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Root attributes governing drought stress adaptation and the associated molecular markers in chromosome segment substitution lines in rice (*Oryza sativa* L.)

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ABSTRACT

The wild relatives of cultivated rice offer crucial resistance genes for combating stresses like drought. Developing rice varieties with drought tolerance is possible through chromosome segment substitution lines (CSSLs), which blend the genetic background of a high-yielding parent with specific chromosome segments from a donor parent. This study aimed to study the effect of drought stress on various root traits of chromosome segment substitution lines (CSSLs) and their relationship with specific molecular markers. Ninety-six genotypes, including 80 chromosome segment substitution lines (Curinga x *O. rufipogon* and Curinga x *O. meridionalis)*, 9 New Rice for Africa (NERICAs) and 7 controls were grown in Basket and PVC pipe methods for phenotyping different root traits. Under drought stress (DS), MER16, MER20, RUF10, RUF16, RUF44, NERICA1, and NERICA3 showed superior performance for most of the root traits. These evaluations were supplemented with association analysis of 17 root trait-linked simple sequence repeat (SSR) markers with root phenotypic traits. The marker RM201 is strongly associated with multiple root traits, found to be independent of three growth conditions (well-watered "WW" under Basket, WW condition and DS conditions under PVC pipe). The marker RM316 is associated with root volume, and the marker RM7424 and RM1054 show maximum root length. In conclusion, these markers can be used in marker-assisted breeding programs, and the lines carrying them can be used as parental lines in variety-development programs for drought tolerance.

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1 Introduction

Rice is consumed as a primary dietary source by 340 million people worldwide, predominantly in southern and south-eastern Asia (Rezvi et al. 2022). In a rainfed ecosystem, standing water quickly disappears once the rain stops, harming rice productivity (Ogura and Forwell 2023). Rice can be grown both in lowland and upland environments. Asia's Rainfed lowland and upland areas are 34 million hectares and 8 million hectares, respectively, for rice cultivation (Anilkumar et al. 2023). The sensitivity of rice to different climatic changes, such as an increase in temperature and an extended drought period (Yoshida et al. 2015), poses complex challenges in securing the future of global food demand. Rice production is severely hampered by drought stress (DS), especially in water-limited upland environments (Ndikuryayo et al. 2022). The situation is worse in rainfed upland areas than in lowland areas, as the crop experiences mild to severe DS between the rainfall events throughout their life cycle, and standing water is rarely available. To avoid DS, the rainfed upland rice varieties develop a more profound and thicker root system that allows the plant access to deep soil-water reserves, thereby maintaining a higher leaf-water status (Zampieri et al. 2023). Higher root volume (RV) and root length (RL) have been reported as a better combination for the selection and development of lines suitable for DS conditions (Dash et al. 2017). Previous studies have focused on rice improvement efforts to promote vigorous deep rooting and enhance yield stability for rainfed uplands (Anilkumar et al. 2023). Deep rooting helps maintain plant-water status in an upland ecology where intermittent drought is common (Sandar et al. 2022). The root traits that affect drought tolerance include root length density, root depth, root thickness, root dry weight, root penetration index, and deep root ratio (Jeyasri et al. 2021). Most current rainfed rice varieties were originally developed for irrigated ecosystems; however, these varieties fail to produce good crops under DS conditions (Venkateshwarlu et al. 2022). It is, therefore, necessary to develop high-yielding varieties with enhanced drought tolerance traits. Developing rice varieties with desirable root traits for upland ecologies by incorporating these traits from landraces into the high-yielding varieties is considered beneficial (Sabar et al. 2019). Attempts have been made to develop varieties using chromosome segment substitution lines (CSSLs) carrying the genetic background of the high-yielding recurrent parent and overlapping donor parent chromosome segments (Pinta et al. 2018; Ding et al. 2022). However, very few reports are available on the genetic improvement of known cultivated varieties for drought tolerance through introgression from wild rice species using the CSSLs approach. Genus *Oryza* has twenty-one wild relatives of the domesticated rice (Vaughan et al. 2003) that serve as a virtually untapped reservoir of genetic diversity and contain many novel resistance genes for biotic and abiotic stresses (Barik et al. 2017; Long et al. 2023). Wild rice prefers various habitats, including wetlands, drylands, and fresh or salty soils. It has a large genetic pool within Oryza that contains characteristics that confer tolerance to many different abiotic stresses. According to Atwell et al. (2014), a study employing a Geographic Information System (GIS) approach that superimposed environmental maps over georeferenced wild rice species occurrences identified many candidate species that warrant additional investigation in the quest for tolerance to cold (1 species), heat (5 species), submergence (4 species) or drought (5 species).

Around 40% of these important alleles in rice were lost from wild to cultivated during domestication. Exploring these favorable alleles of wild rice, through the development of CSSLs from crosses between wild species and cultivated varieties might be a powerful tool by identifying naturally occurring favorable alleles and to overcome yield limitations. CSSL libraries have been developed for many wild rice species, helping identify many quantitative trait loci (QTLs) of biological and economic interest (Subudhi et al. 2015; Zhao et al. 2022). These QTLs have been utilized for their important agronomic traits by effective mapping, cloning, and identification of gene interactions (Bimpong et al. 2011; Li et al., 2023). In the present investigation, 80 CSSLs having chromosome segments of wild rice *O. rufipogon* accession IRGC 105491 (RUF) and *O. meridionalis* accession OR 44(MER) in the genetic background of Curinga, the elite tropical *japonica* upland cultivar from Brazil, were studied.

O. rufipogon and *O meridionalis* possess AA genomes and are cross-compatible, and they are estimated to have diverged around two million years ago (Toulotte et al. 2022). Previous studies showed that *O. rufipogon*, originating in Southern China and presently found throughout Asia, is more genetically diverse than *O. sativa* (Toulotte et al. 2022). *O. meridionalis* is native to Australia, is more drought-tolerant, and is better adapted to arid climates than *O. sativa*. Wild species such as *O. rufipogon* Griff and *O. glaberrima* Steud were extensively used for their improved tolerance to drought stress (Chen et al. 2023).

The recurrent parent Curinga is a commercial rice variety developed by Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA, Goiania, Brazil), tolerant to drought released in 2005 (de-Morais et al. 2005). It is resistant to rice blast leaf scald and tolerant to acidic soils. Nine NERICA lines were derived by crossing *O. glaberrima (*African rice*)* and IR64. *O. glaberrima* is grown in West Africa. They are resistant to African gall midge, nematodes, rice yellow mottle virus (RYMV), DS, acidity, iron toxicity, and have strong weed competitiveness. Understanding the root system structure can promote a second green revolution focusing on crop performance under nutrient and water constraints (Anilkumar et al. 2023).

Since root traits are incredibly crucial, the function of the root under drought stress adaptation creates a hydraulic environment that permits the optimal use of water by plants to maximize the water use efficiency (WUE) under critical conditions (Abdalla et al. 2022; Fonta et al. 2022)

Genetic markers for different QTLs governing root traits relevant to drought tolerance are valuable resources to identify the lines with important QTLs. CSSLs help uncover many desired genes/alleles from wild rice species. Hence, in this study, 80 CSSLs derived from two crosses (Curinga and *O.rufipogon*-IRGC105491; Curinga and *O. meridionalis* OR44) and nine NERICA lines have been selected to characterize their root traits and identify lines with drought tolerance chosen relevant root QTLs. This study aims to identify suitable lines for drought tolerance with desired alleles and phenotypic characteristics. In the current investigation, a set of CSSLs and NERICA lines were phenotyped for root architectural traits, and an attempt was made to identify the lines with desirable root QTLs.

2 Material and Methods

2.1 Plant materials

A set of 96 genotypes, including 80 CSSLs, 9 NERICA lines, and 4 drought-tolerant genotypes collected from the Indian Council of Agricultural Research, National Rice Research Institute (ICAR-NRRI), Cuttack, Odisha, India, and three international control lines were used for this study. The 80 CSSLs included 48 developed lines from the cross between Curinga and *O. rufipogon* IRGC105491 (RUF), and 32 derived lines resulted from the cross between Curinga and *O. meridionalis*-OR44 (MER) (McCouch et al. 2007). Professor Susan McCouch developed these lines at Cornell University, USA. The nine NERICA lines were obtained by crosses between *indica* elite cultivar, IR64 and African rice *O. glaberrima* in the same lab. Four drought-tolerant genotypes, namely Mahulata (a drought-tolerant landrace), Satyabhama (newly released varieties for drought-prone upland areas), CR 143-2-2, and CR 2702, were used as local control lines in this study. The international controls included IR 64 (drought sensitive), Azucena (drought-tolerant) and Curinga (the recurrent parent). The CSSLs and NERICA lines were obtained through a collaborative project on sustainable crop production for international development (SCPRID).

2.2 Phenotyping of root traits for drought stress tolerance using the basket method

Here, we conducted two experiments utilizing the Basket and PVC pipe methods for the root phenotyping of 96 genotypes. The Basket and PVC pipe method experiments, were performed under field conditions and in rainout shelters, respectively. The Basket method approach evaluated the deep-rooted traits from previous research studies with few modifications (Subudhi et al. 2015). This field study was conducted following a complete randomized design (CRD) using two replications. In each replication, six baskets were installed per genotype. The baskets had a 2 mm mesh size and measured 8 cm in height, with top and bottom diameters of 18 cm and 9 cm, respectively. The volume of soil contained in the basket was 5100 cm^3. In each basket, four to five seeds were sown at the center of the basket. Seven days after germination, one plant was retained per basket after thinning. The baskets were buried in the field at 10 cm depth with a 20 cm gap between adjacent baskets and containing soil and sand in a 2:1 (vol:vol) ratio. In the experimental field, 10 cm depth was specified to maintain the ground level and the plants were irrigated daily. The recommended amounts of $N:P_2O_5:K_2O$ @ 80:60:60 kg ha^{-1} were followed when applying urea, DAP, and MOP fertilizers, respectively. Weeds were removed from the field manually as per requirement. During the entire crop growth season, essential plant protection techniques were implemented. Surface irrigation was provided until the completion of root harvest utilizing a jet pipe. The baskets were then soaked in a water tank for 24 hours. The next day, the roots were thoroughly washed with a fine water jet, and the number of roots emerging from the mesh of the baskets was counted. The roots protruding from the basket's sides and bottom were considered deep roots (DR) and shallow roots (SR), respectively. The entire root system present inside and outside of the basket was scanned. Three plants for a single genotype were sampled from each replication for trait evaluation. Seven lines out of 96 genotypes (RUF-35, MER-2, MER-3, MER-5, MER-8, MER-10, and MER-11) showed inferior growth under the basket and were excluded from further root phenotypic evaluation and genotypic study. The following formulae were used for calculating the total roots and ratio of deep rooting (Oyanagi et al. 1993)

$$\text{Total roots (TR)} = \text{DR+SR},$$

$$\text{Ratio of deep rooting (RDR)} = \text{DR/TR}$$

The images of the roots were captured at 400 DPI resolution using an EPSON professional scanner (Magalhães et al. 2011) and analyzed with Win Rhizo Pro 2007a (Regent Instrument Inc., Quebec, Canada) root analysis software.

2.3 Root phenotyping using Polyvinyl chloride (PVC)pipe method

Eighty-nine genotypes were evaluated for root traits in PVC pipes under two different water regimes: well-watered (WW) and drought stress (DS) in two replications for each treatment. Each pipe was filled with 10 kg of sandy clay loam soil collected from the institute field, having moderate acidic pH (4.5-5.5) and a medium organic carbon content (0.50-0.75%). The pipes were

placed in cement pits at a depth of one meter to avoid an increase in temperature due to the direct incidence of solar radiation sunlight on the surface of the pipes. The pipes were one meter long and twenty centimetres in diameter. Before sowing, the pipes were watered daily for 4-5 days till uniform compaction was attained. In each pipe, four to five seeds were sown and 7 days after germination, a single plant was retained after thinning. Thirty-day-old seedlings were exposed to DS through a 20-day stress period (20 DSP) of water restriction. Soil Moisture Content (SMC) was estimated gravimetrically at a soil depth of 50 cm. Since the interval soil sampling might cause root damage, soil sampling was done at the end of the DS. The root and shoot samplings were done at 20 DSP when the tips of the leaf started drying and were completely rolled in the sensitive variety. Under the DS condition, the SMC at a depth of 50 cm was reported as 12%. The pipes were carefully removed and immersed in a water tank for 24 hours for sampling. The roots were then cleaned, following which different root and shoot traits were measured, similar to the prior study (Toorchi et al. 2002). Different traits measured were maximum root length (MRL), shoot length (SL), root volume (RV), root dry wt. (RDW), shoot dry wt. (SDW), total plant dry wt. (TDW), root-to-shoot dry weight ratio (RDW/SDW), root-to-total plant dry weight ratio (RDW/TDW), maximum root-to-shoot length ratio (MRL/SL), specific root length (SRL), the root growth rate in depth (RGRD), the root growth rate in volume (RGRV), root length density (RLD) and the ratio of deep rooting (RDR). Four plants for a single genotype were sampled from each replication under both growth conditions for trait evaluation. Plasticity for each root trait was calculated similarly to understand the previous study (Sandhu et al. 2016).

2.4 DNA isolation followed by PCR amplification and marker visualization linked to root traits

Leaves were sampled from fifteen-day-old seedlings for genomic DNA extraction and molecular screening. The leaves were crushed in liquid nitrogen, and then the total genomic DNA was extracted using the Qiagen kit for DNA extraction (Qiagen, Germany). The extracted DNA was quantified using NanoDrop (Thermo Scientific, 1000-spectrophotometer). The DNA amplification was performed in a 10µl reaction volume containing 0.2mM of dNTP mix, 0.4 picomoles of forward and reverse primers, 30ng of genomic DNA, and one Taq polymerase unit (Mohanty et al. 2019). Amplification was done in a programmable thermal cycler (Eppendorf, USA) with pre-denaturation for 5 minutes at 94^0C, 35 cycles of denaturation for 30s at 94^0C, annealing for 1 minute at 56^0C, extension for 1 minute at 72^0C, and a final extension for 7 minutes at 72^0C. Polymerase Chain Reaction (PCR) products were stored at a temperature of 4^0C. Thirty previously reported root trait-linked SSR markers were used for PCR amplification, 17 of which were found to be polymorphic. The PCR amplification products were mixed with 3 µl of loading buffer, and from this mixture, 10µl was loaded in a 2.5% agarose gel containing 0.5µg/ml of ethidium bromide. Electrophoresis was performed in 0.5X TBE buffer (pH 8.0) at 80 volts (2.5V/cm) for four hrs and photographed using a Gel-Doc System (SynGene, UK). The amplicon size was determined using a DNA ladder of 100 bp

2.5 Genetic Diversity

For each genotype, scoring was performed on the database in the presence or absence of alleles obtained with the primers. A similarity matrix was constructed using Jacquard's coefficients. The Power Marker Ver3.25 software was used to estimate the number of alleles, gene diversity, heterozygosis, allele frequency, and polymorphic information index (PIC) (Lu et al. 2005). Using the software TASSEL 5.0, a general linear model (GLM) and mixed linear model (MLM) were used to investigate the association between SSR markers and root traits.

2.6 Statistical Analysis

The phenotypic and physiological data were calculated using Microsoft Excel. Descriptive analyses, including mean, analysis of variance (ANOVA) and standard deviation (SD) estimates, were calculated on the tested traits over two moisture regimes using CROP STAT ver 7.2. For allele scoring, data were scored according to the presence or absence of the amplified products for each genotype-primer combination. Discrete variables were entered into a binary data matrix. The Power Marker Ver3.25 program determined allele frequency, the number of alleles, gene diversity, heterozygosis, and polymorphic information index (PIC) (Lu et al. 2005). The hypothesis that the SSR markers are associated with root traits was tested by the generalized linear model (GLM) and mixed linear model (MLM) of the TASSEL 5 program (Bradbury et al. 2007).

3 Result

3.1 Phenotyping of root traits using the Basket method

A wide range of variation among the tested lines was observed for all the 14 different root and shoot traits measured after 40 days post-germination under the WW condition. The RDR of 89 genotypes ranged from 30.86% to 87.31%. Three lines, namely, RUF-16, RUF-10, and RUF-44, had a higher RDR and values in other root traits. It was seen that RUF-6 had a shallow rooting (RDR=30.86%), whereas RUF-16 had a relatively deeper rooting (RDR=87.31%). RDR of RUF-16 was significantly higher (p<0.01) than RUF-6 (Figure 1). The highest RLD value of 1.60cm cm^{-3} was recorded from Mahulata, followed by the local tolerant control CR 143-2-2 (1.57 cm cm^{-3}) and RUF-2 (1.51cm cm^{-3}) (Table 1). Among the tested lines, Mahulata, RUF-2, RUF-10, RUF-37, RUF-39, MER-18, MER-23, Azucena and CR 143-2-2 had higher values for multiple root traits under WW condition.

Figure 1a **Figure 1b**

Figure 1 Rooting pattern of contrasting CSSLs showing deep and shallow rooting pattern under well watered (WW) conditions using basket method (a) Deep rooting of RUF-16 (b) shallow rooting of RUF-6

Table 1 Mean (M), range and standard error (SE) of all the measured root traits of 89 genotypes under the basket method

Traits	M± SE	Min.	Max	Range	Best three Lines (values)
Ratio of deep rooting (RDR %)	63.84±0.013	30.86	87.31	56.45	RUF-16 (87.31), RUF-10(84.69), RUF-44(84.52)
Root length density(RLD, cm cm^{-3})	0.72± 0.034	0.16	1.60	1.44	MAHULATA (1.60), CR 143-2-2 (1.57), RUF-2 (1.51)
Maximum root length (MRL, cm)	28.15±0.730	14.15	43.85	29.70	MER-23(43.85),MER-18 (43.70), NERICA-5 (43.50)
Shoot length (SL, cm)	50.26±0.846	29.80	68.50	38.70	RUF-2(68.50), RUF-6 (66.85), RUF-47(65.15)
Root volume (RV, cc)	5.04±0.252	0.60	11.64	11.04	CR 143-2-2(11.64), RUF-2(10.38), MAHULATA (9.48)
Root dry weight (RDW, g)	0.37±0.016	0.13	0.85	0.61	MAHULATA (0.85), AZUCENA (0.74), CR 143-2-2 (0.68)
Root average diameter (RAD, mm)	0.49±0.019	0.20	0.89	0.69	RUF-10 (0.89), NERICA-3 (0.88), CR 143-2-2 (0.85)
Shoot dry weight(SDW, g)	0.94±0.043	0.11	1.80	1.69	CR 143-2-2 (1.80), RUF-10(1.75), RUF-2 (1.64)
Total dry weight (TDW, g)	1.30±0.055	0.31	2.49	2.18	CR 143-2-2 (2.49), RUF-10 (2.39), AZUCENA (2.17)
RDW/SDW (g)	0.44±0.025	0.17	1.85	1.68	RUF-37(1.85), MER-27(1.43), RUF-39(1.19)
RDW/TDW (g)	0.29±0.008	0.14	0.65	0.51	RUF-37(0.65),RUF-39 (0.54), MER-13(0.47)
MRL/SL (cm)	0.57±0.017	0.29	1.11	0.82	RUF-28 (1.11), RUF-31(1.05), MER-23(1.00)
Specific root length (SRL, cm/g)	87.43±2.788	31.73	136.59	104.86	RUF-14(136.59), RUF-4(135.39), RUF-38(134.10)
Total root length (TRL, cm)	3651.65±173.60	804.42	8122.29	7154.41	MAHULATA (8122.29), CR 143-2-2 (7958.83), MER-18 (7764.20)

Table 2 Mean (M), range and standard error (SE) of all the measured root traits of 89 genotypes under the PVC pipe method

Traits	Treatments	Mean± SE	Min.	Max	Range	Best 3 lines
MRL (cm)	WW	78.07±1.597	34.05	100.35	66.30	RUF-16(100.35), MER-26(100.20), MER-32(98.75)
	DS	59.61±1.638	24.05	90.90	66.85	RUF-16(90.90), NERICA-3(88.95), RUF-10(87.30)
SL (cm)	WW	71.65±0.572	54.65	86.05	31.40	RUF-42(86.05), NERICA-1(81.40), RUF-46(81.25).
	DS	58.52±0.644	41.60	72.90	31.30	NERICA-1 (72.90), MER-16(71.20), CR 143-2-2(68.00)
RV (cc)	WW	39.91±2.528	6.00	127.00	121.00	MER-16(127.00), RUF-44(91.50), MER-28 (89.00).
	DS	19.34±1.538	4.00	78.50	74.50	RUF-44(78.50), NERICA-1(66.50), NERICA-3(66.50).
RDW (g)	WW	3.40±0.198	0.99	14.34	13.35	MER-16(14.34), RUF-4(8.86), RUF-24(8.06).
	DS	1.73±0.084	0.45	4.82	4.37	MER-16 (4.83), RUF- 44(3.76), NERICA-4(3.38).
SDW (g)	WW	4.25±0.213	1.30	13.91	12.62	NERICA-4(13.91), NERICA-1(9.20), RUF-4(8.88).
	DS	1.88±0.118	0.37	5.98	5.61	NERICA-1(5.98), NERICA-4(5.76), RUF-23(4.98).
RDW/SDW (g)	WW	0.86±0.051	0.21	3.27	3.06	RUF-28(3.27), RUF-24(2.76), MER-16(2.71).
	DS	1.10±0.068	0.22	3.63	3.41	RUF-28(3.63), MER-20(3.39), MER-16(3.18).
TDW (g)	WW	7.68±0.346	2.73	19.63	16.90	MER-16(19.63), NERICA-4(18.35), RUF-4(17.74).
	DS	3.65±0.177	0.83	9.20	8.37	NERICA-1(9.20), NERICA-4(9.14), RUF-44(7.87).
RDW/TDW (g)	WW	0.43±0.012	0.17	0.76	0.59	RUF-28(0.76), RUF-24(0.73), MER-16(0.73).
	DS	0.49±0.012	0.18	0.78	0.60	RUF-28(0.78), MER-20(0.77), MER-16(0.76).
MRL/SL (cm)	WW	1.10±0.022	0.45	1.47	1.02	RUF-4(1.47), RUF-10(1.44), MER-32(1.44).
	DS	1.04±0.028	0.36	1.67	1.31	RUF-10(1.67), RUF-4(1.60), MER-32(1.47).
SRL (cm/g)	WW	27.28±1.153	6.39	67.80	61.41	RUF-21(67.80), RUF-47(65.85), MER-6(49.07).
	DS	39.52±1.732	17.91	96.00	78.09	RUF-18(96.00), RUF-47(88.67), MER-19(84.04).
RGRD (cm/day)	WW	1.90±0.037	0.83	2.45	1.62	RUF-16(2.45), MER-26(2.44), MER-32(2.41).
	DS	1.45±0.039	0.59	2.22	1.63	RUF-16(2.22), NERICA-3(2.17), RUF-10(2.13).
RGRV (cc/day)	WW	0.95±0.059	0.15	3.10	2.95	MER-16(3.10), RUF-44(2.23), MER-28(2.17).
	DS	0.46±0.035	0.10	1.91	1.81	RUF-44(1.91), NERICA-1(1.62), NERICA-3(1.62).

*WW-well watered, DS-drought stress, Max-maximum, Min-minimum

3.2 Phenotyping of root traits using PVC pipe method

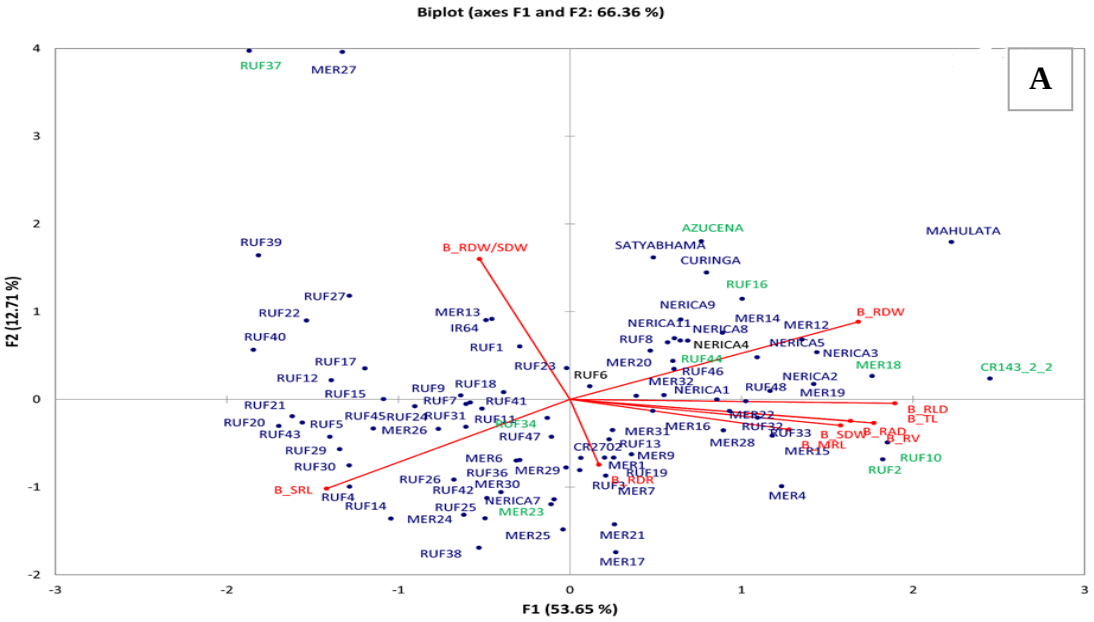
Significant differences ($p<0.05$) were found among the lines for the 12 different root traits and the two treatment conditions, i.e., WW and DS. While comparing the WW to the DS condition, the average values of RV, MRL, RDW, SL, SDW, TDW, MRL/SL, RGRD and RGRV decreased by 51.53%, 23.07%, 47.89%, 18.60%, 56.04%, 52.42%, 5.20%, 23.07% and 51.53%, respectively. However, the average values of SRL, RDW/TDW and RDW/SDW increased by 31.07%, 11.10% and 22.58%, respectively, under the WW condition compared to the DS condition. These results showed that biomass partitioning was more towards the root under DS with a high root length per unit root biomass. Roots grew vertically and horizontally under DS, enabling the plants to access water from larger soil volumes. It was observed that for multiple traits, the lines RUF-10, RUF-16, RUF-28, RUF-44, MER-16, MER-20, NERICA-3, NERICA-1, and NERICA-4 had higher values under the DS condition than the WW condition using the PVC pipe method (Table 2).

The percentage of reduction in SDW, RDW and increment in RDW/SDW were calculated for 89 tested genotypes, of which 10 lines, namely RUF-6, RUF-43, RUF-48, MER-32, RUF-44, RUF-8, RUF-27, RUF-33, RUF-16, and RUF-10 showed less than 30% reduction in SDW and RDW under DS compared to WW. However, 16 lines had a low reduction in RDW and a high reduction in SDW, 20 had a moderate reduction in both SDW and RDW, and 43 had a high reduction in SDW under DS compared to WW. The plasticity of each root trait for the individual lines were estimated by the relative change of a particular root trait under DS

compared with WW condition, and positive plasticity values were obtained for the traits such as RDW/SDW, RDW/TDW, MRL/SL, and SRL, although, most of the root traits showed negative plasticity. The lines RUF-2, RUF-10, RUF-44, and MER-16 showed positive plasticity values for multiple root traits.

3.3 Multivariate analysis and correlation among different root traits

Two different methods, namely the PVC pipe method and the Basket method, were used to construct a genotype vs trait biplot. Here, the varieties for root traits were grown under WW and DS conditions in the PVC pipe method and WW in the Basket method. To reduce noise, the biplot analysis was performed while taking seven traits (RV, MRL, RDW, RDW/SDW, SRL, RGRD and RGRV) from the PVC pipe method and ten traits (RDR, SDW, TL, RV, MRL, RDW, RDW/SDW, SRL, RAD and RLD) from the Basket method. For the PVC pipe method, the plot condensed the information into principal components, out of which the first two components (Figure 2B & C) explained 82.53% and 79.17% of the total variation of the data in WW and DS conditions, respectively. However, for the Basket method, the first two principal components



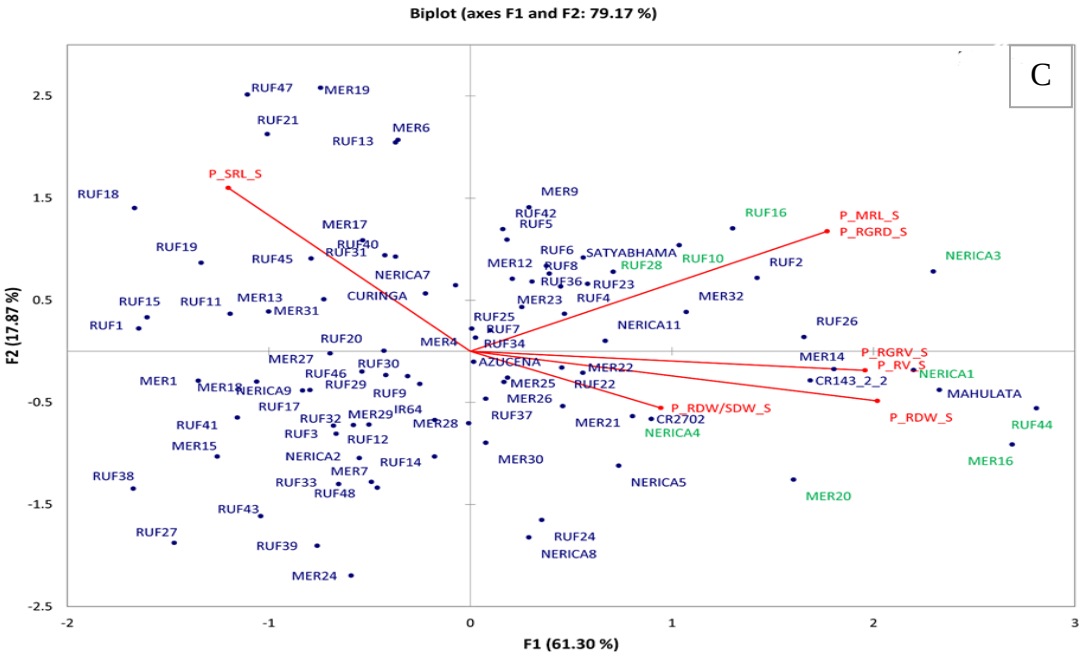


Figure 2 Biplot with two principal components representing the average root traits over two replications for 89 CSSLs under well-watered (WW) condition in basket (a), well-watered (WW) condition (b) and drought stress (DS) condition (c) in PVC pipe method. Variance of each principal component is shown as percentage of the total variance (indicated in axis legend). Black and red colour letters indicate lines and traits, respectively. Mean value was calculated taking two replications for each genotype.

Table 3 Correlation matrix of 12 different root and shoot traits under well-watered (WW) (below) and drought stress (DS) conditions (upper) grown in PVC pipes

	MRL	SL	RV	RDW	SDW	RDW/ SDW	TDW	RDW/ TDW	MRL/ SL	SRL	RGRD	RGRV
MRL		0.125	0.548**	0.666**	0.362**	0.294**	0.576**	0.271**	0.915**	-0.195	1.000**	0.608**
SL	0.112		0.111	0.049	0.198	0.061	0.263*	0.020	-0.271**	-0.150	0.125	0.352**
RV	0.608**	0.352**		0.794**	0.407**	0.277**	0.670**	0.291**	0.438**	-0.484**	0.608**	1.000**
RDW	0.456**	0.260**	0.818**		0.456**	0.456**	0.805**	0.442**	0.539**	-0.714**	0.666**	0.794**
SDW	0.320**	0.086	0.502**	0.409**		-0.462**	0.894**	-0.525**	0.276**	-0.289**	0.362**	0.407**
RDW/ SDW	0.237*	-0.013	0.427**	0.598**	-0.330**		-0.078	0.922**	0.253*	-0.345**	0.294**	0.277**
TDW	0.460**	0.081	0.781**	0.829**	0.849**	0.144		-0.127	0.454**	-0.551**	0.576**	0.670**
RDW/ TDW	0.249*	-0.013	0.421**	0.576**	-0.368**	0.917**	0.108		0.248*	-0.441**	0.271**	0.291**
MRL/SL	0.910**	-0.304**	0.480**	0.424**	0.281**	0.233*	0.417**	0.244*		-0.135	0.915**	0.438**
SRL	-0.144	-0.071	-0.564**	-0.626**	-0.265*	-0.448**	-0.524**	-0.604**	-0.118		-0.195	-0.484**
RGRD	1.000**	0.112	0.548**	0.456**	0.320**	0.237*	0.460**	0.249*	0.910**	-0.144		0.608**
RGRV	0.548**	0.111	1.000**	0.818**	0.502**	0.427**	0.781**	0.421**	0.480**	-0.564**	0.548**	

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

(Figure 2B & C) explained 66.36% of the total variation. In both the treatments, SRL showed a significant negative correlation ($p<0.01$) with all traits except MRL, RGRD in WW and DS conditions in the PVC pipe method and RDW/SDW and RDR in the Basket method.

Among the lines grown in the PVC pipe method under WW and DS conditions, MER-16, RUF-28, NERICA-1, NERICA-3, NERICA-4, RUF-44, and Mahulata had higher values for multiple root traits. Similarly, among the lines grown in the Basket method, MER-4, NERICA-3, RUF-16, RUF-2, RUF-10, MER-18,

Mahulata, Azucena, Satyabhama, Curinga, and CR 143-2-2 recorded higher values for multiple root traits (Figure 2a-c).

Correlations among different root traits for the PVC pipe method under both WW and DS conditions revealed a strong positive correlation among MRL, RV, RDW, SDW, RDW/SDW, TDW, RDW/TDW, MRL/SL, RGRD, RGRV (*p<0.01 or p<0.05 level)*. SRL showed a negative correlation with all the traits but had a significant positive association with RV, RDW, SDW, RDW/SDW, TDW, RDW/TDW, and RGRV under both treatments. SL did not significantly correlate with any of the traits except for a negative correlation with MRL/SL *(p<0.01*) in both the conditions and a positive correlation with TDW *(p<0.05*) and RGRV (*p<0.01*) under the DS condition only (Table 3).

3.4 Marker diversity analysis

Thirty root traits-specific SSR markers were used to genotype the 89 experimental lines, including 73 CSSLs, 9 NERICAs, and 7 tolerant and sensitive controls. Seventeen of the thirty markers were polymorphic, whereas the rest showed no polymorphism among the genotypes. Therefore, these 17 markers were considered for further analysis. The size of the amplicons ranged from 90 to 300 bp (Table 4). A total of 36 alleles were amplified with the 17 SSR markers. The number of alleles per marker varied from 2 to 3, averaging 2.11 per locus.RM219 and RM316 had the highest number of alleles in 89 genotypes. The mean polymorphic information content (PIC) value was 0.2580, with a minimum of 0.0777 (RM201) and a maximum of 0.4954 (RM316). The PIC value indicates the information related to the marker system. The observed heterozygosity (H_o) was zero for all the markers studied. The expected heterozygosis or gene diversity (H_e) ranged from 0.0809 (RM201) to 0.5730 (RM316), with an average of 0.3056. The major allele frequency of these root traits-specific polymorphic markers had an average of 0.7892, ranging from 0.5185 to 0.9577).

3.5 Association of molecular markers with root phenotypic traits

The marker-trait association for all the root traits studied under the Basket and PVC pipe methods was calculated using the general linear model (GLM) and mixed linear model (MLM) of TASSEL5.0 software. Table 5 lists the performance of these models and demonstrates the association between root traits and SSR markers. The squared allele frequency correlation (r^2) values using GLM analysis varied from 0.038 to 0.161, with an average reduced to 0.080, whereas, using MLM analysis, the average was

Table 4 Genetic diversity parameters and details of Simple sequence repeat (SSR) loci used for genotyping of 89 genotypes of rice

S. N.	Marker name	Chromosome number	Min. mol. wt	Max. mol. wt	No of alleles	Major allele frequency	Gene diversity	Heterozygosity	PIC value
1	RM161	1	200	210	2	0.8750	0.2188	0.0000	0.1948
2	RM212	1	180	190	2	0.9213	0.1449	0.0000	0.1344
3	RM1220	1	200	220	2	0.8438	0.2637	0.0000	0.2289
4	RM1282	1	200	210	2	0.7679	0.3565	0.0000	0.2930
5	RM1247	1	200	220	2	0.8679	0.2293	0.0000	0.2030
6	RM525	2	180	190	2	0.9474	0.0997	0.0000	0.0948
7	RM489	3	200	210	2	0.9059	0.1705	0.0000	0.1560
8	RM520	3	110	130	2	0.7978	0.3227	0.0000	0.2706
9	RM567	4	110	130	2	0.8511	0.2535	0.0000	0.2214
10	RM471	4	210	215	2	0.7209	0.4024	0.0000	0.3214
11	RM1054	5	250	300	2	0.6889	0.4286	0.0000	0.3368
12	RM160	9	150	180	2	0.7000	0.4200	0.0000	0.3318
13	RM219	9	180	220	3	0.5976	0.5559	0.0000	0.4910
14	RM7424	9	90	100	2	0.9024	0.1761	0.0000	0.1606
15	RM215	9	190	200	2	0.5185	0.4993	0.0000	0.3747
16	RM316	9	190	210	3	0.5522	0.5730	0.0000	0.4954
17	RM201	9	120	200	2	0.9577	0.0809	0.0000	0.0777
	MEAN					0.7892	0.3056	0.0000	0.2580

Table 5 Association of marker alleles with root phenotypic traits under WW condition in the basket and WW and DS conditions in PVC pipe using the GLM and MLM TASSEL analysis in 89 genotypes

Trait	Condition	Marker name	GLM			MLM		
			F value	*P* value	R^2	F value	*P* value	R^2
RDR	Basket	RM212	5.03624	0.02752	0.04902	1.87802	0.17430	0.02182
		RM489	6.86019	0.01050	0.06541	2.89027	0.09291	0.03359
		RM7424	5.06905	0.02703	0.04933	1.85975	0.17639	0.02161
		RM161	5.10657	0.02649	0.04967	3.16132	0.07911	0.03674
SL	Basket	RM316	5.12609	0.02621	0.05319	2.09271	0.15182	0.02488
		RM1247	5.97914	0.01662	0.06143	5.12266	0.02626	0.06090
		RM1220	5.60082	0.02031	0.05780	3.69655	0.05800	0.04394
		RM525	3.65065	0.05954	0.03853	4.81784	0.03099	0.05727
	Pipe-WW	RM215	5.73004	0.01896	0.05813	4.56076	0.03570	0.05290
	Pipe-DS	RM215	8.59164	0.00437	0.08503	6.00149	0.01642	0.07087
	plasticity	RM215	8.94897	0.00367	0.08803	6.29291	0.01409	0.07432
TL	Basket	RM316	12.12996	7.99E-04	0.08932	11.02210	0.00135	0.09744
MRL	Basket	RM567	7.2526	0.00858	0.06897	4.20459	0.04351	0.04923
		RM1054	5.30390	0.02381	0.05156	3.30835	0.07258	0.03873
	Pipe-WW	RM7424	4.69936	0.03307	0.05105	3.04284	0.08484	0.03588
		RM1247	5.27569	0.02418	0.05693	4.11796	0.04567	0.04856
	Pipe-DS	RM201	5.06364	0.02711	0.05654	4.31065	0.04101	0.04946
RAD	Basket	RM316	5.39851	0.02263	0.05071	4.85640	0.03035	0.05140
RDW/TDW	Basket	RM219	5.73222	0.01894	0.06224	2.22900	0.13928	0.02545
		RM567	11.96688	8.62E-04	0.12131	6.92126	0.01017	0.07903
	Pipe-WW	RM201	16.30910	1.20E-04	0.15277	10.07454	0.00212	0.11937
	Pipe-DS	RM201	17.11390	8.47E-05	0.16169	11.09713	0.00130	0.12954
	plasticity	RM201	16.77223	0.000098322	0.15909	11.04593	0.00133	0.12913
RLD	Basket	RM316	12.11299	8.05E-04	0.08906	11.00674	0.00136	0.09721
RDW	Basket	RM201	6.03276	0.01615	0.05059	3.01442	0.08628	0.03219
	Pipe-WW	RM201	15.39879	1.80E-04	0.14928	12.19985	0.00077	0.14253
	Pipe-DS	RM201	6.41538	0.01322	0.06712	6.13176	0.01533	0.07179
		RM316	6.50533	0.01261	0.06799	5.83887	0.01790	0.06836
RDW/SDW	Basket	RM567	7.06783	0.00943	0.07374	4.36842	0.03971	0.04964
	Pipe-WW	RM489	8.40763	0.00479	0.08912	5.32592	0.02353	0.06340
		RM160	4.58421	0.03524	0.05074	1.48700	0.22618	0.01770
		RM201	15.65058	1.61E-04	0.15359	6.86554	0.01047	0.08173
	Pipe-DS	RM489	5.70485	0.01922	0.06244	4.28527	0.04159	0.05117
		RM201	13.88286	3.56E-04	0.13899	9.97205	0.00222	0.11907

Trait	Condition	Marker name	GLM			MLM		
			F value	*P* value	R^2	F value	*P* value	R^2
TDW	Basket	RM1054	-	-	-	5.09776	0.02661	0.05638
	Pipe-WW	RM201	6.04963	0.01601	0.06663	5.70269	0.01924	0.06663
	Pipe-DS	RM316	6.53788	0.01240	0.06845	5.63716	0.01992	0.06723
MRL/SL	Basket	RM567	5.07825	0.02690	0.05290	2.91232	0.09169	0.03373
		RM1054	9.14598	0.00333	0.09102	3.04926	0.08452	0.03532
	Pipe-WW	RM7424	5.97067	0.01669	0.06000	3.54960	0.06310	0.03642
	Pipe-DS	RM7424	5.56146	0.02074	0.06074	2.75364	0.10085	0.03014
RGRD	Pipe-WW	RM7424	4.67313	0.03355	0.05076	3.02253	0.08587	0.03564
		RM1247	5.22311	0.02487	0.05638	4.07780	0.04672	0.04809
	Pipe-DS	RM201	5.02941	0.02762	0.05617	4.28095	0.04169	0.04910
SDW	Basket	RM567	5.49244	0.02152	0.05378	1.05683	0.30696	0.01188
		RM1054	-	-	-	5.98944	0.01653	0.06732
	Pipe-WW	RM219	4.36835	0.03971	0.04946	4.19798	0.04367	0.04946
	Pipe-DS	RM219	5.61449	0.02016	0.06000	5.36674	0.02302	0.06335
		RM201	6.85431	0.01053	0.07223	7.02003	0.00967	0.08286
RV	Basket	RM316	8.55171	0.00446	0.07720	7.31849	0.00830	0.07474
	Pipe-WW	RM160	0.07432	0.07432	0.07432	4.06693	0.04700	0.04725
		RM201	8.04847	0.00574	0.08649	6.43963	0.01305	0.07482
	Pipe-DS	RM316	15.85299	1.47E-04	0.14516	12.55963	0.00065	0.14161
	plasticity	RM316	15.88086	0.00014	0.14541	12.57342	0.00064	0.14167
RGRV	Pipe-WW	RM160	4.67210	0.03357	0.05216	4.09206	0.04634	0.04754
		RM201	8.08479	0.00563	0.08685	6.46382	0.01289	0.07510
	Pipe-DS	RM316	15.92222	0.00014	0.14569	12.61219	0.00064	0.1423
	plasticity	RM316	15.88086	0.00014	0.1454	12.5734	0.00064	0.1416

WW - well-watered condition, DS - drought stress; GLM - general linear model and MLM - mixed linear model

reduced to 0.064. Among the different comparisons between root traits and markers, 32 were significant at $p < 0.05$ and 22 were significant at p <0.01 while using the GLM analysis. Using the MLM analysis, 28 comparisons were significant at $p < 0.05$, and 13 comparisons were significant at $p < 0.01$.

GLM and MLM analysis were utilized to identify the specific robust markers for various root traits. RM1247, RM567 and RM201 were associated with MRL. RM489 and RM567 with RDW/SDW; RM316 and RM201 with RDW; RM316 with RAD; RM201 and RM160 with RV had higher F-values and lower *p*-values, indicating strong marker-trait association. Plasticity in SL and RDW/TDW were associated with RM215 and RM201, respectively, while plasticity in RV and RGRV were associated with RM316.

Figure 3 shows the Q-Q plot for the smallest log (P-values) using TASSEL 5.0. As shown in the Q-Q plots for root traits in each condition and averaged over all conditions (Figure 3.), the data points observed above the diagonal represent its significance level, and the data points that fell under the diagonal may indicate some overfitting of the model for the traits.

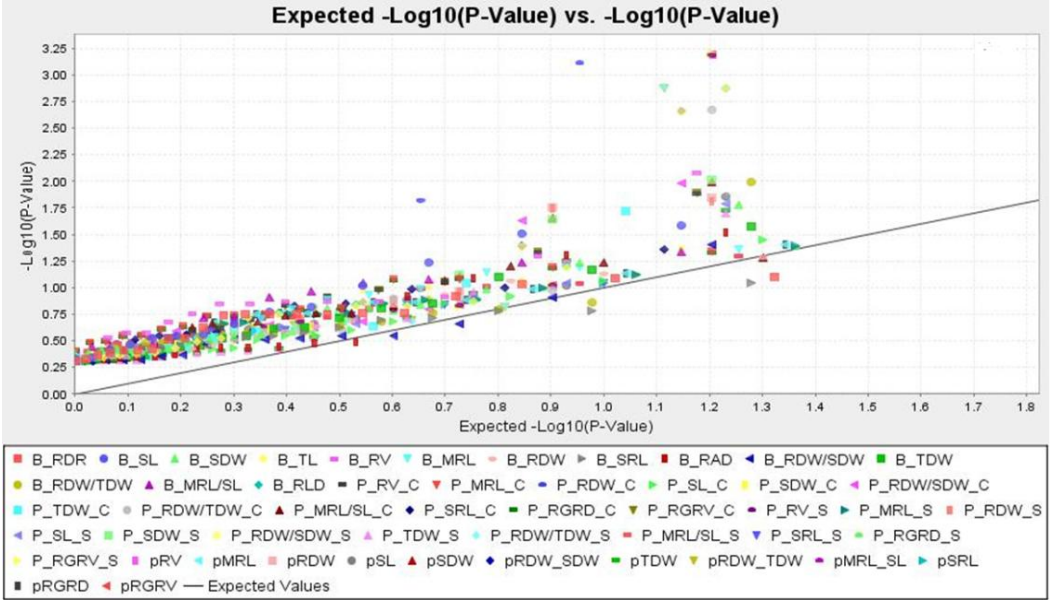


Figure 3 Quantile-Quantile (Q-Q) plot and distribution of marker-trait association from mixed linear model (MLM) analysis; B: Basket experiment under well-watered condition; PC: Pipe experiment under well-watered condition; PS: Pipe experiment under drought stress condition; *p:* plasticity

4 Discussion

The rooting pattern was studied in the Basket and PVC pipe methods in the present experiment. The reduction in SDW was more significant than that in RDW, which was shown by an increase in RDW/SDW (Voetberg and Sharp 1991). Dry matter allocation favoured root growth more than shoot growth (Matsui and Singh 2003). This trend also agreed with our result, showing a higher rate of increase in RDW/SDW. The significance ($p<0.01$) of the correlation between RDW/SDW and RDW suggested that the lines with the highest ratio tend to maintain a higher RDW (Toorchi et al. 2002). Measurement of phenotypic plasticity of root traits revealed that most lines had positive values for RDW/SDW, RDW/TDW, SRL, and MRL/SL. Previous reports showed that genotypes with plastic root traits were better adapted to stress environments (Kadam et al. 2017). RUF-2, RUF-44, RUF-10, and MER-16 had a greater plasticity for multiple root traits and could suggest a better adaptability under DS. From the biplot analysis, these lines outperformed the other lines in all three traits. Improved plant adaptability is generally evaluated by higher production of its shoot biomass under DS. Among the CSSLs, 10 lines had a <30% reduction in SDW and RDW under DS. These lines can be considered drought-tolerant due to their ability to maintain higher shoot biomass under DS since the shoot growth and overall yield production correlate to the morpho-physiological traits of roots (Ghosh and Xu 2014). From the rooting pattern of 89 genotypes studied in the Basket and PVC pipe methods, we observed that some lines with higher root plasticity did not contribute to higher shoot dry matter production under DS as observed in RUF2, RUF26, RUF28, MER16, MER9, MER14, and NERICA3. This might be due to the unfavourable linkages of undesirable traits like tallness, low yield under irrigated conditions, and the traits of interest reported in previous studies (Vikram et al. 2015). However, there is a possibility of breaking the linkage through breeding to develop drought-tolerant high-yielding cultivars (Swamy et al. 2013). Positive plasticity values indicate the trait's increased growth under stress conditions. RUF10, RFU16, and RFU44 had a lower reduction in SDW and RDW with more remarkable root plasticity under DS, which might result in a consistent performance in unpredictable environmental conditions across all seasons. RUF6 despite having a shallow rooting, had the lowest reduction in SDW and RDW under DS. The Basket method looked at the angle, and the PVC pipe method looked at the root growth at a deeper level. Both of these traits were under separate genetic control, evident from the previously studied *DRO*1 lines, which inherited steeper angles but not increased elongation at a deeper level compared to the parent Kinandang Patong (Uga et al. 2013; Singh et al. 2021).

The earlier study indicated that plants adapting to a relatively drier environment have deeper rooting to avoid DS in upland ecology than those growing in WW conditions (Uga et al. 2011; Feng et al. 2012). The TRL and RLD were also determining factors of drought tolerance in rice (Kadam et al. 2015). To meet the moisture demand in plants, RLDs ranging from 0.5 to 1 cm^{-3} were usually considered adequate (de Willigen et al. 2000). In the present study, Mahulata and CR 143-2-2 were recorded with RLD >1.0 cm^{-3}. Besides having a greater RLD and TRL, Mahulata and CR 143-2-2 were drought tolerant, possibly due to the conservative strategies imposed at the stomatal level. An alternative conservation strategy adopted by

RUF10 with more extensive root diameter and lesser RLD allows for better root penetration and exploration of less soil volume, confirmed by the previous findings (Wasson et al. 2012). An effective method to dissect the genetic basis underlying complex traits in plants is association analysis (Vallarino et al. 2023). Root trait-marker association and linkage mapping analyses have previously been reported to determine the genetic basis of certain rice traits (Henry 2013). A wide genetic diversity in root traits was identified in the present study of 89 CSSLs.

In our study, 17 root-linked SSR markers produced 36 alleles with an average gene diversity of 0.3056 and a PIC value of 0.2580. RM201, RM316, RM7424 and RM1054 were associated with 8, 7, 4 and 4 numbers of root traits, respectively. RM201 had the highest association with 8 root traits viz., RDW, TDW, RDW/SDW, SDW, RV, MRL, RDW/TDW, and RGRV. Among these, RDW has the maximum association among them. The RM201 is also associated with the QTLs for basal root thickness, resulting in larger basal root diameter and favouring better soil penetration ability, as suggested by previous findings (Meena et al. 2019). A larger root diameter may enhance the root tissue density and lower SRL, increasing the root biomass (Sandhu et al. 2016). As per the earlier studies of Meena et al. 2019, RM219 and RM316 were associated with the QTL for root number, while RM1054 and RM1247 were associated with MRL. The associated marker alleles can be employed in a molecular breeding program to improve other lines deficient in a particular trait. In the current study, the association of markers with root traits was much more precise, as the lines showed different groups of phenotypes for the root traits. The phenotypic evaluation effectively distinguished the experimental lines into various groups, indicating their heterogeneity towards tolerance to DS. This favoured the presence of linkage disequilibrium and increased the chances of detecting the marker-trait association. In the heterogeneous collection, a similar result showed the potential value of germplasm-detected marker-phenotypic trait association (Zhao et al. 2013; Nachimuthu et al. 2015). The association of markers with most of the traits studied was common between DS and WW conditions of the PVC pipe method, whereas it was uncommon under the WW condition of the Basket method. This difference might be due to the two different plant growth conditions (Basket and PVC pipe) and the duration of the growth period, which was less in the basket (40 days after germination) compared to the PVC pipe method (50 days after germination). Our results from the association of markers RM7424, RM1247, RM201, and RM316 with traits DR, MRL, RL and RV agreed with the previous findings (Uga et al. 2011; Pawar et al. 2012). Also, there is a possibility of the presence of QTLs such as QDrvc9 (root rate volume under control condition) and QMrdc9a (maximum root depth under control), as RM316 is associated with these QTLs (Yue et al. 2006). Similarly, the presence of Dro1 is possible as it is associated with RM7424 (Uga et al. 2015). Therefore, a strongly associated marker, RM201, and the markers indirectly controlling DS tolerance, such as RM7424, RM316 and RM1054, can be used in marker-assisted drought stress tolerance breeding programs.

Molecular marker data and morpho-physiological data exposed to different numerical and taxonomical techniques measure the relationship between the lines. Therefore, combining information from these datasets would be the most effective way to study genetic diversity.

CSSLs are useful in assessing the root responses (Suralta and Yamauchi 2008; Suralta et al. 2010) under transient DS. Studies using the CSSLs have made it possible to evaluate the effect of root growth on shoot dry matter under the DS condition. The finding is less confounding than studies which utilized genetically diverse varieties (Suralta and Yamauchi 2008; Suralta et al. 2010).

Conclusion

Among the 89 genotypes tested, several showed consistent tolerance to DS throughout the study. The drought-tolerant genotypes MER16, MER20, RUF10, RUF16, RUF44, NERICA1, and NERICA3 exhibited superior performance in terms of root traits, specifically in the chromosomal segments on chromosomes 1, 3, 5, 7, and 8, when compared to other genotypes. Certain CSSLs can be further examined to identify the genes responsible for QTL expression. In addition, marker association analysis identified four SSR markers (RM201, RM316, RM7424, and RM1054) that could be used in marker-assisted breeding programs to improve root traits and indirectly enhance DS tolerance. The study highlights the potential of SSR markers in association with multiple root traits for drought tolerance in rice CSSLs. Molecular marker-root trait analysis could be an alternative to linkage mapping for detecting marker-phenotype associations. The findings have significant implications for breeding programs focused on developing new rice lines with high drought tolerance.

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Author contributions

MB and EP have contributed to the research's practical work and prepared the manuscript along with analysis; PS, SKD and MJB have designed the functional problem and supervised the research work, and MB, AP, GKD, and JNM have contributed to the analysis and editing part. All have read the manuscript before publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Conflict of interest

No conflict

Consent to participate

All authors agreed

Consent to publish

All authors agreed to give consent to publishing to the publisher

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