Chapter 4
Conventional and Molecular Breeding Approaches for Biofortification of Pearl Millet

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4.1 Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an essential diet of more than 90 million people in the semi-arid tropics of the world where droughts and low fertility of soils cause frequent failures of other crops. It is an important nutri-rich grain cereal in the drier regions of the world grown on 26 mha by millions of farmers (IFAD 1999; Yadav and Rai 2013). This makes pearl millet the sixth most important crop in the world and fourth most important food crop of the India, next to rice, wheat, and maize with annual cultivation over an area of ~8 mha. Pearl millet is also primary food crop in sub-Saharan Africa and is grown on 15 mha (Yadav and Rai 2013). The significant increase in productivity of pearl millet in India is attributed to development and adoption of hybrids of early to medium duration maturity. More than 120 diverse hybrids/varieties have been released till date for various production environments. The heterosis breeding and improved crop management technologies increased productivity substantially achieving higher increased production of 9.80 mt in 2016–2017 from 2.60 mt in 1950–1951 in spite of declined of area under the crop by 20–30% over last two decades (Yadav et al. 2012).

Over 50% pearl millet grain production in Asia is utilized for food purpose and the 20% is used for feed, while 100% grain is used as food in west and central Africa. The per capita consumption of pearl millet in India is highest among rural population in the western Rajasthan and Gujarat, contributing to more than 50% of cereal consumption in these regions (Parthasarathy Rao et al. 2006). Pearl millet

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grain is also consumed in other parts of Maharashtra and Haryana (Basavaraj et al. 2010). Likewise, the domestic consumption of pearl millet is rising steadily in Africa (Ajetomobi 2008).

Pearl millet is a rich source of energy (361 Kcal 100 g^{-1}) comparable with other cereals such as wheat (346 Kcal 100 g^{-1}), rice (345 Kcal 100 g^{-1}), maize (342 Kcal 100 g^{-1}), and sorghum (349 Kcal 100 g^{-1}) (NIN 2003). The carbohydrates in pearl millet (67.5 g 100 g^{-1}) are lower than in wheat, rice, and sorghum, but higher than in maize, while germ portion of pearl millet is larger than sorghum (NIN 2003). These differences explain pearl millet having lower starch and higher protein content. Pearl millet has high fiber content (1.2 g 100 g^{-1}), lowest glycemic index (55) among cereals (Mani et al. 1993), and has relatively higher methionine and phytochemicals and micronutrients (Mal et al. 2010; Singh et al. 2012). Pearl millet is also rich in calcium, potassium, magnesium, iron, zinc, manganese, riboflavin, thiamine, niacin, lysine, and tryptophan.

Shrinking of food basket to a few fine cereals like wheat and rice largely due to subsidized government price and distribution policies for these two cereals has contributed to inadequate intake of essential micronutrients such as iron (Fe) and Zinc (Zn). Fe deficiency affects more than 30% of the population globally, and highest prevalence of anemia is reported among preschool-age children (47%) and pregnant women (42%) (WHO 2008). An estimated 20% of the global population is at risk of inadequate Zn intake (Wessells and Brown 2012). Thus, deficiency of Fe and Zn is most prevalent worldwide. Although government-supported program in India showed marginal reduction in malnutrition over the decades, the progress is very slow as National Family Health Survey revealed unacceptably high prevalence of anemia (>55%), under-weight (35%), and stunting (38%) among children under 5 years (NFHS 2016). The intake of Fe and Zn appears to be below the recommended dietary allowance for an average Indian adult particularly in the low-income rural households including the pearl millet-consuming regions (ICMR 2002; Parthasarathy Rao et al. 2006). Interestingly, pearl millet serves as a significant source of dietary energy and contributes to 19–63% of the Fe and 16–56% of the Zn intake from all food sources to a vast population in parts of the major pearl millet growing states of India (Parthasarathy Rao et al. 2006).

Addressing the micronutrient malnutrition through supplementations and food fortification has been initiated but has not been found as a sustainable approach in developing countries due to poor purchasing power of the consumers and unsatisfactory delivery infrastructure, especially in the rural areas. Therefore, diversified food uses and biofortified crops provide cost-effective and sustainable options to reduce micronutrient malnutrition in such areas. Dietary diversity is a qualitative measure of food consumption that reflects household access to a variety of foods. However, getting people to eat more nutrient-rich fewer staples is very challenging, and affordability is constrained. Biofortification is the process of increasing the content and bioavailability of essential vitamins and minerals in staple crops, through plant breeding to improve nutritional status. This approach contributes to improving the diet quality of populations, and can be viewed as integral part of dietary diversity. Biofortification program has initiated the development and dissemination of

improved crop cultivars with elevated levels of many micronutrients in several crops including pearl millet (Yadava et al. 2017). Genetic enhancement in pearl millet for increased micronutrients has focused grain Fe and Zn using conventional and molecular breeding approaches and the progress achieved is reviewed here.

4.2 Genetic Enhancement of Grain Quality Traits

The aim of core breeding has long been to increase yield potential of cultivars and has largely been accomplished by increasing grain yield through heterosis and building of resistance genes for various diseases and pests in cross-pollinated crops. Recent addition of improving grain nutritional traits is assumed to be newer area for breeders. Conventional breeding methods in combination with advanced phenotyping and biotechnological approaches enable desirable changes to improve the micronutrient content of new cultivars. The available natural genetic variation for essential nutrient content should permit breeding programs to improve the levels of minerals and vitamins in crops (Cakmak 2008; Monasterio and Graham 2000). In pearl millet, a major initiative toward the development of high-iron pearl millet cultivars has been taken involving the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and National Agricultural Research System (NARS) partners from the public and private sector. Such efforts can only be successful and sustainable when biofortified cultivars maintain high yield productivity along with higher nutrient contents offering a benefit to both grower and consumer. Interestingly, the micronutrient traits in pearl millet (like in other cereals) are relatively more stable than grain yield and its components (Satyavathi et al. 2015; Kanatti et al. 2014a).

The extent of genetic variation for grain Fe and Zn contents in germplasm collection, identification of seed-mineral dense germplasm, nature of genotype × environment interaction, relationships between grain minerals and agronomic traits and genetic control of micronutrients would determine breeding efficiency for developing grain mineral dense cultivars. Therefore, a detailed insight is provided here to assess the progress made so far in these areas.

4.2.1 Targeted Micronutrients and Extent of Genetic Variation

Although pearl millet grain is rich in minerals and proteins, the severity of Fe and Zn deficiency and its associated health consequences show greater importance of these two micronutrients than others. The natural variation for grain Fe and Zn contents has been extensively studied in pearl millet (Table 4.1). In general, the spread of variation for grain Fe content was larger than the grain Zn content in various types of genetic materials that includes breeding progenies, populations, and cultivars. For example, too ambitious variation was observed in some studies: the grain

Table 4.1 Iron (Fe) and Zinc (Zn) content in high-Fe seed parents progenies (data are mean of 6 seasons at Patancheru)

	XRF Fe content (mg kg^{-1})		XRF Zn content (mg kg^{-1})	
Advanced breeding line	Mean	Range	Mean	Range
(EEBC S1-407-1-B-B-B-B-B-1-B-1-B-10-1 × B-bulk (3981-3989 G))-2-4-1	82	57–101	51	35–67
(EEBC S1-407-1-B-B-B-B-B-1-B-1-B-13-1 × B-bulk (3981-3989/S06 G1))-1-2-3	81	68–104	50	29–65
(EEBC S1-407-1-B-B-B-B-B-1-B-1-B-5-1 × B-bulk (3981-3989/S06 G1))-2-1-3	88	61–113	55	31–81
(ICMB 04888 × ICMB 02333)-1-1-3-2	77	68–88	49	33–64
(ICMB 95111 × EEBC S1-407-1-B-B)-17-3-1-B-B-B-B-4-B × 3981-4011 G2}-1-4-2	90	76–104	57	40–72
(ICMB 99555 × ICMB 99111)-2-1-1-B-B-B-1	81	55–101	46	33–57
(NC D2 BC7F4-34-3-1-2-B-2-B × EEBC 407)-12-1-2	86	54–97	56	36–75
(NC D2 BC7F4-34-3-1-2-B-2-B × EEBC 407)-4-2-2-2	86	62–102	52	35–66
{[(843B × ICTP 8202-161-5)-20-3-B-B-3 × B-bulk]-2-B-9 × [(ICMB 96555 × LaGrap C2 S1-32-1)-10 × IP 14758-2-1]-8-2}-1-1-1-2	86	80–92	46	34–53
{[(BESCBPT/91-40 × SPF3/S91-3)-1-2-2-3 × B-bulk]-8-1-1-3-B-B-B-B-3-1 × B-bulk (3981-4011/S06G1)}-1-3-2	104	79–123	57	38–82
AIMP 92901 S1-15-1-2-3-B-3-B-9-2-1	92	71–119	53	30–78
AIMP 92901 S1-296-2-1-1-4-2-B-7-3-1	102	90–118	57	42–72
HHVBC Tall S1-51-1-P1-3-B	100	77–129	53	38–62
ICMR 312 S1-59-1	97	83–118	60	46–77
ICMV 221 S1-366	86	80–92	58	42–73
ICMV 96490-S1-15-1-2-2-1-2	115	95–138	62	41–77
ICTP 8203 S1-386	98	84–124	61	41–83

Fe and Zn contents ranged from 40 to 580 mg kg^{-1} and from 10 to 66 mg kg^{-1}, respectively (Jambunathan and Subramanian 1988). But looking at the size of experimental materials (test entries) that were studied in these studies, varied and only few studies had adequate number of test entries to investigate the variation for grain Fe and Zn contents.

Velu et al. (2007) reported large variability for grain Fe content (30.1–75.7 mg kg^{-1}) and Zn content (24.5–64.8 mg kg^{-1}) in pearl millet breeding lines. Rai et al. (2012) also reported large variation for both Fe and Zn contents: Fe ranging from 18 to 97 mg kg^{-1} and Zn varying from 22 to 69 mg kg^{-1} in the advance breeding lines; and Fe ranging from 52 to 135 mg kg^{-1} and Zn ranging from 40 to 92 mg kg^{-1} in the population progenies. Similarly, two to threefold variation among germplasm collections was reported for both Fe (51–121 mg kg^{-1}) and Zn (46–87 mg kg^{-1}) contents (Rai et al. 2014). Interestingly, most of the high Fe and Zn accessions were from Togo and Ghana that had Fe content of 95–121 mg kg^{-1}and Zn content of 59–87 mg kg^{-1} indicating *iniadi* germplasm as a valuable germplasm resource for genetic improvement of Fe and Zn contents in pearl millet.

The magnitude of variation for these micronutrients among the released and commercial cultivars (18 OPVs and 122 hybrids) in India was studied (Rai et al. 2016). OPVs had a Fe range of 42–67 mg kg^{-1}, and Zn range of 37–52 mg kg^{-1} with ICTP 8203 having the highest Fe content (67 mg kg^{-1}) followed by ICMV 221 (61 mg kg^{-1}) and AIMP 92901 (56 mg kg^{-1}). ICTP 8203 had highest level of Zn content (52 mg kg^{-1}), followed by ICMV 221 and AIMP 92901 (45–46 mg kg^{-1}), whereas Fe content in hybrids varied from 46 to 56 mg kg^{-1} and Zn content from 37 to 44 mg kg^{-1}. Four high Fe and Zn hybrids were identified as Ajeet 38, Proagro XL 51, PAC 903, and 86M86 with 55–56 mg kg^{-1} Fe content and with 39–41 mg kg^{-1} Zn content. These high Fe and Zn cultivars can be readily utilized for expanded cultivation and can also be proposed to be included in development programs.

4.2.2 Micronutrient Phenotyping Protocols

Since pearl millet is a highly cross-pollinated crop, three types of seed samples (selfed, sibbed, and open-pollinated seeds) can be used for the micronutrient analysis. However, open-pollinated seed sampling is the best choice in terms of cost-effectiveness and reliable estimation, provided Al contents of samples are monitored for possible dust contamination (Rai et al. 2015a). The availability of low cost and quick throughput analytical methods for micronutrient screening is a prerequisite for successful biofortification breeding. Although variety of instrumental techniques have been used for plant mineral determination so far, breeders presently rely heavily on Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP). Almost all the analytical laboratories of the National Agricultural Research System (NARS) in India are using AAS and very few use ICP. When a large number of samples are to be screened for a given micronutrient, a simple, rapid, and cost-efficient method can surely save the time and resources, and can increase the breeding efficiency to enhance genetic gain for that trait. Recently, Energy-Dispersive X-ray Fluorescence (XRF) has been used for plant sample analysis. XRF is a relatively non-destructive method for grain Fe and Zn contents estimation and has now been validated for pearl millet (Paltridge et al. 2012). Setting up this table-top machine requires little recurring expenditure and provides non-destructive analysis of 300 samples per day at the cost of <USD 2.0 per sample compared to the ICP method (>18 USD/sample) which takes a month time (Rai et al. 2012). High-throughput XRF facility was established in 2010 at ICRISAT, Patancheru, which enables handling a large number of breeding lines at ICRISAT and its partners' center (15,000–20,000 grain samples per year). Efficiency of XRF over ICP for high-throughput Fe and Zn estimation in pearl millet grain was demonstrated with large samples from several trials (Govindaraj et al. 2016a, b). This study showed that highly significant and positive correlations between ICP and XRF ($r = >0.80^{**}$; $p < 0.01$) for both micronutrients provide the reliable screening technique and breeders can rapidly discard low Fe/Zn genotypes while generation advancement.

4.2.3 Genetics of Grain Iron and Zinc Contents

Understanding the nature of gene action and inheritance patterns of grain micronutrient is crucial for breeders to develop effective biofortification breeding strategies. Several studies in pearl millet using different mating designs showed that the inheritance of grain Fe and Zn contents is largely attributed to additive genetic variance with higher magnitude of heritability, explaining the simple inheritance pattern and simple selection for these micronutrients to be effective (Velu 2006; Arulselvi et al. 2007; Gupta et al. 2009; Govindaraj et al. 2016a, b). In general, variability among the hybrids attributable to general combining ability (σ^2GCA) was 3–4 times greater than the variability attributable to specific combining ability (σ^2SCA) for Fe and Zn contents. This proposition of σ^2GCA over σ^2SCA, in turn, contributes to greater predictability ratio which was always closer to unity for both micronutrients. This indicated that the GCA effect for both Fe and Zn contents were predominantly under additive genetic control in pearl millet (Velu et al. 2011; Rai et al. 2012; Govindaraj et al. 2013; Kanatti et al. 2014a). Highly significant and positive correlations between hybrid performance per se and mid-parental values provided further support for these micronutrients being largely under additive genetic control. In contrary, another study reported predominance of non-additive genetic variance for these micronutrients (Arulselvi et al. 2006).

Differences in reciprocal crosses is a widely used method for estimating maternal effects in trait inheritance. The differences between the direct crosses and reciprocal crosses were non-significant both for the Fe and Zn contents both in genotypes with high- and low-content genetic backgrounds (Kanatti et al. 2018). This indicated that genetics of both the micronutrients are controlled by nuclear determinants of male and female parents which showed the relatively greater importance of both nuclear than cytoplasmic contribution. Further, genetic studies revealed the high grain Fe and Zn parents had positive and significant GCA effects, while parents with low grain Fe and Zn had significant negative GCA effects (Govindaraj et al. 2013; Kanatti et al. 2014a). This pattern of genetic control suggested that the selection for higher grain micronutrients should be commenced in earlier generation while agronomic superiority can be selected in later generations. Interestingly, unlike yield traits, inbreeding has no adverse effect on micronutrient content in pearl millet (Rai et al. 2017).

4.2.4 Conventional Breeding

4.2.4.1 Source of Higher Fe and Zn Contents

Evaluations have been undertaken to identify germplasm sources for high Fe and Zn grain contents. Seed parent and restorer lines of hybrids and advanced breeding lines developed from a diverse range of germplasm have been screened to identify

existing sources for high Fe/Zn in elite agronomic backgrounds. Consequently, large variability was observed for both micronutrients among seed parents progenies and ten lines have been identified having very high Fe and Zn contents (Table 4.1). Except for two progenies that involved a NCD2 (Nigerian Composite Dwarf) progeny as one of the parents in the cross, all other progenies were derived from crosses that had both parents developed from *iniadi* germplasm, with a progeny from Extra-early B-composite (EEBC) involved in most of the crosses. These identified progenies with such high levels of Fe and Zn contents would serve as ready-to-use donor source for B × B crosses to develop counterpart A-line. Similarly, selected sources from restorer progenies for high-Fe and Zn are given in Table 4.2. Previous evaluation studies in pearl millet have also shown that breeding lines, hybrid parents, and improved populations having high Fe and Zn contents were often based largely on *iniadi* germplasm (Velu et al. 2011; Govindaraj et al. 2013; Kanatti et al. 2014a; Rai et al. 2015b). *Iniadi* refers to an early-maturing and large-seeded landrace found in the adjoining parts of Togo, Ghana, Benin, and Burkina Faso and such source also known by various local names such as *nara*, *nata*, *ignati*, *ignate*, *ignie*, *misse*, and *likoun* (Andrews and Kumar 1996).

The positive and significant correlation between per se performance of the parents for micronutrients and their GCA effects indicates that per se performance of the parents, in general, is a good indicator of hybrid performance (Rai et al. 2012).

Table 4.2 Iron (Fe) and Zinc (Zn) content in high-Fe restorer parents progenies (data are mean of 4–8 seasons at Patancheru)

	XRF Fe content (mg kg^{-1})		XRF Zn content (mg kg^{-1})	
Advanced breeding line	Mean	Range	Mean	Range
AIMP 92901 S1-15-1-2-3-B-3-B-9-2-1	92	71–119	53	30–78
AIMP 92901 S1-296-2-1-1-4-2-B-7-3-1	102	90–118	57	42–72
HHVBC Tall S1-51-1-P1-3-B	100	77–129	53	38–62
ICMR 312 S1-59-1	97	83–118	60	46–77
ICMV 221 S1-366	86	80–92	58	42–73
ICMV 96490-S1-15-1-2-2-1-2	115	95–138	62	41–77
ICTP 8203 S1-386	98	84–124	61	41–83
LaGrap C2-S1-14-4-1-3-4-4	89	72–112	57	43–70
MRC HS-130-2-2-1-B-B-3-B-B-B-1-3-1	110	93–128	64	42–76
SDMV 90031-S1-11-1-1-3-3-B-4-B-2-1-B	87	64–107	57	38–79
(EERC-HS-8)-B-2-1-2-1	100	85–119	48	28–78
(MC 94 C2-S1-3-1-3-3-1-2-1 × ICMR 312 S1-3-2-3-2-1-1-B-B)-B-46-P1-1	92	87–96	51	36–69
(MC 94 C2-S1-3-1-3-3-1-2-1 × SDMV 90031 S1-3-3-2-2-2-2-2)-B-8-2-1	76	65–87	62	44–74
(MC 94 C2-S1-3-2-2-2-1-3-B-B × AIMP 92901 S1-488-2-1-1-4-B-B)-B-30-1-3	79	66–105	54	41–67
[(IPC 1617 × SDMV 90031-S1-84-1-1-1-1) × AIMP 92901 S1-296-2-1-1-3-B-1]-4-4-2-1	90	69–121	59	36–79

Using both of these parameters, 863B, ICMB 98222, ICMB 99222, ICMB 02333, ICMB 04999, IPC 1650, IPC 843, IPC 774, IPC 1178, IPC 689, and IPC 735 were identified as moderate-to-high Fe lines and the best general combiners for use in hybrid breeding. The above results suggest that population progenies with higher levels (>70 mg kg^{-1}) are available as donor sources for further genetic enhancement of these micronutrients, and lines with high Fe and Zn can be identified in breeding material with elite genetic backgrounds for their direct use in hybrid parents development.

4.2.4.2 Genotype by Environment Interaction

Interaction between genetic and environmental factors (G × E interactions) affects expression of any quantitative trait. Early breeding efforts in biofortification were hindered by gaps regarding appropriate methods for micronutrient traits assessment and the effects of variable environmental factors on biofortified traits. This was primarily due to perception that biofortified traits are qualitative rather than quantitative in nature. There are now evidences that there are significant G × E interactions in expression of biofortified traits (Reynolds et al. 2005; Govindaraj et al. 2013; Kanatti et al. 2014a). Recent studies have shown significant role of environment and genotype × environment (G × E) interaction in determining the levels of grain Fe and Zn contents in pearl millet (Rai et al. 2016). While G × E interactions for Fe and Zn appear to play an important role, the genetic variance contribution was twice than that due to G × E for these micronutrients (Govindaraj et al. 2016b). Most studies in pearl millet showed that G × E interactions accounted for 10–30% of the variation for Fe and Zn contents and it is possible to identify the genotypes with high and stable mineral content across environments.

Complexity of soil micronutrient status may partly contribute to environmental interaction for expression of these traits. Analyzing soil and grain samples from the target environments explains the underlying factors of G × E interactions and the magnitude of micronutrient trait expression. A large number of multi-location evaluations under biofortification program at ICRISAT indicated that all the locations had sufficient levels of Fe and Zn and other important minerals and did not establish relationship of micronutrient content in the grain with the soil available micronutrient status (Govindaraj et al. 2013; Kanatti et al. 2014a). Differences in soil Fe and Zn contents between contrast environment (rainy and summer) were also not reflected in the grain Fe and Zn contents (Gupta et al. 2009) indicating that soil micronutrient status above critical limits has no influence on grain mineral contents. In spite of G × E challenges, there is a growing evidence that breeding for increased levels of micronutrient across environment is feasible with high yield in pearl millet because of positive correlation between Fe and Zn contents and reported higher heritability of these micronutrients than grain yield (Govindaraj 2011; Govindaraj et al. 2016b).

4.2.4.3 Trait Association

Biofortification aims to address nutrient deficiencies as an integral part of core breeding program, but there is need to understand the potential impact of higher micronutrient contents on other important traits. For instance, selection for increased micronutrient content may be expected to negatively affect yield or other important agronomic and end-use characters. This happens if genes that increase micronutrient content are linked with genes that have a deleterious effect on other desired traits, or it could occur as a consequence of negative trait associations. Association between grain Fe and Zn has largely been studied in pearl millet and other crops and highly positive and significant correlations between Fe and Zn have been revealed (Gregorio et al. 2000; Ozkan et al. 2007; Velu et al. 2011). The correlations between Fe and Zn contents in pearl millet varied from 0.43 to 0.97 (Velu et al. 2011; Rai et al. 2012; Govindaraj et al. 2013 and Kanatti et al. 2014b). Such high positive correlations among micronutrients indicate that improvement in Fe content may simultaneously improve the Zn content owing to similar transport and chelation process affecting the accumulation of both Fe and Zn contents in pearl millet seeds. Studies also reported significant positive association of Fe and Zn contents with grain weight in pearl millet (Velu et al. 2007, 2008a, b; Kanatti et al. 2014b), while other studies in pearl millet observed non-significant association grain Fe and Zn contents with 1000-grain weight (Gupta et al. 2009 and Rai et al. 2012; Yadav et al. 2016). Thus, the genetic enhancement of these micronutrients is possible without compromising on grain size.

The Fe and Zn contents had negative and mostly non-significant correlation with grain yield in pearl millet (Rai et al. 2016; Kanatti et al. 2014b; Yadav et al. 2016). In those studies where correlation was negative, it was weak enough in the magnitude, indicating that if these were the results of adverse genetic associations, high-yielding hybrids with high Fe and Zn contents can be bred by making selection for these traits in larger segregating populations and progenies as compared to those used for breeding for grain yield alone. These weak negative relationships resulted from dilution effects when dealing with selfed seeds where grain yield was reduced and micronutrients were overestimated (Govindaraj et al. 2012). On the other side, this trend could be of unidirectional selections as most correlations so far reported are in those lines/cultivars that were bred exclusively for yield (as target trait). Hence, further research involving random sets of lines derived from random-mated populations constituted from crosses between high-Fe/Zn and low-Fe/Zn lines but high yielding is required to examine the magnitude and direction of association of these micronutrients with grain yield. Commercial hybrids (86M86, XL51, and Ajeet 38) bred for higher yield and widely cultivated in India have higher yield and higher Fe content (>50 mg kg^{-1} Fe) among released cultivars shows the possibility of combining grain yield and micronutrient in cultivars (Rai et al. 2016).

4.2.4.4 Population Improvement

Pearl millet is a highly cross-pollinated crop with 70–80% outcrossing and development of open-pollinated varieties (OPVs) as commercial cultivar is an option. Both Fe and Zn contents being largely under additive genetic control, inter and intra-population improvement is highly effective. Although individual plant selection is not very effective for grain yield, S_1 progenies selection is an effective population improvement method for grain yield in pearl millet. A study confirmed the efficiency of single plant selection for Fe and Zn contents in four diverse OPVs (ICTP 8203, IBV 3, AIMP 92901, and ICMR 312) (Govindaraj et al. 2012). Selfed grains produced from S_0 plants and S_1 progenies were assessed for Fe and Zn content and correlation between the S_0 plants and the mean of S_1 progenies across environments was positive and highly significant in all four populations, both for Fe (r = 0.58–0.75) and Zn content (r = 0.61–0.73). Therefore, individual plant progeny selection is effective for both Fe and Zn contents for intra-population improvement as followed for grain yield improvement. For inter- and intra-population improvement, a study revealed that one cycle of selective random matting had improved grain Fe and Zn in C_1 over C_0 bulks with an increase of 8% (Fe and Zn)) in AIMP 92901 and ICMR 312 (Govindaraj 2011). Interestingly, such selection for high Fe and Zn significantly increased 1000-grain mass by 5–14% in these two populations and had no adverse effect on grain yield. Similarly, ICTP8203-10-2, a population developed by recombining 11S3 progenies, had 71 mg kg^{-1} Fe content (9% higher than original) and 2.2 t ha^{-1} grain yield (11% higher than original). Based on national testing, this population was released as 'Dhanashakti' and is the first biofortified crop cultivar for Fe in public domain in India and few other high-Fe OPVs are under development at ICRISAT with much higher Fe and Zn contents (Rai et al. 2014).

4.2.4.5 Hybrid Breeding

The higher level of outcrossing and heterosis supported with availability of commercially viable cytoplasmic-nuclear male sterility system, hybrids breeding has been very effective in increasing pearl millet productivity in India. Heterosis, defined as the superiority in performance of hybrids over its parents (mostly higher parent), is largely explained either due to dominance or over-dominance effects. There is no better-parent heterosis for Fe and Zn reported so far in pearl millet since predominance of additive gene action in the genetic control of these traits, which indicates that there would be little opportunity to exploit better-parent heterosis for improving these micronutrients. However, development of hybrids with high Fe and Zn contents highly require incorporation of high Fe and Zn genes into both parental lines of hybrids where the mid-parent heterosis of a hybrid is gradually increased. Therefore, to breed high iron/zinc hybrids, all potential parental lines should be characterized for these micronutrients and only selected lines should be hybridized.

Unlike grain yield, performance per se of lines is significantly and positively correlated with general combining ability for Fe and Zn in pearl millet, implying the lines selected for high Fe and Zn will also be high general combiners for these micronutrients (Velu et al. 2011; Govindaraj et al. 2013; Kanatti et al. 2014a, 2016). Development of inbred lines with high Fe and Zn depends on the level of and variability for these micronutrients in the base population (whether F_2s or OPVs or composites), and on the magnitude, direction, and pattern of inbreeding effects. It has been observed that inbreeding had either no significant effect or had marginally increased both micronutrients (Rai et al. 2017). In contrast to the low heritability and inbreeding depression of grain yield, micronutrient contents are highly heritable and hybrids can be readily improved through hybrid parents breeding. So far, the best source of high Fe and Zn contents in pearl millet is found to be *iniadi* germplasm (Velu et al. 2011; Rai et al. 2012; Govindaraj et al. 2013). Considering the additive gene action and one source of germplasm genes introgressed in both parental lines, it is expected to reduce genetic diversity between male and female groups for other important traits. This will also lead to reduced heterosis for yield traits, which are predominantly under non-additive gene control. Thus, genomics approaches for selective introgression of genes for Fe and Zn contents in the parental lines without disrupting the diversity for other traits can play a major role in future biofortification breeding. New sources, other than *iniadi*, of Fe and Zn contents in the germplasm collections are also being explored at ICRISAT for genetic diversification for high Fe and Zn. ICRISAT and NARS have developed several biofortified seed and restorer parents with elite agronomic backgrounds in diverse cytoplasmic systems. The Fe content in these seed parents is 69–110 and 42–55 mg kg^{-1} Zn content while restorer had 74–110 mg kg^{-1} Fe and 41–62 mg kg^{-1} Zn (Table 4.3).

So for biofortification breeding at ICRISAT and NARS is intensively supported by HarvestPlus Challenge Program of the CGIAR which set Fe targets for pearl millet as 77 mg kg^{-1} with increment of 30 mg kg^{-1} over first pearl millet variety (WC-C75). However, a recent study reported baseline for Fe content is 42 mg kg^{-1} among hybrids and thus target would be 72 mg kg^{-1} (Rai et al. 2016). Besides using parental lines with high Fe, hybrids being developed with these targets are being tested at national level. A special hybrid trial at national level is being conducted to encourage mainstreaming of this trait in public and private sector breeding programs. In addition, many more hybrids have been identified (Rai et al. 2016) and are in pipeline for testing. It is important to note that higher adoption of biofortified pearl millet hybrids/varieties in a long run largely depend on higher Fe/Zn contents coupled with high yield, downy mildew resistance and drought tolerance.

4.2.4.6 Improved Cultivars

Identification of appropriate germplasm and populations with highest Fe and Zn contents is very important for demonstrating the biofortification breeding. Use of such materials would continue until parental lines with higher Fe and Zn contents

Table 4.3 High-iron seed and restorer parents developed at ICRISAT (data are mean of four seasons)

Line	50% flowering (days)	XRF Fe content (mg kg^{-1})	XRF Zn content (mg kg^{-1})	1000-grain weight (g)	CMS
Seed parents					
ICMA/B 1501	39	76	42	13.2	A4
ICMA/B 1502	43	92	50	13.6	A1
ICMA/B 1503	43	69	43	15.0	A4
ICMA/B 1504	47	97	55	15.5	A1
ICMA/B 1505 (15222)	41	110	55	15.5	A1
ICMA/B 1506 (15444)	45	96	53	9.9	A4
ICMA/B 1507	43	92	50	10.1	A4
ICMA/B 1508	53	73	44	15.0	A1
Restorer parents					
ICMR 1201	48	79	41	10.5	A1
ICMR 1202	50	89	47	14.2	A1
ICMR 1203	52	101	58	7.9	A4
ICMR 1301	55	91	52	12.7	A1
ICMR 1501	55	86	42	9.9	A1
ICMR 1502	51	110	62	12.4	A1
ICMR 1503	51	99	47	13.7	A4
ICMR 1504	57	96	51	8.8	A1
ICMR 1505	55	74	41	6.9	A4

are developed through targeted breeding for these micronutrients in high-yielding backgrounds. With partnership with NARS, improved version of ICTP8203 was released as Dhanashakti for all India level in 2014 (Rai et al. 2014), and has now been adopted by 60,000 ha. Dhanashakti has been accepted by farmers not only for Fe and Zn content, but also for higher grain yield, earliness and bold seed (Table 4.4). ICMV 221, another popular cultivar, has now been further improved for Fe content and is under testing. The improved version of ICMV 221 has 70 mg kg^{-1} Fe content (11% higher than ICMV 221), 58 mg kg^{-1} Zn content (9% higher than ICMV 221), and grain yield 4.2 t ha^{-1} (5% higher than ICMV 221) (Govindaraj and Rai 2016).

Several A/B pairs with high Fe content (range 65–77 mg kg^{-1}) have been identified with ICMA 98222 and ICMA 99222 as the best general combiner for high-Fe hybrid breeding. By exploiting these lines with advanced high-Fe breeding lines as potential restorers, several high-Fe hybrids have been developed with good yield potential. Based on multi-location and multi-year testing, two hybrids, viz., ICMH 1201 and ICMH 1301 have been identified for commercialization. ICMH 1201 had 75 mg kg^{-1} Fe content and 3.6 t ha^{-1} grain yields. ICMH 1201 flowered only 3 days later than ICTP 8203, so it fits in the early-maturity group and production system. Performance of ICMH 1301 showed 72 mg kg^{-1} Fe content and 3.6 t ha^{-1} grain yield.

Table 4.4 Performance of biofortified pearl millet cultivars for Fe and Zn content, grain yield, and time to 50% flower across environments

	XRF Fe content (mg kg^{-1})		XRF Zn content (mg kg^{-1})		Grain yield (t ha^{-1})		50% flowering (days)	
Cultivar	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Dhanashakti	71	40–106	40	18–71	2.20	0.65–5.65	45	40–55
ICTP 8203[a]	65	31–94	40	19-71	1.97	0.63–4.30	45	38–56
ICMV 221 Fe 11-2	70	62–85	58	53–63	4.24	3.38–5.40	43	40–47
ICMV 221[a]	63	59–69	53	51-58	3.98	3.12–4.81	39	38–41
ICMH 1201	75	47–102	39	18–69	3.58	1.49–6.20	48	41–56
86M86[a]	56	39–71	37	18–61	4.37	2.60–6.47	54	47–64
ICTP 8203[a]	71	50–102	43	19-77	2.58	1.33–5.06	45	38–55
ICMH 1301	77	31–107	42	24–64	3.26	1.32–7.03	52	42–62
86M86[a]	58	39-85	37	18-56	4.10	2.60–6.95	54	44–64
ICTP 8203[a]	75	40–117	44	19-77	2.46	1.28–6.36	45	36–53

[a]Check entry

These hybrids are cultivated by >30,000 farmers using truthfully labeled seed (TLS), mostly in Maharashtra and Rajasthan. Much greater progress in breeding high-Fe hybrids with high-grain yield is expected in the near future by utilizing A- and R-lines that are being developed through targeted breeding for high-Fe content.

4.2.5 Molecular Breeding

4.2.5.1 Availability of Molecular Markers

Pearl millet had been a crop of limited genomic resources in the past. However, since last decade, substantial progress has been made in generation of molecular markers. Like in other crops, PCR-based simple sequence repeats (SSR) markers are the extensively used markers for various genetic and mapping studies of pearl millet due to their abundance in the genome, highly polymorphic nature and easy assay. Transcriptome projects have brought enormous information on expressed sequence tags (ESTs), which were used as targets for the identification of SSR markers known as EST-SSR markers through the computational approach thereby making the SSR marker development rapid, easy, and inexpensive. These markers were subsequently used for various genetic studies in pearl millet. PCR-based screening of a bacterial artificial chromosome (BAC) library constructed using nuclear DNA from pearl millet using five sequence-tagged site (STS), led to the identification of 6 microsatellite markers (Allouis et al. 2001). Twenty-five SSRs were isolated from bacterial artificial clones (BA Clone) of pearl millet without any sub-cloning using 3′ anchored SSR primers and isolation of flanking sequences by suppression PCR (Qi et al. 2001), while screening of a small-insert partial genomic

library with a (CT)15 oligonucleotide probe resulted in the development of 18 SSR markers (Budak et al. 2003). Similarly, 44 markers were developed from a (CA)n-enriched small-insert genomic library (Qi et al. 2004). Yadav et al. (2007) developed 32 SSR markers for pearl millet and assessed them for polymorphism in parental lines of 4–6 mapping populations (Yadav et al. 2008). A set of 58 SSR markers was developed in pearl millet and ESTs made available (Senthilvel et al. 2008). SNPs accounted for about two-thirds of the variation while InDels accounted for one-third of the variation. This demonstrated the use of syntenic information to develop SSCP-SNP markers (Bertin et al. 2005). Subsequently, Rajaram et al. (2013) developed 99 EST-SSR markers from transcriptomics work.

4.2.5.2 Linkage Maps

A genetic/linkage map depicts the arrangement of molecular markers. Linkage map is the basic framework in different linkage groups based on the recombination frequency among the markers, which are essential for the mapping of QTL. The first RFLP linkage map in pearl millet was published by Liu et al. (1994). A linkage map consisting of seven linkage groups and a total of 181 loci was made using an intervarietal F2 population, with an average inter-marker distance of about 2 cM. Qi et al. (2004) developed an integrated consensus linkage map from the genetic maps constructed for four different crosses using 353 RFLP and 65 SSR markers. The mapping of 21 polymorphic SSR markers mapped using existing mapping populations (ICMB 841-P3 × 863B-P2 and 81B-P8 × IPC 804) revealed that most EST-SSR markers map to distal regions of linkage groups to cover the previous gaps (Senthilvel et al. 2008).

A linkage map was constructed using 258 DArT and 63 SSR marker data using a RIL population from cross H 77/833-2 and PRLT 2/89-33 (Supriya et al. 2011). With an objective of developing a linkage map with more evenly distributed markers and greater marker coverage of the gaps in earlier maps, Pedraza-Garcia et al. (2010) constructed a map using 196 PCR-based DNA markers (66 SRAPs, 63 RAPDs, 27 ISSRs, 31 pearl millet, six sorghum, and three maize SSRs) of nine linkage groups with an average genetic distance of 9.2 cM between markers. Rajaram et al. (2013) also constructed Linkage maps using 99 newly developed EST-SSR markers and previously mapped 17 EST-SSR, 53 genomic SSR, and two STS markers.

A consensus map of 174 loci (899 cM) was developed by integrating the individual linkage maps using MergeMap, which showed a well-conserved locus order for nearly all linkage groups. The linkage maps constructed using codominant SSRs and dominant DArTs span 1748.7 cM (ICMB 841 × 863B map consisting of 305 markers) (Kumar et al. 2016). Longest SSR-based skeleton linkage map for F_2-derived F_3 progenies with a length of 1018.7 cM accommodating only 44 well-distributed markers has been reported. Large map using SSRs with map length of 748 cM for an F_2 population was reported by Gulia (2004). Liu et al. (1994) reported by the shortest F_2-based map (287.7 cM) reported so far had 181 RFLP markers.

Yadav et al. (2004) incorporated 91 marker loci in a population of F_2 individuals, with a map length of 617.4 cM using (ICMB 841 × 863B) population. Senthilvel et al. (2008) have used the same F_2 population to obtain a map length of 677 cM with 112 loci. Vengadessan et al. (2013) constructed a linkage map, primarily based on SSCP-SNP markers, using 188 $F_{2:3}$ mapping population progenies produced from a cross between pearl millet inbred lines having diverse parentage.

The skeleton linkage map covered 1019 cM and it comprised of 44 markers distributed across the seven linkage groups. Average adjacent-marker intervals ranged from 14 cM on LG1 to 38 cM on LG6, with an overall mean of 23 cM. Rajaram et al. (2013) developed 99 new EST-SSR markers (IPES series) and constructed linkage maps of four F7 recombinant inbred populations (RIP) based on four crosses along with previously mapped EST-SSR (17), genomic SSR (53), and STS (2) markers. A total of 176 loci detected by 171 primer pairs were mapped among the four crosses. A consensus map of 174 loci (899 cM) detected by 169 primer pairs was constructed using Merge Map to integrate the individual linkage maps.

4.2.5.3 Quantitative Trait Loci (QTL)

Development of a mapping population is the most critical factor in the construction of a molecular map. Decisions on selection of parents and mating design for the development of mapping population and the type of markers used depend upon the objectives of experiments, availability of markers, and the molecular map. An appropriate mapping population, with suitable marker system and the data analyzing software are the key rations for molecular mapping and breeding. Genetic map construction requires: appropriate mapping population; pairwise recombination frequencies calculation on population; establishment of linkage groups; estimation of map distances; and determine map order. Size of mapping population is also essential factor in mapping as limited population sizes used in many QTL detection experiments may have led to underestimation of QTL number, overestimation of QTL effects, and failure to quantify QTL interactions (Beavis 1998; Melchinger et al. 1998; Utz et al. 2000). The size of the population may be determined by the gene effect to be detected as well as the type of population. While the analysis of large population would enable the detection of small-effect QTLs, the basic purpose of mapping would be served if one can detect the major QTL with large effect and this would require, in general, a mapping population of a size 200–300 individuals.

Mapping software packages, such as Mapmaker (Lander and Botstein 1989; Lander et al. 1987), Mapmanager (Manly and Elliott 1991), and Joinmap (Stam 1993) have been developed to analyze the genetic data for map construction. These software packages use genetic data of segregating mapping populations to estimate recombination frequency followed by determination of linear arrangement of genetic markers. Different types of mapping populations that are often used in linkage mapping are: F2 population; F2-derived F3 (F2:F3) populations; Backcrosses; Doubled haploids (DHs); Recombinant Inbred Lines (RILs); and

Near-isogenic Lines (NILs). The F2 mapping population can be developed with possible combinations of parental alleles (Lander et al. 1987). A recombinant inbred line (RIL) can be obtained from an F2 generation by successive self-pollinations using the single seed descent method (SSD) (Burr et al. 1988). The resulting inbred lines are highly homozygous and the segregation ratio for each locus tends to be 1:1 (AA:aa) representing an 'immortal' or permanent mapping family and can thus be used in experiments with replications in several environments allowing for more accurate estimates of genetic components and identification of QTL vs. environment interactions. Disadvantages of recombinant inbred lines are that at least six generations are required to obtain the line and the inability to estimate dominance effects of mapped quantitative trait loci (QTL) due to the absence of heterozygous genotypes.

In pearl millet, several F2:3 and F2:4 mapping population have been developed from diverse inbred lines of Asian, American, and African origin (Hash et al. 2002). Liu et al. (1994) developed the first F2 population of 133 individuals for the study of downy mildew. It was used by Devos et al. (2000) for comparative mapping of pearl millet with foxtail millet and rice. Further to understand the genetic control of domestication trait, Poncet et al. (2000) developed a population of 250 F2 individuals from cultivated and wild F1 hybrid (*P. glaucum* spp. *monodii*). Poncet et al. (2002) developed another F2 population having 168 individuals. Yadav et al. (2002, 2004) developed two mapping population by crosses of H77/833-2 × PRLT 2/89-33 (early maturing inbred line, 150 F2 individuals) and ICMB 841 × 863B agronomically elite inbred seed parent, 106 F6 individuals (Kumar et al. 2016).

The QTL analysis is based on association between trait value and marker allele. Number of studies have been reported for detecting QTLs with traits like downy mildew (Jones et al. 1995, 2002; Gulia 2004), rust and blast (Morgan et al. 1998), drought tolerance (Yadav et al. 2002, 2003, 2004; Bhattacharjee et al. 2002; Bidinger et al. 2007; Kholová et al. 2012), flowering time (Kumar et al. 2017; Saïdou et al. 2009), panicle length (Poncet et al. 2000; Kumar et al. 2017), and 1000-grain mass (Yadav et al. 2002; Bidinger et al. 2007; Kumar et al. 2017). Research on identifying QTLs and candidate genes for elevated levels of Fe and Zn in pearl millet is limited at this time (Manwaring et al. 2016). A recent study has identified the QTLs for higher Fe and Zn contents in ICMB 841 × 863B cross on LG3 (Kumar et al. 2016).

4.2.5.4 Marker-Assisted Selection (MAS)

Despite the fact conventional breeding approaches will continue to make valuable aids to the genetic improvement of important traits in pearl millet, the efficiency of such concerted efforts can be increased extensively through the supplementation of MAS approaches. A number of QTL and associated markers have been identified for downy mildew resistance (Hash and Witcombe 2001; Jones et al. 2002; Hash and Witcombe 2002). NILs of H 77/833-2 introgressed with various putative QTL

regions from PRLT 2/89-33 were used for validation of major drought tolerance QTL in the LG2 target region (Serraj et al. 2005).

The validated QTL on LG3 for higher grain Fe and Zn contents (Kumar et al. 2016) has been the target for marker-assisted breeding (MAB). Using the linked flanking markers, this QTL along with downy mildew resistance QTLs has been moved into genetic background of pollen parent of hybrid HHB 67 Improved. These double QTL introgression lines were crossed with the seed parent of HHB 67 Improved to generate HHB 67 Improved like hybrids. These QTL introgression lines along with the improved test-cross hybrids are being tested in the national testing system in India.

4.3 Future Prospects

Considering pearl millet adaptation traits and productivity gains in the drylands over the decades, it would continue to be an important food crop for India and sub-Saharan Africa. It is an ideal native food crop to expand the Indian and global food basket to meet healthy food and nutritional demand of the growing population. National policy measures such as inclusion of pearl millet under public distribution system are essential, besides promotion of pearl millet in poultry/animal feed and breweries to increase incentives to growers for higher production. Creating public awareness about the nutritional values of pearl millet is urgently needed otherwise consumers are likely to prefer non-native crops for daily energy and nutritional requirements. Major area of future focus in biofortification of pearl millet should include:

- The enhanced nutrient contents of new cultivars have to be achieved without any trade off with higher productivity. This would translate into adding one additional trait in breeding program. Micronutrient traits screening is highly expensive while dealing with larger germplasm. Thus breeding for such additional traits may delay progress for productivity traits when resources are limited to breeders. Micronutrient traits are apparently not affected by genetic erosion and involve little maintenance breeding after the genes are incorporated into elite backgrounds. With availability of XRF tool, cost of biofortification breeding will decrease over time, and micronutrient content built into the gene pool will not affect future breeding for productivity traits. In order to achieve it, a higher investment in breeding would be required on a long-term basis.
- Genomic approaches should now be an integral part of breeding program particularly for nutritional traits to use diagnostic markers given that pearl millet genome has been sequenced now (Varshney et al. 2017). Genomic markers can be used to make the biofortification breeding more efficient through marker-assisted selection in near future. This would also help in improving high-yielding cultivars with low iron and zinc under wider cultivation through introgression of micronutrient genes and become essentially derived variety.

- Seed companies have well established network and dominate the pearl millet hybrid seed market in India. Since, hybrids occupy approximately 90% of the area under improved cultivars, first-biofortified variety (OPV) has limited potential to make a mega impact. To address this, public–private partnership (PPP) model needs to be strengthened by institutional policy of nutrition commitments and special price allocation for mineral-dense seeds with subsidized rates in the markets to promote biofortified cultivars.
- ICRISAT has played a key role in diversifying the hybrid parents and its contribution to achieving higher yield gain at farm level through the PPP model. Seed companies, those have research and development division, capture more than 80% of the pearl millet hybrid seed market in India. Thus, the sustainability of biofortified pearl millet will mainly depend on mainstreaming of biofortification with seed companies, state seed corporations, ICAR institutes, and state agricultural universities. Hence, cultivar product concept of partners requires considering micronutrient as a generic trait in their breeding program and this joint effort will address development of high yielding and micronutrient rich pearl millet cultivars.
- There are good prospects for large scale on-farm field and food product demonstrations through state agricultural universities, agricultural departments and Krishi Vigyan Kendras (KVKs); large-scale production and procurements of biofortified cultivar grains for Anghanwadi (childcare center); and integration of biofortified grains in mid-day meal scheme. Several governments sponsored programs such as National Food Security Mission and Integrated Child Development Program would provide window for PDS system model to address the iron and zinc deficiency.

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