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INTROGRESSION OF DROUGHT TOLERANCE ROOT TRAITS INTO KENYAN COMMERCIAL CHICKPEA VARIETIES USING MARKER ASSISTED BACKCROSSING

A.J. KOSGEI, P.K. KIMURTO[1], P.M. GAUR[2], M.A. YEBOAH[3], S.K. OFFEI[4] and E.Y. DANQUAH[4]

Machakos University, Department of Agricultural Education and Extension, Machakos, Kenya
[1]Egerton University, Department of Crops, Horticulture and Soil Science, Nakuru, Kenya
[2]International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India
[3]The World Vegetable Center, Messa Yaoundé, Cameroon
[4]West Africa Centre for Crop Improvement, University of Ghana, Accra, Ghana
Corresponding author: akosgei@mksu.ac.ke

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ABSTRACT

Roots play critical roles in enhancing drought tolerance, more so under terminal drought conditions. The objective of this study was to introgress drought tolerant root traits into Kenyan chickpea varieties through marker assisted backcrossing (MABC). Eight simple sequence repeat (SSR) markers, linked to quantitative trait loci (QTL) for root traits, were used to screen parents at ICRISAT in India, and 1144 single nucleotide polymorphic (SNPs) markers at Legume Genomics Centre in the United Kingdom. Crosses were made between two selected varieties, ICCV 92944 (*Chania Desi II*) and ICCV 00108 (*LDT 068);* and ICC 4958, *QTL* donor parent. Polymorphic SSR and SNP markers were used to select offspring with root QTL at F_1, BC_1F_1, and BC_2F_1, and later advanced to BC_2F_3. BC_2F_3 families were evaluated for root traits at Egerton University in Kenya in a pot experiment under rain shelter. The BC_2F_3 families were significantly ($P<0.05$) different for root dry weight (RDW), shoot dry weight (SDW), total plant dry weight (PDW), and root to shoot dry weight (R/S) ratio (R/S) for *Chania Desi II* x ICC 4958; while R/S was significantly different for *LDT 068* x ICC 4958. Root length density (RLD) and RDW were positively and significantly ($P<0.05$) correlated with most of the traits, indicating its usefulness in the indirect selection of these traits. The utilisation of MABC is an effective and efficient method of introgressing complex root traits into commercial lines, expected to improve yields under drought. There is need for deployment of marker-assisted breeding in difficult to phenotypically select traits.

Key Words: Polymorphic markers, quantitative trait loci, terminal drought

RÉSUMÉ

Les racines jouent un rôle essentiel dans l'amélioration de la tolérance à la sécheresse, plus encore en cas de sécheresse terminale. L'objectif de cette étude était d'introduire des traits de racine tolérants à

la sécheresse dans des variétés Kenyannes de chickpea par rétrocroisement assisté par marqueurs (MABC). Huit marqueurs de répétition de séquence simple (SSR), liés à des locus de traits quantitatifs (QTL) pour les traits racinaires, ont été utilisés pour sélectionner les parents à l'ICRISAT en Inde, et 1144 marqueurs polymorphes à un seul nucléotide (SNP) au Legume Genomics Center au Royaume-Uni. Des croisements ont été réalisés entre deux variétés sélectionnées, ICCV 92944 (Chania Desi II) et ICCV 00108 (LDT 068) ; et ICC 4958, parent donneur QTL. Des marqueurs SSR et SNP polymorphes ont été utilisés pour sélectionner la progéniture avec un QTL racine à F_1, BC_1F_1 et BC2F_1, puis avancé à BC_2F_3. Les familles BC_2F_3 ont été évaluées pour les traits racinaires à l'Université d'Egerton au Kenya dans une expérience en pot sous abri contre la pluie. Les familles BC_2F_3 étaient significativement différentes (P<0,05) pour le poids sec des racines (RDW), le poids sec des pousses (SDW), le poids sec total de la plante (PDW) et le rapport poids sec des racines sur les pousses (R/S) (R/S) pour Chania Desi II x ICC 4958 ; tandis que R/S était significativement différent pour LDT 068 x ICC 4958. La densité de longueur des racines (RLD) et RDW étaient corrélées positivement et significativement (P < 0,05) avec la plupart des traits, indiquant son utilité dans la sélection indirecte de ces traits. L'utilisation de MABC est une méthode efficace et efficiente d'introgression de traits racinaires complexes dans des lignées commerciales, censée améliorer les rendements en période de sécheresse. Il est nécessaire de déployer la sélection assistée par marqueurs dans les caractères difficiles à sélectionner phénotypiquement.

Mots Clés : Marqueurs polymorphes, locus de caractères quantitatifs, sécheresse terminale

INTRODUCTION

Crop yield fluctuations arising from drought have been a major concern in many parts of the world. Drought has been the most significant factor for yield instability in major chickpea (*Cicer arietinum* L.) producing countries (Tar'an *et al.*, 2013; Devasirvatham and Tan, 2018). According to Varshney *et al.* (2009), abiotic stress causes chickpea yield losses of approximately 3.7 million metric tonnes annually worldwide, amounting to 40 - 50%, with terminal drought reported as the major abiotic constraint in chickpea production (Kashiwagi *et al.*, 2005; Leport *et al.*, 2006). Varshney *et al.* (2019) also reported that drought and heat stress cause yield reduction of over 70% in chickpea. Fang *et al.* (2010) and Onyari *et al.* (2010) have attributed the drought effect to impaired pollen viability and stigma functioning, reduced flowers and pods, and their abortions, and reduced secondary branches; and a decrease in shoot biomass and the number of pods. These unfavourable effects have caused the plants to adapt either by drought avoidance, escape or tolerance (Devasirvatham and Tan, 2018). Drought continues to be a major concern in developing countries as crops are grown during the post rainy season under receding soil moisture, coupled with climate change effects.

Two major strategies used for managing drought are developing early maturing and drought tolerant varieties (Gaur *et al.*, 2008; Kumar *et al.* 2017; Maphosa *et al.,* 2020). Drought tolerant root traits have been considered as the most important attribute that enables the plant to mine water efficiently from deep soil layers under drought (Vadez *et al.* 2008). They play a critical role in dehydration avoidance, as deep and prolific root systems can extract moisture from deeper layers even when the upper layer becomes dry (Serraj *et al.*, 2004; Kashiwagi *et al.*, 2005; Rehman, 2009). Root length density (RLD) and maximum root depth (RDp) were found to positively influence the seed yield under terminal drought environments (Ali *et al.*, 2005; Gaur *et al.*, 2008).Two lines ICC 8261 and ICC 4958, were identified to have the largest RLD and the most prolific and deep root systems (Kashiwagi *et al.*, 2005), and these

have been used in identification of root QTL conferring resistance to drought on linkage group 4 (CaLG04) (Varshney *et al.*, 2013a). The transfer of this QTL region from donor parents into commercial varieties is still low in chickpea, with scanty information in Kenya. Selection of these traits are also difficult.

Given that drought is a complex trait controlled by polygenes, the application of modern breeding technologies such as the use of molecular markers will lead to crop improvement and shorten the breeding cycle. Several strategies such as marker assisted selection (MAS), marker assisted backcrossing (MABC), marker assisted recurrent selection (MARS) and genome-wide selection (GWS) were proposed (Ribaut *et al.*, 2010; Li *et al.,* 2018). Marker assisted backcrossing involves the transfer of a target allele from a donor variety to a popular cultivar by repetitive backcrossing with the help of markers (Nayak *et al.*, 2010) and selection against donor introgressions across the rest of the genome (Tar'an *et al.*, 2013). The application of MABC has been successful in several crops such as introgression of drought tolerant QTL in pearl millet and rice (Serraj *et al.*, 2005; Ramayya *et al.,* 2021), stay green QTL in sorghum (Ngugi *et al.*, 2010), transfer of disease resistance in pepper (Thabius *et al.*, 2004) and quality protein maize (QPM) (Gupta *et al.,* 2013). In chickpea, limited applications of introgression of root drought tolerance traits have been reported.

A root trait from donor parent, ICC 4958, was transferred into JG11 (Varshney *et al.*, 2013a) and recently into three elite Indian chickpea (Bharadwaj *et al.,* 2020); indicating success in managing terminal drought. Chickpea adoption is gaining popularity in dry highlands of Kenya as a relay crop, planted during the short rain season. Further, the crop is also expanding into the semi-arid dryland of Eastern Kenya, which usually receives unreliable and unpredictable rainfall. These conditions expose the chickpea commercial lines to drought, especially terminal drought.

The objective of this study is to introgress root traits into commercial chickpea lines to enhance drought tolerance as a response to the urgent need to develop lines that are adapted to water limited environments in Kenya and indirect selection for root traits.

MATERIALS AND METHODS

Selection of markers. Eight simple sequence repeat (SSR) markers, confirmed to be linked to quantitative trait loci (QTL) for root and yield traits (Varshney *et al.*2013a; Varshney *et al.*, 2014; Thudi *et al.*, 2017, Chahande *et al.,* 2021) were used to screen the parents at ICRISAT, India. Additionally, 1144 single nucleotide polymorphic markers (SNPs) were also used in genotyping of 33 parents. The genotyping services with SNP markers were outsourced from the Legume Genomics Centre (LGC), formerly KBioscience, United Kingdom, where leaf samples were harvested at 14 days after emergence, dried, and shipped. The selection of polymorphic SNP markers was done using Genotypic Data Management Systems (GDMS), version 2.0.7 (ICRISAT, 2014), which is in-built in the Integrated Breeding Management System (IBMS) (Murray *et al.*, 2014).

Selection of parents. Two varieties that have been released in Kenya, *Chania Desi II* (ICCV 92944) and *LDT 068* (ICCV 00108), both being Desi types were used as recurrent parents. These lines are planted mainly during the short rains, exposing the crop to terminal drought. This is because short rains are usually unreliable and chickpea will survive on residual moisture, which eventually affects yield. ICC 4958 was used as donor parent. The ICC 4958 is one of the germplasm from the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), India with high root traits such as root density, root dry weight, and rooting depth. The line was also used in QTL mapping for root and yield traits (Gaur *et al.*, 2008; Varshney *et al.*, 2013a).

Development of backcrossing population. The two recurrent parents, *Chania Desi II* and *LTD 068,* were each crossed to a donor parent, ICC 4958, to generate F_{1s} at Egerton University in Kenya. The hybridity in F_{1s} was checked with SSR markers (TAA170, GA24, and ICCM0249) linked to the root '*QTL-hotspot'* region. The three markers were used for the foreground selection of F_{1s} to ensure the presence of the QTL region. This was done using the GeneMapper software (Applied Biosystems, 2005 USA) by determining the presence of alleles from both parents (heterozygous plants). True F_{1s} were selected for the first generation of backcrossing with the recurrent parents as females, which was maintained throughout the backcrossing. The backcross progenies at BC_1F_1 were tested for heterozygosity using nine markers (TAA170, ICCM0249, NCPGR127, NCPGR21, CaM1903, TA130, TA11, TA113, and TA118) where more markers were added as a result of progress in the identification of marker linked to the '*QTL-hotpot'* region (Varshney *et al.*, 2013a; Thudi *et al.*, 2017).

Five polymorphic markers at BC_1F_1, namely: ICCM0249, CaM0204, NCPGR21, TA113 and TA118, were used to screen BC_2F_1 for *Chania Desi II* x ICC 4958. Similarly, five other markers (NCPGR21, NCPGR127, TA11, TA113, and TA118) were used for screening BC_2F_1 for*LDT 068* x ICC 4958. The BC_2F_1 plants were selected based on foreground SSR and background SNP markers, with the highest percentage recovery of the recurrent parent which was done using the GDMS software programme (ICRISAT, 2014). The selected BC_2F_1 were selfed up to BC_2F_3 and evaluated for root traits in pot experiments under a rainout shelter.

Genotyping with SSR markers. DNA extraction was done using Nucleospin® 96 plant II core kit (Ref: 740468.4) at ICRISAT in India. DNA was extracted from fresh leaves of parental genotypes and F_1s. DNA for backcross progenies (BC_1F_1 and BC_2F_1), on the other hand, were extracted from dried leaf samples harvested at 14 days after emergence and oven-dried at 37 ºC for three days. DNA quality and quantity were checked on 0.8% agarose gel dissolved in 10x TBE (Tris Boric EDTA) buffer. The DNA contents prepared contained, 1µl of DNA, 3 µl of sterilised water, and 2 µl of orange dye, and it was checked against 20 ng of lambda DNA (1µl). This was run in gel electrophoresis (Owl D2 Wide – Thermo Scientific) at 100V for 1 hour. The gel was visualised on a trans-illuminator (Syngene gel documentation system).

The PCR was performed in 10 µl reaction volume. The PCR master mix contained 2 µl of 20 ng DNA, 1.0 µl of 10 x TBE buffer, 0.4 µl of 50 mM $MgCl_2$, 1.0 µl of 2 mM of dNTPs, 1.0 µl each of 2 pmol forward and reverse primers, 0.06 µl of Taq DNA polymerase (Fermentas) 50 µg and 4.56 µl of sterile water. The SSR fragments were amplified in a 384-well PCR machine (GeneAmp® PCR System 9700) using a touchdown programme. The PCR programme consisted of initial denaturation at 94 ºC for 5 minutes, followed by the first 10 cycles consisting of denaturation at 94 ºC for 15 seconds, primer annealing at 60 ºC decreasing by 0.5 ºC for 30 seconds and primer extension at 72 ºC for 30 seconds. This was followed by 40 cycles of the same denaturation, primer extension and primer annealing with a final extension step performed at 72 ºC for 20 minutes. The quality of PCR product using 2 µl of amplified DNA and 3x loading dye were mixed and checked on 1.2% agarose gel against 100 base pairs (bp) lambda DNA of 50 and 100 ng μl^{-1}. The gel was run on a 10x TBE buffer at a constant voltage of 100V for 30 minutes. The amplified PCR product was prepared for Applied Biosystems (ABI) electrophoresis. The ABI mixture contained 20 µl genescan 500 Liz, 800 µl Hi-Di formamide, and 400 µl of water where 10 µl of this mixture was added to 2 µl of amplified PCR product and dispensed in a 96-well plate. This was then separated by capillary electrophoresis using ABI Prism 3730 DNA

Sequencer and analysed using GeneMapper® software (Applied Biosystems, 2005 USA) to identify the segregating plants at every F_1 stage i.e., F_1, BC_1F_1 and BC_2F_1.

Genotyping with SNP markers. The F_1, backcross progenies and parents were planted in a rain shelter at Egerton University. The leaves were harvested at 14 days after emergence and oven-dried at 37 ºC for three days. They were then placed in tubes and shipped to LGC genomics. The principles and procedure of DNA assay were performed according to *KASPar* protocol (http://www.kbioscience.co.uk/reagents/KASP.html) with Chickpea KASPar Assay Markers (CKAMs). The genotyping results from LGC were used to determine polymorphic markers among the parents and these were used for background selection of the progenies to select those with high percentage recovery of the recurrent parents using the GDMS software programme (ICRISAT, 2014).

Evaluation of backcross derived lines for root traits. Root evaluation of BC_2F_3 and their parents were carried out under a moveable rain shelter at Egerton University, Njoro, Kenya. The soil and sand were mixed in a ratio of 1:1, w/w in pots and placed under the moveable rain shelter in a randomised complete block design, to minimise variation due to direction of the sun from the sides of the shelter, with two replications. The pots were filled with the soil-sand mixture and then supplied with water to 70% field capacity to mimic field conditions. Chickpea seeds were then planted and 1.5 liters of water per pot was applied every two days after sowing, until all the plants emerged; after which it was terminated. The rain shelter was always moved to cover the experiment to prevent rainwater from entering and moved out when there were no rains. Roots were sampled 40 days after planting. The shoots were cut off and the roots were washed gently under running tap water to remove three quarters of the soil-sand mixture. The remaining soil-sand mixture was removed by washing the roots under a sieve to minimise root losses. Rooting depth (RDp) was measured using a ruler and the roots were then scanned using image analysis software (WinRhizo Regent Instrument Canada INC., Quebec, Canada) for total root length.

Data collection and analysis. The data collected on the scanned fresh roots included: (a) rooting depth obtained by stretching the roots after washing and the length was measured in cm using a ruler, (b) total root length (TRL) from the WinRhizo analysis results, (c) root length density (RLD) that was calculated as a ratio of total root length to the volume of the pot, (d) shoot dry weight (SDW) where shoots were separated from roots and oven-dried at 80 ºC for 72 hours and their weights recorded, (e) root dry weight (RDW) – the scanned roots were oven-dried at 80 ºC for 72 hours and their weights recorded, (f) root to shoot ratio (R/S) – this was calculated as the ratio of root dry weight to shoot dry weight, and (g) length to root dry weight ratio (LWR) was calculated as total root length/root dry weight

Data analysis was done using PROC GLM with Statistical Analysis Software (SAS), version 9.3. Mean differences were tested using the Least Significant Difference (LSD) test at $P< 0.05$. The model used for analysis of variance (ANOVA) was:

$$Y_{ij} = \mu + t_i + r_j + e_{ij}$$

Where:

Y_{ij} = observation of treatments; μ = overall mean; t_i = i^{th} mean family effect; r_j = j^{th} replication; and e_{ij} = error term.

The correlations among variables were computed using Pearson's correlation using SAS version 9.3.

RESULTS

Polymorphic markers in root traits of chickpea families. Four markers out of eight markers used namely: CaM1903, ICCM0249, NCPGR127, and NCPGR21, were polymorphic for *LDT 068* x ICC 4958 population; while two markers, NCPGR127 and NCPGR21, were polymorphic between *Chania Desi II* x ICC 4958. Markers that failed to amplify parental DNA, namely; GA24, STMS11, TA130 and TA170 were not used when screening progenies for the selection of segregating plants. The results obtained from screening BC_1F_1 lines showed that five markers, ICCM0249, CaM0204, NCPGR21, TA113, and TA118, were polymorphic for *Chania Desi II* x ICC 4958 crosses; while five other markers namely; NCPGR21, NCPGR127, TA11, TA113, and TA118 were polymorphic for *LDT 068* x ICC 4958 crosses. Screening of BC_2F_1 showed that three markers, ICCM0249, CaM204, NCPGR127, were polymorphic for *Chania Desi II* x ICC 4958 and four markers, NCPGR21, NCPGR127, TA11, and ICCM0249, were polymorphic for *LDT 068* x ICC 4958. Two of these markers, ICCM0249 and NCPGR127, were common in the two crosses. The SNP markers screened showed very low polymorphism among the 30 parents in which 18 and 14 markers were polymorphic between Chania *Desi II* x ICC 4958 and *LDT 068* x ICC 4958, respectively.

Development of progenies and selection of heterozygous plants. The results of progenies developed to backcross two (BC_2F_1) and advanced by selfing to BC_2F_3 are represented in Figures 1 and 2. Heterozygous plants were selected in F_1 lines and the backcross F_1 populations (BC_1F_1 and BC_2F_1) using 2-3 polymorphic markers as shown in the Figures 1 and 2. A total of 20 lines of BC_2F_3 from each cross were identified with >85% recurrent parent genome (RPG) recovery evaluated for root traits.

Mean performances of root traits. There was a significant difference in RDW, SDW, PDW, and R/S ratio for crosses from *Chania Desi II* x ICC 4958 (Table 1). The root characteristics of the BC_3F_2 families showed enhanced rooting depth and root mass compared to parents (Fig. 3). Three families, EUC-03-P6-2-2-2-8, EUC-03-P22-1-2-7-8, and EUC-03-P22-1-2-7-13, had the highest TRL, RLD, and RDW compared to their parents, although they did not differ significantly. A significant difference was obtained in R/S ratio in the crosses between *LDT 068* and ICC 4958 (Table 2), with most families in this cross having higher TRL, RLD, RDW, SDW, and R/S ratio than their parents. Three families, EUC-04-P52-1-3-6-2, EUC-04-P39-1-1-1-9, and EUC-04-P52-2-2-2-15 recorded higher total root length of between 21.7 - 23.4 m than their parents *LDT 068* (13.6 m) and ICC 4958 (17.0 m). The overall mean values for most of these root traits were higher for crosses from *Chania Desi II* x ICC 4958 compared to those from *LDT 068* x ICC 4958, except LWR.

Phenotypic correlation estimates of root traits. There was a moderate positive correlation between the rooting depth (RDp) and all the traits except LWR which was negatively correlated for *Chania Desi II* x ICC 4958 (Table 3). TRL had a significantly positive correlation ($P < 0.001$, $r = 1.000$) with RLD and strong significant positive association with RDW ($P < 0.001$, $r = 0.771$), SDW ($P< 0.001$, $r = 0.601$) and PDW ($P < 0.001$, $r = 0.706$). However, it had a low positive significant correlation with R/S ratio and LWR. Root length density (RLD) was positively and significantly correlated with all traits and showed a strong positive correlation with RDW, PDW, SDW, as was the case with TRL. Root dry weight (RDW) was significantly positively correlated with SDW ($P < 0.001$, $r = 0.620$), PDW ($P < 0.001$, $r = 0.791$), and moderately correlated ($P < 0.001$, $r = 0.574$) with R/S ratio. Shoot dry weight was strong

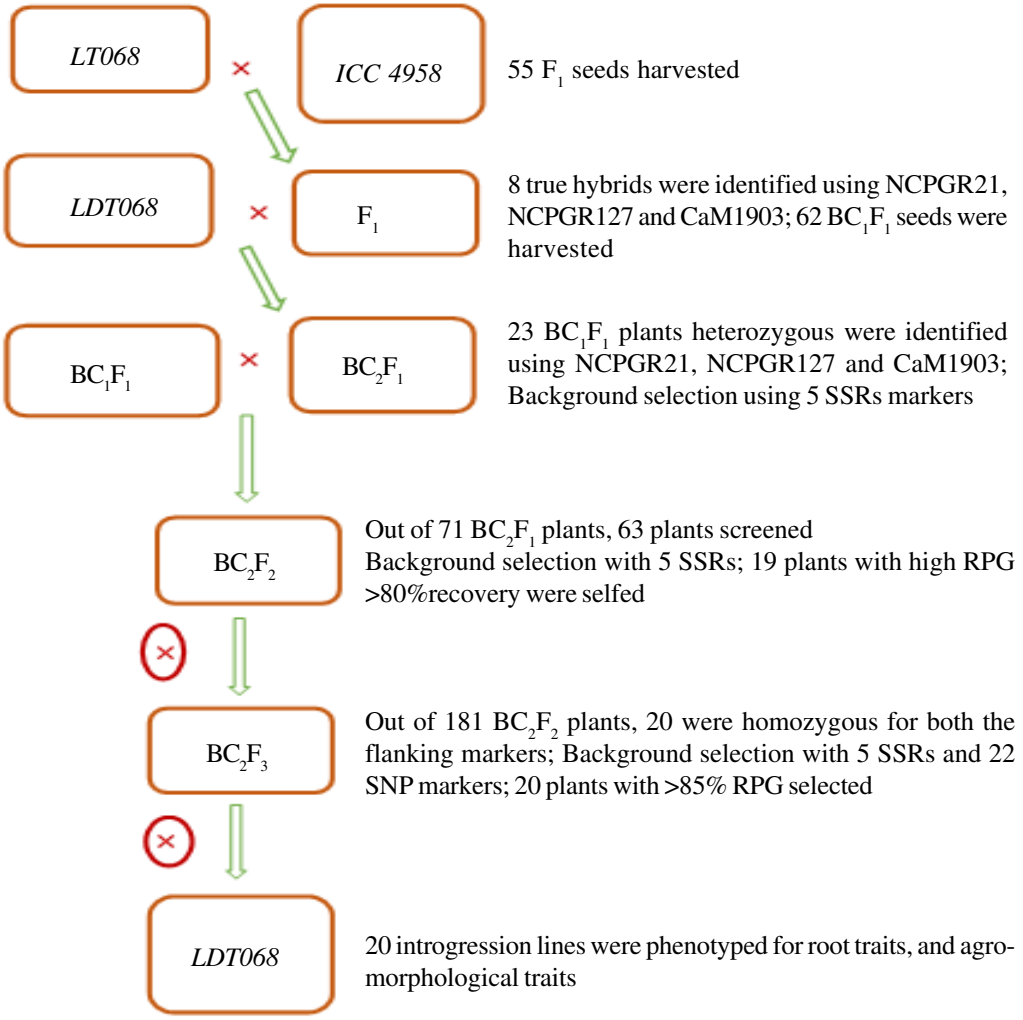

Figure 1. Marker assisted introgression of root traits into commercial line (*LDT068)* from donor parent, ICC4958.

and significantly positively correlated ($P < 0.001$, $r = 0.971$) with PDW and weak but negatively correlated ($P < 0.001$, $r = -0.254$) with R/S ratio. Total plant dry weight (PDW) was negatively correlated with both R/S ratio and LWR. R/S ratio on the other hand had weak negative significant correlation ($P < 0.001$, $r = -0.397$) with LWR. Similar correlations trends were observed for *LTD 068* x ICC 4958 (Table 4). From the results obtained, a positive significant correlation of more than $r = 0.50$ was obtained between SDW and TRL, RLD and RDW from the two populations.

DISCUSSION

Polymorphic markers and polymorphism in chickpea families. Low levels of polymorphism were observed in the local and introduced chickpea parents, and among families. Three SSR markers (ICCM0249, CaM0204, NCPGR127) showed polymorphism for *Chania Desi II* x ICC 4958; while four markers (NCPGR21, NCPGR127, TA11, and ICCM0249) revealed polymorphism for *LDT 068* x ICC 4958 in BC_2F_1 progenies. Two polymorphic markers (NCPGR127 and ICCM0249) were common for the two

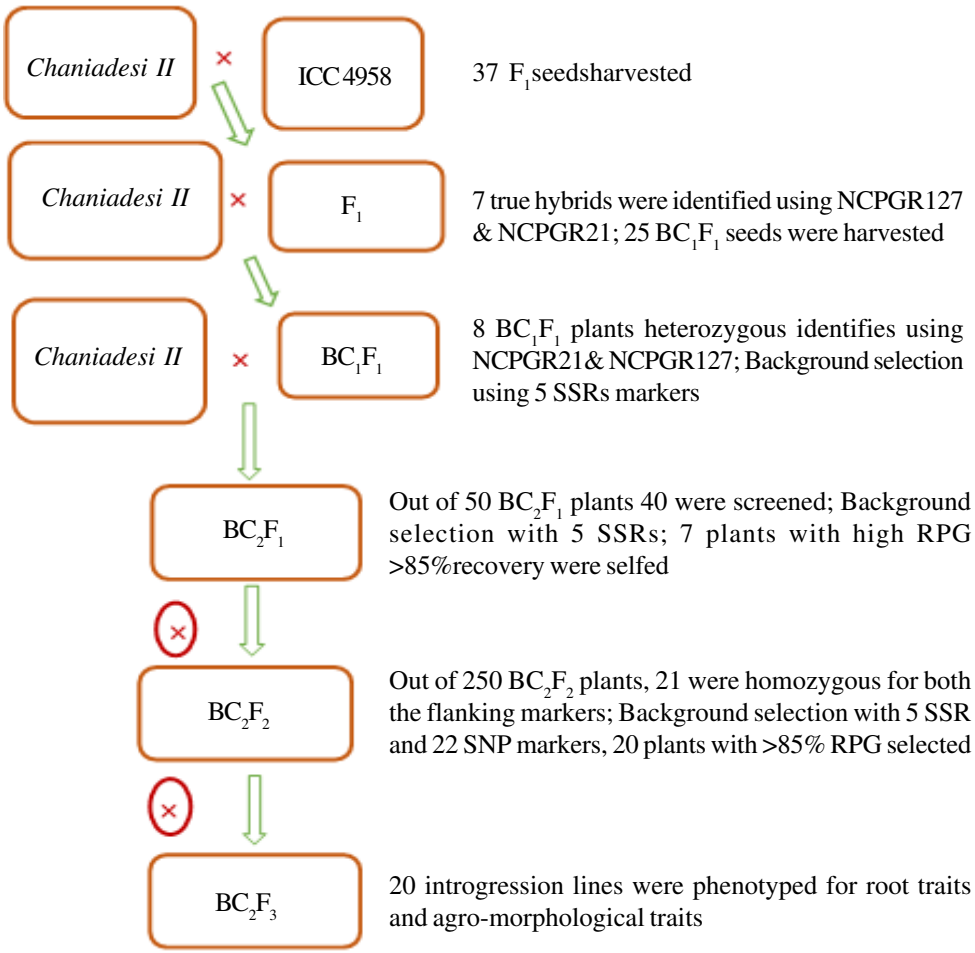

Figure 2. Marker assisted introgression of root traits into commercial line (*ChaniaDesi II)* from donor parent, ICC4958

populations. These markers were within the '*QTL – hotspot'* region on linkage group 4 based on results obtained by Varshney *et al.* (2013a) and Varshney *et al.* (2014). Further, the authors reported that this linkage group (CaLG04) harbours several drought-related traits, including root traits that contributed up to 58.20% of phenotypic expression. Thudi *et al.* (2017), identified 15 markers associated with root dry weight, root length density, root surface area, root volume, and rooting depth, out of which two markers, NCPGR7 (SSR) and DR-237 (SNP), were reported to be associated with more than one trait and the markers could be associated with co-localised QTL. This will be helpful in chickpea improvement as more than one desirable trait can be introgressed from the same region simultaneously and tracked by similar markers.

The low genetic variation obtained in this study is in agreement with reports by Gaur *et al.* (2012) and Chahande *et al.* (2021) among chickpea populations, which limits the sources of novel alleles in addition to chickpeas indeterminate growth habit that allows crop to recovery when conditions are favourable (Maphosa *et al.,* 2020). Similarly, Varshney *et al.* (2019) reported that landraces had a higher number of variations compared to varieties and breeding lines, with breeding lines showing a

TABLE 1. Mean root characteristics of BC_2F_3 families from *Chania Desi II* x ICC 4958 crosses

Genotypes	RDp (cm)	TRL (cm)	RLD (cm cm^{-3})	RDW (g)	SDW (g)	PDW (g)	R/S	LWR (cmg^{-1})
EUC-03-BC_2F_3-P6-2-2-2-8	49.00	2386.35	1.02	0.50	1.06	1.56	0.47	4817.08
EUC-03-BC_2F_3-P22-1-2-7-8	41.73	2012.20	0.86	0.42	0.96	1.38	0.44	4705.89
EUC-03-BC_2F_3-P22-1-2-1-13	36.50	1970.44	0.84	0.47	0.80	1.27	0.60	4187.88
EUC-03-BC_2F_3-P22-1-2-7-41	48.73	1966.10	0.84	0.31	1.20	1.51	0.27	6294.64
EUC-03-BC_2F_3-P6-2-2-2-10	39.50	1940.93	0.83	0.39	0.87	1.27	0.46	4926.14
EUC-03-BC_2F_3-P6-2-1-5-1	45.50	1887.90	0.81	0.34	1.12	1.46	0.32	5477.33
EUC-03-BC_2F_3-P6-1-3-9-2	47.50	1856.88	0.80	0.40	0.85	1.25	0.47	4693.76
EUC-03-BC_2F_3-P22-1-2-7-29	45.73	1855.56	0.79	0.36	0.67	1.03	0.52	5117.69
EUC-03-BC_2F_3-P6-2-1-5-27	31.00	1850.98	0.79	0.39	1.19	1.57	0.33	4782.89
EUC-03-BC_2F_3-P6-2-1-5-11	43.00	1843.71	0.79	0.34	0.93	1.27	0.36	5487.23
EUC-03-BC_2F_3-P6-2-1-5-12	39.00	1821.09	0.78	0.44	0.97	1.41	0.45	4431.00
EUC-03-BC_2F_3-P22-1-2-7-13	38.73	1813.77	0.78	0.34	1.00	1.34	0.34	5308.46
EUC-03-BC_2F_3-P6-1-1-3-12	36.00	1810.28	0.77	0.34	0.78	1.11	0.44	5358.52
EUC-03-BC_2F_3-P6-1-3-9-23	44.00	1810.12	0.77	0.39	1.02	1.41	0.38	4701.47
EUC-03-BC_2F_3-P22-1-2-3-18	33.00	1809.35	0.77	0.26	0.90	1.16	0.29	8751.91
EUC-03-BC_2F_3-P6-2-2-2-14	46.50	1806.45	0.77	0.36	0.82	1.18	0.43	5166.20
EUC-03-BC_2F_3-P6-2-1-5-20	49.00	1799.28	0.77	0.36	1.05	1.41	0.34	5025.92
EUC-03-BC_2F_3-P22-1-2-3-21	37.50	1776.42	0.76	0.34	1.05	1.38	0.31	5401.14
EUC-03-BC_2F_3-P6-2-1-5-4	42.00	1774.78	0.53	0.42	1.21	1.63	0.34	4221.88
EUC-03-BC_2F_3-P6-1-1-3-29	32.00	1756.39	0.72	0.33	1.04	1.36	0.31	5404.26
Chania Desi II (Recurrent)	35.85	1232.00	0.76	0.25	0.68	0.92	0.36	5087.71
ICC 4958 (Donor)	41.32	1685.30	0.75	0.33	1.02	1.34	0.33	5197.37
Mean	39.07	1406.47	0.60	0.27	0.81	1.09	0.34	5343.65
P-values	0.065^{ns}	0.256^{ns}	0.263^{ns}	0.025*	0.001**	0.001**	0.022*	0.501^{ns}

RDp = rooting length, TRL = total root length, RLD = root length density, RDW = root dry weight, SDW = shoot dry weight, PDW = total plant dry weight, R/S = root to shoot ratio, and LWR = length to root dry weight ratio, ns = not significant at 5% level of significance (P>0.05), ns, * and ** = non-significant, significant at 5% probability level (P<0.05) and 1% probability level (P<0.01), respectively

Figure 3. Roots obtained from *Chania Desi II* x ICC 4958 cross at Egerton University in Kenya.

high genetic loss. This was earlier indicated to be due to monophyletic descendant from its wild progenitor *C. reticulatum* in the Fertile Crescent of south-eastern Turkey (Abbo *et al.*, 2003) and probably limited tools available to detect polymorphism (Varshney *et al.*, 2007). Further, farmers are currently adopting new high-yielding varieties and abandoning landraces which also accounts for low genetic diversity. Continuous selection for desirable traits and intercrossing lines with closely related parents to develop superior genotypes has also resulted in low genetic diversity among chickpea. According to Chaturvedi and Nadarajan (2010), inter-mating between lines or inter-varietal crossing was one reason for low polymorphism in chickpea. Additionally, chickpea is a self-pollinated crop with less than 1% out-crossing rate (Singh *et al.*, 2008); hence there is minimal gene contamination from other chickpea varieties in open fields unlike in cross-pollinated crops.

This narrow genetic variation in cultivated chickpea limits molecular marker development and QTL for certain traits (Coram *et al.*, 2007) due to lack of polymorphism of markers among genotypes, where this polymorphism among parental genotypes is a pre-requisite for screening desired genotypes and its application in MAS (Chahande *et al.,* 2021). Wild relatives with traits of interest may be useful in breeding programmes to increase diversity in cultivated chickpea. Although the utilisation of wild relatives has some drawbacks, such as crossing ability barriers (Gaur *et al.*, 2012), this could be overcome with modern breeding strategies such as mutation breeding and with the recent completion of chickpea genome sequencing (Varshney *et al.*, 2013b) offering more opportunities for such studies. Further, using diverse lines in breeding allows recombination which sometimes results in transgressive segregating population with beneficial traits that can be selected for high

TABLE 2. Mean root characteristics of BC_2F_3 families from *LDT 068* x ICC 4958 crosses

Genotypes	RDp (cm)	TRL (cm)	RLD (cm cm^{-3})	RDW (g)	SDW (g)	PDW (g)	R/S	LWR (cmg^{-1})
EUC-04-BC_2F_3-P52-1-3-6-2	36.00	2344.05	1.00	0.33	0.54	0.87	0.61	7124.76
EUC-04-BC_2F_3-P39-1-1-1-9	41.52	2175.33	0.93	0.42	1.20	1.62	0.36	4976.75
EUC-04-BC_2F_3-P52-2-2-2-15	47.48	2166.91	0.93	0.31	0.66	0.97	0.46	7259.08
EUC-04-BC_2F_3-P52-1-1-3-3	52.52	1983.80	0.85	0.28	0.59	0.86	0.48	6881.96
EUC-04-BC2F3-P39-1-1-4-12	41.52	1981.52	0.85	0.35	0.80	1.15	0.44	5452.48
EUC-04-BC_2F_3-P52-1-4-7-2	37.51	1980.73	0.85	0.36	0.89	1.25	0.41	5473.24
EUC-04-BC_2F_3-P53-2-2-2-14	36.52	1918.45	0.82	0.30	0.73	1.03	0.42	6097.25
EUC-04-BC_2F_3-P53-2-2-2-15	40.52	1901.26	0.81	0.28	0.61	0.88	0.46	6673.04
EUC-04-BC_2F_3-P27-1-3-2-3	28.00	1863.44	0.80	0.27	0.77	1.04	0.35	6702.11
EUC-04-BC_2F_3-P6-2-2-3-11	27.52	1823.57	0.78	0.33	1.05	1.38	0.32	5328.81
EUC-04-BC_2F_3-P52-2-2-2-12	37.48	1817.86	0.78	0.28	0.76	1.04	0.36	6615.84
EUC-04-BC_2F_3-P52-1-3-6-5	33.50	1799.78	0.77	0.30	0.91	1.22	0.33	5941.79
EUC-04-BC_2F_3-P53-2-2-2-7	40.52	1757.00	0.75	0.24	0.13	0.37	1.59	7031.88
EUC-04-BC_2F_3-P52-2-2-2-18	32.48	1756.91	0.75	0.26	0.65	0.91	0.38	7088.38
EUC-04-BC_2F_3-P27-1-3-4-21	33.50	1751.47	0.75	0.22	0.49	0.70	0.44	8130.38
EUC-04-BC_2F_3-P39-1-1-4-3	32.00	1742.48	0.75	0.31	0.84	1.15	0.37	5657.39
EUC-04-BC_2F_3-P52-1-1-3-11	31.50	1731.88	0.74	0.32	0.68	1.00	0.50	5629.84
EUC-04-BC_2F_3-P39-1-1-4-1	46.48	1719.91	0.74	0.27	1.03	1.30	0.26	6468.46
EUC-04-BC_2F_3-P27-1-3-4-19	29.50	1716.03	0.73	0.23	0.72	0.96	0.33	7345.23
EUC-04-BC_2F_3-P53-2-2-2-17	36.52	1708.90	0.73	0.28	0.53	0.80	0.53	6014.24
LDT 068 (ICCV 00108) (Recurrent)	33.80	1365.87	0.58	0.23	0.78	1.01	0.31	5968.87
ICC 4958 (Donor)	37.38	1695.49	0.73	0.28	0.87	1.15	0.33	6252.41
Mean	32.38	1216.85	0.52	0.21	0.62	0.83	0.35	6028.54
P –values	0.266^{ns}	0.096^{ns}	0.096^{ns}	0.306^{ns}	0.067^{ns}	0.089^{ns}	0.025*	0.084^{ns}

RDp = rooting length, TRL= total root length, RLD = root length density, RDW = root dry weight, SDW = shoot dry weight, PDW = total plant dry weight, R/S = root to shoot ratio, and LWR = length to root dry weight ratio; ns and * non-significant and significant at 5% probability level ($P<0.05$)

TABLE 3. Phenotypic correlations among the root traits of BC_2F_3 families for *Chania Desi II* x ICC 4958

	[a]RDp (cm)	[b]TRL (cm)	[c]RLD (cm cm^{-3})	[d]RDW (g)	[e]SDW (g)	[f]PDW (g)	[g]R/S	[h]LWR (cmg^{-1})
[a]RDp (cm)	-							
[b]TRL (cm)	0.432***	-						
[c]RLD (cm cm^{-3})	0.432***	1.000***	-					
[d]RDW (g)	0.409***	0.771***	0.771***	-				
[e]SDW (g)	0.356***	0.601***	0.601***	0.620***	-			
[f]PDW (g)	0.402***	0.706***	0.706***	0.791***	0.971***	-		
[g]R/S	0.156**	0.326***	0.326***	0.574***	-0.254***	-0.022^{ns}	-	
[h]LWR (cmg^{-1})	-0.062^{ns}	0.172**	0.172**	-0.414***	-0.094^{ns}	-0.200***	-0.397***	-

[a]Rooting depth, [b]Total root length, [c]Root length density, [d]Root dry weight, [e]Shoot dry weight, [f]Total plant dry weight, [g]Root to shoot ratio and [h]Length to root dry weight ratio

TABLE 4. Phenotypic correlations among the root traits of BC_2F_3 families for *LDT 068* x ICC 4958

	[a]RDp (cm)	[b]TRL (cm)	[c]RLD (cm cm^{-3})	[d]RDW (g)	[e]SDW (g)	[f]PDW (g)	[g]R/S	[h]LWR (cmg^{-1})
[a]RDp (cm)	-							
[b]TRL (cm)	0.614***	-						
[c]RLD (cm cm^{-3})	0.614***	1.000***	-					
[d]RDW (g)	0.561***	0.869***	0.869***	-				
[e]SDW (g)	0.385***	0.542***	0.542***	0.669***	-			
[f]PDW (g)	0.464***	0.675***	0.675***	0.813***	0.977***	--		
[g]R/S	0.087^{ns}	0.274***	0.274***	0.279***	-0.266***	-0.128*	-	
[h]LWR (cmg^{-1})	0.077^{ns}	0.193***	0.193***	-0.261***	-0.254***	-0.275***	-0.024^{ns}	-

[a]Rooting depth, [b]Total root length, [c]Root length density, [d]Root dry weight, [e]Shoot dry weight, [f]Total plant dry weight, [g]Root to shoot ratio and [h]Length to root dry weight ratio

yielding lines with desirable trait combinations (Upadhyaya *et al.*, 2007). Thus, genetic diversity of chickpea is an important resource in breeding for drought tolerance, hence root variations characteristics among the families generated in this study could be useful for future breeding work.

Root trait responses of chickpea families to drought. Root traits showed significant variations among the families (Tables 1 and 2). Genetic variation in root traits has been reported in various recombinant inbred lines (RILs) (Serraj *et al.*, 2004; Kashiwagi *et al.*, 2005; Rehman, 2009). Among the root traits, root dry weight (RDW), shoot dry weight (SDW), plant dry weight (PDW), and root to shoot ratio (R/S) were significantly different among families of the cross *Chania Desi II* x ICC 4958; while R/S ratio was significant for *LDT 068* x ICC 4958. The BC_2F_3 families that had mean total root length values higher than the two parents were EUC-03-P6-2-2-2-8, EUC-03-P22-1-2-7-8, and EUC-03-P22-1-2-1-13 for *Chania Desi II* cross and EUC-04-P52-1-3-6-2, EUC-04-P39-1-1-1-9, and EUC-04-P52-2-2-2-15 for *LDT 068* x ICC 4958. These families had improved total root length of between 40 - 50% compared to the recurrent parents. This is an indication of successful improvement in root traits and such families may be used as donor parents to improve other lines once they are stable at later generations. Root length and rooting depth are important traits for the drought avoidance mechanism. This is necessary as indicated by studies from Kumar *et al.* (2012) that chickpea roots grow deeper to extract moisture from lower soil profiles under rainfed compared to irrigated conditions and avoid drought. In addition to rooting depth, root branching is also an important architectural trait in the uptake of water and nutrients (Lynch and Wojciechowski, 2015) that could be exploited. Similarly, in wheat total root length and rooting depth influenced the distribution of roots in the soil profile and the amount of water absorbed (Manschadi *et al.*, 2006).

Root length density (RLD) and root dry weight (RDW) for most of the top 10 families was higher than those of the parents, an indication that probably they could have better absorption of water from the soil. Root dry weight is also a good indicator of root biomass accumulation which is important in water and nutrient absorption. The RLD represents the root's capability for soil water exploitation; while RDW shows its high biomass accumulation (Kashiwagi *et al.,* 2006; 2008). Research conducted under rain fed conditions indicated that genotypes increased root biomass and rooting depth compared to those under irrigated conditions (Kumar *et al.*, 2010 and Akman, 2021)), which indicates more water uptake that could translate to better yield performance of such lines under drought. Kenyan farmers plant chickpea purely under rainfed conditions, both during long short rainy seasons, hence enhanced root biomass and rooting depth will be suitable for this type of farming. Further, an earlier report indicated that water deficit affects the distribution of root weight density (RWD), root length density (RLD) and rooting depth (RDp) at various soil depths, providing increased water absorption capacity in deeper soil layers to cope with drought (Rehman, 2009 and Purushothaman *et al.,* 2017). This is an indication that root biomass is increased in the deeper soil layers to extract more water. The current research aimed at introgressing root traits in commercial lines of chickpea in Kenya to increase tolerance to drought by increasing root biomass hence water uptake during drought conditions that may increase chickpea yield. Kashiwagi *et al.,* (2015) and Ramamoorthy *et al.* (2017) reported that root traits demonstrated yield advantage in chickpea under terminal drought.

Root length to root dry weight ratio (LWR), which is an important parameter for estimating changes in root densities, was not significantly different among the BC_2F_3 families from the two crosses. However, a low significant increment in LWR in deeper soil layers compared to upper soil layers in stressed environments was reported, which was

attributed to the production of many fine roots (Rehman, 2009). These fine roots are associated with increased water absorption; although under dry conditions, such roots are not common (Krishnamurthy *et al.*, 1998) probably, as a result of drying up due to lack of water.

Some genotypes of chickpea among the families in the present study had high R/S ratios (Tables 1 and 2). These included EUC-03-P22-1-2-1-13 (0.60) and EUC-03-P2-1-2-7-29 (0.52) for *Chania Desi II* x ICC 4958 and EUC-04-P53-2-2-2-7 (1.59) and EUC-04-P52-1-3-6-2 (0.61) for *LDT 068* x ICC 4958, compared to their recurrent parents. High R/S ratio results from inhibition of shoot growth compared to root growth, which is an adaptation mechanism under drought stress (Shaddad *et al.,* 2013; Santos *et al.,* 2020). In chickpea, the root to shoot ratio has been used as an indicator of drought tolerance (Labidi *et al.*, 2009) as more photosynthates are chanelled to roots leading to high root growth, hence increased water absorption. A similar observation was reported in rice where there was an increased R/S ratio under drought stress, that also led to increased proportion of dry matter and soluble root sugars (Xu *et al.,* 2015) which leads to increased water absorption. In maize, root/shoot ratio was predicted as a suitable criterion for classifying genotypes into drought stress tolerant or in susceptibility through exhibiting desiccation or dehydration tolerance (Shaddad *et al.*, 2013).
Correlation estimates of root traits

A positive correlation was found between RLD and all other root traits, as was the case for rooting depth (RDp) with all the other traits, except with LWR (Tables 3 and 4). High rooting depth and large root biomass are important traits for adaptation in a drought environment, allowing for the extraction of moisture from deeper soil depths compared to those with shallow rooting depth. Root biomass and rooting depth were recognised as the main drought avoidance mechanism traits (Kashiwagi *et al.*, 2005; Maqbool *et al.,* 2017). Also, findings by Purushothaman *et al.* (2013), showed that the chickpea root system is well adapted to growth under receding soil moisture due to large numbers of thin xylem tubes that are effective and require less energy for soil moisture absorption. The TRL and RDW were reported to have a significant positive correlation and that TRL was an important criterion for the selection of drought resistant genotypes (Ganjeali and Kafi, 2007; Chen *et al.,* 2017). The correlations between SDW and the three traits namely; TRL, RLD, and RDW, in both crosses in this study were more than 50% (Tables 3 and 4), making SDW useful for indirect selection of root traits, whose measurement is expensive, labour-intensive, and a difficult task especially under field conditions. Thus, indirect selection based on traits that have high correlation and are easy to measure will result in progress in the development of varieties for drought tolerance. Similarly, a linear relationship was observed between root dry weight and shoot dry weight at 35 days after sowing (Serraj *et al.*, 2004). Findings also showed that SDW was significantly positively correlated (approximately 70%) with several important root traits such as RDW, RL (root length), and RLD, but had a low correlation with RD (rooting depth) (0.36) (Nayak *et al.*, 2010). Other research in spring wheat showed that SDW was positively associated with rooting depth, root dry weight, total root length, and root length density (Narayanan and Prasad, 2014). This is an indication that SDW is an important trait in the indirect determination of root traits.

Marker application in chickpea families. Evidently, a good number of BC_2F_3 families had mean RDW, SDW, RDp, TRL higher than the recurrent parents (*Chania Desi II* and *LDT 068*) and donor parent ICC4958. This is an indication of the successful application of marker selection in chickpea for the improvement of varieties with the aid of molecular tools, especially those traits that are

quantitatively inherited. Other previous work by Varshney *et al.* (2014), Mannur, *et al.,* (2019) and Bharadwaj *et al.* (2020), in chickpea and other crops (Thabius *et al.*, 2004; Serraj *et al.*, 2005; Ngugi *et al.*, 2010, Gupta *et al.,* 2013) are among some few successful examples. The application of markers also shortens the breeding cycle as there is less environmental influence on selection, which requires several repeated field trials. In the present study, it was possible to select F_1 and backcross F_1 (BC_1F_1 and BC_2F_1) plants that were heterozygous based on markers linked to the root '*QTL hotpot'* region (foreground selection). It is also possible to directly select a trait of interest using tightly linked markers. With the use of markers, it was possible to identify families with improved root traits at BC_2F_3. Additionally, new molecular technologies using molecular marker(s) tightly linked to the trait of interest improve breeding efficiency (Bharadwaj *et al.*, 2011). The most popular method is marker assisted backcrossing which involves introgression of one or more traits from a donor into an adapted line. It is anticipated that the completion of the sequencing of the chickpea genome (Varshney *et al.*, 2013b) and use of genomics-assisted breeding (GAB) strategies will be crucial in designing future crops (Varshney *et al.,* 2021), leading to continuous improvement of chickpea breeding in terms of time, efficiency and effectiveness. Furthermore, the combination of genome-wide association (GWAS) study and next-generation sequence (NGS) approaches have led to the identification of marker-trait associations (MTAs) for drought and several drought-responsive genes and yield (Thudi *et al.*, 2017; Varshney *et al.,* 2019; Shekari *et al.,* 2021). These methods, when used together with marker assisted backcrossing, will lead to the development of superior drought tolerant varieties in response to current climatic changes due to global warming effects; hence lessen the number of years it takes to release a variety.

CONCLUSION

Low levels of polymorphism were detected in the chickpea parents screened with SSR and SNP markers. Three markers (ICCM0249, CaM0204, NCPGR127) were polymorphic for *Chania Desi II* X ICC 4958; while four markers (CaM1903, ICCM0249, NCPGR127, and NCPGR21) were polymorphic for *LDT 068* x ICC 4958 in BC_2F_1 generation. These markers were within the '*QTL-hotspot'* region. From *Chania Desi II* x ICC 4958 cross, four families namely: EUC-03-P6-2-2-2-8, EUC-03-P22-1-2-7-8, EUC-03-22-1-2-7-13, and EUC-03-P6-1-3-9-2 had higher TRL, RLD, RDp, RDW SDW, and PDW in comparison to the recurrent parent. Similarly, three families, EUC-04-P52-1-3-6-2, EUC-04-P39-1-1-1-9, and EUC-04-P52-2-2-2-15 for *LDT 068* x ICC 4958 had higher TRL, RLD, RDp and RDW mean performance better than the recurrent parent. This may be an indication of early expression of some of the measured traits, which are usually difficult to select due to the complexities of drought-related genes and environmental influence. The identification of the families was possible through the utilisation of linked QTL markers. This may suggest successful introgression of the QTL region in the genetic background of recurrent parents which is expected to improve chickpea yield under drought conditions. Further, families with improved root traits than ICC 4958 could also be identified as donor parents for future breeding. From the study, families identified to be better than the parents need to be evaluated further alongside checks for a possible release of the best lines.

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