DEVELOPMENT OF MAPPING POPULATION AND IDENTIFICATION OF QTLS FOR HIGH ZINC CONTENT IN PIGEONPEA [*Cajanus cajan* (L.) Millsp.]

BASAVARAJESHWARI, R. MATHAPATI

PALB 1044

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
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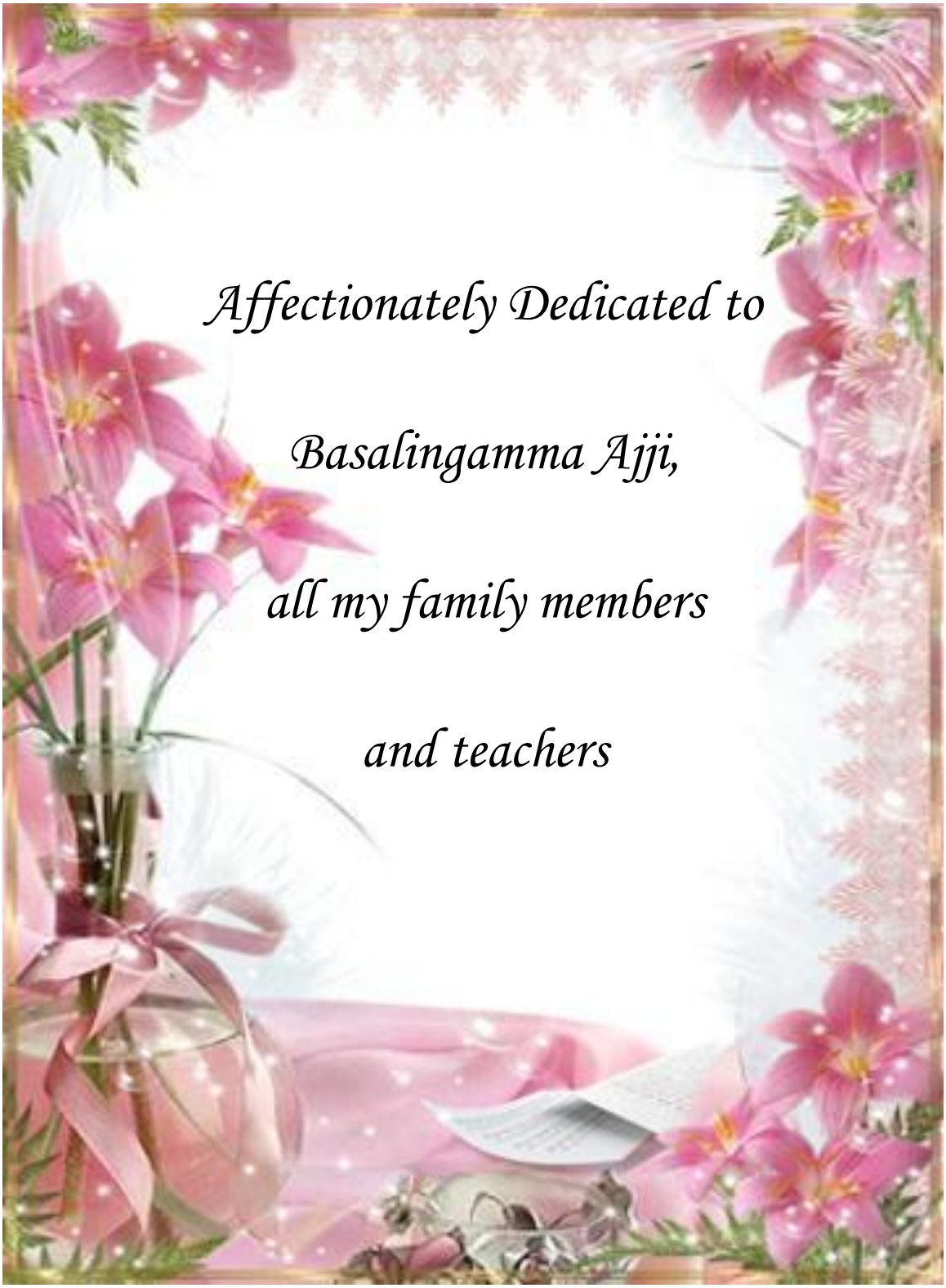
Affectionately Dedicated to
Basalingamma Ajji,
all my family members
and teachers

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU 560 065

CERTIFICATE

This is to certify that the thesis entitled "**Development of mapping population and identification of QTLs for high zinc content in pigeonpea [*Cajanus cajan* (L.) Millisp.]** submitted in partial fulfillment of the requirement for the degree of **Doctor of Philosophy** in **Agriculture**, in **Crop Physiology** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work carried out by **Ms. BASAVARAJESHWARI, R. MATHAPATI, ID No. PALB 1044**, under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bengaluru
April, 2016

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With regardful memories . . . . . .

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DEVELOPMENT OF MAPPING POPULATION AND IDENTIFICATION OF QTLs FOR HIGH Zn CONTENT IN PIGEONPEA [*Cajanus cajan* (L.) Millsp.]

BASAVARAJESHWARI, R. MATHAPATI

THESIS ABSTRACT

Zinc plays key roles in plants and human health regulating several major physiological and biological processes. It has emerged as the most widespread micronutrient deficiency in soils and crops worldwide, resulting in severe yield losses and deterioration in nutritional quality, ultimately leading to human zinc deficiency. The short term interventions to curb human zinc deficiency are mineral supplementation, dietary diversification and food fortification but they are not economically viable in the long run. So, biofortification through genetic or agronomic approaches may be a long lasting solution to cater the needs of both the rural and urban population. In this direction, 30 high and low zinc genotypes selected out of 217 germplasm lines outsourced from ICRISAT were genotyped to analyze their genetic diversity using 50 SSR markers. Cross were conducted between selected lines. Of the crosses, the cross ICP6443 X ICP10960 was used to develop mapping population segregating for seed Zn content and subsequently used for phenotyping, linkage mapping and QTL analysis. Significant phenotypic variability was noticed for the seed zinc and other traits in the population. The polymorphic information of 151 SSR markers were used to construct linkage map. Multipoint linkage analysis resulted in linkage map of 62 markers into 10 linkage groups with a total map length of 1942.8 cM. QTLs for seed zinc, leaf zinc and yield traits were identified using linkage mapping. Several pleiotropic markers were identified for different traits in QTL analysis. Single marker analysis and composite interval mapping resulted in discovery of common markers (AHSSR93, AHSSR118 and ASSR280) for seed zinc content. The study lead to discovery of several transgressive segregates with high seed zinc content, serving as potential genetic resources. An important genic marker bZIP transcription factor (ASSR20) speculated to regulate zinc deficiency response is found to be linked to seed zinc content.

April, 2016

Department of crop physiology,
UAS, GKVK, Bengaluru

A. G. Shankar
(Major Advisor)

ತೊಗರಿಯಲ್ಲಿ ಬೀಜ ಸತು ನಿರ್ದಿಷ್ಟ ನಕ್ಷೆಯ ಅನುವಂಶಿ ಅಭಿವೃದ್ಧಿ ಹಾಗೂ ಅದರ ಬೀಜದಲ್ಲಿ ಹೆಚ್ಚಿನ ಸತುವಿಗೆ ಪರಿಣಾತ್ಮಕ ಲಕ್ಷಣ ಕೇಂದ್ರಗಳನ್ನು (ಕ್ಯೂ.ಟಿ.ಎಲ್.) ಗುರುತಿಸುವಿಕೆ

ಬಸವರಾಜೇಶ್ವರಿ, ಆರ್. ಮಠಪತಿ

ಪ್ರಭಂದದ ಸಾರಾಂಶ

ಸತುವು ಸಸ್ಯಗಳ ಹಾಗೂ ಮಾನವನ ಆರೋಗ್ಯದಲ್ಲಿ, ಹಲವಾರು ಪ್ರಮುಖ ಜೈವಿಕ ಪ್ರಕ್ರಿಯೆಗಳನ್ನು ನಿಯಂತ್ರಿಸುವಲ್ಲಿ ಪ್ರಮುಖ ಪಾತ್ರವನ್ನು ವಹಿಸುತ್ತದೆ. ವಿಶ್ವದಾದ್ಯಂತ ಮಣ್ಣು ಮತ್ತು ಬೆಳೆಗಳಲ್ಲಿ ಸತುವಿನ ಕೊರತೆ ಅತ್ಯಂತ ವ್ಯಾಪಕವಾಗಿ ಕಂಡುಬಂದಿದ್ದು, ಇದು ತೀವ್ರ ಇಳುವರಿ ನಷ್ಟ ಹಾಗೂ ಪೌಷ್ಟಿಕಾಂಶದ ಗುಣಮಟ್ಟ ಕುಗ್ಗುವಿಕೆಗೆ ಕಾರಣವಾಗಿದೆ. ಇದರಿಂದಾಗಿ, ಮನುಷ್ಯರಲ್ಲಿ ಸತುವಿನ ಕೊರತೆ ಕಂಡು ಬರುತ್ತಿದೆ. ನಮ್ಮ ಆಹಾರದಲ್ಲಿ ಸತುವಿನ ಕೊರತೆಯನ್ನು ನಿಗ್ರಹಿಸುವಲ್ಲಿ ಧಾನ್ಯಗಳ ಕೃತಕ ಮೌಲ್ಯವರ್ಧನೆ ಅಥವಾ ಜೈವಿಕ ಮೌಲ್ಯವರ್ಧನೆ (ಬಯೋಫೋರ್ಟಿಫಿಕೇಷನ್) ಸಂಭಾವನೀಯ ಪರಿಹಾರಗಳಾಗಿವೆ. ಜೈವಿಕ ಮೌಲ್ಯವರ್ಧನೆಯು ಬೀಜಾಧಾರಿಕ ತಂತ್ರಜ್ಞಾನವಾಗಿದ್ದು ಗ್ರಾಮೀಣ ಹಾಗೂ ನಗರವಾಸಿಗಳಲ್ಲಿನ ಸತುವಿನ ಕೊರತೆಯನ್ನು ನೀಗಿಸುವುದರಲ್ಲಿ ಸಧೃಡವಾಗಿದೆ ಎಂದು ಭಾವಿಸಲಾಗಿದೆ. ಜೈವಿಕ ಮೌಲ್ಯವರ್ಧನೆಯು ದೀರ್ಘಾವಧಿಯಲ್ಲಿ ಹೆಚ್ಚು ಪರಿಣಾಮಕಾರಿಯಾದ ವಿಧಾನ ಎನ್ನಬಹುದು. ಅತಿ ಹೆಚ್ಚು ಹಾಗೂ ಅತಿ ಕಡಿಮೆ ಸತುವನ್ನೊಳಗೊಂಡಿರುವ ೩೦ ಸಾಲುಗಳನ್ನು, ೨೧೭ ತಳಿಗಳಿಂದ ಆಯ್ಕೆ ಮಾಡಲಾಗಿ, ಅವುಗಳ ಅನುವಂಶಿಕ ವೈವಿಧ್ಯತೆಯನ್ನು ೫೦ ಸರಳ ಅನುಕ್ರಮ ಪುನರಾವರ್ತಿಕ (ಎಸ್. ಎಸ್. ಆರ್) ಗುರುತುಗಳನ್ನುಪಯೋಗಿಸಿ ವಿಶ್ಲೇಷಿಸಲಾಯಿತು. ನಂತರ, ಆಯ್ಕೆ ಮಾಡಿದ ಸಾಲುಗಳ ನಡುವೆ ಸಂಕರಣ ಮಾಡಲಾಯಿತು. ತೊಗರಿ ಬೀಜಗಳಲ್ಲಿನ ಸತುವಿಗೆ ನಿರ್ದಿಷ್ಟವಾದ ನಕ್ಷೆಯ ಅನುವಂಶಿಯನ್ನು ಅಭಿವೃದ್ಧಿಪಡಿಸಲಾಯಿತು. ICP೬೪೪೩ ತಳಿ ಹಾಗೂ ICP೧೦೯೬೦ ತಳಿಗಳನ್ನು ಸಂಕರಣಗೊಳಿಸಿದಾಗ ಉತ್ಪನ್ನವಾದ ೧೪೪ ಎಫ್೨ (F_2) ಪೀಳಿಗೆಯನ್ನು ಸಂಲಗ್ನತೆ ನಕ್ಷೆ ಮತ್ತು ಕ್ಯೂ.ಟಿ.ಎಲ್. ಗುರುತಿಸಿಕೊಳ್ಳಲು ಬಳಸಿಕೊಳ್ಳಲಾಯಿತು. ಈ ಅಧ್ಯಾಯನಕ್ಕಾಗಿ ಬಳಸಿರುವ ೧೫೧ ಎಸ್. ಎಸ್. ಆರ್. ಗುರುತುಕಾರಕಗಳಲ್ಲಿ ೬೨ ಗುರುತುಕಾರಕಗಳನ್ನು ಮಾತ್ರ ೧೦ ಸಂಲಗ್ನತೆ ಗುಂಪುಗಳಾಗಿ ಹೊಂದಿಸಲಾಯಿತು. ಸಂಲಗ್ನತೆ ಗುಂಪುಗಳ ಒಟ್ಟು ಉದ್ದಳತೆ ೧೯೪೨.೪ ಸೆ. ಮಾ. ಕಂಡುಬಂತು. ಸಂಲಗ್ನತೆ ನಕ್ಷೆ ಹಾಗೂ ಲಕ್ಷಣ ಸ್ವರೂಪಿ ಮಾಹಿತಿಗಳನ್ನು ಬಳಸಿಕೊಂಡು, ಬೀಜ ಸತು, ಎಲೆ ಸತು ಹಾಗೂ ಇಳುವರಿಗೆ ಸಂಬಂಧಪಟ್ಟ ಲಕ್ಷಣಗಳಿಗೆ ಕ್ಯೂ. ಟಿ. ಎಲ್. ಗಳನ್ನು ಗುರುತಿಸಲಾಯಿತು. ಪ್ರಸ್ತುತ ಸಂಶೋಧನೆಯಲ್ಲಿ, ಒಂದೇ ಗುರುತುಕಾರಕ ಹಲವಾರು ತೊಗರಿಯ ಸ್ವಭಾವಲಕ್ಷಣಗಳಿಗೆ ಕಾರಣವಾಗಿತ್ತು. ಸಂಯುಕ್ತ ಮಧ್ಯಂತರ ಮ್ಯಾಪಿಂಗ್ ಹಾಗೂ ಏಕ ಗುರುತು ವಿಶ್ಲೇಷಣೆಗಳ ಪರಿಣಾಮವಾಗಿ ಎಸ್. ಎಸ್. ಆರ್. ಗುರುತುಕಾರಗಳಾದ AHSSR೯೩, AHSSR೧೧೪ ಮತ್ತು AHSSR೨೪೦ ಗಳು, ತೊಗರಿ ಬೀಜಗಳಲ್ಲಿನ ಸತುವಿಗೆ ಸಹಚರ್ಯ ತೋರಿಸಿದವು. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿನ ಮುಖ್ಯಾಂಶಗಳು- ತೊಗರಿಯಲ್ಲಿ ಹೆಚ್ಚಿನ ಸತುವುಳ್ಳ ತಳಿಗಳನ್ನು ಕಂಡುಹಿಡಿಯಲಾಯಿತು. ಈ ಸಂಶೋಧನೆಯಲ್ಲಿ, ASSR೨೦ ಎಂಬ ಪ್ರಮುಖ ಗುರುತುಕಾರವು ಬೀಜ ಸತುವಿಗೆ ಸಹಚರ್ಯ ತೋರಿಸಿತು. ಈ ASSR೨೦ ಗುರುತುಕಾರ bZIP ಎಂದು ಕಂಡುಹಿಡಿಯಲಾಗಿ, ಅದು ಸತು ಕೊರತೆ ಪ್ರತಿಕ್ರಿಯೆ ನಿಯಂತ್ರಿಸುವುದರಲ್ಲಿ ಪಾತ್ರ ವಹಿಸುತ್ತದೆಂದು ತಿಳಿದುಬಂದಿದೆ.

ಏಪ್ರಿಲ್, ೨೦೧೬
ಬೆಳೆ ಶರೀರ ಕ್ರಿಯಾಶಾಸ್ತ್ರ
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ಡಾ॥ ಎ. ಜಿ. ಶಂಕರ್
(ಪ್ರಧಾನ ಸಲಹೆಗಾರರು)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Introduction

I INTRODUCTION

Zinc is a trace element found in varying concentrations in all soils, plants and animals and it is essential for the normal healthy growth of higher plants, animals and human beings. Zinc is needed in small quantities. If the amount available is not adequate (critical concentrations), plants and/or animals will suffer from physiological stress brought about by the dysfunction of several enzyme systems and other metabolic functions (Alloway, 2008). Zinc plays a crucial role in structure and function of various proteins, including enzymes, transcription factors, hormonal receptor sites and biological membranes. It has numerous central roles in DNA and RNA metabolism (MacDonald, 2000) and it is also involved in signal transduction, gene expression and apoptosis (Ishimaru *et al*., 2007). Zn metalloenzymes and zinc-dependent enzymes have been identified and are involved in nucleic acid metabolism and cellular proliferation, differentiation and growth (Chesters and Will, 1978). Hence, zinc deficiency would not only reduce the crop quality and nutritive value but also critically affects crop growth and yield (Sinclair and Kramer, 2012), which in turn has put about 49 % of the world's population at risk of zinc deficiency (WHO 2012).

The possible solutions to correct zinc deficiency in humans may be food supplementation, dietary diversification and food fortification or biofortification. The former two programmes require infrastructure, purchasing power, access to market and health care centres and uninterrupted funding, which are constricted in their own ways (Das and Green, 2013). In addition, such programmes favour the urban population with purchasing power, especially in the developing countries. Alternatively, the latter programme, biofortification is the best option for alleviating zinc deficiency. Biofortification is the development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology (Nestel *et al.,* 2006). It will be accessible to both the rural and urban populations. It could be achieved either through genetic biofortification or agronomic biofortification. There is a developing field of research on biofortification of plant foods with zinc. This involves the breeding of new varieties of crops with the genetic potential to accumulate a high density of zinc in edible parts or genetic engineering approaches enabling the development of transgenics for

accumulation of more zinc or reduction of antinutritional factors coming in the way of nutrient absorption (genetic biofortification) and the use of zinc fertilizers to increase zinc density (agronomic biofortification). Although the use of fertilizers is the fastest route to improve the zinc density in diets, plant breeding route is likely to be the most cost-effective approach in the long run (Das and Green, 2013).

Available data indicate that Zn enrichment traits are present within the genomes of crops that could allow substantial increase in the Zn concentration of edible parts without negatively impacting yield. Increasing the amount of Zn in food crops can improve the Zn status of people. Furthermore, the use of Zn-dense seeds results in greater seedling vigour and increased crop yields when the seeds are sown in Zn-poor soils.

Plant genotypes vary widely in their tolerance to soils with low plant-available Zn with respect to both Zn uptake and utilization. Tolerance of plant genotypes to Zn deficiency, as a genetic trait, is usually referred to as Zn efficiency and defined as the ability of a cultivar to grow and yield well in soils that are too deficient in Zn to support a standard cultivar (Sadeghzadeh, 2013). The physiological and molecular mechanisms of Zn deficiency tolerance are just beginning to be understood, and these mechanisms can be exploited in crop breeding programmes (Hacisalihoglu and Kochian, 2003). For example, Zn-efficient genotypes with better Zn utilization may contain higher amounts of chelators that bind Zn and increase its physiological availability at cellular level. A better understanding of the physiological, morphological and genetic bases of Zn efficiency is needed for the development of fast, simple and reliable screening procedures for identifying and breeding genotypes for Zn efficiency. The first step in breeding for Zn efficiency is the assessment of genetic variability for zinc accumulation.

Although lot of information is present for micronutrient variation in bean (Beebe *et al.,* 2000; Islam *et al.,* 2002), rice (Gregorio *et al.,* 2000), wheat (Monasterio and Graham, 2000), maize (Banziger and Long, 2000; Kovacevic *et al.,* 2004), only a few were related to QTL analysis for the micronutrient concentrations (Guzman-Maldonado *et al.,* 2003; Gelin *et al.,* 2007; Gregorio *et al.,* 2000; Stangoulis *et al.,* 2007; Monasterio and Graham, 2000; Tiwari *et al.,* 2009; Vreugdenhil *et al.,* 2004; Waters and Grusak

2008). Availability of useful variability in the germplasm and understanding of its genetic architecture are the prerequisites for a breeding programme aimed at biofortification of crop plants. Realizing the importance of biofortification several studies were undertaken for the evaluation of germplasm and advanced breeding lines for grain Zn content in particular (Cakmak *et al.,* 2000; Monasterio and Graham, 2000; Chhuneja *et al.,* 2006; Morgounov *et al.,* 2007; Rawat *et al.,* 2009, Yamunarani, 2009; Nagarathna, *et al.,* 2010). In our earlier study, progress had been made with respect to screening for genetic variability for Zn concentration in seeds and leaves of pigeonpea.

Pigeonpea, was the crop of choice for this investigation because it is one of the most widely grown and consumed pulse crop and major source of protein for people whose major dietary intake comes from plant based foods. Pigeonpea, supplements the vegetarian diet in developing countries by ensuring high supply of vitamin B, carotene, and ascorbic acid (Miller *et al.,* 1956). Further, enhancing the nutritional status of pigeonpea is considered as highly relevant. A well-framed breeding programme is essential for achieving the goal of biofortification. In view of this, the major goal of the investigation was to discover QTL governing high grain zinc content in pigeonpea, which could be used in marker assisted breeding approaches.

The specific objectives of this investigations are

1. Development of mapping population for seed Zn content in pigeonpea
2. Identification of polymorphic markers between the contrast parents
3. Discovery of QTLs for seed Zn content in pigeonpea

Review of Literature

II REVIEW OF LITERATURE

Zinc, is one of the essential and vital micronutrients required for optimum crop growth because it is part of the functional subunits or cofactor of more than 300 proteins, among them, some are the class of zinc-finger-proteins as well as RNA-polymerases (Juliane and Andres, 2012). In addition, it has been reported to protect plant cells from oxidative stress mediated by reactive oxygen species (Cakmak, 2000) and may act as an intracellular second messenger (Yamasaki *et al.,* 2007). While some soils are capable of supplying adequate amounts of zinc for crop production but a significant proportion of the Earth's arable land is considered zinc deficient (Alloway 2009). Zinc deficiency in soils is attributed to a number of soil and environmental factors including low total zinc content in soil, low soil organic matter, high soil pH, calcareousness, water logging, high phosphate application, high salt concentration and arid climate (Alloway 2008). Since, zinc is an essential trace element for many physiological processes in plants and other organisms (Andreini *et al.,* 2006; Broadley *et al.,* 2007), it has not only resulted in Zn-limited crop growth but has also imposed a widespread problem of zinc deficiency in humans and animals.

As a result mild to moderate zinc deficiency in humans is common throughout the world (Sandstead, 1995), one third of the world population is zinc deficient and the majority of the zinc deficient population which is at high risk live in low-income countries, according to the International Zinc Nutrition Consultative Group (URL http://www.izincg.org). Zinc deficiency leads to impaired growth, immune dysfunction, increased child morbidity and mortality, adverse pregnancy outcomes, and abnormal neuro-behavioural development. Zinc deficiency is directly related to the severity and frequency of diarrhoeal episodes, a major cause of child death (UNICEF, 2012). The body of evidence on zinc deficiency has accumulated to the degree that zinc fortification has been jointly recommended by WHO and FAO (Allen *et al*., 2006).

The question is how agriculture can contribute to sustainable solutions to these malnutrition problems. In this context, it is important to understand the dynamics of Zn accumulation in grains and other edible parts, which has an implication for human

nutrition. As the ultimate source of any mineral nutrient to plants is soil, hence literature has been reviewed in this chapter starting with a brief overview on deficiency of zinc in soil, factors affecting its availability to plants, roles played by zinc in plants, impact of zinc deficiency in human life and various interventions to curb human zinc deficiency are being discussed. Zinc homeostasis has been reviewed to provide insights in Biofortification strategies. Also Biofortification strategies is being discussed in detail where from our objective point of view breeding for zinc efficiency, identifying molecular markers for zinc content are reviewed. Pigeonpea being our crop of research, importance of legumes in general, Pigeonpea in particular and information regarding available genetic resources in Pigeonpea are being highlighted in this review.

2.1 Zinc deficiency in soils

Zinc deficiency in soils and plants is a global micronutrient deficiency problem reported in many countries (Sillanpaa, 1982; Graham *et al.,* 2001; Alloway, 2004; Singh, B., *et al.,* 2005). Low availability of Zn in calcareous soils is one of the most widely distributed abiotic stresses in world Agriculture, particularly in Turkey, Australia, China and India (Cakmak *et al.,* 1999; Kenbaev and Sade, 2002; Brennan and Bolland, 2006). Zinc deficiency is particularly widespread in cereals growing on calcareous soils (Graham, 1991; Graham *et al.,* 1992; Cakmak *et al.,* 1997; Genc *et al.,* 2006). It is the most important micronutrient deficiency in wheat (Cakmak *et al.*, 1998; Kabata-Pendias. 2001; Alvarez and Gonzalez. 2006; Bagci *et al.,* 2007), resulting not only in low production but in the poor nutritional quality of food (Graham and Welch, 1996). In southern Australia more than 18 million hectares of agricultural land are Zn-deficient (Brennan and Bolland, 2006). Similarly, in India alone more than 85 % of the cereal growing area is affected by low Zn. So, availability of Zn appears to be one of the most limiting factors for quality crop production worldwide. Zinc deficiency is common on neutral and calcareous soils, intensively cropped soils, paddy soils and poorly drained soils, sodic and saline soils, peat soils, soils with high available phosphorus and silicon, sandy soils, highly weathered acid and coarse-textured soils (Cakmak *et al.*, 1998; Singh, B., *et al.,* 2005). Factors such as topsoil drying, subsoil constraints, disease interactions and high cost of fertilizer also contribute to zinc deficiency (Sillanpaa, 1982). Thus apart

from the inherent low soil zinc profiles, the extent to which the total concentration of zinc in a soil is available for uptake by plants or movement down the soil profile depends on a range of soil properties.

2.2 Factors affecting the availability of soil zinc to plants

Soils with a low total Zn concentration are often Zn deficient for crop production. Sandy soils, frequently deficient in available Zn, have an inherently low total Zn concentration. For example, Zn deficiency in plants grown on acid soils is generally associated with a low total soil Zn concentration (Sadeghzadeh 2013). These cases of Zn deficiency are related to an absolute Zn deficiency rather than Zn availability.

Thus the term "availability" is commonly used to describe the ability of plants to take up nutrients from the soil (Sadeghzadeh 2013). Several authors have extensively reviewed the factors affecting the solubility of Zn in soils and its availability to plants. Zn availability to plants can be affected by factors such as total soil Zn content, soil pH, soil type, organic matter, soil temperature and moisture regimes, interaction with other elements, root distribution and rhizosphere effects.

2.2.1 Soil pH

With the exception of molybdenum, the availability of micronutrients generally decreases as the soil pH increases. Increasing the soil pH stimulates Zn adsorption to the surface of various soil constituents, such as metal oxides and clay minerals; this results in decreases in the solubility and availability of Zn to plants. A high pH decreases the desorption of Zn from soil surfaces, which also reduces the availability of Zn to plants. Zn can precipitate in the form of Zn(OH), $ZnCO_3$ and Zn_2SiO_4 at high pH (Shukla and Mittal 1979, Saeed and Fox 1977). The Zn concentration in the soil solution is largely dependent on pH; for example, at pH 5.0, the concentration of Zn in the soil solution is approximately 10^{-4} M, whereas at pH 8.0, this concentration is 10^{-10} M. Liming of acid soils resulted in an increase in the soil pH from 5.2 to 6.8 and an approximate 10-fold decrease in the Zn concentration in plants (Shukla and Moris, 1986).

2.2.2 Soil type

Zinc deficiency occurs in a wide range of soil types in many parts of the world; however, tropical regions with highly weathered soils, semi-arid areas with calcareous high-pH soils, sandy-textured soils, and acid soils in several different climatic zones tend to be the most seriously affected because of their low Zn content (Sadeghzadeh, 2013). In acid soils, in contrast, the availability of Zn depends on the amount of soil Zn. In sandy loam, the availability of natural and supplied Zn doubled when the soil pH was reduced from 7 to 5 using ammonium sulfate. The total soil Zn concentration in calcareous and noncalcareous soils is often similar; however, Zn deficiency is frequently reported for calcareous soils (Singh, B., *et al*., 2005). Calcareous soils (pH>7) with moderate-to-high organic matter content (>15g organic C per kg soil) are likely to be Zn-deficient due to high levels of HCO_3^- in the soil solution (Singh, B., *et al*., 2005). In alkaline soils with a low Zn supply, increasing the Zn application increased the Zn concentration in plants and reduced the deficiency symptoms; however, plant growth was only slightly improved. It was concluded that plant growth on the alkaline soil was more responsive to soil alkalinity than Zn deficiency.

2.2.3 Soil organic matter

Soil organic matter content is another factor that contributes to Zn deficiency in crops. Zn availability to plants is often reported to be low in soils with high organic matter content due to increased adsorption of Zn by organic ligands and components (Katyal and Randhawa, 1983). An adequate level of organic matter increases the solubility and diffusion rate of Zn in soils. In the United States, Zn deficiency problems frequently occur in areas where the surface soil has been removed by land levelling (Alloway, 2004). The underlying soil has less organic matter than the topsoil, and in many cases, the subsoil also has a higher pH. Several researchers have shown a positive correlation between extractable Zn and organic matter. Both the diethyltriamine pentaacetic acid (DTPA) extractable Zn and organic matter content decrease with depth in the soil profile (Alloway, 2004). An experiment in wheat showed that the Zn content is positively correlated with the level of soil organic matter.

2.2.4 Soil temperature and moisture

Other factors that contribute to Zn deficiency include low soil moisture and low temperature (Moraghan and Mascagni, 1991). Soil moisture affects the nutrient supply by impairing diffusion to the root surface. Given that Zn diffusion in soils is highly dependent on soil moisture, plant Zn nutrition may be at risk in semiarid and arid areas where soils are usually water-deficient for long periods during the growing season. Accordingly, in Zn-deficient calcareous soils, wheat yield reductions are more severe under rainfed than irrigated conditions (Singh and Abrol, 1986). However, waterlogging alters Zn chemistry in the soil; for example, submerged soils have decreased concentrations of water-soluble Zn compared with well-drained soils. In addition, decreased Zn solubility and low Zn uptake in poorly drained soils is due to the co-precipitation of Zn with soluble iron and aluminium in the soil (Mandal and Mandal 1986). Early in the growing season, Zn deficiency occurs when the soil temperature is still relatively low and subsequently diminishes as the temperature rises. A low soil temperature often increases the incidence and severity of Zn deficiency symptoms (Moraghan and Mascagni, 1991). It was suggested that a colder root zone temperature decreases root colonization with vesicular-arbuscular (VA) mycorrhizae, root growth, Zn uptake and Zn translocation into the shoots (Schwartz *et al.*, 1987; Moraghan and Mascagni, 1991). In barley, shoot Zn uptake was 82% higher in plants grown in solution at 20 °C compared with those grown at 10 °C (Schwartz *et al.*, 1987).

2.2.5 Interaction of Zn with other elements

The interaction of Zn with other elements decreases Zn availability and influences its adsorption, distribution and utilization in plants. These interactions are mainly due to the influences of other cations on the rate of absorption by plant roots rather than to their effects on the availability of Zn or its forms (Loneragan and Webb, 1993). Zn interactions with P and nitrogen (N) are the most important and widespread in soils with limiting supplies of Zn and P or N. A high level of applied N in the absence of Zn can cause Zn deficiency through a dilution effect, changing the pH of the root environment (Loneragan and Webb, 1993) and increasing the shoot-to-root ratio (Loneragan and Webb, 1993). High levels of P in the soil can also occasionally increase the symptoms of Zn deficiency.

Zn and P co-precipitation in the soil may cause the formation of insoluble ZnO_3 $(PO_4)_2$, which decreases the Zn concentration of the soil solution and thus lowers Zn availability. Moreover, under limiting or marginal supplies of Zn and P, the application of P causes Zn deficiency due to the dilution of the plant Zn concentration as a result of growth stimulation. Yang *et al*. (2011) reported that with increasing P application, the proportion of Zn and P content in the wheat grain relative to the whole plant decreased. Moreover, P and Zn acted antagonistically in roots; and excess P inhibited Zn uptake in roots.

2.2.6 Root distribution and rhizosphere effects

A Zn-efficient genotype is able to grow and yield well in soils too deficient for a standard genotype (Graham, 1984). Research efforts over the last decade have resulted in considerable genotypic variation and a better understanding of underlying mechanisms of Zn efficiency. Some studies attributed Zn efficiency to better internal Zn utilization (Hacisalihoglu *et al.,* 2003, 2004), while others attributed it to greater Zn uptake by the root (Graham and Rengel, 1993; Cakmak *et al.,* 1998; Erenoglu *et al.,* 1999; Wissuwa *et al.,* 2006) However, there has been little critical appraisal of root morphological traits such as length, diameter, and surface area, and their relationships to plant growth and Zn uptake in crop species. Dong *et al.,* (1995) showed that a Zn-efficient bread wheat developed longer and thinner roots than a less Zn-efficient bread wheat and a Zn-inefficient durum. This root trait presumably would enable plants to extract more of the slowly diffusible Zn ions from a given soil volume (Rengel and Graham, 1995a, b). A recent study in rice reported that root surface area explained 32 % of the variation observed in Zn uptake (Gao *et al.,* 2005). There has been a study, showing a 0.7-fold increase in root hair density under Zn-deficient conditions compared with Zn-adequate conditions (Ma *et al.,* 2001). Genc Y *et al.,* (2000) reported that the better growth and greater Zn uptake of barley in Zn- deficient soil can be attributed primarily to greater root surface area due to root hairs. Another study, estimated that for a given amount of diethylene triamine penta-acetic acid (DTPA)-extractable Zn, the spatial accessibility of this fraction increases about 2-fold when the root surface area is doubled (Marschner, 1993).

2.3 Zn Uptake and translocation by plants

2.3.1 The long journey from soil to grain

2.3.1.1 Zinc inputs to soils: The primary input of Zn to soils is from the chemical and physical weathering of parent rocks. The lithosphere typically comprises 70–80 µg Zn g^{-1}, whilst sedimentary rocks contain 10–120 µg Zn g^{-1} (Friedland, 1990; Barak and Helmke, 1993; Alloway, 1995). Mean soil Zn concentrations of 50 and 66 µg total Zn g^{-1} soil are typical for mineral and organic soils, respectively, with most agricultural soils containing 10– 300 µg Zn g^{-1} (Alloway, 1995; Barber, 1995). Zinc occurs in rock forming minerals as a result of the non-specific replacement of Mg and Fe with Zn (Barak and Helmke, 1993). Rocks containing weathered Zn minerals, including Zn sulphide (sphalerite, wurtzite), sulphate (zincosite, goslarite), oxide (zincinte, franklinite, gahnite), carbonate (smithsonite), phosphate (hopeite) and silicate (hemimorphite, willemite) minerals, can form 'calamine' soils containing extremely high concentrations of Zn and other metals (Barak and Helmke, 1993). For example, in Plombières in Belgium, soil Zn concentration exceeds 100,000 µg Zn g^{-1} (Cappuyns *et al*., 2006). Such sites are usually localized to a few hectares, although adjacent soils can also have high Zn through water seepage from ore bodies (Chaney, 1993). Secondary natural inputs of Zn to soils arise because of atmospheric processes like volcanoes, forest fires, and surface dusts and biotic processes like decomposition, leaching/wash off from leaf surfaces (Friedland, 1990).

Humans have long influenced Zn inputs to soils. Two thousand years ago, approximately 10,000 tonnes Zn per year was emitted as a result of mining and smelting activities (Nriagu, 1996). Since 1850, emissions have increased 10 fold, peaking at 3.4 Mt Zn per year in the early 1980s, and then declining to 2.7 Mt Zn per year by the early 1990s (Nriagu, 1996). Arctic troposphere Zn concentrations (2 ng Zn m^{-3} in winter months) are yet to reflect this decline (Gong and Barrie, 2005). The ratio of Zn emissions arising from anthropogenic and natural inputs is estimated to be >20:1 (Friedland, 1990). Other anthropogenic inputs of Zn to soils include fossil fuel combustion, mine waste, phosphatic fertilizers (typically 50–1450 µg Zn g), limestone (10–450 µg Zn g^{-1}), manure (15–250 µg Zn g^{-1}), sewage sludge (91– 49 000 µg Zn g^{-1}), other agrochemicals,

particles from galvanized (Zn plated) surfaces and rubber mulches (Chaney, 1993; Alloway, 1995).

2.3.1.2 Zinc behaviour in soils: Soil Zn occurs in three primary fractions: (i) water-soluble Zn (including Zn^{2+} and soluble organic fractions); (ii) adsorbed and exchangeable Zn in the colloidal fraction (associated with clay particles, humic compounds and Al and Fe hydroxides); and (iii) insoluble Zn complexes and minerals (Lindsay, 1979; Barrow, 1993; Alloway, 1995; Barber, 1995).

The distribution of Zn between soil fractions is determined by soil-specific precipitation, complexation and adsorption reactions. The dominant factor determining soil Zn distribution is pH; Zn is more readily adsorbed on cation exchange sites at higher pH and adsorbed Zn is more readily displaced by $CaCl_2$ at low pH. Thus, soluble Zn and the ratio of Zn^{2+} to organic Zn-ligand complexes increase at low pH, especially in soils of low soluble organic matter content. Soil type, soil moisture, mineral and clay types and contents, diffusion and mass flow rates, weathering rates, soil organic matter and soil biota and plant uptake will also affect Zn distribution. Insoluble Zn comprises >90% of soil Zn and is unavailable for plant uptake. Exchangeable Zn typically ranges from 0.1 to 2 µg Zn g^{-1}. Concentrations of water-soluble Zn in the bulk soil solution ($[Zn]_{bss}$) are low, typically between $4x10^{-10}$ and $4x10^{-6}$ M (Barber, 1995), even in Zn contaminated soils (Knight *et al*., 1997). Numerous Zn-ligand complexes can exist in solution which can be difficult to measure directly and speciation models, based on total dissolved concentrations of elements and ligands, their stability constants and mineral equilibria reactions, are often used to infer ($[Zn]_{bss}$) (Barak and Helmke, 1993; Zhang and Young, 2006). Zn^{2+} typically accounts for up to 50% of the soluble Zn fraction and is the dominant plant-available Zn fraction. However, in calcareous soils, Zn may be as low as 10^{-11} to 10^{-9} M and can limit crop growth (Hacisalihoglu and Kochian, 2003).

2.3.1.3 Translocation of zinc from the root to the grain Zinc is acquired from the soil solution primarily as Zn^{2+} but also potentially complexed with organic ligands, by roots which feed the shoots via the xylem. To reach the grain, nutrients enter and leave the symplastic continua of cells within the plant several times. For a divalent cation such as

Zn, the bottlenecks that limit efficient transport seem to be the control points where Zn has to exit living cells. Plant roots contribute to making metal ions more available for uptake by two strategies. First, depending on the nutrient -ATPases, proton pumps in the cell membrane (Palmgren, 2001). An increased proton concentration in the soil results in cation exchange and release of divalent metal ions that are tightly bound to soil particles. Second, roots actively secrete low-molecular-weight compounds that can function as metal chelators in the soil. Depending on species, these include organic acids and phytosiderophores (Romheld and Marschar, 1986; Takahashi *et al.,* 1999). Secreted phytosiderophores are known to facilitate uptake of Fe by graminaceous plants and recently, strong evidence has pointed to a similar role of these compounds in Zn uptake (Suzuki *et al.,* 2008; Suzuki *et al.,* 2006). (i) After metal ions have entered root cells by transport-protein-mediated processes, they migrate by diffusion to xylem parenchyma cells, from where they are actively transported out of the symplast into the dead xylem. In the shoot, they are again taken up by vessel-associated cells in the leaves. (ii) During the period of grain filling, metal ions are remobilized in leaves, from where they are exported and transported via the phloem to the fruit. Here they are exported by transfer cells from the mother plant and are subsequently taken up into the developing seed by specialized metal transport proteins (Palmgren *et al*., 2008).

2.3.1.4 Xylem loading activity and long-distance transport: Long-distance metal transport to the shoot involves symplastic diffusion between interconnected root cells towards the stele and active loading across the plasma membrane of xylem parenchyma into the apoplastic xylem (Sondergaard *et al*., 2004). As a consequence, nutrients to be exported to the shoot have to cross the plasma membrane of root xylem parenchyma cells to reach the vessels (within the vascular tissue) can be very different. Xylem parenchyma cells of *Arabidopsis* are equipped with a plasma membrane proton pump, AHA4, and the xylem parenchyma of barley harbours a plasma membrane electrogenic pump, most likely an H^+ -ATPase, that generates an inside-negative membrane potential (Zhu *et al*., 2007a). An inside-negative membrane potential would provide a strong barrier for the export of a divalent cation and suggests that the committed step in root export of metal ions to the shoot is xylem loading.

2.3.1.5 Other factors influencing metal transport to shoots: Besides the xylem loading activity, at least three additional factors have a key role in determining the rate of translocation of metals from roots to the above-ground parts: (i) the degree of accessibility and mobilization of metals sequestered in the root; (ii) the efficiency of radial symplastic passage through the root and across the endodermis; and (iii) the efficiency by which metals move in the xylem vessels. Metals that are deposited in the vacuole have to be remobilized if they are demanded in other parts of the plant. In cereals, for example, such remobilization occurs in senescing leaves during the grain filling stage, but it is not known whether roots can be triggered to remobilize stored Zn (Palmgren *et al*., 2008).

Efficient translocation of metal ions from cell to cell in the root requires the availability of suitable ligands that can serve as mobile binding partners. A possible cytosolic binding partner for Zn ions could be nicotianamine, which is required for the intercellular movement of Zn in leaves. In roots of *A. halleri*, in which Zn is partitioned primarily into the shoot, nicotianamine concentrations are higher than in roots of *A. thaliana*, in which a major proportion of Zn is retained in the roots. This is consistent with a critical role for nicotianamine in the intercellular movement of Zn inside roots (Takahashi *et al*., 2003).

During long-distance transport in the xylem vessels, metals are chelated by mobile low molecular-weight ligands present in the xylem sap. Organic acids, including phytosiderophores, have been proposed to act as ligands of Zn (Suzuki *et al*., 2008). Despite the multiplicity of processes involved, recent work has shown that transfer of single genes to modify just the xylem loading process has in some cases been successful in significantly altering metal contents in above-ground tissues (Zhu *et al*., 2007b).

2.3.1.6 Unloading of Zn from the xylem and transport to edible parts of the plant: To enter the mesophyll of leaf cells, micronutrients have to be actively taken up by vessel associated cells – living cells surrounding the xylem. From these cells, micronutrients can move symplastically from cell to cell until they reach their final destination in the leaf

(Hanikenne *et al.*, 2008). The leaves of many crops are directly consumed by animals and humans.

2.3.1.7 Loading of Zn into seeds: The embryo and the endosperm of the seed are symplastically isolated from the mother plant and therefore membrane transport steps are required for metal loading (Patrick and Offler, 2001). Thus, to enter specific cells in a seed, a metal ion will first have to exit the phloem (or connected surrounding cells) of the mother plant by transport across the plasma membrane of an efflux cell. Second, it will have to be taken up into the seed across the plasma membrane of an influx cell. For the reasons described further above, the phloem unloading step is likely to be a major bottleneck in this process. Unfortunately, very little is known concerning transporters involved in phloem unloading, either in cereals or in any other plant (Zhang *et al*., 2007). More knowledge is required before we can point at specific transporters essential for grain filling at this initial step in the process.

2.3.1.8 Distribution of metals in seeds: The cereal grain consists of several major depositories for nutrients, namely the testa or pericarp (derived from the mother plant, which is typically diploid) surrounding the seed, the diploid embryo, which also includes the scutellum and the endosperm, which is a genetically distinct triploid organ serving as a nutrient supply for the developing embryo. The aleurone cell layer, which is part of the endosperm, surrounds the starchy internal part of the endosperm. To date, little is known concerning the molecular mechanisms of metal transport between and within these different cell types. Elemental microanalyses of wheat grain sections reveal that phosphate, potassium, calcium, manganese, iron and zinc appear to be distributed in a similar way: the highest concentrations are in the aleurone and the embryo – in particular the scutellum and the lowest concentrations are in the endosperm (Cakmak, 2008; Mazzolini *et al*., 1985).

In the cereal grain and legume seeds, Zn is preferentially stored together with phytate, which is a strong chelator of divalent cations and significantly reduces mineral bioavailability (Bohn *et al.*, 2008). Storage of Zn–phytate complexes takes place in membrane enclosed globoids in the protein storage vacuoles (PSVs) found in the

aleurone and the embryo scutellum. In wheat, specific staining regimes with dithizone (Ozturk *et al*., 2006) illustrate that Zn is present in the highest concentrations in the aleurone and the embryo, whereas there is very little presence of Zn in the endosperm, which explains why Zn is low in milled (i.e. polished) de-embryonated grains.

2.4 The functions of zinc in plants

Once, zinc is translocated to different parts of the plant, it influences the various functions in the plants which are essential for the normal, healthy growth and reproduction of plants. This element is required in small amounts to allow the normal function of several key plant physiological pathways as well as to ensure the structural and functional integrity of membranes. Thus, Zn has important roles in growth regulation, enzyme activation, gene expression and regulation, phytohormone activity, protein synthesis, photosynthesis, carbohydrate metabolism, fertility, seed production and defense against disease (Marschner, 1995). Zn deficiency will impair these physiological functions and compromise the health and productivity of plants, leading to severe reduction in growth, lower yields (or even crop failure) and poor quality crop products (Sadeghzadeh, 2013).

Zinc is the only metal that is required in all six enzyme classes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases). The requirement of Zn for the function of a wide range of enzymes indicates that the metabolism of proteins, carbohydrates and auxin as well as reproductive processes are hampered under Zn deficiency (Romheld and Marschner, 1991). Zn is required for the activity of metalloenzymes that are involved in protein and nucleic acid metabolism.

Zinc is involved in carbohydrate metabolism via its effects on photosynthesis and sugar transformation. Reduced photosynthesis under Zn deficiency can result from a decrease in carbonic anhydrase (CA) activity, the photochemical activity of chloroplasts and chlorophyll content, as well as alterations in chloroplast structure. Low CA may inhibit photosynthetic electron transfer and consequently limit chlorophyll content (Romheld and Marschner, 1991).

Zinc is essential in protein metabolism, and the most important role of Zn in protein synthesis is its involvement in the stability and function of genetic material. Zn is essential in chromatin structure, DNA/RNA metabolism and gene expression. Zn deficiency causes a decrease in protein synthesis (Marschner, 1995) due to RNA degradation (Cakmak *et al.*, 1989), decreased activity of RNA polymerase, ribosomal deformation and a decrease in the number of ribosomes. In Zn-deficient bean leaves, the free amino acid concentration was increased by a factor of 6.5 compared with that of control plants. Upon resupply of Zn, this factor decreased to 5.1 after 24 h, 2.7 after 48 h and 1.4 after 72 h (Cakmak *et al*., 1989).

Zinc has an important physiological role in maintaining the integrity and function of cellular membranes by controlling the generation and detoxification of reactive oxygen species (Sadeghzadeh, 2013). Reactive oxygen species are potentially damaging to membrane lipids and sulfhydryl groups. When these compounds are damaged due to oxidative stress, there is increased leakage of several organic compounds, such as carbohydrates and amino acids, from Zn-deficient root cells. Due to the increased leakage of carbon-containing compounds into the rhizosphere, Zn-deficient plants may be susceptible to diseases such as *Fusarium graminearum* (Sadeghzadeh, 2013).

2.5 Zinc deficiency in plants

Many plant species are affected by Zn deficiency in a wide range of soil types in most agricultural regions of the world. The major staple crops (rice, wheat, barley, maize and sorghum) are all affected by Zn deficiency, together with many different fruits, vegetables and other types of crops, including cotton and flax. Low soil concentrations of plant-available Zn may cause not only a decrease in grain yield but also lead to poor nutritional quality of crop products (Graham and Welch, 1996). However, more severe deficiencies (with leaf symptoms) can reduce yield severely and result in crop failure (Hafeez *et al.,* 2013).

Zinc deficiency can impair many biochemical pathways in crops, which may manifest in visible symptoms such as small and distorted leaves, interveinal chlorosis in young leaves and shortened internodes (Marschner, 1995). In barley, the visual Zn

deficiency symptoms include stunted plants and leaves, chlorotic areas on leaves, necrosis, white spots on leaves and mid-leaf collapse (Genc et al., 2003; Lombnaes and Singh, 2003). In wheat, the symptoms of Zn deficiency are usually observed in young seedlings; later, whitish brown patches and necrotic lesions can be observed on the leaf blades along with mid-leaf collapse (Rengel and Graham, 1995b; Cakmak and Braun, 2001). The appearance of these symptoms can vary with environmental conditions, plant age, deficiency stage and severity as well as the supply of other nutrients.

2.6 Zinc Deficiency in Agricultural Systems and Its Implication to Human Health

Zinc deficiency in soils is a serious global problem that affects many agricultural soils. It is estimated that about half of the cultivated soils in the world contains reduced amounts of bioavailable zinc. This problem is aggravated mainly in arid and semi-arid regions, due to low organic matter and soil moisture as well as high levels of pH and $CaCO_3$ (Cakmak, 2008; Gonçalves Junior *et al.*, 2010). The low availability of this metal in the soil limits zinc uptake by plants, resulting in significant decreases in both productivity and nutritional quality of food and also decreases the dietary intake of zinc when eaten (Henriques A R *et al.*, 2012). Hence, it is not surprising that the well-known zinc deficiency problem in humans occurs predominantly in the countries/regions where soils are low in available zinc, (Fig. 1).

It is estimated that about one billion people in the world do not eat sufficient food to meet their energy requirements and are consequently undernourished (Stein *et al.*, 2007). Many more people suffer from 'hidden hunger': about 3 billion are Zn-deficient (WHO, 2002; Hotz and Brown, 2004; Stein *et al.*, 2007). Zinc acts as a stabiliser of the structures of membranes and cellular components; in human body, most Zn is in the bone and skeletal muscles (Frossard *et al.*, 2000). In addition to its role in enzyme function (Rivera *et al.*, 1998), Zn also plays a major role in gene expression (Sandstrom, 1997). Zn deficiency in humans reduces growth, sexual maturity and the immune defense system (Frossard *et al.*, 2000; Cunnigham-Rundles *et al.*, 2005). Few studies (Ninh *et al.*, 1996; Ruz, 1997; Rivera *et al.*, 1998) reported that increased growth in Zn-supplemented infants and preschool children have lowered the incidence of diarrhoea and respiratory

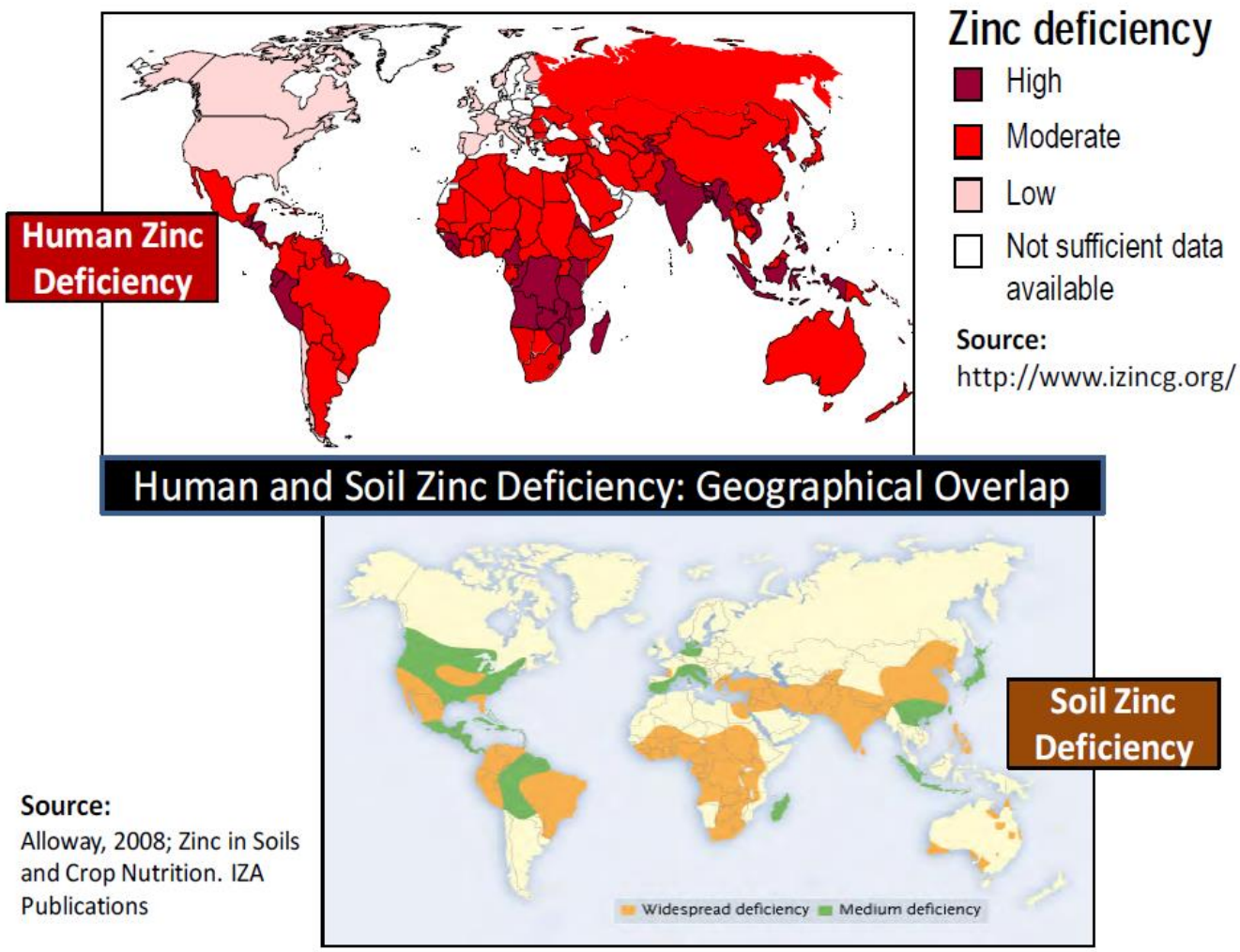


Fig. 1: Zinc deficiency in human and soil

infections. Meat and seafood are good sources of Zn, and most of the Zn in the developed countries' diet is provided by animal products (Sanstead, 1995). However, in many parts of the developing world, most Zn is provided by cereals and legume seeds. These plant foods are high in phytic acid, which is a potent inhibitor of Zn absorption in the human digestive system (Frossard *et al.,* 2000). Among population children and women are mostly affected with Zn deficiency (White and Broadley, 2005, Stein *et al.,* 2007). Given the context of growing world population pressure with limited food supply capacity of arable lands and cropping systems, the holistic sustainable improvements in the entire food systems are required to solve the massive problems of malnutrition and increasing chronic diseases in developed and especially developing countries (Welch, 2005).

2.7 Interventions to curb Human Zinc deficiency

Currently there is a growing concern to address zinc deficiency in humans through different interventions. Typically, these interventions are categorized into four major groups: pharmaceutical supplementation, industrial fortification, dietary diversification and biofortification (Tontisirin *et al.,* 2002; Meenakshi *et al.,* 2007). Zinc pharmaceutical supplementation and industrial fortification are not considered cost-effective and moreover only very few governments can finance such kinds of expensive interventions (Stein, 2007). Several other issues such as frequency of supplementation, selection of food products to be fortified, impact of fortificants on taste, texture, and appearance of food, and availability of fortificant need to be carefully addressed before undertaking these interventions (Shahzad *et al.,* 2014). Dietary diversification offers considerable projection to overcome micronutrient malnutrition (Johns and Eyzaguirre 2007). However, to achieve success on a large scale, poverty alleviation and change in food habits of high-risk groups, the rural poor, children and child bearing women are prerequisites but poor families do not have enough resources to purchase a diversified diet (Shahzad *et al.,* 2014). Thus, considering the limitations linked to the three types of interventions to alleviate zinc deficiencies, the biofortification of staple food crops is considered the most viable strategy to help alleviate zinc malnutrition (Shahzad *et al.,* 2014). While, Biofortification is the development of micronutrient dense staple crops

using the best traditional breeding practices and modern biotechnology (Nestel *et al.*, 2006). It basically aims at either increasing accumulation of micronutrients in edible parts or to improve their bioavailabity. At present two research strategies to achieve this are mineral fertilization and crop biofortification. Mineral fertilization can mainly increase the accumulation of zinc in edible parts, whereas crop biofortification has the potential to accomplish both aims i.e., increase the accumulation as well as the bioavailability (Saltzman *et al.*, 2014).

2.8 Approaches to increase zinc content/Bioavailabity in Plants

The approaches to biofortify crop plants can mainly be divided into two categories viz., Agronomic Biofortification and Genetic Biofortification (Shahzad *et al.*, 2014). Agronomic Biofortification refer to either soil zinc fertilizer application or foliar zinc fertilizer spray. Soil zinc application effectiveness again depends on soil factors while in foliar application the stage of application is tricky and important. Genetic Biofortification studies involve strategies to either increase the zinc accumulation or increase its bioavailabity by decreasing aninutritional factors or enhancing the prebiotics. Thus the genetic Biofortification covers genetic engineering approaches as well as molecular breeding strategies. In recent years, pronounced efforts have been made to increase zinc content and availability in staple crops (http://www.harvestplus.org/).

2.9 Zinc fertilization

It is generally found that zinc deficiency in human beings is associated with zinc-deficient soils (Alloway 2009). This led to numerous studies to identify the effect of soil or foliar zinc fertilization on grain zinc concentration under varied agro-ecological conditions. In wheat Stomph *et al.*, 2011, showed that soil application of zinc resulted in 20 % to 90 % and 60 % to 250 % increase in grain zinc concentration in bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* L.), respectively. A fast and easy solution to prevent zinc deficiency in plants is soil Zn application (Cakmak, 2008). However, there are costs associated with the application, and even though the benefits are wider, this poses a threshold for application. In addition, zinc fertilizer application effect can be impaired by physical and chemical characteristics of soil, which reduce the

availability of Zn to plants, leading to a disappointing experience for farmers. Foliar application of zinc resulted in, even a higher increase in grain zinc concentration than soil application in both bread and durum wheat (Khoshgoftarmanesh *et al.,* 2013). Studies of natural variation revealed the existence of notable differences for zinc accumulation in wheat grains between different wheat cultivars in response to soil and foliar application of zinc (Khoshgoftarmanesh *et al.,* 2013). Various studies focus that determining the timing of foliar application of zinc to be crucial in determining grain zinc content. Usually Foliar application of zinc around flowering time is most preferable (Cakmak *et al.,* 2010). The findings also suggest that both the zinc uptake and the remobilization are important factors that determine the zinc concentration and natural variation for response to zinc fertilization is present in crop plants. An additional benefit of using zinc fertilizers in crop production is their positive effect on yields. Numerous studies have established the positive relationship between provision of trace minerals and crop growth, thus they are recognized as essential when aiming for better grain yields (Xi-wen *et al.,* 2011; Khoshgoftarmanesh *et al.,* 2013). Moreover, zinc fertilization might result in more vigorous seedlings, lower vulnerability to plant diseases, and possibly improved drought resistance (Bouis, 2003). However, Zinc fertilizers may also contain elevated amounts of toxic metals such as cadmium and repeated uses of the fertilizers at high dose over a prolonged period may increase cadmium accumulation in plants (Huang *et al.,* 2003). Thus, a judicious approach is required during the formulation and utilization of these fertilizers in crop production to avoid contamination of soil by toxic metals like cadmium.

2.10 Genetic Engineering approaches

Genetic engineering approaches aim at either increasing zinc accumulation by overexpression of zinc transporters and zinc responsive transcription factors, or increasing zinc bioavailabity by downregulation of antinutritional factors like phytic acid accumulation. In order to provide new insights for the development of crops in areas suffering from low zinc bioavailability, to maintain optimal growth and to design biofortification strategies, understanding the regulatory identity of the zinc homeostasis network in plants and fair knowledge on antinutritional factors and enhancers is very

essential. Thus, such information will help in identifying and manipulating the candidate genes to either increase accumulation or bioavailabity of zinc.

Zinc Homeostasis

Zinc is a transition metal essential for terrestrial life, as already described. However, it is only required at low concentrations, making it function as a micronutrient. Concentrations between 30 and 100 μg zinc g^{-1} DW are enough to support adequate plant growth, whereas zinc toxicity symptoms are observed in concentrations above 300 μg g^{-1} DW for species that are not adapted to high- zinc exposure (Marschner, 1995; Mortel *et al.,* 2006). Therefore, for optimal growth, plants need to keep tight control over zinc homeostasis. Zinc homeostasis requires a complex network of cellular or tissue specific functions to control metal uptake, accumulation, trafficking, and detoxification. The ability to take up Zn of higher plants depends much more on its bioavailability from soil than on the absolute soil concentrations. Zinc bioavailability is modulated by various physical and chemical soil factors. Zinc solubility in soil decreases due to high levels of calcium carbonate, metal oxides, and pH and low levels of organic matter and soil moisture as well as high amounts of phosphate (Robson, 1994; Cakmak, 2011). When available in the soil solution, zinc is absorbed and transported in the divalent ion form (Zn^{+2}) from roots to shoots through the xylem, being easily retranslocated by phloem (Clemens, 2001). This transport of ions and molecules from epidermal and cortical cell to xylem can occur through the symplastic or apoplastic route. Regardless of the chosen path, the solutes that reach the xylem parenchyma cells are transferred to the xylem elements in a tightly-controlled process mediating membrane transport.

Genes involved in zinc homeostasis in plants

Accumulation of zinc or other metallic minerals depends on the uptake capacity and on intracellular binding sites. The metal accumulation rates are affected by the concentration and affinities of chelating molecules and by the presence and selectivity of transport activities (Clemens *et al.,* 2002). Metal transporters are required for metal uptake and efflux or intracellular metal transport in the plant, and metal chelators contribute to metal detoxification by buffering free cytosolic metal concentrations.

Therefore, both play a major role in metal homeostasis. Several studies have focused on the identification and manipulation of genes for zinc transporter proteins and chelator biosynthesizing enzymes.

The zinc regulated transporter (ZRT), iron-regulated transporter (IRT)-like protein (ZIP) family contains many members thought to be involved in transporting zinc into the cytosol across the plasma membrane, which is an important process for plant zinc uptake (Palmer and Guerinot, 2009; Song *et al.*, 2010). ZIP transporters have eight transmembrane domains and a histidine-rich variable loop between transmembrane domains III and IV that appears to be conserved among all family members (Colangelo and Guerinot, 2004). In Arabidopsis, there are 15 ZIP gene members, ZIP1-12 and IRT1-3, of which at least ten members (ZIP1, 2, 3, 4, 5, 9, 10, 11, 12 and IRT3) appear to play a role in plant zinc uptake. Approximately half of the ZIP genes (ZIP1, 3, 4, 5, 9, 10, and IRT3) is induced in response to zinc deficiency (Wintz *et al.*, 2003; Mortel *et al.*, 2006; Assuncao *et al.*, 2010).

The natural resistance associated macrophage protein (NRAMP) is a family of proteins whose function is to take up and transport metal. Research in *Arabidopsis thaliana* (L.) Heynh (*Arabidopsis*) showed that NRAMP transporters have limited metal specificity. *At*NRAMP3 and *At*NRAMP4 are localized to the vacuolar membrane and are involved in the intracellular iron transport (Thomine *et al.*, 2000). These genes show similar expression levels in most tissues (Grotz and Guerinot, 2006), and only double mutants show a mutant phenotype, i.e., they are functionally redundant (Palmer and Guerinot, 2009). Thomine *et al.*, (2003) reported that AtNRAMP3 controls the accumulation of zinc and manganese in roots upon iron starvation.

Another family of transporters involved in zinc efflux is the P_{1B}-ATPase. *Arabidopsis* has eight genes encoding P_{1B}-ATPases that differ in their structure, function, and regulation (Eren and Arguello, 2004). Among these, HMA1, HMA2, HMA3, and HMA4 are involved in zinc transport (Hussain *et al.*, 2004). AtHMA1 localizes in the chloroplast envelop and can contribute to Zn detoxification under excess zinc conditions (Kim *et al.*, 2009). The AtHMA2 gene encodes a Zn^{+2}-ATPase located in the plasma

membrane. Expression is induced by cadmium and zinc (Eren and Arguello, 2004). The AtHMA3protein possibly mediates zinc hyperaccumulation, since zinc hyperaccumulator species show higher expression of HMA3 than the non-hyperaccumulator ones, such as *Arabidopsis* (Becher *et al.*, 2004; Mortel *et al.*, 2006; Hassan and Aarts, 2011).

Recently, HMA3 has been cloned from *Thlaspi carulescens* Alpine Penny-cress (Ueno *et al.*, 2011), and rice (Ueno *et al.*, 2010), therefore it is a vacuolar influx transporter, important for cadmium tolerance in both species. This suggests that different homologs of HMA3 may have different metal-substrate specificity. *At*HMA4, similar to *At*HMA2 and acting alike, plays an important role in translocation of zinc, specifically in loading of zinc into the xylem (Mortel *et al.*, 2006; Waters and Sankaran, 2011). Hussain *et al.*, 2004 reported that both HMA2 and HMA4 are essential to zinc homeostasis and they show a functional redundancy.

Members of the cation diffusion facilitator (CDF) family play an important role in living organisms, as they control cation concentrations in cells through sequestration into internal compartments and efflux from cell (Gustin *et al.*, 2011). MTP1 and MTP3 seem to be involved in the sequestration of zinc in root vacuoles and can act to limit its translocation to the shoot (Arrivault *et al.*, 2006). In addition, when MTP1 is overexpressed in *Arabidopsis*, an increased resistance to zinc and higher zinc content in roots are observed (Kobae *et al.*, 2004). MTP2 is also involved in zinc homeostasis. Under zinc deficiency, MTP2 expression increases suggesting it to play a specific function in counteracting the effect of zinc deficiency (Mortel *et al.*, 2006). MTP8 is another member of the CDF family, which in addition to mediating manganese transport may also function in zinc uptake (Mortel *et al.*, 2006).

Metal chelators are also important for metal homeostasis. Nicotianamine (NA) is a metal-chelating compound, made by the action of NA synthase that binds zinc, as well as iron, copper, and nickel (Curie *et al.*, 2009). NA is thought to be involved in long-distance transport, perhaps also playing a role in the entry of metals into the phloem or xylem through metal-NA chelate transporters of the Yellow Stripe-like (YSL) family (Gendre *et al.*, 2007). NA synthase is encoded by four genes in *Arabidopsis*, AtNAS1-

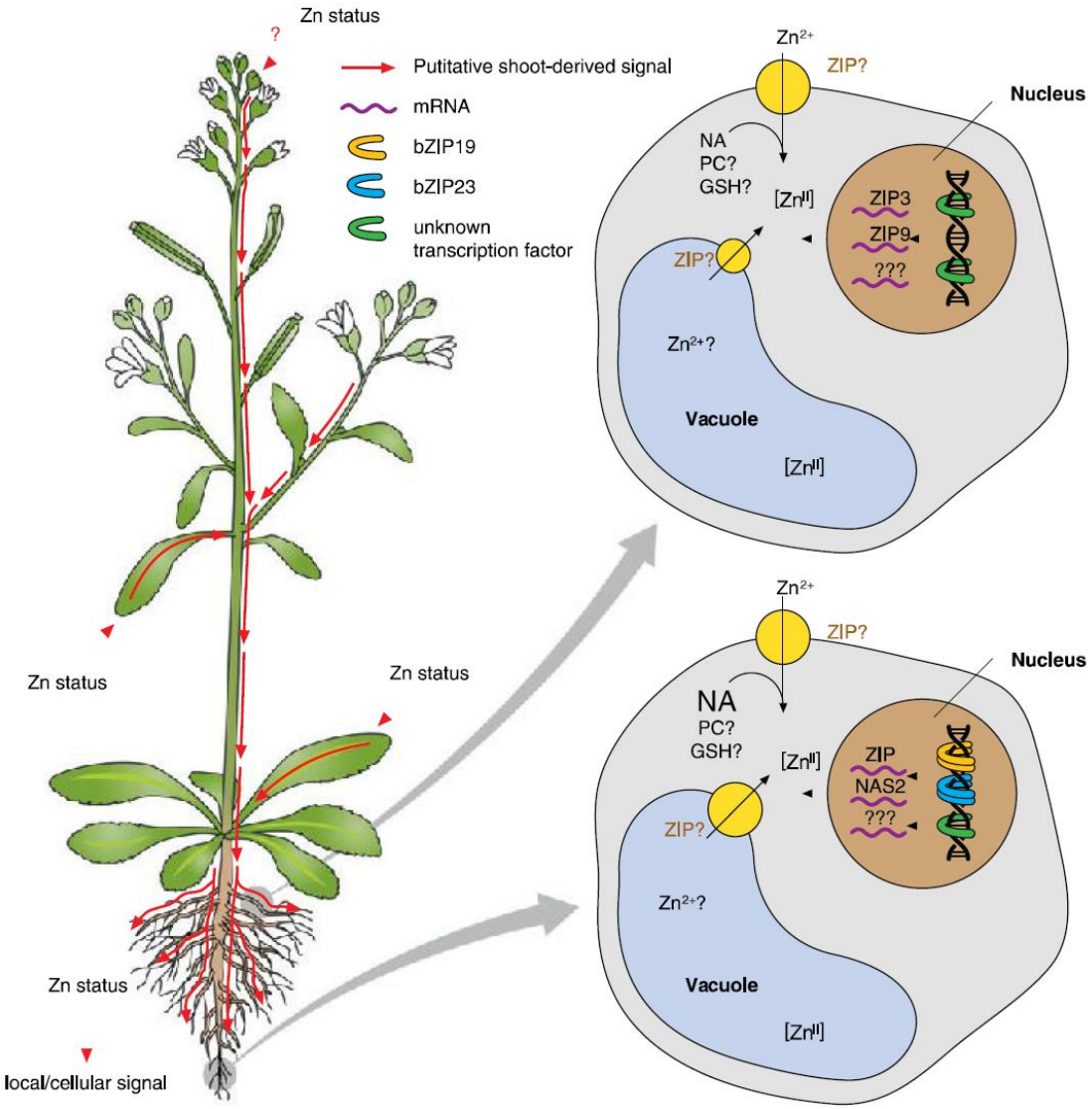
AtNAS4, which act functionally redundant, although they show different expression patterns, suggesting that each NAS gene may have a specialized function (Klatte *et al.,* 2009). As an example, only *At*NAS2 and *At*NAS4 are highly expressed in roots under zinc deficiency (Mortel *et al.,* 2006).

Zinc homeostasis regulation

Recently, two transcription factors, bZIP23 and bZIP19, which are involved in the regulation of zinc deficiency response in *Arabidopsis*, were identified (Fig. 2). These transcription factors recognize 8 to 10 bp palindromic motifs called zinc deficiency response elements, found in tandem in promoters of several zinc homeostasis genes, activation of which constitutes the primary response to zinc deficiency (Assuncao *et al.,* 2010). Modification of these transcription factors to control zinc deficiency tolerance and accumulation is in progress (Sinclair and Kramer, 2012). The simultaneous expression of a set of genes implicated in the same process is a promising strategy to enhance productivity and stress tolerance in plants, when activation of multiple genes at the same time is necessary to obtain an effective stress response. Still, although the listed findings offer interesting options to try and modify plant micronutrient deficiency response, the control of post-transcriptional and translational regulation also deserves attention.

Antinutritional factors

Plants generally contain chemical compounds such as phytate, saponins, tannins, oxalates, trypsin inhibitors and cyanogenic glycosides, which are known as secondary metabolites and are biologically active (Soetan and Oyewole, 2009). However, a substance cannot be classified as an anti-nutritional factor based on the intrinsic characteristic of the compound; an antinutrient leaves its impact generally at the level of the digestive process of humans (Kumar, 1992). Though, most of the staples we consume daily contains many ANFs, from human nutrition perspective Phytic acid is considered most important ANF. Phytic acid (*myo*inositol, 1, 2, 3, 4, 5, 6 hexa*kis*-dihydrogen phosphate) and its salt phytate are widespread in plant seed grains, roots and tubers (Graf, 1986). Phytic acid is generally regarded as the primary storage form of both phosphate and inositol in seeds. Phytic acid phosphorus constitutes the major portion of total



-Sinclair and Kramer, 2012

Fig. 2: Hypothetical model of signalling of zinc deficiency

phosphorus in several seeds and grain. It accounts for 50–80% of the total phosphorus in different cereals (Bohn *et al.,* 2008). The accumulation of phytic acid content in seeds is influenced by cultivar, climatic conditions and time of the year. Legumes have higher phytic acid content up to maximum of 1.75 g/100 g (Raboy 2002).

Enhancers for zinc absorption

Besides containing antinutrients, cereals do contain enhancers of iron and/or zinc absorption such as ascorbic acid and various prebiotics. Numerous studies have shown the dose dependent positive effects of native or supplemented ascorbic acid for absorption of iron (Hurrel and Egli 2010). However, cooking, industrial processing, and storage degrade ascorbic acid and cancels out its mineral absorption effects (Teucher *et al.,* 2004). Prebiotics on the other hand are relatively resistant to such type of losses (Rakha *et al.,* 2010, 2011), hence are of special consideration in breeding programmes aimed at enhancing bioavailability of zinc. Thus, prebiotics are carbohydrates (nondigestible oligosaccharides [NDO] and lactulose) that selectively stimulate the growth and/or activity of a limited number of bacteria (probiotics) in the gastrointestinal tract and thereby exert beneficial effects on the host (Shahzad *et al.,* 2014). A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastro-intestinal microflora that confers benefits upon the host well-being and health" (Roberfroid 2007). With reference to mineral absorption, fructans are the most widely studied prebiotics. Fructans are polysaccharides made up of fructose units linked through ß-(2→1) and/or ß-(2→6) glycosidic bonds. Among fructans, inulins are the simplest and most widely studied (Shahzad *et al.,* 2014).

2.10.1 Genetic Engineering Approaches to Increase zinc Accumulation

A recent study in rice reported the involvement of *OsMT1a* in zinc homeostasis (Yang *et al.,* 2009). *OsMT1a* was found to be predominantly expressed in the roots and was induced by zinc treatment. Overexpression of *OsMT1a* resulted in approximately 1.5- and 2.5-fold increases in zinc accumulation in the *OsMT1a* overexpressing rice lines and yeast, respectively.

The accurate expression of Zinc-Induced Facilitator1 (ZIF1), a vacuolar membrane major facilitator superfamily protein required for basal Zn tolerance, was also shown to be very crucial for both iron and zinc homeostasis (Haydon *et al.*, 2012).

Ramesh *et al*., (2004) over-expressed *Arabidopsis* Zn transporter, *AtZIP1* and *ATZIP3* in barley (*Hordeum vulgare*) by means of a ubiquitin promoter. Transgenic lines were tested in long term growth and short-term uptake experiments. Seeds from transgenic lines grown in soil had higher zinc and iron contents than controls. Short-term uptake rates were higher in the transgenic lines after zinc deprivation. Resupply of zinc after a period of deprivation resulted in the rapid decrease in zinc uptake even in transgenic lines in which a zinc transporter gene was constitutively expressed. They hypothesized that this rapid decrease in zinc transport activity may be caused by the degradation of transporters in response to zinc-sufficient conditions. In the long-term growth experiments, there were no significant differences between transgenic and control lines in leaf zinc content or shoot biomass under zinc-sufficient or -deficient conditions. However, root-to-shoot ratios were higher in the transgenic plants grown under low-zinc conditions; this could impact zinc acquisition under field conditions. Increased seed zinc content by over-expression of a zinc transporter provides a new strategy for increasing the micronutrient content of food crops.

IRT1, IRT2 are members of the *Arabidopsis ZIP* metal transporter family that are specifically induced by Fe deprivation in roots, but short term labeling with Zn^{65} revealed in *irt1* mutant, it responds to Fe and Zn deprivation by altered expression of certain Zn and Fe transporter genes. These data support the conclusion that *IRT1* is an essential metal transporter required for proper development and regulation of Fe and Zn homeostasis in *Arabidopsis* (Henrique *et al*., 2002).

Ishimaru *et al*., (2007), produced transgenic rice plants over expressing *OsZIP4* under 35SCaMV promoter. The Zn concentration in roots of 35S-*OsZIP4* transgenic plants was 10 times higher than in those of vector controls, but it was five times lower in shoots. The Zn concentration in seeds of 35S-*OsZIP4* plants was four times lower compared with vector controls. The results indicated that constitutive expression of

OsZIP4 altered the Zn distribution within rice plants and that *OsZIP4* is a critical Zn transporter that must be strictly regulated. These results suggest that the activity of the zinc transporter might be regulated in planta at the post-translational level. The overexpression of ion transporters in plants provides new opportunities to understand the processes of metal regulation and homeostasis in plants as well as potential biotechnological avenues for increasing the nutritional qualities of foods (Borrill *et al.*, 2014).

A few Zip transporters identified in rice were characterized for their specific role in uptake and translocation. *OsZIP1* and *OsZIP3* have been suggested to be involved in Zn uptake from soil and *OsZIP4*, *OsZIP5* and *OsZIP8* are involved in root to shoot translocation, while *OsZIP4* and *OsZIP8* have been suggested to be important for Zn transport to seed (Lee *et al.*, 2010; Ramesh *et al.*, 2003; Ishimaru *et al.*, 2005; Bashir *et al.*, 2012). Our own lab studies confirmed over-expression of *OsZIP1* transporter gene in finger millet and model plant tobacco under the control of constitutive CaMV35S (35S) and endosperm specific (Bx17) promoters showed higher Zn content in finger millet grains. Ectopic expression of *OsZIP1* in tobacco under 35S and Bx17 improved seed Zn concentration compared to untransformed plants. In addition, finger millet transgenic plants, expressing the *OsZIP1* under 35S and Bx17 promoter, accumulated significantly higher Zn in seeds compared to wild type finger millet plants. Plants expressing the gene under Bx17 promoter accumulated more Zn in seed than 35S plants (Yamuna *et al.*, 2013).

2.10.2 Genetic Engineering Approaches to Reduce Phytate

On the other hand, a very novel and interesting approach has been used in maize and soybean to silence the genes involved in the biosynthesis of PA (Shi *et al.*, 2007). It was found that maize *lpa1* mutants are defective in a MRP ATP-binding cassette (ABC) transporter that is more highly expressed in embryos, but also in immature endosperm, germinating seeds, and vegetative tissues. The expression of this transporter was silenced in an embryo-specific manner. The concentration of PA in seeds of maize transgenic was found to be reduced by up to 87% depending upon the transgenic line, and the transgenic

plants were not affected in grain yield or seed germination in contrast to the *lpa* mutants. Similarly, silencing of MRP transporter in sorghum decreased the PA concentration in seeds by 80% to 86%, and a consequent increase in iron and zinc absorption was observed when analyzed in Caco-2 cell lines (Kruger *et al.*, 2013). Rice transgenics developed by silencing *RINO1*, a gene involved in the biosynthesis of PA, in embryo and aleurone layer-specific manner exhibited up to 67% decrease in PA and were unaffected in grain yield, seed germination, and plant performance (Kuwano *et al.*, 2009). These remarkable findings indicate the possibility to produce GMO crop plants with low PA and without affecting agronomic performance by silencing the expression of transporters involved in the biosynthesis of PA. The difference in agronomic performance between the *lpa* mutants obtained through mutation breeding and low phytate transgenics is thought to be due to the tissue-specific silencing of the genes involved in biosynthesis of PA in case of transgenics (Shahzad *et al.*, 2014).

2.10.3 Genetic Engineering Approaches to Increase Prebiotics

As mentioned earlier, rice and maize varieties analyzed to date contain lower grain fructan, but it is believed that exploring new materials like landraces, wild relatives, and so on may help identify the sources that can be used in breeding programmes to increase grain fructan content. As for now, rice and maize transgenics have been developed that can accumulate high amounts of fructan in grains. Fructans are produced by the combined action of various fructosyltransferases (FTs) (Pan *et al.*, 2009). The enzyme 1-SST catalyzes the initial fructosyl transfer between 2 sucrose molecules. Then FTs (1-FFT, 6G-FFT, 6-SFT, and so on) catalyze chain elongation adding ß-(2→1)- or ß-(2→6)-linked fructose units. Inulin-type fructan biosynthesis in plants is generally believed to occur through the collective action of 2 vacuolar enzymes, 1-SST and 1-FFT. The expression of these enzymes, 1-SST and 1-FFT, from *Jerusalem artichoke* (a high-inulin-accumulating plant) in high-sucrose maize under the control of an endosperm-specific promoter increased the fructan content by 2-fold (Stoop *et al.*, 2007). Moreover, kernel development and seed germination of these transgenic maize plants were not hampered. The overexpression of 1-SST enzyme from *Jerusalem artichoke* as well as *Yacon* (another high-inulin-accumulating plant) in rice under the control of a constitutive

promoter significantly enhanced the production of fructan in plant tissues of transgenic rice (Pan *et al.,* 2009). Constitutively, overexpression of 1-SST enzyme of wheat in rice could increase seed fructan of rice transgenics, with a slight decrease in seed weight (Kawakami *et al.,* 2008). It is likely that increasing fructan content could have a positive effect on zinc absorption.

2.11 Breeding approaches

Plant breeding is an art and science of manipulating plants for the benefit of humans. Plant breeding offers a mechanism for helping to address some of the world's most current concerns. One of the greatest challenges facing modern plant breeders is ensuring global food security in the face of a host of global and local obstacles. The current food supply is expected to be insufficient to support projected population growth, both in quantity and nutritional quality. Before beginning the breeding process, a trait must be defined, along with a system to measure phenotypes (a plant's performance for that trait). The diversity and range of phenotypic values must also be considered. For example, in biofortification the trait of interest is micronutrient content, and phenotypes are analyzed through various assays such as liquid chromatography and spectroscopy.

2.11.1 Variability for zinc content

For any breeding programme existence of variability for the specific trait is important. Field and pot screening studies have revealed significant genetic variation in Zn efficiency in crop plant genotypes, which indicates that selection for improved Zn efficiency is possible.

Chatzav *et al.,* (2010) reported that across total of 154 genotypes, including wild emmer accessions of diverse wheat cultivars, wide genetic diversity was found among the wild emmer accessions for all grain nutrients. The concentrations of grain zinc, iron and protein in wild accessions were about two-fold greater than in the domesticated genotypes. Concentrations of these compounds were positively correlated with one another, with no clear association with plant productivity, suggesting that all three

nutrients can be improved concurrently with no yield penalty. A subset of 12 populations revealed significant genetic variation between and within populations for all minerals.

In our lab grain Zn concentration was estimated in nearly 25 different crop species and 400 germplasm lines of finger millet (Shankar *et al.,* 2006). The range in different species was 5 to 6 fold, the least was in Jowar (1.78 mg/100gm) and Horsegram (1.88 mg/gm) and the highest was in mustard (8.5 mg/100 gm) and gingelly (11.5 mg/100mg). Similarly significant genotypic variation was found in finger millet the range being 3.0 mg to 13 mg/100 gm (Yamuna Rani and Shankar., 2006). Thus, there is exploitable level of zinc variability in crops.

Manoa, 2010; studied 240 germplasm lines of pigeon pea representing around 26 countries consisting of core germplasm lines, local adapted cultivars and released varieties from ICRISAT Hyderabad, and analyzed for their seed zinc content. There was significant variation across the genotypes with zinc content ranging from as low as 1.53 mg/100gm to as high as 6.2 mg/100g. Maximum number of genotypes (109) had zinc content in the range of 2 to 3 mg/100g, and 92 accessions had seed zinc content in the range of 3 to 4 mg/100g accounting for approximately 38 per cent of the germplasm lines. Only 5 germplasm accessions had seed zinc content more than 5 mg/100g. This was our own lab studies. These results suggested that variability do exists for the grain zinc content across the pigeon pea lines, which can be exploited in improving Zn content in pigeon pea seeds.

2.11.2 High throughput screening methodology

Selecting genotypes with higher micronutrient concentrations require fast, accurate, and inexpensive methods of identifying nutrient dense genotypes. Traditionally, elemental analysis was conducted using inductively coupled plasma-optical emission spectrometry (ICP-OES) and Atomic Absorption Spectroscopy (AAS) (Zarcinas *et al.*, 1987), but this method requires expensive equipment, highly trained analysts, contamination free reagents, and extensive sample preparation. Consequently, many biofortification programmes, especially those in more remote locations, have not been able to conduct their own analyses and have had to send samples to better-equipped

laboratories elsewhere. Thus in absence of other advanced technology, estimation of zinc through ICP-OES or AAS would be a better option.

Alternative, colorimetric approaches have been developed for Zn and Fe analysis in different cereal crops (Prom-u-thai *et al.,* 2003; Ozturk *et al.,* 2006; Choi *et al.,* 2007; Velu *et al.,* 2006, 2008). Though simpler to perform than ICP-based methods, these approaches are only semi-quantitative and laborious when applied in large scale screens. Furthermore, energy-dispersive X-ray fluorescence spectrometry (EDXRF) was standardized by Paltridge *et al.,* 2012 for measuring Zn, Fe, and selenium (Se) concentrations in whole grain wheat. The high throughput, low cost XRF screening technique allows screening large number of breeding lines to discard low Zn/Fe lines and the selected high Zn/Fe lines could be tested with ICP for confirmation.

2.12 Molecular Breeding

Progress toward developing mineral-dense seed has mainly relied upon conventional plant breeding approaches, a process that is labour-intensive and time-consuming. Hence, the identification of DNA markers that are diagnostic of Zn efficiency can accelerate the development of cultivars that can remain productive even in Zn-deficient soils. Additionally, these markers may be used in identifying the specific genes responsible for differences in the response of genotypes to Zn deficiency.

2.12.1 Molecular marker system

Molecular markers are heritable differences in nucleotide sequences of DNA at the corresponding position on homologous chromosome of two different individuals, which follow a simple Mendelian pattern of inheritance. Over the last two decades, the advent of molecular markers has revolutionized the entire scenario of biological sciences. DNA-based molecular markers are a versatile tool in the fields of taxonomy, physiology, embryology, genetic engineering, molecular breeding etc. (Schlotterer, 2004). The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker based gene tags, genetic mapping, map-based

cloning of agronomically important genes, genetic diversity studies, phylogenetic analysis, and marker-assisted selection of desirable genotypes etc. (Joshi *et al.*, 2000). Thus, giving new dimensions to breeding and marker-aided selection, that can reduce the time span of developing new and better varieties and the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively. The existence of various molecular techniques and differences in their principles and methodologies require careful consideration in choosing one or more of such marker types. Table 1 gives the list of main type of molecular markers.

Microsatellites markers

Among various genetic markers Microsatelite markers are discussed in details because the strengths of microsatellites include the codominance of alleles, high genomic abundance, and random distribution throughout the genome (Morgante *et al.*, 2002). In general, microsatellites show a high level of polymorphism, so they are very informative markers. The genomes of higher organisms contain three types of multiple copies of simple repetitive DNA sequences (satellite DNAs, minisatellites, and microsatellites) arranged in arrays of vastly differing size (Litt and Luty 1989). They are also known as simple sequence repeats (SSRs), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs) and are the smallest class of simple repetitive DNA sequences. Microsatellites represent tandem repeats but their repeat motifs are shorter (1-6 bp). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20-25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labelled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed (Kesawat and Das 2009). In addition, primers may be used that have already been designed for closely related species. DNA Polymerase slippage during DNA replication, or slipped strand mispairing and unequal crossing over is considered to be the main cause of variation in the number of repeat units of a

Table 1: Overview of 11 main molecular marker technologies

	Allozymes	RFLP	Mini Satellites	PCR Sequencing	RAPD	Micro-Satellites	ISSR	SSCP	CPAS	SCAR	AFLP
Genomic Abundance	Low	High	Medium	Low	High	High	Medium-high	Low	Low	Low	High
Level of Polymorphism	Low	Medium	High	Low	Medium	High	Medium-high	Low	Low-medium	Medium	Medium
Locus-Specifity	Yes	Yes	No/yes	Yes	No	Yes	No	Yes	Yes	Yes	No
Codominance of alleles	Yes	Yes	No/yes	Yes	No	Yes	No	Yes	Yes	No/Yes	No/Yes
Reproducibility	High	High	High	High	Low	High	Medium-high	Medium	High	High	Medium-high
Labour intensity	Low	High	High	Low/high	Low	Low	Low	Low-Medium	Low-Medium	Low	Medium
Technical demands	Low	High	High	High	Low	Low-Medium	Low-Medium	Medium	Low	Low	Medium
Operations costs	Low	High	High	High	Low	Low	Low-Medium	Low-Medium	Low	Low	Low
Development costs	Low	Medium-high	Medium-high	High	Low-Medium	High	Low	High	Medium	Medium	Medium
Quantity of DNA required	-	High	High	Low	Low	Low	Low	Low	Low	Low	Yes
Amenability to automation	No	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes

Kesawat and Das 2009

microsatellite, that can be detected by gel electrophoresis (Matsuoka *et al.,* 2002). They can be used for population genetics studies and gene mapping, ranging from the individual level (e.g. clone and strain identification) to that of closely related species (Jarne and Lagoda 1996). This significantly decreases the analytical costs (Kesawat and Das 2009)

2.12.2 Approaches to discover QTLs

Two approaches are being widely used for the identification of QTLs and genes. One of the trending approach is the association mapping (AM)/ genome wide association studies (GWAS) emerging as a method of QTL detection which surpasses the need for crossing and linkage map construction (Mc Couch and Jung 2013). It focuses on association within the populations of unrelated individuals, which examines a collection of diverse accessions viz., varieties, landraces and breeding lines without generating mapping population. These accessions represent either strong linkage or linkage disequilibrium (LD). Further such populations also reveal significant allele diversity and hence, the population based LD mapping would increase the resolution of the QTL (Flint Garcia *et al.,* 2003; Mc Couch, *et al.,* 2004; Yu and Buckler 2006; Rafalski 2010).

The other approach is the very popular map based QTLs/gene identification which uses a mapping population developed from contrasting parents, they are characterized phenotipically in target environments and genotyped with molecular markers. The linkage analysis results in the identification of trait related genes (QTL) and subsequently, the fine mapping and map based cloning approaches are applied to obtain the candidate genes and linked marker for MAS. The principle disadvantage of association mapping is the spurious marker trait associations that can arise from population structure (Krill *et al.,* 2010; Famoso *et al.,* 2011). While biparental mapping overestimates QTL effects in limited progenies and leads to a wrong predictions, a phenomena called the 'Beavis effect' (Bernardo 2008; Xu 2003). In order to provide a stable and reliable prediction of QTL effects, reasonable population size, replicated field trails from multi-sites and across seasons are usually required along with reliable molecular markers and better statistical techniques (Kearsey and Farquhar, 1998).

QTL analysis

The process of QTL analysis by biparental mapping mainly requires the following four factors:

1. A suitable mapping population of phenotypically contrasting parents

2. A linkage map of molecular markers

3. Mapping methods and software

4. Reliable phenotypic screening methods and generation of phenotypic data

2.12.3 Suitable mapping population

It would be always advantageous using populations of early generations such as F_2, F_3, Back cross population etc. since the development of these populations require only 2 to 3 years compared to the late generation populations such as F_6, F_8, RIL, BC inbred lines etc. which requires about 7 to 8 years (Collard *et al.,* 2005). And development of these early generations are not a costly affair as in case the doubled haploid lines. However predictions made involving early generations would be misleading because of the masking effect of major genes on minor genes in early generations. Continues inbreeding to evolve Recombinant Inbreed Lines (RILs) can eliminate this masking effect. Thus, RILs can remain as the best choice of population for QTL analysis. As an alternative doubled haploid lines (DHLs) can also be used. The inherent homozygosity prevailing in the individuals of these two populations make the RILs & DHLs as immortals and help to have as many replications as required. In contrast, advantage of F_2 population is that it is the most suitable population for preliminary mapping (Datta *et al.,* 2011). It requires less time and effort for development.

2.12.4 Linkage map of molecular markers

Since the development of markers first allowed the construction of saturated linkages maps (Botstein *et al.,* 1980), it has been clear that the technology of quantitative trait locus (QTL) analysis could be usefully employed to analyze the genetics of complex traits.

Genetic map construction requires the following steps

a) Selection of the appropriate mapping population

b) Calculating the pairwise recombination frequencies using these populations

c) Establishment of linkage groups and estimation of map distances

d) Determination of map order.

Large mapping populations are often characterized by different marker systems, so the map construction has become computerized. Computer packages such as Linkage 1 (Suiter *et al.,* 1983), GMendel (Echt *et al.,* 1992), Mapmaker (Lander and Botstein, 1986; Lander *et al.,* 1987) and JoinMap (Stam, 1993) have been developed to aid in the analysis of genetic data for map construction (Staub and Serquen, 1996).

2.12.5 Mapping methods and software

The basis of all QTL detection, regard less of the crop to which it is applied is the identification of association between genetically determined phenotypes and specific genetic markers. The possible methods of analysis to detect QTL include

1) Single Marker Analysis (Marker Trait Method)

2) Interval analysis

QTL mapping methods

QTL mapping methods accounts to 3-step recipe

→ Scan the entire genome with a dense collection of markers

→ Calculate an appropriate linkage statistic S(x) at each position x along the genome

→ Identify the regions in which the statistic ‘S’ shows a significant deviation from what would be expected under independent assortment.

Some of the softwares employed in molecular breeding are given in the Table 2.

Table 2: Softwares and their application in Molecular Breeding

MAPMANAGER	Application	Interval mapping multiple QTL modeling
	Population	F_2 backcross, RIL, DH
	Language	Unix
QTLSTAT	Application	Interval mapping using non linear regression
	Population	F_2 backcross, RIL, DH
	Language	Unix
PGRI	Application	t-test, conditional t-test, linear regression
	Population	F_2 backcross, RIL, DH, F_1, OP
	Language	Unix
QTL cartographer	Application	t-test, Composite Interval mapping, permutation test, bootstrap, jackknife
	Population	F_2 backcross, RIL, DH
	Language	Unix/Mac/PC Windows
MAPQTL	Application	Interval mapping MOM
	Population	F_2 backcross, RIL, DH, F_1
	Language	Vax/Unix/Mac/PC Windows
MAP Manager QT	Application	Interval mapping using regression, MOM
	Population	F_2 backcross
	Language	MAC OS
QGENE	Application	linear regression
	Population	F_2 backcross
	Language	MAC

Datta *et al.*, 2011

The association between quantitative trait variation and marker segregation pattern can be carried out by the following methods:

1. Single Markers analysis (SMA)

SMA is the method used in earliest studies on QTL mapping (Edwards *et al.*, 1987; Weller *et al.*, 1988). In this method one marker is involved at a time to find the QTL – Marker association. This single marker analysis can be implemented as a simple t test, ANNOVA, linear regression and likelihood ratio test and maximum likelihood estimation (Haley and Knott, 1992; Nienhuis *et al.*, 1987; Wang *et al.*, 1994). SMA can be performed using common statistical softwares.

2. Interval Mapping (IM)

IM is considered as a second level of QTL mapping. QTL mapping by this method requires a prior construction of a marker genetic map. The interval mapping approach is based on the joint frequencies of a pair of adjacent markers & a putative QTL flanked by the two markers.

Interval Mapping can be done by the following methods:

Likelihood approach - Lander and Botstien, 1989

Regression approach - Knapp *et al.*, 1990

Combination of likely hood & regression approach - Zeng, 1994

Multiple QTL Mapping:

- Composite Interval mapping

- Multiple Interval mapping

Both SMA and IM are biased when multiple QTLs are linked to the marker or interval being considered. To deal with multiple QTL problems, Jansen 1993, Rodolphe and Lefort, 1993 and Zeng, 1993 independently proposed the idea of combining simple interval mapping with multiple regression analysis in mapping.

Composite interval mapping (CIM)

This method evaluates the possibility of a target QTL at multiple analysis points across each intermarker interval. However at each point it also includes the effect of one or more background markers. The inclusion of a background marker in the analysis helps in one of two ways, depending on whether the background marker & the target interval are linked. If they are not linked, inclusion of a background marker makes the analysis more sensitive to the presence of a QTL in the target interval. If they are linked inclusion of background marker may help to separate target QTL from other linked QTL on the far side of the background marker (Zeng, 1993 and 1994).

Multiple Interval mapping (MIM)

This method uses multiple marker intervals simultaneously to fit multiple putative QTL directly in the model for mapping QTL. With MIM approach the precision and the power of QTL mapping could be improved. Also, epistasis between QTL, genotypic value of the individuals and heritabilities of quantitative traits can be readily estimated and analyzed.

2.13 Review on QTLs related to zinc accumulation and bioavailability in various crops

2.13.1 QTLs for phytate and prebiotics

QTL mapping studies have been performed in *Arabidopsis*. A recombinant inbred lines (RILs) population developed by crossing L*er* and Cvi was characterized for variation in seed phytate concentration and thereby used for QTL mapping (Bentsink *et al.,* 2003). The L*er* × Cvi RILs displayed more than 2-fold variation for phytate concentration. More than 50% of the RILs displayed positive or negative transgression suggesting that new allelic combinations generated by mixing genomes of the 2 individuals offer great potential to reduce seed phytate concentration. Further, the authors mapped QTLs controlling seed phytate and found that one of the QTLs located on top of the chromosome 03 accounted for more than 60% of the total variance. Recently, 3 additional *A. thaliana* RIL populations were used for mapping QTLs of seed phytate concentration (Ghandilyan *et al.*,2009). Positive as well as negative transgression for seed

phytate concentration was consistently observed in all the populations, and QTLs for seed phytate concentration in these populations were also mapped to the top of chromosome 3 that explained more than 30% of the total variance. Thus, top of chromosome 3 in *Arabidopsis* seems very crucial to determine the variation in seed phytate concentration, and high percentage of variance explained by the QTLs in studies conducted under variable environmental conditions suggests that the underlying gene could have major and stable influences on the trait. A similar study was undertaken in rice to map the QTLs of seed phytate concentration and zinc concentration in rice seeds using doubled haploid population (DH) of IR64 × Azucena (Stangoulis *et al.,* 2007). Interestingly, only positive transgression was observed for seed phytate concentration in this DH population in contrast to *Arabidopsis thaliana* RIL populations. In DH population of rice accessions, two QTLs, one on chromosome 05 and another on chromosome 12, respectively, explained 24% and 15% of the total variance.

A study was conducted to identify the QTLs controlling the variation in wheat grain fructan concentration (Huynh *et al.,* 2008) and specifically inulin concentration (Falcon, 2011). In total, 5 QTLs were found to be controlling the variation in grain fructan concentration in a doubled-haploid population of a cross between Berkut and Krichauff, two being the most important ones, located on chromosomes 6D and 7A, which explained 17% and 27%, respectively, of the total phenotypic variance (Huynh *et al.,* 2008). A genetic mapping study performed by Falcon in 2011 using a doubled-haploid population generated from a cross between AC Reed and Grandin showed that the 2 QTLs controlling grain inulin concentration in wheat are located on chromosomes 2B and 5B and explained approximately 20% and 15%, respectively, of the total phenotypic variance. The presence of approximately 4-fold variation in the grain inulin concentration and the modest effect of QTLs controlling this variation indicate that inulin concentration can be significantly be improved in wheat grains through molecular breeding.

2.13.2 QTLs reported for zinc accumulation

One of the approach for the transfer of the seed mineral trait from wild beans to cultivated beans can be through the advanced backcross breeding approach. The goal of this study was to analyze a population of 138 $BC_2F_{3:5}$ introgression lines derived from the very high iron wild genotype G10022 backcrossed into the genetic background of the commercial-type variety 'Cerinza', a large-red seeded bush bean cultivar of the Andean genepool. Seed zinc accumulation trait was measured and the quantitative trait loci (QTL) controlling this trait was also analysed. Interestingly they also tested the adaptation of the introgression lines in two replicated yield trials. The cross was highly polymorphic and an anchored microsatellite map for the population was constructed which was 1,554-cM long and covered all 11 linkage groups of the common bean genome. Through composite interval mapping (CIM) and single point analysis (SPA), they identified associations of markers on b01, b04 and b10 for seed zinc concentration (Blair and Izquierdo 2012).

King *et al.,* 2014 identified seed and leaf QTL for iron and zinc concentration in the soybean population Anoka × A7, 92 $F_{2:4}$ lines. 14 SSR and one morphological marker, was integrated into the original genetic map of the Anoka × A7 used by Lin *et al.,* (1997), which consisted of 82 restriction fragment length polymorphisms (RFLP). There were no significant QTL identified for Zn concentration in 2008, 2009, nor in the combined data. However two suggestive QTL were indentified in 2008, one in 2009, and two in the data set combined over years. Suggestive QTL were those QTL that had peaks that did not exceed the genome-wide threshold but on the chromosome level were close to or were significant at $P = 0.05$ (Tiwari *et al.,* 2009; Willems *et al.,* 2010).The suggestive QTL in 2008 were on chromosomes 12 and 19 and had LOD scores of 3.7 and 3.0, and represented 21.2 % and 16.7 % of the variation, respectively. They were identified in the marker intervals *Sat 334-S12 0711* and *Satt694-Satt143*. In 2009, the suggestive QTL was in the marker interval *Satt175-pK 417H,* had a LOD score of 2.8, and represented 19.0% of the variation. Combined over years, the suggestive QTL were on chromosomes 7 and 18 with LOD scores of 3.0 and 2.9, respectively. These QTL were in the marker intervals *pk 417H-pk 70T* and *pa 890V-K 493H* and represented 23.4% and

18.5% of the variation. Even though these QTL were not significant (LOD = 3.7; P = 0.95), the marker *pk 417H-* was consistent in 2009 and in the combined data. The positive allele for the QTL on chromosome 7 was associated with the inefficient parent Anoka, and on chromosome 18, in the heterozygote.

Several QTLs controlling iron and zinc concentration in wheat grains have been mapped using RIL or DH population issued from crosses of different parents (Distelfeld *et al.,* 2007; Shi *et al.,* 2008; Peleg *et al.,* 2009; Tiwari *et al.,* 2009). Remarkably, 3 of these QTL mapping studies, performed over different years and under various agro-ecological conditions, revealed a common QTL on chromosome 7A to be contributing about 10% of the phenotypic variance to the grain iron and zinc concentration. QTL mapping studies have also been performed in rice using RIL populations (Lu *et al.,* 2008; Garcia-Oliveira *et al.,* 2009; Norton *et al.,* 2010; Anuradha *et al.,* 2012). QTLs affecting both iron and zinc concentration in grains are more often co-localized on chromosome 7 and chromosome 12 in rice. Co-localization of QTLs affecting grain iron and zinc concentration has also been found in maize mapping populations (Qin *et al.,* 2012).

2.14 Genes underlying zinc QTLs

Previously Shi *et al.,* 2008; Peleg *et al.,* 2009 and Tiwari *et al.,* 2009 performed QTL analysis for iron and zinc concentration in wheat with different parental combinations. A common QTL on chromosome 7A around 70 cM, contributing about 10% of the phenotypic variance to the grain iron and zinc concentration was identified in the three studies, performed over different years and under various agro-ecological conditions. Further, positional cloning of Gpc-B1, a wheat quantitative trait locus associated with increased iron and zinc concentration, identified a no-apical meristem (NAM) transcription factor to be responsible for this variation (Uauy *et al.,* 2006).

168 F_7 RILs derived from Madhukar×Swarna were used to map QTLs for iron and zinc concentrations in unpolished rice grains by Anuradha *et al.,* 2012. Iron ranged from 0.2 to 224 ppm and zinc ranged from 0.4 to 104ppm. Genome wide mapping using 101 SSRs and 9 gene specific markers showed 5 QTLs on chromosomes 1, 3, 5, 7 and 12 significantly linked to iron, zinc or both. 14 QTLs were identified for these two traits

where QTLs for iron were co-located with QTLs for zinc on chromosomes 7 and 12. Ten candidate genes known for iron and zinc homeostasis underlie 12 of the 14 QTLs. Another 6 candidate genes were close to QTLs on chromosomes 3, 5 and 7. Thus the high priority candidate genes for high Zn in seeds are OsYSL1 and OsMTP1. OsNAS3, OsNRAMP1, Heavy metal ion transport and APRT were the genes strictly underlying QTLs for both iron and zinc was revealed by genetic mapping studies by Anuradha K *et al.,* 2012. Also several elite lines with high Fe, high Zn and both were identified.

2.15 Pigeonpea crop of choice

Legumes are the primary source of dietary protein in semi-arid tropic (SAT) regions of Asia and Africa. From a food security perspective, legumes provide a highly balanced and nutritious source of calories and protein that is not provided by cereals, especially those commonly grown in semi-arid regions and have a great potential in alleviating protein hunger and malnutrition prevalent amongst the poor in SAT regions (Gowda *et al.,* 2013). The legumes in SAT regions are mostly grown in marginal environments and low fertile soils thus there is an ample of scope for examining the variability for efficient nutrient uptake in different legumes and later using them in breeding programmes to further improve the legume crop quality to meet the nutritional requirements.

Among different legumes, Pigeonpea is the sixth most important legume food crop grown on ~5 million hectares (ha), globally (FAOSTAT, 2012**).** Domesticated >3,500 years ago in India2–4 (Royes, 1976), it is the main protein source for more than a billion people in the developing world and a cash crop that supports the livelihoods of millions of resource-poor farmers in Asia, Africa, South America, Central America and the Caribbean (Mula and Saxena 2010). Besides its primary use as food, it can also be used as forage, fodder, fuel and medicine. Recent findings further show its importance in soil conservation along highways and mountain slopes particularly against soil erosion caused by wind and water (Nsiah, 2012). Since Pigeonpea is considered as poor man's crop, hence by improving nutritional quality of this crop it's possible to meet the nutritional requirements of poor and malnourished population.

2.15.1 Types of populations developed in Pigeonpea for various traits

For instance, as the main objective of the thesis was to identify QTLs governing seed zinc content, thus literature on the plant genetic resources present in Pigeonpea is reviewed. Mapping populations are the pre-requisites for the development of genetic maps and identification of quantitative trait loci (QTLs) for the desired traits. In this respect, several mapping populations have been developed for mapping traits such as resistance to biotic stresses (resistance to FW, SMD and pod borer), abiotic stresses (drought tolerance), agronomically favourable plant type, earliness, fertility restoration etc., Table 3 gives the list of mapping populations available in Pigeonpea, which were developed by various research institutes. These mapping populations were used for identifying QTLs and for generating various inter- and intra-specific genetic maps.

Thus the Pigeonpea mapping population that we developed was first of its kind. It is an F_2 mapping population segregating for grain zinc content.

Table 3: Genetic resources available for mapping different traits in pigeonpea

Trait	Type of population	Population	References
Fusaium wilt resistance	Intra-specific F_2	ICPB 2049 × ICPL 99050	Bohra et al., 2012
Fusarium wilt resistance	F_2	GS 1 × ICPL 87119 GS 1 × ICP 8863	Kotresh et al., 2006
Sterility mosaic disease resistance	Intra-specific F_2	ICP 8863 × ICPL 20097 TTB 7 × ICP 7035	Gnanesh et al., 2011
Fertility restoration	F_2	ICPA 2039 × ICPR 2447 ICPA 2043 × ICPR 3467 ICPA 2043 × ICPR 2671	Bohra et al., 2011
Pod borer	Inter-specific F_2	ICPL 8755 × ICPL 227 ICPL 151 × ICPL 87 ICP 28 × ICPW 94	Saxena et al., 2011
Drought tolerance	Inter-specific F_2	ICPL 8755 × ICPL 227 ICPL 151 × ICPL 87 ICP 28 × ICPW 94	Saxena et al., 2011
Determinancy	F_2	ICPL 85010 × ICP 15774	Mir et al., 2014
Plant type	F_2	TT44-4 × TDT 2004-1	Dhanasekar et al., 2010
Plant height, number of primary and secondary branches, number of pods, days to maturity and days to flowering	F_2	Pusa dwarf × HDM04-1	Kumawat et al., 2012

Material and methods

III MATERIAL AND METHODS

Zinc is an important nutrient in the human diet and its deficiency may lead to a variety of health-related problems. Enhancing plants with zinc may address the present problem. Biofortification of crop plants with zinc is considered as a cost-effective solution to overcome the malnutrition of these minerals in the diet, where biofortification aims at either increasing accumulation of these minerals in edible parts, endosperm, or to increase their bioavailability. Although zinc fertilization management positively influence their accumulation in cereal grains but it has certain soil constraints for efficient uptake. While quantitative genetic studies reveal the existence of ample variation for zinc accumulation in plants as well as the existence of inhibitors or promoters of their bioavailability. However, the genes underlying this variation have rarely been identified and never been used in breeding programmes. Keeping this in view, the present investigations were carried out to discover QTL governing high grain zinc content in pigeon pea. The materials and methods used in this study have been discussed in below sections.

3.1 Selection of plant material

Schematic representation of different experiments conducted in present investigation along with the earlier work is presented in Plate1. Present investigation is continuation work of Manoa (2010) who ascertained the zinc content of 217 germplasm accessions of pigeonpea comprising core germplasm lines, local adapted cultivars and released varieties outsourced from ICRISAT, Hyderabad, Telangana India. The germplasm accessions were scored for zinc content in seeds during two subsequent *kharif* season in 2009 and 2010 under similar soil type and environmental conditions. By interpreting the data of these two seasons, revealed 15 high and 15 low genotypes which showed consistency for seed zinc levels in both the seasons. To examine the extent of genetic diverseness the 15 high and 15 low seed zinc types were genotyped for diversity analysis (Table 4).

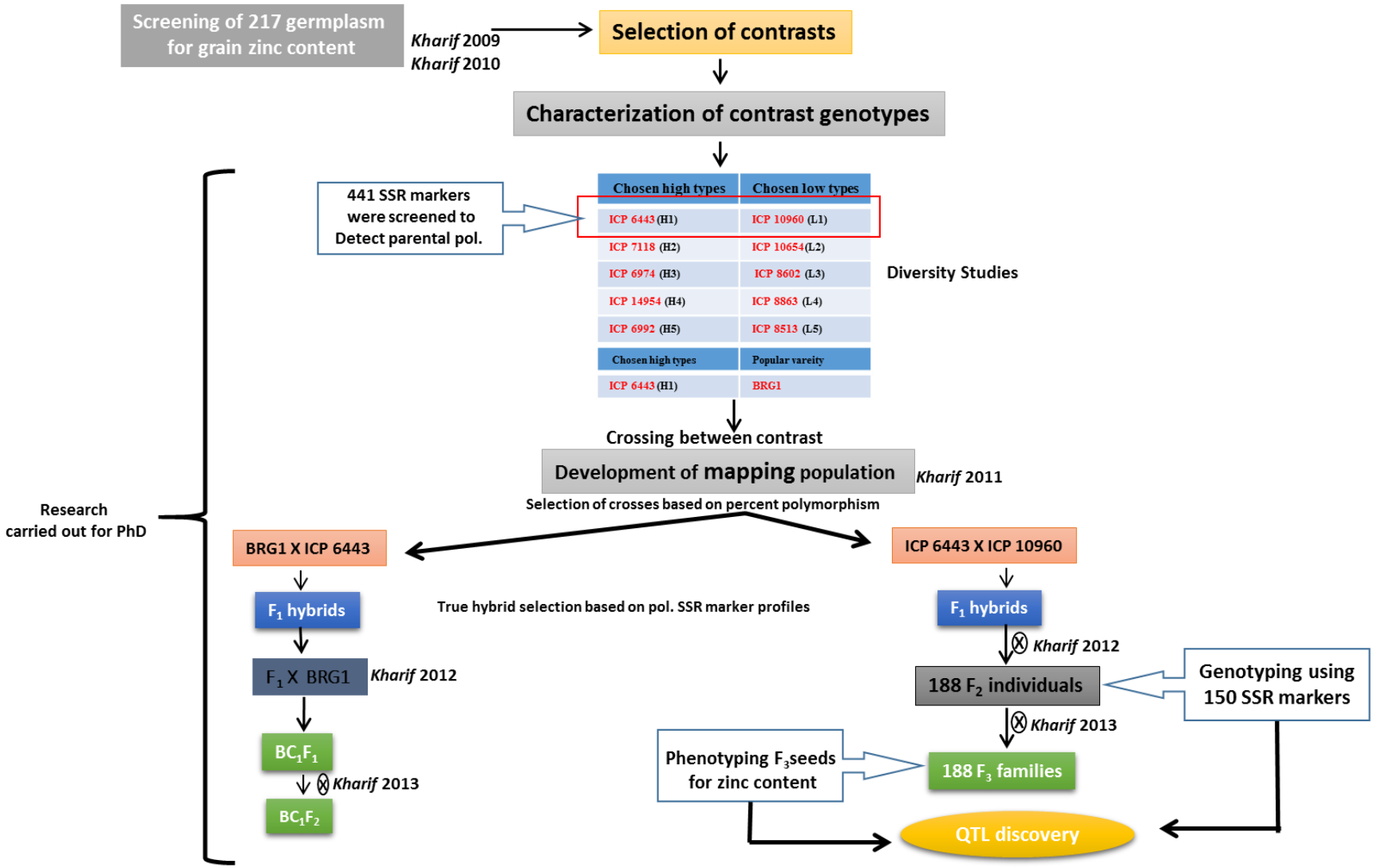


Plate 1: Flow diagram of experiments conducted earlier and in present investigation

Table 4: List of contrast pigeon pea genotypes varying for seed Zn content used for diversity analysis

High Zn types				Low Zn types			
Sl. No.	ICP Acc No.	Zn Content (mg/100 g)		Sl. No.	ICP Acc No.	Zn Content (mg/100 g)	
		2009	2010			2009	2010
1	2059	4.28	3.83	16	7221	2.24	2.65
2	2484	4.35	4.19	17	7623	2.18	2.4
3	2626	4.22	4.1	18	8508	2.15	2.08
4	6370	4.38	3.88	19	8513	1.97	1.74
5	6443	6.2	6.36	20	8516	2.32	2.57
6	6974	5.13	4.54	21	8602	1.94	1.85
7	6992	4.6	4.44	22	8863	1.95	2.1
8	6997	4.62	4.61	23	9336	2.12	2.24
9	7035	5.66	6.11	24	9655	2.36	2.11
10	7118	5.65	5.14	25	9691	2.22	2.43
11	7119	4.21	4.15	26	10654	1.91	1.62
12	7182	4.56	4.23	27	10960	1.91	1.61
13	7314	4.54	4.29	28	11543	2.23	2.18
14	14421	4.19	3.75	29	11946	2.15	2.1
15	14954	4.96	5.14	30	12410	2.17	2.14

3.2 Mapping population

The contrasting five high and five low genotypes and an elite variety BRG-1 were used in the crossing programme. Table 5 gives the list of the genotypes used in the crossing programme.

Table 5: Contrast seed Zn genotypes chosen to carry out crossing

Chosen high types	Chosen low types
ICP 6443 (H1)	ICP 10960 (L1)
ICP 7118 (H2)	ICP 10654 (L2)
ICP 6974 (H3)	ICP 8602 (L3)
ICP 14954 (H4)	ICP 8863 (L4)
ICP 6992 (H5)	ICP 8513 (L5)
Chosen high types	**Elite variety**
ICP 6443 (H1)	BRG-1

3.2.1 Crossing technique

3.2.1.1 Emasculation and pollination

Emasculation is required for artificial hybridization in pigeon pea. The buds most-likely to shed pollen the next day are selected for emasculation (Singh and Oswalt, 1992). The buds which were approximately 65-75 % the size of a mature bud and which were tightly closed and if the corolla of such a bud were greenish yellow in color were selected for emasculation. For best results only two buds on one inflorescence were emasculated and total of two to ten buds were emasculated on a branch, while all other buds were removed (Sharma and Green 1980). Plate 2 gives the steps adopted in Pigeonpea crossing. The pigeon pea stigma is receptive before anthesis; therefore, pollination was done immediately after emasculation. Before emasculation, the flowers in which the anthers had burst and pollens grains were available were kept in Petri-dishes on moist filter papers neatly labeled. While in the bud, pollen remains viable for 42 h at 25-28 °C with relative humidity of 50 % (Prasad *et al.*, 1977). Thus the pollination was carried out in the cool hours of the morning between 7am to 9am. The corolla of the pollen-source bud was removed so that the staminal columns and anthers were uncovered. To transfer pollen, the flower was held in one hand and touched the anthers to the stigma of an emasculated bud. A single flower was being used to pollinate two or three emasculated flowers (Sharma and Green 1980).

Plate 2: Emasculation and crossing technique

a) Give half a slit on the concave side of the bud on the female plant.
b) Gently tip off only the corolla removing the immature anthers.
c) Bud with intact pistil and filaments without anthers
d) Dusting the pollen from the flower of the desired male plant on stigma

3.2.1.2 Pod development

A week after pollination the pods became visible and completed their development in 15—20 days. The seed attained physiological maturity within 30-35 days and were ready for harvest in and around 40 days.

3.2.1.3 Labeling

Individual female-genotype for each cross was sown row wise, hence labeling of individual buds was not carried out. An identification label for the male parent used for crossing was written on tag and tied to the respective female plants. To identify the artificially pollinated buds a small, bright yellow-colored, thin nylon thread was tied to the peduncle of the bud. Seeds were harvested only from such pods.

As pigeonpea is often cross pollinated crop; therefore entire field was covered with the temporary netted structure and a nylon net bag was covered on the plants planted in pots to prevent undesirable cross pollinations (Plate 3).

3.3 Development of F_2 mapping population

Based on the data on zinc content in seed over the two seasons in the field at GKVK, five genotypes with high zinc content in seeds were chosen to cross with five genotypes with low zinc content in seeds during *kharif* 2011. In all the parental lines qualitative traits like flower color, seed, pod characters (Plate 4a & 4b) etc. were noted down so that the observations could help in knowing whether the crossing had been effective or not. After crossing, all the true F_1s were self-pollinated to obtain F_2 seeds during *kharif* 2012. F_2 seeds were sown during *kharif* 2013 and young leaf samples were collected from individual plants of F_2 derived from the cross ICP 6443 X ICP 10960 at 40^{th} day. 188 F_2 were advanced to produce the $F_{2:3}$ seeds for zinc estimation. The F_2 plants were phenotyped for various morpho-physiological traits. Parameters like seed zinc content, seed Iron content and Phytic Acid content were estimated in $F_{2:3}$ seeds. On the other hand one more cross was being maintained, where BRG-1 was crossed to ICP 6443 a high seed zinc type. The resultant F_1 seeds from the cross were back crossed to its recurrent parent, BRG-1 in kharif 2012 to develop BC_1F_1 and subsequently selfed in

Plate 3: Temporary netted structure to prevent undesirable cross pollination

a, b and c : Pigeonpea grown in field covered by temporary netted structure
d: Individual potted plant covered by netted bags

a)

Character	Differences	
Flower colour	Red standard petal L1,H5 	Yellow L2,L3,L4,L5,H1,H2,H3,H4 
Pod size	5 seeded L1 	4 seeded All others 
Plant height	Tall L3,L4 ,H2	Short L1,L2,L5 H1,H3,H4,H5
Seed size	Bold L1	Small All others

b)

Seed and pod characteristics

Plate 4a & 4b: Phenotypic observations in contrasting parents

H series are the high Zn types. H1: ICP6443; H2: ICP7118; H3: ICP6974; H4:ICP14954; H5: ICP6992. L series are the low Zn types. L1: ICP10960; L2: ICP10654; L3: ICP8602; L4: ICP8863; L5: ICP8513

kharif 2013 to develop BC_1F_2 backcross population. This population was developed for future studies for validation of discovered QTLs or development of trait introgressed lines.

3.4 Phenotyping of mapping population

3.4.1 Sample preparation

Seeds and leaves of Pigeonpea were ground with pestle and mortar to make a fine powder. Five ml of concentrated nitric acid (HNO_3) was added to 0.25 g of powdered seed sample and incubated in a digestion hood overnight. The next day, 5 ml of diacid mixture (Nitric: Perchloric acid:: 10:4) was added and placed on a sand bath till all the white fumes evaporated and a colourless liquid was left in the flask. It was allowed to cool and volume was made up to 100 ml using glass distilled water and further dilutions were made if the concentration of the samples was too high. These diluted samples were used for zinc estimation.

3.4.2 Estimation of zinc

Zinc was estimated in the $F_{2:3}$ seed samples and F_2 leaf tissues of Pigeonpea mapping population using Polarised Zeeman Atomic Absorption Spectrophotometer (AAS-2-6100) (Plate5).

Atomic absorption spectroscopy (AAS) determines the presence of metals in liquid samples. It detects Fe, Cu, Al, Pb, Ca, Zn, Cd and many more. It also measures the concentrations of metals in the samples. Typical concentrations range in the low mg/L range. In their elemental form, metals will absorb ultraviolet light when they are excited by heat. Each metal will absorb a characteristic wavelength. The AAS instrument looks for a particular metal by focusing a beam of UV light at a specific wavelength through a flame and into a detector. The sample of interest is aspirated into the flame. If that metal is present in the sample, it will absorb some amount of the light, thus reducing its intensity. The instrument measures the change in intensity. A computer data system converts the change in intensity into an absorbance. The concentration of the element is directly propotional to its absorbance. The calibration curve was constructed by running

Plate 5: Analyst 700 Atomic Absorption Spectrometer facility

standards of various concentrations on the AAS and observing their absorbencies. The Zn and Fe concentration in samples were determined using calibration curve.

Preparation of Standard:

100ppm standard Zn^{2+} solution was prepared using 1000ppm Zn^{2+} atomic absorption standard solution and appropriate dilutions was made to get standard solutions ranging from 0.0 to 1.0 ppm. These standards were subjected to AAS as that of sample to get a standard curve. The samples were then analyzed based on the standard curve.

Protocol:

Suitable dilutions were made from the extract with double distilled water so as to fit their absorbance with the range of standard curve. The diluted samples were subjected to AAS and the concentration was recorded in ppm using the standard curve. The values in ppm were used for calculation of seed zinc content using the following formula:

$$\text{Zinc content} = \frac{\text{Average ppm X Volume of digested sample X Volume made up}}{10^6 \text{ X Weight of sample X Aliquot taken}}$$

Zinc content was expressed in mg/100g of grain sample.

3.4.3 Iron estimation

The same procedure as that of zinc estimation (3.4.1 & 3.4.2) was followed for Iron estimation with a small change in dilution of the digested product.

3.4.4 Phytic acid estimation

Phytic Acid was estimated by using Colorimetric (Wade Reagent) Method. This method was the modification of the method given by Vaintraub and Lapteva in1988 and was modified by Gao *et al.,* 2007.

Modifications applied by Gao *et al.*, 2007 to the Vaintraub and Lapteva (1988) method is given below:

(i) The extraction time was extended to 16 h

(ii) The temperature of centrifugation was reduced to 10 °C, and

(iii) A matrix cleaning step was added to increase PA recovery.

The details of the protocol were as follows. Crude acid extracts were transferred to 2-mL tubes containing 1 m*g* NaCl. The contents were shaken at 350 rpm for 20 min to dissolve the salt and were allowed to settle at 4 °C for 60 min. The mixtures were centrifuged at 1000 *g* at 10 °C for 20 min, and clear supernatants, were collected for colour development. 100 µL of the clear supernatant was diluted 10 times in a 1.5-mL tube by mixing with 900 µL of distilled-deionized (dd) H_2O. One mL of this diluted sample were combined with 333 µL of modified Wade reagent (0.03 % $FeCl_3 \cdot 6H_2O$ + 0.3 % sulfosalicylic acid) in a 1.5-mL tube, thoroughly mixed on a vortex, and centrifuged at 1000 *g* at 10°C for 10 min. A series of calibration standards containing 0, 1.12, 2.24, 3.36, 5.6, 7.84, or 11.2 mg L^{-1} 2 PA-P were prepared from sodium phytate (Sigma, St. Louis, MO). Absorbance of colour reaction products for both samples and standards was read at 500 nm on a spectrophotometer, and for calculation of sample Phytic acid content the method described by Latta and Eskin in1980 was followed.

3.4.5 Morpho-physiological characterization

Plant height (cm)

The plant height was measured at maturity. Total length of plant was measured from ground to the tallest canopy point

Stem girth (cm)

Stem thickness was measured at maturity by measuring the length of the thread that was winded around the stem.

Stem weight (g)

The stem and all the branches of Pigeonpea plant were cut and collected into a brown paper bag, sun dried for a week and then oven dried at 60 °C. After complete drying, the stem weight was recorded separately for each plant

No. of branches

Number of branches refers to the secondary branches which arise from the main stem. For each plant the number of branches that arouse from main stem was recorded.

SCMR (SPAD chlorophyll meter reading)

The chlorophyll meter called Soil Plant Analysis and Development has been developed by Minolta Corporation for determining the chlorophyll content which indicates the Nitrogen status of the crop. The devise is a simple and portable diagnostic tool that measures the greenness or the relative chlorophyll content of the leaves.

SPAD (SPAD-502) utilizes two light emitting diodes, 650 and 940 nm and a photodiode detector to sequentially measure light transmission through the leaves of Red and Infrared light. Red light transmits at 650 nm, the wavelength at which chlorophyll absorbs light and at 940 nm where no light absorption occurs. Upon initial calibration, the Minolta-502 processor converts a current produced by the red (650 nm) and infrared (940 nm) light beam into a voltage and stores the digital values in a unit memory. When the leaf is subsequently measured, the device successively measures the transmission of red and infrared light outputs. A processed value based on the ratio of the measured voltage produced by each wavelength relative to the values stored in the memory.

Since chlorophyll a and b are the dominant pigments absorbing light at 650nm, the ideal SPAD chlorophyll meter reading would be proportional to the chlorophyll concentration in the leaf. Three readings were recorded on three leaflets (9 readings/3 leaflets)

Leaflet length (cm)

Length of the middle leaflet of ten randomly selected leaflets per plant at maturity was measured separately for each plant.

Leaflet width (cm)

Width of the middle leaflet of ten randomly selected leaflets per plant at maturity was measured separately for each plant.

Specific leaf area (SLA) (cm^2g^{-1})

Specific leaf area (SLA) is the one-sided area of a fresh leaf, divided by its oven-dry mass and was computed as follows.

$$SLA = \text{Leaf Area } (cm^2)/\text{Leaf Dry Weight } (g)$$

Pod length (cm)

The length of the pods for ten randomly selected pods per plant was measured at maturity using the measuring scale.

Pod width (cm)

The width of the pods for ten randomly selected pods per plant was measured at the center of the pod at maturity using venire calipers.

No. of seeds/pod

Number of seeds present per pod was counted for each plant separately. Seeds were counted in ten randomly picked pods per plant.

100 seed weight (g)

Weight of 100 seeds was measured for each plant using electronic digital balance.

Shelling percentage (%)

Seed weight to pod weight ratio for each plant were calculated for each plant separately and expressed in percentage.

Seed yield (g plant^{-1})

Total seeds obtained per plant were weighed to record seed yield per plant.

3.5 Genotyping with SSR markers

In the present study, 421 SSR markers were chosen for parental polymorphism survey between the parental genotypes which were used as parents for development of mapping population. Among 441 SSR markers 71 were genic SSR markers (Dutta *et al.,* 2011) and remaining 370 were Genomic SSR markers (Dutta et al., 2013). A 96 well PCR systems (Bio-Rad and Eppendorf) were used for detecting the polymorphism between the parents and individuals of F_2 mapping population.

3.5.1 Sample collection and DNA isolation

Young healthy leaves were collected and frozen in liquid nitrogen from individual plants of F_2 grown in the field during *kharif* 2013. Frozen leaves were used to grind. The fine powder obtained from each sample was used for DNA extraction as per modified CTAB (Cetyl Trimethyl Ammonium Bromide) method of Maroof *et al.,* 1984.

a) The leaves were cut into pieces and homogenized completely with liquid nitrogen using pestle and mortar and leaf powder (0.5g) was transferred to 2ml eppendrof tubes containing 1ml extraction buffer (10 mM Trismabase pH8, 20 mM EDTA Disodium, 25 mM EDTA, 1.4 M NaCl, 4 % CTAB,) and 0.2 % β Mercaptoethanol was added to each tube.

b) The extract was mixed by inverting the tubes several times, then tubes were incubated in a water bath at 65 °C for an hour with constant stirring at an interval of 15 minutes

c) After an hour, 700 µL of chloroform: isoamyl alcohol (24:1) was added to the incubated sample and mixed well by inverting. The tubes were then centrifuged at 6000 rpm for 20 minutes.

d) The aqueous upper phase was carefully transferred using the 1mL cut tips into fresh sterile centrifuge tubes. To this supernatant 0.7 volume (700 µL) of cold isopropanol was added.

e) The tubes were carefully inverted and kept for 5 min in ice. Once DNA precipitation was seen in the form of strands. The tubes were then centrifuged at 6000 rpm for 20 minutes and sedimentation of DNA as a hard pellet was collected.

f) Further the supernatant was decanted gently and the tubes were inverted on a clean filter paper.

g) The pellet was washed twice by suspending in one mL of 70 % ethanol for 5 to 10 minutes and the DNA was centrifuged at 6000 rpm for two minutes.

h) Ethanol was drained of slowly and the pellet was vacuum dried in a desicator for 5 to 10 minutes. The pellet was then dissolved in 500 µL of TE buffer by flicking the tubes. (TE buffer = 0.1 mM Tris + 0.05 mM EDTA)

i) To remove the RNA 5 µL of RNase (10 mg/mL) was added into the DNA solution and is incubated at 37 ˚C in a water bath for 1 hour.

j) Again the DNA solution was cleaned by washing with equal volume (500 µL) of phenol: choloroform:Isoamyl alcohol (25:24:1) by invert mixing several times and centrifuging at 6000 rpm for 15 minutes to separate the two phases.

k) The aqueous upper phase was transferred into a clean 1.5 mL eppendorf tube and twice the volume of 100 % ethanol was added to precipitate the DNA.

l) The pellet was washed twice with 70 % ethanol and dissolved in 500 µL TE. The extracted DNA was quantified using both spectrophotometer and by running in 0.8 % agarose gel.

3.5.2 Quantification of DNA

3.5.2.1 Quantification of DNA by Nanodrop

The Nanodrop has a quartz pedestrel which serves the purpose of cuvettes in a normal spectrophotometer. When the sample is loaded on to pedestrel and closed, column

formation takes place. When the electric beam passes through this column, the instrument measures the absorbance at 260 and 280 nm. The absorbance peak for proteins is 260 nm and for double stranded DNA (dsDNA) is 280 nm. Hence, the quantity of dsDNA in 1 µl of sample is quantified by the instrument based on the absorbance at 280 nm. The ratio of 260 nm/280 nm gives an estimate of the purity of DNA sample. Pure DNA samples are expected to have the ratio of 1.8-2.0. If the sample has a value < 1.8, it is expected to be contaminated by proteins and if the ratio is >2.0, the sample is expected to contain more RNA.

Procedure

The instrument was initialized by pipetting 1 µl of sterile water on to the sample holding platform of the pedestrel. The blank measurements are made using the buffer used to dissolve the DNA sample. After initializing the instrument, 1 µl of DNA sample was pipetted on to the sample holder and the absorbance at 260 and 280 nm were recorded against a buffer blank. The instrument is connected to a computer that has inbuilt data logger sheet where in the reading are automatically recorded in ng/µl. Further the DNA samples were diluted to a final working concentration of 15 ng/µl.

3.5.2.2 DNA quantification on 0.8 % agarose gel

1. 0.8 g of agarose was weighed and taken into a clean 100ml conical flask and 100 ml of 1xTBE buffer was added (TBE buffer 0.89 M Tris base, 0.02M EDTA, 0.89 M Boric acid, pH = 8).
2. Agarose was melted completely by boiling and cooled. 2.5 µl of ethidium bromide (10 mg/ml) was added and mixed well.
3. The ends of gel casting tray were sealed with sticky tape. The melted agarose was poured carefully devoid of any air bubbles and comb was inserted and allowed to solidify.
4. After solidification the tape from either side was removed and the gel was immersed in the buffer tank containing 1x TBE buffer. Then the comb was removed carefully without damaging the wells.

5. To 3 µl of DNA samples 6x loading/tracking dye was added, mixed and then loaded into the well. Around 3 µl of standard uncut λ DNA (25 ng/µl) was used as marker (6x loading/tracking dye – 40 % sucrose, 0.025 % bromophenol blue, 0.25 % xylene cyanol)
6. Electrophoresis was carried out at 90 V for 2 to 3 hours until the bromophenol blue dye migrated to two-third of the gel. The gel tray was removed and the gel was observed under UV transilluminator and documented using Herolab Gel Documentation system, Belgium.
7. The quantity of the DNA was determined based on the intensity of the band relative to uncut λ DNA band.

3.5.3 Genetic analysis using molecular marker system

The mapping individuals were assessed for the genome information using co-dominant (SSR) markers system.

3.5.4 Analysis of simple sequence repeats

Microsatellites are simple sequence repeats (SSRs) of 2-7 nucleotides. They appear to be ubiquitous in higher organisms and conserved across species. They are abundant, dispersed throughout the genome and known to show high level of polymorphism than any other genetic markers. A total of 441 SSR markers were used in this study to test the parental polymorphism. Among 441, 71 were genic SSR markers (Dutta *et al.,* 2011) and 370 were genomic SSR markers (Dutta *et al.,* 2013). The template DNA from the mapping population was amplified using each polymorphic primer from genic and genomic microsatellite markers.

The volume of reaction mixture per one reaction is as follows:

PCRs were carried out in 15 µl volume containing

1.5 µl of 10X reaction buffer,

0.20 µl of 10 mM dNTPs (133 l M),

1.5 µl each of forward and reverse primers (10 pmol),

l µl (62.5 ng) of template genomic DNA and

0.15 µl (0.75 U) of Taq DNA polymerase (Kappa Taq).

3.5.5 Standardization of annealing temperature for SSR primers

The annealing temperature was standardized for each primer by using gradient PCR technique. In this method different annealing temperatures ($T_m \pm 5°C$) were set to each lane of the block and amplification was carried out accordingly. Further, the parents and the progeny were amplified at the standardized T_m.

The PCR conditions followed were.

1) Initial denaturation at 94 °C - 5 min

2) Denaturation at 94 °C - 45 sec

3) Primer annealing for 45 seconds

4) Extension at 72 °C - for 1 min

5) Final extension at 72 °C – 7 min

6) Hold at 15 °C

3.5.6 Fragment analysis

3.5.6.1 Agarose gel preparation and gel electrophoresis

PCR products were separated by electrophoresis in 3 % Agarose gel containing 0.1 µg/ml ethidium bromide in 1X TBE buffer at 130 V for 4 h,. The bands which didn't get resolved in agarose gel, for such products 3 % Metaphor agarose gels containing 0.1 µg/ml ethidium bromide was being used. The gels were visualized and photographed in gel documentation system (Biorad).

3.5.6.2 Microchip based electrophoresis system

MultiNA was employed for PCR products which further did not get resolved on metaphor agarose. Thus fragment analysis for polymorphism among parents and F_2 progeny of mapping population were analyzed on microchip based electrophoresis

system MultiNA (Shimadzu Biotech, Japan) (Plate 6). MultiNA is microchip based technology that is a new electrophoresis analysis platform provides an alternative to agarose gel electrophoresis. This instrument contains microchips (up to 4 chips) made out of quartz. As more is the number of chips used, the shorter is the time required for the overall analysis schedule.

PCR products of different SSR markers differing in expected PCR product were mixed together before fragment analysis which leads to reduce time required and cost of analysis. Those PCR products were mixed with the MCE-202 MultiNA DNA-500 reagent kit (separation buffer and SYBR Gold dye) and size analysis was conducted from 25 bp to 500 bp samples with accuracy of ± 4 bp. The flow chart given in the next page demonstrated the experiment procedure of preparing samples to analysis in MultiNA. The product size was visualized by comparing the sample peak with the ladder DNA-500 (25 bp DNA ladder). Only the highest peak was selected as the PCR product.

3.5.7 Scoring of SSR markers

The product size for each SSR in different Pigeonpea genotypes was scored for diversity analysis. For linkage map construction, the banding pattern of SSR markers was scored as follows:

A= homozygous maternal genotype (ICP 6443)

B= homozygous paternal genotype (ICP 10960)

H= heterozygote genotype

- = Missing value

3.6 Statistical analysis

Different statistical methods employed for analysis are presented below

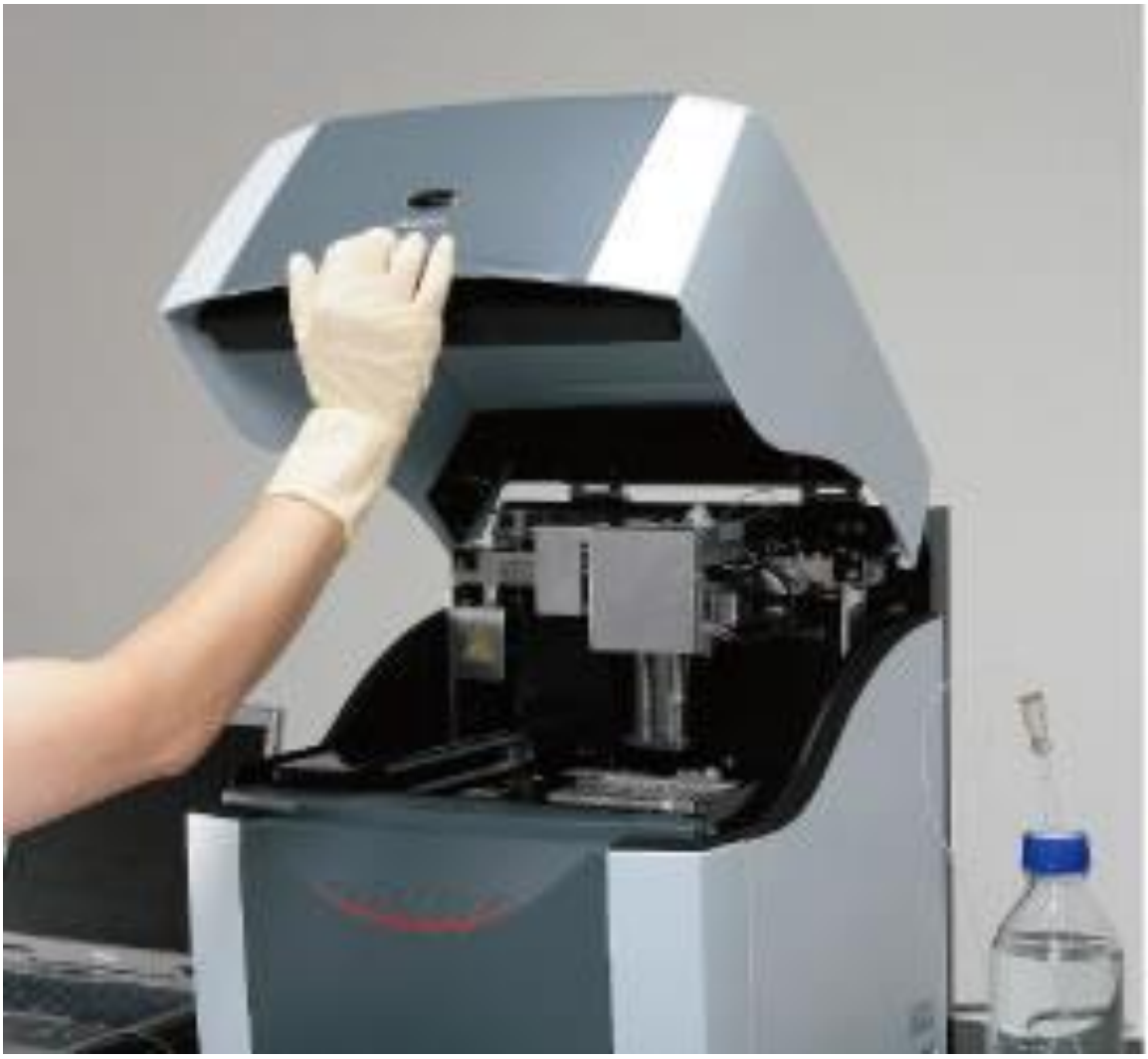
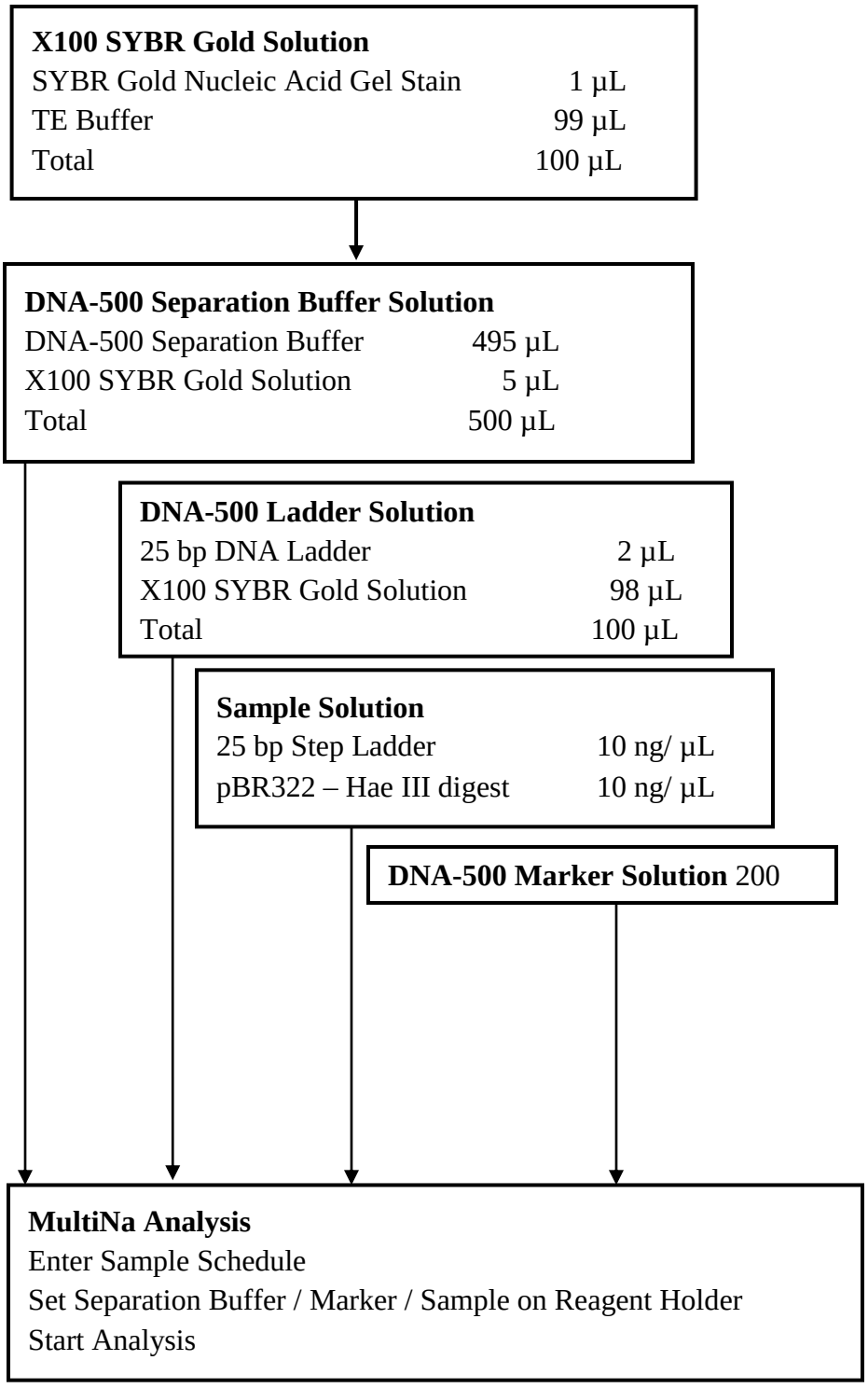


Plate 6: The MultiNA microchip based electrophoresis analysis platform for SSR marker genotyping



Procedure of genotype using MULTINA chip based electrophoresis instrument

3.6.1 Phenotypic data analysis

3.6.1.1 Analysis of variance (ANOVA)

The genotypic variability for seed zinc content and other morpho-physiological traits were assessed using analysis of variance as per Fisher's method. The level of significance was tested at 0.05 probability level in 'F' test. Variance components due to genotype (δ^2 g) and genotype x environment influence (δ^2 ge) were estimated by utilizing the respective mean sum of squares from the variance. This analysis was performed using Genstat® version 12.

3.6.1.2 Descriptive statistics

The following descriptive statistics were calculated as per Sundararaj *et al.,* 1972

Test of normality and frequency distribution: The normal distribution function was determined by the following formula

$$F(x) = 1/[(2*x)^{½} * \sigma]*e*\{-1/2 * (x-\mu)/\sigma^2\}$$

Where,

'μ' is the mean

'σ' is the standard deviation

'e' is Euler's constant (2.71)

'x' is the constant Pi (3.14)

Frequency distribution of F_2 individuals and F_3 families of pigeonpea for various traits was constructed by plotting trait values on X-axis and frequency or counts of population individuals on Y-axis.

Skewness and kurtosis

Skewness, the third degree statistics and kurtosis, the fourth degree statistics were estimated as per Snedecor and Cochran (1994) to understand the nature of distribution of mapping population for seed zinc content and various traits. The values of quantitative traits of mapping population were used to estimate co-efficient of skewness and kurtosis using 'SAS version 9.3'. Kurtosis indicates the relative number of genes controlling the

traits (Robson, 1956). Three types of kurtosis are recognized on the kurtosis value which depends on distribution curve.

If kurtosis value = 3 =Normal curve = Mesokurtic

If kurtosis value >3 =Leaping curve = Leptokurtic

If kurtosis value < 3 =Flat curve = Platykurtic

Similarly, the lack of symmetry *i.e.,* skewness was recognized based on the co-efficient of skewness values which range from -3 to +3. The type of distribution based on the skweness values are as follows.

If skewness value is zero =symmetrical distribution

If skewness value is negative =negatively skewed distribution

If skewness value is positive =positively skewed distribution

3.6.1.3 Correlation analysis

Phenotypic correlations for all the traits were calculated among themselves for entire population using Genstat version 12.

3.6.1.4 Phenotypic and genotypic coefficient of variation

The co-efficient of variability both at phenotypic and genotypic level for seed zinc content and other traits were computed by applying the formula as suggested by Burton and De Vane (1953).

1. PCV % = (P/X) x 100
2. GCV %= (G/X) x 100

Where,

P = Phenotypic standard deviation

G = Genotypic standard deviation

X = Grand mean of the character

PCV = Phenotypic coefficient of variation

GCV = Genotypic coefficient of variation

GCV and PCV were classified as suggested by Robinson *et al.,* 1949

a) Low = 0 -10 %

b) Moderate = 10- 20 %

c) High = > 20 %

3.6.1.5 Heritability

Broad sense heritability (H^2) was estimated as the ratio of genotypic variance to the total phenotypic variance according to Johnson *et al.,* 1955.

$$H^2 (\%) = (Vg / Vp) \times 100$$

Where,

H^2 = Broad sense heritability

Vg = Genotypic variance

Vp = Phenotypic variance

Heritability was categorized into following three classes as suggested by Robinson *et al.,* 1949.

a) Low = 0-30 %

b) Moderate = 30-60 %

c) High >60 %

3.6.1.6 Genetic advance

The extent of genetic advance expected through selection for each character was estimated by using the following formula given by Johnson *et al.,* 1955.

$$\text{Genetic advance (GA)} = K\, h^2\, \sigma_p$$

Where,

K = Selection differential which is equal to 2.06 at 5 per cent selection intensity

h^2_{ns} = Narrow sense heritability estimate

σ_p =Phenotypic standard deviation

Further the GA as percent of mean (GAM) was estimated by using the following formula

$$\text{GA as percent mean (GAM)} = \frac{\text{GA}}{\overline{X}} \times 100$$

Where, GA =Genetic advance estimated

$\overline{X}$ = mean of the population

The genetic advance as percent of mean was categorized as suggested by Johnson *et al.,* 1955 and the same is given below.

Low = 0-10 %

Moderate = 10.1 -20 %

High = >20 %

3.6.2 Genotypic data analysis

3.6.2.1 Diversity analysis

DARwin 5 software was used for diversity analysis of high seed zinc type and low seed zinc type genotypes. Distance based structure of UPGMA (Unweighted Pair Group Method Arithemetic average) was drawn for 30 pigeonpea genotypes based on the genotypic data of 50 SSR markers.

3.6.2.2 Construction of genetic linkage map

A molecular linkage map was constructed using MAPMAKER / EXP 3.0 with a threshold of 3.0 (Lander, 1987). The Mapmaker is an interactive computer package for constructing genetic linkage map and for mapping genes underlying complex traits. It consists of twin programmes namely MAPMAKER/EXP and MAPMAKER/QTL. MAPMAKER/EXP facilitates map construction. It performs full multipoint linkage analysis from primary data for dominant, recessive and co dominant marker in F_2, backcross, F_3, RILs and DHs. Based on Lander Green algorithm it calculates the best map for any given order of loci (Lander *et al.,* 1987). The map was developed based on 120 SSR marker loci using 188 F_2 lines.

3.6.2.3 Association of markers/QTL for seed zinc content and other traits

The DNA markers linked to seed zinc content and other physiological traits were identified by using QTL Cartographer version 2.5. QTL Cartographer is a software programme, which facilitates the mapping of genes controlling polygenic traits in any population. This mapping programme uses dynamic algorithm that allows a multitude of statistical models to be fitted and compared.

Single marker analysis

Markers linked to QTLs for different phenotypic traits were determined using single marker (Tanksley *et al*., 1982) analysis. The linkage data was imported from MAPMAKER/EXP and single marker analysis was performed using QTL cartographer Version 2.5. Association between the markers and various phenotypic traits of the F_2 individuals for various traits was established using simple linear regression model. Different traits were treated as dependent variable and the various molecular marker genotypes as independent variables.

Composite interval mapping by QTL Cartographer

To position the QTLs for seed zinc content and other physiological traits, the linkage map data was imported from MAPMAKER/EXP and the composite interval mapping (CIM) was performed using QTL Cartographer at a LOD ratio of 2.5 at 1000 permutations (Churchill and Doerge, 1994; Doerge and Rebai, 1996). Association between the various markers and the phenotypic performance of the individuals for various traits was also established using multiple regression approach. Different traits were treated as dependent variable and the various molecular marker genotypes as independent variables. The proportion of the total phenotypic variance explained by each marker associated with the QTL (R^2) was calculated as the ratio of the sum of squares examined by the marker locus to the total sum of squares at a probability level $p< 0.05$. The QTL analysis was performed to determine the QTLs associated with different physiological traits.

Experimental Results

IV RESULTS

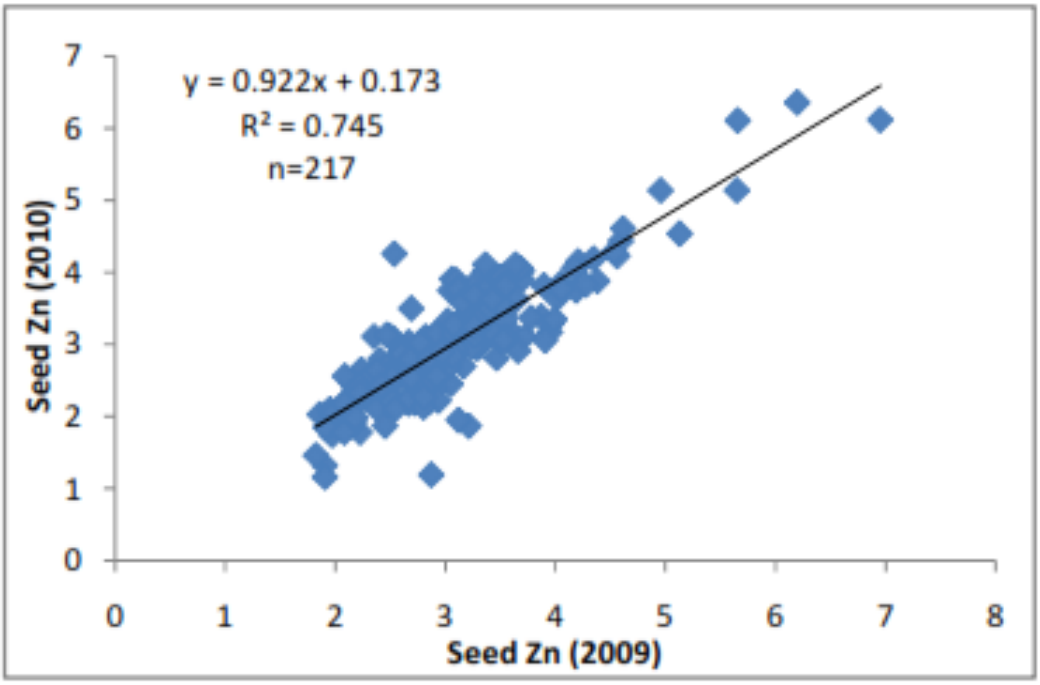
The present investigation was carried out to identify QTLs governing seed Zn content and other physiological traits in pigeonpea. A trait specific mapping population was developed by crossing genotypes contrasting for seed Zn content. This mapping population consisted of 188 F_2 segregating progeny which was extensively characterized for phenotypic and molecular diversity. The results obtained, are presented in this chapter under the following heads.

- Development of mapping population for Zn content in pigeonpea
- Phenotyping of F_2 mapping population
- Genotyping of F_2 mapping population- using genic and genomic SSR markers
- Generation of genetic linkage map and identification of QTLs governing seed Zn content by (a) Single marker analysis and (b) Composite interval mapping
- Congruence of linked markers between SMA and CIM
- Genic markers linked to seed Zn and leaf Zn content

4.1 Development of mapping population for Zn content in pigeonpea

4.1.1 Selection of contrasting zinc genotypes

Manoa (2010) had ascertained the scores for leaf and seed Zn content of 217 germplasm accessions of pigeonpea in *kharif* 2009 and *kharif* 2010. Accessions were analysed to check the consistency for two seasons and found a very strong correlation between the seed Zn content (Fig. 3a). Based on the seed Zn content, 15 genotypes with high seed Zn and 15 genotypes with low seed Zn were selected for further evaluation. Fig. 3b pitches upon consistency of seed Zn content of 30 genotypes (15 high and 15 low). The 30 genotypes were further sub grouped into four types viz., HLHS (high leaf Zn and high seed Zn), HLLS (high leaf Zn and low seed Zn), LLHS (low leaf Zn and high seed Zn) and LLLS (low leaf Zn and low seed Zn) based on the Zn content in leaf and seed (Fig. 4 and Table 6**).** 10 contrasting genotypes were further selected for crossing. Five genotypes with high seed Zn content and high leaf Zn content were



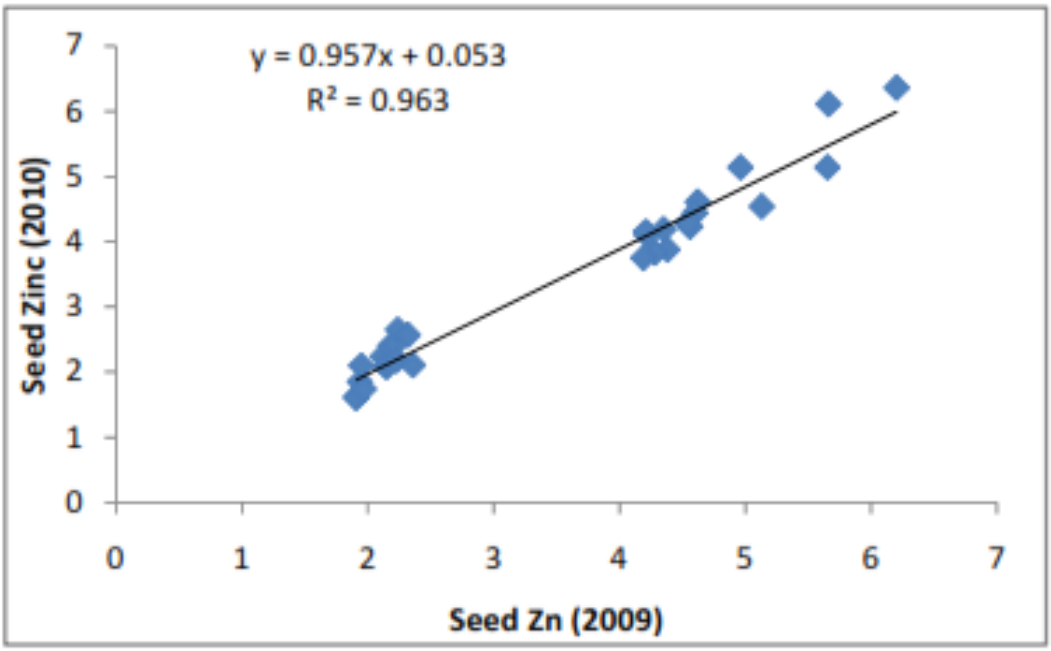


Fig. 3: Correlation between seed Zn content across two seasons

a: Seed Zn content of 217 germplasm accessions

b: Seed Zn content among 15 high and 15 low Zn genotypes

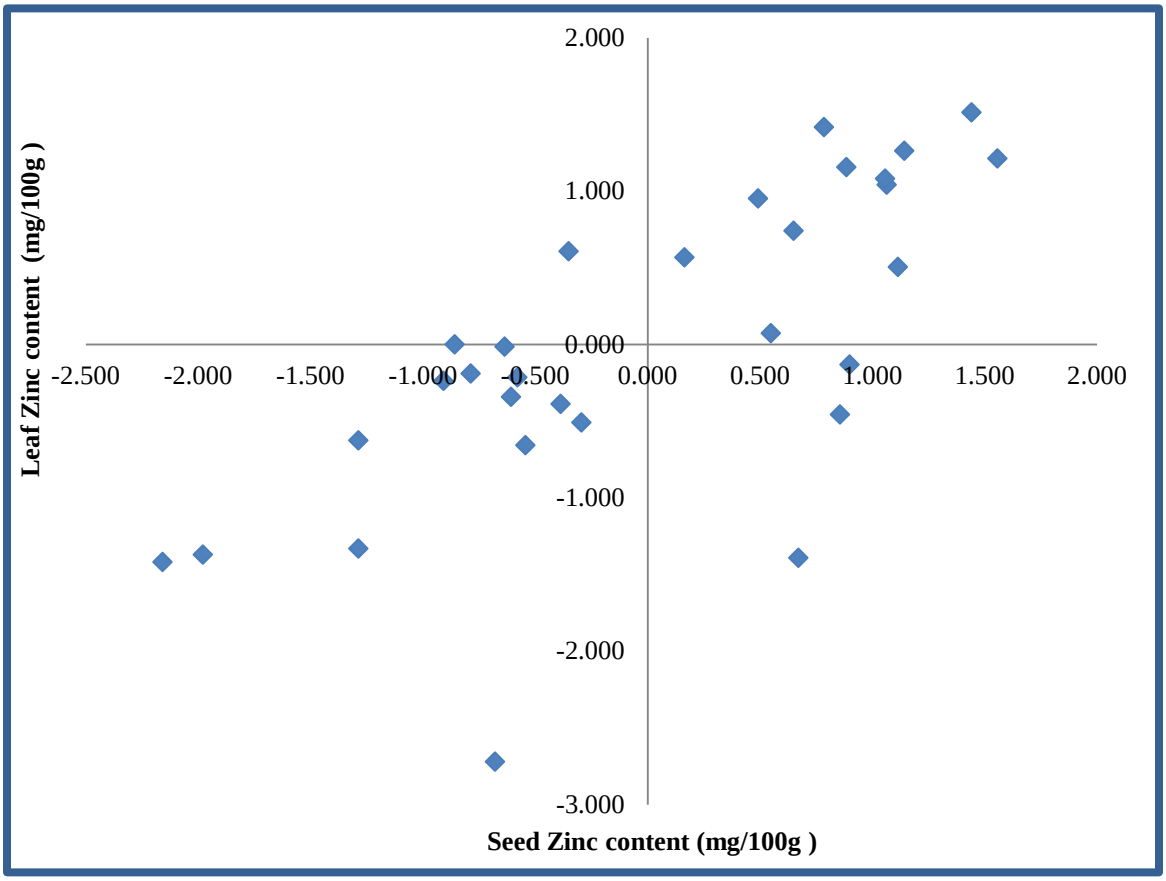


Fig. 4: Z-distribution of seed Zn content versus leaf Zn content

selected from HLHS group and five genotypes having low seed Zn content and low leaf Zn content were selected from LLLS group. The list of contrasting genotypes selected for crossing to generate F_1 crosses is presented in Table 5 of materials and methods.

Table 6: List of genotypes contrast for seed Zn and leaf Zn content

Group	Zn status	Genotypes	Mean Seed Zn (mg/100 g)	Mean Leaf Zn (mg/100 g)
I	**HLHS**	ICP6974, ICP7182, ICP6992, ICP7119, ICP6997, ICP2484, ICP6370, ICP14954, ICP2626, ICP7118, ICP7035, ICP6443, ICP7314	4.64	5.82
II	**HLLS**	ICP8508	2.21	5.75
III	**LLHS**	ICP2059,ICP12410,ICP14421, ICP7623	3.83	4.16
IV	**LLLS**	ICP10654, ICP9336, ICP8513, ICP10960, ICP8863, ICP11946, ICP8602, ICP7221, ICP9691, ICP9655, ICP11543, ICP8516	2.07	3.71

4.1.1 Diversity analysis among the contrasting genotypes

Efficient use of plant genetic resources requires thorough assessment of the genetic variation they comprise. Genetic variation can be measured at two levels: one is phenotype - the combination of individual traits resulting from a genotype and its interacting environment; second being genotype- the particular genetic make-up of an organism (De Vicente and Fulton., 2003). Thirty contrasting genotypes were selected for accessing molecular diversity using 50 SSR markers. PCR profile of AHSSR4, AHSSR305 and AHSSR265 among 30 pigeonpea genotypes is given in plate 7.

These 50 AHSSR markers amplified a total of 192 SSR alleles which were scored from 50 AHSSR markers across 30 accessions. The frequency of alleles amplified ranged from 2 (ASSR1, ASSR108, AHSSR128, ASSR129, ASSR109 and ASSR23) to 8 (AHSSR237) with a mean of 3.86 (Appendix 1). Heterozygosity was observed to some extent as expected in an often cross pollinated crop with an average of 0.13 and the maximum was observed (0.53) for AHSSR21. Polymorphic information content (PIC)

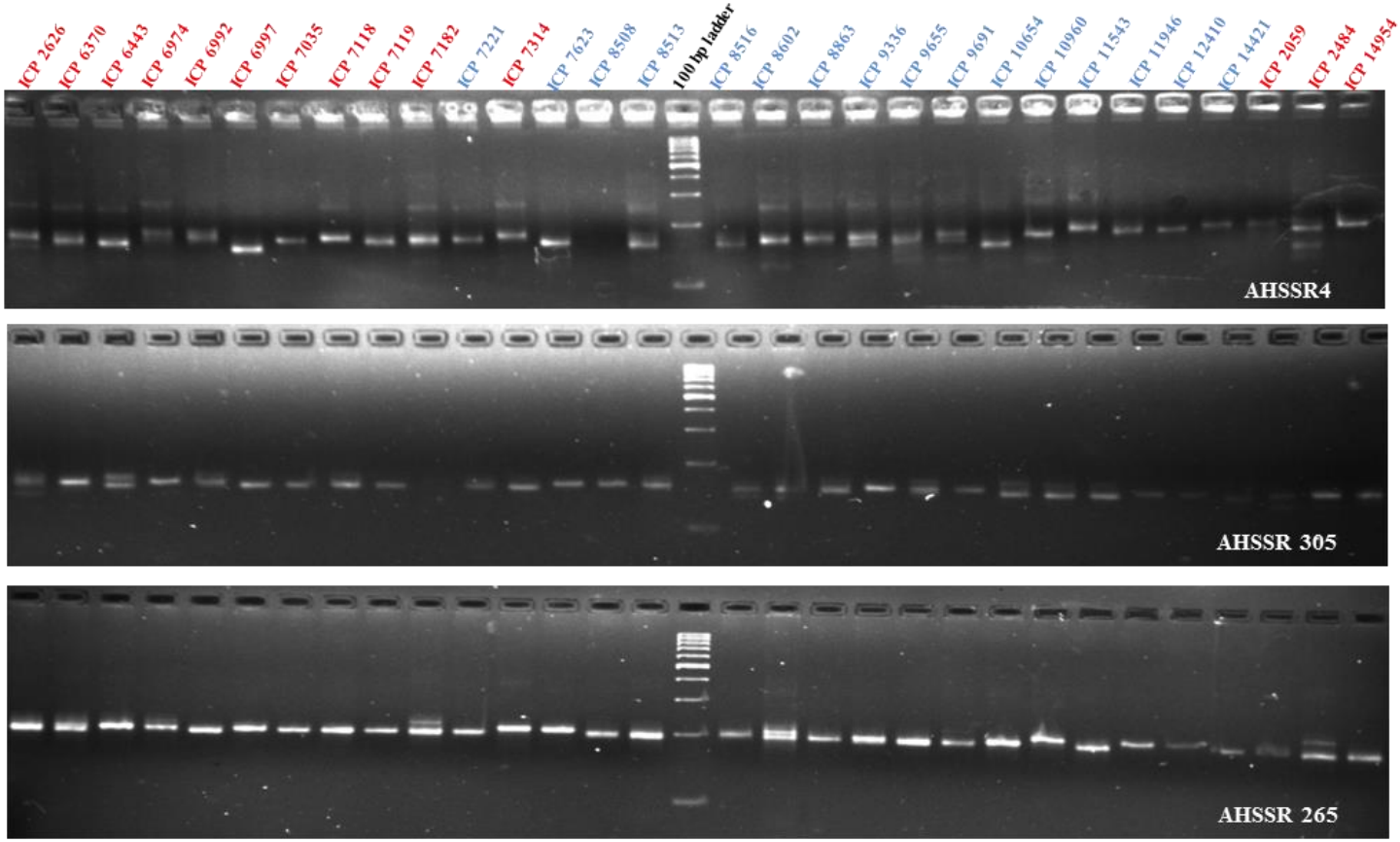


Plate 7: Representative agarose gel showing PCR amplicons of genomic SSR markers for 30 genotypes of pigeonpea

Note: Accession names mentioned in red are the high Zn types and those mentioned in blue are the low Zn types. The top panel is the PCR amplicons for AHSSR4, middle one is for AHSSR305 and bottom panel is for AHSSR265.

was also looked for, which is a measure of the probability of two randomly chosen alleles from population are distinctly different. The PIC value ranged between 0.02 (ASSR108) to 0.76 (AHSSR237) with a mean of 0.39. Around 50 % of the markers were highly informative with PIC values more than 0.50. While 30 % markers were reasonably informative (0.5>PIC>0.25) and rest of the markers had low PIC values of <0.25. Assessment of the molecular diversity is crucial in population genetic studies. Diversity analysis of 30 genotypes using NJ/UPGMA algorithm, grouped them into a single major cluster (Fig. 5). Though there were two sub clusters based on the bootstrap values (range < 80) the accessions had to be considered as single clade. The subcluster-I comprised the maximum of 16 accessions. Among the 30 accessions, ICP7182 and ICP7221 (0.2) were the closest while ICP9336 and ICP14954 (0.86) showed the maximum genetic distance based on UPGMA (DARwin V 5.0). The five high Zn and five low Zn parental genotypes were identified from among the germplasm and used for crossing. These were differentially sub-clustered except for the genotypes ICP6992 and ICP8513 (Fig. 5). The highest genetic distance of 0.73 was observed between the parental lines ICP6443 and ICP10960 (Appendix 2).

4.1.2 Crossing of the contrasts

The five high seed Zn genotypes and five low seed Zn genotypes which were contrasting for most of the physiological parameters and also the whole plant Zn status were selected to make five straight crosses to obtain F_1 seeds. Table 7 gives the list of seeds obtained with the respective crosses. The cross ICP6443 and ICP10960 generated the maximum number of F_1 seeds. Further, BRG1, a high yielding cultivar which had a moderate seed Zn content, though produced less number of seeds, the true F_1 could be used for developing high seed Zn lines in the background of BRG1. This cross form useful pre-breeding lines for trait introgression.

4.1.3 Parental polymorphism studies for selection of a single cross

Among the five crosses made, seeds for three crosses were obtained (Table 7). The crossing of ICP14954 X ICP8863 and ICP6992 X ICP8513 was unsuccessful. In order to develop a F_2 mapping population for QTL study, a single cross had to be selected.

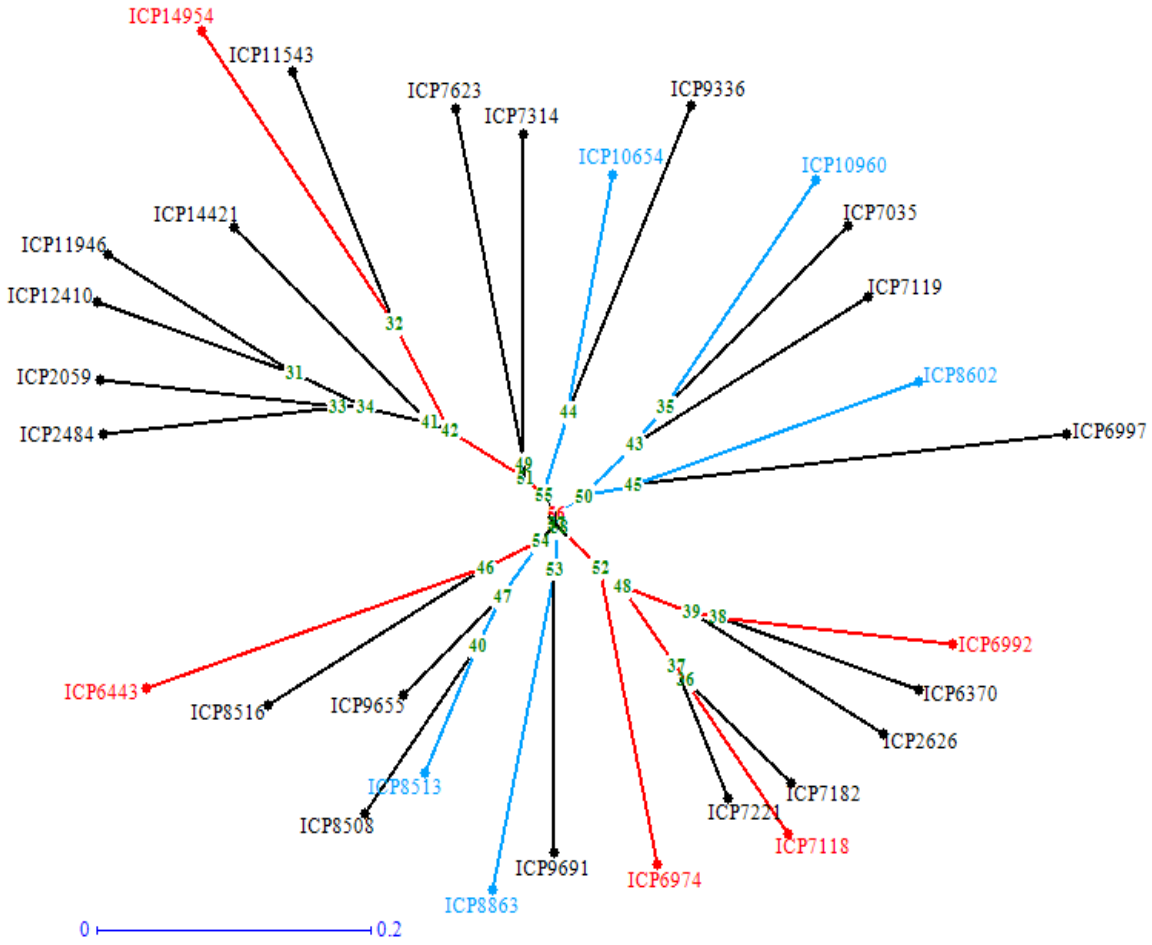


Fig. 5: Distance based structure of UPGMA (Unweighted pair group method arithmetic average) phenogram of 30 germplasm accessions based on genotypic data using DARwin5

Note: Red colour depicts the high Zn content genotypes
Blue colour depicts the low Zn content genotypes

Therefore parental lines were subjected for polymorphism studies using an additional 71 genic SSR markers to select the cross which showed maximum parental polymorphism was selected for advancement. Plate 8 depicts the screening of parental lines on agarose gel for polymorphism detection. A total of 22 markers were polymorphic between ICP6443 X ICP10960 (Appendix 3), followed by 15 in BRG1 X ICP6443 (Appendix 4), eight in ICP7118 X ICP10654 and only four between ICP6974 X ICP8602. Fig. 6 gives the percent polymorphism obtained between the different parental combinations. The crosses having highest parental polymorphism ICP6443 X ICP10960 and backcross population cross BRG1 X ICP6443 were advanced to next generations to develop mapping population.

Table 7: Number of effective crosses between contrasts pigeonpea genotypes

Sl. No.	Crosses	No of seeds (F_1)
1	ICP6443 X ICP10960	263
2	ICP7118 X ICP10654	113
3	ICP6974 X ICP8602	20
4	ICP14954 X ICP8863	0
5	ICP6992 X ICP8513	0
6	BRG1 X ICP6443	32

4.1.4 Advancement of the selected cross to develop mapping population

A total of 205 F_1 seedlings out of 236 seeds of the cross ICP6443 X ICP10960 survived (Plate 9). In order to select the true F_1s, the 22 polymorphic SSR markers were screened in the 205 F_1 plants. Plate 10a depicts a representative agarose gel showing the amplicons obtained in F_1 plants. A total of six true F_1s were selected and selfed to produce F_2 seeds and were sown in next *kharif* season to develop F_2 mapping population of 188 individuals (Plate 11).

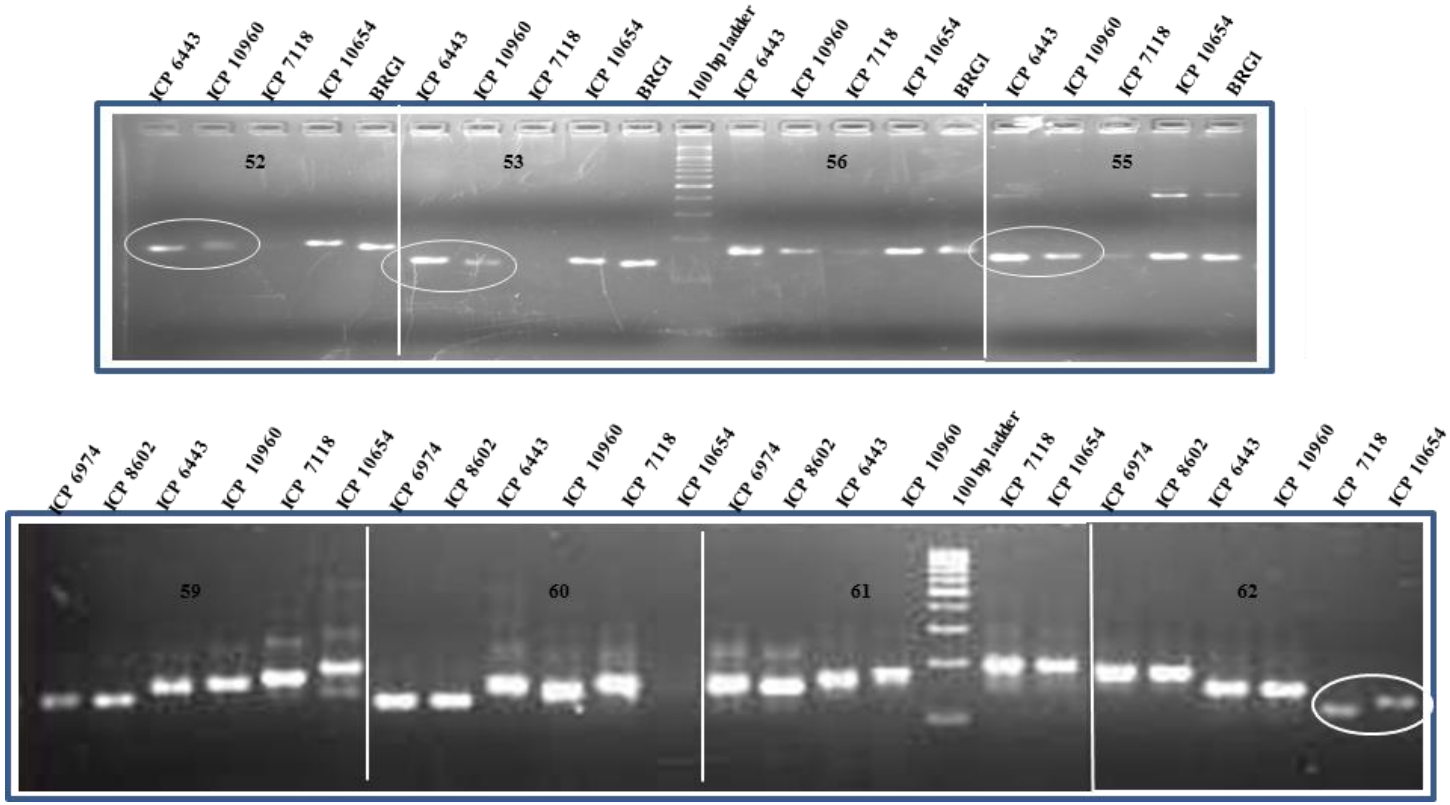


Plate 8: Representative gel showing PCR amplified product of genic SSR markers in the genomic DNA of parental lines

Note: The numeric numbers on the gel picture are the ASSR series of genic SSR markers. ICP6443, ICP7118 and ICP 6974 are the high seed Zn genotypes, while ICP10960, ICP10654 and ICP8602 are the low Zn genotypes. BRG1 is an elite variety with moderate Zn content.

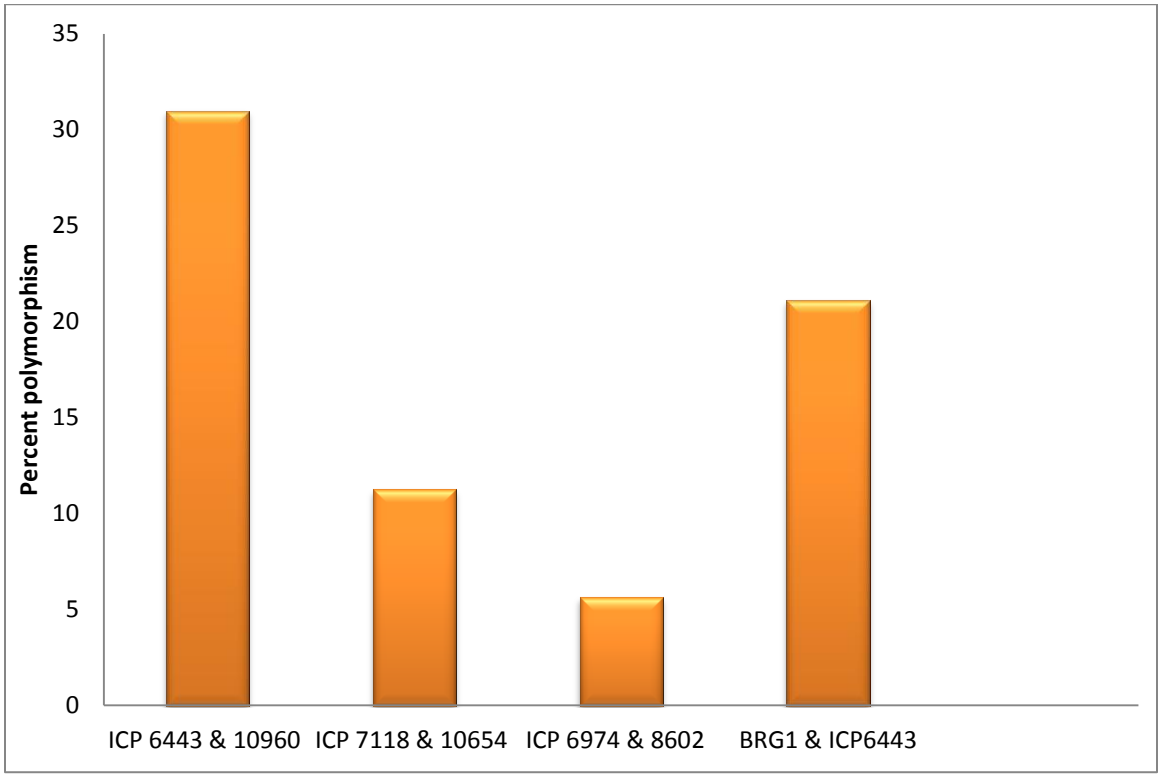


Fig. 6: Number of polymorphic genic SSR markers between the parental lines, expressed in percent polymorphism

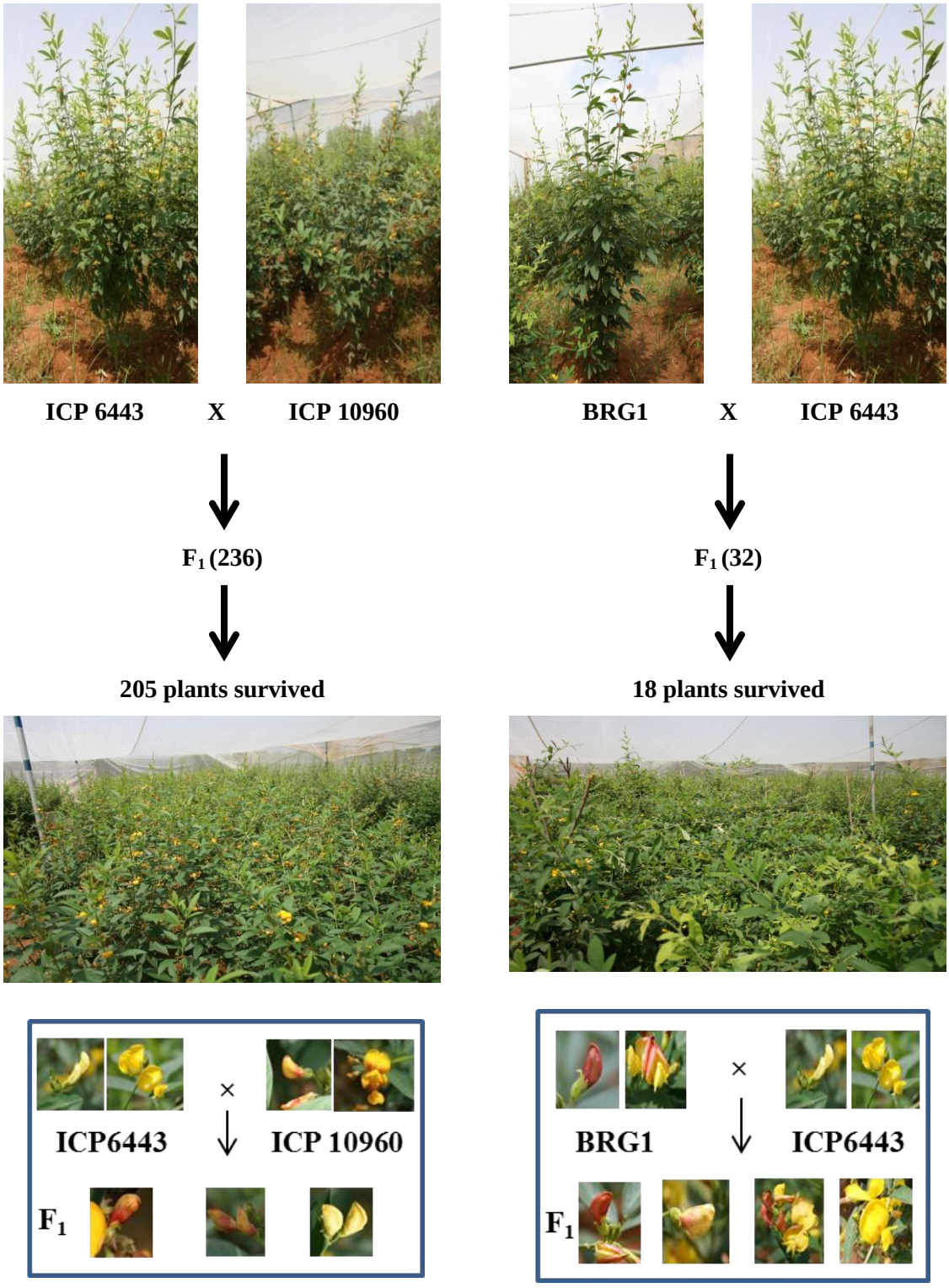


Plate 9: Plant architecture and flower pigmentation in ICP6443, ICP 10960, BRG1 and F_1 plants

ICP 6443, high seed zinc type. ICP10960, low seed Zinc type and BRG1:elite variety with moderate seed zinc content

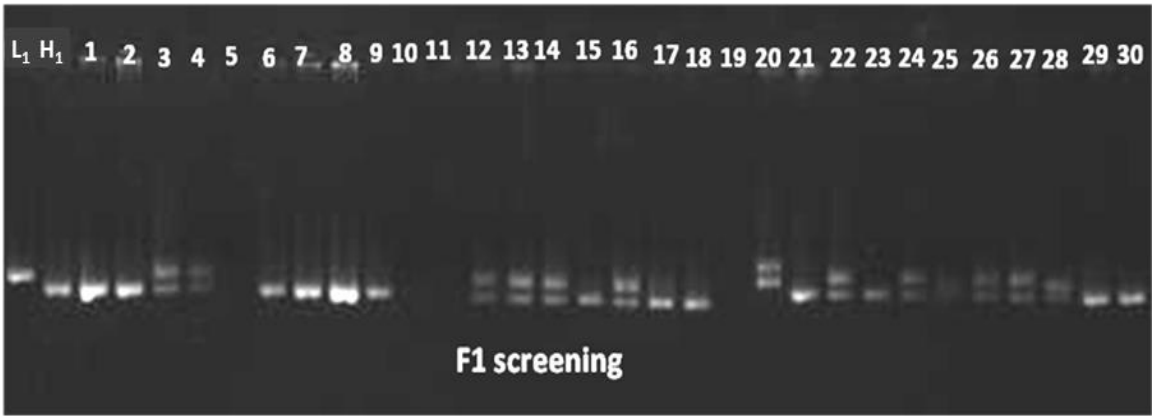


Plate 10a: Representative ethydium bromide gel showing expected PCR amplified product of polymorphic markers in the genomic DNA of F_1 plants of H_1 X L_1 cross

Note: H1 and L1 are ICP6443 and ICP10960 parental lines respectively. While 1 to 30 are the F_1 plants derived from H1 X L1 cross

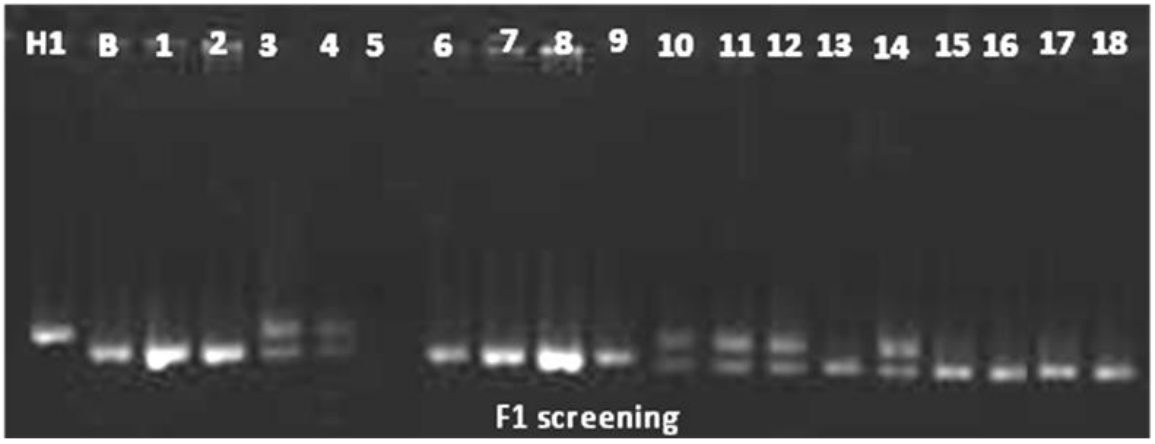


Plate 10b: Representative ethydium bromide gel showing expected PCR amplified product of polymorphic markers in the genomic DNA of F_1 plants of BRG1 X H_1 cross

Note: BRG1 and H1 are the elite variety and ICP6443 parental lines respectively. While 1 to 18 are the F_1 plants derived from BRG1 X H1 cross

Plate 11: F_2 mapping population derived from ICP6443 X ICP10960 in the Field at GKVK, Bangalore

There were 32 F_1 seeds derived from the cross BRG1 X ICP6443 and when sown 18 plants survived (Plate 9). The markers polymorphic between BRG1 X ICP6443 were screened among the 18 plants (Plate 10b) and four true F_1s were identified. These four true F1 were back crossed with BRG1 during *kharif* 2012 to obtain BC_1F_1 seeds. The BC_1F_1 seeds were sown in *kharif* 2013 and self-pollinated to produce BC_1F_2 seeds. In this study a F_2 mapping population (ICP6443 X ICP10960) and a backcross population (BRG1 X ICP6443) were being developed.

For the fulfilment of the subsequent objectives, the F_2 mapping population derived from the cross ICP6443 X ICP10960 was being phenotypically characterized and genotyped to discover QTL for seed Zn content.

4.2 Phenotyping of the F_2 mapping population

4.2.1 Performance of parents and F_2 individuals for seed Zn content and various traits

Mean performance of the parents (ICP6443 and ICP10960) and F_2 individuals for the measured traits under field conditions are given in Table 8. The parental lines differed significantly for most of the traits like seed zinc content, leaf zinc content, plant height, seed yield etc. The parent ICP6443 had higher seed zinc content, leaf zinc content and in addition it also maintained higher seed yield. Where as, ICP10960 stood greater in leaf iron content, SPAD chlorophyll meter reading, pod length and pod width, but these traits were lower for the genotype ICP6443. This suggests the availability of larger genetic variability of traits in parents, which is a preliminary requisite to develop mapping population. For convenient understanding of results, the measured parameters are presented under four sub-headings.

4.2.1.1 Zinc, iron and phytic Acid estimation

The parents ICP6443 and ICP10960 differed significantly for leaf Zn content, seed Zn content, leaf Fe content and Phytic acid content. The leaf Zn content was 7.31 mg/100 g for ICP6443 and 4.92 mg/100 g for ICP10960 and it varied from 2.05 mg/100 g to 10.4 mg/100 g with a mean of 4.72 mg/100 g. The seed Zn also showed significant

variability ranging from 1.2 mg/100 g to 9.55 mg/100 g with the mean of 3.94 mg/100 g in the population. The parents ICP6443 and ICP10960 had 5.93 mg/100 g and 2.03 mg/100 g seed Zn content respectively. Variability for leaf and seed Fe content was also observed in the population, where leaf Fe ranged from 5.51 mg/100 g to 15.78 mg/100 g with a mean of 10.44 mg/100 g and seed Fe ranged from 4.02 mg/100 g to 13.18 mg/100 g with a mean of 9.91 mg/100 g. In addition to Zn and Fe content, Phytic acid content in seeds also varied significantly from 563.39 mg/100 g to 956.25 mg/100 g with a mean of 781.42 mg/100 g (Table 8).

Table 8: Performance of parents (ICP 6443 and ICP 10960) and F_2 individuals for seed Zn and other morpho-physiological parameters under field conditions, at GKVK, Bengaluru

Parameter	P1	P2	F_2 Progenies			
	ICP 6443	ICP 10960	Min	Max	Mean	SD
Leaf Zn (mg/100 g)	7.31	4.92	2.05	10.4	4.72	1.55
Seed Zn (mg/100 g)*	5.93	2.03	1.2	9.55	3.94	1.47
Leaf Fe(mg/100 g)	11.32	13.14	5.51	15.78	10.44	1.86
Seed Fe (mg/100 g)*	10.97	10.01	4.02	13.18	9.91	1.63
Phytic Acid (mg/100 g)*	791.77	845.02	563.39	956.25	781.42	76.45
Plant height (cm)	244	178	62	243	166	36.39
Stem girth (cm)	8.2	9.4	2.9	12.9	6.6	1.58
Stem weight(g)	176	138	15	795	173.17	135.82
No. of branches	21	10	2	19	9.57	3.04
SCMR	56.25	68.43	50.76	70.7	60.21	3.91
Leaf length (cm)	9.77	6.7	5.5	14.3	8.75	1.35
Leaf width (cm)	3.23	1.9	1.97	5.23	3.25	0.61
Specific leaf area	154.72	144.12	74.49	278	145.39	33.09
Pod length (cm)*	4.47	6.27	3.73	8.43	6.04	0.77
Pod width (cm)*	0.97	1.30	0.83	2.3	1.28	0.18
No. of seeds/pod*	4	5	1.5	5.5	3.74	0.73
100 seed weight (g)*	11.8	9.4	9	18.41	12.7	1.83
Shelling percentage*	0.59	0.56	0.34	0.64	0.52	0.07
Seed yield (g)*	70.34	59.96	18.43	180.31	49.07	28.12

Note: *Parameters were measured in $F_{2:3}$ seeds and all other parameters measured in respective F_2 individuals

All the above mentioned traits were normally distributed around the mean, while leaf Zn and seed Fe approximately fit normal distribution and showed continuous variation indicating that the traits are controlled by polygenes. The leaf Zn content and seed Zn were positively skewed indicating the predominance of transgressive segregants towards the superior parent for these traits in the population. The sharper kurtosis peak for seed Zn content indicated that more segregants had values much higher than the mean of the population and also better than the high seed Zn parent (Fig. 7).

4.2.1.2 Shoot traits

Several parameters associated with shoot growth were recorded and significant variability was observed among the population (Table 8). The plant height was observed high in ICP6443 (244 cm) then the recessive parent ICP10960 (178 cm). In F_2 population plant height varied from 62 cm to 243 cm with a mean of 166 cm. The stem girth also varied significantly with the mean of 6.6 cm from 12.9 cm to 2.9 cm. In addition, the stem weight and number of branches per plant varied from 15 g to 795 g and 2 $plant^{-1}$ to 19 $plant^{-1}$ respectively, showing significant variations. The plant height showed relatively symmetric distribution. However, the distribution was platykurtic indicated by negative kurtosis (-0.3). Stem weight exhibited a significant positively skewed distribution (1.81) with a stronger peak (kurtosis =4.09) above the mean indicating maximum number of transgressive segregants having the stem weight more than ICP6443, the better parent (Fig. 8).

4.2.1.3 Leaf traits

Parameters like SPAD chlorophyll meter readings, leaf length, leaf width and SLA were being measured in parents and F_2 progenies (Table 8). The parent ICP6443 had higher leaf length, leaf width and SLA compared to ICP10960, where as ICP10960 had higher SCMR values. In mapping population SCMR varied from 50.76 to 70.7 with a mean of 60.21. Leaf length and leaf width showed a significant variation, where leaf length varied from 5.5 cm to 14.3 cm with a mean of 8.75 cm and leaf width varied from 1.97 cm to 5.23 cm with a mean value of 3.25 cm. SLA also ranged from74.49 cm^2g^{-1} to

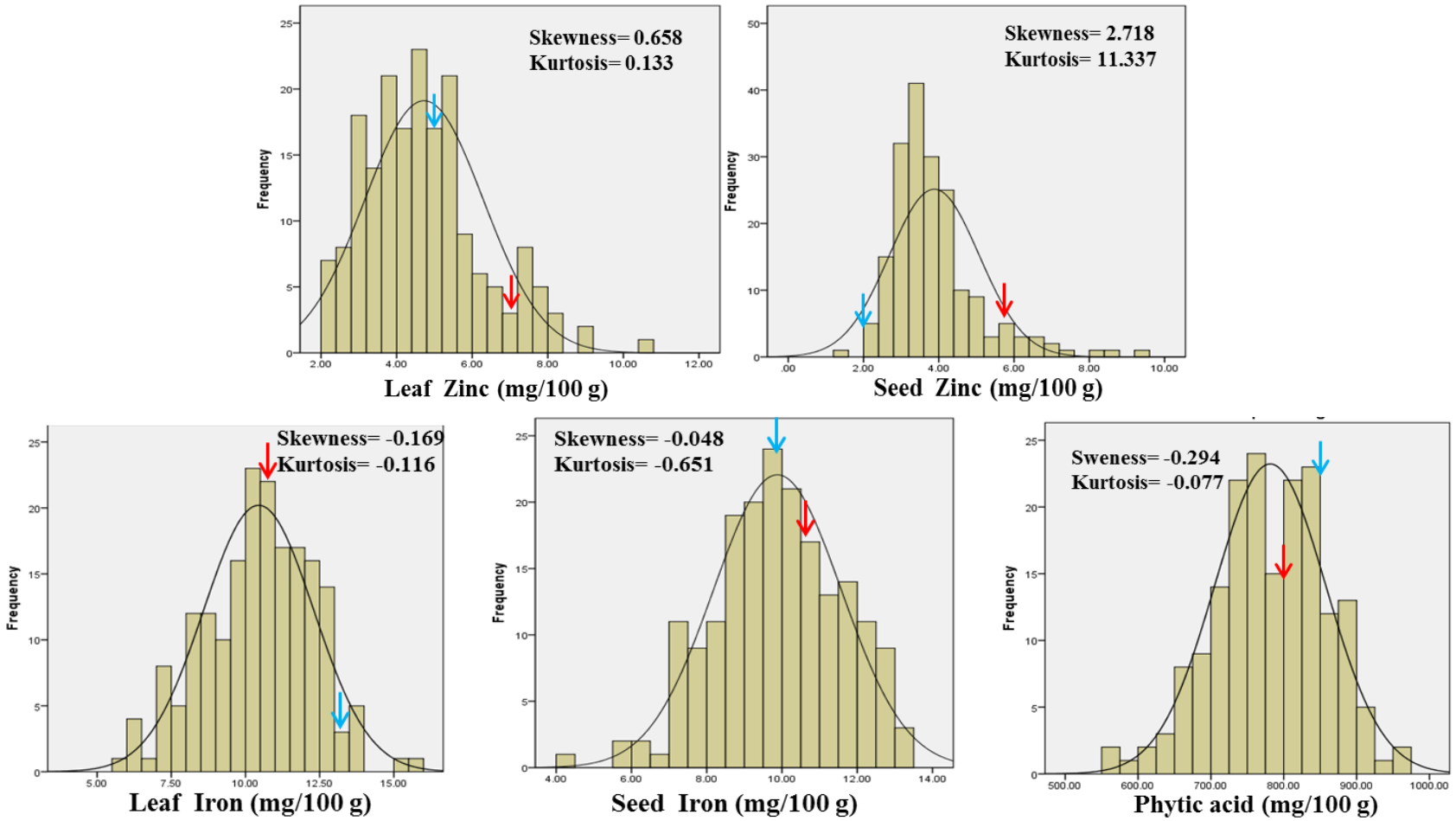


Fig. 7: Frequency distribution of F_2 mapping population derived from the cross ICP6443 and ICP10960 for Zn, Fe and phytic acid

The seed Zn content, seed Fe content and phytic acid content were estimated in the $F_{2:3}$ seeds of respective F_2 individuals. Arrows indicate the parental lines, i.e., red arrow: ICP6443 and blue arrow : ICP10960

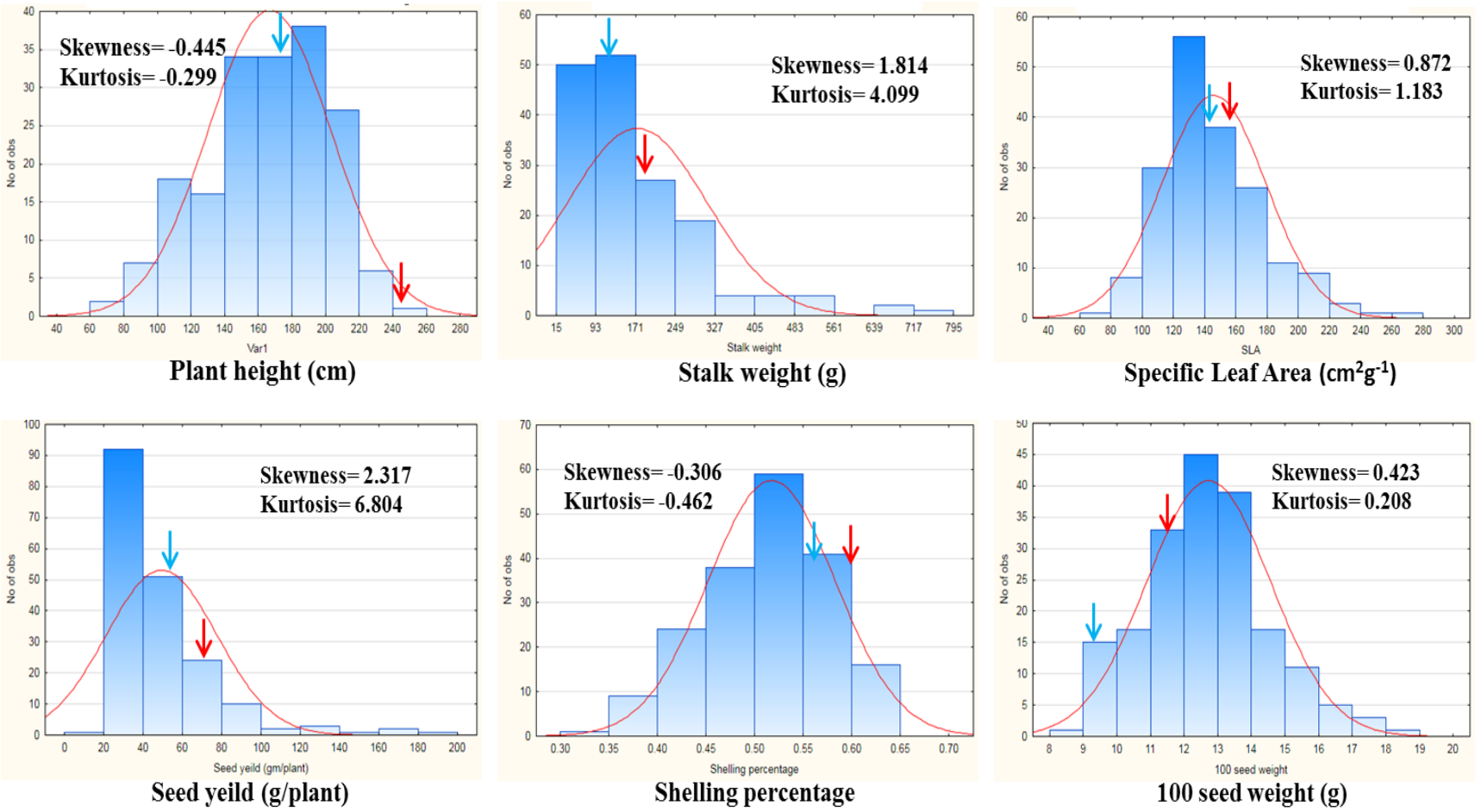


Fig. 8: Frequency distribution of F_2 mapping population derived from the cross ICP6443 and ICP10960 for growth and yield characters

Arrows indicate the parental lines, i.e., red arrow: ICP6443 and blue arrow : ICP10960

278 cm^2g^{-1} and was relatively positively skewed with a platykurtic distribution indicated by positive kurtosis of 1.18 (Fig. 8).

4.2.1.4 Yield related parameters

Pod length, pod width and number of seeds per pod were higher in low seed Zn parent ICP10960, however the test weight, seed yield and shelling percentage were higher in the high seed Zn parent ICP6443 indicating that the ICP6443 had no yield penalty for having higher seed Zn content which is a desirable character. There was a significant variation for all these parameters in $F_{2:3}$ seeds of the respective F_2 individuals. The 100 seed weight and seed yield per plant parameters were positively skewed, where seed yield per plant showed a significantly stronger peak (kurtosis =6.8) above the mean indicating maximum number of transgressive segregants having seed yield more than ICP6443, the better parent (Fig. 8).

4.2.2 Estimation of genetic parameters

The phenotypic characterization of the mapping population revealed that significant variability exists in several parameters distributed normally around the mean. However it is necessary to assess the heritability of the targeted traits before attempting to exploit these traits in breeding programme. An effective phenotypic selection would largely depend on genetic control of the trait. Therefore to know the extent of genotype and environmental effect on phenotype, the genetic parameters like genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), broad sense heritability (h^2) and genetic advance (GA) were estimated for leaf and seed Zn content, leaf and seed Fe content and phytic acid content.

The estimation of genotypic coefficient of variation (GCV) for all the above traits was smaller compared to phenotypic coefficient of variation (PCV). Table 9 indicates the presence of a significant environmental (E) influence on the expression of the traits. GCV for the traits ranged from 8.16 % for phytic acid content to 35.6 % for seed Zn content with the mean of 21.55 % and was found to be highest for seed Zn content followed by leaf Zn, leaf Fe, seed Fe and phytic acid (Table 9). Similar trend was observed in

phenotypic coefficient of variation (PCV) also. Results indicate that, these traits might be governed by additive gene action.

Estimation of heritable variation for all traits provides initial indication for selection of line on phenotypic performance. The heritability estimated ranged from 71 % to 95 % for phytic acid content and seed Fe content respectively. The genetic advance as percent mean ranged from 18.15 in phytic acid content to 70.67 in seed Zn content. Study exhibited higher heritability (>90 %) for leaf Zn content, seed Zn content, leaf Fe content and seed Fe content, while phytic acid content showed less than 90% heritability. High GCV coupled with high heritability is indicative of high genetic gain while selection, similar results were observed for leaf Zn content and seed Zn content. Higher value of h^2 and moderate GA was observed for Phytic acid content. While the vice versa i.e., lower estimate of h^2 and high GA was not observed for any of the parameters in the study. All these above traits showed slightly higher PCV than GCV (Table 9), it confirms the existence of G X E interaction. Though Phytic acid showed high h^2 (>60 %), its GA is moderate. This could be because of complexity of trait and influence of environment on phytic acid biosynthesis. Leaf Zn, seed Zn, leaf Fe and seed Fe had higher h^2 and high GA. Over all, targeted traits which were phenotyped for plants grown under field condition provides indicative information regarding GCV, h^2 and GA and these indices will be useful for selection of promising lines accumulating higher Zn.

Table 9: Estimation of genetic variability in $F_{2:3}$ seeds for seed and leaf Zn content; seed and leaf Fe content and seed phytic acid content under field conditions at GKVK, Bengaluru

	Leaf Zn	Seed Zn	Leaf Fe	Seed Fe	Phytic acid
GCV %	31.19	35.6	16.96	15.84	8.16
PCV %	32.48	36.93	17.55	16.22	9.68
h^2	0.92	0.92	0.93	0.95	0.71
GA	61.69	70.67	43.26	31.85	18.15
Skewness	0.658	2.718	-0.169	-0.048	-0.294
Kurtosis	0.133	11.337	-0.116	-0.651	-0.077

4.2.3 Correlation of seed zinc content with other traits

A total of 136 pair wise combinations were formed among 17 traits of which 35 showed significant ($-0.14 \leq r \geq +0.14$) correlation (Table 10). Seed Zn trait was positively correlated with 100 seed weight and leaf Zn content only. Out of 70 correlation points between nutrient content parameter (Leaf Zn, seed Zn, leaf Fe, seed Fe and Phytic acid content) and morpho-physiological parameters, six pairs of combinations were significant. This suggests that nutrient content parameter is very specific trait and it has to be exploited carefully to obtain lines with higher nutrient content. As seed Zn content showed positive association with 100 seed weight and leaf Zn content. Thus among various traits measured, seed weight and leaf Zn content can be employed in earlier crop stage for selection of genotype with higher seed Zn.

Out of 35 significant correlations, two pairs explained biologically meaningful ($-0.71 \leq r \geq +0.71$) correlation. The two pairs with biological significant correlation were between leaf length and leaf width and the other between pod length and pod width. None of these traits were found to be directly correlated with leaf or seed Zn but were correlated with 100 seed weight, the trait which in turn was positively correlated with leaf and seed Zn.

4.2.4 Correlation between F_2 and F_3 for some physiological parameters

F_3 family plants were phenotyped for various morpho-physiological parameters. Table 11 gives the descriptive statistics, skewness and kurtosis of the parameters measured in the F_3 lines. Like F_2, the F_3 population also significantly varied for all these parameters. A consistency was noticed between the two populations with respect to the morpho-physiological parameters which are represented in the Fig. 9 & 10. Plant height, stem weight, stem girth and seed yield of F_3 followed same trend in population distribution as in F_2.

4.2.5 Classification of F_2 individuals based on the seed zinc content of $F_{2:3}$ seeds

Based on the seed zinc content of F_2 derived F_3 seeds, 15 each of high and low seed Zn transgressive segregants were selected. In these lines the relative content of

Table 10: Pearson's correlation co-efficients of F_2 individuals for seed Zn and other morpho-physiological parameters under field conditions at GKVK, Bengaluru

	SW	SY	PH	NB	SG	SW	LL	LW	SCMR	SLA	PL	PW	LZn	SZn	LFe	SFe	PA
100 seed wt +	1																
Seed yld +	0.09	1															
Plant ht	0.08	0.14	1														
branches	0.09	-0.01	0.17*	1													
Stem girth	0.18*	0.17*	0.42**	0.44**	1												
Stem wt	0.14	0.01	0.38**	0.39**	0.69**	1											
Leaf length	0.17*	0.09	0.42**	0.03	0.48**	0.36**	1										
Leaf width	0.28**	0.06	0.37**	0.04	0.51**	0.37**	0.87**	1									
SCMR	0.04	-0.05	-0.12	0.01	0.08	0.01	0.17*	0.16*	1								
SLA	0.19*	0.03	0.22**	0.09	0.27**	0.23**	0.19**	0.38**	-0.09	1							
Pod length +	0.14	0.10	0.05	0.05	0.18*	-0.02	0.02	-0.01	0.07	0.09	1						
Pod width +	0.21**	0.10	0.04	0.13	0.21**	0.03	0.01	0.02	0.12	0.08	0.78**	1					
Leaf zinc	0.15*	-0.07	0.01	0.04	0.03	-0.01	-0.04	0.01	0.03	0.11	0.14	0.06	1				
Seed zinc +	0.18*	0.10	0.02	0.06	0.01	-0.06	-0.01	0.04	-0.01	0.06	0.11	0.06	0.62**	1			
Leaf iron	-0.01	-0.02	-0.04	-0.01	-0.13	-0.03	-0.08	-0.11	-0.03	0.01	-0.04	-0.05	-0.07	0.02	1		
Seed iron +	0.06	-0.05	0.01	-0.17*	-0.14	-0.09	-0.02	0.03	0.07	0.08	0.02	0.04	0.01	0.02	0.41**	1	
Phytic acid +	0.21**	0.01	0.08	0.08	0.14	0.14	0.11	0.12	-0.01	0.03	-0.01	-0.04	-0.06	-0.05	0.05	-0.02	1

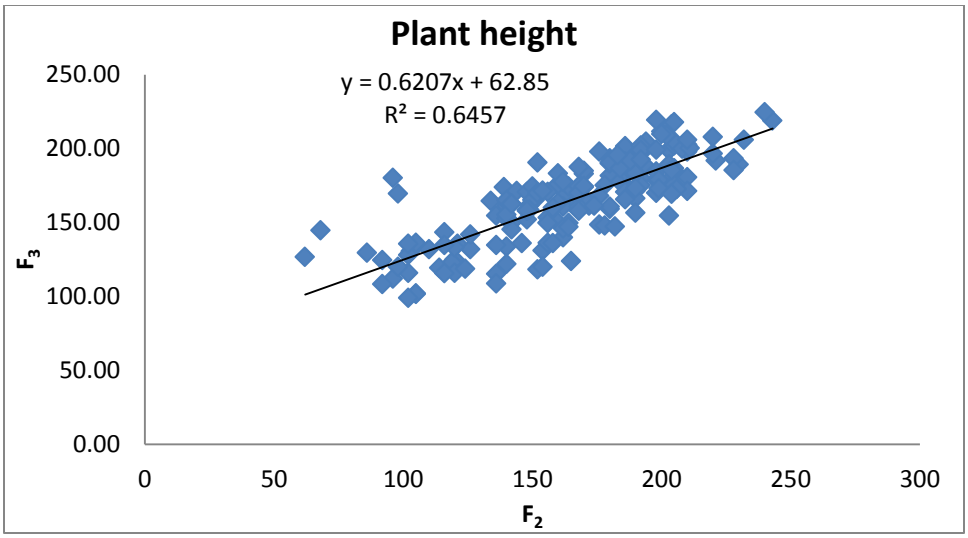
Note: +Parameters were measured in $F_{2:3}$ seeds and all other parameters measured in respective F_2 individuals. * and ** significant P <0.05 and P <0.01 respectively. Abbreviations of the traits expanded below. The traits units are given in methodology section.

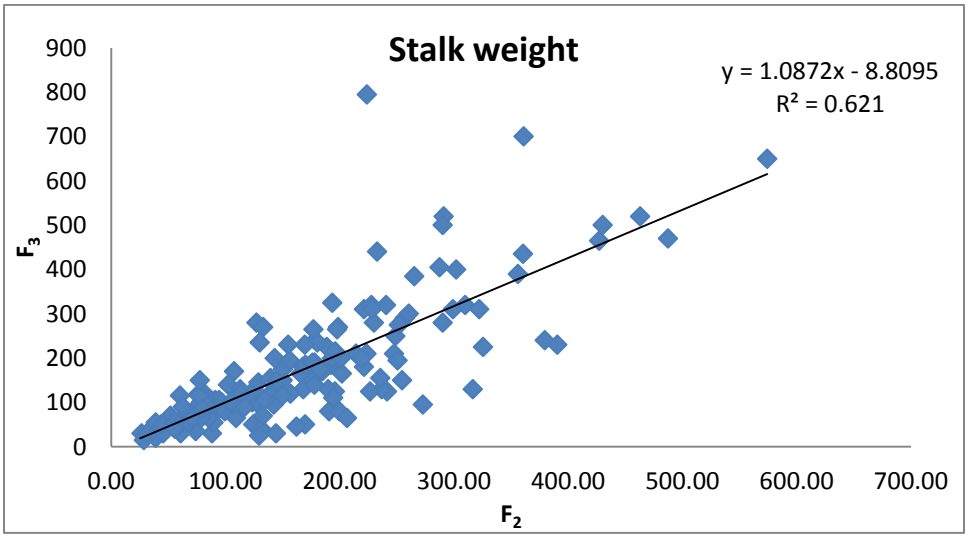
SW: 100 seed weight; SY:Seed yeild; PH: Plant height; NB: No. of branches; SG: Stem Girth; LL: Leaf Length; LW: Leaf width; SCMR:SPAD chlorophyll Meter reading;

SLA: Specific Leaf Area; PL:Pod Length; PW: Pod Width; LZn: Leaf zinc; SZn: Seed zinc; LFe:Leaf iron; SFe:Seed iron; PA: Phytic Acid

Table 11: Performance of F_3 derived from the cross IC6443 X ICP10960 for various morpho-physiological parameters

n=190	Lowest	Highest	Kurtosis	Skewness	Range	Mean	Std. Dev	Std Error	C. V.
plant height (cm)	90.31	233.15	-0.59	-0.19	142.84	165.52	31.59	2.29	19.09
Stem weight (g)	11.73	610.39	2.250	1.19	598.67	165.05	98.69	7.16	59.79
No. of branches	2.91	16.08	0.09	0.14	13.17	9.30	2.39	0.17	25.75
Pod length (cm)	4.07	7.37	-0.29	-0.48	3.3	6.06	0.76	0.056	12.48
Pod width (cm)	0.97	1.44	0.01	-0.14	0.48	1.27	0.08	0.01	6.59
Stem girth (cm)	2.77	12.22	0.37	0.33	9.45	6.43	1.57	0.11	24.41
SCMR	49.43	68.55	-0.49	0.013	19.12	59.72	4.07	0.29	6.81
Seed yield (g/plant)	11.18	149.52	0.92	0.92	138.34	55.51	25.72	1.87	46.35





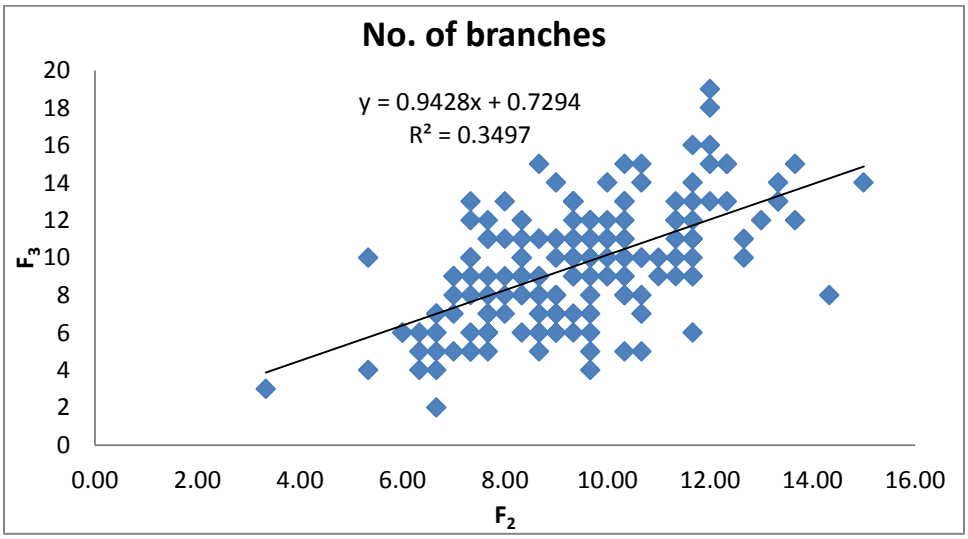
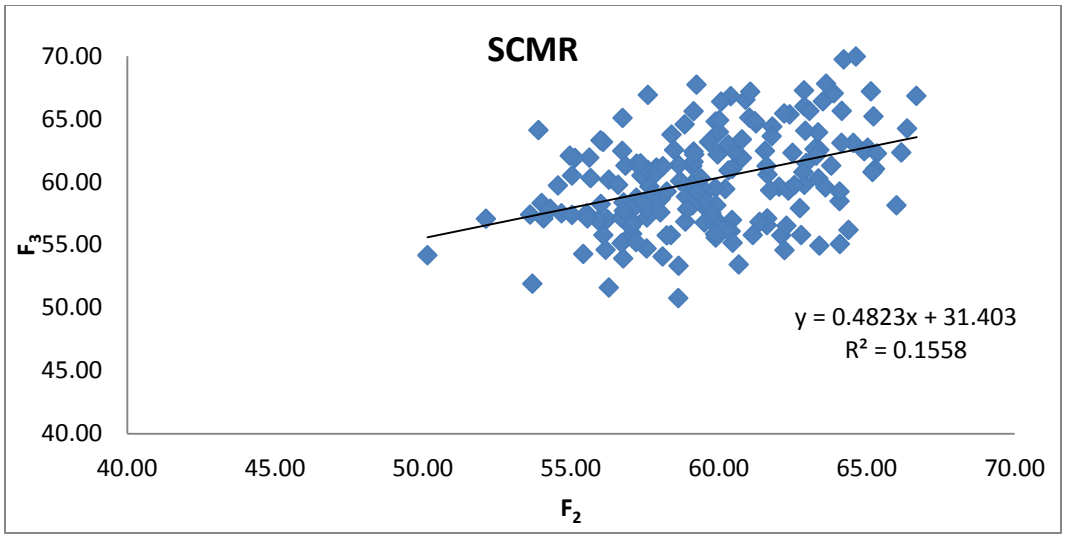
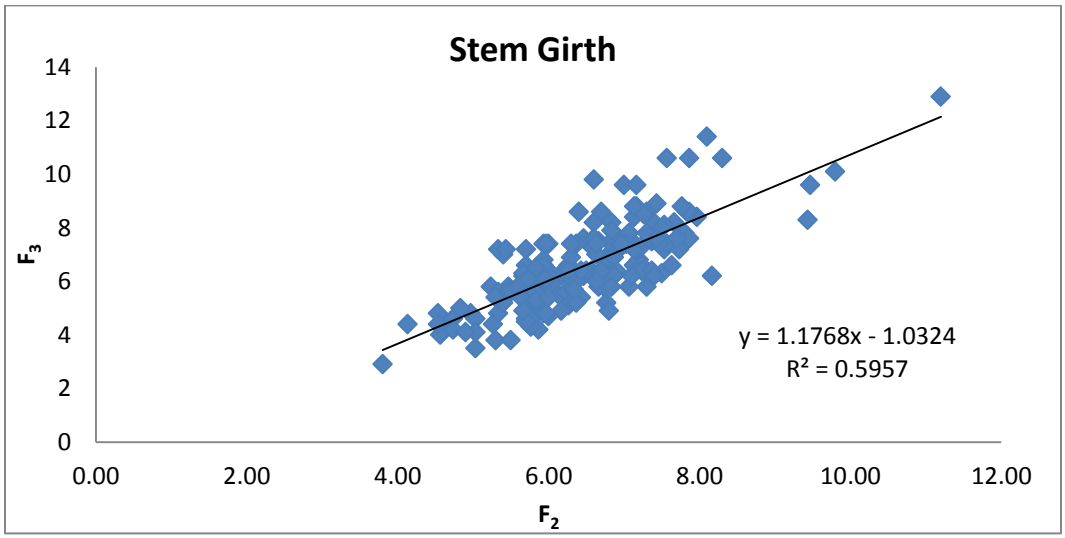


Fig. 9: Correlation of plant height, stalk weight and no. of branches between the F_2 progeny and F_3 families of pigeonpea derived from ICP6443 and ICP10960





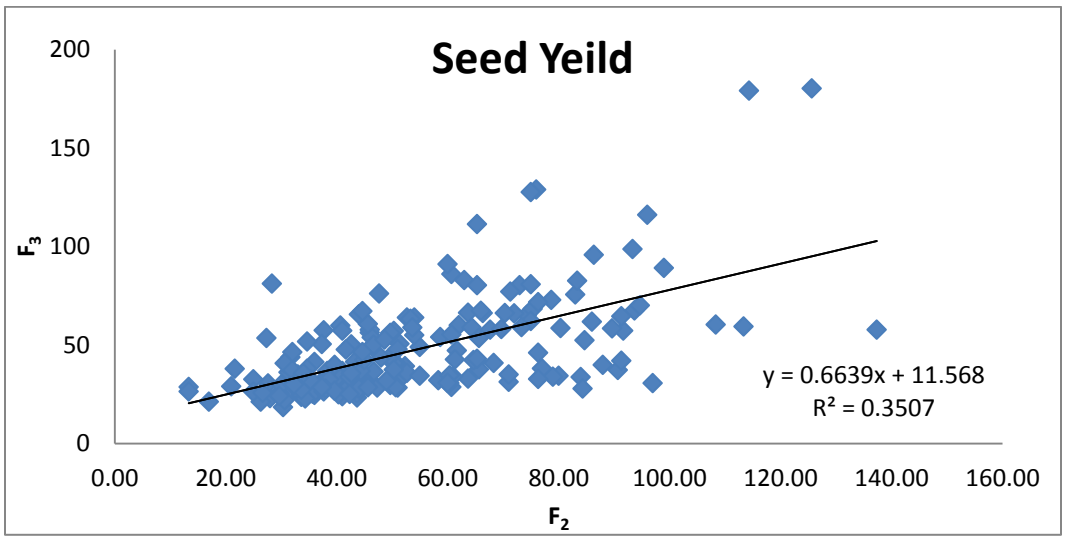


Fig. 10: Correlation of SCMR, Stem girth and seed yield between the F_2 progeny and F_3 families of pigeonpea derived from ICP6443 and ICP10960

divalent cations like Zn, Fe, Ca and Mg and also the Phytic acid in seeds were examined. The results are depicted in the fig. 11. The divalent cations content was relatively high in high Zn lines which were also complemented with relatively higher phytic acid content as well. In the low Zn lines the Fe content was relatively low but the ratio of Iron:Zinc content was relatively higher. Depicting that even though the low Zn lines accumulated less Zn but the Fe they accumulated was relatively higher than the Zn content. Calcium, magnesium and Phytic acid were relatively low in low Zn lines compared to the high Zn lines.

4.3 Genotyping of the F_2 mapping population

4.3.1 Screening of parental lines with SSRs for polymorphism detection

The high seed Zn parent ICP6443 and low seed Zn parent ICP10960 were screened for polymorphism using a total of 441 SSR markers. Among which, 71 genic SSRs were used in initial stages of parental polymorphism screening, while additional 370 genomic SSR markers were further used to increase the marker density in linkage groups. Polymorphism survey revealed a polymorphism of 30.9 % for genic SSRs and 34.8 % for genomic SSRs between the parents ICP6443 and ICP10960 (Table 12). Thus, a maximum number of markers comprising of 22 genic and 129 genomic (Appendix 5) were found polymorphic between ICP6443 and ICP10960 (32.93 %). Plate 12 & 13 are representative agarose gel profile and representative MultiNA gel profile respectively showing the amplified products of AHSSR markers in the genomic DNA of ICP6443 and ICP10960. A total of 151 polymorphic markers were identified between ICP6443 and ICP10960.

Table 12: Number of genic and genomic polymorphic markers detected and percentage of polymorphism between ICP6443 and ICP10960

Parent 1	Parent 2	No. of markers screened		Polymorphic markers		Percent Polymorphism (%)	
		Genic	Genomic	Genic	Genomic	Genic	Genomic
ICP 6443	ICP 10960	71	370	22	129	30.9	34.8

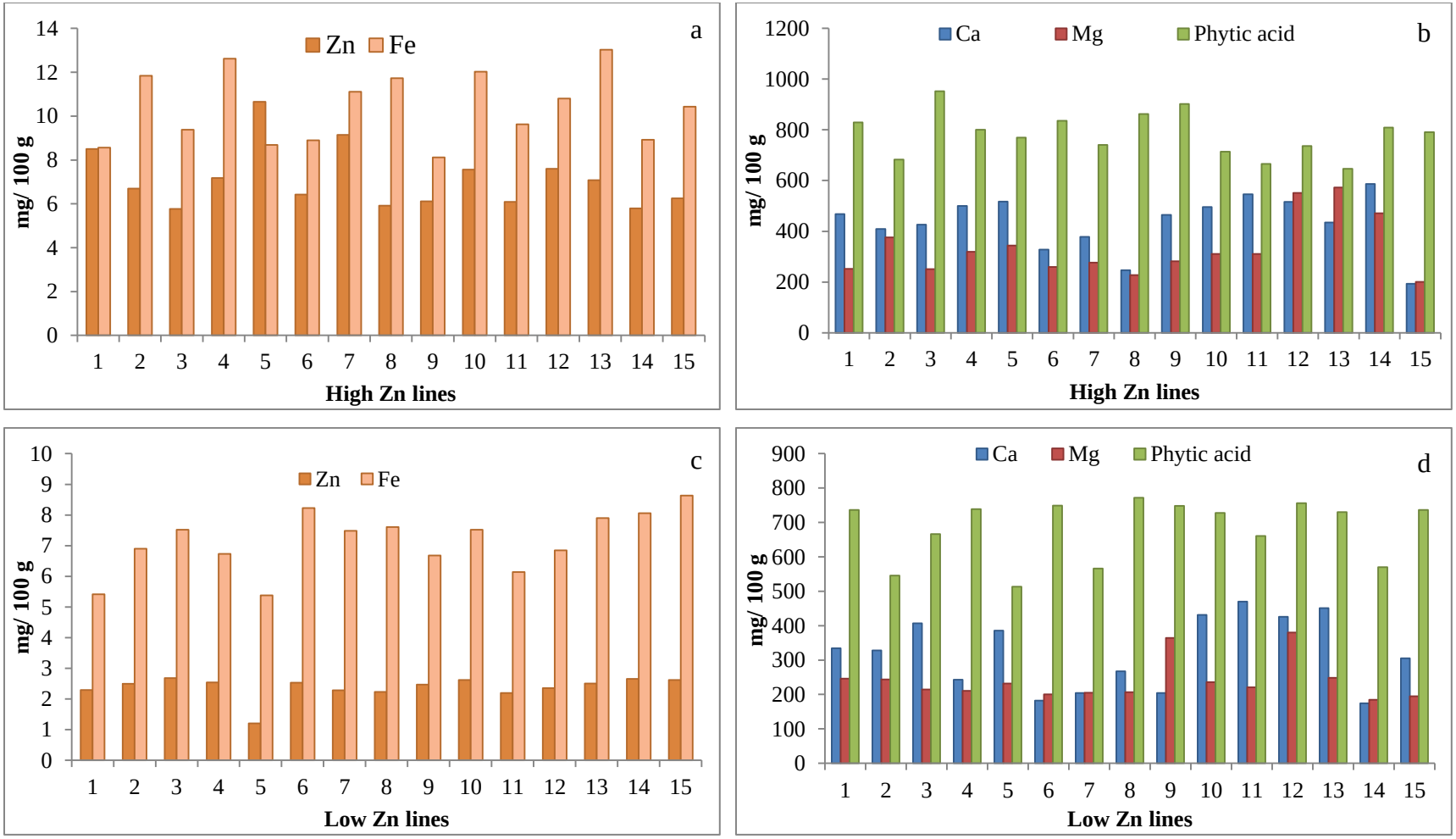


Fig. 11: Histograms depicting the Zn, Fe, Ca, Mg and phytic acid content in the 15 high and 15 low transgressive segregants of $F_{2:3}$ seeds of F_2 mapping population

1 to 15 lines in graph a and b are high seed Zn individuals and 1to 15 lines in graph c and d are low seed Zn individuals

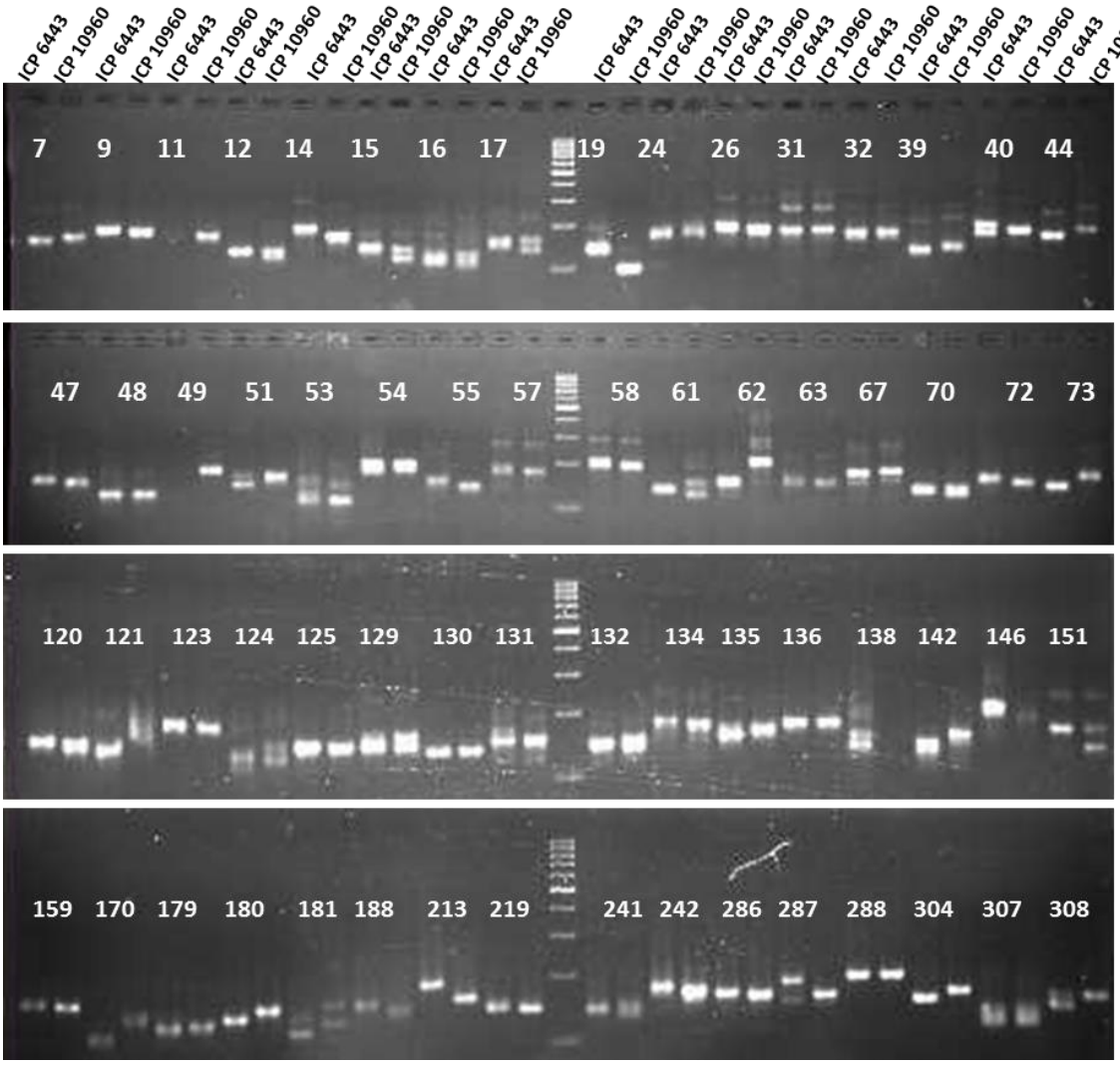


Plate 12: Representative gel showing PCR amplified product of AHSSR markers in the genomic DNA of ICP6443 and ICP10960

Note: The numeric numbers on the gel are the AHSSR genomic SSR marker series. ICP6443 is a high seed Zn parent and ICP10960 is the low seed Zn parent.

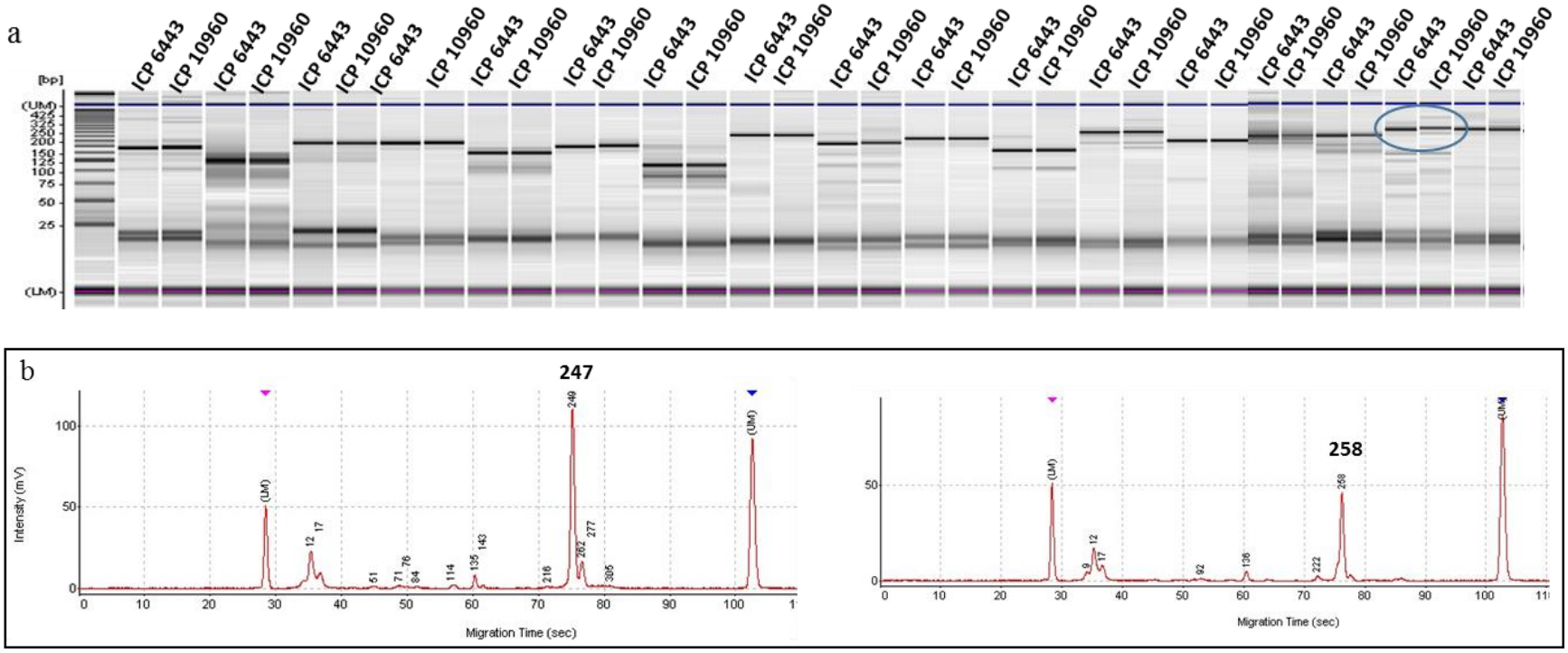


Plate 13: Screening for parental polymorphism between ICP 6443 and ICP 10960 in MultiNA

a. Representative MultiNA gel image of PCR amplified product of AHSSR markers in the genomic DNA of ICP6443 and ICP10960
b. Representative electrophorogram of PCR amplified product of single AHSSR marker in the genomic DNA of ICP6443 and ICP10960

4.3.2 Genotyping of F_2 individuals

A total of 22 genic and 129 genomic SSR markers were employed for genotyping of 188 F_2 individuals. Banding pattern of 188 F_2 individuals were scored as A (ICP6443 type allele), B (ICP10960 type allele), H (hybrid type) and '-' for missing values. Plate 14 & 15 are agarose gel profiles for AHSSR2 and MultiNA gel profile for AHSSR295 respectively showing the banding pattern in the genomic DNA of 188 F_2 individuals and the parental lines.

4.4 Generation of genetic linkage map

Genotypic data obtained from 188 F_2 individuals (derived from the cross ICP6443 X ICP10960) for 151 SSR markers were used for linkage map construction using Kosambi mapping function. Of 151 SSR markers, 62 markers were linked in 10 linkage groups (LG) at logarithm of odds (LOD) 3.0 with a maximum recombination fraction of 0.50. The number of markers mapped per linkage group ranged from 2 to 29 spanning a total distance of 1942 cM. The length of linkage groups ranged from 13.9 cM (LG5) to 1166.7 cM (LG3) with an average interval distance of 37.36 cM (Plate 16). This linkage map was used for mapping of QTLs associated with high seed Zn content.

4.5 Discovery of QTLs for seed zinc content and other parameter

Genetic polymorphism was scored among the mapping population using SSR markers. Single marker analysis (SMA) as well as composite interval mapping (CIM) was employed to find the association of markers to seed Zn and other traits.

4.5.1 Single marker analysis

The common student's t-test was performed between the marker loci and relevant physiological traits using the single marker analysis option in the windows QTL cartographer V.2.5 at 95 % confidence interval.

The results of SMA revealed that markers were associated with eight of all the traits studied in the investigation. A total of three markers were associated with seed Zn content with marker AHSSR93 explaining the maximum phenotypic variance of 6.8 %

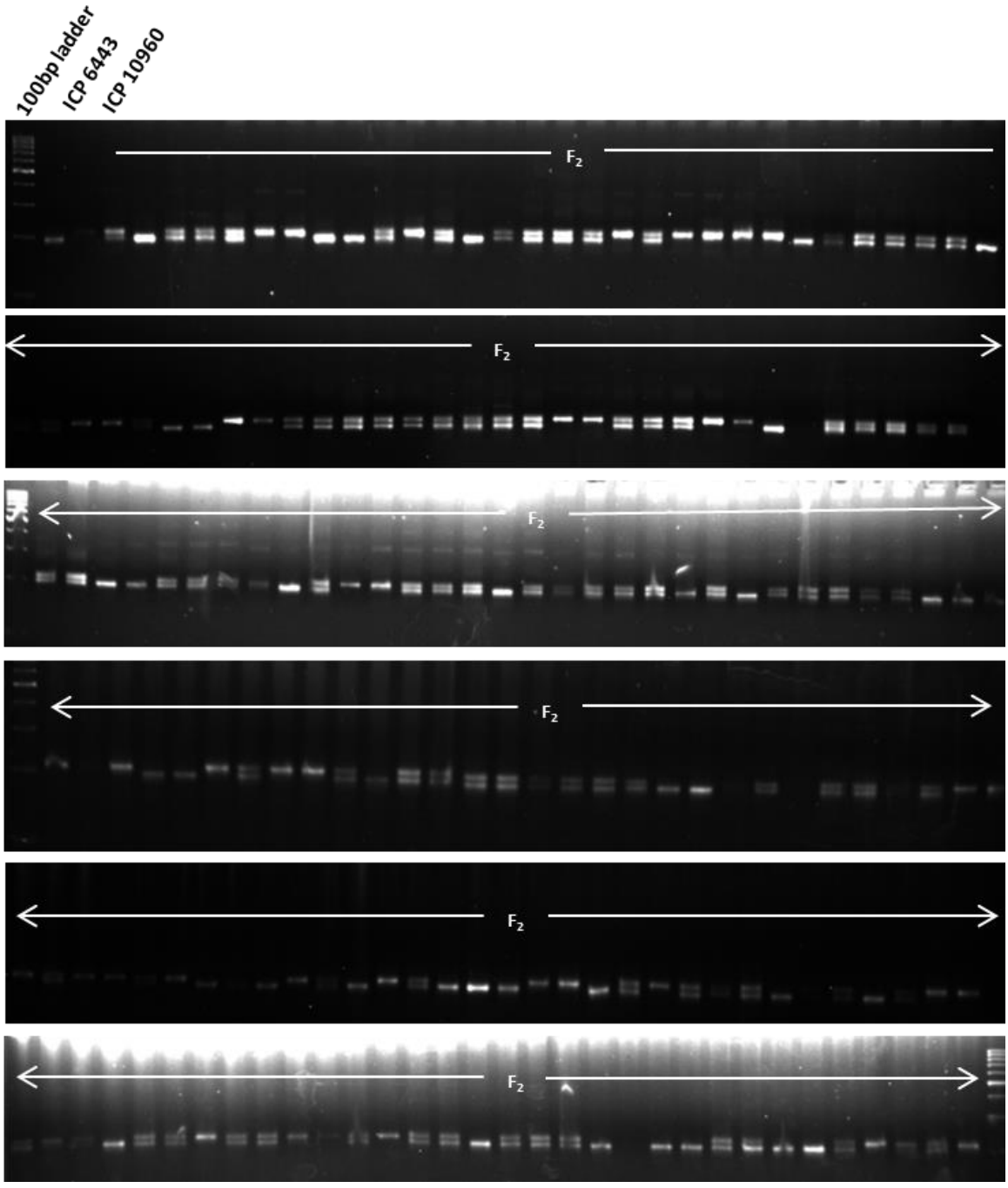


Plate 14: Representative gel showing PCR amplified product of AHSSR2 marker in the genomic DNA of ICP6443 and ICP10960 and its 188 F_2 individuals

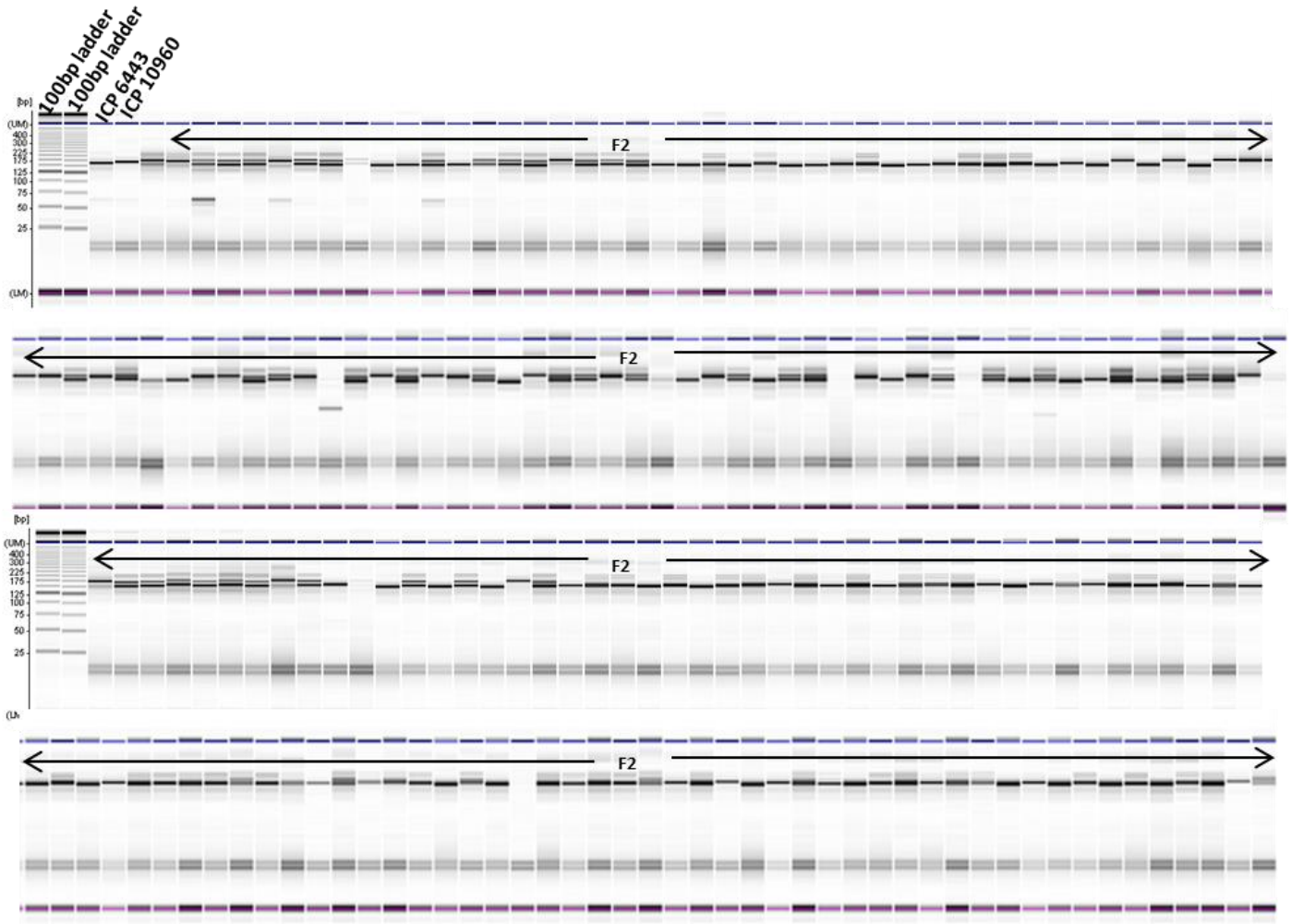


Plate 15: Representative MultiNA gel showing PCR amplified product of AHSSR295 marker in the genomic DNA of ICP6443 and ICP10960 and its 188 F_2 individuals

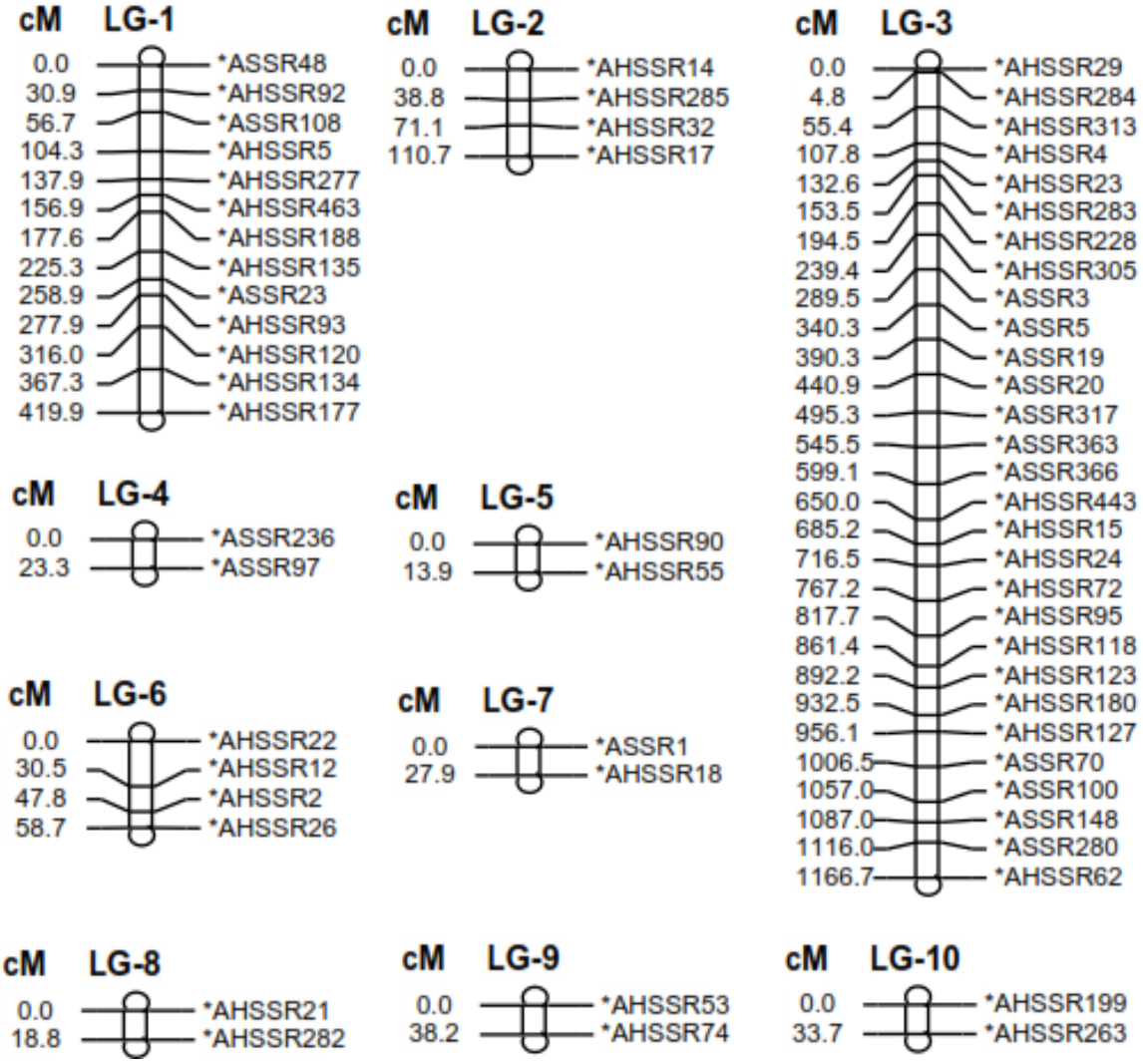


Plate 16: Genetic linkage map of F_2 mapping population of pigeonpea showing marker position on linkage groups

Note: *AHSSR-Arhar Highly variable Simple Sequence repeat
*ASSR- Arhar Simple Sequence repeat

(p=0.012), where as other two markers AHSSR118 and ASSR280 explained 2.9 % of variation each. Marker ASSR280 which was linked to seed Zn was also linked with leaf Zn content explaining the variation of 3 % (p=0.036) (Table 13).

SSR marker AHSSR127 was linked to leaf Fe content explaining 4.5 % variation (p=0.003). A total of four markers i.e., AHSSR277, AHSSR93, AHSSR23 and AHSSR180 were associated with phytic acid content. In which marker AHSSR93 explained a maximum variation of 7.7 % (p=0.0001) and marker AHSSR180 explained about 3.2 % variation (p=0.014) (Table 13).

Table 13: Single-marker analysis (SMA) of markers associated with various traits

Trait	Marker	Linkage Group	P value	R^2
Seed Zn	AHSSR 93	1	0.012*	6.8
	AHSSR 118	3	0.019*	2.9
	ASSR 280	3	0.017*	2.9
Leaf Zn	ASSR 280	3	0.036*	3
Leaf Fe	AHSSR 127	3	0.003**	4.5
Seed Yield	AHSSR 283	3	0.005**	3.8
	ASSR 3	3	0.007**	3.5
	AHSSR 72	3	0.006**	3.6
100 Seed Weight	AHSSR 23	3	0.006**	3.2
	AHSSR 305	3	0.028*	2.5
	ASSR 100	3	0.011*	3.4
	AHSSR18	7	0.015*	3.2
Stem Girth	AHSSR 463	1	0.012*	3.3
	AHSSR 120	1	0.042*	4.6
Phytic Acid	AHSSR 277	1	0.0008***	4.2
	AHSSR 93	1	0.0001***	7.7
	AHSSR 23	3	0.00009****	7.3
	AHSSR 180	3	0.014*	3.2
Plant Height	AHSSR 443	3	0.012*	3.4
	AHSSR 18	7	0.11*	3.5

Two markers each were linked to plant height and stem girth. AHSSR443 and AHSSR18 were associated with plant height showing a variation of 3.35 % (p=0.012) and 3.45 % (p=0.11) respectively. While marker AHSSR463 and AHSSR120 were linked to stem girth explaining about 3.3 % (p=0.012) and 4.6 % (p=0.042) of variation respectively (Table 13).

For yield related traits such as seed yield and 100 seed weight, a total of seven markers were linked. For seed yield AHSSR283, ASSR3 and AHSSR72 showed around 3.5 % of variation. Markers AHSSR23, AHSSR305, ASSR100 and AHSSR18 were linked to seed weight, of which Marker ASSR100 explained the maximum variation of 3.4 % (p=0.011) for seed weight (Table 13).

4.5.2 QTL analysis

QTL identification is primarily based on assumption that there is linkage disequilibrium between markers and chromosome region (QTL) affecting the traits under study. Such linkage disequilibrium is inferred on the basis of likelihood ratio/LOD peaks. The QTLs were mapped on to the genetic linkage map using Composite Interval Mapping through multiple regression approach. The results obtained provide information about the number of QTL associated, linkage group (LG) on which the QTL is located, the interval position (cM), LOD score of the peak, percent phenotypic variance explained by the QTL at the test loci (R^2) and additive effect of the QTL on the trait. In present study, CIM with threshold LOD = 3.0 detected a total of 25 QTL for seed Zn, leaf Zn and other traits (Fig. 12), which were positioned on to four linkage groups. A maximum of 14 QTLs were positioned on linkage group three followed by eight on LG-1, with two on LG-9 and only one on LG-6 (Table 14 and Fig. 12). The QTLs discovered for various traits are presented in this section.

Of the 25 detected QTLs, eight QTLs were detected for seed Zn content and two for leaf Zn content. While seven QTLs were detected for seed yield, three for stem girth, two for 100 seed weight, two for Phytic acid and one for plant height. In a few instances, a given flanking marker region was associated with more than one related or unrelated traits representing the complexity of quantitative traits.

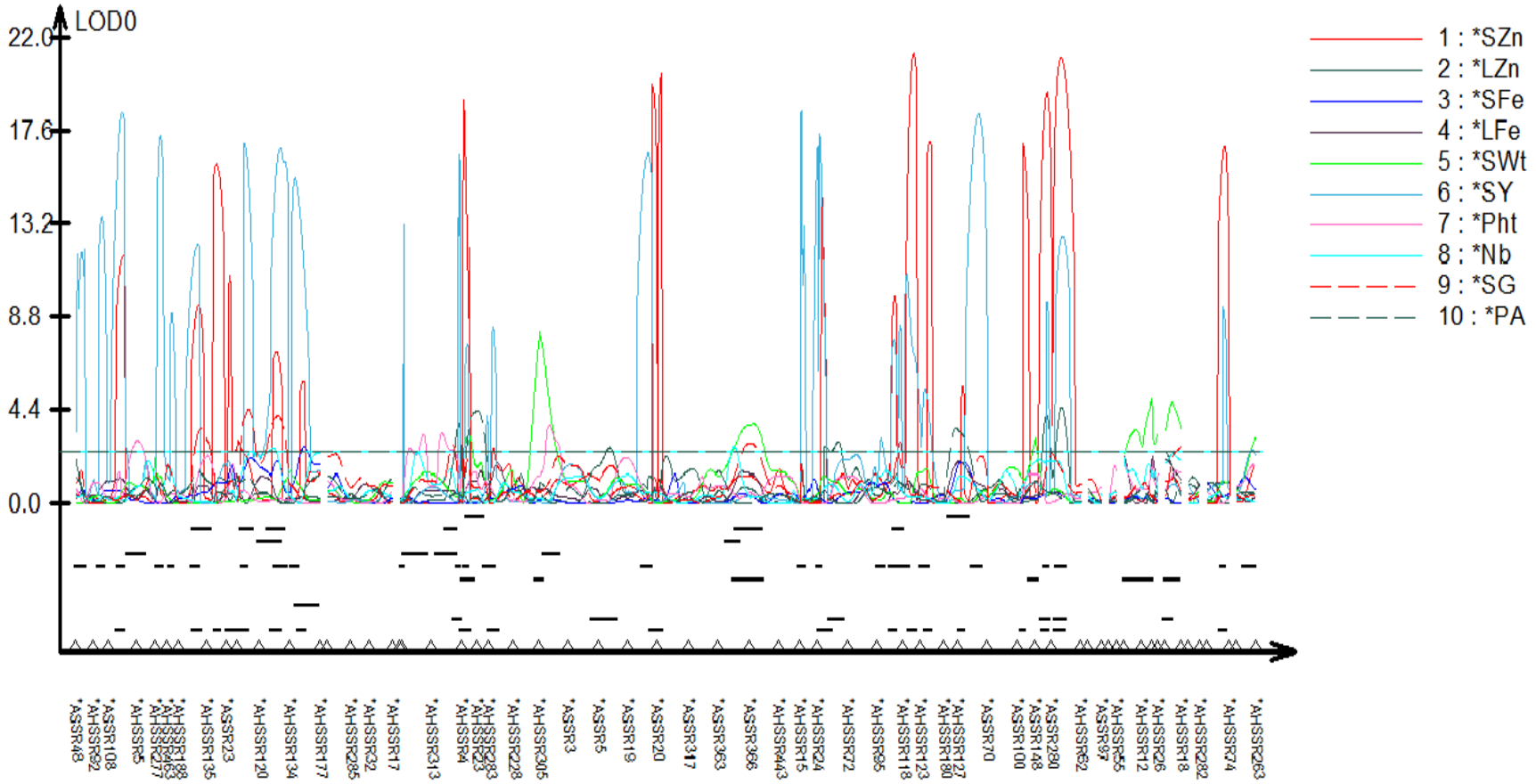


Fig. 12: Composite interval mapping for seed Zn and various other parameters in pigeonpea mapping population derived from the cross between high Zn genotype ICP6443 and low Zn genotype ICP10960

SZn: Seed zinc content; LZn: Leaf zinc content; SFe: Seed iron content; LFe: Seed iron content; SWt: 100 Seed weight; SY: Seed yeild; Pht:Plant height; Nb: Number of branches; SG: Stem girth; PA: Phytic acid

There were three major QTLs for seed Zn content located on the LG 1, 3 and 9 which were responsible for 18.22 %, 60.46 % and 52.35 % of phenotypic variations respectively. The three QTLs qS_Zn1-1, qS_Zn3-2 and qS_Zn9-1 were flanked by AHSSR188-AHSSR135; ASSR120-ASSR313 and AHSSR53-AHSSR74 SSR markers respectively (Table 14 and Fig. 12). For leaf Zn content there were two QTLs (qL_Zn3-1 and qL_Zn3-2) both on LG-3, which explained a variation of 7.5 % in qL_Zn3-1 and 6.6 % in qL_Zn3-2.

Among the seven QTLs which were identified for seed yield in pigeonpea, QTL qS_Y1-3 flanked with AHSSR120-AHSSR124 on LG-1 had a peak of 16.89 and explained 21.47 % variance. For 100 seed weight, the QTL qS_W6-1 with LOD peak of 3.40 was responsible for 8.5 % variation in 100 seed weight. QTL qS_G1-1, explained 58.83 % of variation for stem girth which had a peak of 3.40. The QTL qP_H3-1 anchored by AHSSR305 and ASSR3 was discovered for plant height which had a peak of 4.86 and showed 3.6 % of phenotypic variation. Among the QTLs discovered for phytic acid content QTL qP_A3-1 explained a variation of 14.81 % and had a LOD peak of 4.42 %. The markers anchoring all the QTLs are given in table 14.

4.5.3 Co-location of QTLs

In several instances a given flanking marker region was associated with more than one related or unrelated traits representing the complexity of quantitative traits. Three of the seed Zn QTLs region co-located with QTL region of leaf Zn, seed yield and stem girth. Interestingly, QTL qS_Zn3-3 for seed Zn content and QTL qL_Zn3-1 for leaf Zn content shared common flanking marker region AHSSR24-AHSSR72. AHSSR53-AHSS74 was a common flanking marker region for seed Zn and seed yield present on LG-9. The flanking region AHSSR188-AHSS135 on LG-1 was significantly associated with stem girth and seed yield. Whereas AHSSR305-ASSR3 on LG-1 was associated with 100 seed weight and plant height. Stem girth and seed yield were strongly associated with flanking region AHSSR120-AHSSR134 on LG-1 (Table 14).

Table 14: Summary of significant QTLs identified by composite interval mapping (CIM) in pigeonpea mapping population

Trait	LG	QTL Name	Flanking markers	Position (cM)	LOD	R^2 (%)	Additive Effect
	1	q S_Zn1-1	AHSSR188-AHSSR135	210.6	7.98	60.46	2.39
	1	q S_Zn1-2	AHSSR93-AHSSR120	277.9	3.1	10.78	0.27
	3	q S_Zn3-1	AHSSR283-AHSSR228	159.5	3.4	11.44	-0.05
	3	q S_Zn3-2	ASSR20-ASSR317	447.9	18.22	9.24	2.82
	3	q S_Zn3-3	AHSSR24-AHSSR72	719.5	15.26	9.17	-0.11
	3	q S_Zn3-4	AHSSR118-AHSSR123	879.4	19.87	12.72	3.14
	3	q S_Zn3-5	ASSR280-AHSSR62	1130	21.13	8.14	2.50
Seed Zn	9	q S_Zn9-1	AHSSR53-AHSSR74	30	14.31	52.35	2.79
Leaf Zn	3	q L_Zn3-1	AHSSR24-AHSSR72	754.5	3.5	7.5	0.09
	3	q L_Zn3-2	ASSR148-ASSR280	1106	4.5	6.6	1.65
	3	q S_W3-1	AHSSR305-ASSR3	239.4	8.22	3.4	-0.37
100 Seed weight	6	q S_W6-1	AHSSR22-AHSSR12	17	3.47	8.5	-0.19
Plant Ht	3	q P_H3-1	AHSSR305-ASSR3	255.4	4.86	3.6	-1.63
	1	q S_G1-1	AHSSR188-AHSSR135	213.6	3.4	58.83	0.65
	1	q S_G1-2	AHSSR120-AHSSR134	348	3.63	92.8	1.48
Stem Girth	3	q S_G3-1	AHSSR95-AHSSR118	857.7	3.99	7.66	-0.05
	3	q P_A3-1	AHSSR23-AHSSR283	133.6	4.42	14.81	27.63
Phytic Acid	3	q P_A3-2	AHSSR180-AHSSR127	951.5	3.58	14.65	24.03
	1	q S_Y1-1	ASSR48-AHSSR92	5	11.76	8.12	-42.01
	1	q S_Y1-2	AHSSR188-AHSSR135	208.6	12.06	7.9	-47.44
	1	q S_Y1-3	AHSSR120-AHSSR134	350	16.89	21.47	37.85
	1	q S_Y1-4	AHSSR134-AHSSR177	375.3	14.32	11.14	46.31
	3	q S_Y3-1	AHSSR313-AHSSR4	103.4	13.48	9.11	62.36
	3	q S_Y3-3	AHSSR15-AHSSR24	687.2	17.59	9.12	14.21
Seed Yeild	9	q S_Y9-1	AHSSR53-AHSSR74	27	8.09	8.4	43.96

4.6 Congruence of linked markers between SMA and CIM

The linked markers obtained from the SMA (Single marker analysis) were compared with CIM (Composite interval mapping) results to find if there were any common markers linked to the same traits. After comparison, around seven common markers were found associated with different traits. AHSSR93, AHSSR118 and ASSR280 were found linked to seed Zn content in both strategies. Marker AHSSR305 was linked to 100 seed weight trait and AHSSR23 and AHSSR180 were common in SMA and CIM for phytic acid content in seeds. While the marker AHSSR120 was linked with stem girth in both the methods of linked marker analysis (Table 15).

4.7 Genic markers linked to seed Zn and leaf Zn content

71 genic SSR markers were employed in this study, among which 22 were polymorphic and 15 markers were linked into 10 different linkage groups at different positions along with other genomic SSR markers. During QTL analysis genic markers (ASSR20, ASSR148 and ASSR280) were linked to the trait of interest viz., seed Zn and leaf Zn. These three genic markers were from genic regions which coded for bZIP transcription factor (ASSR20), ethylene response transcription factor (ASSR148) and senescence inducible chloroplast stay green protein (ASSR280). Among these genic markers, ASSR280 was linked to seed Zn as well as to leaf Zn content, while ASSR148 was linked to leaf Zn trait only. Assuncao *et al*., in 2010 identified bZIP23 and bZIP19 TFs involved in the regulation of Zn deficiency response in *Arabidopsis.* Interestingly the QTL analysis performed in this study, resulted in seed Zn content trait getting linked to ASSR20 genic SSR marker (Table 14) derived from the genic region of bZIP transcription factor, where bZIP TF has been reported to have been regulating Zn deficiency responses in plants.

Table 15: Summary of common markers between SMA and CIM

CIM					SMA			
QTL	**LG**	**Marker Interval**	**LOD**	**R^2**	**Trait**	**Marker**	**P**	**R^2**
qS_Zn 1-2	1	AHSSR93 - AHSSR120	3.1	10.78	Seed Zn	AHSSR93	0.012	6.2
qS_Zn 3-4	3	AHSSR118 - AHSSR123	19.87	12.72	Seed Zn	AHSSR118	0.019	2.9
qS_Zn 3-5	3	AHSSR280 - AHSSR62	21.13	8.14	Seed Zn	ASSR280	0.036	3
qS_W 3-1	3	AHSSR305 - ASSR3	8.22	3.4	Seed wt	AHSSR305	0.028	2.5
qP_A 3-1	3	AHSSR23 - ASSR283	4.42	14.81	Phytic acid	AHSSR23	0.001	7.3
qP_A 3-2	3	AHSSR180 - ASSR127	3.58	14.65	Pytic acid	AHSSR180	0.014	3.2
qS_G 1-2	1	AHSSR120 - ASSR134	3.63	92.8	Stem girth	AHSSR120	0.042	4.6

Discussion

V DISCUSSION

Humans require at least 22 mineral elements for their wellbeing (Welch and Graham, 2004; White and Broadley, 2005; Graham *et al.,* 2007). These can be supplied by an appropriate diet. However, it is estimated that over 60 % of the world's 6 billion people are iron (Fe) deficient, over 30 % are zinc (Zn) deficient, 30 % are iodine (I) deficient and 15 % are selenium (Se) deficient. In addition, calcium (Ca), magnesium (Mg) and copper (Cu) deficiencies are common in many developed and developing countries (Frossard *et al.,* 2000; Welch and Graham, 2002, 2005; Rude and Gruber, 2004; Grusak and Cakmak, 2005; Thacher *et al.,* 2006). This situation is attributed to crop production in areas with low mineral phytoavailability and/or consumption of foods with inherently low tissue mineral concentrations, compounded by a lack of animal products in the diet (Welch and Graham, 2002, 2005; Poletti *et al.,* 2004; White and Broadley, 2005; Gibson, 2006; Graham *et al.,* 2007).

Thus, by any standard the magnitude of micronutrient undernutrition is immense. Among all the micronutrients, zinc deficiency has emerged as the most widespread micronutrient deficiency affecting especially women, infants and young children under five years of age (Das and Green, 2013). Zinc deficiency is the fifth leading cause of death and disease in the developing world which has received global attention (UNICEF, 2012). It is also estimated that 60–70 % of the population in Asia and Sub-Saharan Africa could be at risk of low zinc intake (Prasad, 2006). The possible way out of zinc deficiency in humans may be food supplementation, food fortification, dietary diversification or biofortification. The former two programmes require infrastructure, purchasing power, access to market and health care centers and uninterrupted funding, which have their own constraints (Das and Green, 2013). In addition, such programmes will most likely reach the urban population, which is easily accessible, especially in the developing countries. Alternatively, the latter programme, biofortification – fortification of crops with zinc – is the best option for alleviating zinc deficiency. It will cater to both the rural and urban populations thus by leading to a reduction in prevalence of zinc undernutrition (Meenakshi *et al.,* 2007).

Biofortification of food crop can be achieved either by zinc fertilization, genetic engineering approaches or molecular breeding approaches. Sub soil constraints, soil pollution and cost ineffectiveness has limited zinc fertilization from reaching its potential. Genetic engineering would be a good option in the absence of genetic variability for Zn content across the species. Molecular breeding would prove a best option in the presence of genetic variability for Zn content with in the species. Hence our present investigation is based on exploiting the genetic variability of seed Zn content in pigeonpea.

Pigeonpea has a wide range of products, including the dried seed, pods and immature seeds used as green vegetable, leaves and stems used for fodder and the dry stems used as fuel. It also improves soil fertility through nitrogen fixation as well as from the leaf fall and recycling of the nutrients (Snapp *et al*., 2002). It is an important pulse crop that performs well in poor soils and regions where moisture availability is unreliable or inadequate (Kimani, 2000). The crop can withstand low moisture condition and performs well in areas with less than 1000 mm of annual rainfall, depending on the distribution pattern. It serves as a major source of protein to about 20% of the world population (Akande *et al*., 2010). It is a food and cash crops for small hold farmers and National research systems and government are making concerted efforts to promote this crop among poor. Hence focusing research on biofotification in this crop can be expected to reach the poor people and small hold farmers growing this crop.

The primary objective of plant breeding has been to enhance farm productivity, usually by developing crops with higher yields. In contrast, improving micronutrient use efficiencies and increasing nutrient content in plants has rarely been a breeding objective. In fact, crop nutritional problems have mostly been ignored in breeding. Some nutritional problems cannot be easily resolved by altering soil fertility or chemistry, and the application of modern breeding techniques to breed crops that are adapted to soils with a poor nutritional status is required. In the case of micronutrient deficiencies induced by high pH (i.e., Zn, Fe and Mn deficiencies), agronomic solutions (i.e., fertilizers) are not always successful, and a genetic solution is necessary (Cakmak and Braun, 2001). Furthermore, the correction of Zn deficiency induced by subsoil constraints, topsoil drying and diseases is not effective via fertilization (Graham and Rengel, 1993). Hence,

breeding and use of Zn-efficient plant genotypes that can more effectively function under Zn deficiency is an effective and sustainable solution to address Zn deficiency limitations in crop production as well as in humans. Besides plant growth under a given level of soil Zn, the more important factor that determines nutritional security is the amount of available nutrients like Zn and Fe in grains.

It is recognized that combining high yield with high grain Zn is a complex issue where the traits are under polygenic control. Thus, a DNA marker based molecular breeding is being adapted as a potential strategy to achieve genetic enhancement in improving and introgressing such complex traits.

In this investigation an attempt was made to develop a trait specific mapping population segregating for seed Zn content. An extensive molecular and phenotypic characterization of the mapping population lead to the discovery of robust markers linked to seed Zn content and other physiological traits associated with growth in pigeonpea.

5.1 Molecular diversity among the genotypes with contrasting seed Zn content

Significant genetic variability is one of the primary prerequisites for improving Zn acquisition. Such genotypic variations can be exploited in breeding programmes to produce genotypes with higher Zn efficiency. Manoa, (2010) analyzed 217 pigeonpea genotypes procured from ICRISAT for their seed zinc content and observed significant variation across the genotypes with zinc content ranging from as low as 1.53 mg/100 g to as high as 6.2 mg/100 g. Based on seed Zn content these genotypes were grouped into high Zn types and low Zn types (15 in each group). The high Zn group had mean Zn value of 4.77 mg/100 g with range between 4.19 to 6.20 mg/100 g. On the other hand, low Zn group had mean value of 2.12 mg/100 g with a range of 1.91 and 2.36 mg/100 g. These 30 genotypes contrasting for seed zinc content were characterized for molecular diversity with 50 SSR markers. High and low seed Zn (Each five) genotypes were further identified based on the consistent behavior and their molecular diversity. Being an often cross pollinated spp., pigeonpea is reported to have a significant level of residual heterozygosity. Thus, 50 co-dominant SSR markers that were used to decipher the molecular diversity observed a mean heterozygosity of 0.13 among the 30 contrasting

accessions including the parental lines. The SSR markers used were quite capable of detecting polymorphism with a mean PIC value of 0.39 ranging between 0.02 to 0.76. The microsatellite markers revealed moderate genetic diversity among genotypes of pigeonpea, which were clustered into two groups in a single clade. Some of the high Zn genotypes were clustered along with few low Zn genotypes and vice versa (Fig. 5). Therefore, a more elaborate linkage mapping needs to be carried out to identify the causal genomic region controlling seed Zn content.

Sousa *et al.,* 2011, observed low variability, while conducting genetic diversity studies in 77 genotypes of pigeonpea and speculated that low variability in these collections may be due to a narrow genetic base of the original germplasm collection or pre-selection of these genotypes based on some agronomic characteristics. Several other workers also reported a quite narrow genetic diversity (Panguluri *et al.,* 2006), (Yang *et al.,* 2006), (Odeny *et al.,* 2007) in pigeonpea. Narrow genetic base has also been reported due to use of only few genotypes with high degree of relatedness in crossing programmes for the development of new cultivars (Kumar *et al.,* 2004; Yang *et al.,* 2006). Kumar *et al.,* (2004) observed that 16 (34 %) pigeonpea cultivars released in India involved only one or two genotypes as one of the ancestors in their pedigree. To increase genetic diversity of pigeonpea breeding material, new diversity from wild relatives will be extremely useful, although there is substantial variation in existing collections. Also use of genome wide markers could aid in accessing genetic diversity to a greater extent.

Besides the narrow genetic diversity, presence of heterozygosity is yet another factor that potentially slows the progress in crop improvement in pigeonpea. Heterozygocity profile in pigeonpea was as high as 0.53. Although pigeonpea is considered an autogamous species, in the presence of pollinators, the cross-pollination can occur, ranging from 3 % to 26 %. (Reddy *et al.,* 2004).

5.2 Phenotyping of the mapping population

Zn content in the pulse seeds coupled with increased seed production hold the key in achieving nutritional security. Pigeonpea and chickpea are the most widely consumed pulses in India and hence attempt to improve seed Zn content has paramount significance.

It is well perceived that a molecular breeding programme alone can accelerate crop improvement for this important trait. Towards this endeavour, we initiated an elaborate programme to identify QTL governing seed Zn in pigeonpea. Phenotyping of seed and leaf Zn content therefore in pivotal to the QTL discovery programmes.

Zn enhancement in food crop through genetic improvement has been receiving much attention in recent years. As bioinformatics and large-scale genetic tools develop, the quantitative trait loci (QTL) analysis provides a powerful genetic approach in identifying novel genes affecting a certain traits (Vert *et al.,* 2002). Although lots of researches have been taken about micronutrient variation in bean (Beebe *et al.,* 2000; Islam *et al.,* 2002), rice (Gregorio *et al.,* 2000), wheat (Monasterio and Graham 2000), maize (Banziger and Long 2000; Kovacevic *et al.,* 2004), only a few were related to QTL analysis for the micronutrient concentrations (Guzman-Maldonado *et al.,* 2003; Gelin *et al.,* 2007; Gregorio *et al.,* 2000; Stangoulis *et al.*, 2007; Monasterio and Graham 2000; Tiwari *et al.,* 2009; Vreugdenhil *et al.,* 2004; Waters and Grusak 2008).

Availability of useful variability in the germplasm and understanding its genetic architecture are the prerequisites for a breeding programme aimed at biofortification of crop plants. Realizing the importance of biofortification several studies were undertaken for the evaluation of germplasm and advance breeding lines for grain Zn content (Cakmak *et al.,* 2000; Monasterio and Graham 2000; Chhuneja *et al.,* 2006; Morgounov *et al.,* 2007).

QTL discovery can be achieved by assessing phenotypic and molecular segregation pattern in a trait specific mapping population. After extensively analysing a diverse germplasm, contrasts (15 high Zn and 15 low Zn) were identified (Manoa, 2010). Five high and five low seed Zn genotypes were further identified based on the Zn status and molecular diversity. Mapping population segregating for leaf and seed Zn content was developed. In this investigation, extensive phenotyping of mapping population for leaf and seed Zn content was carried out using an Atomic absorption Spectrophotometer (AAS).

The seed zinc content was measured in F_2 derived F_3 seeds. The population showed marked genotypic variation for seed Zn content (Table 8) similar trends were observed in soybean (King *et al.,* 2014), common bean (Blair *et al.,* 2010), wheat (Peleg *et al.,* 2009; Shi *et al.,* 2008; Genc *et al.,* 2009; Tiwari *et al.,* 2009), rice (Gande *et al.,* 2014; Anuradha *et al.,* 2012; Lu *et al.,* 2008) and maize (Qin *et al.,* 2012; Jin *et al.,* 2013).

In several studies on micronutrients in different crops, significantly positive correlations between Zn and Fe concentration have been reported by Blair *et al.,* (2009), Shi *et al.,* (2008), Stangoulis *et al.,* (2007), Gelin *et al.,* (2007), Beebe *et al.,* (2000) and Cakmak *et al.,* (2004). Hence we also measured Iron content in the $F_{2:3}$ seeds and found that the frequency distribution of Fe content showed continuous phenotypic variation as that for zinc content (Table 8). Similar results were reported by Blair *et al.,* in 2010 in common bean RILs and by Jin *et al,*. (2013) in 218 $F_{2:3}$ families. Micronutrients rich crops often seems to have other desirable qualities, such as high yield, good tolerance to nutrition deficiency stress, pest resistance etc. (Gregorio *et al.,* 2000). Particularly Zn is involved in and influences many physiological functions (Hafeez *et al.,* 2013) and ultimately the crop yield (Marschner, 1995). Therefore apart from measuring Zn and Fe in seeds, the population and parental lines were also characterized for other morpho-physiological traits, yield and yield related traits (Table 8) to look for the lines with desirable physiological and yield characters along with high micronutrient content.

Distribution of population

The study of distribution properties such as, co-efficients of skewness and kurtosis provides insight about the nature of the gene action and number of genes involved in the trait expression (Rabson, 1956). All the reported genetic analyses of quantitative traits are based on first degree (gene effects through generation mean analysis) and second degree (components of genetic variances) statistics. Skewness and Kurtosis are more powerful than variances which reveal interaction genetic effects (Choo and Reinbergs, 1982).

The skewed distribution of a trait in general suggests that the trait is under the control of non-additive gene action, especially epistasis and influenced by environmental variables (Kimbeng and Bingham, 1998; Roy, 2000). While, positive skewness is associated with complementary gene interactions, negative skewness is associated with duplicate (additive X additive) gene interactions. The genes controlling the trait with the skewed distribution tend to be predominantly dominant irrespective of whether they have increasing or decreasing effect on the trait. The traits with leptokurtic and platykurtic distribution are controlled by fewer and larger number of genes, respectively. Kurtosis is in the presence of gene interactions (Choo and Reinbergs, 1982).

Frequency distribution of seed Zn in 188 $F_{2:3}$ progenies obtained from the cross between ICP6443 and ICP10960 revealed that it fit normal distribution, while leaf Zn appeared to be skewed towards the high Zn parent ICP6443 was observed for both seed Zn and leaf Zn (Fig. 7), indicating the predominance of transgressive segregants towards the superior parent. The sharper kurtosis peak for seed Zn indicated that more segregants had values much higher than the mean of the population. While the seed Fe did not appropriately fit normal distribution curve (Fig. 7). Absence of normal distribution in the seed Fe content in the mapping population is not uncommon. Blair *et al.,* 2010; Suwarto, 2011 and Anuradha *et al.,* 2012 also reported the absence of normal distribution for seed Fe content in common bean and rice.

Leaf Fe and phytic acid contents also showed continuous variation indicating that the traits are controlled by polygenes. Traits like stem weight and seed yield per plant had significantly stronger peaks indicating maximum number of transgressive segregants having the stem weight and seed yield more than the ICP6443, the better parent (Fig. 7 & 8).

Genetic variability, heritability and genetic advance in $F_{2:3}$ mapping population

In the present investigation, observed values of phenotypic co-efficient of variation (PCV) were higher than the genotypic co-efficient of variation (GCV) (Table 9) for all the traits. Genetic variability estimates viz., PCV and GCV were high for the Zn content in seeds and offer scope for direct selection. Similar findings have been reported

in pigeonpea for seed Zn content (Manoa, 2010) and for grain Zn content in rice (Babu *et al.,* 2012) and for phytic acid content in ragi seeds by Mahesh, 2008. High heritability with higher genetic advance over mean for the seed Zn and other traits indicated the reliability of the estimates of variation between $F_{2:3}$ individuals and effectiveness of selection in this material for this trait. Prajapati *et al.* (2011) reported the higher values of broad sense heritability obtained can be attributed to lesser difference between PCV and GCV. Though phytic acid showed higher h^2 its GA was moderate and this could be because of complexity of trait and influence of environment on phytic acid biosynthesis.

Correlation studies

Factors such as linkage, pleiotropy and environmental effects may result in positive or negative correlations between different traits in a population (Aastveit and Aastveit 1993; Paterson *et al.,* 1988). Seventeen traits were being measured in the mapping population derived from ICP6443 and ICP10960. A total of 136 pair wise combinations were examined for correlations, out of which 35 pairs had significant correlations (Table 10).

The correlations between traits are often an explanation for the interdependence of such traits or are possible when similar causal factors influence the variation in specific traits. In case of nutrients, a large number of genes encode for putative metal transporters, some of which transport multiple metals. For instance, IRT1, an *Arabidopsis* transporter essential for iron uptake also can mediate the accumulation of zinc, manganese and cobalt. Zn could replace iron in some cellular processes under environment stress (Morrissey and Guerinot 2009; Vert *et al.,* 2002). In several studies on micronutrients in different crops, significantly positive correlations between Zn and Fe concentration have been reported by Blair *et al.,* (2009), Shi *et al.,* (2008), Stangoulis *et al.,* (2007), Gelin *et al.,* (2007), Beebe *et al.,* (2000) and Cakmak *et al.,* (2004). The positive correlation between Zn and Fe possibly revealed the common regulated mechanisms for Zn and Fe in plants and the possibility to simultaneously improve the concentration of both elements. On the contrary, Vreugdenhil *et al.* (2004) and Tiwari *et al.* (2009) found no significant correlation between Zn and Fe concentration. Similarly

there was no significant correlation observed between Zn and Fe, neither in seeds nor in leaves in this study (Table 10).

Contrasting opinions are also reported. Nagarathna *et al.,* in 2010 assessed genetic variation in zinc acquisition and transport to seed in diversified germplasm lines of rice (*Oryza sativa* L.) reported that in spite of large variation in leaf Zn, there was no relationship between leaf and seed Zn levels. Similar results were being observed by Manoa, (2010) in pigeonpea, where, there was no significant correlation between seed Zn and leaf Zn. But in the present study a positive and significant correlation between seed Zn and leaf Zn was found among the mapping population. Eva (1993) reported that the seeds accumulating more of heavy metals such as Cd, Cu, Pb and Zn were exponentially correlated with grain size and weight. Accordingly, we observed a positive and significant correlation between seed Zn and 100 seed weight. Thus the seed Zn trait in our study was found correlated with leaf Zn content and 100 seed weight (Table 10).

Kudhsar *et al.,* (2008) studied the changes in growth characters, foliar properties, and Zn-accumulation capacity in pigeonpea genotypes and found Zn influencing the parameters they had measured. As it is well known that Zn influences wide variety of physiological processes in plants, hence we examined for correlation between Zn and other morpho-physiological parameters and found 35 significant correlations, two of which were biologically meaningful correlation (Table 10). Meaningful correlation explains that, more than 50 % of the variation in one trait can be predicted by the other trait (Snedecor and Cochran, 1980). That is, selection of a specific trait leads to an effective selection of the other correlated trait. However, identification of causative correlation would significantly enhance the effective selection of the more relevant dependent trait to improve seed Zn status.

Though Zn is an essential plant nutrient element, the leaf Zn status always need not associate with crop growth rate or grain yield (Wissuwa *et al.,* 2006). Similarly our results also did not reveal any correlation between Zn content and seed yield.

Categorization of genotypes based on Zn content

Classification of genotypes as high leaf high seed (HLHS), high leaf low seed (HLLS), low leaf high seed (LLHS) and low leaf low seed (LLLS) Zn types, that could provide the leads in understanding the basic mechanisms in acquisition and translocation of Zn along with the other factors associated with variability in seed Zn content (Nagaratna *et al.,* 2010). The observed variability could help in identifying specific donor genotypes with high Zn efficiency and high Zn content or a Zn efficient transgressive segregant in case of Zn specific mapping populations. Hence we classified the individuals of the mapping population into four contrasting groups (Table 16). 43 individuals of the mapping population derived from the cross ICP6443 and ICP10960 were categorized into high leaf Zn and high seed Zn containing individuals. Further characterization of these individuals might result in selection of best performing transgressive segregants.

Table 16: Mean and range values for four contrast groups

Zn types	Leaf Zn (mg/100 g DW)			Seed Zn (mg/100 g DW)		
	Range	Mean	SD	Range	Mean	SD
HLHS	4.78-9.4	6.51	1.29	4.01-9.55	5.62	2.00
HLLS	4.73-8.09	5.64	0.80	2.5-3.98	3.37	0.37
LLHS	2.23-4.71	4.19	0.66	4.08-6.69	4.66	0.59
LLLS	2.05-4.65	3.47	0.69	1.2-3.96	3.17	0.51

Note: HLHS are the high leaf Zn and high seed Zn lines; HLLS are the high leaf Zn and low seed Zn lines

LLHS are the low leaf Zn and high seed Zn lines; LLLS are the low leaf Zn and low seed Zn lines

Nevertheless, attempts to improve these traits were not generally successful owing mainly to the tedium involved in determining these traits in large populations and breeding lines. A DNA based molecular marker based breeding approach is expected to bring in significant increase in the precision of trait introgression. Further this approach

would significantly reduce the gestation period of a breeding programme (Sadeghzadeh, 2013).

5.3 Genotyping of the mapping population using SSR markers

Developing mapping population and construction of genetic linkage maps are pre requisite for identifying genomic region linked to trait of interest for molecular breeding programme in pigeonpea. In the present study, a co-dominant marker based genetic linkage map was developed. SSR markers have been useful for both creating and expanding linkage maps in many plant species. The fact that the SSR assay is sensitive to single base changes, contributes to a higher efficiency in screening the entire genomes for polymorphisms. Also SSR are capable of detecting both the alleles of the locus facilitates the precise mapping (Kesawat and Das, 2009).

Towards identification of QTLs conditioning seed Zn and other traits, parental genotypes were initially screened for polymorphism using the 471 genomic and genic SSR primers. After identifying 151 primers polymorphic between parents (Table 12), the primers were screened among 188 F_2 individuals and the polymorphic information was used for construction of linkage map.

5.4 Generation of genetic linkage map for pigeonpea and identification of QTLs governing seed zinc content

Genetic linkage map for pigeonpea

Genetic linkage maps are necessary tools for genome analysis and breeding application. In this study, F_2 mapping population derived from the distinct parents served as a basic genomic tool to identify the genetics of seed Zn content. With the advent of molecular marker technology, based on the segregation pattern, fragment of DNA responsible for trait of interest can be identified by linkage analysis. In the present study, genotypic frequencies among the 188 F_2 for 151 SSR markers were used to develop a linkage map. The SSR markers formed the ten linkage groups. The map covered about 1942.8 cM with 62 markers distributed over 10 chromosomes with an average interval length of 37.36 cM (Plate 15).

The development of genetic linkage map will greatly augment the ability of breeders to tag and follow the introgression of specific chromosome segments linked to desirable traits from different sources into breeding lines. Linkage map indicates the position and relative genetic distances between markers along the chromosomes. The most important use of linkage map is to identify chromosomal locations containing genes and QTLs associated with the trait of interest. Construction of genetic linkage map is necessary and to apply Marker Assisted Selection (MAS) tool in crop improvement programme. The MAS is especially useful for traits which are controlled by recessive alleles and which are difficult for phenotyping. The reports on the construction of genetic linkage map in pigeonpea are of very recent (Bohra *et al.,* 2011; Gnanesh *et al.,* 2011; Yang *et al.,* 2011; Bohra *et al.,* 2012; Kumawat *et al.,* 2012). These authors in their study have used various marker systems like DArT, SNPs, BES-SSR etc. for construction of genetic linkage map.

Single marker analysis

Different statistical methods can determine the linkages between marker loci and QTLs (Liu, 1998; Tanksley, 1993). Among them single marker approach is based on ANOVA, or simple linear regression and performs statistical tests based solely on single DNA marker information, and the calculations are based on phenotypic mean and variances within each of the genotypic classes (Coffman *et al.,* 2003). Genetic map is not a prerequisite for single marker analysis. A serious limitation of this approach is confounding of the effect of one QTL by many others that influence the traits. Thus, single marker approach can explain the significance of the markers in associating to a trait of interest.

SMA in our study revealed that markers were associated with eight of all the traits studied in the investigation. A total of three markers were associated with seed Zn content with marker AHSSR93 explaining the maximum phenotypic variance of 6.8 % ($p=0.012$), whereas other two markers AHSSR118 and ASSR280 explained 2.9 % of variation each. Marker ASSR280 which was linked to seed Zn was also linked with leaf Zn content trait explaining the variation of 3 % ($p=0.036$) (Table 13). Similarly markers

linked to leaf Fe, seed yield, seed weight, stem girth, phytic acid and plant height were obtained (Table 13).

While in rice also, Anuradhha *et al.,* 2012 reported 14 markers for grain Zn located on chromosomes 1, 2, 3, 4, 5, 6, 7 and 10 were linked with Zn concentration. The range of phenotypic variance explained by these 14 loci ranged from 12 to 46 %. Out of 14, 4 markers on chromosomes 3 (RM231, RM514), 6 (RM541) and 10 (RM484) explained high phenotypic variance significant at 1 % (p= 0.006** to 0.009**).

QTL mapping

QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured. A significant difference between phenotypic means of the groups, depending on the marker system and type of population, indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard *et al.,* 2005)

The interval mapping, is the most popular approach for QTL mapping as it allows asymptotically unbiased estimates of QTL location and effect, with the assumption that there is only QTL on chromosome is true (Lander and Botstein, 1989). IM estimates the effects of QTL at any tested location between a given pair of mapped markers. The QTL cartographer walks along chromosomes performing the molecular linkage calculation at regularly spaced intervals (one or two cM apart) and the resulting LOD scores are plotted to reveal candidate QTL locus of highest likelihood.

Composite interval mapping is a combination of simple interval mapping and multiple linear regressions, which increase the precision of QTL mapping by using linked markers as cofactors and reducing the residual variance by eliminating much of the genetic variance by other QTL, thereby increasing the power of QTL detection (Zeng, 1994 and Liu 1998).

QTL mapping in pigeonpea till date was focused on mapping the biotic and abiotic stresses like *Fusarium* wilt resistance (Bohra *et al.,* 2012), sterility mosaic disease resistance (Gnanesh *et al.,* 2011) and drought tolerance (Saxena *et al.,* 2011). Agronomic traits like plant height, days to maturity, days to flowering etc., were also being mapped (Kumawat *et al.,* 2012). The trait, seed Zn content which is being mapped in this study, is first of its kind in pigeonpea.

QTL analysis has been applied to biofortification traits in maize, and proved to be a powerful tool in clarifying the genetic basis and molecular-physiological mechanisms conferring high level of grain micronutrient (Lung'aho *et al.* 2011, Qin *et al.* 2012, Simic *et al.,* 2012, Zhou *et al.,* 2010). In the $F_{2:3}$ mapping population characterized by Jin *et al.,* (2013) discovered QTLs for grain Zn. The results demonstrated that QTL mapping of mineral content in maize grain is feasible and may be a practical approach to proceed with biofortification at the genetic and ultimately molecular level.

In the present study, a total of 25 QTL for seed Zn, leaf Zn and other traits (Table 14), were detected, which were positioned on to four linkage groups. Of the 25 detected QTLs, eight QTLs were detected for seed Zn content and two for leaf Zn content. While seven QTLs were detected for seed yield, three for stem girth, two for 100 seed weight, two for phytic acid and one for plant height. In a few instances, a given flanking marker region was associated with more than one related or unrelated traits representing the complexity of quantitative traits (Table 14). The qS_Zn1-1 QTL explained the maximum variation of 60.46 % for seed Zn content with a LOD score of 7.98. It was flanked by AHSSR188-AHSSR135. For leaf Zn content maximum variation of 7.5 % was explained by qS_Zn3-1 with a LOD score of 3.50 and was flanked by markers AHSSR24-AHSSR72. The QTLs qS_W6-1, qS_G1-2, qP_A3-1 and qS_Y1-3 explained 8.5 % (seed weight), 92.8 % (stem girth), 14.81 % (stem girth) and 21.47 % (seed yield) of variations respectively (Table 14).

Several reports are available on mapping QTL on seed or grain Zn content in various species. Blair *et al.,* (2010) evaluated the inheritance of seed zinc content in recombinant inbred line population of an intra-genepool Mesoamerican X Mesoamerican

to identify quantitative trait loci (QTL) for zinc. The population had 110 lines, derived from a high-seed zinc genotype (G14519) crossed with a low-mineral Carioca-type, (G4825). The QTL near BM158 could be considered to be a single major gene; or alternatively a tight cluster of genes controlling the concentrations of zinc.

In the present study zinc QTLs were collocated with seed yield and stem girth (Table 14). Lu *et al.,* 2008 found that many QTLs for mineral content detected in their study were also associated with QTLs for biomass, yield, and disease tolerance in rice investigated by other scientists with the same population. Lu *et al.,* (2008) explained the rice varieties rich in micronutrients often seems to have other desirable qualities, such as high yield, good tolerance to nutrition deficiency stress, and pest resistance. Moreover Gregorio *et al.,* (2000) found that the high-zinc seeds are nutritious not only for humans but also for the next generation of seedlings, which become more vigorous and better able to withstand weed competition, and pathogen and pest attack. It is also evident from Eva (1993) reports that mean content of Cd, Cu and Pb, per individual grain was exponentially correlated with grain size and weight. Therefore, it is not surprising to find overlapping of Zn QTL (qS_Zn1-1 and qS_Zn9-1) with seed yield QTL (qS_Y1-2 and qS_Y9-1) in the present study (Table 14 & Table 10). The co-localization of QTLs for several constituent traits was also observed by King *et al.,* 2014 in soyabean and Blair *et al.,* 2010 in common bean. It is evident from the observations of Albert *et al*., (1991), Paterson *et al*., (1991) and Xiao *et al*., (1996) that correlated traits often have QTL mapping to the same chromosomal locations. Based on these results we can assume that pleiotropism rather than close linkage of different QTL could be the main reason why QTL for different related traits were detected in the similar marker intervals across locations. Clustering of the QTL for different traits at the same marker intervals was observed and we also found several other traits association in the same marker interval.

Anuradha *et al.,* (2012) used 168 RILs and 101 SSRs to obtain the linked markers for seed Zn trait. They employed both SMA and CIM for linked markers detection, but there was no congruence of linked markers between SMA and CIM results. Interestingly, in the present investigation, SSR markers linked with Zn (AHSSR93, AHSSR118 and

ASSR280) using Single Marker Analysis coincided with the flanking markers of QTLs identified through CIM (Table 15).

The position interval of most of the QTLs in this study was large, which necessitates the need for adding more markers to saturate the QTL region. With the incorporation of more markers and larger population size, additional QTLs might be identified (Hackett *et al.,* 1998). Vales *et al.,* 2005 conducted a study on the effect of population size for the estimation of QTL in barley and reported that number of QTLs increases as the population increase. They also found that QTL with large effects can be detected with small population, but it is necessary to increase the population size to be able to detect QTLs with small effects. A large population size helps to determine locus order because several recombination events are needed to obtain statistical support for complete order. The present study used 188 F_2 lines which is a reasonably high number of lines for QTL analysis.

Different factors that influence the detection of QTLs in segregating population. First is the genetic property of the QTL itself. QTLs with large phenotypic effect will be normally selected. QTL with smaller effect may fall below the significance threshold level of detection. Another genetic factor is the distance between linked QTLs. QTLs that are closely linked (approximately 20 cM or less) may be detected as a single QTL in population below 500 numbers (Tanksley, 1993). Environment has profound impact on expression of QTL and experiment that are replicates across the sites and over the time may provide valuable insight into the environmental influences on traits of interest (Hittalmani et al., 2002; George et al., 2003). Design of the field experiment is crucial and larger the population size the more accurate will be the analysis allowing the detection of QTLs with small effects (Haley and Anderson, 1997). Genotyping error and missing data can affect the order and distance between markers on the linkage maps (Hackett, 2002). A dependable QTL map can be generated from a reliable phenotypic data. Therefore, accuracy of the phenotyping is of utmost importance.

In this attempt to detect QTLs, the composite interval mapping resulted in identifying the specific regions on the pegionpea genome that contributes to seed Zn

content and several other physiological traits. Major effect QTLs have been identified governing all the traits considered in this study. These QTLs can be effectively used in breeding to improve seed Zn content. However it would be more appropriate to saturate these QTL regions by using additional markers and validating their relevance.

5.5 Functional relevance of QTLs

In the present investigation the seed Zn QTL was linked with three genic SSR markers viz., ASSR20, ASSR148 and ASSR280. These are the SSR markers derived from the genes bZIP transcription factor (ASSR20), Ethylene responsive transcription factor (ASSR148) and Senescence inducible chloroplast stay green protein (ASSR280). The role of the bZIP transcription factor is well known for the regulation of Zn homeostasis under Zn deficiency in *Arabidopsis* (Assuncao *et al.,* 2010). These transcription factors (bZIP19 and bZIP23) recognize 8 to 10 bp palindromic motifs called zinc deficiency response elements, found in tandem in promoters of several zinc homeostasis genes like ZIP transporters, NAS etc., activation of which constitutes the primary response to zinc deficiency (Assuncao *et al.,* 2010).

Chandel *et al.,* (2011) identified candidate genes for grain Fe and Zn underlying the target QTL regions in rice. They reported 8 genes related to Fe and Zn from different protein families. In addition, they also found genes belonging to ZIP, zinc transporter family (zinc-regulated transporter/iron regulated transporter proteins), 2Fe–2S iron–sulfur cluster binding domain, and major facilitator super-family on chromosomes 1, 9 and 11.

As the availability of rice genome sequence has opened up the possibility of identifying candidate genes, genomic regions encompassing the QTLs contributing to Fe and Zn in rice were analyzed for the presence of candidate genes by Anuradha *et al.,* 2012. They detected ten genes related to iron and zinc concentrations to be present within selected QTLs on chromosomes 1, 3, 5, 7 and 12. These ten Fe and Zn related genes were OsYSL1, OsARD2, OsIRT1, OsNAS1, OsNAS2, OsMPT1, OsNAS3, OsNRAMP1, Heavy metal ion transporter and OsAPRT belonging to different protein families. The OsYSL genes are known as components of Strategy II of metal transport found in cereals, encoding oligopeptide phytosiderophore transporter proteins (Curie *et al.,* 2001; Gross *et*

al., 2003). OsNRAMP1 showed positive correlation with final Zn concentration in the seeds. OsNRAMP1 functions as a metal efflux transporter participating in the export of metals from the vacuolar compartment to the cytosol, resulting in increased metal concentration available to be transported to the seeds (Sperotto *et al.,* 2010).

Summary

VI SUMMARY

Zinc is a plant micronutrient which is involved in many physiological functions, its inadequate supply will reduce crop yields (Doncheva *et al.,* 2001; Stoyanova and Doncheva, 2002; Di Baccio *et al.*, 2005; Broadley *et al.*, 2007). Besides its role in crop production, Zn plays a part in the basic roles of cellular functions in all living organisms and is involved in improving the human immune system (Aydemir *et al.*, 2006). Amongst micronutrients, zinc deficiency is widespread globally in human population. According to WHO estimates nearly 49% of population is deficient in zinc. Besides fortification with Zn, the most efficient way to provide zinc to people is through the food they consume; from this context biofortification of zinc by developing crop varieties/hybrids with higher seed zinc levels assumes significance. Further it is crucial to improve zinc levels in grains which are often consumed in relatively larger quantities like cereals and pulses. Besides cereals it is also important to improve the seed zinc levels in pulses, especially pigeonpea, which is widely consumed in India.

Zn content is a complex trait and has polygenic inheritance. Little information is available about the genetic control and molecular- physiological mechanisms contributing to high accumulation of micro-nutrients in the grain. QTL analysis proves a powerful tool in agricultural studies, pointing out the chromosomal location of genes suitable for breeding programmes. There is considerable varietal and species variation for Zn uptake and accumulation by plants (Holm *et al.,* 2002). Hence exploiting this natural genetic variation for developing mineral dense crop varieties needs a strategic approach (Pfeiffer and McClafferty, 2007).

In this direction, genetic variability for seed zinc levels in 217 pigeon pea germplasm accessions was accessed (Manoa, 2010). Based on their seed zinc content these genotypes were grouped into high zinc types and low zinc types (15 in each group). All these high Zn and low Zn genotypes were subjected to diversity analysis using 50 SSR markers. Phenogram of 30 genotypes based on genotypic values of SSR markers depicted that the parental lines (Five high Zn and five low Zn) selected for crossing programme were differentially sub-clustered except for the genotypes ICP6974 and

ICP8863. Straight crosses were made between high and low Zn genotypes and also BRG-1 an elite variety was crossed with high Zn parent ICP6443. Ultimately the cross ICP6443 X ICP10960 and BRG1 X ICP6443 were selected based on their percent SSR polymorphism and advanced to F_2 mapping population and BC_1F_2 backcross population respectively. Thus two types of mapping populations were developed, of them F_2 population was used for linkage and QTL analysis.

F_2 derived from ICP6443 and ICP10960, segregating for seed Zn was phenotyped and we observed marked genotypic variation for measured traits. This suggests the availability of larger genetic variability of traits in parents, which is the preliminary requisite to develop mapping population with different allele combinations. The F_2 was characterized for morpho-physiological parameters like plant height, no. of branches, SPAD chlorophyll meter readings, stem weight, stem girth, leaf length, leaf width, and specific leaf area and found a significant variation among the individuals. There were significant positive correlations found between most of the morpho-physiologycal parameters in F_2 and F_3 lines.

The F_2 derived F_3 seeds were used for the characterization of mapping population for seed Zn content, seed Fe content, Phytic acid content, yield and yield related traits. The seed Zn content and other parameters were distributed around the mean confirming their quantitative nature. All the traits were normally distributed and a slightly skewed towards the high Zn parent ICP6443. The Genetic parameters like GCV, PCV, H^2 and GA revealed that seed Zn and other parameters showed higher heritability and high genetic advance, while phytic acid content exhibited relatively lower heritability but a moderate level of genetic advance. Invariably the PCV was higher than GCV for all traits, indicating the presence of a significant environmental influence on the expression of traits.

While in the correlation studies out of 136 pair wise combination of 17 traits, 35 combinations showed significant correlations, while two of the combinations were biologically significant correlations. The seed Zn content was significantly and positively correlated to the leaf Zn content and seed weight in the present investigation.

Transgressive segregation is one of the major factors indicating the performance of the lines. $F_{2:3}$ also enabled the detection and fixation of the transgressive segregants of the magnitude lower or greater than either of the parents as a consequence of congregation of QTLs with negative and positive effects respectively. It was interesting to note that transgressive segregants were observed for all the measured traits revealing the shuffling of parental genomes with respect to performance. Further the whole mapping population was categorized into four contrast groups i.e., high leaf Zn high seed Zn types(HLHS), high leaf Zn low seed Zn types(HLLS) low leaf Zn high seed Zn types(LLHS) and low leaf Zn low seed Zn(LLLS) types. This classification would be useful in selecting the agronomically best performing lines of mapping population with high Zn status from the HLHS contrast group.

With the major objective of identifying the genomic regions (QTLs) associated with these relevant traits, the genomic DNA of 188 F_2 lines were assessed for polymorphism by using co-dominant DNA markers such as genic and genomic SSRs. The polymorphic information content of 151 polymorphic marker loci, were used for construction of linkage map of Pigeonpea. The multipoint linkage analysis performed resulted in specific linkage map of 62 markers in 10 linkage groups with a total map length of 1942.8 cM. The number of marker loci distributed in the linkage group ranged from 2 to 29 (in LG-3) with an average interval distance of 37.36 cM.

Single marker analysis revealed association of 16 markers to various traits. By composite interval mapping 25 QTLs were detected, eight for seed Zn content, and two for leaf Zn content. While seven QTLs were detected for seed yield, three for stem girth, two for 100 seed weight, two for Phytic acid and one for plant height. Three QTLs for seed Zn content explained greater phenotypic variation on the LG 1, 3 and 9 which were responsible for 18.22 %, 60.46 % and 52.35 % of phenotypic variations respectively. The three QTLs qS_Zn1-1, qS_Zn3-2 and qS_Zn9-1 were flanked by AHSSR188-AHSSR135; ASSR120-ASSR313 and AHSSR53-AHSSR74 SSR markers respectively. AHSSR93, AHSSR118 and ASSR 280 were common markers between SMA and CIM linked to seed Zn. Interestingly, some of the genic SSRs viz., bZIP transcription factor

(ASSR20), Ethylene responsive transcription factor (ASSR148) and Senescence inducible chloroplast stay green protein (ASSR280) were linked to seed Zn content.

Salient findings can be summarized as follows

- The mapping population segregating for seed Zn content is first of its kind to be developed in Pigeonpea.
- Significant phenotypic variability was noticed for the seed Zn and other traits in the population under study.
- Several transgressive segregates with high seed zinc content were present which could be potential genetic resources.
- QTLs for seed Zn, leaf Zn and yield traits were identified using linkage mapping.
- Several pleiotropic markers were identified for differently related traits in QTL analysis.
- Single marker analysis and composite interval mapping resulted in discovery of common markers (AHSSR93, AHSSR118 and ASSR280) in both the methods for seed Zn content.
- A potential genic marker bZIP transcription factor (ASSR20), speculated to regulate Zn deficiency response in *Arabidopsis* was found to be linked to seed Zn content.

Future line of work

- Advancement of F_3 population of Pigeonpea to develop RILs.
- Screening of the mapping population across regions for seed Zn content to check the consistency of the lines.
- Saturation of linkage map by adding more markers to identify closely linked marker. SNP genotyping is a desirable option.
- Fine mapping of the QTL region
- Developing populations from other crosses will be highly useful for validation of QTL's mapped in this study.
- In-silico validation of genes and QTLs by comparative genomics.
- BC_1F_2 can be further backcrossed with the recurrent parent to develop trait introgressed lines.

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Appendices

APPENDIX-I

Summary statistics of molecular diversity in 30 pigeonpea genotypes.

Marker	AlleleNo	GeneDiversity	Heterozygosity	PIC
ASSR 1	2.0	0.34	0.07	0.30
ASSR 108	2.0	0.02	0.00	0.02
ASSR 109	2.0	0.03	0.01	0.03
ASSR 11	2.0	0.03	0.00	0.03
ASSR 120	2.0	0.03	0.01	0.03
ASSR 148	3.0	0.43	0.00	0.35
ASSR 17	2.0	0.02	0.00	0.03
ASSR 19	3.0	0.18	0.01	0.15
ASSR 20	3.0	0.14	0.07	0.13
ASSR 23	2.0	0.43	0.23	0.34
ASSR 379	3.0	0.06	0.01	0.06
ASSR 66	3.0	0.04	0.01	0.04
ASSR 77	3.0	0.02	0.00	0.03
ASSR 9	2.0	0.02	0.00	0.03
AHSSR 114	6.0	0.62	0.16	0.54
AHSSR 126	5.0	0.51	0.02	0.46
AHSSR 128	2.0	0.46	0.19	0.35
AHSSR 160	5.0	0.59	0.10	0.55
AHSSR 163	6.0	0.62	0.14	0.57
AHSSR 164	2.0	0.25	0.00	0.23
AHSSR 199	7.0	0.76	0.31	0.75
AHSSR 20	3.0	0.47	0.17	0.34
AHSSR 21	4.0	0.51	0.54	0.40
AHSSR 228	4.0	0.32	0.07	0.29
AHSSR 23	4.0	0.58	0.21	0.50
AHSSR 233	4.0	0.50	0.14	0.42
AHSSR 235	3.0	0.20	0.05	0.18
AHSSR 236	4.0	0.50	0.10	0.40

Marker	AlleleNo	GeneDiversity	Heterozygosity	PIC
AHSSR 237	8.0	0.79	0.20	0.76
AHSSR 239	5.0	0.27	0.23	0.25
AHSSR 249	4.0	0.53	0.31	0.43
AHSSR 263	4.0	0.47	0.22	0.42
AHSSR 265	5.0	0.29	0.04	0.26
AHSSR 269	3.0	0.54	0.33	0.47
AHSSR 271	4.0	0.57	0.16	0.49
AHSSR 29	4.0	0.46	0.14	0.42
AHSSR 301	4.0	0.49	0.28	0.45
AHSSR 302	6.0	0.50	0.15	0.40
AHSSR 303	5.0	0.63	0.44	0.58
AHSSR 305	5.0	0.35	0.10	0.32
AHSSR 42	4.0	0.17	0.01	0.16
AHSSR 45	5.0	0.56	0.30	0.47
AHSSR 59	4.0	0.33	0.03	0.30
AHSSR 89	3.0	0.46	0.20	0.36
AHSSR 90	5.0	0.64	0.18	0.58
AHSSR 92	5.0	0.58	0.12	0.52
AHSSR 95	4.0	0.54	0.10	0.45
AHSSR 99	5.0	0.59	0.17	0.52
Mean	3.9	0.39	0.13	0.34

Note: *AHSSR-Arhar Highly variable Simple Sequence repeat
*ASSR- Arhar Simple Sequence repeat

APPENDIX-II

Dissimilarity indices for contrasting pigeonpea genotypes using DARwin V 5.0.

	ICP6443	**ICP6974**	**ICP6992**	**ICP7118**	**ICP8513**	**ICP8602**	**ICP8863**	**ICP10654**	**ICP10960**
ICP6974	0.54								
ICP6992	0.63	0.50							
ICP7118	0.58	0.37	0.37						
ICP8513	0.46	0.44	0.50	0.46					
ICP8602	0.63	0.58	0.58	0.62	0.46				
ICP8863	0.54	0.44	0.63	0.42	0.46	0.44			
ICP10654	0.62	0.46	0.52	0.50	0.46	0.54	0.48		
ICP10960	0.73	0.63	0.69	0.65	0.62	0.56	0.52	0.60	
ICP14954	0.63	0.73	0.73	0.79	0.62	0.65	0.62	0.73	0.44

APPENDIX-III

List of genic SSR markers polymorphic between ICP6443 and ICP10960

Sl No.	Primer name	SSR motif	Tm
1	ASSR1	(GA)10	55
2	ASSR3	(AGAAAG)5	50
3	ASSR5	(AAATT)6	50
4	ASSR11	(CTC)7	55
5	ASSR13	(ATTAG)5	55
6	ASSR19	(TGTTCA)5	55
7	ASSR20	(AT)11	55
8	ASSR23	(CCTTCT)5	55
9	ASSR48	(AAGAGG)6	55
10	ASSR70	(GGTAGA)6	50
11	ASSR93	(CATTTG)5	55
12	ASSR97	(ATGGAC)8	55
13	ASSR100	(GGT)7	50
14	ASSR108	(GAT)7	55
15	ASSR230	(GAGCAT)9	55
16	ASSR236	(ACTAGC)10	55
17	ASSR280	(TGGCAT)5	55
18	ASSR304	(GTT)7	50
19	ASSR317	(GAGCAT)9	50
20	ASSR363	(GCATCA)5	50
21	ASSR366	(CGT)8	50
22	ASSR416	(TGA)6	50

APPENDIX-IV

List of genic SSR markers polymorphic between ICP6443 and BRG1

Sl. No.	Primer name	SSR motif	Tm
1	ASSR1	(GA)10	55
2	ASSR5	(AAATT)6	50
3	ASSR11	(CTC)7	55
4	ASSR13	(ATTAG)5	55
5	ASSR17	(CCTTCT)6	55
6	ASSR19	(TGTTCA)5	55
7	ASSR20	(AT)11	55
8	ASSR23	(CCTTCT)5	55
9	ASSR48	(AAGAGG)6	55
10	ASSR93	(CATTTG)5	55
11	ASSR97	(ATGGAC)8	55
12	ASSR230	(GAGCAT)9	55
13	ASSR236	(ACTAGC)10	55
14	ASSR280	(TGGCAT)5	55
15	ASSR366	(CGT)8	50

APPENDIX-V

List of genomic SSR markers polymorphic between ICP6443 and ICP10960

Sl. No.	SSR ID	Sl. No.	SSR ID	Sl. No.	SSR ID
1	AHSSR2	44	AHSSR89	87	AHSSR282
2	AHSSR4	45	AHSSR90	88	AHSSR283
3	AHSSR7	46	AHSSR92	89	AHSSR284
4	AHSSR8	47	AHSSR93	90	AHSSR285
5	AHSSR9	48	AHSSR95	91	AHSSR287
6	AHSSR12	49	AHSSR99	92	AHSSR299
7	AHSSR13	50	AHSSR104	93	AHSSR304
8	AHSSR14	51	AHSSR110	94	AHSSR305
9	AHSSR15	52	AHSSR114	95	AHSSR308
10	AHSSR17	53	AHSSR115	96	AHSSR310
11	AHSSR18	54	AHSSR118	97	AHSSR313
12	AHSSR19	55	AHSSR120	98	AHSSR360
13	AHSSR20	56	AHSSR121	99	AHSSR443
14	AHSSR21	57	AHSSR123	100	AHSSR463
15	AHSSR22	58	AHSSR124	101	AHSSR189
16	AHSSR23	59	AHSSR127	102	AHSSR192
17	AHSSR24	60	AHSSR134	103	AHSSR195
18	AHSSR25	61	AHSSR135	104	AHSSR204
19	AHSSR26	62	AHSSR142	105	AHSSR216
20	AHSSR27	63	AHSSR146	106	AHSSR234
21	AHSSR28	64	AHSSR151	107	AHSSR244

Sl. No.	SSR ID	Sl. No.	SSR ID	Sl. No.	SSR ID
22	AHSSR29	65	AHSSR153	108	AHSSR248
23	AHSSR32	66	AHSSR155	109	AHSSR261
24	AHSSR37	67	AHSSR162	110	AHSSR274
25	AHSSR39	68	AHSSR163	111	AHSSR279
26	AHSSR40	69	AHSSR164	112	AHSSR294
27	AHSSR44	70	AHSSR165	113	AHSSR297
28	AHSSR45	71	AHSSR170	114	AHSSR344
29	AHSSR46	72	AHSSR177	115	AHSSR346
30	AHSSR47	73	AHSSR179	116	AHSSR347
31	AHSSR51	74	AHSSR180	117	AHSSR350
32	AHSSR52	75	AHSSR181	118	AHSSR353
33	AHSSR53	76	AHSSR188	119	AHSSR364
34	AHSSR55	77	AHSSR199	120	AHSSR367
35	AHSSR61	78	AHSSR210	121	AHSSR371
36	AHSSR64	79	AHSSR213	122	AHSSR376
37	AHSSR67	80	AHSSR228	123	AHSSR377
38	AHSSR72	81	AHSSR230	124	AHSSR382
39	AHSSR73	82	AHSSR236	125	AHSSR386
40	AHSSR74	83	AHSSR240	126	AHSSR389
41	AHSSR75	84	AHSSR242	127	AHSSR397
42	AHSSR82	85	AHSSR263	128	AHSSR391
43	AHSSR86	86	AHSSR277	129	AHSSR434

Note: Information regarding SSR motif and primer sequences referred from Dutta *et al.*, 2013