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Aavula Naveen
Indian Agricultural Research Institute, New Delhi, India

Patel Supriya
Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

Gampa Mallesh
Indian Agricultural Research Institute, New Delhi, India

Sunaina Yadav
Indian Agricultural Research Institute, New Delhi, India

Thalari Vasanthrao
Professor Jayashankar Telangana State Agricultural University, Hyderabad, Telangana, India

Dharavath Hathiram
Professor Jayashankar Telangana State Agricultural University, Hyderabad, Telangana, India

Hemanth S
Indian Agricultural Research Institute, New Delhi, India

Corresponding Author:
Aavula Naveen
Indian Agricultural Research Institute, New Delhi, India

Biofortification of pearl millet for mineral enhancement: current scenario and prospects for combating hidden hunger

Aavula Naveen, Patel Supriya, Gampa Mallesh, Sunaina Yadav, Thalari Vasanthrao, Dharavath Hathiram and Hemanth S

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Abstract

Pearl millet is a nutritious cereal crop that has the potential to alleviate hidden hunger, a global issue affecting over 3 billion people due to micronutrient deficiencies, particularly iron (Fe) and zinc (Zn). Biofortification, a process of enhancing the nutritional content of staple crops, is a viable solution to address hidden hunger. Pearl millet is an ideal candidate for biofortification due to its superior and balanced nutritional profile compared to other cereals. Conventional breeding relies on germplasm screening, selection, cross-breeding, recurrent selection, and hybrid breeding to develop improved varieties with higher mineral concentrations. Phenotyping techniques such as Atomic Absorption Spectrometry (AAS), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Near-infrared reflectance spectroscopy (NIRS), and Energy-dispersive X-ray fluorescence (ED-XRF) have been used to assess grain mineral content. Genetic studies have revealed significant variability for grain Fe and Zn contents in pearl millet, with additive genetic variance and high heritability, indicating the effectiveness of simple selection for these micronutrients. However, some studies have reported non-additive genetic control and the influence of genotype by environment interactions on grain mineral content. Multi-location trials have been conducted to assess the stability of Fe and Zn contents across different environments. By introducing genes through genetic engineering that enhance mineral uptake and bioavailability, researchers can boost the levels of essential nutrients like iron and zinc in the grain. Key tools such as molecular markers (AFLP, SSR, SNP) and high-throughput genotyping platforms (ddRAD, GBS) have enabled the identification of genetic variations associated with these traits. The development of biofortified pearl millet varieties through conventional breeding and advanced approaches holds great promise in combating hidden hunger and improving the nutritional status of populations in Africa, Asia, and Latin America.

Keywords: Hidden hunger, iron deficiency, zinc deficiency, biofortification, pearl millet, grain minerals, conventional breeding

Introduction

Hidden hunger-the invisible appetite

Unlike macronutrients, which are required in larger amounts for growth and development, micronutrients are needed in trace amounts but are crucial for a variety of physiological functions (Gush *et al.*, 2021) [18]. Although they are only required in small quantities, micronutrients are essential for optimal health and the prevention of deficiency-related disorders. Their roles extend beyond basic physiological processes, affecting immune function, cognitive development, and overall well-being. Vitamins and minerals, for example, contribute to cytokine production, regulate gene expression, and act as coenzymes in metabolic pathways that are vital for immune system maintenance (Ju, 2021; Shetty, 2010) [22, 54]. The term "hidden hunger" refers to the subtle but significant impact of micronutrient deficiencies that may occur even when caloric intake is adequate. These deficiencies do not cause obvious symptoms of hunger but result in various adverse health effects similar to those caused by a lack of macronutrients. For this reason, such deficiencies are described as hidden hunger (Harding *et al.*, 2018) [19].

Global prevalence and impact

More than 3 billion people globally are affected by the "hidden hunger," a situation particularly prevalent in regions such as Africa, Asia, and Latin America. This phenomenon refers to deficiencies in essential vitamins and minerals, known as micronutrients, which are critical for maintaining optimal health. Among these, deficiencies of iron (Fe) and zinc (Zn) are the most widespread, impacting over two billion individuals worldwide, with women and preschool children being the most vulnerable groups (Harding *et al.*, 2018, Kramer and Allen, 2015) [19, 28]. The prevalence of hidden hunger is particularly acute in regions like sub-Saharan Africa and South Asia. Despite some progress in economic growth and agricultural output, these areas continue to struggle with high rates of micronutrient deficiencies. For instance, in South Asia, a significant portion of the population remains reliant on low-cost, energy-dense foods that are often lacking in essential nutrients (Saha *et al*., 2021) [50]. The recent COVID-19 pandemic, combined with the escalating climate crises and ongoing conflicts, has significantly worsened malnutrition in all its forms, particularly in low- and middle-income countries (Bloem and Farris, 2022) [3].

Recommended dietary allowance

The Recommended Dietary Allowance (RDA) for iron varies based on age, gender, and physiological status to ensure adequate intake for the majority of individuals in each group. For adult men aged 19 to 50 years, the RDA is set at 8 mg per day, reflecting their relatively stable iron requirements. However, for adult women in the same age range, the RDA is significantly higher at 18 mg per day, accounting for the substantial iron losses that occur during menstruation. Pregnant women have the highest RDA at 27 mg per day, recognizing the increased demands for iron to support fetal growth and development, as well as the expansion of maternal red blood cell mass (Rana *et al*., 2020) [48]. These gender- and condition-specific recommendations are crucial for preventing iron deficiency and its associated health consequences, such as anemia, fatigue, and impaired cognitive function. It is estimated that 39.8% of anemia in children and 29.9% of anemia in women of reproductive age is caused by iron deficiency (WHO, 2021) [65].

Zinc is an essential mineral that plays a critical role in various physiological functions, including immune response, protein synthesis, DNA synthesis, and cell division (Mcclung and Scrimgeour, 2005) [38].

It is especially important for growth and development during pregnancy, infancy, and childhood. The Recommended Dietary Allowance (RDA) for adults aged 19 years and older, the RDA is 11 mg per day for men and 8 mg per day for women. During pregnancy and lactation, the RDA increases to 11 mg and 12 mg per day, respectively, to support fetal development and milk production (Rana *et al*., 2020) [48]. Over 17% of the global population are at risk of inadequate zinc intake. Zinc deficiency is also the leading cause of stunting, which affects 22% of people globally and more 33% in Africa and in Southeast Asia (Khan *et al.*, 2022) [26]. Zinc deficiency is the cause of stunting, loss of appetite, lowered immunity, increased risk of diarrheal and respiratory diseases.

Possible ways for alleviating hidden hunger

Hidden hunger not only affects a significant portion of the global population but also has far-reaching consequences on public health and economic productivity (Bouis, 2018) [4]. The prevalence of iron and zinc deficiencies highlights the need for targeted interventions, such as food fortification and biofortification programs, to address these nutritional gaps. Understanding the specific dietary requirements for different population groups, as illustrated by the varying Recommended Dietary Allowances for iron and zinc, is crucial in developing effective strategies to combat micronutrient deficiencies.

Food fortification

Enhancing staple foods with essential vitamins and minerals can significantly reduce deficiencies. Fortification occurs during food processing and often results in higher product prices. As a result, these fortified products can become unaffordable for the most impoverished individuals living in remote rural areas. Further, collaboration between governments, food industries, and health organizations is essential to develop and implement effective food fortification strategies that can reach the most vulnerable populations (J Wimalawansa, 2013) [20].

Food supplementation

Providing micronutrient supplements to vulnerable groups, especially pregnant women and young children, can effectively address deficiencies. As a short-term solution, supplementation involves distributing capsules, tablets, syrups, or mineral solutions for immediate intake to quickly alleviate acute mineral shortages. However, while effective in the short term, this approach is not sustainable for large populations and should be transitioned to food fortification as soon as possible (Thompson and Amoroso, 2010) [59].

Diet diversification

Encouraging the consumption of a variety of foods to ensure a more balanced intake of nutrients. Additionally, the integration of underutilized crops as food-based fortifiers is suggested to leverage their nutritional, ecological, and fiscal benefits (Kaur *et al*., 2022) [25]. Dietary diversification is constrained by resource availability for poor households, seasonal availability of fruits and vegetables and limited land availability for growing diverse foods.

Biofortification

Howarth Bouis, a recipient in 2016 of the World Food Prize, pioneered the concept of biofortification in the early 1990s and founded HarvestPlus in 2003. Staple crop biofortification is a practical, viable, proven, demand-led response to hidden hunger—particularly among the hundreds of millions of smallholder farming families who cannot afford nutritionally diverse diets, and are also not easily reached by food fortification or supplementation initiatives. - (Stein *et al*., 2007). Additionally, biofortification offers a practical approach to improving nutrition for malnourished rural communities, especially those with limited access to commercially available fortified foods and dietary supplements (Bouis, 2018) [4].

Pearl millet-an ideal candidate for biofortification

Localization of grain nutrients

In pearl millet grains, minerals are distributed unevenly across different grain tissues, as illustrated by Minnis-Ndimba *et al*. (2015) [40]. Using micro-proton-induced X-ray emission (micro-PIXE), they mapped the mineral distribution in the grain tissue of two pearl millet cultivars. The germ, which includes the scutellum and embryo, and the outer layers of the grain, such as the pericarp and aleurone, are the primary sites where minerals are concentrated. Within the germ, phosphorus (P) and potassium (K) are predominantly stored in the scutellum, while calcium (Ca), manganese (Mn), and zinc (Zn) are mainly found in the embryo. Iron (Fe) has a distinctive pattern, being concentrated at the dorsal end of the scutellum and showing high levels in the outer grain layers. The endosperm, which constitutes most of the grain, has relatively lower concentrations of these minerals (Ndolo and Beta, 2013) [42]. Additionally, the hilar region plays a crucial role in mineral accumulation, particularly for sulphur (S), Ca, Mn, Fe, and Zn, potentially serving as a defense mechanism against infection or damage. This diverse mineral distribution across different grain parts significantly influences the nutritional content and defense mechanisms of pearl millet. Notably, calcium is mainly located in the pericarp, while iron (Fe^{2+}) is dispersed throughout the pericarp and aleurone layer, and zinc is primarily concentrated in the germ (Minnis-Ndimba *et al*., 2015; Ndolo and Beta, 2013) [40, 49, 42].

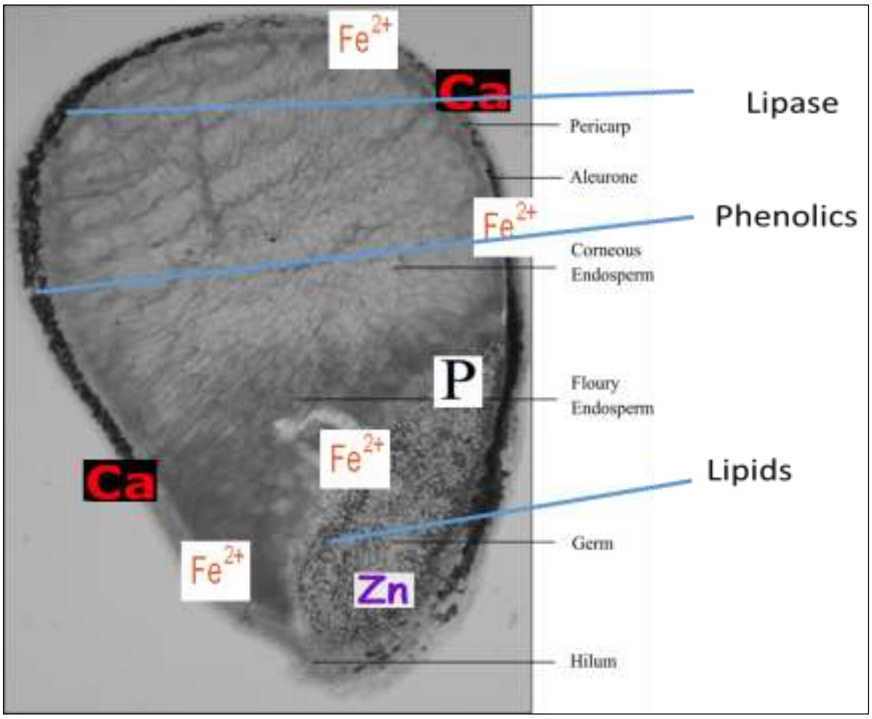

Fig 1: localization and distribution of minerals within pearl millet grain

Nutritional profile of pearl millet grain

Pearl millet is highly nutritious, providing an excellent source of energy, carbohydrates, and proteins. It is rich in crude fibers, including resistant starch, soluble and insoluble dietary fibers, and contains both soluble and insoluble fats with better fat digestibility compared to other major cereals. Additionally, pearl millet is a good source of dietary fiber, antioxidants, iron, and zinc (Satyavathi *et al*., 2021) [51]. It also offers a variety of vitamins, such as riboflavin, niacin, and thiamine, and is abundant in minerals like potassium, phosphorus, magnesium, iron, zinc, copper, and manganese (Malleshi *et al*., 2021) [37]. Pearl millet (*Pennisetum glaucum*) is recognized for its rich mineral content, making it a valuable addition to diets, particularly in regions prone to micronutrient deficiencies. According to the National Institute of Nutrition, India, pearl millet contains significant amounts of essential minerals, including iron (8.0 mg/100 g), zinc (3.1 mg/100 g), calcium (42 mg/100 g), magnesium (137 mg/100 g), and phosphorus (296 mg/100 g). These minerals contribute to the overall nutritional profile of pearl millet, which is also high in dietary fiber and protein. However, the bioavailability of these minerals can be affected by the presence of phytates and other inhibitory factors, which may limit their absorption in the body (Krishnan and Meera, 2018) [29]. Despite these challenges, pearl millet remains a promising source of essential nutrients, particularly for populations in developing countries where it serves as a staple food.

Table 1: Comparison of nutritional composition of pearl millet, rice, wheat and sorghum (per 100 grams of grain)

Nutrient	Pearl millet	Rice	Wheat	Sorghum
Carbohydrates (g)	61.8	78.2	64.7	67.7
Protein (g)	10.9	7.9	10.6	9.9
Fat (g)	5.43	0.52	1.47	1.73
Energy (Kcal)	347	356	321	334
Dietary Fiber (g)	11.5	2.8	11.2	10.2
Calcium (mg)	27.4	7.5	39.4	27.6
Phosphorus (mg)	289	96	315	274
Magnesium (mg)	124	19	125	133
Zinc (mg)	2.7	1.2	2.8	1.9
Iron (mg)	6.4	0.6	3.9	3.9
Thiamine (mg)	0.25	0.05	0.46	0.35
Riboflavin (mg)	0.20	0.05	0.15	0.14
Niacin (mg)	0.9	1.7	2.7	2.1
Folic Acid (µg)	36.1	9.32	30.1	39.4

Source: NIN, 2018

Approaches for biofortification

The mineral content of pearl millet can be enhanced through biofortification strategies, including conventional breeding techniques and genetic engineering approaches (Vinoth and Ravindhran, 2019) [63]. These efforts aim to develop pearl millet varieties with increased concentrations of essential minerals, particularly iron and zinc, to address micronutrient deficiencies in populations relying on this staple crop. Additionally, agronomic practices such as soil management and fertilization can be optimized to improve the mineral content and bioavailability in pearl millet grains.

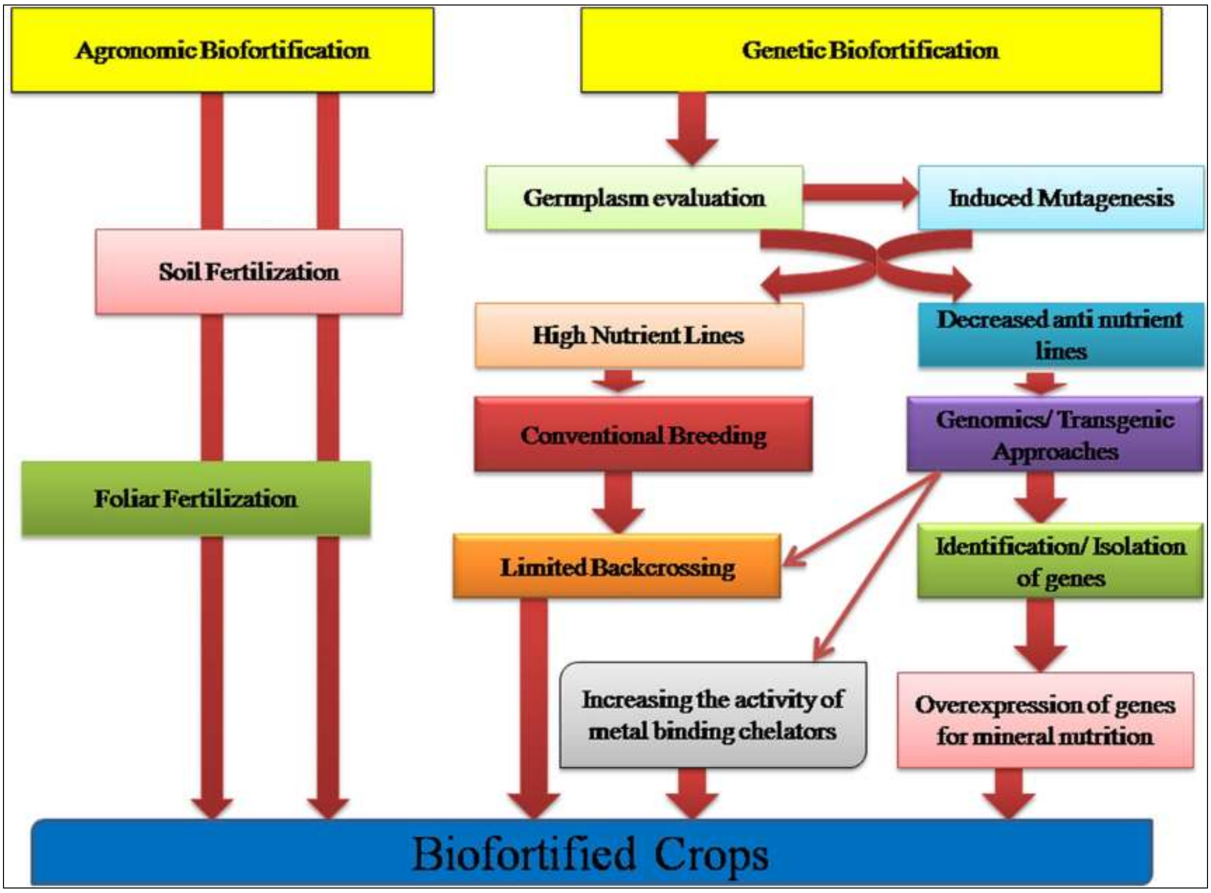

Fig 2: Various approaches for biofortification of crop plants (adopted from Sharma *et al*., 2017

Conventional breeding

Conventional breeding techniques for pearl millet biofortification have focused on germplasm screening to identify lines with high mineral content, followed by cross-breeding to develop improved varieties. This strategy can be further strengthened by leveraging diverse germplasm collections and advanced screening techniques to identify lines with superior nutrient profiles (Govindaraj *et al*., 2020) [15]. Breeding efforts can prioritize the development of pearl millet varieties with increased levels of zinc, iron, and other essential minerals while maintaining or improving agronomic performance and yield potential (Vinoth and Ravindhran, 2019) [63]. Recurrent selection methods have been utilized to incrementally enhance mineral content across multiple generations, while marker-assisted selection has enabled breeders to focus on specific genes linked to higher mineral uptake and accumulation. Furthermore, exploiting heterosis through hybrid breeding has shown promise for creating pearl millet varieties with improved nutritional profiles (Govindaraj *et al*., 2022) [11].

Phenotyping of grain minerals

Phenotyping grain minerals is crucial for evaluating micronutrient content in pearl millet, with various seed types—such as selfed, sibbed, and open-pollinated seeds—being suitable for this analysis. Among these, open-pollinated seed sampling is considered the most flexible for obtaining fast and accurate results (Rai *et al*., 2015) [45]. Several analytical methods are used to determine mineral content, including Atomic Absorption Spectrometry (AAS), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Near Infrared Reflectance Spectroscopy (NIRS), and Energy-Dispersive X-ray Fluorescence (ED-XRF). Notably, there are highly significant and positive correlations between results obtained from ICP and XRF, with a correlation coefficient of $r > 0.80^{**}$ ($p < 0.01$) (Govindaraj *et al*., 2016) [13].

Energy-dispersive X-ray fluorescence (ED-XRF) technology was introduced to agriculture in India by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in 2011 (Govindaraj *et al*., 2016) [13]. This advanced method is employed to measure essential minerals such as iron (Fe), zinc (Zn), calcium (Ca), and others in whole grain samples. At ICRISAT, a vast collection of 23,092 pearl millet accessions, including landraces, cultivars, genetic stocks, breeding lines, and wild relatives have been curated (Vinoth and Ravindhran, 2019) [63]. The use of ED-XRF has provided an opportunity for faster screening of these lines for micronutrient content, aiding in the development of biofortified varieties aimed at addressing malnutrition among populations that depend on pearl millet as a staple food (Govindaraj *et al*., 2016) [13].

Genetic variability for grain iron and zinc

Recent studies on pearl millet have revealed significant genetic variability for iron (Fe) and zinc (Zn) content, key micronutrients targeted for biofortification (Kanatti *et al*., 2014; Pujar *et al*., 2020; Mahendrakar *et al*., 2019) [24, 44, 35]. As summarized in Table 2, Fe concentrations range from 23 to 154 ppm, while Zn varies from 19 to 121 ppm across diverse genetic materials. This variability has been observed in various germplasm types, including inbred lines, commercial hybrids, open-pollinated varieties (OPVs), and mapping populations. Notably, the Iniari germplasm exhibited particularly high Fe (51-121 ppm) and Zn (46-87 ppm) levels (Rai *et al*., 2014) [46], suggesting its potential value in breeding programs. The extensive range of micronutrient concentrations found in different genetic materials, as reported by multiple studies from 2008 to 2020, underscores the rich genetic diversity available for biofortification efforts in pearl millet. This diversity provides a strong foundation for developing varieties with enhanced nutritional profiles to address micronutrient deficiencies in populations relying on this staple crop.

Table 2: Comparison of genetic variability among different genetic materials for iron and zinc

Genetic materials	No. of genotypes	Fe (ppm)	Zn (ppm)	References
Iniari Germplasm Inbreds	191	51-121	46-87	Rai *et al*., 2014 [46]
	45	34-102	34-84	Govindaraj *et al*., 2013 [14]
	28	30-82	27-56	Kanatti *et al*., 2014 [24]
	281	35-116	21-80	Pujar *et al*., 2020 [44]
Commercial hybrids	52	47-85	36-70	Velu *et al*., 2008 [61]
	120	46-56	37-44	Velu *et al*., 2008 [61]
Populations (OPVs)	68	42-80	27-50	Velu *et al*., 2008 [61]
	18	42-67	37-52	Velu *et al*., 2008 [61]
Population progenies	240	29-89	32-71	Govindaraj *et al*., 2016 [13]
	299	31-143	35-82	Govindaraj *et al*., 2019b [12]
Mapping populations	317	23-154	19-121	Mahendrakar *et al*., 2019 [35]
	106	28-124	29-120	Kumar *et al*., 2016 [33]

Genetics of grain iron and zinc contents

The genetics of grain iron and zinc contents is characterized by a combination of additive and non-additive genetic variances, which influence the effectiveness of selection for these micronutrients. Studies indicate that there is a higher magnitude of additive genetic variance with significant heritability, suggesting that simple selection methods can effectively enhance grain iron and zinc levels (Govindaraj *et al*., 2016) [13]. Furthermore, there are strong positive correlations between mid-parental values and F1 performance, indicating that the traits can be reliably predicted from parental lines. Variability among hybrids is primarily attributed to general combining ability (GCA), which is 3-4 times greater than that attributable to specific combining ability (SCA) (Thribhuvan *et al*., 2023) [60]. This suggests that while there is some non-additive genetic control, the overall genetic influence on these micronutrients tends to be additive (Nandaniya *et al*., 2016; Jeeterwal *et al*., 2017; Warrier *et al*., 2020, Thribhuvan *et al*., 2023) [41, 21, 64, 60]. Additionally, a positive and significant correlation exists between the per se performance of the parents for micronutrients and their GCA effects, reinforcing the importance of selecting high-performing parent lines. However, differences in reciprocal crosses were found to be non-significant, indicating that nuclear determinants may play a substantial role in influencing these traits (Kanatti *et al*., 2018) [23].

Genotype by environment interaction

The genetic contribution to grain iron and zinc content in cereals is notably high, often being double that of the genotype by environment (G × E) interaction, as reported by Govindaraj *et al*. (2016) [13]. Typically, G × E interactions account for only 10-30% of the variation in these micronutrients, which is significantly less compared to their impact on grain yield. The complexity of soil micronutrient status may play a role in these environmental interactions, affecting the expression of these traits. However, studies involving multi-location trials and contrasting environments have not consistently demonstrated a strong correlation between grain micronutrient content and soil micronutrient availability above critical levels (Govindaraj *et al*., 2013; Kanatti *et al*., 2014) [14, 24]. These results indicate that breeding for enhanced iron and zinc levels in grains is achievable across various environments, even when aiming for high grain yield. The dominance of genetic variance over G × E interaction underscores the potential for successful selection and improvement of grain micronutrient concentrations in cereal breeding programs (Kanatti *et al*., 2014) [24].

Correlations between micronutrient concentrations in pearl millet

The relationship between grain iron (Fe) and zinc (Zn) content in cereals, particularly pearl millet and wheat, has been the focus of extensive research. Studies consistently report a strong positive correlation between these two micronutrients (Velu *et al*., 2011; Thribhuvan *et al*., 2023) [62, 60]. In pearl millet, the correlation between Fe and Zn content has ranged from 0.43 to 0.97 in various studies (Velu *et al*., 2011; Govindaraj *et al*., 2013; Kanatti *et al*., 2014) [62, 14, 24], indicating that similar mechanisms may govern the transport and chelation of both Fe and Zn in these crops. However, Fe and Zn contents often exhibit a negative and generally non-significant correlation with grain yield (Rai *et al*., 2016; Thribhuvan *et al*., 2023) [47, 60]. Despite this, some commercial hybrids in India, such as 86M86, XL51, and Ajeet 38, have shown that it is possible to achieve high grain yield while maintaining elevated Fe content (>50 mg kg−1 Fe) (Rai *et al*., 2016) [47]. These results are encouraging, suggesting that through targeted breeding, it is feasible to develop cultivars that combine high yield with enhanced micronutrient levels.

Research on other grain minerals in pearl millet

Based on the mineral composition data from Govindaraj *et al*. (2022) [11], a notable variability in mineral content was observed in pearl millet. Potassium (K) and phosphorus (P) were the most abundant minerals, with ranges of 340-725 mg/kg and 275-495 mg/kg, respectively. Magnesium (Mg) showed significant presence at 94-189 mg/100g. Calcium (Ca) content varied widely from 4-40 mg/kg, while sodium (Na) ranged from 3-41 mg/100g. Manganese (Mn) was present in smaller quantities, ranging from 7-23 mg/100g. The overall order of mineral abundance and variability was K > P > Mg > Ca > Fe > Zn > Na > Mn, highlighting the diverse nutritional profile of the studied sample.

Research has shown that iron (Fe) and zinc (Zn) are positively and significantly correlated with calcium (Ca) and manganese (Mn), with correlation coefficients ranging from 0.26 to 0.61 for Ca and 0.24 to 0.50 for Mn ($p < 0.05$). Additionally, high estimates of heritability greater than 0.81 have been observed for these micronutrients, as well as for phosphorus (P), molybdenum (Mo), and magnesium (Mg). The lower magnitude of genotype by environment (G × E) interaction indicates a strong genetic control over most grain minerals, suggesting that breeding programs can effectively enhance these micronutrient levels through selection strategies (Govindaraj *et al*., 2022) [11].

This strong genetic influence highlights the potential for developing cultivars with improved mineral content, which is essential for addressing micronutrient deficiencies in populations reliant on staple grains.

Population improvement for grain mineral enhancement

Selecting individual plant progenies is an effective method for enhancing both iron (Fe) and zinc (Zn) content within a population, like how it is used for grain yield improvement. For both inter- and intra-population enhancement, research has shown that one cycle of selective random mating can increase grain Fe and Zn content, as well as 1000-grain weight, in C1 compared to C0 bulks (Govindaraj, 2011) [10]. An example of this is the population ICTP8203-10-2, which was developed by recombining 11S3 progenies (an intra-population improvement method) and showed an Fe content of 71 mg kg−1, which is a 9% increase over the original population, along with a grain yield of 2.2 t ha−1, which is an 11% improvement. After national testing, this population was released as 'Dhanashakti,' making it the first publicly available biofortified crop variety for Fe in India. Additionally, reciprocal recurrent selection can be utilized to exploit both additive and non-additive genetic variance components (Thribhuvan *et al*., 2023) [60].

Hybrid breeding for biofortification of iron and zinc

While the potential to exploit better parental heterosis for increasing grain iron (Fe) and zinc (Zn) content in pearl millet is currently limited, there are still opportunities for enhancement through mi parental heterosis (Nandaniya *et al*., 2016; Jeeterwal *et al*., 2017; Warrier *et al*., 2020; Thribhuvan *et al*., 2023) [41, 21, 64, 60]. To progressively enhance mid-parental heterosis in hybrids, it is crucial to incorporate genes for these micronutrients into both parental lines. Unlike grain yield, the performance of lines for Fe and Zn content shows a significant positive correlation with general combining ability (GCA), suggesting that selecting parents with high Fe and Zn content will also identify good combiners for these traits (Kanatti *et al*., 2014; Thribhuvan *et al*., 2023) [24, 60]. The variability in micronutrient content among inbred lines depends on factors such as the base population (whether F2s, open-pollinated varieties, or composites) and the degree and direction of inbreeding effects. Research has shown that inbreeding typically has no significant impact or causes a slight increase in Fe and Zn content. Unlike the low heritability and inbreeding depression seen with grain yield, the high heritability of micronutrient content allows for more straightforward improvements through breeding. However, incorporating micronutrient genes into both parents might reduce genetic diversity for other important traits, potentially decreasing heterosis for yield traits, which are mainly controlled by non-additive gene effects (Thribhuvan *et al*., 2023) [60]. Therefore, using genomic approaches to selectively introduce Fe and Zn content genes into parental lines while preserving genetic diversity for other traits could be crucial for future biofortification breeding. Additionally, new sources of Fe and Zn from germplasm collections are being investigated at ICRISAT to broaden the genetic base. ICRISAT, in collaboration with National Agricultural Research Systems (NARS), has developed several biofortified seed and restorer lines with superior agronomic performance across various cytoplasmic backgrounds. For example, the hybrid ICMH 1201, which is high in Fe (75 ppm), is marketed as Shakti 1201 in Maharashtra and Rajasthan. Other biofortified hybrids, including AHB 1200 Fe (ICMH 1202), HHB 299 (ICMH 1203), HHB 311, RHB 233, RHB 234, and Phule Maha Shakti (ICMH 1301), have been released nationally in India (Lakshmi *et al*., 2022).

Genetic engineering

Conventional breeding techniques for biofortification of pearl millet can be complemented by genetic engineering approaches, which offer the potential for more targeted and efficient mineral enhancement. These genetic engineering strategies may involve the introduction of genes that increase mineral uptake, improve translocation to edible tissues, or enhance mineral bioavailability (Grusak, 2002) [17]. Genetic engineering techniques can be used to target specific pathways involved in mineral accumulation, such as overexpressing genes for metal transporters or enzymes involved in phytate degradation (King, 2002) [27]. These approaches could potentially lead to faster development of biofortified pearl millet varieties with enhanced nutritional value compared to traditional breeding methods alone. Additionally, genetic engineering may allow for the simultaneous improvement of multiple nutritional traits in pearl millet, addressing various micronutrient deficiencies in a single crop variety. However, the implementation of genetic engineering approaches in pearl millet biofortification faces challenges such as regulatory hurdles, public acceptance, and the need for extensive safety testing (Grusak, 2002) [17].

Molecular breeding for nutritional enhancement

Molecular markers and mapping

DNA markers have been essential in identifying genetic polymorphism and developing linkage maps in pearl millet. AFLP markers, for example, have been effectively used to evaluate genetic diversity within pearl millet germplasm (Devos *et al*., 1995) [7]. SSR markers have also proven to be highly valuable, especially EST-SSRs, which are often preferred for linkage and association mapping due to their high transferability across related plant species (Srivastava *et al*., 2020) [55]. These markers have played a key role in anchoring comprehensive molecular linkage maps that are densely populated with DArT markers (Supriya *et al*., 2011) [58]. Moreover, thousands of functional SSRs have been developed to support pearl millet genetics and breeding, along with other marker systems such as SSCP-SNP, DArT, CISP, and SNP markers (Supriya *et al*., 2011; Kumar *et al*., 2016) [58, 33]. The development and availability of these various marker resources have greatly enhanced genetic research and breeding efforts in pearl millet.

Linkage maps play a crucial role in pearl millet research and breeding. Initial mapping efforts utilized restriction fragment length polymorphism (RFLP) markers to develop a map with over 180 loci spanning approximately 350 cM, which was later extended to 600 cM through the incorporation of amplified fragment length polymorphism (AFLP) markers (Govindaraj *et al*., 2019a) [16]. Recent advancements have introduced simple sequence repeat (SSR) markers, which are compatible with PCR and facilitate multiplexed genotype analysis (Govindaraj *et al*., 2019a) [16]. These refined linkage maps have significantly contributed to quantitative trait locus (QTL) mapping, enabling the identification of genes linked to key traits. To enhance genetic studies in pearl millet, various forward genetic resources have been developed, such as bi-parental mapping populations created from a wide range of germplasm and association mapping panels like the Pearl Millet Inbred Germplasm Association Panel (PMIGAP). Additionally, reverse genetic resources, including TILLING (Targeting Induced Local Lesions in Genomes) populations, are being established at ICRISAT within the genetic background of the global reference germplasm Tift 23D2B1-P1-P5, aimed at functional genomics research for traits such as iron (Fe) and zinc (Zn) content (Govindaraj *et al.*, 2022) [11]. The combination of these genetic resources with an expanding array of molecular markers offers a robust foundation for advancing pearl millet genetics and breeding efforts focused on mineral enrichment.

Table 3: QTLs and marker trait associations for grain Fe (GFeC) and Zn (GZnC) content

Plant Material/Population	Objective	Markers Used	Number of Identified QTLs/Genes/MTAs	Associated Markers/Candidate Genes	References
210 F6 recombinant inbred lines (RILs) from the cross between PPMI 683 and PPMI 627	Identify QTLs linked to grain iron and zinc content	151 SSR markers	14 QTLs for grain iron content (GFeC) and 8 for grain zinc content (GZnC); 2 QTLs co-localized on LG2 and LG3	5 candidate genes: Ferritin, Al3+ Transporter, K+ Transporters, Zn2+ Transporters, Mg2+ Transporters	Singhal *et al.*, 2021 [54]
Contrast parents from the RIL population: AIMP 92,901 and ICMS 8511	Validate and identify candidate genes affecting GFeC and GZnC	N/A	5 candidate genes: PglFER1, PglZIP2, PglZIP4, PglNramp5, and PglZIP9 involved in GFeC and GZnC	PglFER1 (Ferritin-like gene for Fe), PglZIP and PglNRAMP (for Fe and Zn)	Mahendrakar *et al.*, 2020 [36]
317 F6 RILs derived from the cross ICMS 8511-S1-17-2-1-1-B-P03 × AIMP 92901-S1-183-2-2-B-08	Map QTLs associated with GFeC and GZnC in a pearl millet population	196 markers (177 DArTs and 19 SSRs)	11 QTLs for GFeC and 8 for GZnC; 3 QTLs co-localized for both traits on LG1 and LG7	Fe-Zn: pgpb10531- pgpb9130; Fe: pgpb8427- pgpb13221, pgpb11938- pgpb8987; Zn: Xipes198- pgpb8427, pgpb12329- pgpb9721	Kumar *et al.*, 2018 [32]
Panel of 130 diverse lines (including B-, R-, and advanced breeding lines)	Conduct genome-wide association mapping for Fe and Zn content	267 markers (250 SSRs and 17 genic markers)	Identified SSRs associated with Fe and Zn content on LG3, LG5, and LG7	Xipes 0810 (aspartic proteinase gene), Xpsmp 2261 (intergenic region), Xipes 0096	Anuradha *et al.*, 2017 [1]

High throughput genotyping platforms

High-throughput genotyping platforms have revolutionized genetic research and breeding by enabling rapid and cost-effective identification and analysis of genomic variations (Srivastava *et al.*, 2022) [56]. These platforms employ various sequencing-based approaches to assay large numbers of genetic markers across many samples simultaneously. Some of the most widely used techniques include double digest restriction-associated DNA sequencing (ddRAD), genotyping-by-sequencing (GBS), tunable GBS (tGBS), DArT-seq, and RNase H2 enzyme-dependent amplicon sequencing (rhAmpSeq) (Srivastava *et al.*, 2022) [56]. These methods leverage the power of next-generation sequencing to generate high-density marker data, facilitating applications such as high-resolution linkage mapping, genome-wide association studies for trait-marker associations, identification of candidate genes underlying important agronomic traits, and genomic selection for accelerated genetic gain in breeding programs (Çilingir *et al.*, 2021; Ott *et al.*, 2017) [5, 43]. The circa 1,000 genomes re-sequencing project has provided valuable reference genomes for many crop species, enabling more accurate identification and genotyping of genetic variants using these high-throughput platforms (Kumagai *et al.*, 2018) [30]. The availability of these flexible, high-throughput genotyping tools has significantly advanced our understanding of crop genomes and accelerated the pace of crop improvement efforts worldwide.

Recent developments

Recent developments in pearl millet research have focused on various genomic approaches to enhance the understanding and improvement of iron (Fe) and zinc (Zn) contents. Genome-wide association studies (GWAS) have emerged as a powerful tool for identifying genetic markers associated with these micronutrients, leveraging the availability of extensive genomic resources (Mahendrakar *et al.*, 2019; Srivastava *et al.*, 2022) [35, 56]. Additionally, while transcriptomics and metabolomics have seen very limited applications in this field, they hold potential for future research (Mahendrakar *et al.*, 2019) [35]. Currently, genomic selection and genetic engineering have not yet been explored in depth for pearl millet. Noteworthy advancements include stage-specific comparative transcriptomic analyses that reveal gene networks regulating Fe and Zn content in pearl millet (Satyavathi *et al.*, 2022) [52] and the identification of iron and zinc-responsive genes through a genome-wide RNA-sequencing approach (Goud *et al.*, 2022) [9]. These studies highlight the importance of integrating genomic technologies to improve the nutritional quality of pearl millet, addressing malnutrition in vulnerable populations.

End use quality

End-use quality is crucial for the acceptability of a cultivar by both farmers and consumers, as it directly impacts the nutritional and functional properties of the grain. The presence of certain metabolites, such as phytic acid, polyphenols, and fibers, can affect the bioavailability of essential minerals like iron and zinc (Kumar *et al.*, 2022) [31]. Various processing methods can enhance or reduce the concentration and bioavailability of these micronutrients (Meena *et al.*, 2024) [39]. Therefore, it is essential to consider end-use quality traits, including protein content, grain hardness, and baking properties. Research has shown that processing treatments such as germination, autoclaving, and debranning can effectively reduce levels of phytate, which is known to inhibit mineral absorption (S *et al.*, 2024) [49]. However, some processing methods may lead to a decrease in iron levels; for instance, soaking grains can result in a 25% loss of iron. Understanding these factors is vital for developing pearl millet cultivars that not only meet agronomic needs but also provide optimal nutritional benefits to consumers.

Mainstreaming biofortification

Mainstreaming biofortification is essential for improving the nutritional quality of pearl millet, which can provide up to 80% of daily iron and 100% of daily zinc requirements. In a groundbreaking initiative, In India, the Indian Council of Agricultural Research (ICAR) endorsed minimum standards for iron (42 ppm) and zinc (32 ppm) in pearl millet cultivar release policies starting in 2018 (Govindaraj *et al*., 2019b) [12]. This endorsement is a significant step towards mainstreaming these vital micronutrient traits in the breeding and cultivation of pearl millet. By establishing these standards, ICAR aims to ensure that new varieties not only meet agronomic needs but also contribute to addressing micronutrient deficiencies in populations that rely on pearl millet as a staple food. This approach highlights the importance of integrating nutritional quality into crop improvement programs, ultimately enhancing food security and health outcomes for communities dependent on this resilient cereal crop.

Conclusion

Biofortification of pearl millet is a highly effective and sustainable solution to address malnutrition by enhancing its mineral content. Advances in genetic technologies and a deeper understanding of nutrient pathways support this approach. Collaborative efforts from public and private sectors are essential to promote and expand the market for biofortified pearl millet, ensuring its benefits reach a global audience and contribute to improved nutritional outcomes.

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