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Understanding the Significance of Raffinose Family Oligosaccharides in Seed Physiology

Samrat Rej [a], Sampurna Banerjee [b*], Sandeep K Lal [c] and P R Kumar [a]

[a] ICAR- Indian Agricultural Research Institute, Jharkhand, 825405, India.
[b] Department of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal 741252, India.
[c] Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors have read and approved the final manuscript.

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ABSTRACT

Seed vigour and longevity are pivotal agronomic traits with profound implications for crop yield, food security, and the global economy. Seed longevity, often defined by the duration of seed viability, is crucial for effective gene bank management, influencing seed regeneration cycles and ensuring the long-term conservation of plant genetic resources. The inevitable process of seed deterioration, driven by a complex network of biochemical reactions, leads to altered metabolism and damage to critical cellular components such as membranes, DNA, mitochondria, proteins, and

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**Corresponding author: E-mail: imsampurnabanerjee@gmail.com;*

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the antioxidative defence system. In recent research, Raffinose family oligosaccharides (RFOs) have emerged as key contributors to enhancing seed vigour and longevity. These sugars perform multiple functions in plants, including tolerance to abiotic and biotic stresses, regulation of seed germination, and maintenance of desiccation tolerance, all of which are essential for overall seed health. Studies suggest that RFOs significantly bolster seed vigour and longevity through mechanisms such as cytoplasmic vitrification, water replacement, and osmoprotection in dried seeds. These oligosaccharides are particularly abundant in the seeds of leguminous crops and are also found in roots and specialized storage organs. The glassy state formed by RFOs is vital for protecting cells from oxidative damage caused by reactive oxygen species (ROS), enhancing the stability of enzymes, and preventing deleterious conformational changes in proteins. Furthermore, delaying the degradation of RFOs has been shown to inhibit premature germination, underscoring their critical role in early seedling development and successful crop establishment.

Keywords: Seed longevity; raffinose family oligosaccharides (RFOs); seed vigour; desiccation tolerance; oxidative damage.

1. INTRODUCTION

Ensuring the preservation of seed viability and vigour from the first crop planting to the subsequent sowing, whether it is within the same planting season or beyond, is crucial for the success of agriculture and the subsequent crop yield. Like all living beings, seeds likewise undergo the process of ageing, gradually decreasing their strength and capacity to survive until they eventually perish. Seed life encompasses the duration from the first growth stages to the point where seeds are no longer viable in a dry storage environment. The greatest lifespan achievable by seeds of a certain species may vary significantly from one another under similar conditions in the storeroom. Seed storability refers to the ability of seeds to stay viable during storage, and it is an important characteristic for preserving agricultural goods and germplasm in crops. Seed storability is a multifaceted trait influenced by hereditary and environmental variables throughout the process of seed production, development, and post-harvest.

1.1 Significance of the Seed Storability Research

Seed storability is described as the capacity to stay alive throughout storing and is a critical particularity for agricultural products and germplasm preservation in crops Variation among rice accessions that emerge from various eco-geographic locations. For instance, Indica-type seeds retained their viability longer than Japonica-type seeds

- Adaptive Advantage
- Major Advancement to Gene Banking
- Conservation of inheritable Diversity
- Successful Planning for the future season
- To study the effect of temperature and moisture content on Storage Condition

1.2 Classification of Seeds Based on Longevity

Seed classification based on longevity is essential for understanding their viability and storage potential, particularly in the context of agricultural practices and germplasm conservation. Seeds are typically categorized into three main longevity groups: short-lived, intermediate-lived, and long-lived. Short-lived seeds, such as those from many annual plants, exhibit rapid deterioration and have limited storage potential. Intermediate-lived seeds, like those from some legumes, can maintain viability for several years under optimal conditions. Long-lived seeds, often found in perennials and certain tree species can remain viable for decades or even centuries. The longevity of seeds is influenced by various factors, including their moisture content, the presence of protective compounds like raffinose family oligosaccharides (RFOs), and environmental conditions during storage. RFOs play a crucial role in enhancing seed vigour and longevity by providing osmotic protection and stabilizing cellular structures during desiccation, thereby contributing to improved seed storability and germination rates.

1.3 Rules for Determination of Seed Longevity

Harrington's Thumb Rule: Mean viability period is doubled with each 5% reduction in storage temperature or 1% reduction in moisture content. (This rule is applicable in the temperature range of 0-50^{o} C and moisture range of 5-14%.)

James Rule: The temperature in degrees Fahrenheit + Relative Humidity in per cent = Must not exceed 100.

1.4 Dynamics of Seed Longevity

- For all practical reasons, a seed lot will be regarded rather than an individual seed The viability curve of a seed lot at each time is typically sigmoid indicating a normal distribution.
- The viability curve of a seed batch at each time is generally sigmoid showing a normal distribution.
- In this T is the length after which the seed of a specific variety demonstrates quick and observable reduction in germination.
- Tomato is an excellent storer (T1) as opposed to onion (T2) seen in the figure.
- These results demonstrate that despite the storage environment, disparities exist in lifespan across species stored under the same setting (Singhal, N.C., 2009).

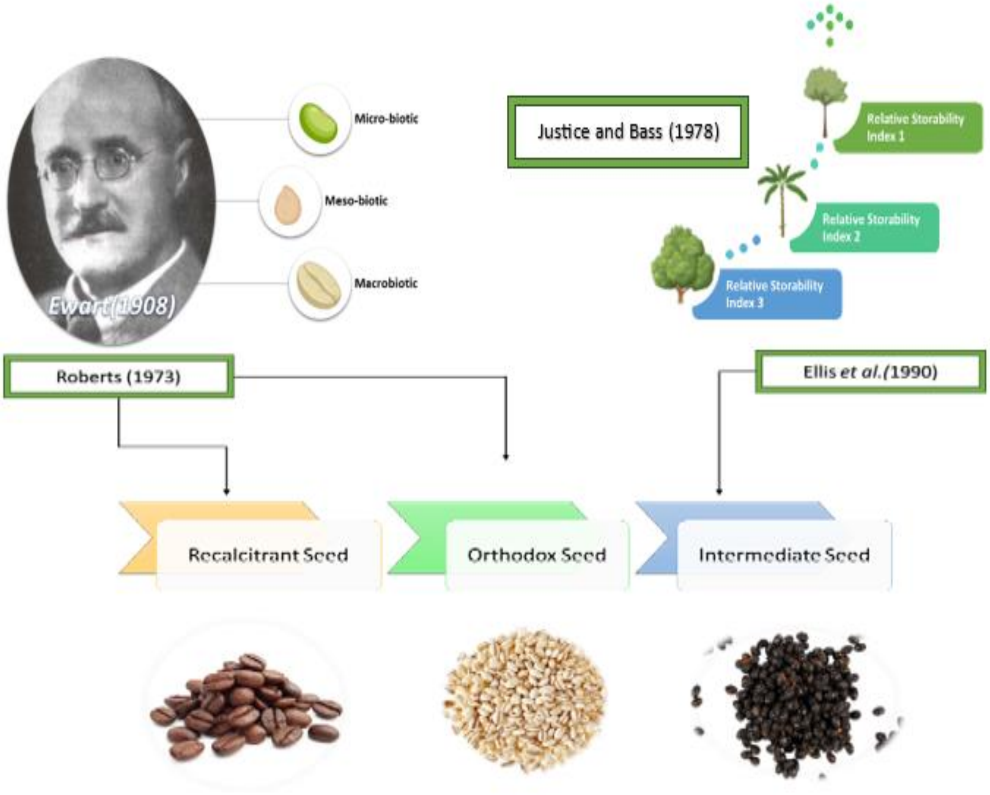

Fig. 1. Classification of Seeds Based on Longevity

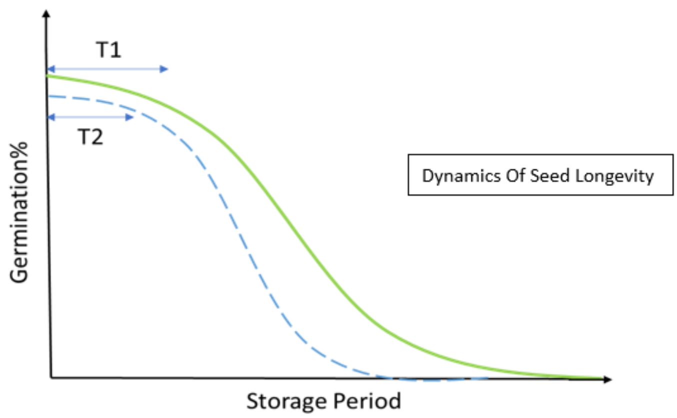

Fig. 2. Classification of Seeds Based on their

1.5 Concept of Seed Ageing

Seed ageing is a frequent physiological process during storage. It is a natural irreversible process that happens and evolves together with the lengthening of the seed storage period. Ageing rates of plant Anhydrobiotes are significantly reliant on the water content and temperature of storage, circumstances (Singhal, N.C., 2009)

2. STORABILITY OVERVIEW and KEY FACTORS REGULATING SEED STABILITY IN STORAGE

2.1 Seed Factors

The sustaining capability of orthodox seeds is associated with their cellular defence mechanism. Following seed development, orthodox seeds experience maturity procedures, which involve a decrease in moisture content plus cessation of metabolic activity [1]. The arid state is considered to be calm, and that helps in the possibility for storing seeds. During late seed maturity, plants develop defensive mechanisms that involve the buildup of anti-oxidants, non-reducing carbohydrates, and protective proteins such as late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), and lipocalins. The DNA in the nucleus is densely organized and chlorophyll is degraded. Seed storage proteins serve as crucial substrates of oxidation, aiding in the regulation of reactive oxygen species (ROS) generated throughout the process of dry storage. Due to the high sensitivity of chlorophyll fluorescence measurements, the fluorescence levels of individual seeds can serve as an indicator of seed maturity for those seeds that have chlorophyll during their entire development, which is the case for most seeds. According to Ranganathan and Groot [2], there is an inverse relationship between the amount of fluorescence and the level of seed maturity. In other words, as the level of fluorescence decreases, the rate of seed maturation increases.

2.2 Function of Chemical Protectants in Different Tissues

The primary pathogen throughout the storage of seeds is oxidative stress that is brought on by reactive oxygen species (ROS). Antioxidants, both molecular and enzymatic, play a critical role in the longevity of seeds. Enzymes have less mobility among molecules in the plasma when conditions are dry, making it impossible for them to access the ROS. Lower molecular weight antioxidants including glutathione, ascorbate (vitamin C), and tocochromanols (tocopherols and tocotrienols) are essential to seeds in these dry conditions. Tocopherols, particularly vitamin E (alfa-tocopherol), are abundant in seeds and are lipophilic antioxidants that have been essential in halting the oxidation of membranes as well as storage lipids. Arabidopsis mutants lacking in vitamin E have significantly shorter seed lives. The two main types of water-soluble antioxidants found in seeds include ascorbate and glutathione. The antioxidant population is going to run out because enzymatic antioxidant replenishment is not possible at the low water content of dried seeds. Elevated relative humidity (RH) or imbibition of seeds facilitates the enzymatic scavenging of reactive oxygen species (ROS) by glutathione-reductase, superoxide dismutases, peroxidases, and catalases. These digestive enzymes are involved in the renewal of molecular antioxidants as well. Studies carried out on barley seeds revealed a decrease in tocopherol and glutathione levels as the seeds grow older, under conditions of regulated deterioration at 45°C and 75% relative humidity as well as arid gene banks to rampage.

Raffinose family oligosaccharides (RFOs), which include verbascose, stachyose, and raffinose, are acquired by the cells during seed formation together with sucrose. It has been proposed that such sugars have a role in the formation of the glassy state, which in turn prolongs seed life. As a primary derivative of raffinose, galactinol is known to be shown to positively correlate with lifespan in Arabidopsis, tomato, and cabbage seeds. This correlation has been confirmed by the shorter life expectancy of seeds derived from Arabidopsis galactinol synthase mutants. In the previous study, the lifespan of tomato and Arabidopsis seeds was assessed at 40°C and 85% relative humidity. In contrast, cabbage seeds were stored in paper bags at 20°C with no RH control. In Arabidopsis leaves, galactinol has also been shown to provide a defence against oxidative stress.

Late Embryogenesis abundant proteins (LEAs) and Heat shock proteins (HSPs) are produced close to the end of the maturation process of seeds. Through maintaining structural proteins, disassembling thylakoids in chloroplasts, condensation of chromatin, and stabilizing the glassy cytoplasm, they contribute towards the preservation of seed survival [3].

The embryo and nutritive tissues are shielded by the seed coat, a type of maternal tissue that creates a layer that provides security which serves both physiological as well as physical purposes. At the end of the seed's development, the cells that make up the seed coat die. The composition and arrangement of the seed coat, as well as the chemical and mechanical protection of the seed and the possibility of longevity, are all regulated by metabolites gathered throughout seed development. The seed coat is made up of flavonoids, lignins and lignans, which are polyphenols. Colourless polymeric substances gather in the vacuoles of the endothelial cells' deepest layer throughout the first stages of seed formation. Afterwards, upon dehydration, polyphenol oxidase oxidizes them to a brown colour, resulting in the creation of flavonoids known as flavonols. By acting as antioxidants and scavenging reactive oxygen species, flavonoids ultimately reduce oxidative stress. During conditions of rapid ageing, the dark-pigmented seeds of Brassica napus, the plant that produces rapeseed, live long. Browning and reduced water penetration of the seed coat may result from the peroxidation of flavonoids that build up in seed coats. Proanthocyanidins (PAs), often referred to as concentrated tannins, are present in the seed coat and may possess antibacterial properties as well, forming a chemical barrier that prevents fungal infections. It additionally demonstrated that PAss was detrimental to bruchid larvae and prevented their invasion of cowpea (*Vigna unguiculata*).

The monolignol units that constitute lignin are abundant in flax seeds. In addition to possessing antioxidant properties, it is speculated to protect the seeds from mechanical damage. Polyphenoloxidases, peroxidases, and chitinases are examples of defence-related proteins that are upregulated in the testa of Arabidopsis and soybeans (*Glycine max*).

2.3 Glassy State of Cytoplasm

- As seeds mature, drying causes the cytoplasm to shift from a fluid state to a glass-like viscosity, disrupting normal crystal structures.
- The glassy state significantly reduces molecular mobility, which halts cellular metabolism and stabilizes cellular components.
- This reduction in deteriorative processes consequently prolongs the lifespan of seeds.
- The transition to the glass phase is crucial for maintaining the seeds' physiological state and their ability to respond to external factors [2].

2.4 Temperature

- Seeds decay more rapidly in warmer environments due to the increased rate of chemical oxidation. As a result, gene banks are recommended to dry and store valuable germplasm at sub-zero temperatures to slow down this process.
- Increasing the temperature lowers the glass transition temperature (Tg) at which the glass phase transition happens.
- Seeds with intermediate storage behaviour, such as those from oil palm, may tolerate desiccation but are sensitive to storage in sub-zero conditions [4].

2.5 Oxygen

- The primary reason for seed deterioration in dry conditions is oxidation. Higher oxygen concentrations accelerate seed ageing [5].
- In dry storage environments, molecular oxygen is a key source of reactive oxygen species (ROS).
- Seeds contain a high concentration of metal ions like Fe^{3+}, Cu^{2+}, and Zn^{2+}.
- When molecular oxygen interacts with these metal cofactors (Fe, Zn, Cu), it leads to the formation of ROS.

2.6 Moisture Content, Water Activity

Dampness is the main factor that leads to seed deterioration in conventional seed preservation. For the majority of chemical and enzymatic activities, water serves as an essential factor. Moisture, oxygen, and temperature cause lipids, proteins, and nucleic acids—essential components that constitute living things—to oxidize more quickly. The oxidative breakdown of unsaturated fatty acids in the oil bodies and membranes is what leads to deterioration in the oily or highly lipophilic portion. Breakdown of the non-oily, or hydrophilic, portion is primarily triggered by macromolecule cross-linking including oxidation of proteins, DNA, as well as RNA. Harrington (1972) established a first rule of thumb for the quantitative impact of humidity upon seed ageing, stating approximately a 1% decrease in seed moisture level increases the expected lifespan of the seeds to double when the percentage of moisture in seeds is between 5% and 14%.

It is important to distinguish among seed moisture quantity and water usage, or storage relative humidity (RH) when researching the effects of moisture upon seed ageing. In the past, statistics on the moisture level of seeds were used to characterize them as part of the seed trade. Additionally, seed technologists were recruited to calculate the moisture level of these seeds based on their fresh or dry mass. The presence of water in the non-oily portion of the seeds, and hence the rates and processes of degradation at which such chemical reactions are occurring, are not specified by the seed moisture level. Assume that the seeds of a castor bean (Ricinus communis) have 50% oil and 10% moisture on a wet basis. This means that the non-oily portion will have 20% moisture. On the other hand, seeds from 2% oil-containing common beans (*Phaseolus vulgaris L.*) would have the identical overall moisture level as seeds but just under ten per cent moisture in the non-oily portion. Because of this, even when two seeds have the same amount of seed moisture, their physiological state will be different. The amount of seed oil varies not just throughout cultivars but also depending on the kind and conditions of manufacturing. The activity of water (aw) is often used within the food industry to determine the moisture content of products, especially seeds. Even though aw is expressed within 0 and 1.0 and the relative humidity (RH) in percentages, the law is roughly closely linked to the RH as long as it is in homeostasis with the average humidity of the ambient air. It was decided that it is preferable to compare seeds depending upon their aw or equilibrium RH (eRH) rather than the amount of moisture they contain for research on seed ageing during the first Seed Longevity Workshop of the International Society of Seed Science (Wernigerode, Germany, July 5–8, 2015) [4]. The Seed Viability Equation states that (even though it is constrained) seed life increases when seed moisture levels decrease. In reality, seed damage may occur more quickly at very low moisture contents, or under conditions known as "ultra-dry storage," which correspond to eRH values lower than approximately 15–20%.

3. ROLE OF RAFFINOSE IN SEED

3.1 Overview of the Raffinose Family of Oligosaccharides

- Soluble carbohydrates are second only to sucrose in their distribution in higher plants [6]. These carbohydrates are abundant in the seeds of many crops, particularly in the legume family, including soybeans (*Glycine max*), lentils (*Lens culinaris*), and chickpeas (*Cicer arietinum*). Soluble carbohydrates are also found in roots and specialized storage organs like tubers.
- The raffinose family of oligosaccharides (RFOs) are *α-D-galactosides* of sucrose, a disaccharide.RFOs include raffinose, stachyose, verbascose, and ajugose, which belong to trisaccharide, tetrasaccharide, pentasaccharide, and hexasaccharide groups, respectively [7]. Structurally, RFOs are α-galactosyl derivatives of sucrose. Raffinose is composed of galactose, glucose, and fructose, while stachyose contains two *α-D-galactose* units, one *α-D-glucose* unit, and one *β-D-fructose* unit.
- Humans lack alpha-galactosidase, an enzyme necessary to break down RFOs, so they are not absorbed or digested in the upper gastrointestinal tract. Instead, RFOs accumulate in the large intestine of the human digestive system [8].

The Raffinose family is comprised of

- Raffinose
- Verbascose
- Stachyose
- Ajugose

4. BIOSYNTHESIS OF RFOS

RFOs are soluble sugars that are mostly found in higher plants, second only to sucrose. RFOs are frequently discovered in the seeds of a wide variety of agricultural crops, particularly those that belong to the Leguminosae family, which includes *Glycine max* (soybean), *Cicer arietinum* (chickpea), and *Lens culinaris* (lentil) [9]. Additionally, they can be gathered from roots and particular storing organs like tubers as well as leaves. For instance, RFO levels in photosynthesizing *Ajuga reptans* leaves and *Stachys sieboldii* (Chinese artichoke) tubers can vary from 25 to 80% depending on their dry mass. α-galactosyl transferases catalyze the synthesis of RFO by facilitating the sequential conversion of galactosyl subunits onto sucrose. The enzyme with the greatest significance in the biosynthesis of RFOs is galactinol synthase (GolS). It catalyzes a process that results in galactinol, and it acts as a galactosyl supplier for the creation of other RFO components [10]. The initial component of RFOs, raffinose, is a trisaccharide produced by the enzyme raffinose synthase.

(RAFS), that utilizes galactinol plus sucrose as inputs [11]. Corresponding to this, stachyose is produced when stachyose synthase (STS) transfers the galactosyl moiety onto raffinose [9]. More galactosyl moiety transference to this chain will end up in greater RFO components like verbascose and ajugose. Verbascose synthase (VES) mediates the synthesis of verbascose by facilitating the binding of galactinol to stachyose. Ajugose is also produced when galactinol is transferred from verbascose to verbascose via STS. The RFO manufacturing mechanism described previously is known as the galactinol-dependent route because galactinol serves as a source for galactosyl at every step of the procedure. Nevertheless, the galactinol-independent route, which is less common than the initial method and has only been identified among two Lamiaceae species—*Coleus blumei* and *Ajuga reptans*—is mediated by a different significant enzyme, galactan-galactosyltransferase (GGT). A higher RFO component is synthesized by the GGT-catalyzed translocation of the galactosyl moiety from one RFO molecule to another. Verbascose and raffinose, for example, would be formed when a galactosyl moiety was transferred over one stachyose to a subsequent stachyose by the reaction of GGT. In this method, the GGT regulates the level of cellular RFO in addition to producing higher members of RFO. RFOs are broken down into galactose and sucrose by α-galactosidases. Sucrose is broken down via invertase and sucrose synthetase into fructose and glucose or UDP-glucose and fructose. The ATP-dependent enzyme galactokinase first phosphorylates glucose to produce galactose-1-P (Gal-1P). A pair of distinct pathways, one called the Lelior pathway while the other an alternative pathway found in plants are used to further break down Gal-1-P. Hexose-1-P uridylyltransferase during the Lelior route releases glucose-1-phosphate when it transports the UMP between UDP-glucose to galactose-1-phosphate, culminating in UDP-galactose. Nevertheless, galactose-1-phosphate is broken down by a different process in crops. Galactose-1-phosphate and UTP are converted to UDP-galactose plus PPi by pyrophosphorylase. UDP-galactose is then converted to UDP-glucose by the NAD-dependent UDP-galactose-4-epimerase.

The major catalysts in the RFO synthesis process in plants are GolS, RAFS, STS, and VES. When these enzymes are altered in crops, other stresses may be acquired and various additional plant metabolic activities can occur [8].

5. GENETICS OF RFOS

GolS Gene found in *Arabidopsis thaliana* and *Cicer arietinium*. *Raf* and *Sta* genes are found in *A. thaliana. Alpha-Gal* genes are found in Beech, *A. thaliana.*

5.1 Process of Regulation of Seed Vigour and Longevity

Dry seeds must be dispersed from the maternal plant to ensure the continuation of plant propagation. As seeds mature, significant physiological, biochemical, and physical transformations take place, ultimately leading to the ability to endure challenging conditions. Dehydration typically happens towards the end of the seed maturation phase, resulting in the accumulation of potentially protective substances, particularly soluble sugars like RFOs and sucrose, along with LEA proteins [12]. Working together, LEA proteins and soluble sugars aid in upholding the structural integrity of proteins and membranes in dry environments by creating a glassy state that hinders deteriorative processes. During maturation and dispersal, seeds undergo desiccation, losing water to prepare for survival in harsh environments. Sugars such as sucrose and RFOs, which are non-reducing, can develop in seeds to prevent desiccation and avoid damage, with research suggesting a role for RFOs in desiccation tolerance. For instance, sucrose and RFOs accumulate in the seeds of *Erythrina speciosa*, a native Brazilian tree, before significant changes in water content, and are relocated from vacuole reserves to the cytosol in the late stages of seed development. It has been suggested that these substances aid in maintaining the liquid crystalline state of cellular membranes in dry conditions, positively impacting desiccation resistance and seed longevity [13]. Conversely, Brazilwood seeds typically exhibit orthodox behaviour, surviving desiccation during maturation due to the presence of sugar alcohols like galactopinitol-A, galactopinitol-B, ciceritol, and lipids. Two main mechanisms involving RFOs have been proposed to govern the desiccation process in seeds. The first mechanism, known as 'vitrification,' involves the significant thickening of a cell solution due to water loss, resulting in a plastic-like solid state that helps maintain cell stability, prevent cellular collapse, and preserve hydrogen bonding. This vitrification state is influenced by LEA proteins, HSPs, and RFOs. The second mechanism, 'water substitution,' entails RFOs' hydroxyl groups replacing water molecules within the cell, maintaining crucial hydrophilic interactions for the stability of macromolecules and membrane structure during dehydration. These RFOs also play a key role in seed germination, protecting embryos from desiccation during development, and enhancing seed viability under harsh conditions. While sugars are commonly viewed as signalling molecules or osmoprotectants, their role and accumulation, particularly RFOs, have been extensively studied for their impact on seed vigour and lifespan. Specifically, RFOs have been demonstrated to assist sucrose in preserving membrane integrity by preventing lipid crystallization and ageing processes.

Sucrose is the most abundant sugar in maize seeds, but its quantity did not correspond with better storage; rather, raffinose as a mass fraction of total sugars demonstrated a large and favourable link with seed vigour and lifespan. In soybeans likewise, it has been proven that an increase in RFO to sucrose ratio as well as alterations in RFO metabolism genes including *GolS* and RAFS has been favourably related to seed maturity, vigour, and lifespan. Additionally, variations in soluble sugar content, notably RFOs, have been related to seed vigour and germinability in Arabidopsis and other species as well [14]. Although the quantity and kind of RFOs that accumulates during seed development differ per species. For example, maize, Arabidopsis, and lettuce collect more raffinose than any other RFOs like stachyose and verbascose whereas castor bean accumulates more raffinose and stachyose but not verbascose. In contrast, galactinol and myoinositol levels have been reported excessively high in seeds across various species. Legumes are the major crops that acquire the greatest RFOs in their seeds. Alpha-galactosides (α-Gal), sucrose-1,6-galactosyl derivatives, are one of the principal complex sugars found in leguminous seeds. Furthermore, RFO accumulation and associated α-GAL activity are connected to ripe and developing chickpea seeds. When compared with the control, preventing RFO breakdown mediated by α-Gal with 1-deoxygalactonojirimycin (DGJ) reduced seed germination by roughly 25% in pea plants. The accumulation of galactinol, and sucrose, occurred during the early stages of chickpea pod formation, whereas the raffinose, and stachyose, accumulate during the later stages of seed maturity, which indicated the accumulation of the higher-order RFOs pathway during seed maturation. However, interestingly, some studies revealed that low RFO genotypes of soybean and chickpea did not display delayed germination, indicating that RFOs had no substantial role in increasing seed germination.

Sucrose is the predominant sugar in maize seeds, but its abundance did not correlate with improved storage conditions; instead, a significant and positive relationship was observed between raffinose levels as a percentage of total sugars and seed vigour and longevity. Similarly, in soybeans, it has been established that an elevated ratio of RFO to sucrose, along with changes in RFO metabolic genes such as GolS and RAFS, are linked favourably to seed development, vigour, and lifespan [15]. Furthermore, fluctuations in the content of soluble sugars, particularly RFOs, have been associated with seed vigour and germination in Arabidopsis and other plant species. While the type and amount of RFOs that accumulate during seed growth differ among species, maize, Arabidopsis, and lettuce tend to amass more raffinose compared to other RFOs like stachyose and verbascose, whereas castor bean accumulates more raffinose and stachyose but not verbascose. Conversely, galactinol and myoinositol levels are notably high in seeds across various plant species. Legumes, in particular, are known to accumulate the highest levels of RFOs in their seeds. Alpha-galactosides (α-Gal), which are sucrose-1,6-galactosyl derivatives, are among the main complex sugars present in leguminous seeds. Moreover, the accumulation of RFOs and the associated activity of α-GAL are linked to the ripening and growth of chickpea seeds. In experiments where RFO breakdown mediated by α-Gal was inhibited using 1-deoxygalactonojirimycin (DGJ), seed germination in pea plants was reduced by approximately 25% compared to the control [16]. Galactinol and sucrose were found to accumulate during the early phases of chickpea pod development, while raffinose and stachyose accumulated during the later stages of seed maturation, indicating the activation of the higher-order RFOs pathway during seed maturation. Interestingly, certain studies have shown that soybean and chickpea genotypes with low RFO levels did not exhibit delayed germination, suggesting that RFOs may not play a significant role in enhancing seed germination [17].

As GolS is a key regulatory enzyme in the production of RFOs, numerous traditional and cutting-edge transgenic methods have been explored to develop plants with enhanced stress resistance, seed vigour, and longevity. While the role of RFOs in plant health is currently under debate, their impact is wide-ranging [18].

6. MULTIFUNCTIONAL ROLE OF RFOS IN PLANT HEALTH

Under conditions of dehydration stress, RFOs provide support to the cell membrane by integrating themselves within the lipid head groups of the bilayer, thereby enhancing RFO levels during desiccation and promoting stability of membrane phospholipids. In situations of abiotic stress, RFOs serve as osmolytes, aiding in the preservation of cell turgor and functioning as antioxidants against reactive oxygen species. While RFOs are a byproduct of the metabolic pathway of inositol, they do not play a direct role in alleviating stress in plants under natural circumstances, unlike other substances derived from the same pathway [19]. A rise in RFO levels, particularly raffinose, has been documented in various instances of abiotic stresses like heat, cold, salinity, or drought [8,20]. Nonetheless, there is limited literature elucidating the specific functional roles of RFOs in enhancing abiotic stress tolerance. Other compounds such as sucrose and proline, known for their contributions to mitigating abiotic stress, also tend to accumulate under similar conditions. Studies have indicated that the removal of biosynthetic enzymes associated with RFOs does not severely impact plant health, offering further evidence in support of the aforementioned assertion.

On the other hand, numerous studies contend that RFOs possess beneficial characteristics as a suitable solute. For instance, Sanyal *et al.* [21] demonstrated that RFOs safeguard the cell membrane during dehydration stress by integrating themselves into the lipid head groups of the membrane bilayer. Farrant (2007) further supported this claim by associating the increase in RFOs during desiccation with the stability of membrane phospholipids. Additionally, their lengthy oligomeric structure could positively impact protective liposomes and function as a scavenger of free radicals [8]. Moreover, several studies propose that the accumulation of RFOs under abiotic stress conditions serves as osmolytes to uphold cell turgor and act as antioxidants against reactive oxygen species [22]. Galactinol synthase (*GolS)* is a vital enzyme involved in RFO production and is linked to abiotic stress [23]. Therefore, manipulating GolS gene expression genetically could provide valuable insights into the role of RFOs in mediating responses to abiotic stressors. These investigations have predominantly focused on Arabidopsis thaliana or tobacco (*Nicotiana tabacum*) plants, as they exhibit increased galactinol and raffinose levels in response to abiotic stressors (Gangola and Ramadoss, 2018. Various forms of *GolS* have been identified in different plant species, each induced under specific abiotic stress conditions. Notably, among the seven known *GolS* genes in *Arabidopsis thaliana*, *AtGolS1* and *AtGolS2* are activated by drought, salt, or heat stress, while *AtGolS3 i*s triggered by cold stress. Manipulation of these genes through overexpression or knockout techniques can be utilized to study RFO physiology. Studies by Sengupta *et al.* (2015) indicated that upregulation of these genes led to increased galactinol (Gol), raffinose (Raf), and stachyose (Sta) accumulation, subsequently enhancing the plant's tolerance to drought, salt, or cold stress. Rensburg [24] also demonstrated that mutant plants lacking *AtGolS1* failed to accumulate heat stress-induced Gol and Raf, suggesting the crucial role of *AtGolS1* in heat stress-induced Raf or Gol accumulation.

Research conducted by Peters *et al.* (2010) utilized a double mutant to reveal that despite enhanced *GolS1* accumulation in *GolS2* mutants, they still exhibited heightened sensitivity to water stress, leading to rapid water loss and reduced enzymatic activity. This indicates their susceptibility to drought conditions. These findings highlight the absence of RFO storage or transportation in Arabidopsis, pointing towards the involvement of various biosynthetic pathways facilitated by different *GolS* isoforms. Additionally, the introduction of the *Medicago falcata Gols (MfGolS1*) gene into tobacco conferred cold temperature tolerance, as demonstrated by Shi *et al.* [25]. elucidated the role of galactinol in signalling RFOs to regulate stress responses, including a response to pathogen invasion, underscoring the contribution of RFOs in defence against biotic stress. GolS was found to enhance the expression of defence-related genes like *PR1a, PR1b, and NtACS1* in tobacco during infections by Botrytis cinerea and *Erwinia carotovora* [26]. Furthermore, GoI was shown to facilitate salicylic acid (SA) signalling post-pathogen invasion, triggering the expression of the PR1a gene to manage disease progression [27]. The presence of W-box cis-elements in the promotors of RFOs (mainly *GolS* and *RafS*), regulated by ABA-inducible WRKY [28], suggests a potential role of RFOs in SA and ABA signalling under both biotic and abiotic stress conditions. Fig. 3 illustrates the significance of RFOs in plant well-being, encompassing their impact on seed germination, seed maturation, desiccation tolerance, as well as biotic and abiotic stress resilience [8].

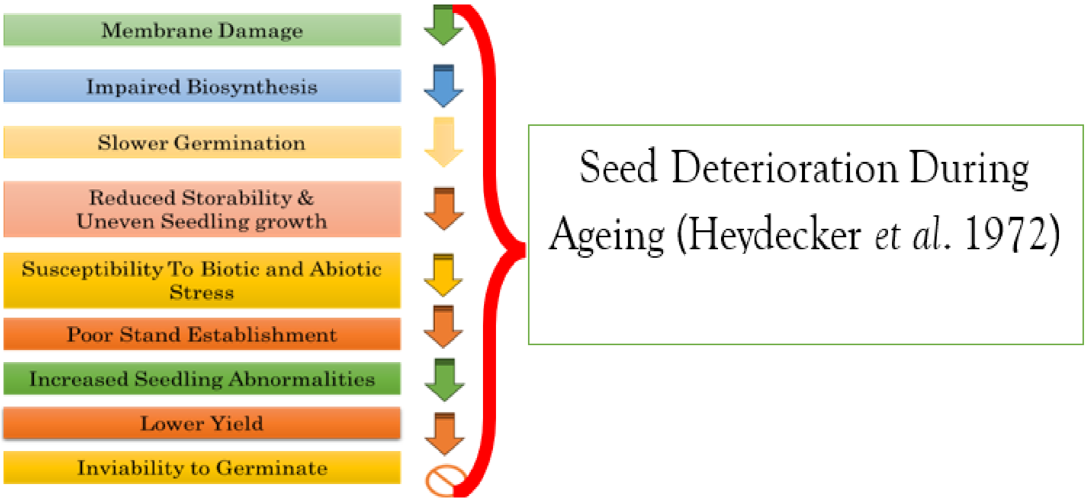

Fig. 3. Physiological Changes occurring while seed deteriorates by ageing

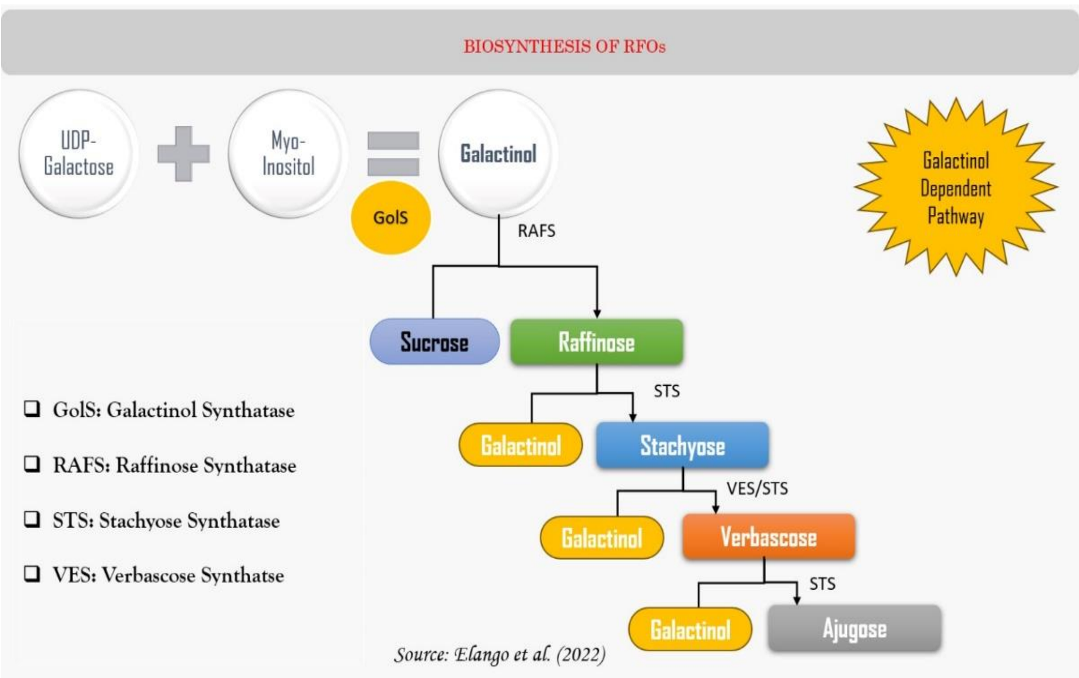

Fig. 4. Biosynthesis of RFO's through Galactinol's Dependent Pathway

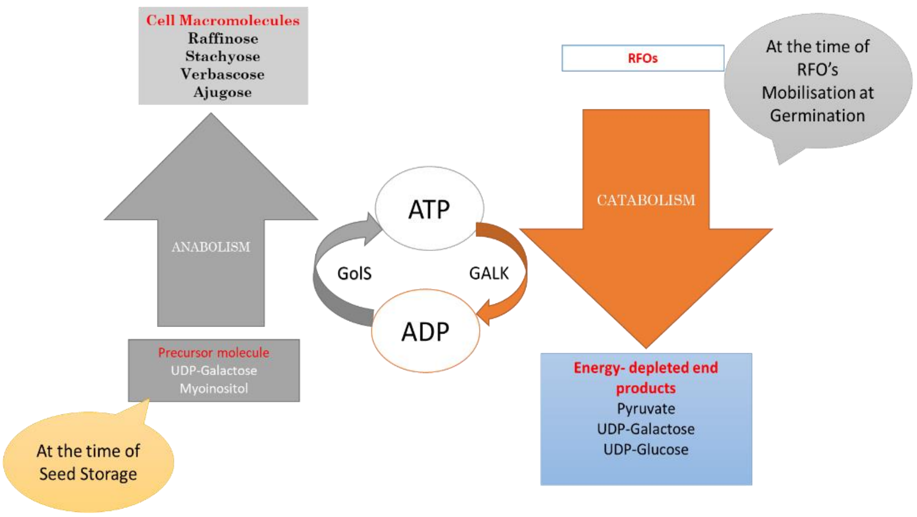

Fig. 5. Dynamics of RFO's Synthesis [10]

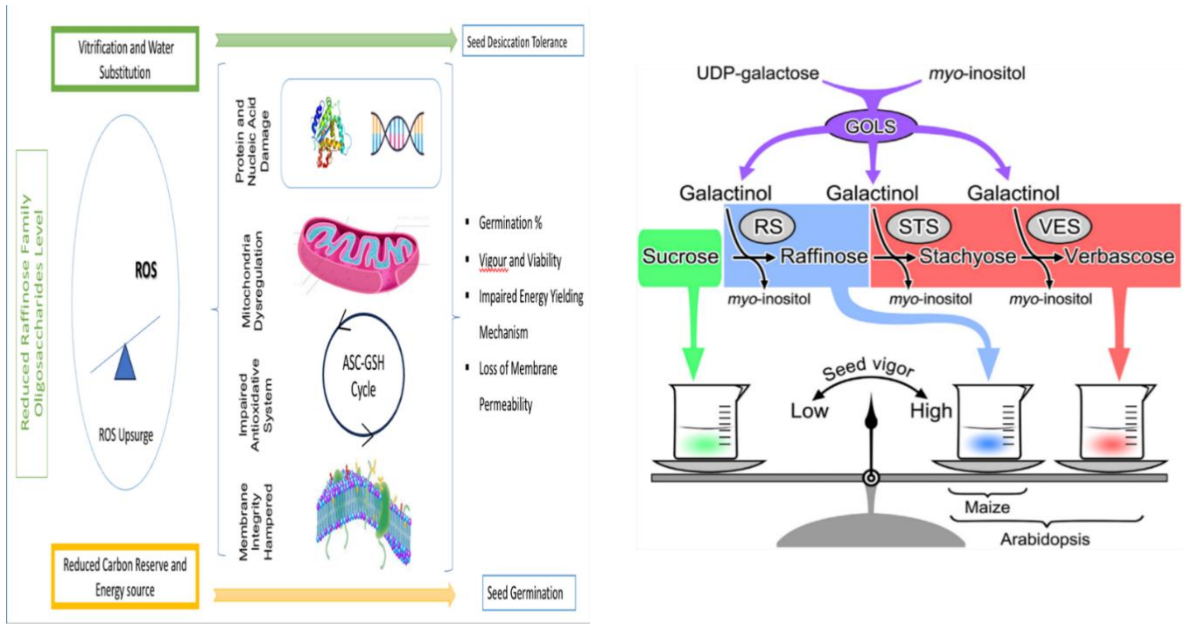

Fig. 6. Multifunctional Role of RFO's (Source: Salvi *et al.*, [10])

7. SUMMARY AND WAY FORWARD

Raffinose family oligosaccharides (RFOs) hold potential as beneficial components in food, though their multifaceted benefits for human and animal health are not yet fully recognized. RFOs positively impact gut microbiota, the large intestines, and colon health and could be used therapeutically to alleviate conditions like inflammation, diabetes, and allergies. However, RFOs are also linked to causing flatulence in both humans and animals, which affects the adoption of crops high in RFOs, such as grain legumes, in food and feed systems [29]. Therefore, finding the optimal concentration of RFOs in crops is crucial to promoting them as functional foods [30]. The exact concentration necessary for human health benefits remains an area for further research. Additionally, apart from Japan, most countries have not yet recognized RFOs as functional foods.

Over the past 50 years, seed storage science has evolved significantly, moving from anecdotal practices to advances grounded in biochemistry, genomics, and biophysics. These advancements have improved our understanding of how seeds achieve cytoplasmic solidification upon drying and how the properties of intracellular glasses relate to the kinetics of ageing. Seed ageing is characterized by the random and continuous oxidative degradation of proteins, lipids, and nucleic acids, which cannot be fully countered by the cells' antioxidant defences. The field of seed storage biology is complex and interdisciplinary, encompassing seed physiology, biophysics, biochemistry, and various 'omics' technologies, including genomics, transcriptomics, proteomics, metabolomics, ionomics, and phenomics. The progress in these areas has enhanced our ability to predict seed storage behaviour and optimize storage conditions to prolong seed viability, thereby supporting agriculture, ex-situ conservation, and the sustainable use of seeds. [31,32].

8. CONCLUSION

In conclusion, Raffinose Family Oligosaccharides (RFOs) play a crucial role in seed physiology, significantly enhancing seed vigour and longevity. These intricate carbohydrates are widely found in the seeds of various crops, particularly legumes, where they have a fundamental role in enhancing stress tolerance, regulating germination processes, and ensuring overall seed well-being. The diverse functions of RFOs encompass cytoplasmic vitrification, aiding in the stabilization of cellular structure by displacing water within the seed matrix. This particular process is essential for preserving cellular integrity during desiccation and preventing harm from osmotic stress.

Furthermore, RFOs offer osmoprotection in desiccated seeds, shielding cells from the adverse impacts of reactive oxygen species (ROS) and ensuring enzyme stability. This protective function is crucial for maintaining the structural and functional soundness of proteins, thereby averting conformational alterations that may compromise seed viability. Through alleviating oxidative stress and bolstering seed resilience to environmental variations, RFOs play a pivotal role in upholding seed longevity and vitality.

Comprehending the significance of RFOs in seed viability and longevity presents noteworthy implications for agricultural methodologies and seed preservation tactics. By harnessing the protective and stabilizing attributes of RFOs, there exists the potential to enhance crop yield, refine seed preservation techniques, and ultimately have a positive impact on the global economy. The scope of RFOs transcends plant physiology, as nascent studies indicate their multifaceted advantages in human and animal well-being. Their functional characteristics as prebiotics and their involvement in regulating gut health suggest a promising avenue for crafting functional foods with health-promoting qualities [33-35].

Hence, further exploration is imperative to comprehensively delve into the diverse advantages of RFOs. Scrutinizing their mechanisms of operation, optimal concentrations for various uses, and potential health benefits will pave the way for innovative applications in agriculture, food technology, and healthcare. By enhancing our grasp of RFOs, novel prospects can be unlocked to boost crop productivity, enhance food security, and contribute to the overall welfare of human and animal populations.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

It is to declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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