CARLOS ALEXANDRE ROCHA DA COSTA

INTERAÇÃO DO PROTEOMA E METABOLOMA DURANTE O DESENVOLVIMENTO DE FRUTOS DE *Caryocar brasiliense* Camb. E *Annona crassiflora* Mart.

LAVRAS – MG
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Prof. Dr. Eduardo Valério de Barros Vilas Boas
Orientador

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RESUMO GERAL

O Cerrado brasileiro é um bioma rico em espécies frutíferas exóticas, como o pequi (*Caryocar brasiliense* Camb.) e o marolo (*Annona crassiflora* Mart.). Para elucidar mecanismos moleculares adjacentes ao desenvolvimento e amadurecimento desses frutos em quatro estádios, foram analisadas a proteômica (UPLC-MS) e um conjunto de metabólitos: vitamina C e carotenoides totais (métodos espectrofotométricos), compostos orgânicos voláteis (HS-SPME-GC-MS) e o perfil de alcaloides e compostos fenólicos (HPLC). No pequi, 234 proteínas foram identificadas e 47 metabólitos foram quantificados. Os resultados indicam que os fatores de transcrição mais influentes atuantes na regulação do amadurecimento são membros da família MAD-box. Proteínas relacionadas ao ciclo da metionina indicaram o alto consumo e reciclagem de metionina. Diferentes proteínas foram correlacionadas com a presença dos metabólitos, como a monodesidroascorbato redutase e a ascorbato peroxidase na reciclagem do ácido ascórbico; piruvato quinase, frutose bisfosfato aldolase e fitoeno sintase com biossíntese de carotenoides; S-adenosilmetionina sintase 1 como doadora de grupos metil na formação de trigonelina. A álcool desidrogenase, enzima fundamental na síntese de ésteres voláteis, aumentou em 7,2 vezes durante o desenvolvimento. No marolo, 993 proteínas foram identificadas e 45 metabólitos foram quantificados. No início do desenvolvimento há uma maior presença de proteínas ribossômicas, transcricionais e pós-traducionais. Foram identificadas as famílias de fatores de transcrição GATA, MADS-Box, MYB, F-box e HD-Zip, que são prováveis coordenadoras dos processos biológicos do marolo. No estádio maduro, há um aumento no acúmulo de celulases, e o sistema antioxidante apresentou um comportamento majoritariamente constante. Portanto, um extenso grupo de proteínas e alguns metabólitos podem servir como biomarcadores de diferentes processos biológicos ou rotas biossintéticas no pequi e marolo.

Palavras-chave: marolo; pequi; frutos do cerrado; crescimento; maturação; isoformas.

GENERAL ABSTRACT

The Brazilian Cerrado is a biome rich in exotic fruit species, such as pequi (*Caryocar brasiliense* Camb.) and marolo (*Annona crassiflora* Mart.). To elucidate molecular mechanisms underlying the development and ripening of these fruits in four stages, proteomics (UPLC-MS) and a set of metabolites were analyzed: vitamin C and total carotenoids (spectrophotometric methods), volatile organic compounds (HS-SPME-GC-MS) and the profile of alkaloids and phenolic compounds (HPLC). In pequi, 234 proteins were identified and 47 metabolites were quantified. The results indicate that the most influential transcription factors acting in the regulation of ripening are members of the MAD-box family. Proteins related to the methionine cycle indicated high methionine consumption and recycling. Different proteins were correlated with the presence of metabolites, such as monodehydroascorbate reductase and ascorbate peroxidase in the recycling of ascorbic acid; pyruvate kinase, fructose bisphosphate aldolase and phytoene synthase with carotenoid biosynthesis; S-adenosylmethionine synthase 1 as a donor of methyl groups in the formation of trigonelline. Alcohol dehydrogenase, a fundamental enzyme in the synthesis of volatile esters, increased 7.2-fold during development. In the marolo, 993 proteins were identified and 45 metabolites were quantified. At the beginning of development there is a greater presence of ribosomal, transcriptional and post-translational proteins. The families of transcription factors GATA, MADS-Box, MYB, F-box and HD-Zip were identified, which are likely coordinators of the biological processes of the marolo. In the mature stage, there is an increase in the accumulation of cellulases, and the antioxidant system showed a mostly constant behavior. Therefore, a large group of proteins and some metabolites can serve as biomarkers of different biological processes or biosynthetic pathways in pequi and marolo.

Keywords: marolo; pequi; cerrado fruits; growth; maturation; isoforms.

INDICADORES DE IMPACTO

Os resultados obtidos no presente trabalho trazem profunda valorização as culturas do pequi e marolo, espécies frutíferas ameaçadas de extinção. Muitos habitantes da região do Cerrado obtêm seu sustento por meio do agroextrativismo, e durante a época de safra, o pequi e marolo são fontes indispensáveis na composição da renda dessas famílias. Entretanto, continuamente tais povos tradicionais sentem o impacto negativo do desmatamento do Cerrado e a diminuição das áreas naturais em que o pequi e marolo são encontrados. Os resultados gerados proporcionam agregação de valor biotecnológico para esses frutos, pois podem promover o interesse de indústrias como a alimentícia e farmacêutica devido ao teor dos metabólitos bioativos e de diferentes grutos de proteínas que podem ter funcionalidades diversas. Com o maior uso biotecnológico desses frutos, medidas de conservação das áreas naturais e até implementação de áreas de cultivo dessas culturas e outras espécies frutíferas do Cerrado podem ser implementadas, dando suporte para obter uma agricultura sustentável por toda a extensão do Cerrado. Tecnologicamente, nesse estudo foi possível elucidar novos aspectos dos mecanismos moleculares do desenvolvimento desses frutos, trazendo compreensão de quais processos biológicos ocorrem e são indispensáveis. A partir de tais entendimentos, futuramente serão possíveis alcançar outros objetivos como realizar melhoramentos genéticos, implementar técnicas que aperfeiçoem a produtividade para obtenção de frutos de maior qualidade na cadeia produtiva. Quanto ao aspecto da saúde, novos dados científicos sobre os benefícios à saúde devido a constituição química do pequi e marolo foram produzidos, evidenciando que consumir tais frutos podem auxiliar na nutrição dos povos tradicionais do Cerrado e dos consumidores em geral. Dentro das áreas temáticas da Política Nacional de Extensão, os impactos desse estudo se encaixam nas áreas de Meio ambiente, Saúde e Tecnologia e produção, além de se alinhar com os Objetivos de Desenvolvimento Sustentável da Organização das Nações Unidas, em especial os objetivos de Fome zero e agricultura sustentável e Trabalho decente e crescimento econômico.

IMPACT INDICATORS

The results obtained in this study bring profound appreciation to the pequi and marolo crops, fruit species threatened with extinction. Many inhabitants of the Cerrado region obtain their livelihood through agroextractivism, and during the harvest season, pequi and marolo are indispensable sources of income for these families. However, these traditional peoples

continually feel the negative impact of deforestation in the Cerrado and the reduction of natural areas where pequi and marolo are found. The results generated provide added biotechnological value for these fruits, as they can promote the interest of industries such as the food and pharmaceutical industries due to the content of bioactive metabolites and different protein groups that can have different functionalities. With greater biotechnological use of these fruits, conservation measures for natural areas and even implementation of cultivation areas for these crops and other fruit species of the Cerrado can be implemented, providing support for achieving sustainable agriculture throughout the Cerrado. Technologically, this study made it possible to elucidate new aspects of the molecular mechanisms of the development of these fruits, providing an understanding of which biological processes occur and are indispensable. Based on such understanding, it will be possible to achieve other objectives in the future, such as carrying out genetic improvements and implementing techniques that improve productivity to obtain higher quality fruits in the production chain. Regarding health, new scientific data on the health benefits due to the chemical composition of pequi and marolo were produced, showing that consuming these fruits can help in the nutrition of traditional peoples of the Cerrado and consumers in general. Within the thematic areas of the National Extension Policy, the impacts of this study fit into the areas of Environment, Health and Technology and Production, in addition to aligning with the Sustainable Development Goals of the United Nations, especially the goals of Zero Hunger and Sustainable Agriculture and Decent Work and Economic Growth.

SUMÁRIO

INTRODUÇÃO GERAL

Ocupando cerca de 23 % do território nacional (2 milhões de km^2), o Cerrado brasileiro é o segundo maior bioma sul-americano e é caracterizado por uma excêntrica aparência savânica. Nesse bioma há a coexistência de árvores e uma camada herbácea-gramínea contínua, onde os padrões de crescimento são orientados pelas estações chuvosa e seca, e por ser uma savana tropical altamente heterogênea, é um dos *hotspots* do mundo, abrigando diversas espécies endêmicas. O Cerrado detém em sua exuberante biodiversidade uma extensa gama de espécies de frutas icônicas e exóticas, como é o caso dos frutos de pequi (*Caryocar brasiliense* camb.) e marolo (*Annona crassiflora* Mart.).

O pequi, considerado o fruto símbolo do Cerrado brasileiro, por isso denominado gentilmente de "ouro do Cerrado", é um fruto não climatérico do tipo drupa globosa de *flavor* intenso e característico, pertencente à família Caryocaraceae. A porção do fruto mais apreciada sensorialmente é o mesocarpo interno, muito utilizado na culinária regional. Sua coloração amarelada se deve à presença abundante de carotenoides, que torna o pequi uma das maiores fontes destes terpenóides superiores. Além dos carotenoides, fitoquímicos outros, como compostos fenólicos, ácidos graxos e vitamina C, bem como outras vitaminas, minerais e fibras têm sido relatados em *C. brasiliense* Camb. O marolo, fruta climatérica pertencente à família Annonaceae, é uma baga subglobosa com cerca de 11 a 15 cm de diâmetro e 1 a 2 kg de peso quando maduro, tendo uma polpa que representa cerca de 50 % da massa total do fruto. A polpa do marolo possui uma coloração branco-amarelada, devido à presença de carotenoides em sua composição fitoquímica. Mas além destes carotenoides, outros fitoquímicos foram investigados na polpa de marolo, como vitamina C, compostos fenólicos, alcaloides e outros terpenoides.

Embora o pequi e o marolo sejam frutos populares no Cerrado, a exploração de ambos ocorre por meio de prática extrativista sustentável, junto a populações nativas, uma vez que não há cultivos comerciais estabelecidos. Essa prática de coleta extrativista apresenta grande relevância socioeconômica e nutricional para populações locais. Regionalmente, o pequi e o marolo são utilizados na culinária em pratos típicos, sejam consumidos *in natura* ou como ingredientes, mas o potencial biotecnológico destes frutos também vem sendo explorado por indústrias alimentícias e farmacêuticas, que utilizam alguns de seus compostos como substituto de corantes artificiais e matéria-prima na formulação de alimentos e medicamentos.

Entretanto, tais espécies tem perdido continuamente espaço físico no Cerrado devido ao descontrole sobre a preservação de áreas nativas. Nos últimos 50 anos, o Cerrado brasileiro teve 53 % de sua vegetação original substituída por monoculturas e pastagens. Neste contexto, o uso de ferramentas tecnológicas e moleculares podem contribuir para a preservação, valorização e ampliação de usos biotecnológicos de *C. brasiliense* Camb. e *A. crassiflora* Mart. Para alcançar tal objetivo, tecnologias da biologia molecular, principalmente ferramentas genéticas e as tecnologias ômicas têm sido cada vez mais empregadas por pesquisadores. O avanço técnico da biologia celular originou várias tecnologias que são utilizadas para produzir bancos de dados que, aliadas a bioinformática, geram informações sobre todos os componentes e mecanismos celulares de um organismo ou um conjunto de organismos.

A proteômica é uma abordagem da biologia molecular que examina de maneira sistemática e dinâmica as variações no acúmulo e as alterações no perfil proteico associadas a diversos processos biológicos em organismos vivos, órgãos, tecidos ou células. As pesquisas nessa área têm desempenhado um papel crucial na elucidação do crescimento fisiológico e da atividade metabólica dos frutos. Por outro lado, a metabolômica é uma técnica molecular que se propõe a analisar, de forma qualitativa, quantitativa e dinâmica, os metabólitos de baixa massa molecular (≤1500 Da) presentes em matrizes biológicas ou químicas. A concentração de proteínas e metabólitos está em constante alteração devido às contínuas reações intracelulares, o que faz com que a análise reflita o conteúdo em um dado momento e sob condições ambientais particulares.

O uso dessas tecnologias está em expansão na identificação de biomarcadores, na análise da relação entre enzimas e substratos, em investigações de bioatividade, na avaliação de vias metabólicas e na compreensão dos processos de crescimento e desenvolvimento vegetal. Por meio dessas abordagens de caracterização molecular e fitoquímica, a integração da proteômica com a metabolômica tem contribuído significativamente para o entendimento bioquímico das transformações que frutos sofrem durante os diferentes estádios de desenvolvimento e amadurecimento.

No tomate, 2607 proteínas foram mais abundantes no fruto maduro do que no fruto verde maduro, evidenciando um aumento principalmente nos processos de fotossíntese e interconversões de pentose e glucuronato. Analisando quatro estádios de cerejas de café arábica, foi confirmado que as vias metabólicas de aminoácidos e ácidos orgânicos são as rotas críticas na formação do sabor durante o amadurecimento. Em três estádios do Sorbus, a

integração das análises permitiu elucidar que a maioria das proteínas diferencialmente abundantes estão relacionadas ao processo de amolecimento, metabolismo de carboidratos e respostas ao estresse, e que enzimas abundantes nos estádios iniciais explicam o acúmulo de açúcares e a redução da acidez durante o desenvolvimento. Na goiaba branca, o comportamento de amadurecimento climatérico foi elucidado, além do aumento de reguladores de crescimento durante o amadurecimento.

O avanço dos diferentes estádios na vida de um fruto envolve modificações de ordem fisiológica, bioquímica, de expressão gênica e estrutural, que influenciam atributos de qualidade como sabor, aroma, textura e aparência, tornando os frutos comestíveis e aptos para o consumo. Portanto, neste presente e inédito estudo, tomamos por objetivo analisar o proteoma e um conjunto de metabólitos bioativos e voláteis de *C. brasilien*se Camb. e *A. crassiflora* Mart. em quatro estádios distintos de desenvolvimento para compreender e gerar *insights* sobre como as mudanças metabólicas podem ter correlações bioquímicas com mudanças no perfil proteico. No primeiro capítulo dessa presente tese, é apresentado o artigo sobre o proteoma e metaboloma do pequi, publicado na Food Research International. No segundo capítulo é apresentado o artigo sobre o protoema e metaboloma do marolo, publicado na Scientia Horticulturae.

ARTIGO 1

Proteome and metabolome of *Caryocar brasiliense* Camb. fruit and their interaction during development

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Proteome and metabolome of *Caryocar brasiliense* camb. fruit and their interaction during development

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ABSTRACT

Considered the symbol fruit of the Brazilian Cerrado, pequi (*Caryocar brasiliense* Camb.) is an exotic and much-appreciated fruit with an internal mesocarp (edible part) with an eye-catching golden yellow color. In an unprecedented way, this study characterized the proteome throughout pequi development. The most influential and essential transcription factors operating in the regulation of pequi ripening identified were members of the MADS-box family. A group of proteins related to the methionine cycle indicates the high consumption and recycling of methionine. However this consumption does not occur mainly for the biosynthesis of ethylene, a process dependent on methionine consumption. In the bioactive compounds presented, different proteins could be correlated with the presence of these phytochemicals, such as monodehydroascorbate reductase and ascorbate peroxidase in ascorbic acid recycling; pyruvate kinase, fructose bisphosphate aldolase and phytoene synthase with carotenoid biosynthesis; S-adenosylmethionine synthase 1 as a donor of methyl groups in the formation of trigonelline and aspartate aminotransferase as a biomarker of initial regulation of the trigonelline biosynthetic pathway; phenylalanine ammonia lyase, chorismate synthesis and chalcone-flavononone isomerase in the biosynthesis of phenolic compounds. Among the volatile organic compounds identified, the majority compound in pequi was ethyl hexanoate ester, with an area of 50.68 % in the ripe fruit, and in this group of esters that was the most representative, alcohol dehydrogenase, a fundamental enzyme in the synthesis of esters, was identified with an increase of approximately 7.2 times between the first and last stages. Therefore, an extensive group of proteins and some metabolites can serve as biomarkers of ripening in pequi, as most were more expressed in the last stage, which is the ripe fruit suitable for consumption.

1. Introduction

The Brazilian Cerrado, the second largest biome in Brazil and South America with edaphoclimatic conditions that resemble savanna characteristics, has an exuberant biodiversity with an extensive range of iconic and exotic fruit species (Arruda, Araújo, & Junior, 2022). Among the eccentric fruits in the Cerrado, one of the most characteristic and representative is the pequi (*Caryocar brasiliense* Camb.), a globose drupe-type fruit with an intense and distinctive flavor. Belonging to the Caryocaraceae family, it is considered the symbol fruit of the Brazilian Cerrado, which is why it is gently called “gold of the Cerrado”. Its exploitation occurs through sustainable extractive practices, with great socioeconomic and nutritional relevance for local populations (Guerra et al., 2023; Ramos-Souza et al., 2023).

Pequi, a non-climacteric fruit (Rodrigues, de Paula, Pinto, & Vilas Boas, 2015), has a thin green exocarp less than 1 mm thick, functioning as the fruit’s interface with the environment. Under is the external endocarp, white or light ivory in color, and the internal mesocarp, golden yellow, which covers a brown, leathery, and spiny endocarp, protecting the seed. The most sensorially appreciated portion of the fruit

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is the internal mesocarp, widely used in regional cuisine. Its yellowish color is due to the abundant presence of carotenoids, which makes pequi one of the largest sources of these superior terpenoids (Carneiro et al., 2023). In addition to carotenoids, other phytochemicals such as phenolic compounds, fatty acids, and vitamin C, as well as other vita- mins, minerals and fiber have been reported in *C. brasiliense* Camb. These compounds have nutritional and bioactive properties, and their ingestion, with pequi as a vehicle, can promote antioxidant, anti-inflammatory, antimicrobial, healing, gastroprotective, and reduction in muscle pain (Cedran, Rodrigues, Sato, & Bicas, 2022; Torres, Santana, Shinagawa, & Mancini-Filho, 2018).

The biotechnological potential of pequi has also been exploited by the food and pharmaceutical industries, which use it as a substitute for artificial colors and as a raw material in the formulation of medicines and skin creams (Carneiro et al., 2023). In addition to these health-promoting effects, the peculiar flavor of pequi makes it an eccentric ingredient in different culinary preparations (Torres et al., 2018). However, even with such an appreciation of the fruit, *C. brasiliense* Camb. is part of several naturally occurring species native or endemic to the Brazilian Cerrado that are continually losing ground to agriculture. In the last 50 years, 53 % of the Brazilian Cerrado′s original vegetation has been replaced by monocultures and pastures (Fonseca, Uagoda, & Chaves, 2021). In this context, technological and molecular tools can contribute to preserving, enhancing, and expanding the biotechnological uses of *C. brasiliense* Camb. and other diverse species. Researchers have increasingly used molecular biology technologies to achieve this goal, especially genetic tools and omics technologies.

Proteomics is a molecular biology tool that systematically and dynamically analyzes differential expression and protein profile changes related to various biological processes in living organisms, organs, tissues, or cells (Zhang et al., 2020). Proteomic studies have significantly contributed to the understanding of the physiological growth and metabolic activity of fruits (Momo et al., 2022). Metabolomics, in turn, is a molecular technique that aims to qualitatively, quantitatively, and dynamically analyze metabolites of small molecular mass (≤1500 Da) from a biological or chemical matrix (Adebo et al., 2021). The concentration of proteins and metabolites constantly changes due to incessant intracellular reactions, and, therefore, they analyze the instantaneous content at a specific time and under a specific environmental condition (Momo et al., 2022; Utpott, Rodrigues, de O. Rios, Mercali, & Floˆres, 2022). The application of these technologies has grown in the detection of biomarkers, enzyme-substrate relationships, bioactivity studies, analysis of metabolic pathways, and the elucidation of plant growth and development mechanisms (Li, Wang, & Suh, 2022). Therefore, in this present and unprecedented study, we aimed to analyze the proteome and a set of bioactive and volatile metabolites of *C. brasiliense* Camb. at four distinct stages of development to understand and generate insights into how metabolic changes can have biochemical correlations with changes in the profile pequi protein.

2. Materials and methods

2.1. Collection and classification

The fruits of *C. brasiliense* Camb. were collected between January and February in the city of Itumirim (21◦ 15′ 57′′ S and 44◦ 50′ 49′′ W), southern region of Minas Gerais, Brazil. The fruits were transported and stored on the same day of each collection to the Laboratory of Post-harvest Physiology of Fruits and Vegetables of the Federal University of Lavras, Lavras, Minas Gerais, Brazil. The fruits were separated into four stages of development, according to the characteristics of the peel and mainly the internal mesocarp (Fig. 1). In the first stage, called fruit set, the fruit was close to its initial stage of development with a size of around 1 cm, beginning the formation of the still whitish internal mesocarp and the woody endocarp. In the second stage, unmature, the putamen was already more developed in size and shape, with the internal mesocarp smoothly beginning the transition to the yellowish color of the fruit. In the third stage, called mature, the putamen showed an increase in overall size and the portion of the internal mesocarp, with an intensification of the yellowish color of the fruit and initial formation of the internal almond. The color of the fruit at this stage, although already close to that of the ripe fruit, still had an opaque appearance. The peel of the first three stages presented marked rigidity, with its opening only possible through sharp instruments. In the last stage, ripe, the peel was very soft, making it possible to break it only by manual pressure. The fruits showed an increase in the portion of the internal mesocarp, with an intense and shiny orange color. After removing the exocarp and external mesocarp, the internal mesocarp was separated using stainless steel knives, immediately frozen in liquid nitrogen, and placed in polyethylene bags, followed by storage in an ultrafreezer at —80 ◦C. After pulping all samples, the frozen fruits were crushed in a food multiprocessor (Varginha, MG, Brazil) to obtain smaller granules and returned to storage at —80 ◦C until analysis.

2.2. Proteomics

2.2.1. Freeze drying

Samples were frozen at —75 ◦C (Coldlab CL 120–86 V, Brazil) for 24 h and then freeze-dried (Edwards, L4KR, Brazil) at —30 ◦C with a vacuum pressure of 0.998 mbar for 72 h in the dark (Meira et al., 2023). Once freeze-dried, the samples were transported to the Vale Techno- logical Institute, located in the city of Bel´em (1◦ 27′ 18″ S, 48◦ 30′ 9″ W), capital of the state of Par´a, northern region of Brazil, where they were subjected to proteomic analyzes.

2.2.2. Protein extraction and quantification

The proteomic analysis was carried out according to the protocol described by do Nascimento et al. (2022). The fruits were macerated in liquid nitrogen until they reached the consistency of a fine powder. To each sample was added 10 mL of buffer containing sucrose (1.5 M), Tris-chloride (1.5 M, pH 8), 10 % sodium dodecyl sulfate (SDS), 100 mM

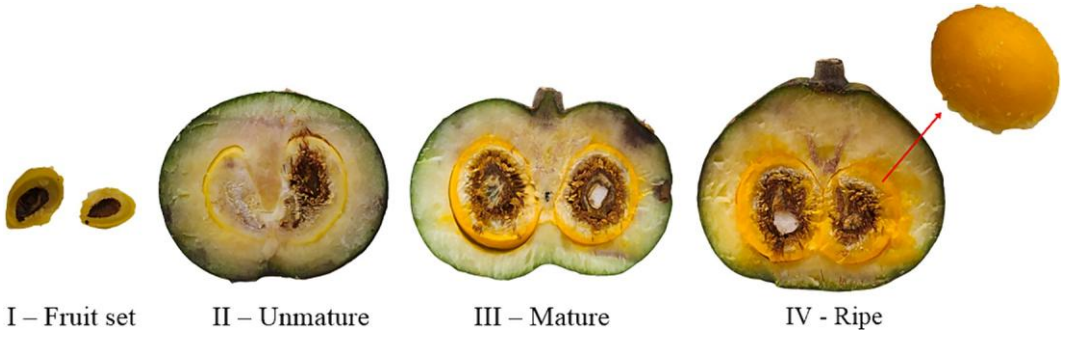

Fig. 1. Four stages of pequi development (*C. brasiliense* Camb.).

methylphenylsulfonyl fluoride (PMSF), polyvinylpolypyrrolidone (PVPP) and ultrapure H_2O, with the addition of 100 μL of protease in- hibitor (Protease Inhibitor cocktail — P8340 Sigma-Aldrich) and 500 μL of β-mercaptoethanol. After a series of five 30 s sonications, the extracts were divided into ten microtubes, each with the addition of 700 μL of phenol per microtube. The samples were vortexed and centrifuged for 8 min at 14,000 rpm twice to separate the phenolic phase and remove the residual aqueous phase or SDS. 1300 μL of ammonium acetate in methanol were added to precipitate the proteins for approximately 24 h at —80 °C. A new centrifugation was carried out at 14,000 rpm for 8 min, after which the supernatant was discarded. The precipitate was transferred to a new microtube and washed with 80 % acetone four times. A final wash was carried out with 70 % ethanol and the precipitate was dried at room temperature in a vacuum concentrator for approximately 7 min. The extracts were solubilized in 200 μL of 0.2 % RapiGest (Waters, Milford, MA, USA) and stored for later analysis.

2.2.3. Protein digestion

Preparation for digestion was carried out by first reducing the proteins with dithiothreitol (DTT, 5 mM), incubating them for 25 min at 56 °C, followed by alkylation with iodoacetamide (IAA, 14 mM) for 30 min. Residual IAA was removed by adding DTT (5 mM) and incubating for 15 min. The samples were diluted in a 1:5 ratio with ammonium bicarbonate (50 mM), and added CaCl (1 mM). Treatment was carried out with trypsin (20 ng μL^{-1}) for 16 h at 37 °C. Trifluoroacetic acid (TFA) was then added at a final concentration of 0.4 % of the volume of the samples to stop the enzymatic reaction. The protein concentration of each sample was measured on the Qubit2.0 fluorometer (Invitrogen, Thermo Fisher Scientific) using the Qubit protein assay kit according to the manufacturer's protocol.

2.2.4. Protein identification and data analysis

Five micrograms of the peptides were analyzed on a NanoACQUITY UPLC ultraperformance liquid chromatography (Waters, Milford, MA, USA), configured for fractionation in two dimensions. For the first dimension, a 5 μm XBridgeTM BEH130 C18 analytical column (300 μm × 50 mm) was used at a flow rate of 2 μL min^{-1}. The second dimension was set up with a 5 μm C18 trap column (180 μm × 20 mm) and a 1.7 μm BEH130TM C18 1.8 μm analytical column (100 μm × 100 mm) at a flow rate of 400 nL min^{-1}. The samples were separated into five fractions from a gradient of 10.8, 14.0, 16.7, 20.4 and 65.0 % acetonitrile. The chromatograph was coupled directly to an ESI-Q-ToF Synapt G2S mass spectrometer (Waters) configured to operate in positive mode and continuous fragmentation (MSE) with collision energy ranging from 5 to 40 eV. Mass spectra were acquired in the 50–1200 Da range, with a 0.5 s scan and a 0.1 s scan interval. The peak width and resolution of the mass spectra were set to automatic mode. The peptide (lockmass) [Glu-1]-fibrinopeptide, with a mass of 785.4827 Da and +2 charges, was used as a reference and read every 30 s (according to the lockmass spray settings).

The data was processed using the Progenesis QI software (Waters) for identification and quantification, using the Uniprot trembl database from UniProt (UniProtKB/swiss-prot, uniprot.org). Protein identification was accepted when the probability of identifying peptides was greater than 90 % and proteins at 95 %. The significance levels of the differentially abundant proteins were determined by applying the ANOVA test (p-value < 0.05) performed by Progenesis QI. The functional annotation of the proteins was carried out using the OmicsBox program version 2.1.14 (Biobam). Principal component analysis (PCA) and the heat map were produced using the Clustvis web tool (https://biit.cs.ut.ee/clustvis/). The Euclidean distance method was used for the heat map. Protein-protein interaction networks were predicted based on functional analysis using STRING software version 12.0 (htt ps://string-db.org/, accessed November 1, 2023), using homologous proteins from *Arabidopsis thaliana* as a background species.

2.3. Vitamin C and carotenoids

Vitamin C content was determined using the spectrophotometric method described by Strohecker and Henning (1967). The results obtained were expressed in mg of ascorbic acid 100 g^{-1}. Total carotenoids were determined using the spectrophotometric method described by Rodriguez-Amaya (2001), with results expressed in μg 100 g^{-1}.

2.4. Theobromine, trigonelline, and phenolic compounds profile

The method used was according to da Costa et al. (2023). Falcon tubes containing 1 g of *C. brasiliense* Camb. pulp samples were supplemented with 10 mL of 70 % HPLC grade methanol. The samples were shaken on a shaker table for 30 min, protected from light, and placed in an ultrasonic bath for 30 min more. Then, the samples were filtered through quantitative filter paper measuring 12.5 cm and a porosity of 0.025 mm. A second filtration was performed with a membrane filter of 13 mm in diameter and porosity of 0.00022 mm and, an aliquot of each filtrate was placed in 1.5 mL vials for injection. High-performance liquid chromatography (HPLC) was carried out in Shimadzu equipment, consisting of a quaternary pump LC-20AT, degasser DGU-20A5, injector SIL-20A, controller CBM-20A, oven CTO-20AC, detector SPDM-20A, detector RID- 10A and fraction collector FRC-10A. The following parameters were adopted: Lc time program: 0.01 min–0 % B, 5 min–20 % B; 25 min–40 % B; 43 min–45 % B; 50 min–80 % B; 55 min–0 % B; 65 min – STOP; Flow: 1 mL min^{-1}; Oven temperature: 35 °C; Injected volume: 0.02 mL; DAD: 280 nm; Shim-pack VP-ODS column 250 mm × 4.6 mm × 0.0005 mm and Shim-pack GVP-ODS pre-column 10 mm × 4.6 mm × 0.0005 mm; Mobile phase A – 2 % solution of glacial acetic acid in ultrapure water; Mobile phase B – Solution consisting of 70 % methanol and 2 % acetic acid in ultrapure water. Fifteen standards were used, namely trigonelline, theobromine, catechin, resveratrol, vanillin and gallic, chlorogenic, ferulic, caffeic, *o*-coumaric, *m*-coumaric, *p*-coumaric, syringic, rosmarinic and *trans*-cinnamic acids. The results obtained were expressed in mg 100 g^{-1} of sample.

2.5. Volatile compounds

Volatile compounds of *C. brasiliense* Camb. samples were analyzed by headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC–MS). Headspace bottles with a capacity of 20 mL containing 2 g of samples were placed in an aluminum block and heated to 40 °C for 30 min. A 50/30 μm DVB/CAR/PDMS fiber was used to extract volatile compounds. Volatile compounds were detected by GC–MS (Shimadzu CG-17 A, Shimadzu, Japan) equipped with an SLB-5MS column 30 m × 0.25 mm internal diameter × 0.25 μm film thickness (bound phase; 5 % diphenyl, 95 % dimethyl polysiloxane). The carrier gas (helium) flow rate was a constant 1.0 mL min^{-1} flow. The initial temperature was 40 °C for 30 min, with a subsequent ramp rate of column by 3 °C per minute until reaching a temperature of 220 °C. The interface temperature for the MS was 240 °C and the ion source 220 °C. The identification of volatile compounds was based on comparing mass spectra using the Willey 8 and NIST libraries.

2.6. Statistic

Analyzes were performed in three replications per stage. Each replication consisted of 24 fruits, totaling 288 fruits in the study. The pulp of the 24 fruits from each replicate was homogenized entirely. Bioactive and volatile compounds were analyzed using SISVAR version 5.8 software. Analysis of variance (ANOVA) and the Scott-Knott method ($p < 0.05$) were used to compare possible significant differences between fruits at the four stages of development. Values were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Proteome of C. Brasiliense camb.

Throughout its development, 234 proteins (Table S1) were identified in the internal mesocarp of *C. brasiliense* Camb. fruits. Of this total, 176 proteins showed differential accumulation with significant values in the four stages. Functional annotation assigned these proteins to 50 biological processes (Fig. 2A). Because they can act on different molecular mechanisms and cellular regions, although with a specific chemical function and structure, some proteins have been attributed to more than one biological process. The variation in abundance (Fig. 2B) and the principal component analysis (PCA) (Fig. 2C) of these 176 proteins in the three replicates of each stage are also presented.

The three biological processes that presented the highest amount of proteins were the organic substances biosynthetic process (62), cellular biosynthetic process (51), and organonitrogen compound metabolic process (49). Of these, 40 proteins were common to all processes, and a general heatmap was developed encompassing the three processes (Fig. 3A). Different background colors highlighted proteins common to the three processes, or shared between two processes or exclusively from one. The proteins attributed to these biological processes are mainly related to energy production, biosynthesis of compounds and tran- scription factors that regulate different metabolic processes.

Fig. 3 B presents the protein–protein interaction analysis, which shows different types of interactions detected or prospected by public databases can be observed.

The fruit ripening process is coordinated by a genetically programmed system of plant hormone signaling pathways, including networks of transcription factors (TFs) and epigenetic modifications that act in combined and independent effects (Li, Lu, Xu, & Liu, 2023). It has been demonstrated that many families of TFs are involved in regulate fruit ripening, such as MADS-Box, MYB, NAC, ARF, and HD-Zip, among others (Liu, Li, Grierson, & Fu, 2022). TFs influence the expression of a range of other genes that encode proteins participating in various metabolic processes (Li, Wang, Zhang, Zhang, & You, 2022b). Among the groups of TFs, MADS-box plays essential roles in almost all aspects of plant growth and development, and evidence suggests that they are the most powerful and central in regulating fruit development and ripening (Liu et al., 2017). MADS-box TFs are involved in the synthesis of polyphenolic compounds and can act as regulators in the flavonoid and carotenoid pathway (S´anchez-Go´mez, Pos´e, & Martín-Pizarro, 2022).

The proteins from the MAD-box family accumulated in the pequi proteome were the MADS-box protein GGM13 and MADS-box transcription factor 25, which showed, respectively, an increase in accumulation by 35.5 times from the third to the fourth stage and 5.5 times from the first to the fourth stage. The MADS-box protein GGM13 belongs to the B_{sister} MAD-box gene subfamily, wich has already been reported to cause a gain of function in fruit growth by increasing cell size (Chen, Deng, Truksa, Peng, & Weselake, 2012). Other TFs were still identified in the proteome, such as three proteins belonging to the HD-ZIP homeobox family, for example. In general, the TFs of the proteome had a similar accumulation pattern, tending to be more present in the mature stage. We conclude that the TFs found in pequi, with emphasis on MAD-box, must be the potent agents that promote changes in fruit ripening, mainly because the fruit is non-climacteric, which has less influence from the action of ethylene.

Two enzymes found in common in the processes of cellular biosynthesis and organic substances are S-adenosylmethionine synthase 1 (SAM1, EC:2.5.1.6) and Gibberellin 20 oxidase 2 (GA20OX2,

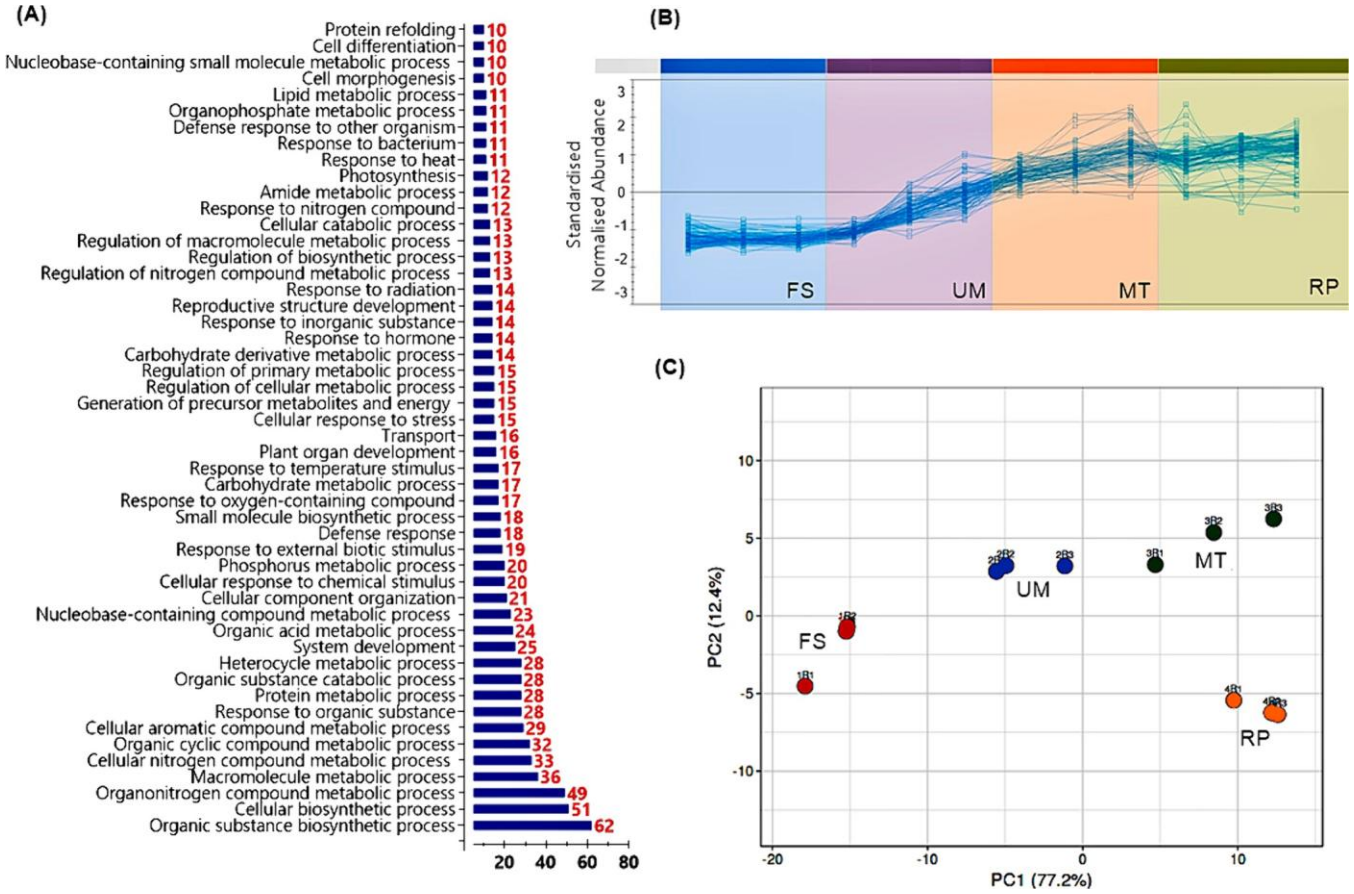

Fig. 2. (A) Functional annotation of biological processes and the respective numbers of proteins for each process. (B) Variation in protein abundance throughout the four stages: fruit set (FS), unmature (UM), mature (MT), and ripe (RP). (C) PCA of the four stages of development.

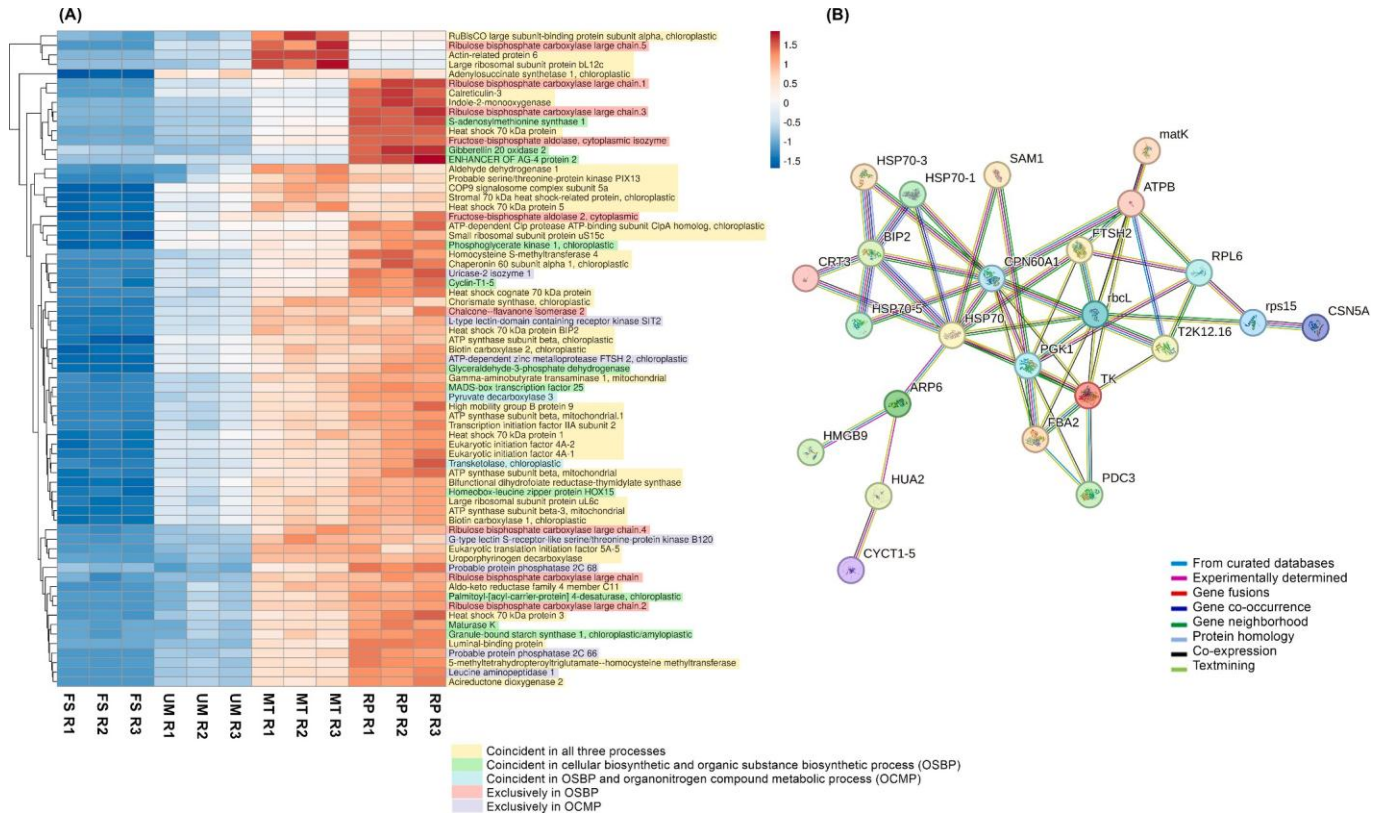

Fig. 3. (A) Heatmap with proteins from the three most abundant biological processes: organic substance biosynthetic process (OSBP), cellular biosynthetic process, and organonitrogen compound metabolic process (OCMP) identified in the pulp of *C. brasiliense* Camb. in four stages: fruit set (FS), unmature (UM), mature (MT), and ripe (RP). Intense red denotes the highest accumulation values, while intense blue the least accumulation values. Related to the background colors of the protein names: Yellow: Coincident in all three processes; Green: Coincident in cellular biosynthetic process and OSBP; Blue: Coincident in OSBP and OCMP; Pink: exclusively in OSBP; Purple: exclusively in OCMP. (B) Protein-protein interactions presented by the list of genes encoding the proteins shown in Fig. 3A compared to homologous proteins from the *A. thaliana* database. Different line colors represent the various types of detected or prospected interactions.

EC:1.14.11), which have increased accumulation between the fruit set and ripe stages, approximately 6 and 5 times, respectively. GA20OX2 is a key enzyme in the biosynthesis of gibberellin, while SAM1 catalyzes the formation of S-adenosylmethionine (AdoMet) using methionine and ATP. From this molecule, several metabolites can be formed following different metabolic pathways, many of which converge to the recycling of methionine, its initial precursor (Sekowska, Ashida, & Danchin, 2018). One of the routes that use AdoMet as a metabolite is ethylene biosynthesis.

Ethylene is a volatile plant hormone closely associated with the ripening of climacteric fruits. Its production route was described by Yang and Hoffman (1984), as well as the recovery cycle of its basic precursor, methionine. Ethylene synthesis is summarized in four steps, namely: Methionine → S-Adenosyl-methionine (SAM) → 1-Aminocyclopropane 1-carboxylic acid (ACC) → ethylene, in reactions catalyzed by the enzymes SAM synthase, ACC synthase and ACC oxidase, respectively (Hoffman and Yang, 1981).

Gibberellins are mainly correlated with cell division and expansion while ethylene, mainly in climacteric fruits, is centrally responsible for regulating ripening (Chen, Wang, Tan, Zhou, & Wang, 2020; Fenn & Giovannoni, 2021; Liu et al., 2020). Fruiting studies revealed that ethylene emissions demonstrated antagonism to the biosynthesis of gibberellins while blocking ethylene perception allowed the accumulation of gibberellins and the induction of parthenocarpy (Chen et al., 2020; Fenn & Giovannoni, 2021; Shinozaki et al., 2015). However, this antagonism is not clearly reported in the development and ripening of fruits, especially fruits with a non-climacteric respiratory pattern, such as pequi (Rodrigues et al., 2015). In the study by Rodrigues et al. (2015), carried out with pequi from the same locality as the pequi collected in this present work, a marked decrease in ethylene production during development was reported, approximately 3 times in an interval of 4 weeks. Therefore, the increase in SAM1 throughout pequi development suggests using methionine in other processes rather than for ethylene production. The crosstalk between gibberellin and ethylene in pequi, and the interaction with other hormones needs to be investigated in particular.

Three other enzymes that act in the methionine recycling cycle were detected in the proteome and are present in Fig. 3A, Acireductone dioxygenase 2 (EC:1.13.11.54), Homocysteine S-methyltransferase 4 (HMT-4, EC:2.1.1.10) and 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase (METE, EC:2.1.1.14). Between the first and last stages, each of these enzymes increased in expression by 4.6, 24 and 9.5 times, respectively. METE is an enzyme that needs folate as a substrate for its action. Another enzyme present in Fig. 3A, Bifunctional dihydrofolate reductase-thymidylate synthase (EC:1.5.1.3), is the key enzyme in folate biosynthesis, and showed minimum and maximum expression in the fruit set and ripe stages, respectively. Therefore, there may be a possible correlation between the synthesis of this set of proteins and their increased expression throughout fruit development specifically in recycling methionine to supply different processes.

Seven Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) isoforms identified (Fig. 3A) were more accumulated between the mature and ripe stages, with six being attributed exclusively to the biosynthesis process of organic substances. RuBisCO is a key enzyme in the photosynthetic process, responsible for fixing atmospheric CO_2 into organic molecules. RuBisCo is one of the proteins with the most isoforms found in nature, and this is an evolutionary strategy due to a catalytic inefficiency of such a protein (Dinh et al., 2019). In tomato ripening, this

protein was expressed and related to carbohydrate biosynthesis. However, its presence is uncommon in non-photosynthetic tissues, such as the internal mesocarp of pequi. The presence of RuBisCO in this tissue may occur due to the mobilization of this protein, probably present in the peel – which presents photosynthetic tissue – and subsequent use of a proportion of its constituent amino acids in the growth of the fruit's internal mesocarp. Throughout fruit development and ripening, the photosynthetic capacity of the fruit is reduced, and enzymes involved in photosynthesis, such as RuBisCO, can be displaced to be catabolized (Walker et al., 2012).

Notably, seven isoforms of 70 kDa heat shock proteins (HSPs) stand out in Fig. 3A and are more accumulated between the mature and ripe stages, with an emphasis on the latter. When discovered, HSP proteins were associated with a unique plant protection mechanism in response to stress from excessive temperatures, and in fact they are effective in cells or tissues exposed to such a condition. However, various protective effects against other types of stress have already been reported, as well as their presence in normal and non-stressed cells (Upadhyay, Tucker, & Mattoo, 2020). HSPs may help newly synthesized proteins fold or protect proteins that may misfold and thus lose their potential functional conformation during a stress event. They are also involved in the growth and development of different parts of plants under normal conditions, including fruits (Ul Haq et al., 2019). In Fig. 3B, five HSPs represented by their coding genes (*HSP70*, *HSP70-1*, *HSP70-3*, *HSP70-5*, and *BIP-2*) showed strong interaction with a chaperonin (*CPN60A1*), an enzyme involved in protein folding.

3.2. Vitamin C, carotenoids, trigonelline, phenolic compounds, and enzymes involved in biosynthetic pathways

Changes in the concentrations of 14 bioactive compounds studied were observed throughout the development of pequi fruit and are described in Table 1.

Table 1
The values shown are the mean and standard deviation of the bioactive compounds identified in the pulp of *C. brasiliense* Camb. at four stages: fruit set, unmature, mature and ripe. The results were expressed in mg 100 g^{-1} for vitamin C, trigonelline, catechin and other phenolic compounds. Carotenoids were expressed in μg 100 g^{-1}. Different letters indicate significant differences ($p < 0.05$) by ANOVA analysis followed by the Scott-Knott test. ND – Not Detected.

	Fruit set	Unmature	Mature	Ripe
Vitamin C mg of ascorbic acid 100 g^{-1}	200.86 ± 4.73^{a}	193.77 ± 9.1^{a}	200.23 ± 5.73^{a}	156.37 ± 4.77^{b}
α-Carotene μg 100 g^{-1}	19.91 ± 0.98^{d}	33.56 ± 2.1^{c}	66.14 ± 3.7^{b}	127.1 ± 1.43^{a}
β-Carotene μg 100 g^{-1}	33.19 ± 0.28^{d}	39.94 ± 0.48^{c}	65.23 ± 1.2^{b}	128.47 ± 4.2^{a}
δ-Carotene μg 100 g^{-1}	15.6 ± 1.55^{d}	24.72 ± 1.32^{c}	41.45 ± 1.33^{b}	83.36 ± 0.75^{a}
γ-Carotene μg 100 g^{-1}	15.3 ± 4.98^{d}	26.15 ± 0.59^{c}	42.94 ± 1.88^{b}	84.13 ± 4.41^{a}
Lycopene μg 100 g^{-1}	13.93 ± 4.06^{d}	26.67 ± 2.76^{c}	41.86 ± 2.17^{b}	83.76 ± 1.33^{a}
Trigonelline mg 100 g^{-1}	36.49 ± 0.12^{b}	36.89 ± 0.61^{b}	32.65 ± 0.57^{c}	77.51 ± 2.91^{a}
Catechin mg 100 g^{-1}	ND	ND	ND	4.20 ± 0.15
Gallic acid mg 100 g^{-1}	8.56 ± 0.15^{b}	7.71 ± 0.08^{c}	12.30 ± 0.33^{a}	4.74 ± 0.01^{d}
Syringic acid mg 100 g^{-1}	0.20 ± 0.00^{a}	0.13 ± 0.01^{b}	0.09 ± 0.00^{c}	ND
Vanillin mg 100 g^{-1}	0.11 ± 0.01^{b}	ND	0.16 ± 0.01^{a}	0.09 ± 0.00^{c}
Ferulic acid mg 100 g^{-1}	ND	ND	ND	0.25 ± 0.01
p-Coumaric acid mg 100 g^{-1}	ND	0.05 ± 0.00^{a}	0.04 ± 0.00^{a}	ND
Resveratrol mg 100 g^{-1}	5.44 ± 0.25^{a}	4.72 ± 0.01^{b}	5.88 ± 0.35^{a}	0.66 ± 0.00^{c}

The vitamin C content of pequi remained stable among the forming, unmature and mature fruits, falling by around 25 % in the ripe fruits. Although with a lower value than in the first three stages, the mature pequi presented a considerable concentration of vitamin C, 156.37 mg 100 g^{-1}. This concentration is higher than that found in oranges, humanity's main source of vitamin C. In a study with orange juices, Randhawa et al. (2020) reported vitamin C levels ranging from 15 mg 100 g^{-1} to 45 mg 100 g^{-1}. The recommended daily dose of vitamin C, also known as ascorbic acid (AsA) or ascorbate, varies from 15 to 120 mg, depending on the age, sex, and health status of the individual, with 75 and 90 mg being proposed for women and men adult, respectively (Lewis et al., 2020; Randhawa et al., 2020).

The decrease in AsA throughout fruit ripening is recurrent and can be attributed in some fruits to its consumption as a metabolite for the synthesis of other compounds, such as oxalic and tartaric acids, for example (Smirnoff, 2018). An AsA recycling mechanism is used as a support strategy to obtain maximum use of this molecule. Pequi in the ripe stage clearly increased the performance of this regenerative pathway, as proteins operating in this process accumulated more between the third and fourth stages when the reduction of AsA occurred. Six isoforms of monodehydroascorbate reductase (MDHAR) and one of ascorbate peroxidase (APX) were identified within the *C. brasiliense* Camb. proteome. All showed lower accumulation in the fruit set stage. Of these, four MDHAR isoforms and APX showed significant accumulation throughout ripening. While APX converts ascorbate to monodehydroascorbate to eliminate H_2O_2, MDHAR reduces monodehydroascorbate back to ascorbate, regenerating this molecule for this antioxidant system to occur again (Zheng et al., 2022). Although only one APX isoform was found, it showed an increase in accumulation that was more significant than MDHAR, approximately six times from the first to the fourth stage. Therefore, we conclude that pequi super accumulates such proteins from the third stage onwards to activate the recycling process when synthetic reduction of AsA is noticed.

The concentrations of carotenoids α-, β-, δ-, γ-carotene and lycopene increased significantly throughout development, reaching maximum values in the ripe fruit. Carotenoids are the pigments that give ripe pequi fruit its golden yellow color. α-carotene and β-carotene presented the highest levels among carotenoids, and from the fruit set to the ripe stage, these compounds increased in concentration by approximately 6.4 and 3.9 times, respectively. The sum of total carotenoids in ripe pequi is 506.82 μg 100 g^{-1}. According to a classification of dietary sources of carotenoid content, this fruit is classified as a source of high content of these secondary metabolites (high content: 500–2000 μg 100 g^{-1} of fresh product) (Elvira-Torales, García-Alonso, & Periago-Castó´n, 2019).

One of the main enzymes of the carotenoid pathway was quantified in the proteomic analysis, phytoene synthase (PSY, EC:2.5.1.32). This enzyme is responsible for a crucial and limiting step in carotenogenesis, in which it catalyzes the condensation of two geranylgeranyl diphosphate (GGPP) molecules derived from the methylerythritol phosphate (MEP) pathway into phytoene, which is the first colorless carotenoid (Zhou et al., 2022). PSY showed minimum and maximum expression in the fruit set and mature stages. Although there was a significant increase between all stages, from the third to the fourth stage there was an exceptional increase in total carotenoid content by 96.73 %. We conclude that in this third stage, pequi, as it is close to full maturity, increases its carotenogenic rate to the maximum, consequently generating the maximum accumulation of PSY to meet this molecular demand. With the fruit completely ripe, PSY begins to naturally reduce. Regarding the other enzymes involved in the carotenogenic pathway, these enzymes that catalyze the pathway's reactions are generally associated with the membrane or integral to the membrane and are not very abundant, which makes their quantification more difficult (Cunningham & Gantt, 2007).

In addition to PSY, other enzymes in our study may correlate with the synthesis of carotenoids by producing essential metabolites for this

route. Our proteome identified two isoforms of pyruvate kinase (PK) and two of fructose-bisphosphate aldolase (FBA), with lower accumulation in the fruit set stage and higher in the last two stages. PK is a key enzyme that catalyzes the last step of the glycolysis pathway, transferring the phosphate group from phosphoenolpyruvate to ADP to produce ATP and pyruvate (Hu et al., 2020). FBA is a key plant enzyme that catalyzes the reversible aldol cleavage of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (GAP) (Feng, Gao, Zheng, Li, & Zhou, 2020). Pyruvate and GAP react and condense, and from this condensation, six further conversions occur to form the isopentenyl pyrophosphate (IPP) molecule, the initial precursor of carotenoids through the plastid MEP pathway. In turn, IPP reacts with dimethylallyl diphosphate (DMAPP) forming geranyl diphosphate (GPP), which is then converted into GGPP, used by PSY in the synthesis of phytoene (Sathasivam, Radhakrishnan, Kim, & Park, 2021). The accumulation of these enzymes in the last stages reiterates that in the third stage the fruit strategically increases the synthesis of carotenoids significantly.

The alkaloid trigonelline and seven phenolic compounds were identified in pequi (Table 1). The trigonelline content did not vary significantly between the forming and unmature fruits, reducing in mature fruits and increasing in ripe fruits (Table 1). In ripe fruits, the trigonelline content was approximately 2.1 times that found in forming and unmature fruits and 2.4 times that found in mature ones. Trigonelline (1-N-methylnicotinic acid) is a polar hydrophilic alkaloid present in many plant species with diverse physiological activities as a defense against biotic stresses (da Costa et al., 2023).

In the pequi proteome, a vital enzyme detected as being active in the synthesis of trigonelline was SAM1. As mentioned, this enzyme generates AdoMet as a metabolite, which reacts with nicotinic acid providing methyl groups for the synthesis of trigonelline (Ashihara, 2015). The highest trigonelline content occurred in the fourth stage simultaneously with the maximum accumulation of SAM1. A possible limiting enzyme for trigonelline biosynthesis detected in the pequi proteome is aspartate aminotransferase (AspAT, EC:2.6.1.1), which deaminates aspartate into oxaloacetate, which is essential in the production of malate used in mitochondria for the Tricarboxylic acid cycle (Han et al., 2021). Aspartate is the initial precursor for the trigonelline biosynthesis cycle (Ashihara, 2015). AspAT had maximum accumulation in the fruit's third stage, which indicates this enzyme's most significant action in deaminating aspartate. Respectively, in this third stage, trigonelline obtained the lowest value among all stages, while in the fourth stage, AspAT showed a reduction in accumulation by 13 %, and trigonelline synthesis obtained the highest result in the entire experiment. Therefore, AspAT may be a biomarker of initial regulation of the trigonelline biosynthetic pathway in pequi.

Among the seven phenolic compounds identified, gallic acid and resveratrol were the only ones observed at all stages of development, with the lowest concentrations found in ripe fruits. Catechin and ferulic acid were found only in ripe fruits, while *p*-coumaric acid was found in unmature and mature fruits. Syringic acid and vanillin were not found in ripe and unmature fruits, respectively, although they were identified in other stages of development.

Gallic acid was the most abundant phenolic compound in pequi, with maximum levels found in the mature fruit, of 12.30 mg 100 g^{-1}. A reduction of approximately 60 % was noted during fruit ripening. The results suggest the predominance of the synthesis of this phenolic until ripening, replaced by degradation during ripening.

In contrast to gallic acid, *p*-coumaric acid was the phenolic compound found in lower concentrations and only in the unmature and mature stages. It is the immediate successor to cinnamic acid in the phenylpropanoids route, and subsequently gives rise to caffeic, ferulic, sinapic, syringic and vanillic acids (da Costa et al., 2023). It can also conjugate with acetyl-CoA, giving rise to coumaryl-CoA, the precursor of flavonoids and resveratrol. The low concentrations, or even absence, of *p*-coumaric acid during the development of pequi can be justified by its use as a precursor of other phenolic compounds, such as ferulic and syringic acids, vanillin, catechin, and resveratrol, also identified in pequi. Ferulic acid was identified only in ripe fruits. Since it precedes syringic acid and vanillin in the phenylpropanoid pathway, its absence in the earlier stages suggests its rapid conversion to its successors. Its accumulation during fruit ripening coincides with the reduction of syringic acid and vanillin, which depend on it for their synthesis. Catechin was identified only in ripe fruits, while resveratrol levels fluctuated in the first three stages of development, falling in ripe fruits to around 12 % of that observed in the previous stages.

Two important enzymes of the phenylpropanoid pathway were identified in the pequi proteome: chorismate synthase (EC:4.2.3.5) and phenylalanine-ammonium lyase (PAL, EC:4.3.1.24). Both had lower accumulation in the fruit set stage, and greater accumulation in the mature and ripe stages, respectively. PAL is considered the key enzyme in the phenylpropanoid pathway and chorismate synthase produces chorismic acid, which can subsequently form three essential aromatic amino acids in the synthesis of secondary metabolites, tryptophan, tyrosine and phenylalanine (Santos-Sa´nchez, Salas-Coronado, Herna´ndez-Carlos, & Villanueva-Can˜ongo, 2019; Zhou, Chen, Xu, Tu, & Tu, 2019). An enzyme active in the initiation of flavonoid formation was also detected, chalcone-flavanone isomerase (EC:5.5.1.6), which is involved in the synthesis of flavonoids such as catechin (Chia, Teh, & Mohamed, 2020). This enzyme showed maximum accumulation in the ripe stage, the same stage in which it showed the presence of catechin. Therefore, pequi appears to develop its phenolic compounds throughout all stages of the fruit, making it difficult to associate the entire group with the behavior of specific proteins, except catechin. The fruit prioritizes the synthesis of this flavonoid when it reaches maturity.

3.3. Pectinesterase

Pectinesterase was an important enzyme identified in the pequi proteome related to cell wall degradation (EC:3.1.1.11). This enzyme catalyzes the breakdown of pectin into polygalacturonic acid and methyl alcohol, subsequently causing the fruit to soften, a hallmark of ripening (Kazimova, Nabiyev, & Omarova, 2021). The action of pectinesterase is essential in the polysaccharide metabolism pathway, where this complex metabolic pathway begins with the decomposition of pectin (Lu et al., 2024). In the pequi proteome, this enzyme had minimum and maximum accumulation in the fruit set and ripe stages, increasing approximately 5.2 times in accumulation during development. Its action has been proven to help complete the ideal characteristics of the ripe fruit, making the fruit soft and suitable for consumption.

3.4. Volatile organic compounds (VOC) and related proteins

33 VOC were detected among the four stages of pequi. However, only 19 compounds were clearly identified compared to the retention index (RI) calculated and tabulated in the literature. Such VOC are presented in Table 2 with their respective percentages of area per stadium

VOC are essential factors that contribute to the quality and acceptance of fruits by animals and humans. These aromatic compounds are molecules with high vapor pressure, moderate hydrophilicity, and low molecular mass, and the simultaneous perception of the volatile profile defines the characteristic odor of food (Jim´enez-Bremont et al., 2024). The VOC profile undergoes several variations according to the stage of maturation, and its biosynthesis occurs through amino acid derivatives, sugars, and compounds derived from fatty acids (Li et al., 2022a; Mostafa, Wang, Zeng, & Jin, 2022). The 19 identified compounds were grouped into three chemical classes in Table 2. Of these, the most representative group was esters with 57.89 % of the total compounds, followed by terpenes, with 31.58 % and, finally, aldehydes (10.53 %). Among the different volatile chemical compounds synthesized by fruits, the most abundant group are volatile esters, which are attributed as responsible for the fruity aroma (Mostafa et al., 2022), in addition to

Table 2
The values shown are the mean and standard deviation of the volatile organic compounds identified in the pulp of *C. brasiliense* Camb. at four stages: fruit set, unmature, mature and ripe. The results obtained were expressed in area percentage. Different letters indicate significant differences ($p < 0.05$) by ANOVA analysis followed by the Scott-Knott test. ND – Not Detected. RI = retention index calculated. RI Lit = retention index found in literature.

Compounds	RI	RI Lit	Fruit set	Unmature	Mature	Ripe
Ester						
Isopropyl butanoate	839	836	0.30 ± 0.00^{b}	0.34 ± 0.03^{a}	0.29 ± 0.02^{b}	ND
Ethyl methylbutyrate	847	846	0.23 ± 0.01^{c}	0.87 ± 0.07^{a}	0.56 ± 0.03^{b}	0.09 ± 0.00^{d}
Ethyl pentanoate	901	901	ND	0.39 ± 0.03^{a}	0.35 ± 0.02^{b}	0.010 ± 0.02^{c}
Methyl hexanoate	923	927	15.54 ± 0.14^{a}	5.43 ± 0.13^{b}	4.92 ± 0.06^{c}	4.93 ± 0.19^{c}
Ethyl hexanoate	999	1002	15.18 ± 0.24^{d}	42.59 ± 1.13^{b}	39.37 ± 0.64^{c}	50.68 ± 1.46^{a}
Ethyl hex-(2E)-enoate	1045	1044	ND	ND	ND	1.31 ± 0.04
Propyl hexanoate	1095	1097	ND	ND	ND	0.28 ± 0.02
Ethyl heptanoate	1097	1098	ND	ND	ND	0.13 ± 0.01
Methyl octanoate	1124	1127	ND	ND	ND	0.40 ± 0.03
Ethyl octanoate	1196	1197	ND	ND	ND	3.37 ± 0.23
Ethyl oct-(2E)-enoate	1248	1249	ND	ND	ND	0.32 ± 0.00
Aldehyde						
2-hexenal	853	854	2.93 ± 0.05	ND	ND	ND
3-(Methylthio)propionaldehyde	908	901	0.69 ± 0.03^{a}	0.07 ± 0.01^{c}	0.09 ± 0.00^{b}	ND
Terpene						
α-pinene	934	939	14.99 ± 0.03^{a}	0.47 ± 0.03^{b}	0.14 ± 0.01^{c}	0.08 ± 0.02^{d}
β-Mircene	990	992	ND	1.18 ± 0.11^{a}	1.10 ± 0.06^{a}	0.14 ± 0.01^{b}
Limonene	1031	1033	4.56 ± 0.38^{a}	0.20 ± 0.02^{c}	0.86 ± 0.07^{b}	0.12 ± 0.01^{c}
α-Phellandrene	1008	1007	1.62 ± 0.03^{a}	ND	0.23 ± 0.02^{b}	ND
β-(Z)-Ocimene	1037	1037	1.20 ± 0.02^{d}	2.52 ± 0.06^{b}	2.87 ± 0.14^{a}	1.54 ± 0.15^{c}
β-(E)-Ocimene	1048	1050	39.77 ± 0.72^{c}	45 ± 0.71^{b}	47.69 ± 0.76^{a}	28.61 ± 0.11^{d}

being critical structural elements and synthetic intermediates (Shang et al., 2009). The majority compound identified in pequi was ethyl hexanoate ester, with an area of 50.68 % in the ripe fruit, followed by the terpene β-(E)-Ocimene (47.69 % in the mature stage).

In our proteome, we identified the presence of ADH (EC:1.1.1.1), which showed minimum and maximum accumulation in the fruit set and ripe stages, respectively. Accordingly, it was in the ripe stage that the highest total concentration of esters was observed compared to the previous stages. Throughout development, ADH synthesis increased approximately 7.2-fold. Therefore, in pequi, when the ripening period is reached, one of the molecular mechanisms that is most accentuated is the massive production of volatile esters caused by the increased accumulation of ADH. This enzyme can be considered a biomarker of pequi ripening and its deficiency could cause substantial damage to the characteristic aroma of ripe fruit.

In addition to possible conversion to esters, aldehydes can also be oxidized by aldehyde dehydrogenases (ALDHs) that transform aliphatic and aromatic aldehydes into carboxylic acids (Islam & Ghosh, 2022). These serve as intermediates or byproducts of crucial biochemical pathways in living cells, such as carnitine biosynthesis, gluconeogenesis, glycolysis, amino acid metabolism, and other physiological processes (Guan, Tanwar, Sobieszczuk-Nowicka, Floryszak-Wieczorek, & Arasimowicz-Jelonek, 2022; Tola, Jaballi, Germain, & Missihoun, 2020). The ALDH identified in the proteome reached maximum accumulation in the fourth stage, increasing 6.15 times between the fruit set and ripe stages. The presence of the two volatile aldehydes identified was more marked in the first stage, as seen in Table 2. Although ALDH may act on the consumption of these aldehydes, it seems less likely when observing the total absence of aldehydes in the fourth stage to the detriment of ALDH expression at this same point in the fruit. ALDH may be involved in the oxidation of other types of non-volatile aldehydes. As an example, in Table 1, a phenolic aldehyde, vanillin, decreased from 0.16 mg 100 g^{-1} to 0.09 mg 100 g^{-1}, and may have been oxidized by the action of ALDH. Therefore, this enzyme limits the presence or accumulation of aldehydes in pequi, which are not fundamental compounds in the volatile composition of the fruit.

All terpenes in Table 2 are monoterpenes (C_{10}), and they share the initial pathway of carotenoids, as they are mainly derived from GPP, formed from the condensation of IPP and DMAPP in the plastid MEP pathway (Huang & Osbourn, 2019). Therefore, the enzymes mentioned for initially acting in the carotenoid pathway, PK and FBA, also correlate with the synthesis of monoterpenes. More specific enzymes for terpene synthesis described in Table 2 were not detected. Regarding the synthetic decrease of all monoterpenes at the ripe fruit stage coinciding with the significant increase in the biosynthesis of all five carotenoids (C_{40}) presented in Table 1, this can be attributed to a prioritization of the biosynthesis of carotenoids, which are superior terpenoids, to the detriment of monoterpenes. In summary, the general results of the detected VOCs point to the priority of ester synthesis to the detriment of monoterpenes and aldehydes in the pequi.

4. Conclusion

This study exhibited the proteome and a set of bioactive and volatile metabolites expressed throughout the development of the pequi fruit. The most influential and essential TFs operating in the regulation of pequi ripening identified were members of the MAD-box family. A group of proteins related to the methionine cycle indicates the high consumption and recycling of methionine. However, due to the non-climacteric respiratory pattern of the pequi, this consumption does not occur mainly for the biosynthesis of ethylene, a process dependent on the consumption of methionine, although this process naturally must arise at a less significant level. In the bioactive compounds presented, different proteins could be correlated with the presence of these phytochemicals, such as MDHAR and APX in AsA recycling; PK, FBA, and PSY with carotenoid biosynthesis; SAM1 as a donor of methyl groups in the formation of trigonelline and AspAT as a biomarker of initial regulation of the trigonelline biosynthetic pathway; PAL, Chorismate synthesis and Chalcona-flavononone isomerase in the biosynthesis of phenolic compounds. Among the VOC identified, the majority compound in pequi was ethyl hexanoate ester, with an area of 50.68 % in the ripe fruit, and in this group of esters that was the most representative, ADH, a fundamental enzyme in the synthesis of esters, was identified with an increase of approximately 7.2 between the first and last stages. Therefore, an extensive group of proteins, as well as some metabolites, can serve as biomarkers of ripening in pequi, as most were more

expressed in the last stage, which is the ripe fruit suitable for consumption.

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CRediT authorship contribution statement

Carlos Alexandre Rocha da Costa: Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Sidney Vasconcelos do Nascimento:** Writing – review & editing, Methodology, Investigation, Data curation. **Rafael Borges da Silva Valadares:** Validation, Data curation. **Luíz Guilherme Malaquias da Silva:** Methodology, Investigation. **Gilson Gustavo Lucinda Machado:** Methodology, Investigation. **Isa Rebecca Chagas da Costa:** Methodology, Investigation. **Sayure Mariana Raad Nahon:** Methodology, Investigation. **Luiz Jose´ Rodrigues:** Writing – review & editing. **Eduardo Vale´rio de Barros Vilas Boas:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114687.

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ARTIGO 2

Proteome and metabolome of *Annona crassiflora* Mart. fruit and their interaction during development

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Research Paper

Proteome and metabolome of *Annona crassiflora* Mart. fruit and their interaction during development

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ABSTRACT

Marolo (*Annona crassiflora* Mart.) is an exotic fruit from the Brazilian Cerrado, rich in various bioactive compounds. This unprecedented study analyzed through different techniques (spectrophotometric methods, UPLC, HS-SPME-GC–MS) the proteomic and metabolic changes in four distinct stages of development, their biochemical correlations, biological processes and how proteins are dynamized promoting such processes and biosynthetic pathways. Among the four stages, 993 proteins were identified and 45 metabolites were quantified. At the beginning of development there is a greater presence of ribosomal, transcriptional and post-translational proteins, to increase the protein synthetic machinery that must coordinate the other biological processes essential for development. The families of transcription factors GATA, MADS-Box, MYB, F-box and HD-Zip were identified, which are likely coordinators of the biological processes of marolo together with phytohormones. At the ripe stage, there is an increase in the accumulation of some cellulases to intensify the softening of the fruit, and the antioxidant system of marolo presented a mostly constant behavior, in order to control the steady states of the naturally produced reactive oxygen species. Several proteins identified are related to the detected metabolites, with emphasis on the production and regeneration of vitamin C and the carotenogenic pathway. The volatile profile of marolo was related to the action of some enzymes and the supremacy of volatile esters. Therefore, proteomics in conjunction with metabolomics allowed us to have a molecular view of how compounds and proteins are produced during marolo development and how the behavior of proteins appears to influence biological processes and pathways.

1. Introduction

Marolo (*Annona crassiflora* Mart.), also known as araticum, cerrado pine or ata, is an exotic fruit from the Brazilian Cerrado belonging to the *Annonaceae* family, widely distributed in tropical and subtropical regions, represented by >3000 species (Ramos et al., 2022, 2023). The fruit is a subglobose berry about 11 to 15 cm in diameter and 1 to 2 kg in weight when ripe. Its pulp is yellowish-white in color, due to the presence of carotenoids in its phytochemical composition, and the pulp accounts for 50 % of the fruit's total mass (Carvalho et al., 2022).

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Traditionally in folk medicine, the fruit is used as a tonic and astringent, and indicated to treat pain and rheumatism (Arruda et al., 2023). Along with the various phytochemicals investigated in marolo pulp, such as vitamin C, phenolic compounds, carotenoids, alkaloids and other terpenoids (Almeida et al., 2024), studies have reported the many health-promoting effects associated with these constituents in marolo, such as antioxidant properties (Arruda et al., 2018; Ramos et al., 2023), anti-Alzheimer's (Lucas dos Santos et al., 2018), anticancer (Carvalho et al., 2022), anti-inflammatory (Rosan Fortunato Seixas et al., 2021) and antibacterial (Stafussa et al., 2021; da Silva et al., 2014) activity.

Although marolo is one of the 20 most popular fruits in the Cerrado region, recognized by 85 % of the local population, its harvest for commercial purposes has been carried out in an extractive manner, from native populations, since there are no established commercial crops (Arruda et al., 2023). There are estimates that indicate a significant productivity of *A. crassiflora* Mart., which can reach 400 fruits per hectare in native areas of the Cerrado, with potential for production of up to 2000 fruits per hectare in commercial crops (Almeida et al., 2024). Marolo is consumed fresh or processed, normally as the main ingredient in the composition of several Brazilian regional products, especially sweets. This fruit outstands for its intense and peculiar aroma, nutritional value and functional appeal. However, despite the great value attributed to the fruit of *A. crassiflora* Mart. and its potential for exploitation by the food and pharmaceutical industries and expansion in the international market, this and other native species of the Brazilian Cerrado biome are progressively losing ground in the face of agricultural expansion. In fact, in the last 50 years, more than half of the original vegetation of the Cerrado has been replaced by monocultures and pastures (Fonseca, Uagoda and Chaves, 2021). In this scenario, the use of technological and molecular tools is crucial for the preservation, improvement and expansion of the biotechnological uses of marolo. Researchers have progressively turned to molecular biology technologies, especially genetic tools and omics technologies, such as proteomics and metabolomics, in order to achieve this goal.

Proteomics is a molecular biology technique that systematically and dynamically investigates the variation and accumulation of proteins associated with various biological processes in living beings, organs, tissues or cells (Tian et al., 2024). Metabolomics, in turn, is the systematic study of metabolite profiles, which recognizes and measures small molecules with molecular mass below 1500 Da, in a specific biological or chemical sample (Utpott et al., 2022). Such technologies have played an important role in expanding the understanding of biological processes in fruits and their use has increased the identification of biomarkers, enzyme-substrate interactions, research on bioactivity, exploration of metabolic pathways and understanding of the mechanisms of growth and development of plants and fruits (Li, Wang and Suh, 2022; Momo et al., 2022).

Through these possibilities of molecular and phytochemical characterization, the integration of proteomics and metabolomics has helped in the biochemical understanding of the changes that occur in fruits during the advancement of development and ripening stages (Tartaglia et al., 2024). In tomato, 2607 proteins were more abundant in the ripe fruit than in the ripe green fruit, evidencing an increase mainly in the processes of photosynthesis and interconversions of pentose and glucuronate (Tang et al., 2020). Analyzing four stages of arabica coffee cherries, it was confirmed that the metabolic pathways of amino acids and organic acids are the critical routes in the formation of flavor during ripening (Li et al., 2023c). In three Sorbus stages, the integration of the analyses made it possible to elucidate that most of the differentially abundant proteins are related to the softening process, carbohydrate metabolism and stress responses, and that enzymes abundant in the early stages explain the accumulation of sugars and the reduction of acidity during development (Tartaglia et al., 2024). In white guava, the climacteric ripening behavior was elucidated in addition to the increase in growth regulators during ripening (Monribot-Villanueva et al., 2022). In four pequi stages, the most influential transcription factors acting in the regulation of ripening were members of the MAD-box family (da Costa et al., 2024a).

Therefore, in this present and unprecedented study, we aimed to analyze the proteome and a set of bioactive and volatile metabolites of *A. crassiflora* Mart. fruits at four distinct developmental stages to understand and generate insights into how metabolic changes may have biochemical correlations with the marolo proteome. We also aimed to identify which biological processes occur throughout marolo development and how proteins are dynamized to promote such processes and biosynthetic pathways.

2. Materials and methods

2.1. Harvest, selection and definition of stages

The fruits of *A. crassiflora* Mart. were harvested in a single day in the town of Paraguaçu (21° 33′ 22′′ S and 45° 44′ 22′′W), southern region of the state of Minas Gerais, Brazil. They were transported, on the same day, to the Post-harvest Fruit and Vegetable laboratory at the Federal University of Lavras, Minas Gerais, Brazil, and sorted into four stages of development, according to the peel and pulp characteristics (Fig. 1).

The marolo is a fruit with no defined description of its development stages, and therefore the description of the stages was processed in two specific ways for this study. The first stage took place in the field with the help of local producers, after a day of research and knowledge exchange. These producers described how they analyze the changes in the fruit, clearly classifying three stages based on external characteristics, which are color and the opening of the peel between the infructescences that together make up the fruit. The second stage was the laboratory evaluation by the research group, analyzing the interior of the fruit together with the overall characteristics of the peel. Both evaluations, by the producers and researchers, were in agreement regarding the definition of the stages and are described below.

In the first stage, called immature, the fruits had a green and extremely rigid peel, and the pulp was firm and whitish. The peel infructescences were very close together, without opening. The overall size of the fruits at this stage is similar to the fruits at the other stages, and is not a differentiating parameter regarding maturation. Marolo fruits present great variability in relation to size. The seeds at this stage presented the same size and dark brown coloration as the other stages. In the second stage, called mature green, the fruits still had predominantly green peel, but with increasing transition to brown, and the peel was softer than the previous stage. The pulp has a color transitioning from white to yellow. The peel infructescences were beginning to open up between themselves. In the third stage, called ripe, the fruit had a soft and brown peel, and the pulp was yellow, juicy and soft. The peel's infructescences had significantly increased the opening between them, a striking characteristic of the fruit considered ripe by producers. The fruits in the last stage, called overripe, were collected on the ground, after natural abscission, with a softer peel texture than in the previous stage, with injuries caused by its fall, which allowed partial visualization of the pulp, yellow in color, less intense than in the previous stage. After removing the peel, the pulps were separated from the seeds manually using stainless steel knives, immediately frozen with liquid nitrogen, placed in polyethylene bags and stored in an ultra-freezer at —80 °C. The pulp of the fruits from each repetition were completely homogenized.

2.2. Proteomics

2.2.1. Freeze drying

Samples were frozen at —75 °C (Coldlab CL 120–86 V, Brazil) for 24 h and then freeze-dried (Edwards, L4KR, Brazil) at —30 °C with a vacuum pressure of 0.998 mbar for 72 h in the dark (Meira et al., 2023). Once freeze-dried, the samples were transported to the Vale Technological Institute, located in the city of Bel´em (1° 27′ 18″ S, 48° 30′ 9″ W), capital of the state of Par´a, northern region of Brazil, where they were

Fig. 1. Four stages of marolo development (*A. crassiflora* Mart).

subjected to proteomic analyses.

2.2.2. Protein extraction and quantification

The proteomic analyses were conducted following the procedure outlined by do Nascimento et al. (2022). The pulp of the fruits were frozen with liquid nitrogen and ground into a fine powder consistency. Each sample received a mixture containing sucrose (1.5 M), Tris-chloride (1.5 M, pH 8), 10 % sodium dodecyl sulfate (SDS), 100 mM methylphenylsulfonyl fluoride (PMSF), polyvinylpolypyrrolidone (PVPP), and ultrapure H_2O. Additionally, 100 µL of protease inhibitor (Protease Inhibitor cocktail - P8340 Sigma-Aldrich) and 500 µL of β-mercaptoethanol were added to each sample. Following five rounds of 30-second sonication, the extracts were split into ten microtubes, each receiving 700 µL of phenol. After vortexing and centrifugation at 14,000 rpm for 8 min twice, the phenolic phase was separated from any remaining aqueous phase or SDS. Finally, 1300 µL of ammonium acetate in methanol were introduced to precipitate the proteins over a period of approximately 24 h at —80 °C. A new round of centrifugation was per- formed at 14,000 rpm for 8 min, leading to the discarding of the supernatant. The resulting precipitate was then moved to a fresh microtube and rinsed four times with 80 % acetone, followed by a final wash with 70 % ethanol. Subsequently, the precipitate underwent a drying process at room temperature in a vacuum concentrator for around 7 min. The obtained extracts were dissolved in 200 µL of 0.2 % RapiGest (Waters, Milford, MA, USA) and stored for subsequent analysis.

2.2.3. Protein digestion

For the digestion preparation, the proteins were first reduced using dithiothreitol (DTT, 5 mM) and allowed to incubate at 56 °C for 25 min. This step was followed by alkylation with iodoacetamide (IAA, 14 mM) for 30 min. To eliminate any remaining IAA, DTT (5 mM) was added once more and incubated for 15 min. The samples were diluted with a 1:5 ratio of ammonium bicarbonate (50 mM), and CaCl (1 mM) was introduced. The treatment with trypsin (20 ng μL^{-1}) was carried out for 16 h at 37 °C. Next, trifluoroacetic acid (TFA) was introduced to the samples at a final concentration of 0.4 % to halt the enzymatic process. The protein levels in each sample were quantified using the Qubit2.0 fluorometer (Invitrogen, Thermo Fisher Scientific), following the manufacturer's guidelines for the Qubit protein assay kit.

2.2.4. Protein identification and data analysis

Subsequently, five micrograms of peptides were analysed on a NanoACQUITY UPLC ultraperformance liquid chromatography system (Waters, Milford, MA, USA), set up for two-dimensional fractionation. The initial dimension utilized a 5 µm XBridgeTM BEH130 C18 analytical column (300 µm x 50 mm) at a flow rate of 2 µL min^{-1}. The second dimension involved a 5 µm C18 trap column (180 µm x 20 mm) and a 1.7 µm BEH130TM C18 1.8 µm analytical column (100 µm x 100 mm) operated at a flow rate of 400 nL min^{-1}. Samples were fractionated into 5 parts based on a gradient of acetonitrile concentrations: 10.8 %, 14.0 %, 16.7 %, 20.4 %, and 65.0 %. The chromatograph was directly connected to an ESI-Q-ToF Synapt G2S mass spectrometer from Waters set to work in positive mode and continuous fragmentation (MS^E), varying collision energy from 5 to 40 eV Mass spectra were captured within the 50 to 1200 Da range, with a scan time of 0.5 s and an interval of 0.1 s. Peak width and mass spectra resolution were adjusted to automatic mode. The peptide [Glu-1]-fibrinopeptide (lockmass), with a mass of 785.4827 Da and carrying +2 charges, was utilized as a reference and checked every 30 s (following the lockmass spray settings).

Data analysis was performed using Progenesis QI software (Waters) for both identification and quantification, with the Uniprot trembl database from UniProt (UniProtKB/swiss-prot, uniprot.org) used. Pro- tein identification was considered valid when the likelihood of accu- rately identifying peptides exceeded 90 %, and for proteins, the threshold was set at 95 %. The levels of significance of the proteins with differential abundance were assessed through the ANOVA test (p-value < 0.05) using Progenesis QI. Functional annotation of the proteins was performed with the OmicsBox program version 2.1.14 (Biobam). Principal component analysis (PCA) and a heat map were generated with the Clustvis web tool (https://biit.cs.ut.ee/clustvis/), where the heat map was constructed using the Euclidean distance method. Prediction of protein-protein interaction networks was based on a functional analysis done with STRING software version 12.0 (http://string-db.org/, accessed March 15, 2024), utilizing homologous proteins from *Arabi- dopsis thaliana* as a reference species.

2.3. Vitamin c and carotenoids

The vitamin C content was determined following the spectrophotometric method outlined by Strohecker & Henning (1967). The extract used was obtained by homogenizing 5 gs of pulp with 45 mL of 0.5 % oxalic acid. After stirring for 30 min, the samples were filtered using quantitative filter paper sized at 15 cm. For the determination, 1 mL of the filtered extract, 3 mL of 0.5 % oxalic acid, 3 drops of 2,6-dinitrophenylhydrazine 2 %, 1 mL of hydrazine and 1 drop of thiourea were added to test tubes. The tubes were placed in a water bath at 37 °C for 3 h, with an ice bath at the end of the period. 5 mL of 85 % sulfuric acid were added to each tube, with the tubes still in an ice bath. After vigorous vortexing and standing for 10 min, the reading was performed at 520 nm. The standard curve was previously obtained with ascorbic acid as

the standard. with the results expressed in mg of ascorbic acid 100 g^{-1}.

Total carotenoids were quantified using the spectrophotometric method described by Rodriguez-Amaya (2001). Initially, 5 gs of sample and 20 mL of cold P.A acetone were added to vials covered with aluminum foil, followed by agitation for 20 min and filtration in Erlenmeyer flasks. The plant material retained on the filter was washed again with acetone until the residue on the paper turned whitish. The filtrate containing the carotenoids extracted in acetone was transferred to separating funnels. In these funnels, 30 mL of petroleum ether and 100 mL of distilled water were added to separate the phases. After the phases were separated, the colorless part was discarded. This procedure was performed 3 times to completely remove the acetone. The remaining extract containing the carotenoids was transferred to 100 mL volumetric flasks, where the volume was completed with petroleum ether. The reading occurred at five absorbances (444 nm = α-carotene; 450 nm = β-carotene; 456 nm = δ-carotene; 462 nm = γ-carotene and 470 nm for lycopene). The following equation was used to determine the carotenoid content:

$$\mu g100g - 1 = \frac{A \times V \times 10^6}{A1cm1\% \times M \times 100}$$

A is the absorbance value obtained, V is the final volume of the solution, A 1cm1 % is the molar absorptivity coeficiente for each carotenoid (2800 = α-carotene; 2592 = β-carotene; 3292 = δ-carotene; 3100 = γ-carotene and 3450 for lycopene). M is the mass of the initial sample. The results were expressed in μg 100 g^{-1}.

2.4. Theobromine, trigonelline and phenolic compounds profile

The technique applied followed the methodology by da Costa et al. (2024a). Falcon tubes with 1 g of *A. crassiflora* Mart. pulp were mixed with 10 mL of 70 % HPLC grade methanol. After shaking for 30 min on a shaker table shielded from light, the samples underwent an additional 30-minute treatment in an ultrasonic bath. Subsequently, the samples were filtered using quantitative filter paper sized at 12.5 cm and a porosity of 0.025 mm. A second filtration step involved a 13 mm diameter membrane filter with a porosity of 0.00022 mm, and a portion of each filtrate was transferred into 1.5 mL vials for injection. High-performance liquid chromatography (HPLC) was carried out in Shimadzu equipment, consisting of a quaternary pump LC-20AT, degasser DGU-20A5, injector SIL-20A, controller CBM-20A, oven CTO-20AC, detector SPDM-20A, detector RID- 10A and fraction collector FRC-10A. The following parameters were adopted: Lc time program: 0.01 min - 0 % B, 5 min - 20 % B; 25 min - 40 % B; 43 min - 45 % B; 50 min - 80 % B; 55 min - 0 % B; 65 min - STOP; Flow: 1 mL min^{-1}; Oven temperature (°C): 35; Injected volume: 0,02 mL; DAD: 280 nm; Shim-pack VP-ODS column 250 mm x 4.6 mm x 0,0005 mm and Shim-pack GVP-ODS pre-column 10 mm x 4.6 mm x 0,0005 mm; Mobile phase A – 2 % solution of glacial acetic acid in ultrapure water; Mobile phase B – Solution consisting of 70 % methanol and 2 % acetic acid in ultrapure water. Fifteen standards were used, namely: trigonelline, theobromine, catechin, resveratrol, vanillin and gallic, chlorogenic, ferulic, caffeic, *o*-coumaric, *m*-coumaric, *p*-coumaric, syringic, rosmarinic and *trans*-cinnamic acids. The results obtained were expressed in mg 100 g^{-1}.

2.5. Volatile compounds

The technique applied followed the methodology by da Costa et al. (2024a). The volatile compounds of the *A. crassiflora* Mart. samples were examined using headspace solid-phase microextraction combined with gas chromatography-mass spectrometry (HS-SPME-GC–MS). Samples weighing 2 g were placed in 20 mL headspace bottles and heated to 40 °C in an aluminum block. Volatile compounds were extracted using a 50/30 μm DVB/CAR/PDMS fiber. Volatile compounds were detected by GC–MS (Shimadzu CG-17 A, Shimadzu, Japan) equipped with an Rtx-5MS column 30 m x 0.25 mm internal diameter x 0.25 μm film thickness (bound phase; 5 % diphenyl, 95 % dimethyl polysiloxane). The carrier gas (helium) flow rate was a constant flow of 1.0 mL min^{-1}. The initial temperature was 40 °C for 30 min, with subsequent ramp rate of column by 3 °C per minute until reaching a temperature of 220 °C. The interface temperature for the MS was 240 °C and the ion source 220 °C. The identification of volatile compounds was based on the comparison of mass spectra using the Willey 8 and NIST libraries.

2.6. Statistic

Data were analysed using the SISVAR 5.8 version software. Analysis of variance (ANOVA) and the Scott-Knott method ($p < 0.05$) were used to compare the possible significant differences among the fruit in four developmental stages. Values were expressed as mean ± standard deviation. The analyses were performed in three replicates per stage. Each replicate was composed of seven fruits, totaling 84 fruits in the study.

3. Results and discussion

3.1. Proteome of A. crassiflora Mart

993 proteins were identified in *A. crassiflora* Mart. fruits during their development (Table S1). Among these, 198 proteins exhibited differential accumulation with statistically significant values at all four stages. These proteins were functionally annotated into 55 biological processes (Fig. 2A). Some proteins were associated with multiple biological processes due to their ability to influence various molecular mechanisms and cellular locations, despite having specific chemical functions and structures. The changes in protein accumulation across stages, represented by proteins that decreased (Fig. 2B) and increased (Fig. 2C), as well as the principal component analysis (PCA) of these 198 proteins among the three replicates at each stage, are also depicted.

The three biological processes that presented the highest amount of proteins were the biosynthesis of organic substances (112), cellular biosynthesis (102), and the metabolic process of organonitrogenous compounds (99). Of these, 68 proteins were common to all processes, and a general heatmap was developed encompassing the three processes (Fig. 3A). Different background colors highlighted proteins common to two processes or exclusive to just one. Fig. 3B presents the analysis of the protein-protein interaction of the proteins described in Fig. 3A, which shows that different types of interactions detected or prospected by public databases can be observed.

In Fig. 3A, it is possible to visualize different clusters formed by the similarity in protein accumulation between stages. In the upper region of the heatmap, a clearly evident cluster is that of proteins most accumulated exclusively in the immature stage. In this cluster, proteins necessary for the translation process stand out. Among these are five ribosomal proteins, 40S ribosomal protein S4, 40S ribosomal protein S18, 40S ribosomal protein S6–2 and two isoforms of 50S ribosomal protein L20. Also in this cluster is the presence of a translation factor, eukaryotic translation initiation factor 5A (eIF5A). Two isoforms of RNA polymerase (EC:2.7.7.6), a transcriptional protein, were also more accumulated in the immature stage. The eukaryotic ribosome, present in the cytosol, is composed of two ribonucleoprotein subunits of unequal sizes, the small 40S subunit and the large 60S subunit (Fakih, Plourde and Germain, 2023), while the chloroplastic ribosome is different, consisting of the 30S and 50S subunits, common to prokaryotes and organelles (Kravchenko et al., 2023). Regarding the main ribosomes in the cytosol, it is in the 40S subunit that mRNA binding occurs at the decoding site, the initial phase of translation, while in the 60S subunit, tRNA molecules catalyze the formation of peptide bonds at the peptidyl transfer site. At the end of translation, the 40S subunit also performs ribosome recycling (Fakih, Plourde and Germain, 2023; Kravchenko et al., 2023). In addition to playing a significant role in ribosome biogenesis, protein synthesis, cell growth, development, apoptosis and

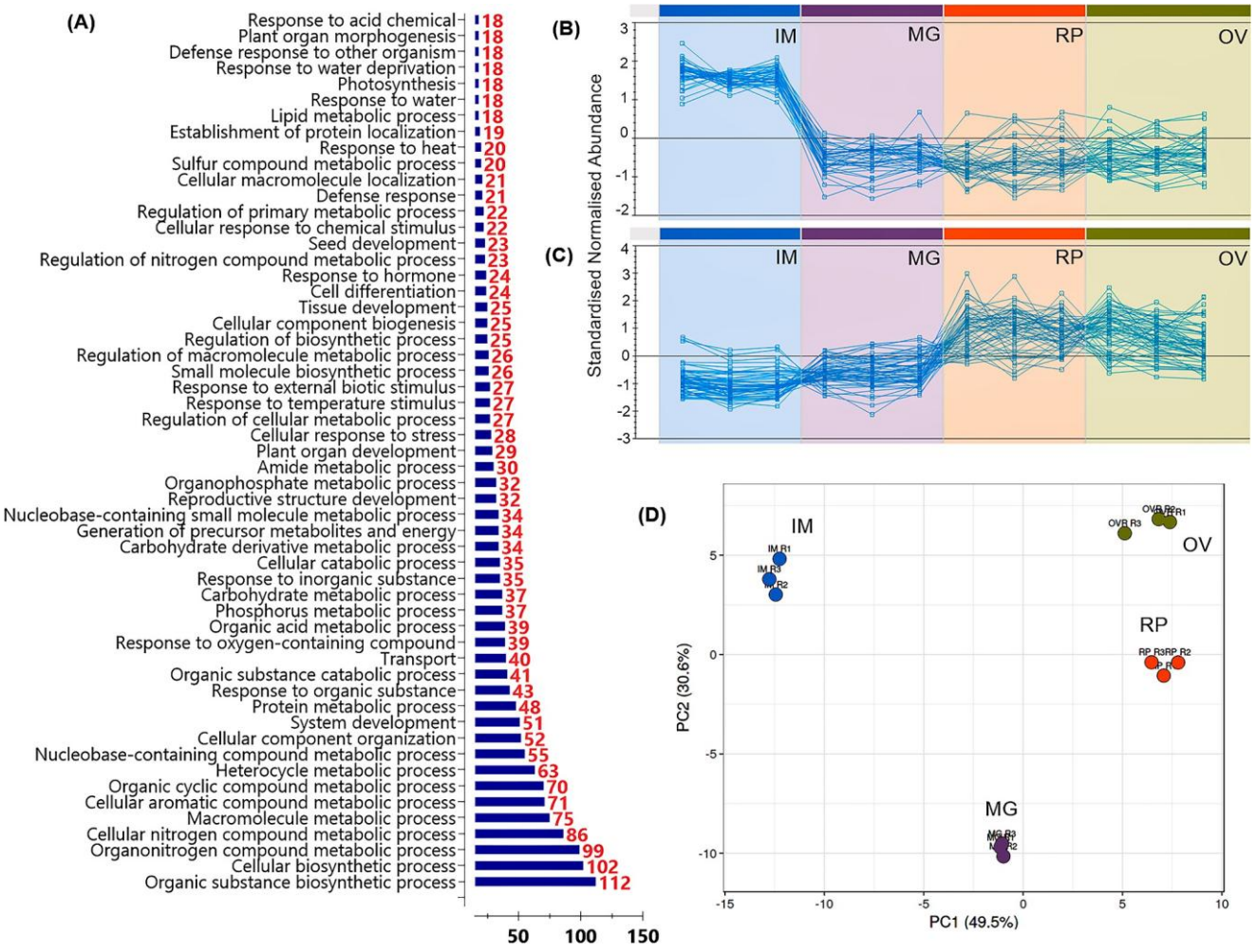

Fig. 2. (A) Functional annotation of biological processes and the respective numbers of proteins for each process. (B) Variation of proteins that decreased in accumulation throughout the stages. (C) Variation of proteins that increased in accumulation throughout the stages. (D) PCA of the four stages of development. Immature (IM), mature green (MG), ripe (RP), and overripe (OV).

maintenance functions, the supply of ribosomal proteins is directly correlated with the rapid growth phase (Saha et al., 2017). eIF5A is a protein that regulates protein synthesis and performs translation elongation and termination as a ribosome quality control cofactor. It also acts by renewing mRNA molecules, in cell proliferation, and in rescuing stalled ribosomes. Its action is reported to be very relevant in the growth of eukaryotic cells, and it has a special peculiarity among eukaryotes, as it is the only protein that contains a rare amino acid, hypusine. Hypusine promotes the activation of eIF5A and its synthesis is directly dependent on the cleavage of spermidine, an important polyamine in climacteric fruits (Park and Wolff, 2018; Pa´lfi et al., 2021). In the first cluster of Fig. 3A, there is the presence of spermidine synthase 1 (SPDSY1, EC: 2.5.1.16), an enzyme that produces spermidine. Spermidine is a key limiting factor for hypusine synthesis and, consequently, for eIF5A activation. Hypusine synthesis exclusively for eIF5A represents one of the most specific post-translational modifications ever reported in cellular studies (Park and Wolff, 2018). Fig. 3A also shows the proteins eukaryotic initiation factor 4A-9 (eIF4A-9) and 60S ribosomal protein L27, both of which are more accumulated in the immature stage. eIF4A-9 is an initiation factor required for mRNA binding to the ribo- some and was accumulated 12.87 times more in the first stage compared to the overripe stage. The combination of these proteins and the behavior of greater accumulation in the first stage shows that one of the most striking events of the immature stage in marolo is the elevation of the cellular machinery that promotes protein synthesis. This must occur to produce more proteins involved in biological processes essential for fruit development. Glycolytic proteins also present at this stage suggest the need for energy production to support all the synthetic processes that tend to increase during fruit development.

As in Fig. 2D, where the immature stage was more distant from the others, in terms of similarity, the first cluster in Fig. 3A, which highlights proteins that were more accumulated in the immature stage, was the largest and most clearly defined among the other clusters. Other smaller clusters in Fig. 3A showed a more dispersed distribution of proteins among the four stages of development of the marolo, with proteins that participate in and regulate distinct routes and reactions. Some of these routes are briefly explained in the topics below.

3.2. Polyamines and S-adenosylmethionine

Polyamines are essential in the regulation of biological processes together with phytohormones, and in the set of most abundant proteins in the first stage of Fig. 3A, another enzyme related to the production of polyamines is Arginase 2 (ARGAH2, EC:3.5.3.1), an enzyme that catalyzes the hydrolysis of arginine into ornithine, subsequently converted into putrescine by the action of Ornithine decarboxylase (ODC, EC:4.1.1.17). ODC was detected in the proteome of marolo in all stages of the fruit, but without statistically significant difference. After the action of ODC, SPDSY1 can then act, converting putrescine into spermidine. Although the function of spermidine as a substrate in the

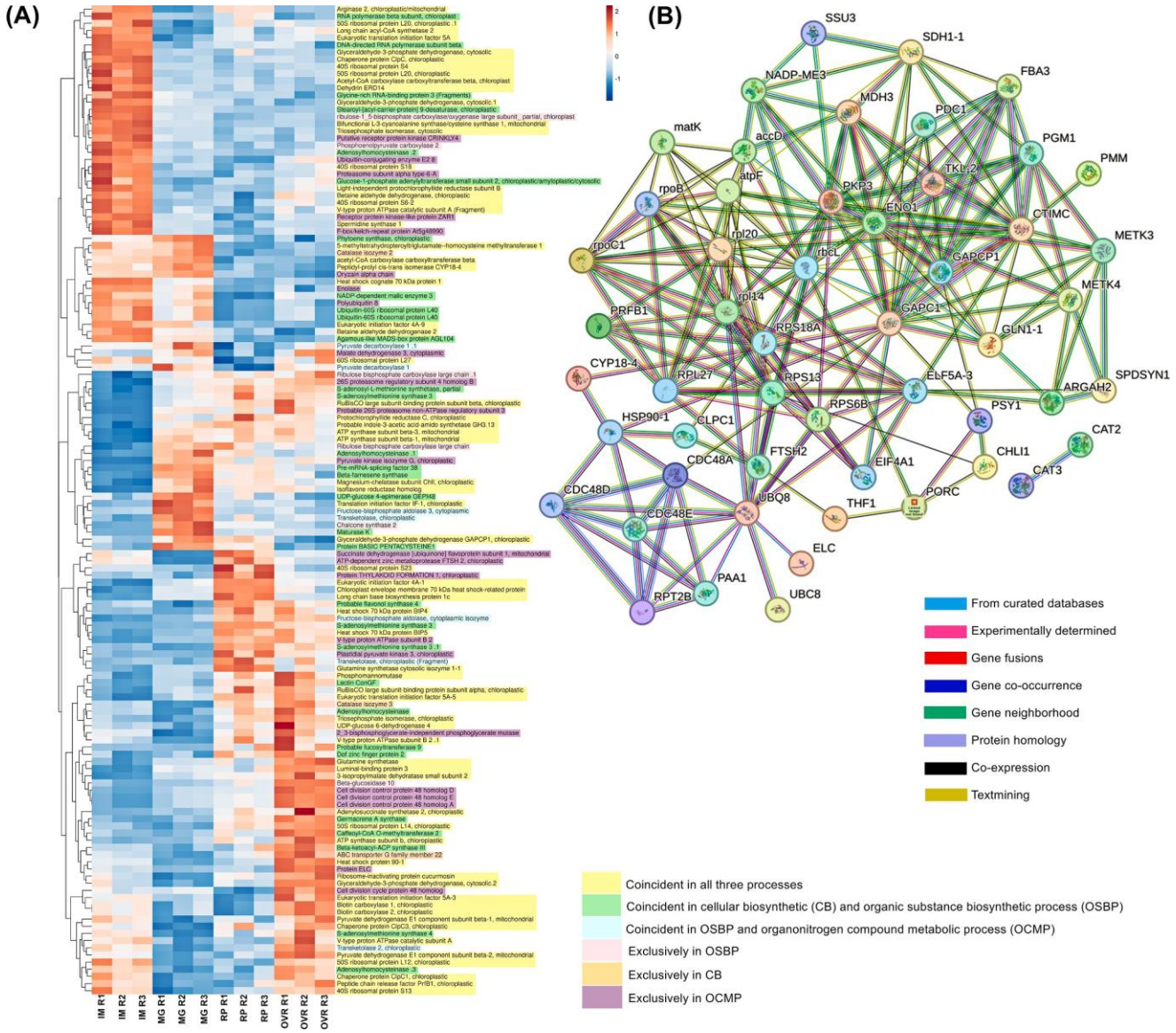

Fig. 3. (A) The heatmap depicts proteins related to the three most prevalent biological processes: organic substances biosynthesis (112), cellular biosynthesis (102), and organonitrogen compounds metabolic process (99) found in the pulp of *A. crassiflora* Mart. across four stages: immature (IM), mature green (MG), ripe (RP), and overripe (OV). Strong red hues indicate high accumulation levels, while deep blue represents lower accumulation. Related to the background colors of the protein names: Yellow: Coincident in all three processes; Green: Coincident in cellular biosynthetic process (CB) and OSBP; Blue: Coincident in OSBP and OCMP; Pink: exclusively in OSBP; Orange: exclusively in CB; Purple: exclusively in OCMP. (B) The protein-protein interactions displayed in the gene list encoding the proteins illustrated in Fig. 3A when compared to equivalent proteins in the *A. thaliana* database. The varying line colors indicate distinct types of recognized or potential interactions.

synthesis of hypusine is reported, most of the spermidine is designated to promote other metabolic processes. Spermidine is an anabolic regulatory molecule, acting as an activation signal for a vast genetic network involved in the regulation of growth and development in fruits (Gao et al., 2021). Therefore, in marolo, the polyamine machinery appears to be more active in the immature stage, aggregating with other development-promoting factors.

The key substrate for polyamine biosynthesis is S-adenosylmethionine (SAM), which is also used for ethylene biosynthesis. Due to the simultaneous occurrence of polyamines and ethylene, there is competition for the same substrate, and depending on the fruit stage, SAM consumption is prioritized between one of these competing pathways (Gao et al., 2021). In marolo, however, 22 different isoforms of S-adenosylmethionine synthase (SAMS), an enzyme that synthesizes SAM, were detected, evidencing an increase in the production of this metabolite to supply the different processes dependent on this substrate. Out of these 22 isoforms observed in marolo, only six showed statistically significant variations among the four stages, all of which exhibited greater accumulation between the third and fourth stages. At the ripe stage, SPDSY1 had the lowest accumulation recorded, allowing the cellular flow of SAM to be directed mainly to ethylene synthesis during marolo ripening. Thus, due to the functions described, the proteins that synthesize polyamines accumulate more in the immature stage to help trigger processes adjacent to fruit development, while in the ripe stage, ethylene must be produced more to intensify the reactions inherent to ripening.

3.3. *Transcription factors*

The process of fruit ripening is controlled by a genetically determined system of plant hormone signaling pathways, which include networks of transcription factors (TFs) and epigenetic modifications that

exert combined and separate effects (Li et al., 2023a). TFs play a role in influencing the expression of various genes encoding proteins involved in different metabolic processes (Li et al., 2022b). Several families of TFs, such as MADS-Box, GATA, F-box, MYB, NAC, ARF, and HD-Zip, have been shown to participate in regulating fruit development and ripening (Forlani, Mizzotti and Masiero, 2021).

A set of proteins from different TF families were detected in the marolo proteome at different developmental stages, with emphasis on the GATA (2), MADS-Box (2), MYB (3), F-box (4), and HD-Zip (5) families. In higher plants, GATA TFs are involved in several biological processes such as response to light, germination, nitrogen metabolism, chloroplast biogenesis, leaf and floral development, phytohormone signaling, and response to many abiotic stresses (Le et al., 2024). However, there are few reports on this family during fruit development. In one of them, GATA TFs, together with F-box and MADS-Box, presented regulatory effects on pepper color formation (Li et al., 2021). Genes from the MYB family, identified in marolo, present pro-carotenogenic activity, in addition to several functions very similar to those of GATA (Yang et al., 2021). MADS-box family TFs are crucial in virtually all aspects of plant growth and development, with evidence indicating that they are particularly influential and central in controlling fruit development and ripening (Liu et al., 2017). MADS-box TFs are engaged in the production of polyphenolic compounds and can act as regulators in the flavonoid and carotenoid pathway (S´anchez-Go´mez, Pose´ and Martín-Pizarro, 2022). The F-box family is one of the most expressive in plants when compared to other eukaryotes. In humans, for example, there are 69 *F-box* genes, while in plants it can exceed 900. Fruits such as apples and pears have 517 and 226 genes from this family, respectively. Due to such diversity, F-box TFs also have broad functions, such as regulating plant growth and development in general, and their action can regulate other TFs, such as MADS-Box (Xu et al., 2021). The HD-Zip family, also found in marolo, follows a similar pattern to the previous superfamilies regarding comprehensive functions in plant growth and development, although they are more closely related to the fruit ripening process when compared to F-box, GATA and MYB TFs. HD-Zip TFs have been correlated with triggering tomato ripening and controlling the ripening process, expression and production of ethylene biosynthesis genes in peach (Lin et al., 2008; Gu et al., 2019).

The individual and joint action of TFs are essential in the development of marolo and in the biosynthesis of various compounds, such as carotenoids and phenolic compounds investigated in this study. The families listed are likely responsible, together with phytohormones, for the central regulation of the development and ripening of *A. crassiflora* Mart. fruits.

3.4. Plant cell wall degradation

One of the most evident physiological changes during fruit ripening and senescence is softening. Biochemically, the polysaccharide networks that make up the plant cell wall are depolymerized by the action of specific enzymes belonging to the cellulase, hemicellulase and pectinase classes.

In the marolo proteome, cellulases stood out with 6 proteins accumulated among all stages, four β-glucosidases (EC: 3.2.1.21) and two endoglucanases (3.2.1.4). The mechanism of enzymatic hydrolysis of cellulose describes the synergistic action of at least three classes of enzymes: endoglucanases, exoglucanases and β-glucosidases or cellobiases. Firstly, endoglucanases act in the internal region of the cellulose fiber, releasing oligosaccharides, while exoglucanases act from the ends of the cellulose, releasing glucose or cellobiose units, and finally, β-glucosidases break the chemical bond that forms cellobiose and oligosaccharides, releasing free glucose units (Sutaoney et al., 2024). Therefore, the action of β-glucosidase is dependent on the above enzymes. Of the six proteins identified, only one endoglucanase and one β-glucosidase showed statistically significant differences between stages. Endoglucanase had maximum and minimum accumulation in the ripe and immature stages, respectively, with a 3.04-fold increase in synthesis between these stages. β-glucosidase showed maximum and minimum accumulation in the overripe and immature stages, respectively, with a 2.24-fold increase in synthesis. It is in the ripe stage that the fruit presents the softening characteristic of the fruit suitable for consumption. Together with the cellulases, three hemicelluloses and one pectate lyase were also identified in the marolo proteome, at practically constant levels during its development. This behavior of the degradative proteins of the cell wall indicates a controlled and measured softening process in the marolo, which is accentuated when the fruit enters the ripe stage, to give it adequate softness. The breakdown of cellulose ap- pears to be the prioritized pathway in marolo. However, the possible role of the aforementioned cellulases in the breakdown of side chains of hemicelluloses and pectins, which contain glucose, cannot be ruled out. The breakdown of these side chains also plays an important role in the softening of fruits.

3.5. Enzymatic antioxidant system

27 Enzymes of the antioxidant system were identified in the marolo proteome, being seven isoforms of superoxide dismutase (SOD, EC 1.15.1.1), 14 isoforms of catalase (CAT, EC 1.11.1.6), one glutathione peroxidase (GPX, EC 1.11.1.9), one glutathione s-transferase (GST, EC:2.5.1.18), three isoforms of monodehydroascorbate reductase (MDHAR, EC:1.6.5.4) and ascorbate peroxidase (APX, EC:1.11.1.11). 18.52 % (five enzymes) of this total showed statistically significant difference between stages. Different processes, such as nitrogen fixation, photosynthesis, respiration and peroxisomal metabolism, constantly generate reactive oxygen species (ROS) from electron transport chains in various organelles (Arias, Feijoo and Moreira, 2022). In order to prevent the toxic accumulation of ROS, plants have developed an antioxidant system that is composed of enzymes and non-enzymatic antioxidant substances, acting as a defense against excess ROS. The oxidative processes involved in the development and ripening of climacteric fruits, such as marolo (da Silva et al., 2013), but also of non-climacteric fruits, result in the production of ROS, which are inevitable byproducts of normal metabolic processes that occur continuously (Huang et al., 2023).

Although they can become toxic if produced uncontrollably, some ROS, such as superoxide radicals and hydrogen peroxide, can play important roles as signaling molecules in cellular processes when present in low concentrations. In these circumstances, antioxidants modulate steady-state concentrations of ROS in order to prevent their toxicity while promoting their beneficial effects. In fruits, antioxidants act to protect tissues against the harmful effects of ROS, reinforcing resistance, preserving nutrients, and extending shelf life after harvest (Huan et al., 2016). Apparently, there were no biotic or abiotic stresses or deregulation of biological processes that could promote oxidative damage in the marolo fruit. Thus, the action of the accumulated antioxidant enzymes probably occurred in controlling the natural production of ROS, with the purpose of keeping them at low levels.

3.6. Vitamin C, carotenoids, alkaloids, phenolic compounds, and enzymes involved in biosynthetic pathways

Changes in the concentrations of the studied bioactive compounds were observed throughout the marolo development (Table 1).

A reduction in vitamin C levels was noted throughout the development of marolo, of the order of 5.7 %, 33.1 % and 41.7 %, in the mature green, ripe and overripe stages, respectively, compared to the immature stage. However, even with such a decrease, ripe fruits, considered ideal for consumption, had a vitamin C content of 96.9 mg 100 g^{-1}. This concentration is higher than that found in oranges, humanity's main source of vitamin C (Randhawa et al., 2020). The Dietary Intake References from the Institute of Medicine (2000) in the United States recommend that for an adult, the dose is 65–90 mg day^{-1}, which suggests

Table 1
Bioactive compounds (mean ± standard deviation) in the pulp of *A. crassiflora* Mart. at four stages of development. Different letters indicate significant differences ($p < 0.05$) by ANOVA analysis followed by the Scott-Knott test. ND – Not Detected.

	Immature	Mature green	Ripe	Overripe
Vitamin C mg of ascorbic acid 100 g^{-1}	144,89^{a} ± 1,99	136,67^{b} ± 0,8	96,9^{c} ± 3,68	84,55^{d} ± 3,77
α-Carotene μg 100 g^{-1}	7,35^{c} ± 0,74	9,53^{b} ± 0,27	14,07^{a} ± 1,23	14,35^{a} ± 2,46
β-Carotene μg 100 g^{-1}	7,94^{c} ± 0,8	10,09^{b} ± 1,02	15,86^{a} ± 1,6	14,73^{a} ± 0,62
δ-Carotene μg 100 g^{-1}	7,26^{b} ± 1,14	7,79^{b} ± 0,74	10,16^{a} ± 1,30	10,69^{a} ± 0,84
γ-Carotene μg 100 g^{-1}	7,7 ^{b} ± 1,21	7,64^{b} ± 0,78	11,2^{a} ± 0,59	10,71^{a} ± 0,89
Lycopene μg 100 g^{-1}	6,35^{b} ± 0,82	8,57^{a} ± 1,79	10,53^{a} ± 0,92	9,63^{a} ± 0,51
Trigonelline mg 100 g^{-1}	12,52^{c}±0,58	14,04^{c}±0,39	26,02^{b}±1,76	61,43^{a}±0,93
Theobromine mg 100 g^{-1}	0,03^{c} ± 0,00	0,06^{b} ± 0,01	0,05^{b} ± 0,01	0,10^{a}± 0,01
Catechin mg 100 g^{-1}	1,18^{b}±0,04	1,01^{b}±0,01	7,99^{a}±0,51	7,62^{a}±0,34
Gallic acid mg 100 g^{-1}	1,66^{c}±0,02	0,89^{d}±0,01	1,89^{b}±0,07	1,95^{a}±0,04
Chlorogenic acid mg 100 g^{-1}	1,57^{d}±0,05	1,91^{c}±0,05	5,72^{a}±0,24	5,31^{b}±0,06
Caffeic acid mg 100 g^{-1}	0,75^{c}±0,04	0,42^{d}±0,02	1,37^{a}±0,03	0,99^{b}±0,02
m-Coumaric acid mg 100 g^{-1}	ND	ND	0,20^{a}±0,02	0,15^{b}±0,01
p-Coumaric acid mg 100 g^{-1}	0,16^{a}±0,00	0,11^{b}±0,01	0,09^{c}±0,00	0,07^{d}±0,01
Rosmarinic acid mg 100 g^{-1}	0,38^{a}±0,03	0,30^{b}±0,02	ND	ND

marolo as an excellent source of this vitamin.

Proteins related to the biosynthesis and recycling pathway of vitamin C, also known as ascorbic acid (AsA) or ascorbate, were detected in the marolo proteome: Phosphomannomutase (PMM, EC:5.4.2.8), Mannose-1-phosphate guanylyl transferase 1 (MPG, EC:2.7.7.13), GDP-mannose 3,5-epimerase (GME, EC:5.1.3.18), three MDHAR isoforms and one APX. PMM, MPG and GME are enzymes of the Smirnoff-Wheeler pathway, the main route of vitamin C synthesis in higher plants (Yu et al., 2021). PMM catalyzes the interconversion of mannose-6-phosphate to mannose-1-phosphate, and MPG converts mannose-1-phosphate to GDP-mannose. Subsequently, through two possible distinct reversible epimerization reactions, GME produces GDP-l-galactose or GDP-l-gulose from GDP-mannose (Dhanalakshmi et al., 2023). Therefore, PMM, MPG, and GME operate sequentially.
While PMM varied significantly ($p < 0.05$) throughout development with maximum and minimum accumulation at the overripe and immature stages, respectively, the latter two remained stable ($p > 0.05$). MDHAR and APX are antioxidant enzymes that recycle AsA during their cellular protection action. APX converts AsA to monodehydroascorbate to eliminate H_2O_2, while MDHAR reduces monodehydroascorbate back to ascorbate, regenerating this molecule so that this antioxidant system can occur again (Zheng et al., 2022). Only one MDHAR isoform varied significantly ($p < 0.05$) throughout development with maximum and minimum accumulation in the immature and ripe stages, respectively.

Therefore, the stability of GME and MPG throughout the development and accumulation of PMM in overripe fruits, despite the degradation of vitamin C (Table 1), does not allow us to conclude whether AsA synthesis is reduced in marolo, or whether this antioxidant is still continuously synthesized, but more consumed as the stages advance. It is noteworthy that the intermediates synthesized in the vitamin C synthesis pathway can also be used in the synthesis of sugars incorporated into the cell wall polysaccharides, such as mannose, fucose, galacturonic acid and glucose. This occurs as part of the strategies to strengthen the plant cell wall, which is naturally degraded during fruit ripening and senescence (Nishigaki et al., 2021). The presence of MDHAR and APX isoforms evidences the occurrence of AsA regeneration, a process that provides greater use of the ascorbate available in the fruit.

The concentrations of carotenoids α-, β-, δ-, γ-carotene and lycopene increased significantly ($p < 0.05$) during marolo development, until the fruit reached the ripe stage, no longer changing until the overripe stage (Table 1). This increase is associated with the appearance of the yellowish color of the pulp at the beginning of maturation and its intensification with ripening (Fig. 1). The most abundant carotenoids were β-carotene and α-carotene, reaching concentrations in the ripe fruit of 15.86 μg 100 g^{-1} and 14.07 μg 100 g^{-1}, respectively.

In the marolo proteome, the following proteins related to carotenogenesis were detected: phytoene synthase (PSY, EC:2.5.1.32), three isoforms of farnesyl pyrophosphate synthase (FPS, EC:2.5.1.1), phytoene desaturase (PDS1, EC:1.3.5.5), two isoforms of 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HMBPPS, EC:1.17.7.1), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HMBPPR, EC:1.17.7.4), eight isoforms of pyruvate kinase (PK, EC:2.7.1.40) and nine isoforms of fructose-bisphosphate aldolase (FBA, EC:4.1.2.13).

PK and FBA do not participate directly in the carotenoid synthetic pathway, but are essential for its occurrence. The PK enzyme catalyzes the final step of glycolysis, transferring the phosphate group from phosphoenolpyruvate to ADP, resulting in the production of ATP and pyruvate (Hu et al., 2020). FBA is responsible for catalyzing the reversible aldol cleavage of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (GAP) (Feng et al., 2020). The interaction between pyruvate and GAP leads to their condensation, and from this process, six successive reactions occur that result in the formation of the isopentenyl pyrophosphate (IPP) molecule, an important precursor of carotenoids in the plastid methylerythritol phosphate (MEP) pathway. HMBPPS and HMBPPR act together in the last two steps preceding the formation of IPP and dimethylallyl diphosphate (DMAPP). HMBPPS converts 2-C-methyl-d-erythritol 2, 4-cyclodiphosphate (ME-2,4cPP) to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP), and then HMBPPR converts HMBPP to IPP and DMAPP (Krause et al., 2023). Next, two IPP molecules combine with DMAPP to form geranyl diphosphate (GPP), which is subsequently converted to geranylgeranyl diphosphate (GGPP) and is used by PSY in the synthesis of phytoene (Sathasivam et al., 2021). This is the synthesis pathway of tetraterpenes (C_{40}), or higher terpenoids, such as the five carotenoids shown in Table 1.

In addition to being produced in plastids by the MEP pathway, carotenoids can also be synthesized in the cytosol by the mevalonate metabolic pathway (MVA). In this pathway, two IPP molecules condense with DMAPP, through the action of FPS, forming farnesyl pyrophos- phate (FPP). FPP is a fundamental branching point of the pathway that can give rise to distinct products, including cholesterol, steroids, doli- chols, and all sesquiterpenoids. The range of structures of these latter compounds is subsequently determined by the action of several sesquiterpene synthases (Park et al., 2021; Wang et al., 2022). Therefore, the presence of FPS isoforms in marolo presupposes the synthesis of other compounds, although not monitored in this study.

The aforementioned enzyme PSY plays a key role in carotenoid production, being crucial in the step in which it converts two GGPP molecules into phytoene, the first colorless carotene (Zhou et al., 2022). The subsequent step is the conversion of phytoene into ζ-carotene, carried out by PDS1 (Gou et al., 2024), also found in this proteome. PSY showed minimum and maximum expression in the overripe and mature green stages, respectively, while PDS1 showed no statistically significant difference between the four marolo stages. The enzymes that catalyze the subsequent steps are usually associated with the membrane and are less abundant and, therefore, more challenging to quantify (Cunningham and Gantt, 2007). The peak accumulation of PSY in the

second stage coincides with the increase in carotenoid content between the second and third stages of development. Therefore, its action in the production of tetraterpenes is essential in the chain and failures in the synthesis of PSY would directly result in a deficiency in the pigmentation of the pulp of *A. crassiflora* Mart.

The alkaloids trigonelline and theobromine showed an increase in their concentrations throughout fruit development ($p < 0.05$; Table 1). The maximum trigonelline content was 61.43 mg 100 g^{-1}, while theobromine was 0.1 mg 100 g^{-1}, both in overripe fruit. Alkaloids, such as trigonelline and theobromine, are compounds that have in common a heterocyclic ring containing an N atom (da Costa et al., 2023). An essential enzyme found to be active in the production of trigonelline was SAMS. This enzyme synthesizes SAM as a metabolite, which interacts with nicotinic acid to supply methyl groups needed for trigonelline synthesis (Ashihara, 2015).

SAMS isoforms with statistically significant variations showed greater accumulation between the third and fourth stages. The most significant rise in trigonelline, a 2.36-fold increase, happened between these two stages. A probable bottleneck enzyme implicated in the production of trigonelline in marolo is aspartate aminotransferase (AspAT, EC:2.6.1.1), responsible for deaminating aspartate into oxaloacetate, a crucial component for malate production utilized in the mitochondria for the tricarboxylic acid cycle (Han et al., 2021). Aspartate serves as the starting material for the trigonelline synthesis pathway (Ashihara, 2015). Three AspAT isoforms were detected, but none with statistically significant differences between stages of development.

Of the 13 phenolic compounds assessed, seven were detected in marolo. In general, there was an increase in the concentrations of catechin and gallic, chlorogenic and caffeic acids and a drop in the concentrations of *m*-coumaric, *p*-coumaric and rosmarinic acids during the fruit's development. It should be noted that *m*-coumaric acid was only identified in ripe and overripe fruit, while rosmarinic acid was identified in immature and mature green fruits. The other phenolics were identified in the fruit at all stages.

Among the enzymes identified in the proteome of *A. crassiflora* Mart. fruits, related to the biosynthetic pathways of phenolic compounds, those of the flavonoid pathway stand out, with 14 specific representatives. Catechin (Table 1) is a flavonoid of the proanthocyanidin class, and was the most abundant phenolic compound observed in this study, reaching a content of 7.99 mg 100 g^{-1} in the ripe fruit. From the group of 14 proteins, five isoforms of chalcone synthase (CHS, EC:2.3.1.74) were identified. CHS is the major and first rate-limiting enzyme in the flavonoid biosynthetic pathway and generates the central metabolite of the flavonoid branch classes, the chalcone naringenin (Liu et al., 2021). The other flavonoid pathway-specific enzymes were three isoforms of flavonol synthase (FLS, EC:1.14.20.6), Flavonol 3-O-glucosyltransferase UGT89B1 (EC:2.4.1.91), two isoforms of Chalcone–flavanone isomerase (CHI, EC:5.5.1.6), and three isoforms of Isoflavone reductase homolog (EC:1.3.1.). In strawberry, *CHS*, *CHI* and *FLS* genes were upregulated by the action of MADS-Box and MYB TFs (Sa´nchez-Go´mez, Pose´ and Martín-Pizarro, 2022).

The reduction in chlorogenic and caffeic acids in overripe fruits may be due to degradation resulting from the onset of senescence. Related to caffeic acid, three Caffeoyl-CoA O-methyltransferases (CCoAOMT, EC:2.1.1.104) and one Caffeic acid 3-O-methyltransferase (COMT, EC:2.1.1.68) were detected in the proteome at all developmental stages of marolo. COMT is responsible for converting caffeic acid to ferulic acid, while CCoAOMT methylates caffeoyl-CoA (a metabolite generated directly from caffeic acid) to feruloyl-CoA and 5-hydroxyferuloyl-CoA to sinapoyl-CoA (Luo et al., 2022). Therefore, only CCoAOMT can obligatorily act when caffeic acid has been converted to caffeoyl-CoA. Only one CCoAOMT isoform showed significant variations in its levels among the four development stages, with maximum and minimum accumulation in the overripe and mature green stages, respectively. The lowest accumulation, observed in the mature green stage, coincided with a 3.3-fold increase in caffeic acid concentration between the second and third development stages. The highest accumulation, observed in the overripe stage, coincided with a reduction in caffeic acid, the immediate precursor of the CCoAOMT substrate. This enzyme is important in strengthening the plant cell wall, being related to the response to wounds or entry of pathogens by increasing the formation of feruloylated polysaccharides bound to the wall (Lepelley et al., 2007). The accumulation of CCoAOMT in the overripe stage can be understood as a defense mechanism, at a stage in which the fruit is highly stressed, weakened and susceptible to microbiological deterioration. To contain the advance of senescence or invaders, molecular strategies such as the action of CCoAOMT and other enzymes that act for this purpose naturally undergo positive regulation. As for the other phenolic compounds studied, proteins directly responsible for their synthesis were not detected in the proteome of *A. crassiflora* Mart. However, their presence suggests health-promoting effects of the marolo, since they are phytochemicals with well-defined bioactive effects (da Costa et al., 2024b; Marcolino et al., 2024).

3.7. Volatile organic compounds (VOC) and related proteins

30 VOC were identified among the four stages of *A. crassiflora* Mart. Such VOC are presented in Table 2 with their respective percentages of area per stadium.

VOC are crucial elements that enhance the quality and appeal of fruits to both animals and humans. These fragrant substances are characterized by their high vapor pressure, moderate hydrophilicity, and low molecular mass. The distinctive smell of food is determined by the combined perception of its volatile profile (Jim´enez-Bremont et al., 2024). The composition of VOC changes throughout the ripening process, and they are produced from amino acid derivatives, sugars, and fatty acid compounds (Li et al., 2022a; Mostafa et al., 2022).

The ester class was the most abundant in the volatile profile of marolo with 17 compounds, representing 51 % of the identified compounds. Next, the class with the most elucidated representatives was terpenes with 5 VOC. The major compounds identified in marolo were ethyl octanoate (33.85 % in overripe fruits), ethyl hexanoate (32.24 % in ripe fruit) and methyl octanoate (21.64 % and 22.02 % for the immature and mature green stages, respectively), all esters. Esters have mainly fruity notes, and in fruits they are the largest group of volatiles synthesized through the esterification of alcohols and acyl CoA derived from the metabolism of fatty acids and amino acids. This reaction is catalyzed by alcohol acyltransferase (Mostafa et al., 2022). Individual esters can act in a coordinated and integrated way, forming what can be identified as an aroma vector. Even with small changes in concentration, very significant changes in flavor perception and intensity can be detected according to the combination of different types and concentrations of esters (Xu et al., 2022).

Proteins involved in the production of volatile esters were detected in the proteome of marolo at all fruit stages: one Linoleate 13S-lipoxygenase 2–1 (LOX2.1, EC: 1.13.11.12) and five isoforms of alcohol dehydrogenases (ADH, EC: 1.1.1.1). Of these, only one ADH isoform varied significantly ($p < 0.05$) throughout development, with minimum and maximum accumulation in the immature and overripe stages, respectively. Lipoxygenases act by degrading linoleic and linolenic acids into volatile aldehydes, such as hexanal (Table 2), while ADH converts volatile aldehydes into alcohols to be subsequently esterified (Yan et al., 2018). Two aldehydes and two alcohols were synthesized in the first stage and then decreased, suggesting a possible conversion into volatile esters. The alcohol 3-hexen-1-ol had a sharp reduction of 99.16 % in area from the first to the second stage, as did the aldehyde 2-hexanal with a reduction of 94.9 % in area, and hexanal was no longer detected. From the immature to the green mature stage, the area of esters increased by 34.31 %, concomitantly with the reduction of their aldehyde and alcohol precursors. This indicates that it is at this stage of development that the production of volatile esters reaches its synthetic peak in *A. crassiflora* Mart.

Table 2
Volatile organic compounds (mean ± standard deviation) in the pulp of *A. crassiflora* Mart. at four stages of development. Different letters indicate significant differences ($p < 0.05$) by ANOVA analysis followed by the Scott-Knott test. RI = retention index calculated. RI Lit = retention index found in literature. ND – Not Detected. NF – Not found.

Compounds	RI	RI Lit	Immature	Mature green	Ripe	Overripe
Esters						
Ethyl butyrate	803	804	ND	0.07±0.01^{c}	0.17±0.01^{a}	0.13±0.00^{b}
Methyl hexanoate	919	921	3.74±0.37^{b}	4.4 ± 0.15^{a}	2.67±0.08^{c}	2.27±0.16^{c}
Ethyl hexanoate	976	998	13.21±0.6^{d}	28.52±0.67^{c}	32.24±0.06^{a}	30.21±0.92^{b}
Hexyl acetate	1013	1014	ND	ND	0.04±0.00^{b}	0.09±0.00^{a}
Ethyl-(3E)-hexenoate	1004	1004	0.19±0.01^{a}	ND	0.02±0.00^{c}	0.08±0.00^{b}
Propyl hexanoate	1112	1097	ND	0.09±0.00^{c}	0.11±0.01^{b}	0.13±0.01^{a}
Methyl octanoate	1124	1123	21.64±1.64^{a}	22.02±0.44^{a}	11.54±0.25^{b}	11.61±0.15^{b}
Ethyl-(4E)-octenoate	1189	1186	ND	ND	1.26±0.06^{a}	1.27±0.01^{a}
Ethyl octanoate	1197	1198	23.09±0.86^{d}	26.22±0.47^{c}	32.76±0.13^{b}	33.85±0.34^{a}
Ethyl (E)¡2-octenoate	1248	1249	ND	ND	0.07±0.00^{a}	0.08±0.00^{a}
Isoamyl hexanoate	1251	1249	ND	0.09±0.00^{c}	0.38±0.03^{b}	0.5 ± 0.00^{a}
Methyl decanoate	1292	1324	ND	0.55±0.01^{a}	0.52±0.04^{a}	0.55±0.00^{a}
cis-3-Hexenyl hexanoate	1353	1380	ND	0.12±0.01	ND	ND
Ethyl decanoate	1398	1398	ND	0.44±0.01^{c}	2.9 ± 0.18^{a}	2.55±0.11^{b}
Hexyl hexanoate	1386	1379	ND	ND	0.13±0.00^{b}	0.18±0.00^{a}
Isoamyl octanoate	1447	1445	ND	ND	0.05±0.00^{b}	0.08±0.00^{a}
4-cyano-2.6-diiodophenyl octanoate	1455	NF	ND	0.58±0.01^{b}	1.91±0.00^{a}	1.98±0.11^{a}
Terpenes						
α-pinene	934	939	0.8 ± 0.06	ND	ND	ND
β-pinene	979	981	0.7 ± 0.01	ND	ND	ND
α-Phellandrene	1008	1007	0.33±0.05^{b}	1.76±0.08^{a}	0.02±0.00^{b}	0.08±0.00^{b}
Menthene	1031	1021	1.63±0.04^{a}	0.08±0.00^{b}	0.15±0.03^{b}	0.12±0.02^{b}
β-ocimene	1047	1038	ND	ND	ND	0.06±0.00
Acids						
Butanoic acid	798	820	ND	ND	ND	0.16±0.01
Octanoic acid	1194	1171	5.92±0.44^{a}	6.49±0.22^{a}	1.44±0.04^{b}	1.74±0.13^{b}
Aldehydes						
Hexanal	801	801	0.95±0.09	ND	ND	ND
2-Hexenal	852	854	1.77±0.11^{a}	0.09±0.00^{b}	ND	ND
Alcohols						
3-Hexen-1-ol	854	858	10.76±0.41^{a}	0.09±0.00^{b}	ND	ND
Hexanol	869	870	0.43±0.03^{a}	ND	ND	0.28±0.00^{b}
Others						
Styrene	888	893	0.53±0.05^{a}	0.1 ± 0.01^{b}	0.06±0.00^{b}	0.05±0.00^{b}
Hexanoic anhydride	1259	NF	ND	4.88±0.2^{b}	7.37±0.33^{a}	7.61±0.39^{a}

Terpenes, the second most abundant class in the volatile profile of marolo, showed more intense accumulation between the two initial stages. It is known that terpenes can act as alarm substances, defensive emissions and defense mechanisms against insects and pathogenic microorganisms at different concentrations and toxicity levels (Ninkuu et al., 2021). Three isoforms of 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS, EC:1.17.7.1) were present in the marolo proteome. This is one of the seven enzymes that participate in the MEP pathway, synthesizing monoterpenes at its end. Of these three isoforms, only one showed statistically significant difference between the four stages, with maximum and minimum accumulation in the immature and ripe stages, respectively. Concomitantly, the immature stage had the highest concentration of terpenes, totaling an area of 3.46 %. Another enzyme involved in the synthesis of many terpenes detected was β-sesquiphellandrene synthase (EC:4.2.3.123). Although this is a sesquiterpene synthase, it can form different compounds using farnesyl diphosphate (FPP) or GPP as substrates. When this enzyme uses GPP, it produces up to nine distinct monoterpenes, including α-phellandrene (Zhuang et al., 2012). This terpenoid was the most abundant detected in this class in marolo, with an area of 1.76 % in the mature green stage. The specific terpene synthases of the other compounds identified in this class were not detected in the marolo proteome.

Although the presence of sesquiterpenes (C_{15}), compounds less volatile than monoterpenes, was not detected in our study, the presence of sesquiterpene synthases was remarkable in all stages of the marolo proteome. τ-cadinol synthase (EC:4.2.3.173), Germacrene A synthase (EC:4.2.3.23), Valerena-4,7(11)-diene synthase (EC:4.2.3.139), Valencene synthase (EC:4.2.3.73) and (E,E)-germacrene B synthase (EC:4.2.3.71) were identified. In addition to these, three Farnesyl pyrophosphate synthases (FPS, EC:2.5.1.1) were detected. FPS forms the initial substrates for the action of sesquiterpene synthases (Li et al., 2023b). The presence of this set of proteins suggests a relevant production of marolo sesquiterns throughout the development of the fruit, although not detected.

Octanoic acid (C_8, Table 2) is a short-chain fatty acid (FA) and a precursor to other compounds. FAs have been increasingly demanded in industries for applications such as fuels, cosmetics, pharmaceuticals, food additives, and as precursors to a variety of industrial chemicals, such as alkanes, α-olefins, and fatty acid methyl or ethyl esters (Chen et al., 2018; Wernig et al., 2021). Short-chain FAs, such as octanoic acid, are especially used directly as food preservatives and dietary supplements (Chen et al., 2018). The first step in FA biosynthesis is catalyzed by acetyl-CoA carboxylase (ACCase), converting acetyl-CoA to malonyl-CoA. In the marolo proteome, five ACCase isoforms were detected, two of which had statistically significant accumulation. These two showed greater accumulation in the immature and mature green stages, and less accumulation in the overripe stage. Octanoic acid had a behavior similar to the accumulation of these enzymes, with higher area values in the two initial stages, followed by a sharp decrease of 77.8 % from the second to the third stage. Octanoic acid is reported as a potential fungicide and is toxic to many insects (Dalcin et al., 2022). It is possible that these protective characteristics are related to the fruit in the development phase as a preventive action against biotic stresses that could negatively influence the development of the marolo. The emission of this volatile compound should inhibit the approach of insects, in agreement with the greater presence of terpenes in the initial stages of the marolo, which act in a similar way.

Regarding the compound hexanoic anhydride (Table 2), little is

reported about this metabolite that is formed by the condensation of two hexanoic acid molecules (Woo et al., 2017). Hexanoic acid has functions similar to octanoic acid regarding protection against biotic stresses in plant cells. Therefore, it is possible that this assignment may be related to hexanoic anhydride, although specific studies are needed.

4. Conclusion

Proteomics and metabolomics of *A. crassiflora* Mart. allowed us to observe biochemical changes throughout development. In the immature stage, the action of ribosomal, transcriptional and post-translational proteins was highlighted, evidencing the focus on increasing the protein synthesis machinery to produce more proteins that should coordinate the other biological processes essential for development. Eukaryotic translation initiation factor 5A, eukaryotic initiation factor 4A-9 and spermidine synthase 1 are perhaps the most representative proteins and biomarkers of this biological process at this stage. The GATA, MADS-Box, MYB, F-box and HD-Zip TFs families are important in the regulation of biological processes in marolo, and other processes such as cell wall degradation and the antioxidant system could also be observed by the accumulation of a set of proteins. The ripe marolo fruit was a rich source of bioactive compounds such as vitamin C, carotenoids, alkaloids and phenolic compounds, and the volatile profile of marolo showed supremacy of volatile esters. Many enzymes could be associated with the synthesis and degradation of these secondary metabolites among the four stages. Therefore, proteomics in conjunction with metabolomics allowed us to have a molecular view of how compounds and proteins are produced in marolo development and how the behavior of these molecules appears to influence biological processes and biosynthetic pathways through enzymatic action.

For the future of molecular research on marolo, as well as other fruits, some suggestions are indicated. The first is to perform other metabolomic analyses covering a greater coverage of metabolite groups, which can be related to more proteins already identified. In addition, the application of other omics, such as transcriptomics in integration with proteomics, can fully elucidate the dynamics of gene expression and deeply analyze the effects of positive or negative gene regulation at the post-translational level. With this set of information, more insights for biotechnological applications and cultivation of marolo can be generated, adding value to this species that is so important for the Brazilian Cerrado.

CRediT authorship contribution statement

Carlos Alexandre Rocha da Costa: Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Sidney Vasconcelos do Nascimento:** Writing – review & editing, Methodology, Investigation, Data curation. **Rafael Borges da Silva Valadares:** Validation, Methodology, Investigation, Data curation, Conceptualization. **Luíz Guilherme Malaquias da Silva:** Methodology, Investigation. **Gilson Gustavo Lucinda Machado:** Methodology, Investigation. **Alice de Paula de Sousa Cavalcante:** Methodology, Investigation. **Sayure Mariana Raad Nahon:** Methodology, Investigation. **Carlos Henrique Milagres Ribeiro:** Methodology, Investigation. **Gre´cia de Andrade Souza:** Methodology, Investigation. **Luiz Jose´ Rodrigues:** Writing – review & editing. **Elisangela Elena Nunes Carvalho:** Methodology, Conceptualization. **Eduardo Vale´rio de Barros Vilas Boas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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CONSIDERAÇÕES FINAIS

A identificação e quantificação dos mais variados grupos de metabólitos é empregada com louvor na ciência vegetal há muitos anos, sempre nutrindo e proporcionando conhecimento sobre a composição fitoquímica de frutos e hortaliças. Contudo, a inserção da proteômica, tecnologia ascendente na ciência e tecnologia de alimentos, trouxe uma perspectiva inovadora para esse presente estudo, possibilitando compreender de modo inédito as mudanças bioquímicas que ocorrem durante o desenvolvimento dos frutos a nível proteômico, e como as células vegetais empregam um sistema coordenado e inteligente para suprir todas as demandas biológicas essenciais para desenvolver os frutos. Acreditamos que nossos resultados irão contribuir significativamente para a disseminação da proteômica dentro da ciência vegetal, trazendo luz a processos biológicos pouco investigados, além de promover novos estudos moleculares em frutos.