Chapter 3

Biofortification: Breeding Micronutrient-Dense Crops

Wolfgang H. Pfeiffer and Bonnie McClafferty

Introduction

In life, minute things can be enormously important. Mineral micronutrients make up a minuscule fraction of the physical mass of a grain, tuber, or fruit; nonetheless, they are crucial to human health. The wide array of micronutrients—more than 20 mineral elements and more than 40 nutrients—necessary for human health, can all be provided by a well-balanced diet. However, the daily diets of large portions of urban and rural populations in the developing world consist mainly of staple foods, such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and cassava (*Manihot esculenta* Crantz), which are good calorie sources, but supply insufficient amounts of the basic micronutrients. Low in minerals, vitamins, and protein from animal and plant sources, poor-quality diets cause micronutrient malnutrition, a burden that afflicts more than one-half of the world's population (UN SCN, 2004).

Micronutrient malnutrition can have disastrous consequences for the more vulnerable members of the human family, especially poor women and preschool children in developing countries. Vitamin A deficiency is the single most important cause of total blindness in developing countries; iron deficiency anemia dramatically reduces the likelihood that mothers will survive childbirth; and even mild levels of micronutrient deficiency can affect physical and cognitive development and lower disease resistance in children. The costs of these deficiencies in terms of diminished quality of life and lives lost are staggering (www.harvestplus.org).

Despite past progress in controlling micronutrient deficiencies through supplementation and fortification, new approaches are needed to expand the reach of these interventions to the rural poor and contribute to sustainable micronutrient deficiency alleviation in burgeoning urban populations. In recent years, an alternative solution is being brought to bear on the problem of micronutrient malnutrition: biofortification of staple crops (Graham and Welch, 1996; Graham et al., 1999, 2001; Bouis et al., 2000; Pinstrup-Andersen, 2000; Underwood, 2000; Bouis, 2003). Biofortification generates nutritionally improved crop varieties through conventional plant breeding and modern biotechnology. Haas et al. (2005) and Van Jaarsveld et al. (2005) demonstrated the feasibility of the biofortification concept from a nutrition perspective. In large-scale human efficacy trials, consumption of diets based on biofortified rice high in iron and orange-fleshed sweetpotato (*Ipomoea batatas* [L.] Lam) high in provitamin A significantly improved human micronutrient status. Hence, biofortified varieties offer the hope that poor at-risk populations in developing countries will be able to meet their micronutrient requirements by consuming the staple crops in their typical diet, at no additional cost.

Plant breeding for micronutrient density began to gain legitimacy when deficiencies in micronutrients, such as iron, iodine, zinc, and vitamins, were recognized as an issue of overwhelming global public health significance and one of the major development challenges of the 21st century. In July of 2003, the Consultative Group on International Agricultural Research (CGIAR) established HarvestPlus: the Biofortification Challenge Program[1] to add food nutritional quality to its agricultural production research paradigm and capitalize on agricultural research as a tool for public health interventions.

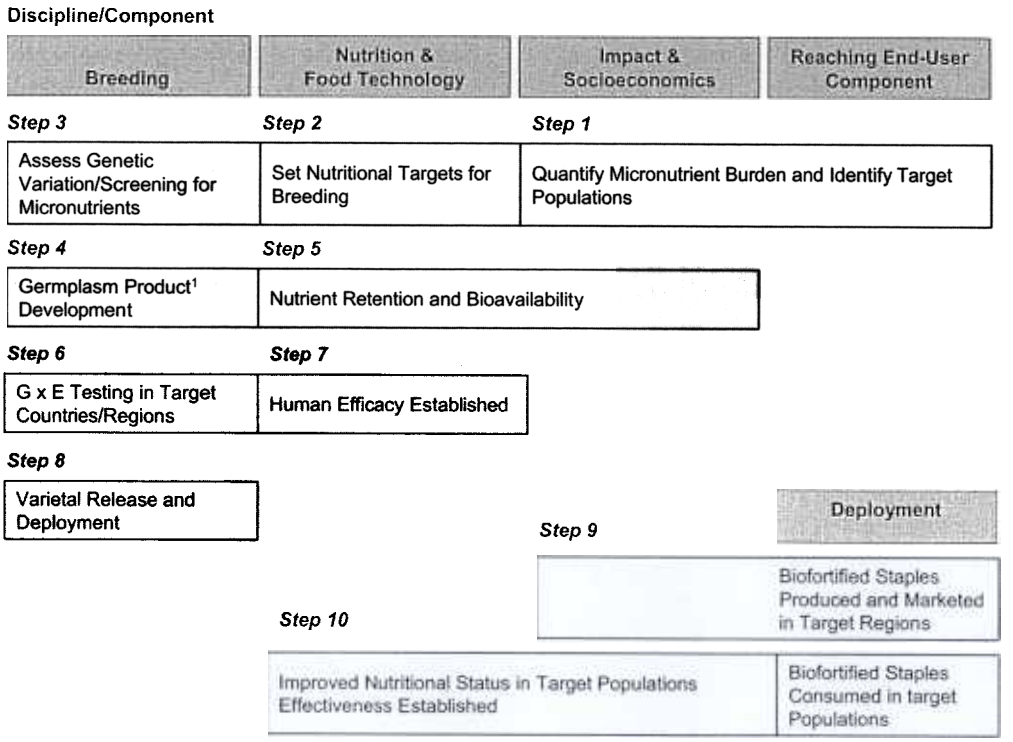
The goal of HarvestPlus is to reduce micronutrient malnutrition among poor at-risk populations in Africa, Asia, and Latin America, thereby improving food security and enhancing the quality of life. HarvestPlus seeks to bring the full potential of agricultural and nutrition sciences to bear on the persistent problem of micronutrient malnutrition. To accomplish this task, HarvestPlus has assembled a multidisciplinary global alliance of more than 150 scientists from CGIAR research centers, private agricultural research institutions, national agricultural research and extension systems (NARES), and non-government organizations (NGOs). Ten CGIAR research centers form the nexus of development of biofortified crops and their NARES partners make up a research alliance that conducts adaptive and participatory breeding as well as participatory variety selection of promising candidate varieties in target zones.

Iron, zinc, and vitamin A, three micronutrients recognized by the World Health Organization as limiting to human health, are the target micronutrients of HarvestPlus. Biofortification research conducted under the auspices of HarvestPlus focuses on a multitude of crops that are a regular part of the staple-based diets of the poor and thus indispensable nutrient sources. Full-fledged plant breeding programs are already underway for six "phase I" crops: the mega-crops—rice, wheat, and maize, plus cassava, common beans, and orange-fleshed sweetpotato. For 10 additional "phase II" crops (bananas/plantains, barley, cowpeas, groundnuts, lentils, millet, pigeon peas, potatoes, sorghum, and yams), pre-breeding feasibility studies have been completed and population development has begun.

Given that discussing the latest research and progress achieved in all these crops is beyond the scope of this chapter, we will give an overview of interdisciplinary research activities currently underway, introduce the underlying principles of breeding micronutrient-dense crops, and address the key issues along the HarvestPlus impact pathway (Fig. 3.1). For the purposes of this chapter, dedicated to highlighting new insights related to breeding micronutrient-dense crops, a clear emphasis will be placed on the current objectives, strategies, results, and activities of the plant-breeding component of this multidisciplinary program.[2]

Biofortification: a new process, a new concept

Biofortification is the process of increasing the bioavailable micronutrient density of staple crops through conventional plant breeding and modern biotechnology to achieve a measurable and positive impact on human health. There are marked differences between traditional plant breeding and crop biofortification. Traditional breeding focuses on improving traits of known economic value and developing product concepts for existing markets. Traits are targeted for selection based on whether they can provide better crop and/or utilization options to farmers, but nutritional value as a trait for selection has been largely ignored. Biofortification breeding, on the other hand, seeks to make an impact on human micronutrient status, an endeavor that entails merging breeding with nutrition and socio-



[1] Includes nutrition genomics.
Source: HarvetsPlus Impact Pathway, 2006.

Fig. 3.1. HarvestPlus Impact Pathway.

economics research to enhance traits that have measurable value in health outcomes. Biofortification breeding accesses the information it needs to identify such traits by linking directly to the human health and nutrition sectors (Nestel et al., 2006), which have become an integral part of crop improvement and product concept development.

At the core of any biofortification breeding program is a product pathway driven by potential impacts of research and nutrition (see Fig. 3.1). Collaboration between plant breeding and socioeconomics allows the exchange of information to identify target populations that consume target crops based on their micronutrient burden (see Fig. 3.1, step 1). The micronutrient burden is the burden imposed on individuals by micronutrient deficiencies and related diseases; it can be quantified using the disability-adjusted-life-years (DALYs) approach (Stein et al., 2005). Measuring the effectiveness of biofortified crops in improving human health provides a benchmark for quantifying the ultimate success of biofortification as a cost-effective public health intervention (see Fig. 3.1, step 10).

For biofortification to be successful, micronutrient levels targeted by breeding programs must be derived from nutrition goals set by nutritionists who understand the complexities of making a measurable impact on human health (see Fig. 3.1, step 2). To set target levels and determine the likely contribution to nutritional status, critical information is needed on the bioconversion/bioavailability of ingested nutrients; retention of the micronutrient after storage, processing, and cooking; human micronutrient

requirements; and potential levels of consumption by the target population. Genotypic differences in retention, post-harvest micronutrient deterioration, and concentrations of antinutrients and promoters that inhibit or enhance micronutrient bioavailability have been established. Throughout crop development, nutrition and food technology (see Fig. 3.1, steps 4–6) are engaged in assessing the magnitude of genetic variation and genotypic differences for these traits; this allows breeders to increase bioavailability (see Fig. 3.1, step 5) and determine the effect of micronutrient-dense crops or candidate varieties on micronutrient status via human efficacy trials (see Fig. 3.1, step 7).

A conceptual framework for breeding biofortified germplasm

Figure 3.2 outlines the key HarvestPlus biofortified germplasm development activities. Different research categories reflect sequentially arranged stages and milestones, and are superimposed upon a decision-tree that allows monitoring progress and making strategic and "go/no-go" decisions when goals and targets cannot be achieved. The role of nutrition, food technology, and socioeconomics in product development is illustrated in Figure 3.2.

Crop improvement activities of HarvestPlus focus, first, on exploring the available

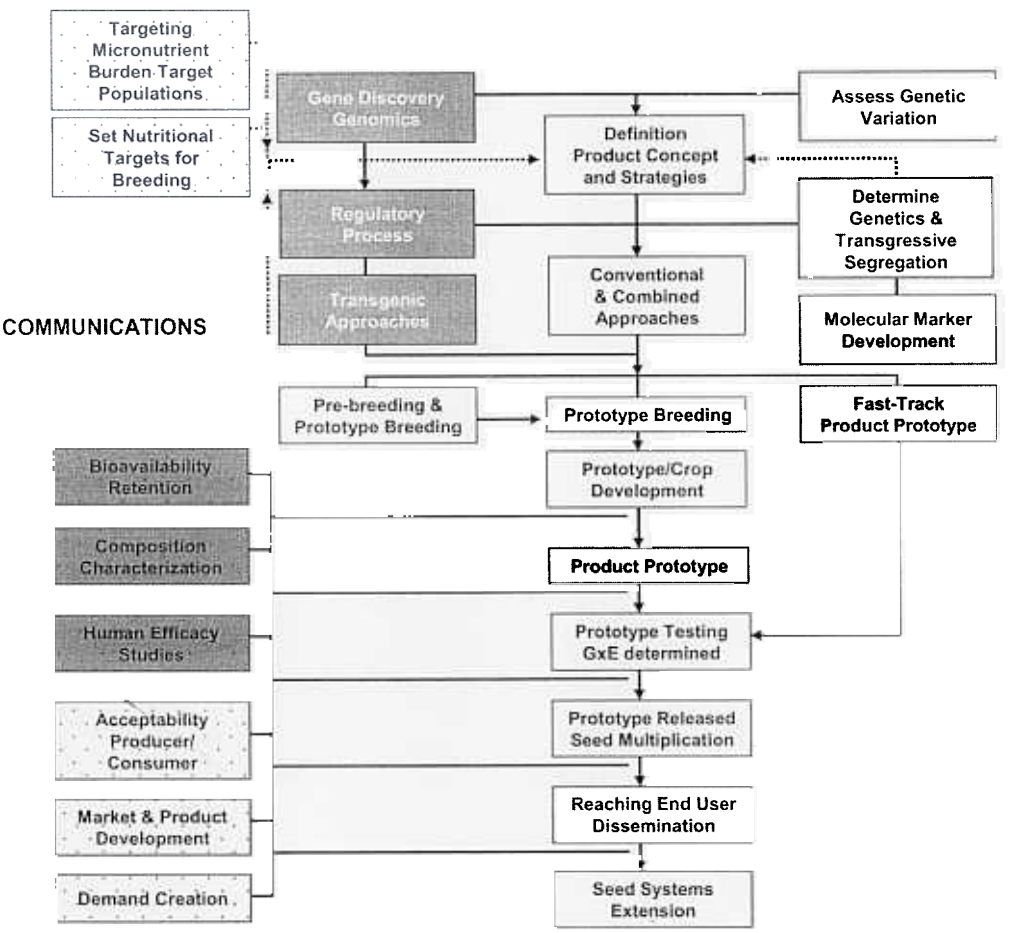


Fig. 3.2. HarvestPlus Breeding Framework.

genetic diversity for iron, zinc, and provitamin A carotenoids. At the same time (or during subsequent screening), agronomic and end-use features are characterized. The objectives when exploring the available genetic diversity are to identify: (1) parental genotypes that can be used in crosses, genetic studies, molecular-marker development, and parent-building, and (2) existing varieties, pre-varieties in the release pipeline, or finished germplasm products for "fast-tracking." Fast-tracking refers to releasing, commercializing, or introducing genotypes that combine the target micronutrient density with the required agronomic and end-use traits so they can be quickly delivered to producers and have an immediate impact on micronutrient-deficient populations.

Identifying the source of genetic variation is essential for the next breeding steps. If variation is present in the strategic gene pool (unadapted trait sources), pre-breeding is necessary prior to using the trait in final product development; if variation is present in the tactical gene pool, by definition, the materials can be used directly to develop competitive varieties. Most breeding programs simultaneously conduct pre-breeding and product enhancement activities to develop germplasm combining high levels of one or more micronutrients. If the available genetic variation suggests that target increments are unlikely to be reached, it is possible to find additional genetic variation through transgressive segregation or by exploiting heterosis. When genetic variation is absent, or micronutrient levels are insufficient to have an impact on human health, a transgenic approach may be one remaining option, e.g., for provitamin A in rice (Khush, 2002; Bouis et al., 2003; Al-Babili and Beyer, 2005). The next breeding steps involve developing and testing micronutrient-dense germplasm, conducting genetic studies, and developing molecular markers to facilitate breeding. Also, genotype × environment interaction (G × E)—the influence of the growing environment on micronutrient expression—needs to be determined at experiment stations and in farmers' fields in the target countries.

Factors related to adoption, commercialization, and product concepts

Activities aimed at reaching the end-users of biofortified crops include delivering seed to farmers and dissemination partners, and, more specifically for HarvestPlus, establishing productive research networks with national program partners and advanced research institutes, and building research capacity to enable sustainable development of biofortified crops within national research systems. However, for biofortified products that contain novel traits and, at times, altered features, product acceptance and marketability need to be assessed prior to deployment throughout an end-user value chain (seed producer, producer/grower, primary processor, manufacturer, distributor, wholesaler/retailer or retail consumer, and consumer) to develop relevant product concepts that will achieve the commercial goal. This information is generally not available for biofortified crops, and, for example, test marketing is not possible without biofortified germplasm products at hand. Hence, product concepts need to be modified and adjusted as part of an iterative process that builds on results generated by crop enhancement, nutrition, food technology, and socioeconomics, as breeding advances through recurrent crop development cycles.

The acceptance of biofortified crops by producers and consumers hinges on developing attractive trait packages without compromising agronomic and end-use characteristics. Crucial to developing such trait packages is research to understand the value farmers place on these traits and, hence, the social, economic, and cultural factors that determine crop adoption. Likewise,

consumer acceptance studies among the undernourished are essential to accurately gauge demand and identify targeted messages and marketing strategies. In many cases, trait packages must take into account the needs and demands of women and mothers as both consumer targets for nutrient-dense food and key guardians of the most nutritionally vulnerable target population, undernourished children. Developing relevant product concepts for biofortified crops relies on feedback and a continuous flow of information from socioeconomics, impact-assessment studies, and marketing and consumer behavior research (see Fig. 3.2). The outcomes of these diagnostic studies contribute to designing communication strategies that are crucial to the diffusion of innovations and product adoption (see Rogers, 1983).

Issues related to trait visibility in micronutrient-dense biofortified crops are crucial for developing product concepts. Higher levels of provitamin A carotenoids will turn endosperm, seed, or tuber color from white or light yellow to dark yellow and orange. Such color changes are important because consumers often prefer products, for example, from white-seeded or white-fleshed varieties. For consumers and producers to accept biofortified (yellow or orange) versions of their staple crops, they would have to be convinced of their health benefits. Therefore, diagnostic studies on the feasibility of achieving acceptability and behavioral changes in a target population are crucial to developing product concepts for biofortified crops, particularly transgenic crops (Chong, 2003).

In contrast to carotenoids, high mineral concentrations are not visible and do not affect sensory traits. However, trait invisibility impedes distinguishing biofortified varieties from regular varieties and raises issues associated with product identity, branding, and procurement. Thus, HarvestPlus has to consider effective formal and informal seed systems, development of markets and food products, effective communications to reach end-users, and demand creation.

Ultimately, biofortified foods and the HarvestPlus Alliance, as their architect, are expanding the existing production-driven agricultural research paradigm to include food quality. This transformation is no small task, for it is forcing traditional agricultural research institutions and scientists to cross disciplines, and human nutritionists to work with crop scientists to develop effective methods, define new protocols, and even create a new lexicon. It is working to re-cast agricultural research as a public health intervention that will reach the most vulnerable and undernourished.

Crop improvement

Existing genetic variation, trait heritability, gene action, associations among traits, available screening techniques, and diagnostic tools are criteria commonly used to identify selectable traits and estimate potential genetic gains. However, for novel traits such as micronutrients, biofortified product concepts have to consider factors associated with probability of success. These factors encompass: (1) technological goals to identify a trait that enables the desired phenotypic and nutritional performance under all production conditions; (2) legal goals to facilitate the development of a final biofortified product unencumbered by intellectual property rights or other legal barriers to development, manufacture, or sale (freedom to operate); (3) production (breeding) goals to generate a plant product containing the trait that enables the desired performance in target populations, target areas, and in all biofortified varieties without compromising agronomic performance, nutrition, or end-use quality; (4) regulatory goals to ensure qualitative traits (in this case, assured seed nutrient density level) and to facilitate the development of a "transgenic event"

embodying the trait or technology in the plant genome that meets all domestic and/or international regulatory requirements for food and feed; and (5) commercial goals to guide the design and delivery of a technology (modified from McElroy, 2004). Achieving each of these product development goals presents their own set of process-specific challenges, costs, and risks (McElroy, 2004). In the following sections, we will focus on: (1) technological and product goals in the context of biofortification; (2) factors relating to genetic advance and the likelihood of success, and their contribution to creating feasible product concepts; and (3) the methodologies used to implement them.

Product concepts

Crop enhancement methodologies and procedures for breeding micronutrient-dense crops follow the standard principles applied to traits with equivalent characteristics (e.g., number of genes and mode of inheritance). In biofortification breeding, as enabling technologies are developed, methodologies have to be tailored according to available trait diagnostics and breeding objectives. Furthermore, product concepts are specific to targeted countries/zones. For example, product concepts for HarvestPlus maize embrace a range of biofortified germplasm products for various target countries or maize-producing areas in Africa, Asia, and Latin America. These include germplasm products enriched with individual micronutrients or combinations of iron, zinc, and provitamin A (according to the micronutrient burden and target countries identified in step 1, Figure 3.1). Elite adapted genetic backgrounds of both conventional and quality protein maize (QPM) are being used as platforms to add micronutrient density. Breeding efforts at CGIAR centers and NARES are focusing on developing hybrids, but they also consider synthetics and open-pollinated varieties on a smaller scale, during a transitional period, until formal and informal hybrid seed systems are established. Agronomic superiority can more easily be achieved by replacing open-pollinated varieties with hybrids. Launching hybrids also overcomes the problem of degeneration of micronutrient density in open-pollinated varieties due to outcrossing. Depending on the target micronutrient(s), product concepts embrace white, mineral-enriched maize, yellow/orange-colored provitamin-A-dense maize, and yellow/orange maize biofortified with both provitamin A and minerals.

Due to a strong cultural preference for white maize for human consumption in Africa, Asia, and Latin America, product concepts for provitamin-A-dense maize have to consider its acceptability to producers and consumers, and feasibility studies are required to guide breeding decisions. If crops are new or non-traditional, color preferences have not usually been established. Provitamin-A-dense maize with orange-colored grain may be perceived as a new product and accepted. Visible traits confer product identity, and added nutritional value may constitute an incentive for adopting biofortified varieties.

For crops biofortified with iron and zinc, the scenario is different because the trait is invisible and does not affect sensory characteristics. Product concepts must consider farmers' criteria for changing varieties, including factors related to food and income security. Farmers usually weigh risk factors against the higher income they would obtain from increased production or improved production efficiency as a result of adopting the new technology.

Because technically genotypes and germplasm can be distinguished on the basis of morphological, biochemical, or molecular characteristics, breeders could incorporate marker traits (e.g., a morphological trait) to distinguish micronutrient-dense genotypes.

However, these markers are impractical for identifying, procuring, and labeling or

branding a product, since they are not directly linked to a mineral and not apparent to growers and consumers. In addition, breeding for these types of markers is not viable, given the added costs and negative impact on genetic progress that can result from breeding for additional traits. Hence, breeding for micronutrient density must consider strategies to keep pace with rates of progress for value-added traits, particularly yield, in non-biofortified germplasm, while simultaneously incorporating additional traits for micronutrient density.

Assessing genetic variation

Developing enabling technologies (e.g., analytical methods and high throughput screening methods to assay micronutrients) and establishing germplasm screening are prerequisites for effectively assessing genetic variation. Inexpensive rapid screening methods boost breeding effectiveness and are crucial for assessing the large number of genotypes in plant population development and coping with screening and sample turnaround requirements for crops with two or more cycles per year. A factor that poses a challenge to sampling and trait diagnostics is rapid post-harvest deterioration, particularly of tuber crops or fruits, which are harvested with high moisture content. In contrast to minerals, provitamin A carotenoids experience greater degradation during storage, drying, milling, and processing.

Initially, the lack of rapid techniques for screening cereals, legumes, and tubers for minerals and provitamin A negatively affected progress in breeding for micronutrient-dense crops. In addition, crop sampling protocols and protocols for conventional analytical methods, including sample preparation, digestion, extraction, and milling procedures, had to be developed and standardized across laboratories. HarvestPlus has made considerable investment in assessing the analytical accuracy of participating laboratories by using external quality assurance programs to allow comparison of results. Tremendous progress has been achieved in this area, and these enabling technologies are currently being validated and implemented at various CGIAR centers and national research institutes. Furthermore, recent studies have found that iron contamination (e.g., from soil), degree of milling/polishing, and seed size/seed shriveling have significant effects on mineral concentrations; earlier research considered these effects only rarely, if at all. In view of the lack of published information on micronutrient analysis and its critical role in breeding, we have included a section that describes in greater detail micronutrient analysis and related research conducted to date.

Micronutrient analysis

Mineral micronutrients make up a minuscule fraction of the physical mass of a grain, tuber, or fruit, with concentrations in parts per million (ppm) or even parts per billion range ($\mu g g^{-1} = mg\,kg^{-1} = ppm$). Typical iron and zinc concentrations in major crops range from $5\,\mu g g^{-1}$ to $150\,\mu g g^{-1}$. In crops that show genetic variation for provitamin A or β-carotene, typical concentrations range from $>1\,\mu g g^{-1}$ to $>400\,\mu g g^{-1}$ (for example, in orange-fleshed sweetpotato). Although sensitive analytical methods are required to accurately determine micronutrients, the sensitivity requirements in applied breeding may vary greatly for pre-screening large segregating populations and characterizing progenitors for crosses or candidate varieties.

Minerals

Table 3.1 contains a summary of precision analysis methods and state-of-the-art high-throughput screening methods (HTMs) that are applied and/or being tested for use in

Table 3.1. Analytical methods for micronutrients.

	ICP-OES: Inductively Coupled Plasma Spectrometer	AAS: Atomic Absorption Spectrometer	XRF: X-Ray Fluorescence Spectrometer	NIRS: Near Infrared Reflectance Spectrophotometer	Modified Perl's Prussian Blue	Modified 2,2 Dipyridal	Modified Zincon
Principle, Throughput, and Practical Considerations							
Principle	Excitation and emissions at various wavelengths	Absorption	X-ray fluorescence	Absorption at wavelengths in the near infra-red	Color reaction	Color reaction	Color reaction
Digestion required	Yes	Yes	No	No	No	Yes	Yes
Sample destructive	Yes		No	No	Yes	Yes	Yes
Throughput	Up to 2.5 min per sample regardless of number of elements analyzed	~2.5 min per element	5–10 min per sample depending on the number of elements analyzed	~2 min per sample	~4 min per sample	~4 min per sample	~4 min per sample
Pros/comments	Total recovery of nutrient achieved but subject to type of digestion procedure used; good sensitivity	Total recovery of nutrient achieved but subject to type of digestion used; good sensitivity; low cost of purchasing equipment	Total recovery of nutrient achieved; good sensitivity with more expensive models; low cost of bench-type equipment; no digestion step	Low cost	Low cost	Low cost	Low cost
Cons/comments	Gas required; high start-up cost; digestion step; destructive	Gas required; digestion step needed; destructive analysis; samples require milling	Problems with Al and Ti sensitivity; samples require milling	Calibration cost can be high; requires ongoing addition of calibrations	Labor-intensive; semi-quantitative	Semi-quantitative	Semi-quantitative
Analytical Capability							
Iron	Yes	Yes	Yes	Yes	Yes; separation into high and low groups	Yes; separation into high and low groups	Yes; separation into high and low groups
Zinc	Yes	Yes	Yes	Yes	No	No	Yes; separation into high and low groups
Contamination indicators	Yes	No	Yes, but sensitivity for Al is inadequate as contaminant indicator	Not tested	No	No	No
Other elements	Yes	Yes	Yes	Yes, although data limited in this area	No	No	No
Other relevant compounds—promoters & inhibitors	No		No	For example, protein, carotenoids, antinutrients	No	No	No

Table 3.1 *Continued.*

	ICP-OES: Inductively Coupled Plasma Spectrometer	AAS: Atomic Absorption Spectrometer	XRF: X-Ray Fluorescence Spectrometer	NIRS: Near Infrared Reflectance Spectrophotometer	Modified Perl's Prussian Blue	Modified 2,2 Dipyridal	Modified Zincon
Precision							
Application	Plant and soil material	Plant and soil materia	Plant and soil material	Plant material	Plant material	Plant material	Plant material
Accuracy for Fe	High	High	High	High	High	High	No
Accuracy for Zn	High	High	High	High	No	No	High
Accuracy for carotenoids	No	No	No	High to medium-high confirmed for different crops	No	No	No
Accuracy for total carotenoids	No	Nc	Nc	High to medium-high confirmed for different crops	Nı	No	Nc
Accuracy for β-carotene	No	No	No	High to medium-high confirmed for different crops		No	No
Accuracy for minerals	Very accurate	Very accurate	Very accurate	Separates out only high- and low-nutrient genotypes	Separates out only high- and low-Fe genotypes	Separates out only high- and low-Fe genotypes	Separates out only high- and low-Zn genotypes
Economics							
Start-up costs (equipment)	$50,000–$300,000 depending on make and model	$10,000–$40,000 depending on make and model	$50,000–$350,000 depending on make and model	$60,000–$90,000		minimal	minimal
Running costs	Varies from lab to lab; between $4.00 and $7.00/sample	Varies from lab to lab; e.g., Argon between $0.22 and $2.00/sample; labor the greatest cost in analysis	$15–$25 AUD/sample for XRF analysis; no cost for gas, just instrument upkeep and labor	$0.5–$2.00/sample; dramatically decreasing costs/measurement as more components are measured	0.5–$1.00/sample	$0.5–$1.00/sample	$0.5–$1.00/sample
Application in Breeding							
Recommended application in breeding	Pre-screening in population development and validation of Fe- and Zn-dense genotypes identified by rapid screening techniques	Pre-screening in population development and validation of Fe- and Zn-dense genotypes identified by rapid screening techniques	Pre-screening in population development and validation of Fe- and Zn-dense genotypes identified by rapid screening techniques	Pre-screening in population development for minerals and provitamins A; precison analysis for carotenoids and β-carotene for certain crops	Pre-screening in population development	Pre-screening in population development	Pre-screening in population development

Source: James Stangoulis, School of Agriculture and Wine, University of Adelaide, Waite Campus.

breeding different crops. For precision analysis, the Inductively Coupled Plasma Argon Optical Emission Spectrometer (ICP), Atomic Absorption Spectrometer (AAS), and X-Ray Fluorescence Spectrometer (XRF) allow identification of a wide range of micronutrients, including elements, such as phosphorus, which is indicative of the antinutrient phytate. The ICP is the current method of choice for quantifying elements, such as aluminum, which has been proposed as an indicator of contaminant iron. Various HTMs are applied in pre-screening to reduce the large number of samples in populations segregating for micronutrient concentration to a more practical number for subsequent, more expensive high precision analyses. Depending on the method used, an approximate 66% proportion for more qualitative colorimetric methods (Modified Perl's Prussian Blue) to a 75–85% proportion for semi-quantitative methods (2,2 Dipyridal) of "lows" can be discarded. The accuracy of semi-quantitative methods can be increased by using computerized systems with image analyzers. Correlations between iron determined by ICP and the 2,2 Dipyridal colorimetric method in rice, wheat, maize, sweetpotato, and cassava ranged between 0.88 and 0.98 (James Stangoulis, personal communication). Colorimetric techniques are simple, fast, and low-cost, but destructive because samples must be milled. Elements indicative of contamination are not determined with color-staining techniques; they need to be quantified during subsequent precision mineral analysis.

The Near-Infrared Reflectance Spectrophotometry (NIRS) method relates a sample's reflectance of near-infrared light to its chemical composition and covers wavelengths between 730 and 2500 nm emitted by major plant compounds, such as oil, starch, cellulose, water, and protein. In breeding, NIRS is routinely used to determine, for example, grain protein, which it can measure with high accuracy. The latest research has shown that NIRS has potential to predict iron and zinc with sufficient precision for pre-screening, although the causality of the association is not yet understood. Correlations between iron and zinc determined by ICP and NIRS in potato, sweetpotato, and beans range between 0.77 and 0.85 (Wolfgang Grüneberg, CIP [International Potato Center], and Steve Beebe, CIAT [International Center for Tropical Agriculture], personal communications), and NIRS has been used to separate high-, medium-, and low-iron barley genotypes (James Stangoulis, personal communication). NIRS is environmentally friendly (no reagents are involved) and easy to operate, but it requires continuing calibration to make sure the calibration set includes samples representative of the genetic variation used in breeding, and it must also account for environmental impact on readings. The section "Provitamin A Carotenoids" later in this chapter elaborates further on NIRS in the context of carotenoid analysis.

Contamination in mineral analyses

Detecting contamination while assaying micronutrient concentration is complicated, and references are not yet available to guide researchers. In the past, contamination has, in general, not been addressed in the literature; extremely high iron concentrations that have been reported are likely due to contamination.

Contamination—for example, by iron—can result from soil, dust, metal parts or paint in threshing equipment, rubber products (particularly silicon and neoprene), sample preparation, or seed handling. Zinc is less subject to be a contaminat than iron. Research to establish protocols and guidelines for determining approximate thresholds and, in particular, for developing corrective measures, is currently underway. However, diagnostics for contamination cannot substitute for validating micronutrient concentrations of selected genotypes

through additional screening. Procedures that can eliminate sources of contamination in experimentation—for example, preventing lodging in cereals or washing tubers before sampling—should be routine. Washing maize grain samples to eliminate iron contamination has been investigated with varying degrees of success (Kevin Pixley, CIMMYT; James Stangoulis, personal communications). Using appropriate spatial experimental designs with replicated standards or checks to estimate error is also warranted, particularly in unreplicated nurseries.

One approach for detecting contamination entails using indicator elements that are: (1) abundantly found in soil, dust, or equipment; (2) uniform in concentration of contaminating fractions (e.g., of soil); and (3) reproducibly released and easily measured. However, contamination-indicator elements must be absent in plants or seed, or present only in trace amounts. Earlier research investigated Al, Ti, and Cr (or a combination of these) for their potential to act as indicator elements, and attempted to establish threshold levels/bands. Other elements may also be suitable, but good indicator elements for soil are, by definition, very hard to determine accurately in plant tissues, if any soil at all is present. To date, research in this area has identified Al as the most suitable indicator element. Effectively correcting for soil/dust contamination is complicated by the large variety of soil types and the varied ratios of Al, Ti, Cr, and Fe and Zn in plant sample analyses, which result, for example, in different recovery rates, depending on particle size/surface area; hence, confidence in such corrections is not high.

Statistical approaches use population parameters to identify values that fall outside the range expected for an assumed normal distribution and are probably erroneously high. The accuracy of detection increases when data from replicated trials or replicated check varieties are available. These two approaches are complementary and should be used in combination.

Data from mineral analyses conducted at Waite Analytical Laboratories (Adelaide, Australia) and from micronutrient screening of cereal, legume, and tuber crops provided by HarvestPlus crop leaders have been studied to establish tentative Al thresholds for iron contamination. The criteria used to examine contamination consisted of analyzing and comparing data subsets with different Al ranges and using replicated check data to validate results in combination with statistical outlier tests and correlations among elements. Results suggest that Al concentrations of more than 5 to 10 $\mu g g^{-1}$ are frequently associated with contaminant Fe. Analyses also revealed that significant correlations between Fe and Al levels in adapted genotypes generally indicate Fe contamination. Eliminating data for samples with Al values >10 $\mu g g^{-1}$ reduced the average correlation between Fe and Al across datasets/crops from r = 0.35 to 0.18; including only data for samples with Al < 5 $\mu g g^{-1}$ further reduced the correlation to r = 0.11. These findings coincided with results from statistical analyses.

Effects of milling/polishing

Minerals in rice, wheat, maize, and other cereals are concentrated in the aleurone and embryo, as shown in Figure 3.3 (Ozturk et al., 2006); mineral concentration in the endosperm is much lower and decreases sharply toward the center of gravity of the kernel. During polishing/milling, the mineral-containing aleurone layer and embryo are completely or partially removed; small differences in wheat flour extraction rates or in rice polishing can, thus, have an over-proportional effect on micronutrient concentration. In wheat, significant portions of non-endosperm particles are retained for flour extraction rates >80%; similarly, mineral concentrations are significantly

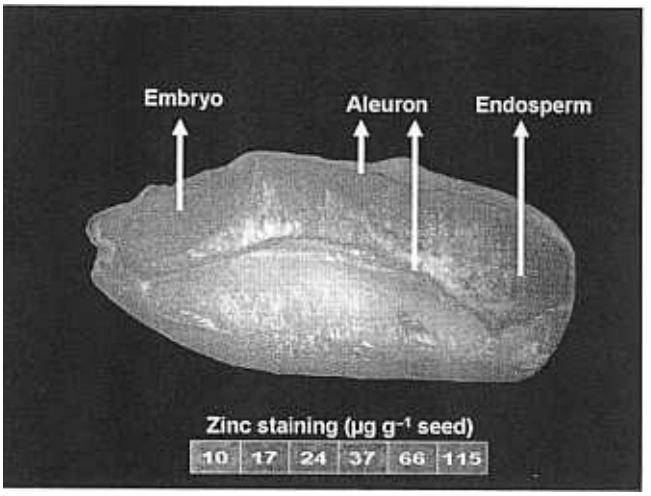


Fig. 3.3. Localization and staining of zinc in wheat seed (*Triticum aestivum* L., cv. Balatilla). Seed was stained with Dithizone (DTZ) 52 days after anthesis and Zn content analyzed with ICP-OES (source: Ozturk et al., 2006).

higher in under-polished rice. Mineral concentrations in wheat grain and endosperm are closely associated, which contrasts with the findings for rice: extensive experience at the International Rice Research Institute (IRRI) revealed a poor association between mineral concentrations in brown rice and polished rice (Gerard Barry, personal communication), although a recent study by Sison et al. (2006) found a close correlation. Research on how the degree of polishing/milling affects micronutrient concentration is complicated, and researchers are simultaneously testing and modifying non-contaminating equipment suitable for milling small samples (such as seed from individual plants). Standardized screening protocols have been developed and are now being validated and implemented in breeding projects to achieve a level of standardization that would permit data comparison.

Micronutrient concentration versus content

Special care in milling is warranted when assessing seed of, for example, wild relatives of crop species, genetic stocks, inbred lines, and unadapted germplasm or genotypes that may have small, shriveled seed and/or incomplete seed set. Biotic and abiotic stress or other production constraints may have the same effect on seed and seed set in adapted genotypes. If the remaining portions of a seed fraction—for example, the embryo—have high micronutrient concentration, concentration levels can be inflated. Furthermore, shriveled seed may require a disproportionate degree of milling or polishing to make processed products that satisfy commercial or laboratory standards.

Seed shriveling, wrinkling, and weathering can have dramatic effects on grain micronutrient density, given that micronutrient concentration in the embryo and seed coat is much higher than micronutrient levels in the endosperm. The seed coat to endosperm ratio is high, which can result in elevated micronutrient concentrations, i.e., the "concentration" effect. The concentration effect can result when fewer grains act as the micronutrient sink because few grains per spike have been produced due to sterility or poor seed set. In plump seed, the seed coat

to endosperm ratio is much lower, causing a "dilution" effect. Because micronutrient concentration is generally determined on whole grain, concentration levels in shriveled seed can be overestimated (Cakmak et al., 2000; Imtiaz et al., 2003).

Figure 3.4 displays iron concentration versus content in 438 synthetic wheat accessions. The variation in content for narrow concentration ranges (e.g., 48–52 $\mu g g^{-1}$) can be crucial in determining micronutrient content (μg seed^{-1} or, in certain cases, μg plant^{-1}) rather than micronutrient concentration ($\mu g g^{-1}$) when characterizing germplasm. In hybrid crops, correlations between mineral content and mineral concentration can be significantly affected by the concentration effect (e.g., in inbred or sibbed lines) and warrant consideration in breeding. There are few reports in the literature regarding content within the context of mineral accumulation in conventional germplasm and transgenic materials.

Grain yield, agronomic performance, and end-use quality attributes (e.g., protein concentration) of large- or plump-seeded adapted genotypes are often compared with those of non-adapted genotypes with small, shriveled grain. Since non-adapted genotypes have higher grain protein concentration due to the concentration effect and regularly produce lower grain yields, correlations between these traits and micronutrient concentration can be overrated. Correlations based on content are usually lower and can partially remove the masking effect of seed size and shriveling. These factors must be taken into account when comparing different types of germplasm or in germplasm selection.

Provitamin A carotenoids

Spectrometric Measurement The quantification of major carotenoids is a challenging task; one of the difficulties results from the

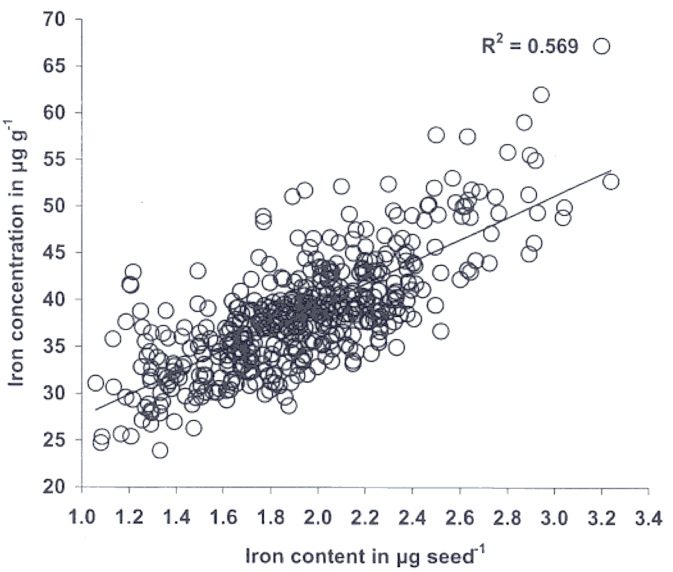


Fig. 3.4. Iron concentration and content measured on 438 hexaploid wheat synthetics accessions (reconstituted wheat from crosses between 4X wheat and *T. tauschii*; AABBDD genome) grown in field experiments at Cd. Obregón, Sonora, Mexico (data source: I. Ortiz-Monasterio, CIMMYT, Mexico).

variation in the carotenoid composition of different crops (Rodriguez-Amaya and Kimura, 2004; Kimura et al., 2007). Knowing a crop's carotenoid composition is essential for determining which measuring method to use. Due to its sensitivity and selectivity, high performance liquid chromatography (HPLC) is the method of choice to quantify individual carotenoids and their isomers. Other methods may not distinguish between individual carotenoids that differ in their provitamin A activity. However, HPLC equipment is more specialized and more expensive to run. For example, cost per sample can range between US$50 and US$70. The low throughput of 15–45 samples per day makes it unsuitable for rapid screening purposes.

Spectrophotometric methods are generally simpler to use. However, they have less capacity to distinguish between different carotenoids and their isomers. For example, a thin-layer chromatography (TLC) method only separates the three different carotenoid groups (β-carotene and α-carotene; β-cryptoxanthin; and lutein and zeaxanthin) but does not detect the presence of trans and cis isomers of β-carotene; cis isomers are known to have lower provitamin A activity and can be present in significant amounts compared with trans isomers.

It may be adequate to use a spectrophotometric method, for example, on cassava or orange-fleshed sweetpotato, where there is typically one major carotenoid—all trans β-carotene. In maize, however, the major carotenoids are zeaxanthin and lutein, which do not show provitamin A activity, but have much smaller amounts of β-carotene and β-cryptoxanthin. With TLC, lutein and zeaxanthin interfere with the detection of other carotenoids, for example, β-carotene. Hence, for maize, use of HPLC is necessary.

Near-Infrared Reflectance Spectrophotometry Carotenoids show absorption in the visible and infrared regions of the electromagnetic spectrum, and NIRS has revealed potential for screening a range of crops for total carotenoids and provitamin A. Brenna and Bernardo (2004) applied NIRS to determine carotenoids and the vitamin A precursors β-carotene and β-cryptoxanthin, which are relevant to breeding biofortified maize. Cross-validation procedures indicated close associations between HPLC values and NIRS estimates for major carotenoids. In sweetpotato and cassava, β-carotene predominates among total carotenoids, and NIRS screening of the two crops revealed correlations between total carotenoids/β-carotene, as determined by HPLC and NIRS estimates, ranging from >0.80 to >0.90 (Wolfgang Grüneberg, CIP; Thomas zum Felde, personal communications).

Based on experience to date, NIRS shows good potential for estimating carotenoids and provitamin A carotenoids with high or medium-to-high precision; determine iron and zinc with the sensitivity required for pre-screening; and predict with high accuracy antinutrients, such as phenolic compounds and promoters, and value-added traits, such as protein, oil, or kernel hardness, which affect flour yield in milling. Since multiple traits can be determined on the same sample, the costs per compound will be proportionately lower. Hence, NIRS may provide a very inexpensive and rapid method for screening large numbers of genotypes for a wide array of traits.

Visual screening of provitamin A carotenoids

The crop-specific variation in the carotenoids profile reflects differences in the association between provitamin A concentration and visual color intensity; it also determines the suitability of using color intensity for visual grading or selection with color charts when pre-screening for provitamin A. Color charts can be used for cassava (Chavez et al., 2005) and orange-fleshed sweetpotato (Zhang and

Xie, 1988; Simonne et al., 1993), and for crops in which β-carotene or provitamin A constitutes the major portion of total carotenoids. For maize, visual color is dominated by the non-provitamin A precursors zeaxanthin and lutein, and inexpensive high-throughput visual selection can only be applied to separate white or light yellow maize grains from dark yellow and orange color types.

Genetic variation—germplasm screening

The availability of genetic variation for micronutrient density is essential for determining the feasibility of achieving meaningful increments through conventional breeding and high rates of genetic progress under selection (G_s) ($G_s = i\sigma_p h^2$, where i is selection intensity, σ_p is phenotypic standard deviation, and h^2 is heritability). Breeders can capitalize on additive gene effects, transgressive segregation, heterosis, and maternal effects to improve micronutrient density. When the required genetic variation is not available, transgenic approaches can provide novel and additional sources of variation to increase provitamin A or iron—via ferritin—in the endosperm and achieve the target micronutrient density (Bouis et al., 2002; Nandi et al., 2002; Matthews et al., 2003; Vasconcelos et al., 2003; Taylor et al., 2004; Al-Babili and Beyer, 2005; Paine et al., 2005; Shewry and Jones, 2005; Sautter et al., 2006; Khalekuzzaman et al., 2006). In the future, breeding will likely combine both conventional and transgenic approaches.

Screening objectives entail assaying representative samples of the genetic diversity for micronutrient density contained in the tactical and strategic gene pools, along with agronomic and end-use quality features of trait-source genotypes. To date, only a relatively small portion of the existing genetic diversity for micronutrients has been evaluated, and an even lower proportion of the genetic diversity for antinutrients and promoters has been assessed. Evaluating all accessions of each relevant species conserved in gene banks is beyond the scope of this chapter, as gene banks at CGIAR centers alone preserve more than 530,000 accessions in-trust (see http://www.cgiar.org/impact/accessions.html). Because of these large numbers of accessions, current screening by breeding programs under HarvestPlus is focusing on core collections. Screening and research on screening methodology need to be expanded to include generic and crop-specific inhibitors and promoters for bioavailability. Future searches for variation outside core collections may employ state-of-the-art geographical information system (GIS) and molecular tools to enhance screening effectiveness. These modern techniques allow targeting accessions that are most likely to possess untapped genetic variation based on, for example, phytogeography and ancestral, genetic, or functional genomic relationships.

Micronutrient concentrations are affected by micro-environmental variation, G × E interaction, germplasm type, and numerous other factors. Consequently, published data on micronutrient genetic diversity reveal significant variation in average values and genotypic variation per se among crops and within crop species. Thus, when interpreting these data, one must consider differences that can produce error such as sampling, milling, analytical protocols, and the type of experimental screening design used.

Ranges in micronutrient concentrations reported in the literature reveal there is significant genetic variation in barley (Ma et al., 2004), beans (Beebe et al., 2000; Nunez-Gonzalez et al., 2002; Wissuwa, 2005), cassava (Simonne et al., 1993; Maziya-Dixon et al., 2000; Chavez et al., 2000), cowpea (Farinu and Ingrao, 1991), maize (Bänziger and Long, 2000; Mi et al., 2004), rice (Gregorio et al., 2000), sorghum

(Reddy et al., 2005; Kayodé et al., 2006) and wheat (Feil and Fossati, 1995; Cakmak et al., 2000; Ortiz-Monasterio and Graham, 2000). In transgenics, there is genetic variation for provitamin A in potato (Taylor et al., 2004; Ducreux et al., 2005), iron in rice (Lucca et al., 2000; Qu et al., 2005; Khalekuzzaman et al., 2006), and β-carotene in rice (Paine et al., 2005; Parkhi et al., 2005). White and Broadley (2005) provided a recent review of genetic variation for minerals.

Maximum micronutrient levels are frequently present in the strategic gene pool, in genetically distant sources, such as wild relative species, landraces, or germplasm unadapted to the agroecology of the target environment (Cakmak et al., 2000; Zeng et al., 2004). The difficulty is accessing the genetic variation in unadapted sources, and the extent to which variation in the tactical gene pool can be recovered depends on factors such as genetic distance, differences in ploidy levels, and trait linkages. In prebreeding, eliminating unfavorable traits associated with the target trait causes "linkage drag," which, depending on its magnitude, adds to product development time and costs. Since trait recovery from gene sources in the strategic gene pool varies, we used the genetic variation present in the tactical gene pool (Fig. 3.5) to predict progress in the shorter term.

Figure 3.5 displays average (baseline) and maximum values for iron (Fig. 3.5a) and zinc (Fig. 3.5b) in adapted germplasm. For rice, HarvestPlus used data for polished rice because brown rice is rarely consumed. For both iron and zinc, the short-term exploitable variation in adapted germplasm is of similar magnitude for cereals and legumes, and lower for tuber crops and rice, regardless of the baseline level. The variation for iron in beans is higher than in other crops; HarvestPlus has found an approximate increase in the maximum of 20 $\mu g g^{-1}$ resulted from a breeding cycle for high-iron beans (Steve Beebe, CIAT, personal communication). Maximum Fe values reported in the literature for certain crops could be up to 10 times higher than those encountered in later studies and likely coincide with the maximum Al values reported. HarvestPlus has found that maximum values for β-carotene (fresh weight basis) are about 9 $\mu g g^{-1}$ in cassava and >300 $\mu g g^{-1}$ in orange-fleshed sweetpotato, whereas for provitamin A in maize (dry matter basis), they are about 15 $\mu g g^{-1}$.

Setting nutritional target levels

The available genetic variation allows the prediction of the magnitude of micronutrient increments that can be added through breeding. However, only a portion of an increment contributes to human micronutrient status; this portion, the bioavailable amount, largely depends on how much nutrient is lost from crop harvest until ingestion and on the bioavailability of a nutrient once ingested. Critical information needed to set nutritional target levels for breeding (for a target country) includes the amount of nutrient retained after storage, processing, and cooking; micronutrient bioconversion/bioavailability in a typical diet once the nutrient is ingested; and nutrient requirements of a target population (Institute of Medicine, 2001; Nestel et al., 2006; White and Broadley, 2005). The daily micronutrient intake supplied by a crop must also be considered when setting target levels. Many of these parameters are interrelated in a highly complex manner, since human micronutrient status, dietary composition, and health status affect bioavailability (for example, for β-carotene: β-carotene absorbed/β-carotene in food) and its components' bioaccessibility (β-carotene freed/micronutrient in food), bioconversion (retinol formed/β-carotene absorbed), and bioefficacy (retinol formed/β-carotene in food). A more detailed discussion of these factors is beyond the scope of this chapter.

As a starting point, when initially setting tentative target levels without detailed

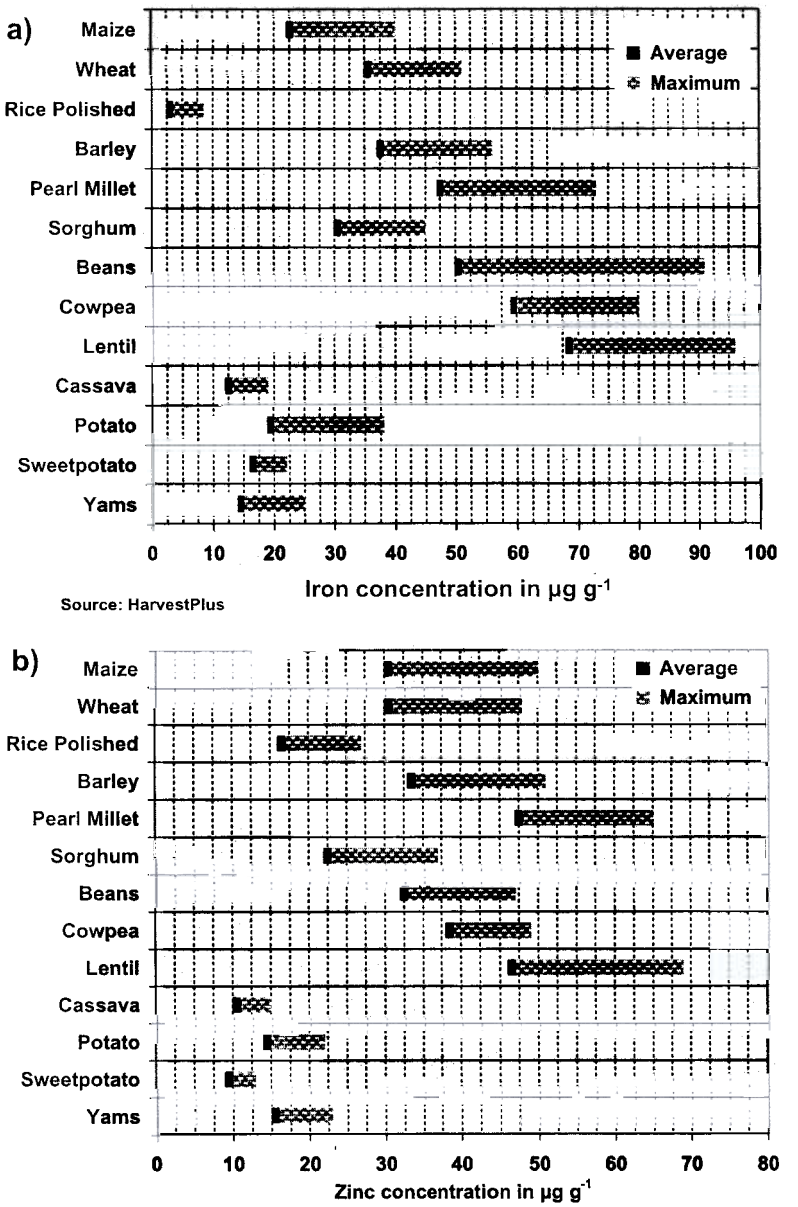


Fig. 3.5. Typical average and maximum concentrations for iron (a) and zinc (b) for adapted genotypes evaluated in field experiments for major cereals, legumes, and tubers (data source: HarvestPlus database; data provided by HarvestPlus crop leaders and published/unpublished sources).

information at hand for accurate assessment, bioavailability of zinc can be assumed to be 25% and bioavailability of iron to be 5% for legumes (e.g., beans, lentil, cowpea) and cereals with significant phytate concentrations (e.g., wheat, maize, sorghum, pearl millet, barley). For tubers (e.g., cassava, potato, sweetpotato, yams) and low-phytate rice, 10% bioavailability can be assumed.

Within a context of general assumptions, Figure 3.6 (a, b, c) illustrates the relationship between micronutrient intake and the micro-

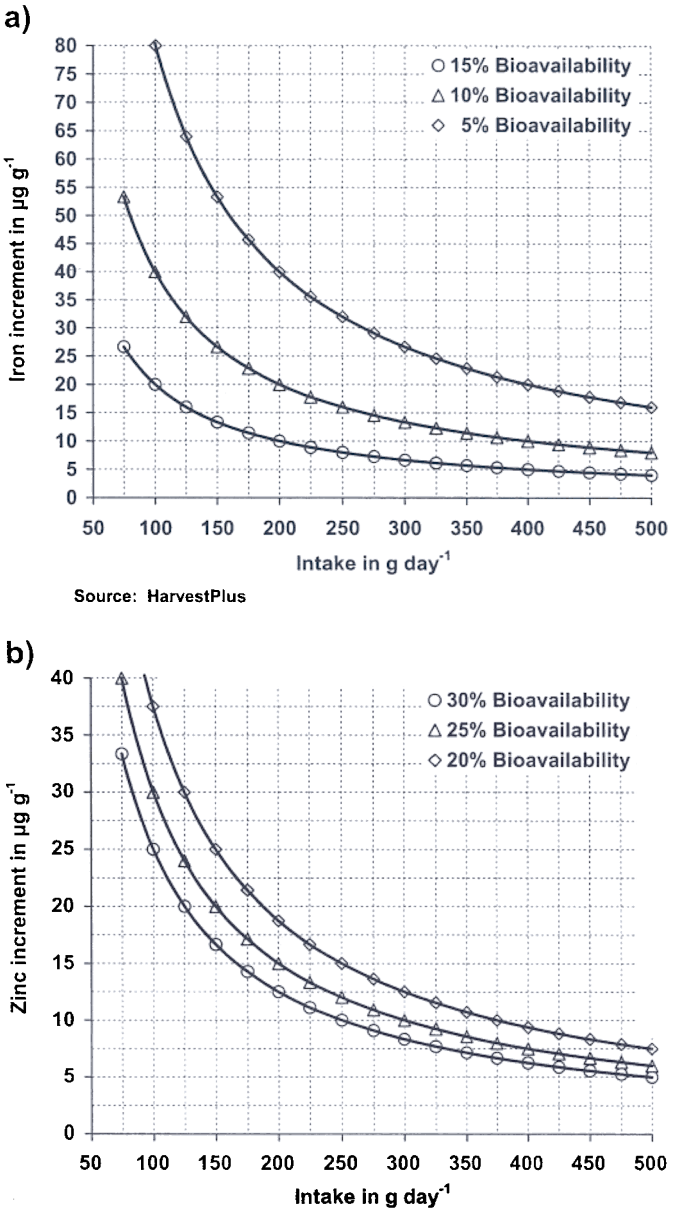


Fig. 3.6. Micronutrient increments from a baseline concentration for a measurable biological impact for women from a public health perspective for various intake levels assuming 100% retention. For iron (**a**), assumed bioavailability is 5% and 10% based on an 8 mg/day^{-1} requirement. For zinc (**b**), assumed bioavailability is 25% based on a requirement of 3 mg/day^{-1}. For β-carotene/provitamins A (**c**), assumed β-carotene/provitamins A:retinol bioconversion rates are 3:1, 6:1, and 12:1, assuming the crop provides 50% of the Estimated Average Requirement (EAR) based on a requirement of 500 mg Retinol Activity Equivalents (RAE)/day^{-1}.

nutrient increment from the baseline concentration needed to make a measurable biological impact on women of childbearing age from a public health perspective (for iron [3.6a], zinc [3.6b], and β-carotene/provitamin A [3.6c]). In Figure 3.6, 100% retention has been assumed to allow generalizations across crops. In Figure 3.6, target

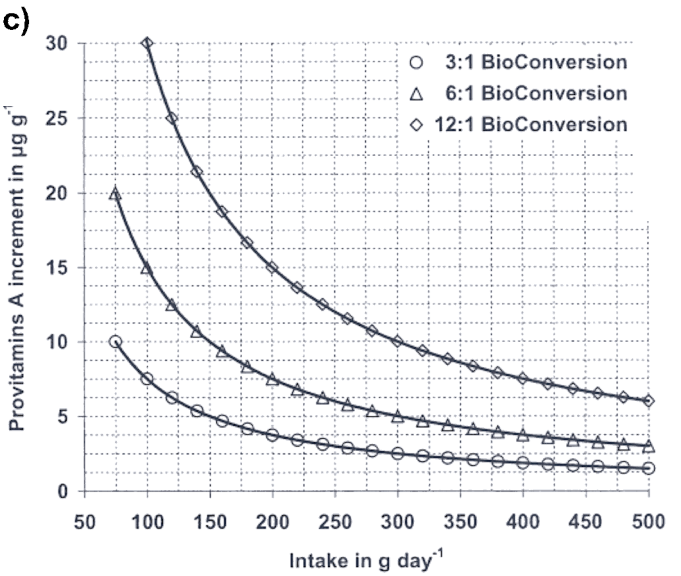


Fig. 3.6. *Continued.*

increment functions are given for iron for 5%, 10%, and 15% bioavailability (Fig. 3.6a), for 20%, 25%, and 30% biovailability for zinc (Fig. 3.6b), and for β-carotene/provitamin A, for bioconversion rates for β-carotene/provitamin A to retinol of 12:1, 6:1, and 3:1 (Fig. 3.6c).

The feasibility of reaching nutritional target increments through conventional breeding largely depends on bioavailability (provitamin A: retinol bioconversion) and intake (Fig. 3.6). There is scope for increasing micronutrient density in the shorter term via transgressive segregation, heterosis, and maternal effects beyond the variation displayed in Figure 3.5, and for discovering higher levels in the tactical gene pool. Figure 3.6 implies, in combination with the variation displayed in Figure 3.5, that in the shorter term, target increments of zinc and provitamin A can likely be reached in most crops. The genetic variation for zinc in varieties, germplasm lines, and parental stocks, especially of cereal and legume crops, is high (see Fig. 3.5b; White and Broadley, 2005). However, due to the substantially lower bioavailability of iron when compared with zinc, significantly higher micronutrient increments have to be added to reach nutritional target levels and achieve a measurable impact on human health.

The bioavailability of iron and zinc is associated with the presence of antinutrients and/or the lack of promoter substances for micronutrients (White and Broadley, 2005). Since an increase in bioavailability translates into a proportional decrease in the nutritional target increment (increasing iron bioavailability from 5% to 10% reduces the target increment by 50%), strategies for breeding micronutrient-dense crops should consider indirect breeding for increased bioavailability, increased retention, or reduced post-harvest micronutrient deterioration, in addition to direct breeding for increased concentration. Although not yet well understood, breeding for increased bioavailability via conventional (Welch et al., 2000; Oikeh et al., 2003) or transgenic approaches (Lucca et al., 2000; 2001; Drakakaki et al., 2005) offers tremendous potential (Hambidge et al., 2004).

Breeding for increased bioavailability

Breeding for micronutrient bioavailability per se is greatly limited by the lack of diagnostic tools for large-scale, rapid germplasm evaluation, such as adequate in vitro and/or animal bioavailability models. Current studies are exploring the feasibility of dissecting overall bioavailability into its causal components, such as antinutrients and promoters, which can be addressed by breeding. Ongoing exploratory research is investigating the feasibility of breeding for inhibitors/enhancers from both the crop improvement and human nutrition perspectives. Breeding studies entail determining the genetic variation for antinutrients and promoters, the magnitude of trait expression/stability through G × E studies, trait heritability, and associations with agronomic and end-use quality traits. Screening methods are being evaluated in a parallel effort, whereas nutrition research and food science are investigating bioavailability and nutritional impact using in vitro and animal bioavailability models and, subsequently, efficacy and retention studies involving human subjects.

Phytate occupied center stage in past and current research on antinutrients (Raboy, 2000). Genetic variation for phytate has been reported in numerous crops: cowpea (Farinu and Ingrao, 1991), beans (Muzquiz et al., 1999), and sorghum (Kayodé, et al., 2006). Low-phytate mutants are available and have been found in barley (Dorsch et al., 2003; Bregitzer and Raboy, 2006), maize (Raboy et al., 2000; Shi et al., 2003; Shukla et al., 2004), and wheat (Guttierie et al., 2004). Transgenic research focuses on the enzyme phytase (potato, Ullà et al., 2003; rice, Hong et al., 2004; maize, Drakakaki et al., 2005). Introduction of phytase in rice (Lucca et al., 2000), in combination with ferritin in maize (Drakakaki et al., 2005), and co-expression of phytase, ferritin, and a metallothionein-like gene in rice (Lucca et al., 2001a,b) significantly increased iron absorption/bioavailability and demonstrated the potential of transgenic approaches to capitalize on higher micronutrient concentration and increased bioavailability.

Lowering phytate concentration can have adverse effects on human health and must be researched by nutrition experts. Safe threshold levels have to be established before addressing the trait in breeding crops for human consumption. The viability of addressing phytate (or heat-stable phytase) in crop improvement via selecting for phytate:zinc or phytate:iron molar ratios also depends on how the crop is consumed. For example, when wheat is milled, there is an over-proportional reduction of phytate (compared with iron and zinc) with decreasing flour extraction rates; in white flour (72% extraction according to international trade standards), iron and zinc concentrations are reduced by approximately 50–60% compared with concentrations in the whole grain, whereas phytate concentration is reduced by about 90%. This over-proportional reduction causes a concomitant decrease in the phytate:mineral molar ratio and, hence, in increased bioavailability. Thus, lowering phytate via breeding, may not have the desired effect if wheat is predominantly consumed as white flour products. Phytate can also be significantly reduced by processing methods (Mamta and Darshan, 2000).

Selecting for increased nutrient retention can increase the micronutrient concentration "on the plate" and lower target increments. Significant genotypic differences in retention that could be exploited in breeding have been found, for example, in cassava and yams for provitamin A and, to a lesser extent, for minerals; evaluation of the genetic variation for micronutrient retention in other crops is also warranted. Micronutrient retention has been related to factors associated with flour extraction rate in wheat (e.g., grain hardness, texture, grain shape) and degree of polishing in rice.

Genetics

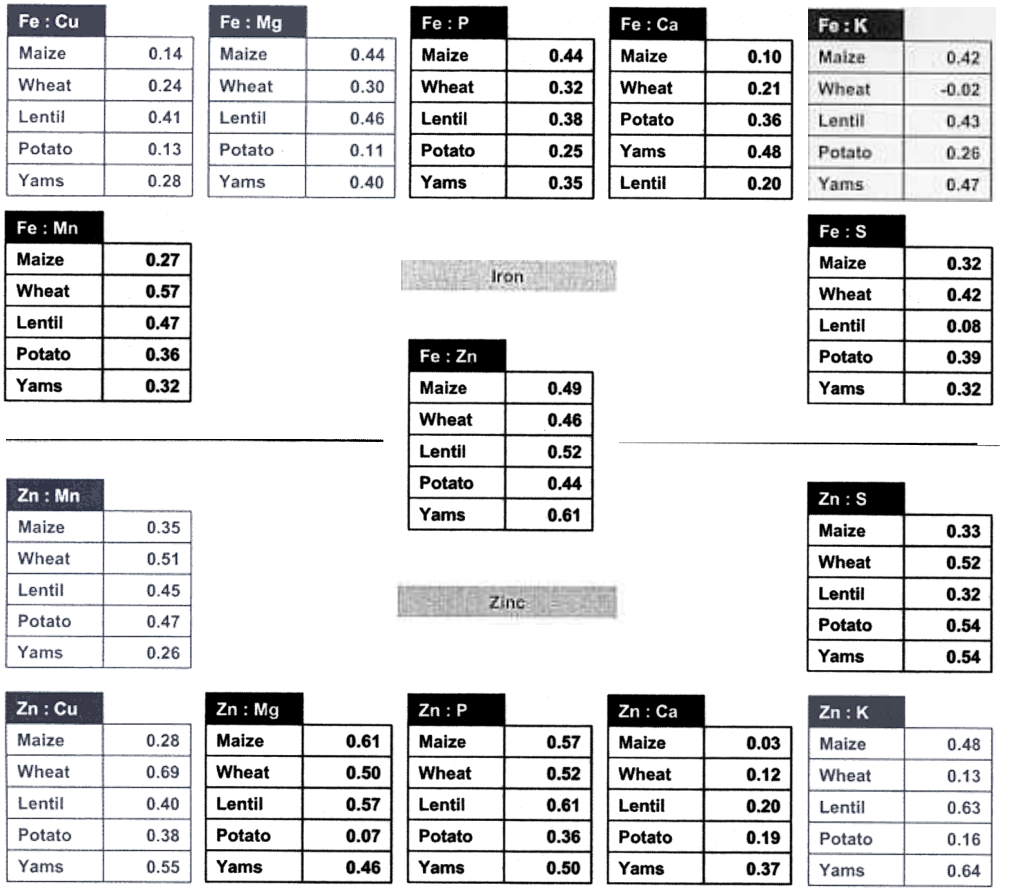
Knowledge of heritability as it relates to genetic progress ($G_s = i\,\sigma_p\,h^2$) and associated genetics is crucial for establishing screening, population development, and G × E testing strategies, and hence, for effective breeding. All plant-breeding components (such as crossing strategies, breeding methodologies, the operational scale with plot size and number of sites and years required for testing) are based on genetic parameters. Furthermore, the potential for developing molecular markers is closely associated with factors such as the number of genes and their individual contributions. Molecular markers and marker-assisted protocols for applied breeding that can be used at early plant stages to select for micronutrient density can greatly increase breeding efficiency; quantitative trait loci (QTL) for micronutrients have been identified in several crops including beans, maize, and rice (Guzmán-Maldonado et al., 2003; Wong et al., 2004; Gregorio and Htut, 2003; Wissuwa, 2005).

Growing evidence from HarvestPlus research supports findings that iron and zinc concentration is controlled by several (2–5) relevant genes, and that mineral heritability is of intermediate magnitude (Maloo et al., 1998; Philip and Maloo, 1996; Gregorio and Htut, 2003; Long et al., 2004; Cichy et al., 2005). Provitamin A appears to be controlled by a few (~2) major genes, and trait heritability is high (Brown et al., 1993; Egesel et al., 2003b; Menkir and Maziya-Dixon, 2004; Grüneberg et al., 2005). However, heritability can be overestimated if studies contrast non-provitamin A and high provitamin A genotypes. For both minerals and provitamin A, additive gene action and general combining ability predominate (Maloo et al., 1998; Egesel et al., 2003a; Egesel et al., 2003b; Gregorio and Htut, 2003; Long et al., 2004). Heterosis has been found for maize and cassava. Also, studies on temperate maize have revealed significant reciprocal (maternal) effects for provitamin A (Egesel et al., 2003a). Transgressive segregation for provitamin A has been encountered, for example, in cassava crossed with wild relatives, for iron in beans, and for zinc in wheat. Although information on transgressive segregation or heterosis is still incomplete, there is growing evidence that complementary genes are present, particularly in genetically distant sources, such as wild relative species. This is not unexpected, given that in the past, breeders did not select for micronutrients, and latent variation may have been lost. Furthermore, in the past, breeders would often select for white endosperm and, hence, against carotenoids (e.g., in maize and wheat), or for lower ash content, which is associated with lower mineral concentration (e.g., in wheat).

Correlations among minerals and value-added traits

Figure 3.7 displays correlations among minerals in cereals (maize and wheat), a legume (lentil), and tubers (potato and yams). The data reveal a generic positive correlation between iron and zinc concentrations, and between iron and zinc and all other nutritionally important minerals and trace elements (Ca, Cu, K, Mg, Mn, P, and S). The substantial positive association among minerals suggests an opportunity for raising levels of a number of micronutrients simultaneously by direct selection for numerous micronutrients or by capitalizing on the indirect selection response (i.e., a parallel increase in micronutrients not targeted in selection). Strain and Cashman (2002) provide an overview of the importance of minerals and trace elements in human nutrition.

Several publications report significant associations between mineral concentrations (in particular zinc) and grain protein concentration in wheat (Peterson et al., 1986; Zebarath et al., 1992; Feil and Fossati, 1995;



Source: HarvestPlus

Fig. 3.7. Average correlations among micronutrient concentrations for maize, wheat, lentil, potato and yams. The average for each crop was calculated as arithmetic mean across different datasets. Data for elements with Al values >10 $\mu g g^{-1}$ were excluded from the analyses. Data for crops comprised between 500 and 2500 micronutrient analyses per crop. Micronutrient analyses were conducted at Waite Analytical Services, Adelaide (data source: HarvestPlus database; data provided by HarvestPlus crop leaders and published/unpublished sources).

Cakmak et al., 2000; Alex Morgounov, CIMMYT, personal communication). A lower magnitude of association has been found in maize (Arnold and Bauman, 1976; Arnold et al., 1977; Bänziger et al., 2000). Due to a negative association between grain protein and grain yield, particularly in wheat, HarvestPlus researchers are now investigating whether these correlations could result in a significant negative association with grain yield and other traits positively correlated with grain yield. However, correlations can be overestimated (see section on micronutrient concentration versus content). Data on HarvestPlus crops (except wheat) have not revealed relevant negative associations between micronutrients and productivity traits (Menkir and Maziya-Dixon, 2004; Mi et al., 2004), but knowledge is still incomplete. Associations between micronutrients and sensory characteristics can be relevant. For example, in sweetpotato, dry matter content and β-carotene concentration are negatively associated (Zhang and Xie, 1988; Grüneberg et al., 2005); this complicates breeding because adult African consumers

prefer sweetpotato with high dry matter content (Tomlins et al., 2004).

Genotype × environment interaction

Our genetic understanding of micronutrient trait expression and trait stability (spatial, temporal, and system-dependent) is limited, and G × E interaction can greatly influence genotypic performance across different crop growing scenarios. Micronutrient trait expression and the extent of G × E interactions in different environments largely determine the screening and breeding methodologies used, as well as the genetic gains achieved from selection. Early biofortification efforts were challenged by knowledge gaps regarding site suitability for trait assessment and the effect of permanent and variable environmental factors, production constraints, and crop management practices on micronutrient concentration. Mineral traits were frequently perceived as qualitative traits until results from multi-environment experiments revealed significant G × E interactions and substantial differences in the suitability of test sites for micronutrient selection in expressing variation and discriminating among genotypes, and, hence, their quantitative nature (Reynolds et al., 2005).

An increasing body of evidence suggests that the expression of provitamin A is relatively stable under different growing conditions (Egesel et al., 2003b; Menkir and Maziya-Dixon, 2004). HarvestPlus researchers have identified cassava, maize, and sweetpotato genotypes with high and stable expression across environments, with G × E interactions predominantly of the non-crossover type. These results agree with findings that β-carotene/provitamin A are controlled by relatively few genes and more simply inherited.

The expression of zinc (and, to a lesser extent, iron) concentration is related to and affected by permanent and variable environmental factors; the higher variation due to G × E when compared with provitamin A reflects the more complex inheritance of iron and zinc, particularly in cereals. However, results from multi-environment trials revealed micronutrient-dense genotypes of cereals, legumes, and tubers with high, stable trait expression in the presence of high G × E interaction.

Differences in genotypic variation for minerals in different environments can be large. In cassava multi-location experiments conducted in Colombia, site means at proximate test locations varied 2–3 fold for zinc, and zinc standard deviations at sites varied 2–4 fold. In contrast, site mean values for iron were comparable to the respective standard deviations. Similar results for zinc have been obtained for wheat in multi-location trials in Kazakhstan (Alex Morgounov, CIMMYT, personal communication). Since soil zinc deficiency is a common problem in major agricultural areas (Cakmak et al., 1990), such results are not unexpected. Due to the complexity of soil mineral dynamics and the interaction with environmental factors, soil mineral status often explains only part of poor iron or zinc expression in the plant. Research aimed at understanding the underlying factors of G × E interactions and micronutrient trait expression by analyzing soil and plant samples is warranted. Such research would also help in understanding the association between soil micronutrient status and crop mineral concentration.

Micronutrient fertilizer can be used to generate repeatable screening and testing environments for minerals, increase breeding effectiveness, and overcome soil mineral deficiency. HarvestPlus is conducting research on the synergistic effects of zinc fertilizer on crop zinc levels in target areas. This research will provide farmers with crop-management recommendations to increase mineral density and reduce spatial

and temporal fluctuations due to G × E interactions.

Microenvironment variation for minerals can be highly significant and, if not adequately sampled, may cause false high positives in mineral screening. Plot size needs to be adjusted to sample microenvironment variation. Standards, repeated checks, replications, and spatial experimental designs are used to take on-site spatial variation into account. Using common checks or standards across experiments allows results from different environments and for different types of germplasm to be compared. Among factors that can influence micronutrient expression are planting date and season: HarvestPlus G × E interaction trials revealed highly significant differences in average mineral concentration and genetic variation between planting seasons for rice and pearl millet, and between different planting dates for wheat. Hence, next to spatial and temporal variation, systems variation caused by differential crop-management practices can have significant effects.

Strategies and approaches for breeding competitive biofortified crops

Malnutrition and micronutrient deficiency frequently coincide in major target areas for biofortified crops in developing countries. In the coming decades, crop production will have to increase to compensate for diminishing per capita land and water resources and keep pace with rising global food demand. Increasing and stabilizing crop production under these circumstances pose one of the greatest challenges for agricultural research of the 21st century, given the fragile and highly variable nature of biofortification target areas and the continued deterioration of natural resources. Thus, at the same time as crop micronutrient concentration is being improved, production efficiency in the different agroecological cropping systems must be maximized while protecting the natural resource base. Within this context, environmental, cultural, and political sustainability is what defines the focus of the research agenda (Pfeiffer et al., 2005a,b).

Breeding for high yield and micronutrient density

Breeding for additional traits not associated with crop productivity or economic yield and, in particular, for novel traits, causes lower rates of progress for productivity traits, if additional resources are not invested. Increasing the operational scale and scope of breeding activities can substantially increase genetic progress, via both genetic variation and higher selection intensity, and avoid compromising yield by breeding for micronutrient density. Other factors that increase breeding efficiency and enhance G_s are breeding and testing in target environments and/or controlled environments that reliably simulate target environments to: (1) increase heritability or the correlation between selection and target environments; (2) intensify testing of experimental germplasm in target environments; and (3) facilitate the use of molecular markers in selection and of molecular marker-assisted background selection to accelerate the introgression of micronutrient density from the strategic gene pool to locally adapted elite germplasm (Guzmán-Maldonado et al., 2003; Wong et al., 2004). The development and implementation of enabling technologies, such as inexpensive high-throughput diagnostic tools, can dramatically increase breeding efficiency.

Introgressing novel traits into the tactical gene pool initially requires additional resources. Novel traits, such as micronutrient density, are therefore being addressed as "specific traits" to accelerate developing micronutrient-rich products for rapid commercialization and/or for immediate impact on micronutrient deficiency alleviation.

Micronutrient traits are presumably not subject to genetic erosion (such as that caused by the evolution of pathogenic races) and require little maintenance breeding once genes have been incorporated. Hence, the cost of breeding for micronutrients decreases across time, and micronutrient density built into the gene pool will not affect future breeding for productivity traits. If micronutrient traits are incorporated into the tactical gene pool, micronutrient concentration can be taken up as a generic trait present in all germplasm products, which is a requirement for biofortification to be sustainable.

Strategies for achieving agronomic superiority

Agronomic superiority and farmer adoption are critical to the success of biofortification. Production increases can originate from various sources (Pfeiffer et al., 2005b): (1) genetic gains in yield potential; (2) genetic gains in tolerance/resistance to abiotic and biotic stresses; (3) improved, sustainable crop-management techniques; and (4) the synergistic effects of all these factors within the context of production economics. Circumstantial evidence indicates that progress in any of these factors will, directly or indirectly, enhance yield.

In reality, there is no environment that is completely free of stress, and breeding for stress tolerance occupies center stage in crop improvement. In practice, indirect selection for tolerance/resistance to key constraints is frequently more efficient for raising genotypic production potential (and, eventually, triggering farmer adoption) than selecting for yield or a specific abiotic stress per se. Breeders can raise productivity by concentrating on improving resistance/tolerance to biotic/abiotic factors and, particularly, resistance to diseases for which they have known and repeatable variation (Pfeiffer et al., 2005a).

Protecting yield through the incorporation of traits that buffer production vagaries and result in higher yield stability can be the key to adoption, given that, in developing countries, farmers' criteria for changing varieties are often based on risk factors related to food and income security.

The agricultural production paradigm that focuses on higher yield to maximize profit has changed along with declining commodity prices and higher proportional costs of crop management and biocides. Farmers have to maximize their revenue by reducing production costs, i.e., by aiming at higher economic yields rather than by increasing crop yields. The possibility of obtaining higher economic returns from varieties with increased input efficiency and/or responsiveness provides an incentive for adoption. Input-use efficiency at low levels of input availability and input responsiveness (i.e., the plant's effectiveness in transforming additional inputs into yield) are under genetic control and can be improved by breeding. Pleiotropic effects associated with high micronutrient content can affect agronomic performance and, hence, agronomic options. For example, seed zinc concentration and micronutrient-dense seeds in wheat are closely associated with greater seedling vigor, increased stand establishment, and higher grain yield, particularly in zinc-deficient soils (Cakmak et al., 1990). In a zinc-deficient soil, seed zinc concentrations of 355 ng seed^{-1}, 800 ng seed^{-1}, and 1465 ng seed^{-1} resulted in grain yields of 480 kg ha^{-1}, 920 kg ha^{-1}, and 1240 kg ha^{-1}, respectively. Product concepts can capitalize on these effects.

The end-use quality of a variety used to produce local and processed food products can be a criterion for variety adoption in subsistence farming systems and market economies. In market economies, higher returns from premium prices for end-use quality traits, such as percent protein, grade requirement, or sensory traits (size, shape,

color, taste), can compensate for any reduction in income due to a yield penalty.

In most developing countries where micronutrient deficiency is prevalent, agronomic superiority can be achieved more easily by replacing open-pollinated varieties (for example, of maize, sorghum, and pearl millet) with hybrids or synthetics. However, if a product concept entails deploying hybrid technologies, it must consider the feasibility of having sustainable seed systems in place.

Adoption often entails implementing a technological package. Improved agronomic practices, such as direct seeding under reduced or zero tillage and stubble retention, along with germplasm adapted to these practices, capitalize on synergies between genetic and agronomic solutions to achieve production and end-use quality objectives. Breeding for adaptation to direct seeding under reduced or zero tillage is feasible (Trethowan et al., 2005). The arsenal of modern crop-management techniques can provide an agronomic platform for successfully producing biofortified varieties and achieving both nutritional and commercial goals.

Acknowledgments

The authors recognize the intellectual contributions made by research partners within the CGIAR, NARES, and nutrition community throughout the world. They also acknowledge the support received from key members of the donor community, including the Asian Development Bank, the Danish Agency for International Development Assistance, the U.K. Department of International Development, the Bill and Melinda Gates Foundation, the United States Agency for International Development, and the World Bank. Special thanks are extended to Alma McNab for her editorial expertise.

Endnotes

1. The HarvestPlus program is funded by the Bill and Melinda Gates Foundation, the World Bank, US Agency for International Development, UK Department for International Development, and the Danish International Development Agency.
2. Challenge programs are time-bound, independently governed programs of high-impact research that target CGIAR goals in relation to complex issues of overwhelming global and/or regional significance, and rely on partnerships among a wide range of institutions to deliver their products. In the case of HarvestPlus, biofortification research is conducted by a global alliance of research institutions and implementing agencies in developed and developing countries, and coordinated by two CGIAR centers, the International Center for Tropical Agriculture (CIAT) and the International Food Policy Research Institute (IFPRI).

References

Al-Babili, S., and P. Beyer. 2005. Golden Rice—five years on the road—five years to go? Trends in Plant Science 10(12):565–573.

Arnold, J.M., and L.F. Bauman. 1976. Inheritance of and interrelationships among maize kernel traits and elemental contents. Crop Sci. 16(3):439–440.

Arnold, J.M., L.F. Bauman, and H.S. Aycock. 1977. Interrelations among protein, lysine, oil, certain mineral element concentrations, and physical kernel characteristics in two maize populations. Crop Sci. 17(3):421–425.

Bänziger, M., and J. Long. 2000. The potential of increasing the iron and zinc density of maize through plant breeding. Food Nutr. Bull. 21:397–400.

Beebe, S., A.V. Gomez, and J. Renfigo. 2000. Research on trace minerals in the common bean. Food Nutr. Bull. 21:387–391.

Bouis, H.E. 2003. Micronutrient fortification of plants through plant breeding: Can it improve nutrition in man at low cost? Proc. Nutr. Soc. 62:403–11.

Bouis, H.E., R.D. Graham, and R.M. Welch. 2000. The Consultative Group on International Agricultural Research (CGIAR) Micronutrient Project: Justification and objectives. Food Nutr. Bull. 21:374–381.

Bouis, H.E., S. Harris, and D. Lineback. 2002. Biotechnology-derived nutritious foods for developing countries: Needs, opportunities, and barriers. Food and Nutrition Bulletin 23(4):342–383.

Bregitzer, P., and V. Raboy. 2006. Effect of four independent low phytate mutations on barley agronomic performance. Crop Sci. 46:1318–1322.

Brenna, O.V., and N. Bernardo. 2004. Application of near-infrared spectroscopy (NIRS) to the evaluation of carotenoids in maize. J. Agric. Food Chem. 52:5577–5582.

Brown, C.R., C.G. Edwards, C.P. Yang, and B.B. Dean. 1993. Orange flesh trait in potato: Inheritance and carotenoid content. Journal of the American Society for Horticultural Science 118(1):145–150.

Cakmak, I., H. Ozkan, H.J. Braun, R.M. Welch, and V. Römheld. 2000. Zinc and iron concentrations in seeds of wild, primitive, and modern wheats. Food Nutr. Bull. 21:401–403.

Cakmak, I., M. Kalayc, H. Ekiz, H.J. Braun, Y. Kılınc, and Y. Yılmaz. 1990. Zinc deficiency as a practical problem in plant and human nutrition in Turkey: A NATO science-for-stability project. Field Crops Res. 60:175–188.

Chavez, A.L., J.M. Bedoya, T. Sanchez, C. Iglesias, H. Ceballos, and W. Roca. 2000. Iron, carotene, and ascorbic acid in cassava roots. Food Nutr. Bull. 21:410–413.

Chavez, A.L., T. Sanchez, G. Jaramillo, J.M. Bedoya, J. Echeverry, E.A. Bolanos, H. Ceballos, and C.A. Iglesias. 2005. Variation of quality traits in cassava roots evaluated in landraces and improved clones. Euphytica 143:125–133.

Chong, M. 2003. Acceptance of Golden Rice in the Philippine "rice bowl." Nature Biotechnology 21(9):971–972.

Cichy, K.A., S. Forster, K.F. Grafton, and G.L. Hosfield. 2005. Inheritance of seed zinc accumulation in navy bean. Crop Sci. 45(3):864–870.

Dorsch, J.A., A. Cook, K.A. Young, J.M. Anderson, A.T. Bauman, C.J. Volkmann, P.P.N. Murthy, and V. Raboy. 2003. Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. Phytochemistry 62(5):691–706.

Drakakaki, G., S. Marcel, R.P. Glahn, E.K. Lund, S. Pariagh, R. Fischer, P. Christou, and E. Stoger. 2005. Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. Plant Molecular Biology 59(6):869–880.

Ducreux, L.J.M., W.L. Morris, P.E. Hedley, T. Shepherd, H.V. Davies, S. Millam, and M.A. Taylor. 2005. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J. Exp. Bot. 409:81–89.

Egesel, C.O., J.C. Wong, R.J. Lambert, and T.R. Rocheford. 2003a. Gene dosage effects on carotenoid concentration in maize grain. Maydica 48(3):183–190.

Egesel, C.O., J.C. Wong, R.J. Lambert, and T.R. Rocheford. 2003b. Combining ability of maize inbreds for carotenoids and tocopherols. Crop Sci. 43(3):818–823.

Farinu, G.O., and G. Ingrao. 1991. Gross composition, amino acid, phytic acid and trace element contents of thirteen cowpea cultivars and their nutritional significance. Journal of the Science of Food and Agriculture 55(3):401–410.

Feil, B., and D. Fossati. 1995. Mineral composition of triticale grain as related to grain yield and grain protein. Crop Sci. 35:1426–1431.

Graham, R.D., and R.M. Welch. 1996. Breeding for staple-food crops with high micronutrient density: Agricultural strategies for micronutrients. Working Paper No. 3; 1996. International Food Policy Research Institute, Washington, D.C.

Graham, R.D., D. Senadhira, S.E Beebe, C. Iglesias, and I. Ortiz-Monasterio. 1999. Breeding for micronutrient density inedible portions of staple food crops: Conventional approaches. Field Crops Res. 60:57–80.

Gregorio, G.B., and T. Htut. 2003. Micronutrient-dense rice: Breeding tools at IRRI. In: T.W. Mew, D.S. Brar, S. Peng, D. Dawe, and B. Hardy, editors. Rice science: Innovations and impact for livelihood. Proceedings of the International Rice Research Conference. Beijing, China, 16–19, September 2002. p. 371–378.

Gregorio, G.B., D. Senadhira, H. Htut, and R.D. Graham. 2000. Breeding for trace mineral density in rice. Food Nutr. Bull. 21:382–386.

Grüneberg, W.J., K. Manrique, Zhang DaPeng, and M. Hermann. 2005. Genotype×environment interactions for a diverse set of sweetpotato clones evaluated across varying ecogeographic conditions in Peru. Crop Sci. 45:2160–2171.

Guttierie, M., D. Bowen, J.A. Dorsch, V. Raboy, and E. Souza. 2004. Identification and characterization of a low phytic acid wheat. Crop Sci. 44:418–424.

Guzmán-Maldonado, S.H., O. Martínez, J.A. Acosta-Gallegos, F. Guevara-Lara, and O. Paredes-López. 2003. Putative quantitative trait loci for physical and chemical components of common bean. Crop Sci. 43(3):1029–1035.

Hambidge, K.M., J.W. Huffer, V. Raboy, G.K. Grunwald, J.L. Westcott, L. Sian, L.V. Miller, J.A. Dorsch, and N.F. Krebs. 2004. Zinc absorption from low-phytate hybrids of maize and their wild-type isohybrids. American Journal of Clinical Nutrition 79(6):1053–1059.

Hong, C.Y., K.J. Cheng, T.H. Tseng, C.S. Wang, L.F. Liu, and S.M. Yu. 2004. Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. Transgenic Research 13(1):29–39.

Imtiaz, M., J.B. Alloway, A.K. Shah, S.H. Siddiqui, M.Y. Memon, M. Aslam, and P. Khan. 2003. Zinc contents in the seed of some domestic and exotic wheat genotypes. Asian Journal of Plant Sciences 2(15–16):1118–1120.

Institute of Medicine. 2001. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. National Academy Press, Washington, D.C.

Kayodé, A.P.P., A.R. Linnemann, J.D. Hounhouigan, M.J.R. Nout, and M.A.J.S. van Boekel. 2006. Genetic and environmental impact on iron, zinc, and phytate in food sorghum grown in Benin. Journal of Agricultural and Food Chemistry 54:256–262.

Khalekuzzaman, M., K. Datta, N. Oliva, M.F. Alam, O.I. Joarder, and S.K. Datta. 2006. Stable integration, expression and inheritance of the ferritin gene in transgenic elite indica rice cultivar BR29 with enhanced iron level in the endosperm. Indian Journal of Biotechnology 5(1):26–31.

Khush, G.S. 2002. The promise of biotechnology in addressing current nutritional problems in developing countries. Food and Nutrition Bulletin 23(4): 354–357.

Kimura, M., C.N. Cobori, D.B. Rodriguez-Amaya, and P. Nestel. 2007. Screening and HPLC methods for carotenoids in sweetpotato, cassava, and maize for plant breeding trials. Food Chemistry 100(4):1734–1746.

Long, J.K., M. Bänziger, and M.E. Smith. 2004. Diallel analysis of grain iron and zinc density in Southern African-adapted maize inbreds. Crop Sci. 44(6): 2019–2026.

Lucca, P., J. Wünn, R.F. Hurrell, and I. Potrykus. 2000. Development of iron-rich rice and improvement of its absorption in humans by genetic engineering. Journal of Plant Nutrition 23(11/12):1983–1988.

Lucca, P., R. Hurrell, and I. Potrykus. 2001a. Approaches to improving the bioavailability and level of iron in rice seeds. Journal of the Science of Food and Agriculture 81(9):828–834.

Lucca, P., R. Hurrell, and I. Potrykus. 2001b. Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theoretical and Applied Genetics 102(2/3):392–397.

Ma, J.F., A. Higashitani, K. Sato, and K. Takeda. 2004. Genotypic variation in Fe concentration of barley grain. Soil Science and Plant Nutrition 50(7):1115–1117.

Maloo, S.R., J.S. Solanki, and S.P. Sharma. 1998. Genotypic variability for quality traits in finger millet (Eleusine coracana (L.) Gaertn.). International Sorghum and Millets Newsletter 39:126–128.

Mamta, P., and P. Darshan. 2000. Effect of pressure and solar cooking on phytic acid and polyphenol content of cowpeas. Nutrition & Food Science 2/3:133–136.

Matthews, P.D., R.B. Luo, and E.T. Wurtzel. 2003. Maize phytoene desaturase and β-carotene desaturase catalyse a poly-Z desaturation pathway: Implications for genetic engineering of carotenoid content among cereal crops. J. Exp. Bot. 54(391):2215–2230.

Maziya-Dixon, B., J.G. Kling, A. Menkir, and A. Dixon. 2000. Genetic variation in total carotene, iron, and zinc contents of maize and cassava genotypes, and ascorbic acid in cassava roots. Food Nutr. Bull. 21:419–422.

McElroy, D. 2004. Valuing the product development cycle in agricultural biotechnology—what's in a name? Nature Biotechnology 22(7):817–822.

Menkir, A., and B. Maziya-Dixon. 2004. Influence of genotype and environment on β-carotene content of tropical yellow-endosperm maize genotypes. Maydica 49(4):313–318.

Mi, G.H., F.J. Chen, X.S. Liu, L. Chun, and J.L. Song. 2004. Genotype difference in iron content in kernels of maize. Journal of Maize Sciences 12(2):13–15.

Muzquiz, M., C. Burbano, G. Ayet, M.M. Pedrosa, and C. Cuadrado. 1999. The investigation of antinutritional factors in Phaseolus vulgaris. Environmental and varietal differences. BASE: Biotechnologie, Agronomie, Société et Environnement 3(4):210–216.

Nandi, S., Y.A. Suzuki, J.M. Huang, D. Yalda, P. Pham, L.Y. Wu, G. Bartley, N. Huang, and B. Lönnerdal. 2002. Expression of human lactoferrin in transgenic rice grains for the application in infant formula. Plant Science 163(4):713–722.

Nestel, P., H.E. Bouis, J.V. Meenakshi, and W.H. Pfeiffer. 2006. Biofortification of staple food crops. J. Nutr. 136:1064–1067.

Nunez-Gonzalez, A., R.K. Maiti, J. Verde-Star, M.L. Cardenas, R. Foroughbakch, J.L. Hernandez-Pinero, S. Moreno-Limon, and G. Garcia-Diaz. 2002. Variability in mineral profile in seven varieties of bean (*Phaseolus vulgaris* L.) adapted in North East of Mexico. Legume Research 25(4):284–287.

Oikeh, S.O., A. Menkir, B. Maziya-Dixon, R. Welch, and R.P. Glahn. 2003. Genotypic differences in concentration and bioavailability of kernel iron in tropical maize varieties grown under field conditions. Journal of Plant Nutrition 26(10/11): 2307–2319.

Ortiz-Monasterio, I., and R.D. Graham. 2000. Breeding for trace minerals in wheat. Food Nutr. Bull. 21:392–396.

Ozturk, L., M.A. Yazici, C. Yucel, A. Torun, C. Cekic, A. Bagci, H. Ozkan, H.J. Braun, Z. Sayers, and I. Cakmak. 2006. Concentration and localization of zinc during seed development and germination in wheat. Physiologia Plantarum 128:144–152.

Paine, J.A., C.A. Shipton, S. Chaggar, R.M. Howells, M.J. Kennedy, G. Vernon, S.Y. Wright, E. Hinchliffe, J.L. Adams, A.L. Silverstone, and R. Drake. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nature Biotechnology 23(4):482–487.

Parkhi, V., M. Rai, J. Tan, N. Oliva, S. Rehana, A. Bandyopadhyay, L. Torrizo, V. Ghole, K. Datta, and S.K. Datta. 2005. Molecular characterization of marker-free transgenic lines of indica rice that accumulate carotenoids in seed endosperm. Molecular Genetics and Genomics 274(4):325–336.

Peterson, C.J., V.A. Johnson, and P.A. Mattern. 1986. Influence of cultivar and environment on mineral and protein concentrations of wheat flour, bran, and grain. Cereal Chem. 63:118–126.

Pfeiffer, W.H., R.M. Trethowan, M. van Ginkel, I. Ortiz-Monasterio, and S. Rajaram. 2005a. Breeding

for stress tolerance in wheat. In M. Ashraf and P. J.C. Harris (eds.). *Abiotic Stresses: Plant Resistance through Breeding and Molecular Approaches.* New York: The Haworth Press, Inc. p. 401–489.

Pfeiffer, W.H., R.M. Trethowan, K. Ammar, and K.D. Sayre. 2005b. Increasing yield potential and yield stability in durum wheat. In C. Royo, M.M. Nachit, N. di Fonzo, J.L. Araus, W.H. Pfeiffer, and G.A. Slafer (eds.). Vol. 2. *Durum Wheat Breeding: Current Approaches and Future Strategies.* New York: The Haworth Press, Inc. p. 531–544.

Philip, J., and S.R. Maloo. 1996. An evaluation of Setaria italica for seed iron content. International Sorghum and Millets Newsletter 37:82–83.

Pinstrup-Andersen, P. 2000. Improving human nutrition through agricultural research: Overview and objectives. Food Nutr. Bull. 21:352–355.

Qu, L.Q., T. Yoshihara, A. Ooyama, F. Goto, and F. Takaiwa. 2005. Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. Planta 222(2):225–233.

Raboy, V. 2000. Low-phytic-acid grains. Food Nutr. Bull. 21:423–427.

Raboy, V., P.F. Gerbasi, K.A. Young, S.D. Stoneberg, S.G. Pickett, A.T Bauman, P.P.N. Murthy, W.F. Sheridan, and D.S. Ertl. 2000. Origin and seed phenotype of maize low phytic acid 1–1 and low phytic acid 2–1. Plant Physiology 124(1):355–368.

Reddy, B.V.S., S. Ramesh, and T. Longvah. 2005. Prospects of breeding for micronutrients and β-carotene-dense sorghums. International Sorghum and Millets Newsletter 46:10–14.

Reynolds, T.L., M.A. Nemeth, K.C. Glenn, W.P. Ridley, and J.D. Astwood. 2005. Natural variability of metabolites in maize grain: Differences due to genetic background. Journal of Agricultural and Food Chemistry 53(26):10061–10067.

Rodriguez-Amaya, D.B., and M. Kimura. 2004. HarvestPlus Handbook for Carotenoid Analysis. HarvestPlus Technical Monograph Series 2.

Rogers, E.M. 1983. *Diffusion of Innovations,* 5th edition. New York: The Free Press.

Sautter, C., S. Poletti, P. Zhang, and W. Gruissem. 2006. Biofortification of essential nutritional compounds and trace elements in rice and cassava. Proceedings of the Nutrition Society 65(2):153–159.

Shewry, P.R., and H.D. Jones. 2005. Transgenic wheat: Where do we stand after the first 12 years? Annals of Applied Biology 147:1–14.

Shi, J.R., H. Wang, Y. Wu, J. Hazebroek, R.B. Meeley, and D.S. Ertl. 2003. The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate. Plant Physiology 131(2):507–515.

Shukla, S., T.T. VanToai, and R.C. Pratt. 2004. Expression and nucleotide sequence of an INS (3) P_1 synthase gene associated with low-phytate kernels in maize (*Zea mays* L.). Journal of Agricultural and Food Chemistry 52(14):4565–4570.

Simonne, A.H., S.J. Kays, P.E. Koehler, and R.R. Eitenmiller. 1993. Assessment of β-carotene content in sweetpotato breeding lines in relation to dietary requirements. Journal of Food Composition and Analysis. 6(4):336–345.

Sison, M.E.G.Q., G.B. Gregorio, and M.S. Mendioro. 2006. The effect of different milling times on grain iron content and grain physical parameters associated with milling of eight genotypes of rice (*Oryza sativa* L.). Phillipines Journal of Science 135:9–17.

Stein, A., J.V. Meenakshi, M. Qaim, P. Nestel, H.P.S. Sachdev, and Z.A. Bhutta. 2005. Analyzing the health benefits of biofortified staple crops by means of the disability-adjusted life years approach: A handbook focusing on iron, zinc and vitamin A. International Food Policy Research Institute (IFPRI) and International Center for Tropical Agriculture (CIAT), Washington, D.C., and Cali (City), Columbia.

Strain, J.J., and K.D. Cashman. 2002. Minerals and trace elements. In M.J. Gibney, H.H. Voster, and F.J. Kok (eds.). *Human Nutrition.* Blackwell Science Ltd. Oxford, U.K. p. 177–224.

Taylor, M.A., W.L. Morris, L.J.M. Ducreux, H.V. Davies, and S. Millam. 2004. Metabolic engineering of carotenoid biosynthesis in potato tubers. Aspects of Applied Biology 72:155–162.

Tomlins, K., E. Rwiza, A. Nyango, R. Amour, T. Ngendello, R. Kapinga, D. Rees, and F. Jolliffe. 2004. The use of sensory evaluation and consumer preference for the selection of sweetpotato cultivars in East Africa. J Sci Food Agric. 84: 791–799.

Trethowan, R.M., M. Reynolds, K.D. Sayre, and I. Ortiz-Monasterio. 2005. Adapting wheat cultivars to resource conserving farming practices and human nutritional needs. Annals of Applied Biology 146(4):405–413.

Ullah, A.H.J., K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, and S. Austin-Phillips. 2003. Fungal phyA gene expressed in potato leaves produces active and stable phytase. Biochemical and Biophysical Research Communications 306(2):603–609.

UN SCN. 2004. 5th Report on the World Nutrition Situation: Nutrition for Improved Development Outcomes. United Nations System Standing Committee on Nutrition (SCN), Geneva.

Underwood, B.A. 2000. Overcoming micronutrient deficiencies in developing countries: Is there a role for agriculture? Food Nutr. Bull. 21:356–360.

Van Jaarsveld, P.J., M. Faber, S.A. Tanumihardjo, P. Nestel, C.J. Lombard, and A.J.S. Benade. 2005. β-Carotene-rich orange-fleshed sweetpotato improves the vitamin A status of primary school children assessed by the modified-relative-dose-response test. Am J Clin Nutr. 81:1080–1087.

Vasconcelos, M., K. Datta, N. Oliva, M. Khalekuzzaman, L. Torrizo, S. Krishnan, M. Oliveira, F. Goto, and S.K. Datta. 2003. Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. Plant Science 164(3):371–378.

Welch, R.M., W.A. House, S. Beebe, D. Senadhira, G.B. Gregorio, and Z. Cheng. 2000. Testing iron and zinc bioavailability in genetically enriched beans (*Phaseolus vulgaris* L.) and rice (*Oryza sativa* L.) in a rat model. Food Nutr. Bull. 21:428–433.

White, P.J., and M.R. Broadley. 2005, Biofortifying crops with essential mineral elements. Trends in Plant Science 10(12):586–583.

Wissuwa, M. 2005. Mapping nutritional traits in crop plants. In: M.R. Broadley and P.J. White (eds.), *Plant Nutritional Genomics*. Ames, Iowa: Blackwell Publishing. p. 220–241.

Wong, J.C., R.J. Lambert, E.T. Wurtzel, and T.R. Rocheford. 2004. QTL and candidate genes phytoene synthase and β-carotene desaturase associated with the accumulation of carotenoids in maize. Theoretical and Applied Genetics 108(2):349–359.

Zeng, Y.W., J.F. Liu, L.X. Wang, S.Q. Shen, Z.C. Li, X.K. Wang, G.S. Wen, and Z.Y. Yang. 2004. Analysis on mineral element contents is associated with varietal type in core collection of Yunnan rice. Rice Science 11(3):106–112.

Zhang, L.Y., and Y.Z. Xie. 1988. Inheritance of flesh colour and its correlation with other traits in sweet potato (Ipomoea batatas). Jiangsu Journal of Agricultural Sciences 4(2):30–34.