Inbreeding Effects on Grain Iron and Zinc Concentrations in Pearl Millet

Kedar Nath Rai,* Mahalingam Govindaraj, Anand Kanatti, Aluri S. Rao, and Harshad Shivade

ABSTRACT
The magnitude, direction, and pattern of inbreeding effects on trait expression in selfing generations have a direct bearing on single-plant and progeny-based selection efficiency. In the present study on a pearl millet [Pennisetum glaucum (L.) R. Br.] biofortification initiative, initial random mated S₀ bulks of three diverse composites and their S₁ to S₄ population bulks derived from four generations of selfing were evaluated for 2 yr under irrigated and terminal drought stress for iron (Fe) and zinc (Zn) concentrations. Both Fe and Zn concentrations were higher under terminal drought than under irrigated condition. Inbreeding had no significant effect on Fe and Zn concentrations in one composite and showed significant though marginal increase of both micronutrients in two composites. This finding, not unexpected, was in conformity with the earlier reports of predominantly additive gene effects and marginal partial dominance of genes determining low concentrations of these micronutrients observed in a low frequency of hybrids. The patterns of genetic changes in Fe concentration due to inbreeding were highly significantly and positively correlated with those in Zn concentration in all three composites. These results indicate that simultaneous single-plant and progeny-based early generation selection for Fe and Zn concentrations is likely to be effective to enhance the breeding efficiency for these micronutrients in pearl millet.


Abbreviations: EBC, Early B-Composite; HHVBC, High Head Volume B-Composite; ICP, inductively coupled plasma optical emission spectroscopy; OPV, open-pollinated variety; SRBC, Smut Resistant B-Composite.

Pearl millet [Pennisetum glaucum (L.) R. Br.] is a highly cross-pollinated crop with 70 to 80% outcrossing (Burton, 1974). This breeding system provides open-pollinated varieties (OPVs) and hybrids as the two broad cultivar options. Hybrids in pearl millet have 25 to 30% grain yield advantage over OPVs (Rai et al., 2006; Yadav et al., 2012b). With the availability of commercially viable cytoplasmic-nuclear male-sterility systems (Burton, 1965; Hanna, 1989; Rai et al., 2001), there has been increasing use of hybrid technology to increase pearl millet productivity in India, which has the largest area of about 8 million ha under this crop in the world, and an estimated 60% of pearl millet area in India is planted to hybrids (Yadav et al., 2012a). Hybrid technology is also being experimented to enhance the productivity of pearl millet in several countries of Africa, which together plant this crop on ~18 million ha. High grain yield potential is an important economic consideration in farmers’ adoption of hybrids. Development of such hybrids depends on combining ability of parental lines and their grain yield potential per se. Given the results of several studies, it has been observed that either there is no correlation between the performance per se of the parental lines and their general combining ability for grain yield, or that

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both are positively correlated (Rai and Virk, 1999). This would imply that high general combiners can be found as frequently or rather more frequently in high-yielding groups than in the other yield groups. High grain yield potential of the parental lines contributes not only to high yield potential of hybrids but is also important from the viewpoint of seed production economy.

Considering the widespread micronutrient malnutrition and its associated adverse health consequences, especially those arising from iron (Fe) and zinc (Zn) deficiencies (Bouis et al., 2011), and the role that pearl millet can play in addressing this issue, selection for high Fe and Zn concentrations has added another dimension to genetic improvement of this crop. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), in alliance with the HarvestPlus Challenge Program of the Consultative Group on International Agricultural Research (CGIAR), has recently undertaken a major initiative to breed high-yielding parental lines of pearl millet with high Fe and Zn concentrations. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), has recently undertaken a major initiative to breed high-yielding parental lines of pearl millet with high Fe and Zn concentrations in pearl millet. It has been shown that, unlike grain yield, performance per se of lines is highly significantly and positively correlated with general combining ability for Fe and Zn concentrations in pearl millet, implying that the lines selected for high Fe and Zn concentrations will also be high general combiners for these micronutrients (Velu et al., 2011b; Govindaraj et al., 2013; Kanatti et al., 2014a, 2016). Development of inbred lines with high Fe and Zn concentrations depends on the level of and variability for these micronutrients in the base population (whether Fe and Zn concentrations depends on the level of and variability for these micronutrients in the base population (whether Fe and Zn concentrations). The Hull Genotypes (Velu et al., 2007, 2008a, 2008b; Gupta et al., 2009; Rai et al., 2012). However, there is no information on inbreeding effects on these micronutrients. The objective of this research was to examine the effect of four generations of selfing on the extent, direction, and patterns of inbreeding effects. Large variability for Fe and Zn concentrations has been reported in pearl millet (Velu et al., 2007, 2008a, 2008b; Gupta et al., 2009; Rai et al., 2012). However, there is no information on inbreeding effects on these micronutrients. The objective of this research was to examine the effect of four generations of selfing on the extent, direction, and patterns of inbreeding effects on Fe and Zn concentrations in three diverse pearl millet composites.

**MATERIALS AND METHODS**

**Experimental Materials**

The basic genetic material for this study consisted of three composites of diverse morphological characteristics: Early B-Composite (EBC), Smut Resistant B-Composite (SRBC), and High Head Volume B-Composite (HHVBC). These composites vary for days to 50% flower (39–56 d), plant height (112–169 cm), tillering ability (1.5–3.5 tillers plant⁻¹), panicle length (17–31 cm), and grain weight (7.5–13.0 g 1000⁻¹ grains). The EBC was developed by random mating 324 potential maintainer lines (B-lines), SRBC by random mating 47 smut [caused by Moesziomyces penicillariae (Bref.) Vanky] resistant potential B-lines, and HHVBC by random mating 38 potential B-lines. These composites were subjected to four generations of selfing in Alfisols at Patancheru to derive self-bulks as follows.

More than 100 random plants in each composite were covered with the parchment paper bags at the initial stage of panicle emergence to produce S₁ seed. One hundred and seven S₁ progenies from each composite were planted in 1-m-long single plots in hills spaced at 10 cm. About 15 d after the emergence, hills were thinned to a single plant per hill. The third plant from the proximal end of each plot was covered with the parchment paper bag to produce S₂ seed in each S₁ progeny. This procedure of planting and selfing was continued for the next two generations to produce S₃ and S₄ progenies (Table 1). Equal quantity of seed from each progeny at a given selfing stage was pooled to produce selfed bulks for each composite.

**Composite Bulk Trial**

The original bulk (S₀) and the four selfed bulks (S₁–S₄) of each composite were planted in split-plot design in Alfisols at Patancheru during the summer seasons of 2010 and 2011 in adjacent strips. One strip was used as an irrigated control (irrigation at 7– to 10-d interval), and the other strip was subjected to terminal drought by holding off the postflowering irrigation (hereafter refer to as drought). There were four buffer rows between the two strips. Each bulk was replicated three times with composites randomized as main plots and the bulks within each composite randomized as subplots. Each entry was planted in four rows of 4-m length spaced 60 cm apart. At about 15 d after the emergence, overplanted plots were thinned to single plants spaced 10 cm apart, which was followed by manual weeding. Basal dose of 100 kg of diammonium phosphate (DAP, contains 18.46% N:P) was applied at the time of field preparation, and 100 kg ha⁻¹ of urea (46% N) was applied as sidedressing after the weeding. Open-pollinated panicles of all plots were harvested at or after physiological maturity, stored in gunny bags, sun dried on tarpouline sheet for 12 to 15 d, and hand threshed to produce grain bulks from which 20 to 30 g of grains were sampled for laboratory analysis.

**Micronutrient Analysis**

The grain samples were analyzed for Fe and Zn concentrations at the Waite Analytical Services Laboratory, University of Adelaide, Australia, using inductively coupled plasma optical emission spectroscopy (Spectro Analytical Instruments), hereafter referred to as ICP analysis, following Wheal et al. (2011). Briefly, grain samples were oven dried overnight at 85°C, ground enough to pass through a 1-mm stainless steel sieve using a Christie and Norris hammer mill, and stored in screw-top polycarbonate vials. The samples were digested with di-acid (nitric-perchloric acid) mixture and the digests were used for Fe and Zn determination using Spectro CIROS Axial ICP. Ten

**Table 1. Number of progenies selfed and advanced from S₁ to S₄ generation in three composites of pearl millet at Patancheru.**

<table>
<thead>
<tr>
<th>Composite†</th>
<th>S₁</th>
<th>S₂</th>
<th>S₃</th>
<th>S₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBC</td>
<td>107</td>
<td>105</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td>HHVBC</td>
<td>107</td>
<td>105</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td>SRBC</td>
<td>107</td>
<td>103</td>
<td>95</td>
<td>93</td>
</tr>
</tbody>
</table>

† EBC, Early B-Composite; HHVBC, High Head Volume B-Composite; SRBC, Smut Resistant B-Composite.
milliliters of nitric acid and 1 mL of perchloric acid were added into a 1.0-g flour sample and stored overnight at room temperature. The samples were heated for 1 h at 120°C, increased to 175°C (until digests turn black in color; if the digests turn black, add nitric acid dropwise until the digest clears), and then further increased to 225°C, which was maintained for ~10 min to allow complete digestion of the sample. To cool, the digests were left at room temperature for 20 min. After cooling, the digests were diluted with 20 mL of 1% nitric acid. Amorphous silica was separated from the digest solution by settling overnight, and then the supernatant was transferred into an auto-sampler test tube before aspirating directly into the plasma for the determination of Fe and Zn. The digested solution was introduced into the plasma using a modified Babington Pneumatic nebulizer. A Gilson Minipuls 2 peristaltic pump with a Tygon red-red (1.14 mm) pump tube was used for solution delivery to the nebulizer. A stabilization time of 30 s was followed by three 20-s integrations. The Fe concentration was read at 259.94 nm and Zn concentration at 213.86 nm in the ICP. Reagent bottles, volumetric ware (plastic and glass), and digestion tubes were cleaned after usage by soaking overnight in 1.42 mol kg⁻¹ HCl, rinsing with water, and oven drying at 60°C. Double distilled water was used for all analytical purposes. In 1 yr during the 2011 summer season, 1000-seed weight was also recorded, which was determined using random sample of 200 grains and then multiplying by a factor of five, both under irrigated control and terminal drought environments.

**Statistical Analysis**

The composite bulk trial was analyzed following fixed model (Gomez and Gomez 1984) using the generalized linear model procedures in SAS 9.3 (SAS Institute, 2009). Analysis of variance was done for individual levels (irrigated and drought stress) and combined across levels with population bulks nested within composites. Assuming no epistasis, only loci with dominance and in heterozygous state would contribute to inbreeding depression. Therefore, population level percentage homozygosity related to the genotypes at such loci would be 0.00% at S₂ stage, 50.00% at S₁ stage, 75.00% at S₂ stage, 87.50% at S₃ stage, and 93.75% at the S₄ stage. The differences among the population bulks at various homozygosity levels were tested for statistical significance following Duncan’s Multiple Range Test at probability of <0.05. The composite bulks at each homozygosity levels were subjected to linear regression analyses, which were tested for statistical significance following Gomez and Gomez (1984) as given below. The residual mean square $S_{xy}^2$ was calculated using the following equation:

$$S_{xy}^2 = \frac{\sum y^2 - (\sum xy)^2}{\sum x^2}$$

where $x$ is the deviations from the mean of the independent variable (i.e., homozygosity level), $y$ is the deviation from the means of dependent variables (i.e., Fe, Zn, and 1000-grain weight), and $n$ is the number of generations. Then, the computed $t$-value $t_b$ was estimated using the following formula:

$$t_b = \frac{b}{\sqrt{\frac{S_{xy}^2}{\sum x^2}}}$$

where $b$ is the regression coefficient, which is tested to be significantly different from zero if the absolute value of $t_b$ is greater than the tabulated $t$-value with $n − 2$ degrees of freedom at the 5 or 1% level of significance. The significant test for difference between control and drought was estimated by $t$-test using the following formula:

$$t = \frac{\bar{d}}{\sqrt{\frac{2EMS_{pooled}}{n}}}$$

where $\bar{d}$ is the mean difference between control and drought at each level of homozygosity (inbreeding generations), as well as averaged over the homozygosity levels. EMS is the appropriate error mean sum of square, and $n$ is the number of observations involved in the value to be tested for statistical significance. If the absolute value of calculated $t$ is greater than the tabulated $t$-value, then the difference between the control and drought would be significantly different.

**RESULTS AND DISCUSSION**

The mean Fe concentration, averaged over the 15 composite bulks and the two treatments (irrigated control and terminal drought), varied from 55 mg kg⁻¹ in 2011 to 74 mg kg⁻¹ (35% higher) in 2010 (data not presented). A similar pattern was observed for Zn concentration, which varied from 48 mg kg⁻¹ in 2011 to 57 mg kg⁻¹ (19% higher) in 2010. The difference between the drought and control was highly significant ($P < 0.01$) for both micro-nutrients (Table 2), and these differences were significant in all three composites (Table 3). The mean Fe concentration, averaged over the five composite bulks and the 2 yr, was 14.7% higher under drought than control in EBC, 9.5% higher in SRBC, and 17.1% higher in HHVBC. Similarly, the mean Zn concentration was 5.0% higher under drought than the control in EBC, 5.7% higher in SRBC, and 13.3% higher in HHVBC. However, although consistently significant differences between the drought and the control were observed for both Fe and Zn concentrations at almost all the homozygosity levels in HHVBC, it was not so in the other two composites. The mean grain weight averaged over the homozygosity levels was highly significantly lower under drought than in the control, varying from 10.4% lower in EBC to 19.1% lower in HHVBC. Earlier studies have also reported terminal drought reducing grain size in pearl millet (Mahalakshmi and Bidinger, 1985; Bidinger et al., 1987; Fussell et al., 1991; Bieler et al., 1993). The reduction in grain size under the drought results largely from reduction in the endosperm component of the grain; thus, outer grain layers constitute a relatively larger proportion of the grain size.
under drought than under the irrigated control. It has been found that both Fe and Zn are mostly concentrated in the germ (consisting of scutellum and embryo) and the outer grain layers (pericarp and aleurone) of pearl millet (Minnis-Ndimba et al., 2015). Thus, reduction in grain size under drought may partly account for higher Fe and Zn concentrations. It has also been observed, though not systematically documented, that terminal drought leads to genotype-dependent variable reduction in seed set in pearl millet. Studies in wheat (*Triticum aestivum* L.) (Morgan, 1980; Dembinska et al., 1992; Weldearegay et al., 2012), maize (*Zea mays* L.) (Herrero and Johnson, 1981), and rice (*Oryza sativa* L.) (Jin et al., 2013) have shown reduction in seed set under terminal drought. It has been found that reduction in seed set increases the concentrations of Fe and Zn in pearl millet (Rai et al., 2015). Thus, possible reduction in seed set may also account, in part, for higher concentrations of these micronutrients under terminal drought.

The differences among the three composites were highly significant (*P* < 0.01), for both Fe and Zn concentrations, whereas their interactions with year and with moisture and their second-order interaction with year and moisture stress were nonsignificant (Table 2).

### Table 2. Mean square for grain iron (Fe) and zinc (Zn) concentrations and 1000-grain weight in pearl millet composites during the 2010 and 2011 summer seasons at Patancheru.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Fe</th>
<th>Zn</th>
<th>1000-grain weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>1</td>
<td>16,977**</td>
<td>3,247*</td>
<td>–</td>
</tr>
<tr>
<td>Replication/Year</td>
<td>4(2)†</td>
<td>177</td>
<td>261</td>
<td>0.91</td>
</tr>
<tr>
<td>Moisture</td>
<td>1</td>
<td>3,222**</td>
<td>751**</td>
<td>57.26**</td>
</tr>
<tr>
<td>Moisture × Year</td>
<td>1</td>
<td>349*</td>
<td>135*</td>
<td>–</td>
</tr>
<tr>
<td>Error 1</td>
<td>4(2)</td>
<td>15</td>
<td>49</td>
<td>0.65</td>
</tr>
<tr>
<td>Composite</td>
<td>2(2)</td>
<td>5,037**</td>
<td>692**</td>
<td>11.15**</td>
</tr>
<tr>
<td>Composite × Year</td>
<td>2</td>
<td>304</td>
<td>37</td>
<td>–</td>
</tr>
<tr>
<td>Composite × Moisture</td>
<td>2</td>
<td>168</td>
<td>92</td>
<td>1.53</td>
</tr>
<tr>
<td>Composite × Moisture× Year</td>
<td>2</td>
<td>50</td>
<td>55</td>
<td>–</td>
</tr>
<tr>
<td>Error 2</td>
<td>16(8)</td>
<td>130</td>
<td>55</td>
<td>0.48</td>
</tr>
<tr>
<td>Bulk/Composite</td>
<td>12</td>
<td>183**</td>
<td>72**</td>
<td>2.32**</td>
</tr>
<tr>
<td>Bulk (EBC)</td>
<td>4</td>
<td>216*</td>
<td>46**</td>
<td>2.86**</td>
</tr>
<tr>
<td>Bulk (SRBC)</td>
<td>4</td>
<td>19</td>
<td>21</td>
<td>2.02**</td>
</tr>
<tr>
<td>Bulk (HHVBC)</td>
<td>4</td>
<td>315**</td>
<td>149**</td>
<td>2.08</td>
</tr>
<tr>
<td>Bulk/Composite × Year</td>
<td>12</td>
<td>83</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>Bulk/Composite × Moisture</td>
<td>12</td>
<td>62</td>
<td>20</td>
<td>1.91**</td>
</tr>
<tr>
<td>Bulk/Composite × Moisture × Year</td>
<td>12</td>
<td>39</td>
<td>27</td>
<td>–</td>
</tr>
<tr>
<td>Error 3</td>
<td>96(48)</td>
<td>60</td>
<td>18</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† Figures in the parentheses indicate df for 1000-grain weight of the 2011 summer season.

### Table 3. Mean grain iron (Fe) and zinc (Zn) concentration and 1000-grain weight in pearl millet composite bulks. Mean of 2 yr, the 2010 and 2011 summer seasons, at Patancheru.

<table>
<thead>
<tr>
<th>Composite‡</th>
<th>Generation</th>
<th>Homozygosity</th>
<th>%</th>
<th>Fe Control</th>
<th>Drought</th>
<th>Mean</th>
<th>Zn Control</th>
<th>Drought</th>
<th>Mean</th>
<th>1000-grain weight†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBC</td>
<td>S0</td>
<td>0.00</td>
<td>57.3</td>
<td>64.2</td>
<td>60.7a§</td>
<td>49.8</td>
<td>51.7</td>
<td>50.8a</td>
<td>12.3</td>
<td>11.1</td>
</tr>
<tr>
<td>S1</td>
<td>50.00</td>
<td>60.2</td>
<td>68.7</td>
<td>64.4b</td>
<td>50.3</td>
<td>53.1</td>
<td>51.7ab</td>
<td>11.7</td>
<td>10.9</td>
<td>11.3a</td>
</tr>
<tr>
<td>S2</td>
<td>75.00</td>
<td>65.2</td>
<td>69.7</td>
<td>67.4c</td>
<td>54.8</td>
<td>54.8</td>
<td>54.8c</td>
<td>11.1**</td>
<td>8.9</td>
<td>10.0b</td>
</tr>
<tr>
<td>S3</td>
<td>87.50</td>
<td>68.0</td>
<td>75.6</td>
<td>71.8d</td>
<td>54.0</td>
<td>56.6</td>
<td>55.3c</td>
<td>11.0</td>
<td>9.7</td>
<td>10.3b</td>
</tr>
<tr>
<td>S4</td>
<td>93.75</td>
<td>59.9**</td>
<td>77.8</td>
<td>68.9c</td>
<td>54.0</td>
<td>56.6</td>
<td>55.3c</td>
<td>11.0</td>
<td>9.7</td>
<td>10.3b</td>
</tr>
<tr>
<td>Mean</td>
<td>62.1**</td>
<td>71.2</td>
<td>66.6</td>
<td>66.6</td>
<td>51.8*</td>
<td>54.4</td>
<td>53.1</td>
<td>11.5**</td>
<td>10.3</td>
<td>10.9</td>
</tr>
<tr>
<td>SRBC</td>
<td>S0</td>
<td>0.00</td>
<td>50.7</td>
<td>55.7</td>
<td>53.2a</td>
<td>46.7</td>
<td>47.2</td>
<td>46.9a</td>
<td>13.3**</td>
<td>9.7</td>
</tr>
<tr>
<td>S1</td>
<td>50.00</td>
<td>48.8**</td>
<td>62.4</td>
<td>55.6a</td>
<td>45.4**</td>
<td>53.5</td>
<td>49.5c</td>
<td>12.6*</td>
<td>11.1</td>
<td>11.9c</td>
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<tr>
<td>S2</td>
<td>75.00</td>
<td>50.3</td>
<td>56.0</td>
<td>53.2a</td>
<td>45.8</td>
<td>49.8</td>
<td>47.8ab</td>
<td>12.2*</td>
<td>10.2</td>
<td>11.2a</td>
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<tr>
<td>S3</td>
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<td>55.3</td>
<td>56.1</td>
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<td>48.9</td>
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<td>50.2c</td>
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<td>54.6</td>
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<td>48.8bc</td>
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<tr>
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<td>72.5</td>
<td>72.5</td>
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<td>55.3</td>
<td>11.0**</td>
<td>8.9</td>
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<td>0.00</td>
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<td>64.9a</td>
<td>47.7*</td>
<td>54.6</td>
<td>51.1a</td>
<td>12.4**</td>
<td>9.6</td>
</tr>
<tr>
<td>S1</td>
<td>50.00</td>
<td>68.2**</td>
<td>80.5</td>
<td>74.4c</td>
<td>51.9**</td>
<td>60.7</td>
<td>56.3b</td>
<td>11.2**</td>
<td>8.4</td>
<td>9.8b</td>
</tr>
<tr>
<td>S2</td>
<td>75.00</td>
<td>70.9**</td>
<td>83.5</td>
<td>77.2d</td>
<td>55.7*</td>
<td>61.4</td>
<td>58.5c</td>
<td>10.2</td>
<td>9.6</td>
<td>9.9b</td>
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<td>71.1*</td>
<td>81.5</td>
<td>76.3cd</td>
<td>54.5**</td>
<td>62.8</td>
<td>58.6c</td>
<td>10.4*</td>
<td>9.0</td>
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<tr>
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<td>93.75</td>
<td>65.5</td>
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<td>11.0**</td>
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<tr>
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<td>78.2</td>
<td>72.5</td>
<td>72.5</td>
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<td>55.3</td>
<td>11.0**</td>
<td>8.9</td>
<td>10.0</td>
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</tbody>
</table>

* Significant between control and drought at the 0.05 probability level.

** Significant between control and drought at the 0.01 probability level.

† According to 1 yr (2011 summer season) of data.

‡ EBC, Early B-Composite; HHVBC, High Head Volume B-Composite; SRBC, Smut Resistant B-Composite.

§ Values within a column followed by the same letter are not significantly different from one another at 0.05 probability.

¶ Standard error to compare control vs. drought at each homozygosity level.

# Standard error to compare differences among homozygosity levels.

†† Standard error to compare means of control vs. drought averaged over homozygosity levels.
among the composite bulks pooled over the three composites was also highly significant, but not their interactions with either year or moisture, for both Fe and Zn concentrations. Further partitioning of this variation showed that the differences among the composite bulks were significant for both Fe and Zn concentrations only in EBC and HHVBC. Given the mean over the years and the two moisture level treatments, as compared with the random mated $S_0$ bulk, there was a significant though marginal increase of 6% Fe concentration in the $S_1$ bulk at 50% homozygosity in EBC, which steadily increased to 18% at 87.5% homozygosity (Table 3). The regression coefficient of Fe concentration over the homozygosity level was positive and significant ($P < 0.05$) (Table 4). There was also a significant though marginal increase of 8% Zn concentration at 75% homozygosity, with no further increase as the homozygosity increased, and the regression of Zn concentration on homozygosity level was not significant. In HHVBC, there was a significant increase of 15% in the Fe concentration at 50% homozygosity level, which further increased to 19% at the 75% homozygosity level. A similar pattern was observed for Zn concentration, which increased by 10% at the 50% homozygosity level, with further increase to 15% at the 75% homozygosity level. However, the regression coefficients of these micronutrients on homozygosity level were not significant. Considering the trends in changes of Fe and Zn concentrations in relation to inbreeding levels, the concentrations of both micronutrients should have either increased in $S_1$ bulks or stabilized at the level of $S_3$ bulks. Surprisingly, and contrary to expectations, there was significant decline of Fe and Zn concentrations in two out of three composites.

The above results showed that either there was no change in Fe and Zn concentrations due to inbreeding (as in SRBC), or there were marginal increases (as in EBC and HHVBC). Inbreeding effects occur due to exposure of recessive alleles at loci having dominance effects. Thus, the lower the level of dominance, the smaller the inbreeding effects. Further, if recessive alleles have negative effects, there would be decline in the trait expression, and the reverse would happen if the recessive alleles have positive effects. Earlier studies in pearl millet have shown both Fe and Zn concentrations predominantly under additive genetic control (Velu et al., 2011b; Govindaraj et al., 2013; Kanatti et al., 2014a). Studies in other cereals, such as rice (Zhang et al., 2004) and maize (Gorsline et al., 1964; Arnold and Bauman, 1976; Brkic et al., 2003; Long et al., 2004; Chen et al., 2007; Chakraborti et al., 2011), have also reported the predominance of additive genetic variance for Fe concentration. Pearl millet studies mentioned above have shown no better-parent heterosis and significant but marginal midparent heterosis only in low frequencies of hybrids, for both Fe and Zn concentrations, which more often were in a negative direction, thereby indicating some degree of partial dominance with dominant alleles having negative effects and recessive alleles having positive effects. This would provide the genetic basis for marginal increases in the Fe and Zn concentrations as a result of inbreeding in EBC and HHVBC. There was a marginal decline in grain weight at the 50% homozygosity level in HHVBC and at the 75% homozygosity level in EBC, and the regression of grain weight on homozygosity was slightly negative, though significant ($P < 0.05$), only in HHVBC (Table 4). There was high negative correlation ($r > -0.71$) between grain size and both micronutrients in EBC, though it was significant ($r = -0.95, P < 0.05$) only between grain size and Zn concentration. Modest negative correlations were observed between Zn concentration and grain size ($r = -0.48$) and between Fe concentration and grain size ($r = -0.67$) in HHVBC. Thus, reduction in grain size may also account, in part, for increases in Fe and Zn concentrations arising from increases in homozygosity. Since inbreeding effect is also highest when the gene frequency at loci displaying dominance is 0.5 (Falconer and Mackay, 1996), the lack of any significant inbreeding effect in SRBC might have resulted from the gene frequency at such loci being closer to unity coupled with predominantly additive gene effects. Given the mean performance over years and the two treatment levels, the changes in Fe concentration at various homozygosity levels were positively and highly significantly correlated with changes in Zn concentration, not only in EBC ($r = 0.89, P < 0.05$) and HHVBC ($r = 0.97, P < 0.01$), but also in SRBC ($r = 0.94, P < 0.05$). Such correlated changes are not unexpected, as several previous pearl millet studies have shown highly significant and high positive correlations between these micronutrients (Velu et al., 2007, 2008a, 2008b; Gupta et al., 2009; Rai et al., 2012, 2013; Govindaraj et al., 2013; Kanatti et al., 2014a, 2014b, 2016). Similar results have also been reported in other cereals, such as maize (Arnold et al., 1977; Oikeh et al., 2003, 2004), rice (Stangoulis et al., 2007; Anandan et al., 2011), wheat (Garvin et al., 2006; Peleg et al., 2009; Zhang et al., 2010; Velu et al., 2011a), and finger millet [Eleusine coracana (L.) Gaertn.] (Upadhyaya et al., 2011). Such trends in association between Fe and Zn concentrations may result due to common and

<table>
<thead>
<tr>
<th>Composite†</th>
<th>Fe</th>
<th>Zn</th>
<th>1000-grain weight‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBC</td>
<td>0.104*</td>
<td>0.041</td>
<td>−0.012</td>
</tr>
<tr>
<td>SRBC</td>
<td>0.017</td>
<td>0.023</td>
<td>−0.011</td>
</tr>
<tr>
<td>HHVBC</td>
<td>0.089</td>
<td>0.047</td>
<td>−0.014*</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 probability level.
† EBC, Early B-Composite; HHVBC, High Head Volume B-Composite; SRBC, Smut Resistant B-Composite.
‡ According to 1 yr (2011) of data.

Table 4. Regression coefficients for grain iron (Fe) and zinc (Zn) concentrations and 1000-grain weight at various homozygosity levels in pearl millet composites at Patancheru.
overlapping quantitative trait loci for Fe and Zn concentrations, as reported in wheat (Peleg et al., 2009; Singh et al., 2010), rice (Stangoulis et al., 2007), common bean (Phaseolus vulgaris L.) (Cichy et al., 2009; Blair et al., 2009), and pearl millet (Kumar 2011).

Single-plant selection for grain yield per se, which has been shown to be predominantly under nonadditive genetic control (Khairwal et al., 1999) and which undergoes a much higher degree of inbreeding depression of the order of 36 to 40%, even after one to two generations of selfing (Khadr and El-Rouby, 1978; Rai et al., 1985), is not very effective. On the contrary, it would appear that single-plant selection for Fe and Zn concentrations, which are predominantly under additive genetic control and which underwent only marginal changes, even after four generations of inbreeding, is likely to be highly effective. A selection study involving four pearl millet composites has shown that correlation between single-plant performance and the performance of their corresponding S₁ progenies was highly significant and as high as the correlation between the performances of S₁ progenies evaluated in two seasons at the same location (Govindaraj et al., 2012). The patterns and magnitudes of changes observed in the present study also showed that plants and progenies selected for high Fe and Zn concentrations are more likely to retain their initial levels or even marginally increase in the subsequent generations.

**Conflict of Interest**
The authors declare that there is no conflict of interest

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