Breeding Cowpea for Quality Traits: A Genetic Biofortification Perspective

P. Dhanasekar, J. Souframanien, and P. Suprasanna

Abstract Cowpea, *Vigna unguiculata* (L.) Walp, is an important arid legume crop cultivated widely in the arid and semiarid tropics of the world mostly by resource-poor farmers involved in subsistence farming. Also known as poor man's meat, this crop is rich in proteins and carbohydrates but does not have appreciable quantities of essential micronutrients. Micronutrient deficiency leading to malnutrition is a major concern that affects one third of the world population. Among various interventions available for alleviating malnutrition, genetic biofortification through plant breeding is considered the most viable, economical, and sustainable approach. Cowpea exhibits considerable genetic variability for important nutritional components such as protein and micronutrient levels, thus offering scope for genetic biofortification. With genetic biofortification breeding programs of primary staples attaining the intended micronutrient level targets, it is high time that similar results are replicated in secondary staples, especially pulses, and in a crop like cowpea that complement the primary staple-based diets. Breeding of cowpea quality traits from a genetic biofortification perspective is discussed with an attempt to provide a comprehensive outlook on priority biofortification traits, their genetic variability and biochemistry, and genomic and analytical tools available. The growing national and international interests of cowpea breeders for pursuing biofortification as a new, complementary intervention to address micronutrient deficiency are expected to result in the development of next-generation biofortified cowpea and ensuring a nourishing future.

Keywords Biofortification · Cowpea · Iron · Legumes · Malnutrition · Micronutrient deficiency · Zinc

P. Dhanasekar (✉) · J. Souframanien · P. Suprasanna
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India
e-mail: sekar@barc.gov.in

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Introduction

Cowpea, *Vigna unguiculata* (L.) Walp, is a proteinaceous arid grain legume crop widely cultivated in the tropical and subtropical regions of the world. They are commonly grown in the semiarid tropics between 35° N and 30° S of the equator, covering Africa, Asia, Oceania, the Middle East, Southern Europe, Central and South America, and the Southern United States (Boukar et al. 2018). The cowpea plant is a herbaceous, warm-season annual legume requiring temperatures of at least 18 °C throughout all stages of its development and having an optimal growing temperature of about 28 °C (Craufurd et al. 2010). Unlike other food legumes, this hardy crop performs well even in the drier regions. Cowpea is a dicotyledon belonging to the order Fabales, family Fabaceae, subfamily Faboideae (Syn. *Papillionoideae*), tribe Phaseoleae, subtribe Phaseolinae, genus *Vigna* Savi (Boudin and Marechal 2011). The pantropical *Vigna* is a highly variable genus encompassing 84 to 184 species (Timko et al. 2007). Cowpea belongs to the section Catiang of subgenus *Vigna* and genus *Vigna.* Section Catiang is comprised of two species, *unguiculata* and *nervosa*. The species *unguiculata* (Latin for "with a small claw," which reflects the small stalks on the flower petals) is further divided into five subspecies with all the cultivated cowpeas being found within the subspecies *unguiculata.* This subspecies is comprised of four cultivar groups: unguiculata, biflora, sesquipedalis, and textilis. All the current evidence suggests that cowpea originated in Southern Africa, although several centers of domestication such as Ethiopia, Central Africa, South Africa, and West Africa have been suggested. Presently, the wild cowpea, *Vigna unguiculata* ssp. *unguiculata* var. spontanea, is supposedly the likely progenitor of cultivated cowpea (Singh 2005). With a view to streamlining and strengthening cowpea breeding programs across the globe, the International Institute of Tropical Agriculture (IITA) was established in 1967 with a mandate to develop improved cowpea varieties for all regions. This nodal agency is maintaining more than 15,100 accessions of cultivated cowpea drawn from over 100 countries and more than 560 accessions of wild cowpeas.

The cowpea, considered to be one of the oldest domesticated crops (Chivenge et al. 2017), probably derived its name due to its use as a fodder crop for cows. It is commonly known by its indigenous or regional names such as "lobia" and "chowlee" in India; "kunde" in East Africa; "beans" and "wake" in Nigeria; "niebe" in francophone Africa; "southern pea," "crowder pea," and "black eye pea" in the United States of America; and "feijão caupe" in Brazil and also by a host of other vernacular names in different countries worldwide. Current estimates indicate that it is grown in about 14.5 million hectares with an annual production of over seven million tons on a global basis (Singh 2014). Over the last three decades, worldwide cowpea production grew at an average rate of 5%, with 3.5% annual growth in area and 1.5% growth in yield, and the area expansion accounted for 70% of the total growth during this period (Fatokun et al. 2012). India is the largest cowpea producer in Asia, and together with Bangladesh, Indonesia, Myanmar, Nepal, Sri Lanka,

Pakistan, Philippines, Thailand, and other far eastern countries, more than 1.5 million ha is under cowpea cultivation (Steele and Mehra 2009).

Cowpea is truly a multifunctional crop, providing food for man and livestock and serving as a valuable and dependable revenue-generating commodity for resource-poor farmers. It is inherently tolerant to drought and heat and has the ability to fix nitrogen (through its symbiotic relation with *Bradyrhizobium* group of nodulating bacteria, can fix 70–350 kg nitrogen per ha) even in very poor soils with a pH as low as 4–5, organic matter below 0.2%, and sand content of over 85% (Kolawale et al. 2000). Its inherent shade tolerance attribute makes it a candidate crop for intercropping with a number of cereals, root, and plantation tree crops. In addition, its quick growth and rapid ground cover has made cowpea an essential component of sustainable subsistence agriculture in marginal drier regions of the tropics where rainfall is erratic and scanty and soils are sandy with little organic matter (Carsky et al. 2001). Its plasticity toward environmental vagaries and its superior nutritional values make it a potent crop under the present context of food, nutritional security, and climate change.

Cowpea seeds provide a rich source of proteins and calories, as well as minerals and vitamins. As a legume in general, its protein content (~25%) is approximately twice that of cereals, and its amino acid (AA) profile, rich in lysine (Lys) and tryptophan (Trp), complements those of cereals, which are rich in sulfurous AAs (Nielsen et al. 1993). With very low fat content and slowly digestible starch (in comparison to cereals), cowpea is highly beneficial for human health. The grain is a rich source of an important vitamin folic acid, which helps prevent neural tube defects in unborn babies. The nutritional richness of cowpea can be comprehended in Table 1. The remnant biomass of the haulm post harvest is a source of quality fodder for ruminant livestock. Cowpea can be consumed as fresh or dry seeds, canned or frozen food, and milled flour in baked goods. In addition, cowpea has been used as an alternative to soybean for people who are allergic to soybean protein (Boukar et al. 2018). Because of its high protein content and largely being cultivated by resource-poor farmers, cowpea is aptly referred to as "poor man's meat."

Agriculture till now has been aimed at producing more calories to negate hunger, but the current scenario in most of the developing countries equally warrants the development of nutrient-rich foods to reduce hidden hunger or malnutrition. Malnutrition results from eating a diet in which one or more nutrients (calories, protein, carbohydrates, fat, vitamins, or minerals) are either not enough (undernutrition) or otherwise (overnutrition) such that the diet causes health problems. Malnutrition (often refers to undernutrition) is more predominant in developing countries with certain groups, in particular pregnant or breastfeeding women and children under 5 years of age being more susceptible. Vitamin and mineral deficiencies result in a myriad of cognitive and health impairments increasing the risk of death. In developing countries, agricultural products are the prime source of nutrients, and the nonavailability or non-affordability of nutrient-rich food grains has deprived the needy poor of these essential nutrients leading to malnutrition. Possible ways to combat those deficiencies encircle dietary diversification (healthy balanced diet), food fortification (nutrient enrichment during processing), biofortification,

Table 1 Nutritional value per 100 g of raw cowpea seeds

Nutrient	Value
Energy	336 kcal (1410 kJ)
Carbohydrates	60.03 g
Sugars	6.9 g
Dietary fiber	10.6 g
Fat	1.26 g
Protein	23.52 g
Vitamins	**Quantity**
Vitamin A equiv.	3 µg
Thiamine (B_1)	0.853 mg
Riboflavin (B_2)	0.226 mg
Niacin (B_3)	2.075 mg
Vitamin (B_6)	0.357 mg
Folate (B_9)	633 µg
Vitamin C	1.5 mg
Vitamin K	5 µg
Minerals	**Quantity**
Calcium	110 mg
Iron	8.27 mg
Magnesium	184 mg
Phosphorus	424 mg
Potassium	1112 mg
Sodium	16 mg
Zinc	3.37 mg
Other constituents	**Quantity**
Water	11.95 g

Source: USDA nutrient database

and supplementation (external nutrient-rich additives) (Ghosh et al. 2019). No single intervention can solve the problem of micronutrient malnutrition, but biofortification complements existing interventions to sustainably provide micronutrients to the most vulnerable people in a comparatively sustainable, inexpensive, and cost-effective manner (Saltzman et al. 2013). Biofortification, the process of increasing nutrient concentration in plant edible parts, can be achieved through three main approaches, namely, transgenic, conventional, and agronomic, involving the use of biotechnology, crop breeding, and fertilization strategies, respectively. Biofortification through conventional breeding is the most accepted method of biofortification. Thus, biofortification through breeding programs aims at increasing the micronutrient dietary intake without changing the diet of those targeted (Gerrano et al. 2017). A number of international initiatives have made impactful success in their sustained efforts for global redressal of malnutrition like Nutrition International, Iodine Network, iZiNCg, Iron Deficiency Project Advisory Service (IDPAS), New Partnership for Africa's Development (NEPAD), UNICEF-Micronutrients, Global

Alliance for Improved Nutrition (GAIN), Helen Keller International, CGIAR Research Program on Agriculture for Nutrition and Health (A4NH), HarvestPlus, etc. In India, various government initiatives have been launched over the years to improve the overall nutrition status in the country. These include the Integrated Child Development Services (ICDS), the National Health Mission, the Janani Suraksha Yojana, the Matritva Sahyog Yojana, the Mid-day Meal Scheme, and the National Food Security Mission, among others. However, concerns regarding malnutrition have persisted despite improvements over the years. It is in this context that the National Nutrition Strategy has been recently released (NITI Aayog, GoI 2017).

Cowpea, a crop of subsistence farming across the world, is therefore an apt crop for breeding quality traits to address the malnutrition. Quality in its broadest sense encompasses a gamut of traits that can be broadly grouped as under:

A. **Morphological and physical quality:** These traits are related to external appearance of the seed. It includes seed shape, seed size, testa color, hilum eye color, seed coat pattern, seed texture, seed weight, etc.
B. **Organoleptic quality:** These traits are related to palatability of the produce. They are easily detected and are very important in consumer preferences**.** It includes seed taste, aroma, flavor, softness, etc.
C. **Biological quality:** The traits included in this group define the actual usefulness of the produce, when consumed. These include protein efficiency ratio, biological value, body weight gain, bioavailability, and digestibility.
D. **Biochemical quality:** It includes protein, vitamins, minerals, carbohydrates, micronutrients, and antioxidants.
E. **Antinutritional quality:** It includes protease inhibitors, phytates, alpha-galactosides (oligosaccharides), tannins, saponins, and polyphenols.
F. **Other quality parameters**: These are important in determining the usefulness of the concerned produce. This includes cooking quality, milling quality, cooking time, and keeping quality.

However, in addressing the malnutrition through biofortification, biochemical parameters, especially micronutrient content, are of prime importance, and hence, this aspect of quality breeding will be elaborated in this chapter. Techniques to increase the total protein and mineral content of cowpea cultivars are considered as an important component of global intervention programs that are focused on alleviating human malnutrition and ensuring food security, especially in semiarid tropical areas (Santos and Boiteux 2013).

Priority Traits for Genetic Biofortification

The United Nations Food and Agriculture Organization has estimated that around 792.5 million people across the world are malnourished, out of which 780 million people live in developing countries (McGuire 2015). Apart from this, around two

billion people across the world suffer from another type of hunger known as "hidden hunger," which is caused by an inadequate intake of essential micronutrients in the daily diet (Hodge 2016) despite increased food crop production (Gould 2017). With increasing incidences of protein malnutrition in developing countries and higher incidence of diabetes, heart problems, and cancer in the developed countries, the consumption of cowpea with superior nutritional quality is expected to increase. A lot of research has gone into the biofortification of primary staple crops such as rice, wheat, maize, cassava, etc., which are consumed in large quantities. Even after decades of research, the biofortified varieties in these crops are not able to meet the entire estimated average requirement (EAR) of nutrients. Hence, it is highly imperative that concerted research efforts have to be directed toward a "food basket approach," providing a range of biofortified food crop options suited to local preferences (Andersson et al. 2017). This approach allows for diversification, both on the plate and in the field. In farmers' fields, different micronutrient-dense crops can be grown in rotation to provide a steady supply of micronutrients throughout the year. The secondary staples like cowpea are usually consumed in lower quantities than primary staples. Consequently, their contribution to daily micronutrient requirements is also lower. Nevertheless, they are an important complement in daily diets and are frequently consumed together with primary staples such as rice or wheat, and any amount of biofortification levels in these crops would help realize the ultimate micronutrient target levels. Therefore, prioritizing traits for genetic biofortification in secondary staples like cowpea has to be in tandem with that of primary staples. Secondly, the target traits for genetic biofortification should be identified such that sufficient and utilizable genetic variation exists in the genetic material for the trait of interest. Thirdly, while deciding the target nutrient levels, the baseline nutrient level has to be determined, and the incremental target level has to be arrived taking into consideration the micronutrient retention after processing, the bioavailability, and the per capita consumption so that the additional percent of EAR is achieved.

The priority traits for genetic biofortification in cowpea are as follows:

(a) **Protein content and quality**: Cowpea is a rich source of proteins (23–25% in dry seeds) and carbohydrates (50–70%), which could meet the increasing consumer demand for healthier and more nutritious food. Unlike soybean, cowpea proteins do not cause allergies and are of higher quality when substituted in diets at equivalent protein contents. In recent years, there has been increasing interest in breeding cowpea cultivars with high seed protein content to improve nutritional quality. Evaluation of seed protein content in cowpea germplasm will help plant breeders select and breed high seed protein content cultivars in breeding programs. Moreover, the amino acid profile of cowpea unlike that of cereals is rich in lysine and tryptophan but lacks in sulfur-containing amino acids. Therefore, the breeding efforts need to be aimed at increasing both the protein content and the proportion of methionine and cysteine amino acids to counter protein malnutrition.

(b) **Iron content (Fe)**: Iron (Fe) is an essential micronutrient for plants and for humans, and it is a constituent of a number of important macromolecules, including those involved in respiration, photosynthesis, DNA synthesis, and metabolism (Briat 2011). Fe deficiency is ranked fifth among the top ten risk factors contributing to disease burden globally. Iron is present in all cells in the human body and has several vital functions, such as carrying oxygen to the tissues from the lungs as a key component of the hemoglobin protein, acting as a transport medium for electrons within the cells in the form of cytochromes, and facilitating oxygen enzyme reactions in various tissues. Too little iron can interfere with these vital functions and lead to morbidity and death (Centers for Disease Control and Prevention 1998). Children, premenopausal women (women of child-bearing age), and people with poor diet are most susceptible to anemia disease caused by the deficiency of Fe. The EAR of Fe in nonpregnant, nonlactating women is 1460 μg/day, while in children of 4–6 years, it is 500 μg/day. Fe retention after processing in cowpea is in the order of 90%, and bioavailability is around 2.5%.

(c) **Zinc content** (Zn): Zinc is an essential micronutrient in biological systems, which is required in small quantities. It is involved in the formation and activation of enzymes that have impact on the growth, development, and production of plants. It also affects pollen viability, flowering, and grain production. In humans, its deficiency is associated with problems of growth and learning capacity in children and increases the risk of infections, cancer, and DNA damage (Veronica et al. 2018). An estimated 17.3% of people worldwide are at risk of inadequate Zn intake (Wessells and Brown 2012), and Zn deficiency leads to estimated annual deaths of 433,000 children under the age of five (WHO 2009). It is present in around one third of the world population, which represents the sixth risk factor for diseases in developing countries (Shahzad et al. 2014). The EAR of Zn in nonpregnant, nonlactating women is 2960 μg/day, while in children of 4–6 years, it is 1390 μg/day. Zn retention after processing in cowpea is in the order of 90%, and bioavailability is around 15%.

(d) **Anti-mineral compound content:** Among the anti-minerals, antinutritional factors present in legumes like cowpea and phytic and oxalic acids are important (Liener and Kakade 1980). Phytic acid, also known as inositol hexakisphosphate (IP6), or phytate when in salt form is the principal storage form of phosphorus in many plant tissues. It is not digestible to humans or nonruminant animals, because these animals lack the digestive enzyme (phytase) required to remove phosphate from the inositol in the phytate molecule. Phytate is well documented to block absorption of not only phosphorus but also of other minerals such as calcium, magnesium, iron, and zinc (Shukkur et al. 2006). Phytic acid and oxalic acid reduce mineral bioavailability that leads to various mineral deficiency diseases, e.g., anemia, or form deleterious complexes with metal ions, e.g., calcium oxalate, which leads to renal damage. But since these antinutritional factors are mainly plant's secondary metabolites, they are involved in a variety of plant metabolic pathways. They are known to be involved in plant defense mechanism against biotic and abiotic stresses, and hence due precau-

tion has to be taken while meddling with these compounds. The breeder has to strike a right balance as to how low these compounds could be reduced without hampering the metabolic or agronomic values of the crop. Therefore, the reduction of anti-mineral compounds leads to increased bioavailability of micronutrients or wholesomeness for consumers and could be construed as a means of biofortification.

Genetic Variability for the Target Traits

The success of genetic biofortification through recombination breeding depends on the genetic variability of the proximate contents of the various target traits. Additive genetic interaction of genes governing the target traits could lead to generation of transgressive segregants enabling the development of varieties with proximate contents greater than that of the donor parents. Therefore, it is imperative to know the extent of genetic variation for various target traits in the existing germplasm of the crop including landraces and wild species, and also the knowledge on the genetics of the trait would enable the selection of suitable breeding method to achieve the target objective. After assessing the genetic variability for the trait of interest and confirming its suitability of genetic improvement, the donor lines with these traits are identified and are used in early-stage product development and parent building. Thereafter, breeding materials with improved nutrient content and high agronomic performance as well as preferred consumer qualities are developed. If necessary, further crosses with locally adapted materials could be attempted to develop final products that meet specific traits required by local producers and consumers. When promising high-yielding, high-nutrient lines emerge, they are tested across a wide range of environments side-by-side with locally preferred varieties. If the trait is lacking in a particular crop, then genetic biofortification through biotechnological interventions like transgenics could be resorted to, provided the legal framework of the country permits.

An analysis of 1541 cowpea germplasm lines (Boukar et al. 2011) revealed that on an average cowpea has 25% protein, 38 mg Zn/kg, 53 mg Fe/kg, 1.9 g Mg/kg, 0.825 g Ca/kg, 5 g P/kg, and 15 g K/kg. The screening of 2000 lines in cowpea (Singh 2016) for studying the genetic variability for major nutritional traits showed existence of wide genetic variability for most of the traits (Table 2).

The range of protein content as reported by various researchers (Asante et al. 2006; Gupta et al. 2010; Itatat et al. 2013; Oke et al. 2015; Ravelombola et al. 2016) falls within the reported range of Singh (2016). However, Afiukwa et al. (2013), Santos and Boiteux (2013), and Dakora and Belane (2019) reported a greater variability of the total seed protein content in excess of 32% up to a maximum of 40% (South African genotype "Bengpla") in cowpea. The broad-sense heritability for seed protein was reported to range from 50.8% to 95%, in various studies (reviewed in Weng et al. 2019) indicating that seed protein content was highly heritable and selection could be rewarding for protein content. But narrow variation in amino acid

Table 2 Genetic variability for quality traits in cowpea germplasm (Singh 2016)

	Range of value	
Parameter	Min	Max
Seed size (g/100)seeds	9	27
Protein (%)	20.9	32.5
Ash (%)	2.9	3.9
Fat (%)	1.4	2.7
Carbohydrate (%)	59.7	71.6
Cooking time (m)	21.1	61.9
Iron (ppm)	51	109
Zinc (ppm)	33	51
Calcium (ppm)	581	1252
Potassium (ppm)	12,084	15,133
Magnesium (ppm)	1611	2052
Phosphorus (ppm)	3867	4922
Sulfur (ppm)	1880	2354

(AA) composition suggests a lesser possibility of improving the contents of specific AAs in cowpea (Muranaka et al. 2016). As far as Fe and Zn are concerned, the former showed more variability in comparison to the latter. Fe content in cowpea ranged from 36.5 to 150 ppm, while Zn content ranged from 33 to 61 ppm (Belane and Dakora 2011; Santos and Boiteux 2013; Singh 2016; Marappa et al. 2016). The cultivar KBC-6 from the University of Agricultural Sciences, Bangalore, was found to have the highest Fe content of 150 ppm. Incidentally, the genotypes which showed high zinc were also associated with stay green trait even after the crop maturity, thus serving as phenotypic markers (Marappa et al. 2016). The variance due to genotype was highly significant (P, 0.01) for crude protein, Fe, and Zn contents. Phytic acid contents ranged from 0.21 to 10.27 mg/g (Garinu and Ingrao 1991; Dhanasekar and Reddy 2017). However, Muranaka et al. (2016) reported phytic acid levels of up to 37 mg/g in IITA lines.

Wide genetic variation and strong correlations among crude protein, Fe, and Zn contents suggest the possibility of improving the concentrations of these nutritional factors simultaneously. There were strong positive genotypic correlations between crude protein and Fe ($r = 0.70$) and Zn ($r = 0.70$) and between Fe and Zn ($r = 0.68$) contents in cowpea (Muranaka et al. 2016). Boukar et al. (2011) also reported strong positive correlations between the contents of crude protein and Fe and of Fe and Zn in their studies with 1541 genotypes reiterating the possibility of simultaneous selection for these traits. Simple correlation coefficient values indicated that selection for high protein and mineral content does not affect grain yield and that it is feasible to obtain new biofortified cowpea cultivars by combining higher levels of protein and essential minerals (Santos and Boiteux 2013). It was also observed that the increase in levels of micronutrients in the grains also favors the agronomic performance of biofortified genotypes in soils that are naturally deficient in these minerals (Welch 2002). In addition, plants with lower concentrations of phytate

improved the bioavailability of zinc and iron (Welch et al. 2000). Therefore, selection for lower levels of natural compounds that reduce the bioavailability of micronutrients in the human diet should also be a novel target for future breeding research aiming to develop biofortified cowpea cultivars.

Mutation Breeding in Genetic Biofortification

The degree of genetic variability for target traits in a crop determines the extent to which the trait of interest could be improved through combination breeding. Low genetic variability is a stumbling block in the genetic improvement, and the potential of mutation breeding in creating genetic variations during situations of low genetic variability has been demonstrated since ages. In genetic biofortification of food crops, mutations affecting various target traits have been reported. By and large, these mutants with the altered biofortification traits have been used in hybridization for transfer of these traits into elite genetic backgrounds. Maize breeders have developed quality protein maize (QPM) with high essential amino acids lysine and tryptophan by incorporating *opaque-2* (*o2*) mutant gene from naturally occurring maize into the maize cultivars (Hossain et al. 2019). Incorporation of *Or* mutant gene from orange cauliflower mutant led to increase in carotenoid level (Lopez et al. 2008). Low phytic acid accumulation is a recessive trait (Maqbool and Beshir 2019), and several losses of function mutations have been reported in various crops like rice, maize, common bean, cowpea (Neeraja et al. 2017; Cominelli et al. 2018; Dhanasekar and Reddy 2017), etc., and are being included in a range of introgressive breeding programs. Mutants could also be helpful in studying the physiological and metabolic pathways; as in maize, the mutant yellow stripe 1 (*ysl*) showed Fe deficiency due to impairment of Fe phytosiderophore uptake and that roots of iron-efficient maize mutants also absorbed more of phytosiderophore-chelated zinc (Von Wiren et al. 1996) probably owing to the involvement of nonspecific Fe transporters. Thus, mutation breeding in tandem with conventional breeding could be a great utility in realizing the biofortification goals.

Biochemistry of the Biofortification Traits

For effective genetic biofortification, knowledge on the biochemistry of the target traits is of immense importance, which would enable to maneuver the trait of interest through manipulating the genes governing the trait.

A. **Iron**: Fe is one of the most essential micronutrient that is required for the proper development of both plants and humans. Plants, as primary producers, are the gateway for iron to enter the food chain. Fe is involved in a variety of metabolic activities such as photosynthesis, mitochondrial respiration, nitrogen assimila-

tion, hormone biosynthesis, production and scavenging of reactive oxygen species, osmoprotection, pathogen defense, and as a limiting factor for biomass production (Briat 2011). Plants obtain Fe from the soil, where Fe exists in either ferrous (Fe^{2+}) or ferric (Fe^{3+}) state. Although Fe is the fourth most abundant element in the Earth's crust, it is not readily available to plant as it binds rapidly to soil particles and forms insoluble complexes under aerobic conditions at neutral or alkaline pH (Gomez-Becerra et al. 2010). Post intake, Fe is complexed with chelators and distributed to sink tissues where it is used predominantly in the production of enzyme cofactors or components of electron transport chains. The processes of iron uptake, distribution, and metabolism are overseen by tight regulatory mechanisms, at the transcriptional and posttranscriptional level, to avoid iron concentrations building to toxic excess. Iron is also loaded into seeds, where it is stored in vacuoles or in ferritin. Iron homeostasis in plants is elaborated in detail by Connorton et al. (2017), and therefore it will be discussed only in brief in this chapter.

(a) **Fe uptake**: Plants adopt different strategies for uptake of low soluble Fe(III) oxyhydrate from the rhizosphere in higher plants: (a) Strategy I (non-Graminaceae) is reduction strategy wherein Fe^{3+} is reduced by ferric reduction oxidase 2 (FRO2) at the plasma membrane before transport across the membrane by iron-regulated transporter 1 (IRT1). In addition, plasma membrane proton pumps help acidify the rhizosphere and increase Fe^{3+} solubility. An array of metabolites including organic acids, phenolics, flavonoids, and flavins may also be exported for reduction of ferric iron. (b) Strategy II (Graminaceae) is the chelation strategy involving secretion of phytosiderophores like deoxymugineic acid (MA) which have high affinity for Fe, and the resulting chelates are imported by oligopeptide transporters like YS1. Some organisms are known to have a combination of both the strategies.

(b) **Iron distribution and storage:** Most iron enters the plant via the root and needs to be transported to the sink tissues where it is required for iron-dependent enzymes. Iron first enters the symplastic pathway through IRT1 found on the outward side of the epidermal cells of the roots. Due to its toxicity and low solubility, iron is translocated as Fe^{3+}chelated complex through a complex cascade involving xylem and phloem loading/unloading, and finally in the leaves it is reduced to Fe^{2+} mainly by FRO proteins. To facilitate this translocation, different chelators such as citrate, MAs, and nicotianamine (NA) play a crucial role. Organelle-specific iron transporters then transport a large proportion of iron into the plastids and mitochondria. Iron is then remobilized from leaf tissues with the help of oligopeptide transporter family proteins like OPT3 and reaches other sink organs through the phloem. Though present in many tissues, the terminal destination of iron is often considered to be the seed, where iron stores are important during germination before the seedling has developed a root and takes up nutrients from the soil. YSL transporters are involved in seed loading, and there is evidence that iron can be delivered to embryos as a Fe^{3+}-citrate/malate com-

plex. Two major storage mechanisms for iron have been proposed: sequestration into vacuoles and into ferritin. The vacuolar iron transporter VIT1 was first identified in *Arabidopsis*. Genes from the *VIT* family are also known to be important for iron localization in grains. Ferritins are important iron storage proteins present across the biological kingdoms. In legume seeds, it is found that 24 subunits of ferritin form a shell capable to store up approximately 2500 Fe^{3+}ions. The proportion of total iron stored in ferritin in seeds varies among species, with approximately 60% in peas but less than 5% in *Arabidopsis* seeds. In cereal grains such as wheat and rice, most iron is present in vacuoles in the aleurone layer which is often removed during grain processing. The way in which iron is stored in seeds can affect its bioavailability when consumed, which is of great importance in biofortification studies. The iron is then used in the biosynthesis of Fe cofactors because of the toxic nature of free iron. The most common forms of iron cofactors are heme, FeS clusters, and di-iron centers.

Plants adapt their root morphology to iron-limiting conditions by increasing the density of root hairs and the number of lateral roots. The greater surface area extends contact between the epidermis and the rhizosphere, and the lateral roots help to explore fresh soil (Li et al. 2016). Great progress has been made in identifying a large number of transcriptional regulators like helix-loop-helix (bHLH) and FER-like iron deficiency-induced transcription factor (FIT) that regulate the iron deficiency response of iron homeostasis. Plants exhibit tight homeostatic control to prevent accumulation of iron where it is not needed, and this may limit iron redistribution to edible tissues such as seeds. Any successful biofortification strategy must bypass these mechanisms without causing physiological damage to the plant.

B. **Zinc:** Zn homeostasis is maintained by a tightly regulated network of low-molecular-weight ligands, membrane transport, and Zn-binding proteins, as well as regulators. Fe and Zn homeostasis interacts as a consequence of the chemical similarity between their divalent cations and the lack of specificity of the major root iron uptake transporter IRT1. A significant proportion of the Earth's arable land is considered Zn-deficient (Alloway 2009). Zn can bind tightly to soil and plant cell wall components and can form precipitates, most commonly in the form of phosphates, carbonates, or hydroxides, in the soil. Like in Fe homeostasis, Zn solubilization in the rhizosphere is thought to occur via plant-mediated acidification and secretion of low-molecular-weight organic chelators. Subsequently, Zn is taken up across the plasma membrane of root cells predominantly as a free ion in a similar fashion as that of Fe. The possible involvements of zinc-regulated transporter and iron-regulated transporter (ZRT-IRT)-like proteins (ZIPs) in cellular Zn^{2+} uptake have been established. The major root epidermal plasma membrane Fe transporter IRT1 mediates the uptake of Zn^{2+} as well as its primary substrate Fe^{2+}. In the cytoplasm of plant cells, Zn is thought to be chelated by low-molecular-weight ligands in order to prevent cytoplasmic precipitation and nonspecific binding to biomolecules. The export

from cells is required for the loading of Zn into the apoplastic xylem and thus for the translocation of Zn from the root to the shoot. Zn^{2+} export from the cytoplasm and further loading into the xylem is accomplished by a subgroup of HMA proteins of the P_{1B}-type ATPase family. A subset of plant metallothioneins is likely to contribute to the buffering or storage of cytosolic Zn. Cation diffusion facilitator (CDF) family of metal cation/proton antiporters, members of which have also been named ZAT (zinc transporter of *Arabidopsis thaliana*) and MTP (metal tolerance protein or metal transport protein), acts in the removal of Zn from the cytoplasm. Inside the xylem, Zn flux into the shoot is mass flow driven. There is some evidence for the chelation of Zn by low-molecular-weight ligands inside the xylem, which could act to prevent Zn retention by metal-binding components of the surrounding cell walls or uptake into cells via Zn^{2+} transporters. Cell vacuoles are the major site for storage and detoxification of excess Zn and a source for Zn remobilization in periods of deficiency. The homeostasis of Zn has been comprehensively reviewed by Sinclair and Kramer (2012).

C. **Phytic acid (PA):** Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) is the most abundant form of phosphorus occurring in seeds (up to 85% of total phosphorus) and other plant tissues. Due to its chemical structure (highly negatively charged at physiological pH), PA easily binds important mineral cations such as iron, zinc, potassium, calcium, and magnesium and makes them unavailable. In plants, PA biosynthesis occurs through two different routes: a "lipid-dependent" (operates in all tissues) and a "lipid-independent" pathway (predominates in seeds). PA biosynthesis begins with the production of *myo*-inositol (Ins) through a highly conserved reaction in which the enzyme d-*myo*-inositol 3-phosphate synthase (MIPS) converts d-glucose-6-phosphate to *myo*-inositol 3-phosphate ($Ins(3)P_1$). *Myo*-inositol 3-phosphate is then dephosphorylated to free Ins by inositol monophosphate phosphatase (IMP). In the "lipid-dependent" pathway, Ins is converted to phosphatidylinositol (PtdIns) by a synthase (PtdIS) and thereafter is sequentially phosphorylated by kinases. The "lipid-independent" pathway consists of sequential phosphorylation of the Ins ring to $InsP_6$, through the action of a number of specific kinases. In rice, a mutation in kinase gene (*OsPGK1*) generates an *lpa* phenotype, while overexpression increases seed $InsP_6$ content, suggesting that *OsPGK1* is a key gene for $InsP_6$ synthesis, being involved in (probably the rate-limiting) step from $InsP_1$ to $InsP_2$. Further, phosphorylation steps, required to convert $InsP_3$ into the more phosphorylated $InsP_4$, $InsP_5$, and $InsP_6$, involve at least three types of inositol kinases (for details, read Sparvoli and Cominelli 2015). Once synthesized, phytic acid is stored as globoids inside the storage vacuoles. Depending on the species, the amount and distribution of phytic acid in different parts of the seed can be quite variable. In the cereals, a large amount (80%) of phytic acid is stored in the aleurone and bran (maternal teguments), while in maize seeds 80% of phytate accumulates in the embryo and scutellum (O'Dell et al. 1972). In case of legume seeds, more than 95% is accumulated in the cotyledons (Ariza-Nieto et al. 2007), while in *Arabidopsis*, it is stored in the embryo (Otegui et al. 2002).

During germination, in order to support seedling growth, phytic acid is then degraded by phytase enzymes to remobilize the phosphorus stored as phytate salts (Raboy 2003).

Therefore, to facilitate an efficient and targeted genetic biofortification for Fe and Zn, five key steps can be addressed: (a) enhanced uptake, (b) increased translocation to seeds, (c) specialization of Fe and Zn storage toward vacuoles, (d) reduction of antinutritional compounds like phytic acid, and (e) increase of bioavailability. Either single approach or combination of multiple approaches can be applied in genetic biofortification.

Analytical Methods

The success of any biofortification program is largely dependent on robust analytical tools that can precisely and rapidly analyze the micronutrient contents for high-throughput screening of a large number of samples from segregating breeding materials in a cost-effective and efficient manner. The key to accurate measurement also depends on the chances of contamination during sample preparation and analysis. Moreover, the tools for analysis should be easily available to the breeders both cost wise and quantity wise and should be as simple as possible without the need for any special expertise. Since the micronutrient contents are very low in the order of ppm, the technology should be highly sensitive to detect accurate variations. Pfeiffer and McClafferty (2007) provide a comprehensive overview of analytical methods and diagnostic tools and also discuss other related issues, such as the varying sensitivity requirements depending on the stage of development, contamination (in the case of minerals), effects of milling/polishing, and micronutrient concentration versus content.

(a) **Protein content determination**: Protein content in cowpea has been widely determined by the age-old Kjeldahl technique or by nitrogen (N)/protein analyzer. The former involves acid digestion, distillation, and titration to determine the nitrogen content. In the latter, the nitrogen content is determined through combustion at high temperature and detection through thermal conductivity (Horneck and Miller 1998). The percent N has also been determined using mass spectrometry (Dakora and Belane 2019) or by dry oxidation (Dumas) method (Gerrano et al. 2017). In all these methods, a factor of 6.25 is typically used to calculate the crude protein content from the N content of legumes, although much lower factors, ranging from 5.32 to 6.03, have also been suggested (Sosulski and Holt 1980; Fujihara et al. 2010).

(b) **Elemental analysis of Fe and Zn**: Spectroscopic methods such as inductively coupled plasma optical emission spectroscopy (ICP-OES) and atomic absorption spectroscopy (AAS) are well established and provide accurate and sensitive results for a range of elements with analytical detection limit ranging from percent to ppm levels. The principle behind both of these methods is based on

the signature spectral absorption/emission of individual elements. AAS involves volatilization of sample by passing through a flame at more than 2000 °C and studying the absorption spectra, while in ICP-OES, constituent atoms are excited at temperatures of up to 10,000 °C and by studying their emission spectra. ICP-OES has been the "gold standard" for micronutrient analysis due to the high accuracy, wide analytical detection range, capability to detect soil contamination, and expansive elemental analysis. However, it is expensive (such as equipment, high-purity reagents required, and consumables), contamination prone, and time-consuming (pre-analysis preparation). AAS is less expensive (both instrument outlay costs and analysis costs), requires greater volumes of digested plant material (compared to ICP-OES), and is generally limited to single element analysis per run (Guild et al. 2017a). ICP coupled with mass spectrometry has also been reported for elemental analysis in cowpea (Dakora and Belane 2019).

For high-throughput qualitative and quantitative elemental screening, spectrometry based on X-ray fluorescence (XRF) is also demonstrated that has proven to be cost- and time-efficient in a wide range of crops including cowpea (Guild et al. 2017b). The XRF technology is less sensitive although it is nondestructive, requires no dissolution (minimal pre-analysis preparation), and has good precision for major elements (Wobrauschek et al. 2010) making it appropriate for the analysis of large samples for multiple elements simultaneously. XRF is based on the principle of elemental excitation using X-rays and the study of secondary "fluorescent" X-ray emission during de-excitation that is characteristic and abundance of the element analyzed. Samples can be screened in either whole grain that reduces sample processing time with reduced contamination risk or flour form that improves the reproducibility and accuracy but increases likelihood of contamination and labor requirement. Therefore, it would be wise to screen a large number of samples with XRF, and later AAS or ICP-OES could be used to confirm nutrient content of the narrowed-down samples. In addition, ED (energy dispersive)-XRF analysis of cowpea indicated that when analyzing flour samples, the results were not significantly different to the reference ICP-MS analysis (average difference of ±1 mg kg^{-1} for both Fe and Zn), while whole grain analysis by XRF gave significant differences and hence is not feasible for screening grains larger than wheat (Guild et al. 2017b).

(c) **Colorimetric analysis of Fe and Zn**: An alternative to ICP and AAS analysis for elemental quantification, colorimetry is a staining technique based on color change caused by chelation of metal ion of interest with specific reagents. This technique has been shown to detect ppm levels of specific elements with added advantage of not requiring expensive equipment or pre-analysis digesting. Since the colorimetric reagent is element specific, this method is predominantly useful when screening for a particular element as in the case of biofortification trials focused on specific micronutrient (i.e., Fe or Zn). Consequently, staining techniques have been used widely to screen for genotypes with high levels of micronutrients. For Fe screening, Perls' Prussian blue (PPB) and 2,2′-dipyridyl stains have been reported, while Zn screening could be achieved by staining

with dithizone (DTZ, diphenyl-thio-carbazone) and Zincon® (2-carboxy-2-hydroxy-5-sulfoformazyl benzene). The intensity of the colored chelate formed by the reaction of the stain with the metal ion determines the concentration of the metal (under optimized conditions). Consequently, it is even visually possible to differentiate nutrient-dense genotypes from those with low levels. The method has been further improved to enable semiquantitative analysis of micronutrient concentrations with the use of image analysis software such as Adobe Photoshop® and ImageJ as demonstrated by Choi et al. (2007) and Duarte et al. (2016), respectively. By using this combination of staining and image processing, it was possible to achieve results correlating color intensity with reference micronutrient analysis (ICP-OES) with $r^2 > 0.8$ for both Fe and Zn (Choi et al. 2007). This enables high-throughput screening even in basic laboratories sans costly analytical equipment.

(d) **Determination of anti-mineral compounds**: The anti-mineral compounds such as phytic acid and polyphenols have been analyzed using UV-Vis spectrophotometer through different methods. In cowpea, the polyphenols could be determined by modified Folin–Ciocalteu method (Singleton et al. 1999), while tannins could be estimated by Vanillin-HCl method as described in Dhanasekar and Reddy (2012). Phytic acid has been estimated following modified Wade's method (Dhanasekar and Reddy 2017). The phenolic compounds could also be studied both quantitatively and qualitatively using high-performance liquid chromatography (HPLC) (Moreira-Araujo et al. 2017).

Molecular Breeding Methods

Molecular markers play an important role in accelerating the pace of selection and therefore the breeding process. The utility of molecular markers depends on the availability of genomic resources in the crop or related crops. The use of molecular markers for genetic biofortification in legumes has been very limited in general and none in cowpea. Although a number of advances in cowpea genetic linkage maps and QTLs associated with some desirable traits such as resistance to *Striga*, *Macrophomina*, *Fusarium* wilt, bacterial blight, root-knot nematodes, aphids, and foliar thrips have been reported (Boukar et al. 2016). Linkage mapping provides a framework for downstream analyses including quantitative trait loci (QTL) identification, map-based cloning, diversity analysis, association mapping, and molecular breeding (Lucas et al. 2011). Now that linkage maps for cowpea with high marker density are available, there are increased opportunities for QTL resolution, map-based cloning, association mapping, and marker-assisted breeding. With the availability of improved consensus genetic linkage maps, physical maps, next-generation sequencing (NGS), and the recent publication of the whole genome sequence in cowpea (Lonardi et al. 2019), molecular markers can play a key role in the identification of QTLs and SNPs for various biofortification traits and also could be invariably put into use for marker-assisted backcross selection (MABC) or marker-assisted

recurrent selection (MARS). About 1100 SNPs mapped on the cowpea genome have been converted to Kompetitive allele-specific PCR (KASP) assays at IITA. Sources of cowpea genomic resources like physical maps, HarvEST:Cowpea, cowpea genomics knowledge space (CGKB), cowpea genomics initiative (CGI), microarray chip, SSR marker kit, consensus genetic linkage map, and software like "SNP selector," "KBioConverter," and "Backcross selector" have been shortlisted by Boukar et al. (2016). Several cowpea breeding programs have been exploiting these resources to implement molecular breeding, especially for MARS and MABC, to accelerate cowpea variety improvement. Molecular markers have been exploited in biofortification breeding in some of the related pulse crops. Several QTLs and/or SNP markers associated with Fe and/or Zn concentrations have been identified in peas (Ma et al. 2017; Gali et al. 2018), chickpeas (Upadhyaya et al. 2016), common beans (Blair et al. 2011), and lentils (Khazaei et al. 2017) that can be used in marker-assisted selection. The detailed discovery of a large number of QTLs for biofortification traits including Fe, Zn, selenium, carotenoids, and folates in different pulse crops is reviewed by Jha and Warkentin (2020).

In recent years, targeted gene-editing technologies using artificial nucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) have given rise to the possibility to precisely modify genes of interest and thus have potential application for crop improvement (Jaganathan et al. 2018). Recently, CRISPR/Cas9 and/or TALEN technologies have been used to generate mutant lines for genes involved in small RNA processing of *Glycine max* and *Medicago truncatula* (Curtin et al. 2018) and for disruption of symbiotic nitrogen fixation (SNF) gene activation in cowpea (Ji et al. 2019). These findings pave the way for applicability of gene-editing technologies for various traits of interest in legumes.

Future Outlook

Much progress has been made toward reaching micronutrient density targets for major primary staple food crops in Africa and Asia. Given the micronutrient malnutrition and hidden hunger among the masses in the developing countries fueled by the poor economy and low purchasing power of farmers involved in subsistence farming in these countries, it becomes highly imperative that genetic biofortification should be included as an inseparable component of national food security missions. By developing more than 150 biofortified varieties that have been released in 30 countries and being consumed by more than 20 million people in developing countries, HarvestPlus and its partners have developed strong evidence that biofortification intervention can help alleviate malnutrition.

With higher incidences of diabetes, heart problems, and cancers in the developing and developed countries, the use of cowpea with high protein content, high fiber, low glycemic index, and high levels of cancer-fighting antioxidants would become

popular. Little efforts have gone into breeding for higher protein and other quality traits. However, recent screenings of cowpea germplasm have shown great variability for protein content and many health-promoting factors. Therefore, there is a need to strengthen breeding efforts to develop cowpea varieties with higher protein and minerals as well as health-promoting factors. The focus on increasing the concentration of micronutrients should go hand in hand with increasing the bioavailability of micronutrients. This can be achieved by enhancing the promoters that stimulate the absorption of minerals and by reducing the concentrations of antinutrients that interfere with absorption. A beginning has been made under the HarvestPlus Biofortification Project wherein the national partner GB Pant University of Agriculture and Technology, Uttarakhand, has successfully released four cowpea varieties with high protein (25–31%), Fe (66 to 109 ppm), and Zn contents (36 to 51 ppm) (Singh 2016), but such efforts should be further concerted and strengthened. New cowpea varieties have fairly high protein content ranging from 27% to 31%, but the cowpea protein, as in other food legumes, is deficient in sulfur-containing amino acids like methionine and cysteine. Conventional breeding for such traits having limited or no genetic variability is not tenable and can be improved through biofortification by genetic engineering. Such efforts should be diligently and unscrupulously encouraged and supported by legal federal policies to pave way for a new era of fortified and safe crop varieties. The cowpea breeders should work closely with biotechnologists to quickly transfer these traits to popularly cultivated varieties in different regions. In addition to improving cowpea varieties through genetic transformation, efforts should be made to develop markers and marker-assisted selection for accelerated genotyping. The possibility of utilizing improved genome editing tools like CRISPR for precise modification within the genome so as to target specific genes of biofortification traits should leverage rapid development of biofortified cowpea varieties. Encouraging success stories of CRISPR from other crops in tweaking the expression of genes by editing the regulatory elements of Fe homeostasis genes should help give a leeway in cowpea biofortification. In a developing country like India, where maximum people do not have sufficient access to commercially fortified foods, diversified diets, and food supplements, biofortification is an acceptable cost-effective way to eliminate malnutrition.

Looking ahead, key investments will help biofortification reach its full potential. Firstly, biofortification traits must be streamlined into conventional breeding programs of secondary staples like cowpea. High micronutrient content must be included as a core trait of breeding programs, by concatenation of micronutrient-dense parental lines. Secondly, investments in high-throughput technologies and development of molecular markers linked with biofortification traits can greatly accelerate genetic gain for these traits. Finally, more investment should be made by the government and private sectors to create awareness among the farmers and consumers to go in for the biofortified crops and products so that micronutrients get bio-concentrated in the human food chain. Given the growing national and international interest for pursuing biofortification as a new, complementary intervention to address micronutrient deficiency, it is hoped that a wider array of partners and

national agricultural research systems synergize in developing the next generation of biofortified crops.

Conclusion

Micronutrients are inevitable components of both human and plant nutrition as they are essential for normal growth and development. Micronutrient deficiency leading to malnutrition is a major concern that affects one third of the world population. Among various strategies, genetic biofortification through plant breeding is considered the most viable, economical, and sustainable approach to tackle micronutrient deficiencies. This universally acclaimed potential approach can reach people living in relatively remote rural areas that have limited access to commercially marketed fortified foods and supplements. With biofortification breeding programs of primary staples attaining the intended micronutrient level targets, it is high time that similar results are replicated in secondary staples like cowpea that complement the primary staple-based diets. Moreover, nutritious crops like cowpea that are widely grown by resource-poor farmers doing sustenance farming are one of the good options for biofortification. In recent years, significant progress has been made with the release of several biofortified crop varieties that are helping to overcome micronutrient deficiencies in the target populations. Improving the nutritional profile of pulse crops like cowpeas that are an important source of protein and energy can significantly increase their consumption. Biofortification to improve the nutritional profile of pulse crops including cowpea has gained momentum in the recent past. However, there are several confrontations and challenges that require to be tackled if the consumption and cultivation of biofortified foods and crops, respectively, are to be maximized effectively.

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