



Biofortification - A promising approach to enhance iron and zinc content in cowpea

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More than half of the world's population is known to suffer from micronutrient malnutrition, which is one of the biggest threats to humanity. Biofortification, a method of breeding nutrients into food crops, is one strategy to address the problem of micronutrient malnutrition. It offers a comparatively affordable, sustainable, and long-term way of providing more micronutrients to rural populations in developing countries. Cowpea is an important pulse crop for global food security and population health due to its high nutritional and nutraceutical values. At present, agronomic, conventional plant breeding, genetic modification and microbial interventions are common methods for biofortification. Agronomic biofortification is the application of fertilizers to increase the micronutrients in edible parts. Due to their distinct structural traits and great efficacy, the use of nanomaterials as fertilizers has become a viable alternative to conventional fertilizers. Breeding procedures involve identifying genetic variation, testing their stability and determining the feasibility of breeding to increase mineral content in edible tissues without altering yields or other quality attributes. When genetic variations are not present then genetic transformation *i.e.*, Biofortification with the help of plant growth-promoting rhizobacteria is microbial biofortification. Among them, agronomic biofortification is simple, easy, inexpensive and quick but in the long term, conventional plant breeding is more stable and cost-effective. Pant lobia-1, 2,3,4,5 are some popular iron and zinc biofortified cowpea varieties developed through conventional breeding. Although some research work has been done in cowpea regarding iron and zinc biofortification, supporting extensive prospective studies on evaluating the results of nutritional fortification is a major challenge.

Keywords: Challenge, Fortification, Malnutrition, Micronutrients, Nutritional Security, Pant lobia-1-5

Introduction

Hidden hunger, or a lack of certain micronutrients, is one of the biggest global problems affecting humanity since it hinders the growth and development of both crops and people. Micronutrient deficits in the soil reduce crop yield and food quality, which together have a detrimental effect on human health and nutrition. Thus, the objective of modern global agricultural practices is to provide enough nutrient-dense food to ensure human health and well-being¹. The primary goal of the green revolution was to increase crop productivity, and little effort was made to improve the quality of these food products. This resulted in the replacement of traditional food crops that provided higher quantities of key micronutrients with less nutritious but higher-yielding crops. Since

the nutritional profile of cereals and tubers is not optimal for human or animal nutrition, a diet mainly of food grains led to a quick increase in micronutrient deficit, hence increasing micronutrient malnutrition among consumers. More than two-thirds of the world's population is assessed to be deficient in one or more mineral nutrients with more than 50% population is deficient in iron (Fe) and more than 30% deficient in zinc (Zn)². In some countries, the success of legume production has resulted in the displacement of cereals with pulse crops, which are nutritionally superior to cereals and can integrate nitrogen into the soil, promoting more sustainable agriculture³.

Pulses, like grains, have a long agricultural history and have been a major component of human diets since about 10,000 BC⁴. Pulses are an important part of our daily diet since they are high in carbohydrates, protein, fiber, vitamins, and minerals. Cowpea (*Vigna unguiculata* L.) is an important pulse crop for global food security and population health due to its high nutritional and nutraceutical values⁵. It is a spring summer and rainy season, an annual herbaceous legume crop from the genus *Vigna* and family

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Abbreviations: AHL, N-acyl homoserine lactone; DAPG, 2,4-diacetyl phloroglucinol; DMDS, dimethyl disulfide; HMB, 3-hydroxy-5-methoxy benzene methanol; IAA, indole-3-acetic acid; ISR=induced systemic resistance; N, nitrogen; Nod=nodulation; PAA, phenylacetic acid; SA, salicylic acid

Fabaceae⁶. It is also known as Black-eye bean, Southern pea, *Lobia* and vegetable meat. Globally cowpea is grown over an area of 14.50 million hectares with a production of 6.20 million tonnes. In India, it is cultivated mainly in Uttar Pradesh, Orissa, Gujrat, Tamil Nadu, Andhra Pradesh and some pockets of Punjab and Haryana⁷. The average yield of cowpea is 10-20 tons/ha of the green pod and 12-15 q/ha of grains.

The term 'biofortification' refers to the phenomenon of nutrient enrichment in the edible parts of crops through conventional breeding approaches, agronomical approaches, microbial interventions and genetic engineering. It provides a long-term and sustainable alternative to offering micronutrient-rich crops to people⁸. Till now, our agricultural system has not been structured to promote human health; rather, it has been focused solely on increasing grain yield and crop productivity. Dietary supplements, food fortification, diet diversification, and supplementation have all been used in the past to combat micronutrient deficiency. Biofortification provides the opportunity to improve crop nutritional value inside the production system with minimal or no cost to consumers⁹.

Success of Biofortification Depends on

- First, a successful breeding program should include high nutrient density as well as high yields and profitability.
- Second, the effect of fortified foods must be confirmed, there should be an enhancement in the micronutrient status of human when consuming the biofortified varieties as normally eaten. Thus, these nutrients must be accessible and preserved in cooking and processing.
- Third, farmers must plant these biofortified foods and reach a considerable percentage of persons suffering from micronutrient deficiency.

Nutritional Qualities of Cowpea

Cowpea crop is primarily grown for pods and seeds. Cowpea seeds are good source of protein (23-32%), carbohydrates (50-60%), dietary fiber (16-20.9 %), fat (1%) as well as vitamins- B₁, B₃, C and are rich in potassium (K), phosphorus (P), sodium (Na), calcium (Ca), molybdenum (Mo) and iron (Fe)¹⁰. The protein content is 2 to 4 times more than cereal and root crops, and high in lysine, leucine, tryptophan, and arginine but low in methionine and cysteine amino acids as compared to cereals. Hence, cowpea should be supplemented with cereals, vegetables, meat, and dairy

products to achieve a balanced diet¹¹. Also, Cowpea grains are rich in phenolic compounds (0.54-0.84 %), tannins, and flavonoids and show considerable antioxidant and radical scavenging activity.

Role of micronutrients in human body

Micronutrients are essential for the metabolism and sustenance of various physiological functions. Micronutrients like Fe, and Zn have crucial roles in nervous system function, wound healing, and guarding of cells against stress damage. Iron is essential to produce haemoglobin and the delivery of oxygen. It is also an important element of several enzyme systems involved in oxidative metabolism, such as cytochromes. Iron deficiency is associated with anaemia and pregnancy complications such as death and poor birth weight¹². The significance of Zn as a vital nutrient for the maintenance of good human health is widely acknowledged. Since zinc plays key structural and functional roles in various enzyme systems involved in gene expression, immune, activities, cell growth & division; an adequate zinc diet is critical for human health. Additionally, Cell-mediated immune functions in humans are mediated by zinc. A deficiency of zinc impairs children's physical growth & development and increases the risk and severity of several infections. The recent pandemic of COVID-19 has also brought zinc into the limelight as it plays a role in antiviral immunity and is suggested to be used in prophylaxis and treatment strategies¹³. This might be a possible reason for the growing interest of the government and public towards zinc biofortified products to tackle nutritional deficiency and associated diseases and syndromes. There is a recommended daily allowance for each micronutrient given in (Tables 1 & 2)^{14,15}.

Bioavailability of micronutrients

Iron and zinc bioavailability ranges from 5-15% and 18-34% of total intake, respectively which leads to a major obstacle for the biofortification strategy.

Table 1 — Recommended daily intake of zinc

Infants		Pregnancy	
0-6 months	2 (mg/day)	<18years	13 (mg/day)
7-12 months	3 (mg/day)	19-50years	11 (mg/day)
Children		Lactation	
1-3years	3 (mg/day)	<18years	14 (mg/day)
4-8years	5 (mg/day)	18-50years	12 (mg/day)
Males		Females	
9-13years	8 (mg/day)	9-13years	8 (mg/day)
14-70<years	11 (mg/day)	14-18years	9 (mg/day)
		19-70<years	8 (mg/day)

Table 2 — Iron daily reference intakes requirements

Infants		Pregnancy	
0-6 months	0.27 (mg/day)	14-18years	23 (mg/day)
7-12 months	11 (mg/day)	19-50years	22 (mg/day)
Children		Lactation	
1-3years	07 (mg/day)	14-18years	10 (mg/day)
4-8years	10 (mg/day)	19-50years	09 (mg/day)
Males		Females	
9-13years	08 (mg/day)	9-13years	08 (mg/day)
14-18years	11 (mg/day)	14-18years	15 (mg/day)
19-70<years	08 (mg/day)	19-50years	18 (mg/day)
		50-70<years	08 (mg/day)

Thus, a significant amount of iron and zinc is required to compensate for their low bioavailability¹⁶. Various factors found in pulses decrease micronutrient bioavailability. The presence of phytate in legumes is responsible for the reduced iron and zinc bioavailability. Biehl *et al.*¹⁷ observed that phytic acid forms complex of low solubility with Ca, Mg, Cu, Fe and thus their bioavailability. Thavarajah *et al.*¹⁸ investigated the effect of temperature on phytic acids in legume seeds and found that concentrations of phytic acid and zinc are higher in areas with higher temperature regimes (8.8 mg/g and 69 mg/kg, respectively) than in areas with lower temperature regimes (6.7 mg/g and 61 mg/kg, respectively). The same trend was found with Fe (116 vs. 113 mg/kg). As a result, when seeds are exposed to cold temperatures, their phytic acid concentration reduces. The findings discussed above are critical for creating new biofortification strategies for decreasing phytic acid in staple crops. Ascorbic acid improves iron (Fe) absorption in plant-based food by generating Fe(III) complexes and converting Fe (III) to more soluble and accessible Fe(II). Thus, enhanced ascorbate levels in plant foods would help to lessen the adverse effect of phytate and polyphenols on Fe bioavailability while simultaneously making these foods an important source of major nutrients and vitamin C¹⁹. Similarly, saponin, lathyragens, protease inhibitors, and -amylase inhibitors are present in legumes, reducing micronutrient bioavailability. Saponin, when consumed in moderation, provides a variety of health benefits, however excessive use might have a negative impact on health in the form diarrhoea, vomiting and abdominal pain²⁰. Traditional techniques of domestic food preparation and processing alter the saponin content originally present in pulses. Soaking, sprouting, and regular cooking are some of these approaches mention in (Table 3)^{21,22}.

Table 3 — Factors present in legumes that inhibit micronutrient bioavailability

1. Phytic acid	Fe, Zn, Ca	All legumes, cereals
2. Fiber	Fe, Zn	All legumes, cereals
3. Haemagglutinins	Fe, Zn	Most legumes, wheat
4. Phenolics	Fe, Zn	All legumes
5. Heavy metals	Zn	Contaminated legumes and leafy vegetables

Biofortification

Although cowpea composition has a number of favourable impacts on humans it still has potential for further improvement in terms of major and minor nutrients. The creation of micronutrient-enriched staple food crops could be an effective and long-term method of increasing micronutrient intake to benefit overall human health. Biofortification is the process of improving the nutritional density of a crop. It is a practical, forthcoming, and cost-effective strategy that combines several strategies such as classical breeding, population mapping, and genetic selection to obtain improved crop lines. As a result, biofortification with highly bioavailable Fe, Zn, Se, and I is urgently needed to combat chronic diseases associated with micronutrient deficiency all over the world. The genetic potential for biofortification of bioavailable Fe, Zn, and provitamin A in the edible portions of several staple food crops, including rice (*Oryza sativa* L.), wheat (*Triticum sp.*), maize (*Zea mays* L.), common bean (*Phaseolus vulgaris* L.), sweet potato (*Ipomoea batatas* L.), and cassava (*Manihot esculenta* C.), has recently been reported. Our agricultural system has not so far been designed to enhance human health; instead, it has focused solely on boosting grain yield and crop productivity. This strategy has resulted in a rise in micronutrient deficit in beans, resulting in micronutrient malnutrition among consumers. Agriculture is currently transitioning from growing more food crops to growing nutrient-rich food crops in sufficient quantities to increase the nutritional quality of various diets²³. Because the edible portions are higher in bioavailable micronutrients, minerals, and vitamins, biofortified pulses have a significant potential to battle hidden hunger. Thus, biofortification will emerge as a less expensive agricultural-based solution for meeting nutritional needs shown in (Fig. 1). When biofortified staple crops are consumed on a regular basis, they produce measurable gains in human health and nutrition. This will help combat "hidden hunger" or "micronutrient malnutrition," especially in impoverished and emerging nations⁸. Agronomic strategies, breeding,

genetic manipulation, and microbial techniques can all be used to boost pulse crops with nutrients²⁴.

Breeding Approaches

Plant breeding programmes, such as traditional, mutation, and molecular breeding approaches, use natural genetic diversity to improve the amount and bioavailability of minerals in staple crops. Breeding procedures involve identifying genetic variation influencing heritable mineral properties, testing their stability under various conditions, and determining the feasibility of breeding to increase mineral content in edible tissues without altering yields or other quality attributes^{25,26}. Traditional breeding relies on successful selection based on additive genetic effects, the phenomena of heterosis in F1 progeny, and transgressive segregation in subsequent generations once a suitable genetic variation is available. In wheat biofortification for micronutrients, distant ancestry

and intermediate values are bred to produce superior transgressive segregants and introgression of genes from wild relatives with higher micronutrient content is standard practice. Molecular breeding, also known as marker-assisted breeding (MAS), makes use of molecular markers that are closely associated with the trait of interest. QTL mapping is used to do genetic analysis of markers linked with the target QTL (quantitative trait loci). Various mapping populations, either mortal (segregating) or immortal (non-segregating), are utilized for QTL mapping. Mortal lines include F2, F2:3, and backcrosses (BC), whereas immortal lines include double haploid (DH) and recombinant inbred lines (RIL) obtained after 6-8 cycles of single seed descent technique (SSD). QTLs, on the other hand, are not stable across environments and have additive and epistatic properties. The meta-QTL method has been presented to discover a few stable and reproducible markers that will be present in a variety of environments. Furthermore, the most recent advancement in genome-wide association studies (GWAS), consisting of variance found in naturally diverse lines (DL) or elite lines (EL), aids in the creation of dense linkage maps shown in (Fig. 2).

Pioneer research on the biofortification of cowpea was initiated by HarvestPlus organisation. Phase -I (2003-2008) of this program was started at the International Institute of Tropical Agriculture (IITA), Nigeria where screening and evaluation of more than 2,000 cowpea lines in replication was done. Screening activities identified iron content ranging from 27-97 ppm and zinc content from 23-62 ppm²⁷.

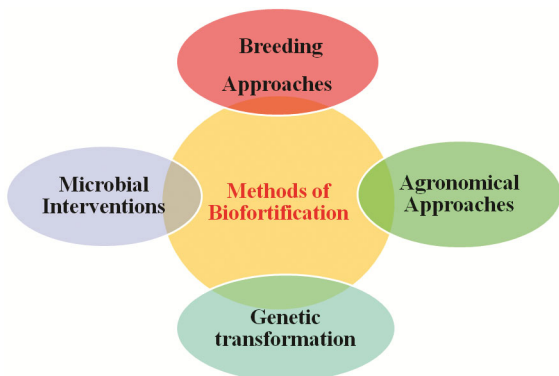


Fig. 1 — Different Methods of biofortification

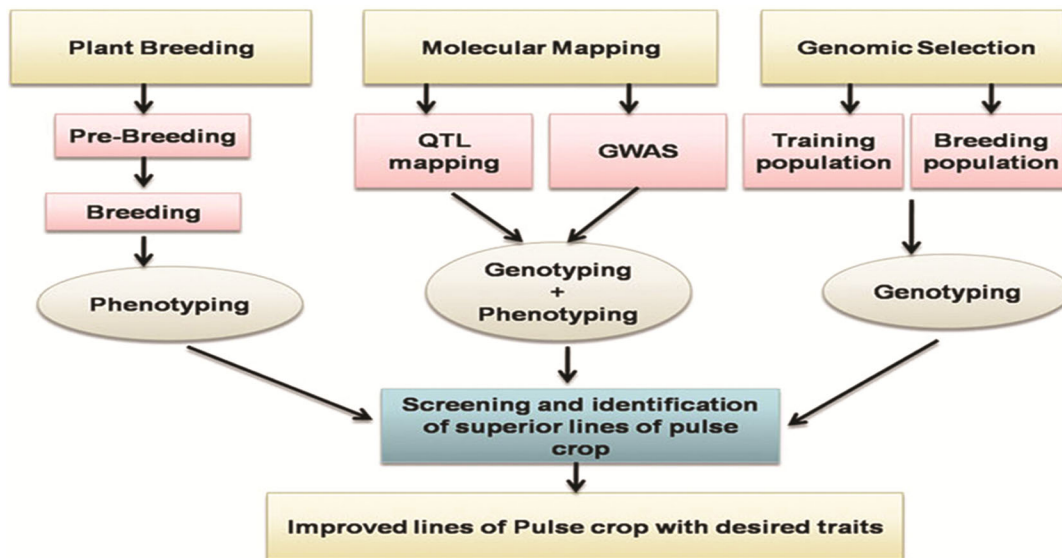


Fig. 2 — The schematic representation of breeding-based strategies of biofortification

Genetic variation for minerals proposes that target increments are possible. In phase II of HarvestPlus, cowpea research was moved to Pantnagar, India's G.B. Pant University of Agriculture and Technology. By using traditional plant breeding, two early-maturing high iron and zinc-fortified varieties—Pant Lobia-1 and Pant Lobia-2 were developed and made available for commercial cultivation in 2008 and 2010, respectively. Pant Lobia-3, Pant Lobia-4 and Pant Lobia -5 were released later in 2013, 2014 and 2015 respectively emphasizing micronutrient biofortification. Brazil also released three varieties of high-iron cowpeas, developed by Embrapa, in 2008-09 listed below in (Table 4)²⁸.

Microbiological interventions

Plant growth-promoting rhizobacteria like *Rhizobium*, *Bacillus*, *Pseudomonas* etc. have the potential to increase the phytoavailability of micronutrients²⁹. The biofortification of crops through the application of PGPR is termed a microbiological approach. PGPRs include beneficial microorganisms that colonize plant roots and promote plant growth through a variety of mechanisms. It is regarded as a supplemental measure that helps to boost micronutrient concentrations in staple crops along with improving yield and soil fertility. Crop yield can be improved up to 10%–40% with the inclusion of PGPR mainly through increases in nutrient uptake³⁰. The use of PGPR in agriculture is continuously expanding because it provides an appealing option to reduce the use of chemical fertilizers, insecticides, and other related agrochemicals³¹. Few studies have demonstrated the potential of legume biofortification for Fe uptake. Inoculation of chickpeas with the siderophore-producer PGPR raised Fe concentrations in grains and shoots by 81% and 75%, respectively³². In chickpeas, inoculation with 19 *Acinetobacter* species boosted overall seed nutritional content, with an increase in Fe composition of 10%–38%³³. Patel *et al.*^{34,35}, demonstrated that inoculating mung beans with a *Pantoea dispersa* MPJ9 isolate increased the Fe content by 3-4-fold. Iron biofortification with PGPR is a possibility. More research is needed to

determine the viability of employing such bacteria on a wide scale and in field situations. Fe biofortification efforts should also incorporate techniques to prevent Fe content loss during postharvest and processing schematic representation in (Fig. 3).

Genetic Transformation

Genetic biofortification is feasible when a species has usable genetic variation, however, transgenic methods are well-suited when there is no genetic variation⁸. Genetic engineering is the advanced biotechnology technique to introduce genes directly into breeding varieties. The genes can come from any source (including animals and microbes). These are designed to improve the mobilizing/ use efficiency of minerals in the soil, reduce the level of anti-nutritional compounds, and increase the level of nutritional enhancer compounds³⁶. However, because it is a GMO crop, obtaining approval for the commercial release of the variety is a huge challenge. Biofortification, on the other hand, is an economically sustainable strategy for feeding Zn and Fe-enriched meals to the undernourished population. A variety of agricultural research projects are under underway to achieve nutritional security. Global organisations such as Harvest Plus and CGIAR are heavily involved in this field³⁷.

Agronomical Biofortification

Although genetic biofortification may be more cost-effective in the long run, agronomic biofortification is a quick and easy way to boost

Table 4 — Iron and zinc content of biofortified cowpea variety

Variety Name	Release Year	Iron Content (ppm)	Zinc Content (ppm)
Pant Lobia-1	2008	82	40
Pant Lobia-2	2010	100	37
Pant Lobia-3	2013	67	38
Pant Lobia-4	2014	51	36

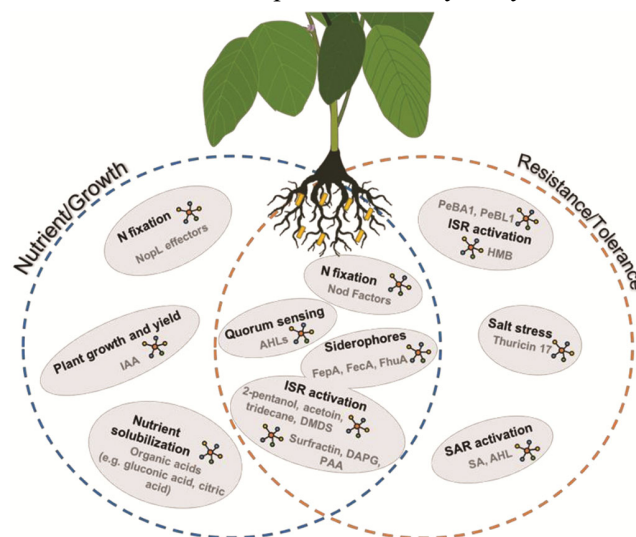


Fig. 3 — Schematic representation of the main processes and relevant molecules underlying plant growth promotion by bacteria

micronutrient concentrations in edible agricultural products. Mineral fertilizers have been applied to soil for hundreds of years to improve the health of their plants. The same approach can also be used to increase mineral accumulation within seeds for nutritional purposes³⁸. The exogenous application of micronutrient-containing mineral fertilizer to the soil and/or plant leaves (foliar) to improve the micronutrient content of the edible part of food crops is known as agronomic biofortification. Micronutrient fertilization works best when combined with NPK, organic fertilizers and improved crop types thus, emphasizing the necessity of integrated soil fertility management³⁹. This method is simple and inexpensive, but it requires knowledge about the source of nutrients, the method of application and the effects on the environment. Foliar application is the most efficient way of application among various methods of fertilizer application⁴⁰. This method can manage immobilization of nutrients in soil and make nutrients available to the crop quickly⁴¹. The highest increases in Fe (154 mg kg⁻¹) and Zn (42 mg kg⁻¹) content of cowpea seeds, were found after foliar fertilization with 2 ppm Fe and 2 ppm Zn by Salih *et al.*⁴². Molina *et al.*⁴³ found that application of zinc chelates (7- & 14-mM L⁻¹ of Zn-EDTA) increased zinc and iron concentration in seeds of cowpea. Morales *et al.*⁴⁴ conducted an experiment to study the impact of agronomical biofortification with zinc on the nutrient content, bioactive compounds and antioxidant capacity of cowpea beans using four edaphic doses (0, 1.25, 0.25 and 0.50 g/plant) and four foliar doses (0, 12.5, 25 and 50 mM/L) of zinc sulphate. They reported that edaphic and foliar application of zinc sulphate increased the grain weight and size, zinc content, total phenols and antioxidant but the highest value for all the observed parameters was noticed with the application of 0.25 g of edaphic and 12.5 mM of foliar doses. According to Marquez-Quiroz *et al.*⁴⁵, applying 100 mM L⁻¹ ferrous sulphate and 50 mM L⁻¹ ferrous chelate raised the iron content of cowpea bean seeds by 29.4 and 32%, respectively, over the control.

Nano-fertilizers: An inventive Approach

New-generation fertilizers based on nanotechnology are termed as nano fertilizers. They have been suggested as viable alternatives to avoid basic agricultural issues related with the application of conventional fertilizers. The size of nanomaterials ranges between 1 and 100 nm. Nanoparticles possess

a high surface-to-volume ratio, thus providing more active sites for absorption/adsorption than bulk materials. In the past few years, nano-fertilizers have attracted extreme attention in agricultural management. Thus, Foliar spray of nanoscale materials as fertilizer carriers or controlled-release products should be done to enhance nutrient use efficiency. Souza *et al.* evaluated mineral composition, chlorophyll fluorescence and zinc biofortification in *Vigna unguiculata* fertilized with bulk and nanoparticulate zinc oxides as soil application using five different doses (0, 100, 300, 500, and 800 ppm). The results showed that 100 ppm ZnO of the bulk and nanoparticulate Zn oxides adequately supplied the Zn concentrations required by cowpea, promoting increases in shoot and grain biomass. At doses higher than 300 ppm of ZnO, bulk ZnO application caused more significant toxicity in plants, promoting decreased biomass and damage to the photosynthetic apparatus. Whereas, Suriyaprabha *et al.* found that copper nanoparticles reveal better seed germination percentage, seedling length and plant height on application of 100 ppm of copper oxide nanoparticles while zinc oxide and iron oxide nanoparticles showed promising responses in plant growth, uptake and bioavailability of seeds at higher doses *i.e.*, 1000 ppm shown in (Fig. 4)⁴⁴⁻⁴⁶.

Criteria of Biofortified crop

The main objective of biofortification is to develop micronutrient-rich staple crops to reduce micronutrient malnutrition which helps in food security, productivity, and quality of life in developing countries²³. Criteria of biofortified crop is explained below: -

Effective: The increased level of micronutrients must have a positive effect on human health.

Stable: The increase level of micronutrients crops must be stable year after year.

High yielding: Productivity of the crop must be sustained after biofortification.

Quality: Crop or variety to be biofortified must be good in taste and cooking quality.

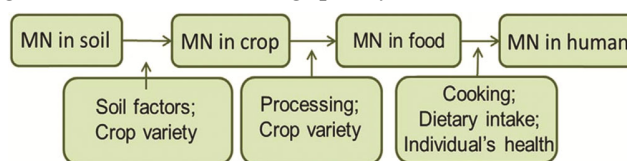


Fig. 4 — Schematic overview of micronutrient (MN) pathway from soil to humans and the factors that influence MN bioavailability to the next level

Cost-effective and sustainable: The technique to be used for biofortification must be cost-effective and sustainable to develop micronutrient rich staple crops to achieve iron and zinc concentrations that can have an assessable impact on nutritional status.

Disease-resistant and climate-resilient varieties: Crops to be biofortified should be resistant to disease and climate change.

Advantages of Biofortification

- Improving the nutritional value of daily diets to lower the risk of diseases associated with dietary deficiencies.
- An increase in genetic variability and an improvement in plant or crop quality²⁹.
- The biofortified crop system has a high degree of sustainability once it is established. Nutrient-rich novel varieties will continue to be grown and consumed year after year.
- Aside from agronomic biofortification, other techniques have the benefit of minimal recurring cost and the ability to transfer germplasm worldwide because one-time investment is made to develop seeds that fortify themselves. This multiplier aspect of plant breeding across time and distance makes it cost-effective.
- Application of biofortified crops would benefit farmers by increasing their income in the long term.
- It is especially important for poor rural communities with finite access to a varied diet, fortified foods or supplements.

Future challenges in Biofortification

- There is need to lower the concentration of anti-nutritional substances like phytic acid, which prevents the gut from absorbing minerals like Fe, Zn, and Ca.
- Strengthening our understanding of how minerals are transported and accumulated from the roots to storage tissues like grain.
- Supporting extensive prospective studies on evaluating the results of nutritional fortification in important staple crops to decrease future malnutrition-related diseases.

Conclusion

In developing nations, a significant proportion of the population suffers from vitamin and mineral deficiencies, primarily due to low concentrations and

decreased bioavailability of the key micronutrients found in most of the foods generally consumed there. Biofortified pulses have a high potential for combating hidden hunger since the edible sections contain a high concentration of bioavailable micronutrients, minerals, and vitamins. Pulses have been targeted for the improvement of inadequate S-containing amino acids, the improvement of Fe and Zn and the reduction of anti-nutrients such as phytic acid. Traditional breeding methods, mutation breeding, and the application of appropriate fertilizers to the leaves or roots have all contributed to some achievements. For some micronutrients, especially Zn, agronomic biofortification can improve yields and nutritional quality. The bioavailability of micronutrients across the entire pathway from soil to plant, food and the human body is crucial to the efficacy of agronomic biofortification. Due to their distinct structural traits and great efficacy, the use of nanomaterials as fertilizers has become a viable alternative to conventional fertilizers. When applied at the proper ratios, micronutrient-enriched fertilizers do not have any significant adverse environmental effects and typically offer agronomic benefits because they increase soil fertility and crop health. Aside from agronomic biofortification, other techniques have the benefit of minimal recurring cost because one-time investment is made to develop seeds that fortify themselves.

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Conflict of interest

All authors declare no conflict of interest.

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