Discovery of DNA markers associated with root traits in Ragi [*Eleusine coracana* (L.) Gaertn] through phenotypic characterization and selective genotyping of a RIL population

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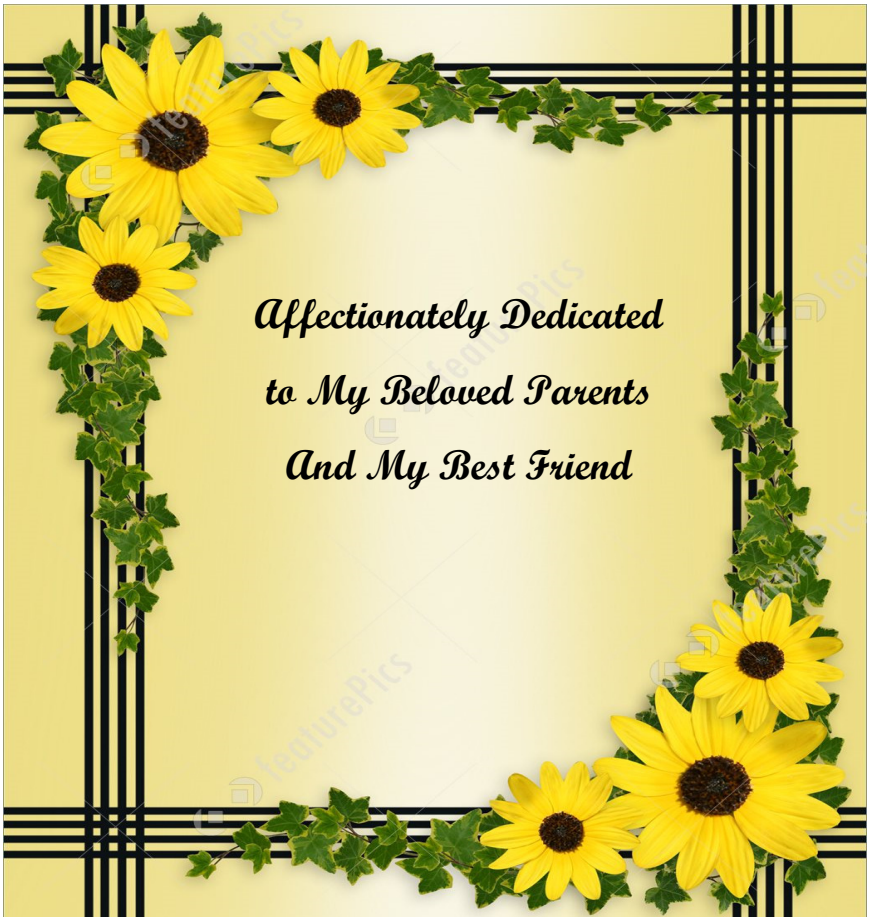
Affectionately Dedicated
to My Beloved Parents
And My Best Friend

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU-560 065

CERTIFICATE

This is to certify that the thesis entitled **"Discovery of DNA markers associated with root traits in Ragi [*Eleusine coracana* (L.) Gaertn] through phenotypic characterization and selective genotyping of a RIL population"** submitted in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture) in CROP PHYSIOLOGY** to the University of Agricultural sciences Bengaluru, is a record of research work done by **Ms. MAHALAKSHMI, M. N. ID.NO.PALB 7225** during the period of her study in this university under my guidance and supervision and that no part of thesis has been submitted for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
August, 2019

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August, 2018 Mahalakshmi, M. N.

Discovery of DNA markers associated with root traits in Ragi [*Eleusine coracana* (L.) Gaertn] through phenotypic characterization and selective genotyping of a RIL population

MAHALAKSHMI, M. N.

Abstract

Ragi is an important millet grown and consumed predominantly in the arid and semiarid regions of the world. Since major area of ragi cultivation is under rainfed conditions its yield is low, increasing its production by improving drought adoptive traits such as root is important. However, screening for these traits is cumbersome. Therefore, our current research was focused on development of high throughput phenotyping methods for measuring root traits. Two genotypes were grown on clerigel, and coirpith to select a best root phenotyping medium. Among these, genotypes grown on the coirpith gave the good result, in which, average root length of parent 1 was 30 cm on 25th day, while the other parent reached only 22.3 cm.

A mapping population comprising of 206 Recombinant inbreed lines were screened for roots by growing them in root structures. At booting stage root traits were measured by dismantling the structure. Root length of the high root parent was 45.29 cm, while it was 36.29 cm for low parent. Root biomass of the RIL population varied from 1.25 g pl^{-1} to 6.35 g pl^{-1} with a mean of 3.36 g pl^{-1}. Bulk segregant analysis was adopted to identify the markers associated with root traits. Among the 102 SSRs polymorphic between the parental lines, three markers UASBFM 85, UASBFM 91 and UASBFM 109 clearly showed specific root related segregation pattern in bulked segregant analysis. These SSR markers can be considered as useful markers for genotyping root traits in ragi.

August,2019

Department of Crop Physiology
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(M. S. SHESHSHAYEE)
Major Advisor

ಫಿಗೋಟೈಪಿಕ್ ಕ್ಯಾರೆಕ್ಟರೈಸೇಶನ್ ಮತ್ತು ರಿಲ್ ಜನಸಂಖ್ಯೆಯ ಆಯ್ದ ಜಿನೋಟೈಪಿಂಗ್ ಮೂಲಕ ರಾಗಿ [*ಎಲ್ಯುಸಿನ ಕೊರಾಕಾನಾ* (ಎಲ್.) ಗೇರ್ಟ್ನ್.] ನಲ್ಲಿನ ಮೂಲ ಗುಣಲಕ್ಷಣಗಳೊಂದಿಗೆ ಸಂಬಂಧಿಸಿದ ಡಿ.ಎನ್.ಎ ಗುರುತುಕಾರಕಗಳ ಆವಿಷ್ಕಾರ.

ಪ್ರಬಂಧ ಸಾರಾಂಶ

ಮಹಾಲಕ್ಷ್ಮಿ, ಎಂ. ಎನ್.

ರಾಗಿ ಒಂದು ಪ್ರಮುಖ ಏಕದಳ ಧನ್ಯವಾಗಿದ್ದು, ಬೇರೆ ಧಾನ್ಯಗಳಿಗೆ ಹೋಲಿಸಿದರೆ ರಾಗಿ ಕಾಳುಗಳು ಅಪಾರವಾದ ಕ್ಯಾಲ್ಸಿಯಂ ಮತ್ತು ಕಬ್ಬಿಣದ ಅಂಶವನ್ನು ಹೊಂದಿರುತ್ತವೆ. ರಾಗಿಯನ್ನು ಪ್ರಮುಖವಾಗಿ ಶುಷ್ಕ ಮತ್ತು ಅರೆಶುಷ್ಕ ಪ್ರದೇಶಗಳಲ್ಲಿ ಬೆಳೆಯಲಾಗುತ್ತದೆ. ಮಳೆಯಾಶ್ರಿತ ಪ್ರದೇಶಗಳಲ್ಲಿ ಬೆಳೆಯಾಗುತ್ತಿದ್ದರಿಂದ ರಾಗಿ ಬೆಳೆಯಲ್ಲಿ ಕಡಿಮೆ ಇಳುವರಿ ಕಂಡುಬರುತ್ತಿದೆ. ಬರ ಪರಿಸ್ಥಿತಿಗೆ ಹೊಂದಿಕೊಳ್ಳುವಂತಹ ರಾಗಿ ತಳಿಗಳನ್ನು ಬೆಳೆಯುವುದರಿಂದ ಉತ್ತಮ ಇಳುವರಿಯನ್ನು ಪಡೆಯಬಹುದಾಗಿದೆ.

ಬೇರುಗಳು ಗಿಡಗಳ ಪ್ರಮುಖವಾದ ಅಂಗವಾಗಿದ್ದು, ಬರ ಪರಿಸ್ಥಿತಿಯಲ್ಲಿ ಭೂಮಿಯ ಆಳದಿಂದ ನೀರನ್ನು ಬಗೆದು ಗಿಡಗಳನ್ನು ಪೋಷಿಸುತ್ತದೆ. ಬೇರಿನ ಗುಣಲಕ್ಷಣಗಳನ್ನು ಆಧುನಿಕ ಮಾಲಿಕ್ಯುಲಾರ್ ತಳಿ ಅಭಿವೃದ್ಧಿ ತಂತ್ರವನ್ನು ಬಳಸಿ ಅಭಿವೃದ್ಧಿಪಡಿಸಬೇಕಾಗುತ್ತದೆ. ರಿಕಾಂಬಿನೆಂಟ್ ಇನ್ಬ್ರೆಡ್ ತಳಿಗಳ ಬೇರುಗಳ ಗುಣಲಕ್ಷಣಗಳನ್ನು ತಿಳಿದುಕೊಳ್ಳಲು ನಾವು ಹೈ ತ್ರೋಪುಟ್ ವಿಧಾನವನ್ನು ಬಳಸಿದ್ದೇವೆ. ಅವುಗಳೆಂದರೆ ಕ್ಲೇರಿಜೆಲ್ ಮತ್ತು ಕಾಇರ್ಪಿತ್ ನಲ್ಲಿ ೨೫ ದಿನಗಳವರೆಗೆ ಗಿಡಗಳನ್ನು ಬೆಳೆಯಲಾಗಿದ್ದು, ಈ ಪ್ರಯೋಗವು ಉತ್ತಮ ಫಲಿತಂಶವನ್ನು ನೀಡಿತು.

ನಂತರ ಎಲ್ಲಾ ರಿಕಾಂಬಿನೆಂಟ್ ಇನ್ಬ್ರೆಡ್ ತಳಿಗಳನ್ನು ಬೇರು ಮಾಪನ ಸೌಲಭ್ಯದಲ್ಲಿ ಬೆಳೆದಿದ್ದು, ಬೇರಿನ ಫೆನೊಟೈಪಿಂಗನ್ನು ಮಾಡಲಾಗಿದೆ. ಈ ನಿಟ್ಟಿನಲ್ಲಿ ಬೇರಿನ ಗುಣಲಕ್ಷಣಗಳಿಗೆ ಹೊಂದಿಕೊಂಡ ಡಿ.ಏನ್.ಏ ಗುರುತುಕಾರಕಗಳನ್ನು Bulked segregant analysis ಮೂಲಕ ಪತ್ತೆಹಚ್ಚಲಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಮೂರು ಗುರುತುಕಾರಕಗಳಾದ UASBFM 85, UASBFM 91, UASBFM 109 ಗಳು ಬೇರಿನ ಗುಣಲಕ್ಷಣಗಳಿಗೆ ಹೊಂದಿಕೊಂಡಿರುವುದು ತಿಳಿದುಬಂದಿದೆ. ಈ SSR ಗುರುತುಕಾರಕಗಳು ಮುಂದೆ ರಾಗಿ ಬೇರಿನ ಗುಣಲಕ್ಷಣಗಳನ್ನು ಜೀನೋಟೈಪಿಂಗ್ ಮಾಡುವಲ್ಲಿ ಸಹಕಾರಿಯಾಗಲಿವೆ.

ಆಗಸ್ಟ್, ೨೦೧೯

ಬೆಳೆ ಶರೀರಕ್ರಿಯಾಶಾಸ್ತ್ರ ವಿಭಾಗ
ಕೃಷಿ ವಿಶ್ವ ವಿದ್ಯಾನಿಲಯ, ಜಿ. ಕೆ. ವಿ. ಕೆ. ಬೆಂಗಳೂರು.

ಎಮ್. ಎಸ್. ಶೇಷಾಶಾಯೀ
(ಮುಖ್ಯ ಸಲಹೆಗಾರರು)

Discovery of molecular markers associated with root system architecture and its component traits through characterization of RIL population by selective genotyping in Ragi [*Eleusine coracana* (L.) Gaertn]

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INTRODUCTION

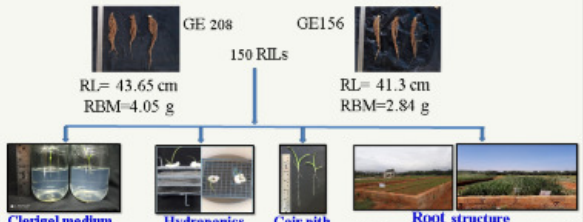
Growth and productivity of crops are often affected by biotic and abiotic stresses. Under drought conditions, tolerant plants show strategic morphological changes like deep root growth to harvest water in the deeper layers of soil to meet transpirational demand. Such root characters are generally being governed by functional QTLs. Identifying such agronomically significant QTLs is important for crop improvement. The major objective of the study is to develop a linkage map and identify QTLs for root and other drought adaptive traits in Ragi. RIL (F5) population segregating for drought adaptive traits is available at small millet improvement programme, UASB,GKVK from the cross between GE 208 (Drought tolerant) and GE156 (Drought susceptible). This population was extensively phenotyped for root and other agronomically important traits in a specialized root structures during Kharif 2018. Genotyping of 150 RILs is being carried out by using genomic SSR markers

OBJECTIVES

1. Development of appropriate HTP (high throughput Phenotyping) approaches for RSA (Root System Architecture) traits.
2. Phenotyping of a Biparental Mapping population to screen for variability in RSA traits.

MATERIALS AND METHODS

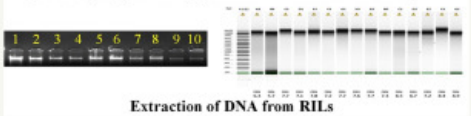
High throughput phenotyping for root system architecture



15 days old seedlings were planted in Root structures

Clerigel, hydroponics and coir pith medium were used for phenotyping for RSA at seedling stage and Root structure at grand growth stage. Root growth pattern and its variations can be mapped by this strategy

Genotyping of RIL population with SSR markers



Extraction of DNA from RILs

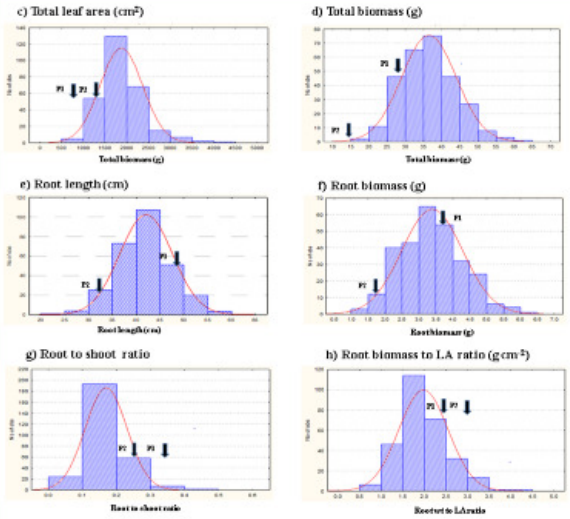
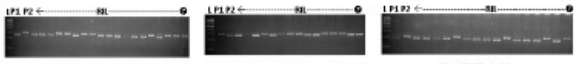


Fig 1: Transgressive segregation in several traits among RILS and parents.

SSR genotyping of RILs :



FMg8SR 73465 UASBFM32 UASBFM 25

Fig 2: Representative gels resolving polymorphic SSR markers among 150 RILs

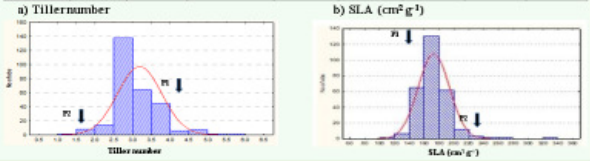
RESULTS

Field phenotyping of RILs:

Table 1: Genetic variability in various growth and drought adaptive traits among RILs

	Tiller number (No.)	SLA ($cm^2 g^{-1}$)	Total leaf area (cm^2)	Total biomass (g)	Root length (cm)	Root biomass (g)	Root to shoot ratio	Root biomass to LA ratio ($g cm^{-2}$)
GE 208	4.00	180.07	1707.14	30.45	47.83	3.57	0.20	1.94
GE 156	1.33	250.82	1715.40	8.17	34.00	1.94	0.19	2.01
Max	5.67	334.43	4382.31	60.88	57.67	6.35	0.48	4.44
Min	1.33	101.64	574.34	17.78	23.67	1.26	0.05	0.63
Mean	3.17	172.35	1863.31	36.40	41.96	3.37	0.17	1.97
SD	0.58	20.96	488.38	7.51	5.54	0.90	0.06	0.57
CV%	18.42	12.64	26.21	20.64	13.20	26.58	35.77	28.63
CD @ 5%	0.61*	31.3*	156.4*	8.3*	5.6*	1.1*	0.03*	0.91*

a) Tillernumber b) SLA ($cm^2 g^{-1}$)



DISCUSSION

Specific morpho-physiological traits associated with water mining and WUE are most relevant to improve crop performance under water limited conditions. Yields of rainfed crops such as ragi can be significantly improved when these traits are introgressed. Introgression of these complex morpho-physiological traits can be best achieved by adopting molecular breeding approaches. RIL population developed by crossing GE208 and GE156 represented a significant variation in root (length and biomass) and canopy traits (SLA and Total leaf area). The RIL population showed normal distribution for these traits with a good numbers of transgressive segregants. RIL with high root biomass and SLA showed high growth rate at comparable leaf area. Genotyping is being done using SSR markers which will lead to marker development to specific drought adoptive traits.

SUMMARY

- Parents of RIL differed in root and other drought adaptive traits.
- RIL segregated for drought adaptive traits revealing quantitative inheritance.
- Several transgressive segregants have been identified.
- DNA polymorphism among RILs is in progress.

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LIST OF ABBREVIATIONS

RIL	Recombinant Inbred Line
QTL	Quantitative Trait Loci
NGS	Next Generation Sequencing
BSA	Bulked Segregant Analysis
HTP	High Throughput Phenotyping
SSD	Single Seed Descent
ARBD	Augmented Randomized Block Design
WUE	Water Use Efficiency
SSR	Simple Sequence Repeats
CTAB	Cetyl Trimethyl Ammonium Bromide

I INTRODUCTION

Millets are major nutria food sources widely consumed in Indian and African subcontinent. Finger millet, [*Eleusine coracana* (L.) Gaertn] called as Ragi is the fourth important and extensively cultivated millet in India after sorghum, pearl and foxtail millet. Its wider adaptability to diverse agro-climatic and water deficit condition allowed its presence ubiquitously in diverse places. Superior nutritive index of finger millet both at whole plant level and in grains makes it better food source for human consumption and fodder for rumens. Among cereals, ragi provides highest quantity of calcium, antioxidants, dietary fibers (18%), protein (6–13%), minerals (2.5–3.5%), phenolics (0.3-3%) and photochemicals (Ishwar *et al.,* 2016).

Finger millet is an allopolyploid with the chromosome number 2n=4x=36 and evolved from a cross between two diploid species, *Eleusine indica* (AA genome donor) and *Eleusine floccifolia*or *E. tristachya* (BB genome donor).

Ragi is an important millet in the southern states of India.The total area coveredunder finger millet cultivation in Karnataka is around 7.5 lakh hectare and with the production of 15.37 lakh tons and the productivity of 2050 Kg/ha (Anon, 2013). This observed yield levels of ragi are far less than the actual yield potential. This gap is due toincreased cultivation under rainfed conditions, which is prone to drought stress at critical growth stages. Plants which are exposed to drought or moisture stress undergo several physiological and metabolic changes. Photosynthetic activity of plants decreases because of closing of stomata and the decreased activity of photosynthetic enzymes.

Water deficit condition causes low and/or unstable yield and theeffectdepends on the growth stages of crop. Water deficit condition causes reduced plant height, culms per plant, leaf area, grain yield components like panicles per plant, filled spikelets per plant and 100-grain weight. Stress during early grain filling stage causes 77% yield loss (Singh, 1991).

Roots, the major organ of a plant which facilitates numerous functions including water and nutrient uptake that make it difficult to overlook its importance to plant productivity. The constitutive traits like root depth, thickness and root system architecture

have been positively correlated with drought tolerance by enhanced water harvesting through deep and fast growing root system (Tardieu, 2011). Breeders are interested to breed plants with superior root traits that improve productivity under drought. Hence, there is a need of know root functional traits and how these traits are related to whole plant strategies to increase crop productivity under drought conditions. There are many root traits which are associated with maintaining plant productivity under drought stress condition, like root length, small fine root diameters, long specific root length, and considerable root length, especially at different soil depths with different levels of available water.

Total root biomass in early growth stages has been shown to significantly contribute to seed yield under terminal drought (Louise *et al*., 2013). Maximizing the absorption of water stored in 15–30 cm soil layer by roots had greater importance as theavailable soil water and root size matches well for the complete use of water from this zone. However, greater exploitation of subsoil water and deeper root systems offers potential for further productivity improvements under terminal drought stress conditions (Kashiwagi *et al*., 2015).

Currently very few number of improved ragi varieties are available for cultivation. There is sufficient scope for identification of the traits associated with drought tolerance which can enhance the yield of the ragi crop. The crop improvement programme involves breeding of superior and high yielding cultivars by utilizing diverse germplasm resources and exploiting the variability in phenotypic characters and associations among them. Improvement in these phenotypic traits depends on the nature and degree of association between the traitsand the genes. Genetic diversity offers opportunities to utilize various genomic resources and technologies to manipulate traits of interest. A rich diversity is existing in land races and these can be explored for their desirable traits and can be further utilized to develop new varieties through molecular plant breeding approaches.

Molecular plant breeding employs various modern approaches like using of molecular makers, which are used to detect variations in the nucleotides of genomic DNA. next-generation sequencing (NGS) technologies has conferred new opportunities

for high-throughput genotyping in various plant species. Recent improvements in high-throughput sequencing have enabled sequences to be used to detect and score single nucleotide polymorphisms (SNPs) by bypassing the time-consuming process of marker development. But still, microsatellite markers are widely employed of their codominance of alleles, high genomic abundance and random distribution throughout the genome (Norton *et al*., 2010). In general, microsatellite show a high level of polymorphism, so they are very informative.

Following approaches are being widely used for the identification of QTLs. One of the trending approach is the association mapping (AM). Genome wide association studies (GWAS) is emerging as a method of QTL detection which surpasses the need for crossing and linkage map construction (Jung and Mc Couch, 2013). It focuses on the association within the populations of unrelated individuals, which includes a collection of diverse accessions viz., varieties, landraces and breeding lines without generating mapping population. (Flint Garcia*et al.,* 2003; Yu and Buckler, 2006; Rafalski,2010). The other equally important approach is QTL identification by using bi-parental mapping population. This gives the QTLs for respective traits and it is usually carried out by genotyping large number of progenies developed by crossing the genotypes contrasting for trait of interest.

Bulked segregant analysis is a simple approach of identifying QTLs associated with a particular trait, which works with selected and pooled individuals which are contrasting for phenotypic characters, which has been extensively used in gene mapping through bulked segregant analysis with biparental mapping populations, mapping by sequencing with major gene mutants and pooled genome-wide association study using extreme variants. Compared to conventional population analysis, bulked sample analysis significantly reduces the scale and cost by simplifying the procedure (Zou *et al*., 2016). Bulked segregant analysis (BSA) serves as an affordable strategy for mapping large effect QTLs by genotyping only selected extreme phenotypes instead of the entire mapping population (Arvindkumar *et al*., 2011).

Hence in the present study, bulked segregant analysis approach was used genotype SSR marker to discover markers associated with root traits. The objectives of the study are listed below

1) Development of appropriate High Throughput Phenotyping approaches for Root System Architecture traits
2) Phenotyping of a biparental mapping population to screen for variability in Root System Architecture traits
3) Selective genotyping of contrasting groups of RILs and marker identification by bulked segregant analysis

II REVIEW OF LITERATURE

Most of the world's millet crops are produced in India, Nigeria, Niger, Mali, Burkina Faso, Chad, and China. Ragi (*Eleusine coracana* (L.) Gaertn), little millet (*Panicum sumatrense* Roth ex Roem. &Schult.), foxtail millet (*Setariaitalica* (L.) P. Beauvois) and proso millet (*Panicum miliaceum* L.) are most commonly cultivated species of various millets. In India, among all the small millets Ragi occupies larger area under cultivation. Ragi is a tropical crop and best suited for dry farming, generally grown under rainfed condition. It is grown in areas with the annual rainfall of 53-75 cm. Ragi is very adaptable and thrives at higher elevations than most other tropical cereals. Cultivated on soils ranging from rich loams to poor shallow upland soils. In India, grown on black cotton soils, but thrives on red lateritic loams (Dinesh *et al*.,2016). Scarcity of water is a severe environmental constraint to plant productivity. Drought stress induced loss in crop yield probably exceeds losses from all other stress conditions, since both the severity and duration of the drought stress are critical for crop growth (Farooq *et al*.,2009).

2.1. Drought: A major challenge to millet cultivation

Most of the millets productivity is being affected by biotic and abiotic stresses. Biotic stresses such as pests and diseases are a cause for severe yield losses to diverse types of millets. However, abiotic stresses are the biggest contributor to yield losses every year. Although, in general, millets perform better than cereals such as wheat and rice in semi-arid environments. In semi-arid and arid environments where millets are the dominant crop, drought or inadequate moisture is the important abiotic stress, which severely decreases grain yield (Muchow, R.C., 1989). Studies in pearl millet showed that drought affects growth, yield, membrane integrity, pigment, osmotic adjustment, water relations and photosynthetic activity (Ajithkumar and Panneerselvam *et al*., 2014).

2.2 Effect of drought on plant growth:

Crop growth and productivity are largely being affected by various abiotic and biotic stresses. These stresses occur mainly due to global climate change. Therefore, developing improved varieties and developing newer approaches for crop improvement

against stress tolerance is becoming very important nowadays. However, most of the crop improvement strategies are directed towards staple cereal crops such as rice, wheat, maize, etc. whereas attention on minor cereal crops such as Ragi [*Eleusine coracana* (L.) Gaertn.] lags far behind. It is an important staple food crop in several semi-arid and tropical regions of the world with excellent nutraceutical properties as well as ensuring food security in these areas even during harsh environment (Sanjay *et al*.,2017).

Plants have developed dynamic responses at the morphological, physiological and biochemical strategies allowing them to escape and/or adapt to unfavorable environmental conditions. Mildest heat and drought stress negatively affect crop yield. Further, several independent studies have shown that increased temperature and drought can reduce crop yields by as much as 50% (Mouna *et al*.,2018).

Drought stress in plants decreases tissue water status thereby it affects the cell growth and protein synthesis. Carbon dioxide diffusion into the leaf tissue and leaf transpiration decrease, but the accumulation of proline and abscisic acid stress increases. Drought stress will also promote stomatal closure thereby it affects carbon dioxide diffusion rate and photosynthesis, it also decreases tissue water level. Thereby it reduces yield by hindering shoot and root growth. (Heidaiy and Moaveni, 2009).

Drought stress at an early period of crop growth severely affects short duration cultivars but late maturing cultivars may have sufficient time to recover (Maurya and O'Toole, 1986). Mild intermittent stress during vegetative stage such astillering to flowering period may not develop any visible stress symptoms. However, due to and stomatal closure reduced leaf area, dry matter accumulation may be affected particularly in most sensitive varieties (Boonjung, 1993). Late stress is a common problem for late maturing cultivars as well as short duration cultivars when planting is delayed due to early drought spell. The reproductive process is severely affected by late season stress (Fukai and Cooper, 1995).

2.2.1 Critical growth stages of ragi in relation to drought:

Water stress condition at the vegetative stage such as tillering to flowering appears to be least detrimental to yield (Boonjung and Fukai,1996). The effect may be

reduced by the growth of additional tillers after water deficit ends. Compensation for decreased tiller number is especially feasible in cultivars with long vegetative phase. The obvious exception to this case is when stress is of severe intensity and duration that kill some plants, thus reducing the number of plants per unit area. (O'Toole and Chang,1979). Moisture deficit condition mainly affects primordial initiation and flowering stage in ragi. The occurrence of drought stress four weeks after sowing resulted in 100% yield loss and over 30% biomass damage (Maqsood andAli,2007). Similarly, yield loss reached up to 77% when the plant experienced drought at the flowering stage (Takele et al.,1997). However, millets produce at least some straw and grain even in bad years unlike drought-intolerant cereals such as wheat and rice which completely fail to produce any yield (Fang and Xiong,2015).

2.3. Mechanism of drought resistance in ragi :

2.3.1. Strategies to drought adaptation or tolerance:

Plants adapt mainly three strategies under drought stress namely, drought escape, drought avoidance, and drought tolerance, they do follow another strategy that is drought recovery which has been identified as the fourth strategy.

2.3.2. Drought escape:

Drought escape refers to the condition in which plants reach maturity before the drought occurs. Traits associated with drought escape are early flowering, high photosynthetic rate, and high leaf nitrogen level (Kooyers,2015). Some study in West Africa indicated that pearl millet matches its phenology to the mean distribution of the rainfall where precipitation is limited and erratic (Shivakumar,1992). The occurrence of an increased period of rain during the main panicle development stage reduces the risk associated with drought events which occur prior to or at the beginning of flowering.

2.3.3. Drought avoidance:

Drought avoidance refers to the ability of the plant to maintain a favorable water balance under water deficit condition in order to avoid water loss or deficit in the plant tissue. Two types of drought avoidance mechanisms have been identified in plants: (i)

those that reduce water loss through transpiration (e.g. reduced leaf and low stomatal conductance) and (ii) those that maintain water uptake during drought period (e.g. high root-to-shoot ratio) (Fang and Xiong,2015, Kooyers,2015, Agriinfo,2015).

2.3.4. Drought tolerance:

Drought tolerance refers to the ability of the plant to produce some yield by withstanding low water potential or even under water deficit condition (Agriinfo,2015). Traits associated with drought tolerance are increased osmo protectants (or compatible solutes such as betaines and amino acids), and osmotic adjustment (i.e. reducing osmotic potential through the accumulation of organic and inorganic substances) (Kooyers,2015, Blum,2005).

2.3.5. Drought recovery:

Drought recovery is a condition in which plants recover from the adverse effects of moisture stress in order to provide some yield and/or biomass. Desiccation□tolerant particularly the wild *Eragrostis nindensis* is the typical example of drought recovery since it stabilizes its cells or membranes at desiccated state (Vander,2004). The above-mentioned strategies which are devised by plants to cope with drought are manifested through changes in some phenotypic or morphological traits. A recent review, (Kooyers,2015) showed that strategies by plants in terms of the life cycle, altered phenotypes or morphology and to the type of drought the plant fits itself. This indicates that the mechanisms and strategies of drought tolerance are interrelated or interconnected.

2.4. Traits associated with drought tolerance mechanisms in millets:

2.4.1. Agronomy-related traits:

Agronomic traits refer to the traits that are commonly known as yield and yield-related components. Among these traits, the number of tillers, number, and size of panicle, seed yield and biomass yield, seed weight, and harvest index are the major ones (Aparna,2014).

2.4.2. Morphology-related traits:

Morphological or anatomical traits are one which plays important roles in drought tolerance including root and shoot length and leaf area (Shao,2008). Flag leaf is the primary source of photosynthesis and changes in any morphological and biochemical properties plays a key role in drought tolerance (Biswaland Kohli,2013). Mechanical properties of the plant also affect drought tolerance in millets. Balsamo *et al*,2006 studied the leaf tensile strength also known as 'force to tear' this is studied in three *Eragrostis* species with different levels of tolerance to drought. According to their findings, drought-tolerant *E. curvula* had higher tensile strength values than the *E. tef* which is a moderately drought-tolerant variety, which in turn had higher values than the drought-susceptible *E. capensis*, this indicates a positive correlation between drought tolerance and leaf tensile strength. Structural investigations of leaves from those three species revealed the presence of extensive lignification of bundle sheath extensions in *E. tef* and *E. curvula* unlike in *E. capensis*. A study in maize indicated that the lignification of the midrib parenchyma and epidermis was directly correlated with increased tensile strength (Balsamo and Orkwiszewski,2008).

2.4.3. Biochemical-related traits:

Reactive oxygen species (ROS) are chemically reactive molecules that are useful in cell signaling at low concentrations but are damaging to cells when present at high concentrations in the cells. The main causes for the high production of Reactive oxygen species (ROS) are environmental stresses such as drought and salinity (Sharma and Dubey,2005). In order to avoid the harmful or damaging effects of ROS, plant produces antioxidants, which include glutathione, ascorbate and carotenoids and ROS scavenging enzymes which include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (AP or APX) (Ajithkumar and Panneerselvam,2014). In little millet, the activity of SOD, CAT, and POD was evaluated under drought conditions to enable the plant to cope up with the unfavorable ROS accumulation (Ajithkumar and Panneerselvam,2014). Similarly, the activity of ascorbate peroxidase (AP) and mono dehydroascorbate reductase (MDAR) increased in plants treated with drought compared to control plants grown under well-watered condition (Smirnoff and Colombe,1998).

2.4.4. Physiology-related traits:

Among the several physiological traits that are differentially regulated during moisture stress condition, osmotic adjustment is a major one which enhances drought avoidance mechanisms to enable the plant to produce some yield. Osmotic adjustment, which refers to the lowering of the osmotic potential or solute potential in the cytoplasm due to the accumulation of compatible solutes such as proline, glycine, betaine and organic acids, these will contribute to maintaining the turgor pressure of shoots and roots (Ajithkumar and Panneerselvam,2014). In little millet, drought stress increased the number of osmolytes like proline, glycine, and betaine in both the root and leaf tissue. Accumulation of free amino acids in millets during a drought might be related to the disruption of protein synthesis, induced proteolysis or its partial hydrolysis or degradation (Ajithkumar and Panneerselvam,2014). Water-use efficiency of the plant, on the other hand, plays a major role as moisture is mostly limited in the areas where millets are extensively cultivated. The experiment using drought-tolerant and drought-sensitive pearl millet genotypes showed that under moisture deficit conditions, the total amount of water extracted by drought tolerant and drought susceptible genotypes were comparable (Vadez *et al*.,2013).However, compared to tolerant genotypes susceptible genotypes extracted more water and tolerant genotypes extracted less water prior to flowering and more water after flowering, these mechanisms enabling drought tolerant genotypes to support the tillers and maintains the stay-green phenotype.

2.5. Improving WUE in Crop Plants :

Water scarcity and the increasing demand for water worldwide in many sectors, including agriculture, has come to a global concern. The rapidly growing world population and the adverse effects of climate change led to growing competition for water use by industrial and urban users for agriculture to secure enough food. Irrigated agriculture is very important which provides a wide range of agricultural products, including fruits, vegetables, grains, cereals, and millets. Effective management for water use is the only way to save water for increasing irrigated agriculture. According to the studies of Boutraa *et al*.,2010 crop with high WUE should have a greater yield than a crop with low WUE.

2.5.1. Water Use Efficiency (WUE) :

Water use efficiency is defined as the ratio of total dry matter to the total amount of water transpired or it is defined as the dry matter produced per unit amount of water transpired.

Water Use Efficiency= Dry matter produced (g)/ Water lost in transpiration (Kg)

Water is the major factor limiting crop growth and an increase in WUE achieved by reducing nonproductive water use or loss will lead to an increase in transpiration and yield (Stanhill., 1986).

WUE = mmol of CO2 fixed / mol of water transpire

Water Use Efficiency is one of the important components in the yield model proposed by Passioura (1982), which states that:

Seed yield (Y) = Water Use (T) x Water Use Efficiency (WUE) x Harvest Index (HI)

Water use efficiency or transpiration efficiency gives the relationship between carbon fixation and water loss. In dryland plants, water evaporates from the interstitial tissues of leaves whenever stomata open for carbon dioxide acquisition. The transpiration efficiency of crop plants will be generally low as they typically lose more amount of water than the equivalent units of carbon fixed during photosynthesis. Under the present situation, increased demand for sustainable water use and increasing agricultural productivity exists so the improvement of transpiration efficiency (TE) of the crop has received much attention (Boutraa, 2010).

2.5.2. Physiological parameters associated with WUE :

Water use efficiency is usually affected by transpiration. Transpiration is the loss of water from the above ground part in the form of water vapour and the transpirational demand will be fulfilled by the roots. The plant that has high ability to draw water from the different soil profiles by virtue of better root system architecture can support a higher stomatal conductance and hence can accumulate higher biomass by maintaining all the physiological activities in the plant.

2.6. Roots :

Roots are the major organ of a plant and are a hidden half of a plant, canopy architecture is well known compared to root architecture and there is a need of knowing root architecture of a plant. Plant roots have two major functions those are the absorption of water and nutrients from different soil profiles and gives anchorage to the plant body. Roots are axial multicellular structures of the sporophytes of vascular plants which usually grows under-ground, have strictly apical elongation growth, and generally have gravitropic responses which ranges from positive gravitropism to diagravitropism, combined with negative phototropism (Raven and Edwards, 2001).

2.6.1. Root system architecture :

Roots are important and specialized part of the plant that has the abilities to overcome many constraints in the soil. The initiation of root in the plant is different from that of shoot of the plant. During root growth and development, meristematic cells in the root tip will divide, elongate and differentiate and only after complete elongation, lateral organs are formed and help in spreading of roots in different profiles of soil. The basic characteristics of root development have been analyzed by diving the root tip into different parts such as root cap, the meristematic zone, elongation zone and maturation zone. Root growth occurs in the root tip which consists of highly dividing meristematic cells. The root apical meristem tissue contains several types of root initials and the quiescent center (QC) is the source of all the tissues of the root. It is stated that the lateral root development occurs some distance back from root apical meristem (Malamy and Benfey,1997).The lateral root growth and development is mainly influenced by endogenous factors like hormones. Among all the hormones, different concentrations of auxin plays a major role in root development and external factors such as water and nutrients availability in soil.

Root system architecture (RSA) refers to the shape and spatial arrangement of a root system within the soil. It is created by modulating rate of growth, the angle, and type of individual roots or lateral roots contributing to the root system. RSA is pivotal for plant anchorage and efficient uptake of water, macro and micro nutrients from the soil and can have a major impact on fertilizer usage and yield in crops worldwide. RSA is

highly plastic in nature, it gets altered whenever the surrounding environment creates stress to the plant and is composed of many root types with specific functions. RSA is shaped by the interactions between genetic and environmental components that establish a framework with which the plant explores the soil and responds to the external environment that dictates future growth patterns. RSA can provide a growth advantage in specific environmental settings (e.g. drought, salinity) and directly influences the above ground parts of the plant that impact yield. Poor soil fertility and environmental stress reduce crop yields in many parts of the world (Rogers and Benfey,2015).

2.6.2. Why is root architecture important in plant productivity?

The importance of root architecture in plant productivity is as said that many soil resources are unevenly distributed in soil or are subject to localized depletion so that the spatial deployment of the root system will in large measure and determine the ability of a plant to exploit those resources. Patches of localized soil phosphorus availability may retain their boundaries within centimeters over some years (Bloom, *et al*,1985). Fluctuation in temperature, oxygen status, pH, water availability, bulked density, and nutrient status commonly occur with soil depth over a scale of centimeters or millimeters. Often such gradients present conflicting constraints and opportunities from the perspective of resource acquisition or absorption; for example, top soils are known to be richer in nutrients but also drier and more subject to temperature fluctuations than subsoils. Spatial heterogeneity of nutrients occurs in soil. Oxisols and ultisols form surface soil where phosphorus and calcium concentration will be more. Desert Aridisols show more spatial heterogeneity in water availability, both because of landscape drainage patterns and depth. Temperate forest soils experience dramatic seasonal fluctuations, as melting snow in the spring season accumulated mobile nutrients such as nitrate through the soil. In this case, roots must respond to the temporal pulse of resources before they are lost to groundwater (Jonathan,1995).

Root architecture has been linked with plant absorption of water and extension of roots helps in the absorption of mobile and immobile nutrients such as Phosphorus. The function of a root system architecture in mechanical support of the shoot is also determined by root architecture (Ennos and Fitter, 1992). An important ecological topic

that has received more attention is how to root system architecture influences root interactions with soil biota and soil processes by determining the biophysical environment of C fluxes and other plant-mediated processes (Wullschleger et al,1994).

The apoplastic pathway plays a major role in plant water uptake and it takes place through aquaporins (Ranathunge et al.,2004). As the root grows it moves into a deeper layer of soil and root surface area also increases. An increased xylem vessel size has been hypothesized to be a useful character to improve water absorption from deeper soil profiles (Yambao et al.,1992). Basal root thickness has been reported to be correlated with yield in upland rice (r2=0.33) (Zichao et al.,2005).

The first plant organ to be sensed by drought stress is the under ground part that is root, and then it conveys the signals produced by stress (drought) to the above-ground parts (Hudak and Patterson,1996). A major drought-resistant trait is fulfilling the evapotranspirational demand of the plants by absorbing water from a deeper layer of soil. The root system architecture is one of the important components of drought resistance. Therefore, understanding of root system architecture and function of mitigating drought is inherently important for selecting root ideotypes for a further breeding program to develop drought resistance cultivar.

2.6.3. The relevance of root traits in imparting drought tolerance mechanism in plants:

Drought resistance may be improved by increasing the ability of the crop to extract water from the different profiles of soil (Wright and Nageswara Rao,1994). Deep rooting, root distribution, root length density, and root angle have been identified as drought adaptive traits that can be used as selection criteria for the development of drought-resistant varieties. Variation among genotypes for shifting root distribution downwards in response to drought in soil has been found in cowpea (Matsui and Singh, 2003), and chickpea (Yusuf Ali *et al*.,2005). This might be achieved by selection for good or large root systems architecture for better adaptation to drought stress conditions (Songsri *et al.,*2008).

Any increase in the crop yield is associated with an increase in transpirational water losses, so the advantage of increased root architecture helps to enhance the soil water recovery and there by increased total biomass and yield (Serraj and Sinclair, 2002). Under drought stress condition, roots can adapt to continue growth while at the same time sending signals to shoot that exhibit growth and development above the ground. O'Toole and Bland, 1987 opined that genotypic variations in root system architecture and reported that plant root systems are an important trait that needs to be exploited improve the drought resistance mechanism in crop plants. A deep root system architecture may also improve yield in less arid climates where occasional long intervals between rains result in depletion of water in upper soil profiles (Taylor, 1980). Maintenance of higher level of transpiration is possible only by improvement of root growth further to reach untapped water in deeper soil profiles (Sashidhar *et al.,*2000).

Some drought tolerant genotypes of wheat have more roots in the crown region with the nodal and seminal roots concentrated close to the soil surface to absorb water from different profiles of soil. The drought susceptible genotypes have fewer roots in the crown regions with the roots concentrated away from the soil surface (Kinyua *et al.,*2003). Ekanayake *et al.,* (1985) reported that root length, number of thick roots, root thickness and root volume were significantly correlated to the field recovery from drought. Further, they also opined that resistance to or tolerance of water stress in crop plants is the combined result of many interacting morphological and physiological characters like roots. Chang *et al.,* (1986) investigated genetic variability in root traits among cultivars and reported that deep thick root systems avoid drought better than those with shallow thin root systems. Improving water access and management is difficult practically as water is a scarce resource, so breeding for drought resistance has been an important strategy in alleviating the problem.

There is a continuous effort for developing new cultivars with improved characters using traditional plant breeding approaches, but limited success has been achieved. Although traditional plant breeding methods based on the phenotypic selection are very effective, it suffers from several limitations such as masking the effect of the environment for complex traits resulting in loss of favorable alleles during the selection

process. The recent advances in omics technologies, i.e., comprehensive and integrated genomics, transcriptomics, proteomics, and metabolomics have the potential to decipher the genetic architecture of plant genomes and disentangle the relationship between genotype and phenotype. The rapid advances in DNA sequencing technology have made whole-genome sequencing (WGS) both technically and economically feasible (Sood *et al*.,2016 and Hittalmani *et al*.,2017).

2.7. Molecular breeding:

Progress toward developing better root system has mainly relied upon conventional plant breeding approaches, a process that is labor-intensive and time-consuming. Hence, the identification of DNA markers that are specific to root architecture traits can accelerate the development of cultivars that can remain productive even drought conditions. The genetic basis of molecular, biochemical, physiological responses to drought involves many gene functions regulated by water availability. Genomics-based approaches provide access to agronomically desirable alleles present at quantitative trait loci (QTLs) that affects such responses, thus enabling us to improve the drought tolerance and yield of crops under water-limited conditions more effectively. Marker-assisted selection is already helping breeders to improve drought related traits. (Tuberosa and Silvio,2006).

2.7.1. Molecular markers :

The discovery of genetic markers created a new era in crop improvement as they enabled polymorphisms to be detected at the individual genomic level. Identification and screening of these markers revolutionized plant improvement techniques. These markers are constantly being developed to assess genetic variation with greater precision, speed, and cost-effectiveness. Genetic markers can be described as DNA sequences or nucleotide sequences that are easily detected and whose inheritance can be monitored (Kumar *et al*.,2009). There are different types of molecular markers for examples Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Cleaved Amplified Polymorphic Sequences (CAPS), Simple Sequence Repeats (SSRs), Inter-Simple Sequence Repeats (ISSRs) Sequence Characterized

Amplified Regions (SCARs), and Single Nucleotide Polymorphisms (SNPs) (Maheswaran,2004). Various researchers have employed the use of different markers types in studying various aspects of Ragi. RAPD markers have been used most frequently in studies on Ragi, probably due to their simplicity and applicability (Bardakci,2000). RFLPs markers have been used to a lesser extent because it has disadvantages like it requires high quality and quantity DNA, time-consuming and expensive. However, these markers are not reproducible and have problems with data scoring.

Among the different types of markers, simple sequence repeats (SSR) have shown to be most promising in studying Ragi, as they are highly reproducible, easy to use, locus-specific and thus offer a nearly unlimited and accessible supply of polymorphism (Tautz and Renz,1984 and Tautz,1989).Microsatellites are being widely utilized in plant genomic studies and in crop breeding (Dib *et al*.,1996). Microsatellites also referred to as short tandem repeats (STRs), simple sequence repeats (SSRs) or simple sequence length polymorphisms (SSLPs). They can be analyzed by PCR owing to the fact that the sequences that flank specific SSRs are conserved within a particular species, across species, within a genus and at the same time across related genera and these conserved areas can be used to design primers for each SSR that is unique, and these markers reveal a high degree of polymorphism (Varshney *et al*.,2002and Parida *et al*.,2009).

Dida *et al*. (2007) developed genomic SSRs by isolating di-and tri-nucleotide SSRs from random genomic *Hind* III, *Pst*I, and *Sal*I libraries of Ragi accession PI 321125 and identified 82 SSR markers and developed the genetic map of Ragi spanning 721 cM of the A genome, 787 cM of the B genome and covered all the 18 Ragi chromosomes using genomic SSRs. In 2008, Dida *et al.,* used 45 genomic SSR markers to evaluate genotypic variation among 79 Ragi accessions belonging to Africa and Asia and some wild species collected in Uganda. In 2013, Musia identified 49 new polymorphic genomic SSR markers from among 92 newly developed SSR in Ragi using next generation sequencing data. Hence employing SSR markers in molecular breeding will hasten the process of plant breeding program.

2.7.2. Approaches to identify markers linked to traits:

The following approaches are being widely used for the identification of QTLs and genes. One of the trending approaches 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 (Jung and Mc Couch, 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; Jung and Mc Couch, 2013; Yu and Buckler,2006; Rafalski,2010).

Bulked segregant analysis (BSA), which works with selected and pooled individuals which are contrasting for phenotypic characters, which has been extensively used in gene mapping through bulked segregant analysis with biparental mapping populations, mapping by sequencing with major gene mutants and pooled genome-wide association study using extreme variants. Compared to conventional population analysis, bulked segregant analysis significantly reduces the scale and cost by simplifying the procedure (Xu*et al*.,2016).QTL mapping gives the respective QTLs for each trait which can be done using mapping populations. And it is carried out by genotyping a large number of progenies, which is labor-intensive, time-consuming and cost-ineffective. Bulked segregant analysis (BSA) serves as an affordable strategy for mapping large effect QTLs by genotyping only selected extreme phenotypes instead of the entire mapping population (Arvindkumar *et al*.,2011).

III MATERIALS AND METHODS

Ragi is an important small millet widely grown in India under irrigated and mostly in rainfed conditions. Though known for drought adaptability and survival under low moisture conditions, there is a huge yield gap between observed and expected yield. Moisture stress at different growth stages has negative consequences, especially during reproductive stage is detrimental with yield penalties up to 77% (Singh, 1991).

Plants withstand drought condition by exhibiting many drought resistant mechanisms like drought escape where plants complete their life cycle before the occurrence of drought. Secondly plants develop deeper roots to take up water from deeper layer of the soil to avoid drought. Third important one is drought tolerance mechanism, where plants survive the drought through osmotic adjustments.

Role of roots is important in drought adaptation apart from nutrient uptake and physical support. Studying the root system architecture will help us understanding these complex drought management strategies. There are many component traits associated with roots contributing to the drought tolerance mechanisms in ragi such as; deep root, more root weight, more lateral roots and also the root angle. Identifying such traits and transferring them to cultivar of interest by conventional breeding methods or accelerated breeding strategies like molecular breeding using markers systems like SNP, SSR etc. will be of more significance for crop improvement for drought tolerance. The RIL population (206) developed by crossing a high root cultivar (GE 208) and low root cultivar (GE 156) were obtained from small millet improvement programme, AICRP, UAS GKVK Bangalore. GE 208 and GE 156 parental plants were used for developing strategies for high throughput phenotyping of root architecture traits. Clerigel, coirpith matrix and field root structures are the different root phenotyping strategies used in this study.

3.1. Development of appropriate High Throughput Phenotyping (HTP) approaches for screening Root System Architecture traits.

Development of appropriate high throughput phenotyping strategies to capture root architecture is crucial for crop improvement for drought associated traits. Ragi is

known for its drought adaptability and the present research focus on capturing root architecture variability in a Recombinant Inbreed Lines. Two parental lines contrast for drought traits viz., GE208 (female) a tolerant phenotype and susceptible male line GE156 lines were grown in three different medium to understand their efficiency in studying root system architectures.

3.1.1 Clerigel medium:

Clerigel is a transparent solidifying nutrient medium widely used in plant tissue culture (Sanjay and Bhagyashri, 2015*).* A clear 0.3% matrix was prepared using quarter strength Hoagland solution. The Hoagland nutrient solution taken in a 200ml culture bottles containing 0.3g clerigel was autoclaved at 121^0C FOR 15 minutes and then allowed to solidify. A single overnight imbibed seeds of the parents were inoculated in clerigel and monitored for root growth till 30 days. Imaging was done periodically until 30days (Plate 1).

3.1.2 Coir pith matrix:

Aerated and loosely packed matrix are suitable medium for characterizing the root system architecture. Coir pith drenched with essential nutrients serves as a perfect medium for growing and measuring root parameters. The coir pith experiment was conducted in a 200ml pots filled with loosely packed coir pith matrix. The matrix was supplemented with quarter strength Hoagland solution. Parental seeds were sown in pots separately and replications were maintained. The seedlings of each lines were removed from the pots and washed thoroughly to get rid of traces of coir strands and other contaminants. Root measurement was conducted on 5^{th} , 10^{th}, 15^{th}, 20^{th} and 30^{th} day after sowing (Plate 2).

3.2. Phenotyping of a Biparental mapping population to screen for variability in Root system architecture traits.

3.2.1. Mapping population

A drought specific mapping population was developed by crossing a drought tolerant (GE 208) and drought susceptible (GE 156) genotype. The population was

available at AICRP, UASB. These populations were advanced to F5 RILs by single seed decent method. Based on specific morphological parameters, the progenies segregating at the expected ratio of 1:1, a total of 206 RILs along with the parents were selected for root system architecture studies.

3.2.2Root structure

Root system architecture of the RILs were studied in a raised field root structures to simulate near field growth conditions. The structures were constructed with a dimensions of 60ft length x 10ft width and 5f height to accommodate the population. A median wall splits the structure length wise to give more room for two rows of planting area. Three structures were made and filled with soil mixed with farm yard manure at the rate of15 t ha^{-1}. The RIL seeds were sown in trays with coir pith medium and grown for 15 days in a polyhouse. Later these seedlings were transplanted to root structures. Augmented randomized block design (ARBD) with 30cm × 10cm inter and intra row spacing was followed according to the package of practice (Plate 3). The parental lines of the RIL population were also sown after every seven rows as check. When the root structure grown plants were 75 days old, the wall of root structure was dismantled and soil was washed off carefully using a jet of water as shown in the (Plate 4). The entire plant was taken carefully from the root structure and washed to measure and record the shoot length, root length, root volume. Root and shoot samples were air dried in the field for 15days and then dried in an oven at 70^0C for 10days. Finally, dry weight was recorded. Different parameters recorded and analysis performed in this experiment are described below.

3.3 Observations recorded

a. Specific Leaf area: The length and breadth of middle portion of leaf from each tiller of a plant was cut. The cut leaf was oven dried and their dry weight was recorded.

The SLA was computed using the equation:
SLA= Area of leaves (cm^2) **/** Weight of leaves (g)

b. Shoot length: The shoot length was measured from the root shoot junction to earhead. The shoots were separated and dried separately for dry weight measurement.

c. Total Leaf Area: All the leaves were collected from each plant separately. 15days air dried leaf samples were transferred to the oven and then oven dried at 70^0C for 10days. After complete elimination of moisture, each leaf sample was weighed to recorded the dry weight. The total leaf area was obtained by multiplying leaf weight with respective SLA and it is obtained using the formula given below.

Total Leaf Area (cm^2 $plant^{-1}$) = total leaf dry weight ($gplant^{-1}$) × SLA (cm^2 g^{-1})

d. Shoot weight: All shoots were collected separately from each plant and air dried for 15days and then oven dried for 10days at 70^0C. After complete drying, dry weight was recorded separately.

e. Root length: The root structures were drenched prior to extraction. The side walls of the structures were removed to ease the extraction procedure. With the help of jet of water intact roots were extracted without damage. After uprooting of each selected plant from the root structure, roots were washed thoroughly to remove the soil. Roots were cut and separated from the main plant and length was measured. Each plant root was collected separately in a labeled brown cover and kept for drying.

f. Root volume: A known volume of water was taken in a graduated glass measuring cylinder, and the roots of each plant was immersed separately and a amount of water displaced was recorded as root volume and it is expressed as cm^3 $plant^{-1}$.

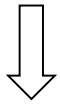
g. Root weight: Root samples were collected separately and air dried for 15days and then oven dried for 10days at 70^0C. After complete moisture removal the weights were recorded.

h. Total biomass: Total biomass was calculated by adding the dry weight of shoot, root and leaf together.

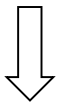
3.3.1. DNA extraction: RILs were grown in a coirpith medium under polyhouse condition to get a uniform growth without any infections from insects and fungus. Leaf samples were collected from 10days old seedlings in liquid nitrogen and stored in -80^0C until extraction. DNA was extracted from the RILs using modified CTAB method (Saghai *et al.,* 1984).

The protocol is as follows:

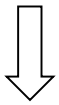
CTAB extraction buffer was prepared using 2% CTAB, 1.4 M NaCl, 20 mM EDTA (Disodium) & 100mM Tris HCL (pH 8), 2%PVP.



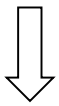
0.3g of fresh young leaf sample was taken in a mortar and finely powdered using liquid nitrogen. Three mL of heated 2% CTAB extraction buffer was added to the powder and buffer mixture was transferred to the 2 mL centrifuge tubes.



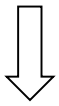
"10 µL of β-mercaptaethanol was added to each tube andthe extract was mixed well by inverting the tubes several times. All tubes were incubatedin a water bath maintained at 65^0C for 45 minutes with constant stirring at an interval of 15minutes".



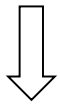
The tubes were then centrifuged at 12000 rpm for 15 minutes. Supernatant was transferred to1.5 ml centrifuge tubesand 4 µL RNAsewas added to remove RNA contamination. The mix was incubated at 37^0C for 15 minutes in a water bath.



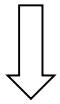
After incubation,750 µL of Chloroform : Isoamylalcohol (24:1) was added to the sample and the contents were mixed well by inverting. The tubes were again centrifuged at 12000 rpm for 20 minutes.



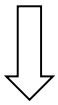
The aqueous upper layer was carefully transferred using 1mL cut tips into fresh 1.5 ml tubes. To this supernatant, 0.7 volume (600 ml) of cold isopropanol was added.



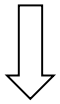
The contents in the tubes were carefully mixed by inverting the tubes and kept overnight in -20^0C for precipitation. The tubes were then centrifuged at 12000rpm for 20 minutes and precipitation of DNA as a hard pellet was seen.



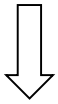
Further the supernatant was decanted gently and the tubes were inverted on a clean tissue paper to remove adhering propanol



The pellet was washed twice by suspending in one mL of 70% ethanol for 5 to 10 minutes and the DNA was centrifuged at 10000 rpm for five minutes.



Ethanol was drained off slowly and the pellet was air dried for 15 minutes. The pellet was then dissolved in 50 µL of nuclease free sterile water by flicking the tubes and stored at 4^0C.



Quality of the DNA was assessed in spectrophotometer and also cross checked on 0.8% agarose gel.

3.3.2.DNA purity assessment

The DNA concentration and purity was measured at 260/280 nm using Nanodrop DNA quantifier (Biospec-nano). The pure DNA samples with required concentration were quantified again on 0.8% agarose gels stained with ethidium bromide.

3.3.3. Preparation of 0.8 % Agarose gel

- 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, 0.89M Boric acid, pH = 8).
- Agarose was dissolved completely by boiling and after cooling 2.5 µl ethidium bromide (10mg/ml) was added and mixed well.

- 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.

- 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.

- To 1µl of DNA samples 4 µl of 1X loading dye was added, mixed and then loaded into the well. Around 3µl of standard uncut λ DNA (25ng/µl) was used as marker. (6x loading/tracking dye - 40% sucrose, 0.025% bromophenol blue, 0.25% xylene cyanol)

- 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 to check the banding pattern and documented using Herolab Gel Documentation system, Belgium.

- The quantity of the DNA was determined based on the intensity of the band.

3.3. Genetic analysis using molecular marker system:

3.3.4. Synthesis and validation 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 codominant in nature, dispersed throughout the genome and known to show high level of polymorphism than any other genetic markers and are easily reproducible in nature.

3.5 Mining for microsatellite markers

The genomic and transcriptome sequence from PR202 was analyzed for the presence of microsatellite regions using GENIOUS PRIME tool. The minimum threshold considered for primer designing was di (2), tri (3), tetra (4), penta (5), hexa (6) and hepta

(7) repeats. The sequences with repeat motifs length between 12 to 65 bases and a final marker length between 15 to 500 were selected for primers designing.

3.6 Development of genic SSR markers.

3.6.1 Genic SSR by characterizing the existing transcriptome library

Simple sequence repeats identified from the ragi transcriptome sequences could function as functional genic SSR marker system as they represent the expressed geneic regions/ coding region of the genome. To develop genic SSRs, transcriptome sequences developed from the stress imposed Ragi variety PR202 was used. These EST sequences were further annotated for the presence of microsatellite regions by the GENEIOUS PRIME program.

3.6.2Primer designing

Transcriptome sequences containing >12 bp length microsatellite region were selected for primer designing using Primer3 (Steve and Helen, 2000) software. The primer designing parameters were considered as GC: 50-60%; TM: 50-63^{o}C; primer length: 16-24bp. The Genic SSR primers designed were named as FMgSSR. The oligos were custom synthesized as desalted oligos (Bioserve). The primers quality was determined using the FAST PCR program and only those primers that would amplify a fragment in the range of 150 and 500 base pairs of template DNA were selected.

3.6.3 Genomic and genic primers annealing temperature standardization

Annealing standardization was carried out using the genomic DNA of PR202 as template to arrive at the optimum annealing temperature for each primer synthesized.

A 15 µl reaction was set up in sterile 0.2ml microfuge tubes. The composition of reaction mixture for one reaction is as follows

10x PCR buffer	1.5µl
2 mM dNTPs	1.5µl
2.5 mM $MgCl_2$	0.3µl
Forward primers (5pMole/µl)	1.5µl
Reverse primers (5pMole/µl)	1.5µl
Taq Polymerase (1U)	0.3µl
Template DNA (20ng)	2.0µl
Sterile water	6.4
Total volume	**15.0 µl**

Amplification was performed in an Eppendorf Master Cycler using the gradient program.

1) Initial denaturation at 94^0C - 5 min

2) Denaturation at 94^0C - 1 min

3) Primer annealing (± 5°C of oligos Tm) for 45 seconds

4) Extension at 72^0C - for 1.30 min

5) Final extension at 72^0C – 8 min

6) Hold at 15^0C

The amplified samples were resolved on 1.5 % agarose gel 1x TBE (Tris-base, Boric acid, EDTA Disodium salt, pH 8.0) buffer electrophoresed at 90 V till the bromophenol blue dye reached the edge of the gel for two hours. The gels were documented using GELSTANE Gel Documentation system.

3.7 Assessment of parental polymorphism

Trait specific mapping populations have been developed using parental lines contrasting for root traits (GE208 x GE 156). Parental polymorphism was assessed using the genomic and genic SSR markers by applying reaction conditions. The volume of reaction mixture per reaction is as follows.

10x PCR buffer	1.5µl
2 mM dNTPs	1.5µl
2.5 mM $MgCl_2$	0.3µl
Forward primers (5pMole/µl)	1.5µl
Reverse primers (5pMole/µl)	1.5µl
Taq Polymerase (1U)	0.3µl
Template DNA (25ng)	2.0µl
Sterile water	6.4
Total volume	**15.0 µl**

The PCR conditions is follows.

1) Initial denaturation step at 94^0C - 5 min

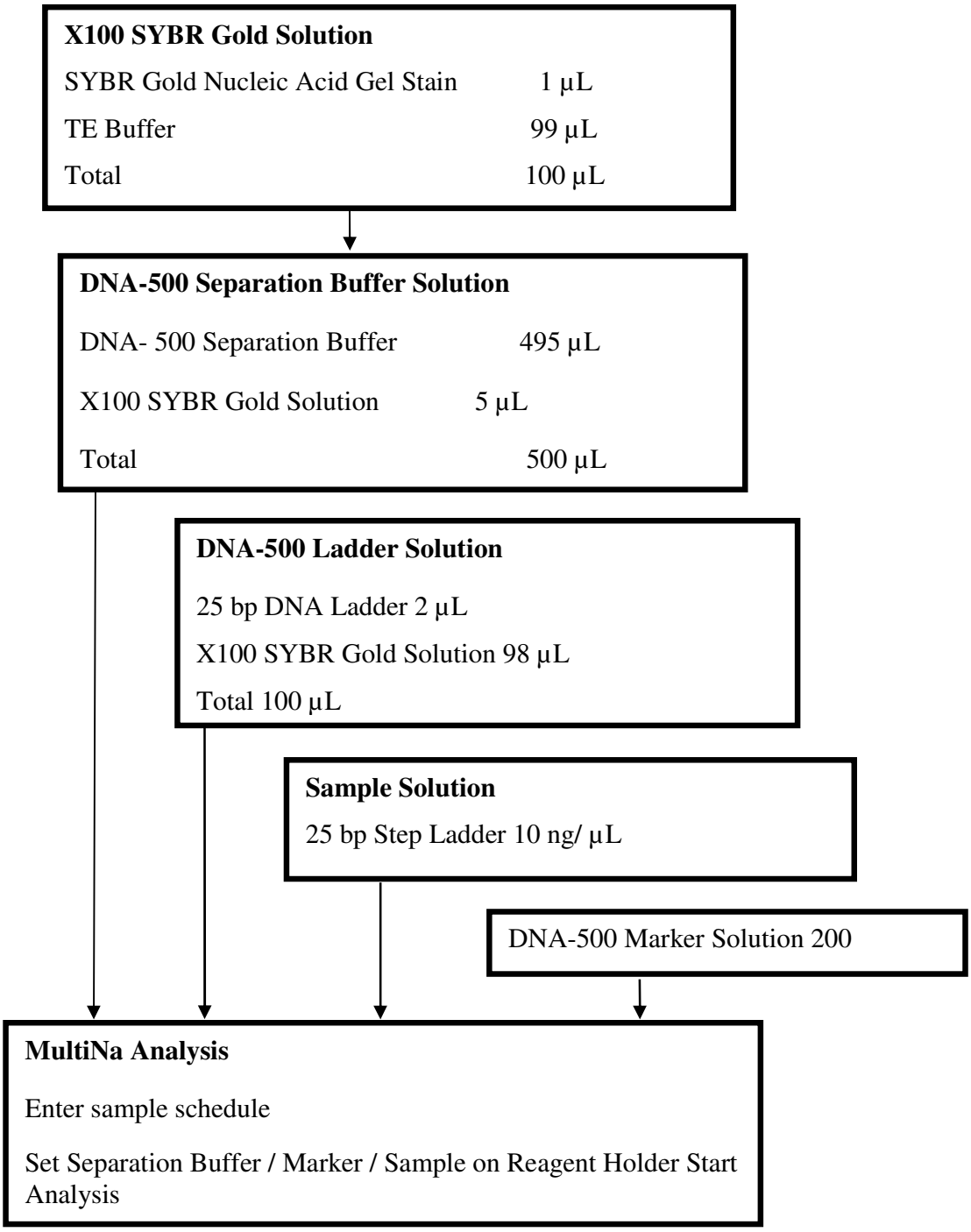
2) Denaturation step at 94^0C - 45 sec

3) Primer annealing step for 45 seconds

4) Extension step at 72^0C - for 1 min

5) Final extension step at 72^0C – 7 min

6) Hold at 15^0C`



Genotyping of bulked DNA samples using multiNA

3.7.1 Fragment analysis

All the amplified products were analyzed on microchip based electrophoresis system MultiNA (Shimadzu biotech, Japan) and the highest peak detected by the fragment analyzer was scored for the presence of the expected band for each primer pair.

3.8 Bulked segregant analysis:

Bulked segregant analysis helps in rapid discovery of DNA markers associated with the trait of interest without the need of phenotyping & genotyping the entire set of mapping population. Grouping of two bulked pools of segregating individuals differing only for the trait of interest / locus. In this study two sets of DNA differing for root trait (20 each from high root and low root types out of 206 RILs) were pooled from the RILs population. These two bulks were tested for locus specificity using the primers polymorphic in parental lines.

3.9 Statistical analysis :

Different statistical methods employed for analysis are presented below

3.9.1 Phenotypic data analysis

3.9.2Analysis of variance (ANOVA)

Augmented analysis was conducted using WINDOSTAT to nullify the effect of environment on the phenotype. The analysed mean were used to calculate the variability in the population. The genotypic variability 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 ($\delta 2$ g) and genotype x environment influence ($\delta 2$ ge) were estimated by utilizing the respective mean sum of squares from the variance.

3.10 Descriptive statistics :

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

The mean, SD and frequency distribution curves were drawn using SPSS software. The

Chi-square test to check the significance of the progenies in the population was tested using Microsoft excel.

3.10.1Skewness 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 root traits and various traits. The values of quantitative traits of mapping population were used to estimate co-efficient of skewness and kurtosis using SPSS software. 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 = Mesokurtic

If kurtosis value >3 = Leptokurtic

If kurtosis value < 3 = Platykurtic

Similarly, the lack of symmetry *i.e.,* skewness was recognized based on the coefficient of skewness values which range from -3 to +3. The type of distribution is based on skewness values and they are

If skewness value is zero = symmetrical distribution

If skewness value is negative =negatively skewed distribution

If skewness value is positive =positively skewed distribution

3.10.2Phenotypic and genotypic coefficient of variation

The co-efficient of variability both at phenotypic and genotypic level for root traits 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 %

IV RESULTS AND DISCUSSION

Ragi is an important millet crop with high grain calcium content and also a rich source of iron and zinc compared any other major cereals. Therefore, it is considered as a nutraceutical crop. But ragi, which is predominantly cultivated as a rainfed crop experiences several stresses and hence its productivity is quite low. Furthermore, with limited conventional breeding efforts and hardly any contemporary research intervention, ragi yield have not been improving noticeably. The current best cultivar for farmer's cultivation, GPU28, was released more than two decades back (1996), suggesting an urgent need for research intervention through adaptation of modern biotechnological and physiological approaches for crop improvement (Wesley *et al*.,2002).

Increasing growth rates as well as drought adaptation indeed appears as a formidable challenge. Maintaining leaf tissue water relations and carbon metabolism are the two ways of sustaining growth rates under water limited conditions. This hypothesis has been successfully tested in our lab previously (Raju *et al*.,2014) where robust QTL's have been identified by association mapping in Rice (Raju *et al*.,2016). Subsequently, these QTL markers were employed in a multi-parent marker associated backcross breeding to introgress traits (Dharmappa *et al*.,2019) associated with water mining and carbon metabolism.

These results strongly imply that when water mining and carbon assimilation capacity are combined, there can be a significant improvement in crop productivity even under water limited conditions (Sheshshayee *et al*.,2018)

Ragi, being a C_4 species is bestowed with superior carbon assimilatory capacity. Hence attempts to enhance water mining through improved root system can definitely enhance growth and productivity of ragi.

4.1. Phenotyping of root traits

Water mining from deeper layer of soil is most often considered as a very useful trait for improving crop performance under water limiting conditions (Sheshshayee *et al*.,2018). However, phenotyping for below ground biomass has always been a challenge

(Sheshshayee *et al*.,2013). Several high throughput methods for phenotyping have been developed that range from physical measurements to imaging of roots (Uga *et al*.,2013).

A simple root structure based root phenotyping technique was developed at our centre (Sheshshayee *et al*.,2013). This method, though tedious, represents one of the most appropriate methods of root phenotyping. In this technique plants are grown in specially designed structures and the plant population is maintained as in the field conditions. This approach therefore provides a near natural phenotypic expression and hence root phenotyping can be accurate. This technique has been extensively used for root phenotyping of germplasm accessions (Raju *et al*.,2014, 2016) and trait introgression lines of rice (Dharmappa *et al*.,2019).

The major limitation of this approach is its inability to determine the initial root growth dynamics. To circumvent this problem, two different media were used to quantify roots.

1. Root measurement using clerigel
2. Root measurement using coir pith

Germinated seeds were planted in these media and root growth was observed periodically. Clerigel is a transparent gel medium that permits visual observation of growing roots (Ramanathan *et al*.,2018). The parental lines viz., GE 208, drought tolerant, and GE 156, drought susceptible lines, were grown in the culture bottles and growth of roots was observed. Despite providing hormones and nutrients, both the parental lines did not respond well (Plate 1).

Coir pith is a simple, low cost and very effective root phenotyping medium. Both the parental lines grew well in coir pith and displayed significant differences in root length. Root growth was recorded periodically at an interval of 5days both in clerigel and coir pith matrix. Root length of long root parent (P1) was 4cm by 5days, while root length of short root parent (P2) was 2cm on the same day. Root growth was monitored for the next 25 days (Plate 2). The rate of growth was linear in case of P1 and it reached a maximum root length of 30cm by 25days, while the low root parent reached only 22.3cm

Plate 1: Root architecture differences of parental lines grown in clerigel medium

Plate 2: Phenotype of 30 day old plants grown in coirpith matrix

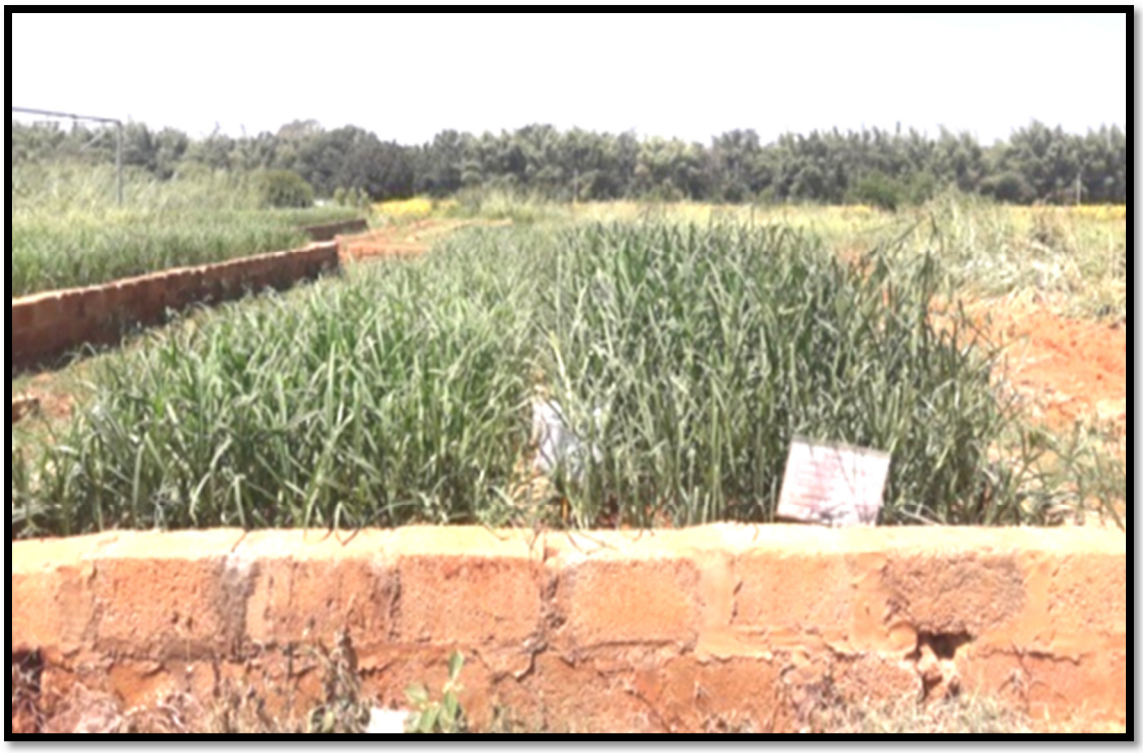


Plate 3 : Ragi RIL population grown in Root structure at 75 days after sowing

Plate 4: Picture depicting the root washing at the time of harvest to study the root diversity in RIL population

on the same day, representing a significant difference between the parental lines (Table 1). Root growth of the parental lines, though was comparable initially for about 10-12 days, was significantly different subsequently, this suggests that the rate of root growth in high root parent, P1, was much faster than that of the low root parent P2. From this study it can be concluded like coirpith medium is the good medium to study the root architecture compared to transparent clerigel medium.

Table 1: Root length differences observed between the two parental lines in coir pith medium. Variation of root length of both the parental lines was measured every five days till 25 days. The significance was tested using student t-test at $P \leq 0.05$ using Microsoft Excel. CD: critical difference.

Root length (cm)			
Days	**P1**	**P2**	**CD %**
5	**4.4**	**2**	**0.6**
10	**10.5**	**6.8**	**2.1**
15	**15.5**	**12.4**	**1.3**
20	**24.2**	**21.4**	**1.6**
25	**30**	**22.3**	**2.3**

To study the extent of root growth in real field condition the plants were grown in a specialized root structure till 75days (flower initiation) after sowing (Plate 3). At the end of the 75th day the length of the root of high root parent (P1) recorded 45.29cm, while it was 36.29cm for low root parent (P2) representing a significant difference (Table 2). Similarly, root weight of high root parent was 4.09g. plant^{-1} while it was 2.34g. plant^{-1} of the low root parent (Fig 1).Presence of significant differences in the root length and root weight both at the early stage and mature stage in the two parents formed them perfect genotypes for developing a mapping population.

It is well known that root growth and root traits have a significant influence on drought adaptation through water mining associated characters. Being a C_4 plant with high photosynthetic efficiency, better root system would apparently impart higher drought adaptability in P1 compared to P2. Therefore, the mapping population developed

from such parents differing for root traits would be an excellent material to study the genetic basis of variation in root traits and drought adaptation subsequently. Similar experiments of developing mapping population using parental lines contrasting for root traits have been done in various other crops like Rice (McCouch *et al*.,2002), Wheat (Song *et al*.,2005), Maize (Sharopova *et al*., 2002), confirming unequivocally root trait has relevance in drought adaptability.

4.2. Phenotyping of a bi-parental mapping population to screen for variability in Root System Architecture traits

4.2.1. Selection of Ragi RIL (F5) population:

Mapping population generated by crossing the high root parent, GE 208 (P1) and the low root parent, GE 156 (P2) comprised of 206 F5 RILs, were used for phenotyping for root and other drought adaptive traits. Initially the genomic DNA of the two contrasting parental lines was screened with 86 SSR markers. These microsatellite markers were developed from the genome sequence of widely used check variety PR202 sequence (Hathakayama *et al.,*2017). The scaffolds generated by whole genome sequencing of the variety was used for identifying single sequence repeats. A drought transcriptome library generated in PR202 by our group was used as a source for developing functional or genic SSR markers. Primers were designed and synthesized for the genomic and genic sequences, and were used for screening two parental lines and mapping population. Among these 102 markers (86 genomic and 16 genic), twelve markers were polymorphic between the parents. Based on the phenotypic data 206 progenies which were showing 1:1 segregation ratio for the major drought tolerant traits like root length, root biomass were selected for further analysis. The major objective of this experiment was to screen for the phenotypic variations and subsequently to identify QTLs governing important drought adaptive traits like root traits.

4.2.2. Phenotypic variations for biometric traits in the RIL population of Ragi

The RILs were grown in the specialized root structures with a population density similar to that of the field conditions. Hence, we believe that this strategy provides a near

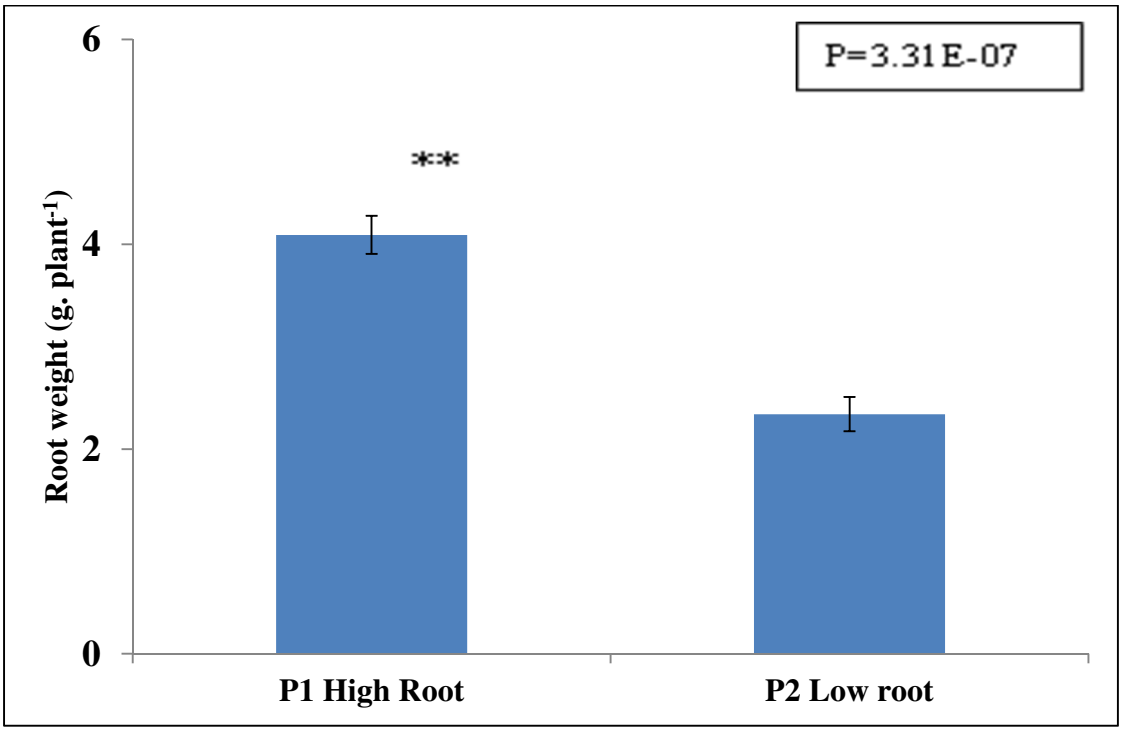


Fig. 1: Differences in root weight of the parental lines, P1 (GE208) and P2(GE156) used for developing RILs. The root weight of the parental lines was measured by growing them in root structures. The significance was tested by student t-test. ** indicate significance at $P \leq 0.01$.

natural phenotypic expression of traits. Therefore, phenotyping by this approach is more reliable. Root traits and the canopy traits were recorded on the 75th day after sowing by dismantling the root structures (Refer methodology section 3.2.2).

Root traits: Average root length of the RILs was 41.93 cm plant^{-1}, which ranged between 26 cm plant^{-1} and 57 cm plant^{-1} representing a significant variation. Similarly, the root weight ranged from 1.25 g plant^{-1} to 6.35 g plant^{-1} with a mean of 3.36 g plant^{-1}. Root volume ranged from 10cm^{3}plant^{-1} to 36.67cm^{3}plant^{-1} with the mean of 21.83cm^{3}plant^{-1} (Table 2).

Canopy traits: The canopy characteristics associated with the leaf parameters also varied significantly in the population. However, the two distinct parents of the mapping population did not show significant difference in the total leaf weight or total leaf area. Hence, this population represents an ideal mapping population for identifying robust QTLs for root characters. Besides the root characteristics, even the canopy traits would have significant influence on biomass and growth rates. One of the canopy trait largely responsible for the differences in the biomass is total leaf area (Table 2).

Total leaf area and total biomass: To understand the influence of other leaf traits on biomass accumulation, we identified a set of 78 RILs with comparable mean leaf area of 1776.2cm^{-2}plant^{-1}. The biomass of this group ranged from 51.69 g plant^{-1} to 26.67 g plant^{-1}. These selected lines did not show any significant regression between total leaf area and total biomass. Indicating that the differences in biomass among these selected lines was due to leaf area. So other parameters of relevance were examined in these selected lines.

Specific Leaf weight (SLW): One of the important leaf parameters that helps in biomass accumulation through increasing photosynthetic rate is leaf thickness. Leaf thickness, as estimated by SLW showed significant variations among the selected RILs. To ascertain the influence of SLW on total biomass, two groups of contrasting lines differing in SLW were selected and the variations in the SLW in these two groups is given in Figure 2A

Table 2: Genetic variability observed for the root traits and associated physiological traits in RIL population of ragi derived by crossing GE 208 and GE 156. Various above ground parameters such as EHD, SLA, LW, Shoot length etc, and below ground parameters like root length, root weight, root volume was measured in the root structure at 75days after sowing.

PARAMETERS	P1 (GE 208)	P2 (GE 156)	MEAN	MIN	MAX	H^2	GCV %	PCV%	CD @5%
EHD (days)	66.29	67.16	63.52	44.00	87.00	84.93	8.9	9.6	8.10
SLA (cm^2 g^{-1})	178.03*	161.96*	172.76	101.64	369.29	89.15	12.13	12.85	24.87
SLW (mg cm^{-2})	5.67*	6.29*	5.89	2.71	10.10	81.41	10.22	11.33	0.98
LW (g)	10.49*	10.14*	10.69	4.78	21.81	88.80	21.96	23.31	2.84
TLA (cm^2)	1849.7	1611.1	1848.25	671.07	4643.77	75.36	24.53	28.26	881.61
SL (cm)	83.32	80.47	88.90	58.67	119.83	86.74	11.83	12.70	13.99
SW (g)	17.63	14.29	22.24	7.39	47.04	94.48	26.21	26.97	4.79
RL (cm)	45.29*	36.92*	41.93	26.67	57.67	69.98	10.63	12.71	9.93
RW (g)	4.09*	2.34*	3.36	1.25	6.35	61.99	22.57	28.67	2.02
RV (cm^3)	25.00	18.27	21.83	10.00	36.67	38.74	16.00	25.71	14.94
TDM (g)	32.43	24.95	36.27	15.10	67.89	89.80	21.71	22.91	9.03
DM/LA (g cm^{-2})	17.80	19.69	20.13	9.79	32.66	29.10	9.39	17.41	13.54
R/S (%)	21.88*	16.77*	15.85	6.85	42.82	49.32	24.74	35.23	13.52
R/LA (g cm^{-2})	2.11*	1.91*	1.90	0.69	3.93	89.29	29.50	31.22	0.66

EHD= Ear head emergence date, SLA= Specific leaf area, SLW= Specific leaf weight, TLW= Total leaf weight, TLA= Total leaf area, SL= Shoot length, SW= Shoot weight, RL= Root length, RW= Root weight, RV= Root volume, TDM= Total dry matter, DM/LA= Dry weight to leaf area ratio, R/S= Root to shoot ratio, R/LA= Root to leaf area ratio, H^2= Broad sense heritability, GCV= Genetic coefficient of variation, PCV= Phenotypic coefficient of variation

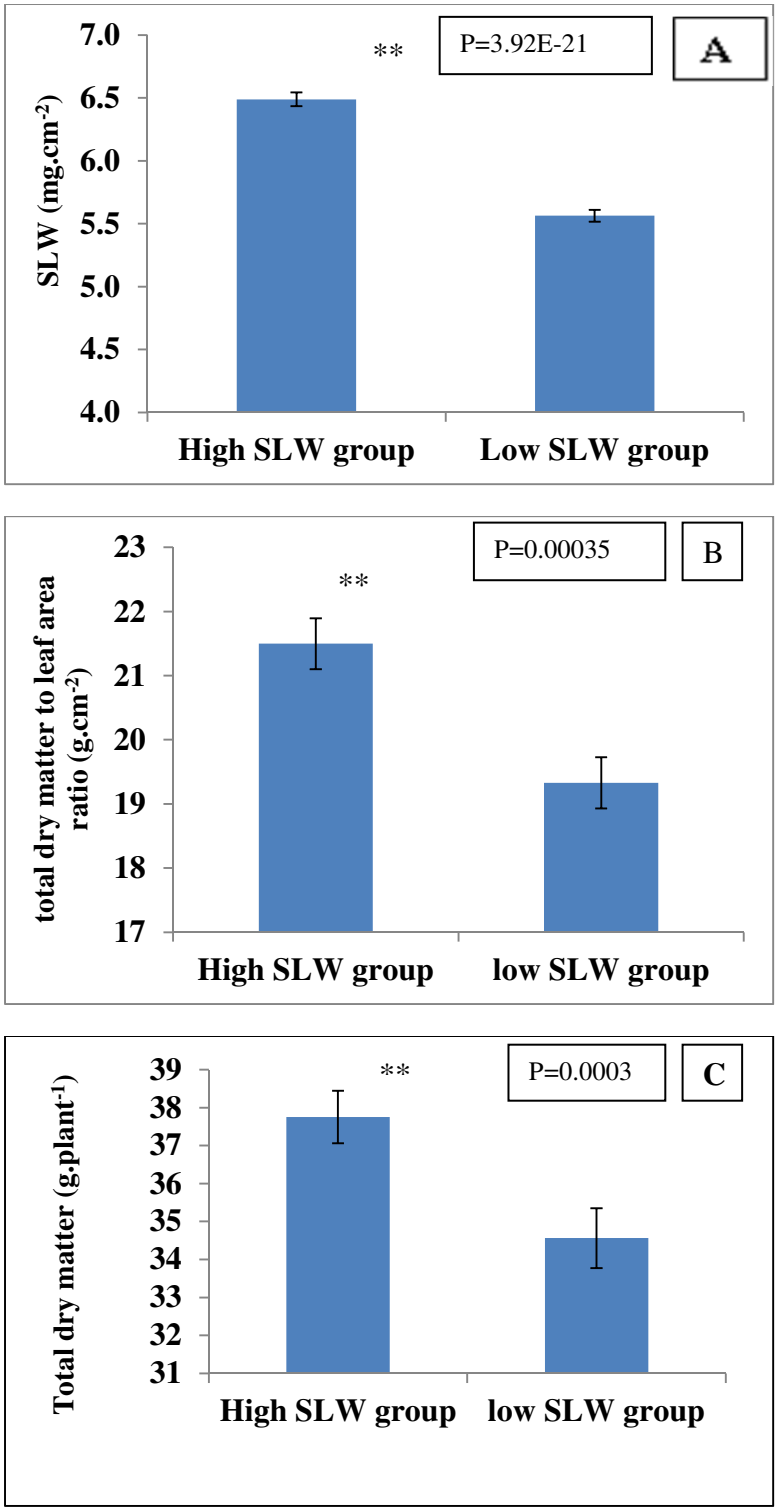


Fig. 2: SLW, DM/LA and TDM differences between high and low SLW group having comparable TLA. The RILs with similar leaf area were separated and the sorted for differences in SLW. All the progenies with high SLW had high DM/LA and TDM. The significance was tested by student t-test. ** indicate significance at $P \leq 0.01$.

Corresponding biomass of contrasting groups of SLW types revealed a significant variation in total biomass (Fig. 2C) emphasizing the relevance of leaf thickness in determining growth rates in ragi. Further, the ratio of DM/LA (an indication of unit leaf area photosynthetic rate) also differed significantly between the two SLW groups. The high SLW representing thicker leaf system had significantly higher photosynthetic capacity than the low SLW group. With comparable leaf area high SLW types would apparently accumulate more biomass because of more photosynthetic rate. It appears that a combination of high SLW with better root system would have much higher biomass production capacity. This when combined with higher partitioning of biomass to the grains can certainly increase productivity in ragi.

4.3. Genetic analysis for various physiological traits

4.3.1. Estimation of GCV, PCV and H^2 for various physiological traits:

The major difference between quantitative and qualitative traits lies in the degree to which they are affected by the environment. Qualitative traits are little or not at all affected by the environment, while quantitative traits are considerably affected. The environmental factors bring about differential expression of the phenotype through its effect on various genes governing the trait. When the number of genes controlling a trait increases, the environmental modulation also increases leading to a continuous variability in the trait. The distribution of the variation among the mean for each trait was computed to ascertain the quantitative behavior of the traits. Root length, root weight, root volume, canopy leaf area and total biomass showed a typical normal distribution for these parameters (Fig. 3). In crop improvement programs we select plants based on their phenotype. The effectiveness of selection would largely depend on the proportion of phenotype due to the genotype. Therefore, to distinguish the extent of genotype and environment effects on the phenotype, the genetic parameters like genotypic coefficient of variation (GCV), and phenotypic coefficient of variation (PCV) were calculated for root traits. Success in introgressing a trait in crop improvement programme depends largely on the availability of exploitable genetic variability for the trait. Based on the genotypic and phenotypic coefficient of variation, heritability and genetic advancement parameters were also computed.

4.3.2. Genetic variability parameters for biometric traits

Heritability denotes the phenotypic variance caused due to genotype. Root traits like root length and root weight showed a high heritability of 69 and 61%, while root volume showed a low heritability of 38%. Total dry matter recorded a highest heritability value of 89% (Table 2).

Root length showed a GCV value of 10.63%, while root weight and root volume showed 22.58and 16.01% GCV, respectively. Highest GCV value was observed for root to leaf area ratio (29.51%). The PCV value ranged from 35.24% for root to shoot ratio to 11.33% for SLW. PCV of root weight and root volume was 28.68% and 25.72%, respectively (Table 2).

Further, the χ^2 analysis of the RILs having trait value similar to high root and low root parent was analyzed. The results given in table 3 indicates that the RILs segregated in 1:1 ratio for root traits and total leaf area. However, total dry matter did not segregate as per expected pattern of RILs. The RILs showed a strong association between total leaf area and total biomass, while a positive but a weak correlation was found between root weight and total dry matter (Fig. 4). This data suggests that the leaf area has a greater influence on total biomass, while the root weight was only responsible for 24% of the variance in total dry matter.

Table 3: Segregation of high root and low root progenies among 206 RILs of ragi.

TRAIT	P1 TYPE	P2 TYPE	χ^2 VALUE
Root length (cm.plant^{-1})	115	91	0.09 (NS)
Root weight (g.plant^{-1})	91	115	0.09 (NS)
Root volume (cm3.plant^{-1})	119	87	0.02 (NS)
Total leaf area (cm2.plant^{-1})	95	111	0.26 (NS)
Total dry matter (g.plant^{-1})	154	52	<0.05 (S)

χ^2 test was performed using MS excel to check the segregation ratio of root and biomass associated traits. All the root traits displayed typical 1:1 segregation ratio for high root and low root parent. NS: Non-significant; S: Significant.

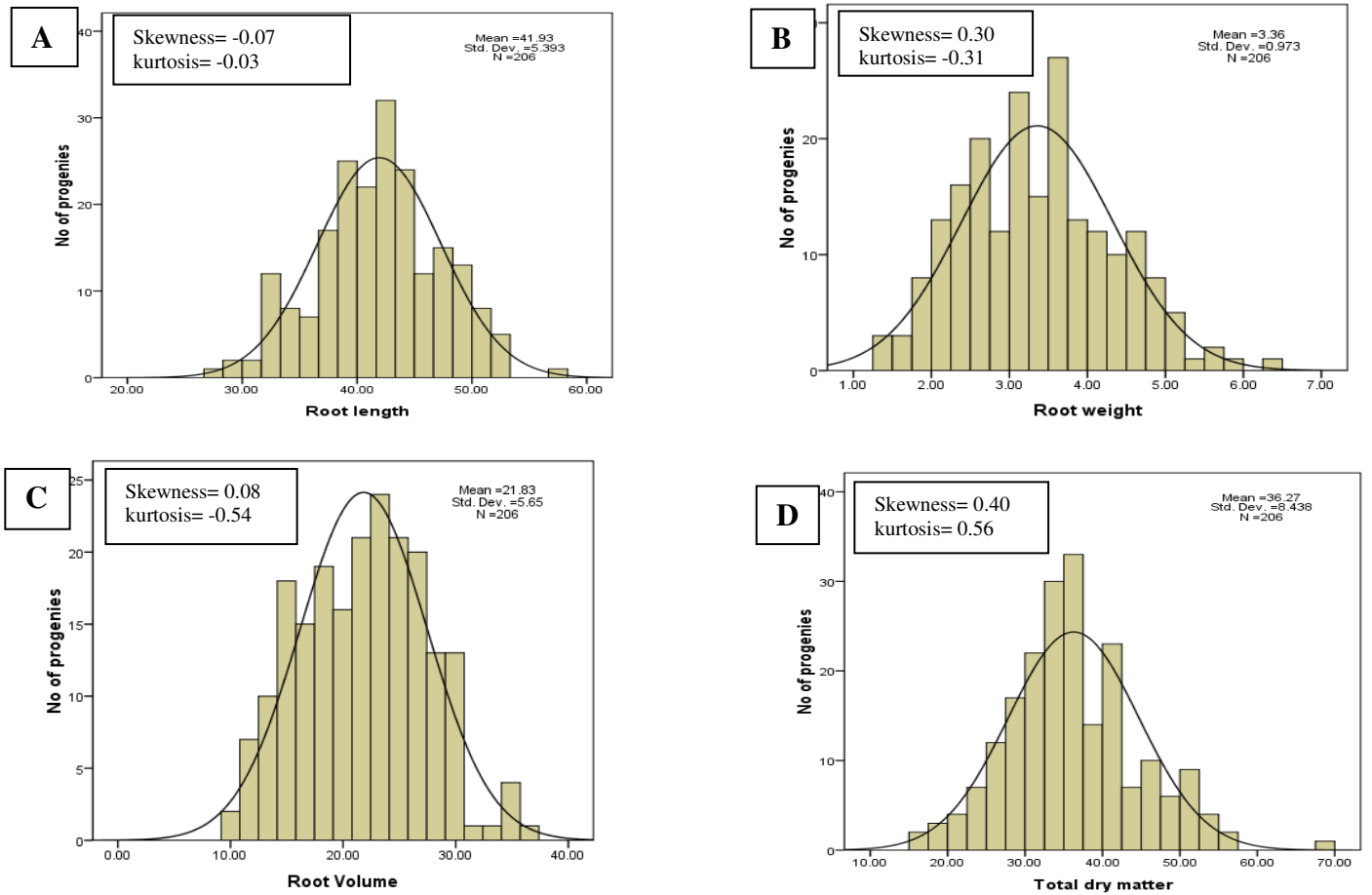


Fig. 3: Histograms showing continuous variability for root traits and total dry matter among the RILs of ragi. Normal distribution in root traits and TDM shows the presence ofcontinuous variability among the RILs for respective traits. All the observations were recorded in 75 day old plants.

4.4. Selective genotyping of contrasting groups of RILs and marker identification by bulked segregant analysis.

4.4.1. Identification of transgressive segregants:

Since the root traits segregated significantly as expected for a mapping population, the number of transgressive segregants having more roots than the high root parent and one that had lower root than the low root parent were selected (Table 4). There were 49 RILs with higher root weight than the high root parent, while 28 RILs had lower root weight than the low root parent. These high root and low root progenies represented 25% and 13% of the RIL population, respectively. Since the transgressive segregants differed significantly in root weight, these selected genotypes had significant variations in the leaf area as well as total biomass. Among these lines a set of 20 progenies having high root weight and 20 having low root weight were selected for bulked segregant analysis (Table 5, 6, 7).

Table 4: The trait values of transgressive segregants selected based on root weight (RW) among the RIL population of ragi. The progenies having high and low RW compared to P1 and P2, respectively were separated at transgressive segregants. The mean trait values of these progenies were compared. Further, 20 high root and 20 low root RILs were selected for BSA analysis.

Two groups of transgressive segregants	Number of progenies	RIL %	TLA (cm^2. $Plant^{-1}$)	TDM (g. $plant^{-1}$)	RW (g. $plant^{-1}$)	SLW (mg. cm^{-2})
Progenies having high root weight compared to parent 1	49	25	2096.94	41.23	4.65	5.75
Progenies having low root weight compared to parent 2	28	13	1517.54	29.05	1.93	5.96

Table 5: The average trait values of high and low root bulks selected for bulk segregant analysis (BSA). Around 20 progenies were selected from each of two groups of transgressive segregants.

Bulks	Number of progenies	TLA (cm2. plant^{-1})	TDM (g. plant^{-1})	RW (g. plant^{-1})	SLW (mg. cm^{-2})
High root bulk (B1)	20	2166.16	41.19	5.11	5.67
Low root bulk (B2)	20	1432.64	27.56	1.86	5.94

Expansion of abbreviation of traits and its units are given in appendix section 2

Table 6: The trait values of all the progenies used as high root bulk (B1). All the progenies in B1 were selected from the transgressive segregants having high root weight compared to parent 1. Equal quantity of DNA from all these progenies were bulked to screen with polymorphic SSR markers. Total leaf area (TLA), total dry matter (TDM), root weight (RW), specific leaf weight (SLW)

RIL NO	TLA (cm2. plant^{-1})	TDM (g. plant-1)	RW (g. plant-1)	SLW (mg. cm^{-2})
RIL - 10	1951.17	33.00	6.35	6.46
RIL - 42	1971.61	42.07	4.95	5.50
RIL - 47	1597.19	34.38	4.16	6.17
RIL - 64	4643.77	45.45	5.17	2.71
RIL - 67	2093.75	46.63	5.16	5.88
RIL - 69	1620.10	42.82	5.14	6.66
RIL - 72	1896.92	44.23	5.09	6.26
RIL - 90	2142.64	40.89	4.91	5.96
RIL - 120	1322.25	38.81	4.81	6.31
RIL - 133	1912.85	36.00	4.99	6.08
RIL - 148	1388.12	32.49	4.73	6.19
RIL - 210	2248.81	42.19	4.93	5.35
RIL - 211	2187.58	40.14	4.69	5.51
RIL - 223	2682.38	50.69	5.69	4.70
RIL - 239	2198.91	44.64	5.15	5.14
RIL - 250	2534.75	37.33	5.75	5.47
RIL - 252	1974.46	51.69	5.27	5.65
RIL - 265	2660.75	50.38	4.77	5.59
RIL - 276	2431.91	36.71	4.85	5.93
RIL - 282	1863.31	33.33	5.65	5.81

Expansion of abbreviation of traits and its units are given in appendix section 2

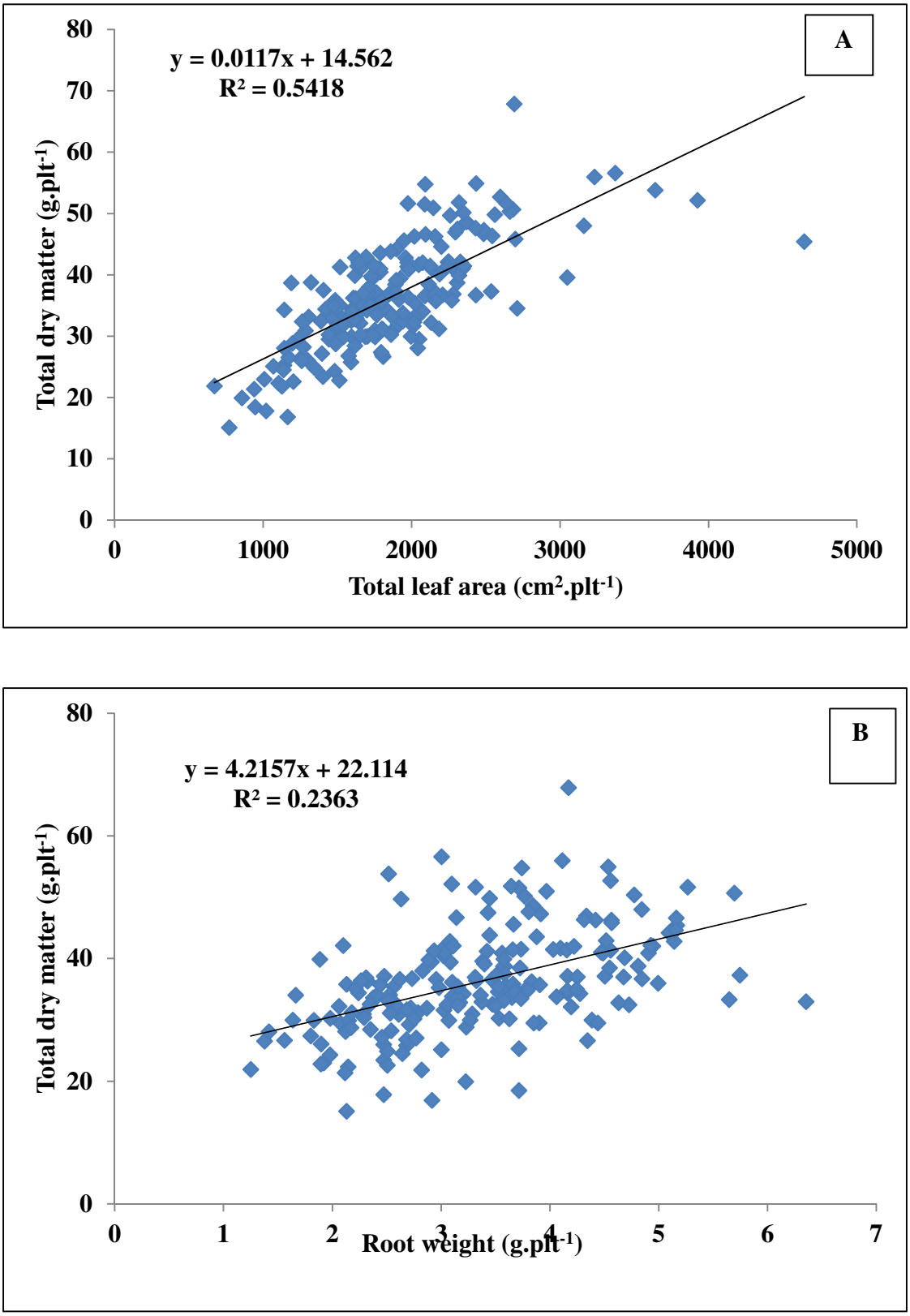


Fig.4 : Relationship between (A) TDM and TLA, and (B)TDM and RW observed in RIL population of ragi. Strong correlation was observed between total dry matter (TDM) and total leaf area (TLA) as well as TDM and root weight

Table 7: The trait values of all the progenies used as low root bulk (B2). All the progenies in B2 were selected from the transgressive segregants having low root weight compared to parent 2. Equal quantity of DNA from all these progenies were bulked to screen with polymorphic SSR markers. Total leaf area (TLA), total dry matter (TDM), root weight (RW), specific leaf weight (SLW)

RIL NO	TLA (cm2. plant^{-1})	TDM (g. plant-1)	RW (g. plant-1)	SLW (mg. cm^{-2})
RIL - 8	1701.15	29.99	1.64	6.38
RIL - 12	1103.59	22.36	2.15	6.05
RIL - 22	1616.71	39.88	1.89	6.62
RIL - 33	1807.69	26.67	1.56	5.55
RIL - 65	1639.88	35.83	2.13	6.05
RIL - 76	1299.26	26.10	1.90	5.04
RIL - 99	1006.86	22.98	1.92	5.76
RIL - 103	1171.34	26.57	1.38	5.85
RIL - 106	1229.51	29.49	2.08	6.22
RIL - 136	938.56	21.38	2.12	5.70
RIL - 141	671.07	21.92	1.25	10.10
RIL - 150	1480.80	24.29	1.98	5.57
RIL - 152	1512.04	22.81	1.89	5.46
RIL - 161	771.07	15.10	2.13	6.19
RIL - 171	1540.66	29.92	1.83	5.48
RIL - 179	2327.10	42.13	2.10	5.55
RIL - 183	1140.91	28.10	2.12	6.17
RIL - 235	1794.59	27.39	1.80	5.16
RIL - 258	1859.94	30.28	1.98	4.85
RIL - 278	2040.00	28.06	1.42	5.13

Expansion of abbreviation of traits and its units are given in appendix section 2

4.4.2. Bulked segregant analysis:

Bulked sample analysis, which works with selected and pooled individuals which are contrasting for phenotypic traits, has been extensively used in gene mapping in bi-parental mapping populations, mapping by sequencing with major gene mutants and

pooled genome-wide association study using extreme variants. Compared to conventional population analysis, bulked segregant analysis significantly reduces the scale and cost by simplifying the procedure (Zou *et al*., 2016).

Bulked segregant analysis is a simple approach to identify markers linked with specific traits. Though not an extensive strategy, BSA certainly provides initial identification of putative markers that are linked with specific traits. Therefore, genomic DNA of each of the 20 lines having high root weight and low root weight were bulked. Equal quantity of the genomic DNA representing each progeny were mixed into one sample. The two bulked DNA samples were screened with 102 SSR markers which consisted of 16genic and 86genomic SSR markers. These markers were also chosen from the whole genome sequence information that was developed at our centre earlier (Hathakayama et al.,2017). The polymorphism between the parents was analysed using a chip based fragment analyser (MultiNA Shimadzu, Japan).

Among the 102 markers screened, only 11% of the markers (12 SSRs) were found to be polymorphic between the parents. The gel images derived from the MultiNA output are given in Fig (5). The list of 12 markers that were polymorphic in parental lines is given along with primer sequence information in Table (8). These 12 markers were used to check the banding pattern in the bulked DNA samples. Of these 12, only three markers viz., UASBFM 85, UASBFM 91 and UASBFM 109 showed perfect parental pattern of the trait segregation in the two bulks (Table 9), indicating a possible linkage of these markers to the root trait.

Further analysis is being carried out to ascertain the mechanism of their function and the genes linked these markers using the whole genome sequencing information available with our group. The gel pictures of the three polymorphic markers showing variation in the bulked DNA samples are given in Fig (6). Since the extent of polymorphism of the genomic markers is hardly 13.5%, this study needs a more elaborate analysis of the DNA polymorphism using more SSR markers. Such low polymorphism percentage might be due to polyploidy nature of finger millet. This kind of anomaly in polymorphism can be overcome when the progenitor DNA are sequenced separately and the A or the B sub-genome SSRs are separately identified.

Table 8: List of SSR markers and their sequence details. These markers were polymorphic between the parental lines and were used for screening the contrasting bulks.

MARKER NAME	SSR motifs	FORWARD PRIMER	REVERSE PRIMER	Annealing temperature	Product size
UASBFM 6	(TATC)18	GATTCTCACGAGGTGTCCCTAC	CTATCCAACCAACCACATTTCA	56 °C	304
UASBFM 7	(AT)37	AAGACATGGAGACAAGAAGCAG	AGATTGTGAGATATACATGAGGGAG	56 °C	223
UASBFM 12	(AT)37	AAGACATGGAGACAAGAAGCAG	AGATTGTGAGATATACATGAGGGAG	56 °C	223
UASBFM 36	(ACG)6	CATCTTCACCTTCACGTACCC	ATGCTTCATCTTACTGACTGCG	56 °C	204
UASBFM 41	(AT)24	GTCCTTTGTGAGTGTGCTGGT	AACCGCCGCTACTTGTTATTT	55 °C	286
UASBFM 47	(AG)10	TGAAAATACCGCCTATGCTTCT	GTTACACCAAATAGTGGCCCTG	55 °C	387
UASBFM 81	(TCA)8	AAGAGCTGATAGGAGCACAAGC	AGAAGAAGAAGGAGGAGGAGGA	55 °C	368
UASBFM 85	(AG)20	GTTGTTTCGTCAAATGTTGTGG	GGGCTACTGTCATGCAATCTTT	55 °C	363
UASBFM 91	(AT)6	CCATCGTTTTCATCCCTAGTTC	CATGTTTGATGTCGGAACAGAT	55 °C	317
UASBFM 98	(GA)7	GCGCACTGAATAATCCTCAATC	CAACGAAGTAACCTGACACCAA	56 °C	371
UASBFM 109	(GAG)7	TGGTGTCGAACTTGTCGTACAT	GATCCTCTCTCTTGTCAGCCAC	55 °C	347
UASBFM 119	(CT)31	CCTTTTCTTCTTTGACCTCTTCC	GAGGGAGGAATCAGCACAATAC	56 °C	245

Table 9: List of markers that showed polymorphism between the contrasting bulks. The banding pattern of these markers was similar to that of the two contrasting parental lines, confirming possible association of these marker with root weight.

MARKER NAME	SSR motifs	FORWARD PRIMER	REVERSE PRIMER	Annealing temperature	Product size
UASBFM 85	(AG)20	GTTGTTTCGTCAAATGTTGTGG	GGGCTACTGTCATGCAATCTTT	55 °C	363
UASBFM 91	(AT)6	CCATCGTTTTCATCCCTAGTTC	CATGTTTGATGTCGGAACAGAT	55 °C	317
UASBFM 109	(GAG)7	TGGTGTCGAACTTGTCGTACAT	GATCCTCTCTCTTGTCAGCCAC	55 °C	347

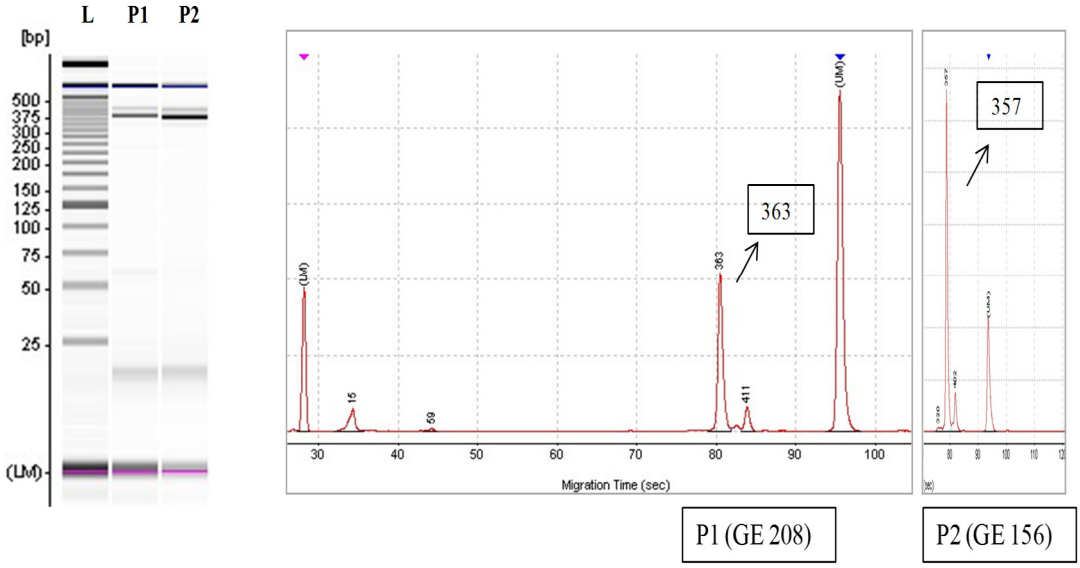


Fig. 5: Representative picture depicting polymorphism between parent 1 (P1) and parent 2 (P2) for the marker UASBFM 85. Both the parental lines were screened with SSR markers. All the polymorphic markers were further used to screen the high root (B1) and low root bulks (B2).

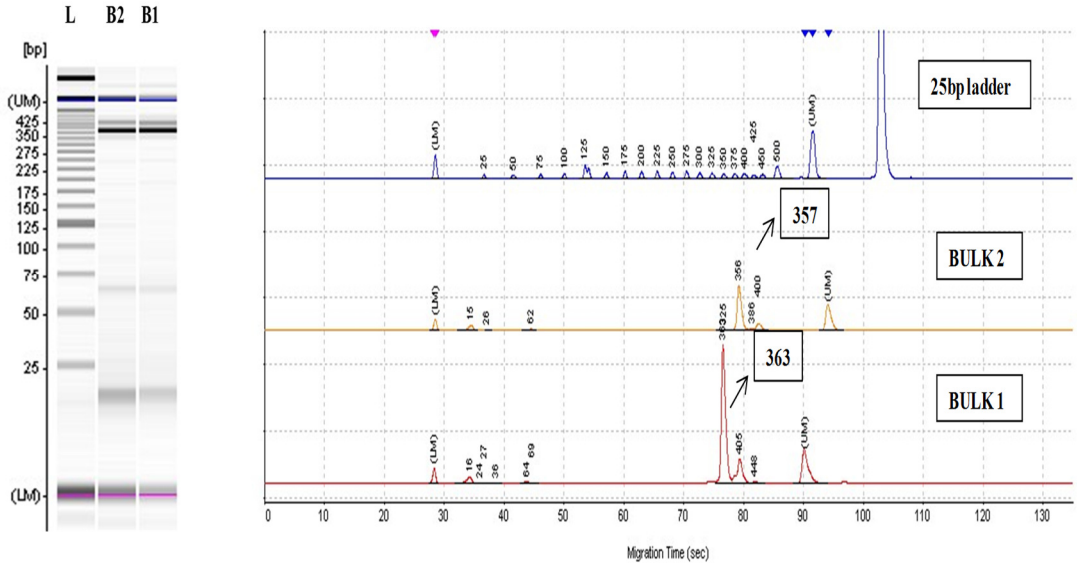


Fig. 6: Representative picture showing amplicons peak of high root (B1) and low root (B2) bulk similar to parental lines for the marker UASBFM 85.

Unfortunately, one of the two progenitors of cultivated finger millet namely *Eleusine floccifolia* is reported to be extinct. Only one progenitor of finger millet namely *Eleusine indica* is available, which has been already sequenced at our centre and analysis is being done to identify progenitor specific SSR markers for further analysis and also to perform genotyping by sequencing of the RIL population.

This study reveals the presence of significant genotypic variation for the traits of interest. The genome analysis by 'genotyping by sequencing' combined with the phenotypic variations would definitely leads to identification of robust QTLs for specific traits associated with drought adaptation in ragi. This can be subsequently used for crop improvement through marker assisted breeding.

V SUMMARY

Ragi [*Eleusine coracana* (L.) Gaertn] is an important cereal grain grown and consumed predominately in the arid and semi-arid regions of the world. It is cultivated under rainfed as well as irrigated conditions. But most of the Ragi growing area is under rainfed conditions where water availability is less. So increasing growth rates as well as drought adaptation indeed appears as a formidable challenge, owing mainly to the complexity of drought stress and an equally complex crop response to drought adaptation.

Drought tolerance is not a single response, but is mostly controlled by many genes and those genes will interact with environment, thus the mechanisms involved in drought tolerance are quite complex in nature. Hence, selection based on the phenotype would be difficult for such traits (Collins *et al*., 2008). Therefore, a DNA based molecular marker assisted approach would strongly complement the conventional breeding method to introgress these relevant traits on to agronomically elite genetic background. Similar hypothesis is also successfully tested in Rice, where we identified robust QTLs by association mapping in Rice (Raju *et al*., 2016). Subsequently, these QTL markers more employed in a multi-parent marker associated backcross breeding to introgress these traits (Dharmappa *et al*., 2019). These results strongly imply that when water mining and carbon association capacity are combined, there can be a significant improvement in crop productivity even under water limited conditions (Sheshshayee *et al*., 2018)

Ragi, being a C_4 species is restored with superior carbon assimilatory capacity. Hence, attempts to enhance water mining through root system can definitely enhance growth and productivity of Ragi.

With this background, an investigation was done using a set of 206 RILs of Ragi developed by crossing a high root genotype (GE 208) and a low root genotype (GE 156) to identify QTLs of associated with these traits. The RIL population (F5) were sown in specialized root structure which represents field condition and were grown till era head emergence date (75DAS). After 75 days plants were harvested and different biometric measurements were recorded.

Variation was noticed in biometric parameters like root length, root weight, root volume, SLW, total leaf area, total biomass, etc. This variation was normally distributed around the mean confirming their quantitative inheritance.

To understand the other important leaf parameters, we identified and selected a set of RILs with comparable leaf area. These selected RILs did not show a significant regression between total biomass and total leaf area. Indicating that the differences in biomass among the selected lines was not due to leaf area. So other parameters of relevance were examined in the same selected lines. The most important parameter which helps in the biomass accumulation through improves photosynthetic rate is the leaf thickness. Leaf thickness, as estimated by SLW showed significant variations among the selected RILs.

To ascertain the influence of SLW on total biomass, two groups of contrasting lines differing in SLW were selected and these lines revealed a significant variation in total biomass. The high SLW group representing thicker leaf system had significantly higher photosynthetic capacity than the low SLW types. With comparable leaf area, high SLW types would apparently accumulate more biomass because of higher photosynthetic rate. By this we can conclude that high SLW with better root system would have much higher biomass production capacity.

With a major objective to identify QTLs governing root traits and other physiological traits, bulked segregant analysis was applied to identify markers linked with specific traits. Among the 88 genomic SSR markers used to screen the parents for polymorphism, only 12 showed polymorphism for both the parents, which were further used to screen the two contrasting bulks differing for root traits. Out of 12 markers only three markers were polymorphic in the bulk.

Though the study displayed significant genotypic variation in the mapping population, the extent of polymorphism observed among genomic markers is hardly 13.5%. However, the genome analysis by genotyping by sequencing (GBS) combined with the phenotypic variations would definitely leads to identification of robust QTLs for specific traits associated with drought adaptation in Ragi.

Future plan of work :

- The population will be screened with more number of markers including SNPs for GBS.
- DNA samples are being used for sequencing and this will be used for construction of linkage and physical

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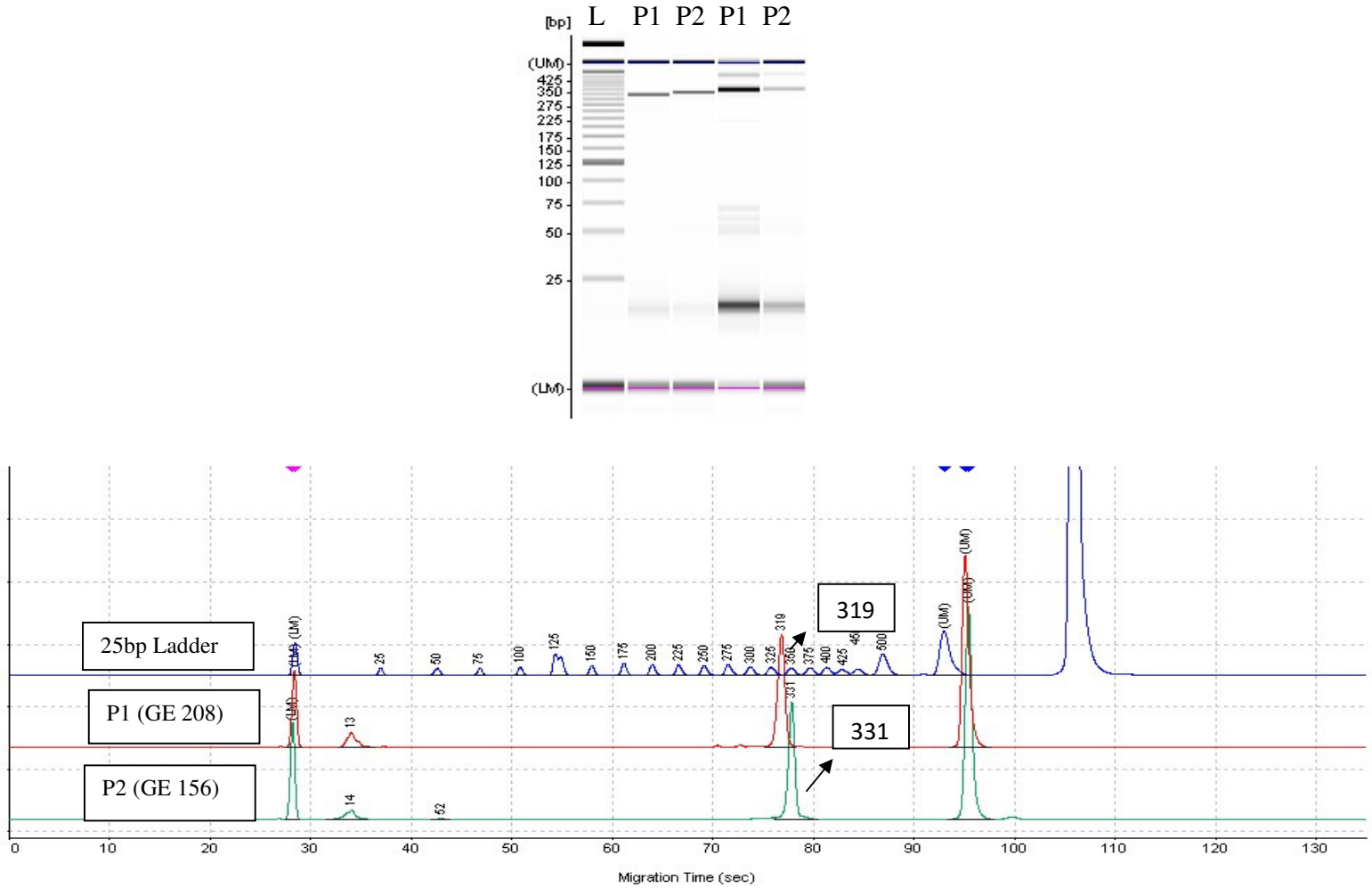
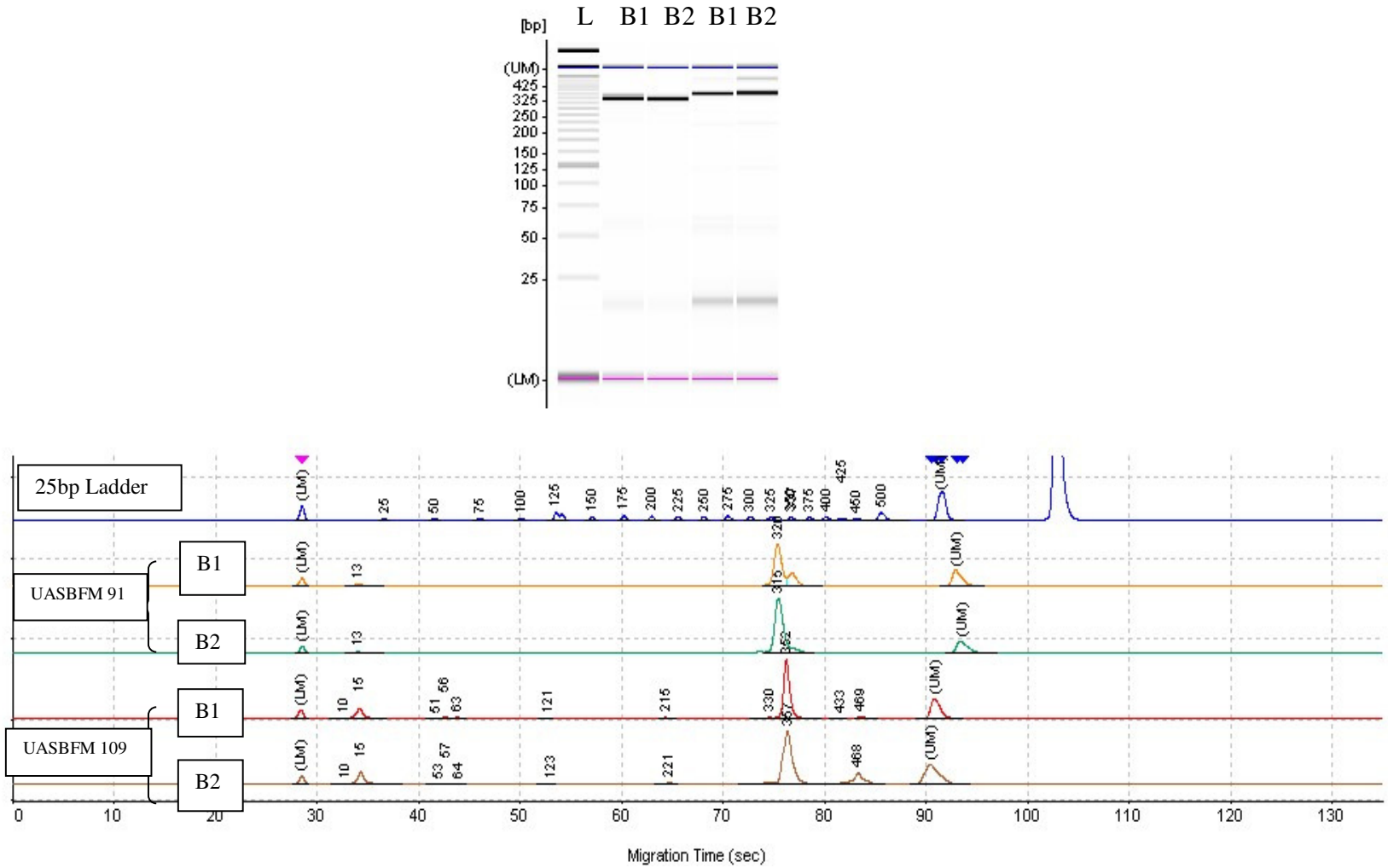
Appendix 2: Details of parameters recorded in root structure with abbreviations and unit

Parameters recorded	**Abbreviations**	**Unit**
Ear head emergence date	EHD	days
Specific leaf area	SLA	$cm^2\ g^{-1}$
Specific leaf weight	SLW	$mg\ cm^{-2}$
Total leaf weight	TLW	g
Total leaf area	TLA	cm^2
Shoot length	SL	cm
Shoot weight	SW	g
Root length	RL	cm
Root weight	RW	g
Root volume	RV	cm^3
Total dry matter	TDM	g
Dry weight to leaf area ratio	DM/LA	$g\ cm^{-2}$
Root to shoot ratio	R/S	%
Root to leaf area ratio	R/LA	$g\ cm^2$

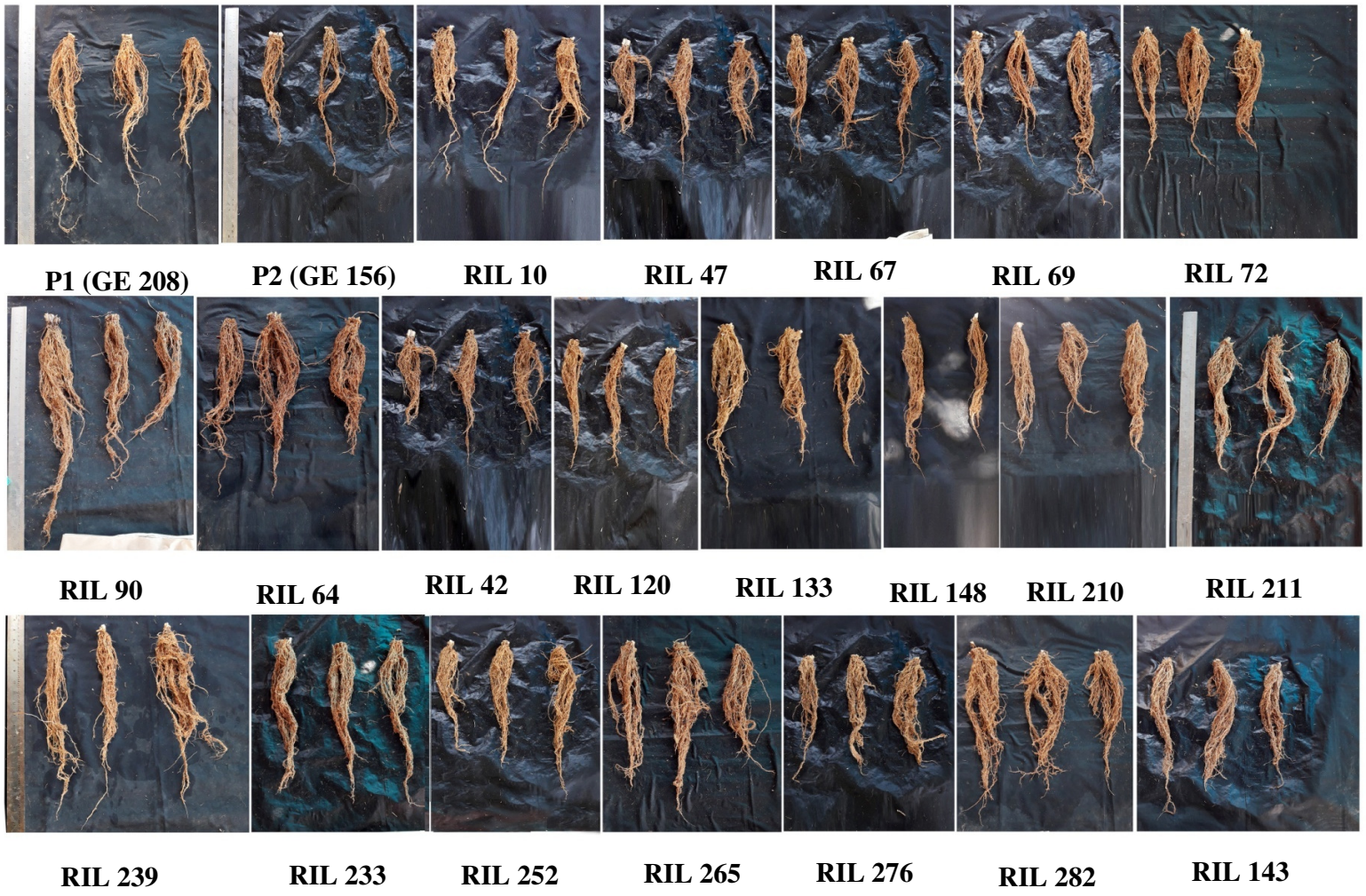
Appendix 3: Representative picture depicting polymorphism between P1 and P2 for the marker UASBFM 91 and UASBFM 109



Appendix 4: Amplicon peak of markers UASBFM 91 and UASBFM 109 in the high root (B1) and low root (B2) bulk



Appendix 5: Root Images of 20 high root RILs selected for BSA



Appendix 6: Root Images of 20 low root RILs selected for BSA