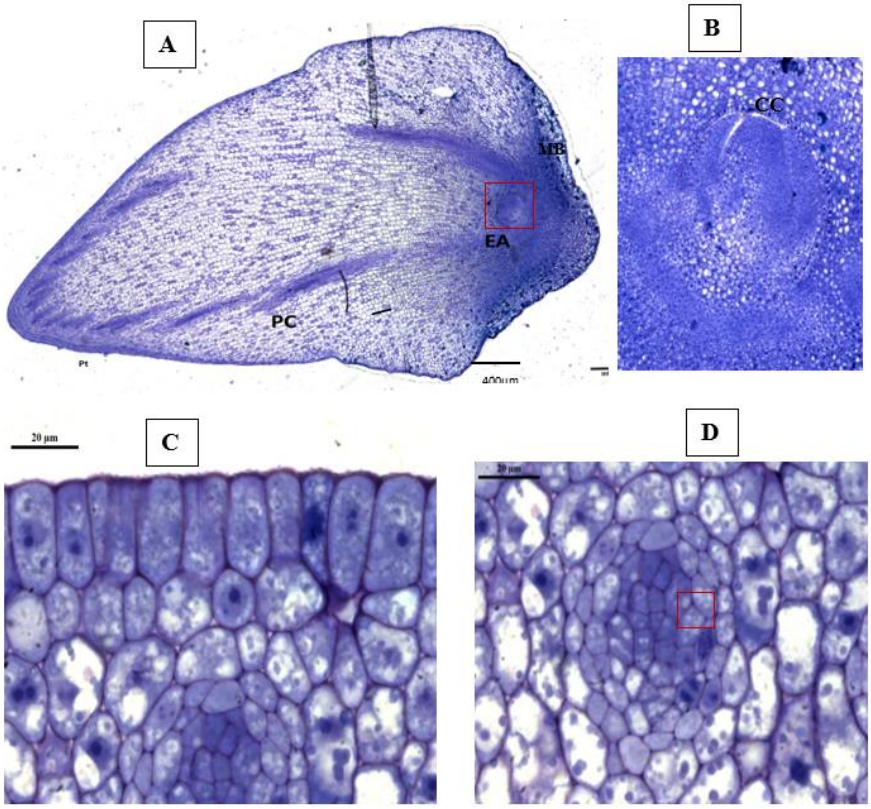
Figure 8- Longitudinal section of mature seed embryo of *C. prunifera*. (A) longitudinal section of the distal and proximal portions of *C. prunifera* mature seed embryo. (B) shows the detailed view of the embryonic axis in the red square. PC, procambium; MB, ground meristem; PD, protoderm; EA, embryonic axis; scale 0.4 mm.



Source: Prepared by author

The anatomical analysis showed that the cotyledonary petiole and haustorium were structures originated from the distal and proximal regions of the embryo respectively. The haustorium was formed by protoderm followed by procambium and ground meristem (Figure 8). These tissues were easily distinguished by the shape of the cells. In the protoderm, the cells were uniseriate, tabular shaped, and showed an evident nucleus. Procambium cells were narrower in comparison to other cells, elongated, and densely stained. Ground meristem cells were vacuolated, isodiametric, being the central region of the haustorium and smaller at the region closer to the procambium. It was the ground meristem cells of the central region that expanded during haustorium development (Figure 8). The cotyledonary petiole kept the embryonic axis composed of closely packed cells.

Figure 9- Longitudinal section of 2DAG embryo of *C. prunifera*. (A) longitudinal section of the distal and proximal portions of *C. prunifera* embryo two days after protrusion of germination button (2DAG). (B) shows the detailed view of the embryonic axis in the red region clearly showing the cotyledonary cavity. (C) showing the cell elongation in the distal haustoria region. (D) shows cell division in the red region and oil droplets. PC, procambium; MB, fundamental meristem; CC, Cotyledonary cavity; EA, embryonic axis; scale 0.4 mm and 0.02 mm.

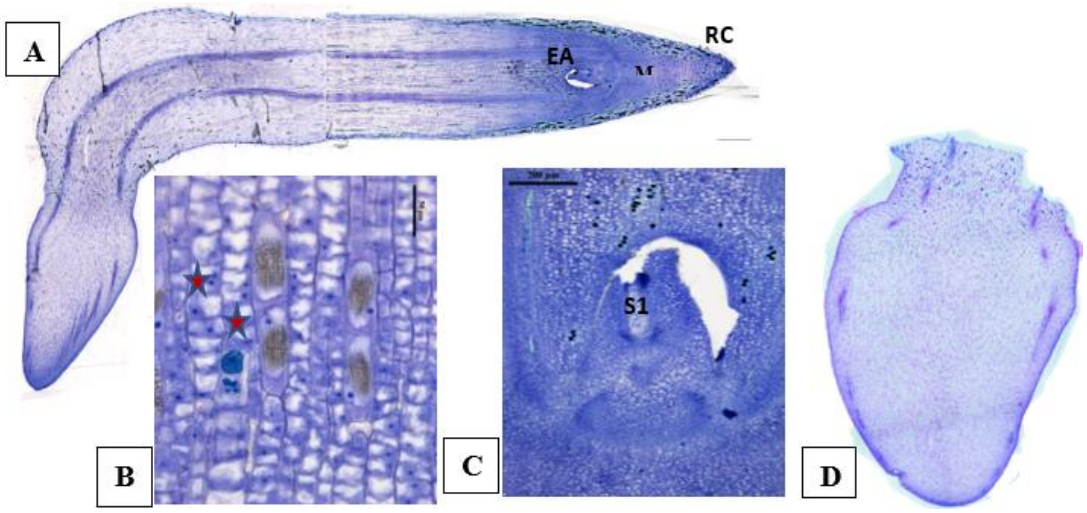


Source: Prepared by author

In the second stage, the emission of a germination button from the seed was evidence of the beginning of the germination process. The germination button emission was the

result of the cell elongation in the central region of the embryo whereas, cell division was observed in the embryonic axis and embryo as a whole. The cotyledonary cavity was also observed to appear as well as the beginning of the appearance of the root meristems and deposition of phenolic compounds in the root-cap-like structure of the cotyledonary petiole region of the developing embryo also appears in this stage. The haustoria region showed the appearance of oil droplets on the surface of the protoderm, which may be evidence of the initiation of absorption of reserves by haustorium from endosperm deposits. whereas cell elongation and division were also observed in the inner procambium region (Figure 9).

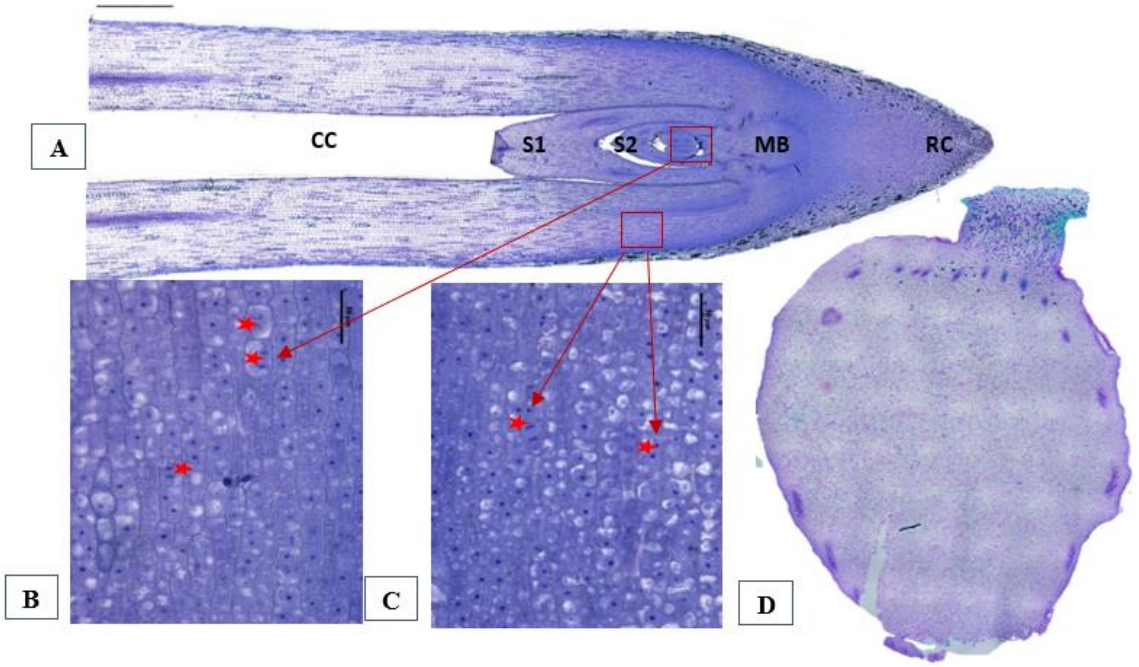
Figure 10- Longitudinal section of 5 days after germination cotyledonary petiole and haustorium of *C. prunifera*. (A) Longitudinal section of the distal and proximal portions of *C. prunifera* embryo five days after protrusion of germination button (5 days after germination). (B) the cell division and brown-colored raphides in the cotyledonary petiole region. (C) detailed view of the embryonic axis, clearly showing the cotyledonary cavity and leaf sheath. (D) the longitudinal section of a cotyledonary haustorium. MB, fundamental meristem; CC, Cotyledonary cavity; EA, embryonic axis; S1, first leaf sheath; Stars indicate cell division; scale 0.4 mm and 0.05 mm.



Source: Prepared by author

In the third stage, 5 days after germination (5 DAG), the cotyledonary petiole elongated and cell division, as well as cell elongation, were also observed in the embryonic axis, radicle, and cotyledonary petiole. The cotyledonary cavity increased in size and the first leaf sheath was also completely distinguishable, whereas raphides (calcium oxalate needle-shaped crystals) were also observed. The haustoria increased and became more globular shaped, the epidermal cells of the haustorium were highly active as indicated by the densely stained cytoplasm, and multi-nucleolated. The vascular bundle was observed from the haustorium to the root tip (Figure 10).

Figure 11- Longitudinal section of 10DAG cotyledonary petiole and haustorium of *C. prunifera.* (A) Longitudinal section of the distal and proximal portions of *C. prunifera* embryo ten days after protrusion of germination button (10DAG). (B) The cell division in the cotyledonary petiole region. (C) The detailed view and cell division in the embryonic axis (leaf sheaths). (D) The longitudinal section of the cotyledonary haustorium. MB, fundamental meristem; CC, Cotyledonary cavity; S1, first leaf sheath; S2, second leaf sheath; Stars indicate cell division; scale 0.4 mm and 0.05 mm.



Source: Prepared by author

The fourth stage which, 10 days after germination (10 DAG), showed the continuous elongation of the cotyledonary petiole whereas the embryonic axis stopped further descending movement within the cotyledonary petiole and continued increasing its size along with a huge increase in the size of the cotyledonary cavity, the appearance of roots meristem becomes more prominent. At that stage, the embryonic axis showed multiple leaf sheaths as well as cell division and elongation. The haustorium continued enlarging and consumed more than half of the endosperm. The densely stained epidermal cells were an indication of the high activity rate of such cells. The columnar shape displayed by the epidermal cells, similar to a secretory palisade, also indicated that such cells are involved in the secretion of substances. At that stage, the haustorium occupied more than half of the seed volume (Figure 11).

In summary, the cotyledonary petiole and haustorium comprised two distinct structures at the morphoanatomical level when studied during the growing embryo. The protoderm, procambium, and ground meristem tissues that made up the haustorium developed at the proximal area. The distal portion gave rise to the cotyledonary petiole, which had an

embryonic axis with tightly packed cells. Cell division in the embryonic axis and cell elongation in the center of the embryo indicated the beginning of germination while the embryonic axis showed continuous growth inside the cotyledonary petiole. Oil droplets on the protoderm in the haustorium indicated that the endosperm reserve had been absorbed. The cotyledonary petiole elongated, the cotyledonary cavity became larger, and the meristem making up the radicle became easier to distinguish with time. As the haustorium enlarged endosperm was consumed as interpreted upon the observation of the epidermal cells.

4.2 Anatomical and morphological findings

Key insights were gained from the anatomical and morphological examination of *C. prunifera's* developmental stages. The mature embryo's proximal region gave rise to the cotyledonary petiole, while the distal region developed into the cotyledonary haustorium. Procambium differentiation and enlargement of protoderm cells both took place in the development of the haustorium. The procambium and ground meristem cell division were both responsible for the haustorium's growth. The endosperm's digested reserves were easier to absorb when the haustorium presented various villi-shaped outgrowth and smooth surfaces. Vascular bundles were observed connecting both the haustorium and embryonic axis, facilitating reserve mobilization. The cotyledonary petiole served as a tube holding the embryonic axis during the remote tubular mode of germination found for *C. prunifera*. Along with roots, eophyll, shoot meristems, and leaf primordia, continuous organogenesis also took place. The embryonic axis left the seed, that is, was carried away from within the seed as the cotyledonary petiole developed and was exposed to the outer side of the seeds. The first leaf appeared, and the cotyledonary petiole had its quantitative proteome analyzed to understand plant stored reserves and mobilization.

The anatomical and morphological analysis of selected developmental stages of *C. prunifera* indicates that the proximal densely stained region of the mature embryo gave rise to the cotyledonary petiole whereas the distal lightly stained region of the embryo gave rise to cotyledonary haustorium. The outermost single-layered, compactly arranged, densely stained, uniseriate protoderm during development becomes elongated cells with an evident nucleus along with procambium which during development become elongated, multi-layered, and differentiated constitute the haustoria structure. During development, it was also observed that cell division in the ground meristem cells found towards its central region may probably cause the expansion of haustorium. However, some cell divisions were also observed in the protoderm. The haustorium had an oval shape structure with one end extended towards the

adjacent endosperm until 5th day after germination, after this, it assumed an undulating structure with a smooth surface entangling the endosperm which may have facilitated the absorption of digested reserves as mentioned by (VERDEIL; HOCHER, 2002). The presence of a vascular bundle from the haustorium towards the embryonic axis concurs with the role of the haustorium in the mobilization of reserves from the endosperm to the growing embryonic axis. The smooth surface of the haustorium implies that the endosperm, which is trapped within it, undergoes degradation through the release of digestive enzymes from the haustorium, which is then absorbed by the haustorium, and protect the direct entrance of digested zone into the haustorium, the morphoanatomical analysis of the current study corroborates the findings presented by Dias et al. (DIAS et al., 2020). The current study will undertake quantitative proteomics analysis only on the developing haustorium while guaranteeing no contamination from the endosperm to evaluate the specificity and accuracy of the hydrolyzing enzyme quantification and identification process. The molecular foundation of our suggested theory is further strengthened by our proteomic investigation of the respective tissues.

The anatomical and morphological analysis revealed that *C. prunifera* germinates by remote tubular mode, which was confirmed by monitoring germination from mature seed to seedling. This shows the emergence of cotyledonary petiole from seed making a long tube holding the embryonic axis inside. It was also confirmed that organogenesis occurs inside the cotyledonary petiole. Unlike date palm in which the growth of the embryonic axis remains paused in the initial stages after germination (XIAO, T. T. et al., 2019) in the current study morphological and anatomical changes such as development and appearance of leaf primordia, cataphyll, euphylls, roots, and shoot meristems were continuously observed from the beginning of germination. A continuous cell division was noticed in the embryonic axis and the surrounding layers along with cell elongation in the cotyledonary petiole which takes away the embryonic axis from the seed during the whole germination process. The developing embryonic axis moves along the cotyledonary petiole and stops its movement after 10 days of germination burying deeply the newly growing organs and meristems. The dark-colored starch granules in the cotyledonary tip were observed along with auxins that play a role in to direct response of the root to gravity (SABATINI et al., 1999; OTTENSCHLÄGER et al., 2003). The continuous growth of leaf and root meristems gives rise to primary roots and the eophyll is protruded out of the soil through enlargement of the cotyledonary cavity which causes rupture of the cotyledonary petiole, such a phenomenon is also observed in the *P. dactylifera* (XIAO, T. T. et al., 2019). Until the protrusion of the first leaf above the soil for photosynthetic independency

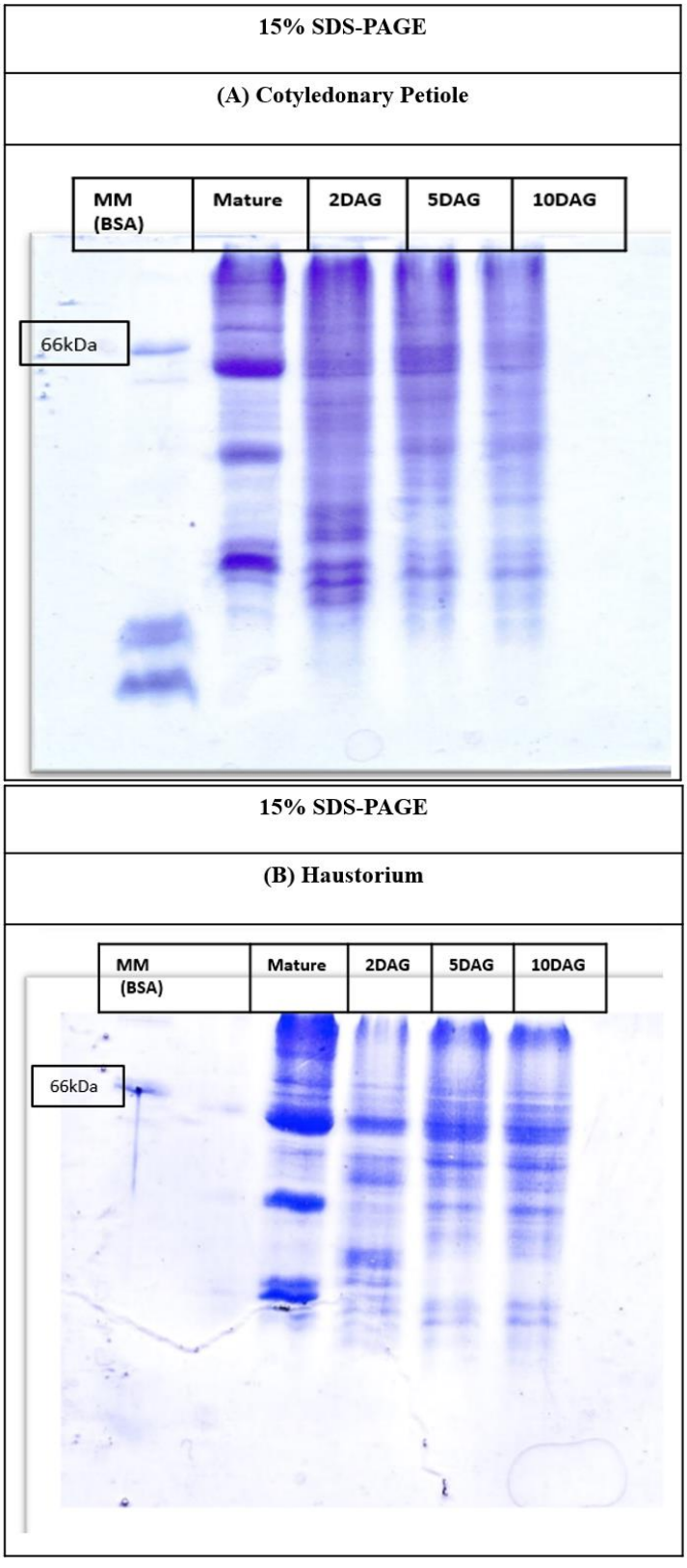
the growing cotyledonary petiole and embryonic axis are connected with the seed utilizing the endosperm storages. For the understanding of storage utilization and mobilization and the factors that are involved in such a unique developmental process, quantitative proteomic analysis was done for the selected stages of cotyledonary petiole containing a growing embryonic axis and haustorium. This will not only provide fundamental knowledge for the biologically important plant *C. prunifera* but also for the family Arecaceae where most of the plants germinate remotely.

In conclusion, the morphological and anatomical examination of the developmental stages of *C. prunifera* sheds light on the formation and structure of the cotyledonary petiole and haustorium. Procambium differentiates and protoderm cells elongate during the development of the haustorium, which contributes to its growth. The haustorium's undulating shape and smooth surface make it easier for the body to absorb the endosperm's digested reserves. Vascular bundles confirm their function in mobilizing reserves. In *C. prunifera*, germination proceeds in a remote tubular pattern, with ongoing organogenesis and cotyledonary petiole lengthening bringing the embryonic axis further from the seed. The cotyledonary petiole's quantitative proteomic analysis provides insight into the use and mobilization of storage.

4.3 1D-SDS-PAGE results

On a 15 percent SDS-PAGE gel, 15 µg of proteins from four stages of the two selected tissues, the haustorium and cotyledonary petiole, were separated. By confirming the extraction technique and the fact that the bands of the majority of the samples were distinct from one another, this methodology corroborated the anatomical investigation that these stages varied in their proteome (Figure 12).

Figure 12- Shows the image of 1D-SDS-PAGE for the selected samples of haustorium and cotyledonary petiole. (A) shows the band pattern of selected stages of cotyledonary petiole. (B) shows the band pattern of selected stages of haustorium. S; standard, MCP; cotyledonary petiole of mature seed embryo; HUM; haustorium of mature seed embryo; HU; haustorium; CP; cotyledonary petiole. The red circle shows the bands which disappear in further stages.



Source: Prepared by author

(Figure 12) demonstrates that some distinct bands in mature stages around about 70kDa are not visible in the other stages, which is an indication that these bands may be storage proteins that are digested along with development either for energy purposes or to synthesize new proteins. Additionally, protein profile changes among the stages. Additional analysis by nLC-MS/MS was conducted to identify these proteins.

4.4 Proteins identification

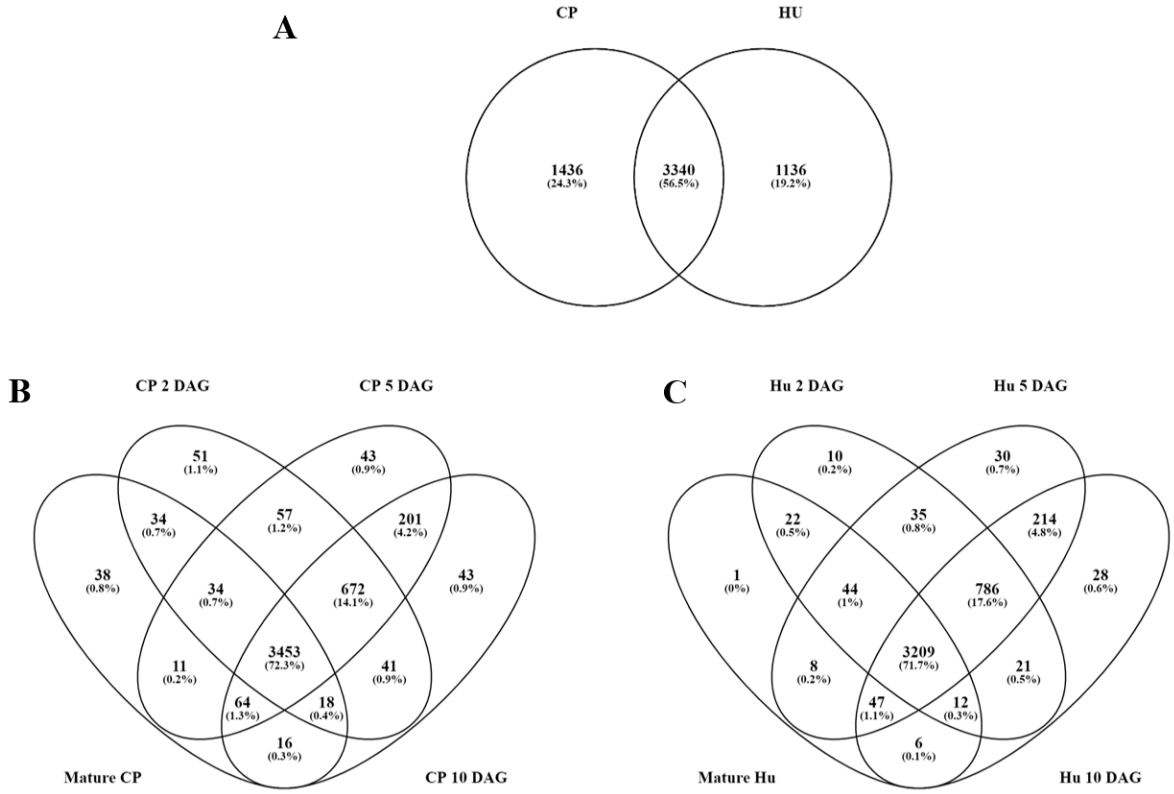
Our analysis led to the identification of a total of 5912 proteins in both tissues, taking into account the presence of a protein in at least two of three biological replicates and one of two technical replicates. Of these, 4776 proteins were found in the chosen stages of the cotyledonary petiole (Supplementary Table I HU, and Supplementary Table III CP). The cotyledonary petiole and haustorium share 3340 proteins altogether. Whereas 1136 and 1436 proteins were exclusive to haustorium and cotyledonary petiole respectively (Table 2 HU, Figure 13A).

Table 2- Shows the number of identified proteins in all the stages of haustorium and cotyledonary petiole.

Tissues	**Cotyledonary Petiole**				**Haustorium**			
Stages	Mature	2 DAG	5 DAG	10 DAG	Mature	2 DAG	5 DAG	10 DAG
Identified Proteins	3668	4360	4535	4508	3349	4139	4373	4323
Total identified proteins in each tissue		**4776**					**4476**	
Total Proteins				**5912**				

Source: Prepared by author

Figure 13- Venn diagram showing the number of unique and shared proteins in selected tissues and their stages. (A) Venn diagram showing the number of unique and shared protein identifications for cotyledonary petiole and haustorium during germination. (B) shows the unique and shared number of identification of proteins in selected stages for cotyledonary petiole and (C) shows the unique and shared number of identification of proteins in selected stages for haustorium.



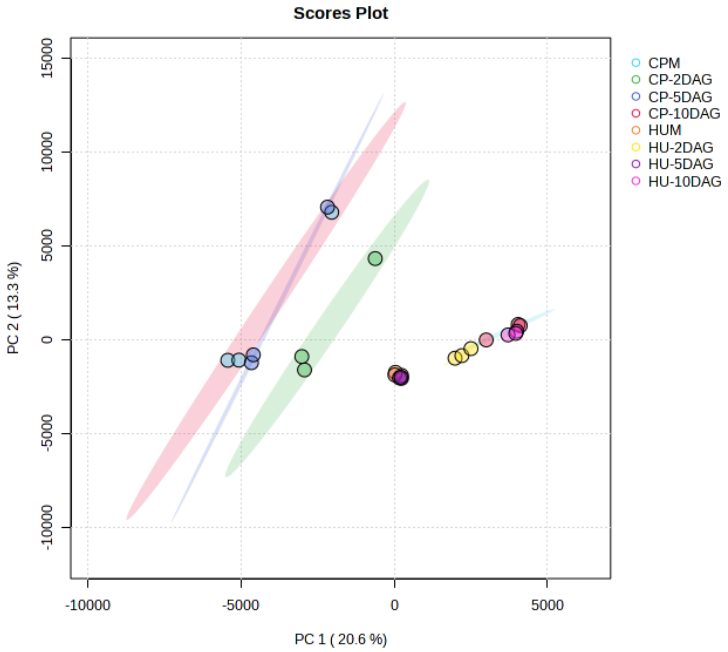
Source: Prepared by author

In conclusion, a significant number of proteins were identified in both the cotyledonary petiole and the haustorium as a result of the study performed using Proteome Discoverer 2.5 and nLC-MS/MS. A total of 5912 proteins were discovered, of which 4776 were only present in specific cotyledonary petiole stages. The 3340 proteins that are shared by the cotyledonary petiole and haustorium suggest that these tissues may have functional ties thanks to their similar protein profiles. 1136 proteins were also discovered to be unique to the haustorium. According to the findings in (Figures 13 B and C), each stage contains its unique proteins in addition to the proteins that are shared by all of the stages, which supports the specificity of these stages. In addition, new proteins are synthesized throughout the entire developmental process.

4.4.1 Principal component analysis of the proteins identified in HU and CP

The Principal Component Analysis (PCA) was implemented using an online tool (https://www.metaboanalyst.ca/) to assess the abundance patterns of identified proteins across all selected stages of haustorium and cotyledonary petiole. Using a significance threshold of FDR 0.05 and p-value < 0.05. The resulting two-dimensional PCA plot, illustrated in (Figure 14), visually represents data patterns. Notably, within both cotyledonary petiole and haustorium, groupings of samples reveal shared abundance trends across developmental stages in both tissues. On the other hand, the clear differentiation between some of the cotyledonary petiole and haustorium groups highlights tissue-specific proteomic differences throughout germination, emphasizing their unique roles.

Figure 14- Shows the principal component analysis (PCA) of all three biological replicates of the selected developmental stages of haustorium and cotyledonary petiole. HM: Mature haustorium; CPM: Mature cotyledonary petiole; DAG: Days after germination.



Source: Prepared by author

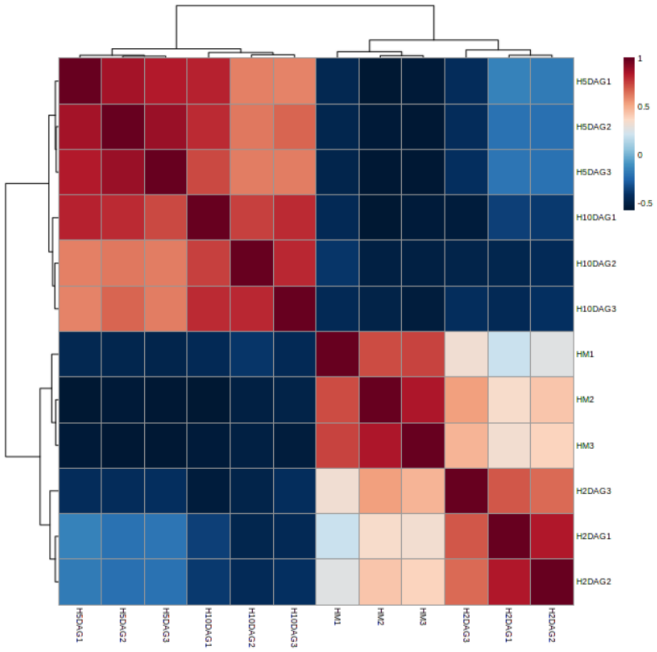
Most of the cotyledonary petiole and haustorium developmental stages showed a general tendency of overlapping data points, indicating some degree of similarity in their proteomes. However, the cotyledonary petiole on the second day after germination (2DAG), which showed clear separation from the other groups, was a striking exception. As seen in this particular location on the PCA plot, the cotyledonary petiole proteome at the 2DAG stage differs significantly from the other developmental stages, possibly indicating particular physiological and structural adaptations during this significant stage of germination. This

unique difference increases the possibility of molecular modifications or regulatory systems that could be vital in guiding the carnauba palm through this critical stage of germination.

4.5 Proteomic analysis of haustorium

The reproducibility of the results was examined using Pearson correlation values. Since all of the values were close to 1, the results were repeatable. A histogram was employed to examine how normalized abundance values were distributed among biological replicates and stages. The specifics are displayed in the following (Figure 15), and the table is provided in the supplemental material (Supplementary Table I).

Figure 15- Shows the Pearson correlation heatmap of different developmental stages and their biological replicates of the haustorium of *C. prunifera*. The correlation values between ±1 are considered to be strong correlations.



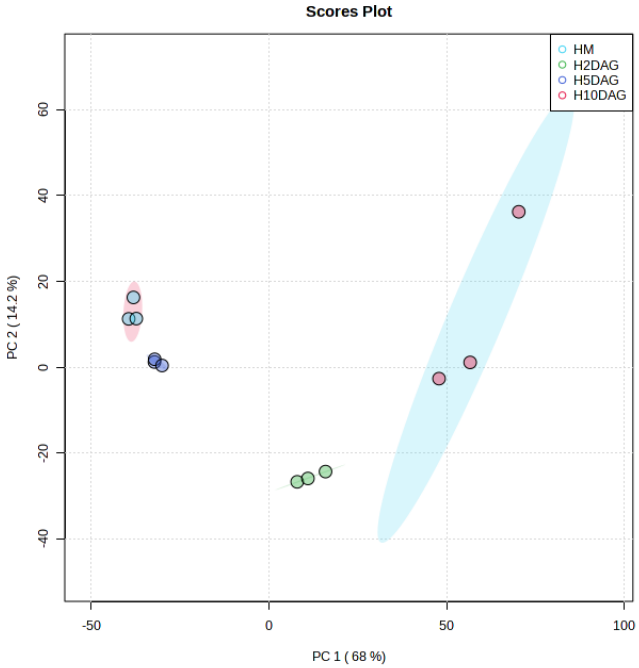
Source: Prepared by author

For biological replicates, the Pearson correlation heatmap revealed strong correlation values close to one, which denotes reproducibility, experimental precision, little biological or technical variability, and well-controlled experimental settings. This indicates that the duplicates' data points are remarkably comparable to one another and have a tendency to change together. Additionally, it guarantees that the methods for sample handling, data analysis, and experimental design were properly thought out to reduce any potential sources of bias that can unintentionally inflate the correlation results.

4.5.1 Principal component analysis of the proteins identified in haustorium

A principal component analysis (PCA) was used, which uses all the identified proteins in all samples and groups the samples in a two-dimensional graph according to similarities in abundance using FDR 0.05 and p-value < 0.05. PCA for different stages of haustorium showed similarities among the group members whereas the groups are distinct from each other (Figure 16).

Figure 16- Shows the principal component analysis (PCA) of all three biological replicates of the selected developmental stages of haustorium. HM: Mature haustorium, DAG: Days after germination



Source: Prepared by author

In Figure 16, the group members are clustered together while the groups themselves are separated, indicating that there is significant variation between the different developmental groups; however, within each group, the protein expression patterns are relatively similar. The separation of the groups in the PCA plot suggests that there are distinct proteomic profiles associated with each developmental group. The proteins identified within each group may exhibit specific expression patterns or abundance levels that differentiate them from proteins in other groups. This indicates that the developmental stage has a substantial impact on the proteomic profile. The tight clustering of group members within each developmental group suggests that the protein expression patterns within the group are relatively homogeneous. The proteins identified within a particular developmental group exhibit similar expression patterns or abundance levels, indicating consistency in the biological replicates. The variance explained

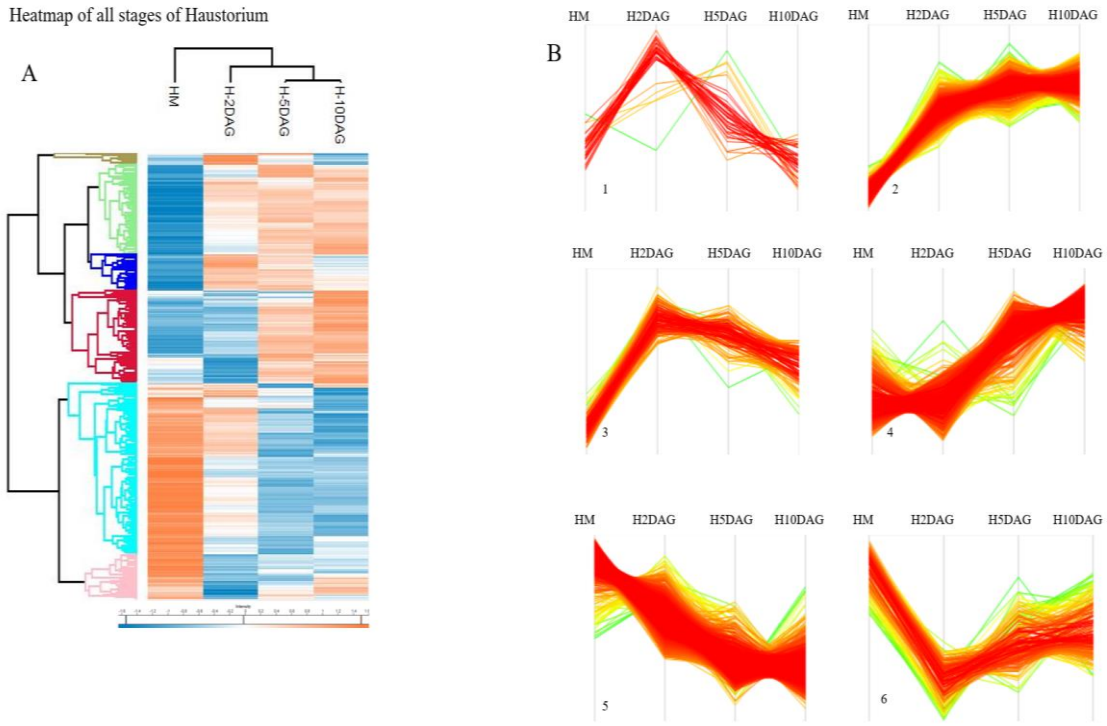
by PC1 (68%) being significantly higher than that explained by PC2 (14.2%) indicates that the majority of the variation in the dataset is captured by the first principal component. PC1 likely represents the major source of variation that distinguishes the different developmental groups from each other. PC2, although explaining less variance, still contributes to the observed separation between groups, but to a lesser extent.

In conclusion, significant differences between various developmental groups may be seen in the analysis of protein expression patterns using PCA. The different grouping of group members and the division of the groups themselves imply that each developmental stage is associated with a certain proteomic profile. The similarities between the protein expression patterns within each group point to group homogeneity. Within a developmental group, there is stability in expression patterns and abundance levels, which supports biological replication. The large variance explained by PC1 (68%) compared to PC2 (14.2%) shows that PC1 can account for the majority of the variation that distinguishes the developmental groups. The observed separation between groups is nevertheless influenced by PC2, albeit to a lesser level.

4.5.2 Analysis of differentially abundant proteins in haustorium

Heatmaps provide a condensed and visual representation of the abundance patterns of differentially expressed proteins, allowing for the identification of distinct clusters and patterns within the dataset. The results of our analysis focused on identifying and visualizing the differential abundance of proteins across the selected developmental stages of the haustorium of *C. prunifera*. The analysis aimed to gain insights into the proteomic changes associated with developing haustorium. Permutation-based ANOVA test with FDR ≤ 5% was used for the quantitative analysis of four developmental stages of haustorium. A total of 1683 proteins were identified as differentially abundant which were grouped in 6 clusters according to their abundance profile in all the selected stages (Figure 17 & Supplementary Table I).

Figure 17- Representation of differentially abundant proteins in developmental stages of haustorium of *Copernicia prunifera* during germination. (A) heatmap of differentially abundant proteins (B) clusters of proteins with similar abundance profiles.



Source: Prepared by author

Cluster one (Supplementary Table V), has 41 proteins identified as differentially abundant which show an increase from the mature stage to the second stage i.e., HU-2DAG, and then started decreasing during germination in the other two stages. In this cluster, nine proteins were involved in carbohydrate metabolism based on the CAZy database. Five proteins were matched in WallProt DB, three peptidases were also identified according to MEROPS DB, and 12 transporters were also identified in this cluster which showed a match with TCDB. Attention was given to Glucomannan 4-beta-mannosyltransferase 2 (*Q9FNI7* CAZy) which is mannose synthase using GDP-mannose as substrate (XU et al., 2021).

The proteins of cluster one were classified into 11 categories of GO biological process (GOBP) annotation, six categories for GO molecular function (GOMF), and 17 categories for cellular component (Supplementary Table Va). A strong emphasis on cellular processes related to protein synthesis and nucleic acid metabolism during germination is suggested by the enrichment of GO terms such as cellular nitrogen compound metabolic process, gene expression, nucleobase-containing compound metabolic process, nucleic acid metabolic process, RNA processing, and ribosome biogenesis. This trend is consistent with the

hypothesis that proteins are among the first molecules to mobilize during germination because they are so important for a variety of cellular functions, such as the mobilization of reserves and seedling growth. To prepare for protein synthesis, the haustorium may activate genes linked to RNA processing, gene expression, and ribosome biogenesis during the first rise in cluster members. Consequently, the sudden drop in cluster members suggests a shift in emphasis toward active reserves mobilization and growth, which calls for the upregulation of metabolic activities involved in converting reserves into vital nutrients for the growing seedling and the upregulation of certain processes related to gene expression.

The importance of binding proteins is shown by the enriched GO function terms in this cluster. These proteins are essential for controlling protein synthesis and gene expression. The terms RNA binding and mRNA binding denote the presence of particular binding proteins that regulate the stability and translation of RNA molecules, both of which are critical for the effective mobilization of reserves and seedling development. The existence of nucleic acid binding terms also suggests interactions with DNA and RNA that help to regulate gene expression as a whole. These binding activities in the haustorium work together to coordinate the molecular procedures necessary for the successful germination and mobilization of reserves in carnauba plants.

In GO cellular component terms, the diversity of intracellular anatomical components during germination demonstrates its complexity and adaptability. The existence of intracellular membrane-bounded and non-membrane-bounded organelles reveals a variety of cellular compartments, each of which contributes to the mobilization of reserves and seedling growth in a different way. The nucleus, the pivotal point of cellular activity and the site of gene expression and DNA control are particularly significant. The existence of complexes involving proteins and ribonucleoproteins emphasizes the importance of macromolecular assemblies in coordinating multiple cellular processes necessary for effective germination.

Cluster two (Supplementary Table V), has 339 proteins with increasing abundance from mature to 10DAG, these are classified into 66 carbohydrates active enzymes (CAZy DB), 38 cell wall (WallProt DB), 38 peptidases (MEROPS DB) and transporter proteins (TCDB) 105 proteins were identified. The identification of glyoxylate/hydroxy pyruvate reductase HPR3 (*A0A8B9A0W2*) is the indication of activation and breakdown of fatty acids in glyoxysomes. In cluster three a total of 133 proteins were identified as differentially abundant proteins which shows an initial increase in abundance along the development. Among these 24 proteins were

identified as CAZy, 11 as WallProt, 19 peptidases (MEROPS DB), and 33 transporters were identified using TCDB.

The proteins of cluster two were classified into 126 categories of GO biological process (GOBP) annotation, 54 categories for GO molecular function (GOMF), and 75 categories for cellular components (Supplementary Table V b). The terms metabolic process, organic substance metabolism, nitrogen compound metabolism, biosynthetic process, and small molecule metabolism suggest active biochemical processes for breaking down and synthesizing molecules in the GO process. Since these activities involve the breakdown of complex molecules like lipids, carbohydrates, and proteins to liberate energy and provide the building blocks for growth, they most likely play a key role in the mobilization of reserves. The use of transport and localization terms emphasizes the molecules' constant mobility throughout the haustorium. The distribution of vital nutrients for seedling growth, the transportation of hydrolyses from the haustorium to the endosperm, and most likely the transportation of reserves from storage sites to sites of consumption all depend on this transport.

The enriched GO function terms provide insight into the molecular processes essential for mobilizing reserves and promoting seedling growth. The existence of hydrolase and catalytic activity indicates that proteins may interact with molecules and catalyze biological events, potentially converting complex reserves into simple forms. Purine ribonucleoside triphosphate binding, nucleotide binding, and nucleotide binding all point to involvement in nucleotide metabolism, which is crucial for chemical synthesis and energy transfer. Carbohydrate derivative binding denotes interactions with molecules involved in the metabolism of carbohydrates and cellular homeostasis.

The enriched GO cellular component terms in cluster two reveal an increase in abundance, and they provide insight into the subcellular organization and localization of key organelles and structures involved in reserve mobilization and seedling development. Chloroplasts, mitochondria, and the endomembrane system are examples of membrane-bounded organelles that are likely important in metabolic activities and energy synthesis during germination. Cytosol and plasmodesma are signs of active cytoplasmic activities, which are essential for mobilizing reserves and facilitating intercellular communication. The importance of cellular membranes in controlling molecular transport and signaling during germination is demonstrated by the plasma membrane, organelle envelope, and cell periphery. The storage and recycling of cellular components by the vacuole probably aids in reserve mobilization. The Golgi apparatus's existence shows that it participates in the processing and classification of

cellular molecules and may even help with the mobilization of reserves and the biosynthesis of cell walls.

The proteins of cluster three were classified into 45 categories of GO biological process (GOBP) annotation, 23 categories for GO molecular function (GOMF), and 25 categories for cellular components (Supplementary Table V c). A consistent collection of essential cellular and metabolic processes involved in mobilizing reserves and seedling development are indicated by the enriched GO process terms in cluster three of the haustorium. In the GO process, the haustorium's emphasis on cellular and nucleic acid metabolism during germination is further highlighted by the enrichment of the cellular nitrogen compound metabolic process and nucleobase-containing compound metabolic process, which likely supports the energy demands of reserve mobilization and seedling development.

The catalytic activity, acting on nucleic acid, ATP-dependent activity, pyrophosphatase activity, and nucleoside-triphosphatase activity enriched GO function terms in cluster three suggest the presence of enzymes involved in the metabolism of nucleic acids and energy-demanding processes during germination. The structural constituent of the ribosome and structural molecular activity point to the participation of proteins with structural functions that may be involved in cellular organization and protein synthesis.

In the enriched GO cellular component Protein-containing complex, ribonucleoprotein complex, and ribosome highlight the presence of macromolecular complexes involved in diverse cellular functions, including protein synthesis and RNA processing during germination. Plant-type cell wall signifies the presence of the cell wall, which provides structural support and protection to the haustorium during germination.

In cluster four (Supplementary Table V), a total of 349 proteins were identified with increasing abundance to 10 DAG. 54 proteins were identified as cell wall proteins (WallProt DB), 63 proteins from different classes were identified for carbohydrate metabolism using (CAZy DB), 43 different peptidases were identified using (MEROPS DB) and 83 transporters were identified from TCDB. The activation of the glyoxylate cycle was confirmed by the identification of citrate synthase (*A0A8B7C8V1*), malate synthase (*A0A8B9B1R0*), and glyoxysomal fatty acid beta-oxidation MF-as (*A0A6I9RSD5* and *A0A8B7D0K0*). Furthermore, the proteins involved in redox metabolism catalyzes (*A0A8B8ZB58, B3TLY5,* and *A0A8B7CNF8*) were classified into cell wall proteins and peroxidases such as L-ascorbate peroxidases and peroxidases (*A0A8B9AV30* and *A0A6I9QTC7*) were also identified in this

cluster. The proteins involved in the dismantling of the cell wall such as beta-glucosidase (*A0A8B9B3N7*) and beta-galactosidases (*A0A8B9ATG7* and *A0A6I9QM50*) demonstrate the production of energy from the breakdown of β-galactosides such as cell wall components like pectin and hemicellulose to release galactose and glucose.

The proteins of cluster four were classified into 200 categories of GO biological process (GOBP) annotation, 76 categories for GO molecular function (GOMF), and 73 categories for cellular components (Supplementary Table V d). The enriched GO process terms for metabolism, organic substance metabolism, cellular metabolism, primary metabolism, and small molecule metabolism point to active biochemical pathways for breaking down the carbohydrates, lipids, and proteins and synthesizing molecules, which are essential for producing energy and providing the raw materials for growth during germination. The haustorium's focus on nitrogen metabolism which is necessary for the synthesis and breakdown of nitrogen-containing compounds during reserve mobilization is indicated by the metabolic processes of nitrogen compounds and organonitrogen compounds.

The presence of enzymes that catalyze different biochemical reactions is suggested by the enriched GO function terms in cluster four, which include catalytic activity, oxidoreductase activity, and hydrolase activity. These enzymes are likely essential for breaking down complex molecules like carbohydrates, proteins, and lipids and producing energy during germination. The term ATP binding refers to interactions with ATP, a molecule that is essential for energy transmission and cellular functions during the mobilization of reserves.

The presence of extracellular region, chloroplast stroma, membrane protein complex, plasmodesma, and mitochondrial inner membrane in the enriched GO cellular component terms in cluster four suggests that particular subcellular regions and structures are involved in communication and metabolic activities during germination. The terms Chloroplast, mitochondrion, peroxisome, and Golgi apparatus refer to vital organelles involved in cellular processes during germination as well as energy production, protein synthesis, and other cellular functions. The terms membrane and protein-containing complex suggest the significance of cellular membranes and protein complexes, which probably take part in signaling, transport, and cellular processes during reserve mobilization. The plasma membrane, Organelle envelope, and Organelle membrane draw attention to the presence of several membranes that divide cellular structures and control molecular transport in the haustorium during germination.

Cluster five (Supplementary Table V), shows 624 proteins with decreasing abundance along the germination in the selected stages. 84 proteins were identified as cell wall proteins (WallProt DB), 8 proteins from different classes were identified for carbohydrate metabolism using (CAZy DB), 63 different peptidases were identified using (MEROPS DB) and 153 transporters were identified from TCDB. This cluster also encompasses vicilin-like seed storage proteins (*A0A8B7BPL4* and *A0A8B7CGP8*) indicating the use of mobilization of storage proteins for the production of nitrogen, carbon, and sulfur during germination.

The proteins of cluster five were classified into 156 categories of GO biological process (GOBP) annotation, 49 categories for GO molecular function (GOMF), and 51 categories for cellular components (Supplementary Table V e). The cellular matrix and the liquid component of the cytoplasm, are represented by the enriched GO cellular component terms in cluster five such as cytoplasm and cytosol, which are essential to a variety of metabolic activities and the mobilization of reserves. Both the terms intracellular organelle and membrane-bounded organelle denote the presence of a variety of membrane-bound organelles, including plastids, mitochondria, and the Golgi apparatus, which are essential for metabolic processes, energy production, and protein transport during germination. Ribosomes are essential for protein synthesis during germination and the mobilization of reserves, and their existence is denoted by the terms cytosolic ribosome, ribosomal subunit, and ribosome.

The enriched GO function terms in cluster five showed catalytic activity, hydrolase activity, lyase activity, isomerase activity, and carbon-carbon lyase activity indicating the presence of enzymes responsible for catalyzing a variety of biochemical reactions, potentially involved in breaking down complex molecules and generating energy during reserves mobilization. structural molecule activity and structural constituent of the ribosome highlight the presence of proteins with structural roles, potentially playing roles in cellular organization and protein synthesis during germination. GTPase activity indicates the involvement of proteins in hydrolyzing GTP, potentially playing roles in regulating cellular processes during germination.

In the GO process, the terms cellular process, metabolic process, primary metabolic process, organic substance metabolic process, cellular metabolic process, and small molecule metabolic process highlight the various cellular and metabolic processes occurring in the haustorium during germination, indicating a high level of metabolic activity and cellular dynamics. Protein synthesis and gene regulation in the haustorium during germination are significant processes that are likely necessary for producing the enzymes and proteins involved

in mobilizing reserves. These processes are denoted by the terms protein metabolic process, gene expression, translation, and peptide metabolic process. Cellular nitrogen compound metabolism, organonitrogen compound metabolism, and cellular nitrogen compound biosynthetic process all imply active nitrogen metabolism in the haustorium, which is essential for the synthesis and breakdown of nitrogen-containing compounds during reserve mobilization. The terms carbohydrate metabolic process, carbohydrate derivative metabolic process, and monocarboxylic acid metabolic process highlight the importance of this process, which is essential for germination since it supplies energy and structural elements.

A total of 169 proteins were identified as differentially abundant proteins in cluster six (Supplementary Table V). In this cluster 24 proteins were identified to be involved in carbohydrates metabolism according to CAZy DB, 17 proteins showed a match in WallProt DB, 24 peptidases were identified using MEROPS DB and 46 transporters were identified on the base of TCDB. Attention was given to the enzymes involved in carbohydrate metabolism such as sucrose synthase (*A5C6H7*).

The proteins of cluster six were classified into 89 categories of GO biological process (GOBP) annotation, 32 categories for GO molecular function (GOMF), and 43 categories for cellular components (Supplementary Table V f). The dynamic cellular and metabolic activities of the haustorium during germination are indicated by the enriched GO process terms cellular process, metabolic process, primary metabolic process, and cellular metabolic process; these activities reflect the mobilization and use of reserves to support seedling growth. The terms carbohydrate metabolic process, carbohydrate derivative metabolic process, and monocarboxylic acid metabolic process highlight the significance of this activity, which most likely supplies the energy and structural components for seedling growth. The terms organic substance metabolic process and organonitrogen compound metabolic process refer to the metabolism of different organic compounds, which may be crucial in the mobilization of reserves. Protein degradation processes suggested by the terms proteolysis and proteasomal protein catabolic process may be important for the mobilization of protein stores during germination.

The enriched GO function terms in cluster six catalytic activity, transferase activity, hydrolase activity, lyase activity, isomerase activity, oxidoreductase activity, and intramolecular oxidoreductase activity suggest the presence of various enzymes that play essential roles in catalyzing a wide range of biochemical reactions, likely involved in the metabolism of carbohydrates, lipids, and other small molecules during reserves mobilization.

The terms oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor, and acting on the aldehyde or oxo group of donors suggest that enzymes are involved in redox reactions and may be involved in metabolic pathways and energy production during germination. Hydro-lyase activity and carbon-oxygen lyase activity indicate the existence of enzymes that break down carbon-oxygen bonds; these enzymes are likely important for carbohydrate catabolism during the mobilization of reserves. Protein synthesis and processing during seedling development may depend on the presence of enzymes involved in protein folding and modification, as indicated by the protein disulfide isomerase activity. Threonine-type endopeptidase activity, Aldehyde dehydrogenase (NAD+) activity, beta-N-acetylhexosaminidase activity, phosphopyruvate hydratase activity, and N-acetyl-beta-D-galactosaminidase activity highlight specific enzymatic activities involved in protein degradation, amino acid metabolism, and carbohydrate processing, essential for reserves mobilization and seedling growth.

The enriched GO component terms for cluster six include protein-containing complex, mitochondrial outer membrane, plasma membrane protein complex, proteasome core complex, and peptidase complex. These terms highlight protein degradation during early germination. The term plant-type cell wall is presumably significant for providing structural support during seed germination and seedling growth.

Through a GO enrichment study using the STRING database, we carefully examined each unique cluster found in the heatmap. The main goal was to investigate any potential molecular interactions and functional enrichments within these clusters. The broader analysis has added to our thorough understanding of the dataset and shed light on the functional and spatial characteristics of proteins involved in reserve mobilization in the haustorium during various developmental stages.

In conclusion, our analysis of the differentially abundant proteins has revealed distinct clusters with unique abundance patterns, shedding light on the proteomic dynamics associated with the development of the haustorium of *C. prunifera*. Through an in-depth exploration of the major proteins within each cluster, we have gained valuable insights into the underlying biological processes and regulatory mechanisms driving the observed abundance patterns. The findings presented here contribute to the broader understanding of the role of haustorium in the mobilization of reserves in the developing seedling of *C. prunifera*.

4.6 Proteins identifications related to reserve mobilization in Haustorium

The activation of particular enzymes and hydrolytic activity is necessary for the complex process of mobilizing reserves. Gaining insights into the physiology and biology of the growing haustorium requires a thorough understanding of the underlying mechanisms and the identification of the proteins involved in this process. We give a thorough investigation of the identified hydrolyses and related proteins, highlighting their contributions to reserve mobilization throughout several haustorium developmental stages. Following is a detailed discussion of several hydrolyses, their substrates, and their significance in mobilizing reserves. The haustorium of the carnauba plant has a two-phase mechanism that involves endosperm nutrients' temporary storage and subsequent mobilization to assist embryonic axis development.

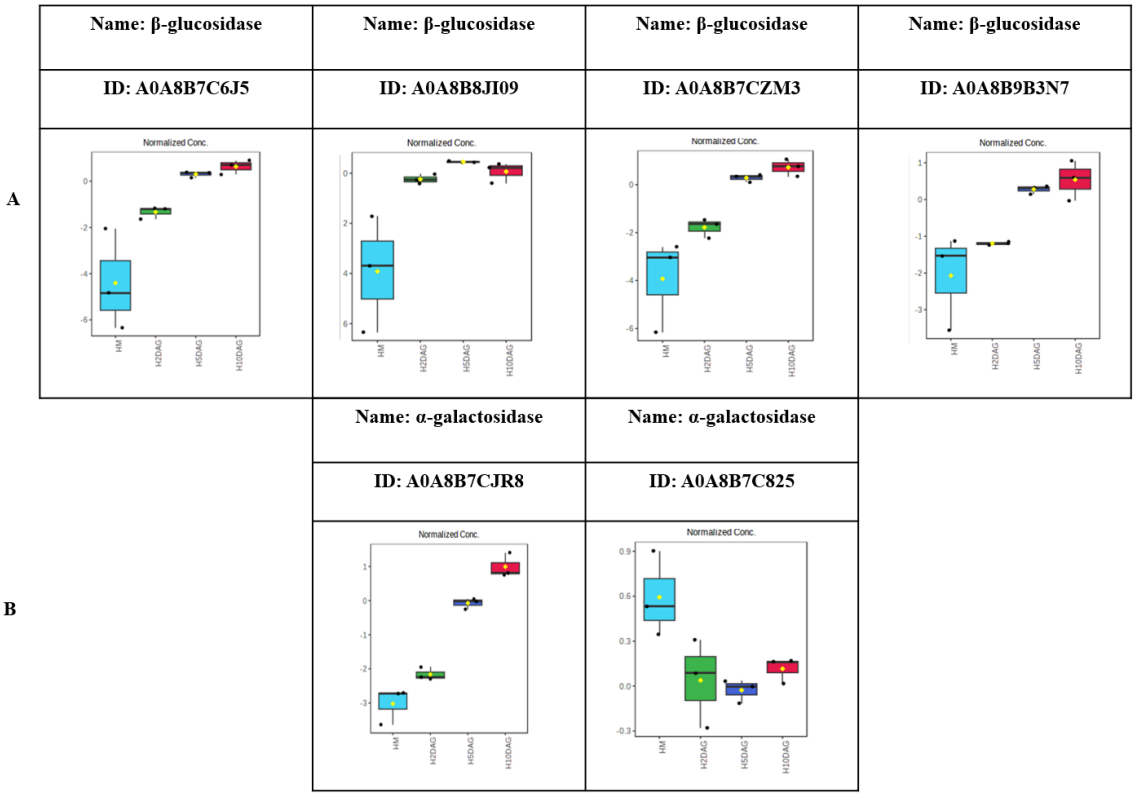
4.6.1 Mannan Reserve Mobilization

Many studies showed that Palm seeds store abundant mannans reserves in their cell wall such as *Euterpe oleracea* Mart (NASCIMENTO et al., 2020) *Elaeis guineensis* (ALANG; MOIR; JONES, 1988)*, Phoenix dactylifera* (DEMASON et al., 1985) and *Acrocomia aculeata* (MAZZOTTINI-DOS-SANTOS; RIBEIRO; OLIVEIRA, 2017). These carbohydrates are commonly stored in cell walls in the form of linear and nonlinear mannans (galactomannans, glucomannans, and galactoglucomannans). In addition, the seed's tough texture also supports the presence of mannans (BUCKERIDGE, 2010).

Studies revealed that the synergistic reaction of several glycoside hydrolase enzymes β-mannanase, β-mannosidase, α-galactosidase, and β-glucosidase brings the hydrolysis of mannans (MALGAS; VAN DYK; PLETSCHKE, 2015). Some mannans hydrolyzing enzymes are previously studied and their sequences are already known i.e. β-mannanase and α-galactosidase however, for the family Arecaceae and *A.thaliana*, the sequences for β-mannosidases and β-mannanase are not available which is making it difficult to identify them by simple proteomics approach (BUCKERIDGE et al., 2000; GHOSH et al., 2013). As this protein is exo-hydrolase it shows significant similarities with β-glucosidase (BAUER et al., 1996; Kaper et al., 2002; MALGAS; VAN DYK; PLETSCHKE, 2015). In *A. thaliana,* the β-mannosidase activity is shown by the β-glucosidase enzyme as one of the functional notations (AHN et al., 2007). Therefore, it is suggested that β-glucosidases are also involved in the cleavage of β-1,4 linked mannosidase, releasing mannose from the non-reducing ends in the mannans and mannose oligosaccharides. The current study identified two β-mannosidases nominated by "mannan endo-1,4-beta-mannosidase" (*A0A6J0PKY5* and

A0A8B8ZML6) which do not vary in abundance in different developmental stages however, it is identified after germination with good coverage (2 and 10 respectively), and unique PSM whereas in the mature embryonic stage, it wasn't identified. As for α-galactosidase, four proteins were identified (*A0A8B7C825*, *A0A8B7CJR8, A0A8B7CX02,* and *A0A6I9QK56*) among which attention was given to *A0A8B7CJR8*, and *A0A8B7C825* which shows differential abundance pattern among all the selected developmental stages this protein was identified with high coverage (16 and 11 respectively), PSM and unique peptides (4 and 5) (Fig 18 B). The 1,4-β-D-glucopyranoses at the non-reducing ends released from galactoglucomannans and glucomannans are hydrolyzed by an exo-enzyme β-glucosidases are also identified in the proteome of the haustorium. A total of seven β-glucosidases are identified in the selected developmental stages among which four (*A0A8B7C6J5, A0A8B8J109, A0A8B7CZM3,* and *A0A8B9B3N7*) were found in increasing in abundance during development (Figure 18 A).

Figure 18- Shows the abundance pattern of different mannan hydrolyses and their abundances during different developmental stages. (A) shows the abundance pattern of beta-glucosidase increasing abundance in different developmental stages (B) shows the increasing abundance of alpha-glucosidase in selected developmental stages of haustorium of *C. prunifera*.



Source: Prepared by author

The identification of α-galactosidase and β-glucosidases in increasing abundance during development indicates that oligosaccharides released from the hydrolysis of hetero-

mannans from endosperm are hydrolyzed in the haustorium and their increase in abundance in haustorium also shows that they are synthesized in the haustorium, thus the role of haustorium is important in the mobilization of seed reserves and act as absorption, storage, conversion, and transport of hydrolysis products to the seedling the current findings are similar to (MAZZOTTINI-DOS-SANTOS et al., 2020; MAZZOTTINI-DOS-SANTOS et al., 2017; NASCIMENTO et al., 2020). The decrease in the abundance of one isoform of α-galactosidase (*A0A8B7C825*) indicates that haustorium may be relying less on mannans as an energy source or substrate, either as a result of the completion of particular growth-related activities or the availability of substitute nutritional sources and shift of metabolic processes.

In conclusion, the present study identified two β-mannosidases, *A0A6J0PKY5* and *A0A8B8ZML6*, which were found to be active following germination but undetected during the mature embryonic stage. Particular focus was placed on *A0A8B7CJR8* and *A0A8B7C825*, two of the four β-galactosidases identified (*A0A8B7C825, A0A8B7CX02*, and *A0A6I9QK56*), as they shown increased abundance throughout all chosen developmental stages. Seven β-glucosidases were also identified, four of which (*A0A8B7C6J5, A0A8B8J109, A0A8B7CZM3*, and *A0A8B9B3N7*) showed an increase in abundance during development. α-galactosidase and β-glucosidase may be involved in the hydrolysis of oligosaccharides produced by the breakdown of hetero-mannans in the haustorium given their presence and rising abundance. This suggests that by absorbing, storing, converting, and conveying hydrolysis products to assist seedling growth, the haustorium plays a critical role in mobilizing seed reserves. These results support earlier research and highlight how crucial haustorium is for using seed reserves.

4.6.2 Mobilization of other carbohydrate reserves

Supplementary Table I shows a variety of proteins that are involved in the digestion of cell wall carbohydrates indicating that a combined action of all these enzymes brings about the release of mannose reserves from the cell wall. Many studies reveal that 30-50% of the primary cell wall is composed of pectin except in grasses (CAFFALL & MOHNEN, 2009; ROPARTZ & RALET, 2020). However because of the limited tools its physical state is not yet fully defined (TEMPLE et al., 2021). In the current study, a variety of enzymes involved in the degradation of pectins like pectinesterase and polygalacturonase were identified in developing haustorium.

Xyloglucans constitute a part of the hemicellulose of the primary cell wall in most land plants (SCHELLER & ULVSKOV, 2010; PARK & COSGROVE, 2015), xyloglucans are

degraded into oligosaccharides by the action of β-galactosidases is also identified in the proteome of the haustorium whereas the enzymes β-glucosidases and α-xylosidases involve in the breakdown of this oligosaccharide into glucose and xylose were also identified in different developmental stages of haustorium (SAMPEDRO et al., 2017). Besides all these several enzymes involved in the hydrolysis of cellulose, callose, galactanose, galactomannanos, and glycoproteins were also identified.

It can be concluded that the haustorium has a wide variety of enzymes that are engaged in the breakdown of cell wall carbohydrates. Pectinesterase and polygalacturonase have been identified as pectin-degrading enzymes, which suggests that they play a part in the breakdown of this important primary cell wall constituent. Furthermore, the presence of enzymes that break down xyloglucans into oligosaccharides, glucose, and xylose, such as β-galactosidases, β-glucosidases, and α-xylosidases, suggests that these enzymes are engaged in this process. The discovery of enzymes that hydrolyze cellulose, callose, galactanose, galactomannanos, and glycoproteins further suggests that several different pathways and processes are involved in the digestion of cell wall carbohydrates. In general, the abundance of carbohydrate-degrading enzymes emphasizes the importance of carbohydrate metabolism and mobilization in the haustorium, underlining its function as a crucial site for nutrient absorption, storage, and utilization during plant growth and development.

4.6.3 Mobilization of protein bodies and lipid bodies

Several proteins involved in the breakdown of protein bodies and programmed cell death were discovered by the proteome analysis of the haustorium. It was observed that proteins from the peptidase family, including *A0A8B7CEG0, A0A6I9RG95,* and *A0A8B7CJU8*, are involved in the breakdown of protein bodies. Furthermore, serpin-ZXA proteins (*A0A8B8ZEH0* and *A0A8B7C665*) that inhibit metacaspase-9, a crucial regulator of programmed cell death, were discovered. According to these findings, the haustorium mobilizes protein stores, and the amino acids that are released may be used to create new enzymes or structural proteins. As a result of the identification of the enzymes responsible for the hydrolysis of triacylglycerols (lipids) and subsequent oxidation of fatty acids via beta-oxidation, lipid mobilization was also seen in the haustorium. Involvement of gluconeogenesis via the breakdown of long-chain fatty acids is indicated by the identification of lipases, peroxisomal fatty acid beta-oxidation multifunctional proteins, and glyoxysomal fatty acid beta-oxidation multifunctional proteins. The glyoxylate cycle, which helps to produce non-reducing carbohydrates like sucrose that may

be used in the haustorium or delivered to the growing embryonic axis, is nourished by this mechanism.

Three proteins (*A0A8B7CEG0, A0A6I9RG95,* and *A0A8B7CJU8*) corresponding to (A01.020;) peptidases family (MEROPS) are involved in the degradation of protein bodies (MAZORRA-MANZANO et al., 2010) Both Mazzottini-dos-Santos et al. (2020) and Souza Dias et al. (2018) claim that the development of vacuoles as a result of protein breakdown is observed and found it essential for the mobilization of lipid bodies (MAZZOTTINI-DOS-SANTOS et al., 2020; SOUZA DIAS et al., 2018). The mobilization of reserves is mostly brought about by the process of programmed cell death in Angiosperm (MOYANO et al., 2018). Two proteins serpin-ZXA (*A0A8B8ZEH0* and *A0A8B7C665*) are involved in the inhibition of metacaspase-9 which is involved in the control of programmed cell death (FLUHR; LAMPL; ROBERTS, 2012). The proteins that are responsible for the degradation of proteins such as 26S proteasome and alpha and beta subunits of proteasome complex were also identified in the proteome of haustorium which indicates that protein reserves are mobilized and the amino acids may either be translocated to the developing embryonic axis for the production of new enzymes or structural proteins or may be utilized in the haustorium for the production of a variety of different enzymes including hydrolases involved in the mobilization of other reserves (ROSENTAL; NONOGAKI; FAIT, 2014). The synthesis of new proteins is essential for germination, as the amino acid reserves are not enough for the production of new proteins, this deficiency is compensated by the mobilization of seed-reserved proteins (BEWLEY et al., 2013).

For the support of seedling establishment seeds commonly store lipids in the form of triacylglycerols (YANG AND BENNING, 2018). The lipases hydrolyze to fatty acids which are oxidized to acetyl-CoA in the peroxisomes by beta-oxidation (CAI et al., 2020). Which provides a substrate to the glyoxylate cycle for gluconeogenesis (MAESHIMA & BEEVERS, 1985:Y. YANG & BENNING, 2018). In the haustorium, 15 GDSL esterase/lipase, 4 3-ketoacyl-CoA thiolase 2, 3 peroxisomal fatty acid beta-oxidation multifunctional protein-like and 3 glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a are identified which are involved in the gluconeogenesis by the β-oxidation of long-chain fatty acids during seedling development and germination (RONIQUE GERMAIN et al., 2001; RYLOTT et al., 2006)

The enzymes involved in the glyoxylate cycle have also been identified in the haustorium proteome supplementary table I. The identification of two proteins as isocitrate

lyases (*A0A8B7CNY0* and *A0A6J0PKT8*), two malate synthases (*A0A8B9B1R0* and *Q9LZC3*), three glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a (*A0A8B7D0K0, A0A6I9RSD5,* and *A0A8B7BT48*), two fumarate hydratase (*A0A8B8ZLU5* and *A0A8B7C3D9*) and four succinate dehydrogenases (*A0A6I9SEI1, Q9ZPX5, A0A6I9RJU9,* and *A0A8B7CIJ8*) and their subunits indicates that free fatty acids can be processed in haustorium for the production of transportable non reducing sugars such as sucrose by the glyoxylate cycle which can be utilized in the haustorium or transport to developing embryonic axis and cotyledonary petiole (RENNIE; TURGEON, 2009).

The haustorium's proteomic study has revealed important details about the wide variety of proteins involved in the mobilization of protein reserves, adding support to the idea that storage proteins serve as the growing embryo's first source of energy. Notably, aspartic proteinase oryzasin-1-like isoform X1 (*A0A8B7CJU8*), which corresponds to Q42456 (MEROPS DB), was discovered and its abundance pattern highlights its role in the mobilization of protein reserves. The idea that storage proteins play a crucial role as an important energy source during early seedling growth is further supported by our results, which support earlier studies on *Euterpe oleraceae* and *E. precatoria.*

The identification and abrupt decrease in the abundance pattern of aspartic proteinase oryzasin-1-like isoform X1 (*A0A8B7CJU8*) corresponding to *Q42456* (MEROPS DB) which is involved in the mobilization of protein reserves indicates that storage proteins are the first source of energy which is utilized by the developing embryo which adds weight to the previous findings of our a work on *Euterpe oleraceae* (NASCIMENTO et al., 2020) and similar results were shown by a study on *E. precatoria* (ERREIRA et al., 2020). The identification of proteins involved in the mobilization of protein reserves, dismantling of the primary cell wall, and regulation of PCD (supplementary table I) indicates the possible synthesis and/or secretory role of haustorium as studied showed no existence of programmed cell death in the haustorium (SOUZA DIAS et al., 2018).

Finally, the proteomic investigation of the haustorium has shown that it contains proteins that are involved in the mobilization of protein reserves, degradation of the primary cell wall, and control of programmed cell death (PCD). The discovery of aspartic proteinase oryzasin-1-like isoform X1 (A0A8B7CJU8) and its abundance pattern highlights the relevance of storing proteins as the primary energy source used by the developing embryo. Additionally, the discovery of enzymes linked to the glyoxylate cycle and lipid mobilization raises the

possibility that lipid reserves are used for gluconeogenesis and the synthesis of transportable non-reducing sugars like sucrose.

4.7 Haustorium as a possible synthesis site for hydrolases

The diversity of cell wall carbohydrates involved in the remodeling of the cell wall, which is essential for the structural growth and extension of the haustorium, has been shown by the proteomics study of several developmental phases of the haustorium. Although endo-1,4-beta-mannosidase nine and another mannan enzyme were found in the haustorium, there were no appreciable differences in their abundances. However, the discovery of four beta-glucosidases (*A0A8B7C6J5, A0A8B8J109, A0A8B7CZM3,* and *A0A8B9B3N7*) and two differentially abundant alpha-galactosidases (*A0A8B7C825* and *A0A8B7CJR8*) suggests their production and secretion in the haustorium's digesting zone. These results are consistent with earlier investigations on the secretory activity of hydrolases in haustoria like *Cocos nucifera, Elaeis guineensis,* and *Euterpe oleraceae*. They, however, go against the conclusions of a study on the evolution of haustoria's ultrastructural examination.

Diversity of cell wall carbohydrases were identified in the proteomics analysis of different developmental stages of haustorium. Which were mainly involved in the remodeling of the cell wall which is necessary for the structural growth and expansion of the haustorium. Two mannan enzymes endo-1,4-beta-mannosidase 9 were identified in the haustorium but both of them were not differentially abundant. However, the identification of two Alpha-galactosidase (*A0A8B7C825* and *A0A8B7CJR8*) corresponding to (*Q5QLK3* and *P14749*) CAZy families as differentially abundant proteins and the identification of 4 β-glucosidases (*A0A8B7C6J5, A0A8B8J109, A0A8B7CZM3,* and *A0A8B9B3N7*) corresponding to (*Q2MV11, Q6UY81,* and *A8TVR1*) CAZy families in cluster 2 and 4 with increasing abundance during different developmental stages of haustorium suggest that these hydrolases may probably synthesized in the haustorium and secreted into the digestion zone. The current findings are in accordance with the previous findings of haustoria as the site of synthesis of hydrolases for *Cocos nucifera* (SUGIMUMA; MURAKAMI, 1990); (VERDEIL; HOCHER, 2002), *Elaeis* guineensis (OO; STUMPF, 1983) and *Euterpe oleraceae* (NASCIMENTO et al., 2020). The current findings do not agree and showed opposite results with the findings of a study on haustoria ultrastructural analysis during development where no secretory activity has been mentioned (MAZZOTTINI-DOS-SANTOS et al., 2020).

In conclusion, the significance of these enzymes for structural growth and expansion by demonstrating the presence of a wide variety of cell wall carbohydrases involved in the remodeling of the cell wall. While the alpha-galactosidases and beta-glucosidases were differentially abundant compared to the mannan enzymes, this suggests that the digesting zone of the haustorium is where these enzymes are secreted. These results lend support to earlier research on hydrolases' secretory activities in several plant species.

4.7.1 Transient starch biosynthesis in the haustorium

The presence of starch is identified in the haustorium of hemi parasites (ZHOU et al., 2021a). Studies showed that haustoria of Arecaceae does not show any traces of starch before germination and it is believed that the starch is formed from endosperm as the germination initiates (MOURA; VENTRELLA; MOTOIKE, 2010). In the current study, some proteins such as 1,4-alpha-glucan branching enzyme (*A0A6I9SB78* and *A0A8B8J0X0*) corresponding to (*A5HSI0* and *A2TIS0*) of CAZy families, seven different types of fructokinases (1,2,4 and 6) corresponding to (D3EAV7 and E3E4E3) of CAZy families, glucose-1-phosphate adenylyl transferase (*A0A8B7C9U1*) and starch synthase, chloroplastic/amyloplastic (*A0A8B7BFJ3*) corresponding to B5THH5 CAZy family demonstrate the role of haustorium as the site of starch biosynthesis, storage and transport to the developing embryo which is already reported in the family Arecaceae (OLIVEIRA et al., 2013).

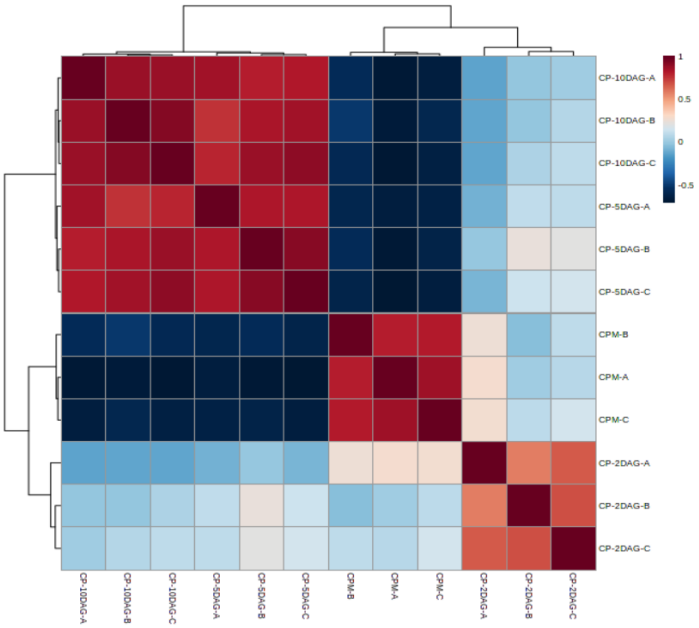
In conclusion, our study's investigation of the haustorium has shown that starch and proteins involved in its synthesis are present, indicating that the haustorium serves as a place for starch production, storage, and transportation to the developing embryo. According to earlier research, Arecaceae species' haustoria do not contain starch before germination; instead, it is thought that starch is generated from the endosperm as germination begins. The 1,4-alpha-glucan branching enzyme, fructokinases, glucose-1-phosphate adenylyl transferase, and starch synthase are just a few of the proteins that the current research shows are present and involved in starch production. These proteins provide credence to the idea that starch is actively synthesized and stored in the haustorium before being transferred to the growing embryonic axis.

5 PROTEOMIC ANALYSES OF COTYLEDONARY PETIOLE

In this section, we describe a thorough proteomics analysis of the cotyledonary petiole of *C. prunifera*, with a particular focus on the characterization of protein expression dynamics during four different developmental stages. We used proteomics methods to examine protein abundance and expression patterns across the four chosen developmental stages. Gene Ontology (GO) annotation was performed by the STRING tools to gain insights into the functional roles and cellular localization of the identified proteins across the four developmental stages of the cotyledonary petiole of *C. prunifera*. Based on the abundance profiles of the identified proteins, PCA enables the display and investigation of general trends and similarities/differences between developmental stages. We distinguish various grouping patterns and trends using PCA, and these findings offer preliminary insights into the dynamic proteome alterations taking place in the cotyledonary petiole of *C. prunifera* development. We further provide the findings of the heatmap analysis of 318 proteins with varied levels of abundance. To gain a deeper knowledge of the abundance profiles and connections between the various proteins across the developmental stages, the heatmap visualization enabled us the identify six clusters and patterns within the dataset. We delve into specific proteins that are known to be involved in growth and development processes to find pathways and mechanisms that are conserved and contribute to the characteristic way of growth and development of *C. prunifera*. We also explored the exclusive proteins found in the cotyledonary petiole that may be special or have cotyledonary petiole-specific functions. We also analyzed the proteins that are frequently expressed in both the cotyledonary petiole and the haustorium. The proteome profiles of these two tissues in the same developmental stages were compared to find common molecules and possible connections.

Using Pearson correlation scores, the results' repeatability was evaluated. The results could be reproduced because all of the values were near 1 (Supplementary Table III). A histogram was used to look at the distribution of normalized abundance values among biological replicates and stages (Figure 19). These results demonstrate that the analysis proceeded with good reproducibility.

Figure 19- Shows the Pearson heatmap of different developmental stages and their biological replicates of the cotyledonary petiole of *C. prunifera*. The correlation values between ±1 are considered to be a strong correlation.



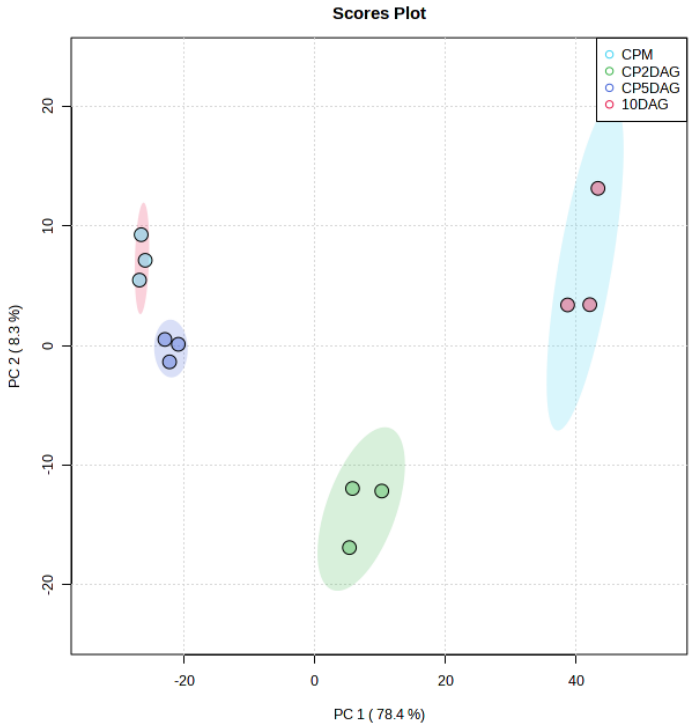
Source: Prepared by author

The Pearson correlation heatmap for biological and duplicates showed significant correlation values near one, which indicates reproducibility, experimental precision, limited biological or technical variability, and carefully controlled experimental conditions. This suggests that the data points of the duplicates are very similar to one another and have a propensity to alter simultaneously. Additionally, it ensures that the procedures for handling the samples, analyzing the data, and planning the experiment are well-considered to minimize any potential sources of bias that can accidentally inflate the correlation results.

5.1 Principal Component Analysis

A principal component analysis (PCA) was used, which uses only identified proteins in all samples and groups the samples in a two-dimensional graph according to similarities in abundance. PCA for different stages of cotyledonary petiole showed similarities among the group members whereas the groups are distinct from each other (Figure 20).

Figure 20 - Shows the principal component analysis (PCA) of all three biological replicates of the selected developmental stages of the cotyledonary petiole of *C. prunifera*.



Source: Prepared by author

Figure 20 shows that while there is significant variance between the various developmental groups, the protein expression patterns within each group are generally comparable. Group members are grouped while the groups themselves are segregated. The division of the groups in the PCA plot implies that each developmental group is connected with a unique proteomic profile. The proteins within each group may have unique expression patterns or abundance levels that set them apart from proteins in other groups. This suggests that the proteome profile is significantly influenced by the developmental stage. Each developmental group's members are tightly clustered together, which shows that the protein expression patterns are rather uniform within each group. Similar expression patterns or abundance levels are shared by the proteins identified within a given developmental group (biological replicates), suggesting consistency in the biological replicates. The fact that the variance described by PC1 (78.4%) is much higher than that explained by PC2 (8.3%) shows

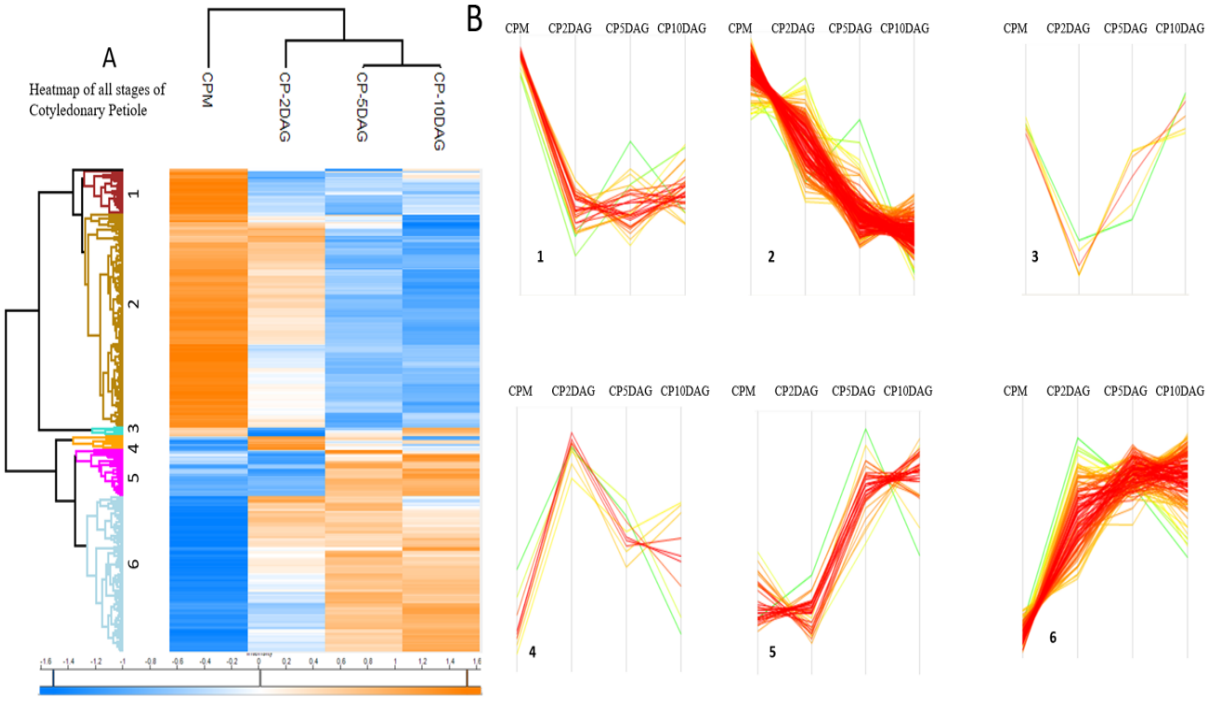
that the first principal component accounts for the majority of the variation in the dataset. The primary source of variation that separates the various developmental groups from one another is probably found in PC1. Even though PC2 explains less variance, it still helps explain some of the observed group distinctions.

As a result, Figure 20's analysis shows that there is a large amount of diversity among the various developmental groups, revealing unique proteomic profiles related to each stage. The division of the groups in the PCA plot reveals that the developmental stage affects the proteome profile. The tight clustering of group members suggests that the protein

5.2 Differentially abundant proteins in cotyledonary petiole

To identify distinctive clusters and patterns within the dataset, heatmaps offer a condensed and visual representation of the abundance patterns of differentially expressed proteins. The outcomes of our investigation concentrated on identifying and visualizing the varied protein abundances throughout the chosen cotyledonary petiole developmental stages of *C. prunifera*. The investigation sought to understand the proteome alterations connected to the development of the cotyledonary petiole. A total of 318 proteins were identified as differentially abundant proteins using a permutation-based ANOVA test with FDR ≤ 5% and Tukey's HSD test (Tukey's Honest Significant Difference) was used for the quantitative analysis of four developmental stages of cotyledonary petiole of *C. prunifera* the same as used for haustorium. The significantly abundant proteins were grouped in six clusters according to their abundance profile in all the selected stages (Figure 21, Supplementary Table III).

Figure 21- Representation of differentially abundant proteins in the developmental stages of the cotyledonary petiole of *Copernicia prunifera* during germination. (A) heatmap of differentially abundant proteins. (B) Clusters of proteins with similar abundance profiles.



Source: Prepared by author

Cluster 1 (Supplementary Table VI CP) comprises a of total 29 proteins which shows an abrupt decrease from the mature stage to 2DAG and remains decreased along all stages of germination stages. 7 proteins were identified to be involved in the metabolism of carbohydrates using CAZy DB, 7 proteins were identified as cell wall proteins (WallProt DB), 4 different types of peptidases were also identified by using MEROPS DB, whereas 5 different types of transporters were identified by TCDB, the identification of proteasome subunit beta (*A0A8B7D1E9*) which is characterized by its ability to cleave peptides bonds with very broad specificity such as with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving groups and as an ATP-dependent proteolytic activity, and the presence of leucine aminopeptidase 2 *(A0A8B9A6N5)* which is involved in the release of an N-terminal amino acid. Indicates that protein reserves are first to mobilize during germination. Whereas the identification and abundance pattern of triosephosphate isomerase, cytosolic (*A0A8B7CN34*) which plays a crucial role in the independence of seedlings from seed reserves indicates that glycolysis is active (CHEN; THELEN, 2010). The decrease in abundance along the development of oxoglutarate dehydrogenase (succinyl-transferring) (*A0A6I9R929*) which impacts carbon-nitrogen metabolism and modulates plant growth is allowing the developing plant to increase its growth (CONDORI-APFATA et al., 2021). The identification of alpha-xylosidase 1

(*A0A8B7CEW2*) corresponding to the (*Q9LGC6*) CAZy DB, also got attention in this cluster which is involved in affecting germination both physically by affecting cell wall integrity and also chemically by affecting the genes expression of auxins and abscisic acid hormones (SHIGEYAMA et al., 2016). In the GO enrichment analysis, this cluster shows only the enrichment of two terms, cytoplasm, and cytosol (Supplementary Table VI a). The presence of proteins in the cytosol in GO enrichment analysis suggests that these proteins are involved in metabolic pathways that drive the conversion of nutrients, allowing the embryonic axis to grow quickly. Furthermore, the abundance of proteins in the cytoplasm emphasizes their importance for cellular processes other than reserve mobilization. These proteins might be involved in the synthesis, degradation, and control of numerous signaling pathways. These activities become essential for coordinating cellular responses and maintaining proper growth and differentiation of tissues and organs when emerging organogenesis occurs and the embryonic axis grows. They probably aid in the distribution of nutrients to growing organs, assisting in the development of leaves, stems, and roots. To further ensure a balanced and coordinated developmental trajectory, the complex interactions between these proteins and regulatory factors might affect the time and degree of organ development.

In cluster 2 (Supplementary Table VI CP) there is a total of 139 identified proteins with decreasing abundance from mature to 10DAG in which 10 proteins were recognized as carbohydrates metabolism proteins by using the CAZy database, 18 proteins were recognized as cell wall proteins with the help of WallProt DB, 9 peptidases from different classes were also identified using MEROPS database and a total of 36 different types of transporters were identified with the help of TCDB. The storage proteins vicilin seed storage proteins (*A0A8B7BPL4, A0A8B9A5W3, A0A8B7CGP8* and *A0A6I9SEK1*), cocsin1 (*A0A6J0PPI1, A0A8B7CS47, A0A6J0PPG2, A0A6J0PS65* and *A0A6I9RK14*) and 63 kDa globulin-like protein (*A0A6I9QBZ9*) were included in this cluster. The identification of histone H4 (*A0A6I9QVY9*) protein indicates that cell division is higher in the initial stages but gradually decreases in the older stages but its existence means that cell division is still occurring in all the stages (XIAO, T. T. et al., 2019). Aquaporin TIP3-1-like (*A0A8B7CJ95*) corresponding to the *P26587* TCDB (1.A.8.10.1 family), was also identified in this cluster which corresponds to the transport of ions and water in response to stress and is also an important component for development and growth (WANG et al., 2020). Ras-related protein RIC1 (*B3TLR9*) corresponds to the *P32939* (9.A.63.1.1 family in TCDB), which regulates negatively abscisic acid signaling and positively regulates auxin signaling (CHOI et al., 2013). The decrease in abundance of these enzymes

indicates that during development the reaction goes in the opposite direction, in other words, abscisic acid signaling will increase in comparison with auxin signaling. Another important protein thiamine thiazole synthase (*A0A8B8ZZI3*) was also identified in this cluster which acts as a cofactor for various enzymes involved in important biological processes such TCA cycle, glycolysis, kelvin cycle, carbohydrates metabolism, and synthesis of the branched amino acids (BOCOBZA ET AL., 2013; LINKA & WEBER, 2010). Nitrogen is one of the essential element for growth and development which is initially provided by the metabolism of nitrogen-rich reservoir arginine by arginase 1 (*A0A8B7BSS5*) is also making part of this cluster and its abundance pattern indicates the depletion of arginine reserves in the seed during development (SIDDAPPA; MARATHE, 2020). Arginase is also involved in the biosynthesis of essential plant metabolite putrescine by converting arginine to agmatine and putrescine which play a vital role in plant stress response (PATEL, J 2017). Cysteine desulfurase (*A0A6I9SLU5*) is involved in the maturation of plants chloroplast iron-sulfur protein was also identified in this cluster (VAN HOEWYK et al., 2007)

The proteins of cluster two were classified into 17 categories of GO biological process (GOBP) annotation, 4 categories for GO molecular function (GOMF), and 15 categories for cellular components (Supplementary Table VI b). Small molecule metabolic process and reaction to organic substance enrichment in the GO process suggest continuing metabolic reconfiguration. As resources become scarcer, the plant may be adjusting its metabolic pathways to maximize energy expenditure and ensure effective resource usage. Changes in the production and utilization of energy could be reflected in processes like the carboxylic acid metabolic process and the glycolytic process.

In the GO components organelles like the chloroplast and mitochondrion are enriched, indicating how important these components are to biosynthesis and energy production. These organelles may experience modest reorganizations as their quantity declines, guaranteeing a balanced energy supply and the synthesis of essential chemicals during the seedling growth phases. The vacuole and plant-type vacuole enrichments, in particular, imply important functions in storage. These vacuoles might adjust their roles to improve nutrient storage as their abundance declines, in line with the plant's shifting developmental requirements. The enrichment of internal lipid droplets and non-membrane-bounded organelles suggests that the cell's specialized structures have been carefully arranged. As their abundance declines, these elements might experience specific modifications that support lipid metabolism, signaling, and storage.

The complicated regulation of vital cofactors by the plant is indicated by the enrichment of metal ion binding in the GO function, which includes magnesium ion binding. The modification of metal ion interactions implies a control regulation of metabolic pathways, cellular processes, and enzymatic activities important for growth and development. Unfolded protein binding is a sign of increased attention to protein homeostasis. In response to shifting cellular needs, the plant may prioritize appropriate folding and stabilization of proteins as their abundance declines. This underlines how crucial it is to keep the proteome balanced during development. The nutrient reservoir activity enrichment provides information on the plant's resource allocation strategy. This function probably helps with nutrient storage and release optimization when abundance declines, which is consistent with the plant's development.

Cluster 3 (Supplementary Table VI CP) consists of six proteins in which one protein was identified to be involved in carbohydrates metabolism (*A0A8B9AS87* corresponding to *A0A060C4J4* in CAZy DB belonging to GH92 family) and one to be involved in seed storage processes through its helical domain (*A0A8B7BWV0* corresponding to *AT2G45180.1* WallProt DB), whereas two proteins were identified for TCDB and MEROPS. In this cluster, attention was given to aquaporin PIP2-6 (*A0A6I9RVP9*) which is involved in ion movement and water transport facilitation (GAMBETTA et al., 2017) which indicates that along with using seed storages, developing seedlings has started its independency process by absorption of environmental water and nutrients.

In cluster 4 (Supplementary Table VI CP) a total of nine proteins were identified of which two proteins (*A0A8B8ZNB8* and *A0A8B7C2L3* corresponding to *B9EUX8* and *Q7XUQ8* CAZy DB) were identified to be involved in carbohydrate metabolism, two (*A0A6I9S942* and *A0A8B8ZNB8* corresponding to *BAS81739* and *CSS0026846* WallProt DB) were identified as cell wall degrading proteins, whereas one transporter (*A0A8B7CLM4* corresponding to *Q96662*) was also identified based on TCDB. Phosphoenolpyruvate carboxykinase (*A0A6I9QEH1*) which along with being involved in the gluconeogenesis pathway has a prominent role in the growth of germinating seedlings and roots elongation, in tomato the suppression of (PEPCK) suppressed seedling germination and roots growth (HUANG et al., 2015).

Cluster 5 (Supplementary Table VI CP) shows 29 proteins of which six proteins were identified to be involved in carbohydrate metabolism, eight proteins were identified to be involved in cell wall degradation, one peptidase, and four different types of transporters were also identified in this cluster. The identification and abundance pattern of persulfide

dioxygenase ETHE1 homolog (*A0A6I9Q7Y2*) which catalyzes the oxidation of persulfides in the mitochondrial matrix and is essential for early embryo development indicates its role in the development of the embryonic axis which starts as its abundance increases (KRÜSSEL et al., 2014). The identification of coniferyl alcohol acyltransferase (*A0A8B9A2H0*) which catalyzes the substrate of coniferyl alcohol to coniferyl acetate, which is an important substrate for synthesizing eugenol. The studies showed that eugenol inhibits early root growth through ROS-mediated oxidative damage, despite activation of the antioxidant enzyme machinery (AHUJA et al., 2015). This can be evident in the absence of roots up to a certain length of cotyledonary petiole.

Due to their small number of proteins, clusters three and four might not have displayed significant GO enrichment, whereas cluster five only showed enrichment in two functional terms (Supplementary Table VI c). The specific activities' GO function enrichment points to a crucial involvement in redox control and energy dynamics at this developmental stage. The presence of NADPH dehydrogenase (quinone) suggests that the plant uses NADPH as a reducing agent in processes involving quinone. Quinones are thought to play a role in energy production and electron transfer inside the growing embryonic axis because they are essential parts of electron transport chains and redox activities. Similar to the last example, the embryonic axis's dependency on these particular enzymatic activities for electron transfer is highlighted by oxidoreductase activity, which acts on NAD(P)H, quinone, or a related molecule as an acceptor. The maintenance of cellular redox balance, a basic process that controls a variety of cellular functions, including signal transduction, metabolic regulation, and stress responses, may depend on this activity.

In cluster 6 (Supplementary Table VI CP) a total of 103 proteins were identified of which 33 were involved in carbohydrate metabolism, 17 were identified to be involved in cell wall degradation, eight different types of peptidases, and 23 different transporters were also identified by using the MEROPS database. The identification of malate dehydrogenase (*A0A6I9R4U7*) is an indication of the activity of the glyoxylate cycle. Proteins involved in the mobilization of reserves and dismantling of cell walls like sucrose synthase (*A0A8B7BQ46* and *A0A6I9S437*) and beta-glucosidase (*A0A8B7MUD2* and *A0A8B9B1M9*) were also identified in this cluster. 5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (*A0A6I9QGV3* and *A0A8B7BJZ2*) and cysteine synthase (*A0A8B8ZGW1*) plays a vital role in the formation of cysteine by methyl group transfer to homocysteine from 5-methyltetrahydrofolate, cysteine is reported to be used as growth regulator which shows a

promising increase in growth and development of plants especially in drought stress, its increasing abundance along with developmental stages support the theory of its increasing abundance along with developmental stages support the theory of (YOU et al., 2019). Actin-101 isoform X2 (*A0A6I9R951*) is also identified in this cluster which plays a vital role in several physiological processes in plants such as growth, development, cytokinesis, and cell division (SZYMANSKI; STAIGER, 2018). Its increase in abundance indicates its role in *C. prunifera's* growth in development. Nitrogen is a key component in plant growth and development, the identification and abundance pattern of glutamine synthetase (*A0A6I9QGX3*) shows that it plays a vital role in nitrogen metabolism, also plays an important role in plant growth, development, and stress tolerance (temperature, drought, and salinity) (YIN et al., 2022). Both cluster five and cluster six exhibit an increasing abundance pattern throughout all developmental stages. However, the members of cluster five show an abrupt increase from stage two (2 DAG) to stage three (5 DAG), indicating that these proteins may be responsible for the possible morphoanatomical changes of stage three (5 DAG).

The proteins of cluster six were classified into 49 categories of GO biological process (GOBP) annotation, 2 categories for GO molecular function (GOMF), and 12 categories for cellular components (Supplementary Table VI d). Highlighting the dynamic nature of the biochemical reactions sustaining cellular functions are the terms metabolic process, cellular metabolic process, organic substance metabolic process, and primary metabolic process. The prevalence of the terms cellular biosynthetic process, organic substance biosynthetic process, and small molecule biosynthetic process further emphasize the active production of vital growth-supporting components by the plant. These procedures probably aid in the development of vital cellular elements that enable the growth and differentiation of tissues within the embryonic axis of the carnauba. The enrichment of the Phosphorus metabolic process and organophosphate metabolic process indicates that phosphorus-containing molecules are used by the plant, which may be important for signaling and energy transmission. Nucleotide metabolism is also highlighted by the terms nucleobase-containing compound metabolic process and nucleobase-containing small molecule metabolic process, which may be crucial in the synthesis of DNA and RNA during developmental phases.

Catalytic activity and oxidoreductase activity, two enriched GO function terms, shed light on important molecular mechanisms regulating the growth and development of the carnauba palm. The catalytic activity emphasizes the plant's ability to accelerate important chemical processes, possibly facilitating the transformation of substrates necessary for a variety

of cellular functions. Oxidoreductase activity, on the other hand, denotes the participation of redox mechanisms important for energy production and signaling. These enzymatic activities might be crucial for cellular metabolism as the embryonic axis of the carnauba palm develops, facilitating effective energy transfer and preserving redox equilibrium. Catalytic and oxidoreductase activities are probably key to the molecular mechanisms that drive growth, adaptability, and differentiation within the embryonic axis as their abundance increases.

It is clear from the inclusion of cytoplasm, cytosol, and intracellular anatomical structure that these compartments are crucial for housing important cellular functions and molecular interactions. Plasmodesma and plant-type cell walls are also present, pointing to the plant's structural support system and intercellular communication pathways, which probably support tissue integrity and nutrition exchange as the embryonic axis progresses through developmental stages. Peroxisome, Plastid, and Vacuole are enriched terms that refer to specialized organelles that may participate in diverse metabolic routes and storage functions. These organelles might be essential for energy synthesis, nutrition storage, and cellular detoxification. The existence of intracellular organelle and intracellular membrane-bounded organelle highlights the sophisticated subcellular organization that underlies the complicated cellular processes that drive growth and development.

In conclusion, different protein clusters with varied abundances were found throughout the germination process of *C. prunifera* in four developmental stages of the cotyledonary petiole. These protein groups included elements of cell wall dynamics, transport mechanisms, and development and growth. Aspects of the molecular processes governing seed germination and early seedling growth are revealed by the discovery and abundance patterns of certain proteins. The considerable decline in protein abundance associated with reserve mobilization in Cluster 1 shows that these proteins were used up quickly during germination. Notably, proteins involved in peptidase activity, transporters, cell wall composition, and glucose metabolism were identified, highlighting the significance of these activities in promoting seedling growth and development. Proteins involved in the metabolism of carbohydrates, cell wall dynamics, storage proteins, and transporters were found in Cluster 2, which showed a tendency toward decreasing abundance. The discovery of storage proteins brings attention to their function in supplying vital nutrients in the early stages. Additionally, the presence of histone H4 signals that cell division is occurring continuously, and the presence of aquaporins and transporters shows that nutrients and water are being absorbed throughout germination and development. Proteins involved in the breakdown of cell walls, the metabolism

of carbohydrates, and phosphoenolpyruvate carboxykinase (PEPCK) were found in Cluster 4. Given its function in gluconeogenesis and growth, PEPCK is likely crucial for seedling growth and root elongation. Persulfide dioxygenase ETHE1 homolog and coniferyl alcohol acyltransferase were abundant in cluster 5, indicating that they were involved in the development of the embryonic axis and the control of root growth, respectively. These findings offer important new understandings of the molecular processes involved in *C. prunifera* seed germination and early seedling growth. The identified proteins and their patterns of abundance provide information about how reserves are used, how cell walls remodel dynamically, how metabolic pathways are activated, and how growth processes are regulated.

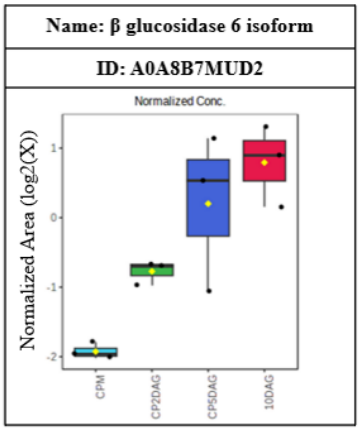
5.3 Protein identifications related to reserve mobilization in cotyledonary petiole

Different proteins regarding reserve mobilization were identified in developing cotyledonary petiole which are mentioned below.

5.3.1 Mannan Reserve Mobilization

In cotyledonary petiole, five α-galactosidases (*A0A8B7C825, A0A8B7CJR8, A0A8B7CX02, A0A8B8ZHT0,* and *A0A6I9QI77*) were identified with high coverage, PSM and unique peptides but none of them were found as differentially abundant during developmental stages. While 11 β-glucosidases (*A0A8B7C6J5, A0A8B8J109, A0A8B7MUD2, A0A8B9B3N7, F4JAB7, A0A6I9R8G3, A0A6I9QKX7, A0A8B9AN67, A0A6I9R4B0, A0A6I9QEE2* and *A0A6I9SHT4*) were identified in which only *A0A8B7MUD2* shows as increasing abundance during developmental stages. All of them were identified with greater coverage, PSM, and unique peptides (Figure 22).

Figure 22- The figure shows the increasing abundance of beta-glucosidase 6 isoform in selected developmental stages of *C. prunifera*



Source: Prepared by author

The quantification of β-glucosidases and α-galactosidases indicates that the mannans present as seed reserves are not only linear mannans but can also be non-linear mannans i.e. (glucomannans, galactomannans, and glucogalactomannans). In linear mannans like glucomannans, β-glucosidases are enzymes that catalyze the hydrolysis of β-1,4-glucosidic bonds. On the other hand, α-galactosidases are enzymes that break down the α-1,6-galactosidic linkages that are typically present in the non-linear mannans galactomannans and glucogalactomannans. The presence of both β-glucosidases and α-galactosidases suggests that the seed reserves may have a combination of mannans with various linkages and structures, such as linear glucomannans, linear galactomannans, and branched glucogalactomannans.

Besides these, a variety of enzymes that are involved in the digestion of cell wall carbohydrates such as cellulose, callose, xyloglucans, galactanos, galactomannans, glycoproteins, and pectins were also identified in the cotyledonary petiole of *C. prunifera* which are mentioned in supplementary table III. The identification of the enzymes in all the selected developmental stages indicates that seed reserves might not be completely metabolized in haustorium but are also transported to the developing cotyledonary petiole which might further be metabolized by these enzymes.

Insights into the presence and quantity of several enzymes involved in the digestion of cell wall carbohydrates, particularly α-galactosidases, and β-glucosidases, have been gained from the proteomic study of the cotyledonary petiole in *C. prunifera.* Five α-galactosidases were discovered, which is interesting because none of them had variable abundance across developmental stages. On the other hand, only one (*A0A8B7MUD2*) of the eleven β-glucosidases discovered showed increasing abundance throughout the developmental stages.

The cotyledonary petiole was also shown to contain a wide variety of enzymes involved in the digestion of different cell wall polysaccharides, such as cellulose, callose, xyloglucans, galactanos, galactomannans, glycoproteins, and pectins. Since these enzymes are present at all of the aforementioned developmental stages, the seed reserves may be transferred to the growing cotyledonary petiole and undergo additional metabolism there rather than being completely digested within the haustorium.

5.3.2 Mobilization of protein and lipid bodies

In the cotyledonary petiole of developing *C. prunifera* different types of proteases were identified which most prominent are serine and cysteine proteases. Along with its role in programmed cell death cysteine proteases are also involved in hormone synthesis and degradation, protein maturing, and nutrient mobilization (LEMOS ROCHA et al., 2017). The identified proteases in cotyledonary petiole were not identified as differentially abundant, In morphological studies there is no evidence of PCD in early stages however later stages show PCD in some specific locations, however During the developmental process dPCD (developmental programmed cell death) the functionality of hollow xylem tubes for the transport of waters is related to controlled and precise death of these tissue, which is a key requirement for the terrestrial habitats colonization (ESCAMEZ; TUOMINEN, 2014). The periodic cell death on the specialized location of the root-cap-like structure of cotyledonary petiole resembles a study on *A. thaliana* which hypothesizes that cell death in root caps is an indication of where the lateral roots will originate in the future (XUAN et al., 2016).

To sum up, proteome analysis of the cotyledonary petiole in growing *C. prunifera* has shown the presence of a variety of proteases, especially serine and cysteine proteases (caspases). The discovery of these proteases supports their involvement in several activities, including programmed cell death, hormone synthesis and degradation, protein maturation, and nutrition mobilization, even if their differential abundance was not found. Early morphological studies did not reveal any indication of programmed cell death, but subsequent stages revealed programmed cell death in particular areas. The precise and regulated death of tissues, such as the hollow xylem tubes, which are vital for water transport and the colonization of terrestrial habitats, depends on this developmentally programmed cell death. PCD is a mechanism involved in various developmental and physiological processes in plants, and it can play a role in tissue remodeling and the formation of specialized structures during seed development. The cotyledonary cavity formation during seedling development may be due to programmed cell death.

5.4 Growth Regulators Synthesizing proteins

Plants' growth hormones are chemically a diverse group of compounds and their slight increase and decrease has a very high impact on the growth and development of the plant. Many proteins that are involved in the biosynthesis of different growth hormones were identified only in the cotyledonary petiole of selected developmental stages of *C. prunifera*. As these proteins are not identified as differentially abundant among the proteome of the selected developmental stages of the cotyledonary petiole of *C. prunifera* (supplementary table IV), this might be because of the high abundance of other proteins which makes its slight change in abundance un-noticeable. Studies showed that in palm trees (macaw palm) hormones like GA, cytokinin, and ABA play a crucial tissue-specific role in dormancy control, seed germination, and cotyledonary petiole development (RIBEIRO et al., 2015).

Salicylic acid (SA) is derived from chorismate by the action of phenylalanine ammonia-lyase (PAL) (SHINE et al., 2016). The enzyme involved in the conversion of trans-cinnamic acid to benzoic acid which further leads to SA was not identified in the present study, however, PAL (phenylalanine ammonia-lyase) catalyzes the deamination of phenylalanine to produce trans-cinnamic acid was identified in the current study, which indicates the possible biosynthesis of SA (LEFEVERE, H et al., 2020). Besides its role in plants' defense against biotic stress, it plays an active part in abiotic stress conditions such as drought, chill, and salinity (BAGAUTDINOVA et al., 2022). Recent studies have also shown its involvement in cell expansion and division during growth and development (LI et al., 2022). In the current study phenylalanine ammonia-lyase 3 (*A0A0R6HKZ0*) was identified in the later three stages which indicates its possible role in cell division and expansion in newly developing cotyledonary petiole and embryonic axis.

Allene oxide synthase 1 (*A0A6I9QLG1* and *A0A8B7C668*) and allene oxide synthase 2 (*A0A6I9QU69* and *A0A8B7BJC6*) were identified in all the stages of developing cotyledonary petiole which is involved in the first step of biosynthesis of jasmonic acid from lipoxygenase derived hydroperoxides of free fatty acids (SIVASANKAR; SHELDRICK; ROTHSTEIN, 2000). A key enzyme for jasmonate acid biosynthesis *Allene-oxide cyclase* (*A0A8B7C604* and *A0A6I9RDF5*) was also identified. Which plays an active role in biotic and abiotic stress conditions (SUN et al., 2020). This lipid-derived plant growth hormone is involved in the regulation of growth in both biotic and abiotic stress conditions (SIDDIQI; HUSEN, 2019). Jasmonic acid is also involved in plant growth and development (WASTERNACK, 2007). A study on soybeans showed that plants with overexpressed jasmonic

acid gene showed narrow and elongated leaves whereas the lateral roots development was also stimulated (XUE; ZHANG, 2007) Which indicates the involvement of jasmonic acid in plant morphologic regulation.

Spermidine (a specific type of polyamine with three amino groups) is synthesized from putrescine by the action of spermidine synthase (*A0A8B7BKF1*), which upon the action of spermine synthase (*A0A8B7BRG2, A0A6J0PFR0*) is converted to spermine (EFROSE et al., 2008). Polyamines are not only a protective compound to be involved in the defense of plants against biotic and abiotic stress but also play some role in plants' growth and development by its involvement in cell division and differentiation especially in roots apexes and during the formation of lateral and adventitious roots (CHEN et al., 2019). Polyamines (PA) are involved in diverse processes in plants including cell proliferation, cell signaling, gene expression, and membrane stabilization, their depletion can cause decreased root growth and can even be lethal for the plants (KUSANO et al., 2008). *A. thaliana* mutated arginine decarboxylases which are a precursor for PA biosynthesis were observed with stunt growth of seedlings, which upon treatment with PA was reverted (SÁNCHEZ-RANGEL et al., 2016). All these findings indicate the crucial role of PAs in the growth and development of seedlings and their identification in the developing cotyledonary petiole further justifies its function.

Ethylene is synthesized by the oxidation of 1-aminocyclopropane 1-carboxylic acid, this reaction is catalyzed by 1-aminocyclopropane-1-carboxylate oxidase (POLKO; KIEBER, 2019). In the current study, three different isoforms for 1-aminocyclopropane-1-carboxylate oxidase (*A0A8B7BP73, A0A8B9AHC4,* and *A0A8B7CQC1*) were identified. This gaseous hydrophobic plant hormone can easily enter all types of cells and is responsible for growth and developmental inhibition and acceleration depending upon its concentration as well as plant species. Slow-growing plants *Poa* (*Poa alphina* and *Poa compressa*) showed more sensitivity to its concentration by inhibition of leaf elongation while in low concentration the faster-growing plants showed slight inhibition in their leaf elongation but slow-growing plants showed an accelerating effect on their leaf elongation (FIORANI et al., 2002). The morphoanatomical analysis of the developmental stages of *C. prunifera* shows the elongation of the cells in the cotyledonary petiole and the embryonic axis also shows cataphyll elongation along the time, furthermore, the ethylene synthesizing proteins were not identified as significant abundant proteins during quantitative proteome analysis of these stages which favors the findings of (FIORANI et al., 2002). Similar results were also found when ethylene over-producing *A. thaliana* plants were found dwarf with reduced growth, but increased growth as

observed upon the mutation of ethylene signaling pathway positive regulators (FENG; LIU; XIAO, 2015).

In bacteria tryptophane is converted to 3-indole propionic acid (IPA), which upon the action of indol-3-pyruvate decarboxylate is converted to indol-3-acetaldehyde, the oxidation of indol-3-acetaldehyde (IAD) by the action of indole-3-acetaldehyde oxidase forms indol-3-acetic acid (MANO; NEMOTO, 2012). The identification of indole-3-acetaldehyde oxidase (*A0A8B7BG33*) in all the developmental stages and indole-3-acetaldehyde oxidase (*Q7G192*) in the first two stages of *C. prunifera* indicates the possible synthesis of (IAA) by (IPA) pathway, which is for the first time reported in this study for family Aceraceae. This first discovered auxin is involved in all types of developmental processes including root development and embryogenesis (ZHAO, 2018). The level of this plant growth regulator is higher in undifferentiated cells and is involved in cell elongation, division, and differentiation (BALZAN; JOHAL; CARRARO, 2014). A low concentration of (IAA) promotes seed germination and development while high concentrations inhibit germination (HAIWEI et al., 2016).

Cytokinin is activated by the action of 5'-nucleotidase (*A0A6J0PBJ1*, *A0A6I9QVQ7,* and *A0A8B7CTI5*) (FRÉBORT et al., 2011). Conditional overexpression of LOG genes in *A. thaliana* showed an increase in cell division in embryonic and leaf vascular tissues with delayed leaf senescence (KUROHA et al., 2009). During the early stages of leaf formation, the shoot apical meristem growth capability is sustained by cytokinin and provide stem cell to leaf primordia. It also promotes cellular expansion and cell division during leaf development and expansion stages (WU et al., 2021). It has been discovered that some symbiotic microorganisms also secrete this adenine-derived small molecule phytohormone which is involved in almost all the growth and developmental processes to alter plant metabolism as it has a regulatory role in plant interaction with the biotic environment (NEIL EMERY; KISIALA, 2020). To become photosynthetically independent, the greening of cotyledon is one of the most important post-germinative events, cytokinin plays a vital role in this phenomenon by antagonizing abscisic acid (GUAN et al., 2014). In the case of *C. prunifera* the cataphyll emergence and its greening might be the evidence of this function.

Xanthine dehydrogenase (XDH) (*A0A8B9A9L8*) also plays an important role in plant growth and development by taking part in a variety of physiological reactions (KATALIN BARABÁS et al., 2000). The inactivation mutation of xanthine dehydrogenase shows low growth and senescence during the tiller initiation stage (XU et al., 2022) indicating its role in

growth and senescence. This approximately 300 kDa homodimer protein catalyzes pterins, purines, and aldehydes along with their natural substrates xanthine and hypoxanthine, and is also involved in various cellular processes and cell death, to provide ROS for all these processes XDH has the ability for the production of H_2O_2 ions and superoxide ions, all these activities were noticed in senescence pea leaves by (PASTORI; DEL RÍO, 1997). The identification of XDH in the cotyledonary petiole of developing seedlings of *C. prunifera* indicates its role in stress tolerance and plant defense and it might be involved in the senescence of root-cap-like structure of developing cotyledonary which was observed in the anatomical analysis.

In *A. thaliana* and tomato (*S. lycopersicum*) it was shown that geranyl geranyl diphosphate synthase (*A0A6I9Q9M2*) is involved in the biosynthesis of gibberellins and that the pathway of other terpenoid molecule is different from gibberellins biosynthesis (VAN SCHIE; HARING; SCHUURINK, 2013). The *AtGPS* silencing in tomato and *A. thaliana* caused a decline in gibberellic acid content and the ultimate results were extremely dwarf plants, indicating the role of GPS in the biosynthesis of gibberellic acid (VAN SCHIE et al., 2007). The silencing of farnesyl pyrophosphate synthase two genes (*FPS1* and *FPS2*) in *A. thaliana* causes the death of the embryo, however silencing after germination causes a delay in developmental processes and chlorosis in leaves and roots indicating its role in seedling development (MANZANO et al., 2016). The tetracyclic diterpenes synthesized by gibberellin have a strong influence on stem elongation, leaf expansion, and seed germination (YAMAGUCHI, 2008). The deactivation of GA is a complex process and the enzymes involved in this process are known as GA 2-oxidases, The identification of three enzymes of 2-oxoglutarate-dependent dioxygenase AOP1.2 (*A0A8B7C6Z1, A0A6I9RFQ4,* and *A0A8B9AVY4*) is the indication that GA is not completely functional and that they are synthesized for the later use (LESTER et al., 1999). GA is believed to have a direct influence on plant shoot elongation by strong regulation of cell growth (CASTRO-CAMBA et al., 2022). The breakage of seed dormancy and initiation of seed germination and seedling development is an important stage in plant livelihood in which GA plays a vital role. In monocots especially in palms the complex embryo has a very special way of germination and post-germination pattern in which seed germination and seedling development are important stages of plant life. In *A. aculeata* the dormancy breaking was associated with the removal of the operculum and an increase in abundance of GA and CKs with a decrease in ABA in cotyledonary petiole (RIBEIRO et al., 2015).

In conclusion, the analysis of particular cotyledonary petiole developmental stages in *C. prunifera* showed the presence of several proteins essential to the synthesis and control of plant growth hormones. Their small differences in abundance were likely covered up by the samples' high abundance of other proteins. Several key enzymes related to the biosynthesis of different growth hormones were identified, indicating their potential roles in cell division, expansion, and differentiation during the development of the cotyledonary petiole and embryonic axis. These enzymes included phenylalanine ammonia-lyase (PAL), allene oxide synthase (AOS), allene oxide cyclase (AOC), spermidine synthase, 1-aminocyclopropane-1-carboxylate oxidase (ACO), indole-3-acetaldehyde oxidase (IAAO), and 5'-nucleotidase.

5.5 CP exclusive proteins involved in growth and adaptation

Along with proteins involved in growth regulators biosynthesis and the proteins which are differentially abundant in selected developmental stages of *C. prunifera* some exclusive proteins in cotyledonary petiole are involved in important biological processes, some of which are mentioned below.

Different isoforms of tubulins alpha and beta chains (*A0A8B8ZY68, A0A6I9R0S5*, and *A0A8B7BXP1*) were identified which are involved in the formation of microtubules and play a vital role in cell division, intracellular trafficking and communication and cell wall deposition (BREVIARIO; GIANÌ; MORELLO, 2013a). which is the indication of continuous cell division in cotyledonary petiole which was also confirmed in morpho-anatomical analysis.

Four pyruvate kinase isoforms (*A0A8B7BIU4, Q9FFP6, A0A8B8J5E0,* and *A0A8B7BVZ6*) which are involved in biosynthesis of essential metabolites for growth and development by the catabolism of storage reserves, PKP1 mutants in *A. thaliana* showed accumulation of soluble sugars, impaired storage oil metabolism and seed germination inhibition (ANDRE; BENNING, 2007). This indicates its presence and importance for the developing seedling.

Serine hydroxymethyltransferase (*A0A8B7BHE2*) catalyzes the biosynthesis reaction of glycine from serine which plays a key role in one-carbon metabolism (NONAKA et al., 2019). Which is a series of interlinked reactions providing C1 units involved in the biosynthesis of amino acids, creatine, and DNA (CLARE et al., 2019). Glycine betaine is also involved in combating stress by reactivating dysfunctional metabolic pathways thus playing a vital role in plant growth during stress conditions (ANNUNZIATA et al., 2019). The

identification of these enzymes is the indication of plants' adaptability to stress conditions from the beginning.

A set of eight different types of alcohol de hydrogenases were identified in the cotyledonary petiole of selected developmental stages of *C. prunifera* which play a vital role in plants' growth, development, and adaptations (JIN et al., 2016). These enzymes are involved in the production of ethanol by catalyzing acetaldehyde with the oxidation of $NADH^{+}$ to NAD. Ethanol production is believed to be involved in minimizing the harmful effects caused by the accumulation of acetaldehyde (GONÇALVES; LIMA; FERNANDES; BORGES; BUCKERIDGE, 2010). Cell wall lignification is a very complex process that involves different routes one of them is monolignol biosynthesis (VAVILALA; GHAG; D'SOUZA, 2019). This biosynthesis is completed by the synthesis of cinnamyl alcohol from cinnamaldehydes by the action of cinnamyl alcohol dehydrogenases (*A0A8B8ZU34*, *A0A8B8ZII9,* and *A0A6I9RA95*) (TRONCHET et al., 2010). A study on *G. soja* suggested the indirect involvement of cinnamyl alcohol dehydrogenases in the biosynthesis of salicylic acid and thus taking a part in plant resistance (XUN et al., 2022). Leaf radish-brown color is the indication of its reduced activity (PILATE; DEJARDIN; LEPLE, 2012). This class of enzymes plays a vital role in plant survival and seed germination under stress and anaerobic conditions (SHEN et al., 2021). The identification of this group of proteins in selected developmental stages is an indication of their diverse role in plant growth, germination, and resistance.

Three isoforms of prohibitin (*A0A6I9QLV1*, *A0A6I9RYU0,* and *A0A8B8ZHT9*) were also identified in the present study. This is a two-subunit protein, In *N. benthamiana* it was observed to be involved in mitochondrial biogenesis, senescence, cell cycle progression, and apoptosis. The suppression of this gene resulted in a decrease in mitochondrial number and mass with growth inhibition, cell death, yellowing of leaves, and a 10-20fold increase of reactive oxygen species (AHN et al., 2006). In *A. thaliana* its expression was observed in roots and shoots proliferating tissues. Its loss of function causes swelling and a decrease in mitochondrial number, deliberate cell division, a decline in meristematic cell production, and reduced cell expansion (VAN AKEN et al., 2007). Its role as a positive regulator of genes that are involved in seedling growth in the presence of ethylene was also observed in a study on *A.* thaliana (CHRISTIANS; LARSEN, 2007). The cell elongation that has been observed in the morpho-anatomical analysis of cotyledonary petiole may probably be correlated with the function of this enzyme.

Coatomer is another set of protein which consist of seven subunits. In the current study, two isoforms for alpha, beta, and gamma subunits were identified. In *N. benthamiana* the virus-induced gene silencing for these proteins showed an indication of cell death in leaves along with irregular cell plate formation during cell division (cytokinesis) and altered structure of Golgi complex which indicates its direct influence on plant growth (AHN et al., 2015). This group is supposed to be involved in protein transport throughout the membrane system (PAUL; FRIGERIO, 2007). It is also believed that they are maintaining the specific proteins in their respective organelles thus retaining the original property of the organelles (GAO et al., 2014). The loss of function analysis of the beta subunit of this protein in *A. thaliana* showed high sensitivity to salt stress and overall impaired growth under standard conditions. Furthermore, its relation with its bi-directional protein transport between the Golgi complex and endoplasmic reticulum was also observed (SÁNCHEZ-SIMARRO et al., 2020). The identification of these proteins showed its role in the transport of proteins inside and within the cells thus showing its association in overall seedling development.

The biosynthesis of flavonoids which is considered one the most important plant secondary metabolites is initiated by the action of *chalcone synthase* (WANG, P. et al., 2018), (*A0A6I9QGE2* and *A0A6I9RNW1*) was also identified. This enzyme plays a vital role in plant development and growth regulation. The overexpression of this enzyme in tobacco showed improved seed germination under drought stress (HOU et al., 2022). Keeping in view the natural habitat of *C. prunifera* the presence of this enzyme shows its involvement in seed germination and seedling development under stress conditions. In a study on *Capsicum annuum L.*, the *chalcone synthase* showed an increase in concentration upon treatment with drought and UV-B treatment further confirming its role in stress adaptation (RODRÍGUEZ-CALZADA et al., 2019). Proteomic analysis of tea plants under drought stress showed down-regulated *chalcone synthase* which also indicates its role in stress adaptation (GU et al., 2020).

Six different isoforms of *Xyloglucan endotransglucosylase/hydrolase* were identified in the selected developmental stages of the cotyledonary petiole proteome. This enzyme is involved in modifying the cell wall by grafting of xyloglucan chains to oligosaccharides or other glucans thus playing a vital role in plant growth and developmental regulation (MARIS et al., 2011). A study on *A. thaliana* revealed that this enzyme is involved in cell proliferation and tissue reunion in incised stems (PITAKSARINGKARN et al., 2014). In *Zea mays L.* it was observed that the aerenchyma causes cell wall degradation followed by cell lysis which was associated with the high concentration of *Xyloglucan*

endotransglucosylase/hydrolase. On these bases, it was also hypothesized that this protein is involved in development and response to environmental stimuli (SAAB; SACHS, 1996). The role of *Xyloglucan endotransglucosylase/hydrolase* in xyloglucan mobilization of seed was also reported (FANUTTI; GIDLEY; GRANT REID, 1993). In a study on *C. arietinum* the function of this protein was noticed in the elongation of parenchyma and vascular tissues furthermore its role in epicotyl elongation and developing tissues was also noticed (JIMÉNEZ et al., 2006). A study on the germinating seed of *Lycopersicon esculentum* Mill. Showed high expression of *Xyloglucan endotransglucosylase/hydrolase* mRNA which disappeared after the complete emergence of radicle, this study also predicted the role of this enzyme in the digestion and weakening of endosperm cap during germination (CHEN; NONOGAKI; BRADFORD, 2002). In the cell wall of elongating cells of the epidermis and developing roots high action of *Xyloglucan endotransglucosylase/hydrolase* was observed (VISSENBERG et al., 2003). The identification of this enzyme indicates its role in the growth and development (ZHANG et al., 2022) of selected developmental stages by its diverse role in cell elongation, cell wall assembly (SHARPLES; NGUYEN-PHAN; FRY, 2017), and other behaviors.

A set of ten different isoforms of subtilisin-like proteases were also identified in the developing cotyledonary petiole. These enzymes also known as subtilases or serine proteases perform a broad-spectrum biological activity in plants ranging from stress management and response to signal cascades and plant development (FIGUEIREDO; SOUSA SILVA; FIGUEIREDO, 2018). It was also reported in a study on rice (mutant apical panicle abortion 1331) plant that these proteins are also involved in the biosynthesis of cuticles in ROS-mediated PCD and biosynthesis of spikelet (ALI et al., 2022). Serine proteases are one of the important classes of proteases that play a vital role in the defense, stress adaptation, physiology, and development of the plant (FIGUEIREDO; MONTEIRO; SEBASTIANA, 2014). Aspartate, serine, and histidine are the catalytic triad of serine proteases (DODSON; WLODAWER, 1998). This multifunctional protein is most common in the plants of the Arecaceae family which take part in many processes such as cell wall manipulation, seed development, development of epidermis and its pattern formation, PCD, peptide growth factors processing, response to biotic and abiotic factors and adoption of specific physiologic roles in plants (SCHALLER; STINTZI; GRAFF, 2012). In a study on exploiting the secondary growth of *A. thaliana* subtilisin-like serine proteases were found to play an important role in the differentiation of xylem (ZHAO et al., 2000). Which is justifying the same role of this enzyme in the developing cotyledonary petiole.

Actin bundles not only play a vital role in providing structural integrity to the cell but also play an important function in the cellular cytoskeleton, mechanosensing, and intracellular transport (CHANDRASEKARAN; UPADHYAYA; PAPOIAN, 2019). In the current study, two actin-bundling proteins villin-2 and villin-3-like isoform X1 (*A0A8B7C3P5* and *A0A8B7C1G4*) were identified. Actin plays an essential role in the growth and development of the plant by taking a vital part in various physiological processes such as cell growth, cytokinesis, cell division, and intracellular trafficking (SZYMANSKI; STAIGER, 2018). In *A. thaliana* double mutant of villin-2 and villin-3 showed twisted roots, stems, and leaves which were associated with the differences in cell length thus indicating its role in the regulation of cellular elongation and expansion (VAN DER HONING et al., 2012). The presence of these proteins shows that they are critical for the cell shape and physiology (BARTLES, 2000) and may probably perform their function in cellular elongation and expansion and overall cellular organization in developing the cotyledonary petiole and embryonic axis.

The protein *Methylmalonate-semialdehyde dehydrogenase* (*A0A6I9S508*) which is regulated by gibberellin was also identified in the proteome of developing cotyledonary petiole. The expression analysis of this protein showed 7 folds of more protein in roots and leaf sheath and areas that are undergoing growth and tissue differentiation than wild type. Transgenic plants showed slight inhibition in leaf sheath than control plants which indicates its role in the elongation of leaf sheath and root development (TANAKA et al., 2005). The identification of this protein provides a better understanding of the unique growth pattern of leaf sheath (embryonic axis) in the cotyledonary of *C. prunifera*. This protein plays a vital role in the synthesis of acetyl-CoA and propionyl-CoA from methylmalonate-semialdehyde and malonate-semialdehyde respectively. Its knock-out showed poor cell expansion and seed germination (MACIEJ SERDA et al., 2013).

Plant growth, development, and defense are strictly controlled by the *Mitogen-activated protein kinase* (MAPKs) cascade (JIANG et al., 2022). In the current study, 3 *Mitogen-activated protein kinases* were identified (*A0A6I9R805*, *A0A8B7C2I9*, and *Q9FJV0*). These proteins also take part in controlling cell division and differentiation thus taking an active part in plant growth and development(XU; ZHANG, 2015). Another important function of MAPKs is receiving and translating extracellular stimuli and transferring them to target cellular and developmental responses (KOMIS et al., 2018). The role of MAPKs cascade in defense response has been reported largely (ZHANG et al., 2018). The identification of these proteins

indicates the ability of *C. prunifera* to the biotic and abiotic stress and makes it well adapted to their environmental conditions.

The natural habitat of *C. prunifera* exposes it to drought stress. In *N. tabacum L.* the overexpression of *Major latex proteins like protein-423* showed increased drought tolerance which was associated with an increase in abscisic acid transcription level, decreased ROS accumulation, and membrane damage whereas overexpression of stress-related genes (LIU et al., 2020b). In the current study, two *Major latex proteins like protein-423* (*A0A8B8ZM75* and *A0A6I9QQ86*) are also identified. In *A. thaliana* leaf curling responsiveness (LCR) and F-box proteins were regulated by micro-RNA394 which showed alteration in the organization of epical meristems and polarity of leaves. The proteomics analysis of altered apices showed several identifications of MLP-423. The reduction of these MLPs in transgenic *A. thaliana* showed various developmental problems along with altered leaf morphology and patterning, defects in the shoot apex, and ultimately premature death of the plant (LITHOLDO et al., 2016). This indicates the vital role of MLP-423 in plant growth and development.

The Kinesin superfamily is responsible for the unidirectional transport of mRNA, protein complexes, and membranous organelles. These proteins play an important role in signal transduction, morphogenesis, mitosis, cell growth, and cell division and play a vital role in the biosynthesis of gibberellins (LI; XU; CHONG, 2012). In the current study, three *kinesin-like protein KIN-7I isoform X2* were identified (*A0A8B9A6U6*, *A0A6I9SK02,* and *A0A6I9RMF3*). A study on MDKIN2 mutants of *A. thaliana* showed an overall mal development including embryo, endosperm, and pollens. This indicates its important role in cell division and overall plant development (GALINDO-TRIGO et al., 2020). These proteins were observed to be tightly bound with mitochondria in *A. thaliana* and were believed to have associated functions, furthermore, their effect on growth and development was also noticed (NI et al., 2005). The double mutant genes for these proteins in *A. thaliana* showed abnormal pollen formation and impaired cytokinesis and cell division which was associated with its role in the transport of vesicles during the formation of the cell plate (PAN; LEE; LIU, 2004). The identification of these proteins shows their vital role in organelle trafficking and role in physiology, cell elongation, growth, and overall development (CAI; CRESTI, 2012) of the cotyledonary petiole and embryonic axis.

Expansin is a protein involved in the loosening of cell wall polymers by reducing the adhesion of adjacent polysaccharides during cytokinesis and cell division thus playing a vital role in defining cell shape, size, and function (MAROWA; DING; KONG, 2016). In the

current study, three different isoforms of *Expansin* (*A0A8B7CWU5, A0A8B8ZRD7,* and *A0A8B7BEJ6*) were identified. Besides its role in softening or breakdown of the cell wall, it is also involved in abscission, pollination, fruit ripening, and seed germination (LI; JONES; MCQUEEN-MASON, 2003). The transgenic plants showed that this broad-ranged biologically active protein has an important role in cell expansion, growth and development, and abiotic stress response (CHOI; CHOB; LEE, 2006). Besides the loosening of the cell wall by weakening glucan-glucan binding, it is believed to be involved in the cell expansion and growth of root hairs (COSGROVE et al., 2002). This enzyme may hydrolyze the breakage of the cell wall by breaking hemicellulose-cellulose or cellulose-cellulose hydrogen bonds. Being the acidic PH optima, this protein may be involved in the softening of ripped fruit (HANDA; TIZNADO-HERNÁNDEZ; MATTOO, 2011). The developing cotyledonary petiole undergoes continuous cell elongation and cell division in all the parts including the embryonic axis as observed during morphoanatomical analysis, the identification of this enzyme, and its role and participation in this development.

Fasciclin-like arabinogalactan protein plays a variety of physiological functions in plants including cell wall biosynthesis, growth, development, and defense against abiotic stress. In the present study, five different isoforms of Fasciclin-like arabinogalactan were identified. The real-time qPCR analysis of seedlings, leaves, and roots of the *Cannabis* genome showed the differential expression in younger regions and regions of elongation during this study it was also deducted that this enzyme is involved in the formation of secondary cell wall (GUERRIERO et al., 2017). A study on *Nicotiana benthamiana* showed the up-regulation of the gene responsible for this protein family in qPCR analysis during turnip mosaic virus (TuMV) which suggested the role of Fasciclin-like arabinogalactan in pathogenic response (WU et al., 2020). In *A. thaliana* mutant *fla16* showed an alteration in the biochemical process including reduced length of the stem and reduced level of cellulose (LIU et al., 2020). In *A. thaliana* the phytochrome abscisic acid causes a rapid decrease in the abundance of this protein due to its role in ABA-mediated signaling pathways and possible significance for plant development and stress tolerance, FLA1 mRNA abundance declines sharply in response to ABA. Furthermore, during the development of shoot and callus the transcripts of these proteins differed which indicates their involvement in shoot development and competence acquisition (JOHNSON et al., 2003). The identification of this class in the developing cotyledonary petiole of *C. prunifera* is an indication of its involvement in secondary cell wall synthesis and development of the embryonic axis.

SKP1-like protein targets ubiquitinate and plays a vital role in plant biology. In *A. thaliana* seed germination and development this protein was found significantly regulated whereas found up-regulated during abiotic stress. Apart from its participation in the formation of SCF complex (SKP-cullin-F-box protein) its knockout and overexpression showed a positive influence on seed germination and seedling growth and development (RAO et al., 2018). The microprojectile bombardment with N-terminally truncated LSK1-LSK3-GFP (green fluorescent protein) showed significant retardation in the elongation of the pollen tube which shows its vital role in 26s-proteosome mediated pathway which an important role in pollen tube elongation (CHANG et al., 2009). In *G. max L.* this enzyme showed high expression in roots but was simultaneously induced by salicylic acid (SA), jasmonic acid, and abscisic acid (ABA), drought, and low-temperature treatment. Also, transgenic *N. tabacum* overexpression showed greater tolerance to drought and high salinity (CHEN et al., 2018). The identification of this protein in the current study indicates its critical role in the overall growth, development, and stress tolerance of the plant through its participation in a wide range of cellular processes and pathways.

Laccases are copper-containing phenol oxidases that are distributed widely in plants' genomes and produce lignin from the oxidation of monolignols which plays a vital role in plant growth, development, and resistance to biotic and abiotic stress (BAI et al., 2023). In the current study, 3 isoforms of Laccases were identified (*A0A8B7CQI5, Q9FJD5,* and *A0A6I9R289*). In *A. thaliana* alternation in the Laccase gene causes severe growth arrest, indehiscent anthers, roots with a narrow diameter, and arrest in vascular development with a lake of lignification (ZHAO et al., 2013). This copper-containing protein is involved in the reduction of multi-copper oxidases to water without the production of any harmful substance thus contributing to the protection and development of the plants (JANUSZ et al., 2020). High expression of Laccases was noticed in the roots and stem of *C. sinensis* and its expression increased upon the treatment with gray blight. Furthermore, the increase in expression of this protein was noticed with abiotic (drought and cold) and biotic stress (fungus and insects) via transcriptome analysis (ZHU et al., 2023). This indicates its important role in growth, development, and stress tolerance in *C. prunifera*.

UBX domain-containing protein (*A0A8B7D4Y2* and *A0A6I9S190*) is involved in the negative regulation of GAs. Thus, controlling seed germination, cell elongation, and division. Loss of function mutants in *A. thaliana* showed a rapid increase in germination, flowering, and root elongation (HAUVERMALE et al., 2022). This AAA-ATPase is also

known to be involved in the regulation of the structure and function of oligomeric structure in *A. thaliana* AtCD4C8 (RANCOUR et al., 2004). This protein is also involved in the regulation of cell division control proteins 48 and has a fast response mechanism (ZHANG; VANCEA; AROLD, 2022). This protein is essential for plants' quick adaptation to environmental changes and protein homeostasis. The identification of this protein in the proteome of *C. prunifera* indicates its role in plant adaptation in the rapidly changing habitat of the plant Furthermore, it is an inhibitor of GAs, which means that it has some role in emergent organogenesis and controlled development of embryonic axis inside the cotyledonary petiole.

To sum up, the proteins shown to be involved in the growth and development of the cotyledonary petiole in *C. prunifera* have a variety of roles that they play in the growth, metabolism, and adaptation of plants. These proteins play important roles in a variety of biological functions, including cell division, intracellular trafficking, metabolism of store reserves, stress response, protein transport, production of secondary metabolites, cell wall modification, and cytoskeletal architecture. There are a total of 1437 proteins that are exclusive to cotyledonary petiole compared with haustorium and are involved in various important biological functions contributing directly or indirectly to the growth and development of the developing seedling.

5.6 Common differentially abundant proteins in CP and Haustorium

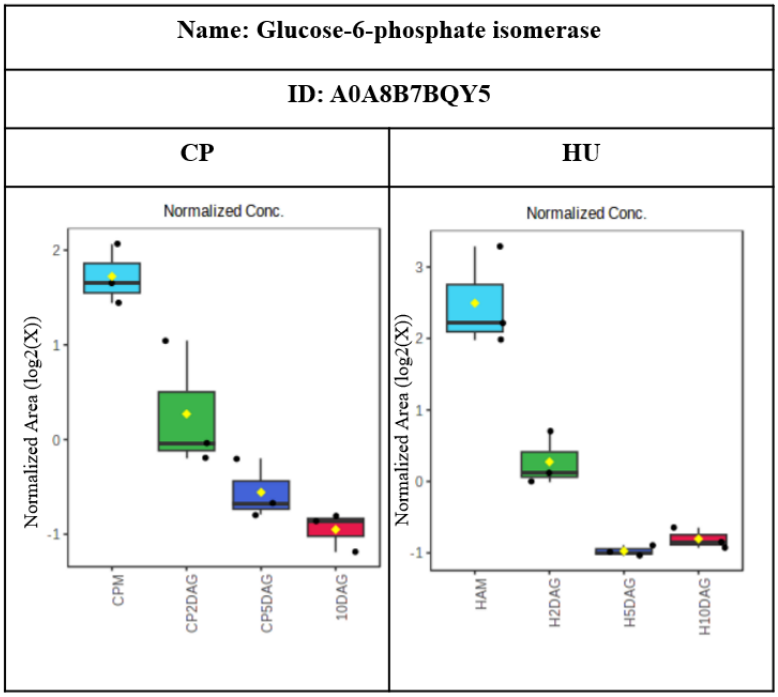
The common differentially abundant proteins present in the cotyledonary petiole and haustorium of *Copernicia prunifera* will be discussed and analyzed in this section. By contrasting the abundance patterns in the selected stages within these two different tissues.

5.6.1 Enzymes of the glycolytic pathway

In plants during glycolysis, the hexoses are oxidized for the production of ATP and pyruvate which is used as a precursor for different anabolic processes (ANOMAN et al., 2016). In the second step of glycolysis, fructose-6-phosphate is produced by the action of *Glucose-6-phosphate isomerase* (*A0A8B7BQY5*) on glucose-6-phosphate (FERMO et al., 2019). The decreased abundance of *Glucose-6-phosphate isomerase* in these tissues could be a result of the depletion of endospermic reserves, which would then cause a fall in the enzyme's levels (Figure 23). During the oxidative pentose phosphate pathway (OPPP) the same enzyme is involved in glucose-6-phosphate regeneration (BAHAJI et al., 2015). In *Fusarium graminearum* reduced ATP production, glucose metabolism, pyruvate biosynthesis, septa formation, and hyphal growth were observed upon the mutation of the gene responsible for the production of glucose-

6-phosphate isomerase (ZHOU et al., 2021). Targeting plastids G6P of *N. tabacum* by *A. thaliana* cytosolic G6P causes disturbance in the starch degradation and accumulation. This enzyme acts as a one-way valve by preventing G6P backflow in the Calvin cycle (PREISER et al., 2020).

Figure 23- Shows the decreasing abundance of *Glucose-6-phosphate isomerase* in the selected developmental stages of cotyledonary petiole and haustorium of *C. prunifera*.



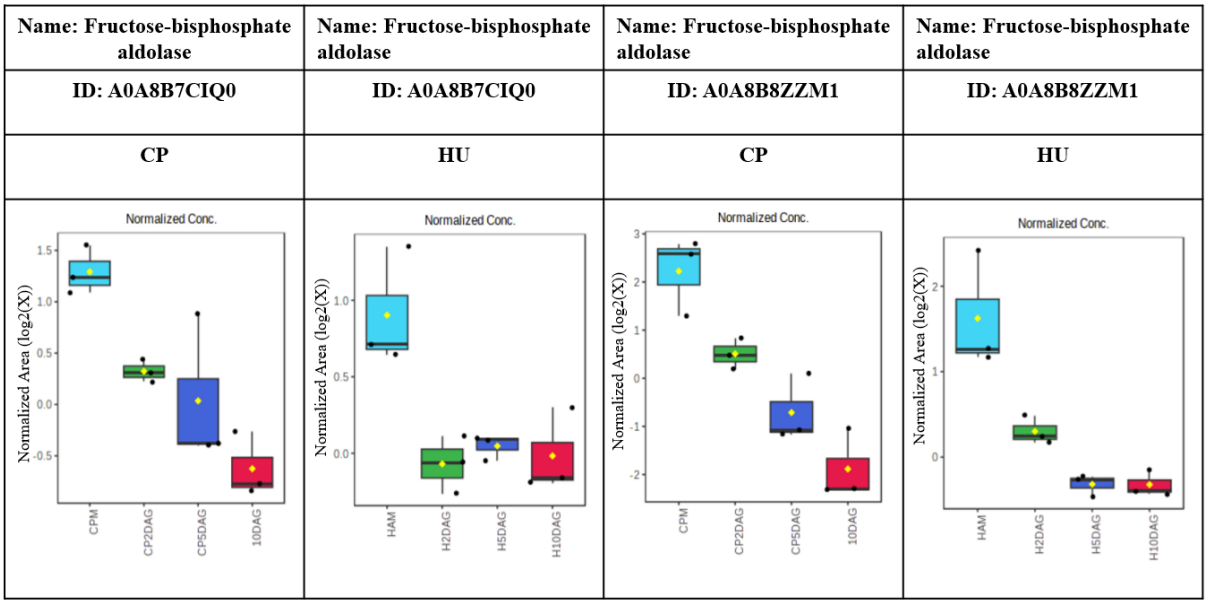
Source: Prepared by author

In conclusion, plants' oxidative pentose phosphate pathway (OPPP) and glycolysis both depend on the enzyme glucose-6-phosphate isomerase. It facilitates glycolysis by catalyzing the transformation of glucose-6-phosphate into fructose-6-phosphate. Additionally, it plays a role in the OPPP's regeneration of glucose-6-phosphate. Numerous metabolic activities, including ATP generation, glucose metabolism, pyruvate biosynthesis, septa development, and hyphal growth in fungi, have been linked to this enzyme, according to studies.

Fructose-bisphosphate aldolase (*A0A8B7CIQ0* and *A0A8B8ZZM1*) is another enzyme that is involved in glycolysis, Calvin-cycle, and gluconeogenesis thus playing a vital role in plant growth and development (LV et al., 2017). This enzyme is involved in the synthesis of dihydroxy-acetone-phosphate and glyceraldehyde-3-phosphate from fructose 1,6-diphosphate (RÓPOLO; FELIZIANI; TOUZ, 2019). In wheat, *Triticum aestivum* L. increased expression of this enzyme was noticed in roots and under biotic and abiotic stress. This enzyme is also believed to play a vital role in regulating the growth and developmental processes of

plants (LV et al., 2017). The identification and abundance pattern of these enzymes in all the selected developmental stages and tissues indicates not only their vital role in glycolysis but also their role in the overall growth and development of the plant (Figure 24).

Figure 24- Shows the decreasing abundance of fructose-bisphosphate aldolase in the selected developmental stages of cotyledonary petiole and haustorium of *C. prunifera*.



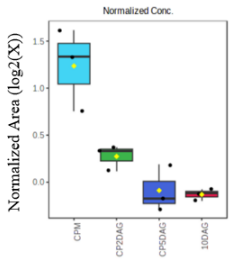
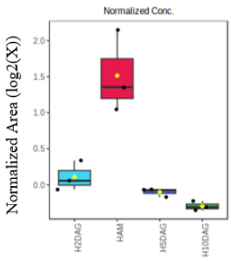
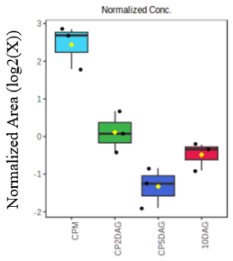
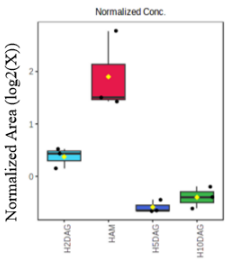
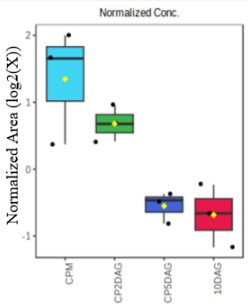
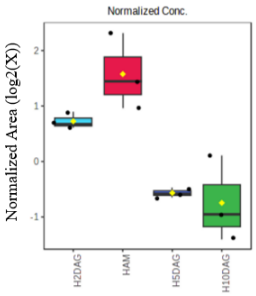
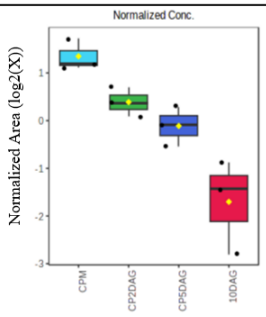
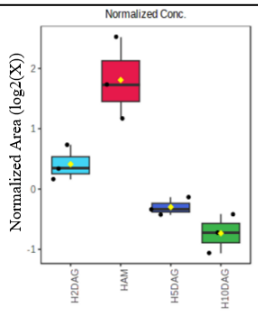
Source: Prepared by author

In conclusion, fructose-bisphosphate aldolase is a significant enzyme that is engaged in the Calvin cycle, glycolysis, and gluconeogenesis and is essential for the growth and development of plants. It contributes to crucial metabolic processes by catalyzing the synthesis of dihydroxy-acetone-phosphate and glyceraldehyde-3-phosphate from fructose 1,6-diphosphate. Additionally, it is thought that Fructose-bisphosphate aldolase is essential for controlling plant growth and development. Its significance in not just glycolysis but also the general growth and development of plants is further highlighted by the detection and consistent abundance pattern of this enzyme throughout all the selected developmental stages.

Pyruvate kinases (*A0A8B7CN09, A0A8B7BHH0, A0A6I9RCY2,* and *A0A6I9R5D3*) are involved in the biosynthesis of ATP and pyruvate thus playing a vital role in energy synthesis and cell metabolism regulation. Mutant *A. thaliana* showed poor embryo development and seed germination (BAUD et al., 2007). In plants besides being the important precursor for various products pyruvate also plays an important role in cellular respiration in the dark. Pyruvate can be made available for energy synthesis in three possible ways i.e., mitochondrial can synthesize its pyruvate from malate, it can be transported as pyruvate from the cytosol by mitochondrial pyruvate carriers or it can be converted to alanine in the cytosol and reverted to

pyruvate in mitochondria which shows its importance for plant life and that is the reason that by mutating any of two pathways in *A. thaliana* it didn't show any effect on the growth (LE; LEE; HARVEY MILLAR, 2021). In *A. thaliana* this enzyme controls the carbohydrate flow via a glycolytic pathway by its unique pattern of expression, interaction with different enzyme subgroups, and other regulatory properties (LE; LEE; HARVEY MILLAR, 2021). Mobilization of seed reserves for biosynthesis of essential metabolites and energy is important for seed germination and early growth. In *A. thaliana* mutants for *pkp1* showed impaired oil storage mobilization, hypocotyl elongation, and less chlorophyll and tocopherol (ANDRE; BENNING, 2007). In potato *S. tuberosum* mutant for *Pk* showed a decrease in alternative oxidases and pyruvate which upon external feeding with pyruvate was recovered, indicating the crucial role of this enzyme in the regulation of pyruvate and indirectly on alternative oxidases proteins (OLIVER et al., 2008). The gradual decrease of these enzymes shows the gradual decrease in the carbohydrate reserve of the seed (Figure 25).

Figure 25- Shows the decreasing abundance of pyruvate kinases in the selected developmental stages of cotyledonary petiole and haustorium of *C. prunifera*.

Name: Pyruvate kinases	**Name: Pyruvate kinases**	**Name: Pyruvate kinases**	**Name: Pyruvate kinases**
ID: A0A8B7CN09	**ID: A0A8B7CN09**	**ID: A0A8B7BHH0**	**ID: A0A8B7BHH0**
CP	**HU**	**CP**	**HU**
			
Name: Pyruvate kinases	**Name: Pyruvate kinases**	**Name: Pyruvate kinases**	**Name: Pyruvate kinases**
ID: A0A6I9RCY2	**ID: A0A6I9RCY2**	**ID: A0A6I9R5D3**	**ID: A0A6I9R5D3**
CP	**HU**	**CP**	**HU**
			

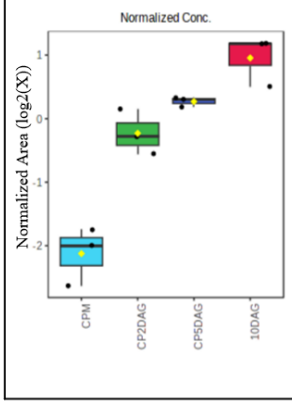
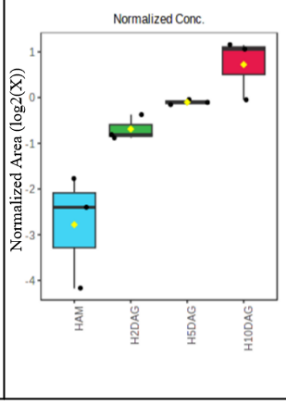
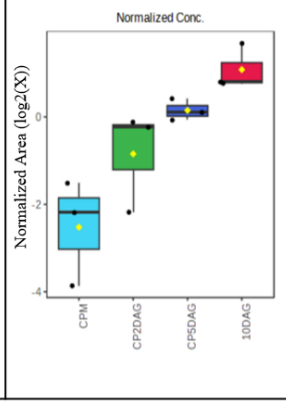
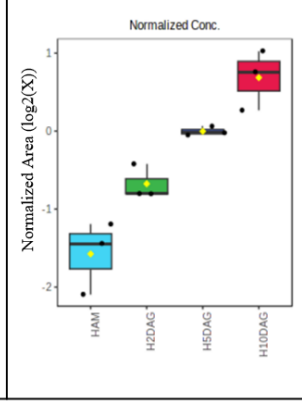
Source: Prepared by author

In conclusion, because they are involved in the synthesis of ATP and pyruvate, pyruvate kinases are crucial for the production of energy and the control of cell metabolism. They play a crucial role in many facets of plant growth and development. Pyruvate kinases are important for embryonic development, seed germination, oil storage mobilization, hypocotyl elongation, and the control of chlorophyll and tocopherol levels, according to mutant studies in Arabidopsis thaliana. Pyruvate kinases have a function in more than just energy generation; they also interact with different subgroups of enzymes and participate in several other regulatory processes. As a critical chemical involved in cellular respiration and a precursor for numerous metabolites, pyruvate is also regulated and made available by pyruvate kinases. Pyruvate kinases gradually drop during seedling development, indicating that the reserves of carbohydrates are being depleted.

5.6.2 Cytoskeleton proteins

Tubulins are involved in the synthesis of microtubules which play a vital role in growth, cell division, communication and intracellular trafficking, and cell wall deposition (BREVIARIO; GIANÌ; MORELLO, 2013). Microtubules play a vital role in cell division by guiding phragmoplast and spindle fibers function (ECKARDT, 2006). Microtubules also play an important function in morphogenesis by maintaining the organelles in their specific cellular locations and providing proper shape to the cell relevant to its function and tissues, They also help in cell wall assembly by controlling enzyme delivery for the synthesis of cellulose, it is also involved in transporting vesicles to the apex which are involved in growth (ONELLI; IDILLI; MOSCATELLI, 2015). In the current study tubulin alpha (*A0A6I9SG99* and *A0A8B7CQT9*) and beta (*A0A8B8ZPM6, A0A6I9SJ21, A0A6I9SEC3,* and *P29515*) were identified in all the selected stages of both cotyledonary petiole and haustorium. These subunits polymerize and form a heterodimer which results in the formation of microtubules thus playing a vital role in the formation of two daughter cells by contributing to cytoskeleton formation and cell division (MOTTA; SCHNITTGER, 2021). The increase in abundance in both tissues along the development indicates its role in continuous cell division and tissue differentiation (Figure 26).

Figure 26- Shows the increasing abundance of tubulin alpha chain in the selected developmental stages of cotyledonary petiole and haustorium of *C. prunifera*.

Name: Tubulin alpha	**Name: Tubulin alpha**	**Name: Tubulin alpha**	**Name: Tubulin alpha**
ID: *A0A6I9SG99*	**ID: *A0A6I9SG99***	**ID: *A0A8B7CQT9***	**ID: *A0A8B7CQT9***
CP	**HU**	**CP**	**HU**
			

Source: Prepared by author

In conclusion, tubulins are crucial proteins involved in the synthesis of microtubules, which are important for several cellular functions including growth, cell division,

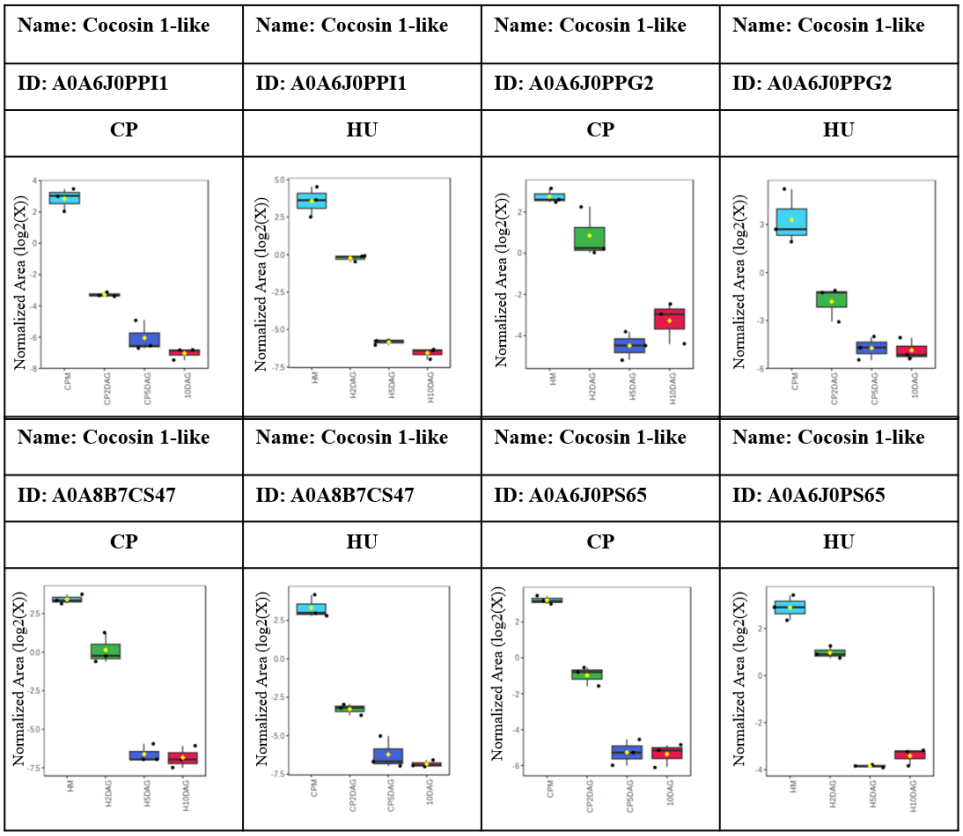
intracellular trafficking, and the formation of cell walls. By directing the activity of the phragmoplast and spindle fibers, ensuring appropriate chromosome segregation, and resulting in the production of two daughter cells, microtubules aid in cell division. Microtubules play a crucial role in morphogenesis as well as in retaining organelles in their precise cellular positions, giving cells structure, and facilitating tissue organization. By controlling the distribution of enzymes involved in cellulose synthesis, they also contribute to the construction of cell walls. Tubulin alpha and beta subunits have been found in both the cotyledonary petiole and the haustorium, indicating that they are continuously involved in cell division, tissue differentiation, and cytoskeleton creation. Both tissues' increased abundance during development provides more evidence for their critical function in these processes.

5.6.3 Storage proteins

Seed is not only the organ of propagation but also during development accumulates reserves for germination and post-germination seedling growth and development (MOUZO et al., 2018). In the present study four different isoforms of *Cocosin* 1 (*A0A6J0PPI1, A0A8B7CS47, A0A6J0PPG2,* and *A0A6J0PS65*) two isoforms of *vicilin-like seed storage protein* (*A0A8B7BPL4* and *A0A8B7CGP8*) and 63 kDa *globulin-like protein* (*A0A6I9QBZ9*) with decreasing in abundance in all the selected developmental stages of both cotyledonary petiole and haustorium of *C. prunifera* (Figure 27). The decrease in abundance of these proteins indicates their mobilization during germination which was also associated with seed vigor in a study on *Phaseolus vulgaris* (EHRHARDT-BROCARDO; COELHO; SOUZA, 2022). As the germination proceeds the abundance of hydrolases increases to utilize these reserves for the generation of carbon and nitrogen for post-germination processes (NASCIMENTO et al., 2020). The initial mobilization of these proteins starts with imbibition and their three-dimensional structure is usually cleaved at specific sites by cysteine proteases (TAN-WILSON; WILSON, 2012). In *C. prunifera* seed germination is a crucial process, The imbibed seed upon breaking the dormancy and post-germination period shows high sensitivity to its natural environmental conditions thus embryonic axis takes a long time in the cotyledonary petiole before the appearance of the first photosynthetic leaf. During this period growth and development are completely dependent on utilizing these seed reserves. A study on soybean mutants for seed storage proteins showed aberrant seedling establishment, post-germination processes, and delayed germination (WEI et al., 2020). In *J. curcas* pentose phosphate pathway, TCA cycle, glyoxylate cycle, and gluconeogenesis were found to be involved in the

mobilization of seed reserves mobilization (YANG et al., 2009). Most of the enzymes for these pathways were also identified in the current study which confirms this hypothesis.

Figure 27- Shows the abundance pattern of different isoforms of cocosin 1-like protein in different developmental stages of haustorium and cotyledonary petiole of *C. prunifera.*



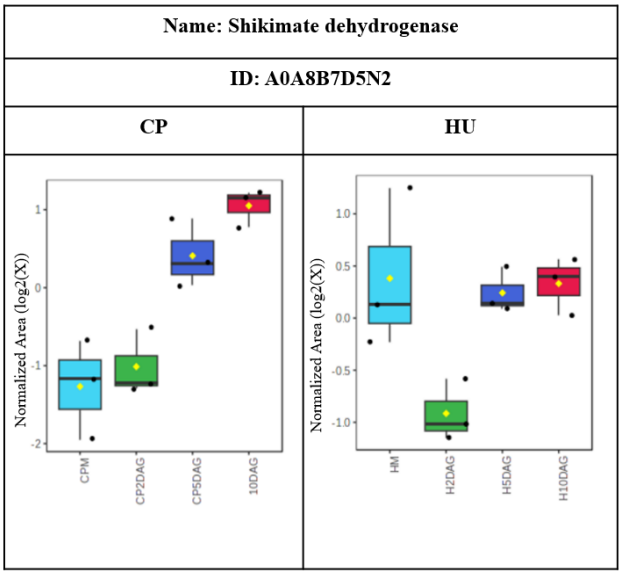
Source: Prepared by author

To sum up, the cotyledonary petiole and haustorium of *C. prunifera* included many seed storage proteins, which were discovered in the current study and showed a decrease in abundance during various developmental stages. These proteins act as crucial reserves for post-germination seedling growth and development, hence this decrease suggests that they were mobilized during germination. A vital mechanism that delivers carbon and nitrogen for the developing seedling is the mobilization of seed reserves. These proteins can be used more easily since cysteine proteases can break them down. In *C. prunifera*, where the germinated seedling depends on them for a protracted period before the appearance of the first photosynthetic leaf, the dependence on these seed reserves is particularly significant. The discovery of enzymes involved in the pentose phosphate pathway, the TCA cycle, the glyoxylate cycle, and gluconeogenesis provides additional evidence in favor of the idea that these processes are essential for the mobilization of seed storages in *C. prunifera*.

5.6.4 Other proteins

Shikimate dehydrogenase (*A0A8B7D5N2*) plays a vital role in protein biosynthesis through the production of aromatic amino acids via the shikimate pathway as it is the central enzyme of this pathway (CARRINGTON et al., 2018). The identification and increasing abundance pattern of this protein show the synthesis of essential proteins and activation of basic metabolic pathways in the selected developmental stages (Figure 28). *Shikimate dehydrogenase* is involved in the biosynthesis of gallic acid which is also a precursor for hydrolyzable tannins the shikimate act as a precursor for the biosynthesis of anthocyanins (HABASHI et al., 2019). In tobacco plants, the gene for this enzyme was suppressed by RNAi which led to a severe decrease in aromatic amino acid contents and a drop in growth and development (DING et al., 2007). A wide range of precursors for vital metabolites and protein biosynthesis are produced through the shikimate pathway which shows its pivotal contribution in growth and post-germination development. The aromatic amino acids like phenylalanine, tryptophan, and tyrosine which are the building blocks for the synthesis of various proteins are synthesized in the shikimate pathway (HUANG et al., 2019). Its increase in abundance indicates the activation of this pathway along the developmental process. Some of the important amino acids like phenylaniline, tryptophane, and tyrosine are synthesized exclusively in plants through the shikimate pathway (KAYSER; AVERESCH, 2015), which not only plays an important part in the biosynthesis of various essential proteins but also provide precursors for important secondary metabolites and plays a vital role in the growth and development (TZIN; GALILI, 2010).

Figure 28- Shows the abundance pattern of shikimate dehydrogenase in selected developmental stages of haustorium and cotyledonary petiole of *C. prunifera.*



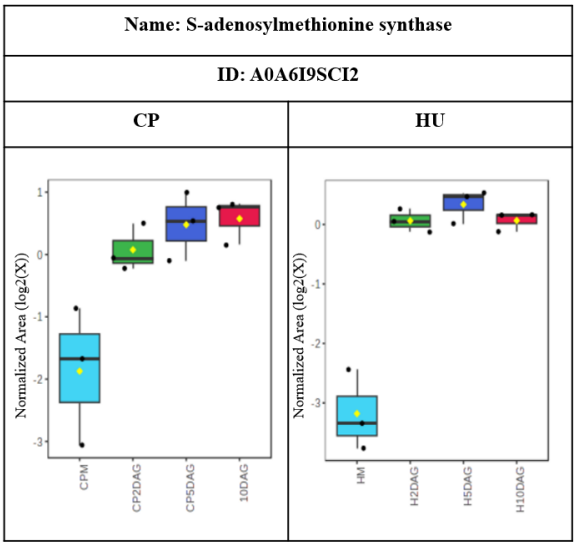
Source: Prepared by author

In conclusion, the discovery of shikimate dehydrogenase (A0A8B7D5N2) and its pattern of increasing abundance in several developmental stages of *C. prunifera* point to its critical function in protein production via the shikimate pathway. The synthesis of aromatic amino acids, which are necessary for protein synthesis and the activation of fundamental metabolic pathways, is facilitated by this enzyme, which acts as the pathway's key component. The large spectrum of precursors for vital metabolites and proteins that the shikimate pathway offers emphasizes the pathway's crucial role in growth and post-germination development. The rise in shikimate dehydrogenase abundance during development shows that this pathway is activated and crucial amino acids are synthesized, aiding *C. prunifera's* growth and developmental processes.

S-adenosylmethionine is produced by the action of S-adenosylmethionine synthase (*A0A6I9SCI2*) which is an important co-factor for various methylation reactions (SEKULA; RUSZKOWSKI; DAUTER, 2020). It plays a vital role in the synthesis of flavonoids, phenylpropanoids biosynthesis, and other metabolic pathways (JOSHI; CHIANG, 1998). It is very important for cell survival and normal function as it plays a key role in the synthesis of polyamine, transsulfuration, and transmethylation. Cysteine is the important precursor for glutathione and taurine which is produced by the transsulfuration of sulfur of this enzyme through various enzymatic reactions. Whereas polyamine is essential for normal cell growth and development (LU, 2000). A study on *C. sativus L.* suggested that these enzymes are important for plant development and stress tolerance (HE et al., 2019). In *A. thaliana* this

enzyme is involved in the methionine metabolic pathway, further analysis on mutants for S-adenosylmethionine synthase showed accumulation of methionine and impaired pollen tube growth (CHEN; ZOU; MCCORMICK, 2016). The grafting of transgenic tomato plants showed increased accumulation of polyamines, stronger root system, increased element absorption, and maintainability of nutrients (GONG et al., 2014). Upon exposure to abscisic acid, the tomato S-adenosylmethionine synthase showed a decrease in expression which shows its involvement in plant growth and development as it plays a pivotal role in various important biological processes including biosynthesis of ethylene and polyamine (HEIDARI et al., 2020). The identification and increasing abundance pattern during post-germination development show its importance in the growth and development of *C. prunifera* (Figure 29).

Figure 29- Shows the abundance pattern of S-adenosylmethionine synthase in different developmental stages of cotyledonary petiole and haustorium of *C. prunifera.*



Source: Prepared by author

In conclusion, the discovery of S-adenosylmethionine synthase (*A0A6I9SCI2*) and its pattern of increasing abundance in the post-germination development of *C. prunifera* demonstrate the enzyme's critical function in plant growth and development. An essential co-factor for numerous methylation processes, S-adenosylmethionine, is produced by this enzyme. The synthesis of flavonoids, phenylpropanoids, and other metabolic pathways depends on S-adenosylmethionine, which helps ensure optimal cell survival and function. Polyamines, which are essential for cell growth and development, are also produced by S-adenosylmethionine synthase. S-adenosylmethionine synthase appears to be active in promoting growth and

developmental activities in *C. prunifera*, as evidenced by the rising quantity of the enzyme throughout post-germination development.

6 FINAL CONSIDERATIONS AND PERSPECTIVES

The proteomics analysis of different developmental stages of haustorium of *C. prunifera* showed the presence of various hydrolases that are involved in the digestion of cell wall polysaccharides which indicates that haustorium may probably be involved in the synthesis of these hydrolases which might be used for the digestion of storage reserves in the endosperm. Similar work was conducted on the haustorium, endosperm, and digestive zone of *E. oleracea* which showed the presence of hydrolases in all three tissues (NASCIMENTO et al., 2020). The hypothesis of haustorium being the site of synthesis of cell wall hydrolases and their secretion to the endosperm was also proposed by (BUCKERIDGE, 2010). However, for further confirmation of target proteomics techniques, PRM (Parallel Reaction Monitoring) will be suggested for haustorium in addition to endosperm of selected developmental stages, which will help us to accompany the quantification and identification of proteins of our interest as mentioned by (LIN et al., 2023). This strategy will help to compare the level of abundance of cell wall hydrolases in haustorium and endosperm to provide a better understanding. To confirm the hypothesis of haustorium as the site of synthesis of cell wall hydrolases and their secretion to endosperm the in-situ expression technique is suggested to analyze the expression of the genes involved in the synthesis of these enzymes and compare between endosperm and haustorium during different developmental stages of *C. prunifera*. The development of the cotyledonary petiole and embryonic axis involves the division, expansion, and differentiation of cells. Several proteins in the cotyledonary petiole proteome have been linked to the synthesis and regulation of plant growth hormones, indicating their critical roles in these processes. Several enzymes that may have an impact on growth processes in the early stages of seedling development have been found, including spermidine synthase, 1-aminocyclopropane-1-carboxylate oxidase, indole-3-acetaldehyde oxidase, and 5'-nucleotidase. The cotyledonary petiole's ability to break down cell wall polysaccharides was another important discovery. The breakdown of several cell wall components, including cellulose, callose, xyloglucans, galactanos, galactomannans, glycoproteins, and pectins, is carried out by a variety of hydrolases, including α-galactosidases and β-glucosidases, which were identified by the proteomic analysis. During seed germination and the first stages of growth, these enzymes are essential for the mobilization of seed reserves and nutrient metabolism. These enzymes are present in the cotyledonary petiole at every selected stage of development, which suggests that seed reserves may be transported and further metabolized in this tissue. Future studies should concentrate on target proteomics to quantify particular proteins and learn more about how they influence emergent organogenesis and nutrition mobilization. Instead of the whole

cotyledonary petiole, focusing solely on the developing embryonic axis will make it possible to identify the active proteins responsible for emergent organogenesis. Integrating proteomics data with other omics approaches will further validate the roles of specific proteins.

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