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Germination in Pulse Seeds: Comprehensive Insights via Biochemical, Metabolic, Proteomic, and Immunohistochemistry analysis

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Abstract

Germination *sensu stricto* is a complex biological process that induces major metabolic changes and activates the accumulation of nutrients required for seedling growth. Germination begins with the absorption of water by the seeds and ends with the elongation of the radicle. During this process, the seeds accumulate free amino acids, vitamins, and sugars contributing to an enhanced nutritional profile. In addition, germination reduces the content of antinutritional factors (α-galactoside and phytate), which are responsible for reducing pulse consumption. The aim of this study was to improve the nutritional profile of lentils and cowpeas by determining the optimal germination conditions. In addition, this study aimed to understand and identify the main metabolic pathways activated during germination. A preliminary study was conducted to analyze the effect of soaking on the α-galactoside content, with the purpose of differentiating the impact of soaking from that of germination. To evaluate the impact of the germination process, an experimental plan was developed to analyze the effects of temperature, light, and water content on the levels of thiamine, folates, amino acids, α-galactosides, and phytate. This initial exploratory phase was completed by metabolomic and proteomic which identified the key metabolites and enzymes driving the accumulation of amino acids and the initiation of sugar metabolism during germination. The final phase of the work focused on studying the spatial distribution of the compounds of interest (i.e. thiamine and folates), their evolution and the microstructural modifications induced during germination. The cross-sectional approach employed in this study enabled us to gain a better understanding of the microstructural changes, compound localization, and metabolic pathway orientation. This elucidation of the complex interactions that occur during germination not only improves our understanding of the processes but also lays the foundation for the food industry to adopt optimized germination processes for pulse processing.

Keywords: Germination, Pulses, Nutritional, Metabolomics, Proteomics, Immunohistochemistry

Résumé

La germination *sensu stricto* est un processus biologique complexe qui induit des modifications métaboliques majeures et active l'accumulation des nutriments nécessaires à la croissance des plantules. La germination commence par l'absorption d'eau par la graine et s'achève avec l'élongation de la radicule. Pendant ce procédé l'accumulation d'acides aminés libres, vitamines et de sucres contribue à l'amélioration du contenu nutritionnel des graines. De plus, la germination permet de réduire la teneur en facteurs antinutritionnels (α-galactosides et phytate) qui constituent un frein à la consommation des légumineuses. L'objectif principal de cette étude était d'améliorer les propriétés nutritionnelles de la lentille et du niébé en déterminant les conditions optimales de germination. Par ailleurs, cette étude visait à comprendre et à identifier les principales voies métaboliques activées au cours de la germination. Une étude préliminaire a été réalisée afin d'analyser l'effet du trempage sur la teneur en α-galactosides, dans le but de dissocier l'impact du trempage par rapport à celui de la germination. Pour évaluer l'impact du procédé de germination, un plan expérimental a été réalisé pour analyser les effets de la température, de la lumière, et de la teneur en eau sur la concentration en thiamine, en folate, en acides aminés, en α-galactoside et en phytate. Cette première phase exploratoire s'est vue compléter par des études métabolomique et protéomique permettant d'identifier les principaux métabolites et enzymes impliqués dans les voies de production des acides aminés libres et dans le métabolisme des sucres initiées durant la germination. La dernière phase du travail s'est focalisée sur l'étude de la distribution spatiale des composés d'intérêt (i.e. thiamine et folates) et leur évolution concomitamment aux modifications microstructurales induites pendant la germination. L'approche transversale employé lors de cette étude nous a permis de mieux comprendre les changements microstructuraux, la localisation des composés et l'orientation des voies métaboliques. Cette élucidation des interactions complexes qui se produisent au cours de la germination améliore non seulement notre compréhension des processus à l'œuvre au cours de ce procédé biologique, mais jette également les bases pour que l'industrie agroalimentaire puisse adopter des procédés de germination optimisés en vue de transformer les légumineuses.

Mots clés : Germination, Légumineuses, Nutrition, Métabolomique, Protéomique, Immunohistochimie

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Liste des abréviations

GI: Glycemic Index

AA: Amino Acids

EAA: Essential Amino Acids

NEEA: Nonessential Amino Acids

CEAA: Conditionally Essential Amino Acids

IVPD: in vitro Protein Digestibility

PDCAAS: Protein Digestibility Corrected Amino Acid Score

SSP: Seed Storage Proteins

PTMs: Post-Translational Modifications

RDS: Rapidly Hydrolyzed Starch

SDS: Slowly Hydrolyzed Starch

RS: Resistant Starch

HI: Hydrolysis Index

eGI: estimated Glycemic Index

SDF: Soluble Dietary Fiber

IDF: Insoluble Dietary Fiber

TDF: Total Dietary Fiber

RDA: Recommended Daily Intake

TMP: Thiamine Monophosphate

TDP: Thiamine Diphosphate

TTP: Thiamine Triphosphate

Thia-HCL: Thiamine Hydrochloride

PDH: Pyruvate dehydrogenases

ρABA: ρ-Aminobenzoic Acid

PteGlu: Folic acid

H_4folate: Tetrahydrofolate

5-CH_3-H_4folate: 5-methyltetrahydrofolate

5-CHO-H_4folate: 5-formyltetrahydrofolate

10-CHO-PteGlu: 10-formylfolic acid

DFE: Dietary Folate Equivalents

RFOs: Raffinose Family Oligosaccharides

IHC: Immunohistochemistry

LED: light emitting diode

LMICs: low- and middle-income countries

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I. Introduction

According to the Food and Agriculture Organization (FAO) and Codex Alimentarius Commission, dried legumes used for human consumption or animal feed can be called pulses. Pulses are distinguished from leguminous oil seeds by their low-fat content (FAO & Alimentarius, 2019). The United Nations General Assembly (UNGA) declared 2016 the International Year of Pulses (IYP) to celebrate, raise awareness, and promote the consumption of pulses around the world. This event highlights the importance of pulses in a healthy diet, since they are rich in proteins, complex carbohydrates, minerals, and vitamins, and low in fat content. Pulses are linked to positive human and environmental health impacts (Didinger & Thompson, 2020). Additionally, the IYP emphasized the contribution of pulses production to soil fertility and biodiversity making pulses economically accessible and environmentally friendly. Pulses are responsible for fixing atmospheric nitrogen into the soil because of symbiosis with N_2 fixing bacteria (rhizobia), reducing their reliance on synthetic fertilizers (Calles *et al.,* 2019; Heldt & Piechulla, 2011). Nevertheless, given their low productivity in relation to cereals, they are underrepresented in many countries. However, introducing pulses with cereals and oilseeds into cropping systems (crop rotation) can increase the gross margins of subsequent crops (Preissel *et al.,* 2015; Reckling *et al.,* 2016). This system increases above- and below- ground biodiversity, and consequently reduces weeds, pests, and diseases, and improves soil structure (Watson *et al.,* 2017). The worldwide production of pulses was estimated at 92.4 million megatons (MT) in 2018, with a remarkable increase of 36 million MT over the period 1998-2018 (Rawal & Navarro, 2019). The chickpeas (*Cicer genus*), beans (*Phaseolus and Vigna genus*), lentils (*Lens genus*), peas (*Pisum genus*), and vetch (*Vicia genus*) are the main cultivated pulses. In Europe, pulse production area declined over the last 5 decades from 5.8 million ha in 1961 (4.7% of the arable area) to 2.0 million ha in 2014 (1.6% of the arable area). This decrease is due to several factors, such as the availability and low costs of synthetic nitrogen fertilizers relative to farm product prices, imported protein feed such as soybean, intensification of livestock farming, and political support for cereal production because of their value in the market (Voisin *et al.,* 2014). However, the increasing knowledge in Europe that pulses improve the agronomic performance of farming systems and provides protein-rich food and feed has recently heightened the political debate to increase the productivity of these crops (Watson *et al.*, 2017). For instance, between 2015 and 2019, the production of lentils and peas increased by 46% and 22%, respectively (FAOstat, 2021). Worldwide the major producers of lentil are Canada (1.4 million tons), India (1.0 million tons), and Turkey (0.45 million tons) (FAO, 2023). Pulses are of great importance for livelihoods in

southern countries, especially Africa. Their production and processes are responsible for the main source of income for farmers, as they fetch a good price in local and international markets (Snapp, 2018). The most produced pulses in Western Africa are cowpeas (*Vigna unguiculata*, 7 MT of production), Bambara beans (*Vigna subterranea*, 149 kT of production), and chickpeas (*Cicer arietinum*, 2.2 kT of production) (FAOstat, 2021). Cowpeas is one of the most economically important pulses for farmers, providing an affordable economic source and making a major contribution to the householder's food security (Paliwal *et al.*, 2020).

Pulses have great health benefits and are excellent suppliers of complex carbohydrates, proteins, fibers, minerals, and essential vitamins such as those of the B complex (Bojňanská *et al.*, 2016). Their nutritional benefits can contribute to the diversification of the diet of a society and help eradicate hunger and malnutrition worldwide (Considine *et al.*, 2017). However, pulses have antinutritional factors, such as tannins, phytic acid, α-galactosides, lectins, protease, and α-amylase inhibitors, impacting their nutritional value and reducing pulses consumption. To reduce or eliminate these compounds, food processing, such as soaking, cooking, and extrusion, is required. These food processes are extremely used by the industrialist and householders. They are responsible for reducing or eliminating antinutritional compounds by leaching out of these compounds into soak medium (diffusion) and/or enzymatic activation (hydrolysis) (Chitra *et al.*, 1996; Hall *et al.*, 2017). However, they have a negative nutritional effect by leaching out water-soluble vitamins, such as vitamins from the B complex, and by thermally degrading these vitamins. Changing these processes to a biological process, such as germination, or adding it as a pretreatment, presents a nutritional advantage. Because of metabolic activation during germination, seeds exhibit major biochemical, nutritional, and sensory characteristics modification. During this process, B-vitamins are synthesized, the amino acid composition is modified, starch and protein *in vitro* digestibility increases, and fats and carbohydrates are broken down. In addition, it reduces the amount of some antinutritional factors such as polyphenolic compounds, phytic acid, α-galactosides, lectins, protease, and α-amylase inhibitors (Prodanov *et al.*, 1997; Vidal-Valverde *et al.*, 2002). The germination process is a biological and low-tech food transformation, which is simple and less expensive than other food processing, where any food industry worldwide can develop.

To trigger the germination process, seeds must be placed in a controlled growth-conductive environmental conditions, with adequate temperature, light, and relativity humidity. The germination process in food industry requires specific equipment under controlled environmental conditions (Mayer & Poljakoff-Mayber, 1982; Prodanov *et al.*, 1997). Therefore, it is important that the scientific community shows an interest in optimizing

germination parameters in order to encourage its use by the food industry. The aim of this study was to determine the optimal germination conditions, including temperature, lighting, and water content to improve the nutritional profile of lentils and cowpeas. In addition, we aimed to understand and identify the main metabolic pathways involved in germination, and investigate whether these pathways are initiated as a result of structural variations induced by the germination process. To do this, we employed a multidisciplinary approach involving various methodologies. The study was divided in four sections:

(i) carry out a preliminary study to analyze the effect of the soaking process on the levels of the antinutritional factor, α-galactosides. This will facilitate to identify the specific impact of soaking before germination;

(ii) develop germination equipment to optimize the germination process through biochemical quantification response (thiamine, folates, and α-galactosides). This will provide insights into the optimal germination conditions, such as temperature, lighting, and water content, to enhance the nutritional quality of pulses;

(iii) perform metabolic and proteomic profiling to gain deeper insights into the germination process of pulses. This will allow to explore the metabolic pathway, identify the key proteins involved, and offer valuable understanding into the biochemical changes that occur during germination;

(iv) perform immunochemistry and cytology analyses to localize *in situ* nutritional components (thiamine and folates) and follow microstructural modifications. This will enable investigation of the impact of the germination process on the microstructure of pulses and how the localization of specific compounds changes during germination.

The combination of multidisciplinary approaches, such as physiology, biochemical quantification, metabolomic/proteomic, and immunochemistry, can establish connections between microstructure changes, compound localization, and metabolic pathways during germination. This will provide valuable insights into enzymatic interactions with substrates, leading to the synthesis or degradation of target compounds. By employing this comprehensive approach, we hope to enhance our understanding of the pulse germination process and identify strategies to optimize it for improved nutritional outcomes.

To do so, two model seeds were chosen for the study: lentils and cowpeas. Lentils were chosen due to their economic and consumption importance in northern countries, while cowpeas were chosen for their importance in African countries. Besides, these seeds are a great source of proteins and vitamins. The results of this work are presented successively in the form of articles (published or in progress), in successive chapters of this manuscript. These are preceded by a review of the literature (published article) covering all the aspects addressed in this work, but focusing more specifically on the nutritional value of pulses and the impact of the various elaboration process on this value.

The outcome of this project will increase the knowledge related to metabolic orientation in pulses during germination, the localization of major substrates/products and activities during pulses germination, and the nutritional benefit of the germination process. This can help to expand the germination process for pulses processing and encourage the scientific community, industry, and government to invest in research and development to increase germinated pulses-based food, to replace other products, or to develop new ones. Pulses flour can actually be incorporated into cereal flour to prepare pasta, breads, ready-to-eat breakfast cereals, snack foods, texturized vegetable protein, pet foods, dried soups, dry beverage mixes, and digestible baby foods (Prodanov *et al.*, 1997; Tiwari *et al.*, 2011; Sadohara *et al.*, 2022).

II. **Literature Review**

(based on the review article "Improving the nutritional quality of pulses via germination" (Avezum *et al.*, 2022)

The germination of pulses is a traditional food process that has been commonly practiced in the Orient for centuries. However, in Western countries, this practice has only been accepted in recent years, perhaps due to increasing recognition of its flavor and nutritional benefits (Prodanov *et al.*, 1997). Germination causes important changes in the biochemical, nutritional, and sensory characteristics of pulses. For example, *in vitro* digestibility of starch and protein increases, fats are broken down, and the content of water-soluble vitamins and amino acids increases. In addition, it reduces the amount of antinutritional factors such as phytic acid, α-galactosides, trypsin inhibitors, and lectins (Prodanov *et al.*, 1997; Vidal-Valverde *et al.*, 2002). Germinated pulses can be used to produce new foods with a high nutritive value, such as pulse flour and isolate proteins that can be incorporated into the food industry. However, the germination process has several limitations. The pulses need to be placed in a growth-conductive environmental conditions, with adequate temperature, light, relative humidity, and oxygen pressure, which for an industrial scale requires specific equipment with fine control of environmental conditions (Mayer & Poljakoff-Mayber, 1982; Prodanov *et al.*, 1997). For this purpose, it is important that the scientific community works on the optimization of germination parameters.

This literature review aimed to illustrate the advantages of the germination process in enhancing the nutritional quality of pulse seeds. First, a complete description of the seed structure, maturation, and germination process was discussed. Subsequently, we explored a critical discussion on one of the primary concerns associated with germination: the microbiological risk. The review then shifted its focus to a comprehensive examination of the nutritional profile of pulse seeds, encompassing both their nutritional and antinutritional aspects, as well as the numerous health advantages linked to pulse consumption. Given that this project focuses on germination *sensu stricto*, and the transformation of germinated pulses into edible forms necessitates thermal food processing, we then discussed how various food processing methods (soaking, cooking, and germination) influence the nutritional and antinutritional compounds within pulses. In the context of our present study, which adopts a multidisciplinary approach, the final section highlighted innovative technologies for food science, such as multi-omics and immunohistochemistry. We examined their mechanisms, functions, applications in food science, and their contributions to our understanding of food processes.

I. Seed structure

For a better understanding of the germination process, it is important to describe seed structure. In angiosperms, seeds contains three compartments, namely the zygotic diploid embryo, triploid endosperm, both originating from the double fertilization event, and testa (seed coat), derived from maternal tissue (Baroux & Grossniklaus, 2019; Linkies *et al.*, 2010). Pulses are mainly non-endospermous dicotyledon seeds, as the endospermic tissue is completely consumed by the growing embryo during developmental stages and is almost absent in dry mature seeds (Yan *et al.*, 2014). The embryo is the result of fertilization of the egg cell in the embryo sac by a male pollen tube nucleus. Thus, seed storage compounds are mainly deposited in the cotyledons of the embryo, which accounts for almost all of the seed mass. Most pulses comprise the embryo with massive and reserve rich-cotyledons (1), embryonic axis (2), testa (3) or seed coat, hilum (4), and micropyle (5) (Figure 1). The seed coat is made up of an integument around the ovule and protects the seed from the external environment. The protective physical and chemical nature of the seed coat can discourage insect predation, provide a water-retaining barrier, restrict oxygen uptake, and exchange gases between the embryo and the environment (Bewley & Black, 1994). Seed filling, acquisition of desiccation tolerance, dormancy/germination, and nutritional quality are mainly controlled by zygotic genes and are influenced by the mother plant environment (Scheelbeek *et al.*, 2018). During the seed development of *M. truncatula,* major proteome and transcriptome changes were observed. Genes involved in carbohydrate, glycolysis, and amino acid metabolism are among the most regulated genes. In young developing seeds, starch transiently accumulates and later disappears, probably due to the donation of carbon skeletons for the synthesis of other reserve compounds during seed development. This could explain the low carbohydrate content of the total seed mass (Gallardo *et al.*, 2007; Yan *et al.*, 2014).

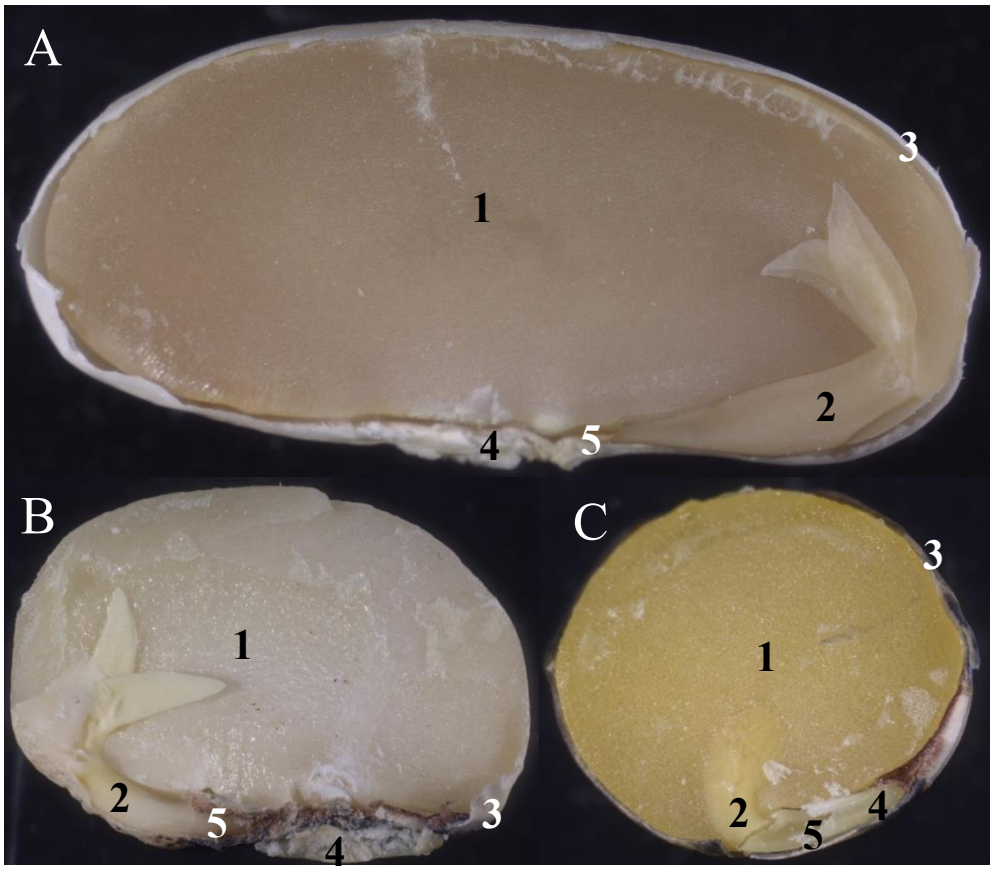


Figure 1. Symmetric longitudinal seed sections of Phaseolus vulgaris (A), Vigna unguiculata (B), and Lens culinaris (C). The structure comprises the cotyledon (1), the embryonic axis (2), the seed coat or testa (3), the hilum (4), and the micropyle (5)

II. Seed maturation and germination process

In scientific literature, the term “germination” may vary from a “physiological/biologist” or “processing/technical” point of view. In the context of processing and technical aspects, there may not be a major difference between the germination and sprouting processes. However, it is crucial to distinguish between these two processes to ensure a fair comparison of comparable factors, as they can exhibit differences in seed properties and metabolic activities. On the other hand, from a physiologist/biologist perspective, germination and sprouting processes are distinct. For instance, the germination process begins with water uptake by the seed and ends with elongation of the radicle following testa rupture. After this phase, the germination process

is completed and seedling growth is marked by post-germination events such as mobilization and dilution of major storage reserves (Rajjou *et al.,* 2012). Germinated pulses maintain the properties of the seed, which requires further processing, such as cooking, drying, or roasting before consumption (Benincasa *et al.,* 2019). Making this distinction allows for a more accurate understanding and analysis of the specific stages and events that occur during these processes, which is important for research.

For pulses, the germination process marks the end of seed quiescence, a rest state established by the seed at maturity, with low moisture content (5-15%) and metabolic activity almost stagnant (Bewley & Black, 1994). At the end of the maturation program, seeds experience a major loss of water, leading to a dry period called desiccation (Angelovici *et al.,* 2010). Seed maturation involves the end of seed development, including the period of reserve accumulation with a reorganization of metabolism and synthesis of storage compounds (*i.e.* starch, proteins, and lipids), acquisition of desiccation tolerance, and both germination and longevity potential (Angelovici *et al.,* 2010). Before the quiescent stage, the seed is responsible for the synthesis of storage proteins owing to its high metabolic activity (Oracz & Stawska, 2016). The main amino acid synthesized during this phase is methionine (a sulfur-containing amino acid), which is responsible for synthesizing storage proteins during seed filling. The enzymes Met synthase and AdoMet are responsible for methionine synthesis in the seed coat and embryo. During seed development, enzyme levels are limited and compartmentalized in tissues. These changes allow the embryo to store all nutrients and indicate a metabolic shift from a highly active to quiescent state (Gallardo *et al.*, 2007; Oracz & Stawska, 2016).

Germination potential (seed vigor) is a complex physiological trait that ensures rapid and uniform radicle protrusion and seedling establishment under a wide range of environmental conditions (Rajjou *et al.,* 2012; Wu *et al.,* 2017). The lifespan of seeds (*i.e.* longevity) is an important aspect of seed quality and depends on genotypes and storage conditions (Rajjou *et al.*, 2012). For instance, seeds stored under high temperature and high moisture conditions lose their germination capacity and their viability more quickly. Seed lots with high vigor are of paramount importance for proper germination, seedling emergence, increasing crop yield, and reducing the cost of agricultural production by prolonging their storage time (Wu *et al.*, 2017). Seed storage compounds, dynamic regulation of gene expression, and hormone pathways (especially abscisic acid and ABA) influence seed vigor (Wu *et al.,* 2017). Reactive oxygen species (ROS), lipid peroxidation, loss of cellular membrane integrity, enzyme inactivation,

weak energy metabolism, and DNA degradation contribute to seed deterioration during storage (Kumar *et al.,* 2021; Wu *et al.,* 2017).

Before the germination process begins, seeds can be in a physiological state called dormancy, in which they not germinate even under optimal conditions (Rajjou *et al.,* 2012). Seed dormancy provides ecological advantages by adjusting progeny dispersal to the favorable growth period but represents a major drawback in agriculture as it limits rapid and uniform germination. Therefore, this trait was targeted by plant domestication and modern breeding programs to remove seed dormancy in crops, including pulses (Ladizinsky, 1987). The duration of the dormancy state is extremely variable and is genetically determined and influenced by the environment of the mother plant (Prodanov *et al.,* 1997; Penfield & MacGregor, 2017). Germination can be prevented either because the embryo is limited by its surrounding structures (coat-enhanced dormancy), or by embryo dormancy (Bewley, 1997). In pulses, it has been widely documented that dormancy is mainly due to seed coat water impermeability and hardness and is defined as physical dormancy (Baskin *et al.,* 2000; Smykal *et al.,* 2014). The seed coat is an important source of phytohormones for seed development (Verdier *et al.,* 2013). Seed dormancy and its inhibitory effect on the germination process are mainly triggered by the phytohormone abscisic acid (ABA), which is responsible for sustaining the maturation process (Ohanenye *et al.,* 2020; Rajjou *et al.,* 2012). Seeds are considered non-dormant when ABA anabolism is deficient or if ABA signaling is disrupted (Bewley, 1997). To begin the germination process, the seed must be placed in growth-conductive environmental conditions, which means a suitable temperature, light, adequate composition of the soaking and rinsing media, and composition of the gases in the atmosphere (Mayer & Poljakoff-Mayber, 1982; Prodanov *et al.,* 1997). Each plant species and variety has its requirement for these conditions, which are determined by their genome and physiological state (*i.e.,* dormant, non-dormant, aged), and the interactions between genotype and environmental conditions (Bewley, 1997).

The germination process begins with the imbibition of water by quiescent and non-dormant dry seeds and ends with testa rupture and cell elongation of the embryo, which is marked by radicle protrusion (Bewley & Black, 1994; Weitbrecht *et al.,* 2011). Post-germination events include mobilization of the major storage reserves, cell division, nutrient dilution (*e.g.,* carbon, nitrogen, sulfur, phosphorus), and growth of the seedling to the surrounding area. The germination process involves three-phase water uptake by mature dry seeds (Figure 2). Phase I is a rapid (*i.e.,* a few hours) initial water uptake, where the solutes of the seeds will reach out to the medium and the cell repair systems will be activated. This process is determined by the

composition and permeability of the seed coats. In pulses, the testa is largely impermeable to water and can restrict the metabolism and growth of the inner tissues, which can impact the germination process. However, when placed under appropriate environmental conditions, water enters through the micropyle, the area around the hilum, and through the testa (Figure 1) (Bewley, 1997; Turner, 2010). Imbibition causes a swelling effect in the seed, which produces changes in imbibition pressure and osmolarity. Phase II is characterized by a limited water uptake (lag phase) and strong metabolic reactivation (Prodanov *et al.*, 1997). It is influenced by protein turnover and synthesis/degradation of regulatory hormones (Bewley, 1997; Weitbrecht *et al.*, 2011). ABA hormone is rapidly degraded upon imbibition during the early phase of germination. Nine-cis-epoxycarotenoid dioxygenase (NCEDs) and ABA 8'-hydroxylases (CYP707As) are the major enzymes involved in ABA biosynthesis and degradation, respectively (Weitbrecht *et al.*, 2011). To trigger the germination process, seeds must establish specific catabolism and biosynthesis inhibition to reduce ABA levels and synthesize other hormones such as gibberellins (GAs), which are essential for cell elongation and inhibit ABA actions. ROS also control the germination process and are formed in the dry state of seeds (Rajjou *et al.*, 2012; Weitbrecht *et al.*, 2011). ROS are involved in regulating the ABA/GA ratio by promoting ABA catabolism and GA biosynthesis (Rajjou *et al.*, 2012). Seed vigor depends on the ABA/GA ratio, and germination can only occur when GA biosynthesis is preferred (Ohanenye *et al.*, 2020). Therefore, hormone regulation and interactions play an important role in determining the physiological state of the seed and in regulating the germination process (Weitbrecht *et al.*, 2011). Other hormones, such as ethylene, brassinosteroids, salicylic acid, cytokinin, auxin, jasmonic acid, and oxylipins, also influence germination (Ohanenye *et al.*, 2020; Rajjou *et al.*, 2012; Shu *et al.*, 2016). Ethylene acts in the late phase of germination, whereas GA is important in the early and late phases of germination (Weitbrecht *et al.*, 2011). GAs stimulate the synthesis and activity of amylases, proteases, and β-glucanases, resulting in the seed germination. Additionally, some important environmental conditions can stimulate germination metabolism, including nitric oxide gas (NO) and nitrate (NO_3^-) (Rajjou *et al.*, 2012; Weitbrecht *et al.*, 2011). Throughout germination *sensu stricto* (phases I and II), from water uptake to radicle protrusion, seeds present a heterotrophic metabolism depending on their storage materials to survive without incoming and residual outcoming of biomass constituents. A further increase in water uptake (phase III) occurs only after germination is completed, when nutrients are released from the reserves and lead to the embryo (Turner, 2010). All the hormonal modifications and nutrient reserves accomplished in phases I and II will be mobilized by the seedling establishment of photosynthetic activity (Botha *et al.*, 1992). This triggers cell

multiplication, DNA synthesis, and elongation of embryonic axes. Radicle extension through the structures surrounding the embryo terminates germination and marks the beginning of seedling growth (phase III, post-germination) (Bewley, 1997; Turner, 2010). After seed germination is recognizable by radicle protrusion, the embryo grows to establish a seedling displaying photosynthetic competence. This late step is characterized by a heterotrophic-to-autotrophic metabolic transition accompanied by dilution and conversion of seed storage compounds into simpler metabolites (*e.g.,* sugars, amino acids, and free fatty acids), which migrate quickly and feed the new cells for developing tissues (Ha *et al.,* 2017).

From a food science and processing perspective, the term "germination" may sometimes include seedling growth (the post-germination process). Under these conditions, seedling/sprouts no longer have seed properties and can be consumed immediately as ready-to-eat food products. This double meaning for the germination word may confuse the scientific community and create an obstacle in comparing scientific results. For example, in the literature, the impacts of the germination process on nutritional and antinutritional factors from 24 h to 6 days have been reported. In these cases, the authors used the word germination to describe two different processes: germination *sensu stricto* and post-germination. As discussed previously, both seed germination and seedling growth induce different metabolic pathways and result in different food properties (seeds and seedlings). It is important to highlight that, under most conditions, short periods of seed imbibition represent a process suitable for the germination *sensu stricto,* whereas a process requiring a long period of seed imbibition is suitable for seedling production and metabolic specificities of post-germination growth. This misunderstanding of the terminology disrupts the research and knowledge of the real impact of the germination process on nutritional and antinutritional factors. For this purpose, it is important to raise awareness in the scientific community to use proper terminology to describe complex processes, such as germination. Further, reducing the germination time enables the

reduction of microbiological risk when compared with sprouting, as discussed in the next section.

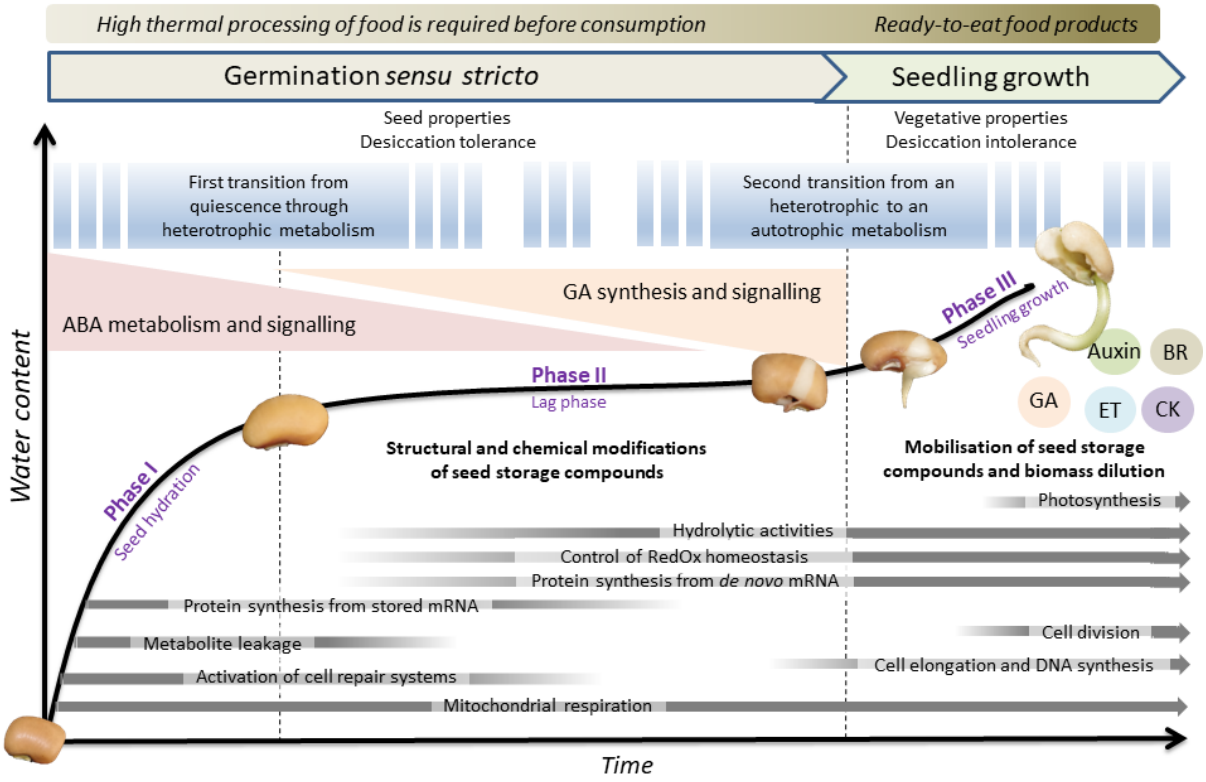


Figure 2. Seed water uptake associated with major molecular and metabolic events during germination *sensu stricto* and seedling growth. Phase I has strong water activity, active abscisic acid (ABA) catabolism and turn off ABA signalling. Phase II (lag phase) represents structural and chemical modifications of seed storage compounds. These phases are related to germination *sensu stricto* process mainly driven by gibberellins (GA). Phase III represents the seedling growth process with some hormone activation, such as gibberellins (GA), ethylene (ET), brassinosteroids (BR), auxin, and cytokinin (CK).

III. Microbiological risk

The sprouting and germination process may present microbiological risks for consumers, given the temperature and humidity used during the processes and the availability of nutrients from the seeds. The sprout-related outbreaks were caused by bacterial pathogens such as

Salmonella spp. and *Escherichia coli*. (Miyahira & Antunes, 2021). In Europe, Regulation (EC) 2073/200513 establishes several microbiological criteria relevant to the production of ready-to-eat sprouted seeds. There are no EU legislative microbiological criteria for the germination *sensu stricto* process as it is not considered a ready-to-eat food product. The food safety and process hygiene criteria for sprouted seeds in Regulation (EC) 2073/200514 indicate the absence of *Salmonella* in 25 g of sprouted seeds and 1000 CFU/g for *E. coli* populations (EFSA, 2011; The Commission of the European Communities, 2010).

To reduce the microbiological risk of germinated and sprouted seeds, it is important to have good seed quality control and hygiene practices during pre-harvest, post-harvest, and germination/sprouting activities. For instance, in pre-harvest, irrigation water quality, manure management, and the health and hygiene of farm workers should be controlled. In post-harvest, seeds require sanitization procedures (hot water, sodium hypochlorite, and ethanol disinfection) and good storage (dry, insect-free, and hygienic) (Miyahira & Antunes, 2021; Santos *et al.*, 2020). The post-harvest sanitization process is important to reduce the microbiological charge in seeds as many microorganisms can survive the dry conditions used for storage. For instance, Bari *et al.* (2010) showed that hot water treatment at 85°C for 40s in mung bean seeds reduced *Salmonella* and *E. coli* to undetectable levels without reducing the germination rate. Integrating chemical treatment, such as soaking in a 2,000-ppm hypochlorite solution for 2 h, into hot water treatment reduced the bacterial populations in the seeds. This combination reduced bacterial growth during germination (Bari *et al.*, 2010). During germination/sprouting, water quality should be controlled, and potable water should preferably be used. After sprouting, seeds should be refrigerated to prevent the growth of pathogenic bacteria (Miyahira & Antunes, 2021). As mentioned previously, after the germination process, seeds need to undergo thermal treatment before consumption. Adding thermal treatment may reduce the number of microbiological pathogens in germinated seeds. For instance, Santos *et al.* (2020) showed that treat contaminated sprouted seeds with *E.coli* with hot water at 80 °C for 90 s reduced its microbiological charge from $2.7x10^8$ to $2.7x10^7$ UFC/mL. Other chemical, biological, and physical seed disinfection protocols can also be used to reduce the microbiological charge of germinated and sprouted seeds. Physical methods, such as heat, high pressure, irradiation, and UV light, are most commonly used because they are more adapted for commercialization and are easily adapted for any food industry (Miyahira & Antunes, 2021).

IV. Nutritional quality of pulses

Pulses are considered essential in the diet to cope with nutritional deficiencies and are known for being a great source of food protein. They are an excellent complement to cereals, which are the largest source of calories in diets worldwide, as they provide a well-balanced essential amino acid profile (Bessada *et al.*, 2019; Boye *et al.*, 2010; Snapp *et al.*, 2018). Additionally, pulses are rich in fiber and have a lower glycemic index (GI) than cereals (Abeysekara *et al.*, 2012; Mudryj *et al.*, 2012). Consumption of pulses provides a diet rich in fiber without increasing caloric intake, favoring the reduction of diet-related diseases (Neacsu *et al.*, 2017; Ohanenye *et al.*, 2020). For instance, *Cajanus genus* represents the pulse with the lowest GI, which is a great food source for patients with diabetes. Meanwhile, *Cicer genus* has the highest GI, which can help malnourish patients (Sandhu & Lim, 2008). The *Lens*, *Vigna*, and *Phaseolus genus* have the highest protein concentration, with an average of 25, 24, and 23 g/100 g of dry matter, respectively (Table 1). In addition, they have a low-fat concentration of less than 6.5%.

Table 1. Nutritional content in raw pulses (g/100 g of dry matter) (Avezum *et al.*, 2022)

	Protein	**Carbohydrates**	**Fat**	**Fiber**	**Ash Content**
	g/100g				
Cajanus genus	19	63	2.0	6.4	-
Cicer genus	20	51 - 61	5.0 – 6.	16 - 28	1.8 - 3.5
Lens genus	25	34 - 59	0.7 – 3.6	19 – 35	2.1 - 3.2
Phaseolus genus	19 - 27	43 - 75	2.0 – 3.5	14 - 25	4 - 4.9
Pisum genus	14 - 31	55 - 72	1.0 – 4.0	3.0 - 20	2.3 - 3.7
Vigna genus	17 - 28	54 - 63	1.0 - 6.5	3.9 - 6.2	2.9 – 5.0

IV.1. Protein composition

The recommended human protein intake should represent 10 – 30% of the total daily calorie intake (or 0,8 g of protein per kg of body weight) (Ohanenye *et al.*, 2020). The nutritional quality of pulses proteins is determined by their composition of essential amino acids, digestibility, and bioavailability. Their characteristics can determine their use in different food products and intake in human nutrition (Boye *et al.*, 2010; Gulzar, 2017). The protein content and composition of raw pulses depend on the plant genotype and metabolism stage, which in turn depends on the pulses development phase and its interaction with the mother plant environment.

Proteins are complex polymers built from different amino acids (AA) sequences connected through amide-peptide bonds. The structural and functional properties are determined by the AA sequence and the length of the formed peptides. Twenty proteinogenic amino acids are classified in human nutrition as essential amino acids (EAA – lysine, isoleucine, leucine, methionine, phenylalanine, valine, threonine, histidine, and tryptophan), nonessential amino acids (NEAA – alanine, aspartic acid, asparagine, glutamic acid, and serine), and conditionally essential amino acids (CEAA – arginine, cysteine, glutamine, glycine, proline, and tyrosine) (Bessada *et al.*, 2019). EAA are not synthesized by human cells and are necessarily taken up by the diet. This category may differ from person to person depending on the developmental stage, health or pathological conditions, pregnancy, lactation, stressing environmental factors, and intestinal microbiota. On the other hand, the NEAA are synthesized in the human body and are not necessarily obtained from the diet. Depending on the developmental stage, health, or pathological conditions, the endogenous synthesis of CEAA cannot meet metabolic needs, and additional dietary sources are required. In addition, food protein quality depends on the profile and bioavailability of AA. Pulses contain all 20 AA; however, the AA distribution pattern and its concentration are less optimum in pulses than in animal food. Pulses have higher concentrations of lysine and leucine and lower concentrations of methionine, cysteine, and tryptophan than those recommended for human needs. Meanwhile, cereals are rich in some of these EAA. Therefore, mixing pulses and cereals provides balanced EAA intake (Bessada *et al.*, 2019). For example, lysine content in lentils (*Lens genus*) and cowpeas *(Vigna genus*) represent 7 and 7.5% of total protein, respectively (Table 2).

Table 2. Amino acid composition of pulses (% of total protein) (Avezum *et al.*, 2022)

	Essential Amino Acids								
	Lysine	**Isoleucine**	**Leucine**	**Methionine**	**Phenylalanine**	**Valine**	**Threonine**	**Histidine**	**Tryptophan**
Cajanus genus	1.4	1.2	1.5	0.2	1.7	1.0	0.7	-	0.1
Cicer genus	7.2	4.8	8.7	1.1	5.5	4.6	3.1	3.0	0.9
Lens genus	7.0	4.1	7.8	0.8	5.0	5.0	3.5	2.2	0.7
Phaseolus genus	1.6	1.1 - 1.3	1.9	0.2 - 0.3	1.2 - 1.3	1.3	1.0	0.7	2.0
Psium genus	8.1	4.5	7.4	1.1	5.2	5.0	3.8	2.4	0.8
Vigna genus	7.5	4.5	7.7	2.2	7.5	5.0	3.8	3.1	0.7

	Non-Essential Amino Acids				Conditionally Essential Amino Acids				
	Alanine	**Aspartic acid and Asparagine**	**Glutamic acid**	**Serine**	**Arginine**	**Cysteine**	**Glycine**	**Proline**	**Tyrosine**
Cajanus genus	-	-	-	-	1.5	0.3	-	-	0.6
Cicer genus	5.0	11	17	3.7	8.3	0.6	3.7	3.8	2.8
Lens genus	4.2	12	22	5.2	7.8	0.9	3.6	3.5	32
Phaseolus genus	1.0	2.8	3.9	1.4	1.5	0.2	1.0	0.9	0.8
Psium genus	5.2	11	18	5.1	7.2	1.7	4.5	3.8	3.7
Vigna genus	4.2	11	17	4.5	7.5	5.0	3.8	4.0	3

The major proteins found in pulses are globulins and albumins, although other proteins such as prolamins and glutelins can be found in smaller amounts, less than 5 % (Boye *et al.,* 2010; Shevkani *et al.,* 2019) (Table 3). Proteins are recognized by their solubility properties; globulins are salt-soluble, albumins water-soluble, glutelins alkali-soluble, and prolamins alcohol-soluble. Storage proteins include globulins, albumins, and glutelins. In pulses, the major storage proteins are globulins (80% of the total proteins), which are dispersed in cytoplasmic cells, but they can also aggregate and form specialized structures called protein bodies. Studies have shown that protein bodies are generated directly from the rough endoplasmic reticulum (ER) or from protein storage vacuoles during seed maturation (Bewley, 1997; Bollini & Chrispeels, 1979; Müntz *et al.,* 2001). During germination, these proteins are hydrolyzed, thus providing nitrogen and sulfur, and storing carbon units for the developing seedlings. Globulins have a globular structure with no catalytic activity (Bessada *et al.,* 2019). Globulins comprise two classes, termed 11S or legumins, and 7S, usually called vicilin, but their names depend on their species of origin (Boye *et al.,* 2010; Wang *et al.,* 2003). Legumin is a hexameric oligomer of 330-410 kDa. The six polypeptides presented are linked by a disulfide bond and are composed of one basic and one acidic subunit (Mession *et al.,* 2012). Vicilin is a trimeric oligomer of 150-190 kDa containing three monomers of 50 kDa linked by non-covalent bonds (Bessada *et al.,* 2019). Enzymatic hydrolysis is limited by its globular structure (Sánchez-Vioque *et al.,* 1999). The major secondary structures of globulins proteins are β-sheets and α-helices, with small numbers of β-turns and random coils (Yu, 2005). Protein-protein interactions and aggregate formation are due to the hydrophobic character of β structures, which reduce their digestibility by lowering their accessibility to proteolytic sites (Carbonaro *et al.,* 1997). Vicilin is the major storage protein in kidney beans and cowpeas, accounting for up to 88 % of the total globulins. On the other hand, chickpeas present legumins as their major globulin, while field peas contain both vicilin and legumins as storage proteins. Albumins constitute only 10 to 20 % of the total seed proteins, and they include many "housekeeping" proteins, lectins, and lipoxygenases (Bessada *et al.,* 2019; Shevkani *et al.,* 2019; Wang *et al.,* 2003). They are considered proteins with biological activity that comprise enzymatic proteins, proteases, and amylase inhibitors (Bessada *et al.,* 2019; Boye *et al.,* 2010; Shevkani *et al.,* 2019). Pulses albumins are generally low in molecular weight and are the most nutritive proteins in terms of amino acid composition (higher in cysteine and methionine) (Shevkani *et al.,* 2019).

Table 3. Protein fractions of pulses (Avezum *et al.*, 2022)

	Proteins fractions (%)			
	Globulins	**Albumins**	**Glutelins**	**Prolamins**
Cicer genus	53 - 60	8.0 - 12	19 - 25	3.0 – 7.0
Lens genus	49	17	11	3.0
Phaseolus genus	54 – 79	12 - 30	20 – 30	2.0 – 4.0
Pisum genus	50 – 85	15 - 25	12	-
Vicia genus	70 – 78	22 - 30	-	-
Vigna genus	50 - 70	11 - 25	15 - 24	0.2 – 5.0

Protein digestibility is another important characteristic that determines food protein quality. It is determined by the percentage of protein or AA intake absorbed by the digestive tract (Bessada *et al.*, 2019). Proteins with higher digestibility are desirable because they have higher nutritional value. Differences in digestibility may be due to differences in pulse variety, growth conditions, and processing (Boye *et al.*, 2010).

There are two main types of digestibility depending on which part of the gastrointestinal tract is considered. Ileal digestibility concerns the small intestine (from the mouth to the terminal ileum), while fecal digestibility concerns the entire digestive tract (Boye *et al.*, 2012). In the evaluation of ileal digestibility, different methods can be applied to correct endogenous losses, resulting in various protein quality indicators such as apparent, standardized, or true digestibility, as listed above (Bessada *et al.*, 2019). The FAO/WHO 1991 Expert Committee assessed that ileal digestibility provides better results; however, this type of method is complex to implement. Therefore, the standardized rat fecal-balance has been advocated (Boye *et al.*, 2012). In practice, *in vitro* methods to measure protein digestibility are widely used as they are easier to conduct than animal experiments. Different methods using multi-enzyme systems based on pH variation have been developed. A good correlation between these methods and *in vivo* digestibility has been previously reported (Boye *et al.*, 2012). However, Boye *et al.* (2012) reported that *in vitro* digestibility values could be higher than *in vivo* values for some pulses.

All methods have their own advantages and limitations, and must be chosen according to the study context.

The most commonly used methods are *in vitro* digestibility (IVPD) and the Protein Digestibility Corrected Amino Acid Score (PDCAAS) (Bessada *et al.*, 2019; Boye *et al.*, 2012). It should be noted that the IVPD does not measure true digestibility as the digestibility is measured by enzymatic degradation and does not account for the concentration and availability of EAA. True digestibility encompasses not only the breakdown of proteins into smaller peptides and amino acids, but also their absorption and utilization by the body. Therefore, while the IVPD assay provides valuable information about the susceptibility of proteins to enzymatic degradation, it does not provide a comprehensive assessment of the overall protein digestibility in terms of EAA concentration and bioavailability (Boye *et al.*, 2012). Therefore, the FAO/WHO 1991 Expert Committee recommends characterizing proteins based on their content in digestible AA and measures each AA as an individual nutrient (Bessada *et al.*, 2019). The PDCAAS method addresses these issues because it is based on protein digestibility, amino acid profile, and reference requirements for the human diet. To calculate the PDCAAS, the limiting AA score was multiplied by protein digestibility (true fecal digestibility), which provides a value for protein quality corrected for digestibility (Bessada *et al.*, 2019). Some disadvantages of the PDCAAS have been highlighted: the impact of antinutritional factors associated with proteins is not included, protein digestibility does not precisely represent the bioavailability of each AA, and proteins with scores higher than 100% are truncated to 100%; therefore, the additional nutritional benefits of these proteins are not covered (Boye *et al.*, 2012; Nosworthy & House, 2017). Other less commonly used methods to measure protein digestibility are the Amino Acid Score (AAS), Apparent Protein Digestibility (APD), Corrected (or true) Protein Digestibility (CPD), Nitrogen Balance (NB), Protein Efficiency Ratio (PER), Net Protein Ration or Retention (NPR), Net Protein Utilization (NPU), Protein Rating (PR), Relative Nutritional Value (RNV), Apparent, True and Relative Biological Value (ABV, TBV, rBV), and Digestible Indispensable Amino Acid Score (DIAAS) (Bessada *et al.*, 2019; Boye *et al.*, 2012). Pulses protein digestibility was not analyzed in this study. It was considered that PDCAAS and ileal digestibility are the best analyses; however, they cost time and money. Additionally, IVPD does not satisfactorily quantify protein digestibility, as it is far from measuring the real human protein digestibility due to all factors in human digestibility that it is not integrated into the IVPD quantification.

The protein digestibility of uncooked food pulses ranged from 34 % to 80 %, and can increase through food processing, such as soaking, cooking, and germination (Rehman & Shah, 2005) (Table 4). The values for PDCAAS reflect the lack of EAA on pulses, such as methionine, tryptophan, and cysteine. The average for IVPD is 70% for all pulses, as shown in Table IV. This value is lower than the IVPD of raw animal products, which is 92.4% for beef and 95.2% for chicken (Boye *et al.,* 2012). The secondary structure of pulse proteins (as mentioned previously) is one of the reasons for the reduction in pulse proteins digestibility. Carbonaro *et al.* (2012) observed a strong negative correlation between the amount of β-sheets structures and the *in vitro* digestibility. They suggested that the hydrophobic character of the β structures was responsible for the stabilization of the native protein structure. Hydrophobic forces have been suggested to enhance protein-protein interactions and aggregate formation, which leads to lower accessibility to proteolysis sites (Carbonaro *et al.,* 1997). In addition, β-sheets are ordered structures with many hydrogen bonds between sheets. In this type of structure, as well as in random coils, the accessibility of residues is low for digestive enzymes, and thus digestibility is low (Yu, 2005). Another reason for the reduced protein digestibility of pulses is the presence of antinutritional factors, such as protease inhibitors (trypsin and chymotrypsin inhibitors) and phytic acid. These antinutritional factors will be discussed later.

Table 4. In vitro protein digestibility (IVPD) and Protein Digestibility Corrected Amino Acid Score (PDCAAS) in raw pulses (Avezum *et al.,* 2022)

	IVPD (%)	**PDCAAS (%)**
Cajanus genus	69	-
Cicer genus	34 - 76	69*
Lens genus	76 - 77	-
Phaseolus genus	71 - 78	28
Pisum genus	78 - 80	59 - 62
Vicia genus	-	56
Vigna genus	72 - 75	56 - 80

*values for *Cicer genus* flour

Protein bioavailability and functionality (*i.g.*, ability to form gels, emulsions, and hold water) may also be affected by oxidative damage to proteins. In a pro-oxidative environment and under oxidative stress, carbonyl moieties are formed and/or introduced into proteins. This process is irreversible and is known as carbonylation. Reduced protein functionality is due to the fact that protein oxidation can alter protein-water interactions, changing the isoelectric point of proteins and forming protein aggregates. Additionally, protein oxidation may form irreversible protein-insoluble aggregates via carbonyl-amine crosslinks, reducing protein digestibility. On the other hand, mild and progressive protein oxidation may facilitate protein functionality by stabilizing the gel and may favor partial unfolding of the protein structure, which facilitates enzymatic approach, recognition, and action on the substrate (Estévez *et al.*, 2022). Lipid oxidation in food systems and its impact, principally on the deterioration of oils, meat, and dairy products, has always draw attention from the scientific community. However, the impact of protein oxidation on food and human health has rarely been studied in the last few years. This could be due to the complexity of protein oxidation chemistry and the necessity for precise methodologies to analyze and quantify protein oxidation (Estévez & Luna, 2017).

It is noteworthy that during seed germination proteases are both activated and synthesized inhibitory factors (e.g. phytic acid and protease inhibitor) are degraded, and thus strengthen the enzyme's activity and increasing protein digestibility (Ghavidel & Prakash, 2007; Müntz, 1996; Poncet *et al.,* 2015; Tsuji *et al.,* 2013). Additionally, due to post-translational modifications (PTMs), seed storage proteins (SSP) display a large isoforms diversity involved in the modulation of relevant biological processes (Arc *et al.*, 2011; Mouzo *et al.*, 2018). Notably, a modification in the phosphorylation status of 7S globulin was observed in germinating common bean seeds (López-Pedrouso *et al.,* 2014). Indeed, an increase of phosphorylated isoforms promotes 7S globulin degradation in germinating seeds supporting the idea that phosphorylation-dependent proteolysis could play an important role in the mobilization of seed storage proteins (SSP). Phosphorylation may induce conformational modifications and promote protease accessibility and digestibility of SSP in germinating seeds (Arc *et al.*, 2011).

IV.2. Carbohydrate composition

Pulses carbohydrates contain monosaccharides (*e.g.*, ribose, glucose, galactose, and fructose), disaccharides (*e.g.*, sucrose and maltose), oligosaccharides (*e.g.*, raffinose, stachyose, and verbascose), and polysaccharides (mainly starch). Their amount goes up to 60-65% of seed weight (Tiwari *et al.*, 2011). The carbohydrates present in pulses offer human health benefits, including a reduction in glucose release, inducing to a low glycemic index, and acting as a probiotic by providing substrates for microbiological fermentation in the large intestine (Tiwari *et al.*, 2011). Oligosaccharides represent 14% of the seed weight, whereas monosaccharides generally constitute less than 1%. The oligosaccharide family is the most studied carbohydrates in pulses. They are non-digestive in humans because of the β-glycosidic bond that links the monosaccharides (Hall *et al.*, 2017).

Starch is one of the most abundant plant polysaccharides and represents the major fraction of the total carbohydrate in almost all pulses and energy in the human diet (Ratnayake & Jackson, 2008; Tiwari *et al.*, 2011). Pulses starch granules vary in size, shape, and molecular arrangement depending on the species, cultivar, variety, and genetic-environment interactions. They are composed of d-glucopyranose-based polymeric structures in which individual glucose units are linked by either α (1-4) linkages or α (1-6) glycosidic bonds (Ratnayake & Jackson, 2008). Starch consists mainly of amylose (24-65%) and amylopectin, the content of which is due to genetic differences, the physiological state of the seed, or the amount of amylose complexed within lipids (Tiwari *et al.*, 2011). Amylose is a linear polymer with mainly α (1-4) linkages, and amylopectin is a branched polymer with both α (1-4) and α (1-6) bonds (Ratnayake & Jackson, 2008).

Pulses starches are less digestible than cereal starches, which may be due to their botanical origin, granule size, amylose/amylopectin ratio, starch crystallinity, amylose-lipid complexes, antinutrients (enzyme inhibitors, lectins, saponins, phytates, and tannins), and amylopectin chain-length distribution. To this end, the incorporation of pulses in diets has shown health benefits because it can induce a low glycemic index and consequently prevent chronic diseases (diabetes, coronary heart diseases, and cancers) (Chung *et al.*, 2008[a]; Oyeyinka *et al.*, 2021). Starch can be classified as soluble (rapidly hydrolyzed by digestive enzymes or RDS), insoluble (slowly hydrolyzed by digestive enzymes or SDS), or resistant starch (RS) (not hydrolyzed or

digested as it passes through the gastrointestinal tract). RS is beneficial to human colonic health, considering it as a probiotic, which acts as a substrate for microbiological fermentation, produces short-chain fatty acids (SCFAs), methane, carbon dioxide, and in lesser degree, impacts lipid and glucose metabolism (Hoover *et al.*, 2010). Dietary intake of RS varies considerably worldwide. For instance, in low-income countries, the intake is between 30 and 40 g/day, and in the European Union, between 3 and 6 g/day (Tiwari *et al.*, 2011). SDS is responsible for stabilizing glucose metabolism and inducing satiety; however, studies on their health benefits are scarce. To this end, due to the potential health benefits of SDS and RS, it is advantageous to focus research efforts on enhancing our knowledge of the relationship between the molecular structure of pulses starches and their rate and extent of digestibility. Such investigations could be of great interest to starch chemists, food industry, and nutritionists (Hoover *et al.*, 2010).

Starch digestibility in pulses is commonly measured by determining the percentage of RDS, SDS, and RS. There are basically two methods of analysis used in the literature that were developed by Englyst *et al.* (1992) and by the Approved methods of the American Association of Cereal Chemists, (2000). Their method represents the primary principle for determining starch digestibility, wherein the enzymatic hydrolysis of starch is controlled, and the released glucose is measured using glucose oxidase. The RDS is measured after incubation with pancreatic amylase and amyloglucosidase at 37 °C for 20 min, while the SDS is measured after 100 min. RS is the starch not hydrolyzed after 120 min of incubation (AACC, 2000; Englyst *et al.*, 1992). The hydrolysis index (HI) is measured by dividing the area under the hydrolysis curve of the sample by the area obtained for white bread (control). The estimated glycemic index (eGI) is calculate using the following equation: eGI = 8.198 + 0.862HI (Chung *et al.*, 2008[b]; Sandhu & Lim, 2008). The RDS content of pulses starches ranged between 4.2% and 29.9%, with the lowest being found in pigeon pea starch and the highest in chickpea (Table 5). The SDS content in pulses starches ranged from 16.9 - 57.7%, while RS contents ranged from 8.1 – 78.9%. The pigeon peas represent the pulse with the lowest percentage of eGI. Thus, it can be a good food source for patients with diabetes. Meanwhile, chickpeas have the highest eGI percentage, which can help malnourish patients. In general, pulse starches have lower HI and GI values than cereal starches, which confirms their health benefits discussed previously (Sandhu & Lim, 2008).

Through the seed germination process, amylases catalyze amylose and amylopectin to simple sugars (glucose, maltose, and sucrose), thereby increasing digestibility (Benincasa *et al.*,

2019). For instance, microscopy analyses showed that germinated seeds had some dents or potential holes on the surface, whereas the raw seeds had a smoother surface. This indicates that germination modifies the starch granules and activates enzymatic activity, which cuts glucan chains into shorter lengths, increasing digestibility (Ma *et al.*, 2018). This result is supported by another study that showed maximum α-amylase activity after 3 days of germination (Ghavidel & Davoodi, 2011). However, microscopic analyses of starch in raw and germinated mung beans showed similar round and elliptical shaped granules, thus proving that germination does not affect the shape of mung beans starch granules (Liu *et al.*, 2020). The divergence in the results shows the complexity of the germination process in terms of the influence of starch structure in pulses. Therefore, is of interest to invest in research on the starch structure of germinated pulses, as most studies have been conducted on cereals (Li *et al.*, 2017; Li *et al.*, 2012).

Table 5. Starch fractions (%RDS, %SDS, and %RS), hydrolysis index (HI), estimated glycemic index (eGI) and the content of Soluble (SDF), Insoluble (IDF) and Total Dietary Fiber (TDF) (g/100g dry matter) (Avezum *et al.*, 2022)

	RDS (%)	**SDS (%)**	**RS (%)**	**HI**	**eGI**	**Soluble Dietary Fiber (SDF)**	**Insoluble Dietary Fiber (IDF)**	**Total Dietary Fiber (TDF)**
Cajanus genus	4.2	17	79	8.2	44	-	-	6.4
Cicer genus	22 - 30	46 - 58	8.1 - 18	70 - 74	69 - 72	4.3 - 5.0	13 – 24	16 - 18
Lens genus	16 - 17	58 - 62	13	67	66	1.8 – 3.8	17 – 31	19 - 35
Phaseolus genus	8.2	32	59	33	58	5.9	20 – 27	14 – 32
Pisum genus	18 - 24	54 - 59	8.1 - 13	69 – 72	68 - 70	5.6	9.7	3 – 20
Vigna genus	9.5	30	61	17	49	-	-	3.9 – 6.2

Dietary fiber (DF) is the edible part of plants that is resistant to digestion and absorption by human endogenous enzymes with complete or partial fermentation in the large intestine. DF is a combination of chemically heterogeneous substances or a complex mixture of polysaccharides (*e.g.*, cellulose, hemicellulose, pectin, and gums), waxes, and lignin as plant cell wall materials (Tiwari *et al.*, 2011). They are classified into two types (insoluble and

soluble) based on their solubility in a pH-controlled enzyme solution (Tosh & Yada, 2010). Insoluble dietary fiber, or IDF, (*e.g.*, cellulose, lignin, and some hemicelluloses) promotes the movement of material through the digestive system. Soluble dietary fiber, or SDF, (*e.g.*, pectins, galactomannan gums, mucilage, and some hemicelluloses fractions) are natural gel-forming fibers. Pulses are a high source of dietary fiber and the concentration of the insoluble fraction is dominant, accounting for 63 - 90% of the total dietary fiber (TDF) (Table 5) (Tiwari *et al.*, 2011; Tosh & Yada, 2010). Unfortunately, data on SDF, IDF, and TDF for raw pulses and comparisons between pulses classes or cultivars are limited in the literature (Tosh & Yada, 2010).

IV.3. Minerals composition

Minerals are considered essential micronutrients for human health, because they play an important role in human metabolism. The mineral content of pulses depends on variety, growth, and soil mineral composition (Hall *et al.*, 2017). Pulses are rich in iron (Fe), magnesium (Mg), zinc (Zn), and potassium (K). The recommended daily intakes (RDA) of Fe, Mg, Zn, and K in adults are, respectively, 10-15 mg/day, 350 mg/day, 12-15 mg/day, and 2000 mg/day (National Research Council, 1989) (Table 6). Among the pulses, lentils and chickpeas present the highest amount of iron, with an average of 70 µg/g and 68 µg/g, respectively. Chickpeas are the main source of Mg, with a concentration of 1176 µg/g. Lentils and beans have the highest zinc concentrations of up to 70 µg/g and 50 µg/g, respectively. Cowpeas have the highest potassium concentration of 1280 mg/100 g.

Despite the fact that pulses are considered to be rich in some minerals, the intake of 100 g of raw pulses does not reach the RDA, except for cowpeas, which reached 323% for copper (Table 7). However, eating 100 g of lentils provides 50% of the RDA for iron, 47% for zinc, and 49% for phosphorus. The low bioavailability of pulses minerals is related to the high amount of phytic acid (phytate). This compound is discussed in section V.5.2.

Table 6. Mineral content in pulses (Avezum *et al.*, 2022)

	Chromium	**Copper**	**Iron**	**Magnesium**	**Manganese**	**Zinc**	**Sodium**	**Potassium**	**Phosphorus**	**Calcium**
	µg/g						mg/100 g			
Cicer genus	0.09 - 0.3	3 - 5	65 - 70	1176	26	37 - 43	101	1155	251	197
Lens genus	0.12 - 0.6	2 - 3	65 - 75	922	13	45 - 70	79	874	394	120
Phaseolus genus	0.08 - 0.2	3 - 3.5	62 - 67	1166	12	39 - 50	-	-	-	-
Pisum genus	0.05 - 0.1	1.5 - 2	19 - 26	42	22	33 - 40	111	1021	283	110
Vigna genus	-	97	26	48	17	51	102	1280	303	176

Table 7. Percentage of mineral intake for 100 g raw pulses (Avezum *et al.*, 2022)

	Chromium	**Copper**	**Iron**	**Magnesium**	**Manganese**	**Zinc**	**Sodium**	**Potassium**	**Phosphorus**	**Calcium**
Cicer genus	13	16	47	34	52	29	20	58	31	25
Lens genus	30	10	50	26	26	47	16	44	49	15
Phaseolus genus	11	12	44	33	24	34	-	-	-	-
Psium genus	5.0	6.3	17	1.2	44	27	22	51	35	14
Vigna genus	-	323	17	1.4	34	34	20	64	38	22
RDA (mg/day)	0.2	3	15	350	5	15	500	2000	800	800

IV.4. B vitamins in pulses

Vitamins are organic compounds that are essential to the diet in small amounts (µg or mg) for physiological function (*e.g.*, maintenance, growth, and metabolic integrity). Vitamins are distinct from fats, carbohydrates, and proteins, as they do not serve structural functions, nor provide significant energy to the human body, and from minerals, as they are inorganic compounds (Bender, 2003). Vitamins are synthesized by microorganisms and plants, and higher animals must match their demands by dietary intake. The deficiency or the absence of vitamins in the human body results in deficiency diseases. These nutrients have an important physiological role, as they work as co-enzymes in many metabolic functions and are responsible for numerous oxidoreduction reactions (Combs & McClung, 2017; Hall *et al.*, 2017).

The B vitamins complex is the main source of vitamins in pulses. They are water-soluble and are not stored in the body, a continuous daily supply is required in our diet. Foods containing B vitamins require proper storage and preparation to minimize vitamin loss, as they are easily destroyed or washed out during food storage or preparation. The B complex group includes eight B vitamins: thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8), folate (B9), and cobalamin (B12) (Moore, 2012). A deficiency in B vitamins can cause numerous diseases such as beriberi, pellagra, dermatitis, oral and genital lesions, and chronic diseases, which are a major public health problems in many countries (FAO *et al.*, 2013). Pulses are a great source of B vitamins, especially B_1, B_2, and B_9 (Table 8). Thiamine and folate are the main vitamins in the *Vigna genus*, with amounts of 771 µg/100 g and 420 µg/100 g, respectively. This means that consuming 100 grams of raw *Vigna genus* provides more than 70% of the daily intake of thiamine and 100% of folate. In general, 100 g of pulses do not provide a considerable daily intake of riboflavin, with the highest intake being only 26% from *Phaseolus* and *Lens genus*.

Table 8. Vitamins contents in pulses (µg/100 g) and their respective daily intake percentage for 100 g of raw pulses (%) (Avezum *et al.*, 2022)

	Thiamine	**Riboflavin**	**Folates**
Cajanus genus	-	-	174 (43)
Cicer genus	453 (41)	173 (16)	150 (37)
Lens genus	560 (51)	280 (26)	146 - 290 (55)
Phaseolus genus	750 (68)	280 (26)	131 - 140 (35)
Pisum genus	740 (67)	150 (14)	102 (25)
Vigna genus	771 (70)	250 (23)	420(105)

IV.4.1. Thiamine

Thiamine (B1 vitamin) is composed of a pyrimidine (4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate, HMP-PP) ring linked to a thiazole (4-methyl-5-β-hydroxyethylthiazolium, HET-P) ring by a methylene bridge. It can be present in a free form (unphosphorylated) in living organisms and also in its phosphorylated derivatives, such as thiamine monophosphate (TMP), diphosphate (TDP), and triphosphate (TTP) (Figure 3) (Goyer, 2010). These forms with corresponding enzymes constitute the system of thiamine metabolism (Makarchikov, 2009; Schmidt *et al.*, 2017). Pyrimidine and thiazole moieties of thiamine are produce in the chloroplasts and mostly in green tissues. The pyrophosphorylation to TDP takes place exclusively in the cytosol (Figure 4). Thiamine diphosphate, or thiamine pyrophosphate, functions as a co-enzyme and is found in cells at the highest concentration, constituting up to 90 % of the total thiamine and its derivatives (Goyer, 2010). TDP plays a crucial role in various cellular compartments, including cytosol, mitochondria, and chloroplasts, and is involved in key metabolic pathways such as glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, and mitochondrial and chloroplastic pyruvate dehydrogenases (PDH). It regulates amino-acids metabolism, as well as the transformation of carbohydrates, including pentoses required for nucleotide synthesis. Additionally, phosphorylated derivatives of thiamine exert control over cell metabolism through allosteric regulation of enzymes, transmission of nerve signals in synapses, and regulation of protein synthesis via riboswitches in microorganisms and plants (Tylicki *et al.*, 2018). Moreover, studies have demonstrated that thiamine acts as an activator of the plant defense system, contributing to its importance in various physiological processes (Ahn *et al.*, 2005).

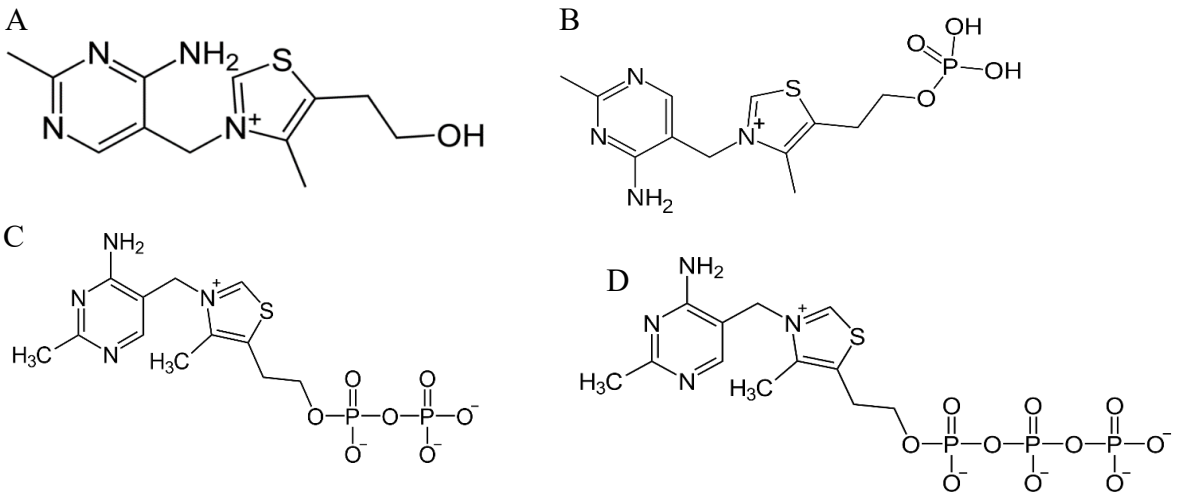


Figure 3.Molecular structure of unphosphorylated thiamine (A), TMP (B), TDP (C), and TTP (D)

The insufficient intake of thiamine affects the proper functioning of nervous, cardiovascular, and locomotive systems due to an overall decrease in bioenergetic processes, carbohydrate metabolism, and its interconnection with amino acid metabolism. In developing countries, its deficiency results in a disease called beriberi, which occurs in milk-fed infants whose nursing mothers are deficient (Chunming *et al.,* 2001). Thiamine deficiency may also occur in cases of dietary deficiencies and excessive use of drugs or alcohol abuse, which reduce vitamin absorption. The risk of thiamine deficiency includes the elderly, patients after major surgery, pregnant and breastfeeding women, smokers, diabetes patients, and youth who prefer a high-carbohydrate diet (Tylicki *et al.,* 2018). The recommended daily intake of thiamine is 1.1 mg/day for women and men (Elmadfa *et al.,* 2009).

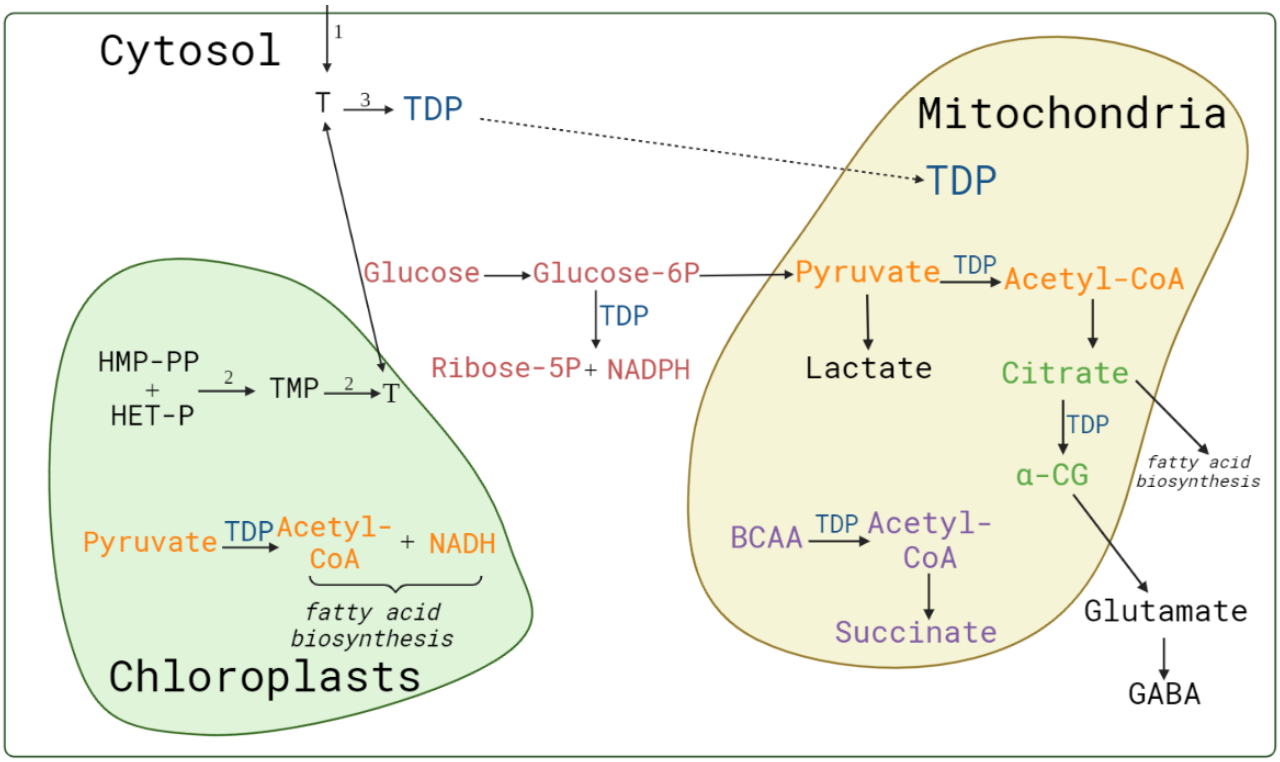


Figure 4. Thiamine transportation and metabolic pathway in plant cell. Black arrows indicate biosynthetic steps and dotted arrows indicate mobilization of compounds. Thiamine (T) absorption takes place through passive diffusion by protein carrier (1). In chloroplasts, 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate (HMP-PP) and 4-methyl-5-β-hydroxyethylthiazole phosphate (HET-P) synthesizes thiamine monophosphate (TMP) mediate by TMPPase enzyme (2). TMP uses TMPPase to coverts to thiamine (T). In cytosol, T is phosphorylated by thiamine pyrophosphokinase (3) into thiamine diphosphate (TDP). TDP is used as co-enzyme in pentose phosphate cycle in cytosol (red); as coenzyme in pyruvate dehydrogenase complex (orange); branched chain amino acid (BCAA) catabolic pathway (violet); tricarboxylic acid cycle (TCA)(green) in mitochondria; and as coenzyme in pyruvate dehydrogenase in chloroplasts (orange) (Goyer, 2010; Makarchikov, 2009; Marcé-Grau *et al.*, 2019)

IV.4.2. Folates

Folate molecules are composed of pterin, ρ-aminobenzoic acid (ρABA), and a glutamate chain with 1 to 14 glutamate moieties. They differ chemically according to the oxidation state of the pterin ring (di- or tetrahydrofolate), the oxidation state of the carried one-carbon (C1) unit, and the number of glutamate moieties (Rébeillé *et al.,* 2006). The principal folates forms present in the seeds are folic acid (PteGlu), tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), and 10-formylfolic acid (10-CHO-PteGlu) (Figure 5). In seeds, folates is not equally distributed between the embryos and cotyledons. Jabrin *et al.,* (2003) reported that dry pea embryonic axis contained 7.5 nmol folate g^{-1} fresh weight against only 0.27 nmol folate g^{-1} fresh weight in cotyledons. When the seeds were soaked for 18 h, the folate content increased only in the cotyledons, whereas in the embryonic axis, the folate concentration decreased by 50% (Jabrin *et al.,* 2003). Folate levels increase during imbibition due to the *de novo* synthesis of H_4folate. Recent studies have indicated that the germination of various pulses leads to a 2.5-4-fold rise in the overall folate content (Sallam *et al.,* 2021). Furthermore, a cowpea seed study revealed that soaking at various temperatures resulted in alterations in the behavior of folate within the seeds (Coffigniez *et al.,* 2019[b]). During germination, the seeds undergo major metabolic transformations, characterized by a transition from quiescent to active metabolic state. Thus, the increase in folate concentration during germination is not surprising, as H_4folate is a central cofactor for many cellular processes, such as C1 and amino acid metabolism, synthesis of nucleic acids, methionine, pantothenate, glycine, and serine, and mitochondrial and chloroplast protein biogenesis (Jabrin *et al.,* 2003). In plant cells, H4folate is synthesized in the mitochondria and transported to other cell compartments, such as cytosol and chloroplasts, before the final glutamylation step (Figure 6) (Rébeillé *et al.,* 2006). H4folate synthesizes other folate forms in the mitochondria, cytosol, and chloroplasts for further be use in C1 metabolism and methionine cycle (Rébeillé *et al.,* 2006). 5-CH_3-H_4folate is the main form found in cowpeas, chickpeas, and fava beans (Coffigniez *et al.,* 2019[b]; Hefni & Witthöft, 2013). This form provides the methyl group required to transform homocysteine into methionine (Rébeillé *et al.,* 2006).

Since humans cannot synthesize folates, they depend on plants, animal-based food, or dietary supplements. Pulses contain significant amounts of dietary folate (Table 8) (Zhang *et*

al., 2018). Folate content varies among species, the origin of the plant tissue, and its maturation process. This strongly indicates that folate synthesis is tightly related, controlled, and modulated as a function of the metabolic requirements of seeds and plants (Rébeillé *et al.*, 2006). In addition, the content of folate in plant structures can be negatively affected by factors such as heat, oxygen exposure, and light intensity (Scott *et al.*, 2000). Due to folate solubility in water, and thus its leaching into the surrounding water, processes such as washing, blanching, canning, or cooking can reduce the folate content.

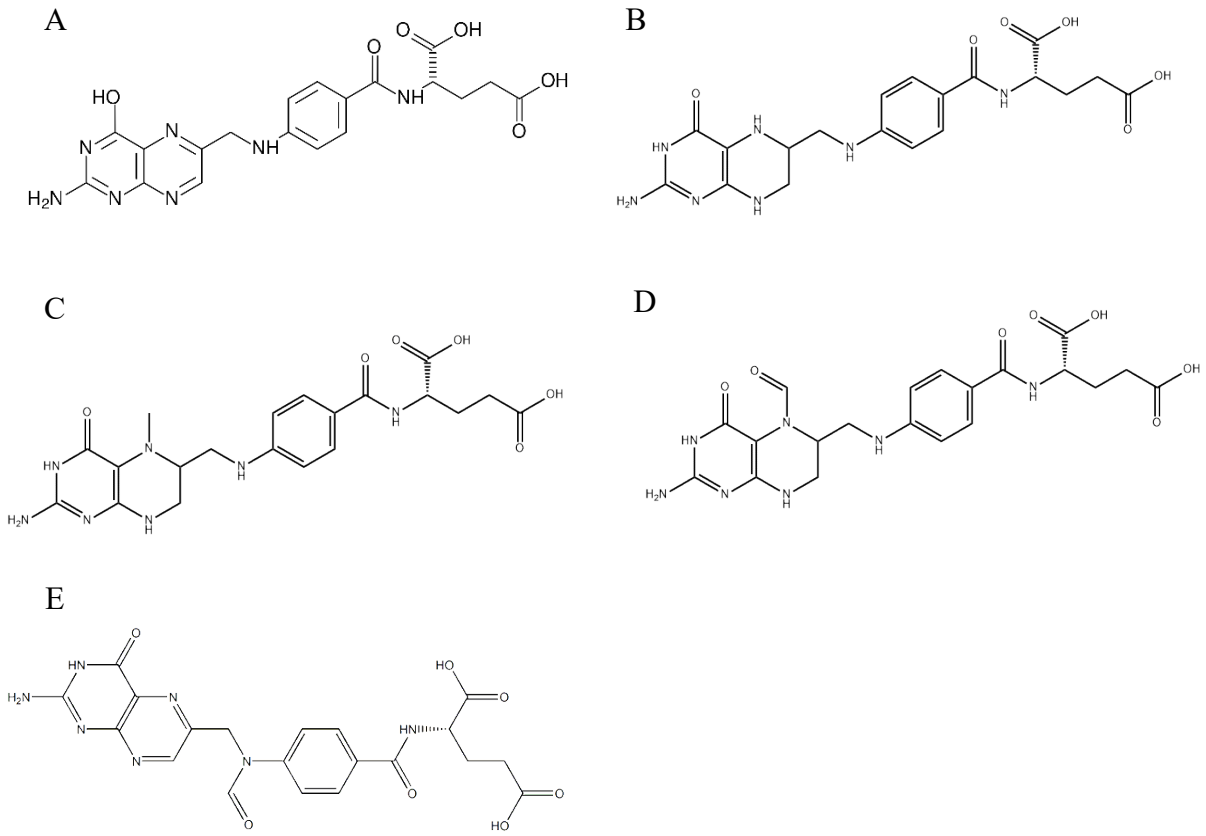


Figure 5. Molecular structure of folic acid (A), tetrahydrofolate (B), 5-methyltetrahydrofolate (C), 5-formyltetrahydrofolate (D), and 10-formylfolic acid (E)

The requirements and recommendations for folate intake are expressed as dietary folate equivalents (DFE). The daily recommended folate intake varies from 200 and 400 µg for adults according to age and living conditions (pregnancy and lactation, rapid growth in adolescence, and patients on kidney dialysis) (Krawinkel *et al.*, 2014; Moll & Davis, 2017). Folate deficiency is strongly linked to the development of neural tube defects in fetuses and to an increase risk of chronic diseases such as cardiovascular disease, childhood leukemia, and certain types of cancer (Moll & Davis, 2017; Zhang *et al.*, 2018). One of the major causes of folate deficiency is

inadequate dietary intake, which is associate with old age, alcohol abuse, and poverty as risk factors (Moll & Davis, 2017).

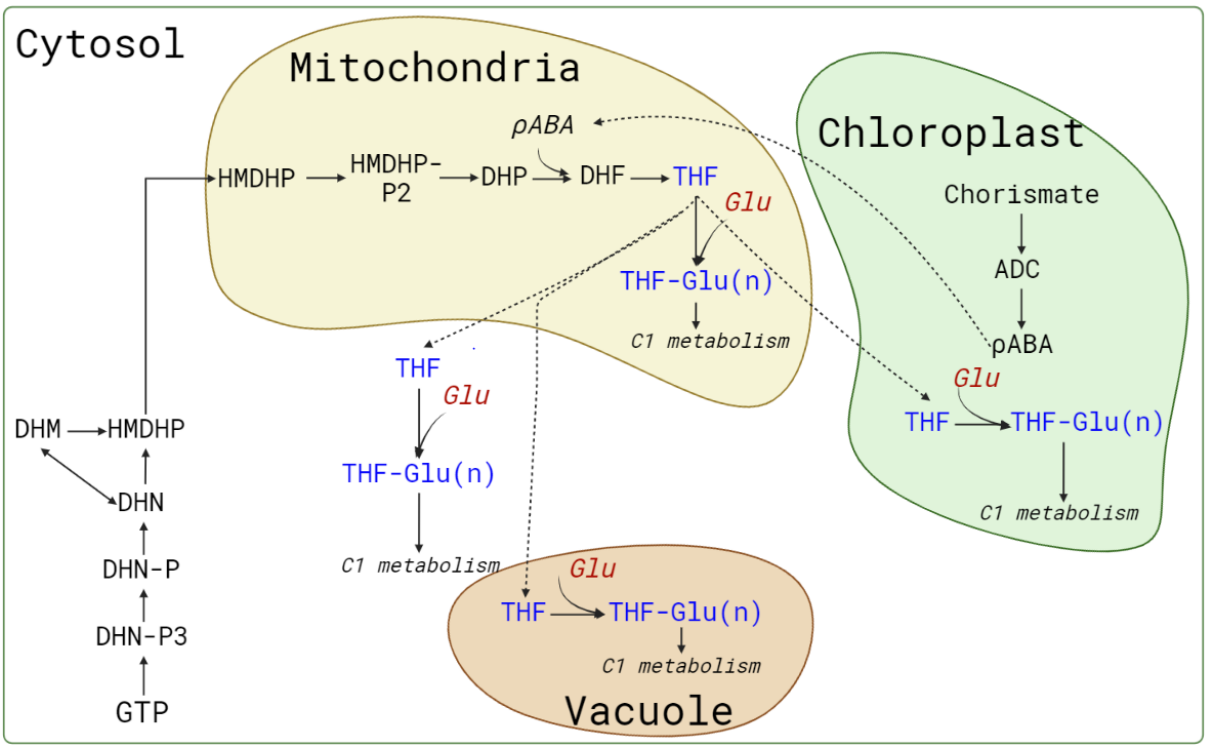


Figure 6. Folate transportation and metabolic pathway in plant cell. Black arrows indicate biosynthetic steps and dotted arrows indicate mobilization of compounds. Precursors: guanosine triphosphate (GTP); dihydroneopterin pyrophosphate (DHN-P3); dihydropteroate (DHP); tetrahydrofolate (THF); tetrahydrofolate polyglutamate (THF-$Glu_{(n)}$); aminodeoxychorismate (ADC); para-aminobenzoic acid (pABA) (Gorelova *et al.*, 2017; Rébeillé *et al.*, 2006; Scott *et al.*, 2000)

IV.5. Antinutritional factors in pulses

Pulses are a great source of protein and vitamins, but unfortunately, they also contain antinutritional factors, such as phenolic compounds, phytic acid, oligosaccharides, lectins, and protease and amylase inhibitors, which can affect their nutritional value. Antinutritional factors can be divided into two major categories: those that are sensitive to high temperatures, such as lectins, protease, and amylase inhibitors, and others that are stable or resistant to high temperatures, including polyphenolic compounds (Soetan & Oyewole, 2009). Although they

are considered antinutritional factors, these compounds present health benefits. For instance, they can reduce the risk factors for coronary heart disease, manage type 2 diabetes, reduce cholesterol, and act as an antioxidant, anti-obesity, and anti-cancer agent (López-martínez *et al.*, 2017; Campos-Vega *et al.*, 2010).

Tannins represent the lowest amount of antinutritional factors in pulses, with an average amount of 3.5 g/kg (Table 9). The pulses have a higher concentration of phytic acid. For instance, phytic acid content goes up to 12 g/kg in pigeon peas, 9.8 g/kg in cowpeas, and 11 g/kg in chickpeas. The *Pisum genus* is the pulse with the highest oligosaccharides content, with a maximum amount of 52 g/kg. Among pulses, chickpeas and kidney beans present the highest amount of phenolic acid, with gallic acid concentrations of 83 and 64 µg/g, respectively. Catechin is the main flavonoid found in lentils, black beans, and pearl beans with an amount of 78, 110 and 101 µg/g, respectively (Shahidi & Yeo, 2016). However, due to differences in the analysis methods of each study, including different compounds analyzed, it is difficult to compare the results with each other.

Table 9. Antinutritional factors contents in raw pulses (Avezum *et al.*, 2022)

	Tannins	**Phytic acid**	**α-galactosides**	**Lectins**	**Trypsin inhibitors**	**α-Amylase inhibitors**
	g/kg	g/kg	g/kg	HU/mg	TIU/mg	AIU/mg
Cajanus genus	15 - 29	12	35	-	8.1 - 12	0.02 - 0.03
Cicer genus	4.9	1.2 – 14	25 - 42	2.7 - 6.2	6.7 - 19	0.1 - 8.7
Lens genus	3.0	6.6 – 17	28 - 30	11	3.0 - 8.0	3.3
Phaseolus genus	4.0 – 28	6.4 – 19	6.1	75 - 89	3.1 - 25	0.2 - 1.4
Pisum genus	28 – 31	4.0 – 12	52	5.6	2.9 - 15	-
Vigna genus	2.0 - 26	4.3 - 9.8	26 - 47	0.01 - 11	1.1 - 30	3.8

HU = hemagglutinin activity

TIU = trypsin inhibitor units

AIU = amylase inhibitor units

IV.5.1. Polyphenolic compounds

The major polyphenolic compounds in pulses are tannins, phenolic acids, and flavonoids. These are divided by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (Michalak, 2006). The seed coat is composed of the epidermis, hypodermis, chlorenchyma, palisade, parenchyma, and endothelium cells, all of which contain most of the organs, such as vacuoles, which contain high amounts of phenolics in both the soluble and insoluble-bound forms (Shahidi & Yeo, 2016). Tannins are water-soluble polyphenolic compounds present in different plants. They are responsible for precipitating proteins, leading to a reduction in protein digestibility and availability of essential AA (Jasman, 1993; Bessada *et al.*, 2019; Rehman & Shah, 2005). In addition, because of their astringent properties, which are caused by the formation of complexes with salivary glycoproteins, they affect the palatability of pulses (Jezierny *et al.,* 2010). Tannins can be separated into two tannin sub-groups: hydrolysable tannins and non-hydrolysable or condensed tannins. Hydrolysable tannins are readily hydrolyzed by acids, alkalis, or enzymes. Condensed tannins are also referred to as flavones and procyanidins. Tannins are found in dry beans, peas, chickpeas, fava beans, cowpeas, and lentils. In most pulses, tannins are present as condensed tannins and are mostly situated in the seed coat (Jasman, 1993). In general, lower amounts of tannins are found in light-colored seeds than dark-colored brown pulses. For instance, white beans contain almost negligible tannin amounts, compared with colored varieties that are richer in tannin content (Jain *et al.,* 2009). Although they are considered an antinutritional factor, they also have health benefits for human consumption (López-martínez *et al.,* 2017). This topic will be discussed in more detail in section 2.3.6 (Health Benefits of Pulses).

After complex germination and post-germination processes, the total phenolic compound content and its antioxidant activity may either decrease or increase (Xu *et al.,* 2020). The early stage of germination initiates the synthesis of natural phenolic compounds from glucose or aromatic amino acids via the oxidative pentose phosphate, glycolytic, and shikimate pathways. These phenolic compounds are further polymerized or bound to macromolecular nutrients, such as polysaccharides, proteins, and lipids, stored in cell walls or vacuoles, thus decreasing their content. Continuing the germination process, the macromolecular nutrients are metabolized by enzymes, resulting in the release of phenolic compounds from their bound form (Xu *et al.,* 2020). At the beginning of the post-germination process, new plant cells proliferate, and the

synthesized soluble phenolics can be secreted to the cell wall to form new bound phenolics, thus increasing their content (Gan *et al.*, 2017). To this end, controlling the germination process is important for regulating the polyphenolic compounds in pulses.

IV.5.2. Phytic acid

Phytic acid (myo-inositol 1,2,3,4,5,6 – hexakisdihydrogen phosphate), known as phytate, represents a group of phosphorus compounds widely found in pulses, cereals, and vegetables. It accounts for approximately 78% of the total phosphorus in pulses and is stored in protein bodies, in the aleurone layer, and in scutellum cells (Campos-Vega *et al.*, 2010). The major structure of phytate is the IP6, which is a cyclic compound containing six phosphate groups ($C_6H_{18}0_{24}P_6$). In seeds, phytate is also present in its reduced forms, such as IP5, IP4, IP3, IP2, IP1, and unphosphorylated myo-inositol, although in lower quantities (Chitra *et al.*, 1996; Shi *et al.*, 2004). IP6 concentration tends to be higher in raw dry beans, black-eyed peas, and pigeon peas than in lentils, green and yellow split peas, and chickpeas (Campos-Vega *et al.*, 2010). Phytate is responsible for a strong chelation affinity with cations, principally Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mn^{2+}, and Cu^{2+}, reducing their solubility and availability for human absorption (Ali & Elozeiri, 2017). Also, they can bind to digestive enzymes, such as protease, alpha-amylases, and trypsin, leading to their inhibition and interfering with the digestion process (Chitra *et al.*, 1996). On the other hand, phytates have beneficial effects on human health, such as their anticarcinogenic action and their ability to decrease the risk of heart diseases or diabetes (Campos-Vega *et al.*, 2010). Phytate in germinating seeds is hydrolyzed by the enzyme phytase, releasing phosphate, cations, and inositol, which are used for physiological and metabolic requirements (i.e., enzymes of starch metabolism and ATP synthesis) (Ali & Elozeiri, 2017). To this end, as the germination process advances, phytate content decreases; thus, the bioavailability of phosphorus and minerals increases, enhancing the nutritional value of germinated pulses (Benincasa *et al.*, 2019).

IV.5.3. α-Galactosides

α-Galactosides are low-molecular-weight non-reducing sugars derived from sucrose, and contain 1,3-galactose units linked by α (1 → 6) bonds (Chibbar *et al.*, 2016; Martínez-Villaluenga *et al.*, 2008). They belong to the raffinose family oligosaccharides (RFOs) and are mainly represented by galactinol, raffinose, stachyose, and verbascose. They represent approximately 5% of the dry weight of the pulses and are soluble in water and alcohol-based solutions (Bessada *et al.*, 2019). Stachyose is present in higher amounts in the cotyledon and embryonic axe fractions of the seeds. Verbascose is the major α-galactoside in the seed coat (Njoumi *et al.*, 2019). α-Galactosidase is an enzyme responsible for the degradation of α-galactosides generating galactose and sucrose as the end products (Veldman *et al.*, 1993). Verbascose is composed of 3 galactose units, while stachyose has 2, and raffinose has 1 (Figure 7). The degradation of 1 mol of verbascose produces 1 mol of stachyose, and 1 mol of stachyose produces 1 mol of raffinose, which in turn is degraded to galactose and sucrose (Coffigniez *et al.*, 2018[b]). Because of the absence of α-galactosidase in the human and animal intestinal mucosa, the RFOs escape digestion and are metabolized by colon bacteria, producing hydrogen, carbon dioxide, and methane, causing flatulence and gut gas production (Bessada *et al.*, 2019; Naczk *et al.*, 1997). However, α-galactosides have a prebiotic effect in the intestine, and daily doses (less than 3 g/day) may be beneficial to human health (Elango *et al.*, 2022; Martínez-Villaluenga *et al.*, 2008).

During the germination process, the disappearance of RFOs is accompanied by an increase in the levels of sucrose, glucose, and galactose, which are used as energy by the seedling (Akinlosotu & Akinyele, 1991; Oboh *et al.*, 2000; Vidal-Valverde *et al.*, 1992). This shows the

high metabolic activity in the germinated pulses, promoting the synthesis and activity of the α-galactosidase enzyme (Coffigniez *et al.*, 2018[a]).

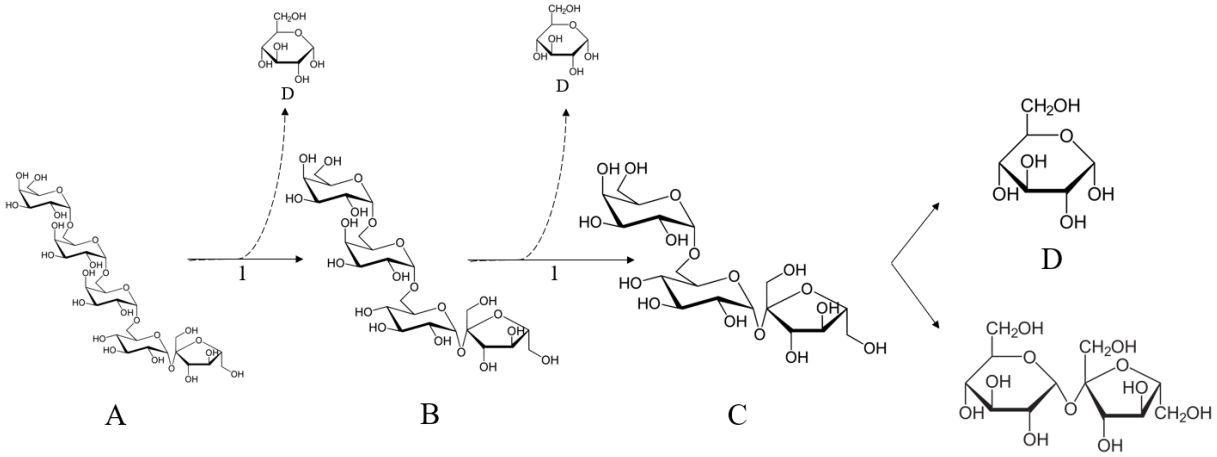


Figure 7. Chemical structure and degradation of verbascose (A), stachyose (B), raffinose (C) into galactose (D) and sucrose (E) by alpha-galactosidase enzyme (1)

IV.5.4. Lectins

Lectins (hemagglutinins or phytohemagglutinins) are proteins found in most plant; however, pulses are the main sources in human food. They are originally defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells and/or precipitate glycoconjugates (Hirsch, 1999). Depending on the species and variety, pulses account for 2 - 10 % of lectins (Abbas & Ahmad, 2018). The *Phaseolus* and *Lens genus* are important sources of lectins, as they are the major proteins found in these pulses (Campos-Vega *et al.*, 2010). Lectins form complexes with specific sugars (mono- and oligosaccharides) and other proteins; therefore they reduce the bioavailability and absorption of these nutrients (Derbyshire & Delange, 2011; Jain *et al.*, 2009). However, lectins also have beneficial effects as they may aid in obesity treatment and tumor growth (Campos-Vega *et al.*, 2010).

IV.5.5. Protease and α-amylase inhibitors

Protease inhibitors are proteins found everywhere in nature. In pulses and cereals, the amount increases from 1 to 10% of the total protein content. Their main function in seeds is defense-related, and they act against insect attack (Parca *et al.,* 2018). In human health, protease inhibitors have anticarcinogenic benefits and can control blood clotting (Parca *et al.,* 2018; Tiwari *et al.,* 2011). However, given their resistance to pepsin and the stomach's acidic pH, they have some adverse effects on human health, for example the reduction of protein absorption in the human digestive tract (Bessada *et al.,* 2019; Jain *et al.,* 2009). They are generally classified into two families: *Kunitz* and *Bowman-Birk*. The Kunitz type acts specifically against trypsin (trypsin inhibitor). The Bowman-Birk type inhibits trypsin and chymotrypsin (chymotrypsin inhibitor) simultaneously at independent binding sites. Protease inhibitors of the Bowman-Birk family have been characterized in *Phaseolus*, *Vigna*, and *Lens genus* (Campos-Vega *et al.,* 2010). The protease inhibitor content in whole seeds with a dark seed coat is higher than that of decorticated seeds with a light seed coat (Singh, 1988). Therefore, the levels of these antinutritional factors are considered important parameters to determine the nutritional quality of pulses (Bessada *et al.,* 2019).

Amylase inhibitors are responsible for reducing starch digestion; however, they also have a therapeutic role in obesity and diabetes control as they reduce blood glucose and insulin levels (Champ, 2002). The *Phaseolus genus* are the second largest group of seeds after cereals, which have been reported as natural sources of α-amylase inhibitors (Campos-Vega *et al.,* 2010). Amylase inhibitors are known for their heat-labile. In the *Cicer genus*, when extracts are boiled for 10 min, amylase inhibitors become inactive (Singh, 1988). Additionally, in lentils, soybeans, peas, winged beans, and lima beans, α-amylase inhibitors are undetected, and field beans, black-eyed peas, and chickpea contain low levels of α-amylase inhibitors (Campos-Vega *et al.,* 2010). To this end, since pulses have low α-amylase inhibitors content and α-amylase inhibitors are inactivate at low cooking times, it is not of practical importance to further study this antinutritional component, except if pulses are consumed unheated.

V. Health Benefits of pulses

According to FAO, in 2019, 650.3 million people worldwide (8.4 % of the world's population) were undernourished. In other words, they were unable to meet their daily dietary energy and nutritional need. Most undernourished people live in Asia (418 million) and Africa (282 million) (FAO, 2021). Another major public health threat worldwide linked to malnutrition is overweight and obesity, which has immediate impacts on health and increases the risk of diseases such as diabetes, cardiovascular diseases, and cancer. In 2020, 5.7% (38.9 million) of children under five years were overweight. In 2016, 13% of the adult population were obese. The main regions affected by obesity are North America (36%), Western Asia (30%), and Australia and New Zealand (29%) (FAO, 2021).

To reduce diet-related diseases, increasing fiber intake without increasing caloric intake is recommended (Ohanenye *et al.,* 2020). Plant-based protein diets can help to achieve this goal, since they are rich in fiber, have a lower glycemic index (GI) than cereals, and thus can help regulate blood sugar and lipid levels in both healthy and unhealthy individuals (Derbyshire & Delange, 2011). The soluble and insoluble fibers present in pulses are fermented by the gastromicrobiota and specifically produce butyric acid (SCFA), which is absorbed from the large intestine, and alters hepatic and muscle metabolism, thereby decreasing cholesterol synthesis in the liver (Marinangeli & Jones, 2012). Gao *et al.* (2009) showed that butyric acid supplementation increases hepatic and muscle energy use, as well as fat oxidation, mitochondrial oxidation, and biogenesis in mices. Additionally, pulses contain resistant starch (RS), which inhibits digestion in the small intestine and is highly fermentable in the colon. Diets containing RS increase endogenous fat oxidation (Higgins *et al.,* 2004). Given the nutrients found in pulses, it can be suggested that they can regulate macronutrient metabolism and increase fat oxidation (Marinangeli & Jones, 2012). This information may be valuable for future research on pulses consumption and its impact on reducing lipid levels in humans and decreasing the risk factors of overweight and obesity. Additionally, clinical research has shown that a pulses-based diet is effective in reducing total and LDL-cholesterol in older adults (> 50 years), the age at which the risk of cardiovascular disease (CVD) is elevated. The pulses-based diet decreased total cholesterol by 8.3% and LDL-cholesterol by 7.9%. A reduction in total cholesterol is estimated to reduce the risk of congenital heart disease (CHD) by 17 - 25% (Abeysekara *et al.,* 2012). Another study analyzed the effect of pulses consumption on nutrient

intake in adults (≥ 19 years). The survey divided participants into non-consumers of pulses and consumers of pulses. The study showed that a higher consumption of pulses increases energy per day by 11% to 16%, carbohydrate by 13% to 24%, protein by 12% to 19%, and fiber by 34% to 85%, compared to the average values for non-consumers. Compared to non-consumers, pulses-based diets increased micronutrient intake, such as folate (45%), phosphorus (20%), magnesium (35%), and zinc (28%). However, the study did not collect information on specific diets, such as low-carbohydrate, vegetarian, or vegan diets, which can lead to misleading results (Mudryj *et al.,* 2012).

Pulses also have other beneficial properties that are related to their content in biologically active components, called polyphenolic compounds (discussed on section IV.5.1). Although they can be considered antinutritional factors, they also offer health benefits due to their antioxidant and phytochemical activity. Studies have reported that the total antioxidant activity of pulses is dominated by phenolic compounds. However, other studies have shown that proteins also exhibit antioxidant activity. In cowpeas, the antioxidant activity of proteins is attributed to peptides with a molecular mass below 3 kDa. In addition, some branched-chain amino acids (leucine, isoleucine, and phenylalanine), aromatic amino acids (tyrosine, phenylalanine, and tryptophan), and sulfur-containing amino acids (cysteine) can act as antioxidants owing to their ability to donate protons to free radicals (Jayathilake *et al.,* 2018). Antioxidant and phytochemical compounds are responsible for regulating the metabolic syndrome and glycemic index in diabetes, and they have anti-inflammatory and antioxidant activities. To this end, they can reduce the risk factors for coronary heart disease (CHD), side effects of menopause, cancer, and age-related disorders (Tiwari *et al.,* 2011; Shahidi & Yeo, 2016; López-martínez *et al.,* 2017; Xu *et al.,* 2020). Antioxidant capacity and its constituents in food have become an interesting research topic in the food and health sciences (Huang *et al.,* 2005). In food, polyphenols are responsible for reducing lipid oxidation and preventing rancidity and are responsible for decreasing the adverse effects of reactive oxygen species (ROS). They can inhibit the formation of ROS, or act as scavengers to stop radical chain reactions (Huang *et al.,* 2005). Flavonoids scavenge ROS in the presence of electrons or hydrogen atoms. Additionally, the antioxidant activity of phenolic compounds is due to their hydroxyl and carboxyl groups, which chelate metals such as iron and copper. Another mechanism responsible for their antioxidant ability is their ability to inhibit lipid peroxidation by trapping lipid alkoxyl radicals. This action depends on the structure of the molecules and the position and number of the hydroxyl groups (Michalak, 2006). Hanson *et al.*, (2013) was

interested in investigating the effects of pulses-based diets on hypertension, arterial stiffness, and vascular remodeling in spontaneously hyperactive rat (SHR), which is the most common animal model of human essential hypertension. The study concluded that a lentil-based diet could decrease the rise in blood pressure in rats. This may be due to phenolic compounds that exhibit antioxidant effects when assayed *in vitro* (Hanson *et al.,* 2013).

The high consumption of animal proteins is linked to certain health disorders, such as overweight, diabetes, heart attack, cancer, bone diseases, and premature death (Ohanenye *et al.*, 2020). In the United States, it was reported that the substitution of red meat with plant-based diets lowered the risk of type 2 diabetes by 16% to 35% (Ohanenye *et al.,* 2020). Due to the health benefits of pulses demonstrated previously, it is interesting to increase research on pulses to better understand their mechanism and incorporate plant-based food into the staple diet of the population to help reduce malnutrition worldwide.

VI. Impact of food processing on pulse composition and nutritional quality

As described before, pulses are a great nutritional source rich in protein, high dietary fiber, minerals, and vitamins. There is growing interest in processing pulses to be used as an ingredient in the food industry, either using pulses alone or in combination with cereals. Traditional processing methods include dehulling, soaking, cooking, germination, sprouting, fermentation, and roasting (Figure 8). More recent processing techniques used exclusively by the food industry include micronization, extrusion, canning, puffing, and flour milling (Malcolmson & Han, 2019). Different analyses are used to verify the impact of food processing, such as omics technology by LC-MS, biochemical quantification by UPLC or HPLC, and imaging techniques by immunolocalization and µtomography. The particularity of omics and immunolocalization will be discussed latter in session VIII, IX, and X. In this recent study, we were interested in investigating the impact of the germination process on the nutritional quality of pulses. As shown in figure 8, to produce edible pulses as an end-product throughout the germination s*ensu stricto* process, a combination of soaking and cooking processes is necessary. To this end, we will analyze the impact of these three processes (soaking, cooking, and germination) on the nutritional and antinutritional compounds in pulses.

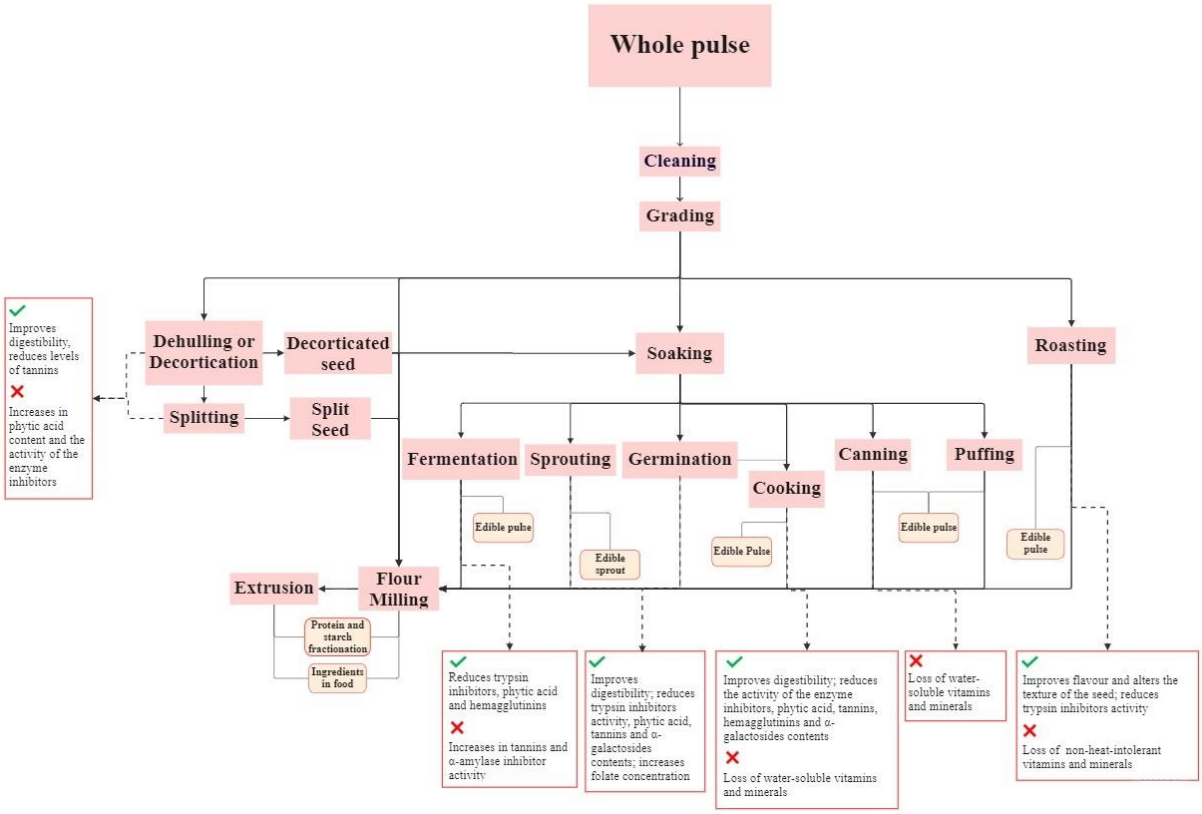


Figure 8. Flowchart of pulses food processing. The indicates different food processing to obtain the final product indicated in . The indicates the advantage (✓)and disadvantages (✗) of each process. The germination process requires a further high-thermal process (such as cooking) before consumption and the sprouting process gives an edible sprout as a ready-to-eat food product.

VI.1. Impact of food processing on protein digestibility

As previously mentioned, the average IVPD of raw pulses is 70%, which can be related to the secondary structure of proteins, particularly those rich in β-sheets. Food processes, such as soaking, germination, and cooking, can affect protein digestibility by the hydrolysis of seed proteins and by the reduction or elimination of different antinutrients (Bessada *et al.*, 2019; Chitra *et al.*, 1996; Jood *et al.*, 1989) as well as thermal phase changes in proteins (Rui *et al.*, 2011). Heat treatment in the presence of water results in the denaturation of inter and intramolecular bonds in proteins (Carbonaro *et al.*, 1997). Consequently, the tridimensional structure of the protein unfolds, and intramolecular cleavage sites become more accessible for

enzymatic hydrolysis. Different thermal stabilities have been observed between pulses because of the different ratios of specific proteins (Barbana & Boye, 2013). The thermal stability of legumin is higher than that of vicilin because of the presence of disulfide bonds. Covalent bonds are more stable, so they need higher energy to be cleaved compared to non-covalent bonds (Sirtori *et al.,* 2012). Therefore, the protein composition of the pulses is an important parameter for thermal denaturation and protein digestibility. Protein denaturation is followed by the aggregation of unfolded polypeptides (Carbonaro *et al.,* 1997). This thermal coagulation is due to the formation of intermolecular β-sheet structures (Rui *et al.,* 2011). Hydrophobic AA (such as phenylalanine, tryptophan, and tyrosine) and charged basic AA (lysine and arginine) are involved in such aggregates, so the accessibility of those AA is thus restricted. The emergence of protein aggregates can reduce digestibility in the small intestine because of the specific cleavage sites of trypsin and chymotrypsin (Carbonaro *et al.,* 2012).

Processing pulses can effectively increase *in vitro* protein digestibility and, consequently, their nutritional value (Carbonaro *et al.,* 2012). Previous studies showed that cooking under pressure (autoclaving) at 121 °C for 10 min improves protein digestibility by 96% to 105% when compared to raw pulses for black grams, chickpeas, lentils, and red and white kidney beans (Rehman & Shah, 2005). Nevertheless, an increase in cooking time at this temperature decreased protein digestibility, perhaps because of a decrease in the availability of some amino acids, such as lysine. This result appears to reflect the increase in protein aggregates observed by Carbonaro *et al.* (2012). For chickpeas, IVPD improved after boiling, autoclaving, and microwave cooking. The last two cooking techniques induced a greater increase in IVPD (Alajaji & El-Adawy, 2006). Protein digestibility was also higher when grains were soaked instead of unsoaked (Setia *et al.,* 2019). Germination also increases IVPD depending on the seed variety and germination conditions. Pigeon peas, kidney beans, and fava beans germinated at 25 °C for 72 h increased 23%, 110%, and 110%, respectively, in the IVPD (Alonso *et al.,* 2000; Chitra *et al.,* 1996). However, Setia *et al.* (2019) showed that germinated yellow peas and fava beans for 72 h only increased IVPD by 1.3% and 2.4%, respectively. To this end, it is obvious that the cooking process is advisable to further improve the IVPD in pulses.

VI.2. Impact of food processing on starch digestibility

Three structural factors are known to contribute to the enzymatic resistance of native starch from pulses: the compactness of the granular structure (low porosity), specific branch-chain distribution of amylopectin, and high amylose content favoring retrogradation (Ren *et al.*, 2021). With a sufficient amount of excess heat and water, starch undergoes an irreversible phase transition, namely gelatinization. Such order-disorder transitions associate with the granular diffusion of water, amorphous background region water uptake, granule hydration and swelling, heat uptake, crystalline order loss, and structural granular disorganization leads to amylose leaching (Hoover *et al.*, 2010). As previously illustrated by Toro *et al.* (2015), the gelatinization process also leads to a significant reduction in the resistant starch (RS) fraction with an increase in food product digestibility. The degree of starch gelatinization and starch digestibility can be modulated according to the processing conditions used. Physical and biological processes are known to change the structure and digestibility of starch and starch-based products (Hoover *et al.*, 2010; Magallanes-Cruz *et al.*, 2017; Singh *et al.*, 2015; Tiwari *et al.*, 2011). Consequently, nutritional intake and health benefits can be modulated.

Specific unit operations usually applied to cereals and pulses, such as soaking, germination, and cooking have been shown to significantly increase the *in vitro* digestibility of pulses starch, with a significant reduction in the RS fraction (Bishnoi & Khetarpaul, 1993; Bravo *et al.*, 1998; Erba *et al.*, 2018; Setia *et al.*, 2019). The sprouting process applied to chickpeas and green peas kept their well-preserved and ordered cell wall structure, with native and swollen starch granules (Erba *et al.*, 2018). No significant variation in the insoluble starch (SDS) fraction (55 vs. 62%, 39 vs. 49%, respectively) was highlighted between the raw and germinated seeds of both species. A complementary investigation conducted to investigate the impact of short-term germination of fava beans and yellow peas confirmed that the RS fraction was relatively low in native flours (14% and 26%, respectively), probably due to grinding (Setia *et al.*, 2019). A germination duration of 24 h to 72 h induced a significant reduction in the RS fraction of fava bean and yellow pea native flours (8% to 5% and 15% to 9%, respectively) (Setia *et al.*, 2019). Moreover, as expected, the cooking process applied to the flours (10 min) induced a major increase in starch digestibility, with a RS fraction from 3 to 1% in fava beans, and from 7 to 1% in yellow peas. Thus, considering that many raw pulses starches exhibit a high RS fraction in the 59% to 81% range, cooking is an effective process to reduce RS to below 11% (Ma *et*

al., 2017). The last studies illustrate that the cooking process is needed to obtain a high starch digestible fraction for human consumption, even in the cases where soaking and germinating unit operations are also used to lower the antinutritional compounds of pulses.

VI.3. Impact of food processing on vitamins B content

Soaking and cooking can decrease the amount of vitamins in pulses due to chemical destruction and leaching out into the medium due to their water-soluble characteristics (Table 10). For example, soaking cowpeas at 30 °C for 14 h decreased the total folate content by 13%. Moreover, cooking cowpeas at 95 °C for 2 h decreased the total folate content by 50% (Coffigniez *et al.*, 2019[b]). Cooking chickpeas at 100 °C for 90 min retained only 34% and 48% of thiamine and riboflavin, respectively (El-Adawy, 2002). These results show that the major loss of B-group vitamins is mainly due to leaching into the soaking water medium and not oxidation or other degradation pathways. Therefore, when the cooking medium is consumed as soup, no losses in vitamins B content need to be accounted for (Coffigniez *et al.*, 2019[b]; Delchier *et al.*, 2012; Hefni & Witthöft, 2013). However, it is not recommended to consume soaking or cooking medium, as it may contain antinutritional factors due to diffusion into the medium. Compared to soaking and cooking, germination can present more satisfactory results by increasing the vitamins content in the seed without compromising its nutritional quality, as it also reduces antinutritional factors. For example, sprouting of chickpeas at 25 °C for 3 days in the dark retained 63% and 16% of thiamine and riboflavin content, respectively (El-Adawy, 2002). Additionally, sprouting of lentils at room temperature for 6 days retained 72 to 89% of thiamine and 138 to 420% of riboflavin content, depending on the variety (Prodanov *et al.*, 1997). It is interesting to mention that these last studies mentioned the term "germination process" but they were referring to the "post-germination process." In this case, it is not possible to determine the positive impacts of the germination process on the content of these compounds in pulses, and further studies are needed for a better conclusion. Germination of chickpeas at 25 °C for 24 h, 48 h, and 72 h increased folate content by 65%, 39%, and 142%, respectively (Hefni & Witthöft, 2013). From these studies, it is possible to conclude that the sprouting process is the best treatment to increase thiamine and riboflavin content, and the germination process has the advantage of increasing folate content.

Table 10. Impacts of food processing on B vitamins content in pulses (% of retention) (Avezum *et al.*, 2022)

Treatment	Seeds	Time	Temperature (°C)	Thiamin retention (%)	Riboflavin retention (%)	Folate retention (%)
Soaking	*Vigna genus*	9 -38 min	Room temperature	-	-	81
		14 h	30	-	-	87
Cooking	*Cicer genus*	90 min	100	34	48	-
	Vigna genus	2 h	95	-	-	50
		45 min	100	31	-	-
Germination	*Cicer genus*	24 h	25	-	-	165
		48 h	25	-	-	139
		72 h	25	-	-	242
		3 days	25	63	16	-
	Lens genus	6 days	Room temperature	72-89	138 - 420	-
	Vigna genus	4 days	30	-	-	200
	Vicia genus	24 h	25	-	-	131
		48 h	25	-	-	172
		72 h	25	-	-	177
		6 days	20	72	353	-

VI.4. Impact of food processing on antinutritional factor content

Soaking, germination, and cooking processes can induce leaching into the soak water and induce thermal and/or chemical changes, leading to a degradation, formation, and/or conversion of antinutritional factors. Thus, it can reduce the content of condensed tannins, phytic acid, α-galactosides, lectins, protease, and α-amylase inhibitors in pulses (Table 11) (Alonso *et al.*, 2000; Khandelwal *et al.*, 2010; Njoumi *et al.*, 2019; Veldman *et al.*, 1993).

The effect of antinutritional factors loss during soaking depends on the cultivar, soaking solution, period, temperature, variety, and enzymatic activity of each pulse; however, satisfactory results have not been obtained with soaking alone (Shi *et al.*, 2004). During the soaking process, the reduction in antinutritional compounds may be due to leaching into soaking medium or enzymatic activity. Phytic acid, lectins, protease inhibitors, and α-amylase inhibitors may be reduced by diffusion into the soaking medium (Egli *et al.,* 2002; Shi *et al.,* 2017, 2018). However, soaking pulses had very little influence no reducing these antinutritional compounds. For example, Egli *et al.* (2002) showed that soaking at 25 °C for 16 h retained 87% of the phytic acid content in lentils, 92% in cowpeas, and 85% in chickpeas. Shi *et al.* (2018) showed that peas, lentils, fava beans, chickpeas, and navy beans soaked for 4 h at room temperature only had a lectin reduction ranging from 0.5 to 3%. Similarly, Shi *et al.* (2017) showed that soaking *Phaseolus genus* at room temperature for 4 h reduced the α-amylase inhibitors content by 4 to 10%. These results may be explained by the low enzymatic activity under these soaking conditions and by the cellular structure of the intact seed, which could limit the removal of antinutritional factors by leaching out into soaking medium. It is noteworthy that an analysis of the soaking medium under these conditions must be conducted to confirm this hypothesis (Shi *et al.,* 2018). On the other hand, α-galactosidase reduction by diffusion to soaked water or by enzymatic degradation depends on the soaking conditions (Coffigniez *et al.,* 2018[a]). For example, soaking cowpeas at 30 °C reduces α-galactosides by enzymatic degradation and not by leaching out into the soaking medium. Soaking cowpeas for 38 h at this temperature, decreased stachyose content by 35% (Coffigniez *et al.,* 2018[a]). However, with an increase in temperature (60 °C), α-galactosidase is thermally degraded, and the losses of α-galactoside is transferred to the soaking medium. When cowpeas were soaked for 14 h at 60 °C, the concentrations of verbascose, stachyose, and raffinose were reduced by 63%, 76%, and 74% of their initial values, respectively (Coffigniez *et al.,* 2018[a]). The loss of tannins may result from leaching into soak medium or by the enzymatic activity of polyphenol oxidase (Shi *et al.*, 2018). Further analyses of the soaking medium and/or polyphenol oxidase activity are needed to better explain tannin loss.

By increasing the process temperature (cooking) and/or performing a biological process (germination), the cellular structure of the seeds can be weakened, making it easier for substances to diffuse into the soak water. Additionally, these processes can enhance the metabolic activity, leading to increased enzymatic activity. To this end, it would be interesting to invest in these food processes to reduce the antinutritional content in pulses to a greater

extent. Cooking can reduce the antinutritional factors contents in pulses by leaching into soaking water or by the heat-lability of the compounds. This last may result in the breakdown of their structures into subunits or other unknown changes in their conformational structure (Shi *et al.*, 2004). Diffusion into the soaking water is responsible for reducing tannins and α-galactosides. For example, cooking pigeon peas, chickpeas, and lentils under pressure at 121 °C reduced tannins content by 27%, 35%, and 36%, respectively (Khandelwal *et al.*, 2010). Cooking cowpea seeds at 95 °C for 3 h reduced the concentrations of verbascose, stachyose, and raffinose by 69%, 61%, and 63%, respectively (Coffigniez *et al.*, 2018[a]). It is noteworthy that in these cases, if cooking medium is consumed, there is no decrease in tannins and α-galactosides content. Phytic acid, lectins, protease and α-amylase inhibitors are known to be heat-labile (Shi *et al.*, 2004; Shi *et al.*, 2018; Shi *et al.*, 2017). Cooking under pressure at 121 °C for different periods (10, 20, 40, 60, and 90 min) and 128 °C for 20 min, reduced the phytic acid content in black grams, chickpeas, lentils, red kidney beans, and navy beans by 28 to 52% (Rehman & Shah, 2005). Cooking in different varieties of chickpeas, beans, fava beans, lentils, and peas for 1 h at 95 °C reduced the lectin content by 94 to 99% . Grant *et al.*, (1995) showed that α-amylase inhibitors in kidney beans presented a large thermal resistance, and processing at 80 °C for 30 min did not significantly reduced the level of this compound. However, cooking kidney beans at 100 °C for 30 min completely removed α-amylase inhibitor activity (Grant *et al.*, 1995). Other studies also agree with these results (Alonso *et al.*, 2000; Shekib *et al.*, 1988; Shi *et al.*, 2017). Cooking has been reported to be effective in inactivating antinutritional factors in several pulses (Udensi *et al.*, 2007). However, excessive heating reduces the nutritional value of pulse proteins and vitamins. For instance, some limiting essential amino acids in pulses, such as methionine, have been reported to undergo nutritional damage when heated. Therefore, it is important to establish the optimum heat condition or to combine different processing (*e.g.* germination and cooking) to best benefit the nutritional qualities of pulses (Singh, 1988).

The decrease in antinutritional factors in pulses during a complex biological process, such as germination, may be due to the activation of metabolic pathways that induce an increase in enzymatic activity, thus degrading antinutritional compounds. For example, during germination, tannins may be reduced by the activation of polyphenol oxidase and other catabolic enzymes. Germination of chickpeas at room temperature for 24 h reduced tannin content by 43%, and was not detected in pigeon peas and lentils. This represents the greatest loss of tannins compared to pressure-cooked and soaked pulses (Khandelwal *et al.*, 2010). Similarly, seeds use phytate as a source of inorganic phosphate and increase phytase activity

during the germination process (Reddy *et al.*, 1982). After 6 days of post-germination, the amount of IP4 and IP3 increased, indicating a high enzymatic activity (Vidal-Valverde *et al.*, 2002). After 72 h of germination, the phytic acid content reduced by 66%, 61%, and 30% in pigeon peas, fava beans, and common beans, respectively (Alonso *et al.*, 2000; Chitra *et al.*, 1996). However, another study showed that after 72 h of germination at 25 °C, white beans, chickpeas, lentils, and peas reduced the phytic acid content by 24%, 14%, 5%, and 2%, respectively. In the case of cowpeas, phytic acid content increased during germination (Egli *et al.*, 2002). Germination also has the advantage of reducing α-galactosides content (Vidal-Valverde *et al.*, 2002). After 24 h of germination at room temperature, stachyose and raffinose contents were reduced by 100% in cowpeas (Ibrahim *et al.*, 2002). However, another study with cowpeas did not show the same results under the same conditions, where verbascose and raffinose were eliminated only after 48 h and stachyose after 72 h (Akinlosotu & Akinyele, 1991). Alonso *et al.* (2000) showed that germination can further reduce α-amylase inhibitors compared with soaking. For instance, kidney beans and fava beans germinated at 25 °C for 72 h reduced α-amylase inhibitors content by 34% and 37%, respectively. Neither soaking nor germination reduces the level of lectins in kidney beans and fava beans (Alonso *et al.*, 2000). The final result for the antinutritional amount in germinated pulses depends on the conditions of germination, such as temperature, time, oxygen availability, moisture, light, seed variety, or differences in the analysis methods used that may vary (Nelson *et al.*, 2013).

Table 11. Impacts of food processing on antinutritional factor in pulses (% of reduction) (Avezum *et al.*, 2022)

Treatment	Seeds	Time	Temperature (°C)	Polyphenolic compounds	Phytic acid	α-galactosides	Lectins	Protease inhibitors	α-amylase inhibitor
Soaking	*Cajanus genus*	6-12 h	25	15	-	8.0 - 24	-	-	-
	Cicer genus	12 h	Room temperature	-	-	51 - 67	-	-	-
		6-12 h	25	16 - 22	7.0	7 - 37	-	-	-
		16 h	25	-	15	40	-	-	-
	Lens genus	4-12 h	25	24	-	33-37	-	-	-
		16 h	25	-	13	10	-	-	-
	Phaseolus genus	4-12 h	Room temperature (25)	-	18	33 - 45	1.0	6.0 – 9.0	-
		6-12 h	25	-	-	6 - 44	-	-	-
		12 h	30	24	6	-	0	5.0 - 15	11
	Vicia genus	6-12 h	25 - 30	4.0 - 48	9 - 33	8 - 39	0	4.5	15
	Vigna genus	12h-1	20 - 100	-	8.0	15	-	7.0 - 61	-
		9-38 min	Room temperature	-	0	15 - 20	-	-	-
		4h-12	Room temperature	23	14	13 - 32			
		8-16h	Room temperature	-	0.2-10	8 - 100	-	0.5 - 24	-
		4-16 h	25 - 29	-	8.0	9 - 41	-	-	-
		6-12 h	28	-	-	25 - 92	-	-	-
		14 h	60	-	-	71	-	-	-
		1-4 h	80	-	-	83 - 86	-	-	-
Cooking	*Cajanus genus*	45 min	100	-	-	75-84	-	-	-
		150-180 min	100	-	-	47-88	-	-	-
		-	121	27	-	-	-	-	-

	Cicer genus	45-60 min	100	-	4.0	50 - 60	-	82	-
		40-95	100	-	-	63 - 71	-	-	-
		90 min	100	-	29	46	100	82	-
		35 min	121	35	41	46	100	84	-
	Lens genus	15 min	100	-	36	-	-	-	-
		60-20 min	100 - 120	-	-	46 - 65	-	-	-
		-	121	36	-	-		-	-
	Phaseolus genus	15-90 min	100	-	-	9.0 - 13	-	93 - 100	-
		35 min	100	-	25 - 65	49 - 66	89 - 100	25 - 65	
		45 min	100	-	-	56 - 70	-	-	-
		150-180 min	100			66 - 68			
		60-20 min	100 - 120	-	-	47 - 69	-	-	-
	Psium genus	45 min	100	-	5.0	-	-	-	-
		10 min	121	11 - 62	6.0 - 13	-	-	-	-
	Vicia genus	45 min	100	-	-	67 - 78	-	-	-
	Vigna genus	3 h	95	-	-	64	-	-	-
		until soft	100	-	-	16 - 70	-	-	-
		20-50min	100	-	-	7 - 40	-	-	-
		30-45 min	100	-	-	67 - 97	-	-	-
		40 min	100	-	-	38 - 41	-	-	-
		15-60 min	100	-	32 - 68	22 - 68	38 - 76	33 - 52	-
		45-20 min	100 - 120	-	7.0 - 33	42 - 82	-	87 - 93	-
		60-20 min	100 - 120	-	-	46 - 62	-	-	-
		15-60 min	120	-	7.0 - 19	22 - 84	42 - 58	97 - 100	-
Germination	*Cajanus genus*	24 h	Room temperature-30	ND	-	65 - 75	-	-	-
		48 h	30	-	-	66 - 81	-	-	-

	72 h	Room temperature-30	-	66	66 - 100	-	-	-
Cicer genus	24 h	Room temperature-30	27 - 43	15 - 40	53	-	-	-
	48 h	Room temperature-30	37	15 - 64	35 - 70	-	-	-
	72 h	25 - 30	43	5.0 - 60	100	-	-	-
Lens genus	24 h	Room temperature	ND	-	-	-	-	-
	72 h	25	-	24	-	-	-	-
	2 days	20	-	-	100	-	-	-
Phaseolus genus	24 h	25 - 30	43	35 - 73	15 - 70	0 – 7.0	1.0 - 12	24
	48 h	Room temperature-30	-	11 - 87	32 - 91	0 - 15	16 - 22	32
	72 h	25 - 30	72	2.0 - 89	55 - 100	0 - 18	15 - 29	34
Vicia genus	24 h	25 - 30	9-56	47	35	0	6.0 – 9.0	27
	48 h	Room temperature - 30	18	57 - 59	80	0	11 - 16	33
	72 h	25 - 30	21-60	61 - 63	100	0	12 - 25	37
Vigna genus	24 h	Room temperature	40	17 - 38	25 - 100	-	31	-
	48 h	Room temperature	50	23 - 48	62 - 100	-	54	-
	72 h	Room temperature	57	55	88 - 100	-	-	-

VII. Economic and societal importance of lentils and cowpeas

In the preceding sections, we explored the significant health benefits of pulse seeds. These seeds are rich in protein (more than cereals), complex carbohydrates, and fiber and possess a lower glycemic index than cereals. They also contain polyphenolic compounds, which serve as antioxidants in the human body. Given their health importance, we aimed to further investigate the impact of food processing on lentils and cowpeas. We deliberately selected these pulses because of their economic, nutritional, and societal importance in both the northern and southern regions.

Lentils are an important food crop given their global production trade and popularity among consumers (Kaale *et al.,* 2023). It originated in the Middle East and subsequently developed in Asia, the Indian subcontinent, and South and North America (Muehlbauer & McPhee, 2005). Lentils, being cool season crops, exhibit favorable growth when cultivated in winter or early spring, thereby maximizing vegetative expansion and increasing yield (Sarker & Erskine, 2006; SPG, 2012). The principal abiotic constraints of lentil production are inadequate moisture levels and elevated temperatures in spring, whereas in regions of high elevation, winter exposure to cold temperatures poses a challenge (Sarker & Erskine, 2006). Given their indeterminate growth habit, lentils will frequently continue flowering as long as the conditions for vegetative growth are favorable. Therefore, inducing low-moisture or low-nitrogen stress is necessary to accelerate maturation (SPG, 2012). The cultivation of lentils covers six million hectares worldwide, yielding a total production of six million tons. Asia accounts for 52% of this output, whereas the Americas account for 38%. The major producers of lentil are Canada with 1.4 million tons of production, and India with 1.0 million tons (FAO, 2023). In high-income countries such as Canada, Australia, and the U.S., lentil crops are highly valued as export commodities. Like other pulse seeds, lentils can fix nitrogen into the soil, improving soil fertility, and consequently bolster cereal crop yields. Employing the technique of crop rotation between lentils and cereals increased the yield in spring and durum wheat. Moreover, incorporating cereals and oilseeds into crop rotations has been effective in reducing the risk of soil-borne pathogens, which are particularly detrimental to lentil cultivation (SPG, 2012). The genus Lens comprises seven taxa in four species: *L. culinaris* Medikus, *L. ervoides* (Brign.) Grande, *L. nigricans* (M. Bieb.) Godron, *L. lamottei* Czefr. The subspecies of *L. culinaris* Medikus include *culinaris, orientalis, tomentosus,* and *odemensis.* (Muehlbauer & McPhee,

2005). Given their small size (40 - 50 g per 1000 seeds), lentils offer a quicker and simpler preparation process than other pulses (Kaale *et al.*, 2023; SPG, 2012). Traditional cooking processes are the predominant transformation approaches in households and industries. The divergent consumption patterns observed worldwide primarily comes from variations in lentil varieties rather than in processing or prepared products (Kaale *et al.*, 2023). The color of seed coats ranges from clear to green, tan, brown, gray, blotched purple, or black, whereas cotyledons range from yellow, red, or green (SPG, 2012). The most common lentil types consumed across many nations are red and green lentils, whereas yellow and brown lentils are favored in some countries. Green lentils are normally consumed whole or in their dehulled form, whereas red lentils undergo dehulling before being cooked and consumed. Nowadays, lentil flour is used in baking and extruded products as a substitute for wheat flour, which potentially yields gluten-free products. However, the incorporation of lentil flour requires precise analysis and understanding of its functional characteristics and impact on the sensory traits of the final products. Adding lentil flour to these food items can significantly increase the protein and dietary fiber content, exceeding that of conventional 100% wheat-based food products (Kaale *et al.*, 2023). In fact, lentils have a protein content of 25-27%, surpassing the protein content of cereals by more than two-fold (Avezum *et al.*, 2022).

The origin and domestication of cowpeas occurred near Ethiopia, and subsequently developed in southern Africa. Recognized as one of the oldest cultivated crops, cowpeas adapt to high temperatures and drought, surpassing many other crop varieties (Gómez, 2004). Although it has achieved global cultivation, Africa remains the predominant production region. Nigeria and Niger stand out as the largest producers and consumers of cowpea, producing around 2.7 million and 1.2 million tons annually, respectively (FAO, 2023). In African production, cowpeas are often intercropped with sorghum, pearls, millet, maize, cassava, or cotton. Thanks to their ability to fix nitrogen in the soil, cowpeas improve soil fertility, leading to increased cereal crop yields (Ehlers & Hall, 1997; Gómez, 2004). Cowpeas are essential for rural families in Central and West Africa because they offer great flexibility, food, animal feed, and cash income. Rural households have the flexibility to adjust their approach during production, opting to invest more resources to increase cowpea yield or alternatively allow plants to produce more foliage for animal feed. Therefore, a higher supply of feed for livestock translates into increased meat and milk production, balancing the lower cowpea yields. This flexibility renders cowpeas an exceptional choice for cultivation within the challenging climatic conditions that African farmers often confront (Gómez, 2004). Cowpeas are resilient to abiotic

stress factors, such as drought and high temperatures (Ehlers & Hall, 1997). Because of its deep taproot and ability to grow in arid environments, cowpeas demonstrate greater drought tolerance than soybeans or mung beans, which can flourish with as little as 300 mm of rainfall. However, its susceptibility to cold stress induces the fact that its geographic range is lower than that of soybeans (Gómez, 2004). Dry grains are the primary product of the cowpea plant used for human consumption; however, leaves and fresh green pods are also consumed (Ehlers & Hall, 1997). Pods generally hold 8-20 seeds of varying sizes, shapes, and colors. Ranging from reniform to red, brown, black, speckled, blotched, eyed (the hilum-central line is white surrounded by a dark ring), or mottled, cowpea seeds measure 2-12 mm in length and weighing 5-30 g per 100 seeds (Gómez, 2004). The nutritional profile of cowpeas closely aligns with that of common beans, but they have elevated carbohydrate and folate contents (Avezum *et al.*, 2022). Cowpeas are classified into four cultivar groups: *i) unguiculata*, the common form; *ii)* biflora or catjang, characterized by small erect pods that are mainly found in Asia; *iii)* sesquipedalis or yard-long bean, which is prevalent in Asia and characterized by its very long pods; and *iv)* textilis, native to West Africa and historically used for fiber extraction from its long peduncles (Ehlers & Hall, 1997).

VIII. Advancing the understanding of the germination process thanks to omics and imaging technologies

Seed germination is a complex biological process that involves the interaction of various internal and external factors. It activates various metabolic pathways that promote important changes in the biochemical, nutritional, and sensory characteristics of the pulses. As stated in session VI (impact of food processing), germination offers distinct advantages compared to soaking and cooking. While soaking and cooking can decrease antinutritional compounds through diffusion or enzymatic degradation, they also lead to a reduction in vitamins due to a diffusion into the medium. However, germination proved to be advantageous as it increases vitamin content while simultaneously reducing antinutritional compounds through enzymatic degradation. Now, the question arises: How does the germination process produce this nutritional advantage? What are the specific mechanisms by which germination conditions impact the metabolic pathways? Further exploration and investigation are required to truly understand the effects of germination on the nutritional quality of pulses. In all the studies

mentioned above, biochemical quantification was used to analyze the impact of food processing on vitamins and antinutritional compounds. While previous studies have provided valuable insights into the effects of the germination process and its nutritional benefits, innovative technologies such as multi-omics combined with immunohistochemistry can further enhance our understanding of this process.

The next sections of our study will explore these subjects in greater detail. By utilizing both proteomics and metabolomics, we can unravel the complex metabolic pathways and molecular processes underlying the changes that occur during germination (Prakash *et al.*, 2020). Through immunohistochemistry, we can gain deeper insights into cell specific metabolism, facilitating the understanding of metabolic modification and compartmentation in different tissues in various plant structures, such as seeds, fruits, leaves, stems, and roots (Walker *et al.*, 2001). By answering these questions through multi-omics and immunohistochemistry approaches, we can gain valuable insights into the potential of germination as a promising food processing method for enhancing the nutritional value of pulses.

VIII.1. Omics technology

Omics technologies are a set of scientific fields associated with measuring biological molecules in a high-throughput way, including genomics, transcriptomics, proteomics, or metabolomics. It integrates different approaches such as chemical analytical methods, bioinformatics, computational analysis, and many disciplines in biology (Prakash *et al.*, 2020). Omics help to understand the complex links between genes, proteins, and metabolites within the resulting phenotype. The use of omics techniques in agricultural studies is known to improve agricultural yields (Van Emon, 2016). Genomics provides for example insights into the annotations and functions of genes related to the regulation of specific traits of interest such as salt, heat, and humidity resistance. Through the analysis of genes exhibiting varying response patterns, transcriptomics can identify genes that play a role in controlling plant quality traits. Proteomics and metabolomics are powerful tools for studying plant and seed biology and can provide insights into the molecular mechanisms of growth and development, the effects of

environmental factors on plant metabolism, and the genetic and physiological basis of seed metabolism. The integration of omics technologies can provide a more comprehensive view of the plant system, leading to the development of smart strategies for optimizing crop breeding and improving the nutritional value of seeds (Yang *et al.*, 2021; Zhang *et al.*, 2022). Moreover, omics techniques help to characterize the quality, safety, taste, and nutritional composition of food crops by: *i)* analyzing protein quality and quantity through proteomics; *ii)* quantifying vitamins, fatty acids, organic compounds, sugars, antinutritional compounds, and volatile compounds through metabolomics; *iii)* improving shelf life prediction by identifying pathogens through proteomics; and *iv)* reducing allergens (detection by peptide markers) (Van Emon, 2016). In this regard, it makes sense to integrate omics studies into the food industry (foodomics) to study food and nutrition using integrated omics approaches (Valdés *et al.*, 2021; Ahmed *et al.*, 2022). Foodomics helps to understand food metabolic networks, allowing better insight into the molecular details of food characteristics (flavor, color, texture, aroma, and added value nutrition) and food-related matrixes. In food science, proteomics has mainly been applied to detect allergenic proteins and pathogenic microorganisms, and metabolomics to identify biomarkers that confirm the quality of food products (Rizo *et al.*, 2020). The use of these technologies in food science is discussed in the following section. It would be interesting to integrate proteomics and metabolomics in the food chain because they provide insights into the biochemical, molecular, and cellular mechanisms that underlie the effects of certain food processes and bioactive food components. Additionally, this approach enables to determine how bioactive food constituents impact crucial molecular pathways, whether they have beneficial or adverse effects (García-Cañas *et al.*, 2012).

Bioinformatics, popularly known as computational biology, plays an important role in food safety. Novel methods and software can be used as tools for processing, analyzing, and interpreting biological data obtained from various biochemical and omics approaches (genomics, transcriptomics, proteomics, and metabolomics) to decipher the mechanistic investigation of biological processes that connect the cell-chromosome-DNA-RNA-protein-metabolite. Thus, the collective application of computational approaches to experimental science could generate new hypotheses about the functioning of living systems.

VIII.1.1 Metabolomics

Metabolites are small molecules displaying low molecular weight (<1.5 kDa) in a biological system (Prakash *et al.*, 2020). Metabolomics involves the study of metabolites, which are the end products of cellular processes and provide a snapshot of the biochemical composition of a cell system. In other words, metabolomics determines whether the accumulated proteins are metabolically active and identifies the biochemical processes and roles of the resulting metabolites in cell metabolism (Van Emon, 2016; Yang *et al.*, 2021).

The metabolome is dynamic and denotes the complete set of metabolites synthesized via metabolic pathways in plant systems (Van Emon, 2016; Yang *et al.*, 2021). This may be influenced by the environmental and internal conditions (Van Emon, 2016). Metabolites can be analyzed in three different ways: target analysis, metabolic profiling, and metabolic fingerprinting. Target analysis is based on the quantitative measurement of selected analytes. Metabolic profiling study a specific metabolic pathway or a group of related metabolites. Metabolic fingerprinting analyzes metabolites between samples; it does not identify all the involved metabolites, but it aims to detect the considered biomarkers (Ibáñez *et al.*, 2013). Gas/liquid chromatography-mass spectrometry (GC/LC-MS) is one of the most commonly used methods for metabolite quantification. This is a sensitive, selective, and time-consuming technique. Other commonly used techniques include liquid chromatography-electrochemistry mass spectrometry (LC-EC-MS), thin layer chromatography (TLC), direct infusion mass spectrometry (DIMS), nuclear magnetic resonance (NMR), Fourier-transfer infrared (FT-IR), and capillary electrophoresis-liquid-chromatography mass spectrometry (CE-MS) (Yang *et al.*, 2021).

In food science, monitoring changes in metabolites can help to increase the quality of nutrition, assess food safety and traceability, and improve plant health. This is achieved by quantifying the desired bioactive compounds, conducting food profiling, and establishing unique food fingerprints (Prakash *et al.*, 2020). For instance, Farag *et al.* (2021) used GC-MS and UPLC-MS techniques to identify primary and secondary metabolites in chickpeas, fenugreeks, fava beans, and lentils sprouts. This study showed a positive correlation between flavonoid content and antioxidant activity of sprouts. Balestra *et al.* (2015) used GC-MS and electronic nose technique to characterize volatile metabolites impacting flavoring of wheat and KAMUT Khorasan flour on fermented dough. This study provides a deeper understanding of

the consequences of acidification on sourdough fermentation. Baek *et al.* (2010) and Namgung *et al.* (2010) used metabolomics technique to analyze metabolite profiling of fermented soybean paste. These studies showed how the fermentation process can impact different metabolites in seeds and change the taste and flavor of fermented foods. Sayre-chavez *et al.* (2022) used UPLC-MS to analyze metabolites from cooked flour of cowpeas, pigeon peas, and common beans. This study identified novel cowpeas and pigeon peas food metabolites that could be nutritionally beneficial for human dietary intake. Moreover, metabolomics can also analyze the changes in the metabolome pathway of undesirable microorganisms influenced by intrinsic (food) and extrinsic (stockage and processing) conditions (Ibáñez *et al.,* 2013; Prakash *et al.,* 2020; Yang *et al.,* 2021). When food is contaminated, the metabolic activity of pathogenic microorganisms can result in food spoilage. To address food safety concerns, scientists have explored the use of microbial volatile organic compounds as indicators of microbial contamination in food products under different conditions and analyzed the metabolic profiles of different pathogens. This alternative approach has been successfully employed in various food categories such as dairy products, meat, fish products, fruit, fruit preserves, and beverages (Rizo *et al.,* 2020). In summary, these results show the importance and utility of metabolomics in food science. Analyzing the metabolites in food products is crucial for understanding the influence of food processing on various aspects, including sensory attributes, nutritional quality (carbohydrates, amino acids, antioxidant properties, and antinutritional factors), and microbiological safety. This valuable information enhances our understanding of the composition and properties of foods.

The integration of proteomic and metabolomic (metabo-proteomic) techniques offers a powerful tool for understanding metabolic networks, identifying novel biomarkers, and elucidating cellular mechanisms. For instance, Wu *et al.* (2020) used integrated proteomic and metabolomic analyses to understand the formation mechanism of non-volatile chemical constituents during oolong tea manufacturing processes. This study showed an association between the abundance of multiple enzymes and the production of flavonoid pathways during the oolong tea manufacturing process (Wu *et al.,* 2020). Metabo-proteomics can be used to characterize the source, function, metabolic pathway, and other information on proteins using bioinformatics techniques. The use of integrated proteomic and metabolomic technologies in food science has increased in recent years. The combination of these two technologies has been used to analyze various traditional fermented foods and beverages, including cheese, sourdough bread, kimchi, kefir, and wine (Yang *et al.,* 2020).

VIII.1.2. Proteomics

Plant proteins play important functional roles in the texture, yield, flavor, and nutritional value of food. Proteomics is a technique used to analyze the proteome, which comprises profiling of total accumulated proteins in a biological system at a given time (Ibáñez *et al.*, 2013; Colgrave, 2017; Yang *et al.*, 2021). In food science, proteomics helps ensure food quality, food security, and safety, by detecting food pathogens and allergens (Ibáñez *et al.*, 2013; Van Emon, 2016). The first step in the analysis of plant proteins is protein extraction. The major difficulty in protein extraction is due to the different physicochemical properties of proteins and the presence of other compounds in plants, such as the cell wall (prevents protein diffusion), storage polysaccharides, lipids, and phenolic compounds, which bind to proteins and prevent protein solubilization (Ibáñez *et al.*, 2013; Van Emon, 2016).Thomas Burr Osborne, an American chemist, developed a classification of plant proteins based on their solubility in a series of solvents, which is known as the Osborne classification (Osborne, 1924). Thus, proteins are classified according to their solubility properties as salt-soluble, water-soluble, alkali-soluble, and/or alcohol-soluble (Bessada *et al.*, 2019). For better extraction, proteins should be solubilized to break the interactions between protein aggregates, such as disulfide bonds, hydrogen bonds, Van der Waals forces, and ionic and hydrophobic interactions. If this process is not carried out well, proteins can aggregate or precipitate, resulting in sample loss. Protein extraction should be performed in solubilization solutions containing chaotropic agents, detergents (classified as nonionic, zwitterionic, anionic, or cationic), reducing agents, buffers, salts, and ampholytes (Gilbert-López *et al.*, 2017). Phenol extraction, followed by methanol/ammonium-acetate precipitation, is the preferred method for protein removal in proteomic studies. This method is known for its efficiency in removing nucleic acids that interact with proteins and provide poor resolution. Phenol interacts with proteins via hydrogen bonding and causes them to become denatured and soluble in the organic phase (Hervé *et al.*, 2006). Another protein extraction method that has been proven to be effective is thiourea/urea extraction (Luche *et al.*, 2003; Vâlcu & Schlink, 2006). However, their performance depends on the plant biochemistry, physical characteristics, and concentrations of thiourea and urea. Both are chaotropic agents responsible for breaking up hydrogen bonds and hydrophobic interactions between and within proteins. They destroy the secondary protein structure, triggering the solubilization of proteins (Gilbert-López *et al.*, 2017).

Traditionally, gel electrophoresis, immunoassays, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been used to identify and quantify proteins. However, recent advancements in technology, such as mass spectrometric methods like MALDI-TOF and LC-MS/MS, have enabled more precise detection of specific protein abundance. Today, these new technologies are considered the best methodology for protein separation and quantification and have been widely used by scientists (Aebersold and Mann, 2003; Prakash *et al.,* 2020; Shuken *et al.,* 2023). Prior to protein analysis by LC-MS/MS, food samples need to undergo fractionation, purification, and/or separation because of the different nature and extreme complexity of proteins found in biological and food samples. Moreover, sample clean-up is a crucial step before protein analysis to reduce contaminants, such as salts, stabilizers, or detergents used during the extraction process. These contaminants can interfere with the LC-MS/MS results, resulting in the quantification of low-abundant proteins. To this end, proper sample preparation is crucial to ensure high accuracy and reliability of LC-MS analysis (Gilbert-López *et al.,* 2017).

In food science, proteomics approach provides valuable insights into protein structure, abundance, function, and interaction with other bioactive components present in the food matrix. This technique can be used to characterize, identify, and quantify foodborne toxins and pathogenic microbes, such as bacteria and molds, by analyzing their specific proteins (Prakash *et al.,* 2020; Rizo *et al.,* 2020). For example, Gajdošik *et al.* (2013) used proteomics to investigate the inhibitory activities of pyridinium oximes against *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli,* and *Yersinia enterocolitica*. This study identified key proteins crucial for protein turnover and bacterial metabolism in foodborne pathogens (Gajdošik *et al.*, 2013). This information offers insights into the proteome of food pathogens, facilitating the detection of potential biomarkers for tracing the routes and sources of food contamination. Proteomics is an efficient technology for analyzing changes in protein abundance and patterns during fungal adaptation to variable conditions. Zhao *et al.* (2020) used proteomics to identify *Aspergillus oryzae* (a starter in the soy sauce industry) stress adaptation proteins under low-temperature and high-salt stress conditions and established a correlation with flavor compounds. Other authors have used label-free quantitative proteomic analysis to investigate the microbial interactions between *Lactobacillus hordei* and *Saccharomyces cerevisiae* in water kefir (Xu *et al.,* 2019). This study reported the potential of proteomics to analyze microbial interactions in fermented food, elucidating new strategies to optimize the production of water kefir. Proteomics can provide insights into the complex interactions among

microorganisms, environmental stress, and flavor formation in fermented foods. Proteomics has been used to study seed germination in various plant species, including pulses (Han *et al.*, 2021) These advancements hold significant promise for ensuring safer and more nutritious food products, benefiting the food industry and consumers.

VIII.2. Immunolocalization

Immunohistochemistry (IHC) is a technique used to detect molecular, cellular, or tissue constituents (antigens) based on the specific recognition of this antigen by an antibody. An antigen is a molecule that has an antigenic determinant groups or epitopes which is specifically recognized by an antibody directed against this epitope. These sites are composed of a small number of amino acids and monosaccharide units (Suvarna *et al.,* 2019). Antibodies, also known as immunoglobulins, are found in the blood and tissue fluids. Its basic unit is a monomer composed of two heavy (antibody-specific) and two light (common to all antibodies) chains (Figure 9). Five types of antibodies are found in the blood of higher vertebrates: IgA, IgD, IgE, IgG, and IgM. IgG is the most common and frequently used antibody for IHC and is composed of polypeptide chains linked by disulfide bonds to form a Y-shaped structure. The terminal regions (heavy and light chains) of each arm vary in their amino acid sequences, which allows the recognition of a particular antigen epitope. The variation in amino-acids enables the antibody to bind specifically to the antigen against which it is raised (Suvarna *et al.,* 2019). The amino acid side chains of the antibody form a geometric and chemically cavity-like site complementary to an antigen epitope molecule (Figure 9). The specific binding forces of antigen-antibody is held together by a combination of hydrogen bonds, electrostatic interactions, hydrophobic interactions, and Van der Waals forces(Suvarna *et al.,* 2019).

Numerous IHC techniques are available for *in situ* localization molecules and tissues antigens. The most common techniques are the traditional direct and two-step indirect techniques. The protocol is selected based on the specimens under investigation. The direct technique was demonstrated by Coons *et al.* (1941), using a primary antibody labeled with a fluorescent dye (Suvarna *et al.,* 2019). The primary antibody that recognize the antigen of the targeted molecule is directly labelled with a fluorochrome, and visualization is performed using a fluorescence microscope (Kabiraj *et al.,* 2015). This technique is easy and quick to use, but

provides less signal with a risk of loss of fluorescence (fluorochrome degradation) during storage of the primary antibody (Suvarna *et al.*, 2019). In the two-step indirect technique, the primary antibody binds to the antigen of interest. However, the primary antibody itself is not directly labeled with a fluorescent dye. To localize the antigen-antibody complex a secondary antibody labeled with a fluorescent dye is used (Figure 10). This secondary antibody is specifically directed towards the immunoglobulin of the animal species in which the primary antibody is raised thereby allowing for the detection and visualization of the target antigen (Figure 10). This technique is more sensitive in term of signal detection because the secondary antibodies may react with different antigenic sites on the primary antibody, increasing signal amplification, and is more versatile as the same labeled secondary antibody can be used with different primary antibodies raised from the same animal (Kabiraj *et al.*, 2015; Suvarna *et al.*, 2019).

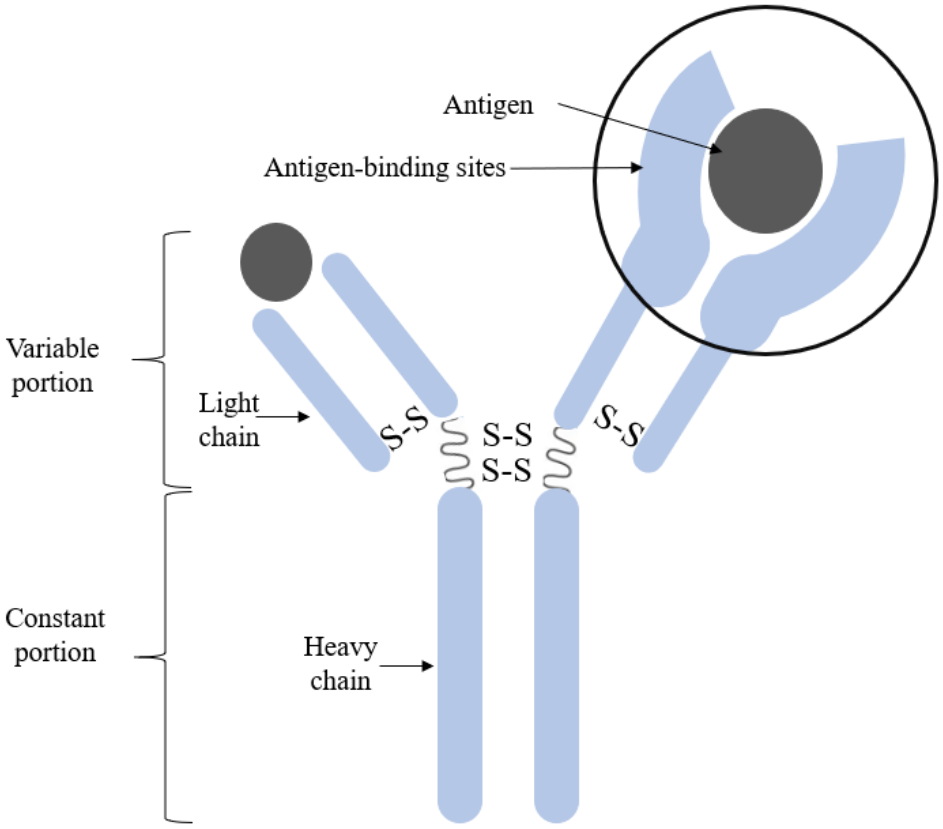


Figure 9. Antibody structure

(Huber, 1980; Ma & O'Kennedy, 2015; Psimadas *et al.*, 2012).

Primary antibodies are selected based on their molecular specificity and the likelihood that they would react with the antigens and secondary antibody. There are monoclonal and polyclonal antibodies. The monoclonal antibody binds to a single epitope on an antigen, which

is highly specific. In contrast, polyclonal recognize multiple isoforms of the target molecule (Kabiraj *et al.*, 2015).

For visualization, antibodies can be labelled with enzymes or fluorochromes. Horseradish peroxidase (HRP) is the most widely used enzyme because it is easily obtained in a highly purified form, is stable, and remains unchanged during manufacture, storage, and application. The use of HRP in combination with a chromogen substrate by a standard histochemical method results in the formation of a stable, colored reaction end product that precipitates and can be observed under a wide field microscope. This method offers flexibility in terms of enzyme and chromogen selection, allowing the user to choose the desired color for the final reaction product (Suvarna *et al.*, 2019). Fluorochromes are fluorescent labels that, when conjugated to an antibody, absorb visible light of a particular wavelength. When excited by the wavelength, fluorochrome electrons gain energy, creating an unstable excited state (Figure 10). As electrons return to the ground state, the fluorochrome subsequently emits light of a longer wavelength than the excitation wavelength (Suvarna *et al.*, 2019). In the past, the most widely used fluorochrome in immunofluorescent microscopy was fluorescein isothiocyanate (FITC), which has a maximum absorption of 494 nm, maximum emission of 518 nm, and a green color (Suvarna *et al.*, 2019). The fluorescent microscope should deliver light of a specific wavelength to cause specific excitation of the fluorochrome and then collect the emitted light. But the fluorescence of FITC is unstable over time and today the fluorochromes of the Alexa family (such as Alexa 488) are the most used because of their stability over time which can go up to several months.

IHC is an ideal technique for identifying and analyzing modifications in molecules, cellular metabolism, or tissue constituents of food matrices during food processing. IHC technic is used in biology either for the *in situ* localization of a molecule of interest in a biological matrix or for the quantification of molecules by ELISA (enzyme-linked immunosorbent assay), after extraction of the biological matrix. IHC is extensively used as a valuable technique in the field of plant biology, however, its application in food science is not as prevalent. This is primarily due to the challenges associated with analyzing the complex structure of food and the considerable time and financial resources required to implement IHC in food-related studies (Kaláb *et al.*, 1995). However, in some studies, IHC has been used to identify contamination or adulteration in foods, investigate the presence of allergenic proteins, analyze enzyme distribution, and explore eventual structural changes that occur during processing. Coffigniez

et al. (2019[b]) used IHC to localize 5-methyltetrahydrofolate in cowpeas during soaking at 60 °C and 95 °C. This investigation allowed them to visualize the distribution and potential changes in the localization of 5-methyltetrahydrofolate within different seed tissues under varying thermal conditions. Costa *et al.* (2015) used an immunosorbent assay (ELISA) for the detection and quantification of hazelnuts (allergic risk) in model chocolates, after extraction from the matrix. Additionally, Nagaraj *et al.* (2016) developed a sandwich ELISA immunoassay to detect specific "non-classical" enterotoxins associated with staphylococcal food poisoning. Other studies have used ELISA for folate detection using monoclonal and polyclonal antibodies to folic acid (Finglas & Morgan, 1994; Kong *et al.*, 2016). Zeng *et al.* (2020) were the first to produce a specific anti-thiamine monoclonal antibody and develop an immunoassay for its detection and quantification in energy drinks and infant milk powder. While traditional methods for analyzing vitamin B1 involve microbiological assays, high-performance liquid chromatography (HPLC), and liquid chromatography tandem mass spectrometry (LC-MS/MS), these techniques come with high instrument costs and involve complex separation and extraction steps (Zeng *et al.*, 2020). In contrast, Zeng *et al.* (2020) opted for ELISA analysis, which offers an efficient, sensitive and high-throughput method for quantifying vitamins, simplifying the process. Although not widely used in the food industry, the development and use of specific and sensitive techniques such as IHC and ELISA play a crucial role in enhancing food safety and quality. IHC allows the verification of the location and distribution changes of various compounds during food processing, facilitate the detection of allergenic proteins in complex food matrices, and identify pathogens in food products. Furthermore, this technique provides valuable tools for the food industry to understand the impact of food processing on compounds, food microstructures, compartmentalization, and cell metabolism ensuring accurate package labeling, and effectively control contamination. These specific and sensitive techniques serve as valuable tools for promoting food safety and quality and ultimately safeguarding consumer health and well-being.

The last-mentioned techniques, omics and immunolocalization, offer substantial potential for analyzing nutritional value, metabolic pathways, and *in situ* localization in germinated pulses. In the context of this project, it is coherent to apply these technologies to understand the impact of germination and its variables on the nutritional aspects and microstructure of pulses. This multidisciplinary approach can provide valuable insights into enzymatic interactions with substrates, leading to the synthesis or degradation of target compounds, and their *in situ* localization within the seed. By employing this comprehensive approach, we aimed to deepen

our understanding of the mechanisms that drive pulse metabolism during germination and identify strategies to optimize it, ultimately leading to improved nutritional outcomes.

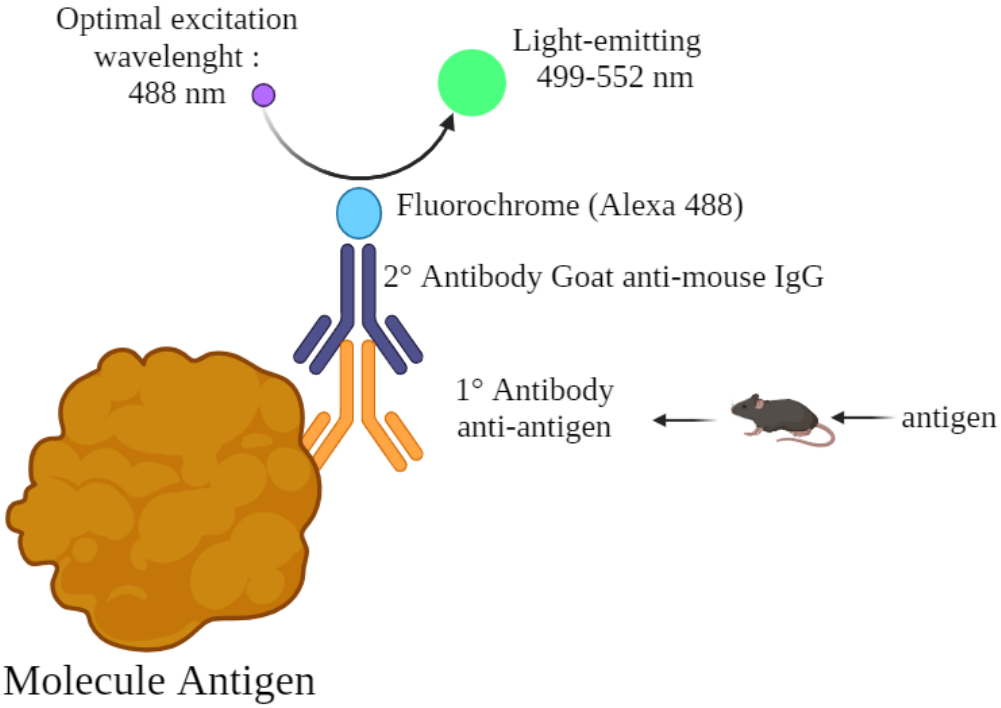


Figure 10. Two-step indirect immunohistochemical mechanism.

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III. Hydrolysis and diffusion of raffinose oligosaccharides family in pulses under different pH and temperature soaking conditions

Soaking is the primary step that triggers germination, initiating major metabolic processes and setting the stage for the subsequent germination process. Soaking involves rapid initial water uptake, leaching out solutes from the seeds to the medium, and activation of cell repair systems (Bewley & Black, 1994). Additionally, it marks the beginning of protein synthesis from the stored mRNA and activates mitochondrial respiration. The transition from soaking to germination marks the first metabolic shift from quiescence to heterotrophic metabolism. Therefore, further investigation into the soaking process is essential. The aim of the next study was to analyze the effects of different temperatures and pH on α-galactosides content in chickpeas, lentils, and beans soaking. Analysis of α-galactosides is crucial because they are the main reason why some individuals avoid consuming pulses. The absence of α-galactosidase activity in the intestinal mucosa of humans and animals leads to the escape of α-galactosides from digestion, and they are anaerobically fermented by bacteria to produce hydrogen, carbon dioxide, and methane. This fermentation process causes flatulence in the intestinal tract, leading to discomfort, abdominal rumblings, cramps, pain, and diarrhea (Martínez-Villaluenga *et al.*, 2008; Naczk *et al.*, 1997). Therefore, it is of interest to analyze how food processing can effectively reduce the levels of this antinutritional compound. Soaking is a traditional practice widely used by many households and can be effective in reducing α-galactosides, depending on the specific conditions employed. Although many studies have analyzed the effects of temperature and basic pH (Akissoé *et al.*, 2021; Coffigniez, *et al.*, 2018[a]; Coffigniez, *et al.*, 2018[b]; Madodé *et al.*, 2013; Vidal-Valverde *et al.*, 1992; Vijayakumari *et al.*, 2007), none have investigated the impact of acidic pH on the reduction of antinutritional factors in pulses. In fact, α-galactosidases are also active at lower pH between 2 and 5. Therefore, it is worthwhile to explore how acidic pH affects the α-galactosidases activity. Moreover, existing literature has shown that the temperature and pH conditions during soaking and cooking can impact the permeability of the seed coat and cell wall. This study provides valuable insights into enhancing the nutritional quality of pulses by reducing the α-galactosides content through the soaking process. This study is the subject of preliminary experiments aimed to distinguishing the effects of soaking from those of germination before delving into a comprehensive analysis of germination itself.

Hydrolysis and diffusion of raffinose oligosaccharides family in pulses under different pH and temperature soaking conditions

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Abstract

Soaking pulses in water is a traditional practice widely used both by many households and by the food industry, and depending on the specific conditions used, can effectively reduce α-galactosides. Monitoring changes in α-galactoside content in pulses under different steeping conditions can provide insights into the degradation mechanisms and help overcome the barrier to consumption caused by digestive problems. In this study, we analyzed the impact of steeping at different temperatures (30, 45, 60, 75, and 90 °C) and at different pH (4.0, 5.0, and 6.0) on α-galactosides content in chickpeas, lentils, and beans. Our results showed that the lower the pH, the faster the α-galactosides were reduced. Moreover, steeping at lower temperatures (30 °C and 45 °C) favored hydrolysis of α-galactosides, whereas steeping at higher temperatures (60, 75, and 90 °C) favored diffusion. Soaking at 45 °C at a pH of 4 for 3 h resulted in acceptable levels of α-galactosides, *i.e.* a reduction of up to 65% in chickpeas, 85% in lentils, and 52% in beans.

Keywords: α-Galactosides; Pulses; Hydrothermal; Diffusion; Enzyme reaction.

I. Introduction

α-Galactosides belong to the raffinose family oligosaccharide (RFO) and are mainly represented by galactinol, raffinose, stachyose, and verbascose. α-Galactosides accumulate in pulses during maturation and play a role in desiccation tolerance in embryos. They are a form of stored carbohydrates of low molecular weight, are non-reducing sugars, and are soluble in water and hydroalcoholic solutions (Naczk *et al.,* 1997). α-Galactosides are derived from sucrose and contain 1,3-galactose units linked by α (1→6) bonds (Martínez-Villaluenga *et al.,* 2008).

Consuming more than 3g/day of α-galactosides has negative effects on health (Martínez-Villaluenga *et al.,* 2008). Due to the absence of α-galactosidase in human and animal intestinal mucosa, α-galactosides escape digestion and are anaerobically fermented by bacteria to produce hydrogen, carbon dioxide, and methane. This causes flatulence in the intestinal tract resulting in discomfort, abdominal rumbling, cramps, pain, and diarrhea (Martínez-Villaluenga *et al.,* 2008; Naczk *et al.,* 1997). Other negative health effects of α-galactosides include reducing dietary net energy and producing osmotic effects in the intestine that disrupt the digestion of other nutrients including amino acids and glucose (Martínez-Villaluenga *et al.,* 2008). However, α-galactosides also have a prebiotic effect in the intestine, and daily doses of less than 3 g/day may be beneficial to human health (Elango *et al.,* 2022; Martínez-Villaluenga *et al.,* 2008). In general, pulses contain higher concentrations of α-galactoside (2 to 10 g/100 g dry basis [db]) than cereals (0.1 to 2 g/100 g db) (Das *et al.,* 2022; Pedrosa *et al.,* 2021). Chickpeas (*Cicer genus*), lentils (*Lens genus*), and common beans (*Phaseolus genus*) are among the most widely consumed legumes in European countries and all contain high concentrations of α-galactosides, ranging from 0.61 to 4.2 g/100 g db (Avezum *et al.,* 2022).

Food processing, including steeping, fermentation, germination, and cooking, can reduce α-galactoside contents via diffusion into the steeping water and/or due to enzymatic hydrolysis that takes place in seeds and water (Das *et al.,* 2022; Pedrosa *et al.,* 2021). Previous studies showed that soaking chickpeas at 25 °C for 16 h reduced α-galactosides by 40% (Njoumi *et al.,* 2019). At 25 °C, enzymatic activity led to hydrolysis of α-galactosides, as evidenced by a significant (1000%) increase in galactose content in chickpeas, whereas at 95 °C, the α-galactosides in cowpeas was transferred to the steeping water, resulting in a nearly constant

mass balance, and the α-galactosides were not thermally degraded. At high temperatures, the diffusion effect is prioritized as thermal degradation of enzymes occurs (Coffigniez *et al.*, 2018[a]). The optimum temperature range for the action of α-galactosidases has been shown to be between 20 °C and 65 °C and the optimum pH between 4.7 and 6.1. Previous studies have shown that enzymatic activity is favored at low temperatures while diffusion is favored at high temperatures (Coffigniez *et al.*, 2018[b]; Dey *et al.*, 1983). The effects of alkaline pH and temperature on α-galactosides degradation have already been described (Akissoé *et al.*, 2021; Coffigniez *et al.*, 2018[a]; Coffigniez *et al.*, 2018[b]; Madodé *et al.*, 2013; Vidal-Valverde *et al.*, 1992; Vijayakumari *et al.*, 2007), including a slight, but not significant, decrease in α-galactosides when the pulses were soaked at basic pH. Abdel-Gawad, (1993) showed that basic pH can soften the seed coat and cotyledons, favoring the diffusion of α-galactosides into the steeping medium. However, few authors specifically investigated the impact of acid pH on enzymatic hydrolysis and on the diffusion of α-galactosides. It can be hypothesized that, at acidic pH, α-galactosidases are more activated, favoring the hydrolysis of α-galactosides. Additionally, acidic pH might change the microstructure of the seed coat and cotyledons, thereby favoring the diffusion process.

The aim of the present study was to test these hypotheses by investigating the impact of different steeping temperatures (30, 45, 60, 75, and 90 °C) and different pH (4.0, 5.0, and 6.0) on the concentration of α-galactosides in chickpeas, lentils, and beans. Knowing whether changing temperature and pH reduces α-galactosides content by hydrolysis and diffusion will advance our understanding of α-galactosides degradation, which, in turn, will help model and optimize the steeping process. Ultimately, this research could help reduce the presence of antinutritional compounds in pulses, making them more appealing for consumption not only by reducing antinutritional factor but also by shortening preparation time.

II. Material and Methods

II.1. Material

Chickpeas (*Cicer arietinum*, var. Elvar), green lentils *(Lens culinaris*, var. Anicia), and navy beans (*Phaseolus vulgaris*, var. Linex) were purchased from Moulin Marion (Saint-Jean-sur-Veyle, France), Cibèle (Saint-Georges-Sur-Arnon, France), and Cavac (La Roche-sur-Yon, France), respectively. The lentils were purchased in 2020, and the chickpeas and beans in 2018. All the pulses were stored in plastic bottles at -20 °C in the dark until use.

Galactose, raffinose, stachyose, and verbascose standards (purity ≥ 98%) were purchased from Sigma (USA). Ultrapure water was used in all the experiments (Simpak MilliQ System, Millipore, USA). Ethanol (purity ≥ 99.8%) was purchased from Honeywell Riedel-de-Häen (Germany).

II.2. Soaking experiments

In the soaking experiments, 30 g of pulses were heated in 120 mL of thermally pre-equilibrated water under agitation at 30, 45, 60, 75, or 90 °C, at a pH between 4.0 and 6.0 (Table 1). In addition, the pulses were soaked in 120 mL water mixed with respectively, 500 µL and 20 µL of acetic acid, for pH 4.0 and 5.0, at 30 and 45 °C. The soaking time was adjusted according to the soaking conditions (pH and temperature). After soaking, the pulses were quickly removed from the soaking water and centrifuged at 7 x g for 5 min at 25 °C. The water medium was aliquoted and stored at -20 °C until analysis. The pulses were freeze-dried and ground in an ultra-centrifugal mill 3100 (Perten Instruments, Sweden) grid diameter 0.5 mm. The pulse flour was stored at -20 °C in the dark until analysis. Each soaking experiment was performed in duplicate.

Table 1. Experimental soaking conditions used for all three pulses.

pH	Temperature (°C)	Time (h)
4	30	1, 3, 7, 14
	45	1, 3, 7, 14
5	45	1, 3, 7, 14, 18, 24
6	30	1, 2, 4, 8, 14, 18, 24
	45	1, 3, 7, 14
	60	0.5, 1, 2, 3, 4
	75	0.25, 0.5, 1, 2, 3
	90	0.17, 0.25, 0.5, 1, 1.5

II.3. α-Galactosides extraction and detection

α-Galactosides was extracted following the methodology described by Coffigniez *et al.* (2018)[a] with some modifications. α-Galactosides were extracted from 80 mg of pulse flour placed in 10 mL of 80% (v/v) ethanol at 80 °C for 1 h. α-Galactosides were separated by high-performance anion exchange chromatography using the ICS-5000 $^{+}$ HPIC model from Thermo Scientific ^{TM} Dionex ^{TM}. The sugars were separated in a 4 x 30 mm *Dionex CarboPac PA210-Fast-4µm* pre-column and a 4 x 150 mm *Dionex CarboPac PA210-Fast-4µm* column (Dionex, Germany) at room temperature. The injection volume was 10 µL. The mobile phase was a 12 mM potassium hydroxide solution at a dose of 0.8 mL/min. Galactose, raffinose, stachyose, and

verbascose were quantified using calibration curves with external standards and are expressed as g/100 g db. For each soaking condition, extraction was performed in triplicate.

II.4. Mass balance

Water content ($WC\ (t)$, g/100 g db) of soaked pulses was measured as a function of soaking time (t, h) according to the following equation (Eq. (1)):

$$WC\ (t) = \frac{m_w(t) - m_d(t)}{m_d(t)} * 100 \quad (1)$$

where m_w is the water mass of the wet pulses (g), m_d is the water mass of dried pulses (g) at time t (h).

III. Results

III.1. Water uptake

All the pulses studied reached equilibrium after 4 h of soaking at 30 and 45 °C (Figure 1). The water content at equilibrium was higher at 45 °C (~ 140 g/g db) than at 30 °C (~ 110 g/g db). When pulses are soaked at a higher temperature, equilibrium is expected to be reached more rapidly; for this reason, at 75, and 90 °C soaking lasted between 10 min and 1.5 h (Table 1). Equilibrium was reached in 1-2 h at 75 °C, but it was not possible to set the equilibrium at 90 °C within 1.5 h. Soaking lentils at 75 °C for 2 h resulted in an equilibrium of 134 g/100 g (db) versus a maximum value of 194 g/100 g (db) at 90 °C after 1.5 h.

1

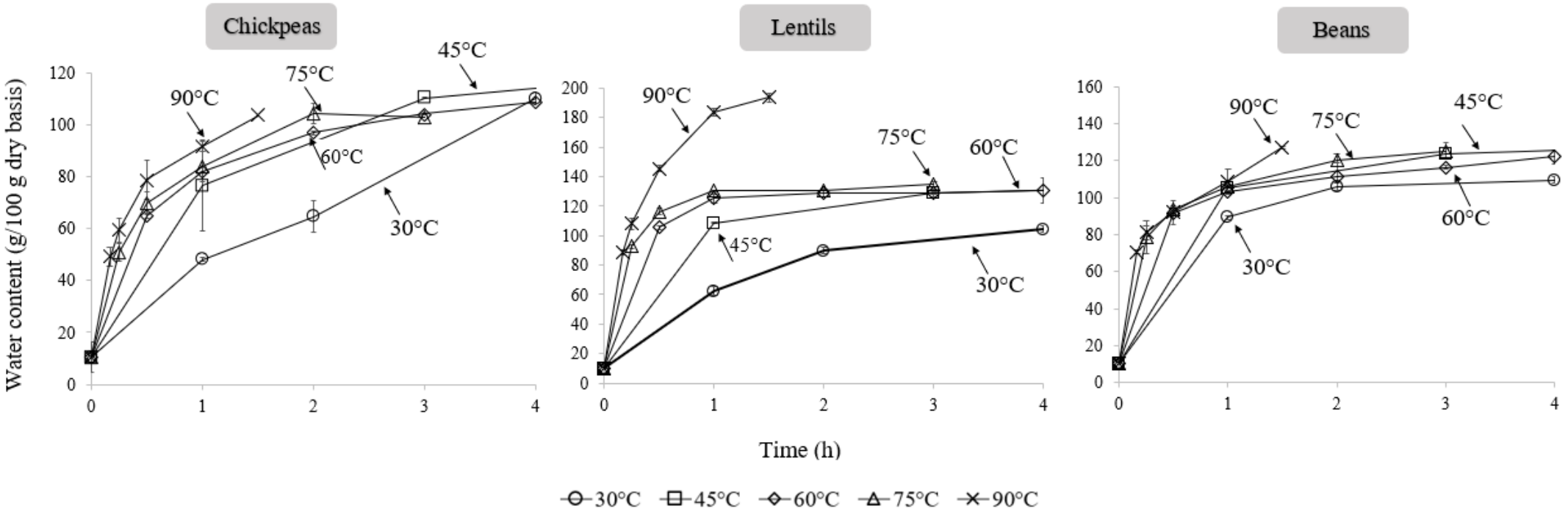


Figure 1. Water content (g/100 g dry basis) of chickpeas, lentils, and beans during soaking at 30 °C (○), 45 °C (□), 60 °C (◊), 75 °C (Δ), and 90 °C (**x**) for periods of different length (h)

III.2. Distribution of α-galactosides and galactose after the grains were soaked in water at different temperatures

Raw lentils had the highest α-galactoside contents (3.84 g/100 g db), followed by raw beans (3.70 g/100 g db), and raw chickpeas (2.80 g/100 g db) (Table 2). Stachyose was the principal main α-galactoside, it accounted for 74%, 81%, and 88% of the total α-galactoside contents in raw chickpeas, lentils, and beans, respectively. Raffinose accounted for from 8% to 24% and verbascose from 2% to 9%.

Table 2. Galactose, raffinose, stachyose, verbascose and total α-galactoside contents in g/100 g on a dry basis in raw chickpeas, lentils, and beans.

			α-Galactosides (g/100 g db)		
	Galactose	Raffinose	Stachyose	Verbascose	Total α-galactosides
Chickpeas	0.07 ± 0.004	0.67 ± 0.06	2.07 ± 0.08	0.05 ± 0.0009	2.80 ± 0.15
Lentils	0.06 ± 0.05	0.40 ± 0.01	3.10 ± 0.07	0.34 ± 0.0003	3.84 ± 0.05
Beans	0.06 ± 0.04	0.31 ± 0.002	3.27 ± 0.06	0.13 ± 0.02	3.70 ± 0.04

Soaking at 30 °C for 24 h reduced the concentration of α-galactosides by 42% in chickpeas, 46% in lentils, and 31% in beans (Figure 2). However, total α-galactoside contents were very low in the steeping water after 24 h at 30 °C: 0.17 g/100 g, 0.011 g/100 g, and 0.09 g/100 g in chickpeas, lentils, and beans, respectively, and represented only 0.3% to 6% of the initial α-galactosides contents.

Soaking at 45 °C for 14 h reduced the concentration of α-galactosides in grains by 84% in chickpeas, and by 92% in lentils, but only by 62% in beans. In all three pulses studied, the release of α-galactosides into the steeping water was quite high after 7 h (representing 30% of the initial content), but then decreased and represented only 9% after 14 h. At this temperature (45 °C), hydrolysis of α-galactosides was also significant and galactose release accounted for 35%, 7%, and 13% of the total reduction in α-galactosides contents after 14 h in chickpeas, lentils (Figure 4), and beans, respectively.

Soaking at 60 °C for 4 h reduced the α-galactoside contents by 80% in chickpeas, 82% in lentils, and 65% in beans. The concentrations of verbascose, stachyose, and raffinose in chickpeas were reduced by 91%, 80%, and 79%, respectively. In this case, the reduction in α-galactosides was mainly due to diffusion into the steeping water: 47% to 65% of α-galactosides was recovered in the steeping water. In lentils, the diffusion appeared to be almost completed after 2-3 h at 60 °C (Figure 2), and the concentrations (g per g of water) of α-galactosides were very similar in the steeped pulses and in the remaining steeped water. In addition, α-galactosides were hydrolyzed and galactose recovered in pulses and in water representing 16-29% of the initial α-galactosides content (the example of lentils is shown in Figure 4). A very similar pattern was observed at 75 °C, just a little more rapidly. At 90 °C, diffusion was fastest and was complete after 1 h, but no enzymic degradation and release of galactose was observed.

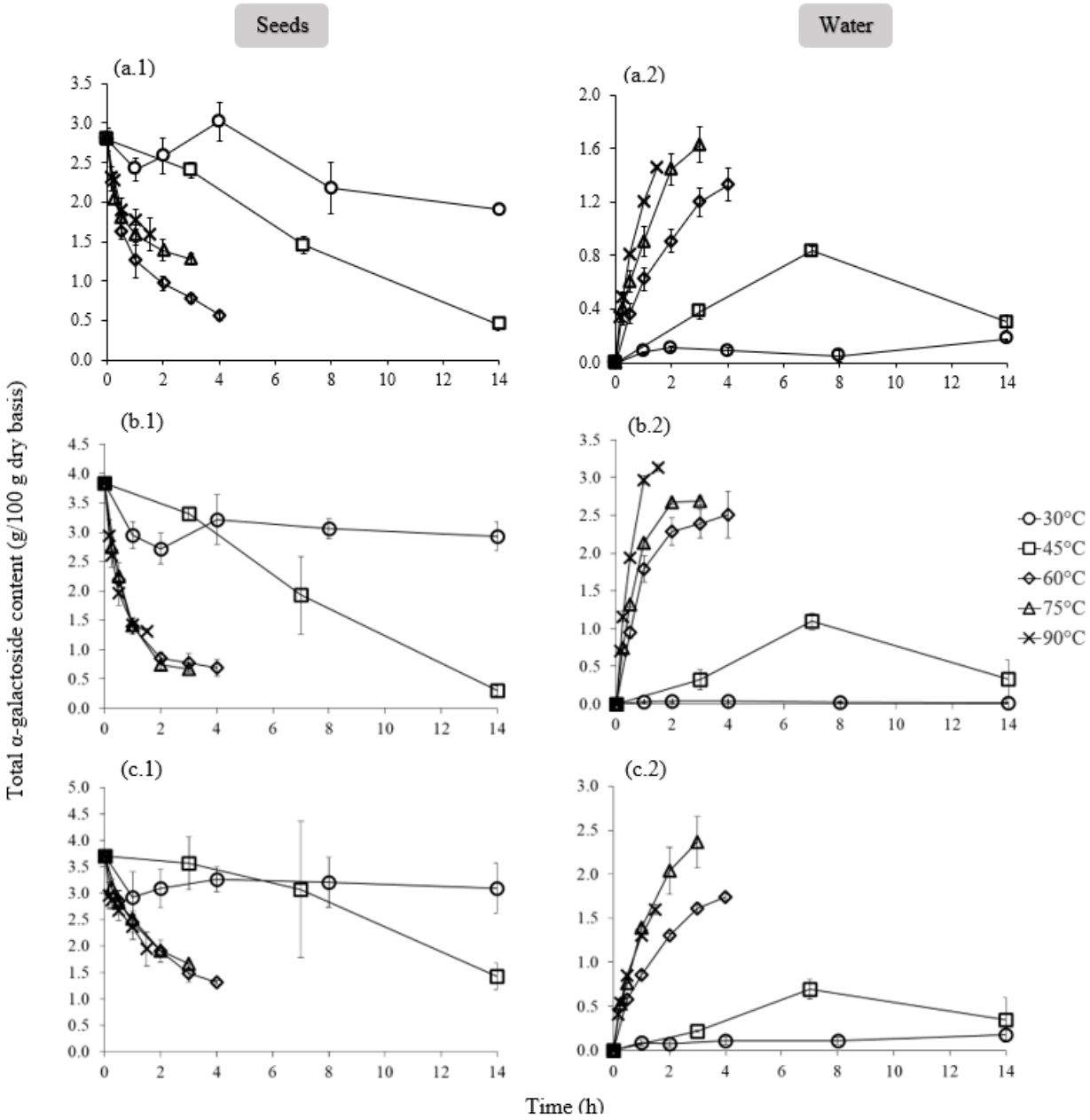


Figure 2. Total α-galactoside contents (g/100 g dry basis) in pulses and in soaking water in chickpeas (a), lentils (b), and beans (c) at 30 °C (○), 45 °C (□), 60 °C (◊), 75 °C (Δ), and 90 °C (x) and pH 6.

III.3. Effect of pH on the diffusion and degradation of α-galactosides

At 30 °C, lowering the pH of steeping water from 6.0 to 4.0, after 18 h the concentration of α-galactosides in the pulses was reduced from 43% to 73% in chickpeas, from 56% to 70% in lentils, and from 35% to 59% in beans (Figure 3). Similarly, when soaked at 45 °C, the reduction in α-galactoside contents in pulses was significantly more rapid at lower pH. For instance, soaking lentils at 45 °C for 7 h reduced α-galactoside contents by 50% at pH 6.0, by 76% at pH 5.5, and by 87% at pH 4.0 mainly linked to diffusion, but also to degradation. Indeed, total galactose (in both the pulses and in the steeping water) increased from 10% of the initial α-galactosides content after 7 h at pH 6.0 to 21% at pH 5.5 and 33% at pH 4.0.

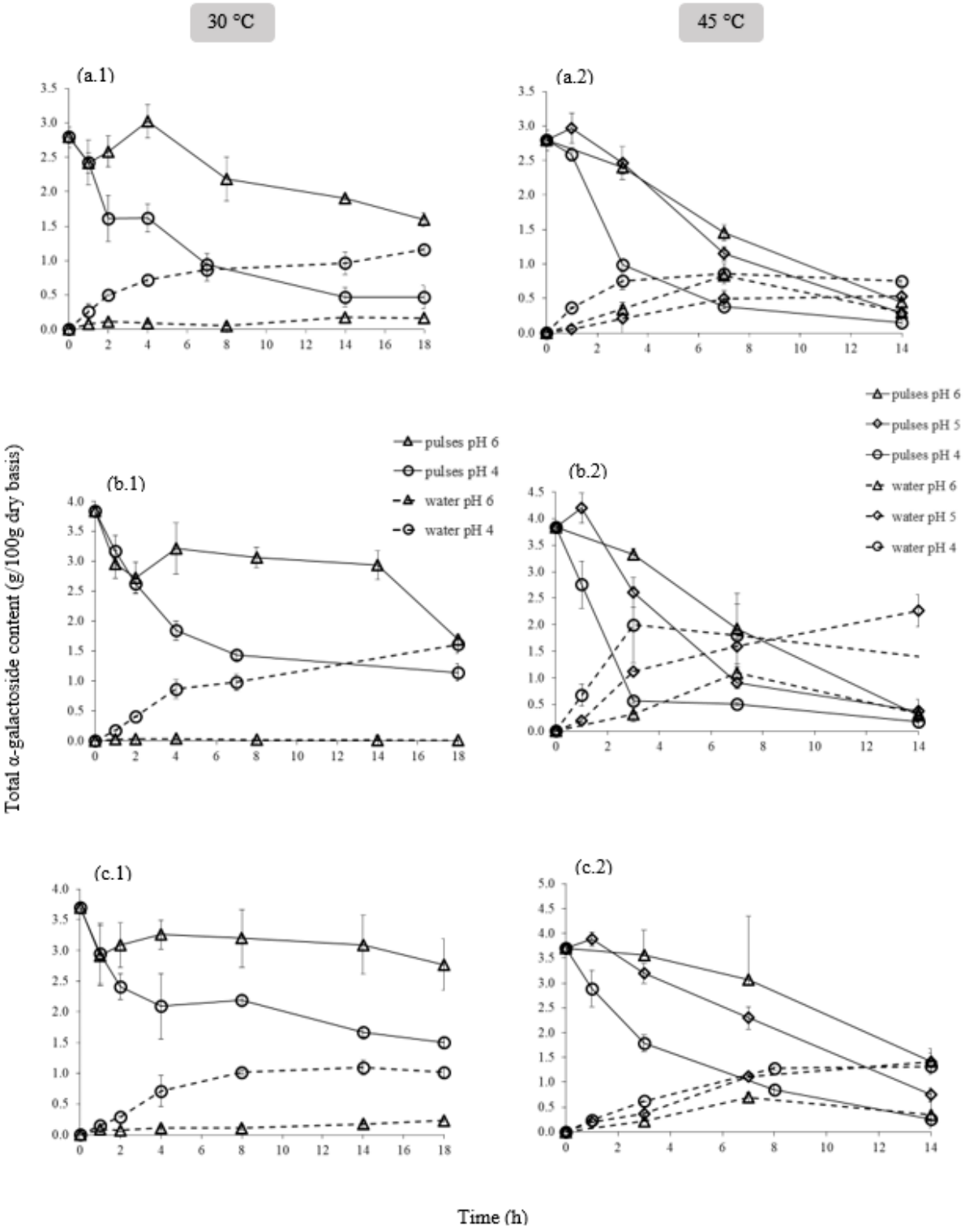


Figure 3. Total α-galactoside contents in pulses (solid line) and in soaking water (dashed line) on g/100 g on dry basis in chickpeas (a), lentils (b), and beans (c). Graphs on the left correspond to soaking at 30 °C pH 6 (Δ) and pH 4 (○). Graphs on the right correspond to soaking at 45 °C pH 6 (Δ), pH 5 (◊), and pH 4 (○)

IV. Discussion

Equilibrium water uptake was reached in 2-4 h between 30 and 75 °C, but was not observed at 90 °C within 1.5 h. Siguemoto *et al.* (2023) and Coffigniez *et al.* (2018[a]) also reported relatively low equilibrium water uptake, i.e. below 80 °C for 4 h, but a rapid and notable increase in water uptake at 95 °C and above. This result can be attributed to gelatinized starch whose water absorption capacity in particular, is greater than that of native starch (Lefèvre *et al.*, 2021); water has been shown to migrate from protein fiber and native starch to gelatinized starch (Siguemoto *et al.*, 2023) when beans are soaked at or above 70 °C, suggesting that at 90 °C, water equilibrium should be reached after approximately 3 h of soaking. In our study, the concentration of α-galactosides in raw seeds was slightly higher than the concentrations reported in the literature. The concentrations of α-galactoside in raw lentils and chickpeas reported by Njoumi *et al.* (2019) were, respectively, 23% and 11% lower than those we measured in our study. Another study reported 29% less α-galactoside content for lentils and 32% for beans (Abdel-Gawad, 1993). The observed differences could be due to variations in the pulse genotypes and in the agronomic conditions under which the seeds were produced. Stachyose was also the main α-galactosides found in the above-mentioned studies, (Abdel-Gawad, 1993; Njoumi *et al.*, 2019).

After steeping at 30 °C for up to 24 h, the final loss of α-galactosides measured in the grains was between 31% and 42%, but the concentration of α-galactosides in the steeping water was very low (6% or less): diffusion was indeed very low. In addition, the mass balance, which represents the sum of the α-galactosides content in the pulses and steeping water (Figure 4) did not appear to be linked to direct hydrolysis of α-galactosides to galactose; indeed we measured no increase in galactose content. We thus hypothesize that α-galactosides and galactose are used by the seeds for other metabolic reactions. Njoumi *et al.* (2019) showed that soaking lentils and chickpeas at 25 °C for 24 h reduced total α-galactoside contents by 10% and 40%, whereas Abdel-Gawad (1993) and Shimelis & Rakshit (2007) reported a decrease from 36% to 45% for common beans and kidney beans steeped at room temperature for 12 h while Vidal-Verde *et al.* (1992) reported 66% loss from lentils steeped at room temperature for 9 h. Most previous studies provided no information about the concentration of α-galactoside in the steeping water, although Vidal-Verde *et al.* (1992) reported that only 1-10% of the reduction in the concentration of α-galactoside in grains during steeping was linked to leaching into the soaking

solution, and Njoumi *et al.* (2019) also reported very low concentrations of α-galactosides in soaking water at 25 °C; and in our study, we also found very low concentrations of α-galactosides in the steeping water at 30 °C.

Increasing the soaking temperature to 45 °C improved the reduction of α-galactosides which also took place in less time (14 h). The concentration of α-galactoside in the steeping water reached a peak at 7 h, then decreased. The reduction in α-galactoside contents in the steeping water after 14 h can be attributed to enzymatic hydrolysis, as evidenced by the increase in galactose content in steeping water between 7 and 14 h. Garro *et al.* (1993) showed that the optimum temperature for α-galactosidase activity was 45 °C. Their finding supports our observation of a greater reduction in α-galactoside contents at 45 °C than at 30 °C, pointing to enhanced enzymatic activity at 45 °C. A larger proportion of galactose was recovered at 45 °C, but the sum of α-galactosides and galactose was lower at the end of the steeping period at 45 °C (Figure 4) indicating the use of galactose by metabolized reactions during steeping (Vidal-Verde *et al.*, 1992)

The reduction in α-galactoside contents in pulses at 60 °C was attributed to enzymatic hydrolysis to galactose and diffusion to the steeping water. Indeed, 47-65% of α-galactosides was recovered in steeping water, nevertheless, the mass balance decreased over time and galactose content increased. Galactose recovered in pulses and in water (respectively, 5-11% and 12-23%) produced by α-galactosidase represented 16-29% of the initial concentration of α-galactosides. In all three types of pulses studied here, the sum of α-galactosides and galactose remained constant during steeping at 60 °C (see example of lentils in Figure 4) in contrast to observations made at 30 °C and 45 °C. This should mean that diffusion of α-galactosides and of galactose continued during steeping at 60 °C, but that degradation and use of galactose stopped at 60 °C. In the case of lentils, diffusion appeared to be almost complete after 2-3 h at 60 °C (Figure 2), and the concentrations (g per g of water) of α-galactosides and galactose were very similar in the steeped pulses and in the remaining steeping water. In contrast, after 4 h, the concentration of α-galactosides in the steeping water was two times lower than that in the steeped pulses. Coffigniez *et al.* (2018)[a] reported that soaking cowpeas at 60 °C mainly reduced α-galactosides via diffusion into the steeping medium. In their conditions, soaking for 14 h reduced the concentrations of verbascose, stachyose, and raffinose by respectively, 63%, 76%, and 74% (Coffigniez *et al.*, 2018)[a].

Increasing the soaking temperature largely inactivated α-galactosidase activity while accelerating the diffusion of α-galactosides into the steeping water. After soaking at 75 °C and 90 °C, the concentration of galactose did not increase in the pulses or in the water associated with degradation of α-galactosides. The only exception was in lentils after 2-3 h at 75 °C, when the concentration of galactose in the steeping water accounted for 20% of α-galactosides. Additionally, α-galactosides were not thermally degraded, as indicated by the fact the mass balance remained close to 100%. However in lentils, the diffusion of α-galactosides into the steeping water (Figure 2 and 4) was much faster and more efficient when the temperature of the steeping water was increased: 83% after 3 h at 75 °C and 66% after 1.5 h at 90 °C versus 54 - 55% and 43 - 48%, respectively, for chickpeas and beans, under the same conditions. The higher transfer from lentils may be linked to its ten-times smaller size (3.2-6.8 g/100 g) as reported by Joshi *et al.* (2010), versus 22.5 to 48 g/100 g for chickpeas and beans (Avola & Patanè, 2010; Paredes-Lopez *et al.*, 1989). Previous studies have shown equivalent losses of α-galactosides in pulses after cooking: 50% loss in lentils, beans and cowpeas after boiling for 60 min (Abdel-Gawad, 1993), 60% loss after cooking cowpeas at 95 °C for 3 h (Coffigniez *et al.*, 2018[a]), but up to 81-100% after boiling cowpeas for 40 min (Ibrahim *et al.*, 2002; Onyenekwe *et al.*, 2000).

There is strong evidence for a role of the seed coat and of cell wall permeability in the diffusion of α-galactosides (Abdel-Gawad, 1993; Bassett *et al.*, 2021; Coffigniez *et al.*, 2019). The temperature and pH conditions during soaking and cooking conditions can influence the permeability of the seed coat and cell wall. For instance, Abdel-Gawad, (1993) reported that increasing the pH by adding NaHCO3 solution softened the seed coat and cotyledons, thereby reducing the concentration of α-galactosides through diffusion into the steeping medium in common bean, fava bean, lentil, and cowpea seeds. Coffigniez *et al.* (2019) showed a slight disruption of cowpea cotyledon cell walls at 30 °C, while higher cell wall degradation occurred at 60 °C and 95 °C. These results show that higher temperatures increase cell wall permeability, thereby facilitating the diffusion of cellular contents, including α-galactosides, into the intercellular space. To further investigate and validate these hypotheses, microscopy and µtomography analyses are needed to explore the microstructural changes that occur during the soaking/cooking process. This would further elucidate the hydrolysis and diffusion of α-galactosides in pulses during processing.

In addition to temperature, the pH of steeping water can also influence the reduction of α-galactoside contents in both pulses and water, due to the effect of diffusion and the action of

enzymes, particularly of α-galactosidase. The mass balance of α-galactosides (linked to enzymatic degradation of α-galactosides) only decreased by 5-10% (from 63-80 to 59-69) from pH 6.0 to pH 4.0. This suggests that α-galactosidase activity at 30 °C was only slightly favored at lower pH. The bigger decrease in α-galactosides in pulses after steeping at pH 4.0 was thus not associated with higher enzymatic degradation but rather with increased diffusion into the steeping water. Indeed, the recovered α-galactosides in steeping water after 18 h at pH 4.0 accounted for 45% of the loss of α-galactosides in pulses (Figure 3), but for less than 10% when steeping took place at pH 6.0.

When pulses were soaked at 45 °C for 14 h, enzymatic degradation of α-galactosides was favored at a pH of 4.0. Under these conditions, the concentration of galactose in both the pulses and the steeping water represented more than 50% of the initial concentration of α-galactosides in lentils (Figure 4) and in beans, and 30% in chickpeas. It should also be noted that at pH 4.0, the concentration of α-galactosides in pulses was lower than the concentration of α-galactosides in the steeping water in less than 3 h in lentils, and in 14 h for chickpeas and beans. It is also worth underlining that α-galactosides have potential health benefits as prebiotics, and doses of less than 3 g/day are well tolerated and do not cause flatulence (Elango *et al.*, 2022; Martínez-Villaluenga *et al.*, 2008). To this end, soaking pulses at 45 °C at pH 4 for 3 h was sufficient to reduce the concentration of α-galactosides to a sufficiently low level to reduce its negative impacts on health. However, the soaking medium must be discarded before cooking. Previous studies have shown that two α-galactosidases are activated at a pH between 2 and 8 (Coffigniez *et al.*, 2018[b]; Dey *et al.*, 1983). Dey *et al.* (1983) reported that the optimum pH for α-galactosidases I and II in lentils was 6.1 and 4.7, respectively, in agreement with our results, considering that α-galactosides enzymatic hydrolysis is much more effective and rapid at lower pH (pH 4.0) and at 45 °C. Coffigniez *et al.* (2018)[b] reported that α-galactosidase activity in cowpeas was higher at pH 4.0 than at pH 6.0.

To our knowledge, to date no studies have assessed the impact of acidic pH on the hydrolysis and diffusion of α-galactosides in pulses. The majority of existing studies analyzed the impact of basic pH when NaHCO3 was added to the soaking medium. For instance, Vidal-Valverde *et al.* (1992) showed no significant difference between lentils soaked at room temperature for 9 h in distilled water, or in a solution of citric acid (0.1% w/v), or sodium bicarbonate (0.07% w/v). These authors reported that soaking lentils can reduce stachyose content by up to 64%, in agreement with our findings. However, the reduction is not only due to diffusion into the soaking water but also to α-galactosidase activity, due to an increase in

glucose and fructose content in the pulses and in the steeping water (Vidal-Valverde *et al.*, 1992). Other authors have shown a slight, but non-significant decrease in α-galactosides when pulses were soaked with NaHCO3 (Akissoé *et al.*, 2021; Madodé *et al.*, 2013; Vijayakumari *et al.*, 2007). However, in the two latter studies, we do not know whether the reduction in α-galactosides was due to leaching out or to α-galactosidase activity, since information on the mass balance or galactose content was not provided.

Our study proved that the hydrolysis and diffusion of α-galactosides depend on the soaking conditions (temperature, pH, and duration). Depending on the soaking conditions, two mechanisms can occur: an increase in α-galactosidase activity, favoring the hydrolysis of α-galactosides, or a change in the permeability of the seed coat and cell wall, facilitating the diffusion of α-galactosides. Our results showed that hydrolysis of α-galactosides was favored at lower temperatures (30 °C and 45 °C), while diffusion was favored at higher temperatures (60, 75, and 90 °C). The reduction in α-galactoside contents in pulses was significantly more rapid at lower pH, via diffusion at 30 °C, and via hydrolysis and diffusion at 45 °C.

The most effective steeping condition to reduce the total α-galactoside content in chickpeas, lentils, and beans was soaking at 45 °C for 14 h that resulted in a reduction of 84%, 92%, and 62%, respectively. However, reducing the soaking time to 3 h at the same temperature and at a pH of 4.0 also led to a significant reduction in α-galactosides content, and reached a level at which its benefits as a prebiotic still function. Under these conditions, the α-galactosides content in the pulses was lower than its concentration in the steeping water. Again, it is important to note that steeping water has to be discarded after soaking, i.e. before cooking.

To further advance our understanding of α-galactosides hydrolysis/diffusion in pulses during food processing, microscopy and µtomography analyses should now be performed, as these techniques provide insights into the microstructural changes that take place during the soaking/cooking process.

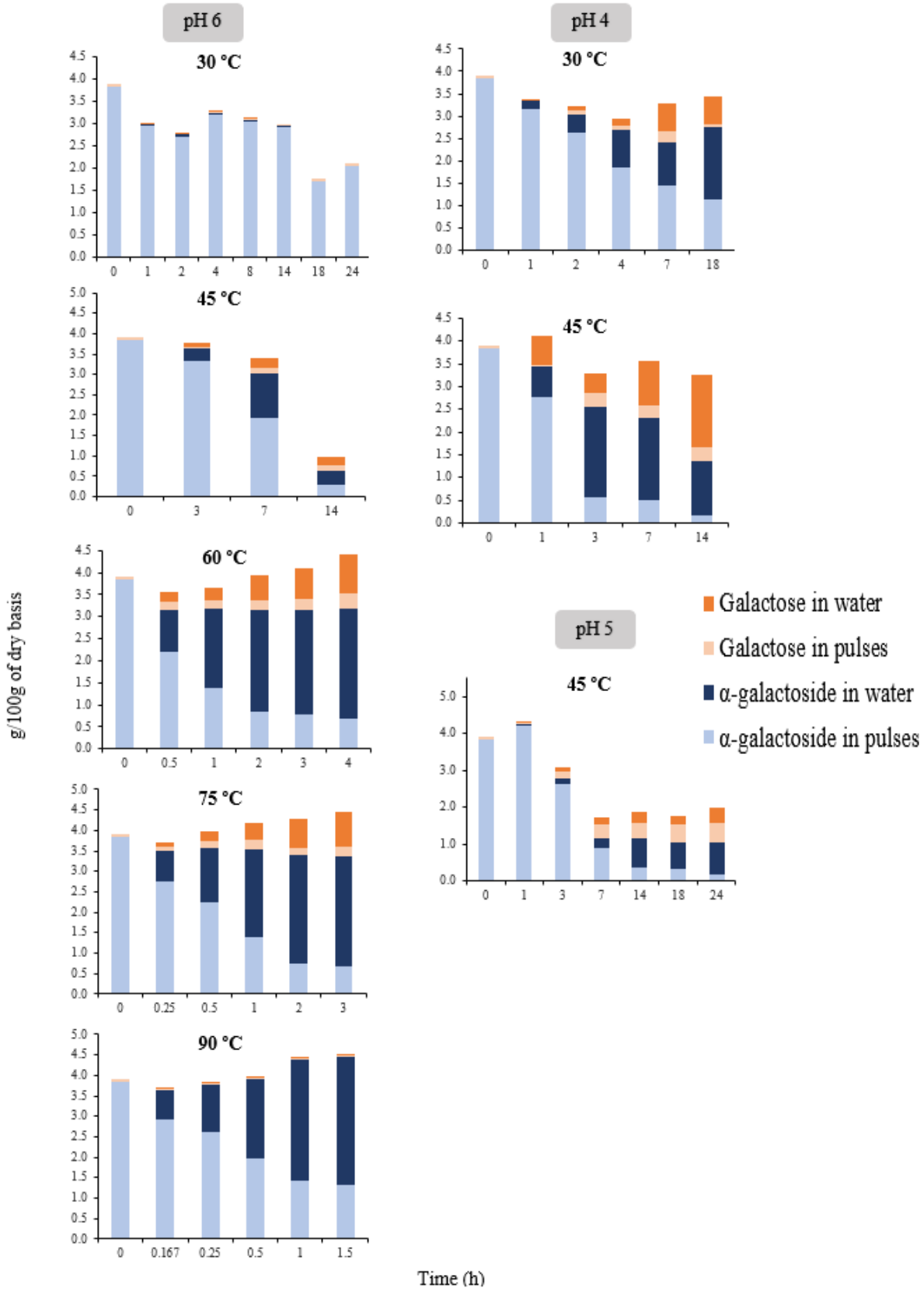


Figure 4. Total α-galactoside and galactose contents in lentils and steeping water in g/100 g on a dry basis at 30 °C, 45 °C, 60 °C, 75 °C, and 90 °C pH 6; at 30 °C and 45 °C pH 4; at 45°C pH 5. The dark and light orange bars represent galactose contents in water and in the pulses, respectively. The dark and light blue bars represent α-galactoside contents in water and in the pulses, respectively.

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IV. New insight into the rapid germination process of lentil and cowpea seeds: high thiamine and folate, and low α-galactoside content

While soaking is an effective process to decrease α-galactosides through diffusion or hydrolysis, it also leads to a reduction in vitamins due to their diffusion into the medium. Adding the germination process increases the vitamin content while simultaneously reduces antinutritional compounds through enzymatic degradation. Actually, the germination process causes important changes in the biochemical, nutritional, and sensory characteristics of pulses. However, to get the most out of the germination process, pulses need to be placed in growth-conductive environmental conditions with adequate temperature, light, and relative humidity, which for an industrial scale requires specific equipment. Therefore, the aim of the next study was to optimize the germination *sensu stricto* process to increase the nutritional quality of pulses. We investigated the influence of temperature, light, and water content on the levels of thiamine, folate, and α-galactosides in lentils and cowpeas. Most studies on germination reported in the literature analyse the effects of temperature and light on the nutritional content of pulses (Coffigniez *et al.*, 2021; Ghavidel & Davoodi, 2011; Joy *et al.*, 2022; Sallam *et al.*, 2021). However, no study has analysed the influence of seed water content during germination. At the laboratory scale, our study is the first specifically designed to simultaneously assess the impact of temperature, light, and water content on the nutritional value of pulses during germination *sensu stricto.* Thiamine and folate were the chosen vitamins for analysis due to their significant presence in both pulses and their essential role in human health. For instance, thiamine deficiency results in a disease called beriberi, which occurs in milk-fed infants whose nursing mothers are deficient. Folate deficiency is strongly linked to defects in the development of neural tube of fetus. The results of this study can be valuable for scientists in integrating data for germination modeling and can serve as insights to add water content as novel parameter when analyzing the effect of the germination process. Furthermore, it could provide guidance for industrialists seeking to innovate pulse processing and integrate them as ingredients to produce new foods with a high nutritive value.

New insights into the rapid germination process of lentil and cowpea seeds: high thiamine and folate, and low α-galactoside content

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Highlights

- Germination increased the thiamine content and changed the vitamer profile in lentil.
- Germination had a more positive effect on folate content in cowpea than in lentil.
- Germination considerably reduced the total α-galactoside content in lentil and cowpea.
- In lentil, low temperature, light, and water content are recommended for germination.
- In cowpea germination higher temperature, light, and water content should be promoted.

Abstract

During germination *sensu stricto* in pulses, an increase in the content of thiamine (B1) and folate (B9) vitamins is expected, along with a reduction in α-galactoside levels. The aim of our study was to optimize germination to increase the nutritional quality of lentils and cowpeas. An experimental design was carried out at 12 h and 24 h of imbibition to analyze the effects of temperature, light, and water content on thiamine, folate, and α-galactoside content. Germination increased thiamine content by 152% in lentils, while in cowpeas, the increase was only 10%. Folate content in cowpea increased by 33%, while α-galactoside content decreased by 99% in cowpeas and by 48% in lentils. Germination *sensu stricto* can be safely implemented by any food company worldwide as it is simple and involves less sanitary risk than sprouting. This opens up opportunities for enhancing food nutrient content and new ways of processing pulses.

Keywords: Germination, Pulses seeds, Nutritional compounds, Vitamins, Antinutritional compounds

I. Introduction

Germination *sensu stricto* is a complex biological process that leads to major metabolic changes and activates pathways for the synthesis of the nutrients required for seedling growth (sprouting). Seed imbibition refers to the process of water absorption by the dry seed. This uptake of water triggers the resumption of metabolic activity in the quiescent dry seed. The uptake of water by a dry mature seed is divided into three phases. Phase I is characterized by rapid initial uptake, followed by phase II, which is a lag phase where water uptake slows down, accompanied by high metabolic modifications in the seed (Galland *et al.,* 2014). Phase III occurs only after germination is completed when the embryonic axe starts elongating, leading to a further increase in water uptake. Germination is considered complete when the embryonic axe elongates, indicating that the seedling is actively growing and developing. The passage mentions that radicle extension, which is the elongation of the primary root, through the structures surrounding the embryo, is the event that terminates germination *sensu stricto* and marks the beginning of seedling growth (Bewley & Black, 1994; Nonogaki *et al.,* 2007; Rajjou *et al.,* 2012). This event is what we refer to as "sprouting," where the radicle emerges from the seed coat and starts growing into a seedling. Some semantic mistakes have been made in the description of germination *sensu stricto* and sprouting. During germination *sensu stricto,* seeds have a heterotrophic metabolism and are still considered as seeds containing large quantities of storage compounds. Depending on the genotype of the seed and environmental conditions, germination can be completed within 24 h, but further thermal processing may be required before consumption. In contrast, sprouting involves autotrophic metabolism and sprouts are classified as ready-to-eat food products. Even if the word ‘germination’ is used, most studies in the literature actually report the effect of sprouting (Hefni *et al.,* 2015; Shohag *et al.,* 2012). The use of incorrect terms can interfere with the advance of knowledge on the advantages of germination. A short germination period of 24 h and a thermal post-germination process may reduce the microbiological risk of germinated products (Avezum *et al.,* 2022; Bresciani & Marti, 2005). Most studies on germination reported in the literature are carried out between 20 °C and 30 °C, with 12 h to 16 h of light (Coffigniez *et al.,* 2021; Ghavidel & Davoodi, 2011; Joy *et al.,* 2022; Sallam *et al.,* 2021). However, all the studies were conducted at water saturation, and no changes in seed water content during germination were reported. Germination and sprouting at 25 °C for 24 h to 6 days have been shown to increase the concentrations of water-soluble vitamins and *in-vitro* digestibility of starch and protein in

pulses and cereals, while simultaneously reducing the levels of antinutritional factors, such as phytic acid, α-galactoside, trypsin inhibitor, and lectins in the cotyledon (Prodanov *et al.*, 1997; Vidal-Valverde *et al.*, 2002).

Among seeds used for germination, pulses are excellent sources of carbohydrates, proteins, fibers, minerals, and B vitamins (especially thiamine and folate) (Bojňanská *et al.*, 2016; Mudryj *et al.*, 2014). Thiamine (B1) is a cofactor for multiple enzymes that catalyze bioenergetic reactions, control amino acid metabolism, and convert some carbohydrates. Thiamine may be present in a free form, or in its phosphorylated derivatives, namely thiamine monophosphate (TMP), diphosphate (TDP), triphosphate (TTP), and adenosine thiamine triphosphate (Tylicki *et al.*, 2018). In pulses, folates are present in different forms, including folic acid (PteGlu), 10-formylfolic acid (10-CHO-PteGlu), 5-formyltetrahydrofolate (5-CHO-H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), and tetrahydrofolate (H_4folate). Folate co-enzymes mediate two major interrelated metabolic cycles that are responsible for the synthesis of thymidylate, purines, and methionine (Moll & Davis, 2017). The recommended daily intake (RDI) of thiamine and folate for adults, is respectively, 1100-1500 µg/day and 200-400 µg/day (Chunming *et al.*, 2001; Krawinkel *et al.*, 2014; Moll & Davis, 2017). In 100g, raw lentils contain 51% of the RDI of thiamine and 70% for cowpea; and respectively, 55% and 105% of RDI for folate (Coffigniez *et al.*, 2021; Hall *et al.*, 2017). However along with their health benefits, pulses also contain antinutritional factors, such as α-galactoside, in the form of raffinose, stachyose, and/or verbascose (Bessada *et al.*, 2019). Due to the lack of α-galactosidase in human and animal intestinal mucosa, these oligosaccharides escape digestion and are metabolized by colon bacteria, thereby producing gut gas in the form of hydrogen, carbon dioxide, and methane, and resulting in flatulence (Bessada *et al.*, 2019; Naczk *et al.*, 1997).

At laboratory scale, our study is the first specifically designed to simultaneously assess the impact of temperature, light, and water content on the nutritional value of pulses during germination *sensu stricto*. Few studies have analyzed the effect of temperature on the antioxidant and bioactive compounds in cereals and pulses during germination (Domínguez-Arispuro *et al.*, 2018; Paucar-Menacho *et al.*, 2017; Rico *et al.*, 2020). In this study, we investigated whether optimizing germination *sensu stricto* enhances vitamins content and reduces α-galactosides content of lentil (*Lens culinaris*) and cowpea (*Vigna unguiculata*). The

outcome will allow food companies to better understand the advantages of germination, while improving the nutritional status of pulses.

II. Material and Methods

II.1. Material

Two pulses were chosen as models and analyzed: lentil (*Lens culinaris*) and cowpea (*Vigna unguiculata*). Berry green lentil seeds were purchased in 2020 from CIBELE Company, France. White cowpea seeds were purchased directly from the producer in south-east Benin. Cowpea seeds were sowed in November 2021 and harvested in February 2022. Both seeds were stored at -20 °C until use.

II.2. Germination

To trigger germination, 80 g of seeds were soaked in 320 mL water (ratio 1:4; w/v) pH 6.0 under agitation at 30 °C, lentils for 3 h and cowpeas for 5 h. The soaked seeds were removed from the soaking water, immediately drained and placed in a chamber with controlled temperature and relative humidity for germination. The whole process took 12 h and 24 h (including soaking time). For lentil germination, a BIA Climatic chamber with 25 °C – 35 °C temperature regulation and 90% relative humidity was used. The seeds were placed in a rectangular perforated weighing pan (361 cm^2) equipped with an integrated balance to monitor the seed weight and, consequently, its water content. A spray was automatically activated when the calculated water content dropped below the expected level and stopped after 10 minutes; spraying was activated roughly once an hour. To verify the water content, 5 g of wet seeds were sampled in five different locations across the rectangular weighing pan, and dried at 105 °C for 48 h. Alpheus LED (France) light was used which corresponds to sunlight. The cowpea seeds were germinated in Benin in a BINDER-KMF 240 chamber (~ 95% relative humidity). In this

case, spraying was done manually after 18 h of germination using 60 mL of water, for a targeted water content of 110%, or using 120 mL for 140% water content (db). The spraying was carefully carried out to ensure that the desired water content levels were achieved and maintained under control. The samples were weighed once an hour to monitor the water content. The light used was a normal white LED (Light Emitting Diode). None of the lentil or cowpea seeds, in either of the experimental locations, developed a radicle (i.e. germinated) in the first 12 h. At this stage, the samples were considered as non-germinated, and all the seeds were analyzed. After 24 h of germination, the seeds were sorted into non-germinated (no radicle), germinated (radicle < 5 mm), and sprouted seeds (radicle > 5 mm). At 24 h, only the germinated seeds were analyzed. Lentil seeds were collected and frozen in liquid nitrogen and stored at -80 °C. Cowpea seeds were collected and directly stored at -40 °C. All the seeds were freeze-dried and then ground in a QIAGEN – TissueLyser II ball mill grinder to obtain a fine flour. The flour was freeze-dried and stored at -20 °C until further analyses.

II.3. Experimental design

Response surface methodology (RSM) was used to analyze the effect of germination parameters on the nutritional quality of the pulses and to determine the optimal conditions for enhancing the nutritional quality of germinated lentil and cowpea flour via a desirability analysis. An experimental design with two levels and three factors was used (Table 1). The impact of temperature, light, and seed water content on germination rate and nutritional quality of pulses was analyzed. The center point 9 (C) was performed in triplicate. Preliminary trials and data from the literature were used to select the boundaries of the experimental design. The same experiment design was implemented for lentil and cowpea. The desirability function was used to optimize temperature, light, and water to reach the maximum desirable response. The variables considered were germination temperature (T), light (L), and water content (W). The response variables were thiamine (Thia), folate (F), and α-galactoside (G). To optimize the process, the linear equation was applied (1):

$$Y = \beta_0 + \beta_T T + \beta_L L + \beta_w W + \beta_{T,L} TL + \beta_{T,W} TW + \beta_{L,W} LW + \beta_{T,L,W} TLW + \sum \in \quad (1)$$

where Y is the estimated response, β_0 and β_X represent the constant and coefficients of linear and interactive effects, respectively, and ϵ represents the error. Non-significant variables ($p \geq 0.1$) were deleted from the linear equation.

Table 1. Experimental design for lentils and cowpeas germination

Assay	Temperature (°C)	Light (h)	Lentil Water Content (% db)	Cowpea Water Content (% db)
1	25	0	80	80
2	35	0	80	80
3	25	12	80	80
4	35	12	80	80
5	25	0	120	140
6	35	0	120	140
7	25	12	120	140
8	35	12	120	140
9 (C)	30	6	100	110

II.4. Germination rate

Given the large number of lentil seeds used for germination, it was not feasible to manually count each individual germinated seed. Instead, 5 g of wet seeds were sampled in five different locations in the rectangle weighing pan (one sample in each corner and one in the center). All

germinated and sprouted seeds were counted and the mean and its standard deviation were calculated. Samples were collected at the same locations in the pan in all the experiments. For cowpea germination, the germination rate was calculated by counting all germinated and sprouted seeds. In both cases, the germination rate was calculated according to equation (2).

$$Germination\ rate\ (\%) = \frac{\Sigma\ of\ germinated\ seeds + \Sigma\ \text{of}\ sprouted\ seeds}{\Sigma\ of\ total\ seed} \times 100 \quad (2)$$

II.5. Thiamine extraction and detection

Thiamine was extracted and quantified as described by Schmidt *et al.* (2017) with some modifications. The thiamine was extracted from 0.5 g of seed flour in 40 mL of 0.1 M HCL (Hydrochloric Acid) solution for 1 h under agitation. Samples were centrifuged at 8000 rpm for 15 min at 4 °C. The samples were oxidized to fluorescent thiochrome in absence of light. The resulting solutions were filtered through a 0.45 µm and 0.2 µm filter. The thiamine was quantified on an Ultra Performance LC (UPLC™) system equipped with an Acquity™ fluorescence (FLR) detector. The column was operated at a flow rate of 0.40 mL/min. The total cycle was set to 6 min until the following injection. Thiamine diphosphate (TDP), thiamine monophosphate (TMP), and thiamine hydrochloride (Thia-HCL) were quantified using a calibration curve with external standards. The thiamine equivalent (thia-eq) was calculated according to equation 3 and 4.

All samples were prepared in duplicate.

$$\text{Thia} - \text{eq} = \frac{(\text{nTDP} + \text{nTMP} + \text{nThiaHCL}) \times \text{MM Thiamine}}{\text{M}} \quad (3)$$

Where M is the mass (g) of the sample, n_X is the number of mols of X (X = TDP, TMP or Thia-HCL) in mol with:

$$n_X = \frac{[X] \times V}{MM_X} \times 10^6 \quad (4)$$

[X] = Concentration of X in g/mL

V = Extraction volume in mL

MM_X = molar mass of X in g/mol

II.6. Folate extraction and detection

The folates were extracted and quantified using the methodology described by Coffigniez *et al.* (2021) and Striegel *et al.* (2018). Folic acid (PteGlu), Tetrahydrofolate (H4folate), 5-methyl-tetrahydrofolate (5-CH3-H4folate), 5-formyl-tetrahydrofolate (5-CHO-H4folate), and 10-formylfolic acid (10-CHO-PteGlu) were quantified by stable isotope dilution assay with LC-MS/MS detection using isotopologues of the analytes as internal standards. Both extraction and measurements were performed in duplicate.

II.7. α-Galactoside extraction and detection

α-Galactoside was extracted using the methodology described by Coffigniez *et al.* (2018) with slight modifications. α-galactoside was extracted from 80 mg of flour placed in 10 mL of 80% (v/v) ethanol at 80 °C for 1 h. The supernatant was collected and filtered through a 0.45 µm filter. α-galactoside was separated by high-performance anion exchange chromatography using a Dionex ICS-5000 + Ion Chromatography System (Thermo Scientific, France). The sugars were separated at room temperature in a 4 x 30 mm Dionex CarboPac PA210-fast-4µm pre-column and a 4 x 150 mm Dionex CarboPac PA210-fast-4µm column (Dionex, Germany). The injection volume was 10 µL. The mobile phase was 12 mM potassium hydroxide solution. The flow rate was 0.8 mL/min. Raffinose, stachyose, and verbascose were quantified using a calibration curve with external standards. Extraction was done in duplicate.

II.8. Statistical analyses

The thiamin, folate, and α-galactoside contents were analyzed using TIBCO Statistica software (StatSoft). For each nutritional compound, an ANOVA of temperature, light, water, and its interaction was performed. For a better understanding of the impact of germination conditions on the nutritional quality, 12 h and 24 h germination periods were statistically analyzed separately. Tukey-HSD test was performed to check for significant differences between samples for each compound.

III. Results and discussion

III.1. Germination rate

The highest germination rate for lentils, 51.4%, was reached after 24 h at 25 °C, including 12 h of lighting and under water saturation (120%) (Table 2). In many of the experiments (assay 1, 3, 5, 7, 11), the standard deviation of the germination rate was high (> 10%) underlying the heterogeneity of the samples. This heterogeneity may be due to the use of the automatic spraying system, as the effect on the support was not even. However, the heterogeneous germination of the lentil seeds did not directly affect the final nutritional parameters, since only the germinated seeds were analyzed. Another study reported that, at 22 °C for 24 h, the germination rate of the lentils was 80% (Vasilean *et al.*, 2018). This discrepancy may be due to the difference in germination methodology used in the two studies (lighting and HR were not mentioned in the Vasilean *et al.* study), seed varieties, and seed vigor.

The cowpea germination rate was higher (83.3 %) than the lentil germination rate with the same lighting conditions and water content but at a temperature of 35 °C. Cowpea germination rates at 28 °C for 48 h have been reported to range from 60% to 99% (Ravelombola *et al.*, 2017). Coffigniez *et al.* (2021) reported that the germination rate of cowpea at 30 °C reached 75% after only 48 h, while under the same germination conditions, but after 24 h, the germination rate was between 40% and 50% (Coffigniez *et al.*, 2021). These results are in agreement with the data we found at the central point (30 °C) for which the average of germination rate was 45.6%.

In our study, in lentil, only temperature had a negative significant impact ($p < 0.05$) on the germination rate. In cowpea, the temperature and water content had a positive significant impact ($p < 0.01$). Thus, lower temperatures favored lentil germination and higher temperatures and water content favored cowpea germination. The effect of temperature showed that the seeds used in our experiments are adapted to their environment. Lentils are cultivated in regions with milder temperatures, *e.g.* North America, Europe, India, and Australia, while cowpeas are cultivated in tropical regions like West Africa.

Table 2. Germination rate in lentils (mean ± SD) and cowpeas (total rate) after 24 h.

T (°C)	Light (h)	Lentil Water Content (% db)	Cowpea Water Content (% db)	Mean germination rate for lentil (%)	Total germination rate for cowpea (%)
25	0	80	80	29 ± 26	0
35	0	80	80	0.38 ± 0.005	23
25	12	80	80	21 ± 25	0
35	12	80	80	3.8 ± 5.5	33
25	0	120	140	45 ± 19	41
35	0	120	140	7.6 ± 4.2	76
25	12	120	140	51 ± 24	60
35	12	120	140	2.9 ± 0.014	83
30	6	100	110	32 ± 0.22	47
30	6	100	110	43 ± 0.2	50
30	6	100	110	31 ± 14	40

III.2. Nutritional analyses

III.2.1. Thiamine content

Germination increased thiamine content and changed the vitamer profile in lentil seeds. Dry lentil seeds contained 154 µg/100 g (db) thiamine-equivalent (thia-eq) (Table 3). The thiamine content in lentil reported in the literature is higher, ranging between 433 µg/100 g and 510 µg/100 g (db) (Ghavidel & Prakash, 2007; Prodanov *et al.*, 1997). The difference is probably due to the use of different varieties, seed conservation, and thiamine extraction methods. In the present study, soaking increased the total thia-eq in lentil by 109%. Between dry lentils and

lentils germinated for 24 h (35 °C, 12 h of lighting, and 80% water content) the thia-eq content increased by 169%. However, no significant impact ($p > 0.05$) of the studied factors was found on the total thia-eq in germinating (12 h) and fully germinated (24 h) lentil seeds. Ghavidel & Prakash, (2007) reported different results, *i.e.* an increase of up to 33% after 36 h of germination in lentil (Ghavidel & Prakash, 2007), and a study by Vidal-Valverde *et al.* (2002) showed no significant variation in thiamine content when lentils, peas, and beans were germinated at 20 °C for between 2 to 4 days. Under the same germination conditions but for 6 days, Prodanov *et al.* (1997) reported a significant reduction (11% to 14%) in thiamine content in lentils. Thus, a short germination period may increase or at least maintain the thiamine content in lentils while thia-eq may decrease after 6 days of sprouting, as it is used by the plant for seed and seedling metabolism. In both the above-mentioned studies, the seeds were no longer in germination *sensu stricto* but in the seedling growth stage.

Table 3. Average total concentration of thiamine equivalent, folate content in µg/100 g and total α-galactoside content in g/100 g on a dry basis for lentil. The percentage increase in thiamine and folate and the percentage of reduction in α-galactoside content is calculated between dry seeds and each condition

Temperature (°C)	Light (h)	Water Content (% db)	Time (h)	Total thia-eq in lentil [µg/100 g db]	% increase	Total folate content in lentil [µg/100 g db]	% increase	Total α-galactoside content in lentil [g/100 g on db]	% reduction
Dry				154 ± 3.4		147 ± 7.4		5.56 ± 0.25	
Soaked				322 ± 5.1	109%	122 ± 1.2	-17%	4.65 ± 0.56	16%
25	0	80	12	345 ± 16	124%	124 ± 1.8	-16%	4.41 ± 0.007	21%
			24	388 ± 11	152%	157 ± 1.3	6%	2.97 ± 0.17	47%
35	0	80	12	345 ± 9.4	125%	128 ± 7.8	-13%	4.14 ± 0.20	26%
			24	371 ± 3.8	141%	146 ± 7.2	-1%	3.56 ± 0.22	36%
25	12	80	12	343 ± 6.8	123%	123 ± 5.3	-17%	3.92 ± 0.45	30%
			24	392 ± 5.1	155%	148 ± 1.9	1%	3.12 ± 0.024	44%
35	12	80	12	348 ± 13	126%	120 ± 0.87	-18%	3.92 ± 0.0004	30%
			24	418 ± 11	169%	158 ± 6.5	8%	3.88 ± 0.059	30%
25	0	120	12	351 ± 2.3	128%	120 ± 0.34	-18%	3.99 ± 0.011	28%
			24	377 ± 16	145%	147 ± 2.4	0%	3.39 ± 0.37	39%
35	0	120	12	346 ± 13	125%	111 ± 3.4	-24%	3.93 ± 0.16	29%
			24	385 ± 15	150%	155 ± 9.6	5%	3.35 ± 0.037	40%
25	12	120	12	358 ± 13	132%	113 ± 0.35	-24%	4.09 ± 0.59	26%
			24	373 ± 4.7	142%	147 ± 13	0%	3.00 ± 0.12	46%
32	12	120	12	350 ± 2.5	128%	115 ± 6.9	-22%	4.11 ± 0.045	26%

			24	366 ± 7.8	137%	148 ± 1.5	1%	3.18 ± 0.034	43%
30	6	100	12	347 ± 7.9	125%	98 ± 1.3	-33%	4.03 ± 0.75	27%
			24	367 ± 6.3	138%	145 ± 8.5	-2%	3.24 ± 0.26	42%
30	6	100	12	346 ± 5.3	125%	122 ± 1.6	-17%	3.97 ± 0.22	29%
			24	379 ± 14	146%	146 ± 6.8	-1%	3.02 ± 0.33	46%
30	6	100	12	353 ± 12	129%	123 ± 9.4	-17%	4.07 ± 0.19	27%
			24	373 ± 2.5	142%	147 ± 5.0	0%	2.92 ± 0.20	48%

In addition to the increase in thia-eq in lentils after soaking, the vitamer profiles changed during germination (Figure 1). The changes in the vitamer profile followed the same trend regardless of the germination conditions. Figure 1 shows the average of each vitamer in all 11 study conditions for 12 h and 24 h. In dry lentils, thia-HCL and TDP accounted for 86% and 11% of the total vitamers, respectively. Soaking and germination induced a higher increase in TDP content than thia-HCL. Soaking increased thia-HCL by 91% and TDP content by 321%. The increase in the percentage of TDP may be the result of the metabolic activation during germination and its production for use by the seedling. TDP is the active form of thiamine and is required for the performance of enzymes linked to carbohydrate metabolism. Temperature and water had a negative significant ($p < 0.01$) effect on thia-HCl content (Table 4), whereas temperature had a positive significant ($p < 0.01$) effect on TDP.

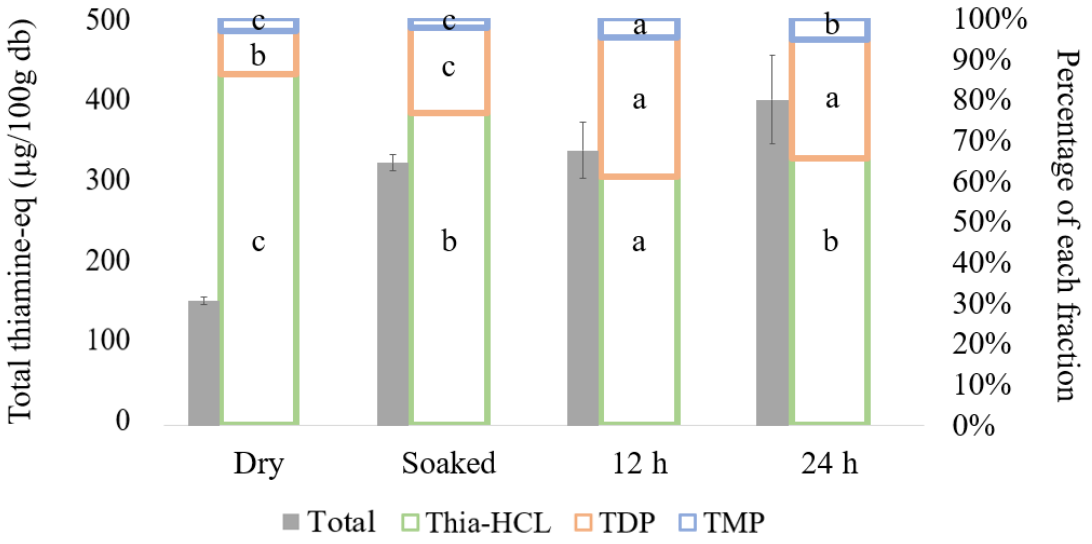


Figure 1. Total thiamine-eq content (gray) in µg/100 g (db) and the percentage of each thiamine fraction, thiamine hydrochloride (Thia-HCl) (green), thiamine diphosphate (TDP) (orange), and thiamine monophosphate (TMP) (blue), in dry, soaked, germinating (12 h) and germinated (24 h) lentils. Letters indicate statistically (Tukey-HSD test) significant difference ($p < 0.05$) within each compound in comparison with each sample.

Based on an amount of 932 µg/100 g (db), dry cowpea seeds were 6 times richer in total thia-eq than lentil seeds (Table 5). Other authors found lower thiamine content (144 µg/100 g, 640µg/100 g and 870 µg/100 g) in cowpeas (Akinlosotu & Akinyele, 1991; Ghavidel & Prakash, 2007; Joy *et al.*, 2022). This variation may be linked to the thiamine extraction method used, the variety of seed, and the timing of harvest. Soaking and germination had less effect on

total thia-eq in cowpeas than in lentils. For instance, soaking only resulted in a 4% increase in total thia-eq in cowpeas and germination only to an increase of between 1% to 10%. Like in lentils, we found no significant impact ($p > 0.05$) of the factors we studied on the total thia-eq in germinating (12 h) and germinated (24 h) cowpea seeds. Ghavidel & Prakash (2007) reported similar results to ours: after 36 h, the thiamin content of germinated cowpea increased by 8% (Ghavidel & Prakash, 2007). As mentioned above, 36 h of germination in fact corresponds to sprouting and the seed is no longer in the germination *sensu stricto*. However, Akinlosotu & Akinyele, (1991) reported different results: germinating cowpea at room temperature for 24 h reduced thiamine content by 6.9%, and increasing the germination time to 72 h reduced thiamin content to 51% (Akinlosotu & Akinyele, 1991). In the present study, TDP was the main vitamer found in cowpea and represented 89% of total vitamers. Unlike in lentils, in cowpeas, we found no significant difference ($p > 0.05$) in any vitamer percentage in any of the conditions analyzed.

Table 4. Statistical summary of ANOVA results for thiamine diphosphate (TDP), thiamine monophosphate (TMP), thiamine hydrochloride (Thia-HCl), and thiamine equivalent (thia-eq) in germinated lentil seeds for 24 h.

	TDP	TMP	Thia-HCL	Thia-eq
	R^2=0.8954	R^2=0.9774	R^2=0.9722	R^2=0.8490
Temperature	+++		- - -	
Light		++		
Water		++	- -	
Light*Water		+++		+
Temperature*Light*Water		++		

+ is for proportional correlation. + (0.05 < pvalue < 0.1); ++ (0.01< pvalue < 0.05); +++ (pvalue < 0.01)
- is for disproportional correlation. - (0.05 < pvalue < 0.1); - - (0.01< pvalue < 0.05); - - - (pvalue < 0.01)

III.2.2. Folate content

In dry lentils, the total folate concentration was 147 µg/100 g (db) (Table 3). In the literature, a value of 244 µg/100 g of fresh weight was reported for brown lentil (Sallam *et al.,* 2021). In our study, folate content decreased by 17% after soaking and remained in the same range after partial germination at 12 h. However, after 24 h germination, the total folate content increased back to the original folate content of dry lentils whatever the germination conditions. This shows that in the first hours of germination, lentil seeds used the folate for their metabolism. With a longer germination period, the seeds produce folate to prepare for the next germination stage, which is the seedling. The factors (temperature, light, water content) we studied had no significant impact ($p > 0.05$) on the total folate content in either germinating and germinated lentil seeds.

The vitamin profile of dry lentil seed was diverse: 5-CH3-H4folate was the main vitamer present accounting for 35% of the total content, followed by H4folate (29%), 5-CHO-H4 folate (24%), PteGlu (6%), and 10-CHO-PteGlu (5%) (Figure 2). However, Sallam *et al.* (2021) identified 10-CHO-PteGlu as the main folate form in lentil, accounting for 27% of total folate content. The changes in vitamer profiles followed the same trend, irrespective of the germination conditions. Since the factors we studied had the same impact on the total folate content, figure 2 shows the average of each vitamer in all 11 study conditions at 12 h and at 24 h. During soaking, the 5-CH3-H4folate content increased by 60% and 5-CHO-H4folate and H4folate content decreased by 54% and 71%, respectively. The changes in vitamer profiles are given by the conversion of 5-CHO-H4folate and H4folate into 5-CH3-H4folate to be used for subsequent seedling metabolism (Coffigniez *et al.,* 2021). In all germination conditions, PteGlu and 10-CHO-PteGlu accounted for a minimum percentage of the total folate content ranging from 2% to 9%. 5-CH3-H4folate was the main vitamer found in lentils, and so the impact of germination on its content was analyzed. Results of ANOVA showed a positive significant ($0.05< p <0.1$) impact of temperature on the concentration of 5-CH3-H4folate when the lentils were germinated for 24 h. Sallam *et al.* (2021) reported that when brown lentils were germinated at 26 °C (during the day) and 21 °C (at night) the maximum folate content (a three-fold increase) was reached on the fifth day. Like in the present study, the vitamer most present in germinated seeds was 5-CH3-H4folate (Sallam *et al.,* 2021).

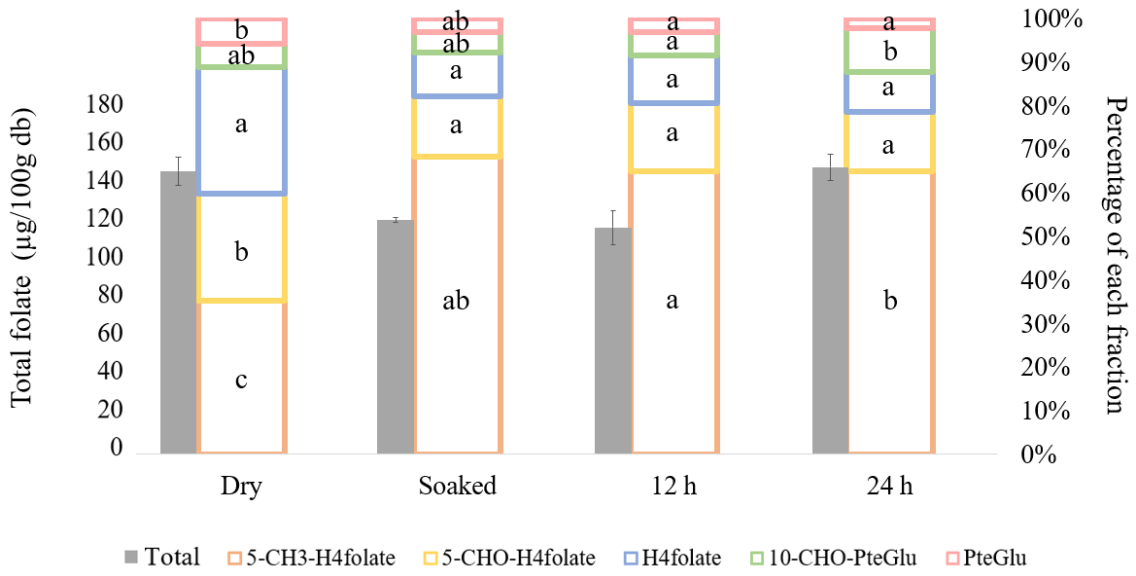


Figure 2. Total folate content (gray) in µg/100 g (db) and the percentage of each folate fraction, 5-methyl-tetrahydrofolate (5-CH3-H4folate) (orange), 5-formyl-tetrahydrofolate (5-CHO-H4folate) (yellow), tetrahydrofolate (H4folate) (blue), 10-formylfolic acid (10-CHO-PteGlu) (green), and folic acid (PteGlu) (red), in dry, soaked, germinating (12 h) and germinated (24 h) lentils. Letters indicate statistically (Tukey-HSD test) significant difference ($p < 0.05$) within each compound in comparison with each sample.

In contrast to what happens to thiamine metabolism, soaking and germination had a more positive effect in cowpeas than in lentils; germination indeed increased total folate content and changed the vitamer profile in cowpea seeds. Cowpea was twice as rich in folate as lentil, with a concentration of 329 µg/100 g (db) (Table 5). Coffigniez *et al.* (2021) found total folate contents of 420 µg/100 g in brown cowpea, and a similar concentration (329 µg/100 g, fresh weight) was found by Sallam *et al.* (2021) in the same variety. In cowpea, soaking increased the total folate content by 10% and germination by up to 33%. Between germinating and germinated seeds, the total folate content increased by 3% to 20%. Temperature was the only factor that had a significant ($p < 0.05$) positive effect on total folate content in germinating cowpea (12 h) (Table 6).

In dry cowpea seeds, the 5-CHO-H4folate was the main vitamer present, accounting for 44% of the total content, followed by 10-CHO-PteGlu (23%), PteGlu (15%), H4folate (10%), and 5-CH3-H4folate (8%) (Figure 3). Coffigniez *et al.* (2021) showed a folate profile close to that found in our study: 5-CHO-H4folate was still the main folate found with a percentage of 54%, but 5-CH3-H4folate, PteGu, 10-CHO-PteGlu, and H4folate represented 11%, 16%, 16%,

and 3%, respectively (Coffigniez *et al.*, 2021). However, Sallam *et al.* (2021) found 5-CH3-H4folate (30%) to be the main form of folate in cowpea. These different results indicate that folate vitamers can differ depending on both genetic and environmental conditions.

The changes in vitamer profile followed the same trend independently of the germination conditions. Soaking significantly affected the vitamin profile in cowpeas (Figure 3). To trigger germination, seeds produce 5-CH3-H4folate, mostly from 5-CHO-H4folate, to use for their metabolism (Coffigniez *et al.*, 2021). Between dry and soaked cowpea seeds, the 5-CH3-H4folate content increased by 731% whereas 5-CHO-H4folate content decreased by 77%. The total folate concentration and the vitamer profile did not differ significantly ($p > 0.05$) between soaked and germinated seeds. Temperature was the only factor that had a significant ($p < 0.05$) positive impact on the concentration of 5-CH3-H4folate (Table 6). The sum of PteGlu, H4folate, and 10-CHO-PteGlu accounted for less than 20% of total folates in germinated cowpea. Coffigniez *et al.* (2021) reported that germinated cowpea seeds at 30 °C for 48 h did not significantly increase the total folate content. However, the folate content doubled between 48 h and 96 h of germination (Coffigniez *et al.*, 2021). Germinated cowpeas at 26 °C (during the day) and 21 °C (at night) induced a 2.8 fold increase in total folate content after 5 days (Sallam *et al.*, 2021). Like in this present study, the main vitamer that accumulated during germination was 5-CH3-H4folate, which reached maximum (490.4 µg/100 g) on the fifth day (Coffigniez *et al.*, 2021; Sallam *et al.*, 2021). The difference in results is due to the duration of germination. As already mentioned, 48 h and 5 days of germination correspond to sprouting and the seed is no longer in the germination *sensu stricto*.

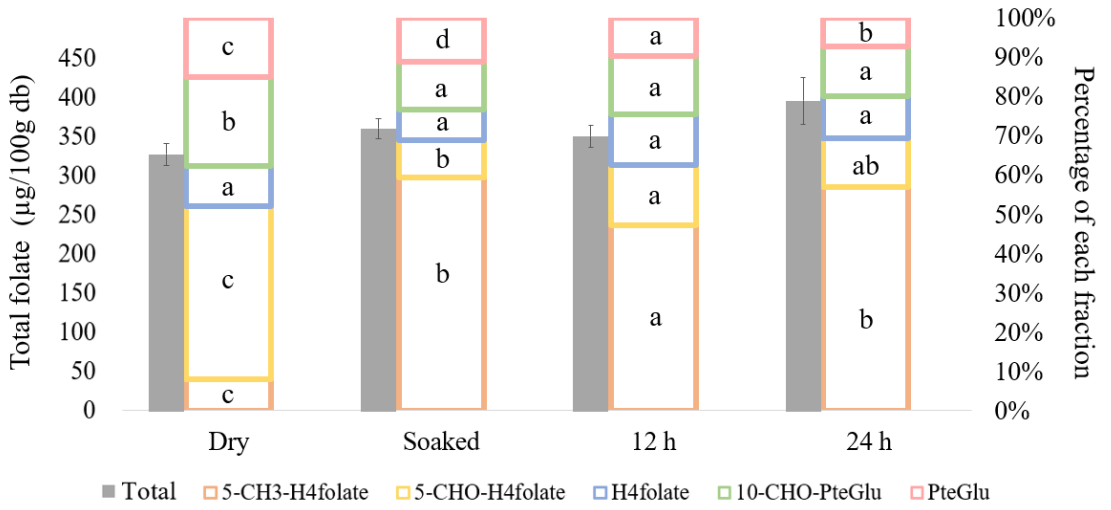


Figure 3. Total folate content (gray) in µg/100 g (db) and the percentage of each folate fraction, 5-methyl-tetrahydrofolate (5-CH3-H4folate) (orange), 5-formyl-tetrahydrofolate (5-CHO-H4folate) (yellow), tetrahydrofolate (H4folate) (blue10-formylfolic acid (10-CHO-PteGlu) (green), and folic acid (PteGlu) (red), in dry, soaked, germinating (12 h) and germinated (24 h) cowpeas. Letters indicate statistically (Tukey-HSD test) significant difference ($p < 0.05$) within each compound in comparison with each sample.

Table 5. Average total concentration of thiamine equivalent, folate content in µg/100 g and total α-galactoside content in g/100 g on a dry basis for cowpea. The percentage increase in thiamine and folate and the percentage reduction in α-galactoside content is calculate between dry seeds and each condition.

Temperature (°C)	Light (h)	Water Content (% db)	Time (h)	Total thia-eq in cowpea [µg/100 g db]	% increase	Total folate content in cowpea [µg/100 g db]	% increase	Total α-galactoside content in cowpea [g/100 g on db]	% reduction
Dry				932 ± 27		329 ± 14		7.41 ± 0.43	
Soaked				967 ± 7.1	4%	362 ± 13	10%	5.42 ± 0.07	27%
25	0	80	12	980 ± 9.6	5%	334 ± 8.0	2%	6.60 ± 0.85	11%
			24	964 ± 16	3%	345 ± 8.0	5%	5.69 ± 0.21	25%
35	0	80	12	976 ± 28	5%	359 ± 12	9%	4.61 ± 0.09	38%
			24	947 ± 33	2%	381 ± 5.2	16%	0.18 ± 0.01	98%
25	12	80	12	927 ± 35	-1%	328 ± 4.6	0%	6.05 ± 0.34	18%
			24	971 ± 6	4%	355 ± 3.5	8%	2.73 ± 0.04	63%
35	12	80	12	987 ± 20	6%	356 ± 8.4	8%	3.16 ± 0.27	57%
			24	941 ± 16	1%	394 ± 9.6	20%	0.12 ± 0.02	98%
25	0	120	12	980 ± 7.5	5%	335 ± 5.2	2%	6.29 ± 0.29	15%
			24	1023 ± 5.2	10%	383 ± 11	16%	0.69 ± 0.08	91%

35	0	120	12	985 ± 16	6%	368 ± 1.2	12%	2.72 ± 0.08	63%
			24	955 ± 2.9	2%	420 ± 6.3	28%	0.22 ± 0.02	97%
25	12	120	12	995 ± 5.9	7%	351 ± 6.2	7%	0.94 ± 0.22	87%
			24	989 ± 1.4	6%	393 ± 2.8	19%	0.57 ± 0.13	92%
32	12	120	12	975 ± 15	5%	366 ± 0.34	11%	2.57 ± 0.30	65%
			24	979 ± 19	5%	437 ± 16	33%	0.07 ± 0.002	99%
30	6	100	12	985 ± 10	6%	354 ± 6.9	8%	3.18 ± 0.14	57%
			24	973 ± 12	4%	426 ± 17	29%	0.12 ± 0.006	98%
30	6	100	12	999 ± 9.5	7%	361 ± 1.0	10%	3.33 ± 0.31	55%
			24	999 ± 22	7%	426 ± 6.3	29%	0.19 ± 0.03	98%
30	6	100	12	997 ± 32	7%	357 ± 2.5	9%	3.30 ± 0.24	56%
			24	1016 ± 6.1	9%	421 ± 12	28%	0.10 ± 0.01	99%

Table 6. Statistical summary of ANOVA results for folic acid (PteGlu), tetrahydrofolate (H4folate), 5-methyl-tetrahydrofolate (5-CH3-H4folate), 5-formyl-tetrahydrofolate (5-CHO-H4folate), verbascoce, stachyose, raffinose, and total α-galactosides content in germinated cowpea seeds for 24 h.

	PteGlu	H4folate	5-CH_3-H4folate	5-CHO-H4folate	Verbascose	Stachyose	Raffinose	Total α-galactosides content
	R^2=0.9912	R^2=0.9374	R^2=0.9162	R^2=0.9619	R^2=0.8889	R^2=0.9038	R^2=0.8722	R^2=0.9043
Temperature	++	-	++	- -	+	++	+	++
Light	+++							
Water	+	+++		-	+	+		+
Temperature*Water	+++			- -	+	+		+
Light*Water	+++			- -				

III.2.3. α-Galactoside content

Germination considerably reduced total α-galactoside content in both lentil and cowpea seeds. The α-galactoside content of dry lentils was 5.56 g/100 g (db) (Table 3). This value is higher than that reported in the literature, which is between 2.72 g/100 g (db) and 2.96 g/100 g (db) (Abdel-Gawad, 1993; Njoumi *et al.*, 2019; Vidal-Valverde *et al.*, 2002). In the present study, soaking lentils for 3 h at 30 °C pH 6.0 reduced α-galactoside content by 16%. Independently of the germination time and condition, there was always a decrease in α-galactoside content in lentils. For instance, the total α-galactoside content decreased by up to 29.5% and 47.5% after 12 h and 24 h of germination on lentil seeds. The ANOVA showed that the interaction of light and water had a significant ($p < 0.05$) negative effect on the total α-galactoside content when lentils were germinating for 12 h; α-galactoside decreased under light and with high water content. α-galactoside content decreased after germination for 24 h, but no significant impact of parameters was found ($p > 0.05$). Vidal-Valverde *et al.* (2002) reported that germinating lentils at 20 °C reduced the total α-galactoside content by 90% after 2 days, but no effect of light was evidenced during germination.

In the present study, the total α-galactoside content in cowpea seeds was 7.41 g/100 g (db) (Table 5). In the literature, α-galactoside content in cowpea ranged between 2.56 and 4.66 g/100 g (db) (Akissoé *et al.*, 2021; Coffigniez *et al.*, 2018; Ibrahim *et al.*, 2002; Nnanna & Phillips, 1988). However, Akinlosotu & Akinyele, (1991) found a total α-galactoside content of 9.54 g/100 g, which is in agreement with our findings. Soaking cowpea seeds for 5 h reduced α-galactoside content by 27%. Germination had a more positive effect in cowpeas than in lentils, with an up to 99% reduction in the total α-galactoside content. Between 12 h and 24 h of germination, there was a decrease of up to 97% in α-galactoside. These results show germination is an effective way to reduce α-galactoside content in pulses, with evidence that the longer the process, the greater the reduction. Ibrahim *et al.* (2002) showed that germinated cowpea seeds at room temperature for 24 h reduced total stachyose and raffinose contents by 100%, which is in agreement with our results. However, other authors obtained different results: for instance, a 25% reduction in total α-galactoside content after 24 h of germination at room temperature (Akinlosotu & Akinyele, 1991). However, when cowpea seeds were germinated

for 48 h, the authors reported similar results to ours (-92%), (Akinlosotu & Akinyele, 1991). The difference in the results is due to the different germination methodology and temperature conditions used. The results of ANOVA showed that temperature, light, water, and their interaction had a significative negative impact ($p < 0.05$) on the total α-galactoside content in germinating cowpea (12 h). These results indicate that increasing the values of the factors studied reduced the total α-galactoside content. The temperature had a significant ($p < 0.05$) negative impact on the total α-galactoside content in cowpea germinated for 24 h. We can thus conclude that the conditions used in the present study favor the reduction of α-galactoside in cowpea, and higher temperatures should be preferred.

The α-galactoside profiles of dry, soaked, germinating (12 h) and germinated (24 h) lentil and cowpea seeds were homogenous (Figure 4). Stachyose was the main α-galactoside identified accounting for more than 80% of the total concentration. The α-galactoside profile followed the same trend, independently of the germination conditions. Figure 3 shows the average of each vitamer at 12 h and 24 h of germination in all 11 conditions studied. In the literature, stachyose was also the main α-galactoside found in lentil and cowpea seeds (Akissoé *et al.*, 2021; Coffigniez *et al.*, 2018; Ibrahim *et al.*, 2002; Njoumi *et al.*, 2019; Vidal-Valverde *et al.*, 2002).

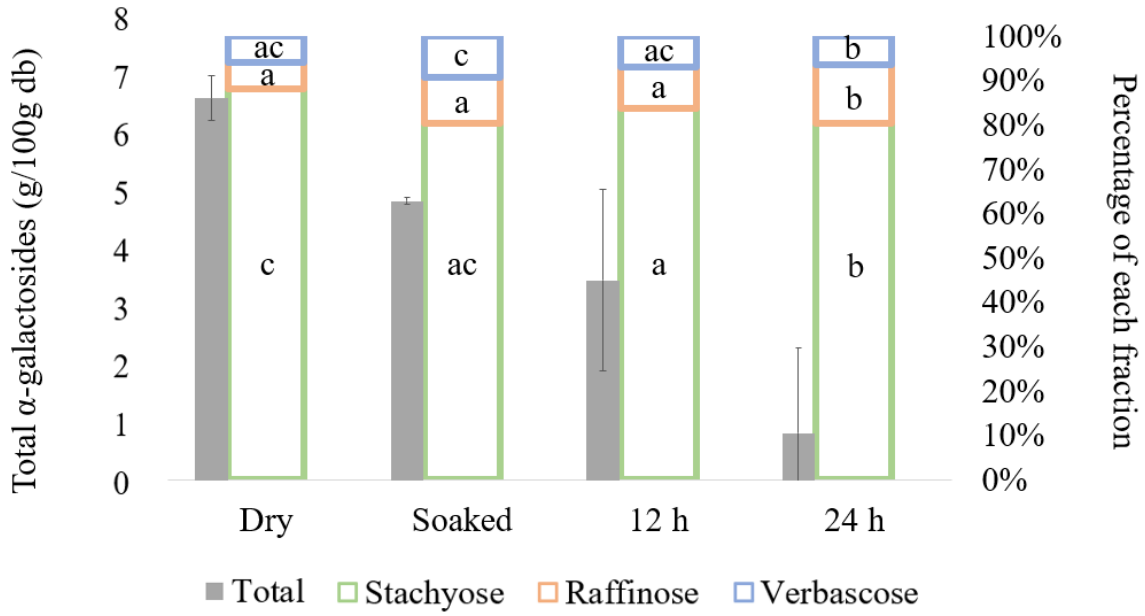


Figure 4. Total α-galactoside content (gray) in g/100 g (db) and the percentage of each fraction, stachyose (green), raffinose (orange), and verbascose (blue), in dry, soaked, germinating (12 h) and germinated (24 h) cowpea seeds. Letters indicate statistically (Tukey-HSD test) significant difference ($p < 0.05$) within each compound in comparison with each sample.

III.3. Optimal germination process

Using the RSM models, it was possible to optimize the parameters during germination in lentil and cowpea seeds. For this purpose, the principal vitamer and α-galactoside was used for each seed variety. By maximizing thiamine (Thia-HCL) and reducing stachyose content at 24 h of germination, the optimal germination condition for lentil was shown to be 25 °C, without light, and with 80% water content. In these conditions, the thiamine, folate, and α-galactoside contents were 388 µg/100 g (db), 157 µg/100 g (db), and 2.97 g/100 g (db), respectively (Table 7). Thiamine and folate contents increased by 152% and 6%, respectively, while total α-galactoside content decreased by 47%. The recommended daily intakes (RDI) of thiamine and folate are 1100-1500 µg/day and 200-400 µg/day for adult, respectively (Chunming *et al.*, 2001; Krawinkel *et al.*, 2014; Moll & Davis, 2017). In the above-mentioned conditions, consuming 100 g of germinated lentils will cover 26% and 39% of the RDI of thiamine and folate, respectively. In addition, the low water content may help reduce microbiological risk during germination. By maximizing the 5-CH3-H4folate and reducing the total α-galactoside content at 24 h of germination, the optimal germination condition for cowpeas was shown to be at 35 °C, with 12 h of light, and 140% water content. In these conditions, the thiamine, folate, and α-galactoside contents were respectively, 979 µg/100 g (db), 437 µg/100 g (db), and 0.07 g/100 g (db) (Table 3). Between dry and germinated seeds, the thiamine and folate content increased by 5% and 33%, respectively, and the total α-galactoside decreased by 99%. Under these germination conditions, eating 100 g of germinated cowpea seeds will cover 65% and 109% of the RDI of thiamine and folate, respectively.

In our study, we optimized the germination *sensu stricto* to increase the nutritional quality of lentil and cowpea. Germination *sensu stricto* has an advantage because it requires less time (24 h), than sprouting (2 to 6 days). We suggest that reducing germination time increases vitamin content and reduces microbiological risks. For instance, sprouts are highly perishable and harbor bacterial pathogens such as *Salmonella* and *Escherichia coli* (Miyahira & Antunes, 2021). It is known that cell populations of these bacteria increase with an increase in the length of the germination period (Liu *et al.*, 2018). To confirm this hypothesis and the safety of germination, microbiological analyzes should now be undertaken. Seeds that undergo germination *sensu stricto* must undergo thermal treatment before being edible. We suggest that cooking also helps to reduce the microbiological risk of germinated seeds, in comparison to

sprouting. Moreover, adding germination as a pre-treatment reduces the negative impact of thermal treatment. For instance, folate content is 52% higher in germinated-canned fava beans than conventional canned (Hefni *et al.*, 2015). The results of our study could be used to model the nutritional behavior of lentil and cowpea seeds during food processing. The impact of other kinds of food processing, such as soaking/cooking/autoclaving, should also be modeled. A first model to predict folate and α-galactoside activity during soaking-sprouting-cooking in cowpea seeds was recently published (Coffigniez & Briffaz, 2023), with sprouting at 30 °C for 72-92 h, followed by cooking (95 °C or 100 °C) for less than 30 min. This type of model will advance our understanding and check the effects and benefits of germination *sensu stricto* as a pre-treatment before processing of pulses into food.

Table 7. Final thiamine-equivalent, total folate, and total α-galactoside contents in lentil and cowpea after soaking and optimized germination.

	Lentil				***Cowpea***			
	Temperature, light, water content, duration	Thiamine-eq µg/100 g	Folate µg/100 g	α-galactoside g/100 g	Temperature, light, water content, duration	Thiamine-eq µg/100 g	Folate µg/100 g	α-galactoside g/100 g
Soaking	30 °C, 0 h, 3 h	322 (+ 109%)	121 (-17%)	4.65 (-16%)	30 °C, 0 h, 5 h	967 (+ 4%)	362 (+ 10%)	5.42 (-27%)
Germination	25 °C, 0 h, 80%, 24 h	388 (+ 152%)	157 (+ 6%)	2.97 (-47%)	35 °C, 12 h, 140%, 24 h	979 (+ 5%)	437 (+ 33%)	0.07 (-99%)
RDI		1100 – 1500 µg/100 g/day	200-400 µg/100 g/day			1100 – 1500 µg/100 g/day	200-400 µg/100 g/day	

RDI = Recommended daily intake

IV. Conclusion

Our study was the first to simultaneously analyze the impact of temperature, light, and water on the nutritional quality of lentil and cowpea. Temperature and water showed a more significant impact on the nutritional quality when compared to light. This finding could provide valuable insights for researches to consider including water content as a critical factor in their germination analyses. The use of RSM models allowed for the optimization of the technological parameters. For lentils, low temperature, no light, and low water content should be used. On the other hand, cowpea germination should be at higher temperature, light, and water content. Optimized germination increased thiamine content by 152% in lentils and by 5% in cowpeas. In addition to increasing the total thiamine content, germination changed its vitamer profile in lentil by increasing TDP through a metabolic shift. Germination increased folate content by up to 6% and 33% between dry and germinated lentil and cowpea seeds, respectively. The 5-CH3-H4folate content increased in both seeds during germination. This vitamer is the main form of folate found in germinated lentil and cowpea and is produced for seed metabolism. The α-galactoside content decreased by up to 47% in germinated lentil seeds and by 99% in germinated cowpea seeds. In both seeds, stachyose was the main form of α-galactoside found at all the germination times and conditions. To further advance our understanding of germination, we now need to analyze the metabolomics changes that take place during germination to identify the different metabolites used by the seed to produce nutrients. This will provide more accurate information about the different chemical pathways used during germination. This information can be used to improve the nutritional quality of pulses, even by industrial food companies as a novel strategy to process pulses.

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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V. Enhancing nutritional value through seed germination: an integrated proteomics and metabolomics insight into cowpeas

In the previous chapter, we observed that germination *sensu strico* effectively increased thiamine and folate levels, while reducing α-galactoside content in lentils and cowpeas. The remaining question is how temperature, light, and water content influence the metabolic pathway during pulse germination and specifically, which metabolites and enzymes are synthesized or degraded during this process to induce these metabolic changes. To address this, we performed a metabolic and proteomic analyses. The aim of the next study was to gain a comprehensive insight into germination *sensu stricto*, identify key metabolites and enzymes involved in the germination process, and elucidate the metabolic pathways associated with amino acids and sugars. Furthermore, we investigated the effects of temperature, light, and water content on the metabolic and proteomic outcomes. We also evaluate the alterations in the levels of essential amino acids, α-galactoside, and myo-inositol during cowpea germination. While the integration of omics studies has found extensive application in model plants, such as *Arabidopsis,* investigations on pulse crops of agronomic importance, especially in tropical and subtropical regions, remain relatively limited. In this context, our study stands out as the first to employ integrated metabolomics and proteomics technologies to investigate how germination conditions, including temperature, light, and water content, affect the metabolic pathways and nutritional compounds of cowpea seeds. These findings facilitate the optimization of germination conditions to enhance the nutritional quality of pulses. Additionally, they contribute to a deeper comprehension of the complex impacts of germination on seed metabolism. This valuable information serves as a foundation for subsequent investigations in this field, illuminating the complex correlation between germination and seed nutritional value. The outcome will benefit the food industry in developing new processing technologies and new food products.

Enhancing nutritional value through seed germination: an integrated proteomics and metabolomics insight into cowpea

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Abstract

Cowpea (*Vigna unguiculata*), originated from southern Africa, is a robust and adaptable pulse crop that grows in arid and tropical regions. It serves as a vital source of nutrition, serving as an excellent source of proteins, carbohydrates, fibers, minerals, and vitamins. This pioneer study used integrated metabolomics and proteomics to investigate the effects of germination conditions (*i.e.* temperature [25 °C to 35 °C], light exposure [0 -12h], and water content [80% - 140%]), on the content of essential amino acids, α-galactosides, and phytic acid levels in cowpea seeds. Additionally, conducting a joint-pathway analysis enabled the identification of pivotal metabolites and proteins involved in essential amino acid and sugar metabolisms. Some key identified proteins, including dehydrin ERD14 (early response to dehydration 14), glutathione S-transferase (GST), protein SLE1, SLE2, and betaine aldehyde dehydrogenase 1 (BADH1), may played a significant role in influencing cowpea germination responses and sensitivity to abscisic acid, temperature stimulus, and water deprivation. Germinated cowpeas at 35 °C with 140% of water content (WC) displayed an increase in essential amino acids by 2.5 - 17-foldchange. Under these conditions, as well as at 25 °C with 140% WC, raffinose content reduced by 5.2-foldchange and stachyose content was not detected. In addition to reducing α-galactoside content, germination was an effective process for reducing phytate content, as indicated by the observed 3.5-fold increase in myo-inositol content. These insights can be valuable for optimizing the germination conditions to enhance the nutritional quality of pulses. Additionally, it can provide a deeper understanding of the impact of germination on seed metabolism, benefiting the food industry and consumers.

Keywords: Germination, Cowpea, Metabolic, Proteomics, Amino acids, Antinutritional compounds

I. Introduction

Cowpea (*Vigna unguiculata*), originated from southern Africa, is a diploid member of the Fabaceae family with a 2n = 22 chromosome and a previously estimated genome size of 640 Mb (Lonardi *et al.,* 2019). This versatile and resilient pulse crop grows in arid and hot regions of the tropics and subtropics, such as West Africa and northeastern Brazil (Singh, 2014). Nigeria and Niger stand out as the largest producers and consumers of cowpea, producing around 2.7 million and 1.2 million tons annually, respectively (FAO, 2023). Cowpeas, one of the major pulse crops in low- and middle-income countries (LMICs), ensure food and nutritional security. In addition to its economic benefits, cowpea seeds have remarkable nutritional qualities, serving as an excellent source of protein, carbohydrate, fiber, minerals, and vitamins. Furthermore, this pulse exhibits low glycemic index and fat content (Abebe & Alemayehu, 2022; Avezum *et al.,* 2022). In LMICs, conventional diets are centered around cereals, yet they frequently lack sufficient protein and essential amino acid content (Harris *et al.*, 2022; Karanja *et al.,* 2022; Mann *et al.,* 2007). Cereal grains contain a lower amount of protein than pulses, ranging between 7% and 18% dry matter (Poutanen *et al.,* 2022). Cowpea seeds contain high amounts of protein, ranging from 17% up to 40%, representing more than two to four times the total protein content found in cereals and tubers (Dakora and Belane, 2019). Incorporating cowpeas into conventional cereal-based diets in LMICs contributes to a more balanced intake of essential amino acids (EAA) while also enhancing protein content.

Similar to other pulses, cowpea seeds contain antinutritional factors that diminish their nutritional quality. A notable example is α-galactosides, which is derived from sucrose and contains 1,3-galactose units linked by α (1→6) bonds (Martínez-Villaluenga *et al.*, 2008). They belong to the raffinose family oligosaccharides (RFOs) and are mainly represented by galactinol, raffinose, stachyose, and verbascose. They accumulate in seeds during maturation, and constitute approximately 5% of their dry weight (Bessada *et al.*, 2019; Naczk *et al.*, 1997). The enzyme α-galactosidase breaks down α-galactosides into galactose and sucrose (Veldman *et al.,* 1993). However, because of the absence of α-galactosidase in the intestinal mucosa of humans and animals, RFOs escape digestion and are metabolized by colon bacteria, producing hydrogen, carbon dioxide, and methane, causing flatulence and gut gas production (Bessada *et al.*, 2019; Naczk *et al.*, 1997). Phytic acid is another major antinutritional compound found in pulse seeds. This compound consists of myo-inositol hexakisphosphate and serves as the

principal phosphorus reservoir in plants, accounting for up to 85% of total phosphorus content (Campos-Vega *et al.,* 2010; Oatway *et al.,* 2001). Phytic acid binds to minerals, proteins, and starch, altering their solubility and functionality, ultimately reducing their availability and digestibility (Oatway *et al.,* 2001).

Germination is an effective process for increasing the nutritional quality of pulses by releasing free amino acids and reducing antinutritional compounds (Avezum *et al.,* 2022; Benincasa *et al.,* 2019). For plant physiologists, germination *sensu stricto* begins with water uptake by the dry seed and ends with the start of elongation of the embryonic axis, resulting in testa (seed coat) rupture and radicle protrusion (Bewley and Black, 1994; Ribeiro-Oliveira *et al.,* 2021). During germination, major metabolic modifications occur that change the biochemical, nutritional, and sensory characteristics of the seeds. Upon imbibition, seed initiates a series of metabolic events, including the activation or synthesis of hydrolytic enzymes. As a result, stored compounds such as proteins, starch, lipids, hemicellulose, α-galactosides, and phytic acid, are hydrolyzed into forms that can be readily available for the embryo metabolism. For instance, the activation of proteolytic enzymes during germination breaks down seed storage proteins into small peptides and free amino acids which are then utilized by the embryo for *de novo* protein synthesis and various other metabolic processes. Indeed, several studies have reported that germination *sensu stricto* and sprouting (*e.g.* post-germinative processes including seedling growth) increase the free amino acid content by up to 450% (Gu *et al.,* 2017; Wu *et al.,* 2020; Wu *et al.,* 2023). Moreover, during germination, RFOs are hydrolyzed to release sucrose, glucose, and galactose, which are used as energy by seedlings (Akinlosotu & Akinyele, 1991; Oboh *et al.,* 2000; Vidal-Valverde *et al.,* 1992). Phytic acid is hydrolyzed into myo-inositol (P5-P1) by phytase (*i.e.* phosphatase enzymes) to release free myo-inositol and inorganic phosphorus compounds. Myo-inositol is used during germination to meet the biosynthetic needs of growing tissues and cellular formation (Oatway *et al.,* 2001). Germinated pulses reduced the content of RFOs by 62 - 100%, while reducing phytic acid content by 17 - 47% (Ibrahim *et al.,* 2002; Mehanni *et al.,* 2021; Sofi *et al.,* 2020; Wu *et al.,* 2020).

Our study is the first to use combined metabolomic and proteomic approaches to explore the impact of germination conditions (*i.e.* temperature, light, and water content) on the nutritional profile of cowpea seeds. Our primary objective was to analyze whether germination enhances the content of essential amino acids while simultaneously reducing α-galactosides and phytic acid levels in cowpeas. Moreover, our aim was to gain a comprehensive insight into

germination *sensu stricto* and to identify key metabolites and enzymes involved in the germination process, as well as the metabolic pathways of amino acids and sugars. A previous study used metabolomics and transcriptomics to investigate the accumulation of anthocyanins and flavonoids in cowpea pods (Li *et al.*, 2020). However, this study did not investigate the effects of germination. While the integration of omics studies has been extensively used in model plants such as Arabidopsis, investigations in pulse crops of agronomic significance, particularly within tropical and subtropical regions, remain relatively underexplored (Ramalingam *et al.,* 2015). Nevertheless, there is growing interest in the use of omics technologies to enhance breeding program, increase productivity and strengthen the resilience of these crops (Ali *et al.,* 2022; Nayak *et al.,* 2021). Within this context, the use of combined metabolic and proteomics has allowed us to better understand the metabolic pathway, identify the key proteins involved, and provide insights into the biochemical changes that occur during cowpea germination.

II. Materiel and methods

II.1. Biological Material

Cowpea (*Vigna unguiculata* [L.] Walp.) were purchased directly from the producer in South East Benin. Cowpea seeds were sowed in November 2021 and harvested in February 2022. Seeds were stored at -20 °C until use.

II.2. Germination process

The experiment followed the method as described by Avezum *et al.* (2023). In brief, cowpea seeds (80g) were soaked in 320 mL of water (1:4 w/v ratio, pH 6.0) at 30°C for 5 hours to complete the water uptake phase and initiate germination. After soaking, the seeds were drained and placed in a controlled environment for germination. The study employed a factorial

experimental design with three variables (*i.e.* temperature, light, and water content) at two levels (Tabla 1). The conditions for cowpea germination were maintained in a BINDER-KMF 240 chamber with 95% relative humidity. Water was manually sprayed after 18 hours, with 60 mL for 110% water content (db) and 120 mL for 140% water content (db), ensuring precise control of moisture levels. Weight measurements were taken hourly to track water content. A white light emitting diode (LED) light source was used. After 24 h, the seeds were sorted into non-germinated (no radicle), germinated (radicle < 5 mm), and sprouted seeds (radicle > 5 mm). It is worth noting that the -omic analyses focused exclusively on germinated seeds. Cowpea seeds were collected and directly stored at -40 °C. All the seeds were freeze-dried and then ground in a QIAGEN – TissueLyser II ball mill grinder to obtain a fine flour. The flour was freeze-dried and stored at -20 °C until further analyses. The label used for the germinated samples follows this pattern: g_temperature conditions_light duration_water content_24 h of germination (Table 1).

Table 1. Experimental plan for cowpea germination over a 24-hour period

Assay	Temperature (°C)	Light (h)	Cowpea WC (% db)	Label germinated samples
1	25	0	80	g_25_0_80_24
2	35	0	80	g_35_0_80_24
3	25	12	80	g_25_12_80_24
4	35	12	80	g_35_12_80_24
5	25	0	140	g_25_0_140_24
6	35	0	140	g_35_0_140_24
7	25	12	140	g_25_12_140_24
8	35	12	140	g_35_12_140_24
9 (C)	30	6	110	g_30_6_110_24

II.3. Metabolome Analysis by Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

Metabolite samples were prepared from three replicates of 20 mg of dry, soaked or germinated cowpea flour (section II.2). Each sample was placed in 2 mL Safelock Eppendorf tubes (Eppendorf AG, Hamburg, Germany). The frozen ground samples were resuspended in 1 mL of water/acetonitrile/isopropanol (2:3:3) at -20 °C, supplemented with ribitol at 4 µg/mL, and extracted at 4 °C with shaking at 1400 rpm for 10 minutes in an Eppendorf Thermomixer (Eppendorf). Centrifugation at 20,000× g for 5 minutes was performed to remove insoluble material. Twenty-five microliters of the resulting extract were collected, dried for 150 minutes using a SpeedVac (Savant SPD131DDA, Thermo Fisher Scientific, Waltham, MA, USA), and stored at -80 °C. Prior to further analysis, the samples were thawed at room temperature for 15 minutes and then dried for an additional hour in the SpeedVac before adding 10 µL of 20 mg/mL methoxyamine in pyridine to the samples, blanks, amino acid, and sugar standards. The reaction was conducted for 90 minutes at 28 °C with continuous shaking in the Eppendorf thermomixer. Subsequently, 90 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were added, and the reaction was continued for 30 minutes at 37 °C. After cooling, 50 µL of the sample were transferred to an Agilent vial for injection. Two hours after the derivatization process, 1 µL of the sample was injected in splitless mode into an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer. The column used was a Rxi-5SilMS from Restek (30 m with 10 m integra-guard column). Before each series of analysis, the liner (Restek # 20994) was changed, and 10 cm of the column was cut. The oven temperature ramp started at 70 °C for 7 minutes and then increased by 10 °C/min to 325 °C, which was held for 4 minutes (total run length: 36.5 minutes). The helium constant flow was set at 1.52 mL/min. The following temperatures were used: injector at 250 °C, transfer line at 290 °C, source at 250 °C, and quadripole at 150 °C. The samples and blanks were randomized during the analysis. Amino acid standards were injected at the beginning and end of the analysis for monitoring the derivatization stability. Additionally, an alkane mix (C10, C12, C15, C19, C22, C28, C32, and C36) was injected into the middle of the queue for external retention index (RI) calibration. A data acquisition rate of five scans per second was employed. The raw Agilent data files were converted into NetCDF format and analyzed with AMDIS (http://chemdata.nist.gov/mass-spc/amdis/). Metabolite identification was performed using a home retention indices/mass spectra library. Peak areas were then determined using the QuanLynx software (Waters

Corporation, Milfords, MA, USA) after converting the NetCDF files into MassLynx (Waters) format.

II.4. Shotgun Proteomics

II.4.1. Protein Extraction

The protein extraction procedure was conducted in accordance with Hervé *et al.* (2006), with slight modifications described here. The phenol solution was obtained from Amresco-Interchim, Biotechnology Grade, saturated with Tris-HCL at pH 7.9. The extraction buffer was prepared using 0.5 M Tris-HCL at pH 7.5, 0.7 M sucrose, 50 mM EDTA, 0.1 M KCL, and 10 mM Thiourea. This solution was stored at 4 °C during the analysis. Prior to extraction, 2% (v/v) 2-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride (PMSF) were added to the extraction buffer. The precipitation solution was prepared using 0.1 M ammonium acetate in cold methanol and stored at -20 °C. The protein solubilization buffer was prepared with 6M urea, 2 M thiourea, 10 mM Dithiothreitol, 30 mM Tris-HCL at pH 8.5, and 0.1% RapiGest SF (Waters).

The protein extraction procedure started by suspending 50 mg of seed flour in 5 mL of extraction buffer within a 30 mL tube, which was then thoroughly mixed using vortexing. The solution was incubated on a shaker at 2500 rpm for 5 min at 4 °C to well hydrate the sample. Following this, an equivalent volume of Tris-buffered phenol was introduced, and the solution was placed on the shaker for a 30 min at 4 °C. To separate insoluble material, for aqueous and organic phases, the sample was centrifuged for 30 min at 6000 rpm at 4 °C. The phenolic phase, which naturally rises to the top due to the use of saccharose, was carefully recovered to prevent contact with the interphase. This recovered phase was transferred into a new 30 mL tube, then, the phenol phase was back-extracted with 5 mL of extraction buffer. The sample was shaken at 2500 rpm for 30 min at 4 °C, and centrifuged for 30 min at 6000 rpm at 4 °C. The phenol phase, still on the top of the tube, was carefully recovered, transferred into a new 30 mL tube and 20 mL of precipitation solution was added. The tube was shaken by inverting, and the sample was incubated overnight at -20 °C. Proteins were finally pelleted by centrifugation at 6000 rpm for

30 min at -10 °C. After, the solution was removed and the pellet was washed with cold methanol. After centrifugation at 6000 rpm for 5 min at -10 °C, the solution was finally washed with cold acetone and recentrifuged at 6000 rpm for 5 min at -10 °C. Lastly, the pellet is dried under the hood at room temperature. The analysis was performed in triplicate for each sample.

II.4.2. Protein Quantification

The final pellet was resuspended in 300 µl of protein solubilisation buffer. The pellet was broken up using a spatula and the tube was shaken at 2500 rpm at room temperature. Finally, after centrifugation at 6000 rpm for 25 min, the supernatant was transferred to a new tube. Protein concentration was estimated using 2D Quant Kit (Cytiva Life Sciences™) using bovine serum albumin as standard.

II.4.3. LC-MS/MS Analysis

The protein concentration was adjusted to 4 µg/µL, and 40 µg of protein were digested with trypsin in solution as described in Balliau *et al.* (2018). After equilibrating at room temperature for 30 minutes, the sample was alkylated by adding 2 µL of 300 mM iodoacetamide in 50 mM ammonium bicarbonate (resulting in a final concentration of 50 mM iodoacetamide in the sample) for 1 hour in the dark. It was then diluted with 90 µL of 50 mM ammonium bicarbonate. Next, 800 ng of trypsin was added and the mixture was incubated overnight at 37 °C. Digestion was stopped by adding TFA to a final concentration of 1%. The protein digest was desalted using C18 solid phase extraction cartridges (Strata™-XL 30 mg, Phenomenex) following these steps: first, the cartridges were conditioned with 500 µL of acetonitrile and equilibrated three times with 500 µL of a solution containing 2% acetonitrile and 0.6% acetic acid in water. Next, the sample was diluted with a solution containing 2% acetonitrile and 0.6% acetic acid in water and loaded onto the cartridges. The cartridges were then rinsed four times with 500 µL of a

solution containing 2% acetonitrile and 0.6% acetic acid in water. Peptides were eluted using 2 aliquots of 300 µL of a solution containing 70% acetonitrile, 0.6% acetic acid in water, and then dried using a SpeedVac SPD111V concentrator (Thermo Scientific). The peptides were solubilized in a solution containing 2% acetonitrile and 0.1% formic acid to achieve a concentration of 0.1 µg/µL based on the amount of digested protein. The peptides were injected into a Q Exactive Plus mass spectrometer (Thermo, Waltham, MA, USA) coupled to a nanoHPLC nano ultra 2D (SCIEX). A total of 400 ng of peptides were loaded onto a trap column (NanoSeparation Biosphere C18, 5 µm, 120Å, 360/100 µm, 20 mm) and rinsed for 8 minutes with a solution containing 2% acetonitrile and 0.1% formic acid in water, at a flow rate of 5 µL/min. The peptides were then eluted onto a C18 column (NanoSeparation Biosphere C18, 3 µm, 120Å, 360/75 µm, 300 mm) at a flow rate of 300 nL/min, using a linear gradient from 5% of 0.1% formic acid, 99.9% acetonitrile (buffer A) to 35% of buffer B (95% 0.1% formic acid, 99.9% water). The column was subsequently regenerated with 5% of buffer A in 95% buffer B for 10 min, and then equilibrated with 95% of buffer A in 5% of buffer B for another 10 min. The eluted peptides were ionized using electrospray at 1600 V with a non-coated capillary needle (non-coated capillary silica tips, 360/20-10, New Objective Inc., Woburn, MA, USA). Data were acquired using Xcalibur v4.0 in data-dependent mode. Full MS was performed at a resolution of 70,000, with an AGC target of $3x10^6$ for ions between 350 and 1400 m/z. MS2 scans were conducted for the eight most intense ions, with a threshold of $8.3x10^3$ for ions with charges between 2 and 4. Dynamic exclusion was set to 50 seconds, and peptide match was set to the preferred mode. Fragmentation was performed with a normalized collision energy of 27, isolation windows set to 1.5 m/z, and AGC target to $1x10^5$ with a maximum ion time of 120 ms. The scan resolution was set to 17,500. Rawdata were converted into mzXML file using ProteoWizard 3.0.18344 and centroided using vendor algorithm option. Protein identification was performed with X!Tandem version 2015.04.01.1 (Craig and Beavis, 2004) against *Vigna unguiculata* database (NCBI genome assembly ASM411807v2 ; https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_004118075.2/) and a custom contaminant database (trypsin, keratine …). Enzymatic cleavage was set to trypsin mode with proline exclusion in strict mode allowing for 1 potential missed cleavage. In the first pass, cysteine carbamidomethylation was set as a static modification, methionine oxidation as a potential modification, Protein N-terminal methionine excision with or without N-terminal acetylation, pyroglutamic acid formation from N-terminal glutamine or glutamic acid, and N-terminal pyro-carbamidomethylated cysteine. A refinement was performed allowing for up to 5 potential missed cleavages. Identification filtering and protein inference were performed

using I2masschroq (Langella *et al.*, 2017) version 0.4.70. For a protein to be considered, it must have been identified with an E-value lower than 0.00001 and with two different peptides, each with an E-value lower than 0.01. The resulting FDR was 0.2 at the peptide level and 0.08 at the protein level. Peptide quantification by ion intensity was conducted using MassChroQ V2.4.15 (Valot *et al.*, 2011). Chromatogram alignment was executed using a sample composed of a mixture of all samples in the experiment, by comparing deviations of identified peptides. XIC extraction was performed for 80% of the theoretical natural isotopic profile. Only the theoretical isotope with the highest abundance was used for quantification. Protein abundance for XIC ion quantification was performed only with specific peptides with less than 10% missing data, a standard deviation of retention time less than 20 seconds, and a correlation for peptides of the same protein greater than 0.5. The protein abundance was computed as the sum of peptides after normalization as described in (Lyutvinskiy *et al.*, 2013). Only proteins quantified by at least two peptides were considered.

II.5. Statistical analysis

Metabolic and proteomic data analyses were carried out using R software. To detect differences between two conditions ($p < 0.05$), ANOVA and pairwise Tukey tests were performed using R package "multcomp" (https://cran.r-project.org/web/packages/multcomp/index.html). Significant metabolites and proteins were visually represented using principal component analysis (PCA) through the "FactoMineR" package (http://factominer.free.fr/index_fr.html). Hierarchical clustering was performed using the "pheatmap" function in R (https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topics/pheatmap). Clustering in metabolic and proteomic data analysis groups a set of metabolites or proteins together based on their similarities. Essentially, it organizes them into groups (clusters), where the members within the same cluster are more similar to each other in terms of their properties or behaviors than to those in other clusters.

Differential metabolites were quantified using log2 fold change compared to dry seed and utilized for generating volcano plots using the R packages "gridExtra" and "purr". In the volcano plot, we use log10(p-value) as a measure to assess the statistical significance of each

data point. By taking the logarithm (base 10) of the p-value, we transform it into a more visually interpretable scale.

Proteomic clustering enrichment was conducted using "compareCluster" (clusterProfiler, v3.10.1, https://guangchuangyu.github.io/software/clusterProfiler/). Enrichment analysis was used to identify proteins that exhibited significantly upregulation and downregulation in one sample compared to another, particularly in critical biological functions. In our study, we conducted comparisons between dry seeds and soaked or germinated (specifically the sample g_35_0_140_24, representing the optimal germination conditions as indicated in our last study (Avezum *et al.*, 2023)). Additionally, we conducted the enrichment analysis between each germination conditions to analyze the effect of temperature and water (light effect was neglected). Please note that not all graphs generated in this study are included in this article. The Metaboanalyst website (https://www.metaboanalyst.ca/) was used for joint-pathway investigations. The joint-pathway analysis was based on the corresponding protein ID of *Arabidopsis thaliana* (*thale cress*).

III. Results

III.1. Germination Rate

In our last study, we demonstrated that temperature and water content had a significant positive impact ($p < 0.01$) on the germination rate of cowpeas (Avezum *et al.*, 2023). The highest germination rate for cowpea was reached at 35 °C and under water saturation (140%) (g_35_0/12_140_24) (Table 2). Under low temperatures (25 °C) and limited water content (80%), the cowpea seeds did not germinate. Cowpeas are mostly cultivated in tropical regions, such as West Africa and northeastern Brazil. Therefore, our results indicated that the cowpea genotype used in our experiments are well suited to their natural environment and are mostly susceptible to chilling stress and drought stress.

Table 2. Germination rate in cowpeas (total rate) after 24 h (Avezum *et al.,* 2023).

T (°C)	Light exposure (h)	Cowpea WC (% db)	Total germination rate for cowpea (%)
25	0	80	0
35	0	80	23
25	12	80	0
35	12	80	33
25	0	140	41
35	0	140	76
25	12	140	60
35	12	140	83
30	6	110	45 ± 5.1

III.2. Metabolomics

Through metabolomic quantification, we quantified a total of 244 different metabolites, out of which 212 were significantly different ($p < 0.05$) in their accumulation levels. Of the significant quantified metabolites, 40% were not identified. After eliminating the unidentified metabolites (127), the most abundant metabolite class were organic acid (17%), free amino acids (15%), and carbohydrates (9.4%). The remaining identified metabolites were distributed as follows: phosphoryl compounds (3.3%), sterols (3.8%), free fatty acids (1.9%), flavonoids (0.9%), and others compounds (9.4%). These results provide valuable insights into the metabolic profile of the samples and highlight the prominence of organic acids, amino acids, and carbohydrates among the identified metabolites. Further investigation of the unidentified metabolites could offer deeper understanding of the complex metabolic processes occurring during germination process in cowpea. The 212 significantly metabolites were used to plot

Principal Component Analysis (PCA) (Figure 1). The PCA is useful to provide an understating of the overall metabolite differences between groups and germination conditions and the variability between samples within a group. In figure 1 the distance between two circles indicates the correlation of two treatment effects. The first dimension (Dim1) explained 44% of the total variance, while the second (Dim2) dimension explained 13.2%. The results showed that triplicate was closely clustered (three trial and the mean), indicating that the experiment was reproducible and reliable. On Dim1 the dry, soaked, and germinated (25 °C with 80% WC, with or without light) seeds were plotted on the left side and separated from the others germination conditions. On the left side of the plot, no germination was observed (Avezum *et al.*, 2023), indicating a similar metabolite profile pattern. It was thus clearly observed that increasing water content had an impact on the metabolism of cowpea seeds, favoring the germination process. There was no light variance in Dim1 nor in Dim2, but temperature clearly influenced the metabolism of cowpea seeds as evidenced on Dim2: low temperature seedling was plotted on the low side of Dim2.

In according to these results, the heatmap analysis showed the shift of metabolism during germination; 9 classes of compounds were plotted (Figure 2). Various metabolites (classes 1-3) were highly expressed in dry, soaked, and germinated seeds at 25 °C with 80% WC (db), regardless of light exposure whereas classes 4-9 were less expressed within these samples. These same metabolites (classes 4-9) showed higher expression levels when cowpeas were germinated at high temperature and water content.

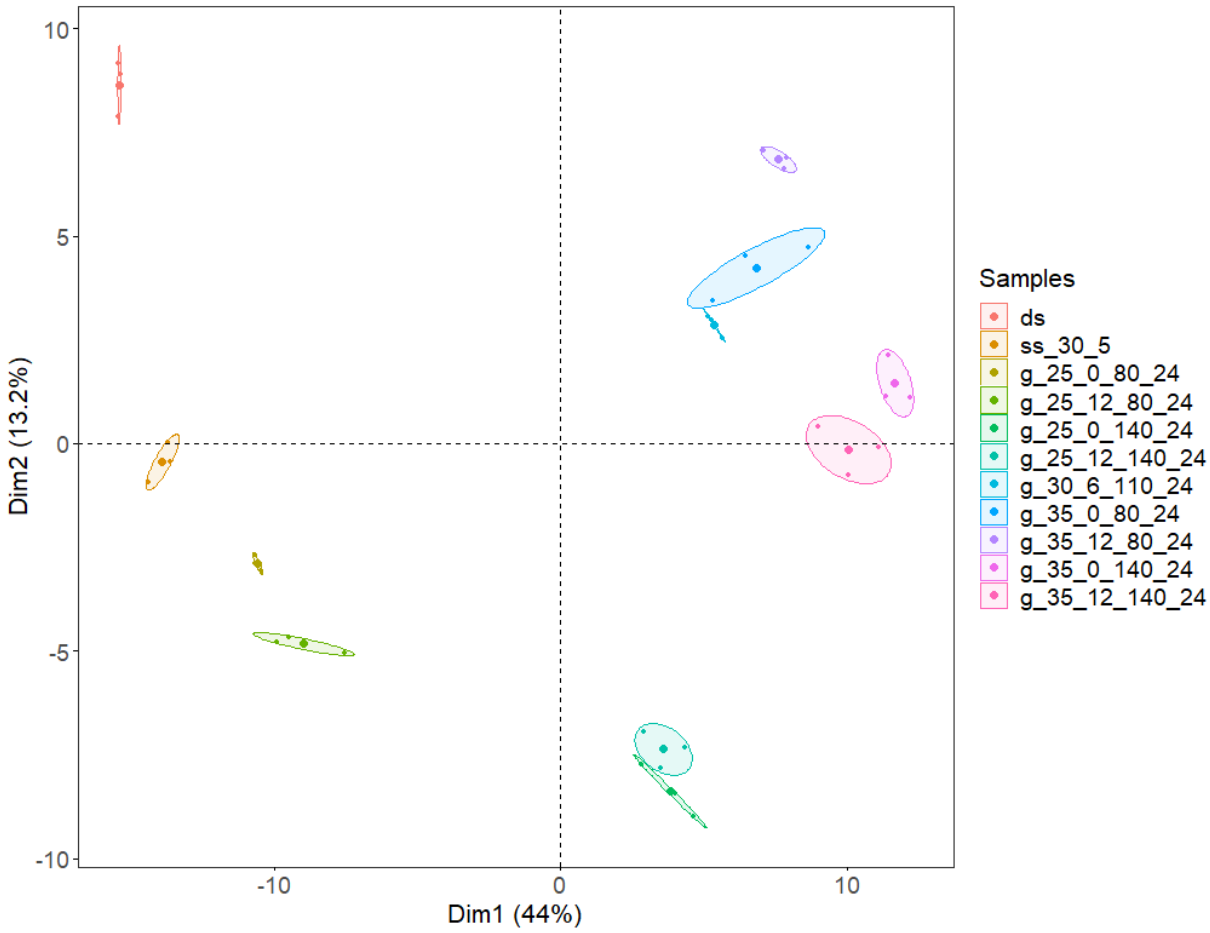


Figure 1. Principal Component Analysis (PCA) of metabolites in cowpea seeds. The percentage of explained variance is labeled along each axis. Prior to PCA computation, all values were centered and scaled. The plot is based on 212 significantly differentially accumulated metabolites, offering insights into the overall metabolite variations among groups and germination conditions, as well as the variability within sample groups. Dry seeds (ds), soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination).

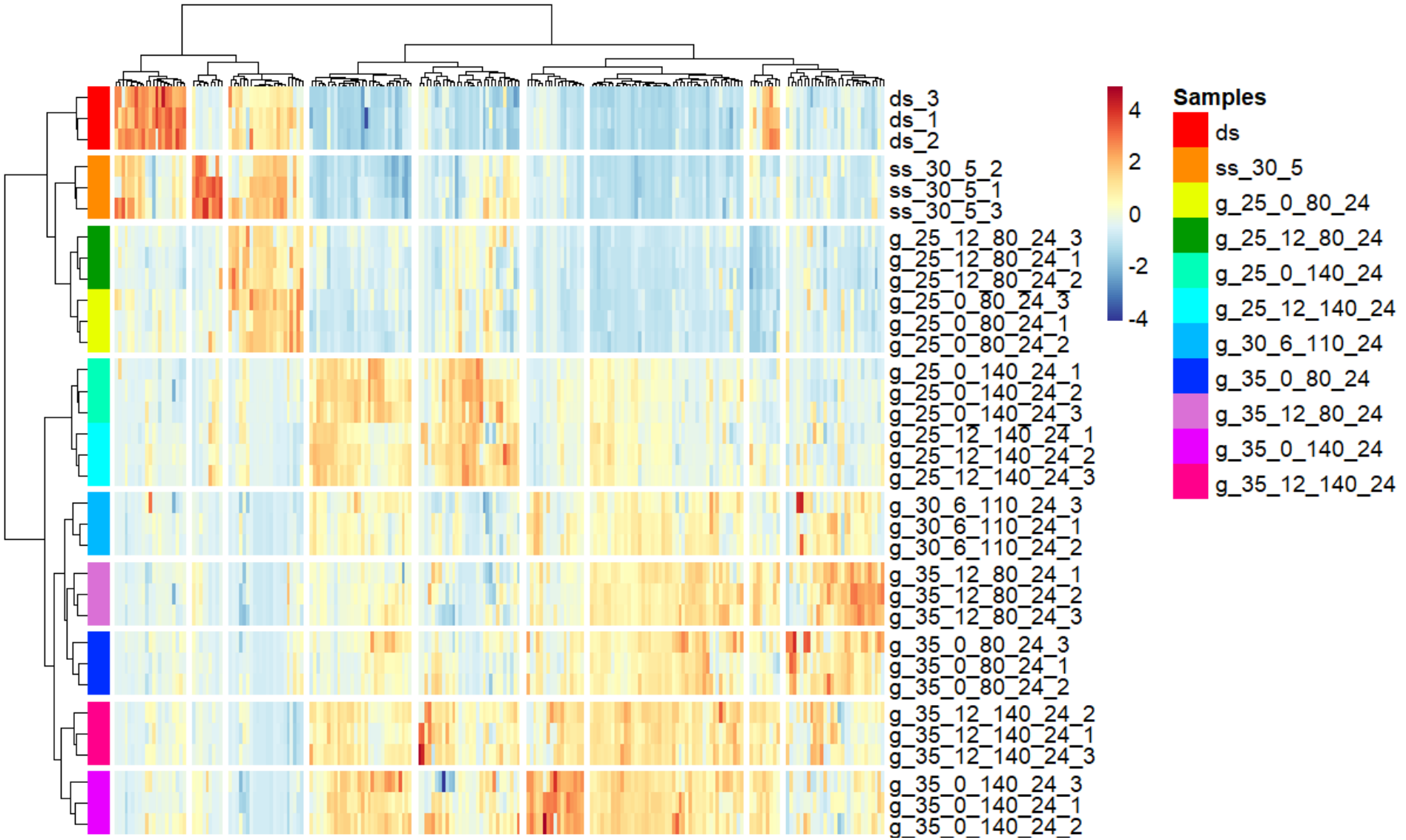


Figure 2. Heatmap depicting identified cowpea metabolites under various germination conditions using GC-MS. The heatmap was generated by R with ggplot2. The blue (low levels) and red (high levels) colors represent the relative abundance of metabolites in cowpea seeds for each condition. Dry seeds (ds), soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination).

To further investigate which classes of metabolites were more accumulated in germinated seeds, we calculated the log2foldchange (log2FC) for each metabolite class between dry and soaked and all germinated conditions. Subsequently, we visualized these differences using a volcano plot (Figure 3). Regardless the germination condition, the major metabolites that experienced an increase were primarily from the amino acids and sugars class. Increasing germination temperatures (30 °C and 35 °C) and water content (110% and 140% db) showed a more pronounced variation in the log2FC, indicating a substantial increase in the relative abundance of most metabolites. Moreover, when seeds were soaked and germinated at 25 °C with 80% water content, regardless the light condition, amino acids presented a high value of -log10(p-value) combined with a low value of log2FC. This indicates a subtle but statistically significant difference in the amino acids' abundance between these conditions and the dry seed. Given to the importance of amino acids and sugars in human food, we further investigated the effect of soaking and germination conditions on the metabolism, accumulation and decrease, of essential amino acids (lysine, isoleucine, leucine, methionine, phenylalanine, valine, threonine, histidine, and tryptophan), and carbohydrates metabolism that corresponds to the hydrolysis of antinutritional compounds, such as α-galactosides (stachyose, raffinose, and its derivatives; sucrose, fructose, glucose, and galactose), and phytate content (myo-inositol).

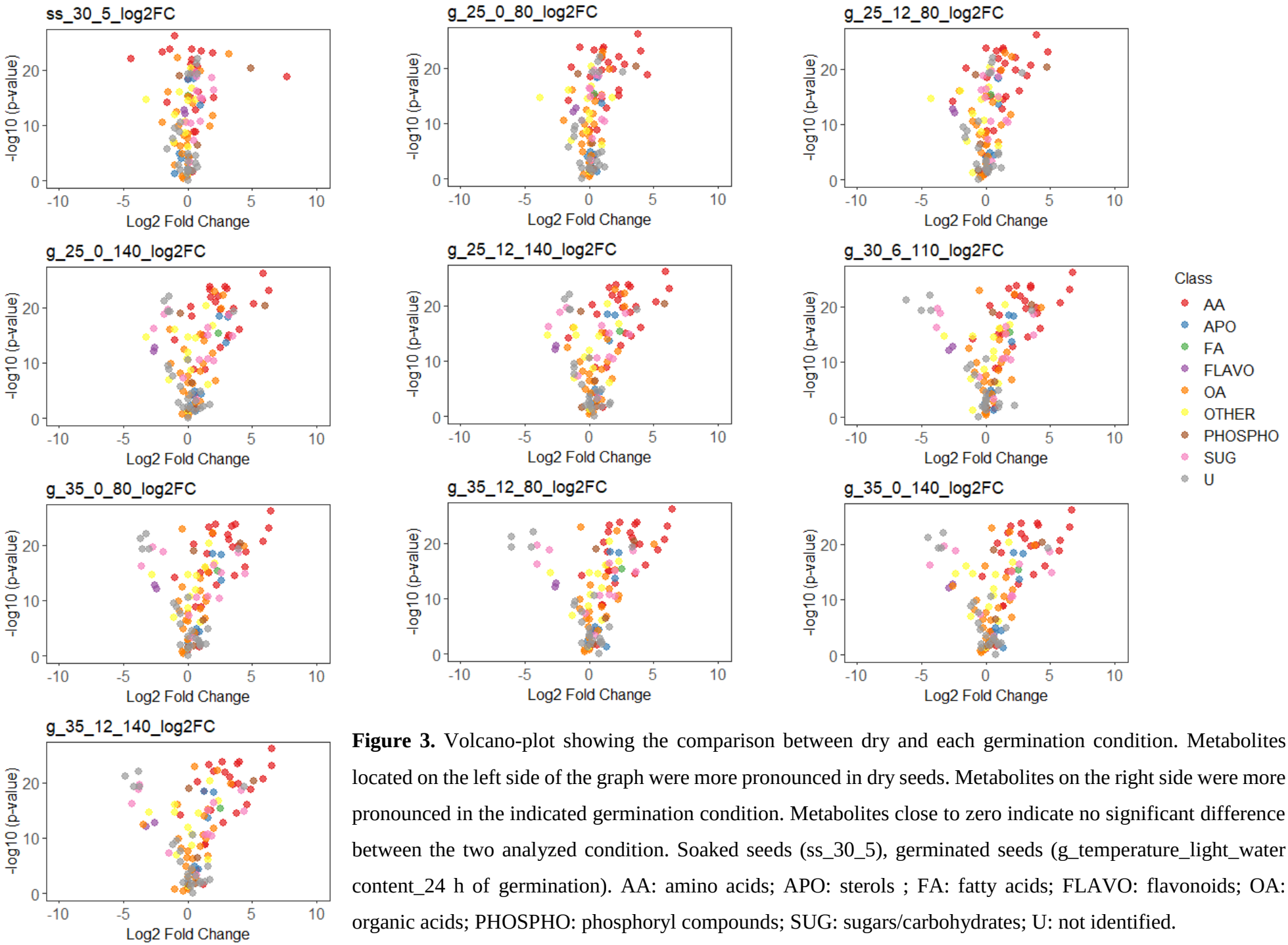


Figure 3. Volcano-plot showing the comparison between dry and each germination condition. Metabolites located on the left side of the graph were more pronounced in dry seeds. Metabolites on the right side were more pronounced in the indicated germination condition. Metabolites close to zero indicate no significant difference between the two analyzed condition. Soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination). AA: amino acids; APO: sterols ; FA: fatty acids; FLAVO: flavonoids; OA: organic acids; PHOSPHO: phosphoryl compounds; SUG: sugars/carbohydrates; U: not identified.

Soaking cowpea for 5 h showed a significant ($p < 0.05$) increase in all essential amino acids, compared to dry seeds, except for phenylalanine. This indicates that the soaking process activated key metabolic pathways essential for cowpea germination, leading to an improvement in amino acid availability. Additionally, there was an increase in the relative abundance of cysteine (4-foldchange) and lysine (1.1-foldchange) (Table 3). Despite the positive effect on amino acid content, the soaking time was insufficient to reduce antinutritional compounds. For instance, there were no significant differences in the relative abundance of stachyose and galactose, between dry and soaked seeds. After soaking process, the raffinose, stachyose, and glucose content increased by 1.6, 1.2, and 3.6-foldchange, respectively. There was no significant difference in the relative abundance of myo-inositol, suggesting that soaking time was not efficient in reducing the phytate content. Although the soaking process contributed to an increase in essential amino acid content, the germination process proved to be more effective. The highest increase in essential amino acids was observed in the synthesis of methionine, reaching up to 25-foldchange after germination (Table 3). Contrarily to soaking, germination process effectively reduced α-galactoside content, by reducing raffinose content by up to 80% and by a complete elimination of stachyose. Additionally, germination process increased fructose content by up to 35-foldchange and glucose by up to 32-foldchange (Table 4). It is important to note that galactose and sucrose did not showed any significant increase during this process. Myo-inositol increased by up to 247% after 24 h of germination as an indicator of phytic acid hydrolisis.

The results of PCA and heatmap analysis provided clear evidence that temperature and water content were the main factors influencing the metabolite profile in germinated cowpea seeds after 24 h. To further investigate, we compared two temperatures, 25 °C and 35 °C, while fixing water content of 80% (db) and 140% (db). We also compared two water contents, 80% (db) and 140% (db), while fixing temperatures at 25 °C and 35 °C. In all analyses, the light effect and the central point were neglected. The temperature effect between 25 °C and 35 °C with 80% or 140% of WC showed a significant (p <0.05) increase in the relative abundance of essential amino acids at 35 °C, except for histidine. Moreover, germinated cowpea at 35 °C showed a significant reduction in α-galactoside and phytate content in cowpea seeds, as evidenced by the reduction of the relative abundance of raffinose (-80%) and stachyose (-100%), while increasing the relative abundance of fructose (35-foldchange), glucose (32-foldchange), and myo-inositol (3.5-foldchange) (Table 4). Regarding the impact of water content, a slight effect was observed at 35 °C, where only the relative abundance of sucrose

presented a significant increase when cowpea was germinated at 80% WC. On the other hand, with 140% WC there was a significant increase in the relative abundance of isoleucine, leucine, fructose, and cysteine. These results indicated that at 35 °C water content had minimal influence on metabolites, as the majority of them did not show any significant difference. On the other hand, water content had a major influence when cowpea seeds were germinated at 25 °C. For instance, there was a significant increase in all essential amino acids at 25 °C with 140% WC, except for tryptophan. Increasing water content at 25 °C favored the reduction of antinutritional compounds, as it significantly reduced the relative abundance of raffinose and stachyose, while significantly increase myo-inositol content.

Given to these results, germination at 35 °C, whether with 80% or 140% WC, significantly improved the nutritional potential of germinated cowpea. This improvement was evidenced through the increased levels of essential amino acids, such as lysine, isoleucine, leucine, methionine, phenylalanine, valine, threonine, and tryptophan. Furthermore, germination at 35 °C led to a reduction in antinutritional compounds, such as α-galactoside and phytate.

Table 3. Concentration (µg/mg) of free essential amino acids in dry, soaked, and germinated cowpea seeds (g_temperature_light_water condition_ 24 h of germination).

Essential amino acids (µg/mg)									
	Lysine	Isoleucine	Leucine	Methionine	Phenylalanine	Valine	Threonine	Histidine	Tryptophan
Dry	0.007 ± 0.001	0.01 ± 0.001	0.01 ± 0.002	0.004 ± 0	0.09 ± 0.013	0.03 ± 0.003	0.04 ± 0.001	0.3 ± 0.03	0.03 ± 0.001
Soaked	0.02 ± 0.001	0.02 ± 0.001	0.03 ± 0.002	0.007 ± 0	0.10 ± 0.004	0.04 ± 0.002	0.06 ± 0.004	0.40 ± 0.02	0.05 ± 0.006
g_25_0_80_24	0.01 ± 0.002	0.03 ± 0.003	0.03 ± 0.005	0.02 ± 0.003	0.11 ± 0.009	0.06 ± 0.003	0.14 ± 0.006	0.40 ± 0.04	0.05 ± 0.002
g_35_0_80_24	0.06 ± 0.006	0.16 ± 0.006	0.15 ± 0.005	0.09 ± 0.005	0.26 ± 0.02	0.34 ± 0.028	0.59 ± 0.02	0.57 ± 0.16	0.07 ± 0.01
g_25_12_80_24	0.02 ± 0.001	0.03 ± 0.004	0.04 ± 0.007	0.02 ± 0.001	0.11 ± 0.001	0.07 ± 0.01	0.19 ± 0.02	0.27 ± 0.01	0.04 ± 0.004
g_35_12_80_24	0.08 ± 0.01	0.16 ± 0.008	0.17 ± 0.006	0.09 ± 0.009	0.24 ± 0.02	0.35 ± 0.009	0.57 ± 0.05	0.46 ± 0.22	0.07 ± 0.001
g_25_0_140_24	0.06 ± 0.003	0.09 ± 0.004	0.12 ± 0.006	0.07 ± 0.007	0.19 ± 0.02	0.18 ± 0.01	0.46 ± 0.02	0.44 ± 0.02	0.05 ± 0.002
g_35_0_140_24	0.09 ± 0.01	0.20 ± 0.003	0.24 ± 0.001	0.10 ± 0.006	0.28 ± 0.02	0.36 ± 0.02	0.59 ± 0.02	0.52 ± 0.33	0.09 ± 0.02
g_25_12_140_24	0.07 ± 0.002	0.10 ± 0.003	0.13 ± 0.009	0.06 ± 0.005	0.21 ± 0.008	0.19 ± 0.01	0.48 ± 0.02	0.62 ± 0.05	0.05 ± 0.003
g_35_12_140_24	0.10 ± 0.004	0.19 ± 0.008	0.21 ± 0.009	0.10 ± 0.005	0.29 ± 0.009	0.36 ± 0.03	0.58 ± 0.02	0.69 ± 0.03	0.09 ± 0.003
g_30_6_110_24	0.07 ± 0.009	0.13 ± 0.007	0.16 ± 0.005	0.08 ± 0.01	0.21 ± 0.019	0.27 ± 0.006	0.57 ± 0.05	0.47 ± 0.14	0.06 ± 0.001

Table 4. Concentration (µg/mg) of free carbohydrates in dry, soaked, and germinated cowpea seeds (g_temperature_light_water condition_ 24 h of germination).

	Carbohydrates (µg/mg)												
	Fructose	Fructose 6-phosphate	Galactose	Galactosylglycerol	Galactinol	Glucose	Glucose-6-Phosphate	Glycerol	Myo-Inositol	Raffinose	Sorbitol	Stachyose	Sucrose
Dry	0.06 ± 0.01	0.003 ± 0	0.10 ± 0.03	0 ± 0	0.52 ± 0.09	0.04 ± 0.006	0 ± 0	0.27 ± 0.03	0.28 ± 0.03	1.7 ± 0.14	0.15 ± 0.01	0.88 ± 0.16	38 ± 3.0
Soaked	0.13 ± 0.01	0.09 ± 0.01	0.14 ± 0.01	0 ± 0	0.81 ± 0.04	0.13 ± 0.006	0.23 ± 0.02	0.33 ± 0.04	0.32 ± 0.02	2.8 ± 0.26	0.09 ± 0.005	1.0 ± 0.11	45 ± 3.4
g_25_0_80_24	0.06 ± 0.001	0.04 ± 0.005	0.11 ± 0.007	0 ± 0	0.68 ± 0.04	0.06 ± 0.005	0.08 ± 0.003	0.30 ± 0.05	0.31 ± 0.02	2.5 ± 0.20	0.05 ± 0.004	0.65 ± 0.12	88 ± 1.2
g_35_0_80_24	1.3 ± 0.15	0.05 ± 0.004	0.11 ± 0.01	0.002 ± 0	0.08 ± 0.001	0.57 ± 0.07	0.09 ± 0.001	1.2 ± 0.06	0.87 ± 0.02	0.46 ± 0.03	0.38 ± 0.005	0 ± 0	203 ± 30
g_25_12_80_24	0.06 ± 0.01	0.08 ± 0.004	0.11 ± 0.005	0.001 ± 0	0.71 ± 0.02	0.07 ± 0.002	0.28 ± 0.003	0.33 ± 0.01	0.35 ± 0.009	1.5 ± 0.13	0.04 ± 0.004	0.45 ± 0.10	123 ± 7.1
g_35_12_80_24	0.76 ± 0.12	0.03 ± 0.003	0.08 ± 0.003	0.002 ± 0	0.03 ± 0.002	0.40 ± 0.02	0.08 ± 0.005	1.3 ± 0.05	0.82 ± 0.02	0.21 ± 0.06	0.35 ± 0.03	0.004 ± 0.007	151 ± 18
g_25_0_140_24	0.67 ± 0.1	0.19 ± 0.01	0.08 ± 0.005	0.001 ± 0	0.18 ± 0.003	0.33 ± 0.02	0.51 ± 0.02	1.0 ± 0.09	0.75 ± 0.02	0.48 ± 0.10	0.07 ± 0.01	0.04 ± 0.02	149 ± 16
g_35_0_140_24	2.1 ± 0.17	0.06 ± 0.004	0.07 ± 0.004	0.001 ± 0	0.06 ± 0.006	1.2 ± 0.04	0.16 ± 0.002	1.2 ± 0.09	0.96 ± 0.06	0.33 ± 0.04	0.05 ± 0.003	0 ± 0	142 ± 8.8
g_25_12_140_24	0.53 ± 0.02	0.20 ± 0.002	0.05 ± 0.002	0.003 ± 0	0.16 ± 0.005	0.26 ± 0.02	0.56 ± 0.02	1.3 ± 0.07	0.73 ± 0.02	0.35 ± 0.01	0.07 ± 0.03	0.01 ± 0.009	135 ± 18
g_35_12_140_24	1.2 ± 0.04	0.10 ± 0.007	0.04 ± 0.002	0.002 ± 0	0.04 ± 0.003	0.66 ± 0.04	0.29 ± 0.008	1.4 ± 0.01	0.90 ± 0.01	0.12 ± 0.04	0.07 ± 0.003	0.02 ± 0.03	138 ± 14
g_30_6_110_24	0.81 ± 0.27	0.04 ± 0.005	0.07 ± 0.02	0.002 ± 0	0.04 ± 0.001	0.49 ± 0.10	0.06 ± 0.01	1.0 ± 0.16	0.80 ± 0.04	0.15 ± 0.02	0.30 ± 0.05	0.009 ± 0.01	143 ± 15

III.3. Proteomic

To complete the metabolic analysis of cowpea seed germination, we conducted a differential proteomic analysis. The proteins were quantified by spectral counting method (SC) and by ion intensity (XIC). The SC method allows to identify large amounts of protein compared to XIC. However, XIC method is more accurate than SC (Rozanova *et al.*, 2021). In our study, we identified 2830 different proteins by SC, out of which 414 were significantly different ($p < 0.05$) in their accumulation levels. Through XIC methodology, we quantified 677 different proteins, of which 407 were significantly different ($p < 0.05$). Since XIC provides more accurate data, we decided to perform the PCA and heatmap analyzes with this dataset.

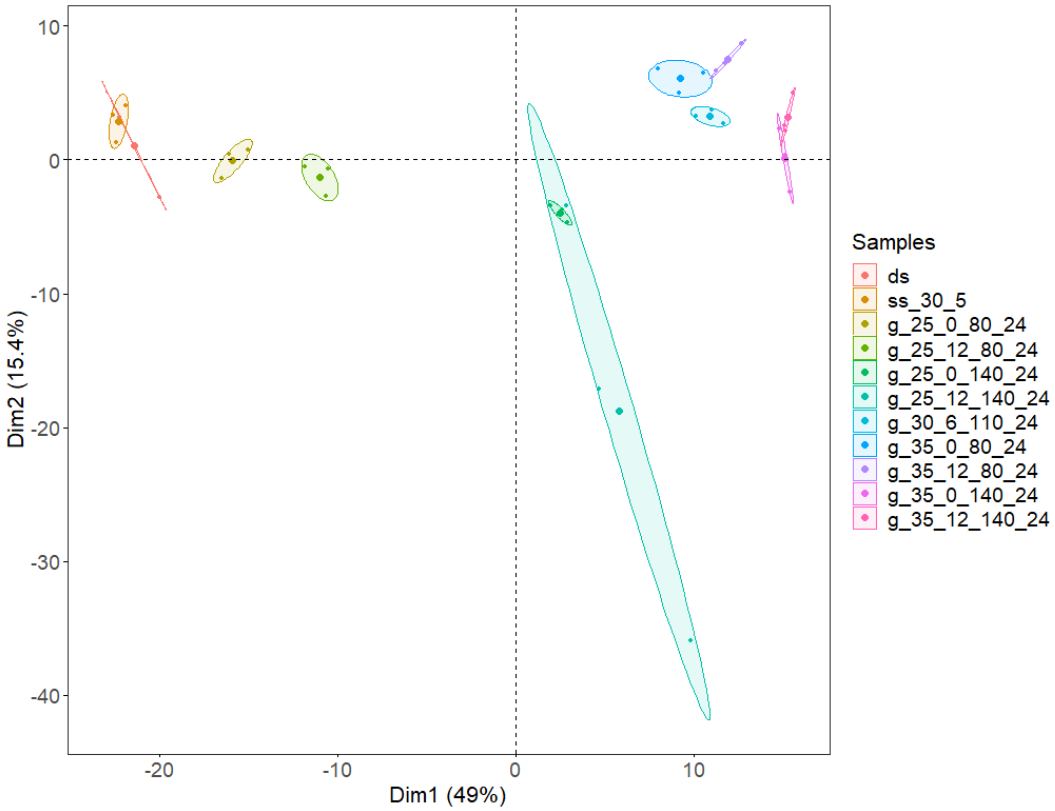


Figure 4. Principal Component Analysis (PCA) of proteins in cowpea seeds. The percentage of explained variance is labeled along each axis. Prior to PCA computation, all values were centered and scaled. Proteomics enabled the quantification of 677 distinct proteins, with 407 showing significant differences ($p < 0.05$). Dry seeds (ds), soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination).

PCA results showed that the first dimension (Dim1) explained 49% of the total variance, while the second dimension (Dim2) explained 15.4% (Figure 4). Besides to the sample germinated at 25°C, with 12 h of light and with 140% water content (g_25_12_140_24), the results showed that triplicate was closely clustered, indicating that the experiment was reproducible and reliable. The sample g_25_12_140_24 was an outlier, probably due to technical trouble. Therefore, as the light had minor impact on protein profile, this sample was excluded from the dataset for further analysis. On Dim1 the dry, soaked, and germinated (25 °C with 80% WC, with or without light) seeds were plotted on the left side, similar to what was observed in the metabolic analysis. Higher temperature and water content significantly impact the protein profile in cowpea. For instance, the germinated samples at 30 °C and 35 °C, regardless the light and water condition, were closely grouped together, showing a similarity of protein abundance. There was no light variance in Dim1 nor in Dim2. As in metabolomic analysis, temperature and water were the parameters which most impacted the protein abundance in cowpeas. These results were also observed when the outlier sample was eliminated (Figure 5). In this context, there was no significant influence of temperature or water content on Dim2, which explained only 8.3% of variance. In according to these results, the heatmap analysis showed that proteins in clusters 1-4 were less abundant in dry, soaked, and germinated seeds at 25 °C with 80% WC (db), regardless of light exposure (g_25_0/12_80) (Figure 6). These same proteins showed higher accumulation levels when the germination temperature and water content increased. On the other hand, proteins in clusters 5-9 showed higher abundance in dry, soaked and g_25_0/12_80 seeds, and lower accumulation levels when germination temperature and water content were increased. Similar to the findings in metabolomics, temperature and water content were the primary factors influencing protein accumulation in cowpea seeds. To this end, the light effect was neglected in the subsequent analyses.

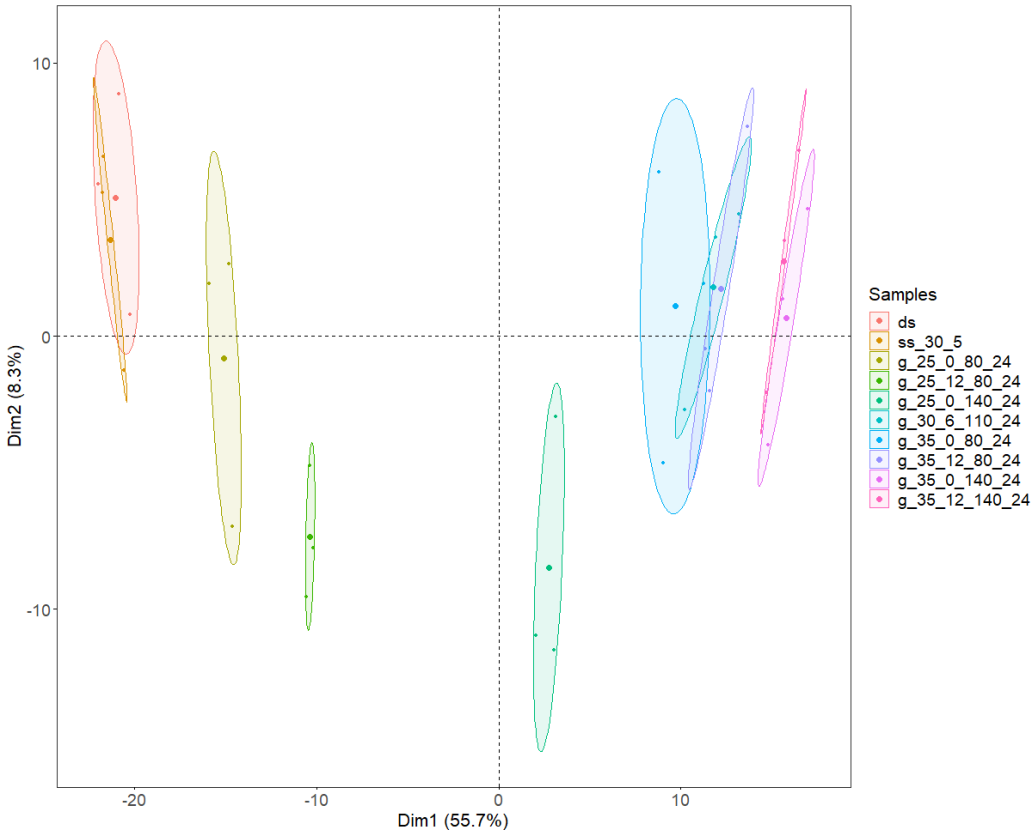


Figure 5. Principal Component Analysis (PCA) of proteins in cowpea seeds without the outlier sample g_24_12_140_24. The percentage of explained variance is labeled along each axis. Prior to PCA computation, all values were centered and scaled. Proteomics enabled the quantification of 677 distinct proteins, with 407 showing significant differences ($p < 0.05$). Dry seeds (ds), soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination)

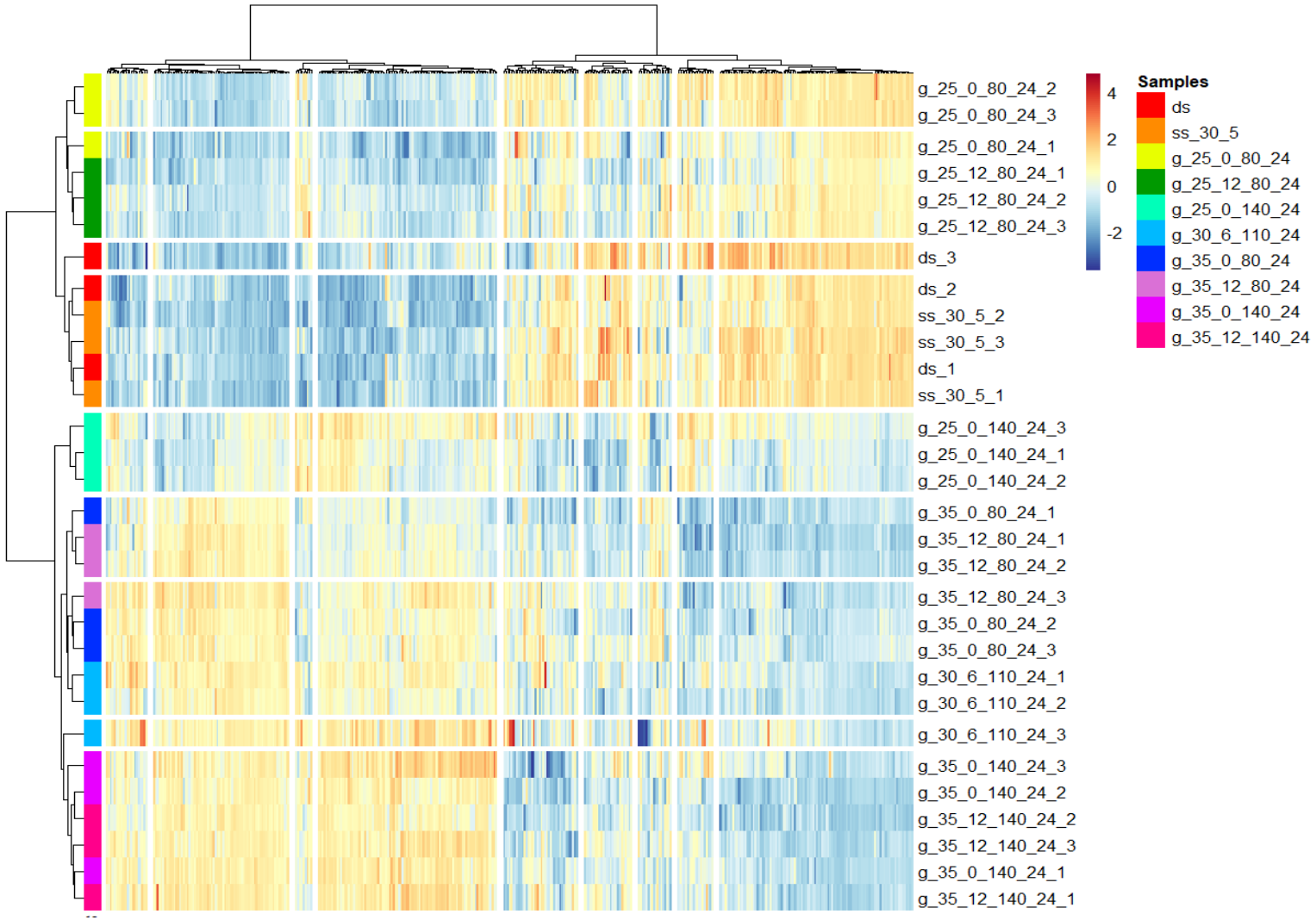


Figure 6. Heatmap depicting identified cowpea proteins under various germination conditions using LC-MS. The heatmap was generated using R with ggplot2. The blue (low levels) and red (high levels) colors represents the relative abundance of proteins in cowpea seeds for each condition. A total of 407 significantly differentially abundant proteins are represented ($p < 0.05$). Dry seeds (ds), soaked seeds (ss_30_5), germinated seeds (g_temperature_light_water content_24 h of germination).

Through the protein analyses we identified key proteins involved in metabolism. For instance, we identified 167 proteins involved in proteases activity, in which 19 had a significant difference between our samples (Table 5). Out of these 19 proteins, 13 proteins significantly increased their values during germination. The proteins subtilisin-like protease SBT1.4, subtilisin-like protease SBT1.7, serine carboxypeptidase-like 40, thiol protease aleurain-like, protein aspartic protease in guard cell 1-like, and E3 ubiquitin-protein ligase ZNF598-like were undetectable in dry, soaked, and no germinated seeds. However, their levels significantly increased and became detectable during germination (25 °C with 140% water content, and at 30 °C and 35 °C, regardless the light and water condition). Additionally, germination increased puromycin-sensitive aminopeptidase isoform X1 values by up to 125%, aspartyl protease At4g16563 by up to 133%, and ubiquitin-conjugating enzyme E2 7 by up to 248%. Moreover, 4 proteins displayed variations in their values depending on the specific germination condition. For example, aminopeptidase P1 increased by 20% when cowpea seeds were germinated at 35 °C, with 12 h of lighting and 140% water content. Conversely, this same protein reduced by 20% when seeds were germinated at 25 °C (under the same light and water content conditions). Despite the overall increase in protease activity during the germination process, it is worth noting that the values of two proteases actually decreased. Specifically, the ubiquitin receptor RAD23c-like which reduced by up to 32% after germination, and the CO_2-response secreted protease-like which completely disappeared once germination occurred.

In addition to the proteins associated with protease activity, we have identified 11 proteins involved in essential amino acid metabolism (Table 6) and another 11 proteins associated with carbohydrate metabolism (synthesis of myo-inositol, starch, and sucrose, and degradation of α-galactosides) (Table7). Regarding essential amino acid metabolism, after 24 h of germination, the enzymes cysteine synthase (OASA1) and aspartate aminotransferase (ASP3), which are involved in cysteine synthesis, exhibited a significant increase in their content by up to 252% and 67%, respectively. Moreover, 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (ATMS1), an enzyme involved in methionine synthesis, reported an increase by up to 24% after germination. However, the remaining eight identified proteins involved in essential amino acid metabolism reported a decrease in their content once germination was completed. For instance, dihydroxy-acid dehydratase (DAD) and branched-chain amino acid aminotransferase (ATBCAT-5), both involved in isoleucine synthesis, reported a decrease in their content by up to 27% and 50%, respectively. Concerning carbohydrate metabolism, after 24 h of germination, raffinose synthase (SIP2) and stachyose synthase (STS), both involved in

the degradation of α-galactosides, increased their content by up to 12-foldchange and 1.7-foldchange, respectively. However, α-galactosidase content was reduced by up to 57% after germination. Moreover, β-glucosidase (BGLU44) and sucrose synthase (SUS4), which are implicated in sucrose metabolism, increased their content by up to 84% and 546%, respectively. The content of inositol phosphate phosphatase (VTC4), a key player in inositol metabolism, increased by up to 55% after 24 h of germination.

Table 5. Relative abundance of proteases-related factors in dry, soaked, and germinated cowpea seeds (g_temperature_light_water content_24 h of germination).

Samples	26SPRS	PSAIX1	26SS10BB	26S10BA	SPSBT1.4	AMIP1	URRAD23	U40SRPS27	UNEDD8	SPSBT1.7	SC40	IP	TP	AP	PAP	ATPDP	CORSP	UCEE27	E3UPL
Dry	12 ± 1.2	8 ± 0	12 ± 0.6	12 ± 1.2	0 ± 0	10 ± 1	6 ± 1.5	15 ± 1.5	14 ± 2.1	0 ± 0	0 ± 0	2 ± 1.5	0 ± 0	2 ± 0	0 ± 0	1 ± 1	2 ± 1	1 ± 0.6	0 ± 0
Soaked	17 ± 2.6	9 ± 1	13 ± 2.1	11 ± 2	0 ± 0	9 ± 1	7 ± 1	18 ± 2.3	17 ± 3.1	0 ± 0	0 ± 0	1 ± 0.6	0 ± 0	3 ± 1.7	0 ± 0	2 ± 0	1 ± 0.6	1 ± 0.6	0 ± 0
g_25_0_80_24	13 ± 1	10 ± 2	11 ± 0.6	9 ± 0.6	0 ± 0	8 ± 0.6	7 ± 0.6	19 ± 0.6	18 ± 1	0 ± 0.6	0 ± 0	2 ± 1.2	0 ± 0	3 ± 0.6	0 ± 0	1 ± 0	0 ± 0.6	1 ± 0	0 ± 0.6
g_35_0_80_24	15 ± 1.5	11 ± 1.5	14 ± 1.2	12 ± 1	0 ± 0	11 ± 0.6	7 ± 0.6	20 ± 2	19 ± 1	0 ± 0.6	0 ± 0	2 ± 0.6	0 ± 0	3 ± 1.2	0 ± 0	1 ± 0	0 ± 0.6	0 ± 0.6	1 ± 0.6
g_25_12_80_24	14 ± 0.6	13 ± 1.5	16 ± 1.2	14 ± 0	1 ± 1.5	11 ± 0.6	7 ± 0.6	21 ± 1	21 ± 1.5	5 ± 2.1	3 ± 1	3 ± 1.2	0 ± 0	4 ± 0.6	0 ± 0.6	1 ± 0	0 ± 0.6	2 ± 0.6	1 ± 0
g_35_12_80_24	16 ± 2.9	12 ± 2.5	11 ± 2.1	10 ± 2.1	2 ± 1.5	8 ± 3	4 ± 1.5	25 ± 2.6	23 ± 3	5 ± 2	2 ± 1	3 ± 1.7	0 ± 0	4 ± 0.6	2 ± 0.6	0 ± 0.6	1 ± 0.6	1 ± 0.6	1 ± 0.6
g_25_0_140_24	15 ± 2.1	15 ± 3.1	13 ± 1.5	12 ± 2.1	15 ± 1	11 ± 0.6	6 ± 1	21 ± 4.9	21 ± 4.9	6 ± 0.6	4 ± 3	1 ± 0.6	4 ± 2.3	3 ± 0	3 ± 2.1	1 ± 0	1 ± 0.6	1 ± 1.1	1 ± 0
g_35_0_140_24	14 ± 2.1	13 ± 1.2	14 ± 1.7	13 ± 1.2	13 ± 2.6	12 ± 1.5	5 ± 0.6	21 ± 1.7	22 ± 1.5	5 ± 1.2	1 ± 0	2 ± 0.6	3 ± 2.1	5 ± 0.6	1 ± 1	2 ± 0.6	0 ± 0.6	1 ± 1	1 ± 0
g_25_12_140_24	16 ± 1	14 ± 2.1	15 ± 1.5	12 ± 1.2	17 ± 0.6	12 ± 2	7 ± 0.6	19 ± 0.6	19 ± 1.5	6 ± 1	3 ± 0.6	4 ± 1.5	8 ± 1	3 ± 0.6	3 ± 1	1 ± 0.6	0 ± 0	1 ± 0.6	0 ± 0.6
g_35_12_140_24	17 ± 2	18 ± 2	15 ± 0.6	14 ± 1	11 ± 1.5	10 ± 1.2	5 ± 1	21 ± 2.5	20 ± 3.1	5 ± 0.6	6 ± 0.6	3 ± 1.5	1 ± 0.6	4 ± 0.6	2 ± 1	1 ± 0.6	1 ± 0	2 ± 0.6	1 ± 0.6
g_30_6_110_24	13 ± 1.5	14 ± 2.6	13 ± 2.6	11 ± 1.5	15 ± 2.6	11 ± 0.6	6 ± 1	22 ± 1.7	22 ± 2.6	6 ± 0.6	6 ± 0.6	3 ± 0.6	6 ± 1.7	4 ± 1	2 ± 1.5	1 ± 0	0 ± 0	2 ± 0.6	1 ± 0.6

Data set from SC. 26SPRS: 26S proteasome non-ATPase regulatory subunit 1 homolog A-like; PSAIX1: puromycin-sensitive aminopeptidase isoform X1; 26SS10BB: 26S proteasome regulatory subunit S10B homolog B; 26S10BA: 26S proteasome regulatory subunit 10B homolog A-like; SPSBT1.4: subtilisin-like protease SBT1.4; AMIP1: aminopeptidase P1; URRAD23: ubiquitin receptor RAD23c-like; U40SRPS27: ubiquitin-40S ribosomal protein S27a; UNEDD8: ubiquitin-NEDD8-like protein RUB2; SPSBT1.7: subtilisin-like protease SBT1.7; SC40: serine carboxypeptidase-like 40; IP: lon protease homolog 1, mitochondrial; TP: thiol protease aleurain-like; AP: aspartyl protease At4g16563; PAP: protein ASPARTIC PROTEASE IN GUARD CELL 1-like; ATPDP: ATP-dependent Clp protease proteolytic subunit 5, chloroplastic; CORSP: CO(2)-response secreted protease-like; UCEE27: ubiquitin-conjugating enzyme E2 7; E3UPL: E3 ubiquitin-protein ligase ZNF598-like.

Table 6. Relative protein abundance of enzymes acting in amino acids pathways in dry, soaked, and germinated cowpea seeds (g_temperature_light_water content_24 h of germination)

Relative protein abundance in amino acids pathways											
	OASA1	ASP3	HMT2*	ATMS1*	SAM1	IIL1	ACS	DAD	ATBCAT-5	ASD	DPE
Dry	-0.45 ± 0.7	-1.38 ± 0.29	3.3 ± 1.5	14 ± 1.7	1.02 ± 0.35	0.52 ± 0.09	0.66 ± 0.8	0.93 ± 0.56	0.94 ± 1.43	0.35 ± 0.72	0.83 ± 0.93
Soaked	-0.31 ± 1.02	-1.2 ± 0.11	2.3 ± 0.6	14.7 ± 2.3	1.37 ± 0.37	0.49 ± 0.5	1.27 ± 1.28	1.66 ± 0.69	0.07 ± 0.17	-0.08 ± 0.55	1.05 ± 0.98
g_25_0_80_24	0.64 ± 1.29	-0.93 ± 0.1	1.3 ± 0.6	13.7 ± 2.1	0.82 ± 0.2	-0.5 ± 0.66	-0.3 ± 0.65	0.72 ± 0.52	-0.27 ± 0.19	1.25 ± 1.09	1.08 ± 1.16
g_35_0_80_24	-0.28 ± 0.99	0.67 ± 0.32	0.7 ± 1.2	15 ± 3	-1.31 ± 0.37	-0.45 ± 0.09	-0.15 ± 1.48	-0.93 ± 0.52	-0.85 ± 0.11	-0.5 ± 1.1	-0.48 ± 0.4
g_25_12_80_24	-0.14 ± 1.15	-0.85 ± 0.08	2.3 ± 2.1	16 ± 1	0.7 ± 0.51	-0.47 ± 0.13	-0.4 ± 1.52	0.14 ± 0.63	-0.08 ± 0.46	-0.09 ± 0.29	0 ± 0.54
g_35_12_80_24	-0.13 ± 1.25	0.9 ± 0.24	2.3 ± 1.2	15.7 ± 0.6	-1.5 ± 0.43	-0.39 ± 0.13	-0.34 ± 0.33	-0.75 ± 0.52	-0.57 ± 0.67	-0.22 ± 0.33	-0.71 ± 0.55
g_25_0_140_24	-0.3 ± 1.05	-0.25 ± 0.26	2 ± 0	17.3 ± 3.1	0.12 ± 0.26	-0.29 ± 0.54	-0.56 ± 0.26	-0.12 ± 0.39	-0.01 ± 0.92	-0.38 ± 1.93	-0.1 ± 0.57
g_35_0_140_24	-0.52 ± 0.58	1.38 ± 0.55	1 ± 1.7	16 ± 0	-0.58 ± 0.3	-0.3 ± 0.24	-0.52 ± 0.32	-0.27 ± 0.38	0.24 ± 1.7	-0.34 ± 0.79	-1.01 ± 1.21
g_25_12_140_24	0.68 ± 1.32	-0.22 ± 0.32	2 ± 1.7	22 ± 4.6	0.42 ± 0.95	1.44 ± 3.06	0.55 ± 1.62	0.52 ± 1.06	1.58 ± 0.91	-0.99 ± 1.56	-0.34 ± 1.48
g_35_12_140_24	-0.17 ± 1.23	1.2 ± 0.04	1.3 ± 0.6	16.3 ± 3.1	-0.65 ± 0.52	0.12 ± 0.19	-0.21 ± 0.43	-1.2 ± 0.86	-0.22 ± 0.82	0.37 ± 0.22	-0.06 ± 0.36
g_30_6_110_24	0.99 ± 0.46	0.68 ± 0.3	2 ± 1	15.7 ± 1.5	-0.41 ± 0.2	-0.17 ± 0.26	0 ± 0.84	-0.7 ± 0.09	-0.83 ± 0.19	0.63 ± 0.33	-0.26 ± 0.81

*relative proteins abundance from spectral counting method (SC); OASA1: cysteine synthase; ASP3: aspartate aminotransferase; HMT2: homocysteine S-methyltransferase; ATMS1: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase; SAM1: S-adenosylmethionine synthetase; IIL1: 3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit; ACS: acetolactate synthase I/III small subunit; DAD: dihydroxy-acid dehydratase; ATBCAT-5: branched-chain amino acid aminotransferase; ASD: aspartate-semialdehyde dehydrogenase; DPE: diaminopimelate epimerase.

Table 7. Relative protein abundance of enzymes acting in carbohydrate pathways in dry, soaked, and germinated cowpea seeds (g_temperature_light_water content_24 h of germination)

Relative protein abundance in carbohydrate pathways											
	α-Gal	SIP2	STS	VTC4	BGLU44	SUS4	SPS1F	GBSS1	ISA3	BAM5	α-GP
Dry	1.36 ± 0.54	-1.24 ± 0.21	0.4 ± 0.17	-0.69 ± 0.96	-0.66 ± 0.65	-0.89 ± 0.08	0.8 ± 0.12	1.87 ± 0.48	-0.14 ± 0.01	0.94 ± 0.56	0.68 ± 1.55
Soaked	1 ± 0.23	-1.3 ± 0.04	0.24 ± 0.21	-0.45 ± 0.36	-0.57 ± 0.2	-0.93 ± 0.03	0.03 ± 1.02	1.58 ± 0.57	-0.16 ± 0.03	0.91 ± 0.53	0.34 ± 1.36
g_25_0_80_24	1.13 ± 0.7	-0.7 ± 0.09	0.03 ± 0.25	1.21 ± 1.23	-1.1 ± 0.17	-0.69 ± 0.23	0.95 ± 0.29	0.78 ± 0.1	-0.16 ± 0.05	0.25 ± 0.18	-0.01 ± 0.95
g_35_0_80_24	-0.3 ± 0.11	-0.09 ± 0.15	0.79 ± 2.55	-0.12 ± 0.92	0.44 ± 0.69	-0.24 ± 0.07	-0.67 ± 0.95	0.1 ± 0.17	-0.17 ± 0.03	-0.47 ± 0.09	0.18 ± 1.16
g_25_12_80_24	0.57 ± 0.36	-0.28 ± 0.15	0.12 ± 0.08	1.22 ± 0.15	-0.96 ± 0.24	-0.81 ± 0.09	0.18 ± 1.24	-0.14 ± 0.15	-0.16 ± 0.02	-0.22 ± 0.28	-0.8 ± 0.42
g_35_12_80_24	-0.74 ± 0.32	-0.31 ± 0.15	0.52 ± 2.39	-0.51 ± 0.7	0.48 ± 0.18	-0.29 ± 0.05	0.03 ± 1.12	-0.24 ± 0.03	-0.18 ± 0.02	-0.53 ± 0.08	0.41 ± 0.75
g_25_0_140_24	0.27 ± 0.37	2.02 ± 0.31	-0.27 ± 0.16	0.63 ± 0.71	-0.4 ± 0.4	0.18 ± 0.18	0.07 ± 1.02	-0.69 ± 0.06	-0.18 ± 0.01	-0.36 ± 0.13	-0.08 ± 1.18
g_35_0_140_24	-0.47 ± 0.1	0.43 ± 0.74	-0.54 ± 0.24	-0.75 ± 1.43	0.69 ± 0.34	1.3 ± 0.21	-0.75 ± 1.32	-0.81 ± 0.04	-0.2 ± 0.01	-0.71 ± 0.14	-0.77 ± 0.59
g_25_12_140_24	-1.45 ± 1.15	0.98 ± 0.93	-0.13 ± 0.45	0.01 ± 1.12	0.59 ± 2.52	1.36 ± 2.18	-0.69 ± 1.06	-0.46 ± 0.15	1.72 ± 3.34	1.1 ± 2.84	-0.43 ± 1.7
g_35_12_140_24	-0.55 ± 0.19	0.06 ± 0.24	-0.72 ± 0.06	0.05 ± 0.63	0.6 ± 0.25	0.95 ± 0.06	-0.09 ± 1.2	-1.33 ± 0.02	-0.19 ± 0.02	-0.62 ± 0.36	-0.05 ± 0.29
g_30_6_110_24	-0.81 ± 0.85	0.44 ± 0.59	-0.44 ± 0.18	-0.6 ± 0.21	0.9 ± 0.26	0.05 ± 0.12	0.15 ± 1.09	-0.65 ± 0.09	-0.18 ± 0.02	-0.27 ± 0.28	0.52 ± 0.58

α-Gal: α-galactosidase; SIP2: raffinose synthase; STS: stachyose synthase; VTC4: inositol phosphate phosphatase; BGLU44: β-glucosidase; SUS4: sucrose synthase; SPS1F: sucrose-phosphate synthase; GBSS1: granule-bound starch synthase; ISA3: isoamylase; BAM5: β-amylase; α-GP: α-glucan phosphorylase.

To investigate the effect of soaking and germination in the up-accumulation and down-accumulation of proteins in biological functions, we conducted enrichment analyses between dry and soaked seeds, and dry and g_35_0_140_24, representing the optimal germination conditions (Avezum *et al.,* 2023). When comparing dry and soaked seeds, the enrichment analysis did not reveal significant upregulation or downregulation of proteins in any biological functions. However, comparing the dry seed with the germination process (g_35_0_140_24), the enrichment analysis revealed 136 significantly different proteins involved in 20 key biological functions (Figure 7). The germination process favored responses to abscisic acid, alcohol, cellular aldehyde metabolic process, glyoxylate cycle, glyoxylate metabolic processes, tricarboxylic acid cycle, and citrate metabolic process, resulting in the up-accumulation of 23 proteins acting in these processes. Moreover, compared to the germinated seeds, dry seeds downregulated starch biosynthetic process, starch metabolic process, glucan biosynthetic process, polysaccharide metabolic processes, DNA strand elongation involved in DNA replication, and cellular aldehyde metabolic process, acting by down-accumulation of 40 proteins (Figure 7).

To investigate the effect of germination conditions in the up-accumulation and down-accumulation of proteins in biological functions, we conducted enrichment analyses between two temperatures, 25 °C and 35 °C, while fixing water content of 80% (db) and 140% (db). We also compared two water contents, 80% (db) and 140% (db), while fixing temperatures at 25 °C and 35 °C. Light conditions and the central point were not taken into consideration. The enrichment analyses between germination condition showed a temperature effect more pronounced at 80% WC, with 90 enrichment proteins at 80% *vs* 29 at 140% (Appendix 1). The upregulated pathways at 35 °C with 80% water content included responses to abscisic acid, alcohol, protein stabilization, response to water deprivation, regulation of membrane permeability, and cold acclimation. These results demonstrated the metabolic response of the seed to the low-water stress. At 35 °C, the proteins identified to be involved in responses to abscisic acid and water deprivation were dehydrin ERD14, glutathione S-transferase, and betaine aldehyde dehydrogenase 1. Through the enrichment analysis, the water content effect was more pronounced at 25 °C, with 76 enrichment proteins *vs* 13 at 35 °C (Appendix 2). The upregulated pathways at 25 °C with 140% water content was isopentenyl diphosphate biosynthetic process, isopentenyl diphosphate metabolic process, glyceraldehyde-3-phosphate metabolic process, pyruvate metabolic process, and cellular aldehyde metabolic process.

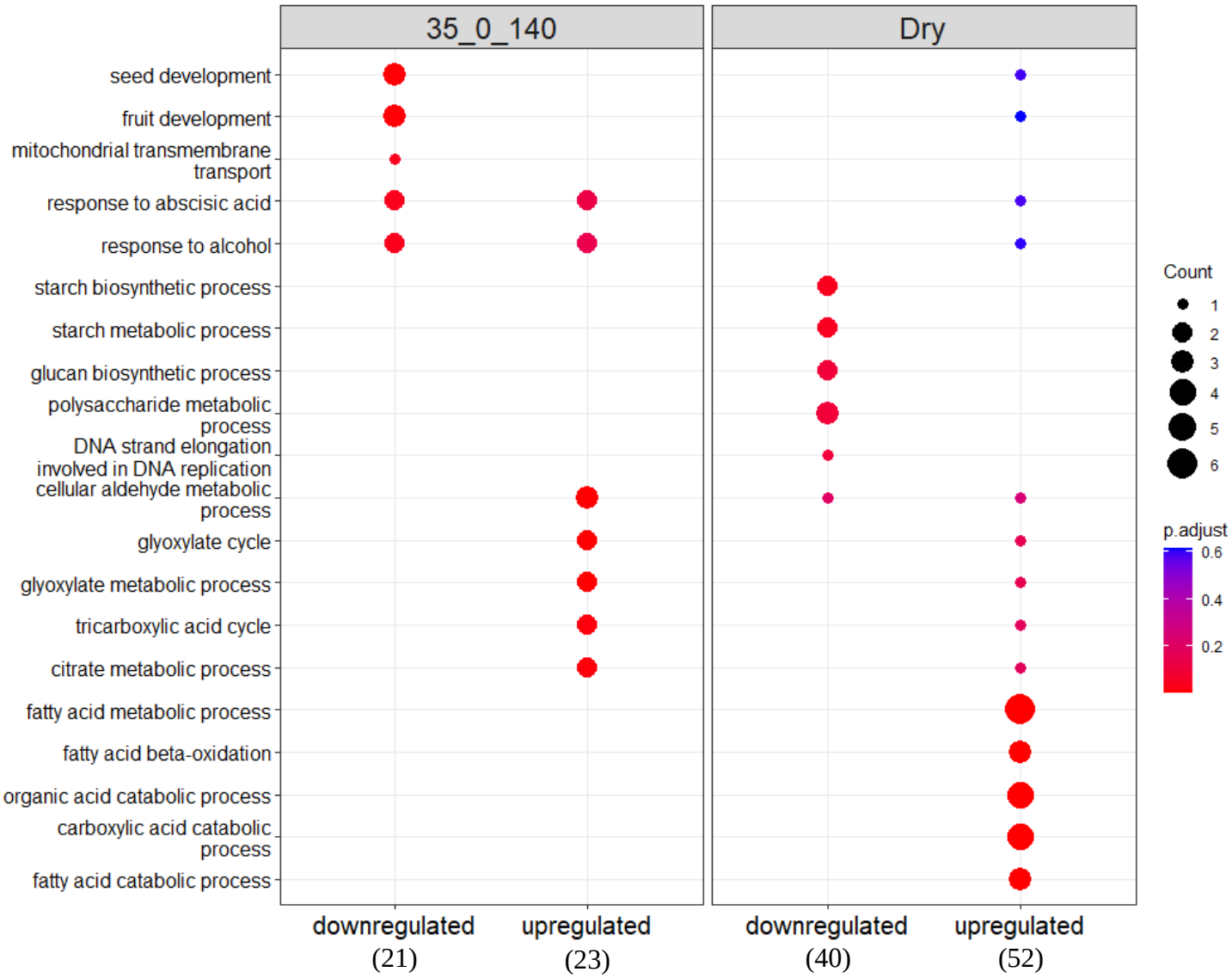


Figure 7. Proteomic enrichment between dry and germinated seeds at 35 °C, without light, with 140% water content

IV. Discussion

Seed germination is a complex cellular metabolism process. However, no prior studies in the literature have investigated the combined effects of temperature, light, and water content on cowpea germination from a nutritional perspective, using a multi-omics approach encompassing both metabolomics and proteomics. Our study, is the first to address this issue and to discuss the differences during germination condition at the multi-omics level.

In our last study, we demonstrated that the germination rate ranged from 23% to 83%, depending on the temperature and water condition (Avezum *et al.*, 2023). Other studies analysed the germination rates in cowpea seeds. For instance, cowpea germination rates at 28 °C for 48 h have been reported to range from 60% to 99% (Ravelombola *et al.*, 2017). Coffigniez *et al.* (2021) reported that the germination rate of cowpea at 30 °C reached 75% after 48 h, while under the same germination conditions, but after 24 h, the germination rate was between 40% and 50% (Coffigniez *et al.*, 2021). In our last study, we observed a significant reduction in the germination rate when cowpea seeds were germinated at low temperatures (25 °C) and at limited water content (80 %). These conditions induced chilling and drought stresses, which influenced plant metabolic activity and functionality of crucial enzymes, resulting in lower germination rates, compromised seedling emergence, seed growth, and cell division. It is important to note that our germination *sensu stricto* process was specifically designed for food processing purposes. In this context, when referring to water content or deprivation, we are actually discussing the effects of our spraying system and seed water content, rather than the soil's water content. Similarly, the temperatures mentioned correspond to the settings within the climatic chamber used for our experiments, and not the ambient environmental temperature. Abiotic stress, including temperature and drought conditions, can constraint crop productivity by affecting seed germination, plant photosynthesis, and overall yield (Omomowo & Babalola, 2021). Abscisic acid (ABA) is the most critical hormone involved in regulating germination potential, tolerance to abiotic stress, and play a key role in temperature and drought tolerance. This hormone is involved in the regulation of stomatal closure and the induction of stress-responsive genes (Lamaoui *et al.*, 2018). Interestingly, some ABA-induced proteins such as late embryogenesis abundant (LEA) proteins are also over-accumulated under temperature and drought stress and play a crucial role in protecting cellular structures and stabilizing membranes (Lamaoui *et al.*, 2018). In our present study, we identified two major proteins involved in

regulating the response to abscisic acid and temperature stimulus: dehydrin ERD14 and glutathione S-transferase. These proteins were significantly up-accumulated at high temperatures (35 °C). Dehydrins belong to the group 2 of the LEA protein family and are characterized by their high phosphorylation status (Hanin *et al.*, 2011; Kosová *et al.*, 2014). Numerous studies reported the positive correlation between the accumulation of dehydrins protein and plants' responses and adaptation to dehydration caused by drought, cold, freezing, or salinity (Hanin *et al.*, 2011; Kosová *et al.*, 2014; Maszkowska *et al.*, 2019). Moreover, other studies have shown that dehydrin ERD14 was identified in *Arabidopsis* as response to dehydration and salinity stress (Kovacs *et al.*, 2008; Maszkowska *et al.*, 2019). Glutathione S-transferase is another enzyme recognized to act against oxidative stress in plants. Xu *et al.* (2015) reported that transgenic plants expressing genes for glutathione S-transferase improved drought and salinity tolerance in *Arabidopsis*. Nguyen *et al.* (2020) reported that dehydrin ERD14 has a protective role against oxidative damage, by activating glutathione S-transferase Phi9 catalysis in *Arabidopsis thaliana* under osmic stress. Additionally, we identified other key proteins associated with the abscisic acid response: protein SLE1 and SLE2, belonging to the Em-like Groupe-1 LEA gene family. These proteins showed a down-accumulation in germinated seeds under conditions of 25 °C and 140% WC, as well as at 35 °C, regardless of the water content. However, they exhibited an up-accumulation in dry and soaked seeds. Regarding drought stress, the protein betaine aldehyde dehydrogenase 1 (BADH1), chloroplastic-like played a critical role in responding to water deprivation. BADH1 was observed to be up-accumulated under water scarcity conditions (80%) for both temperatures, 25 °C and 35 °C.

Soaking process is a critical step preceding the germination process. During soaking, there is activation in metabolism, setting the stage for the subsequent germination process. Upon imbibition, it is generally assumed that the dry seed already contains the necessary structures and enzymes to initiate the resumption of metabolic activity (Bewley, 1997). Indeed, based on our proteomics analysis focused on amino acids and sugars pathways, the majority of enzymes presented high levels in dry, as well as in those that were soaked or remained un-germinated (25 °C with 80% water content) (Tables 6-7). However, there were no significant differences in metabolic changes between dry and soaked seeds, as indicated by the PCA and heatmaps analyses. This finding is consistent with the results reported by Wu *et al.* (2020) which showed the limited metabolic changes in mung-bean seeds during the first 6 h of imbibition at 26 °C. In our study, dry and soaked cowpea seeds presented relatively high concentration of

phenylalanine, threonine, and histidine. On the other hand, Jirapa *et al.* (2001) showed that cowpea seeds presented high isoleucine and lysine content.

As the germination time increased, metabolic changes were more pronounced and increased the relative abundance of essential amino acids compared to dry seeds, specially methionine. The significant increase in methionine content was attributed to the enhanced enzymatic activity of 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (ATMS1), which is responsible for the conversion of homocysteine to methionine (Table 6 and figure 8). This enzyme showed a high concentration levels during germination at 35 °C, regardless of water and light condition, as well as at 25 °C with 140% water content. However, another enzyme involved in the homocysteine to methionine conversion, homocysteine S-methyltransferase (HMT2), presented lower levels during germination at 35 °C. Contrastingly, it presented high levels in dry, soaked, and germinated at 25 °C with 80% WC. Pulses are commonly recognized for their low methionine content (Bessada *et al.,* 2019; Ohanenye *et al.,* 2020). For instance, Jirapa *et al.* (2001) reported that cowpeas presented low concentration of sulfur amino acids, such as methionine and cysteine. Our results highlighted that germination serves as a promising food processing to significantly increase the levels of methionine. Methionine is a nutritionally indispensable amino acid due to its functions as a building block for protein synthesis and as a primary methyl donor for S-adenosylmethionine (AdoMet) production via the enzyme S-adenosylmethionine synthetase (SAM1). Methionine plays a crucial role in the synthesis of several essential metabolites, such as biotin, creatine, and phosphatidylcholine (Elango, 2020; Rajjou *et al.,* 2012).

Our results showed that the best germination condition to increase essential amino acids in cowpea was at 35 °C with 140% of water content, regardless the light. Under this condition, the content of lysine, isoleucine, leucine, methionine, phenylalanine, valine, threonine, and tryptophan significantly increased by 2.5 - 17-foldchange. The histidine level did not change significantly. The identified key enzymes dihydroxy-acid dehydratase (DAD) and branched-chain amino acid aminotransferase (ATBCAT-5) participates to the biosynthesis of isoleucine, leucine, and valine. They presented lower values when germination rate was higher, while high levels in dry, soaked, and germinated at 25 °C with 80% WC. This observation indicates that these enzymes are already available to initiate metabolic pathways even before the germination process begins. The ready availability of these enzymes in the dry and soaked state might reflect their crucial roles in early metabolic responses and essential preparatory steps leading up to

germination. Additionally, the high increase in essential amino acids after germination could be due to the increase of proteases activity, which break down proteins and releases free amino acids. As previously demonstrated, 13 proteases reported significantly increases in their values during germination. Wu *et al.* (2020) reported that germinated mung-beans in the dark at 26 °C for 18 h increased isoleucine, leucine, phenylalanine, valine, histidine and tryptophan content by 32 - 467%. On the other hand, Jirapa *et al.* (2001) reported that germinated cowpeas in the dark at room temperature for 24 h reduced lysine and valine content by 9% and 8%, respectively. Other studies showed that sprouting process increased the amino acids content in soybean by 1.3 times when compared to raw seeds (Gu *et al.*, 2017; Wu *et al.*, 2023). The difference in results is related to the longtime of sprouting process. During germination, seed releases free amino acids which serve as essential co-factors in various metabolism process, such as valine, leucine, and isoleucine biosynthesis/degradation, lysine degradation, and glycine, serine, and threonine metabolism. As germination progresses into sprouting, seeds will consume these amino acids, leading to a reduction in their content. Therefore, it becomes advantageous to perform a short germination process to profit the seeds' metabolism which synthesize beneficial nutrients for human consumption.

In relation to galactose metabolism, α-galactosides (stachyose and raffinose) were reduced and converted into galactose, glucose, fructose, and sucrose as germination time, temperature, and water content increased (Figure 9). The results of the hydrolysis of α-galactosides were used as an energy source by the seedling during germination. Interestingly, our results reported that the soaking process was not effective in reducing α-galactosides content; instead, it led to an increase in stachyose and raffinose content. However, our previous study reported a 27% reduction under the same soaking conditions (Avezum *et al.*, 2023). This difference can be attributed to the distinct α-galactosides extraction and quantification method used in the previous study. The germination process significantly reduced the α-galactoside content as germination rates increased (high temperature and water content). Our results are in agreement with previous studies. Ibrahim *et al.* (2002) reported that germinated cowpeas in the dark at room temperature for 24 h reduced raffinose and stachyose content by 100%. Wu *et al.* (2020) reported that germinated mung beans in the dark at 26 °C for 18 h reduced raffinose family oligosaccharides (raffinose, stachyose, and verbascose), by 66%; while increased the sucrose content by 169%, α-glucose by 567%, and malate by 108%. On the other hand, Nnanna & Phillips, (1988) reported that germinated cowpea in the dark at 35 °C for 24 h increased raffinose, maltose, and glucose content, by 109%, 222%, and 50%, respectively; while reduced

stachyose content by 62%. The α-galactoside reduction was facilitated by the action of key enzymes, such as α-galactosidase, raffinose synthase, and stachyose synthase (Figure 9). In our study, α-galactosidase and stachyose synthase showed high values in dry, soaked, and germinated at 25 °C with 80% WC, reaching the end of their enzymatic activity as germination temperature and water content increased. Arunraj *et al.* (2020) also reported higher levels of α-galactosidase in mature and dry chickpea seeds than in germinated for 24 h. However, Grant Reid & Meier (1973) reported that α-galactosidase activity increased after 24 h of germination in the endosperm. These findings highlight the complexity of seed germination and the metabolism alterations that occur during germination/sprouting. Furthermore, they demonstrate how germination can be an effective process to reduce α-galactoside content in pulses.

Through inositol metabolism it was observed that germination process increased myo-inositol content. Other authors showed same results than ours, Gu *et al.* (2017) reported that germinated soybeans for 4-day increased myo-inositol content by 2-foldchange. Phytic acid is composed of myo-inositol hexakisphosphate, and its hydrolyzation generates myo-inositol (P5-P1) through the action of phytases enzymes (Campos-Vega *et al.,* 2010). The reduced form of phytate, including inositol 1-phosphate, myo-inositol 4-phosphate, and 1D-myo-inositol 3-phosphate, releases free myo-inositol through the enzymatic activity of inositol-phosphate phosphatase (Figure 8). This degradation provides phosphorus for the development of seeds and young seedlings uses the free myo-inositol for cellular formation (Oatway *et al.,* 2001). This indicates a correlation between hydrolysis of phytic acid and high myo-inositol levels. Ibrahim *et al.* (2002) reported that germinated cowpeas in the dark at room temperature for 24 h and 48 h reduced phytic acid content by 17% and 23%, respectively. Similarly, other studies reported that germinated fava beans, chickpeas, cowpeas, and soybeans at room temperature for 24 h reduced phytic acid content by 38 - 47% (Mehanni *et al.,* 2021; Sofi *et al.,* 2020). All mentioned studies indicated that the longer the period of germination, the greater the reduction in phytic acid content. In according to these results, other studies showed that sprouting pigeon peas and soybeans for 72 h reduced phytic acid content by up to 90% *(*Anaemene & Fadupin, 2022*;* Wu *et al.,* 2023). The observed increase in myo-inositol content in our results, combine with the reduction in phytic acid as documented in previous studies, confirms that germination and sprouting are an effective process for reducing phytic acid content. It is important to highlight that in the last mentioned studies (Ibrahim *et al.,* 2002; Mehanni *et al.,* 2021; Sofi *et al.,* 2020), the germinated seeds were oven-dried after germination process, as opposed to our method of freezing germinated samples for conservation. This may potentially influence the

reduction and analysis of nutritional and antinutritional compounds, masking the true effect of germination process.

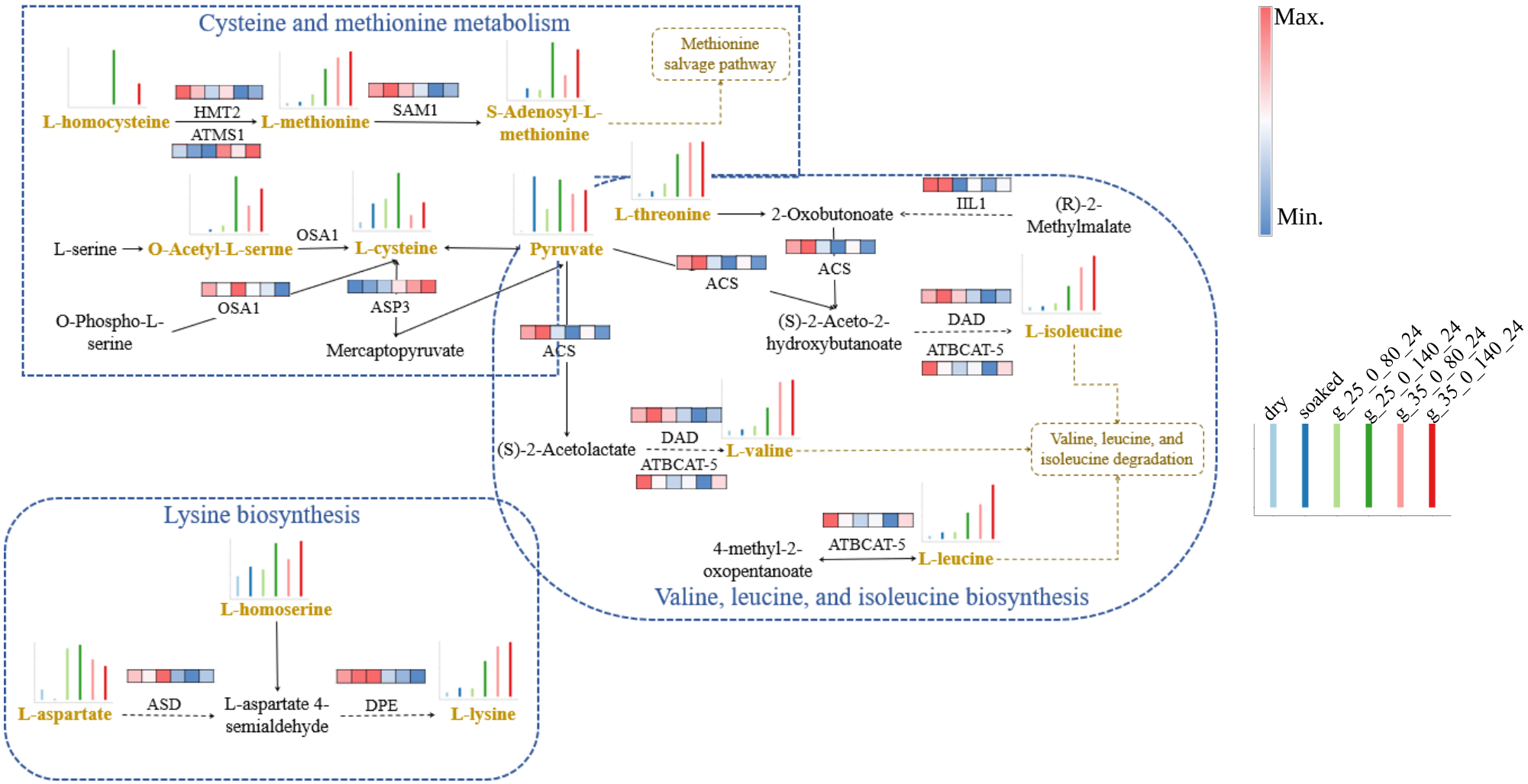


Figure 8. Amino acids pathway with major and identified metabolites and relative proteins accumulation during germination process of cowpea seeds. The bar plots represent the relative content of metabolites at dry, soaked, g_25_0_80, g_25_0_140, g_35_0_80, and g_35_0_140 respectively from left to right. The bar heatmap was plotted using protein abundance at dry, soaked, g_25_0_80, g_25_0_140, g_35_0_80, and g_35_0_140, respectively from left to right. OASA1: cysteine synthase; ASP3: aspartate aminotransferase; HMT2: homocysteine S-methyltransferase; ATMS1: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase; SAM1: S-adenosylmethionine synthetase; IIL1: 3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit; ACS: acetolactate synthase I/III small subunit; DAD: dihydroxy-acid dehydratase; ATBCAT-5: branched-chain amino acid aminotransferase; ASD: aspartate-semialdehyde dehydrogenase; DPE: diaminopimelate epimerase.

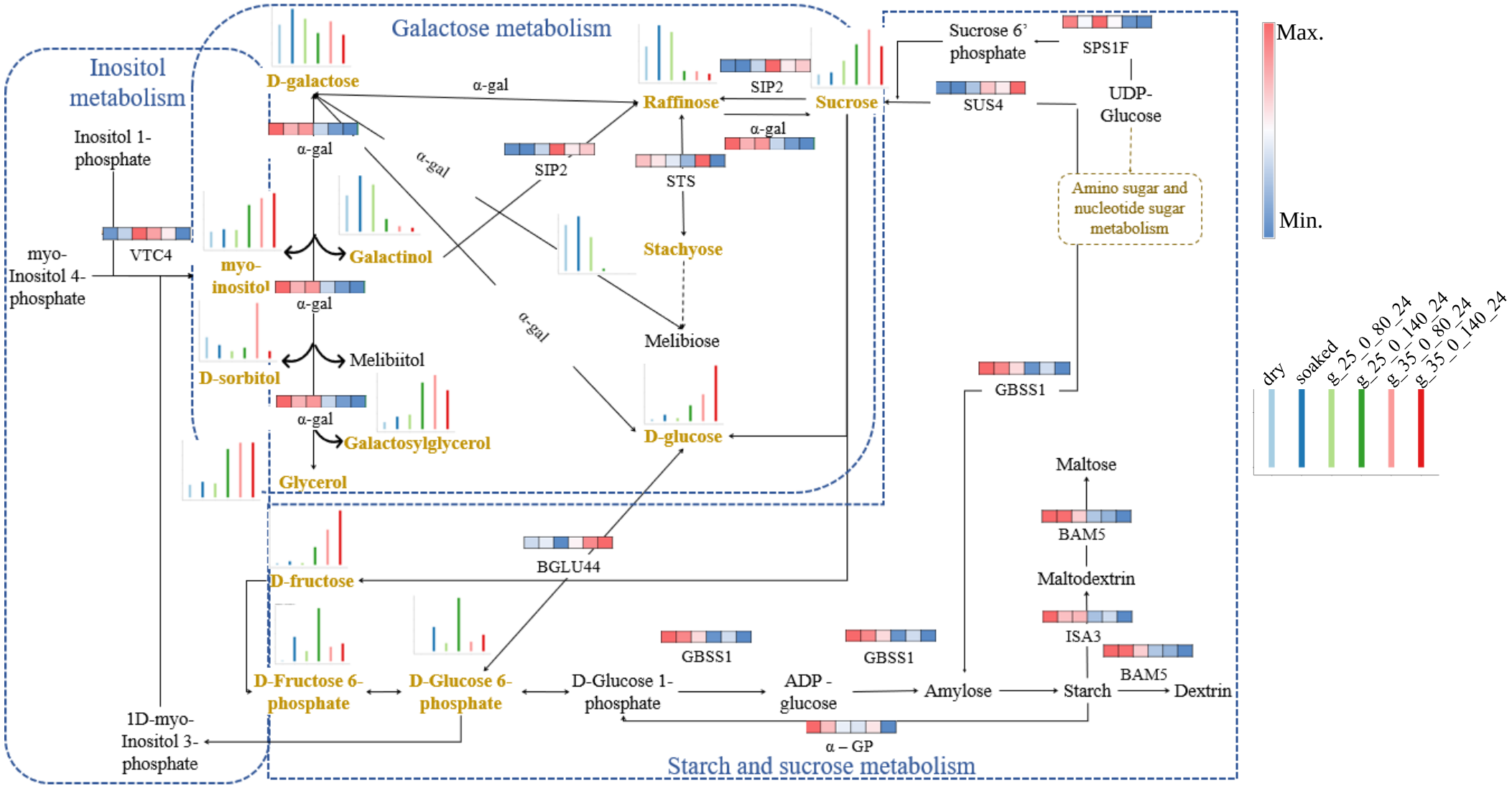


Figure 9. Carbohydrates pathway with major and identified metabolites and relative proteins accumulation during germination process of cowpea seeds. The bar plots represent the relative content of metabolites at dry, soaked, g_25_0_80, g_25_0_140, g_35_0_80, and g_35_0_140 respectively from left to right. The bar heatmap was plotted using protein abundance at dry, soaked, g_25_0_80, g_25_0_140, g_35_0_80, and g_35_0_140, respectively from left to right. α-Gal: α-galactosidase; SIP2: raffinose synthase; STS: stachyose synthase; VTC4: inositol phosphate phosphatase; BGLU44: β-glucosidase; SUS4: sucrose synthase; SPS1F: sucrose-phosphate synthase; GBSS1: granule-bound starch synthase; ISA3: isoamylase; BAM5: β-amylase; α-GP: α-glucan phosphorylase.

V. Conclusion

Germination is a complex biological process that results in major changes in metabolism, reshaping of metabolic pathways, and modifications in the nutritional composition of pulses. Our study represents a pioneering effort using metabolomics and integrated proteomic technologies to explore the influence of germination conditions on the nutritional profiles of cowpeas. Our results revealed the crucial roles of temperature and water content during germination, illustrating how they shape the nutritional composition of cowpea seeds. Germination under higher temperatures and water content resulted in a higher germination rate (83%), increased content of essential amino acids, and reduced α-galactosides and phytic acid in cowpeas. We identified key enzymes, including dehydrin ERD14, glutathione S-transferase, proteins SLE1 and SLE2, and betaine aldehyde dehydrogenase 1, which regulate germination responses and sensitivity to abscisic acid, temperature stimulus, and water deprivation. Germinated cowpeas at 35 °C with 140% of water content increased essential amino acids by 2.5 - 17-foldchange, signifying the most pronounced enhancements. The identified key enzymes involved in amino acid metabolism were 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (ATMS1), homocysteine S-methyltransferase (HMT2), dihydroxy-acid dehydratase (DAD), and branched-chain amino acid aminotransferase (ATBCAT-5). Moreover, we identified the enzymes α-galactosidase, raffinose synthase, and stachyose synthase, which participate in the hydrolysis of α-galactosides. Germinated cowpea at 25 °C with 140% WC, and at 35 °C, regardless the water and light condition, reduced raffinose content by 5.2-foldchange and stachyose content was undetected. In addition to reducing α-galactoside content, germination was an effective process for reducing phytate content, by increasing myo-inositol content (up to 247%) through inositol phosphate phosphatase activity.

These findings highlight the importance of temperature and water content during germination in molding the nutritional landscape of cowpea seeds. Such insights not only help optimize germination conditions to enhance the nutritional quality of pulses, but also contribute to a deeper understanding of the complex impact of germination on seed metabolism. This knowledge lays the groundwork for further research in this area, illuminating the complex

correlation between germination and seed nutritional value, resonating beneficially within the food industry, and for consumers.

VI. References

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VI. Thiamine and folate immunolocalization in germinating lentil seeds: a microstructural investigation

In previous studies, we explored and demonstrated the efficacy of soaking in reducing α-galacatosides content, as well as how germination increases nutritional quality by enhancing vitamins and essential amino acids, while reducing antinutritional compounds, including α-galacatosides and phytate. Moreover, we discuss how germination factors influence germination rates and the nutritional quality of pulses. Our study used several quantitative techniques, including HPLC, UPLC, LC-MS, and GC-MS. These techniques were utilized to collect data that, after analysis, facilitated the identification of metabolic pathways triggered by germination conditions and contributed to the determination of optimal conditions for enhancing the nutritional quality of pulses. This compilation of information holds significant value in elucidating the complex metabolic processes that shape the nutritional profile of seeds and how food processing impacts the nutritional value of food products. Although these technologies and information are accurate and indispensable for quantifying compounds within the food matrix, they lack in the capability to provide insights into their spatial distribution in cotyledons and cells. Given the structural complexity of seed storage tissues, it becomes imperative to understand the *in-situ* localization of compounds and ascertain how food processing influences their compartmentation and de-compartmentation. The questions that remain are: how the microstructure of pulse seeds is modified through the germination process? Where are located the essential compounds, including thiamine and folate, in the seed structure? How the germination process modifies the localization of these compounds?

In our next study, we used cytology and immunohistochemistry techniques to answer these questions. Our aim was to employ immunohistochemistry to both localize and quantify *in-situ* thiamine and folates in dry, soaked, and germinated lentil seeds. The use of this technique provides valuable insights into the local distribution of these compounds and how their locations are altered during germination. Moreover, we performed cytology analysis to understand the microstructural changes during lentil germination. We expect that the use of these two techniques would provide comprehensive qualitative and quantitative insights into the spatial distribution of these compounds. This approach will help answer our research question and provide essential information for a deeper comprehension of the seed metabolism process. Furthermore, this study will aid in elucidating the diffusion and degradation of vitamins during food processing, thereby enriching our knowledge of these essential compounds.

Thiamine and folate immunolocalization in germinating lentil seeds: a microstructural investigation

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Abstract

Thiamine and folate are extremely important for human and vegetal health, and act as cofactors in many metabolic pathways. Thiamine is involved in the pyruvate dehydrogenase reaction and in carbohydrate and amino acid metabolism. On the other hand, folate is essential for DNA synthesis and provides a C1 group for the methyl cycle, which converts homocysteine to methionine. Germination has been shown to be an effective process for increasing the thiamine and folate contents in lentils. High-performance liquid chromatography (HPLC) is a widely used method to quantify vitamins in different food matrices. However, although it is precise, it does not provide information about the localization of compounds in cotyledons and cells during germination. Immunohistochemistry (IHC) can solve this problem by localizing the modification of metabolism and compartmentation in different plant tissues. In this pioneering study, we aimed to use IHC technique to localize and quantify *in situ* thiamine and folate in dry, soaked, and germinated lentil seeds. Image analysis revealed heterogeneous distribution of vitamins in the cotyledons and cells. Moreover, thiamine and folate are localized around starch granules in dry seeds, whereas in soaked and germinated seeds they are dispersed in the cytoplasm and around protein bodies. Through this original approach, we obtained both quantitative and qualitative information on the impact of germination on the average vitamin contents. This valuable information enhances our understanding of seed metabolism and facilitates explanations of the diffusion and degradation of vitamins during food processing.

Keywords: Immunohistochemistry, Thiamine, Folate, Lentil, Germination

I. Introduction

Germination *sensu stricto* begins with water uptake by the seeds and ends with the elongation of the radicle through testa. During this process, there are major metabolic changes and activation pathways that hydrolyze nutrients, such as carbohydrates, proteins bodies, and vitamins, which are mobilized to ensure radicle growth until the plantlet becomes fully autotrophic (Rajjou *et al.,* 2012). Contrary to fermentation, germination is rarely conducted at the industrial scale in pulses due to microbiological risks and lack of knowledge regarding the benefits of germination (Bresciani & Marti, 2005). Among the seeds used for germination, pulses provide health benefits, being excellent suppliers of carbohydrates, proteins, fibers, minerals, and B vitamins (Benincasa *et al.,* 2019). Several studies reported that germination is an effective process to increase the thiamine and folate content in pulses (Avezum *et al.,* 2022; Benincasa *et al.,* 2019). Thiamine is used as a cofactor for various enzymes and is present in a free form or in its phosphorylated derivatives, namely, thiamine monophosphate (TMP), diphosphate (TDP), triphosphate (TTP), and adenosine thiamine triphosphate (Tylicki *et al.,* 2018). The active form of thiamine (TDP) is used as a cofactor in many seed metabolic processes: *i)* mitochondrial and chloroplast pyruvate dehydrogenase reaction, *ii)* response to biotic and abiotic stress, *iii)* defense system responses, and *iv)* carbohydrate and amino acid metabolism (Goyer, 2010). Folates are present in different forms in pulses, such as folic acid (PteGlu), tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), and 10-formylfolic acid (10-CHO-PteGlu). The most important cellular function is their implication in the synthesis of DNA by PteGlu, 5-CH_3-H_4folate, 5-CHO-H_4folate, and 10-CHO-PteGlu. The 5-CH3-H4folate provides a C1 group for the methyl cycle to be used by methionine synthase to convert homocysteine to methionine. In plants, H_4folate is involved in subcellular compartmental photorespiration (Gorelova *et al.,* 2017). In seeds, thiamine and folate contents depend on the cultivar, origin of the plant tissue, and stage of seed development (Rébeillé *et al.,* 2006).

Shrivas *et al.* (2018) reviewed various analytical techniques used for the determination of thiamine in pharmaceutical and food samples. Among the simple and effective methods, high-performance liquid chromatography (HPLC) has been extensively used to quantify the vitamers contents of thiamine in various unmalted and malted cereals (Hucker *et al.,* 2012). Among other recent techniques, localized surface plasmon resonance (LSPR) is a promising technique for

colorimetric detection of thiamine in rice filtrate samples (Shrivas *et al.,* 2018). Biospecific methods for folate detection include Enzyme-protein binding assay (EPBA) and Biomolecular Interaction Analysis (BIA) were reported according to Ball, (2006). Attractive high-throughput detection methods, such as Enzyme-linked immunosorbent assay (ELISA), is also mentioned for folate detection (Finglas & Morgan, 1994; Kong *et al.,* 2016), using monoclonal and polyclonal antibodies to folic acid. A recent article also reported the first production of a specific monoclonal antibody to thiamine, and mentioned the development of an immunoassay for its detection in energy drinks and infant milk powder (Zeng *et al.,* 2020). According to these biospecific methods for the detection of thiamine and folate, the innovations push back the technical boundaries, with lower limits of detection and specificity in unstructured food matrices.

However, given the structural complexity of seed storage tissues such as cotyledon in pulses, and cellular organization of biological materials, the bottleneck remains to understand the *in-situ* compartmentation and decompartmentalization that occurs after hydration to mobilize nutrients from the seed's reserve forms. Furthermore, comprehending the underlying mechanisms that govern the dynamic evolution in metabolites during physiological and technological processes remains a complex task. In particular, the use of immunohistochemistry (IHC) technique can facilitate the understanding of the modification of metabolism and compartmentation in different tissues in many plant structures, such as the vasculature developing seeds, fruits, leaves, stems, and roots (Walker *et al.,* 2001). Apart from some attempts dedicated to localization of some phenolic compounds in living coffee plants and vanilla fruit by fluorescence (Talamond *et al.,* 2015) and to recent observation by immunostaining of the diffusion of folate in cowpea seeds during the soaking-cooking process (Coffigniez *et al.*, 2019), the localization of thiamine and folate in ungerminated and germinated lentil seeds has never been done through image analysis, while taking benefits of immunostaining specificity, and limit of detection.

Taking benefits from the recent description of the germination process of lentils using the optimal germination conditions for thiamine and folate production (Avezum *et al.,* 2023), the aim of this study was to use IHC technique to localize and quantify *in-situ* thiamine and folate in dry, soaked, and germinated lentil seeds. Moreover, we used cytology technique to follow microstructural modifications, especially in starch granules, protein bodies and mitochondria, during germination. Beyond the original methodological approach, the utilization of the IHC

technique enables qualitative and quantitative information on the impact of germination on the average vitamin B1 and B9 contents. Moreover, it provides valuable insights into the local distribution of these compounds and how their locations are modified during the germination process. Utilizing image analysis to explore the impacts of processing on the microstructure of a complex food matrix can greatly enhance our understanding of the diffusion and degradation processes of vitamins and other compounds.

II. Materiel and methods

II.1. Material

Green lentils (*Lens culinaris*, var. Anicia) seeds were purchased in 2020 from CIBELE Company, France. The seeds were stored at -20 °C until use. Phosphate buffer (PBS), bovine serum albumin (BSA), and paraformaldehyde (PFA) were purchased from Euromedex (France). The monoclonal anti-Vitamin B1 - CABT-L4600 primary antibody from mouse for thiamine detection was purchased from Creative Diagnostics (USA). Anti-5-methyltetrahydrofolic acid primary monoclonal antibody from mouse was purchased from Sigma-Aldrich (USA). An anti-mouse secondary antibody labeled with Alexa 488 from goat (second antibody used for the detection of the complex antigen-primary antibody) was purchased from Invitrogen, USA (Cat. # A-11029).

II.2. Germination process and dehydration

Germination process was described by Avezum *et al.* (2023). To activate germination, 80 g of lentil seeds were soaked in 320 mL water (ratio 1:4; w/v) under agitation at 30 °C for 3 h. Soaked seeds were removed from the soaking water, immediately drained and placed in a BIA Climatic (France) chamber at a temperature of 35 °C and a relative humidity of 90% for 24 h of germination. Seeds were placed in a rectangular perforated weighing pan (361 cm^2) equipped

with an integrated balance to monitor seed weight and, consequently, its water content. A spray was automatically activated when the water content dropped below 80% dry basis, and stopped after 10 minutes. The light used was an Alpheus LED (France) which corresponds to sunlight, with a photoperiod of 12 h. According to our last study, this germination condition represents the optimal condition for increasing thiamine and folate content in lentils (Avezum *et al.*, 2023). After soaking or germination, seeds were fixed by immersion in a 4% PFA solution in PBS (phosphate buffer) overnight. The seeds were then washed two times for 15 min in glycine/PBS (0.1 M) solution and two times in PBS. The seeds were dehydrated using a vacuum system with 50% and 70% ethanol for 30 min each, before being stored in 70% ethanol at 4 °C until further analysis (cytology and immunohistochemistry).

II.3. Cytohistology

The seeds were dehydrated in increasing concentrations of ethanol (70%-100%); and impregnated with butanol 100% and resin (Technovit 7100 from Kulzer). All steps were carried out in Histos 5 rapid microwave histoprocessor (Milestone, Denmark). Longitudinal sections of 4 µm were serially cut using a microtome (Leica RM2255). Sections were stretched in a distilled water bath and serially mounted on glass microscope slides. Sections were cytochemicaly stained to visualize major cell components using: periodic acid-Schiff's stain (PAS) for both starch and pectocellulosic cell walls (pink), and naphthol blue-black (NBB) for protein (blue) (El Maâtaoui & Pichot, 1999; Fisher, 1968). Sections were visualized in wide field microscopy using a Nikon Eclipse NI-E at 20x Plan Apo λ 0.75 NA (quantification) and 40x Plan Apo λ 0.75 NA (visualization) magnification.

II.4. Immunohistochemistry

The fixed/dehydrated dry, soaked, and germinated seeds were sliced into sections of 80 µm thickness using a vibratome. Some cross-sections for each condition were set aside as control

samples and were not included in the labeling protocol. The sections for labeling protocol were immersed in a blocking solution containing 5% BSA in PBS overnight at 4 °C. The sections were washed three times with PBS buffer for 10 min. The primary antibody, anti-vitamin B1 for thiamine localization or anti-5-methyltetrahydrofolic acid for folate localization, was diluted in PBS to a final concentration of 10 µg/mL and incubated at 4 °C overnight. Thiamine and folate labeling was performed on separate cross sections. The sections were washed three times with PBS buffer for 10 min. The secondary antibody (Alexa 488, Invitrogen, France) was added at a final concentration of 4 µg/mL and incubated for 1h. Finally, the sections were washed three times with PBS buffer for 10 min. The sections were mounted on microscopic slides with 50% glycerol (w/v) and visualized using a confocal Zeiss LSM880. The excitation wavelength was 488 nm and the emission wavelength were 500 – 571 nm. Each immunostaining experiment for each vitamin was performed in triplicates. Cross-sections of seeds were analyzed by Tile Scan method (quantification) in confocal microscopy (Zeiss LSM880) using the Zen software from Zeiss. Cotyledon cells were observed under a 10x Plan Apo 0.45 NA and 40x LD C-Apo 1.1NA W objectives (visualization).

II.5. Mitochondria labeling and localization

Mitochondria labeling was performed following the protocol provided by the supplier (Invitrogen™) using MitoTracker™ Red CMXRos. First, a stock solution of lyophilized MitoTracker was prepared by diluting it in anhydrous dimethyl sulfoxide (DMSO) to achieve a final concentration of 1mM. The stock solution was diluted in growth medium to obtain a final concentration of 200 nM for labeling the mitochondria. Soaked seeds were sliced into sections of 130 µm thickness using a vibratome and incubated with Mitotracker probe for 45 min. The sections were mounted on microscopic slides with 50% glycerol (w/v) and visualized using a confocal Zeiss LSM880. The excitation wavelength was 561 nm and the emission wavelength were 590 – 618 nm. The cross-section was fixed with a 4% PFA solution for 15 min and washed three times with PBS buffer for 10 min each. To label mitochondria, it is essential to ensure that the sample cells are alive, and the fixation and vitamins labeling process should be conducted after Mitotracker labeling. The immunohistochemical protocol described in section 2.4 was then followed.

II.6. Quantification and statistical analyses

Cytohistology images were analyzed using ImageJ software. After manual thresholding, starch granule count per cell, circularity, area, and hilum count were determined for all experimental conditions (surface with magnification of 20x). All data were statistically analyzed using ANOVA ($p < 0.05$) and TukeyHSD test.

The immunolocalization of thiamine and folate was quantified on tile scan images using ImageJ software. The mean gray value intensity of the cross-section was measured for the control (without labeling) and labeled samples for each condition. The threshold applied (25 – 255) and image acquisition were the same for all samples. The labeled intensity values were subtracted from the control intensity values to calculate the actual intensity labeling in our samples. The percentage of labeling area was calculated to indicated the proportion of labeled area in relation to the total tissue area. All data was statistically analyzed by ANOVA ($p < 0.05$) and by TukeyHSD test.

III. Results and Discussion

III.1. Cytology

Dry seeds presented intercellular spaces, which decreased in soaked, but significantly expanded during germination due to water saturation causing increase in pectin interstitial gaps (Figure 1). This suggests that in dry state, cellular dehydration leads to a contraction in cell size and preserves cellular morphology. In a similar matter, Atudorei *et al.* (2021) reported that germinated seeds presented larger intercellular spaces between then compared to non-germinated ones. The cell walls of lentils, rich in pectocellulosic compounds, remains largely intact after soaking and germination for 24h (Figure 1). This suggested low hydrolytic enzyme activity may occur. Atudorei *et al.* (2021) showed that 48 h sprouted lentils, beans, chickpeas, lupines, and soybeans presented higher disruption of the cellular walls, due to an increase of the hydrolytic enzyme activity. The observed difference in cellular integrity is attributed to the

more advanced seed metabolic activity at 48 h than at 24 h, with high enzyme activity, which degrades pectin in the cell wall and other cell compartments (Atudorei *et al.*, 2021).

Pink-stained starch granules were the most representative structures on the lentil cotyledon section-surface, surrounded by protein bodies colored in blue, and enveloped by intact cell walls (Figure 1). The area of each starch granule for the dry seeds was 345 μm^2, while it was 549 μm^2 for soaked grains, and of 586 μm^2 for germinated seeds. The significant difference ($p < 0.05$) between dry and soaked/germinated seeds starch granule area are most probably related to the water uptake after soaking that increases the starch granule volume, given its hygroscopicity. Other studies have also shown that starch granules are major compounds in cells of cowpeas, lentils, chickpeas, and common beans (Atudorei *et al.*, 2021; Coffigniez *et al.*, 2019[a]; Marconi *et al.*, 2000). Starch granules had a globular surface with circularity of 0.78 ± 0.03, 0.79 ± 0.01, and 0.80 ± 0.02 in dry, soaked, and germinated seeds, respectively. The hilum is apparent in the core of the starch granules and is the starting point for starch granule growth (Ang *et al.*, 2010). In dry seeds, the hilum was noticeable in 22% of starch granules, whereas in the soaked seeds, it was visible only in 3% (Table 1). Soaking for 3 h was insufficient for the starch granules to fully uptake water and may hide the presence of all hilum. This could be due to the competition between starch and protein water uptake during the short soaking period (Lefèvre *et al.*, 2022); or to the limited water uptake during soaking due to the integrity of the cell walls. After 24 h of germination, the hilum is no more visible (0% of the total starch granules) due to complete hydration. Advancing from soaking to germination allowed starch and proteins to become saturated, resulting in the complete swelling of starch and potentially in the dissimulation or the apparent elimination of the hilum. These results indicate that as the seeds absorbed water, the starch granules area increased (up to 1.7-fold) in size while hilum space decreased (soaked) or disappeared (germinated). The phenomenon of complete starch hydration is likely facilitated by the swelling of cell walls as well as solubilization of pectin during hydrothermal treatments, which alters the cell walls porosity and facilitates water uptake by starch granules (Ajala *et al.*, 2023; Junejo *et al.*, 2021; Li *et al.*, 2019). It is noteworthy mentioned that cell wall stability, integrity, and the absence of pectin solubilization can reduce the bioavailability and digestibility of starch and proteins. Actually, the cell wall act as a physical barrier that inhibits the swelling of starch granules, consequently preventing gelatinization during cooking (Junejo *et al.*, 2021; Li *et al.*, 2019). Additionally, this physical barrier can slow down the enzymatic breakdown of starch and protein bodies, making them less readily accessible for digestion (Do *et al.*, 2020). Studies have shown that *in vitro* starch

digestion is correlated to cell size, shape, thickness of cell walls, number of starch granules, fiber content, and hydration rate (Ajala *et al.*, 2022; Do *et al.*, 2020; Xiong *et al.*, 2018). Therefore, studying the impact of food processing on the seed microstructure is crucial to understand the underlying mechanisms that occur during these processes. This investigation can help to explain the variations in digestibility, hydration, diffusion, and degradation of compounds that occur during food processing.

Protein bodies were dispersed in cytoplasm and were better identified in cotyledon cells after 24 h of germination (Figure 1). Blue-black naphthol is sensitive to the concentration of proteins. Proteins that are highly concentrated and condensed, as found in reserve protein bodies, result in a darker blue-black coloration. Conversely, the less concentrated proteins, such as soluble cytoplasmic proteins, appear as a lighter shade of blue. Indeed, in the dry seeds, soluble proteins in the cytoplasm are more concentrated due to the retraction of the dehydrated cytoplasm and cell content. This could explain that when using naphthol blue-black staining it becomes challenging to distinguish between proteins present in protein bodies and those dispersed in the cytoplasm, as the staining will color both in dark blue. However, during soaking and germination, the cytoplasm absorbs water making proteins less concentrated. Following staining under these conditions, the staining solution can show a clear distinction between protein bodies (dark blue) and proteins dispersed within the cytoplasm (light blue). Moreover, the protein bodies in germinated pulses (Figure 1- c) exhibited a lighter blue color than the protein bodies in soaked pulses (Figure 1 – b). This change in coloration could be attributed to the initial unfolding of reserve proteins linked to their rehydration and to the early stages of protein hydrolysis. As previously discussed in our prior study (refer to Chapter V), germinated cowpea seeds increased the relative abundance of proteases, which could explain the hydrolysis of protein, leading to the liberation of free amino acids used in seed metabolism. Atudorei *et al.* (2021) showed that shorter germination period leads to a higher compactness of the protein bodies structure. In contrast, as mentioned by other authors, sprouting process leads to a more open structure of protein bodies and depletion in starch and protein granules due to hydrolytic enzymes activity (Atudorei *et al.*, 2021; Di Stefano *et al.*, 2019). These findings align with our observation that a longer germination process corresponds to higher enzymatic activity of proteases, resulting in the hydrolysis of proteins and the opening of their structure.

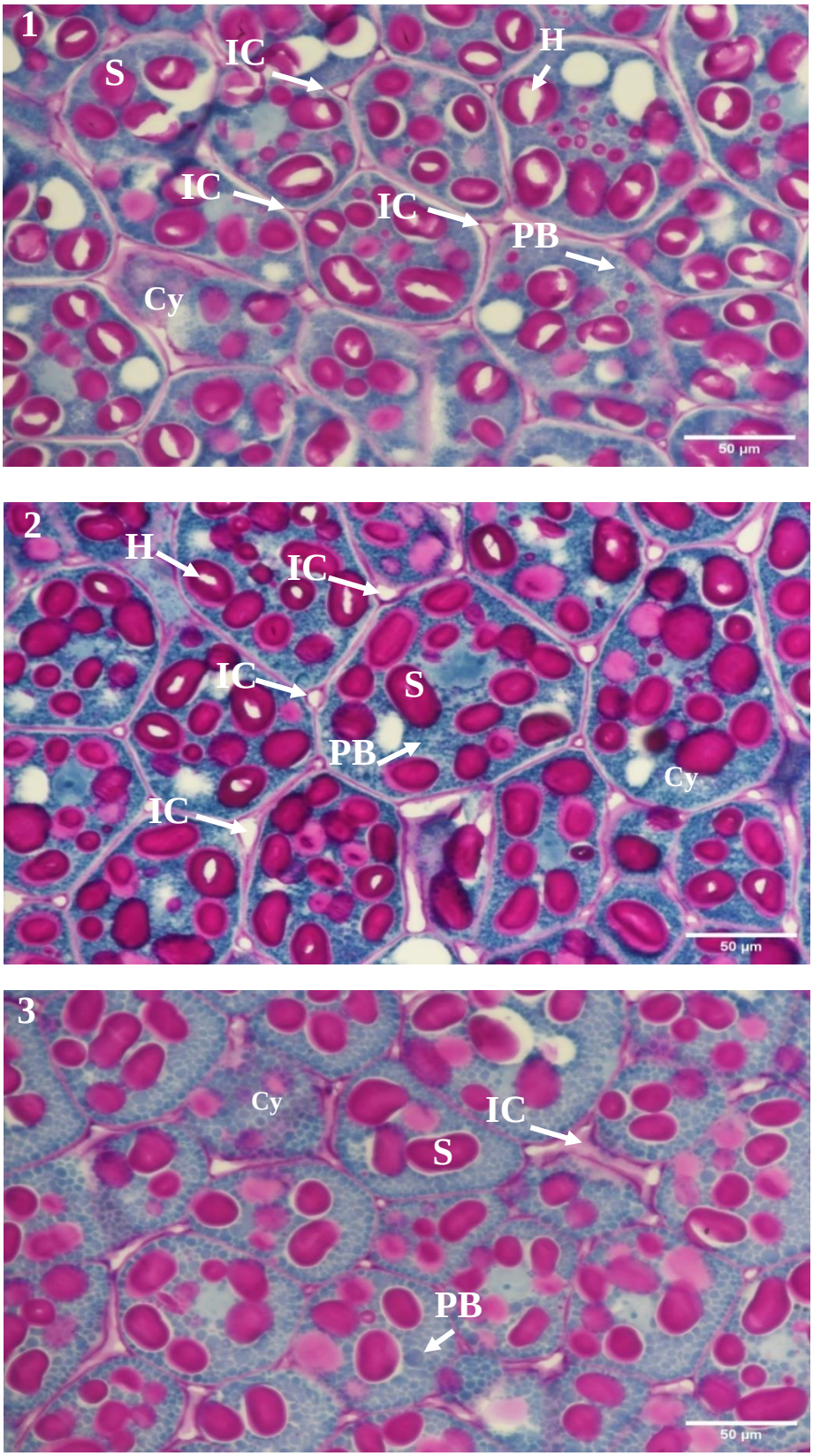


Figure 1. Microstructure of dry (1), soaked (2), and germinated (3) lentil seeds at 40x magnification. Sections were stained with PAS-NBB. S: starch granule; H: hilum; PB: protein bodies; Cy: cytoplasm; IC: intercellular space. Quantification was performed at 20x magnification in one cross-section for each condition.

Table 1. Quantification of starch granules number, area, and circularity and hilum number in cotyledon cross-section at 20x magnification using ImageJ Software.

Condition	Number of starch granule per cell	Starch Granule Circularity	Starch granule area (µm2)	Hilum Number	% of presence of hilum
Dry	5.9 ± 0.71	0.78 ± 0.03	345 ± 95.9	107 ± 16.1	22 ± 0.04
Soaked	6.2 ± 0.79	0.79 ± 0.01	549 ± 29.6	13 ± 14.8	3 ± 0.03
Germinated	5.4 ± 0.32	0.8 ± 0.02	586 ± 83.2	0 ± 0	0 ± 0

III.2. Immunolocalization of thiamine and folate

Thiamine and folate appeared concentrated around starch granules in dry seeds and dispersed in cytoplasm and around protein bodies in soaked and germinated seeds (Figures 2 and 3). One hypothesis is that due to the low water content in dry seeds, thiamine and folate tend to be more concentrated in specific regions, such as around the starch granules. However, during soaking and germination, the cells take up water, resulting in solubilization of vitamins in the cytoplasm. Moreover, the activation of metabolic activity during these processes favors the synthesis of thiamine (+ 169%) and folate (+ 8%) (Avezum *et al.*, 2023). This could favor the dispersion of thiamine and folate throughout the cytoplasm, making them readily available for metabolic processes and energy generation essential for germination.

Another hypothesis is that thiamine and folate my bind to protein bodies during the soaking and germination processes. Actually, unphosphorylated thiamine binds to high-affinity protein sites present in some globulins (vicilin) (Gołda *et al.*, 2004; Watanabe *et al.*, 1998). The results of the present study, along with our previous research, indicated that dry seeds have low levels of thiamine and probably unphosphorylated thiamine, which leads to minimal binding to protein sites. However, as the germination process begins and metabolic activity is activated, there was a significant increase in total thiamine content (Avezum *et al.*, 2023). Unfortunately, in our previous study, we did not directly analyze unphosphorylated thiamine. However, we can presume that the increase in thiamine diphosphate and monophosphate may also indicate an increase in the level of unphosphorylated thiamine (Avezum *et al.*, 2023). As a result, we observed a greater presence of thiamine closer to proteins during soaking and germination.

However, Gołda *et al.* (2004) showed a progressive decay in thiamine-binding activity during the germination and sprouting for 1 to 6 days in maize, oats, fava beans, and pea seeds. Another study also showed a reduction in thiamine-binding proteins during germination and sprouting for 1 to 4 days in brown rice seeds (Mitsunaga *et al.,* 1987). The study suggested that thiamine-binding proteins was broken down by proteolytic enzymes to produce amino acids, which are essential for synthesizing new proteins during germination, and subsequently released thiamine to produce the phosphorylated form (Mitsunaga *et al.,* 1987). However, within a short germination period such as 24 h, it is possible that there were less proteolytic enzymes responsible for releasing thiamine from protein bodies. This could potentially explain the observed accumulation of thiamine around protein bodies during germination *sensu stricto* process. Folates are relatively unstable molecules; however, studies have shown that binding folates to proteins increases folate stability, reducing its susceptibility to oxidative damage (Rébeillé *et al.,* 2006; Jones & Nixon, 2002; Hutchinson *et al.,* 2000). During germination and post-germinative process, seeds require high amounts of methyl groups to participate in Ado-Met and Met metabolism, which is central to seed germination (Rajjou *et al.,* 2012; Gallardo *et al.,* 2007; Gallardo *et al.,* 2001). 5-CH3-H4folate is the main folate form used by the seeds to acquire the methyl group. Therefore, stabilizing folate by protein-binding during germination may be important to preserve methyl groups for the post-germinative event.

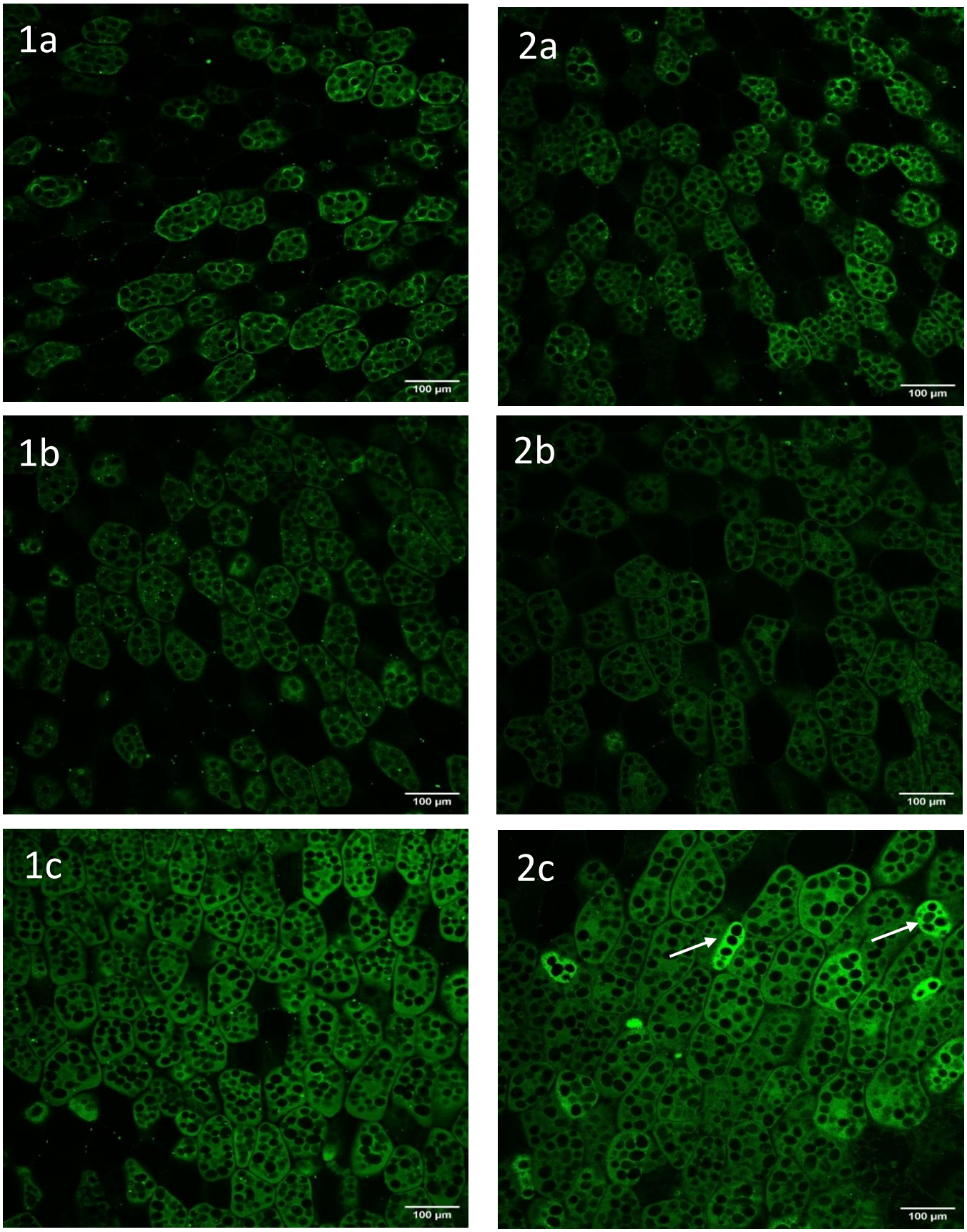


Figure 2. Immunolocalization of thiamine (1) and folate (2) for dry (a), soaked (b), and germinated (c) seeds with 10x/0.45 magnification

For better visualization, the brightness and contrast of images were adjusted to 0 - 40 000 for thiamine (1) and 0 – 20 000 for folate (2)

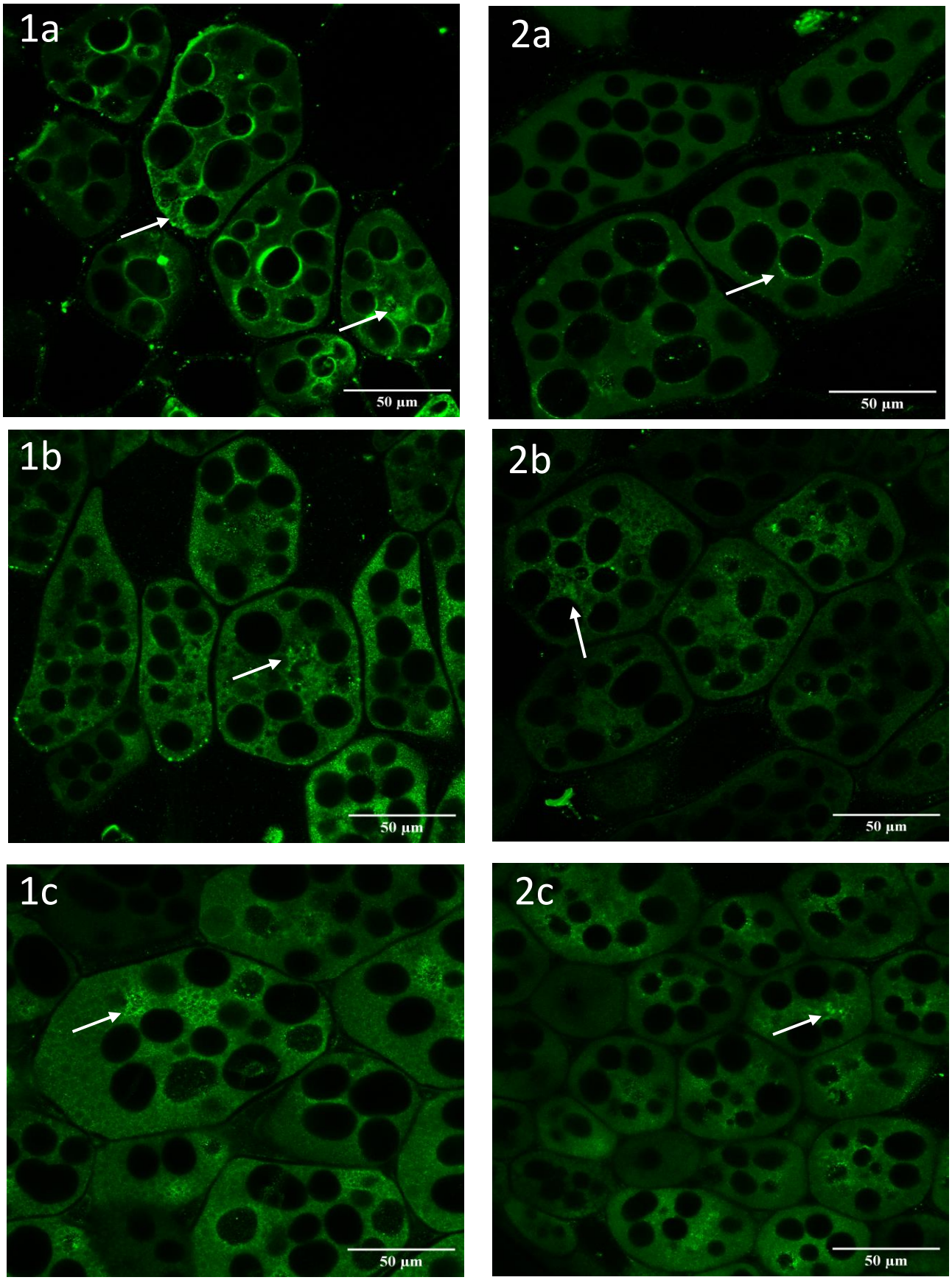


Figure 3. Immunolocalization of thiamine (1) and folate (2) for dry (a), soaked (b), and germinated (c) seeds with 40x/1.1 magnification

Arrows indicates the high-intensity signals of possible colocalization with mitochondria

A high heterogeneity of thiamine and folate labeling was observed in lentil cotyledon and cells through 10 x/0.45 and 40x/1.1 magnification. For instance, Figure 2 – 2c (arrows) showed different folate labeling intensities among different cells. This phenome was more evident in germinated seeds than dry or soaked. Additionally, heterogeneity in signal distribution within cells was also observed, with the presence of a uniformly distributed signal and points where the signal was much more intense (arrows in Figure 3). This heterogeneity was observed for thiamine and folate and in all studies conditions. This indicated that some cells have higher thiamine or folate content than others, highlighting the variability within the tissue. Jabrin *et al.* (2003) showed that in dry pea seeds, folate is present unequally between the embryos and the cotyledons. Coffigniez *et al.* (2019)[b] showed that the fluorescence signal of folates was higher in embryonic axes than in the cotyledons, because folate is largely consumed in the diving cells to synthesize thymidylate and purine. Additionally, the synthesis of thiamine and folate is regulated and modulated according to the specific tissue and metabolic requirements during seed development (Rébeillé *et al.,* 2006). This adaptive control mechanism explains the observed heterogeneity of thiamine and folate levels in cotyledon and cells in dry, soaked, and germinated seeds.

Given the high heterogeneity observed within cells in figures 2 and 3, and the highly intense signals seen in thiamine and folate localization, we investigated deeper the underlying factors. In plants, the active form of thiamine, thiamine diphosphate (TDP), is used as cofactor in mitochondrial and chloroplast pyruvate dehydrogenases reaction (Goyer, 2010); and the tetrahydrofolate (H4folate) is synthesized in mitochondria and further transported to other subcellular compartments (Coffigniez *et al.*, 2019[b]; Gorelova *et al.*, 2017; Rébeillé *et al.*, 2006). Therefore, we strongly believe that the observed high-intensity signals correspond to the colocalization of mitochondria with thiamine and folate. To answer this question, we performed a mitochondrial labeling followed by vitamin labeling. Unfortunately, the fixation step and subsequent immunolocalization protocol likely caused interference or interaction with mitochondria label, resulting in reduced signals for both labels when cross-section was analyzed using a confocal microscope. This reduction in signal intensity prevented us from calculating the colocalization between vitamins and mitochondria, as well as to determine whether the signals associated with vitamins coincided with the mitochondria in the same location, different locations, or independent locations (*i.e.*, colocalization, anti-colocalization, and non-colocalization, respectively) in cotyledon cells. However, through the mitochondrial labeling image (Figure 4), a striking similarity can be observed in the labeling patters of mitochondria

and vitamins, suggesting a potential colocalization. Rébeillé *et al.* (2006) showed that 40% of folate is present in mitochondria, the resting of folate is unequally distributed in cytosol (30%), vacuoles (20%), and plastids (10%). In mitochondria, the main folate pool found is H4folate and 5-formyl derivatives (Rébeillé *et al.*, 2006). Our images analysis and this last study, confirms the hypothesis of the colocalization of vitamins and mitochondria. However, it is advisable to revise and improve the Mitotracker and immunolocalization protocol when performed mutually. This may involve to increase label reagents concentrations or extending the duration of the incubation period. These adjustments could potentially enhance the labeling signals and improve the feasibility of assessing the colocalization between mitochondria and the target vitamins.

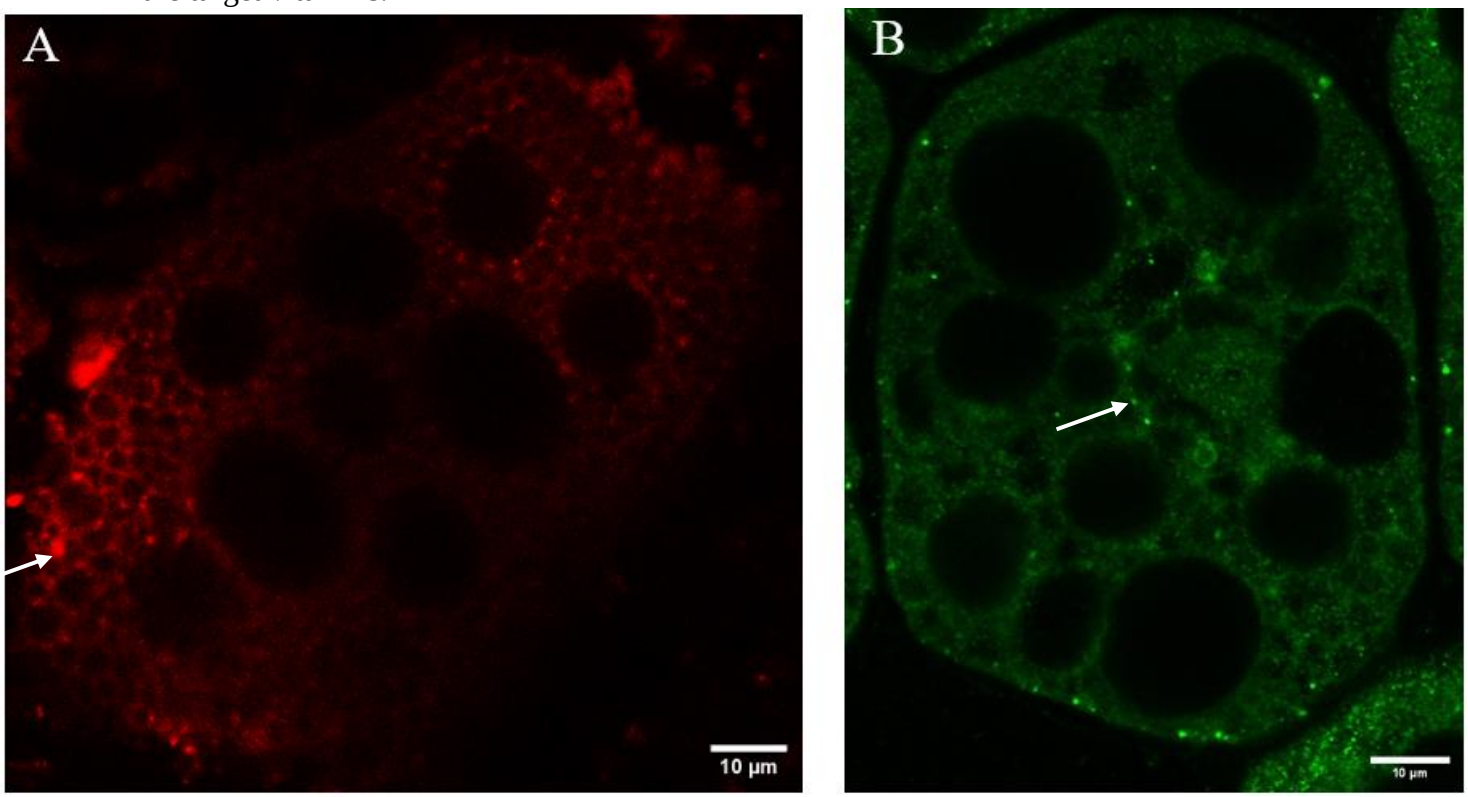


Figure 4. Mitochondria label (A) and thiamine localization (B) in soaked lentil seeds

III.3. Quantification

The mean labeling intensities of thiamine in dry, soaked, and germinated seeds were 1.0e+6, 2.6e+6, and 3.6e+6, respectively (Table 2). Thiamine intensity increased by 160% (dry *vs.* soaked) and by 256% (dry *vs.* germinated). Under the same conditions, Avezum *et al.* (2023) showed by a biochemical quantification that the thiamine content increased by 109% (dry *vs.* soaked), and by 169% (dry *vs.* germinated). Comparing our results to the biochemical quantification, it was noticeable that the image quantification showed quite similar trend but with some overrepresentation of thiamine content under all conditions (Table 3). As mentioned above, thiamine and folate localization were heterogeneous in the cotyledon. The mean percentage area of labelled thiamine in dry, soaked, and germinated seeds were 16% ± 0.07, 19% ± 0.04, and 36% ± 0.05, respectively (Table 2). These results showed an increase in the localization of thiamine labeling in germinated seeds. The higher intensity signals observed in germinated seeds may arise from the elevated thiamine content. Additionally, this phenomenon might be attributed to the greater accessibility of the vitamins epitopes to the antibody compared with dry seeds, suggesting that they might be more readily available to participate in metabolic processes.

Table 2. Quantification of intensity by mean gray value and percentage of label area in control, B1 and B9 label samples.

Condition	Thiamine labeling intensity	% thiamine label area	Folate labeling intensity	% folate label area
Dry	1.0E+06 ± 0.59	16%	2.3E+06 ± 0.66	28%
Soaked	2.6E+06 ± 0.51	19%	2.2E+06 ± 0.52	17%
Germinated	3.6E+06 ± 0.56	36%	4.6E+06 ± 0.5	46%

Labeling intensity corresponds to intensity of samples minus the intensity of control samples. The percentage of label area represents the total labeled area in relation to the overall surface area of the cross-section in each sample (cotyledon and germ). Data in a column with different letters are significantly different ($p < 0.05$) by the TukeyHSD test.

The mean labeling intensity of folate in dry, soaked, and germinated seeds were 2.3e+6, 2.2e+6, and 4.6e+6 (Table 2). The folate intensity does not change after soaking (decreased by 1%, dry *vs.* soaked) then increased by 103% after germination (dry *vs.* germinated). Through biochemical quantification, Avezum *et al.* (2023) showed that folate content decreased by 17%

after soaking, and then increased by 8% with germination. Similar to thiamine, image quantification overrepresented folate content. Our image analysis showed a similar decreasing and increasing trend, which aligns with the findings of biochemical quantification (Avezum *et al.*, 2023). However, in a study conducted by Coffigniez *et al.* (2019), the fluorescence signal indicated a lower relative concentration of 5-CH3-H4folate than the biochemical quantification of 5-CH3-H4folate. According to the study, this is due to the fact that part of the folate had diffused outside the embryonic axis, but had not yet let the seeds. In our study, the mean percentage area label of folate in dry, soaked, and germinated seeds were 28% ± 0.02, 17% ± 0.02, and 46% ± 0.06, respectively. Again, the higher intensity signals observed in germinated seeds could be attributed to the greater accessibility of their epitopes to the antibody compared with dry seeds.

Through image quantification, germinated seeds were found to have higher folate concentrations. However, this is not in agreement with the biochemical quantification, which showed a higher thiamine content but not a higher folate content (Avezum *et al.*, 2023). Qualitative results agree with biochemical quantification, as thiamine content was higher than folate in dry, soaked, and germinated seeds. The visual and quantification differences were attributed to the limited distribution across the entire histogram for folate quantification. This influenced the intensity values detected for folate by confocal microscopy, which overrepresented the results. The low-intensity values increase the background noise, making it more challenging to distinguish the true signal from the noise. To reduce this background noise, the antibody concentration must be increased to improve the dynamics of the intensity histogram.

Table 3. Mean of thiamine and folate labeling quantification and their respective percentage of increase with soaking and germination.

Condition	Mean of thiamine labeling	% of increase	Thiamine biochemical quantification (µg/100 g) *	% of increase	Mean of folate labeling	% of increase	Folate biochemical quantification (µg/100 g) *	% of increase
Dry	1.0E+06		154		2.3E+06		147	
Soaked	2.6E+06	160%	322	109%	2.2E+06	-1%	122	-17%
Germinated	3.6E+06	256%	418	169%	4.6E+06	103%	158	8%

* Reference values from Avezum *et al.* (2023)

IV. Conclusion

Our study was the first to use image analysis to localize and quantify thiamine and folate content within cotyledon and cells in lentil seeds during germination process. The results of our image analysis revealed a heterogeneous distribution of vitamers and a distinct pattern in vitamin localization. This distinction manifests between dry seeds (concentrated around starch granules) and soaked/germinated seeds (dispersed in the cytoplasm and around protein bodies). Our observations showed uniformly distributed signals, as well as areas where the signal exhibited significantly higher intensity within the cells. This phenomenon was evident in both thiamine and folate labeling under all experimental conditions. We strongly believe that some vitamins colocalize with mitochondria; however due to technical limitations, we were unable to perform calculations for colocalization, anti-colocalization, and non-colocalization. Our image analysis revealed a striking similarity in the labeling patterns of mitochondria and vitamins, suggesting potential colocalization. Quantitative image analysis indicated an increase in vitamin intensity in soaked and germinated seeds for thiamine, and in germinated seeds for folate. The higher intensity signals observed could be due to the elevated vitamin content within the seeds, or from the greater accessibility of their epitopes to the antibody, compared to the dry seeds.

This original approach has provided comprehensive qualitative and quantitative insights into how germination may affect the localization and content of vitamins within lentils. The use of IHC technique has contributed to elucidating the complex processes of vitamin diffusion and degradation during food processing. This methodology has the potential to further illuminate the complex interactions between compounds and the microstructures of food products, facilitating the construction of models for food processing.

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VII. Key takeways and prospective trajectories

In this study, we demonstrated that germination effectively enhances the nutritional quality of pulse seeds by increasing vitamins and essential amino acids and reducing antinutritional factors, such as α-galactosides and phytate. We used a combination of multidisciplinary techniques, including biochemical quantification, metabolomic/proteomic analysis, and immunolocalization, to comprehensively investigate the impact of germination on the nutritional quality and microstructure of lentils and cowpeas. The conclusions of this project provided a number of key insights:

- Soaking pulses at acidic pH further reduced α-galactosides content in chickpeas, lentils, and beans. For instance, soaking pulses at 45 °C with pH 4.0 for 3 h reduced α-galactosides content to an acceptable level.
- Germination at lower temperatures (25 °C) favored lentil germination, whereas higher temperatures (35 °C) promoted cowpea germination.
- Temperature and water content were the primary factors shaping nutritional profile and germination rate in lentil and cowpea seeds.
- Light exposure did not significantly affect the germination rate or nutritional profile in lentil and cowpea seeds.
- Germination effectively increased thiamine and folate levels, while reduced α-galactosides content. Optimal conditions: lentil germination at 25 °C and with 80% water content; cowpea germination at 35 °C and with 140% water content.
- Higher germination temperatures and water content increased essential amino acid content and reduced α-galactosides and phytic acid levels in cowpeas.
- Key enzymes, including dehydrin ERD14, glutathione S-transferase, protein SLE1, SLE2, and betaine aldehyde dehydrogenase 1, played a crucial role in regulating cowpea germination responses to abscisic acid, temperature stimulus, and water deprivation.
- Vitamins exhibited heterogeneous distribution in lentil seed cotyledons and cells.
- Thiamine and folates were grouped around starch granules in dry seeds and were dispersed in the cytoplasm and around protein bodies in soaked and germinated seeds.
- Qualitative images illustrated an increase in thiamine and folates intensity from dry/soaked to germinated seeds.

In this study, we analyzed how germination factors, such as temperature, light, and water content, impact the nutritional profiles of lentils and cowpeas. While previous studies have explored the influence of germination temperature and light on pulse nutritional content (see Chapter II), no study has focused on investigating the seed water content during germination.

As previously highlighted, water content played a crucial role in shaping the nutritional composition of lentils and cowpeas. Our findings showed that the germination process did not require excessive water supply to achieve a satisfactory nutritional composition in pulses (especially in lentils). We expect that this discovery will encourage researchers to incorporate water content as a critical factor in their germination analyses. We strongly believe that the germination *sensu stricto* process reduces microbiological risk compared to sprouting. Employing shorter germination duration and lower water content can effectively reduce microbiological contamination. Moreover, as discussed in the Chapter II in the section "*Seed maturation and germination process"*, germinated pulses must undergo high-thermal food processing to render them suitable for consumption. Adding the thermal food processing can effectively reduce the microbiological load, further ensuring their safety for consumption. Unfortunately, in this study, we did not analyze the microbiological charge present in germinated seeds after 12 h and 24 h of processing. Exploring the microbiological charge would provide substantial evidence to support this hypothesis, and it is strongly recommended for future studies. Additionally, it would be interesting to investigate the impact of combining germination with various thermal food processing methods, including cooking, extrusion, canning, and drying. Such an analysis could reveal the combinations that most optimize nutritional quality of the final product, providing new trends for the food industry.

During the germination process, our analysis focused on the impact of lighting duration rather than the specific light source. Various studies have reported that the nutritional composition of sprouts can be modified based on the type of light source used, whether UV or visible spectrum LED (Artés-Hernandez *et al.,* 2022). Artés-Hernandez *et al.* (2022) reported that factors such as light direction, source, photoperiod, and intensity play a crucial role in shaping metabolic pathways, including respiration, senescence, photosynthesis, sprouting, and growth in sprouts. However, surpassing plant tolerance can lead to adverse effects. Rather than acting as an abiotic elicitor, it can result in DNA damage, slow photosynthesis, reduced flowering and pollination, and impact seed development (Artés-Hernandez *et al.,* 2022). However, the last-mentioned review reported the impact of light sources on sprouts, but not on germinated seeds. Given our short 24 h germination period and the heterotrophic metabolism during this phase, lighting did not exert any significant influence on the nutritional profile of lentils and cowpeas. On the other hand, sprouts have an autotrophic metabolism, and as the leaves develop, they engage in photosynthesis. The influence of light might play a crucial role in shaping metabolic pathways during sprouting process and leaves development rather than

germination. However, investigating different light sources could be of interest to verify if this factor results in a distinct nutritional composition during germination *sensu stricto*.

Our project was unable to comprehensively evaluate numerous pulse seed varieties or genotypes due to time constraints. Exploring the impact of the germination on other seed varieties, such as chickpeas, common beans, and peas, could be interesting. Moreover, certain genotypes naturally exhibit greater nutritional value, with higher protein and vitamin contents, and low antinutritional factors. For instance, Madodé *et al.* (2012) reported differences in protein, fat, fiber, mineral content, and flour functionality among 22 different genotypes of cowpeas, categorized as pigmented or unpigmented. Their study highlighted that unpigmented genotypes had lower fiber and phenolics content, and reduced water absorption capacity. However, protein, calcium, phytate, iron, and zinc content are similar across genotypes (Madodé *et al.*, 2012). These findings suggest the potential benefits of analyzing various seed genotypes within the same category to identify those with superior nutritional profiles. Therefore, investigating the impact of germination on these specific genotypes could provide valuable insights into how genotypes influence nutritional content. This approach could highlight lesser-consumed pulse genotypes, contributing to the availability of more nutritious food options for society. Additionally, it will support biodiversity enhancement and dietary diversity.

Integrating germination as a preliminary step in cooking, extrusion, and flour production, for ingredient use, holds significant promise for the food industry. This project expanded our understanding of the impact of germination factors on the nutritional quality of pulses and its potential use in pulse processing. The use of germinated pulse flour as an ingredient in the food industry has increased in recent years. Several studies have reported the influence of incorporating germinated pulse flours on flour quality, texture, dough characteristics, and baking performance during bread baking (Millar *et al.*, 2019; Setia *et al.*, 2020). These studies reported that the addition of pulse flour leads to a significant increase in the protein content of the final product. However, it also results in a reduction in the gluten performance index, as well as alterations in density, volume, and crumb texture (Millar *et al.*, 2019; Setia *et al.*, 2020). Moreover, pulses have the potential to replace existing products or introduce innovative options. Pulses flour can be incorporated into cereal flour to create a wide array of products, presenting a promising solution to reduce meat consumption while also generating gluten-free food products. The development of new food products using germinated pulse flours poses a

series of challenges, as these flours have the potential to alter the functionalities, microstructure, and texture of the final product. Therefore, it is important to conduct a deeper investigation into the functionalities of pulse flour, including water and fat absorption capacity. These studies serve to deepen our comprehension of the ways in which the pulse flour influence the characteristics of the final product. Additionally, the utilization of microscopy, cytology, immunochemistry, and μtomography techniques emerges as a highly valuable approach. These methods explore the structural adjustments resulting from the incorporation of pulse flour and offer valuable insights into structural modifications.

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VIII. **Synthèse des résultats clés du projet**

Les légumineuses sont des graines sèches issues exclusivement des plantes de la famille des Fabacées. Elles se distinguent des graines oléagineuses par leur faible teneur en matières grasses (FAO & Alimentarius, 2019). Les légumineuses sont réputées pour leur contribution à la fertilité des sols, la promotion de la biodiversité et à la protection de l'environnement. Elles sont responsables de la fixation de l'azote atmosphérique dans le sol, grâce à leur symbiose avec des bactéries fixatrices de N_2 (rhizobia), ce qui réduit la nécessité d'utilisation des engrais synthétiques dans leur culture (Calles *et al.,* 2019; Heldt & Piechulla, 2011). En raison de leur faible productivité par rapport aux céréales, elles sont sous-représentées dans de nombreux pays. Cependant, l'introduction de légumineuses avec des céréales et des oléagineux dans les systèmes de culture (rotation des cultures) peut augmenter les marges brutes des cultures suivantes (Preissel *et al.,* 2015; Reckling *et al.,* 2016). Les principales légumineuses cultivées sont les pois chiches (genre *Cicer*), les haricots (genres *Phaseolus* et *Vigna*), les lentilles (genre *Lens*), les pois (genre *Pisum*) et la vesce (genre *Vicia*).

Les légumineuses sont reconnues pour leur importance dans l'alimentation en raison de leur richesse en protéines, en glucides complexes, en fibres, en minéraux et en vitamines essentielles, telles que celles du complexe B (thiamine and folate) (Bojňanská *et al.,* 2016). Leurs avantages nutritionnels peuvent contribuer à la diversification du régime alimentaire et aider à éradiquer la faim et la malnutrition dans le monde (Considine *et al.,* 2017). Cependant, les légumineuses présentent des facteurs antinutritionnels, tels que les tanins, l'acide phytique, les α-galactosides, les lectines, et les inhibiteurs de protéases et de l'α-amylase, qui ont un impact sur leur valeur nutritionnelle et restreindre la consommation de légumineuses. Pour réduire ou éliminer ces composés, il est nécessaire de procéder à des traitements tels que le trempage et/ou la cuisson. Ces procédés permettent de réduire ou d'éliminer les composés antinutritionnels par leur lessivage dans le milieu de trempage (diffusion) et/ou par activation enzymatique (hydrolyse) (Chitra *et al.,* 1996; Hall *et al.,* 2017). Cependant, le trempage et cuisson présentent un effet nutritionnel négatif en induisant une diffusion des vitamines hydrosolubles dans l'eau, telles que les thiamines et folates, et en dégradant thermiquement ces vitamines. Le fait d'ajouter un processus biologique, tel que la germination, en complément du trempage et de la cuisson présente un avantage nutritionnel. En raison de l'activation métabolique au cours de la germination, les graines présentent des modifications majeures des caractéristiques biochimiques, nutritionnelles et sensorielles. Au cours de ce processus, les vitamines B sont synthétisées, la composition en acides aminés est modifiée, la digestibilité *in*

vitro de l'amidon et des protéines augmente, les graisses et les hydrates de carbone sont décomposés, et les facteurs antinutritionnels diminuent (Prodanov *et al.*, 1997; Vidal-Valverde *et al.*, 2002). Afin de déclencher le processus de germination, les graines doivent être soumises à des conditions environnementales contrôlées favorables à leur croissance, incluant une température, une lumière et un taux d'humidité adéquates. L'objectif de notre étude est d'optimiser le processus de germination, notamment la température, la lumière, et la teneur en eau, afin d'augmenter la qualité nutritionnelle de la lentille et du niébé. De plus, nous avions pour objectif de comprendre et d'identifier les principales voies métaboliques impliquées dans la germination, et de déterminer si ces voies sont initiées à la suite de variations structurelles induites par le processus de germination. Pour ce faire, nous avons utilisé une approche multidisciplinaire faisant appel à différentes méthodologies. L'étude était divisée en quatre sections :

(i) la réalisation d'une étude préliminaire pour analyser l'effet du processus de trempage sur la teneur en facteur antinutritionnel, notamment les α-galactosides. Cela permettra de mieux comprendre l'impact spécifique du trempage avant la germination ;

(ii) la mise au point d'un dispositif de germination pour optimiser le processus en fonction des réponses biochimiques (thiamine, folate, et α-galactosides). Cela fournira des informations sur les conditions optimales de germination, telles que la température, l'éclairage et la teneur en eau, pour améliorer la qualité nutritionnelle des légumineuses ;

(iii) l'utilisation des techniques métabolomiques et protéomiques pour une meilleure compréhension de la germination du niébé. Cela permettra d'explorer la voie métabolique et d'identifier les protéines et métabolites clés impliquées pendant la germination ;

(iv) l'application de techniques d'immunohistochimie pour localiser *in situ* les composants nutritionnels (thiamine et folate) dans la lentille. Cela permettra d'étudier l'impact du processus de germination sur la microstructure de la lentille et la manière dont la localisation de composés spécifiques change au cours de la germination.

Cette approche pluridisciplinaire peut aider à établir des liens entre les changements de microstructure, la localisation des composés et les voies métaboliques qui se produisent au cours de la germination. Pour ce faire, deux modèles de graines ont été choisies pour l'étude : la lentille et le niébé. La sélection a été établie en tenant compte de l'importance économique et de la consommation dans leur pays respectif, la France et le Bénin. Les résultats de ce projet

contribueront à étendre le processus de germination pour la transformation des légumineuses et encourager la communauté scientifique, l'industrie et le gouvernement à investir dans la recherche et le développement afin d'augmenter la place des aliments à base de légumineuses germées.

Le trempage est la première étape, déterminante pour déclencher le processus de germination, initiant les processus métaboliques essentiels et préparant le terrain en vue de la croissance future de la jeune plantule. Cette phase implique une absorption initiale rapide d'eau, la diffusion des solutés des graines vers le milieu de trempage, ainsi que l'activation des mécanismes de réparation cellulaire (Bewley & Black, 1994). En conséquence, il est essentiel d'approfondir les recherches sur le processus de trempage. L'objectif de notre première étude visait à analyser les effets des températures variées (30, 45, 60, 75, et 90°C) ainsi que du pH (4,0 ; 5,0 et 6,0) sur la teneur en α-galactosides dans les pois chiches, les lentilles et les haricots. Nous avons démontré que l'hydrolyse et la diffusion des α-galactosides sont influencées par les conditions de trempage (température et pH). Selon les paramètres de trempage, deux mécanismes distincts peuvent se produire : une augmentation de l'activité des α-galactosidase, favorisant l'hydrolyse des α-galactosides, ou une modification de la perméabilité du tégument et de la paroi cellulaire, facilitant la diffusion des α-galactosides. Nos résultats soulignent que l'hydrolyse des α-galactosides est privilégiée à des températures plus basses (30 °C et 45 °C), tandis que la diffusion est favorisée à des températures plus élevées (60, 75 et 90 °C). En plus, les résultats ont révélé que la réduction de la teneur en α-galactosides dans les légumineuses est nettement plus rapide à un pH inférieur (4,0). Cette réduction est le résultat d'une diffusion accrue à 30 °C, ainsi que d'une combinaison d'activité enzymatique et de diffusion à 45 °C. La condition de trempage la plus efficace pour réduire la teneur totale en α-galactosides dans les pois chiches, les lentilles et les haricots s'est avérée être à 45 °C pendant 14 heures, aboutissant à une réduction respective de 84 %, 92 % et 62 %. Notamment, la réduction du temps de trempage à 3 heures, tout en maintenant la température à 45 °C et le pH 4,0, a entraîné une réduction significative de la teneur en α-galactosides, atteignant ainsi des niveaux considérés comme acceptables pour préserver ses propriétés prébiotiques. Il est important de noter que l'eau de trempage doit être éliminée après le processus. Pour une compréhension plus approfondie de l'hydrolyse et de la diffusion des α-galactosides dans les légumineuses au cours de la transformation alimentaire, l'application de techniques de microscopie et µtomographie s'avère pertinente. Ces techniques permettront d'explorer les changements microstructuraux au sein des légumineuses lors des étapes de trempage et de cuisson. Cette étude a apporté des

indications précieuses pour l'amélioration de la valeur nutritionnelle des légumineuses en réduisant les α-galactosides grâce à l'étape de trempage.

Si le trempage s'avère être un procédé efficace pour réduire la teneur en α-galactosides par diffusion ou dégradation enzymatique, il conduit également une réduction des vitamines due à leur diffusion dans l'eau. En intégrant la phase de germination, la teneur en vitamines peut être augmenté tout en réduisant les composés antinutritionnels par dégradation enzymatique. En effet, la germination induit des changements importants au niveau des propriétés biochimiques, nutritionnelles et sensorielles des légumineuses. La seconde partie de cette étude visait donc à optimiser le processus de germination *sensu stricto* afin d'améliorer la qualité nutritionnelle des légumineuses. A l'échelle du laboratoire, notre étude est la première spécifiquement conçue pour évaluer simultanément l'impact de la température, de la lumière et de la teneur en eau sur la valeur nutritionnelle des lentilles et du niébé pendant la germination *sensu stricto* (12 h et 24 h). À partir de nos résultats, nous avons pu déterminer les conditions optimales pour la germination des lentilles et du niébé. En ce qui concerne les lentilles, les conditions idéales sont une température de 25 °C, l'absence de lumière et une teneur en eau de 80% en base sèche. En revanche, pour le niébé, une température de 35 °C, une exposition à la lumière pendant 12 h et une saturation en eau à 140% en base sèche son recommandées. En conséquence de notre démarche d'optimisation de la germination, nous avons observé une augmentation de la teneur en thiamine de 152 % pour les lentilles et de 5 % pour les niébés. En plus d'augmenter la teneur totale en thiamine, la germination a modifié le profil de vitamines dans la lentille en augmentant la concentration du diphosphate de thiamine grâce à un changement métabolique. La germination a aussi permis d'augmenter la teneur en folate de 6 % pour les lentilles et de 33 % pour les niébés. La teneur en 5-méthyl-tétrahydrofolate a augmenté dans les deux graines au cours de la germination. Ce vitamère est la principale forme de folate utilisée par le métabolisme des graines au cours de la germination. Parallèlement, la teneur en α-galactoside a diminué de 47 % dans les lentilles germées et de 99 % dans les niébés germés. Cette étude nous a permis d'obtenir des informations plus précises quant à l'optimisation du procédé de germination, et elle peut permettre de fournir des préconisations pour son application au sein de l'industrie agroalimentaire.

Afin d'approfondir notre compréhension du processus de germination, il est désormais essentiel d'analyser les changements métabolomiques et protéomiques qui se produisent au cours de cette phase. Notre étude est pionnière dans l'utilisation d'approches combinées de

métabolomique et de protéomique pour explorer l'impact des conditions de germination, tels que la température (25 °C à 35 °C), la durée d'exposition à la lumière (0 - 12 h) et la teneur en eau (80% - 140%), qui influent sur les voies métaboliques et le profil nutritionnel des graines de niébé. L'objectif de cette approche était de dresser un panorama exhaustif de la germination *sensu stricto*, d'identifier les métabolites et les enzymes clés impliquées dans ce processus, et d'élucider les voies métaboliques liées aux acides aminés et aux sucres. Alors que l'intégration des études omiques a connu une large application dans des plantes modèles, telles qu'*Arabidopsis*, les recherches portant sur les légumineuses d'importance agronomique, en particulier dans les régions tropicales et subtropicales, restent relativement limitées. Nous résultats ont révélé le rôle crucial de la température et de la teneur en eau pendant la germination, illustrant comment ils façonnent la composition nutritionnelle des graines de niébé. La germination à des température et à une teneur en eau plus élevées a entraîné un taux de germination plus élevé (83%), une teneur accrue en acides aminés essentiels et une réduction des α-galactosides et de l'acide phytique dans le niébé. Parmi les enzymes clés identifiées qui régulent les réponses à la sensibilité à l'acide abscissique, au stimulus de la température et à la privation d'eau pendant la germination, il est possible de mentionner la déshydrine ERD14, la glutathion S-transférase, les protéines SLE1, SLE2 et la bétaïne aldéhyde déshydrogénase 1. Le niébé germé à 35 °C avec une teneur en eau de 140 % a présenté une augmentation de la teneur en acides aminés essentiels, allant de 2,5 à 17 fois, ce qui représente les améliorations les plus prononcées. L'augmentation de la teneur en acides aminés est due en partie à l'action des protéases et des enzymes impliquées dans le métabolisme des acides aminés. Grâce aux analyses de protéines, nous avons identifié 167 protéines impliquées dans l'activité des protéases, dont 19 présentaient une différence significative. De ces 19 protéines, 13 ont significativement augmenté leurs valeurs au cours de la germination. Les protéines protéase de type subtilisine SBT1.4, protéase de type subtilisine SBT1.7, sérine carboxypeptidase-like 40, thiol protéase de type aleuraine, protéine aspartique protéase de type cellule de garde 1 et E3 ubiquitine-protéine ligase ZNF598-like étaient indétectable dans les graines sèches, trempées et non germées. Cependant, leurs niveaux ont significativement augmenté et sont devenus détectables pendant la germination (25 °C avec 140% d'eau, et à 30 °C et 35 °C, quelles que soient les conditions de lumière et d'eau). Les enzymes clés identifiées impliquées dans le métabolisme des acides aminés étaient la 5-méthyltétrahydropteroyltriglutamate-homocystéines méthyltransférase (ATMS1) (+ 24% après 24 h de germination), l'homocystéine S-méthyltransférase (HMT2) (- 70% après germination), la dihydroxyacide déshydratase (DAD) (- 27% après germination) et l'aminotransférase d'acide aminé à chaîne ramifiée

(ATBCAT-5) (- 50% après germination). De plus, le niébé germé à 25 °C avec 140% de teneur en eau, et à 35 °C, indépendamment des conditions d'eau et de lumière, a réduit la teneur en raffinose de 5,2 fois, tandis que la teneur en stachyose n'était plus détectable, grâce à l'action des enzymes identifiées. Parmi celles-ci, on retrouve les enzymes α-galactosidase (-57% après germination), la raffinose synthase (12 fois plus après germination) et la stachyose synthase (1,7 fois plus après germination). En plus de la réduction des α-galactoside, la germination s'est avérée un processus efficace pour réduire la teneur en acide phytique. Cette réduction a été rendue possible grâce à une augmentation significative de la teneur en myo-inositol, multipliée par un facteur de 3,5, grâce à l'activité de l'inositol phosphate phosphatase. Ces résultats vont permettre d'envisager l'optimisation des conditions de germination afin d'améliorer la qualité nutritionnelle des légumineuses. Par ailleurs, elles contribuent à une meilleure compréhension des interactions complexes entre la germination et le métabolisme des graines.

Les méthodes employées dans les premières phases de notre étude ne permettent pas d'appréhender la distribution spatiale des composés d'intérêt dans les cotylédons et les cellules. Étant donné la complexité structurelle des tissus de stockage des graines, il est impératif de saisir la localisation *in situ* de ces composés et de déterminer comment la transformation alimentaire impacte leur compartimentation et leur décloisonnement. Dans le cadre d'une dernière phase de notre étude, nous avons fait appel à des techniques de cytologie et d'immunohistochimie pour apporter des réponses aux questions suivantes : Où se situent précisément les composés essentiels, tels que la thiamine et le folate, au sein de la structure de la graine ? Comment la localisation de ces composées est-elle altérée par le processus de germination ?

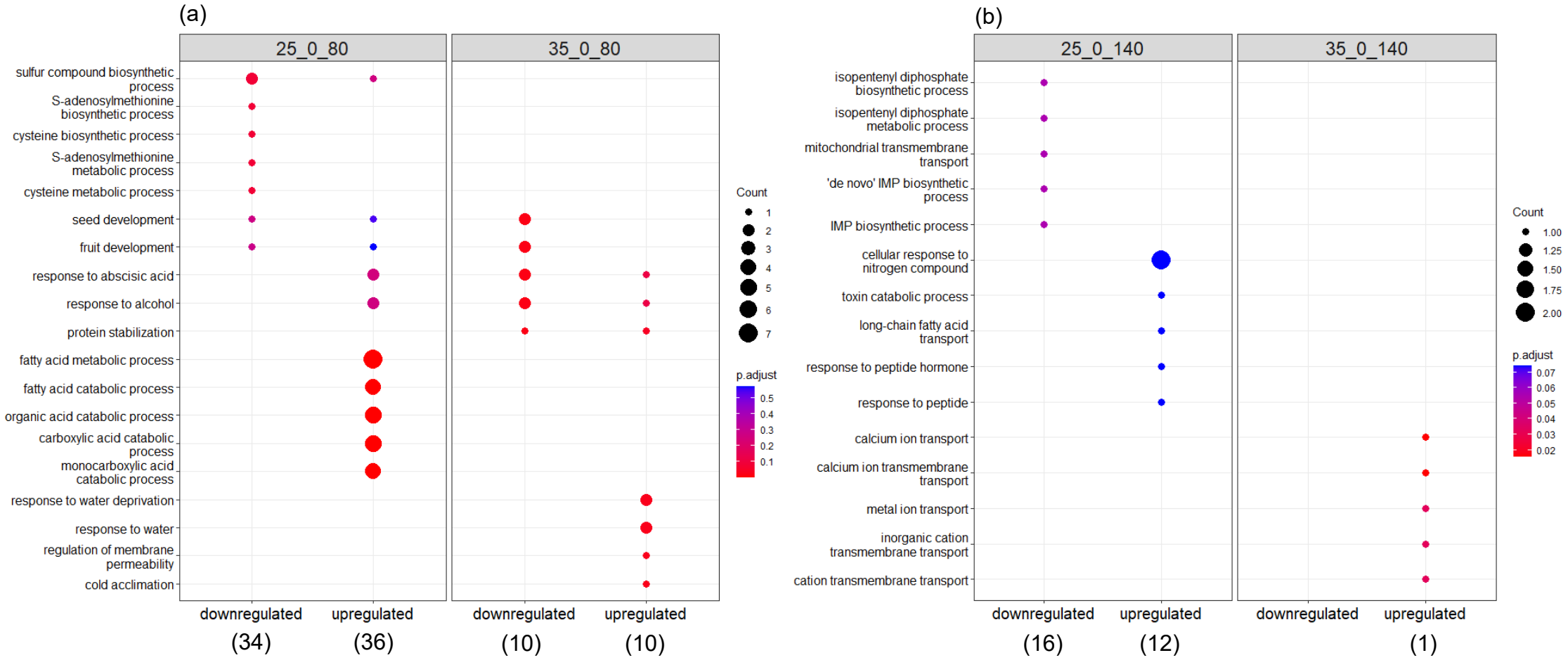
Notre objectif était de localiser et de quantifier la thiamine et le folate *in situ* dans des graines de lentilles sèches, trempées et germées, en utilisant l'immunohistochimie (IHC). Cette approche nous a procuré des données renseignant sur la distribution locale de ces composés ainsi que sur les altérations de leur localisation engendrées par le processus de germination. Notre étude s'est distinguée en tant que pionnière dans l'utilisation de l'analyse d'images pour localiser et quantifier la teneur en thiamine et en folate au sein des cotylédons et des cellules des graines de lentilles pendant le processus de germination. Les résultats de l'analyse d'images ont mis en évidence une distribution hétérogène et évolutive des vitamines selon les conditions (graine sèche, trempée, ou germée). Cette différenciation est nette entre les graines sèches (vitamines concentrées autour des granules d'amidon) et les graines trempées/germées

(vitamines dispersées dans le cytoplasme et autour des corps protéiques). Par ailleurs, l'analyse d'images a mis en évidence une similitude frappante entre les motifs de marquage générés par les mitochondries et celui des vitamines, suggérant une possible colocalisation de ces deux cibles. L'analyse quantitative des images a indiqué une augmentation de l'intensité des vitamines dans les graines trempées et germées pour la thiamine, et dans les graines germées pour le folate. Les signaux d'intensité plus élevée observés pourraient être liés soit à une teneur effectivement plus élevée de vitamines dans les graines ou à un biais associé à une meilleure accessibilité de leurs épitopes à l'anticorps comparativement aux graines sèches. Cette approche originale a fourni une compréhension exhaustive, à la fois qualitatives et quantitatives, de la manière dont la germination influence la localisation et la teneur en vitamines des lentilles. L'utilisation de la technique IHC a grandement contribué à élucider les processus complexes de diffusion et de dégradation des vitamines lors de la transformation alimentaires. Cette méthodologie a le potentiel d'éclairer davantage les interactions complexes entre les composés et les microstructures des produits alimentaires, facilitant ainsi la construction de modèles pour la transformation des aliments.

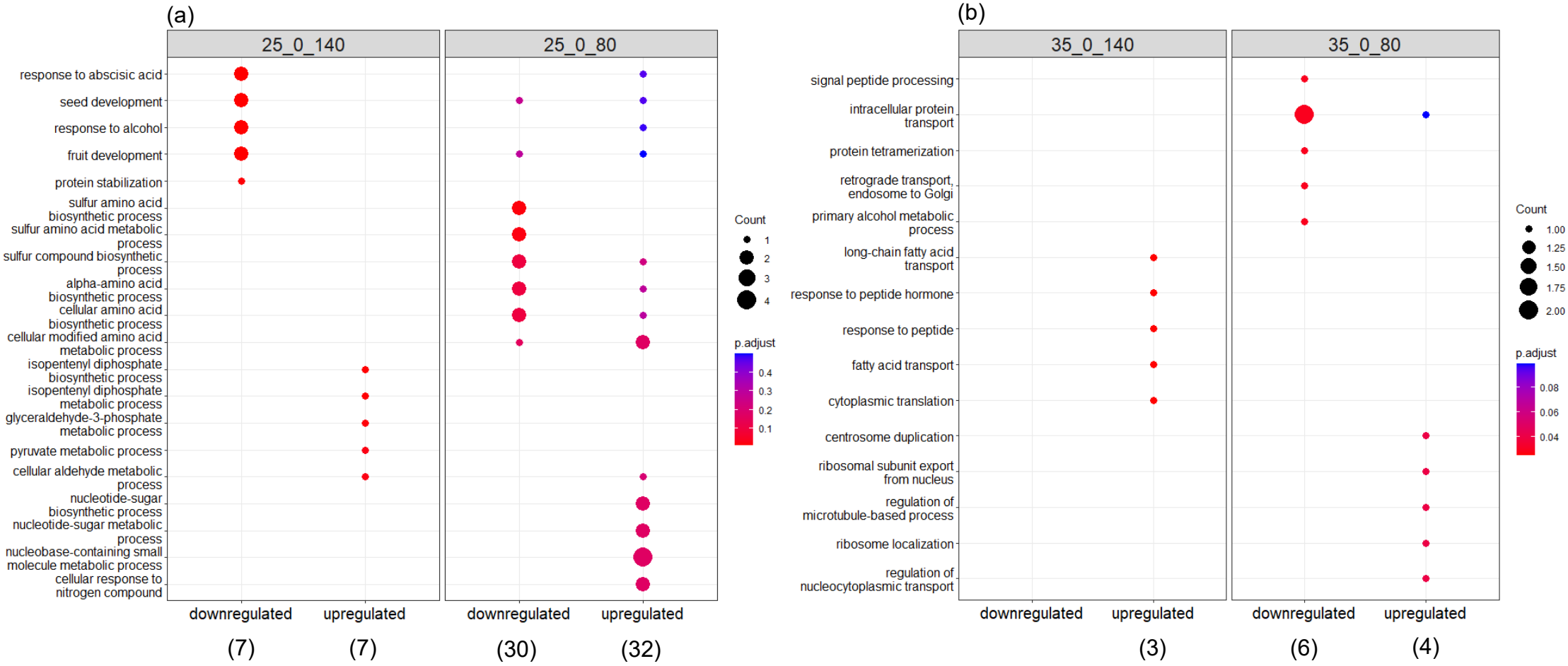
De façon global, cette étude a permis de mettre en évidence le fait que la germination constitue une stratégie efficace pour améliorer la qualité nutritionnelle des légumineuses en augmentant les teneurs en vitamines et en acides aminés essentiels, tout en réduisant la présence de facteurs antinutritionnels, tels que les α-galactosides et les phytates. L'intégration de multiples techniques multidisciplinaires, allant de la quantification biochimique à l'analyse métabolomique/protéomique en passant par l'imagerie, nous a permis d'explorer de manière exhaustive les effets de la germination sur la qualité nutritionnelle des lentilles et du niébé. Cette approche a permis d'initier l'établissement de connexions entre les changements microstructuraux, la localisation des composés et les voies métaboliques mises en œuvre, apportant ainsi des éclaircissements essentiels sur les interactions complexes qui se produisent au cours de la germination.

APPENDIX

Appendix 1. Proteomic enrichment between two germination temperatures, 25 °C and 35 °C, while fixing water content of 80% (db)(a) and 140% (db) (b).



Appendix 2. Proteomic enrichment between two germination water content, 80% (db) and 140% (db), while fixing temperatures at 25 °C (a) and 35 °C (b).





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