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REVIEW

Iron biofortification in cereal crops: Recent progress and prospects

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Abstract

Micronutrient malnutrition is one of the major causes of human disorders in the developing world. Iron (Fe) is an important micronutrient due to its use in human metabolism such as immune system and energy production. Estimates indicate that above 30% of the global population is at risk of Fe deficiency, posing a particular threat to infants and pregnant women. Plants have adapted various strategies for uptake, transport, accumulation, and storage of Fe in tissues and organs which later can be consumed by humans. Biofortification refers to increase in micronutrient concentration in edible parts of plants and understanding the pathways for Fe accumulation in plants. Conventional plant breeding, transgenics, agronomic interventions, and microbe-mediated biofortification are all potential methods to address Fe deficiency. This review article critically evaluates key aspects pertaining to Fe biofortification in cereal crops. It encompasses an in-depth analysis of the holistic presence of Fe, its significance in both human and plant contexts, and the diverse strategies employed in Fe uptake, transport, accumulation, and storage in plant parts destined for human consumption. Additionally, the article explores the bioavailability of Fe and investigates strategies for biofortification, with a specific emphasis on both traditional methods and recent breakthroughs aimed at enhancing the Fe content in food crops. Keeping in view the significance of Fe for human life, appropriate biofortification strategies may serve better to eliminate hidden hunger rather than its artificial supplementation.

KEYWORDS

biofortification, genetic engineering, human health, iron deficiency, plant metabolism

1 | INTRODUCTION

Micronutrient malnutrition, often referred to as hidden hunger, is a widespread nutritional disorder (Nair et al., 2013) that affects approximately one-third of the global population (FAO, 2013). This silent epidemic occurs when daily food intake falls short of the recommended daily allowances (Jha & Warkentin, 2020) and remains overlooked unless the deficiency symptoms are diagnosed (Majumder et al., 2019). The annual economic cost of deficiencies in both micronutrients and macronutrients is estimated to be around 2 trillion US dollars (Hoddinott, 2016; Panel, 2016). Consequently, enhancing nutritional health poses a significant challenge for many countries (Maqbool et al., 2020). Iron is an essential nutrient for living organisms, playing a vital role in various metabolic processes in plants and animals. In humans, Fe is vital for growth, development, and several physiological processes, including the transportation of oxygen from the lungs to tissues, the development of immune system, and the synthesis of oxygen transport proteins such as hemoglobin and myoglobin (Jomova et al., 2022). Globally, Fe deficiency is the most prevalent micronutrient disorder, impacting over 2 billion people (World Health Organization [WHO], 2019). It gives rise to significant health complications, including increased mortality risk, limited physical and mental development, weakened immunity, anemia, and fatigue (Black et al., 2008). Pregnant women are particularly vulnerable to the consequences of Fe deficiency, which can lead to adverse outcomes such as premature births, low birth weight babies, impaired growth and development in infants, and compromised cognitive abilities (Bailey et al., 2015; Lozoff et al., 2008).

Iron is crucial for fundamental processes in plants, encompassing respiration, photosynthesis, and antioxidant defenses. It also plays a vital role in numerous biochemical pathways, including those related to hormones and secondary metabolism, making it an essential micronutrient (Kobayashi et al., 2019; Vigani & Murgia, 2018; Therby-Vale et al., 2022). Its involvement extends to various chemical forms, such as Fe-heme groups, Fe-S clusters, and nitrosyl-Fe complexes (Ramirez et al., 2011). Iron is crucial in symbiotic nitrogen fixation, aiding in synthesizing the Fe-molybdenum cofactor (FeMo-co) of the nitrogenase enzyme, which is essential for activating and catalyzing nitrogen fixation (Rubio & Ludden, 2008).

Human Fe nutrition is closely tied to the concentration of Fe in plants, which, in turn, relies on the Fe content present in the soil (Cakmak, 2012; Li et al., 2023; Shukla et al., 2014). However, calcareous soils, which are widespread across more than 30% of the Earth's land surface, particularly in arid and semi-arid regions, typically exhibit a pH range between 7.4 and 8.5. Within this pH range, the presence of inorganic Fe forms is minimal (Prasad & Djanaguiraman, 2017; Shenker & Chen, 2005), the concentration of Fe is less than 10^{-10} M (Frossard et al., 2000; White & Broadley, 2009); which has drawn attention in last few years at the global level. The transformation of Fe into the insoluble Fe-hydroxyl complex leads to natural deficiencies of Fe in saline, alkaline, sodic, and calcareous soils. This limitation restricts the roots' capacity to absorb and uptake Fe. Conversely, in acid sulfate soils with a pH below 5, Fe is excessively available, facilitating its transport to plants (Fageria et al., 2002, 2008). Additionally, the removal of Fe-containing aleurone and embryonic tissues during postharvest processing of staple crops such as rice, wheat, and corn significantly contributes to the low intake of Fe from diets (Connorton & Balk, 2019).

Given the widespread prevalence of Fe deficiency and its associated health risks, it is crucial to implement necessary practices to enhance Fe content in human food consumption. In recent years, several strategies, such as increased food production, supplementation, food fortification, and biofortification, have been employed to address Fe deficiency. Biofortification, specifically enhancing Fe levels in plants, is a promising approach to improve the nutritional value of crops and mitigate Fe deficiency in humans (Monika et al., 2022). The objective of this review is to elaborate on the role of Fe in humans and plants, its dynamics in soil, factors affecting its bioavailability, and its uptake as well as translocation in plants. Moreover, it will critically discuss biofortification strategies, especially conventional approaches and recent modifications for Fe enrichment in cereal crops. Given the critical importance of Fe for human health, biofortification strategies may prove more effective in addressing hidden hunger than artificial supplementation.

2 | IRON AND HUMAN HEALTH

Iron is an essential component found in every human cell, playing a crucial role in numerous biological functions within the body. It is involved in vital processes such as DNA and protein synthesis, the formation of connective tissues, and the enhancement of the immune system (Abbaspour et al., 2014). Approximately 85% of the Fe in the human body is utilized in the production of crucial heme proteins, namely hemoglobin and myoglobin, which play a vital role in facilitating the transportation of oxygen (Bell & Dell, 2008; Tak et al., 2013). Iron plays a crucial role as both a necessary component and activator in numerous enzymes that participate in electron flux and redox reactions (Gharibzahedi & Jafari, 2017). Furthermore, it contributes to energy production through cellular respiration, aids in the synthesis of bile acids and steroid hormones in the liver, and is involved in the production of neurotransmitters such as dopamine and serotonin, which regulate brain signaling (Charles, 2012; Stoltzfus et al., 2003).

Iron deficiency is a critical global health concern, ranked as the sixth most perilous factor among 10 primary threats to human race (Fageria et al., 2012; Uzoh & Babalola, 2020). Its consequences include stunted growth, mental retardation, weakened immunity, fatigue, and reduced work efficiency (Bharadva et al., 2019; Black et al., 2008). Children and pregnant women are particularly at a higher risk of Fe deficiency, with susceptibility rates of 40% and 38%, respectively, due to their increased Fe requirements (Camaschella, 2015; Pasricha et al., 2013; Rahal & Shivay, 2016; Stevens et al., 2013), leading to over 60,000 deaths annually during pregnancy or childbirth (Tak et al., 2013). Furthermore, inadequate Fe intake can cause anemia, with 50% of global anemia attributed to Fe malnutrition, as reported by WHO (2019). The daily requirement of Fe based on age and sex, with men, women, and children needing 1.46, 1.05, and 0.71 $\mathrm{mg\,day^{-1}}$, respectively (FAO/WHO, 2004). It is noteworthy that the Fe content in cereal grains varies widely, ranging from 15 to 115 $\mu\mathrm{g\,g^{-1}}$ (Uzoh & Babalola, 2020), while the tolerable upper limit is set at 45 $\mathrm{mg\,day^{-1}}$ (Rahal & Shivay, 2016).

3 | ROLE OF IRON IN PLANT METABOLISM

Iron is the third most limiting nutrient for plants, which plays a vital role in their growth and physiological functioning (Rout & Sahoo, 2015; Therby-Vale et al., 2022). The adequate range of Fe in plant tissues ranges from 50 to 250 ppm (Rengel et al., 1999). Iron plays a crucial role in plant metabolism, primarily due to its involvement in the formation of enzymes (Rawashdeh & Florin, 2015). It actively participates in vital processes such as DNA synthesis, electron transport system, N-fixation, photosynthesis, and respiration, making it essential for plant growth and productivity (Briat et al., 2015; Mazaherinia et al., 2010; Rout & Sahoo, 2015; Tripathi et al., 2018). Nearly 80% of Fe in plant tissues is distributed in the photosynthetic apparatus, with direct involvement in photosystem II (PS-II), Photosystem I (PS-I), the cytochrome complex, and ferredoxin (Abbaspour et al., 2014; Ma et al., 2021; Varotto et al., 2002). The biosynthesis of chlorophyll and the development of chloroplast are also reliant on Fe (Layer et al., 2010; Yadavalli et al., 2012).

Iron is a fundamental element necessary for numerous biosynthetic processes in plants. It plays a critical role in the production of essential molecules such as cytochromes and heme, which are integral components involved in the synthesis of chlorophyll (Briat et al., 2007; Hansch & Mendel, 2009). Fe also plays a role in the synthesis of Fe–S clusters in the chloroplasts, which are involved in electron transport within thylakoid membranes and serve as cofactors for several protein complexes (Balk & Schaedler, 2014; Couturier et al., 2013; Eberhard et al., 2008; Ma et al., 2021). Additionally, it is an integral component of chlorophyllide, an oxygenase enzyme, involved in conversion of chlorophyll *a* to chlorophyll *b* during chlorophyll synthesis (Eggink et al., 2004; Tanaka & Tanaka, 2006).

4 | IRON IN SOILS AND ITS DYNAMICS

Iron is abundant in lithosphere, ranked as the fourth most plentiful element (Ma, 2005; Rout & Sahoo, 2015) constituting up to 5% of agricultural lands; while its concentration ranges from 22.4 to 112 $\mathrm{tons\,ha^{-1}}$ in the plow layer (Rawashdeh & Florin, 2015). In soil, Fe exists in four different pools, including primary and secondary minerals, bioavailable pools, and organically bound Fe (Colombo et al., 2013). Soil minerals contain Fe in ferrous ($\mathrm{Fe^{2+}}$) and ferric ($\mathrm{Fe^{3+}}$) oxidation states, which are released through chemical reactions. However, the subsequent fate of released Fe depends on various physiochemical and biological soil properties such as soil texture, aeration, temperature, pH, $\mathrm{CaCO_3}$ content, organic matter, and soil phosphorous content (Carrillo-Gonzáles et al., 2006; Robin et al., 2008).

Among all factors, pH and redox potential (Eh) are the most significant factors, influencing Fe bioavailability in soil. Under high pH-Eh soil system, Fe bioavailability is low (Briat, 2005; Schulte, 2004) due to rapid oxidation of released Fe from primary minerals, resulting in precipitation as oxides, hydroxides, and oxyhydroxides (Borggaard, 2002; Hinsinger, 2001; Robin et al., 2008; Rout & Sahoo, 2015). Goethite (α-FeOOH) and hematite ($\alpha\text{-Fe}_2\mathrm{O}_3$) are the predominant minerals in well-aerated soils, known for their low solubility and stability (Colombo et al., 2012). This condition is exacerbated in calcareous soils with high pH, leading to Fe precipitation (de Santiago & Delgado, 2006). Under such conditions, Fe is 100 times less available than the required concentration for optimum plant growth (Uzoh & Babalola, 2020), as approximately 30% of cultivable lands exhibit high pH levels that hinder the uptake of Fe (Babalola & Glick, 2012; Kobayashi et al., 2019). Under high pH and oxic soil conditions, the total bioavailable fraction of Fe is about 10^{-10} M (Boukhalfa & Crumbliss, 2002) against the total concentration of 20–40 $\mathrm{g\,kg^{-1}}$ (Cornell & Schwertmann, 2003); while the required concentrations for optimum plant growth is about 10^{-8} mol L^{-1} (Eskandari, 2011). In contrast, low pH-Eh soil systems enhance Fe solubility (Johnson et al., 2012) by reducing Fe^{3+} to Fe^{2+} form, facilitating its entry into the available pool through adsorption on soil exchangeable sites (Hochmuth, 2011).

Soil organic matter plays a critical role in regulating the availability of Fe in the soil. High organic matter content enhances the bioavailability of Fe by forming soluble complexes with it (Hochmuth, 2011). This formation of soluble complexes helps protect Fe from potential precipitation, which would otherwise reduce its solubility (Cesco et al., 2000; Varanini & Pinton, 2001). Additionally, humic substances exhibit chelating and redox reactive properties (Olaetxea et al., 2018) and contain phenolic groups that facilitate the reduction of Fe^{3+} to Fe^{2+}. These reduced forms of Fe can either remain in the soil solution or become adsorbed onto exchangeable sites (Heitmann et al., 2007; Kögel-Knabner et al., 2008). Tipping (2002) demonstrated that within pH range of 6.5–8.5, the concentration of organically bound Fe was more than twice the concentration of all inorganic Fe species. However, the process of Fe^{3+} reduction by organic substances is strongly influenced by pH (Chen et al., 2003). As pH increases, humic substances tend to bind more frequently to metal cations, resulting in a reduced ability to carry out the reduction of Fe^{3+} to Fe^{2+} (Chen et al., 2003).

5 | LOADING AND ACCUMULATION OF IRON IN SEEDS

The vasculature of the mother plant and the provasculature of the embryo lack continuity. Consequently, nutrients, including Fe, must be discharged from the phloem and subsequently absorbed by the embryo. In *Arabidopsis* and other dicotyledonous plants, the chalaza and nucellus are the sites where nutrients are released into the embryo sac fluid. Subsequently, the embryo absorbs these nutrients from the fluid. Similarly, in cereals, Fe is transported from plant roots and aging leaves to developing seeds through the phloem. This movement occurs because the upward flow of xylem sap, driven by transpiration, typically results in slow or even negligible transport of minerals through the xylem to reproductive tissues that are not directly exposed, such as fruits and seeds (Figure 1; Morrissey & Guerinot, 2009; Stacey et al., 2008). Thus, the transport of Fe to these tissues occurs solely through the phloem (Stacey et al., 2008). Furthermore, mineral nutrients, including Fe, require active and selective transport mechanisms and are not directly unloaded from the seed coat into the endosperm (Grillet, Mari, et al., 2014; Grillet, Ouerdane, et al., 2014). This suggests a potentially crucial role for transporter proteins in the loading of nutrients into the seed.

AtYSL1 expression has been identified in both the funiculus and the chalazal endosperm (Le Jean et al., 2005). Examination of Fe speciation in the embryo sac fluid during pea seed development indicates the presence of ferric Fe bound to citrate and malate in this extracellular compartment (Grillet, Mari, et al., 2014; Grillet, Ouerdane, et al., 2014). Consistent with this finding, the citrate efflux transporter FRD3 is active in the peripheral cell layer of the embryo and the tegument cell layer facing the embryo sac throughout seed development (Roschzttardtz et al., 2011). FRD3's secretion of citrate in the embryo sac is indicative of its role in maintaining Fe solubility and availability for the embryo's uptake. In dicotyledonous plants, Fe is absorbed in its ferrous form, necessitating its reduction before uptake by the embryo. Despite efforts, genetic analyses in Arabidopsis have not pinpointed a crucial membrane-bound ferric chelate reductase among FRO2 homologues for Fe acquisition by the embryo. Conversely, an examination of pea embryo sac fluid revealed a substantial concentration of ascorbate, effectively reducing Fe(III) to Fe(II) before the embryo takes it up (Grillet, Mari, et al., 2014; Grillet, Ouerdane, et al., 2014). Notably, Arabidopsis vtc mutants lacking ascorbate biosynthesis show reduced seed Fe content, aligning with the importance of ascorbate in this process. The speciation of Fe in the embryo sac mirrors that of other extracellular plant compartments like xylem sap. Interestingly, the use of ascorbate for Fe reduction in the embryo sac contrasts with the employment of membrane-bound ferric chelate reductases in roots, leaves, and intracellular organelles such as mitochondria and plastids (Jain et al., 2014; Jeong et al., 2008). While the transporters responsible for secreting ascorbate and Fe in the embryo sac remain unidentified, in the case of zinc (Zn), Heavy Metal pumping P-type ATPases HMA2 and HMA4 releases Zn from mother tissues for subsequent embryo uptake (Olsen et al., 2016). Concerning Fe, the plasma membrane Fe efflux transporter IREG1/FPN1 might play a role, but no defect in Fe supply to the embryo has been reported in IREG1/FPN1 mutants so far (Morrissey et al., 2009). In wheat and barley, a single vascular strand along the ventral crease is responsible for providing nutrients, including Fe, to the grain (Borg et al., 2009). Iron is primarily supplied by the phloem and traverses several specialized cell layers, namely the crease vascular parenchyma, the pigment strand, and the nucellar projection. The ultimate destination is the transfer cells, which face the embryo. These transfer cells, akin to the modified aleurone cells adjacent to the embryo, feature highly invaginated plasma membranes that facilitate the release and reabsorption of nutrients (Borg et al., 2009).

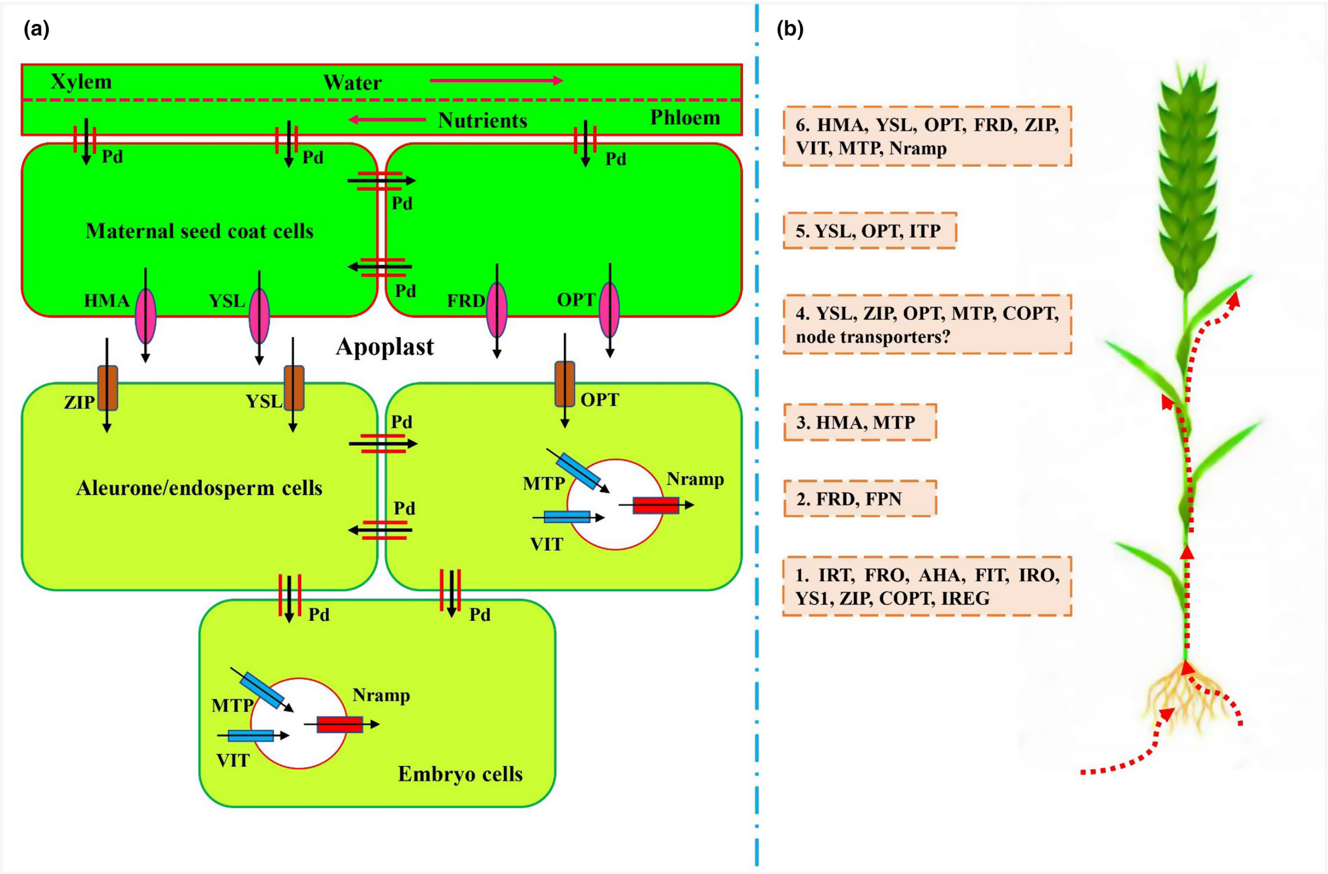

FIGURE 1 Model of location and pathways of Fe uptake or transport genes discussed in this review. (a) Detail of seed loading. Gene families potentially involved in seed mineral micronutrient transport are pictured in hypothetical or known localizations. Maternal tissues are shown in green, and filial tissues are shown in gold. Efflux transporters are shown in light blue, plasma-membrane localized uptake transporters in dark blue, vacuolar uptake transporters in gray, vacuolar efflux transporters in red. Pd, plasmodesmata. (b) Model of wheat plant showing the following translocation steps to the seed: (1) uptake from the rhizosphere; (2) xylem loading; (3) root-to-shoot transfer; (4) distribution to the leaves or seed-covering tissues; (5) phloem loading for movement to seed; (6) loading into the seed (Conceived from Waters & Sankaran, 2011). AHA, Arabidopsis H+-ATPase; COPT, copper (Cu) transporter; FIT, FER-like Fe deficiency-induced transcription factor; FPN, ferroportin; FRD, ferric reductase defective; FRO, ferric reduction oxidase; HMA, heavy metal ATPase; IREG, iron-regulated gene; IRO, iron-responsive operator; IRT, iron-regulated transporter; ITP, iron transport protein; MTP, metal tolerance proteins; Nramp, natural resistance-associated macrophage protein; OPT, oligopeptide transport; Pd, plasmodesmata; VIT, vacuolar iron transporter; Ys1, yellow stripe1; YSL, yellow stripe-like; ZIP, ZRT- and IRT-like protein.

Iron accumulation and speciation within these structures exhibit distinct patterns in mature wheat grains. The nucellar projection, where Fe is concentrated, shows co-localization with sulfur. In contrast, the modified aleurone accumulates Fe to a lesser extent, likely acting as a distribution source for other aleurone cells, the embryo, and the endosperm (De Brier et al., 2016). X-ray absorption spectra suggest that Fe in the nucellar projection is mainly associated with NA, while in the modified aleurone, it is linked to phytate (De Brier et al., 2016).

Barley homologs of AtVIT1, responsible for driving Fe influx into the vacuole where phytate is localized, are highly expressed in the aleurone (Borg et al., 2009; Connorton, Jones, et al., 2017; Kim et al., 2006; Lott & Spitzer, 1980). Conversely, genes encoding NAAT, NAS, and YSL, promoting Fe mobility, exhibit high expression levels in transfer cells (Borg et al., 2009; Curie et al., 2009). In *Arabidopsis*, the absence of Nicotianamine Synthase 4 (NAS4) has been found to inhibit the translocation of Fe into flowers and seeds (Klatte et al., 2009). Likewise, Kruger et al. (2002) identified an iron transporter protein (ITP) in the phloem of castor bean plants. They found that nicotianamine (NA) is involved in mobilizing and transporting Fe within and out of the phloem. The transport of Fe in the phloem is facilitated by a 17 kDa iron-binding protein. Ishimaru et al. (2006) observed the expression of the OsIRT1-GUS gene in both the phloem cells of roots and shoots in rice plants. The gene expression was triggered due to

Fe deficiency, particularly in companion cells. It was reported that OsIRT1 facilitates the transport of Fe2+ into the phloem cells, where it is chelated by NA (Ishimaru et al., 2006). In the future, it will be crucial to elucidate the specific expression patterns and roles of each gene involved in Fe transport within this intricate structure. This effort should aid in identifying key transporters responsible for releasing Fe to the extracellular space from transfer cells and those involved in its uptake by the embryo in modified aleurone cells. In rice plants, approximately 4% of the Fe in the shoots is remobilized to the seeds, while in wheat, this percentage is higher at 77% (Morrissey & Guerinot, 2009). During grain development, phloem cells are responsible for transporting Fe into the maternal seed coat. Simultaneously, the translocation of Fe into the grain apoplast involves various types of influx and efflux transporters, including yellow stripe-like (YSL), ZRT- and IRT-like protein (ZIP), and natural resistance-associated macrophage protein (Nramp) (Tauris et al., 2009).

The distribution of Fe in seeds varies among species and developmental stages. This distribution, along with subcellular localization, is closely tied to Fe speciation, which refers to the nature of the ligand binding Fe and influencing its bioavailability (Clemens, 2014). For instance, Fe phytate complexes stored in vacuoles are known for their low bioavailability (Hallberg, 2001). In contrast, ferritin-bound Fe stored in plastids serves as a highly bioavailable source of Fe (Briat, 1999). Limited information exists regarding the generation of Fe distribution patterns during embryo development. The pronounced expression of VIT1 throughout seed development aligns with the early emergence of the Fe pattern at the torpedo stage, particularly in the central region of the cotyledons where provascular tissue is set to differentiate (Kim et al., 2006; Roschzttardtz et al., 2009).

In graminaceous species, such as grains, the distribution of Fe differs significantly from that observed in Brassicales (Mari et al., 2020). Unlike Arabidopsis, where the embryo constitutes a substantial volume of the seed, in grains, particularly in wheat (*Triticum turgidum* L.), the embryo represents a small portion, with the major part consisting of the starchy endosperm. The endosperm is enveloped by the aleurone cell layer, which, during germination stimulated by gibberellins, becomes active, releases enzymes for carbohydrate digestion stored in the endosperm, and eventually undergoes programmed cell death (Fath et al., 1999; Jones, 1969). In grains, a significant portion of Fe is concentrated in the aleurone layer, with another pool found in the embryo (De Brier et al., 2016; Iwai et al., 2012; Singh et al., 2013). Notably, in wheat, the highest Fe concentrations are observed in the aleurone layer and the scutellum, the absorptive structure in the embryo akin to the human placenta, responsible for nutrient uptake from the endosperm (Singh et al., 2013). Separate measurements of wheat flour and bran revealed that nearly 60% of grain Fe is in the bran, encompassing the aleurone layer and representing the primary Fe reservoir in grains (De Brier et al., 2015). Within the aleurone layer, Fe is concentrated along with phosphorus and other minerals in globoids within the protein storage vacuole, resembling the subcellular localization observed in Arabidopsis (Lott & Spitzer, 1980).

In situ X-ray absorption spectroscopy analyses have revealed that within the aleurone layer, the majority of Fe is bound to phytate, existing as either Fe(II) or Fe(III), consistent with its subcellular localization (De Brier et al., 2016; Singh et al., 2013). Another pool of Fe is bound to citrate, potentially playing a role in transport from modified aleurone cells in the crease to aleurone cells (De Brier et al., 2016). In rice, Fe accumulates in the aleurone layer along with phytate and in the scutellum, mirroring observations in wheat (Iwai et al., 2012; Takahashi et al., 2009). However, the precise speciation of Fe in the embryo and endosperm remains undetermined in these tissues, which have lower Fe concentrations, likely due to limitations in the sensitivity of X-ray absorption spectroscopy. Based on element co-occurrence, it is suggested that Fe is bound to phosphate, potentially as phytate, in the wheat scutellum and to other ligands in different parts of the embryo and endosperm (De Brier et al., 2016, Singh et al., 2013). These ligands may encompass proteins or smaller molecules like NA, present in grains and seeds of nongraminaceous species (Le Jean et al., 2005; Lee et al., 2009). In wheat flour, corresponding to the starchy endosperm, NA serves as the primary ligand for Fe (Eagling et al., 2014).

Limited information exists regarding the mechanisms governing the pattern of Fe localization in grains. A developmental study in rice grains revealed that throughout grain development, Fe co-localizes with phosphorus in the aleurone cell layer, suggesting that Fe is likely bound to phytate upon storage (Iwai et al., 2012). Mutations in OsVIT1, OsVIT2, and mitochondrial iron transporter (MIT) disrupt Fe localization within the embryo (Bashir, Takahashi, Akhtar, et al., 2013; Zhang et al., 2012). OsYSL9 exhibits strong expression in the scutellum and contributes to Fe storage in the embryo (Senoura et al., 2017). The phytosiderophore efflux transporter TOM2 is expressed in the dorsal vascular bundle, epithelium, and the scutellum, potentially playing a role in Fe distribution (Nozoye et al., 2015). Silencing TOM2 gene expression did not impact seed total Fe content. Utilizing techniques such as sXRF, Perls staining, or seed dissection for Fe distribution analysis could offer further insights into its function during rice grain development.

6 | AGRONOMIC BIOFORTIFICATION

Micronutrients, which are essential for human health, are naturally present in varying amounts in different parts of plants and are typically absorbed from the soil (Bhardwaj et al., 2022). Agronomic based biofortification approach implicates the supplementation of nutrient-rich fertilizers aimed to enhance their content in edible plant parts; thus, enhancing their consumption by individuals. This is considered the traditional way to enrich grains of food crops with vital micronutrients (Figure 2). The pathway through which nutrients move from the soil to plants, food, and eventually into the human body is a complex process that is influenced by their bioavailability (de Valenca et al., 2017; Zulfiqar, Maqsood, & Hussain, 2020; Zulfiqar, Maqsood, Hussain, & Anwar-ul-Haq, 2020). Several factors play a crucial role in determining the effectiveness of agronomic biofortification, as there is a substantial loss of nutrients during the transitional phases from soil to plant, plant to food, and ultimately from food to humans (Daud et al., 2016, 2017). Several factors contribute to the success of agronomic biofortification in addressing Fe deficiency in human beings. These factors depends on the availability of Fe at different stages: the presence of available Fe for crop uptake (soil to crop), the localization and mobilization of Fe into the edible harvested food (crop to food), the availability of Fe in the ready to eat food for humans, and the health condition of the human body, which determined its ability to absorb and assimilate Fe (food to human).

7 | SOIL TO CROP

The bioavailability of nutrients from the soil to plants is influenced by various factors such as soil pH, organic matter content, soil moisture and aeration, and interaction with other mineral nutrients and by the crop variety that defines the structure and function of the root system (Alloway, 2009; de Bang et al., 2020; Jalil et al., 2023). Some plants have the ability to modify the rhizosphere by secretion of H^+ ions or organic acids that improve the availability and uptake of micronutrients (Zhang et al., 2010). Rhizosphere acidification plays a crucial role in facilitating Fe absorption by plants. Studies have shown that the solubility of Fe decreases exponentially by a factor of 1000 for every one-unit increase in pH within the range of 4–9 (Zhou et al., 2018). Multiple beneficial microorganisms have been identified to facilitate the absorption of Fe by plants through various mechanisms, including chelation, reduction, acidification, and induction (Castulo-Rubio et al., 2015). Acidification enhances the mobility of Fe^{3+}, and microbial-induced rhizosphere acidification promotes plant Fe uptake. Microbes play a role in this process by producing organic acids in their extracellular metabolites. For example, *Bacillus amyloliquefaciens* GB03 emits volatile organic compounds (VOCs) such as glyoxylic

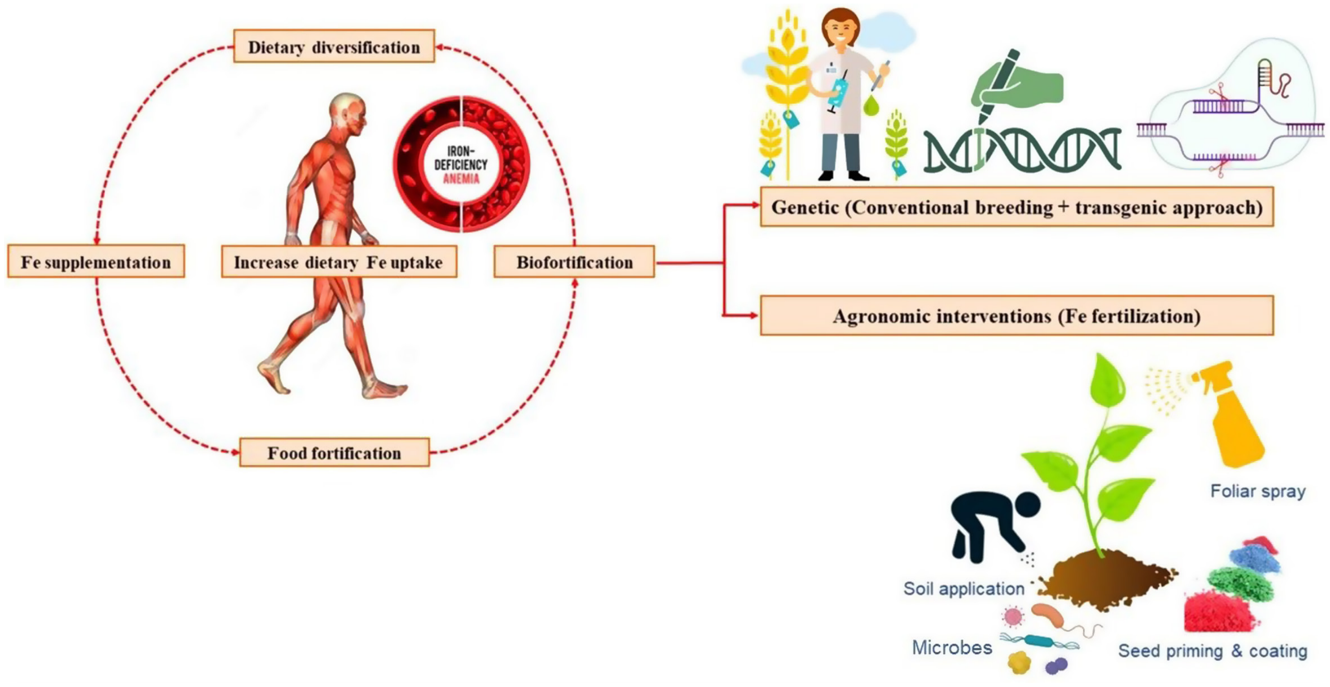

FIGURE 2 Iron biofortification in cereals.

acid, 3-methyl-butanoic acid, and diethyl acetic acid, leading to direct rhizosphere acidification (Farag et al., 2006; Zhang et al., 2009). Plant roots produce siderophores, enhancing Fe availability by efficiently chelating Fe^{3+} and solubilizing Fe from mineral or organic compounds (Miethke & Marahiel, 2007). Typically, siderophores exhibit a strong 1:1 affinity for complex formation with Fe^{3+}. Both gram-positive and gram-negative bacteria absorb these complexes through their cell membranes, resulting in the reduction of Fe^{3+} to Fe^{2+}. Subsequently, the cell membrane expels these ions from the siderophores into the cell through a mechanism known as "gating," involving the linkage of inner and outer membranes (Boukhalfa & Crumbliss, 2002). This process facilitates the solubilization of Fe from otherwise unavailable minerals or organic compounds under Fe-limiting conditions (Indiragandhi et al., 2008). Microbial biofertilizers also improve the availability and uptake of micronutrients in the soil (Kaur et al., 2020).

Interaction between elements significantly influences the bioavailability for root uptake. The uptake of elements by plants relies not only on the presence of those elements in the soil solutions but also on the roots' nutrient uptake capacity. An imbalance of P, either deficiency or excess, can impact the Fe homeostasis of plants (Shi et al., 2019; Zheng et al., 2009). Iron and P can interact at various levels, including in the soil or growth medium, on the root surface, and within plant systems (Rai et al., 2015). Elevated concentrations of Fe have been observed in P-deficient plants, attributed to the activation of Fe-responsive genes in response to P deficiency (Zheng et al., 2009). However, this phenomenon is not observed in a high P medium (Hirsch et al., 2006). In experimental conditions, the absence of P increased the Fe concentration in seedling shoots, while the Fe concentration in the roots remained unaffected, indicating an antagonistic relationship between P and Fe (Chaiwong et al., 2018). Consequently, the regulation of P homeostasis significantly influences the availability of Fe (Bournier et al., 2013).

The interaction between Fe and Zn occurs due to the chemical similarity between their divalent cations and basic transporter proteins (Sinclair & Krämer, 2012). While an antagonistic relationship between Fe and Zn is commonly reported in many plants (Jalil et al., 2023; Saenchai et al., 2016), some exhibit a more complex dynamic in their uptake and distribution. In *Arabidopsis thaliana*, an increase in Zn concentration led to a decrease in Fe concentration in the shoots, but the roots of *A. thaliana* were unaffected in terms of Fe concentration. The Zn concentration in plant shoots showed a positive correlation with the Zn concentration in the growth medium and remained unaffected by Fe concentration (Shanmugam et al., 2012). Despite *Thlaspi caerulescens* having high Zn concentration in the leaves, the root Zn content was low (van de Mortel et al., 2008). The decrease in Fe content in *T. caerulescens* shoots was attributed to a high concentration of Zn in the growth medium. Conversely, increasing the Fe concentration in the medium resulted in a decrease in Zn concentration in the shoots (Pineau et al., 2012). It was observed that Zn interferes with the uptake and translocation of Fe. Similarly, there is an antagonistic relationship between Fe and Mn. When present at equimolar concentrations, Mn interferes with the uptake and transport of Fe (Gayomba et al., 2015). The application of sulfur-based fertilizers alters soil properties, that is, pH and stimulates Fe bioavailability and uptake (Ramzani et al., 2016).

8 | CROP TO FOOD

Iron absorption is significantly influenced by the physical state of Fe, specifically its presence as ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms (Piskin et al., 2022). In the diet, nonheme Fe is primarily found in the oxidized or ferric form, although ferrous Fe is more readily absorbed by enterocytes. Ferric Fe precipitates at pH levels above 3, whereas ferrous Fe remains soluble under neutral pH conditions. As a result, ferric Fe needs to be first solubilized and chelated in the stomach to be absorbed in the less acidic proximal small intestine (Conrad & Umbreit, 2002). This chelation process occurs rapidly due to the presence of other components in food, as iron is released into the intestinal lumen. These chelators, acting as enhancers (ascorbic acid and animal tissues) or inhibitors (phytic acid, polyphenols, calcium, and oxalic acid), play a significant role in influencing iron absorption by affecting iron solubility (Clemens, 2014). Consequently, the composition of the diet is a critical factor in determining the absorption of nonheme Fe (Sharp, 2010). The presence of ascorbic acid in the diet has been shown to enhance the absorption of nonheme Fe (Teucher et al., 2004). Ascorbic acid facilitates Fe absorption by forming a chelate with ferric iron (Fe^{3+}) at the acidic pH of the stomach, maintaining solubility even at the alkaline pH of the duodenum, the initial part of the small intestine. Additionally, ascorbate, the salt of ascorbic acid, acts as a free radical scavenger and donates an electron, thereby reducing Fe oxidation states to Fe^{2+}. This reduction is crucial since Fe^{2+} is the bioavailable form for enterocyte cells (Smirnoff, 2018). It is important to note that Fe^{2+} is the only form of Fe that can be absorbed through the Fe transporters present in intestinal enterocyte cells (Gulec et al., 2014). The effect of vitamin C on Fe absorption relies on the dosage and the simultaneous consumption of both nutrients (Cook & Reddy, 2001; Davidsson et al., 1998). Iron absorption shows an increase from 0.8% to 7.1% when supplementing a meal containing 4.1 mg of nonheme Fe with 25–1000 mg of ascorbic acid. Consuming 500 mg of ascorbic acid alongside food enhances Fe absorption by six-fold, whereas taking it 4–8 h prior to the meal has a diminished effect (Cook & Monsen,1977).

Phytate and polyphenols in plant-based foods inhibit Fe absorption by forming complexes with dietary Fe in the gastrointestinal tract (Schönfeldt et al., 2016). Phytate, found in plants, significantly reduces the bioavailability of minerals (Me et al., 2009). Humans lack the necessary enzymes (endophytases) to digest phytate, so it remains unabsorbed in the small intestine, making chelated minerals inaccessible (Wilson et al., 2015). Hallberg et al. (1989) studied the inhibitory effects of sodium phytate on Fe absorption in humans consuming wheat rolls with varying phytate levels (2–250 mg). Phytate's inhibitory effect depended on the dose: 2 mg inhibited Fe absorption by 18%, while 250 mg led to an 82% inhibition. Additionally, the study found that the addition of ascorbic acid counteracted the inhibitory effect of phytates (Hallberg et al., 1989).

Nutrient bioavailability in food is influenced by crop variety and processing techniques. In case of rice, the outer layer, which contain Fe, is often removed during processing, resulting in lower Fe levels (Majumder et al., 2019). Paraboiling, a method used to particularly boil rice, can effectively retain and even enhance nutrient contents, particularly when micronutrients are added to water during paraboiling. This process tends to-mobilize nutrients from the outer layer to the endosperm (Hotz et al., 2015). In contrast, wheat contains Fe and Zn in the endosperm, which remains unaffected by processing (removal of seed coat and aleurone layer during milling and bread making) (Ajiboye et al., 2015). While food processing can lead to nutrient losses, it also reduces the levels of antinutrients, thereby enhancing the bioavailability of micronutrient. Soaking cereals in water can reduce phytate availability and increase the bioavailability of Zn, Fe, and other micronutrients (Hotz & Gibson, 2007). However, conventional cooking methods may still result in nutrient loss. To ensure an adequate intake of micronutrient, it is crucial to breed crop varieties that concentrate these nutrients in the edible parts of the plant.

9 | FOOD TO HUMAN

The bioavailability of a nutrient refers to the proportion of the ingested substance that becomes accessible for utilization and storage within the body (Gibson, 2007). The supply of nutrients to the human body does not only depends on the amount of the nutrient in a food but also on its bioavailability (Melse-Boonstra, 2020; Schönfeldt et al., 2016). It encompasses their absorption by improving accessibility in the intestinal lumen as well as their maintenance, absorption, and uptake in the body ultimately influencing their utilization by the body (Fernández-García et al., 2009; Hambidge, 2010). Indeed, the bioavailability of nutrients is correlated with their bioaccessibility and bioactivity, as bioaccessibility focuses on the release of compounds during digestion, while bioactivity relates to the biological effects or actions of those compounds once they are absorbed into the body (Rodrigues et al., 2022).

The inhibiting factors are known to reduce nutrient bioavailability by binding the nutrient in consideration into a form that is not recognized by the uptake systems while enhancing factors can act in different ways such as keeping a nutrient soluble or protecting it from interaction with inhibitors (Schönfeldt et al., 2016). Different nutrients (including protein, iron, and vitamin A) and the forms, in which they exist in the ingested medium, will react in different ways to inhibit or enhance the minerals absorption depending on their ingested quantity. In addition, the bioavailability can be affected by the concentration of a nutrient, dietary factors, chemical form, supplements taken separately from meals, nutrition and health of the individual, excretory losses, and nutrient–nutrient interactions (Quintaes et al., 2015). Bioavailability of iron, for example, is known to be influenced by various dietary components, which include both inhibitors and enhancers of absorption (Hemalatha et al., 2007). In the case of minerals, the amount available for absorption (bioaccessible or soluble) depends on the composition and physical characteristics of the diet, the content and the chemical nature of the mineral, the presence of ligand promoters or inhibitors of the absorption, luminal gastrointestinal secretions, and interactions that occur as a result of the interplay of these factors (Drago, 2017).

10 | AGRONOMIC APPROACHES TO MANAGE IRON IN FOOD CROPS

Iron deficiency in mineral soils is rare due to limited solubility caused by high soil pH and HCO_3 content in calcareous soils (Aciksoz et al., 2011). In acidic soils, where Fe is abundant, the presence of Fe^{2+} can pose a significant toxicity risk to plants. Iron toxicity is frequently observed in rice-growing environments, characterized by adverse conditions like inadequate drainage, highly reducing conditions, and elevated sulphide levels (Mahender et al., 2019). Limited solubility and availability is one of the reason of lower grain Fe concentration, consequently affecting human Fe intake (Grillet, Mari, et al., 2014; Grillet, Ouerdane, et al., 2014). Agronomic methods offer short-term solutions compared to breeding approaches

(Zulfiqar, Hussain, Ishfaq, Ali, Ahmad, et al., 2021; Zulfiqar, Hussain, Ishfaq, Ali, Yasin, et al., 2021). Soil application is less effective due to fixation and poor mobility in the phloem. However, the use of Fe chelates can help minimize fixation and prove useful as soil Fe fertilizers (Cakmak et al., 2010; Cakmak & Kutman, 2017; Rengel et al., 1999). Different Fe sources, such as $FeSO_4 \cdot 7H_2O$, Fe-EDTA, Fe-EDDHA and Fe nanoparticles, have been utilized as fertilizers for various crops (Aciksoz et al., 2011; Alsamadany et al., 2024). In a field study, the application of $FeSO_4 \cdot 7H_2O$ at 50 and 100 kg ha^{-1} as basal dose resulted in an improvement in grain Fe concentration the bread wheat by 14.9% and 19.1%, respectively (Yadav et al., 2016). Regarding flooded rice, the basal application of $FeSO_4 \cdot 7H_2O$ led to a 9.6%–14.9% increase in grain Fe concentration (Yadav et al., 2016). Similarly, when $FeSO_4 \cdot 7H_2O$ was applied to the soil at a rate of 67 mg kg^{-1}, the grain Fe concentration increased in direct-seeded aerobic rice by 25% (Meena et al., 2016). Agronomic biofortification has proven successful in different cereal crops as detailed in Table 1.

Fe can be effectively applied through foliar application to address visual deficiencies in plants and improve the micronutrient status of crops, thereby assisting in the achieving biofortification goals (Fageria et al., 2009; Therby-Vale et al., 2022). The foliar application of Fe-AA (Fe-amino acid) significantly enhanced the Fe concentration in brown rice, with a 14.5% higher Fe concentration recorded in foliar-applied treatments compared to the control (Yuan et al., 2013). Zhang et al. (2010) demonstrated that foliar application of ferric citrate, ferric citrate + $ZnSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$ increased wheat grain Fe concentration by 16.8%, 19.1%, and 34.7%, respectively. Similarly, foliar application Fe-EDTA and Fe-EDDHA improved grain Fe concentration by 13.3% and 14.8%, respectively (Aciksoz et al., 2011). Foliar application of different Fe sources, such as nano Fe oxide, Fe chelates, and $FeSO_4$, improved grain Fe accumulation in wheat by 7%–38% (Ghafari & Razmjoo, 2015). Likewise, in flooded rice, foliar application of 2% solution of $FeSO_4$ enhanced the grain Fe concentration by 16% compared to the control (Yadav et al., 2013). Foliar application of

TABLE 1 Fortification levels of Fe achieved in various cereal crops using different application techniques.

Application technique	Crop	Biofortification level (%)	Reference
Soil application	Maize	66	Saleem et al. (2016)
	Sorghum	5–12	Singh et al. (2016)
	Finger millet	17.8	Teklu et al. (2023)
Foliar application	Rice	8	Prom-U-Thai et al. (2020)
	Wheat	22	Aziz et al. (2019)
	Wheat	8	Pahlavan-Rad and Pessarakli (2009)
	Soybean	36	Sharma et al. (2019)
	Wheat	6–85	Narwal et al. (2012)
	Maize	52	Saleem et al. (2016)
	Wheat	80	Ramzan et al. (2020)
	Wheat	58–65	Zulfiqar, Maqsood, Hussain, and Anwar-ul-Haq (2020)
	Rice	37	Zulfiqar, Hussain, Maqsood, et al. (2021)
Nutripriming	Wheat	70	Sundaria et al. (2019)
	Wheat	15	Zulfiqar, Maqsood, Hussain, and Anwar-ul-Haq (2020)
	Wheat	20–121	Rizwan et al. (2019)
	Rice	31	Zulfiqar, Hussain, Maqsood, et al. (2021)
Seed coating	Wheat	11	Zulfiqar, Maqsood, Hussain, and Anwar-ul-Haq (2020)
	Rice	21	Zulfiqar, Hussain, Maqsood, et al. (2021)
Soilless cultivation	Rice	51	Chen et al. (2017)

$FeSO_4$ at 25 μM L^{-1} improved Fe concentration and bioavailable Fe contents in cowpea seeds (Marquez-Quiroz et al., 2015). Similar improvement in Fe concentration in grains in grains of cowpea (Salih, 2013) and mungbean (Ali et al., 2014) were also observed.

Micronutrients can be directly delivered to plants during germination through seed treatments such as priming and coating, which offer cost-effective alternatives to soil and foliar applications (Farooq et al., 2012, 2019). Seed priming involves presowing hydration, allowing the seed to initiate pre-germination activities without radical protrusion (Bradford, 1986), while seed coating applies nutrients to the seed surface using a sticky agent, affecting the soil–seed interface, and potentially altering nutrient availability (Farooq et al., 2012). However, the effectiveness of micronutrient delivery can be influenced by factors such as micronutrient source, sticky agent, nutrient-seed ratio, soil type, and fertility (Halmer, 2006). Iron pulsing is another promising and cost-effective technique to improve grain Fe concentration without incurring environmental costs (Dey et al., 2021). In an experiment, rice seeds were subjected to Fe pulsing ($FeSO_4$ and $FeCl_3$ treatment for 72 h), leading to improved rice productivity and plant Fe status (Dey et al., 2019).

11 | INCREASING IRON BY CONVENTIONAL BREEDING

The breeding of food crops to obtain desired traits has been practiced for centuries. However, the availability of genetic diversity for exploitation through breeding varies among different crops. For instance, crops like maize rich in pro-vitamin A have been successfully bred due to a clear understanding of the genetics behind pro-vitamin A accumulation (Gebremeskel et al., 2018). On the other hand, progress has been slower for traits such as Fe accumulation. Biofortification efforts require overcoming the tightly regulated mechanisms of Fe homeostasis to achieve Fe accumulation in targeted tissues (Connorton, Balk, et al., 2017). In transgenic strategies, known genes involved in Fe homeostasis are specifically targeted, whereas in traditional breeding methods, the focus is on inheriting the phenotype with high Fe content along with specific gene markers (Rommens, 2007).

Compared to their wild counterparts and primitive races, modern cultivars exhibit a lower degree of genetic diversity. The NAC transcription factor (NAM-B1) encoded by the ancestral wild wheat allele expedites senescence and enhances nutrient remobilization from leaves to developing grains. In contrast, contemporary wheat varieties harbor a nonfunctional NAM-B1 allele. Employing RNA interference to decrease RNA levels of various NAM homologs resulted in a senescence delay of more than 3 weeks and a reduction of over 30% in wheat grain protein, Zn, and Fe content (Uauy et al., 2006). Additionally, in wheat, the contemporary cultivars have been observed to possess lower levels of Fe (Esquinas-Alca'zar, 2005). Moreover, the downward trend of Fe accumulation has been observed as the yield increases (Fan et al., 2008). Iron accumulation in different crops was also affected by environmental factors including an increase in atmospheric CO_2 (Leakey, 2009; Myers et al., 2014). A study investigated the impact of elevated atmospheric CO_2 on nutrient levels in edible portions of different crops (Myers et al., 2014). The findings revealed that higher CO_2 levels were associated with significant declines in Fe concentrations among all C_3 grasses. For instance, wheat grains cultivated under elevated CO_2 had 5.1% less Fe compared to those grown in ambient CO_2 conditions. Similarly, rice exhibited a reduction of 5.2% in Fe concentration when exposed to elevated CO_2 compared to the control group. The C_4 plant, maize, also showed a similar pattern, with a 5.8% decrease in Fe concentration observed under elevated CO_2 conditions. These results suggest that increased atmospheric CO_2 levels can negatively impact Fe levels in these crops (Myers et al., 2014). Old varieties of rice have more Fe content compared to new varieties, but yield may be reduced (Anandan et al., 2011). Breeders are now exploring the possibility of incorporating desirable genes from wild ancestors into modern cultivars (Palmgren et al., 2015). Inserting chromosomes from the wild ancestor Aegilops has been successful in doubling the Fe content in wheat grains (Neelam et al., 2011; Tiwari et al., 2010).

Genome-Wide Association Studies (GWAS) and QTL mapping have been quite helpful in the identification of parts of chromosomes and alleles closely linked with high Fe content in crops. The assessment of various single nucleotide polymorphisms (SNPs) can be done by GWAS in an extraneous population (Mitchell-Olds, 2010). It is helpful in the study of all the existing forms of a particular gene and the identification of single genes that have a role in increasing Fe content.

GWAS and QTL mapping simultaneously help in the refinement of chromosomal regions and identification of specific genes which is otherwise a very difficult step. Meta-QTL analysis is particularly useful in collecting information from various studies and accounting for environmental factors that may affect QTL studies (Garcia-Oliveira et al., 2018; Wu & Hu, 2012). In South and Middle America, research conducted in seven experiments found two QTLs for high Fe content and eight QTLs for high Zn and Fe content in common beans (Izquierdo et al., 2018). Twelve candidate genes from different families, that is, ferric chelate reductases, bZIP transcription factor, and metal transporters (MATE, NRAMP, and ZIP) were identified in these studies (Izquierdo et al., 2018). In rice, the genes OsVITI and OsVIT2 are crucial for Fe homeostasis. OsVITI transports Fe to the vacuole (Zhang et al., 2012), whereas OsVIT2 distributes Fe to various tissues through vacuolar sequestration (Che et al., 2021). The gene OsNAS3 has been found within the QTL that regulates the natural variation of grain Fe accumulation (Talukdar et al., 2022). A recent GWAS study in wheat found 137 SNPs correlated with Fe content in grains ranging from 24 to 52 $\mathrm{mg\,kg^{-1}}$ (Alomari et al., 2019). The identity and precise functions of numerous genes containing SNPs are yet to be fully elucidated. Nevertheless, certain genes are situated within an NAC family transcription factor known as NAM-B1. These genes play a crucial role in mineral remobilization within wheat, significantly influencing the nutrient composition of grains (Uauy et al., 2006).

QTL mapping has successfully identified loci associated with high Fe content in crops such as maize, rice, and wheat. In rice, QTL with significant additive effects were found on chromosomes 1 and 5, which coded for candidate genes OsYSL1 and OsNAS3 (Anuradha et al., 2012). In wheat, QTL on chromosomes 7DS and 4A in wheat explained 14.5% and 21% variation in grain Fe content, respectively, whereas QTL on chromosome 5 in maize explained 16% of the variation in grain Fe content (Crespo-Herrera et al., 2016, 2017; Jin et al., 2013). The presence of combined Fe- and Zn-associated QTL is not surprising since mineral translocation chelators can bind to both cations (Benes et al., 1983). Breeding experiments can benefit from targeting QTL that improve both Zn and Fe accumulation, as both minerals are important for crop improvement.

12 | INCREASING IRON USING TRANSGENICS

The identification of genes involved in Fe regulation is of great importance to determine their potential for overexpression, aiming to enhance Fe content and its storage in desired tissues without negatively impacting crop yield. Genes related to Fe homeostasis have been studied individually or in combinations throughout the entire process (Bashir, Takahashi, Nakanishi, et al., 2013; Masuda, Aung, et al., 2013; Vasconcelos et al., 2017). Investigating these genes with different promoters has helped in the development of optimal transgenic techniques for enhancing Fe accumulation. Plant-based proteins play a crucial role in the bioavailability and storage of Fe in a vegan diet (Kawakami & Bhullar, 2018). Various enzymes, such as proton ATPases, ferric reductases, coumarins, and phytosiderophores, are responsible for enhancing the solubility of Fe hydroxides in the soil (Connorton & Balk, 2019; Lemanceau et al., 2009; Paffrath et al., 2024). In dicots, IRT1 acts as a mediator for the transport of Fe from the apoplast to the symplast (Jeong et al., 2017), while in grasses, proteins of the yellow stripe family are responsible for Fe transport (Curie et al., 2009). The supply of Fe to different parts of the plant is facilitated by specific transport proteins, while biosynthetic enzymes aid in the incorporation of Fe into Fe–S clusters or heme groups. Moreover, Fe can be stored in vacuoles or bound to Fe-binding proteins like ferritin in plastids (Eroglu et al., 2019). Genes associated with any of these proteins hold promising potential as targets for biofortification efforts.

In a study by Masuda et al. (2012), three transgenic approaches were employed to enhance Fe content in rice. The first approach involved controlling the expression of ferritin, an Fe-binding protein, using specific endosperm promoters. The second approach focused on overproducing the metal chelator NA to increase Fe deposition. Last, two promoters, namely a specific endosperm promoter and a sucrose transport promoter, were used to regulate the expression of the Fe (II)-NA transporter OsYSL2. Under greenhouse conditions, Fe content was enhanced up to six times, while field planting yielded a fourfold increase. The introduction of multiple genes was found to be more beneficial for Fe biofortification compared to single gene introgression. In another study, the ferritin gene from common bean was introduced in to the rice plant using transgenic approach. The gene was controlled by the glutelin promoter, resulting in a twofold increase in Fe accumulation (Lucca et al., 2001). Goto et al. (1999) utilized agrobacterium-mediated transformation to introduce the SoyferH1 gene from soybean into rice endosperm. The gene was placed under the control of the GluB-1 promoter, leading to a threefold increase in Fe accumulation. Qu et al. (2005) developed transgenic rice by incorporating the SoyferH1 gene from soybean, controlled by both the glutelin (GluB-1) and globulin (Glb-1) gene promoters. Additionally, they used a soybean Fe binding protein under the control of the Glb-1 promoter. This multi-gene approach resulted in a threefold increase in Fe accumulation. In a study by Tan et al. (2015), the MxIRT1 gene from the apple tree was introduced into rice, resulting in a threefold increase in Fe and Zn levels.

13 | INCREASING IRON UPTAKE

A broad array of genes are involved in Fe uptake (Brumbarova et al., 2015; Connorton, Balk, et al., 2017). However, only a few genes have been studied as targets for biofortification (Brumbarova et al., 2015; Connorton, Balk, et al., 2017). Plants utilize two strategies for Fe uptake: a chelate-based strategy found in grasses and a reductive strategy employed by other plant species. Several key genes involved in the uptake of Fe through this mechanism have been recognized, including iron-regulated transporter 1 (IRT1) (Eide et al., 1996), ferric-chelate oxidase 2 (FRO2) (Robinson et al., 1999), and the HC-ATPase (HA) genes (Kobayashi & Nishizawa, 2012). The overexpression of the divalent transporter IRT1, crucial for the reductive uptake of Fe, has been explored. This overexpression led to an increase in Fe accumulation in leaves, up to 1.7 times, and only 1.1 times in rice grains. These results suggest that when sink capacity is inadequate, Fe starts to accumulate in vegetative parts of plants (Lee & An, 2009). To enhance Fe deposition, the overexpression of IRT1, in combination with PvFER1, was employed in the endosperm of rice, resulting in a four-fold increase in Fe deposition (Boonyaves et al., 2017). Gómez-Galera et al. (2012) developed transgenic rice with elevated expression of the barley Fe(III)-MA transporter gene HvYS1, utilizing the CaMV35S promoter for control. The study proposed that the enhancement of HvYS1 expression could potentially improve the uptake of Fe from the rhizosphere.

Recent findings propose that the formation of a protein complex involving AHA2-FRO2-IRT1 could play a crucial role in optimizing Fe uptake at the cell membrane (Martín-Barranco et al., 2020). All three proteins were observed to co-localize on the cell surface, with AHA2 distributed evenly and FRO2 and IRT1 showing polar localization, enriched at the outer plasma membrane facing the rhizosphere (Barberon et al., 2014; Dubeaux et al., 2018; Martín-Barranco et al., 2020). The close proximity of FRO2 and IRT1 may facilitate the efficient transport of Fe by coordinating reduction and transport. This complex likely aids in Fe uptake in aerobic soil environments by preventing the re-oxidation of ferrous Fe produced by FRO2. The presence of AHA2 may create a local acidic pH environment around FRO2, mitigating the adverse effects of high pH or bicarbonates on ferric reduction. The functional significance of this protein complex is still unclear, pending the identification of factors or residues in AHA2-FRO2-IRT1 crucial for its formation. Whether AHA2, FRO2, and IRT1 must form an obligatory complex for efficient Fe transport remains uncertain. Intriguingly, increasing the expression of IRT1 or FRO2 alone has been shown to enhance Fe uptake (Barberon et al., 2011; Connolly et al., 2003). The limitation of FRO2 and IRT1 for Fe acquisition argues against the necessity of a stoichiometric complex between AHA2, FRO2, and IRT1 for efficient Fe uptake. This implies that a pool of free FRO2 or IRT1 at the plasma membrane may contribute to Fe import into root epidermal cells. While FRO2 and IRT1 were previously reported to strictly co-localize at the outer plasma membrane domain of root epidermal cells (Martín-Barranco et al., 2020), the resolution limits of confocal microscopes hinder definitive conclusions. The development of super-resolution imaging techniques with FRO2 and IRT1 is expected to aid in visualizing free and complex-loaded FRO2 and IRT1 proteins at the cell surface.

14 | FACILITATING IRON DISTRIBUTION

The Fe is primarily available in the form of chelates, mainly malate and citrate, in the xylem, and NA and its derivatives in the phloem. YSL transporters, that is, YSL2 in case of rice, facilitate the transport of NA and Fe complexes across cell membranes (Ishimaru et al., 2010). NA is involved explicitly in divalent metals transport; hence, it is of the main focus for biofortification. It can also be easily synthesized by using NA synthase with substrate S-adenosyl methionine. Overexpression of NAS genes with strong promoters such as ZmUBIQUITIN and Cauliflower Mosaic Virus 35S has been observed to increase NA levels up to 10 times in leaves and even higher in seeds, resulting in a significant enhancement of Fe content, up to two times, in rice grains. NA is further converted to deoxymugineic acid (DMA) by NA aminotransferase and DMA synthase (Bashir et al., 2006). Plant roots also secrete DMA, which aids in the uptake of Fe. In rice, the overexpression of NAS1 and NAAT together led to a 29-fold increase in DMA concentration and a 4-fold increase in Fe concentration (Banakar et al., 2017). The OsSUT1 promoter has been employed to manipulate Fe distribution in rice seeds (Ishimaru et al., 2010). Plant ferritin genes, when expressed under endosperm-specific promoters, have been used to enhance Fe content in rice and wheat grains. In rice, the expression of soybean FERH1 resulted in a three-fold increase in grain Fe content (Goto et al., 1999). In contrast, expressing PvFER1 or TaFER1-A in wheat, using the OsGLUB or Ta-GLUB-1D-1 promoter, led to only a 1.5-fold increase in Fe content (Borg et al., 2012; Singh, Gruissem, et al., 2017).

In a genetic transformation experiment, introducing ferritin and mugineic acid biosynthetic genes, including SoyferH2, HvNAS1, HvNAAT-A, HvNAAT-B, and IDS3, into rice (Masuda, Kobayashi, et al., 2013) led to a four-fold or greater increase in Fe content in polished grain in genetically modified rice lines cultivated in both normal and calcareous soils. Similarly, expressing the soybean ferritin gene in bread wheat resulted in a 60% rise in total Fe levels in wheat grains (Bhati et al., 2014). However, X-ray fluorescence imaging indicated that Fe is lost during milling, accumulating in the bran rather than the starchy endosperm of the grain (Neal et al., 2013). As an alternative strategy, the utilization of the Fe storage protein ferritin, coupled with NAS overexpression, was adopted to enhance grain micronutrient content (Neal et al., 2013). Elevating the expression of the endosperm-specific ferritin gene TaFer1-A, using the 1Dx5 promoter in wheat seeds, resulted in a 50%–80% increase in Fe levels (Harrington et al., 2023). Wheat contains the VIT family of genes, such as TaVIT1, TaVIT2, and TaVIT3, as well as VTL genes like TaVTL1, TaVTL2, TaVTL4, and TaVTL5 (Harrington et al., 2023). Overexpression of TaVIT2-D using the wheat endosperm-specific high molecular weight glutenin D1 (HMWG-D1) promoter in transgenic wheat led to a consistent more than two-fold increase (from 8 to 20 $\mu g\,g^{-1}$) in Fe content in white flour, excluding the aleurone (Ram et al., 2021; Sheraz et al., 2021). This approach achieved an increase in Fe content within the range of required fortification levels in cereal grains (Gupta et al., 2021), surpassing what could be attained by using NAS genes alone. A threefold increase in Fe content, reaching approximately 25 $\mu g\,g^{-1}$, was observed in highly pure white flour composed of endosperm. This significant increase was achieved in two genetically engineered hexaploid wheat cultivars, where TaVIT2-D expression in the endosperm was combined with OsNAS2 expression. Although the distribution of grain Fe in these transgenic lines did not improve, OsNAS2 expression resulted in redistributing grain Fe to the endosperm rather than the aleurone, demonstrating that combining the two transgenes into a single cassette provides nutritional advantages beyond what can be achieved through conventional breeding methods.

15 | ENHANCING IRON STORAGE

Iron in plants is stored in vacuoles or in the form of ferritin. Genes that control plant ferritin have been successfully overexpressed in crops such as maize, wheat, and rice using specific endosperm promoters (Connorton & Balk, 2019). For biofortification purposes, the soybean gene FERH1 has been utilized in rice and maize. This resulted in a significant enhancement of Fe accumulation, up to threefold in rice. However, the outcomes in maize were not as desired (Kanobe et al., 2013; Oliva et al., 2014). Similarly, when the FER1 gene from common bean was expressed in wheat, there was a 1.5-fold increase in Fe content (Borg et al., 2012; Neal et al., 2013; Singh, Gruissem, et al., 2017; Singh, Keller, et al., 2017). Additionally, when the TaVIT2 gene, which is responsible for Fe transport to the vacuole, was overexpressed with the GLU-1D-1 endosperm promoter, more than a twofold enhancement of Fe content was observed (Connorton, Jones, et al., 2017).

In cereals, Fe is primarily stored in vacuoles, making it a crucial focus for Fe storage compared to ferritin storage (Tanin et al., 2024). The reduced expression of VIT1 or VIT2 gene in rice has been shown to increase Fe content in embryo, resulting in a 1.3-fold enhancement of total Fe content (Bashir, Takahashi, Akhtar, et al., 2013; Zhang et al., 2012). Mutant VIT lines exhibited lower Fe content in shoots and roots compared to wild types. Plants tend to redirect Fe to seeds and embryos when the storage capacity of these parts is reduced. While individual gene transformation can effectively enhance Fe storage, the strategy for vacuolar or ferritin storage may differ depending on the species (Gupta et al., 2021). Successful multigenic approaches have been employed to enhance Fe absorption, transportation, and storage simultaneously. Global regulators involved in Fe homeostasis can be modified to alter the expression of multiple genes. For example, in brown rice, the transcription factor OsIRO2, when overexpressed, activated several genes related to Fe absorption, resulting in a threefold increase in Fe accumulation (Ogo et al., 2011). Genes HRZ1 and HRZ2, which negatively regulate the transcriptional response to Fe deficiency, can be targeted for biofortification. In mutant lines, these genes have led to increased transcription of genes involved in Fe absorption and transport, resulting in a 3.5-fold increase in Fe content in rice seeds (Kobayashi et al., 2013). In maize, overexpression of the phytase gene from *Aspergillus* under the control of the rice endosperm promoter Gt-1, along with increased ferritin protein from soybean, has been effective in increasing Fe content and its bioavailability (Drakakaki et al., 2005).

16 | IMPROVING IRON BIOAVAILABILITY

Bioavailability connotes the extent of nutrients that can be absorbed from food into the blood and help the body to function properly. In food plants, the bioavailability of Fe is typically less than 15%, making it a crucial factor to consider in the biofortification of cereal crops. Bioavailability is influenced not only by the chemical composition of the nutrient but also by factors such as age, gender, life stage, and individual nutrient status. Human studies are necessary to assess the improved Fe nutrition resulting from biofortification efforts. Caco-2 cell analysis could be utilized to evaluate bioavailability by measuring Fe absorption in these cells, which resemble the enterocyte lining of the intestinal absorptive cells but lack the mucosal layer. The total amount of ferritin formed after digestion is measured to determine the level of Fe absorption (Glahn et al., 1998). The Caco-2 cells can be employed to investigate the impact of components that inhibit nutrient absorption such as phytic acid. While animal models such as mice and chickens have been used to study Fe bioavailability, they do not provide comprehensive information about Fe uptake in the human gut. Certain biophysical methods have been employed to explore the available Fe in food and provide insights into its bioavailability (Narayanan et al., 2019). It is important to note that cooking or digestion of food can alter the chemical form of Fe, negatively impacting its bioavailability. However, there are contradictory observations regarding the impact of cooking or digestion on Fe availability, as Mamiro et al. (2017) stated that the process of cooking plays a crucial role in facilitating mineral absorption during digestion in humans by reducing the binding of minerals, making them more readily available for physiological uptake. On the other hand, some studies indicated that heating the food matrix can release ferritin Fe hydroxides as Fe^{2+} or Fe^{3+} ions, which can bind with phytic acid and reduce bioavailability (Hoppler et al., 2008; Moore et al., 2018; Perfecto et al., 2018).

In cereal foods, the molar concentration of phytic acid to Fe should be <1:1 to ensure effective absorption of Fe (Hurrell & Egli, 2010). Therefore, the concentration of phytic acid can serve as an indicator to assess bioavailability. Some whole-grain cereals and pulses have a phytic acid to Fe ratio of 10:1, resulting in negligible bioavailability when analyzed using Caco-2 cells (Rodriguez-Ramiro et al., 2017), whereas isotope analysis in young women showed absorption rates of up to 4%–6% (Petry et al., 2013). Breeders are working toward developing crops with reduced phytic acid content, although a significant reduction may lead to lower yields. Caco-2 cell studies have demonstrated improved Fe bioavailability with up to a 60% reduction in phytic acid in peas (Liu et al., 2015; Warkentin et al., 2012) and reduced phytic acid in common beans resulted in increased Fe levels in young females (Petry et al., 2013). In maize, the expression of fungal phytase enhanced available Fe levels by up to three times (Drakakaki et al., 2005). Phytic acid is typically more concentrated in the bran than in the endosperm of cereals. Therefore, targeting Fe accumulation in the endosperm can help mitigate the phytic acid problem (Connorton, Jones, et al., 2017). However, differences in Fe bioavailability are not solely related to phytic acid levels. The overlapping of QTL controlling grain Fe content with QTL for bioavailability, particularly through the combination of three large QTLs, has been shown to enhance Fe bioavailability (Lung'aho et al., 2011). Furthermore, some studies have reported improved bioavailability by adding NA to meals (Zheng et al., 2010) or increasing NA levels through the overexpression of NAS genes in wheat and rice grains, as confirmed by Caco-2 cell analysis or in mice (Beasley et al., 2019; Zheng et al., 2010). However, experiments involving human subjects have not yet been conducted.

17 | GENETIC ENGINEERING VIA OVEREXPRESSION OF GENES, RNAI, CRISPR/CAS9

Recent advancements in biotechnology have opened up possibilities for the identification and characterization of target genes, making genetic engineering for biofortification a feasible approach. Techniques, such as metabolite profiling, gene expression analysis, physical mapping, and whole-genome sequencing, have greatly aided in the identification of desirable genes and their transfer into target organisms. Through genetic modification, the transfer of genes of interest and development of desired varieties with anticipated traits have become more accessible and efficient compared to traditional breeding methods.

Transgenic experiments have been conducted in significant agricultural crops with the goal of accumulating micronutrient and protein in specific target tissues. Proper coalescence of omics data, that is, proteomics, genomics, and transcriptomics, is essential to manipulate genes using transgenics (Chaudhary, Deshmukh, Mir, et al., 2019; Chaudhary, Alisha, et al., 2019; Deshmukh et al., 2014). A comprehensive understanding of transgenic approaches aids in the development of the desired crops for cultivation. Golden rice stands as a well-known genetically engineered biofortified crop (Ye et al., 2000). Moreover, transgenic approaches have been employed to enhance Fe and Zn content in rice (Trijatmiko et al., 2016). Scientists are actively working on developing plants with high levels of Zn and Fe by improving their uptake and translocation in plant parts (Rana et al., 2019; Zhu et al., 2007). Transgenic varieties hold immense nutritional potential, but progress has been limited due to national biosafety regulations. However, the emergence of genome editing techniques, such as CRISPR/Cas, has facilitated the bypassing of regulatory processes to develop desired transgenic varieties (Mushtaq et al., 2020; Vats et al., 2019). CRISPR/Cas has already been employed in rice for genome editing to enhance biofortification (Vats et al., 2019). These studies often target known genes with well-understood molecular mechanisms.

Mutation breeding is a low-cost and convenient method for crop improvement, although it relies chance-driven process (Bansal et al., 2019; Chaudhary, Deshmukh, & Sonah, 2019; Kumawat et al., 2019). For biofortification, the probability of success is higher if a single gene controls the desired trait. In mutation breeding, traits that are negatively regulated may exhibit more significant improvements compared to positively regulated traits. Sevanthi et al. (2018) conducted a study where mutants were selected from a pool of 87,000 rice mutants with EMS-induced mutations and screened for levels of Fe and Zn in grains. Mutants with enhanced Zn and Fe levels have been identified in various studies. For example, a rice mutant with sequence variation in NA aminotransferase resulted in increased Fe content (Cheng et al., 2007). Jeng et al. (2012) also identified mutants with enhanced Zn and Fe content in milled rice from the IR-64 variety.

18 | MICROBE-ASSISTED BIOFORTIFICATION

Microbes play a crucial role in maintaining the environment's biogeochemicals (Gadd, 2010; Lugtenberg, 2015). Some microorganisms can enhance plant growth and nutrient use efficiency by promoting nutrient uptake (Pang et al., 2023; Xu et al., 2018; Yadav, Rastegari, et al., 2020; Yadav, Singh, et al., 2020). These microbes employ various mechanisms, such as solubilization, oxidation, nitrogen fixation, and phytohormone production, to increase nutrient availability for plants (Kour et al., 2020; Pang et al., 2023; Yong et al., 2014). For instance, inoculating both *Bradyrhizobium* strain and *Streptomyces griseoflavus* together resulted in enhanced nodulation, nitrogen fixation, and nutrient uptake in *Glycine max* (Htwe et al., 2018). Microbes produce siderophores to increase Fe bioavailability in soil, especially in aerobic calcareous soils (Schalk et al., 2011). The synthesis of siderophores and their solubilization mechanism in Fe hydroxides is a significant microbial activity that improves Fe acquisition (Jin et al., 2010). Inoculation with siderophore endophytes, such as *Enterococcus hirae* DS-163 and *Arthrobacter sulfonivorans* DS-68, significantly increased grain Fe concentration in high and low Fe accumulating wheat genotypes by up to 67% (Singh, Gruissem, et al., 2017; Singh, Keller, et al., 2017). Likewise, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* are capable of producing two types of siderophores, pyoverdine and pyochelin. These siderophores facilitate the uptake of Fe (Shen et al., 2013). Furthermore, various studies have identified several rhizobacteria capable of producing siderophores, including *Klebsiella*, *Bacillus*, *Bradyrhizobium*, *Streptomyces*, *Serratia*, and *Rhizobium* (Mustafa et al., 2019). Inoculating wheat with *Providencia* sp. PW5 resulted in increased grain Fe contents, while a combination of *Providencia* sp., *Ochrobactrum anthropic*, and *Brevundimonas diminuta* boosted rice grain Fe levels by 13%–16% (Rana et al., 2015; Rana, Joshi, et al., 2012; Rana, Saharan, et al., 2012). Microbes can play an important role in biofortification of deficient micronutrients like Fe and Zn, where other techniques of improving nutrient uptake encountered limitations due to soil properties, organic matter content, and micronutrient form in soil. Plants and microbes produce siderophores to chelate insoluble Fe in soil and improve Fe uptake (Sharma & Johri, 2003). Siderophores increase Fe bioavailability by chelating Fe^{3+} to Fe^{2+} under varying pH conditions. Inoculation of arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* in maize significantly enhanced Fe uptake (Liu et al., 2000). Fungi have various mechanisms for Fe uptake, such as siderophore-Fe chelates, low-affinity Fe chelates, heme, hemoglobin, and transferrin (Philpott, 2006). In a study, 51 rhizospheric bacteria were evaluated for their capacity to produce siderophores and promote Fe uptake in plants under Fe-limited conditions. In maize, inoculation with *Arthobacter globiformis* led to enhanced plant biomass and increased Fe uptake (Sharma et al., 2016). In another study, *Burkholderia phytofirmans* and *Enterobacter* sp. were found to increase grain Fe contents by 10.14% compared to a control treatment (Yaseen et al., 2018). Soil pH has a significant impact on Fe availability, with a decrease in each unit of pH reducing Fe availability up to 1000-fold (Guerinot & Yi, 1994). The acidification of the rhizosphere through the nitrification process of ammonium-containing fertilizers can increase Fe availability. Ammonia-oxidizing microbes can catalyze soil nitrification (Jetten et al., 1997). In calcareous soils, P-solubilizing microbes such as *Penicillium bilaji* and *P.* cf. *fuscum* can lower the soil pH in the presence of NH_4^+, resulting in increased Fe solubility (Illmer & Schinner, 1995).

19 | CONCLUSIONS AND PROSPECTS

Micronutrient malnutrition leads to health issues and economic problems, including mortality, impaired development, reduced productivity, and increased healthcare costs. Biofortification offers a solution to reduce Fe and other micronutrients deficiencies, promoting global welfare in terms of improved food quality. Historically, food production has prioritized quantity over quality to meet the growing population's demands. However, with growing concerns about hidden hunger, research has shifted toward genetic and agronomic biofortification for sustainable solutions. It is important to note that these biofortification methods work in tandem to achieve maximum impact. The future of biofortification lies on several fronts; in gene editing, with a focus on genes that enhance Fe uptake, translocation, and bioavailability, while reducing antinutrient accumulation in cereals. The role of certain soil microbes in improving Fe availability to plants may be crucial, but, further research is needed to identify suitable microbial species and mixtures that can enhance Fe availability through various mechanisms in the rhizospheres. Another strategy is the development of specific genetically modified microbes or manipulation of endophytes and rhizosphere microbiome through bioengineering to enhance their traits. Postharvest management plays a vital role in delivering nutrient-rich foods to consumers. However, essential nutrients like Fe and Zn are often removed during processing, limiting their availability to humans. Improved whole grain processing can address this issue by increasing the dietary and nutritional value of products. Moving forward, genetic engineering tools are the most promising strategy to improve the Fe content of cereal crops and enhance the process of biofortification. These tools have significant potential for improving the biofortified Fe content of food crops. Conversely, it is important to acknowledge that genetically modified food crops may face challenges in receving societal acceptance.

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