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DEVELOPMENT OF STRUCTURED POPULATIONS AND BREEDING LINES FOR TRAIT ANALYSIS AND IMPROVED VARIETIES IN BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* L. VERDC)

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Abstract

Underutilised crop species have the potential to contribute significantly to increased crop diversity and to improved food and nutrition security worldwide. Bambara groundnut [*Vigna subterranea* (L.) Verdc.] is an underutilised, protein-rich and self-pollinating legume, which can withstand high temperatures and drought stress, and mainly grown in semi-arid Africa. The crop is still largely grown as landraces (a mixture of genotypes) and has limited established structured populations and breeding lines due to a lack of genetic improvement activities and commercial interests.

Twelve genotypes of bambara groundnut collected from East, West and Southern Africa and Southeast Asia were used to evaluate the variation in phenotypic traits and the correlation between the observed variation and the landraces' geographical origins in randomised complete block design (RCBD). All phenotypic traits in the twelve genotypes were significantly influenced ($p < 0.01$) by genotypes. Principal component analysis (PCA) showed that PC1 accounted for 97.33% of the variation and was associated with four genotypes collected from East and Southern Africa. PC2 accounted for 2.48% of the variation and was associated with five genotypes collected from East, West and Southern Africa. The variation observed within the twelve genotypes of bambara groundnut provides a breeding resource pool for use in controlled crossing to develop ideotypes with desirable phenotypic traits, i.e., high *harvest index, 100-seed weight,* early *days to flowering* or short life cycle.

Two F_2 bi-parental segregating populations of bambara groundnut derived from different geographical origins, IITA-686 (Tanzania, East Africa) × Tiga Nicuru (Mali, West Africa) and S19-3 (Namibia, Southern Africa) × DodR (Tanzania, East Africa) were developed to obtain structured populations and breeding lines for genetic analysis and trait dissection. Transgressive segregation for a number of traits was observed in the two F_2 bi-parental populations, as some

individual lines in the segregating populations showed trait values greater or less than their parents. The variability between the two F_2 bi-parental segregating populations and the negative relationship between morphological traits and yield-related traits provide resources for development of structured populations and selection of breeding lines for bambara groundnut breeding programme.

Assessment of segregating populations for their ability to withstand drought stress conditions is one of the best approaches to develop breeding lines and drought-tolerant varieties. The genotype S19-3 exhibits short life cycle and is considered as drought resistant landrace while DodR is reported to have comparatively high *100-seed weight* and yield. A total of 114 individual lines derived from S19-3 × DodR were advanced into F_3 and F_4 segregating populations and examined in a rainout shelter to identify superior lines under drought stress. Drought stress significantly reduced ($p < 0.05$) *shoot dry weight, seed weight per plant, harvest index, shelling percentage*, *chlorophyll content index* and *quantum yield PSII photochemistry* (F_V/F_M) in the F_3 and F_4 segregating populations of bambara groundnut. *Stomatal conductance, photosynthesis rate, transpiration rate* and *intracellular CO_2* were significantly reduced ($p < 0.05$) while *leaf water use efficiency* was significantly increased ($p < 0.05$) towards the end of the drought stress period in the F_4 segregating population. Individual lines with higher *chlorophyll content index, quantum yield PSII photochemistry* (F_V/F_M), *relative water content, stomatal conductance, leaf water use efficiency, seeds weight per plant* and *harvest index* were identified. These individuals could be selected as superior lines for genetic analysis and variety development for drought adaption.

In order to dissect the complexity of drought resistance, the inheritance of yield-related and morphological traits and to use genomic tools for yield enhancement of bambara groundnut under drought-stressed conditions, a genetic linkage map covering 1,040.92 cM across 11 linkage groups

was constructed using 228 DArTseq markers in the F_2 segregating population derived from S19-3 × DodR. Significant QTLs for *shoot dry weight* were mapped on LG10 accounting for 15.5% of the phenotypic variation explanation (PVE) under well-watered conditions and a putative QTL for the same trait mapped on LG10 with reduced PVE (10.10%) under drought-stressed conditions in the F_3 segregating population. Significant QTLs associated with *number of seeds per plant, number of double-seeded pod per plant, seed weight per plant* and *pod weight per plant* were mapped on LG4 (nearest marker: 4181663 and 4175954) with overlapping confidence intervals and explaining 21.9%, 21.8%, 23.5% and 19.9% of the PVE, respectively, under well-watered conditions in the F_4 population, which could be considered as major QTL involved in the control of these traits. Fourteen QTL loci that were found to be consensus QTLs for yield-related, morphological and physiological traits across LG1A, LG2, LG3, LG4, LG5, LG7A, LG7B, LG10 and LG11.

This study provides a pipeline for the development of breeding resources, including structured populations and breeding lines, for genetic analysis, trait dissection and potentially development of new improved varieties. The study also provides fundamental knowledge of QTLs associated with yield components, morphological and physiological traits under well-watered and drought-stressed conditions in bambara groundnut, which is also essential for yield improvement of bambara groundnut in response to drought stress.

List of Publications

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°C	Celsius	GEM map	Gene expression-based genetic map
100SW	100-seed weight	GS	Genomic selection
A	Photosynthesis rate	gs	Stomatal conductance
ABA	Abscisic acid	GW	Genome wide
AFLP	Amplified fragment length polymorphism	HI	Harvest index
AGL-83	Amylo-Alpha-1, 6-Glucosidase, 4-Alpha-Glucanotransferase	IL	Internode length
ANOVA	Analysis of variance	IM	Interval mapping
AP2	APETALA2	LA	Leaf area
ATAUX2-11	Auxin responsive protein	LEA	Late embryogenesis abundant
BC	Backcross	LEN	Seed length
bZIP	Basic leucine zipper	LGs	Linkage groups
CCI	Chlorophyll content index	LOD	Logarithm of odds
Ci	Intracellular CO_2	LWUE	Leaf water use efficient
CID	Carbon isotope discrimination	MOP	Muriate of potash
cM	Centi-Morgan	MOP	Muriate of potash
COMT	Beta-fructofuranosidase, Catechol-O-methyltransferase	MYB	Myeloblastosis
CRD	Completely randomized design	NAC	N-acetyl cysteine
DArT	Diversity array technology	NF-Y	Nuclear factor Y
DArTseq	Diversity array technology sequencing	NID	Delta N15 isotope analysis
DNA	Deoxyribonucleic acid	NL	Number of leaves per plant
DS	Drought-stressed	NP	Number of pods per plant
DTF	Days to flowering	NPK	Nitrogen phosphorous potassium
E	Transpiration rate	NS	Number of seeds per plant
ERF	Ethylene-responsive element binding factors	P/I	Petiole internode ratio
EST-SSRs	Expressed sequence tags SSRs	PAL1	Phenylalanine ammonia-lyase-1
F_V/F_M	Quantum yield of PSII photochemistry	PCA	Principal component analysis
g-SSRs	Genomic SSRs	PCR	Polymerase chain reaction
G*E	Interaction between treatment and genotypes	PER	Seed perimeter
GBS	Genotyping-by-sequencing	PH	Plant height

PL	Petiole length	SNP	Single nucleotide polymorphism
PW	Pod weight per plant	SP	Shelling percentage
QTL	Quantitative trait loci	SPD	Single plant descent
RAPD	Random amplified polymorphic DNA	SSD	Single seed descent
RCBD	Randomised complete block design	SSR	Simple sequence repeat
RILs	Recombinant Inbred Lines	SW	Seed weight per plant
ROS	Reactive oxygen species	TSP	Triple super phosphate
RR7	Proline-rich protein 7	WID	Seed width
RWC	Leaf relative water content	WLR	Seed width length ratio
SCC	Seed coat colour	WW	Well-watered
SD	Standard deviation	Δ13C	Carbon Isotope Discrimination
SDW	Shoot dry weight	µL	Microlitre, equal to 10^{-6} litre
SLA	Specific leaf area		

Chapter 1 General Introduction

1.1 Food supply challenge in the face of climate change

The Green Revolution was a major success in safeguarding global food security and saved millions of lives (Pingali 2012). The large-scale modifications during the Green Revolution, which included the development of high-yielding varieties of cereal grains, expansion of irrigation infrastructure, the introduction of mechanization techniques and use of huge quantities of synthetic fertilizers and pesticides have been well documented and are implicated in global change processes (Pingali 2012). The Green Revolution provided food to millions of people and ensured food security, however, it also introduced hazardous pesticides, caused soil fertility deterioration due to excessive fertilization and compaction, and unsustainable agriculture system caused by a tremendous increase in world food production and distribution, particularly of rice (*Oryza Sativa* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) in low- and middle-income countries (Pellegrini and Fernández 2018; Armanda et al. 2019; Karunarathne et al. 2020). This calls for new approaches (new Green Revolution technologies) to tackle global food insecurity in the face of increasing population growth and demographic change, rising average incomes, resource competition and scarcity, environmental change and the need to reduce greenhouse gas emissions (Godfray and Garnett 2014).

Agriculture is inherently sensitive to climate variability and change (Tito et al. 2018). Climate change is expected to bring warmer temperatures, changes to rainfall patterns, increased frequency of heat waves, droughts and intense rain events, and overall unpredictable weather conditions (Res and Trenberth 2011). In addition to extreme weather events, atmosphere CO_2 concentrations is

also projected to change (Sutton et al. 2011). Climate change poses a range of direct and indirect effects on global food security. These include alteration in climatic suitability for the cultivation of specific crops, influence on food distribution and nutritional adequacy (Wheeler and von Braun 2013; Myers et al. 2017; Tito et al. 2018; Asseng et al. 2019). As a major crop and eaten by billions, 50% of average yield losses in rice production have been reported due to water shortage and insufficient rainfall during the growth season (Mohanty et al. 2013; Elert 2014). Global wheat yield could be increased by introducing warmer temperature adaptation genotypes, but grain quality probably may not benefit from yield increase (Asseng et al. 2019). The decline in maize production (21% - 29%) and crop quality have also been reported and these were mainly associated with warmer temperatures and greater incidence of pests (Tito et al. 2018).

Several researchers have reported that food production must be increased by 2050 to cope with climate change and population explosion (Dixit et al. 2014; Massawe et al. 2016). There are at least 50,000 species of plants that are suitable for human consumption but only around 20 plant species including three major crops (rice, wheat and maize) currently supply 90% of the world's calories (Jacques and Jacques 2012; Massawe et al. 2016). Reports show that over 7,000 crop species have been used as a source of food (Padulosi et al. 1999; Williams and Haq 2000). Relying predominantly on the three major crops cannot solve food insecurity challenges and potentially make agriculture even more vulnerable to major threats, such as diseases and environmental stresses (Pingali 2012; Stamp et al. 2012; Massawe et al. 2016). In addition, the Green Revolution was not necessarily appropriate in low-income countries, where population densities were low, market infrastructure was poor and depended largely on orphan crops rather than the three main cereals (Pingali 2012; Massawe et al. 2016). Crop diversity is essential to make future agriculture

sustainable, resilient, and suitable for local environments and soils under climate change and growing population, which also plays an important role in global food security (Pingali 2012; Massawe et al. 2016).

1.2 Underutilised crops - a crop diversification solution for food security and nutrition

The potential for underutilised and neglected crops to improve food security and the crop diversity system has been long recognised (Aliyu and Massawe 2013; Sarkar et al. 2019). Underutilised, minor, orphan, neglected, marginal, niche, lesser used, lesser-known, under-researched, underfunded, promising or traditional crops are labels often applied to indigenous plants and often cultivated by woman or smallholder farmers for subsistence purposes (Padulosi and Hoeschle-Zeledon 2004; Mayes et al. 2012; Aliyu et al. 2015). The term 'underutilised crops' has been accepted in general to represent all the labels above (Jaenicke and Höschle-Zeledon 2006; Mayes et al. 2012; Stamp et al. 2012; Massawe et al. 2016).

Underutilised crops are those grown in their centres of origin or centres of diversity and considered to be adapted to marginal, harsh environments, and play a significant role in food security, nutrition, income generation and cultural functions for people who grow them (Padulosi and Hoeschle-Zeledon 2004; Mayes et al. 2012; Ahmad 2013). The promising underutilised crops have environmental adaptation, market potential and a broad genetic base (Stamp et al. 2012; Massawe et al. 2016). Thousands of potential plant species could fit into the definition of underutilised species, including bambara groundnut, aibika (*Abelmoschus manihot*), pitpit (*Setaria palmifolia/Saccharum edule*), pummelo (*Citrus maxima*), jackfruit (*Artocarpus heterophyllus*), tamarind (*Tamarindus indica*) and taun (*Pometia pinnata*) (Jaenicke and Höschle-Zeledon 2006;

Mayes et al. 2012). Padulosi and Hoeschle-Zeledon (2004) defined underutilised species as "those non-commodity crops, which are part of a larger biodiversity portfolio, once more popular and today neglected by users' groups for a variety of agronomic, genetic, economic, social and cultural factors."

Some organisations and institutions such as the Food and Agriculture Organization of the United Nations (FAO), the Global Facilitation Unit for Underutilised Species (GFU), Biodiversity International of the Consultative Group on International Agricultural Research (CGIAR) and Crops For the Future (CFF) have been engaged in several initiatives aimed at enhancing the use of underutilised species to realise social and economic benefits (Padulosi and Hoeschle-Zeledon 2004; Stamp et al. 2012; Ahmad 2013; Mayes et al. 2015; Massawe et al. 2016; Gregory et al. 2019). These organisations and other scientists worldwide, have conducted research and development activities aimed at improving and promoting a wider utilisation of underutilised crops.

Crop diversification that involve cropping sequence diversification such as rotation or intercropping of diverse crop species provides a potential approach to reduce yield variations, improve resilience to multiple environmental stresses and increase farmers' income (Gaudin et al. 2015; Makate et al. 2016; Mustafa et al. 2019; Sarkar et al. 2019). Moreover, crop diversification improves soil fertility in agriculture, reduce the need for fertilizer if leguminous crops were included in rotation or intercropping, control pests and diseases by protecting crops from parasites and provides habitats to beneficial insects (Makate et al. 2016; Mustafa et al. 2019).

Intercropping systems have often shown higher productivity than sole systems mainly due to effective resource utilization, such as water, light and nutrients (Dahmardeh et al. 2010). Alhassan and Egbe (2012) reported that intercropping maize and bambara groundnut was productive mainly due to the greater grain yield of the maize component. In a study conducted in the Southeast of Iran, intercropping maize with cowpea (*Vigna unguiculata* L. Walp) was observed to improve grain yield and soil fertility compared to sole crop (Dahmardeh et al. 2010). Intercropping taro (*Colocasia esculenta* L. Schott) and bambara groundnut was found to improve production of taro than sole cropping under rainfed conditions and contributed to the diversity of the local agro-system in KwaZulu-Natal, South Africa (Mabhaudhi and Modi 2014).

1.3 Plant adaptation mechanisms in response to drought stress

Drought is one of the major environmental stresses limiting plant growth and crop productivity worldwide (Haake 2002; Kang et al. 2002; Liu et al. 2012; Chai et al. 2016; Muhammad et al. 2016; Khan et al. 2017). Generally, plants have developed mechanisms to cope with drought stress. These mechanisms can be categorised into three drought resistance mechanisms: drought escape, drought avoidance and drought tolerance (Acquaah 2007).

Drought escape allows the plants to complete its life cycle by early flowering and/or maturity to escape the drought stress period (Delzon 2015). For example, short-season cultivars of soybean (*Glycine max*) are planted in March or April and set pods in late May to escape the possible drought period in July to August in the Early Soybean Planting System in the southern USA (Heatherly and Elmore 2004).

Drought avoidance consists of mechanisms that increase water uptake via the root system, and maintain high water status during periods of stress, for example, stomatal closure, leaf rolling or leaf area reduction (Turner et al. 2001; Chaves and Oliveira 2004; Kavar et al. 2008).

Drought tolerance allows plants to maintain cell turgor and metabolism by osmotic adjustment, xylem vascular system and/or stomatal regulation via abscisic acid (ABA), reactive oxygen species (ROS) scavenging to withstand low tissue water potential in plants (Nguyen et al. 1997; Buchanan et al. 2005; Delzon 2015). Different plant species use varied mechanisms to resist water-deficit stress conditions.

1.3.1 Flowering time

Time to flowering is one of the important developmental events which is strongly influenced by drought stress in plants (Kenney et al. 2014; Shavrukov et al. 2017). Bodner et al. (2015) reported that plants with early flowering were selected by breeders because early flowering limit plant vegetative growth and enable reproduction before drought stress onset. In *Brassica rapa*, plants that flowered earlier had fewer leaf nodes and lower water use efficiency to maintain high stomatal conductance, which allowed them to rapidly gain carbon for growth (Franks 2011).

Moreover, plants adjust the flowering time to escape or adapt to drought stress conditions through either a plastic response by altering their phenotype or the evolutionary shift to early flowering (Franks 2011; Kenney et al. 2014). Bambara groundnut landraces, namely Red, Brown and Light Brown geographically collected from South Africa, had an average 38 days flowering duration (17% – 35% reduction) and average 146 days to maturity (9% – 15% earlier) under water deficit conditions (Mabhaudhi and Modi 2013). However, the limited vegetative growth can constrain

grain yield accumulation through limited photosynthetic production and pod filling process (Radhika and Thind 2014).

1.3.2 Photosynthate and water use efficiency

Three common bean (*Phaseolus vulgaris* L.) elite lines (NCB 226, SER 78, SER 125) showed superior levels of adaptation to drought stress conditions by remobilizing photosynthate to increase grain yield (Rao et al. 2017). Drought stress caused significant changes in photosynthesis, relative water content, root and shoot dry weight, which are good indicators of drought monitoring in chickpea (*Cicer arietinum* L.) (Farooq et al. 2009; Pang et al. 2017; Khan et al. 2019), and in cowpea, the photosynthetic machinery is sensitive to water deficit (Souza et al. 2004). For example, Pingo de Ouro 1-2 (PO), a drought-tolerant cowpea cultivar, maintained higher photochemical activity and leaf gas exchange during water deficit for a longer period than the drought-sensitive cultivar, Santo Inácio (SI) does (Rivas et al. 2016). As SI cultivar had a larger leaf area than PO, PO plants had higher photosynthetic performance under water deficit and faster recovery of photosynthesis after water stress than SI plants, which may be among the mechanisms enabling plants to overcome stressful conditions (Bastos et al. 2011; Rivas et al. 2016). Groundnut (*Arachis hypogaea*) genotypes use two mechanisms, increasing root proportion to uptake soil water and decreasing transpiration to reduce water loss, to respond to water deficit under pre-flowering drought conditions (Jongrungklang et al. 2013).

Studies of drought stress response mechanisms in bambara groundnut has been reported (Jørgensen et al. 2010; Berchie 2012; Mabhaudhi and Modi 2013; Chai et al. 2016; Massawe et al. 2016). It was shown that S19-3 landrace from Namibia experienced reduced respiration and stomata closure at a comparatively lower water threshold coupled with fast phenological

development, short life cycle and early maturing proved to be among the mechanisms to ameliorate drought conditions (Massawe et al. 2005; Jørgensen et al. 2010). Three landraces of bambara groundnut collected from South Africa i.e., Brown, Red and Light Brown were reported to have reduced stomatal conductance of 1% – 8%, reduced *chlorophyll content index, plant height, leaf number*, reduced *leaf area index* and biomass accumulation of 5% – 8% and yield loss of 50% under water defict conditions (Mabhaudhi and Modi 2013). Landrace Brown and Red showed higher *emergence rate, stomatal conductance*, *chlorophyll content index* and yielded more than Light Brown in response to water deficit conditions (Mabhaudhi and Modi 2013). In bambara groundnut, dark-coloured seeds performed better than light-coloured seeds under drought stress conditions due to the tannins present in dark-coloured seeds which are polyphenols and act as antioxidants under stress conditions (Chibarabada et al. 2015).

1.3.3 Grain yield

Drought is also major abiotic stress that affects productivity in common bean (Polania et al. 2016). Seven common bean lines showed greater root vigour and higher grain yield under drought stress, and these lines could be selected as parental lines for drought resistance breeding improvement in common bean (Polania et al. 2016).

Chickpea, an important component of the subsistence farming in drought-prone areas, thrives in low input marginal lands, but still suffers about 50% yield losses due to drought stress, and its drought tolerance reactions and adaptation mainly depended on the root growth and water-use efficiency of genotypes (Varshney et al. 2014; Purushothaman et al. 2016). An interesting finding is that early drought stress which occurs during the pod and seed formation stages sometimes increases yield of groundnut (Puangbut, et al. 2009; Jongrungklang et al. 2013). This could be due

to (1) longer roots and high root density and root mass during water deficit period that allows for sufficient water uptake from soil; (2) increased partitioning of phosythates to root mass, leading to reduced mass allocation to the shoot and hence reduced leaf area index (LAI) and could conserve water in that way; (3) high transpiration process under water deficit conditions would increase canopy photosynthesis to maintain reasonably yield (Reddy et al. 2003; Jongrungklang et al. 2013).

Compared to the irrigated treatment, drought treatment plants showed higher *stomatal density* and reduced *100-seed weight, harvest index* and *leaf area* in an F_5 segregating population of bambara groundnut derived from Tiga Nicuru × DipC (Chai et al. 2016). Yield losses of 45% and reductions in the rate of leaf area expansion, final *canopy size* and *total dry matter, pod dry matter, pod number*, reduction of 16% in *seed weight* and 15% in *harvest index* have also been reported in bambara groundnut landraces, i.e. S19-3, DipC and UN when the plants were subjected to drought stress (Mwale et al. 2006). Landrace S19-3 performed better than DipC and UN under drought stress conditions mainly because of its shorter life cycle (110 DAS without significant loss of yield) and higher *harvest index* in response to water deficit (Mwale et al. 2006).

1.3.4 Proline accumulation

Proline accumulation in plants has been observed under environmental stress conditions such as drought stress and high temperature (Sairam et al. 2002). Drought stress increased proline content by tenfold in three varieties of chickpea (drought-tolerant Bivaniej and ILC482 and drought-sensitive Pirouz) (Mafakheri et al. 2010). The proline content was higher in the drought tolerant variety ILC482 compared to the drought-sensitive variety Pirouz under both drought-stressed and irrigated conditions (Mafakheri et al. 2010).

In a study of an untargeted metabolomic profiling conducted in drought-sensitive chickpea variety Punjab Noor-2009 (G1) and drought-tolerant variety 93127 (G2) under drought stress to identify genetic variations in chickpea varieties (Khan et al. 2019), a number of metabolites were identified. Some metabolites, such as allantoin, proline, tryptophan, histidine, tyrosine, isoleucine, and arginine were identified as potential biomarkers for drought stress tolerance improvement in chickpea (Khan et al. 2019). Seven genotypes in cowpea showed a general decrease in germination and seedling growth and an increase in proline content under drought stress, which are helpful for drought resistance improvement breeding (Carvalho et al. 2019).

Drought led to *shorter plant height, canopy diameter, internode diameter, stem length* and *leaf length*, smaller the *number of leaves, flowers, stems* and *internodes, lower canopy weight* and *root weight* and a decrease in the *leaf chlorophyll contents*, but a slight increase in the *leaf proline contents* of twelve bambara groundnut genotypes collected from Indonesia (Fatimah et al. 2020). In a study involving four landraces (DodR Tz, SB 4-2, Uniswa Red and S19-3) of bambara groundnut, proline concentration increased under water-deficit conditions when subjected to drought stress (Nautiyal et al. 2017). The higher proline accumulation in drought-tolerant landraces S19-3 than other three landraces of bambara groundnut under moderate drought stress conditions, would suggest that proline content could be used as a selection tool for drought adaptation breeding programme (Gibon et al. 2000; Nautiyal et al. 2017).

1.3.5 Drought-related genes and transcription factors

A fatty acid elongase gene, KCS1 gene, showed potential to improve stress tolerance in drought susceptible groundnut cultivar K-6, which was also reported in Arabidopsis (Lokesh et al. 2019)

and apple (*Malus domestica Borkh*) (Albert et al. 2011). The transgenic groundnut plants overexpressing *AhKCS1* gene showed drought stress tolerance by preventing non-stomatal water loss and improved traits, such as enhanced epicuticular wax accumulation, reduction in cuticular transpiration, lower membrane damage, high cell membrane stability, and high free proline content (Lokesh et al. 2019).

Drought causes osmotic stress to plants which result in the production of abscisic acis (ABA). The transcription factors (TFs) involved in ABA-responsive gene expression such as APETALA2 (AP2), ethylene-responsive element binding factors (ERF), basic leucine zipper (bZIP), N-acetyl cysteine (NAC), WRKY, nuclear factor Y (NF-Y) and myeloblastosis (MYB) transcription factor families, reactive oxygen species (ROS) scavengling, protein kinase, late embryogenesis abundant (LEA) proteins, are the key regulators of ABA signaling (Cutler et al. 2010; Nakashima and Yamaguchi-Shinozaki 2013; Quach et al. 2015; Yoshida et al. 2015; Tripathi et al. 2016).

Two genotypes (DipC and Tiga Nicuru (TN)) derived from landraces of bambara groundnut under drought stress showed the differential expression of transcriptions factors, the high levels of expression four genes namely, phenylalanine ammonia-lyase-1 (PAL1), Beta-fructofuranosidase, Catechol-O-methyltransferase (COMT) and UBC-2 (Khan et al. 2017). However, with the XSpecies hybridisation approach (Affymetrix GeneChip microarray), the two genotypes showed contrasting transcriptional behaviour in response to drought stress, the DipC showed more TFs than TN of bambara groundnut (Khan et al. 2017). WRKY40, a well-known member of plant drought-response networks, showed the most co-expressed genes in DipC, whereas CONSTANTS-like 1 and MYB60 being the most significant expression in TN bambara groundnut

(Singh and Jain 2015; Khan et al. 2017). Proline-rich protein 7 (PRR7), auxin responsive protein (ATAUX2-11), CONSTANS-like 1, MYB60, amylo-Alpha-1, 6-Glucosidase, 4-Alpha-Glucanotransferase (AGL-83), and a Zinc-finger protein may be considered as drought-related genes and involved in different drought response mechanisms in bambara groundnut (Khan et al. 2017).

1.4 Nutrition values of underutilised crops

The majority of underutilised crops are used as cereals, oil, spice, fruits and vegetable and fodder (Williams and Haq 2000). One of these is quinoa (*Chenopodium quinoa*), an Amaranthacean, deemed the 'Queen of Superfoods'. The crop is considered as a stress-tolerant and underutilised grain of the Andean region, containing far high amounts of certain essential amino acids than wheat and can be grown under different environmental conditions (Vega-Gálvez et al. 2010; Vurayai et al. 2011). Teff [*Eragrostis tef* (Zucc.) Trotter], a gluten-free and main cereal crop in Ethiopia and Eritrea, is another important crop that could be grown under arid and semi-arid areas prone to drought and heat, and waterlogged soil conditions (Cheng et al. 2017). The genome of teff has been sequenced and could be an ideal model plant for future food crops (Abraham et al. 2014).

Other underutilised plants such as Oca (*Oxalis tuberosa*), ulluco (*Ullucus tuberosus*) and mashwa (*Tropaeolum tuberosum*) are rich in Vitamin A and Vitamin C; the leaves of black nightshades (*Solanum nigrum*) contains minerals, Vitamins A and C and proteins (Padulosi and Hoeschle-Zeledon 2004). These plants only represent a small part of underutilised crop species. Research and development of underutilised crops have a long way to go. Policy makers and scientists should

be aware of the importance of protecting and utilising the underutilised species to realise benefits from local agrobiodiversity (Padulosi and Hoeschle-Zeledon 2004; Massawe et al. 2016).

1.5 Bambara groundnut

Bambara groundnut (2x = 2n = 22) is an African indigenous protein-rich and self-pollinating legume and has ability to fix atmospheric nitrogen, withstand high temperatures and drought stress and grown predominantly in semi-arid Africa (Massawe et al. 2005; Mabhaudhi and Modi 2013; Atoyebi et al. 2017; Sarkar et al. 2019). The seed of bambara groundnut contains approximately 24% protein (rich in the essential amino acids lysine, methionine and cysteine), 64% carbohydrates (53% starch, 10% dietary fibre), and 18% oil (predominantly oleic, palmitic and linolenic acids), providing nutrition and a balanced diet for human (Minka and Bruneteau 2000; Suwanprasert et al. 2006; Okpuzor et al. 2010; Halimi et al. 2020). The bambara groundnut seed makes a complete food, as it has been concluded that the seed is a useful ingredient for different food products e.g. vegetable milk, flours, snack (pudding or steamed-paste) and different beverages (Massawe et al. 2005; Eltayeb et al. 2011; Mohammed et al. 2015).

Bambara groundnut is considered as a drought-tolerant leguminous crop capable of growing in marginal and low-input environments and mainly cultivated in semi-arid tropics (Massawe et al. 2005; Basu et al. 2007). As the third most important food legume crop in semi-arid Africa after groundnut and cowpea (York and Garden 1994; Olaleke et al. 2005), it has the potential to assist with providing nutrition and food security in the dry areas all over the world (Ahmad 2013; Mohammed et al. 2015).

The most likely centre of origin of bambara groundnut is North-Eastern Nigeria and Northern Cameroon in West Africa (Kerstingiella and Hepper 1963). Bambara is the name of a tribe, who now lives mainly in Mali, West Africa (Nwanna et al. 2005). The pods usually develop underground, similar to groundnut (Kerstingiella and Hepper 1963), but the two species belong to two different genus. The crop is distributed and grown in most parts of Africa, in Northern Australia, Asia, and South America, but the present degree of cultivation outside Africa is negligible (Kerstingiella and Hepper 1963; Suwanprasert et al. 2006).

Bambara groundnut plant life cycle ranges from 90 to 150 days and starts to form pods around 30 to 40 days after fertilisation (Table1-1; Figures 1-1 and 1-2; Massawe et al. 2005; Basu et al. 2007). Flowering in bambara groundnut starts 30 to 45 days after emergence and may continue until the end of life cycle depending on landraces and the environment (Berchie et al. 2010). Bambara groundnut forms nodules on the roots to fix atmospheric nitrogen, which is an important trait for crop rotation and intercropping (Figure 1-3; Karikari and Tobana 2004). Some other important traits, such as flower number, days to maturity, leaf number, pod development, yield, photoperiod and response to sowing date differ significantly among the landraces (Massawe et al. 2005; Sesay et al 2008).

Table 1-1 The description of Bambara groundnut growth stage.

Growth Stage			**Description**
Vegetative	Emergence	VE	The cotyledon pokes through the soil.
	Cotyledon	VC	Cotyledons are flat and open at soil surface.
	First Trifoliate	V1	The first trifoliate is fully expanded
	Nth Trifoliate	VN	The Nth trifoliate is fully expanded.
Reproductive	Beginning flowering	R1	Plant has one flower open at any node on the stem.
	Beginning pod development	R2	Plant has one pod development at any node on the stem. Plant has flower open on the stem.
	Full pod	R3	One fully-expanded pod, to dimensions characteristic of the cultivar.
	Beginning seed	R4	One fully-expanded pod in which seed cotyledon growth is visible when the fruit is cut in cross-section with a razor blade (Past the liquid endosperm phase).
	Full seed	R5	One pod with cavity apparently filled by the seeds when fresh.
	Beginning maturity	R6	One pod showing visible natural coloration or blotching of inner pericarp or testa.
	Harvest maturity	R7	2/3 or 3/4 of all developed pods reach mature colour

VE: The cotyledon pokes through the soil.

VC: Cotyledons are flat and open at soil surface.

V1: The first trifoliate is fully expanded

Figure 1-1 Illustration of bambara groundnut vegetative growth stages.

R1: Plant has one flower open at any node on the stem

R2: Plant has one pod development at any node on the stem. Plant has flower open on the stem.
R3: One fully-expanded pod, to dimensions characteristic of the cultivar.

R7: 2/3 or 3/4 of all developed pods reach mature colour

Figure 1-2 Illustration of bambara groundnut reproductive stages

Figure 1-3 Nodules formed on the roots of bambara groundnut.

1.6 Germplasm and structured population in bambara groundnut

There are around 5,000 accessions of bambara groundnut mainly collected from African countries and these collections are held by international or regional seed banks (Table 1-2). The major germplasm collection held by the International Institute of Tropical Agriculture (IITA) and gathered from 25 African countries have been characterized and evaluated (Begemann and Engels 1997). The crop is still largely grown as landraces and the variations harboured by these landraces is a great asset for breeding programmes (Olukolu et al. 2012; Kendabie et al. 2015; Mayes et al. 2015; Massawe et al. 2016). Phenotypic descriptors (Begemann and Engels 1997), biochemical markers (Pasquet et al. 1999), molecular markers including AFLP markers (Massawe et al. 2002), RAPD (Massawe et al. 2003), SSR markers (Molosiwa et al. 2013; Aliyu and Massawe 2013; Redjeki et al. 2020), DArT markers (Olukolu et al. 2012) and SNP markers (Redjeki et al. 2020) have been used to assess genetic diversity within the available germplasm of bambara groundnut.

Table 1-2 Bambara groundnut accessions held by countries or institutions (*Begemann and Engels 1997; Muhammad et al. 2020)*.

Country/institution	No. of accessions
Benin	3
Botswana	26
Burkina Faso	143
Departement of Agricultural Research (DAR)	338
France, Office de la Recherche Scientifique et Technique d'Outre-Mer (ORSTOM)	1416
Ghana, Plant Genetic Resources Unit (PGRC)	166
Ghana, Savanna Agricultural Research Institute (SARI)	90
Ghana, University of Ghana	80
Guinea	43
Kenya, Kakamega Regional Research Centre (KARI)	2
Kenya, National Genebank	6
Kenya, National Museums	2
Mali	70
Mozambique	12
Namibia	23
Niger	79
Nigeria, International Institute of Tropical Agriculture (IITA)	2035
Plant Genetic Resources Research Institute (PGRRI)	296
South Africa, Department of Agriculture	20
South Africa, Grain Crops Institute	198
South Africa, Institute for Veld and Forage Utilization	117
Tanzania, National Plant Genetic Resources Committee (NPGRC)	222
Zambia, National Plant Genetic Resources Committee (NPGRC)	232
Zambia, University of Zambia	463
Zimbabwe	129

Controlled crossing protocols have been established in bambara groundnut (Massawe et al. 2005; Suwanprasert et al. 2006; Kendabie et al. 2015) and these have been used successfully in artificial hybridisation efforts. For example, Basu et al. (2007) reported on a single F_2 population, derived from a domesticated landrace from Botswana (DipC; female parent) crossed with a wild accession

collected in Cameroon (VSSP11; male parent) which was developed to investigate the inheritance of 'domestication' traits in bambara groundnut (Basu et al. 2007). The results of this work suggested that traits including *leaf area, specific leaf area* (SLA), *carbon isotope discrimination* (CID), and *100-seed weight* are controlled by several genes while internode length, stems per plant, days to emergence and seed eye pattern around the hilum are likely to be under largely monogenic control (Basu et al. 2007). Chai (2015) evaluated an F_5 breeding population derived from Tiga Nicuru × DipC to evaluate the effects of mild drought stress on the morpho-physiological characteristics in bambara groundnut. Strong genotypic variation was observed for many traits, including *100-seed weight, harvest index,* stomatal density and leaf area (Chai et al. 2016). Five segregating populations have also been developed from crosses involving photoperiod-sensitive (Ankpa4 and LunT) and less-sensitive (S19-3, DipC, DodR and IITA-686) parental genotypes to exploit germplasm and accelerate breeding for improved varieties in bambara groundnut (Kendabie et al. 2015; 2020). These populations include: Ankpa4 × IITA-686 (reciprocal), Ankpa4 × DodR, Ankpa4 × DipC, S19-3 × Ankpa4 and IITA-686 × LunT (Kendabie et al. 2015). Two F_2 bi-parental segregating populations derived from IITA-686 × Tiga Nicuru and S19-3 × DodR were developed and advanced to obtain structured populations and breeding lines for genetic analysis and trait dissection including *days to flowering, harvest index* and *100-seed weight* to support bambara groundnut breeding programmes (Gao et al. 2020).

1.7 Molecular marker and breeding resources in bambara groundnut

Most of the plant breeding programmes aim to develop an ideal plant that combines a maximum number of desirable characteristics, such as disease and insect resistance, abiotic stress tolerance, high yield and other specific traits to improve crop yield, quality and adaptation to the environment (Jain and Brar 2009). As a self-pollinated and underutilised crop, methods for bambara groundnut

breeding could include mass selection, pure line selection and hybridization (pedigree, bulk population and backcross) (Acquaah 2007). The pure lines selected through mass selection or pure line selection could be used as varieties, parental lines for crossbreeding or mutation study. The objective of hybridization is to combine desirable traits from two or more contrasting parents into a single variety with superior traits compared to the parental lines. However, conventional breeding approaches depend on the phenotypic selection and may require additional time and expense (Moose and Mumm 2008). Molecular plant breeding such as marker-assisted breeding (MAB) using DNA markers is a novel strategy for crop improvement (Moose and Mumm 2008; Jiang 2018). Random amplified polymorphic DNA (RAPD) markers, amplified fragment length polymorphism (AFLP) markers, simple sequence repeat (SSR or microsatellite) markers, and more recently DArTseq markers and single nucleotide polymorphism (SNP) markers have been developed and applied in several bambara groundnut studies (e.g. Massawe et al. 2003; Ntundu et al. 2004; Somta et al. 2011; Molosiwa et al. 2015; Ahmad et al. 2016; Chai et al. 2017; Ho et al. 2017; Redjeki et al. 2020).

1.7.1 Molecular markers

1.7.1.1 RAPD markers

Random amplified polymorphic DNA (RAPD) markers use random decamer primers (10 bp in length) to produce PCR fragments. The technique is simple, rapid and inexpensive as no knowledge of DNA sequence of the targeted genome is required for PCR (Williams et al. 1990). RAPDs were used to assess genetic diversity and to identify variation between and within landraces in species such as bambara groundnut germplasm (Amadou et al. 2001; Massawe et al. 2003). Massawe et al. (2003) reported high polymorphism levels among 12 landraces of bambara groundnut using 16 RAPD primers and Amadou et al. (2001) found high genetic variation among

25 African accessions of bambara groundnut using 15 RAPD primers. However, this technique is comparatively less reliable due to the low reproducibility of RAPD markers (Jones et al. 1997; Massawe et al. 2003). Moreover, the allelic variants cannot be detected as RAPD markers are dominant (Williams et al. 1990).

1.7.1.2 AFLP markers

Amplified fragment length polymorphism (AFLP) markers are PCR amplified fragments and used to identify genetic variation in species (Vos et al. 1995). AFLP markers are dominant as RAPD markers but the technique is more reproducible and sensitive compared to RAPD markers (Mueller and Wolfenbarger 1999). In a study to determine genetic variation among a diverse group of 100 bambara groundnut landraces from Tanzania, two major clusters were identified using 11 AFLP primers, which generated a total of 49 polymorphic fragments across the bambara groundnut accessions (Ntundu et al. 2004). In another study, Massawe et al. (2003) used seven AFLP markers and generated 504 amplification products, ranging from 50 to 400 bp in 16 cultivated bambara groundnut landraces. The AFLP has a number of disadvantages, it needs to be purified and high molecular weight DNA for standard protocol and it is a relatively labour-intensive method (Paun and Schönswetter 2012).

1.7.1.3 SSR markers

Microsatellite markers, or simple sequence repeats (SSRs) markers are widely used for population genetics studies and plant breeding projects because they are easy to score and highly polymorphic (Squirrell et al. 2003; Narum et al. 2014; Meyer et al. 2017). A microsatellite is a tract of repetitive DNA motifs, ranging from 1 – 6 bp in length and typically 5 – 50 times in plant genomes (Richard et al. 2008).

Aliyu and Massawe (2013) used microsatellite markers alongside characterisation of morphological features to analyse the level of genetic diversity in a small collection of ten Ghanaian bambara groundnut landraces. Eighty individual genotypes of the ten landraces were clustered into seventeen units. Genetic distances both inter and intra between landraces of bambara groundnut using SSR markers were in the range of 0.48 – 0.90, which is consistent with previous reports (Massawe et al. 2003) obtained using RAPD markers. The limitations of SSR markers include: high development cost, low density throughout the genome, complex mutational patterns and the possible presence of homoplasy and null alleles and confusion between parameters and estimators, and the identity of various statistics (Putman and Carbone 2014; Meyer et al. 2017).

High-throughput next-generation sequencing has enabled researchers to develop novel SSR markers, such as genomic SSRs (g-SSRs) and expressed sequence tags SSRs (EST-SSRs), which are less costly, faster, easier, contain a higher level of genetic diversity and transferability compared to traditional approaches (Taheri et al. 2018). Meyer et al. (2017) identified 13 genomic SSRs and 13 EST-SSRs to characterize the genetic diversity and population dynamics of native and invasive species in *Ambrosia artemisiifolia* populations. A total of 65%, 75% and 40% of these markers were transferred from *Ambrosia artemisiifolia* to other closely related *Ambrosia* species (*Ambrosia psilostachya*, *Ambrosia tenuifolia* and *Ambrosia trifida*), which showed transferability of cross-species marker (Meyer et al. 2017).

1.7.1.4 DArTseq markers

DArTseq technology combines Diversity Arrays Technology (DArT) with next-generation sequencing platforms (Courtois et al. 2013; Cruz, et al. 2013). Diversity Arrays Technology is a

species independent high-throughput genotyping method, which is based on microarray hybridization that detect the presence versus absence of individual fragments in genomic representations (Jaccoud et al. 2001). Next-generation sequencing is a high-throughput sequencing technique, which is quick, relatively inexpensive, and readily applicable and available for crops with a low level of investment and without a sequenced genome (Metzker 2010).

DArTseq is an efficient genotyping-by-sequencing (GBS) platform for genome wide marker discovery through restriction fragments sequencing and genome complexity reduction (www.diversityarray.com). DArTseq generates two types of data: (1) SilicoDArTs, which scores for presence/ absence (the 0/1 scores) dominant markers; and (2) Single nucleotide polymorphisms (SNPs) in fragments present in the representation (http://www.diversityarrays.com/). Single nucleotide polymorphisms markers are single nucleotide replaced at a specific position in the genome with more than 1% frequent abundance (Brookes 1999).

DArTseq is a relatively new molecular marker technique, more comprehensive in terms of molecular variation underlying the polymorphisms with affordable price, has been reported in several species, such as rye (*Secale cereale* L.) (Gawronski et al. 2016), watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] (Ren et al. 2015), Yellowtail kingfish (*Seriola lalandi*) (Nguyen et al. 2018) and bambara groundnut (Ho et al. 2017; Redjeki et al. 2020). The DArTseq genotyping in rye was reported to generate 6,177 polymorphic markers and 515 DArT sequences were incorporated into publicly available rye genome zippers (Gawronski et al. 2016). A total of 4,808 SNPs were identified from the DArTseq based genotyping systems in watermelon, resulting in a high-density genetic linkage map (Ren et al. 2015). DArT sequencing technology was also

used for genomic prediction of traits of commercial importance in yellowtail kingfish, whose variation is of polygenic nature (Nguyen et al. 2018).

Olukolu et al. (2012) identified a relatively high genetic diversity using 554 DArT markers among 40 landraces of bambara groundnut collected from East Africa (Kenya, Madagascar, Tanzania and Zambia), West Africa ((Nigeria, Ghana, Burkina Faso and Republic of Benin), and Central Africa (Cameroon). More recently, a total of 170 bambara groundnut accessions collected from Indonesia, East Africa, West Africa, Central Africa, and Southern Africa were used to evaluate the genetic diversity among landraces using 170 SSR markers and 168 DArTseq markers (Redjeki et al. 2020). The results of this study suggest that the current Indonesian accessions were likely introduced from Southern Africa (Redjeki et al. 2020).

1.7.2 Genetic linkage map construction and QTL mapping

As an important component of both fundamental research and practical application in many studies of plants, animals and microorganisms, a genetic linkage map represents the relative order of genetic markers along a chromosome and the relative distance between them determined by recombination frequency (Liu et al. 1998; Yeboah et al. 2007). Understanding the genetic basis and identification of molecular markers for target traits are prerequisites for deploying molecular breeding for developing superior genotypes (Kullan et al. 2012). The construction of a linkage map includes: (1) Grouping markers, which is placing markers into linkage groups based on their linkage relationships; and (2) Ordering the markers within each group, which is based on some criteria, such as Recombination fraction, LOD (Logarithm of odds) score (base-10 log likelihood ratio) and significant P-value (Liu et al. 1998). High-density genetic linkage maps are also used to analyse the inheritance of target genes and for map-based cloning (Kullan et al. 2012).

The first bambara groundnut genetic linkage map was constructed by 67 AFLP and 1 SSR markers, consisting of 20 linkage groups and 516 cM in length using an F_2 segregating population derived from a cross between a wild accession, VSSP11 and a cultivated accession, DipC (Basu et al. 2007). QTL analysis in the F_2 population identified a range of QTLs associated with agronomic traits including *internode length, leaf water use efficiency* (LWUE), *Carbon Isotope Discrimination* (Δ13C), *seed weight* and *testa colour* (Basu et al. 2007). Another bambara groundnut genetic map was constructed using 269 polymorphic markers which included 236 DArT and 33 SSR markers in an F_3 segregating population of bambara groundnut derived from a narrow cross between DipC and Tiga nicuru (Ahmad et al. 2016). The genetic map consists of 21 linkage groups (LGs) with a total genetic distance of 608.3 cM, a total of 36 significant QTLs associated with various important phenotypic traits in bambara groundnut were detected (Ahmad et al. 2016). In addition to the linkage map construction, two stable QTLs were mapped for the internode length (LG4, 3.0 cM; LOD 7.9 and 7.1) and growth habit (LG4, 0.0 cM; $p < 0.0005$) and explained by more than 40% of phenotypic variation in the F_3 populations under controlled environment glasshouse and field conditions (Ahmad et al. 2016).

The first expression-based genetic map (GEM map) in the F_5 population of bambara groundnut was developed for QTL analysis using 527 markers and covered 982.7 cM and 13 linkage groups (Chai et al. 2017). QTLs associated with stomatal conductance, carbon isotope discrimination analysis and stomatal density were largely mapped on LG2 (Chai et al. 2017). QTLs for (Delta N15) isotope analysis (NID) mapped on LG1 linked with *internode length, pod number per plant, pod weight per plant* and *seed number per plant*, showing a significant co-expression relationship

between nitrogen assimilation and biomass in plants (Chai et al. 2017). The major QTL related to *internode length* and *peduncle length* on LG1 and minor QTL in LG8A in both drought and irrigated populations suggested these two traits are probably controlled by a single gene or two closely-linked genes (Chai et al. 2017).

1.7.3 XSpecies (cross-species) microarray

For the crops without the whole genome sequencing data, XSpecies (cross-species) microarray approach can be adopted to compare across experiments between a given species (e.g. bambara groundnut) and another related species (e.g. soybean) (Mayes et al. 2013). XSpecies hybridisation that combines the Affymetrix GeneChip microarray approach and Next-generation sequence technique is an inexpensive and efficient approach to transfer genomic and transcriptomic data to other related and low investment species, which have been successfully used in bambara groundnut, banana (*Musa* spp.) and blueberry (cultivar Bluecrop (*V. corymbosum*) and Tifblue (*V. virgatum*) (Davey et al. 2009; Die and Rowland 2013). Investigating the genetic linkage map in related crops and emphasizing shared synteny among these species might help to identify the markers closely linked to traits of interest in plant species (Ahmad 2013).

As an underutilised and orphan crop, bambara groundnut lack of investment from funding bodies such as government, research institutes and companies. Genetic map that is developed based on markers derived from closely-related species and QTL analysis have been successfully adopted in bambara groundnut to detect syntenic loci and potential candidate genes that control important agronomic traits (Mayes et al. 2012; Bonthala et al. 2016; Khan 2016; Chai et al. 2017; Ho et al. 2017). As bambara groundnut has no microarray chip, Soybean Affymetrix GeneChip was

utilised to detect genes and gene modules related to low-temperature responses in a bambara groundnut genotype, S19-3 (Bonthala et al. 2016). A total of 375 and 659 differentially expressed genes ($p < 0.01$) was detected under 23°C and18°C, respectively (Bonthala et al. 2016). Cross-species hybridisation to the soybean microarray was also conducted to identity the transcriptomics related to drought response mechanisms in two contrasting bambara groundnut genotypes, DipC and Tiga Nicuru (Khan et al. 2017). Several candidate genes involved in the regulation of drought stress was identified including *WRKY40, PRR7, ATAUX2-11, CONSTANS-like 1, MYB60, AGL-83*, and a Zinc-finger protein. The first expression-based genetic map (GEM map) in bambara groundnut was constructed using XSpecies hybridisation with the Affymetrix Soybean Genome GeneChip microarray, to identify intrinsic and drought-related quantitative trait loci (QTL) in an F_5 segregating population of bambara groundnut (Chai et al. 2017). Significant QTLs associated with *pod number per plant* and *harvest index* were reported to be mapped on LG1 with 33.9% and 24.5% of phenotypic variation explained, respectively under irrigated treatment, while putative QTL was detected on LG1 with the reduced to 18.2% and 16.1% of phenotypic variation explained, respectively under drought treatment for these traits (Chai et al. 2017).

1.8 The Future of Bambara groundnut - molecular breeding approaches

Bambara groundnut is an indigenous and underutilised legume crop, grown in subsistence and small-scale agriculture in Africa and in some parts of Southeast Asia e.g. Indonesia and Thailand (Basu et al. 2007; Mayes et al. 2018). The main aim of any bambara groundnut improvement programme should be to enhance the genetic potential and ensure the crop sustainability and reliability, alongside the high yields and quality (Massawe et al. 2005). The breeding strategies for bambara groundnut can be simplified into three categories: (i) Evaluation of variation of bambara groundnut landraces based on observed variants and molecular markers; (ii) controlled mating

from different parents by the selection of desirable traits; (iii) selection of recombinants by specific genes or marker profiles after monitoring the inheritance of with-genome variation. The first genome sequence was released (Chang et al. 2018) by the African Orphan Crops Consortium (AOCC), when the genome is fully assembled and annotated, it will greatly facilitate bambara groundnut improvement programmes. Integration of modern breeding strategies into conventional breeding programmes will be needed to support bambara groundnut future improvement programmes.

1.8.1 Marker-assisted selection

Marker-assisted selection (MAS) is an indirect and effective selection method and depends on the association of phenotypic variation with genetic diversity at specific locus surveyed by particular markers (Ribaut and Hoisington 1998; Reynolds et al. 2001). A reliable marker system, knowledge of the associations between markers and the traits of interest and genetic linkage map are critical factors for marker-assisted breeding (Jiang 2013). Molecular markers namely, RAPD, AFLP, SSR and DArTseq markers and genetic linkage map have been successfully developed in bambara groundnut. Markers that are closely correlated with associated phenotypic traits and QTLs will reliably improve the target traits and gain confidence in the value of a particular locus (Miles et al. 2008). However, QTLs information is difficult to directly transfer from one species to another due to both genetic and environmental effects (Wang et al. 2014). Taking a single cross as an example, the general procedure for MAS involves eight integral steps (Figure 1-4).

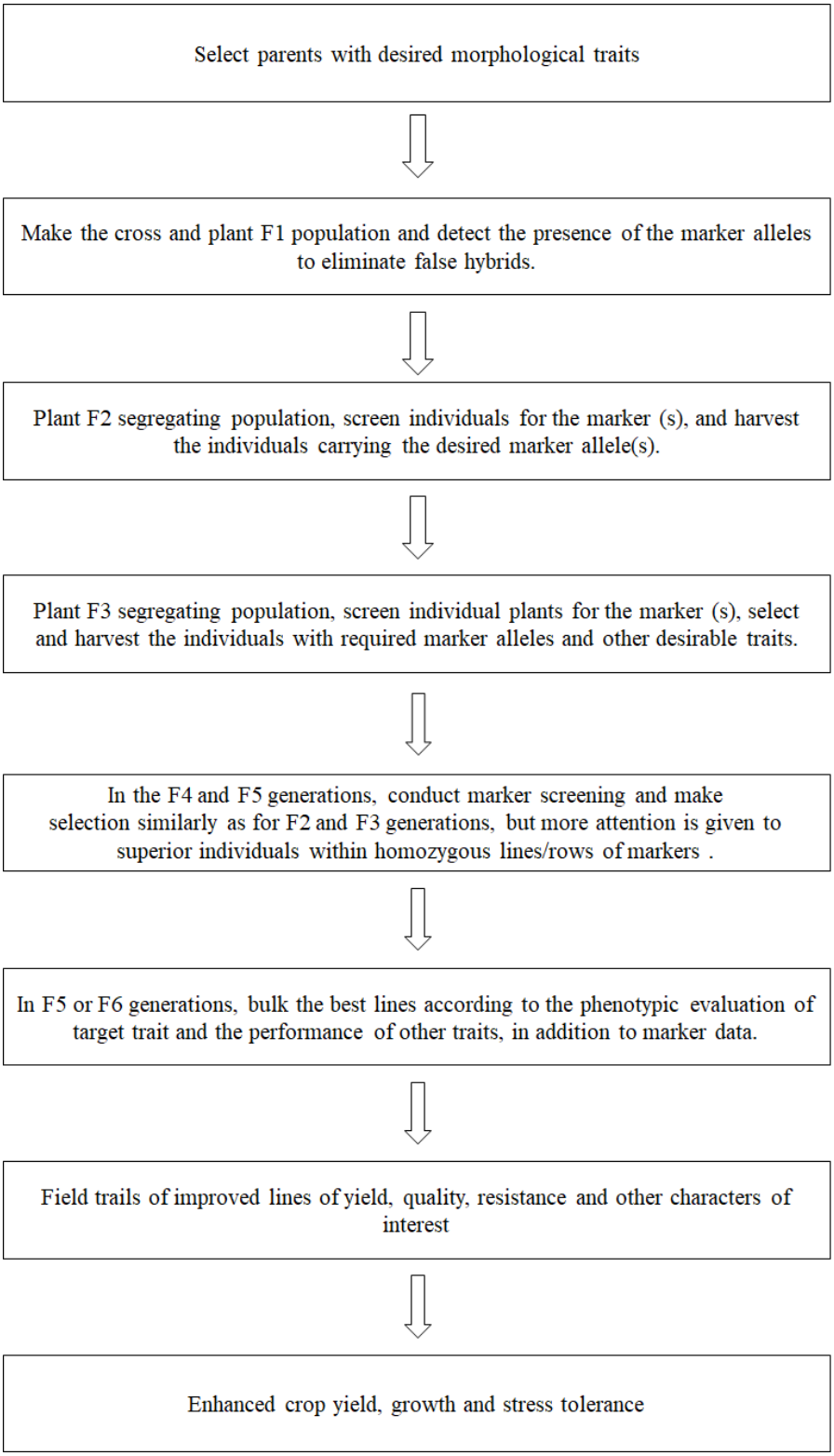


Figure 1-4 The general procedure for marker-assisted selection (MAS) for abiotic stress tolerance in plants (taking a single cross as an example) (*Adapted from Jiang (2013)*).

1.8.2 Genomic selection

Genomic selection is a form of MAS in which a very large number of markers obtained by Single Nucleotide Polymorphims (SNPs), covering the whole genome are used so that all quantitative trait loci (QTL) are closely linked in linkage disequilibrium with at least one marker (Goddard and Hayes 2007). Compared to the phenotypic selection, genomic selection has high selection accuracy, reduced selection duration, greater genetic gain per unit time and more reliable results, which could accelerate improvement breeding process (Desta and Ortiz 2014; Bhat et al. 2016).

Both genomic selection and traditional MAS rely on association between marker and target traits (Arruda et al. 2016). Genomic selection is expected to be more efficient and reliable than marker-assisted selection in terms of complex traits as genomic selection uses genome-wide and densely distributed molecular markers that account for all QTLs associated with phenotypic traits (Goddard and Hayes 2007; Jannink et al. 2010). The rapid advances in sequencing technology such as next generation sequencing has led to higher throughput and quality markers for improvement breeding especially in non-model and low investment crops and crops with complex genomes (Arruda et al. 2016). The general procedure for genomic selection involves three main parts (Figure 1-5).

Zhang et al. (2016) compared the prediction accuracy of MAS and genomic selection for seed weight using 31,045 single nucleotide polymorphisms in a genome-wide association study. The results showed both MAS and genomic selection are applicable when training and validation populations were related or had similar genetic components, and genomic selection outperform MASwhen training and validation populations were unrelated panels (Zhang et al. 2016). To date,

genomic selection for breeding in bambara groundnut has not yet been reported. The released genome sequence will provide a good resource for molecular breeding in bambara groundnut once well assembled and annotated.

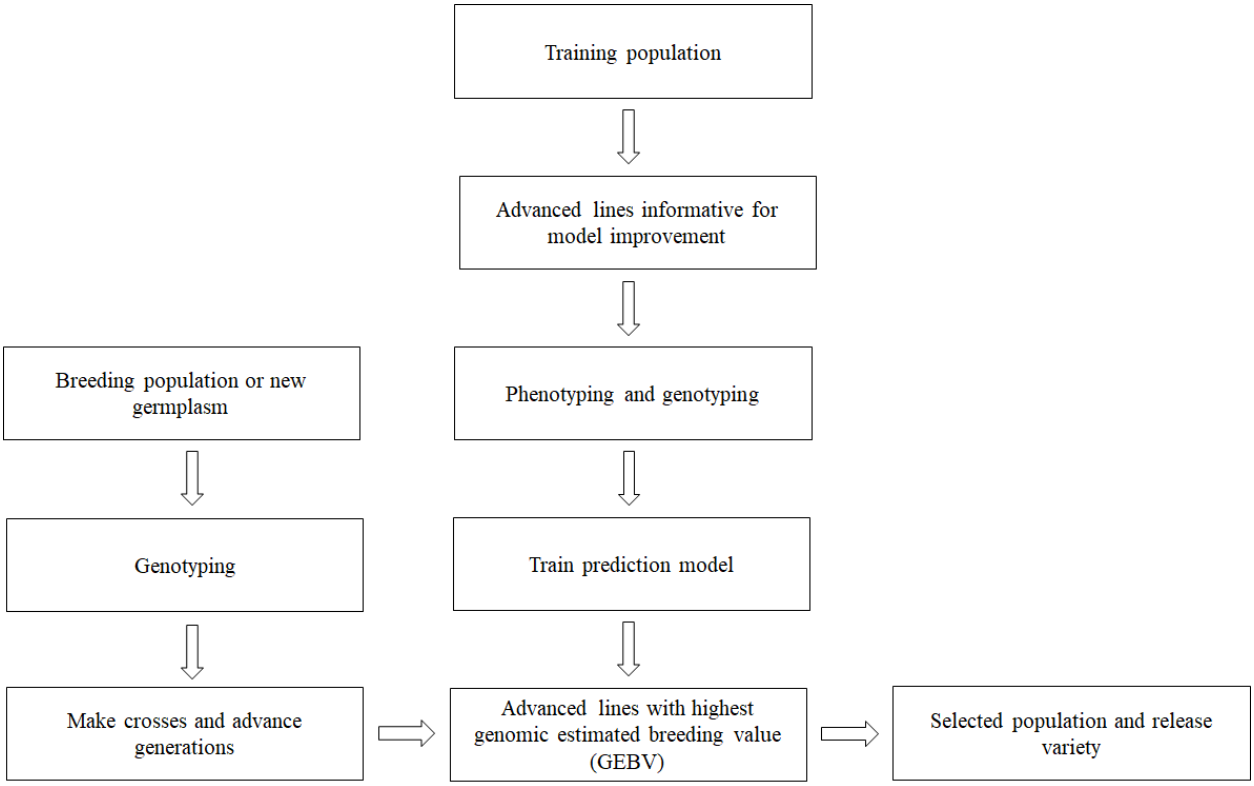


Figure 1-5 The general steps for genomic selection for crop improvement breeding programme (*Adapted from Bhat et al. 2016; Heffner et al. 2009*).

1.8.3 Multi-parent advanced generation inter cross strategy

Typical populations for conventional QTL mapping is bi-parental populations such as the F_2, backcross (BC) or recombinant inbred populations (RILs), in which only two alleles are analyzed and that genetic recombination in these populations is limited (Bandillo et al. 2013). Multi-parent advanced generation intercross (MAGIC) strategy has been proposed by intercrossing over multiple generations before selfing to generate inbred lines to combine all the genomes of founders

(normally 4, 8 or 16 parental lines) in a single line (Cavanagh et al. 2008; Huang and Han 2014). Multi-parent populations combine targeted traits from each of the founders selected to make multi-parent crosses, and the increased recombination in MAGIC populations can lead to genotypic diversity and precision in QTLs detection (Cavanagh et al. 2008; Scott et al. 2020). In addition to the selection of multi-parent founders, MAGIC requires genome sequence data for the founders and resource populations to construct a high-density map for QTLs detection (Pascual et al. 2015).

A MAGIC population for cowpea has been developed from eight diverse founder parents differing in abiotic and biotic stress resistance ability, seed quality and agronomic traits (Huynh et al. 2018). However, this breeding strategy have not been reported in bambara groundnut. Bambara groundnut is a type of automatic self-pollination crop with flower buds ranging in size from ≤ 1 mm (buds) to 9 mm (opened flower) (Bhavya 2018). The artificial cross involved emasculation by opening the flower, pulling out the anthers with a pair of forceps and pollination by putting the pollen from the male parent flower on the stigma of each flower one by one (Massawe et al. 2003), which offers opportunity to conduct MAGIC strategy for bambara groundnut breeding programme.

1.9 Project overview, Aims and Objectives

This project aims to develop structured populations and breeding lines as resources for genetic analysis and trait dissection and variety improvement in bambara groundnut. Twelve genotypes and two F_2 segregating populations, S19-3 × DodR and IITA × Tiga Nicuru crosses, were developed to evaluate genetic variability and phenotypic varation in bambara groundnut. As the informative generation for genetic analysis, the F_2 segregating population derived from S19-3 × DodR was used to construct genetic linkage map. The subsequent F_3 and F_4 segregating generations were then also subjected into drought stress experiment to evaluate phenotypic

variation among the individual lines and to select breedling lines with traits of interest for variety development. QTLs controlling phenotypic traits of interest under drought-stressed and well-watered conditions in the F_3 and F_4 segregation populations were identified and localised prior to identification of candidate genes controlling traits, allowing fundamental understanding of genetic mechanisms of the variation.

The aims/objectives of the study are:

(1) To investigate the phenotypic variations among twelve genotypes of bambara groundnut collected from different geographical locations.

(2) To identify phenotypic variations in two F_2 segregating populations (S19-3 × DodR and IITA × Tiga Nicuru) of bambara groundnut for crop variety development.

(3) To construct genetic linkage maps in the F_2 segregating populations derived from S19-3 × DodR of bambara groundnut.

(4) To assess the effect of water stress on important phenotypic traits in the F_3 and F_4 segregating populations derived from S19-3 × DodR of bambara groundnut

(5) To conduct QTLs analysis for important agronomic traits in response to drought stress in the F_3 and F_4 segregating populations derived from S19-3 × DodR to identify potential genomic regions involved in the regulation of key traits.

1.10 Structure of the Thesis

Chapter 1: Presents a brief introduction and an extensive literature review covering the current food supply challenge, the importance of underutilised crops, plant adaptation mechanisms in response to drought stress. This chapter also introduces bambara groudnut and covers relevant

topics including the development of structured populations, molecular markers and breeding resources mainly used in bambara groundnut. Aims and objectives of the PhD programme and the structure of the thesis are presented in this chapter.

Chapter 2: Reports on the phenotypic variations in twelve bambara groundnut genotypes and two F_2 bi-parental segregating populations (IITA-686 × Tiga Nicuru and S19-3 × DodR). Relationship between morphological traits and yield components, and phenotypic variations in the segregating populations are also presented.

Chapter 3: Reports the effect of drought stress on yield components, morphological traits and physiological traits in the F_3 and F_4 segregating population derived from S19-3 × DodR of bambara groundnut. The genetic variation among lines in the structured population and potential breeding lines with good drought resistance ability and high yield for development of improved varieties are reported in this chapter.

Chapter 4: Presents the genetic linkage mapping, QTLs and qualitative trait analysis for important traits in response to drought stress in the F_3 and F_4 bambara groundnut populations. Major QTLs and potential genes associated with agronomic traits under drought-stressed and well-watered conditions are reported in this chapter.

Chapter 5: Covers the general discussion and conclusions and provides an overview of the present results and implications of the whole project. The impacts of the findings and suggestions for future work are also discussed.

Chapter 2 Variation of phenotypic traits in twelve bambara groundnut (*Vigna subterranea* (L.) Verdc.) genotypes and two F_2 bi-parental segregating populations

2.1 Introduction

Bambara groundnut is an underutilised legume crop, mainly grown by subsistence farmers in Africa (Mayes et al. 2012; Massawe et al. 2016; Atoyebi et al. 2017; Mabhaudhi et al. 2018). Underutilised crops are still grown in their centres of origin or centres of diversity and are adapted to local conditions and marginal environments, and play a significant role in food security, nutrition, income generation and cultural functions for people who grow them (Padulosi and Hoeschle-Zeledon 2004; Jaenicke and Höschle-Zeledon 2006; Massawe et al. 2016).

As with most of the underutilised and neglected crop species which lack established breeding programmes, landraces of bambara groundnut have remained the main source of planting materials used by farmers and the crop is still largely grown as landraces (Basu et al. 2007; Olukolu et al. 2012; Aliyu et al. 2015; Massawe et al. 2016; Mayes et al. 2019). A major crop improvement programme is needed to enhance the genetic potential of bambara groundnut and to ensure sustainability and resilience, along with reasonable yield and quality. Development of improved varieties will bring into the market new materials and desirable traits such as early maturity, high yield and protein content, large pods and fast cooking to boost production and utilization of bambara groundnut (Massawe et al. 2003; Aliyu et al. 2015). Hybridization approaches for bambara groundnut have been reported and optimised (Massawe et al. 2003; Suwanprasert et al. 2006; Kendabie et al. 2015). The development of breeding resources that

contribute towards variety development can also serve as material for genetic studies related to abiotic and biotic stress adaptive mechanisms.

In the present study, twelve genotypes from East, West and Southern Africa and Southeast Asia and two F_2 bi-parental segregating populations generated from four genotypes, IITA-686 × Tiga Nicuru, S19-3 × DodR were characterised. This study specially investigated phenotypic variations among twelve genotypes collected from different geographical locations, the relationship between morphological traits and yield components, and explored phenotypic variations in the segregating populations for potential contribution to development of improved crop varieties.

2.2 Materials and Methods

2.2.1 Plant materials and growing conditions

Twelve genotypes developed through single plant descent (SPD) in bambara groundnut and two F_2 bi-parental segregating populations generated from four genotypes, IITA-686 × Tiga Nicuru (156 individual lines) and S19-3 × DodR (114 individual lines) were evaluated for phenotypic traits in a rainout shelter at the School of Biosciences, the University of Nottingham Malaysia (2°56′46.74″N; 101°52′24.35″E) with mean 31 ± 4°C / 25 ± 1°C day / night air temperature in February to June 2017 (Appendix 2). These twelve genotypes were collected from Africa and Southeast Asia, namely S19-3, Uniswa Red, DipC and AHM from Southern Africa, IITA-686, DodR and TAN385 from East Africa, LunT, Tiga Nicuru, Ankpa-4 and Getso from West Africa, Gresik from Southeast Asia (Table 2-1). Ten replicates of twelve genotypes were arranged in a randomised complete block design (RCBD). The two F_2 bi-parental segregating populations, were planted in separate plots with their parental lines.

The seeds of the twelve genotypes and the two segregating populations were soaked in distilled water at room temperature (approximately 28°C) for one day before sowing. For the trials, a planting distance of 50 cm × 30 cm was established between the rows and between the plants. Fertilizer including 1.86 kg of nitrogen (N), phosphorus (P) and potassium (K) NPK (15:15:15) (133 kg/ha), 0.662 kg of triple superphosphate (TSP) fertilizer (44 kg/ha) and 0.933 kg of muriate of potash (MOP) (67 kg/ha) was applied two weeks after sowing. All the other agronomic procedures, such as watering, weeding and spraying of pesticides were carried out as and when necessary. Soil analysis was carried out by a registered soil science laboratory (Applied Agriculture Resources (AAR), Malaysia). In summary, the soil was found to acidic, sandy clay in texture and low in organic carbon (Table 2-2).

Table 2-1 Geographic origins and distinctive characteristics of twelve bambara groundnut genotypes.

Geographical Origin	Landraces	Collected country	Annual rainfall (mm)	Distinctive characteristics
East Africa	DodR	Tanzania	1000 (Camberlin et al. 2007; Siebert 2014)	Quantitative for short days, high 100-seed weight and yield (Redjeki et al. 2013; Kendabie et al. 2020)
	IITA-686	Tanzania	1000 (Camberlin et al. 2007; Siebert 2014)	Quantitative for long days, shallow and highly branched root growth habit (Kendabie et al. 2020; Mateva et al. 2020)
	TAN385	Tanzania	1000 (Camberlin et al. 2007; Siebert 2014)	-
West Africa	LunT	Sierra Leone	>2000 (Mateva et al. 2020)	Quantitative for short days, shallow and highly branched root growth habit (Kendabie et al. 2020; Mateva et al. 2020)
	Tiga Nicuru	Mali	450 (Camberlin et al. 2007; Jørgensen et al. 2010)	Quantitative for short days, bunchy growth habit, early maturity (Mwale, et al. 2007; Kendabie et al. 2020)
	Ankpa4	Nigeria	>2000 (Siebert 2014; Mateva et al. 2020)	Qualitative for short days (Kendabie et al. 2020)
	Getso	Nigeria	>2000 (Siebert 2014; Mateva et al. 2020)	Quantitative for short days (Kendabie et al. 2020)
Southern Africa	S19-3	Namibia	365 (Jørgensen et al. 2010; Siebert 2014)	Quantitative for long days, early maturity, drought tolerant long taproots and great root length distribution (Mwale et al. 2007; Jørgensen et al. 2010; Kendabie et al. 2020; Mateva et al. 2020;)
	Uniswa red	Kingdom of Eswatini	1390 (Jørgensen et al. 2010)	Quantitative for long days, long growth cycle (Mwale, et al. 2007; Kendabie et al. 2020)
	DipC	Botswana	500 (Mateva et al. 2020)	Quantitative for long days, long taproots and great root length distribution (Kendabie et al. 2015; Mateva et al. 2020)
	AHM	Namibia	365 (Siebert 2014; Jørgensen et al. 2010)	-
Southeast Asia	Gresik	Indonesia	>2000 (Mateva et al. 2020)	Quantitative for short days, shallow and highly branched root growth habit (Kendabie et al. 2020; Mateva et al. 2020)

Table 2-2 Physical and chemical properties of the soil in the rainout shelter of The University of Nottingham Malaysia.

Chemical Properties		Exchangeable cations (cmol/kg)		Physical properties (%)	
pH (H_2O)	5.26	K	0.28	Sand	48
Acid fluoride soluble P (mg/kg)	9.21	Ca	4.25	Silt	11.43
Carbon (%)	0.61	Mg	0.38	Clay	40.57
N (%)	0.08	Cation exchange capacity	4.06	Soil texture	Sandy clay

2.2.2 Traits recorded

Phenotypic traits i.e., *days to flowering, number of leaves per plant, petiole length, internode length, petiole internode ratio, plant height, 100-seed weight, harvest index* and *shelling percentage*, were recorded based on the bambara groundnut descriptor list (IPGRI 2000) (Appendix 1) with minor modification. Measurements included:

Days to flowering, number of leaves per plant, petiole length, internode length and *plant height*.

Petiole internode ratio (P/I) was recorded based on the classification, Bunch type (P/I = > 9); Semi-bunch type (P/I = 7 – 9) and Spreading type (open) (P/I = < 7).

Yield data included *100-seed weight, harvest index* and *shelling percentage* recorded after pods were dried in a high-volume oven (Memmert, Germany) at 40ºC for 14 days.

2.2.3 Data analysis

Normality of trait data was examined using Shapiro-Wilk normality test and data transformation (logarithm base 10) was performed for non-normally distributed trait data for twelve genotypes using 18th edition Genstat Statistical package (18th edition, VSN International, UK). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were carried out, while Pearson's correlation coefficient analysis was conducted to analyse the

relationship between phenotypic traits, Principal components analysis (PCA) was conducted to analyse the relationship between phenotypic trait variations among genotypes and their collected origins using 18th edition of Genstat Statistical package (18th edition, VSN International, UK). Moreover, phenotypic traits of the two F_2 bi-parental segregating populations were subjected to Frequency distribution, Pearson's correlation coefficient test was conducted to analyse the relationship between phenotypic trait and Linear Regression test was used to visualize the relationship between two phenotypic traits using 18th edition Genstat Statistical package (18th edition, VSN International, UK).

2.3 Results

2.3.1 Phenotypic Trait Variation in Twelve Bambara Groundnut Genotypes

Plant height, petiole length, internode length and *100-seed weight* showed normal trait distribution in the twelve genotypes ($p > 0.05$). All phenotypic traits were significantly influenced ($p < 0.01$) by genotypes (Table 2-3). Comparing among twelve genotypes, Tiga Nicuru showed the earliest *days to flowering,* the shortest *plant height*, *petiole length* and *internode length* while IITA-686 showed the fewer *number of leaves per plant* but high *harvest index*. DodR was reported to have long *internode length*, the high *number of leavers per plant* and *100-seed weight*, while S19-3 had the fewer *number of leaves per plant*, but high *harvest index* and *shelling percentage*. Growth habit ranged from the bunch (LunT, Tiga Nicuru and Uniswa Red) to spreading types (DodR) and most of the genotypes classified as semi-bunch types (AHM, Ankpa4, Getso, Gresik, IITA-686, S19-3 and TAN385). The spreading growth habit type showed not only the longest *internode length* and but also the highest *plant height* in comparison to other growth habit types.

Table 2-3 Characterization of phenotypic traits in twelve genotypes of bambara groundnut.

Traits	DTF	NL	PH	PL	IL	P/I	HI	100SW	SP
	(days)		(cm)	(cm)	(cm)	(ratio)		(g)	(%)
AHM	35.50 ^{cd}	532.80 ^{a}	35.20 ^{a}	26.13 ^{ab}	3.76 ^{ab}	7.08 ^{d}	0.09 ^{cd}	21.45 ^{efg}	77.70 ^{a}
Ankpa4	42.40 ^{b}	198.20 ^{b}	33.79 ^{ab}	23.49 ^{abc}	2.86 ^{c}	8.23 ^{cd}	0.04 ^{d}	20.09 ^{fg}	39.58 ^{d}
DipC	38.57 ^{bc}	250.40 ^{b}	34.50 ^{a}	27.04 ^{a}	2.49 ^{cde}	10.89 ^{ab}	0.14 ^{cd}	29.03 ^{cdef}	75.10 ^{a}
DodR	32.10 ^{de}	266.50 ^{b}	34.35 ^{a}	21.60 ^{c}	4.04 ^{a}	5.55 ^{e}	0.28 ^{abc}	45.49 ^{cdef}	78.01 ^{a}
Getso	30.80 ^{e}	82.10 ^{c}	31.05 ^{abc}	15.23 ^{de}	1.83 ^{ef}	8.49 ^{cd}	0.16 ^{bcd}	52.07 ^{a}	62.74 ^{bc}
Gresik	51.19 ^{a}	159.60 ^{b}	31.30 ^{abc}	15.23 ^{de}	2.11 ^{de}	7.20^{d}	0.26 ^{abc}	33.32 ^{cd}	74.54 ^{a}
IITA-686	32.50 ^{de}	66.00 ^{c}	28.15 ^{bcd}	14.79 ^{de}	2.09 ^{de}	7.15 ^{d}	0.38 ^{ab}	24.68 ^{def}	75.73 ^{a}
LunT	33.00 ^{de}	184.00 ^{b}	34.44 ^{a}	25.36 ^{abc}	2.60 ^{cd}	9.65 ^{bc}	0.26 ^{abc}	37.26 ^{bc}	71.53 ^{ab}
S19-3	33.40 ^{de}	84.80 ^{c}	27.33 ^{cd}	17.44 ^{d}	2.46 ^{cde}	7.30 ^{d}	0.47 ^{a}	27.47 ^{cdef}	77.87 ^{a}
TAN385	33.50 ^{de}	768.10 ^{a}	34.50 ^{a}	23.07 ^{bc}	3.15 ^{bc}	7.42 ^{d}	0.03 ^{d}	13.04^{g}	61.74 ^{c}
Tiga Nicuru	27.13 ^{f}	81.10 ^{c}	22.71 ^{d}	12.52 ^{e}	0.96 ^{g}	13.36 ^{a}	0.21 ^{bcd}	24.58 ^{def}	74.08 ^{a}
Uniswa Red	33.44 ^{de}	80.30 ^{c}	28.33 ^{bcd}	14.64 ^{de}	1.13 ^{fg}	13.24 ^{a}	0.18 ^{bcd}	31.05 ^{cde}	73.21 ^{a}
Mean	35.30	229.42	31.31	19.71	2.46	8.80	0.21	29.95	70.15
F pr	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: DTF days to flowering, NL number of leaves per plant, PH plant height, PL petiole length, IL internode length, P/I petiole internode ratio, HI harvest index, 100SW 100-seed weight, SP shelling percentage. Different letters indicate significant difference at $p < 0.05$ level (Tukey's multiple comparison test), mean values ($n = 10$), F pr = F-probability ($p < 0.01$).

Number of leaves per plant ($r = 0.64$, $p < 0.05$), *plant height* ($r = 0.79$, $p < 0.01$) and *petiole length* ($r = 0.74$, $p < 0.01$) showed a strong linear relationship with *internode length* (Table 2-4). Both *number of leaves per plant* ($r = 0.61$, $p < 0.05$) and *plant height* ($r = 0.86$, $p < 0.01$) showed a strong linear relationship with *petiole length*. The negative correlation was observed between *harvest index* and *number of leaves per plant* ($r = -0.60$, $p < 0.05$). Yield related traits, namely *100-seed weight, harvest index, shelling percentage* showed a negative correlation with the key vegetative growth indices, *number of leaves per plant, plant height, petiole length, internode length* and *petiole internode ratio* ($r < 0.04$). A positive correlation between *harvest index* and *shelling percentage* ($r = 0.59$, $p < 0.05$) was observed.

Table 2-4 The correlation coefficient analysis of phenotypic traits in twelve genotypes of bambara groundnut.

Traits	DTF	NL	PH	PL	IL	P/I	HI	100SW	SP
Days to flowering (DTF)	–	–	–	–	–	–	–	–	–
Number of leaves per plant (NL)	0.05	–	–	–	–	–	–	–	–
Plant height (PH)	0.37	0.62 *	–	–	–	–	–	–	–
Petiole length (PL)	0.17	0.61 *	0.86 **	–	–	–	–	–	–
Internode length (IL)	0.15	0.64 *	0.79 **	0.74 **	–	–	–	–	–
Petiole internode ratio (P/I)	−0.28	−0.35	−0.50	−0.27	−0.77 **	–	–	–	–
Harvest index (HI)	−0.15	−0.60 *	−0.49	−0.45	−0.18	−0.18	–	–	–
100-seed weight (100SW)	−0.13	−0.50	0.04	−0.21	−0.07	−0.07	0.31	–	–
Shelling percentage (SP)	−0.23	−0.12	−0.25	−0.18	−0.04	0.03	0.59 *	0.22	–

*Note: * = Significant at (p = 0.05), ** = Significant at (p = 0.01). Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation: 0.70-0.90, 0.50-0.70, −0.60-−0.80, −0.40-−0.60.*

2.3.2 Principal Components Analysis for Twelve Genotypes Based on Phenotypic Traits

A principal component analysis (PCA) was carried out in order to investigate whether the trait variation observed among genotypes was influenced by the geographical locations where these genotypes were originally collected from. The first two principal components (PC), PC1 and PC2, accounted for 97.33% and 2.48% of the variation, respectively, with a cumulative variation of 99.81% (Figure 2-1). PC1 was associated with four genotypes collected from East and Southern Africa, i.e., TAN385 and DodR from East Africa and AHM and DipC from Southern Africa. The genotype TAN385 contributed 73% of the variation in the PC1. PC2 was associated with five genotypes from East, West and Southern Africa, i.e., IITA-686 from East Africa, Tiga Nicuru and Getso from West Africa, S19-3 and Uniswa Red from Southern Africa.

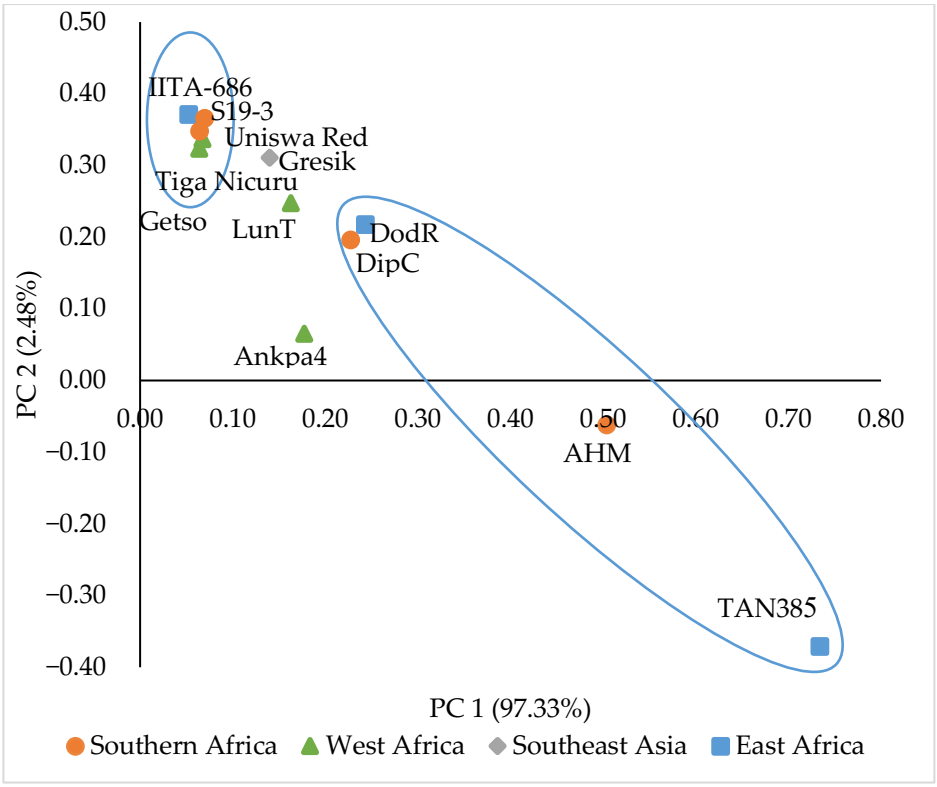


Figure 2-1 Principal component analysis (PCA) graph and loading scores for each component (PC1 and PC2) from latent vectors (loading), performed with Genstat Statistical package (18th edition, VSN International, Hemel Hempstead, UK) using phenotypic traits data of twelve genotypes. Data was coloured based on geographical collection origins.

2.3.3 Phenotypic variations in the F_2 bi-Parental Segregating Populations

Plant height, petiole length, 100-seed weight and *harvest index* showed the normal trait distribution in the F_2 bi-parental segregating population, IITA-686 × Tiga Nicuru and S19-3 × DodR ($p > 0.01$) (Tables 2-5 and 2-6). Transgressive segregation for traits was observed in the F_2 bi-parental segregating populations, IITA-686 × Tiga Nicuru and S19-3 × DodR (Figures 2-2 and 2-3). For example, the F_2 individual lines derived from IITA-686 × Tiga Nicuru had a *petiole length* that ranged from 11.83 cm (Line-66) to 30.67 cm (Line-125) whereas the *petiole length* in IITA-686 ranged from 10.10 cm to 19.75 cm (IITA-686 mean, 14.79 ± 0.87 cm; s.d. 2.73; $n = 10$) and Tiga Nicuru ranged from 10.60 cm to 15.57 cm (Tiga Nicuru mean, 12.52 ± 0.53 cm; s.d. 1.51, $n = 10$) (Table 2-5 and Figure 2-2). The F_2 individual lines derived from

S19-3 × DodR had a *100-seed weight* that ranged from 10.79 g (Line-29) to 41.75 g (Line-96) whereas the *100-seed weight* in S19-3 ranged from 16.70 g to 39.58 g (S19-3 mean, 27.47 ± 2.95 g; s.d. 7.81; *n* = 10) and DodR ranged from 37.88 g to 52.70 g (DodR mean, 45.49 ± 1.57 g; s.d. 4.96, *n* = 10) (Table 2-6 and Figure 2-3).

Table 2-5 Summary phenotypic traits of the F_2 bi-parental segregating population derived from IITA-686 × Tiga Nicuru and their parental genotypes.

							IITA-686		Tiga Nicuru	
Traits	**Mean**	**Min**	**Max**	**SD**	**Variance**	**Normality**	**Min**	**Max**	**Min**	**Max**
Days to flowering	34.98	31.00	61.00	3.54	12.51	**	29.00	34.00	27.00	28.00
Number of leaves per plant	87.24	17.00	297.00	42.56	1811.00	**	28.00	114.00	43.00	116.00
Plant height (cm)	26.18	16.00	44.00	4.69	22.01	*	18.00	34.50	19.50	27.00
Petiole length (cm)	19.72	11.83	30.67	3.50	12.23	ns	10.10	19.75	10.60	15.57
Internode length (cm)	2.03	0.85	4.90	0.69	0.48	**	1.53	2.75	0.73	1.13
Petiole internode ratio	11.22	5.21	22.90	3.24	10.48	ns	5.52	8.85	9.71	16.91
Harvest index	0.24	0.01	0.46	0.11	0.01	ns	0.06	0.76	0.06	0.36
100-seed weight (g)	21.85	4.69	45.56	7.70	59.31	ns	15.67	29.50	19.90	35.21

*Note: * = Significant at (p = 0.05), ** = Significant at (p = 0.01). ns, not significant, SD, standard deviation.*

Table 2-6 Summary phenotypic traits of the F_2 bi-parental segregating population derived from S19-3 × DodR and their parental genotypes.

Traits	Mean	Min	Max	SD	Variance	Normality	S19-3		DodR	
							Min	Max	Min	Max
Days to flowering	36.41	31.00	42.00	2.81	7.90	**	29.00	36.00	29.00	36.00
Number of leaves per plant	77.67	15.00	325.00	50.61	2561.00	**	6.00	133.00	95.00	396.00
Plant height (cm)	28.34	13.30	46.50	5.66	32.02	*	18.30	33.00	27.00	44.00
Petiole length (cm)	16.20	10.83	23.33	2.54	6.43	ns	7.17	24.50	18.10	24.50
Internode length (cm)	2.48	0.73	4.50	0.77	0.59	ns	1.17	3.55	3.00	5.00
Petiole internode ratio	7.09	3.89	16.36	2.15	4.64	**	5.63	9.37	3.81	7.50
Harvest index	0.40	0.16	0.56	0.10	0.01	ns	0.32	0.85	0.16	0.40
100-seed weight (g)	28.72	10.79	41.75	6.40	40.91	ns	16.70	39.58	37.88	52.70

*Note: * = Significant at (p = 0.05), ** = Significant at (p = 0.01). ns, not significant, SD, standard deviation.*

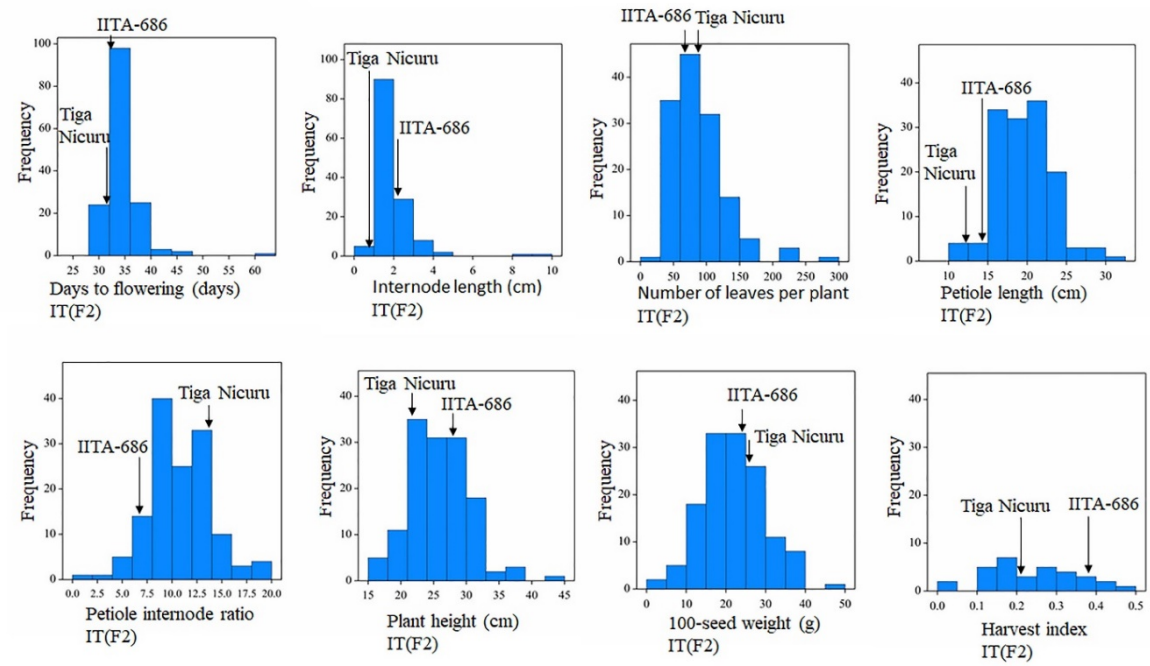


Figure 2-2 The frequency distribution of phenotypic traits in the F_2 bi-parental segregating population, IITA-686 × Tiga Nicuru and their parental lines. IT (F2), F_2 individual lines derived from IITA-686 × Tiga Nicuru.

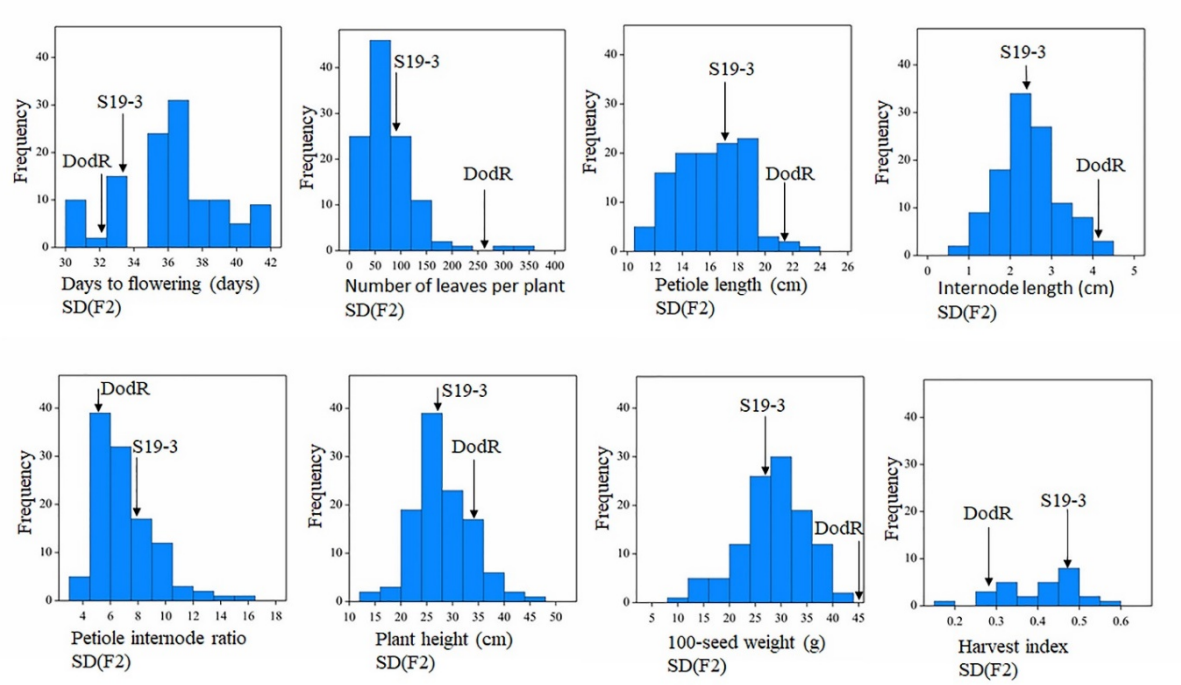


Figure 2-3 The frequency distribution of phenotypic traits in the F2 bi-parental segregating population, S19-3 × DodR and their parental lines. SD (F2), F2 individual lines derived from S19-3 × DodR.

2.3.4 Correlation Coefficient Analysis of Phenotypic Traits in the F_2 bi-Parental Segregating Populations

Number of leaves per plant showed positive correlation with *plant height* and *internode length* in the two F_2 bi-parental segregating populations, IITA-686 × Tiga Nicuru ($r = 0.54$, $p < 0.01$; $r = 0.53$, $p < 0.01$) and S19-3 × DodR ($r = 0.62$, $p < 0.01$; $r = 0.51$, $p < 0.01$) (Tables 2-7 and 2-8). *Harvest index* and *100-seed weight* showed strong positive linear relationship ($r = 0.81$, $p < 0.05$) in the F_2 segregating population derived from IITA-686 × Tiga Nicuru (Table 2-7, Figure 2-4A), and weak correlation ($r = 0.40$, $p < 0.05$) in the F_2 segregating population derived from S19-3 × DodR (Table 2-8).

Harvest index showed a negative correlation with the key vegetative growth indices, *number of leaves per plant* ($r = -0.50$, $p < 0.01$), *plant height* ($r = -0.70$, $p < 0.01$), *petiole length* ($r = -0.49$, $p < 0.01$), *internode length* ($r = -0.69$, $p < 0.01$) and positive correlation with *petiole*

internode ratio (r = 0.44, p < 0.05) (Table 2-8). The negative linear relationships between *harvest index* and both *internode length* and *plant height* were observed in the F_2 segregating population derived from S19-3 × DodR (Figure 2-4B, C).

Table 2-7 Correlation coefficient analysis of phenotypic traits in the F_2 bi-parental segregating population derived from IITA-686 × Tiga Nicuru.

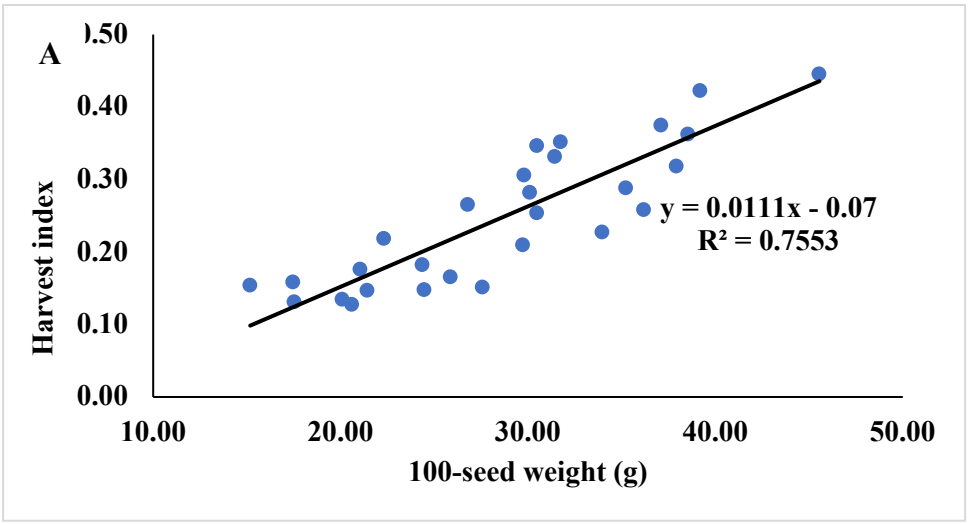
Traits	DTF	NL	PH	PL	IL	P/I	HI	100SW
Days to flowering (DTF)	–	–	–	–	–	–	–	–
Number of leaves per plant (NL)	−0.19	–	–	–	–	–	–	–
Plant height (PH)	0.33	0.54**	–	–	–	–	–	–
Petiole length (PL)	0.19	0.53**	0.83**	–	–	–	–	–
Internode length (IL)	0.10	0.53**	0.30	0.48**	–	–	–	–
Petiole internode ratio (P/I)	−0.10	−0.26	−0.09	−0.20	−0.89**	–	–	–
Harvest index (HI)	−0.10	−0.17	−0.26	−0.33	−0.31	0.21	–	–
100–seed weight (100SW)	0.10	−0.21	−0.15	−0.26	−0.37*	0.25	0.81*	–

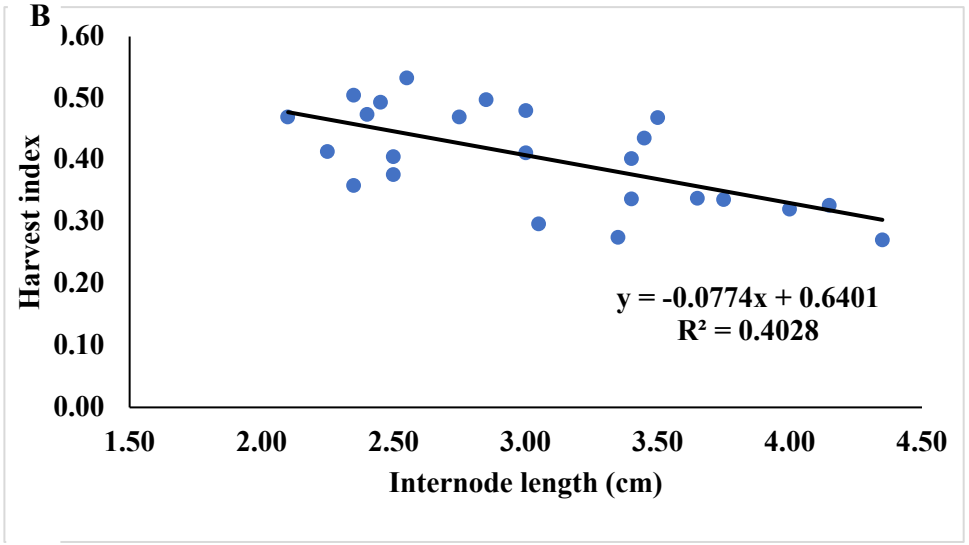
*Note: * = Significant at (p = 0.05), ** = Significant at (p = 0.01). Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation: 0.70-0.90, 0.40-0.70, −0.60–−0.90, −0.30–−0.60.*

Table 2-8 Correlation coefficient analysis of phenotypic traits in the F_2 bi-parental segregating population derived from S19-3 × DodR.

Traits	DTF	NL	PH	PL	IL	P/I	HI	100SW
Days to flowering (DTF)	–	–	–	–	–	–	–	–
Number of leaves per plant (NL)	−0.01	–	–	–	–	–	–	–
Plant height (PH)	−0.18	0.62**	–	–	–	–	–	–
Petiole length (PL)	−0.28	0.36	0.53**	–	–	–	–	–
Internode length (IL)	0.05	0.51**	0.63**	0.38	–	–	–	–
Petiole internode ratio (P/I)	−0.18	−0.31	−0.38	0.12	−0.85**	–	–	–
Harvest index (HI)	0.10	−0.56**	−0.70**	−0.49**	−0.69**	0.44*	–	–
100–seed weight (100SW)	0.01	−0.27	−0.24	−0.09	−0.08	0.01	0.40*	–

*Note: * = Significant at (p = 0.05), ** = Significant at (p = 0.01). Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation: 0.50-0.70, 0.40-0.50, −0.70–−0.90, −0.40–−0.70.*





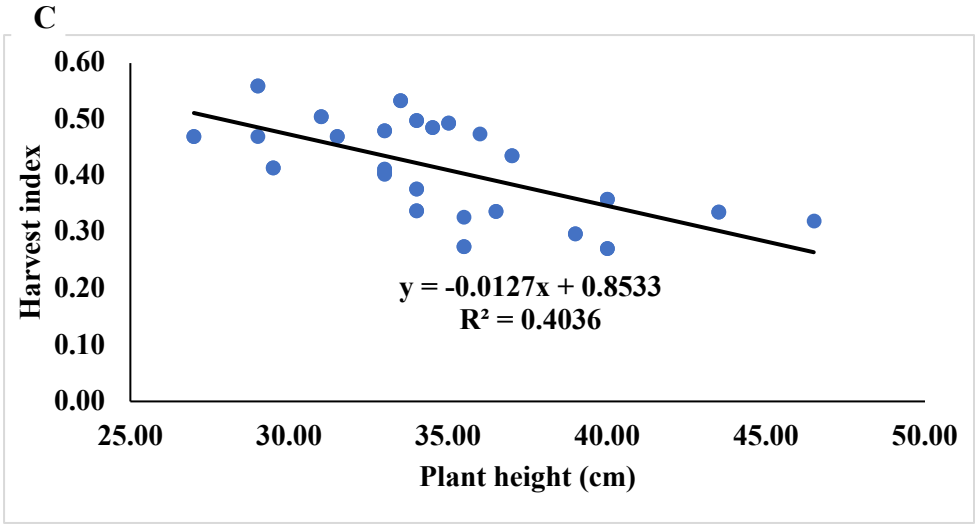


Figure 2-4 Regression for (**A**), *harvest index* and *100-seed weight* (g) in the F_2 bi-parental segregating population derived from IITA-686 × Tiga Nirucru; (**B**), *harvest index* and *internode length* (cm) and (**C**), *harvest index* and *plant height* (cm) and in the F_2 bi-parental segregating population derived from S19-3 × DodR.

2.4 Discussion

The significance of SPD in the context of deploying various short-to-medium term variety of development strategies within bambara groundnut breeding programmes have been highlighted (Massawe et al. 2003; Aliyu et al. 2015; Molosiwa et al. 2015). High inbreeding coefficient and heterozygosity (Ho) below 5% observed in 119 landrace-derived genotypes (through SPD) of bambara groundnut studied by Molosiwa et al. (2015) indicates that these cleistogamous landraces are likely to be composed of a series of inbred lines.

In the present study, PCA showed a total of 99.81% of the variation across the twelve genotypes based on *days to flowering, number of leaves per plant, petiole length, internode length, petiole internode ratio, plant height, 100-seed weight, harvest index* and *shelling percentage*, and the distribution was suggested to be related to geographic origins (Figure 2-1). PC1 was associated with high loadings in genotypes collected from moderate annual rainfall areas (600-1000 mm annual rainfall) and semi-arid areas (200-600 mm annual rainfall) in Africa, including TAN385 and DodR from Tanzania, East Africa with 1000 mm mean annual rainfall, and AHM from Namibia and DipC from Botswana, Southern Africa with less than 600 mm mean annual rainfall (Camberlin et al. 2007; Siebert 2014). PC2 was associated with high loadings in genotypes from different origins with semi-arid areas, moderate and high (above 1000 mm annual rainfall) annual rainfall in East, West and Southern Africa. Tiga Nicuru was collected from Mali, West Africa and S19-3 was collected from Namibia, Southern Africa with less than 450 mm mean rainfall per year (Jørgensen et al. 2010; Siebert 2014). IITA-686 was collected from Tanzania, East Africa with 1000 mm mean annual rainfall (Camberlin et al. 2007; Siebert 2014). Getso collected from northern Nigeria, West Africa with more than 2000 mm mean annual rainfall (Siebert 2014; Mateva et al. 2020). Uniswa Red was collected from the Kingdom of Eswatini, Southern Africa with 1390 mm mean annual rainfall (Jørgensen et al.

2010). Furthermore, the high genotypic variability between landraces of bambara groundnut allows breeders to select parents for the controlled crossing to develop and release new improved varieties with desirable traits. S19-3, TAN385, ZAM696, AHM753 and BOTS1 were recommended as the best performing genotypes with good yield component traits in Botswana (Karikari 2004; Molosiwa et al. 2015). Tiga Nicuru and S19-3 are likely to avoid terminal drought stress by early maturity or reduced respiration and stomata closure at a comparatively lower water threshold coupled with fast phenological development (Massawe et al. 2005; Mwale et al. 2007; Pedercini et al. 2012; Chai et al. 2016) and longer tap roots, as well as greater root length distribution in deeper (60–90cm) soil depths (Mateva et al. 2020). The variation of genotypes provides opportunities to develop ideotypes with drought-tolerant, high yield, short life cycle or other favourable traits in breeding programmes of bambara groundnut.

In the present study, the negative correlation between the morphological traits, i.e., *number of leaves per plant* (NL), *petiole length* (PL), *internode length* (IL), *petiole internode ratio* (P/I), *plant height* (PH) and yield-related traits, i.e., *100-seed weight* (100SW), *harvest index* (HI) and *shelling percentage* (SP), would suggest that fewer leaves, reduced PL and IL and shorter plants could lead to high 100SW and HI. Similar findings have been reported in pea (*Pisum sativum* L.) that the increased generative shoots and fruiting nodes had a negative impact on the harvest index (Klimek-Kopyra et al. 2018). Furthermore, plant height was negatively correlated with a key yield component, number of tillers in a bushy rice mutant (Xing and Zhang 2010). Flowering time, typically after the vegetative stage, is a decisive trait for yield improvement in crop plants under different environmental conditions (Mathan et al. 2016). Mabhaudhi et al. (2013) reported that early flowering to escape drought stress can lead to early maturity but has a yield penalty (reduced seed yield) in bambara groundnut. However, no

significant correlation between flowering time and other phenotypic traits was observed in the twelve genotypes and two F_2 segregating populations in the present study. From the present study and previous report (Ntundu et al. 2006), the semi-bunch growth type was the most common growth habit type (58.3%), followed by the bunch (33.3%) and spreading (8%) (Table 2-3). The significantly negative correlation between *internode length* and *petiole internode ratio* in the present study, suggests *internode length* is the most critical trait to determine the plant growth habit type in bambara groundnut, similar to Basu et al. (2007).

The two sets of parental genotypes in the present study had contrasting traits and was selected for the controlled crossing to develop two segregating populations for the selection of breeding lines for variety development and to act as mapping populations for genetic studies. Results showed high variability and transgressive segregation in the F_2 bi-parental segregating lines (Chai et al. 2016). Transgressive segregation identified for trait values including *days to flowering, number of leaves per plant, petiole length, internode length, plant height, petiole internode ratio, 100-seed weight, harvest index*, and *shelling percentage* in the two F_2 bi-parental segregating populations, provides an opportunity for selection of superior individuals for breeding purposes. The significant and negative correlation between *harvest index, plant height* and *internode length* in the F_2 segregating population, S19-3 × DodR (Table 2-8), which was also confirmed by regression analysis (Figure 2-4B, C), suggested the possibility of developing individual lines with high yield through a selection of target genotypes with short internode or height for breeding improvement. For example, some individual lines, i.e., 6, 38, 44, 48, 50 in the F_2 bi-parental segregating population, IITA-686 × Tiga Nicuru and 51, 73, 86, 108, 111 in the F_2 bi-parental segregating population, S19-3 × DodR, showed combined desirable traits, such as earlier flowering, higher *harvest index, 100-seed weight*, shorter *plant height* and *internode length* than average of the population and are recommended for further

field investigation to develop improved varieties (Appendix 4 and 5). However, it is worth taking note that the final yield is rather complex due to the possible interaction between genetic and environmental factors, which could contribute to the high variability observed between genotypes and within segregating lines (Chai et al. 2016; Karikari 2004; Mickelbart et al. 2015). As offspring segregate for agronomically important traits, breeders can select target lines that are adapted to the target environments based on their breeding and selection plan. In addition to selection of lines, the development of structured populations and breeding lines provide resources for genetic analysis and trait dissection, i.e., genetic mapping and identification of regions of the genome correlated with phenotypic traits.

2.5 Conclusions

The present study provides initial results from the two F_2 structured populations and clears a path to develop the first-ever advanced structured populations and improved varieties of bambara groundnut. The variation within twelve genotypes of bambara groundnut provides a breeding resource pool for use in controlled crossing to develop ideotypes with desirable phenotypic traits, i.e., high *harvest index, 100-seed weight,* early *days to flowering* or short life cycle. Two F_2 bi-parental segregating populations of bambara groundnut derived from different geographical origins, IITA-686 (high *harvest index*, collected from a moderate annual rainfall area, Tanzania, East Africa) × Tiga Nicuru (early *days to flowering*, collected from semi-arid area, Mali, West Africa) and S19-3 (high *harvest index*, collected from semi-arid area, Namibia, Southern Africa,) × DodR (high *100-seed weight*, collected from moderate annual rainfall area, Tanzania, East Africa), were developed to obtain structured populations and breeding lines for genetic analysis and trait dissection. The negative correlation between the morphological traits, i.e., *number of leaves per plant, petiole length, internode length, petiole internode ratio* and *plant height* and yield-related traits, i.e., *100-seed weight, harvest index* and *shelling*

percentage, would suggest a competition for assimilates between vegetative development and yield accumulation. Therefore, a balanced development of vegetative growth and yield accumulation is a critical strategy to obtain improved varieties for breeding programme. Individual lines in the segregating populations with higher *harvest index* or *100-seed weight*, earlier flowering and shorter *plant height* and *internode length* could be selected as potential high yield genotypes for improved variety development. Further studies would focus on advanced generations to investigate the correlation between morphological traits, yield-related traits and final yield and to identify potential genomic regions involved in the regulation of key agronomic traits in bambara groundnut.

This chapter has been published - please see:

Gao X, Bamba ASA, Kundy AC, Mateva KI, Chai HH, Ho WK, Musa M, Mayes S, Massawe F. Variation of phenotypic traits in twelve bambara groundnut (*Vigna subterranea* (L.) Verdc.) genotypes and two F_2 bi-parental segregating populations. *Agronomy* 2020. 10, 1451; doi:10.3390/agronomy10101451

Chapter 3 Evaluation of the F_3 and F_4 bambara groundnut (*Vigna subterranea* (L.) Verdc) segregating populations under drought stress

3.1 Introduction

Drought is one of the major abiotic stresses negatively impacting plant growth and crop yield worldwide (Zhu 2011; Farooq et al. 2017). Studies on chickpea have shown yield losses of up to 50% due to drought stress (Varshney et al. 2014; Purushothaman et al. 2016) and a reduction of canopy temperature, canopy biomass accumulation, photosynthate remobilization, pod production, and 50% – 55% of grain yield losses have also been reported in 24 common bean genotypes under water deficit conditions (Polania et al. 2016). Plant roots play a vital role in the absorption of water and nutrients to support plant growth, and adaptation to drought conditions (Polania et al. 2017).

Bambara groundnut is an underutilised and drought-resistant leguminous crop. Landraces with different drought response abilities provide resources for breeders to select and breed for drought resistance varieties with high yield in bambara groundnut (Jørgensen et al. 2010; Mabhaudhi et al. 2013; Nautiyal et al. 2017). Similar to most of the underutilised and neglected crop species which have limited established breeding programmes, bambara groundnut is far behind in terms of effective screening techniques, genetic improvement activities and commercial interest in breeding varieties and landraces (a mixture of genotypes) have remained as the main source of planting material (Massawe et al. 2005; Olukolu et al. 2012; Mayes et al. 2018; Muhammad et al. 2020). Development and assessment of segregating populations for their ability to withstand drought stress conditions is one of the approaches to develop and select drought resistant varieties for drought prone semi-arid regions.

Golabadi et al. (2006) reported that it is possible to obtain drought tolerant lines by developing the F_3 and F_4 segregating population of durum wheat derived from a cross between drought tolerant genotype, Oste-Gata and susceptible genotype, Massara-1. The results showed genetic diversity of lines and differential response of genotypes for drought tolerance, drought susceptibility index, mean productivity and geometric mean productivity when plants in the segregating populations were subjected to water deficit (Golabadi et al. 2006).

S19-3 is considered as a drought resistance landrace (Jørgensen et al. 2010) and exhibits reduced transpiration and stomata closure at a comparatively low water threshold coupled with fast phenological development and short life cycle/early maturing (Massawe et al. 2005; Mwale et al. 2007; Jørgensen et al. 2010). On the other hand, landrace DodR is reported to have higher *100-seed weight* and yield compared to other six African landraces including LunT, AHM753, SB165A, S19-3, DIPC and Uniswa Red, but showed unstably performance in three experimental sites for *50% flowering, days to maturity, pod number per plant* and *100-seed weight* (Redjeki et al. 2013). Landraces, DodR, S19-3, Uniswa Red and SB4-2 exhibited drought tolerance by a smaller reduction in biomass, higher pod weight and proline accumulation compared to other landraces, including S165A, AHM 753, DodC, AS 17, DIPC and GabC under water-deficit, while S19-3 was classified as water saving and DodR was classified as water spending (Nautiyal et al. 2017). This study was carried out to evaluate the effect of drought stress on yield components, morphological and physiological traits in the the F_3 and F_4 segregating populations derived from S19-3 × DodR. Genetic variation among lines in the structured population, not only offer opportunities to select superior and drought resistant lines for improved varieties, but also provides a better understanding of mechanisms involved in drought resistance.

3.2 Plant material and Experimental design

3.2.1 Plant material and experimental design in the F_3 and F_4 segregating populations

A total of 114 individual lines in the F_3 segregating population derived from a cross between S19-3 and DodR were evaluated in a rainout shelter at the University of Nottingham Malaysia (2°56'46.74"N; 101°52'24.35"E) with a mean air temperature of 29°C/24°C day/night and relative humidity of 75%/95% day/night from November 2018 to February 2019. This was followed by the evaluation of 114 individual lines in the F_4 segregating population derived from the same cross in the rainout shelter with a mean air temperature of 36°C/25°C day/night and relative humidity of 58%/91% day/night from April to July 2019, respectively.

Both experiments were carried out in a completely randomized design (CRD) with three replicates and two treatments (drought-stressed and well-watered treatments). Each of the replicates was represented by one plant from each of the individual lines. Drought treatment was imposed on drought treatment plots after 100% flowering was observed at 47 days after sowing (DAS) until early pod-filling stage at 74 DAS. Irrigation of plants was resumed at 74 DAS.

3.2.2 A subset of measurements in the F_4 segregating population

In the F_4 segregating population, a total of 114 individual lines were clustered into four groups using a "Non-hierarchical Cluster Analysis" (Genstat Statistical package (18th edition, VSN International, UK) namely group 1 (25 lines), group 2 (27 lines), group 3 (33 lines), group 4 (29 lines), according to the variation observed in *harvest index* and *100-seed weight* under well-watered conditions in the F_3 segregating population (Table 3-1). A sample of nine individual lines from each of the cluster groups (total of n = 36 individual lines) (Table 3-2) were selected as a subset for taking detailed measurements of photosynthetic parameters, including *leaf*

relative water content, quantum yield, leaf area, photosynthesis rate, stomatal conductance, transpiration rate and *intracellular CO_2* in response to drought stress. For yield components, all of the 114 individual lines were subjected to trait measurements.

Table 3-1 Four groups consisting of a total of 114 individual lines were clustered according to *harvest index* and *100-seed weight* under well-watered conditions in the F_3 segregating population.

Group	No. of lines	*Harvest index*	*100-seed weight*
1	25	0.29	20.43
2	27	0.35	26.32
3	33	0.37	31.13
4	29	0.41	35.41

Table 3-2 A sample of nine individual lines from each of the cluster group were selected as a subset in the F_4 segregating population.

No.	Group	Line	*Harvest index*	*100-seed weight*
1	1	5	0.27	19.75
2	1	7	0.33	22.47
3	1	10	0.29	20.77
4	1	17	0.13	16.30
5	1	18	0.20	20.64
6	1	27	0.30	20.42
7	1	29	0.47	18.11
8	1	31	0.17	23.01
9	1	47	0.35	19.57
1	2	2	0.39	28.20
2	2	3	0.30	27.44
3	2	8	0.34	25.26
4	2	13	0.25	26.37
5	2	14	0.46	26.60
6	2	15	0.32	26.03
7	2	19	0.34	28.02
8	2	23	0.47	26.95
9	2	24	0.35	25.80
1	3	4	0.35	32.60
2	3	6	0.36	32.85
3	3	9	0.30	32.36
4	3	20	0.39	30.47
5	3	21	0.39	32.40
6	3	22	0.35	32.62
7	3	25	0.28	32.47
8	3	30	0.29	29.52
9	3	33	0.41	31.54
1	4	1	0.40	35.08
2	4	11	0.37	36.87
3	4	12	0.40	35.80
4	4	26	0.41	35.55
5	4	28	0.34	36.36
6	4	32	0.49	36.25
7	4	34	0.41	36.01
8	4	37	0.36	43.69
9	4	40	0.36	35.40

3.2.3 Field management

Trickle irrigation system was set to irrigate the plants at 0700 and 1900h for 10 minutes with a flow rate of 2 L/hr. A planting distance of 40 cm × 30 cm was implemented, and a mixture of

single and compound fertilizer was applied at a rate of N:P:K 20:40:60 kg/ha, i.e., 133 kg/ha of nitrogen (N), phosphorus (P) and potassium (K) NPK (15:15:15), 44 kg/ha of triple superphosphate (TSP) fertilizer and 67 kg/ha of muriate of potash (MOP) as a basal application before seed sowing and after emergence. All other agronomic procedures, such as weeding and spraying of pesticides were carried out when necessary.

3.2.4 Soil moisture content

Two evenly spaced PR2 profile access tubes (Delta-T Devices Ltd., Cambridge, UK) were inserted into the centre of each of the treatment plots. There were 12 access tubes in total. Three PR2 readings %Vol (volumetric water content as a percentage) were taken three times a week between 0900 and 1100h at soil depth of 100, 200, 300, 400, 600 and 1000 mm from seed sowing until maturity.

3.2.5 Chlorophyll content index (CCI)

Leaf chlorophyll content index was estimated using the chlorophyll meter SPAD-502 (Spectrum Technologies, Inc., Aurora, Illinois, USA). Three readings were taken per leaf and averaged to give a final reading.

Readings were taken on the middle leaflet of one most fully expanded leaf between 0800 and 1200 h for all 114 individual lines in the F_3 segregating population before drought treatment was imposed at 46 DAS and during drought period at 60 and 74 DAS. For 114 individual lines in the F_4 segregating population, readings were taken before drought treatment was imposed at 46 DAS, during drought period at 57, 64 and 71 DAS, and after irrigation was resumed, at 78 and 86 DAS.

3.2.6 Leaf relative water content (RWC)

Leaf relative water content (RWC) was calculated as:

$$RWC = [(Fw – Dw) / (Tw – Dw)] \times 100$$

where FW = fresh weight of leaves, TW = turgid weight of leaves after incubating leaves in distilled water for 24 h, and DW = dry weight of leaves after oven drying at 80 °C for 48 h.

The middle leaflet of one most fully expanded leaf per plant was harvested between 0800 and 1200 h for all 114 individual lines in the F3 segregating population, before the drought treatment was imposed at 46 DAS and during drought period at 60 and 74 DAS. In the F4 segregating population, the middle leaflet was harvested for a subset of 36 individual lines before drought treatment was imposed at 46 DAS, during drought period at 57, 64 and 71 DAS, and after irrigation was resumed, at 78 and 86 DAS.

3.2.7 Quantum yield PSII photochemistry (F_V/F_M)

Quantum yield was estimated from dark-adapted leaves for 30 minutes using FlourPen FP 100 (PSI, CZ, Czech Republic). Photosystem II quantum yield is equivalent to ratio of variable fluorescence/maximal fluorescence (F_V/F_M) in dark-adapted samples.

Readings were taken on the middle leaflet of one most fully expanded leaf between 0800 and 1200 h for all 114 individual lines in the F_3 segregating population, before drought treatment was imposed at 46 DAS and during drought period at 60 and 74 DAS. In the F_4 segregating population, the readings were taken on the middle leaflet for a subset of 36 individual lines before drought treatment was imposed at 46 DAS, during drought period at 57, 64 and 71 DAS, and after irrigation was resumed, at 78 and 86 DAS.

3.2.8 Yield components and morphological traits

The standard descriptor for bambara groundnut (IPGRI 2000) was used as a guide for all data collection in the F_3 and F_4 segregating populations. They were *days to flowering, number of leaves per plant, petiole length, internode length, petiole-internode ratio, plant height, shoot dry weight, number of seeds per plant, number of pods per plant, pod weight per plant, seed weight per plant, harvest index, 100-seed weight* and *shelling percentage* (Appendix 1).

3.2.9 Photosynthetic parameters in the F_4 segregating population

Photosynthetic parameters, i.e., *photosynthesis rate* (*A*), *stomatal conductance* (*gs*), *intracellular* CO_2 (*Ci*) and *transpiration rate* (*E*) were measured using LI-6400XT Portable Photosynthesis System (Li-Cor, Lincoln, USA). Readings were taken on the middle leaflet of one most fully expanded leaf between 0800 and 1200 h in a subset of 36 individual lines in the F_4 segregating population, starting from 50% flowering observed at 46 DAS before the drought treatment was imposed, during drought period at 57, 64 and 71 DAS, and after irrigation was resumed, at 78 and 86 DAS.

3.2.10 Seed size in the F_4 segregating population

Seeds were analyzed from scanned images using WinSEEDLE™ Pro software (Regent Instruments Inc., Quebec, Canada) for all harvested seeds of 114 individual lines under drought stress and well-watered treatment in the F_4 segregating population. The following seed traits were measured: *seed length* (mm), *seed width* (mm), *seed width-length ratio* and *seed perimeter* (mm).

3.2.11 Leaf area in the F_4 segregating population

Leaf area was measured at 71 DAS for a subset of 36 individual lines in the F_4 segregating population. The pictures of three leaflets per line were captured, calibrated and measured using

Image J (64-bit) software (http://imagej.nih.gov/ij/) (LOCI, University of Wisconsin, USA). The individual readings were then averaged to give a final reading.

3.2.12 Data collection and analysis

Analysis of variance (ANOVA), W-west normality test, Non-hierarchical Cluster Analysis, Repeated Measures - Analysis of variance (ANOVA) tests, Pearson correlation coefficient analysis and principal components analysis were carried out using 18th edition of Genstat Statistical package (18th edition, VSN International, UK). Data transformation was performed for non-normally distributed data.

3.3 Results

3.3.1 Soil moisture content

3.3.1.1 Soil moisture content in the F_3 segregating population

Soil moisture content under drought-stressed treatment declined by 47.53 % from 47 DAS to 74 DAS. On average, soil moisture content declined by 0.49% per day at depth 200 mm and 0.35% per day at depth 100 mm over 28 days of drought (Figure 3-1). There was a significant difference ($p < 0.01$) between drought-stressed and well-watered treatments at depths 100 mm, 200 mm and 300 mm. No significant difference was observed between drought-stressed and well-watered treatments at depths 400 mm, 600 mm and 1000 mm (Figure 3-2).

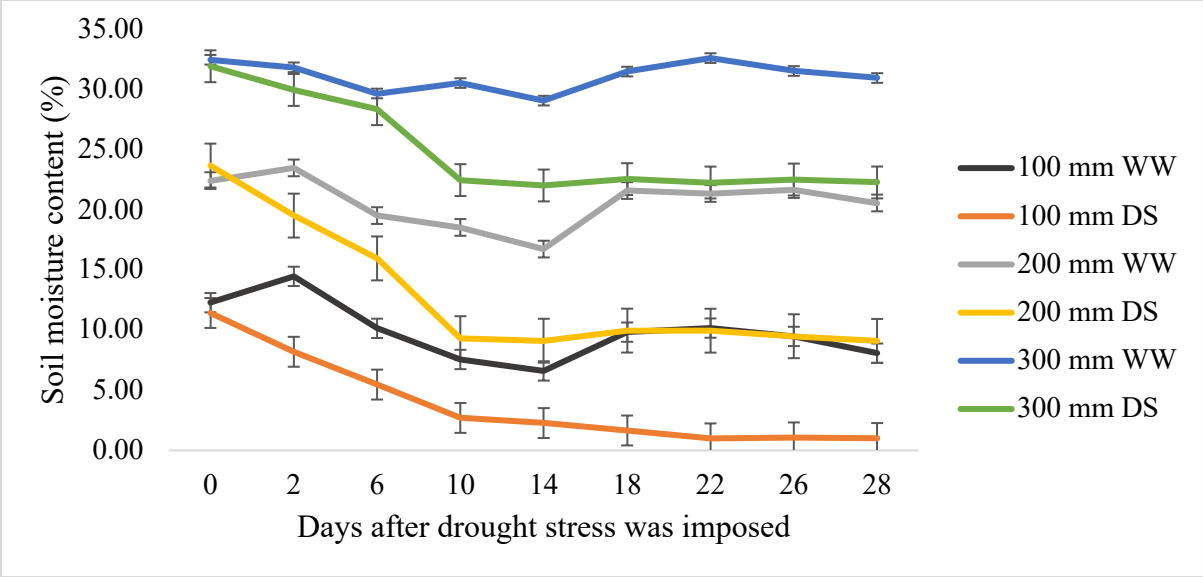


Figure 3-1 Soil moisture content measurements at depths 100 mm, 200 mm and 300 mm based on PR2 reading (% vol) under drought-stressed (DS) treatment plots and well-watered (WW) treatment plots. Data represent mean values of soil moisture content during plant growth season in 2018; n = 6. Data represent mean values ± standard error.

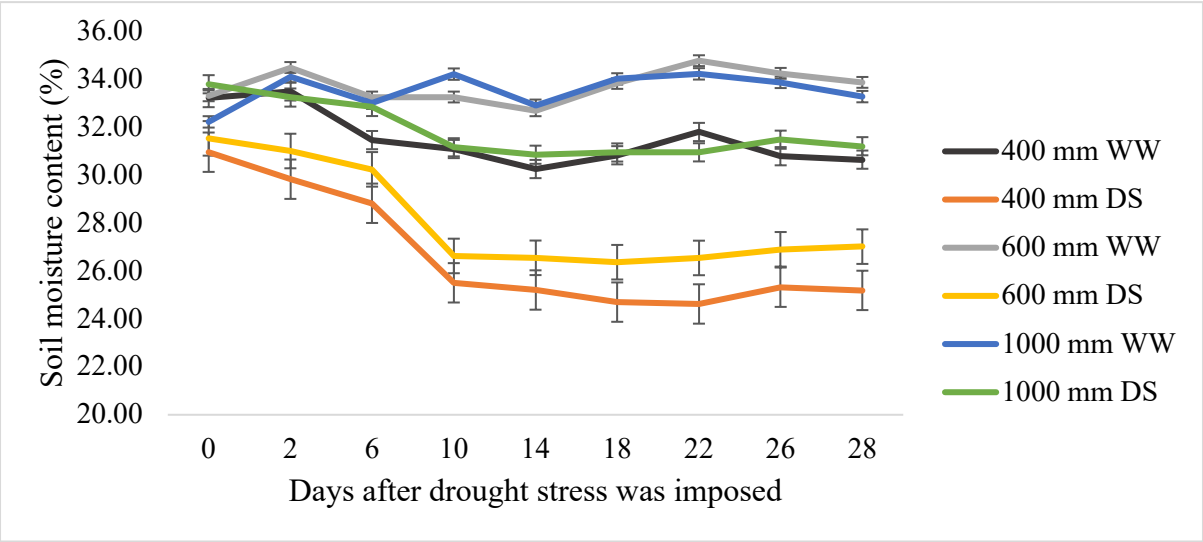


Figure 3-2 Soil moisture content measurements at depths 400 mm, 600 mm and 1000 mm based on PR2 reading (% vol) under drought-stressed (DS) treatment plots and well-watered (WW) treatment plots. Data represent mean values of soil moisture content during plant growth season in 2018; n = 6. Data represent mean values ± standard error.

3.3.1.2 Soil moisture content in the F_4 segregating population

Soil moisture content under drought-stressed treatment declined by 41.61 % from 47 DAS to 74 DAS. On average, soil moisture content declined by 0.41% per day at depths 300 mm and 0.31% per day at depth of 400 mm over the 28 days of drought imposition (Figure 3-3). A significant difference ($p < 0.01$) was observed between drought-stressed and well-watered treatment at depths 100 mm, 200 mm and 300 mm. No significant difference was observed between drought-stressed and well-watered treatments at depths 400 mm, 600 mm and 1000 mm (Figure 3-4).

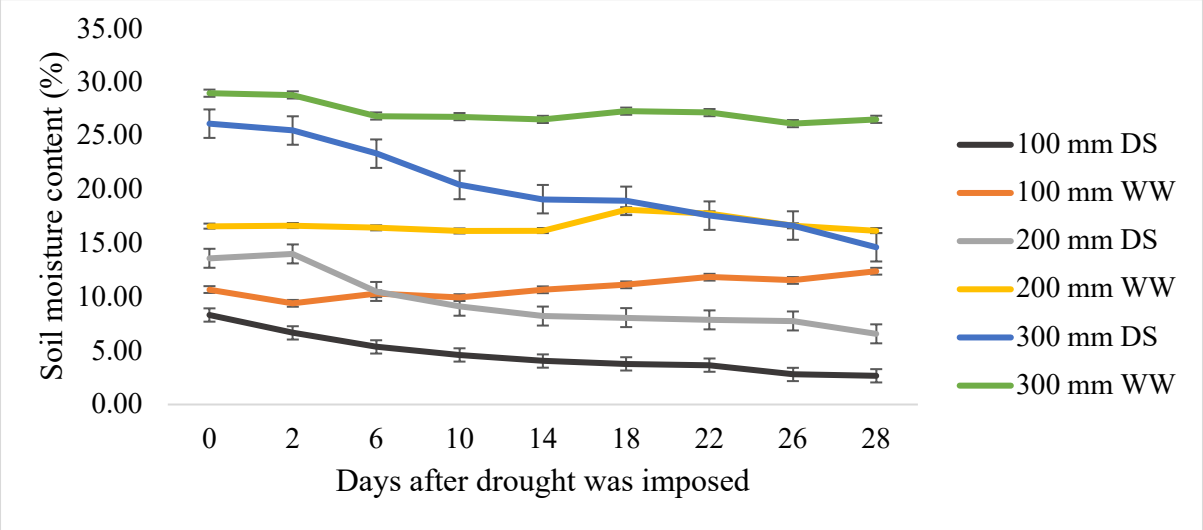


Figure 3-3 Soil moisture content measurements at depths 100 mm, 200 mm and 300 mm based on PR2 reading (% vol) under drought-stressed (DS) treatment plots and well-watered (WW) treatment plots. Data represent mean values of soil moisture content during plant growth season in 2019; n = 6. Data represent mean values ± standard error.

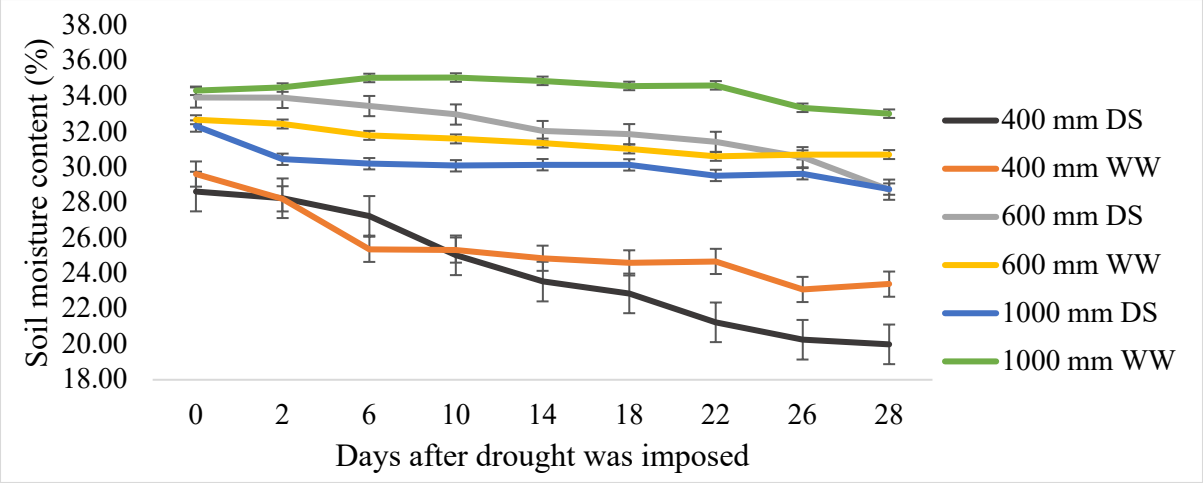


Figure 3-4 Soil moisture content measurements at depths 400 mm, 600 mm and 1000 mm based on PR2 reading (% vol) under drought-stressed (DS) treatment plots and well-watered (WW) treatment plots. Data represent mean values of soil moisture content during plant growth season in 2019; n = 6. Data represent mean values ± standard error.

3.3.2 Chlorophyll content index (CCI)

3.3.2.1 CCI in the F_3 segregating population

Chlorophyll content index declined significantly ($p < 0.01$) by 12.6% over 28 days after drought treatment was imposed from 47 DAS to 74 DAS (Figure 3-5). *Chlorophyll content index* declined significantly ($p < 0.01$) by 8.8% at 74 DAS in drought-stressed treatment compared to well-watered treatment (Figure 3-5). A significant difference ($p < 0.05$) was observed among the individual lines during drought period at 60 DAS.

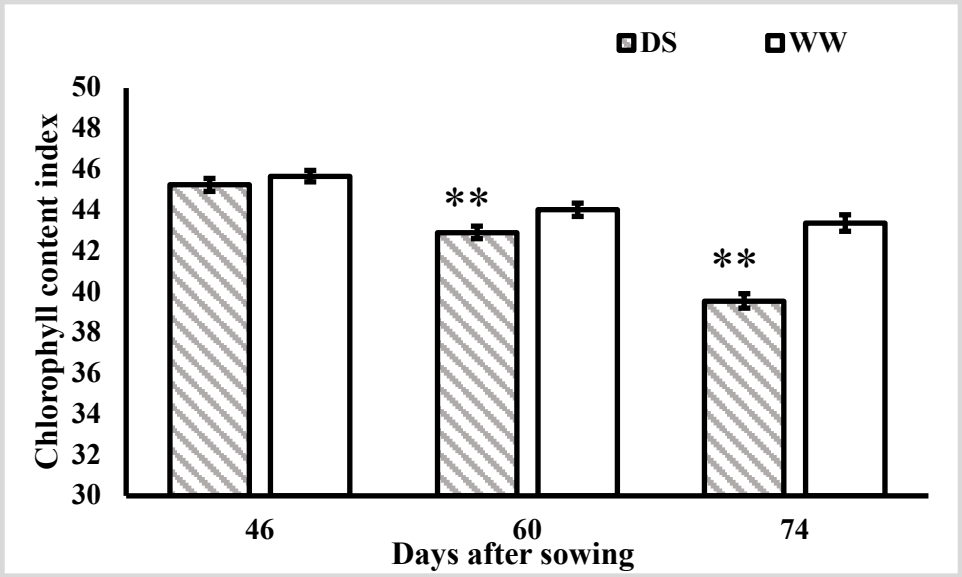


Figure 3-5 The effect of drought treatment on chlorophyll content index in the F_3 segregating population, n = 114. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. * = Significant at (p = 0.05), ** = Significant at (p = 0.01).

3.3.2.2 CCI in the F_4 segregating population

Chlorophyll content index declined by 17.9% over 28 days (p = 0.193) after drought treatment was imposed from 47 DAS to 74 DAS (Figure 3-6). C*hlorophyll content index* reported in drought-stressed treatment showed 2.5% significant reduction ($p < 0.05$) at 71 DAS compared to well-watered treatment (Figure 3-6). A significant difference was observed among the individual lines during drought period at 71 DAS ($p < 0.01$), and after irrigation was resumed from 78 DAS to 86 DAS ($p < 0.05$). The interaction between individual lines and water treatment was significant ($p < 0.01$) at 71 DAS.

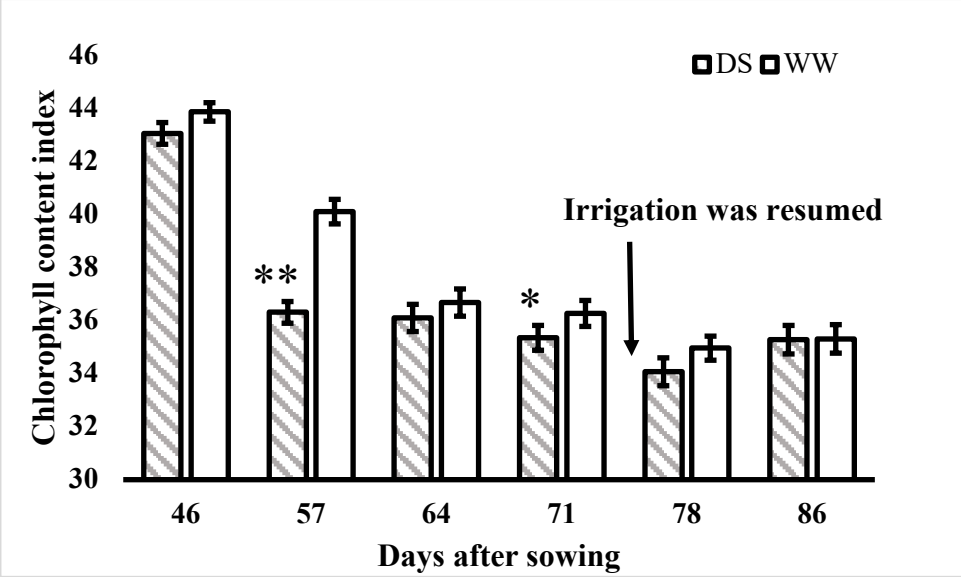


Figure 3-6 The effect of drought treatment on chlorophyll content index in the F_4 segregating population, n = 114. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. * = Significant at (p = 0.05), ** = Significant at (p = 0.01). Arrow irrigation was resumed at 74 DAS.

3.3.3 Leaf relative water content (RWC)

3.3.3.1 RWC in the F_3 segregating population

Leaf *RWC* was maintained at around 74% in drought-stressed treatment during the drought period. Significantly ($p < 0.01$) lower leaf *RWC* was reported in drought-stressed treatment compared to well-watered treatment at 60 DAS but there was no significant difference (p = 0.57) for drought treated plants at 74 DAS (Figure 3-7). The interaction between individual lines and water treatment was significant ($p < 0.01$) at 74 DAS.

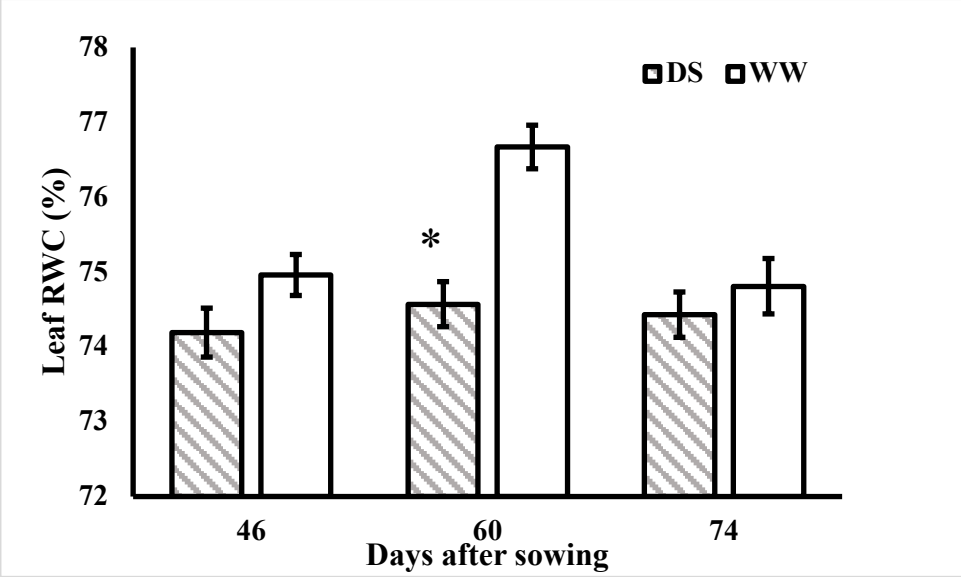


Figure 3-7 The effect of drought treatment on relative water content (RWC) in the F_3 segregating population, n = 114. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. * = Significant at (p = 0.05), ** = Significant at (p = 0.01)

3.3.3.2 RWC in the F_4 segregating population

Leaf RWC was reduced by 7.55% under drought-stressed treatment from 81.98% at 57 DAS to 75.79% at 71 DAS, with significant difference ($p < 0.05$) observed between drought-stressed and well-watered treatment at 71 DAS and after irrigation was resumed at 86 DAS (Figure 3-8). A significant difference was observed among the individual lines during drought period at 57 DAS ($p < 0.01$) and after irrigation was resumed at 78 DAS ($p < 0.05$). The interaction between individual lines and water treatment was significant ($p < 0.05$) after drought stress was imposed at 57 DAS.

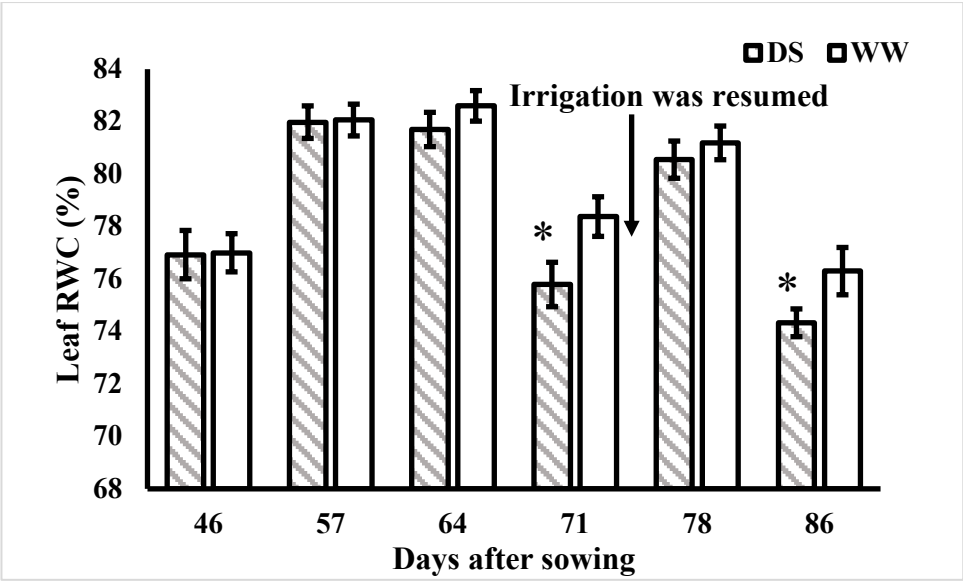


Figure 3-8 The effect of drought treatment on relative water content in the F_4 segregating population, n = 36. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. * = Significant at (p = 0.05), ** = Significant at (p = 0.01). Arrow irrigation was resumed at 74 DAS.

3.3.4 Quantum yield of PSII photochemistry (F_V/F_M)

3.3.4.1 F_V/F_M in the F_3 segregating population

Quantum yield of PSII photochemistry (F_V/F_M) declined gradually (p = 0.713) by 5.97% during drought period from 0.67 at 47 DAS to 0.63 at 74 DAS (Figure 3-5). Drought stress significantly ($p < 0.01$) reduced F_V/F_M by 2.9% (F_V/F_M: 0.65 under WW conditions and 0.63 under DS conditions) at 74 DAS (Figure 3-9). The interaction between individual lines and water treatment was also significant ($p < 0.05$) at 74 DAS.

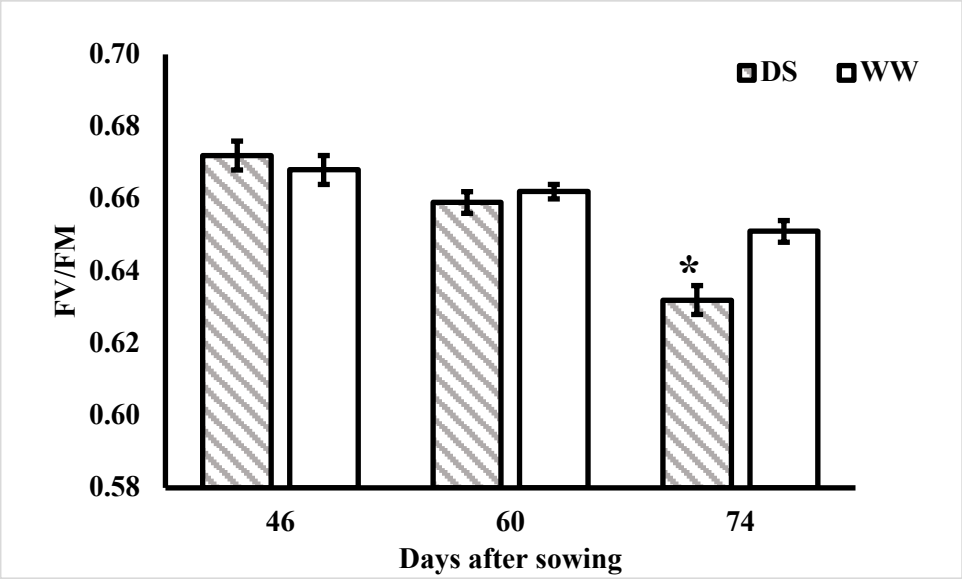


Figure 3-9 The effect of drought treatment on quantum yield (F_V/F_M) in the F_3 segregating population, n = 114. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. * = Significant at (p = 0.05), ** = Significant at (p = 0.01).

3.3.4.2 F_V/F_M in the F_4 segregating population

Quantum yield of PSII photochemistry (F_V/F_M) declined gradually (p = 0.208) by 8.48% during drought period from 0.66 at 57 DAS to 0.60 at 71 DAS (Figure 3-10). Drought-stressed plants recorded reduced ($p < 0.01$) F_V/F_M of 7.69% (F_V/F_M: 0.65 under WW conditions and 0.60 under DS conditions) at 71 DAS (Figure 3-10). An increase in F_V/F_M value up to 5.62% was also observed after irrigation was resumed starting from 74 DAS, with significantly higher ($p < 0.05$) F_V/F_M observed at 78 and 86 DAS in well-watered treatment. A significant difference was observed among the individual lines before drought treatment was imposed at 46 DAS ($p < 0.01$), during drought period at 64 DAS ($p < 0.01$) and after irrigation was resumed at 78 and 86 DAS ($p < 0.05$). The interaction between individual lines and water treatment was significant ($p < 0.05$) after drought stress was imposed at 64 and 78 DAS.

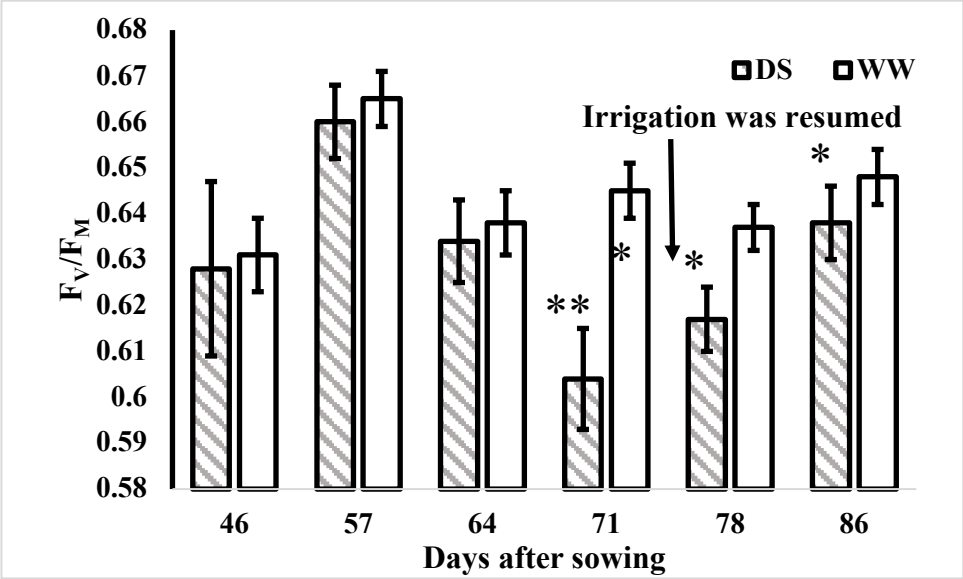


Figure 3-10 The effect of drought treatment on quantum yield (F_V/F_M) in the F_4 segregating population, n = 36. Data represent mean values ± standard error. DS, drought-stressed; WW, well-watered. Significant at (p = 0.05), ** = Significant at (p = 0.01). Arrow irrigation was resumed at 74 DAS.

3.3.5 Photosynthesis parameters in the F_4 segregating population

On average, *photosynthesis rate* (*A*) declined from 36.24 µmol m^{-2} s^{-1} to 18.61 µmol m^{-2} s^{-1} (by 48.6%) was observed under drought-stressed treatment from 47 DAS to 71 DAS followed by recovery to 23.3 µmol m^{-2} s^{-1} (by 25.20%) at 78 DAS after irrigation was resumed, with significant difference ($p < 0.01$) observed between DS and WW treatments at 71 DAS (Figure 3-11a). A significant difference ($p < 0.05$) was also observed for *A* among the individual lines during drought period at 64 and 71 DAS and at 78 and 86 DAS after irrigation was resumed. The interaction between the individual lines and water treatment was significant ($p < 0.05$) during drought period at 61 and 71 DAS.

On average, *stomatal conductance* (*gs*) declined significantly ($p < 0.01$) from 0.497 mol m^{-2} s^{-1} to 0.203 mol m^{-2} s^{-1} (by 59.2%) while *leaf water use efficiency* (LWUE) increased

significantly ($p < 0.01$) from 79.97 µmol mol^{-1} to 142.7 µmol mol^{-1} (by 55.9 %) under drought-stressed treatment from 47 to 74 DAS. On average, *gs* then was observed to recover to 0.276 mol m^{-2} s^{-1} (by 35.96%) and LWUE to 116.5 µmol mol^{-1} (by 18.36%) at 78 DAS after irrigation was resumed (Figure 3-11b & 3-11e). A significant difference was observed for *gs* among the individual lines before drought was imposed at 46 DAS ($p < 0.01$), during drought period from 57 to 71 DAS ($p < 0.01$), and at 78 and 86 DAS after irrigation was resumed ($p < 0.05$). LWUE exhibited significant difference ($p = 0.079$) among individual lines similar to *gs,* but they were not significantly different ($p = 0.141$) during drought period at 64 DAS and after irrigation was resumed at 86 DAS. Stomatal conductance, *gs* exhibited significant interaction ($p < 0.05$) between individual lines and water treatment before drought treatment was imposed at 46 DAS, during drought period at 57, 64 DAS and at 78 and 86 DAS after irrigation was resumed. LWUE exhibited significant interaction between individual lines and water treatment similar to *gs,* but not significantly different ($p = 0.051$) during drought period at 64 DAS.

Similar to *gs,* on average, *intracellular* CO_2 (*Ci*) significantly ($p < 0.01$) declined by 50.37% from 273.6 µmol m^{-1} to 135.8 µmol m^{-1}, after drought treatment was imposed at 47 DAS and recovered to 186.6 µmol m^{-1} (by 37.41%) at 78 DAS after irrigation was resumed, with significant difference ($p < 0.01$) observed between DS and WW treatments during drought period from 57 to 71 DAS and at 78 and 86 DAS after irrigation was resumed (Figure 3-11d). A significant difference was observed for Ci among the individual lines during drought period at 71 DAS ($p < 0.05$) and at 78 and 86 DAS ($p < 0.01$) after irrigation was resumed. The interaction between individual lines and water treatment for *Ci* was significant ($p < 0.01$) before drought stress was imposed, at 46 DAS and during drought period, 57 DAS.

On average, *transpiration rate* (*E)* significantly ($p < 0.01$) declined from 12.47 mol m^{-2} s^{-1} to 3.54 mol m^{-2} s^{-1}, a reduction of 71.61%, under drought-stressed treatment from 46 to 71 DAS

and recovered to 4.98 mol m^{-2} s^{-1} (by 40.68%) at 78 DAS after irrigation was resumed, with significant difference ($p < 0.01$) observed between DS and WW treatments during drought period from 57 to 71 DAS and at 78 DAS ($p < 0.05$) after irrigation was resumed (Figure 3-11c). A significant difference ($p < 0.05$) for *E* was observed among individual lines and interaction between individual lines and water treatment at 71 DAS.

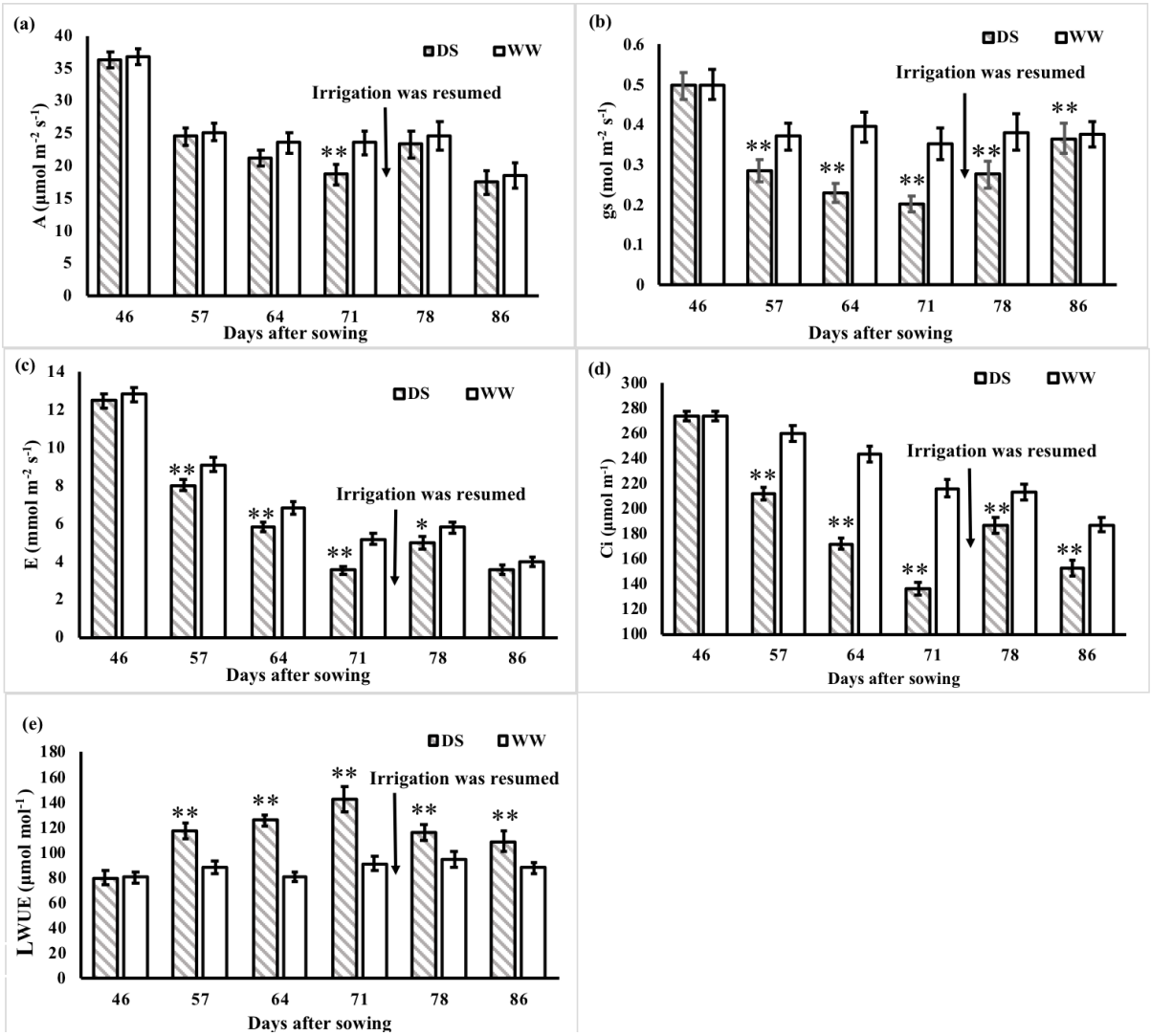


Figure 3-11 Comparison of (a) *photosynthesis rate*, *A* (b) *stomatal conductance, gs* (c) *transpiration rate, E* (d) *intracellular* CO_2, *Ci* and (e) *leaf water use efficiency,* LWUE between individual lines under drought-stressed (DS) and well-watered (WW) treatment in the F_4 segregating population. Mean and standard error are indicated at the time of measurement. n = 36. * = Significant at ($p = 0.05$), ** = Significant at ($p = 0.01$). Arrow irrigation was resumed at 74 DAS.

3.3.6 Seed size in the F_4 segregating population

Seeds harvested from the drought-stressed treatment had significantly larger seed width ($p < 0.05$; by 21.59%), length ($p < 0.05$; by 37.79%) and perimeter ($p = 0.214$; by 2.5%), but seed width-length ratio was significantly ($p < 0.05$; by 1.0%) lower compared to seeds under well-watered treatment (Figure 3-12). A significant difference ($p < 0.01$) was observed among individual lines and also interaction between individual lines and water treatment ($p < 0.05$) for seed width, length, width/length ratio and perimeter.

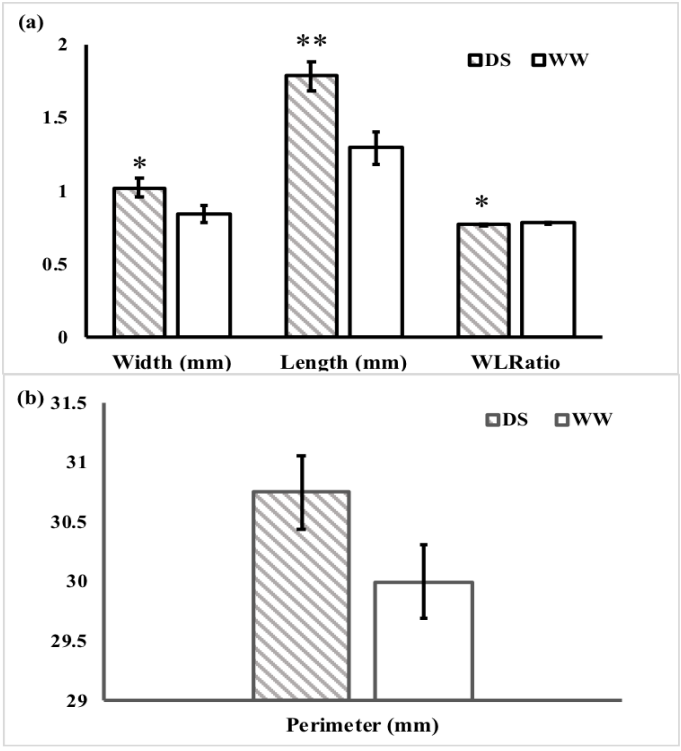


Figure 3-12 Comparison of (a) *seed width, length* and *width/length ratio* and (b) *seed perimeter* between individual lines under drought-stressed (DS) and well-watered (WW) treatment in the F_4 segregating population. Mean and standard error are indicated at the time of measurement. n = 114. Significant at ($p = 0.05$), ** = Significant at ($p = 0.01$).

3.3.7 Leaf area in the F_4 segregating population

Drought stress reduced leaf area by 39.7% with significant difference ($p < 0.01$) observed among the F_4 individual lines and interaction between treatment and individual lines (Figure 3-13).

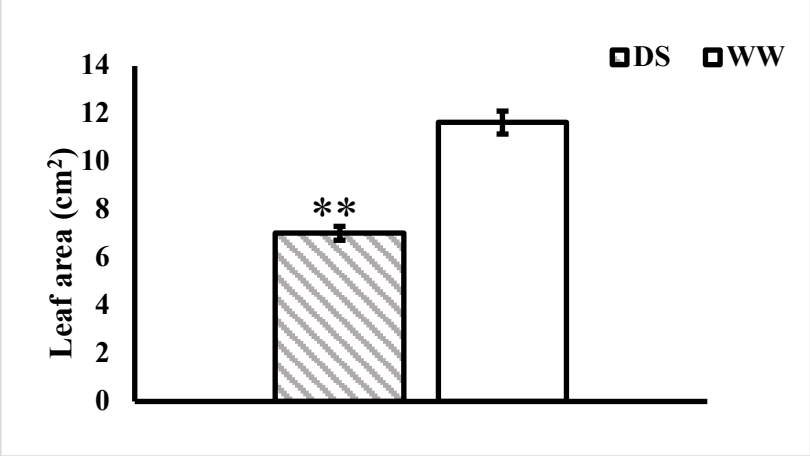


Figure 3-13 Comparison of leaf area between individual lines under drought-stressed (DS) and well-watered (WW) treatment in the F_4 segregating population. Mean and standard error are indicated at the time of measurement. n = 36. Significant at ($p = 0.05$), ** = Significant at ($p = 0.01$).

3.3.8 Yield components and morphological traits

3.3.8.1 Yield components and morphological traits in the F_3 segregating population

The two parental lines differed significantly ($p < 0.05$) for yield components and morphological traits (*number of seeds per plant, number of pods per plant, 100-seed weight, internode length, petiole internode ratio* and *plant height*) when grown under well-watered and drought-stressed treatments. Under drought-stressed conditions, DodR had significantly ($p < 0.05$) heavier seeds *(100-seed weight)* and taller plants *(plant height)* than S19-3. On the other hand, drought-stressed plants from S19-3 landrace showed significantly higher values for *number of seeds per plant, number of pods per plant, internode length* and *petiole internode ratio* than DodR ($p < 0.05$).

The average results showed a significant reduction ($p < 0.05$) of 14.8% in *number of seeds per plant,* 10.5% in *seeds weight per plant,* 16.1% in *pods weight per plant,* 5.4% in *harvest index* and 9.8% in *plant height* under drought-stressed conditions compared to well-watered conditions in the F_3 segregating population (Table 3-3). All yield components and morphological traits showed significant difference ($p < 0.05$) among individual lines except *number of double-seeded pods per plant, 100-seed weight, shelling percentage and plant height.* The interaction between individual lines and treatment was significant ($p < 0.05$) for *number of double-seeded pods per plant, seed weight per plant, pod weight per plant, shelling percentage, days to flowering, number of leaves per plant* and *petiole length*.

Transgressive segregation was observed in the F_3 segregating population. *Harvest index* showed a significant difference ($p < 0.01$) under different treatment conditions and among individual lines. For example, *harvest index* ranged from 0.02 to 0.55 under drought-stressed conditions (S19-3 mean, 0.36 ± 0.04, sd 0.12, n = 9; DodR mean, 0.32 ± 0.04, sd 0.09, n = 9), and 0.03 to 0.69 (S19-3 mean, 0.30 ± 0.03, sd 0.12, n = 9; DodR mean 0.26 ± 0.02, sd 0.06, n = 9) under well-watered conditions.

Positive correlations amongst yield components, i.e., *shoot dry weight, number of seeds per plant, number of pods per plant, seed weight per plant* and *pod weight per plant*, *100-seed weight* and *harvest index* were observed under drought-stressed and well-watered treatments (Table 3-4). Yield components, i.e., *number of seeds per plant, number of pods per plant, seed weight per plant* and *pod weight per plant* showed strong positive linear relationship under drought-stressed and well-watered conditions, while the overall correlation under well-watered treatment ($r = 0.88 – 0.91$, $p < 0.01$) was higher than those under drought-stressed treatment (r

= 0.73 – 0.86, $p < 0.01$) (Table 3-4). A moderate positive correlation was observed amongst morphological traits i.e., *number of leaves per plant, petiole length, internode length* and *plant height* under both water regimes ($r = 0.32 – 0.60$, $p < 0.01$) (Table 3-4). *Chlorophyll content index* positively ($p < 0.05$) correlated with *shoot dry weight, pod weight per plant, seed weight per plant, 100-seed weight, harvest index* and *plant height* under well-watered treatment. *Leaf relative water content* negatively ($p < 0.05$) correlated with *number of seeds per plant, number of pods per plant, number of double pods per plant, seed weight per plant, pod weight per plant* and *chlorophyll content index* under well-watered treatment. F_V/F_M negatively ($p < 0.05$) correlated with *seed weight per plant, pod weight per plant* and *number of leaves per plant* under well-watered treatment.

Table 3-3 Comparison of yield components and morphological traits under well-watered (WW) and drought-stressed (DS) treatments in the F_3 segregating population derived from S19-3 ×DodR and their parental lines.

Traits	Treatment	Normality	Median/ Mean	Range	95% confidence interval		Interquartile range / standard deviation	F-probability			S19-3		DodR	
Yield components					lower	higher		Treatment	Genotypes	G*E	Min	Max	Min	Max
SDW (g)	DS	**	11.31	24.51	10.86	12.18	7.91	0.79	**	0.09	5.02	20.98	5.04	14.08
	WW	**	11.16	34.32	11.26	12.82	7.30				4.32	30.13	6.30	13.96
NS	DS	**	23.00	68.00	23.44	26.77	18.75	**	**	0.15	9.00	42.00	9.00	23.00
	WW	**	27.00	114.00	27.81	32.92	23.00				11.00	72.00	11.00	18.00
NP	DS	**	31.00	97.00	30.37	34.49	22.00	0.17	**	0.15	17.00	46.00	17.00	28.00
	WW	**	31.00	112.00	32.39	38.16	27.00				11.00	95.00	13.00	33.00
NDP	DS	**	0.00	10.00	0.08	0.28	0.00	0.55	0.35	0.07	0.00	0.00	0.00	2.00
	WW	**	0.00	4.00	0.10	0.26	0.00				0.00	3.00	0.00	0.00
SW (g)	DS	**	7.15	27.43	7.16	8.48	6.90	**	**	*	2.25	11.27	1.48	12.00
	WW	**	7.99	32.35	8.45	10.18	9.47				2.10	18.36	2.80	5.35
PW (g)	DS	**	8.53	34.20	8.76	10.38	8.48	**	**	*	2.98	14.11	1.97	13.33
	WW	**	10.17	40.56	10.76	12.93	11.73				2.60	26.20	3.51	7.00
100SW (g)	DS	ns	29.76	51.93	28.41	31.11	10.84	0.77	0.20	0.28	16.98	28.30	16.44	52.17
	WW	ns	30.02	57.21	28.83	31.22	9.35				10.39	25.50	15.56	43.82
HI	DS	**	0.35	0.52	0.33	0.36	0.13	**	**	0.19	0.20	0.48	0.21	0.44
	WW	**	0.37	0.66	0.35	0.37	0.14				0.16	0.47	0.18	0.33
SP	DS	**	0.82	0.61	0.81	0.82	0.06	**	0.71	0.08	0.71	0.83	0.75	0.90
	WW	**	0.79	0.63	0.77	0.79	0.05				0.64	0.81	0.69	0.84
Morphological traits														
DTF	DS	**	33.00	12.00	32.89	33.58	3.00	0.29	*	**	31.00	40.00	29.00	37.00
	WW	**	31.00	7.00	31.01	31.55	3.00				30.00	34.00	28.00	33.00
NL	DS	ns	51.71	86.00	49.60	53.81	16.91	**	**	**	36.00	72.00	4.50	85.00
	WW	**	44.50	86.00	45.62	49.97	22.50				31.00	59.00	32.00	59.00

PL (cm)	DS	**	16.17	19.57	15.98	16.82	3.79	**	**	**	13.50	19.50	14.40	21.57
	WW	**	15.41	14.83	15.45	16.22	3.90				13.33	20.00	13.93	19.03
IL (cm)	DS	ns	2.23	4.10	2.21	2.40	0.75	0.08	**	0.51	1.70	2.93	1.90	4.07
	WW	ns	2.24	4.17	2.16	2.34	0.72				1.03	2.30	2.47	3.87
P/I	DS	**	7.16	34.36	7.49	8.44	2.58	0.60	**	0.71	6.00	9.26	4.14	9.65
	WW	**	7.09	21.33	7.31	7.99	2.87				6.78	15.81	4.92	6.82
PH (cm)	DS	**	23.00	34.50	23.34	24.78	7.43	**	0.06	0.23	20.10	29.50	23.00	40.50
	WW	**	25.50	28.00	25.27	26.52	5.90				22.00	26.70	21.00	31.50

*Note: SDW shoot dry weight, NS number of seeds per plant, NP number of pods per plant, NDP number of double-seeded pods per plant, SW seed weight per plant, PW pod weight per plant, 100SW 100-seed weight, HI harvest index, SP shelling percentage, DTF days to flowering, NL number of leaves per plant, PL petiole length, IL internode length, P/I petiole internode ratio, PH plant height, SD standard deviation, G*E interaction between treatment and genotypes, F pr = F-probability, * = Significant at (p = 0.05), ** = Significant at (p = 0.01).*

Table 3-4 Pearson correlation coefficient among mean variables for yield components and morphological traits in the F_3 segregating population derived from S19-3 × DodR.

	SDW	NS	NP	NDP	SW	PW	100sw	HI	SP	DTF	NL	PL	IL	P/I	PH	CCI	RWC	F_V/F_M
SDW	–	0.73**	0.70**	0.10	0.73**	0.74**	0.36**	0.24*	–0.01	–0.16	0.79**	0.55**	0.46**	–0.18	0.51**	0.07	–0.04	0.08
NS	0.63**	–	0.90**	0.20*	0.86**	0.86**	0.28**	0.59**	0.02	–0.22*	0.63**	0.37**	0.44**	–0.24*	0.34**	0.12	–0.16	0.03
NP	0.68**	0.97**	–	0.24*	0.73**	0.74**	0.15	0.44**	–0.06	–0.19	0.62**	0.30**	0.38**	–0.22*	0.25**	0.12	–0.17	0.00
NDP	0.12	0.37**	0.29**	–	0.14	0.16	0.05	0.14	–0.11	–0.08	0.04	0.08	0.04	0.00	0.05	–0.09	–0.15	–0.08
SW	0.70**	0.90**	0.88**	0.26**	–	0.98**	0.65**	0.71**	0.10	–0.24*	0.56**	0.40**	0.44**	–0.23*	0.43**	0.19*	–0.11	0.08
PW	0.71**	0.91**	0.90**	0.27**	0.99**	–	0.62**	0.65**	–0.09	–0.21*	0.55**	0.40**	0.41**	–0.21*	0.42**	0.17	–0.14	0.07
100SW	0.42**	0.24*	0.24*	0.01	0.57**	0.55**	–	0.71**	0.13	–0.17	0.23*	0.40**	0.31**	–0.12	0.45**	0.13	0.01	0.13
HI	0.11	0.60**	0.55**	0.23*	0.64**	0.63**	0.50**	–	0.23*	–0.20*	0.20*	0.23*	0.30**	–0.18	0.22*	0.12	–0.13	0.08
SP	0.14	0.15	0.10	0.01	0.30**	0.20*	0.33**	0.28**	–	–0.05	0.06	0.05	0.15	–0.11	0.09	0.06	0.13	0.15
DTF	–0.17	–0.07	–0.13	0.12	–0.15	–0.16	–0.11	0.06	0.03	–	–0.18	0.09	–0.16	0.27**	–0.16	–0.01	0.11	0.19*
NL	0.82**	0.59**	0.61**	0.02	0.57**	0.59**	0.28**	0.11	0.09	–0.18	–	0.46**	0.37**	–0.12	0.43**	–0.05	–0.05	0.03
PL	0.56**	0.42**	0.41**	0.21*	0.42**	0.43**	0.21*	0.08	–0.02	0.01	0.45**	–	0.32**	0.23*	0.47**	0.09	0.02	0.10
IL	0.57**	0.34**	0.32**	0.16	0.35**	0.36**	0.26**	0.08	–0.01	0.17	0.44**	0.60**	–	–0.80*	0.47**	0.05	–0.06	–0.04
P/I	–0.29**	–0.12	–0.09	–0.06	–0.13	–0.13	–0.17	–0.04	–0.05	–0.18	–0.26**	–0.08	–0.80**	–	–0.21*	–0.05	0.06	0.09
PH	0.54**	0.39**	0.40**	0.10	0.38**	0.38**	0.18	0.04	–0.02	–0.03	0.40**	0.58**	0.53**	–0.21*	–	0.02	0.01	–0.03
CCI	0.27**	0.19	0.18	0.18	0.28**	0.26**	0.31**	0.14*	0.16	–0.14	0.11	0.13	0.09	0.09	0.26**	–	0.03	0.14
RWC	0.01	–0.27**	–0.23*	–0.19*	–0.23*	–0.22*	–0.05	–0.25	–0.08	0.03	0.01	–0.03	–0.04	0.06	0.04	–0.23*	–	0.08
F_V/F_M	–0.13	–0.16	–0.16	0.15	–0.19*	–0.20*	–0.17	–0.13	0.01	0.13	–0.20*	0.02	–0.06	0.07	–0.01	0.03	–0.11	–

Note: : SDW shoot dry weight, NS number of seeds per plant, NP number of pods per plant, NDP number of double–seeded pods per plant, SW seed weight per plant, PW pod weight per plant, 100SW 100-seed weight, HI harvest index, SP shelling percentage, DTF days to flowering, NL number of leaves per plant, PL petiole length, IL internode length, P/I petiole internode ratio, PH plant height, * = Significant at (p = 0.05), ** = Significant at (p = 0.01), Values below diagonal are correlation coefficients among traits under well-watered treatment; values above diagonal*

are correlation coefficients among traits under drought-stressed treatment. Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation: *0.70-0.99,* *0.50-0.70,* *−0.60-−0.90.*

3.3.8.2 Yield components and morphological traits in the F_4 segregating population

The two parental lines showed a significant difference between well-watered and drought-stressed treatment conditions for yield components and morphological traits, including *shoot dry weight, 100-seed weight, number of leaves per plant, petiole length* and *plant height* ($p < 0.05$) (Table 3-5). Under drought-stressed conditions, DodR showed significantly ($p < 0.05$) higher values for *number of leaves per plant, petiole length* and *plant height* than S19-3.

The average results showed a significant reduction ($p < 0.05$) of 41.5% in *shoot dry weight,* 41.2 % in *number of seeds per plant,* 45.8% in *number of pods per plant,* 47.9% in *seeds weight per plant,* 47.6% in *pods weight per plant,* 12.5% in *harvest index,* 40.5% in *number of leaves per plant* and 4.9% in *petiole length* between well-watered and drought-stressed conditions in the F_4 segregating population (Table 3-5). All yield-related and morphological traits showed significant differences ($p < 0.05$) among individual lines except on the *number of double-seeded pods per plant.* The interaction between treatments and F_4 individual lines was significant ($p < 0.05$) for all traits except *number of pods per plant, 100-seed weight* and *days to flowering*.

Transgressive segregation was also observed in the F_4 segregating populations. *Harvest index* showed a significant difference ($p < 0.01$) among individual lines, under different treatment conditions and interaction between individuals and treatment. *Harvest index* ranged from 0.01 to 0.48 under drought-stress conditions (S19-3 mean, 0.19 ± 0.04, sd 0.08, n = 9; DodR mean, 0.21 ± 0.04, sd 0.10, n = 9), and 0.05 to 0.77 (S19-3 mean, 0.18 ± 0.04, sd 0.07, n = 9; DodR mean 0.16 ± 0.02, sd 0.05, n = 9) under well-watered conditions.

Yield components, i.e., *number of seeds per plant, number of pods per plant, seeds weight per plant* and *pods weight per plant* showed strong positive linear relationship under both conditions, in which overall correlation under well-watered conditions (r = 0.90 – 0.97, p < 0.01) was higher than under drought-stressed conditions (r = 0.73 – 0.82, p < 0.01) (Table 3-6). *Shoot dry weight* positively correlated with *pod weight per plant, number of leaves per plant* and *plant height* under both conditions (Table 3-6). *Harvest index* positively correlated with *number of seeds per plant* (r = 0.57, p < 0.01), *seeds weight per plant* (r = 0.82, p < 0.01) and *pod weight per plant* (r = 0.80, p < 0.01), and negatively correlated with *number of double-seeded pods per plant* (r = –0.64, p < 0.01) under drought-stressed treatment. A moderate positive correlation was observed among morphological traits i.e., *number of leaves per plant, petiole length, internode length* and *plant height* under both water regimes in the F_3 generation (drought-stressed conditions: r = 0.32 – 0.47, p < 0.01; well-watered conditions: r = 0.40 – 0.60, p < 0.01) and *petiole length, internode length* and *plant height* under both water regimes in the F_4 generation (drought-stressed conditions: r = 0.63 – 0.78, p < 0.05, p < 0.01; well-watered conditions: r = 0.70 – 0.74, p < 0.01) (Table 3-6).

Table 3-5 Effect of water treatments on yield components and morphological traits under well-watered (WW) and drought-stressed (DS) conditions in the F_4 segregating population derived from S19-3 × DodR and their parental lines.

Traits	Treatment	Normality	Median/ Mean	Range	95% confidence interval		Interquartile range / standard deviation	F-probability			S19-3		DodR	
Yield components					lower	higher		Treatment	Genotypes	G*E	Min	Max	Min	Max
SDW (g)	DS	**	8.41	31.47	8.63	10.07	5.35	**	**	**	5.25	15.01	5.97	11.71
	WW	**	14.38	44.30	14.65	17.96	11.16				4.68	13.39	12.49	38.39
NS	DS	**	10.00	74.00	10.72	14.34	12.25	**	**	0.05	6.00	30.00	1.00	16.00
	WW	**	17.00	97.00	17.88	23.04	13.75				1.00	30.00	12.00	28.00
NP	DS	**	13.00	70.00	14.66	19.02	16.00	**	**	0.19	9.00	56.00	1.00	16.00
	WW	**	24.00	125.00	24.12	30.39	19.50				3.00	43.00	14.00	41.00
NDP	DS	**	0.00	11.00	0.01	0.27	0.00	0.76	0.20	*	0.00	0.00	0.00	0.00
	WW	**	0.00	2.00	0.10	0.28	0.00				0.00	1.00	0.00	1.00
SW (g)	DS	**	1.55	20.07	2.06	2.88	2.73	**	**	*	1.18	5.37	0.03	3.36
	WW	**	2.98	28.51	3.52	4.88	3.56				0.07	5.70	2.89	7.55
PW (g)	DS	**	2.52	27.08	3.06	4.19	3.74	**	**	*	1.68	11.42	0.16	4.40
	WW	**	4.81	42.08	5.41	7.31	4.88				0.15	10.43	3.52	13.07
100SW (g)	DS	ns	19.08	42.17	17.95	20.22	7.47	0.34	**	*	11.08	19.89	3.00	21.00
	WW	ns	19.62	22.54	18.75	20.48	4.82				6.38	19.00	23.57	26.96
HI	DS	*	0.14	0.47	0.15	0.18	0.17	**	**	**	0.13	0.30	0.01	0.21
	WW	*	0.16	0.72	0.17	0.21	0.11				0.01	0.22	0.08	0.23
SP (%)	DS	*	0.70	0.79	0.63	0.68	0.20	0.21	**	**	0.44	0.70	0.19	0.76
	WW	ns	0.66	0.51	0.64	0.68	0.12				0.38	0.69	0.36	0.82
Morphological traits														
DTF	DS	**	33.00	18.00	33.70	34.67	4.00	0.57	**	0.96	33.00	41.00	31.00	40.00
	WW	**	33.00	18.00	33.80	34.85	3.00				32.00	42.00	32.00	40.00
NL	DS	**	44.00	125.00	45.48	51.22	22.25	**	**	**	24.00	54.00	24.00	69.00

	WW	**	74.00	386.00	75.21	93.75	46.00				29.00	63.00	61.00	220.00
PL (cm)	DS	**	13.17	18.40	12.79	13.42	2.51	*	**	**	11.17	13.67	12.33	15.03
	WW	ns	13.85	10.94	13.45	14.25	2.23				11.67	14.53	12.47	17.37
IL (cm)	DS	**	1.72	4.50	1.72	1.91	0.78	0.12	**	**	1.07	1.67	1.50	2.83
	WW	**	1.87	4.10	1.87	2.10	0.96				1.00	1.77	1.70	2.77
P/I	DS	**	7.59	14.50	7.52	8.27	2.96	0.46	**	**	8.04	10.75	4.53	10.02
	WW	**	7.07	15.15	7.14	8.04	3.21				7.29	13.47	5.43	8.57
PH (cm)	DS	ns	22.23	16.00	21.70	22.76	3.47	0.10	**	**	17.00	20.70	20.00	26.00
	WW	**	23.00	28.60	22.71	24.38	5.88				19.00	24.50	21.00	32.00

*Note: SDW shoot dry weight, NS number of seeds per plant, NP number of pods per plant, NDP number of double-seeded pods per plant, SW seed weight per plant, PW pod weight per plant, 100SW 100-seed weight, HI harvest index, SP shelling percentage, DTF days to flowering, NL number of leaves per plant, PL petiole length, IL internode length, P/I petiole internode ratio, PH plant height, SD standard deviation, G*E interaction between treatment and genotypes, F pr = F-probability, * = Significant at (p = 0.05), ** = Significant at (p = 0.01).*

Table 3-6 Pearson correlation coefficient among mean variables for yield components and physiological traits in the F_4 segregating population derived from S19-3 × DodR.

	SDW	NS	NP	NDP	SW	PW	100sw	HI	SP	DTF	NL	PL	IL	P/I	PH
SDW	–	0.71**	0.71**	–0.21	0.80**	0.78**	0.13	0.38	0.38	0.09	0.88**	0.43	0.57*	–0.62*	0.70**
NS	0.43	–	0.99**	–0.27	0.82**	0.77**	–0.15	0.57*	0.16	0.36	0.83**	–0.15	–0.04	–0.18	0.19
NP	0.52	0.93**	–	–0.2	0.77**	0.73**	–0.24	0.49	0.22	0.36	0.82**	–0.15	–0.03	–0.19	0.22
NDP	0.13	0.51	0.43	–	–0.45	–0.46	–0.27	–0.64*	0.11	–0.3	–0.22	0.03	0.12	–0.19	0.2
SW	0.47	0.97**	0.90**	0.5	–	0.98**	0.37	0.82**	0.08	0.27	0.79**	0.13	0.17	–0.27	0.49
PW	0.57*	0.95**	0.90**	0.48	0.97**	–	0.4	0.80**	0.04	0.32	0.73**	0.13	0.21	–0.32	0.53
100SW	0.60*	0.52	0.5	0.19	0.65*	0.62*	–	0.44	–0.5	0.04	–0.07	0.39	0.3	–0.12	0.41
HI	–0.50	0.42	0.28	0.21	0.4	0.23	0.12	–	–0.08	0.22	0.5	–0.2	–0.18	0.06	0.14
SP	–0.17	0.36	0.31	0.2	0.37	0.19	0.39	0.76**	–	–0.06	0.42	0.2	0.25	–0.27	0.21
DTF	–0.25	–0.24	–0.21	–0.23	–0.08	–0.16	0.32	0.26	0.38	–	0.07	0.01	–0.23	0.38	–0.19
NL	0.81**	0.32	0.41	–0.08	0.31	0.4	0.54	–0.34	–0.06	–0.03	–	0.12	0.29	–0.41	0.48
PL	0.78**	0.32	0.4	0.2	0.41	0.47	0.56*	–0.44	–0.11	0.16	0.74**	–	0.78**	–0.42	0.63*
IL	0.51	0.32	0.26	0.36	0.4	0.41	0.56*	–0.17	0.1	0.12	0.44	0.70**	–	–0.85**	0.75**
P/I	0.04	–0.15	0.02	–0.37	–0.19	–0.10	–0.23	–0.24	–0.33	0.01	0.16	0	–0.69**	–	–0.67**
PH	0.63*	0.22	0.36	0.1	0.33	0.45	0.46	–0.41	–0.26	0.33	0.56*	0.74**	0.28	0.39	–

Note: : SDW shoot dry weight, NS number of seeds per plant, NP number of pods per plant, NDP number of double–seeded pods per plant, SW seed weight per plant, PW pod weight per plant, 100SW 100-seed weight, HI harvest index, SP shelling percentage, DTF days to flowering, NL number of leaves per plant, PL petiole length, IL internode length, P/I petiole internode ratio, PH plant height, * = Significant at (p = 0.05), ** = Significant at (p = 0.01), Values below diagonal are correlation coefficients among traits under well-watered treatment; values above diagonal are correlation coefficients among traits under drought-stressed treatment. Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation:*

0.70-0.90, 0.50-0.70, –0.70–-0.90, –0.50–-0.70.

3.3.9 Relationship between photosynthetic parameters and drought-related traits in the F_4 segregating population

A positively correlated with *E* ($r = 0.56$, $p < 0.05$) and *Ci* ($r = 0.67$, $p < 0.01$), negatively correlated with RWC ($r = –0.69$, $p < 0.01$) under well-watered treatment, and negatively correlated with seed width-length ratio ($r = 0.63$, $p < 0.05$) under drought-stressed treatment (Table 3-7). *Ci* negatively correlated with RWC ($r = –0.63$, $p < 0.01$) under well-watered treatment (Table 3-7). *Seed length* and *seed width* showed negative correlation with CCI, RWC and F_V/F_M under drought-stressed treatment ($p < –0.40$) (Table 3-7). LWUE and *gs* showed negative linear relationship under well-watered treatment ($r = –0.79$, $p < 0.05$), and under drought-stressed treatment ($r = –0.63$, $p < 0.01$) (Fig. 3-14). *A* and *Ci* showed positive linear relationship under well-watered treatment ($r = 0.67$, $p < 0.01$) and under drought-stressed treatment ($r = 0.42$, $p < 0.05$) (Figure 3-15).

Table 3-7 Correlation coefficient analysis of photosynthetic parameters, seed size and physiological traits under drought-stressed and well-watered conditions in the F_4 segregating population.

	CCI	RWC	F_V/F_M	*A*	*E*	*Ci*	*gs*	LWUE	*Seed width*	*Seed length*	*Seed WLRatio*	LA
CCI	–	0.45	0.49	–0.20	0.36	–0.06	–0.15	–0.10	–0.40	–0.60*	–0.31	0.03
RWC	–0.10	–	0.38	–0.12	–0.2	0.18	–0.13	0.25	–0.63*	–0.62*	0.05	0.12
F_V/F_M	–0.15	0.13	–	0.05	0.12	–0.04	–0.06	0.3	–0.56*	–0.42	–0.35	–0.28
A	0.05	–0.69**	0.15	–	0.17	0.42*	0.36	–0.03	–0.05	0.01	–0.63*	0.02
E	0.03	–0.49	0.3	0.55*	–	–0.02	0.07	–0.25	0.01	0.04	–0.36	0.26
Ci	0.01	–0.63*	–0.1	0.67**	0.44	–	0.45	–0.32	–0.26	–0.28	0.1	0.29
gs	0.04	–0.38	–0.16	0.65**	0.31	0.46	–	–0.63**	0.23	0.22	0.14	–0.43
LWUE	–0.19	0.09	0.59*	0.25	–0.08	–0.01	–0.79*	–	–0.26	–0.11	–0.21	0.09
Seed width	0.14	0.33	–0.39	–0.22	–0.22	–0.09	0.12	–0.42	–	0.88**	0.01	–0.32
Seed length	0.66*	0.24	–0.11	–0.34	–0.33	0.08	–0.26	0.07	0.17	–	–0.01	–0.24
Seed WLRatio	–0.14	0.19	–0.59*	–0.33	–0.41	–0.27	0.09	–0.39	0.61*	–0.02	–	–0.03
LA	–0.11	–0.22	0.34	0.55	0.22	–0.13	–0.08	0.45	–0.03	–0.29	–0.09	–

*Note: CCI chlorophyll content index, RWC leaf relative water content, F_V/F_M quantum yield of PSII photochemistry, A photosynthesis rate, E transpiration rate, Ci intracellular CO_2, gs stomatal conductance, LWUE leaf water use efficient, Seed WLRatio seed width-length ratio, LA leaf area, * = Significant at (p = 0.05), ** = Significant at (p = 0.01), Values below diagonal are correlation coefficients among traits under well-watered treatment; values above diagonal are correlation coefficients among traits under drought-stressed treatment. Highlighted values represent highly significant correlation between phenotypic traits. Different colour represent the highlighted correlation:*

0.70-0.90, 0.50-0.70, −0.70−−0.90, −0.50-−0.70.

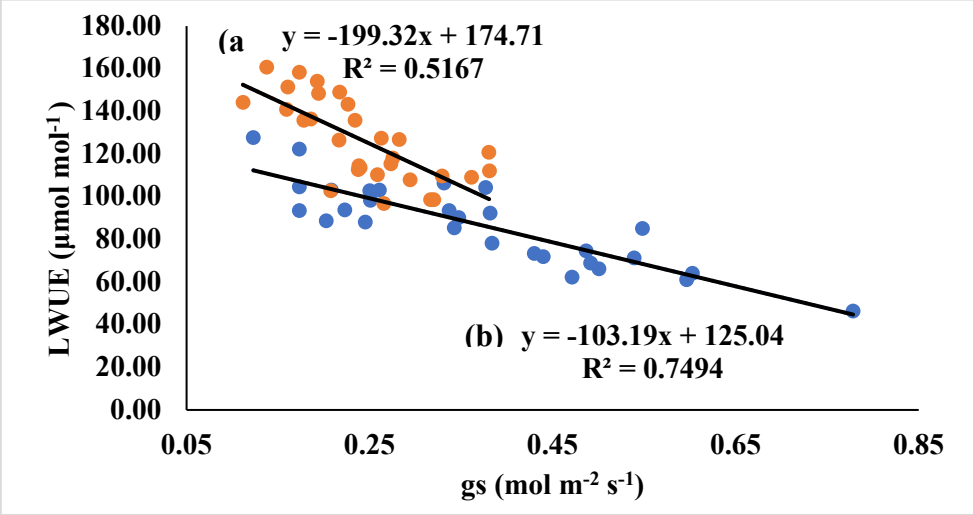


Figure 3-14 Regression for *stomatal conductance* (*gs*) and *leaf water use efficiency* (LWUE) under (a) drought-stressed and (b) well-watered treatment in the F_4 segregating population.

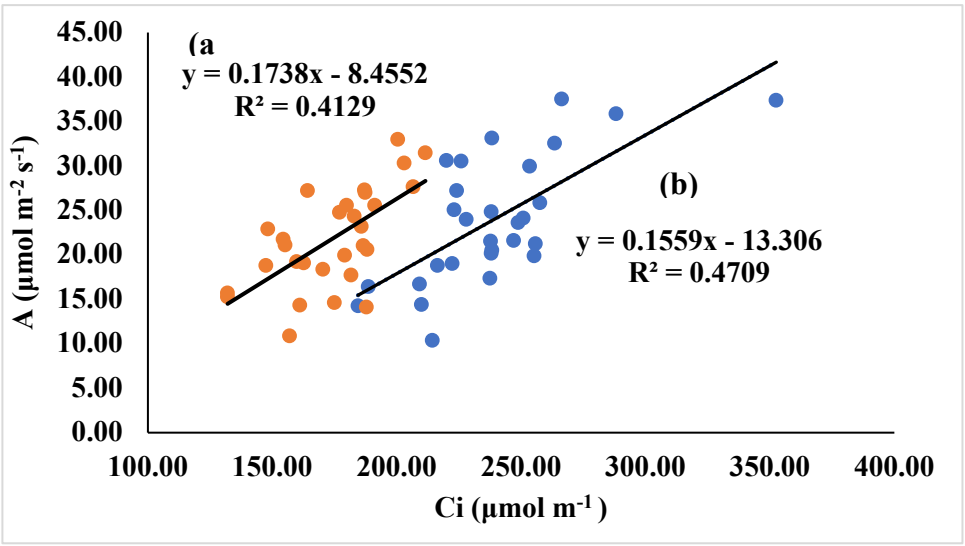


Figure 3-15 Regression for *intracellular* CO_2 (*Ci*) and *photosynthesis rate* (*A*) under (a) drought-stressed and (b) well-watered treatment in the F_4 segregating population.

3.3.10 Principal components analysis in the F_4 segregating population

A principal component analysis (PCA) was carried out to investigate the variation across 28 traits. The first two PCs explained 83.11% traits variation under well-watered treatment and 74.58% under drought-stressed treatment (Table 3-8). PC1 explained the majority of this

variation, 64.96% under well-watered treatment and 41.25% under drought-stressed treatment (Table 3-8). PC1 associated with high loadings were NL, LWUE*, Ci* and *A* under well-watered conditions, and NP, *Ci*, NS, and NL under drought-stressed conditions. PC2 associated with high loadings were LWUE, NP, NS and PH under well-watered conditions, and NP, NL, NS, and LWUE under drought-stressed conditions. PC3 associated with high loadings were LWUE and *Ci* under both conditions.

Table 3-8 Principal component analysis for 28 traits measured in the F_4 segregating population of bambara groundnut derived from S19-3 × DodR under well-watered and drought-stressed treatment.

	Well-watered			Drought-stressed		
	PC1	PC2	PC3	PC1	PC2	PC3
Latent roots	19175	5358	2631	7511	6069	2930
Percentage variation	64.96	18.15	8.91	41.25	33.33	16.09
A	**0.16**	–0.15	0.05	0.02	–0.02	0.11
gs	0.00	–0.01	0.00	0.00	0.00	0.00
Ci	**0.19**	**–0.79**	**0.52**	**0.32**	–0.36	**0.85**
E	0.01	–0.03	0.00	0.03	0.03	0.00
LWUE	**0.32**	**0.52**	0.75	–0.79	0.34	**0.46**
CCI	–0.01	–0.02	–0.02	0.02	0.02	–0.02
RWC	–0.02	0.05	0.01	0.00	0.02	0.05
F_V/F_M	0.00	0.00	0.00	0.00	0.00	0.00
SDW	0.16	0.03	–0.13	0.01	0.11	0.01
NS	0.07	0.12	0.05	**0.25**	**0.37**	0.09
NP	0.10	**0.20**	0.01	**0.40**	**0.55**	0.14
NDP	0.00	0.01	–0.01	0.00	0.00	0.00
PW	0.03	0.04	0.02	0.01	0.06	0.04
SW	0.02	0.03	0.02	0.01	0.05	0.02
HI	0.00	0.00	0.00	0.00	0.00	0.00
100SW	0.04	0.01	0.02	–0.14	0.03	0.04
SP	0.00	0.00	0.00	0.00	0.00	0.00
DTF	0.00	0.02	0.06	0.02	0.01	0.08
NL	**0.89**	–0.04	–0.38	0.14	**0.54**	0.05
PL	0.04	0.02	–0.02	–0.03	0.02	–0.02
IL	0.00	0.00	–0.01	–0.01	0.01	–0.01
P/I	0.01	0.00	0.04	0.03	–0.05	0.04
PH	0.05	0.07	0.04	–0.03	0.08	–0.01
Seed width	0.00	0.00	–0.01	0.00	–0.03	–0.07
Seed length	0.00	0.00	0.02	–0.02	–0.03	–0.08
Seed perimeter	0.03	0.04	0.06	–0.07	–0.01	0.00
Seed WLRatio	0.00	0.00	0.00	0.00	0.00	0.00
LA	0.05	0.05	–0.04	0.01	0.02	0.06

A photosynthesis rate, gs stomatal conductance, Ci intracellular CO_2, E transpiration rate, LWUE leaf water use efficient, CCI chlorophyll content index, RWC leaf relative water content, F_V/F_M quantum yield of PSII photochemistry, SDW shoot dry weight, NS number of seeds per plant, NP number of pods per plant, NDP number of double-seeded pods per plant, PW pod weight per plant, SW seed weight per plant, HI harvest index, 100SW 100-seed weight, SP shelling percentage, DTF days to flowering, NL number of leaves per plant, PL petiole length,

IL internode length, P/I petiole internode ratio, PH plant height, Seed WLRatio seed width-length ratio, LA leaf area.

3.4 Discussion

3.4.1 Physiological traits associated with improved drought resistance

Chlorophyll content and quantum yield PSII photochemistry (F_V/F_M) are non-stomatal limiting factors and capture light energy for plant photosynthesis (Chaves and Oliveira 2004; Keyvan 2010). In the present study, drought stress significantly reduced ($p < 0.05$) chlorophyll content index (CCI) and F_V/F_M in both the F_3 and F_4 segregating populations, which suggests that the ability of bambara groundnut plants to capture light energy for plant photosynthesis is significantly curtailed by drought conditions. Similar to these findings, Mafakheri et al. (2010) reported that drought significantly reduced total chlorophyll content ($p < 0.05$) under drought stress during vegetative growth in three chickpea cultivars. Rahbarian et al. (2011) also reported that drought stress reduced F_V/F_M in two drought-tolerant genotypes and two drought-sensitive genotypes of Chickpea. Additionally, in a study involving three bambara groundnut landraces, Mabhaudhi and Modi (2013) also showed that *chlorophyll content index* was lower under water-deficit compared to irrigated conditions. The F_V/F_M value was also reported to have declined by 25% at the end of drought stress trial involving three bambara groundnut landraces (Muhammad et al. 2016). Individual lines with higher CCI and F_V/F_M under both water regimes than the population mean, or parental lines are recommended as superior lines for genetic analysis and variety development (Appendix 5 and 6). These individual lines also have the potential for drought stress adaptation and with a further selection of superior lines done in advanced generations. Similar findings have been reported in maize that high correlation between F_V/F_M and leaf chlorophyll with yield, these parameters can be used for

evaluating the stress intensity and selecting the most tolerant genotype (Gholamin and Khayatnezhad 2011).

Leaf relative water content (RWC) is an indicator of plant water status revealing the stress intensity (Lawlor and Cornic 2002). In the present study, RWC increased in the initial stage of drought, declined gradually until the end of drought period, suggesting some individual lines (e.g., 6, 12, 20, 21 and 37) have the ability to adapt to drought stress. The present results are similar to those reported by other authors. For example, RWC was reported to have decreased by 21% – 24% with time after water deficit and increased by 13% – 17% after irrigation was resumed (Muhammad et al. 2016). Keyvan (2010) reported wheat cultivars with high RWC under drought stress conditions to be resistant. In the present study the following lines were identified as superior for RWC under drought conditions and will form a basis for genetic analysis and variety development (Appendix 7). However, only line 12 showed high CCI, F_V/F_M in the F_4 segregating generation and RWC in both the F_3 and F_4 segregating generations, suggesting further evaluation are needed in multiple generations or environments.

3.4.2 Yield components as indicators of drought resistance

Yield components and correlations between yield components and morphological traits could be used as surrogates in the selection of superior genotypes and breeding lines in crop improvement programmes (Xing and Zhang 2010; Ghaffari et al. 2012; Klimek-Kopyra et al. 2018). Ghaffari et al. (2012) reported that *plant height* positively correlated seed yield in both normal and drought stress conditions and *plant height* is an important determinant of seed yield in sunflower (*Helianthus annuus* L.). In the present study, positive correlations between yield components and morphological traits observed under both conditions in the F_3 segregating population, would suggest that more leaves, higher PL, IL and PH could lead to high seed yield. The negative correlation was observed between morphological traits and yield-related traits

including *100-seed weight* and *harvest index* in the twelve genotypes and two F_2 segregating population (Chapter 2). However, no consistent correlation was observed between morphological traits and *100-seed weight* and *harvest index* under both water regimes in the F_3 and F_4 segregating generations.

Seed weight per plant and *pod weight per plant* were positively correlated with *chlorophyll content index* under both conditions in the F_3 segregating population, which is consistent with reports in common bean (Ambachew et al. 2015), maize (Chen et al. 2016) and cotton (*Gossypium hirsutum* L.) (Karademir et al. 2009). In the F_3 segregating population, *seed weight per plant* and *pod weight per plant* negatively correlated with *leaf relative water content* under both conditions. Similar findings have been reported in snap bean (Omae et al. 2005). Kumar et al. (2018) also reported that *chlorophyll content index, leaf relative water content* and F_V/F_M could be reliable indicators for screening of drought tolerance in chickpea.

In the present study, a number of yield components were affected by drought including a reduction in *seed number*, *pod number, seed weight, pods weight and harvest index.* These results are similar to those reported by Mwale et al. (2007) in three bambara groundnut landraces. Chai et al. (2016) also reported that *100-seed weight* and *harvest index* decreased under drought stress in an F_5 bambara groundnut segregating population. In the present study, *harvest index* and *100-seed weight* were reduced by drought stress and the two positively correlated with *shoot dry weight, number of seeds per plant, number of pods per plant, seed weight per plant* and *pod weight per plant* suggesting that *harvest index* and *100-seed weight* could be considered as surrogates in breeding programme for drought tolerance in bambara groundnut (Chai et al. 2016). The significant differences observed among individual lines ($p < 0.05$) and the interaction between treatment and individual lines for yield components and

physiological traits, would suggest that individual lines in the segregating populations harbour genetic diversity and selection for lines with superior performance under multiple environmental conditions is feasible (Zhao et al. 2016). Individual lines with high *harvest index* and *100-seed weight* under both conditions could be selected as superior lines to develop new and adapted varieties for multiple locations (Appendix 8 and 9). Line 20 showed a high *harvest index* and *100-seed weight* in the F_3 segregating generation and RWC in both the F_3 and F_4 segregating generations, while line 33 and 50 showed a high *harvest index, 100-seed weight,* F_V/F_M and CCI in the F_3 segregating generation, which suggesting genetic diversity and phenotypic variation exist among individual lines in the segregating populations and these selected lines need further validation for improved variety selection in breeding programme.

3.4.3 Drought stress impacts photosynthesis and LWUE

Stomatal closure usually happened during the initial stages of drought stress, which results in the reduction of transpiration in plant leaves, a decrease in CO_2 flow into leaves, a decline in net photosynthesis, and ultimately reduced plant growth (Mafakheri et al. 2010; Ashraf and Harris 2013; Mabhaudhi et al. 2013).

In the present study, *leaf water use efficiency* (LWUE), calculated as *A/gs*, increased after drought stress was imposed, then declined gradually after irrigation was resumed under drought-stressed treatment in the F_4 segregating population. Singh and Reddy (2011) reported that LWUE increased under drought stress in 15 cowpea genotypes, suggesting that stomatal regulation was a major limitation to photosynthesis and plant growth. The positive correlation between *A* and *Ci* under well-watered treatment ($r = 0.67$, $p < 0.05$) and under drought-stressed treatment ($r = 0.42$, $p < 0.05$) suggests that lower internal CO_2 accumulation concentration during drought is responsible for the reduction in photosynthesis (Lidon and Cebola 2012). LWUE is regulated by *gs* and multiple factors including the available energy impinging on the

leaf, vapour pressure deficit, and aerodynamic exchange (Hatfield and Dold 2019). The negative correlation between LWUE and *gs* under well-watered treatment ($r = –0.79$, $p < 0.05$) and under drought-stressed treatment ($r = –0.63$, $p < 0.01$) suggests that *gs* decreases faster than *A*, leading to increased LWUE under drought stress (Chaves and Oliveira 2004). Genotypes with high *stomatal conductance* and LWUE in response to drought stress were suggested to have good drought tolerance and adaptation ability (Singh and Reddy 2011). For example, a drought-tolerant cowpea cultivar (PO) maintained higher photochemical activity and leaf gas exchange under water deficit and showed faster recovery of photosynthesis after irrigation was resumed than the drought-sensitive cultivar (SI), revealing possible mechanisms enabling plants to overcome stressful conditions (Bastos et al. 2011; Rivas et al. 2016). Plants maintain high water status by reducing stomatal conductance during periods of drought stress, which involves either drought avoidance or tolerance or both mechanisms (Turner et al. 2001; Chaves and Oliveira 2004; Kavar et al. 2008). In the present study, we identified individual lines with higher LWUE and higher *gs* than population mean or parental lines under both conditions during drought period, which could be recommended as drought resistant lines for genetic analysis and variety development in breeding programmes (Appendix 10 and 11). Some overlapped recommended individual lines were observed among the different groups, for example, Line-20 showed high *harvest index* and *100-seed weight* in the F_3 segregating generation, RWC in both the F_3 and F_4 segregating generations and high *gs* in the F_4 segregating generation, Line-12 showed high CCI and F_V/F_M in the F_4 segregating generation, RWC in both the F_3 and F_4 segregating generations and high LWUE in the F_4 segregating generation. These selected lines can be used as candidate lines to develop improved varieties in the breeding programme of bambara groundnut.

3.4.4 Drought stress can have an impact on seed size

Seed size is a major factor associated with food and seed quality including germination rate and seedling establishment (Arellano and Peco 2012; Kesavan et al. 2013). In the present study, although drought led to a reduction in *seed number per plant, seeds weight per plant* and *100-seed weight,* a significant increase in *seed width*, *seed length* and *seed perimeter* was observed in seeds harvested from drought-stressed plants. This contradicts previous findings in various crops that drought stress decreased seed size e.g. in soybean (Samarah et al. 2004), reduced seed width and thickness in wheat (Konopka et al. 2007), seed weight and seed size in cowpea (Ahmed and Suliman 2010). The effect of drought stress on seed size has conducted in the F_4 segregating population for one season, this observation warrants further studies in multiple seasons and locations to determine the impact of drought on seed size and especially seed width and length in bambara groundnut.

3.5 Conclusions

A significant reduction in *shoot dry weight, seed weight per plant, harvest index, shelling percentage*, *chlorophyll content index* and *quantum yield PSII photochemistry* (F_V/F_M) was observed under drought conditions both in the F_3 and F_4 segregating populations. However, some individual lines showed superior performance and these (Tables 3-9, 3-10, 3-11, 3-12 and 3-13) have been recommended for further advancement into RILs (recombinant inbred lines) and breeding lines for variety development. The RILs would be important resources for genetic analysis of important traits especially targeting drought, yield and yield components. Yield components including *seed weight per plant* and *pod weight per plant* was positively correlated with *chlorophyll content index*, while negatively correlated with *leaf relative water content* under both conditions. *Seeds weight per plant* and *harvest index* in particular could be used as surrogates yield component traits for drought resistance breeding.

Stomatal conductance, photosynthesis rate, transpiration rate and *intracellular CO_2* were significantly reduced while *leaf water use efficiency* significantly increased towards the end of drought period. Additionally, a strong linear correlation was observed between *stomatal conductance* and *leaf water use efficiency, photosynthesis rate* and *intracellular CO_2 accumulation*. Individual lines with higher *gs* and/or LWUE were selected (Tables 3-14 and 3-15) for further studies in order to identify drought resistant adaptive traits relevant for the cultivation of bambara groundnut in drought-prone areas.

Chapter 4 Genetic linkage mapping and identification of QTLs associated with drought resistance in Bambara groundnut (*Vigna subterranea* (L.) Verdc)

4.1 Introduction

Bambara groundnut is an underutilised and drought-resistant leguminous crop with high protein content (16% – 25%). The crop is mainly grown by subsistence farmers and served as an edible protein source in Africa (Cleasby et al. 2016; Massawe et al. 2016; Atoyebi et al. 2017; Halimi et al. 2019). Genetic maps with reliable markers are useful tools to identify QTLs and potential candidate genes that regulate complex traits, accelerating the marker-assisted breeding process and shorten the breeding cycle (Conson et al. 2018). Understanding the genetic basis of bambara groundnut and identification of molecular markers for traits of interest are prerequisites for deploying molecular breeding to develop superior genotypes (Kullan et al. 2012). However, to date a limited number of studies have been reported to focus on mapping quantitative and qualitative loci to a location on the chromosomes of bambara groundnut (Ahmad et al. 2016; Chai et al. 2017; Ho et al. 2017).

The first genetic map reported in bambara groundnut consisted of twenty genetic linkage groups, which were identified using AFLP markers and SSR markers in the F_2 segregating population derived from a 'wide' cross between domesticated type (DipC) and wild type (VSSP11), and covered 516 cM (centimorgan) of bambara groundnut genome (Basu et al. 2007). First intraspecific genetic linkage map consisting of 21 linkage groups with a total genetic distance of 608.3 cM was constructed using 209 DArT dominant and 29 co-dominant SSR markers in the F_3 segregating population derived from two domesticated landraces, Tiga Nicuru × DipC, in bambara groundnut (Ahmad et al. 2016). Two stable QTLs were mapped

for *internode length* and *growth habit*, respectively, under controlled environment and field conditions (Ahmad et al. 2016).

The first expression marker-based genetic map using gene expression markers (GEMs), which were developed after cross-hybridisation of bambara groundnut leaf RNA to the Affymetrix Soybean Genome GeneChip using 65 F_5 segregating population derived from Tiga Nicuru × DipC, was reported to consist of 13 linkage groups containing 218 GEMs and covered 982.7 cM of bambara groundnut genome (Chai et al. 2017). Chai et al. (2017) identified co-localised QTLs mapped on LG11 in GEM map for agronomic traits including *internode length, peduncle length, pod number per plant, seed number per plant, pod weight per plant, seed weight per plant* and *harvest index* in the irrigated F_5 segregating population of bambara groundnut, which suggests these traits are probably controlled by the same underlying genes. QTLs associated with *pod number per plant* and *harvest index* in GEM map in bambara groundnut have also been reported to be affected by drought stress (Chai et al. 2017). This GEM map presented the possibility of translating information and resources from major and model plants to underutilised crops. In addition, Ho et al. (2017) demonstrated the use of limited genetic information in bambara groundnut but linked to well characterised closely related legumes i.e., common bean, adzuki bean (*Vigna angularis*), mung bean and soybean to identify potential candidate genes underlying traits of interest in bambara groundnut through conserved syntenic locations of QTLs in the sequenced and well-annotated genomes of closely related species. A combination of population-specific and pre-selected common markers were used to construct two individual intraspecific genetic maps in bambara groundnut from the two crosses: genetic map of IITA686 × Ankpa4, which was derived from 263 F_2 segregating population, gave 11 linkage groups comprising of 223 DArTseq markers and covered 1,395.2 cM while a genetic map of Tiga Nicuru × DipC, derived from 71 F_3 segregating population, showed 11 linkage

groups consisting of 293 DArTseq markers and covered 1,376.7 cM in bambara groundnut (Ho et al. 2017). A significant QTL for *internode length* mapped on LG2 (50.6 cM; flanking markers between 47.6 – 54.4 cM), explaining 33.4% phenotypic variation observed in this cross, showed syntenic blocks at *Pv03* (38.4 – 39.1 Mbp; common bean), *Va11* (12.5 – 17.4 Mbp; azuki bean) and *Vr07* (39.4 – 43.5 Mbp; mung bean) (Ho et al. 2017). Close correspondence observed between bambara groundnut and common bean allows sequenced genomes of common bean to be used as an initial 'pseudo' physical map for bambara groundnut and identify potential genes underlying this corresponding region in bambara groundnut for *internode length*. Constructing genetic maps are essential approaches to identify genetic architecture and QTL responsible for phenotypic variation in bambara groundnut breeding programme (Chai et al. 2017). The first whole genome sequence of bambara groundnut, which was released recently by the African Orphan Crops Consortium (AOCC) (https://bioinformatics.psb.ugent.be/orcae/aocc/overview/Vigsu), also provided a better understanding of potential candidate genes involved in agronomic traits regulation (Chang et al. 2018). Moreover, these identified candidate genes and their related QTLs will speed up molecular marker-assisted MAS in bambara groundnut breeding programmes.

Varshney et al. (2014) identified nine QTL clusters from two mapping populations – ICCRIL03 (ICC 4958 × ICC 1882) and ICCRIL04 (ICC 283 × ICC 8261) under drought conditions in chickpea. One QTL Cluster 5 on CaLG04 showed high potential to enhance drought tolerance in chickpea, which contains stable and consistent QTLs for drought tolerance-related traits, namely, *100-seed weight, root length density, days to 50% flowering, days to maturity, biomass, plant height, pods/plant, harvest index, root dry weight/total plant dry weight ratio, shoot dry weight, seeds/plant* and *yield* with up to 58.20 % phenotypic variation explanation (Varshney et al. 2014). Dramadri et al. (2019) identified eighteen significant QTLs for *days to*

flowering, days to maturity, harvest index, pod-partitioning index, pod weight per plant and *seed yield per plant* under drought stress and non-stress conditions in a recombinant inbred line (RIL) mapping population of common bean. Significant QTLs for *seed yield per plant* co-located with *pod weight per plant* on Pv01 and on Pv02, with *days to flowering* and *days to maturity* on Pv03, and *harvest index* on Pv06 under drought stress conditions (Dramadri et al. 2019). One QTL, SY 3.3 on Pv03 for *seed yield per plant* has been reported to be consistent with the previous report in Mesoamerican common bean (Hoyos-Villegas et al. 2016), which should be useful to improve seed yield of Andean common beans under drought stress conditions (Dramadri et al. 2019).

In the present study, we mapped QTLs for yield-related and morphological traits under well-watered conditions in the F_2 segregating population, under drought-stressed and well-watered conditions in the subsequent F_3 and F_4 segregating populations of a bambara groundnut cross, S19-3 × DodR. This study provided critical insights into how genetic features control these traits in bambara groundnut in response to drought stress.

4.2 Materials and Methods

4.2.1 Mapping population

A total of 114 individual lines of F_2 segregating population derived from a cross between a drought-tolerant single genotype (S19-3, collected from Namibia) and presumed drought susceptible single genotype (DodR, collected from Tanzania) were used to evaluate QTLs involved in yield components and morphological traits (refer to Chapter 2). Eighty-six of F_2 lines and parental lines were selected for Diversity Arrays Technology sequencing (DArTseq) prior to development of genetic linkage map. In addition, 114 individual lines each in the subsequent F_3 and F_4 segregating populations were evaluated under drought stress (refer to

Chapter 3) to determine the impact of drought on yield components, morphological and physiological traits and were mapped to evaluate QTLs involved in these traits.

4.2.2 Leaf DNA extraction

One to two fresh and young leaflets from the same leaf of all 114 F_2 segregating lines and parental lines were collected individually and flash-frozen in liquid nitrogen. Leaf samples were kept in a –80°C freezer until they were used. Approximately 0.1 g of freeze-dried leaf samples were ground into fine powder by EPPI-pestle for homogenising in Eppendorf ® -type 1.5ml/2.0ml reaction tubes (exact fit) (Eppendorf, Germany) in a 2µl pre-chilled microcentrifuge tube with liquid nitrogen.

Genomic DNA was extracted from freeze-dried leaf samples following the instruction manual from DNeasy Plant Mini Kit (Qiagen GmbH, Hilden Germany). The quantity and quality of DNA was estimated visually on a 1% agarose gel with ethidium bromide staining, NanoDrop 1000 (Thermo Scientific, NH, USA). and test digestion using a restriction enzyme, *Hind*III (NEB, USA).

One restriction endonuclease (RE), *Hind*III (NEB, USA), was used for RE digestion to check the quality of DNA samples. A total volume of 10 µl was prepared in 0.2 ml tube containing 2 µl of 50 ng/µl genomic DNA, 0.2 µl of *Hind*III enzyme (10,000 units), and 7.8 µl nuclease-free water. RE digestion was carried out with 1 hour of incubation at 37^{o}C, and then subjected to 1% agarose gel electrophoresis. High-quality DNA samples were used for DArTseq analysis (Fig. 4-1).

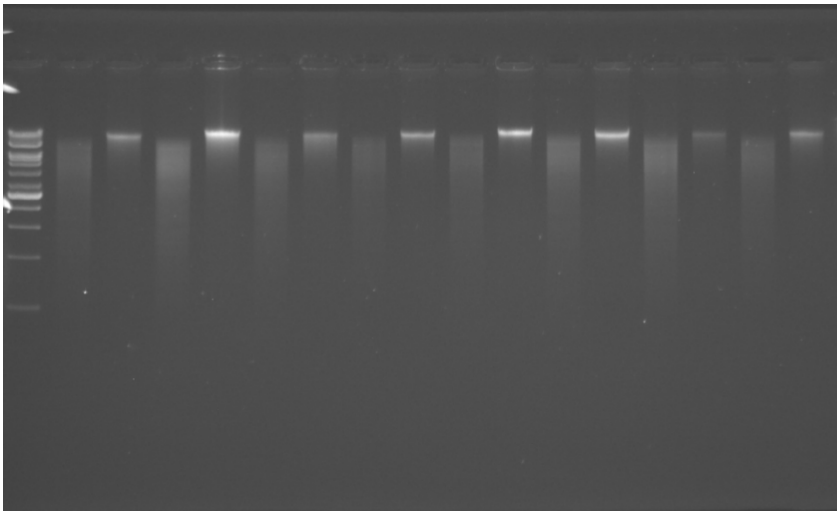


Figure 4-1 Example of restriction endonuclease (RE) *Hind*III digestion products and DNA samples for DArTseq analysis in the F_2 segregating population (S19-3 × DodR) in bambara groundnut.

DNA concentration was then adjusted to 50 ng/µl prior to DArTseq genotyping. Around 100 ng DNA samples of 86 F_2 segregating lines and parental lines (S19-3 and DodR) were sent to DArT Pty. Ltd. (Canberra, Australia) for DArTseq genotyping. This latter consisted of a genomic complexity reduction by RE combinations (PstI and TaqI) that digest genomic DNA, followed by ligation of adapters, PCR amplification of fragments digested by both RE, and their sequencing on Illumina Hiseq 2500. Processing of generated sequences, and SNP calling were then carried out within the DArT analytical pipeline (Kilian et al. 2012).

4.2.3 DArTseq markers selection and construction of genetic linkage map

Appendix 12 presented the linkage map group, position, trimmed sequence and SNP of DArTseq markers. The presence or absence (0/1) scoring of co-dominant DArTseq markers for each individual line in the F_2 segregating population was converted into genotype codes, either (a, c) or (b, d) by comparison with the parental lines. DArTseq markers were assigned as ‘a’, ‘b’ and ‘h’ as appropriate in each individual line according to the scoring pattern in both parental lines. The markers that were scored as 1:1 and/or 0:0 in parental lines, which were considered as monomorphic markers or unreliable, were filtered out.

A Chi-square goodness-of-fit test in JoinMap v4.1 (Van Ooijen et al. 2009) was used to evaluate any discrepancy from the expected segregation ratios (1:2:1 for F_2 population) at a significance level of $p < 0.05$. A total of 843 polymorphic DArTseq markers were pre-selected from 6,396 DArTseq markers and a total of 48 from 843 DArTseq markers showing distorted segregation ($p < 0.05$) from expected Mendelian ratios were excluded. A total of 795 population-specific DArTseq markers were selected and 86 F_2 individual lines were included to construct the genetic linkage map using JoinMap v4.1 (Van Ooijen et al. 2009). Markers were sorted to linkage groups with the *Create Groups Using the Grouping Tree* function of JoinMap 4 (Van Ooijen 2006). The grouping of markers between the logarithm of the odds (LOD) 2.0 and 10.0 with a step of 0.5 and the Independence LOD option was adopted. The Haldane mapping function with default calculation settings (recombination frequency < 0.4 and LOD > 1.0, ripple value = 1, jump in goodness-of-fit threshold = 5) was selected to calculate genetic distances on the basis of recombination frequencies. The markers that showed double cross-over events between two neighbouring markers within a map distance of 1 to 3 cM were manually removed. The nearest neighbour fit, the nearest neighbour stress (Fit & Stress) and plausible positions produced by the maximum likelihood (ML) mapping algorithm were used as indicators whether a locus fitted well between its neighbouring loci. The optimal positions of each marker in the final genetic map were used for QTL analysis.

4.2.4 Trait measurements

Phenotypic traits (Table 4-1) were measured using F_2 segregating population under well-watered conditions (refer to Chapter 2), and also using F_3 and F_4 segregating populations under well-watered and drought-stressed conditions (refer to Chapter 3).

Table 4-1 Evaluation of traits in the F_2, F_3 and F_4 segregating populations.

Traits	Trait abbreviation	Evaluation method	Measured population and time
Days to flowering	DTF	From sowing date to the first open flower	F_2, F_3, F_4 generation at flowering stage
Plant height (cm)	PH	From the ground level to the tip of the highest point, including the terminal leaflet	F_2, F_3, F_4 generation at harvest
Petiole length (cm)	PL	The average length of three leaves	F_2, F_3, F_4 generation at harvest
Internode length (cm)	IL	The average length of three stems	F_2, F_3, F_4 generation at harvest
Number of leaves per plant	NL	One leaf including three leaflets	F_2, F_3, F_4 generation at harvest
Shoot dry weight (g)	SDW	Above-ground after dried in oven at 70ºC for 3 - 5 days	F_2, F_3, F_4 generation at harvest
Number of pods per plant	NP	Pod number per plant	F_2, F_3, F_4 generation at harvest
Number of seeds per plant	NS	Seed number per plant	F_2, F_3, F_4 generation at harvest
Pod weight per plant (g)	PW	Pod weight per plant	F_3, F_4 generation at harvest
Seed weight per plant (g)	SW	Seed weight per plant	F_2, F_3, F_4 generation at harvest
Petiole internode ratio	P/I	Petiole length / internode length	F_2, F_3, F_4 generation at harvest
100-seed weight (g)	100SW	Seed weight / number of seeds per plant *100	F_2, F_3, F_4 generation at harvest
Harvest index	HI	Seed weight / (pod weight + shoot dry weight)	F_2, F_3, F_4 generation at harvest
Shelling percentage (%)	SP	Seed weight / pod weight *100	F_3, F_4 generation at harvest
Leaf chlorophyll content index	CCI	Three readings were taken per leaf and averaged to give a final reading by chlorophyll meter SPAD-502 (Spectrum Technologies, Inc)	F_3, F_4 generation (drought was imposed after 100% flowering until early pod-filling stage)
Leaf relative water content (%)	RWC	RWC = [(fresh weight – dry weight) / (turgid weight – dry weight)] ×100	F_3, F_4 generation (drought was imposed after 100% flowering until early pod-filling stage)
Quantum yield PSII photochemistry (F_V/F_M)	F_V/F_M	Dark-adapted leaf samples for 30 minutes were estimated by FlourPen FP 100 (PSI, CZ)	F_3, F_4 generation (drought was imposed after 100% flowering until early pod-filling stage)
Seed perimeter (mm)	PER	Analyzed from scanned images	F_5 seeds at harvest
Seed length (mm)	LEN	Analyzed from scanned images	F_5 seeds at harvest
Seed width (mm)	WID	Analyzed from scanned images	F_5 seeds at harvest
Seed width length ratio	WLR	Analyzed from scanned images	F_5 seeds at harvest
Seed coat colour	SCC	Black and red	F_4 and F_5 seeds at harvest

4.2.5 QTLs detection

Genetic linkage map and phenotypic data from F_3 and F_4 segregating populations were subjected to QTL analysis using MapQTL 6.0 software (Van Ooijen et al. 2009). The

significant threshold of the Genome-Wide (GM) logarithm of odds (LOD) threshold was obtained from permutation testing using 1,000 repetitions at $p < 0.05$ (5%). Interval mapping (IM) was carried out following the permutation test and the LOD values from IM was compared with GW LOD threshold at $p < 0.05$ from the permutation test. Significant QTLs were detected if the LOD score was equivalent or higher than GM LOD threshold. Putative QTLs were detected if the LOD score was lower than GM LOD threshold by up to a 1-LOD interval.

The non-parametric Kruskal-Wallis (KW) test was performed to determine the significant level of all marker loci associated with the quantitative traits under both water regimes in the F_3 and F_4 generations. Non-parametric KW analysis was used for non-normal distributed quantitative traits. KW test ranks all individuals according to the quantitative trait and classifies them according to their marker genotype (Van Ooijen and Maliepaard 1996). MapChart 2.3.2 (Voorrips 2002) was used to depict the linkage groups and QTLs.

4.3 Results

4.3.1 Linkage map and marker distribution

At LOD > 3.5 of grouping independence in regression mapping approach, 795 of 843 polymorphic markers were assigned into eleven linkage groups. The final genetic linkage map was constructed by 228 DArTseq markers after pre-selection and covered 1,040.92 cM of the genome with an average marker density of 5.23 cM (Table 4-2). Among the linkage groups, LG3 with 27 DArTseq markers was the longest group covering 171.67 cM followed by LG2 with a length of 152.07 cM and LG5 with a length of 119.70 cM (Table 4-2). LG1B with four DArTseq markers was the shortest group covering 4.90 cM, followed by LG6B with a length of 8.10 cM. LG7A has the longest average distance of 8.38 cM and the second-longest distance of 35.45 cM between two adjacent markers (Table 4-2).

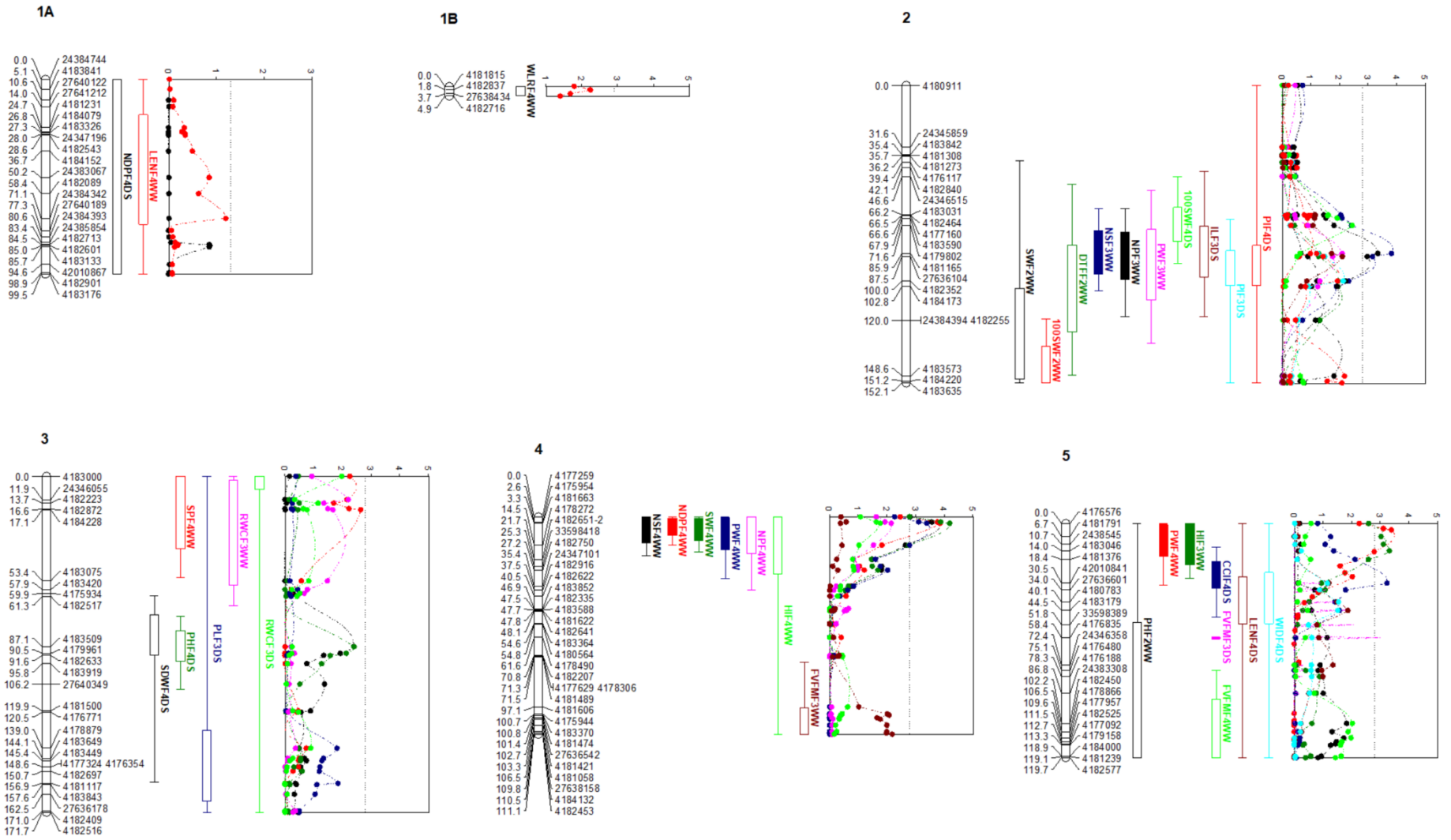
Table 4-2 DArTseq marker distribution and distance within individual linkage groups on the genetic linkage map of F_2 segregating population derived from S19-3 × DodR in bambara groundnut.

Linkage group	Number of markers	Length (cM)	Average marker interval (cM)	Max distance between adjacent markers (cM)
LG1A	22	99.53	4.52	13.51
LG1B	4	4.90	1.23	1.85
LG2	22	152.07	6.61	31.58
LG3	27	171.67	6.36	36.32
LG4	32	111.14	3.47	25.54
LG5	24	119.70	4.60	15.34
LG6A	18	56.53	3.14	20.60
LG6B	5	8.10	1.62	3.82
LG7A	9	75.43	8.38	35.45
LG7B	7	10.95	1.56	8.94
LG8	21	63.86	2.78	19.15
LG9	13	78.10	6.01	14.35
LG10	11	43.76	3.98	15.89
LG11	13	45.18	3.23	23.34
Total	228	1040.92	57.49	265.68
Mean	20.73	94.63	5.23	24.15

4.3.2 Detection of QTLs associated with yield components, morphological and physiological traits under well-watered and drought-stressed conditions in the segregating populations

Significant and putative QTLs for fourteen yield components and morphological traits, namely *shoot dry weight* (SDW), *number of pods per plant* (NP), *number of seeds per plant* (NS), *pod weight per plant* (PW), *seed weight per plant* (SW), *100-seed weight* (100SW), *harvest index* (HI), *shelling percentage* (SP), *days to flowering* (DTF), *plant height* (PH), *petiole length* (PL),

internode length (IL)*, number of leaves per plant* (NL) and *petiole internode ratio* (P/I) have been detected under well-watered conditions in the F_2 segregating population and under well-watered and drought-stressed conditions in the F_3 and F_4 segregating populations (Fig. 4-2). Most QTLs for yield components and morphological traits were distributed in LG2, LG3, LG4, LG5, LG7A and LG10. A total of two significant and seven putative QTLs for three physiological traits, *namely leaf chlorophyll content index* (CCI), *quantum yield PSII photochemistry* (F_V/F_M) *and leaf relative water content* (RWC) were detected under well-watered and drought-stressed conditions in the F_3 and F_4 segregating generations (Fig. 4-2). Six putative QTLs for *seed perimeter* (PER), *seed length* (LEN), *seed width* (WID), and *seed width-length ratio* (WLR), were distributed on LG1A, LG1B, LG5, LG6 and LG11 under well-watered and drought-stressed conditions in the F_4 segregating generation (Fig. 4-2).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28.0 24347196
28.6 4182543
36.7 4184152
50.2 24383067
58.4 4182089
71.1 24384342
77.3 27640189
80.6 24384393
83.4 24385854
84.5 4182713
85.0 4182601
85.7 4183133
94.6 42010867
98.9 4182901
99.5 4183176
NDPF4DS
LENF4WW
1B
0.0 4181815
1.8 4182837
3.7 27638434
4.9 4182716
WLRF4WW
2
0.0 4180911
31.6 24345859
35.4 4183842
35.7 4181308
36.2 4181273
39.4 4176117
42.1 4182840
46.6 24346515
66.2 4183031
66.5 4182464
66.6 4177160
67.9 4183590
71.6 4179802
85.9 4181165
87.5 27636104
100.0 4182352
102.8 4184173
120.0 24384394 4182255
148.6 4183573
151.2 4184220
152.1 4183635
SWF2WW
100SWF2WW
DTFF2WW
NSF3WW
NPF3WW
PWF3WW
100SWF4DS
ILF3DS
PHF3DS
PIF4DS
3
0.0 4183000
11.9 24346055
13.7 4182223
16.6 4182872
17.1 4184228
53.4 4183075
57.9 4183420
59.9 4175934
61.3 4182517
87.1 4183509
90.5 4179961
91.6 4182633
95.8 4183919
106.2 27640349
119.9 4181500
120.5 4176771
139.0 4178879
144.1 4183649
145.4 4183449
148.6 4177324 4176354
150.7 4182697
156.9 4181117
157.6 4183843
162.5 27636178
171.0 4182409
171.7 4182516
SPF4WW
PHF4DS
SDWF4DS
PLF3DS
RWCF3WW
RWCF3DS
4
0.0 4177259
2.6 4175954
3.3 4181663
14.5 4178272
21.7 4182651-2
25.3 33598418
27.2 4182750
35.4 24347101
37.5 4182916
40.5 4182622
46.9 4183852
47.5 4182335
47.7 4183588
47.8 4181622
48.1 4182641
54.6 4183364
54.8 4180564
61.6 4178490
70.8 4182207
71.3 4177629 4178306
71.5 4181489
97.1 4181606
100.7 4175944
100.8 4183370
101.4 4181474
102.7 27636542
103.3 4181421
106.5 4181058
109.8 27638158
110.5 4184132
111.1 4182453
NSF4WW
NDPF4WW
SWF4WW
PWF4WW
NPF4WW
HIF4WW
FVFMF3WW
5
0.0 4176576
6.7 4181791
10.7 2438545
14.0 4183046
18.4 4181376
30.5 42010841
34.0 27636601
40.1 4180783
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51.8 33598389
58.4 4176835
72.4 24346358
75.1 4176480
78.3 4176188
86.8 24383308
102.2 4182450
106.5 4178866
109.6 4177957
111.5 4182525
112.7 4177092
113.3 4179158
118.9 4184000
119.1 4181239
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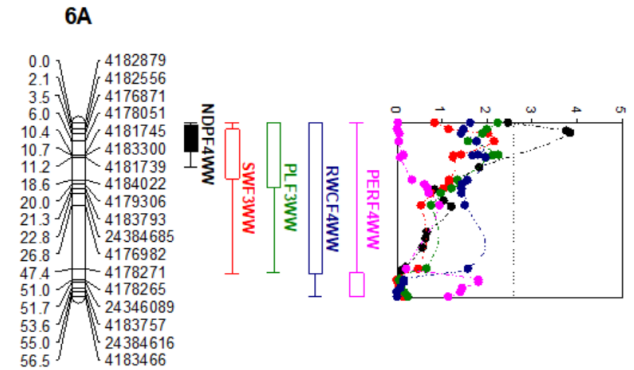
PWF4WW
HIF3WW
CCIF4DS
FVFMF3DS
FVFMF4WW
LENF4DS
WDF4DS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18.6 4184022
20.0 4179306
21.3 4183793
22.8 24384685
26.8 4176982
47.4 4178271
51.0 4178265
51.7 24346089
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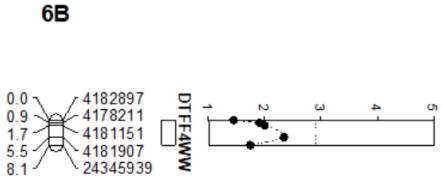
SWF3WW
PLF3WW
RWCF4WW
PERF4WW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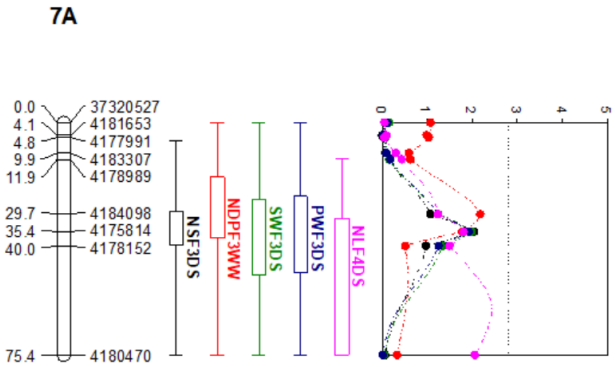
0.9 4178211
1.7 4181151
5.5 4181907
8.1 24345939
DTFF4WW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11.9 4178989
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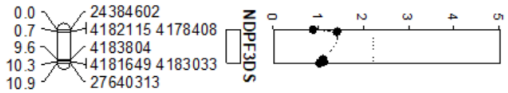
40.0 4178152
75.4 4180470
NSF3DS
NDPF3WW
SWF3DS
PWF3DS
NLF4DS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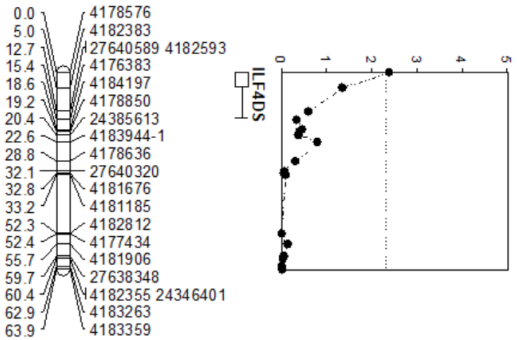
NDPF3DS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22.6 4183944-1
28.8 4178636
32.1 27640320
32.8 4181676
33.2 4181185
52.3 4182812
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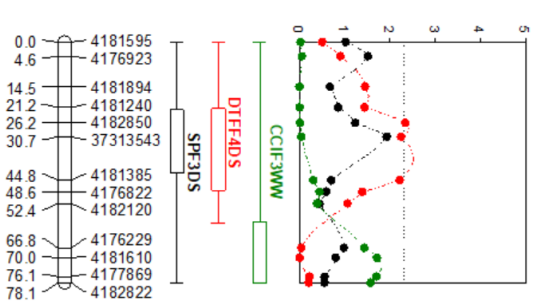
63.9 4183359
ILF4DS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48.6 4176822
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66.8 4176229
70.0 4181610
76.1 4177869
78.1 4182822
SPF3DS
DTFF4DS
CCIF3WW

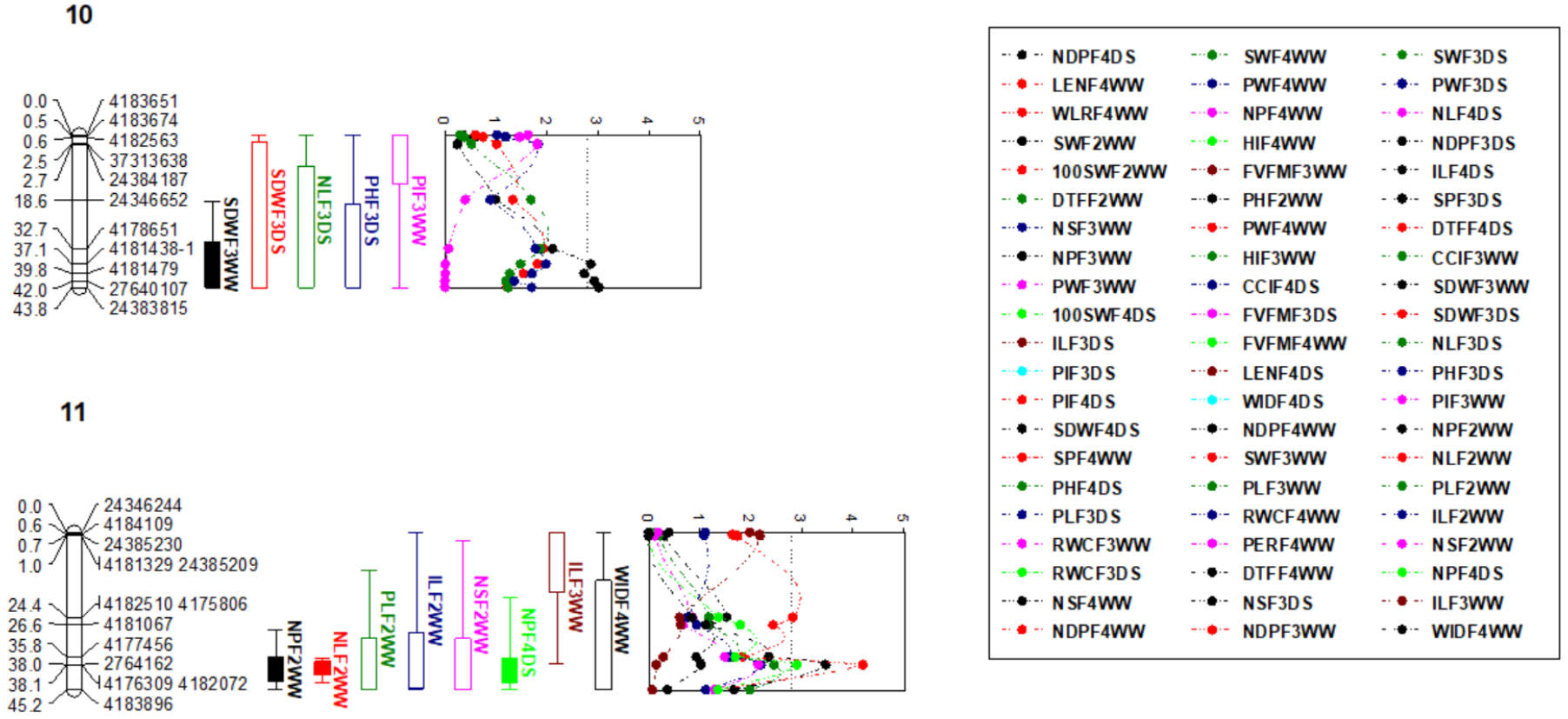


Figure 4-2 Map position of the quantitative trait loci (QTL) under well-watered (WW) and drought-stressed (DS) in the F_2, F_3 and F_4 segregating populations developed from S19-3 × DodR. Right: positions of markers (cM); left: name of the markers. Rectangular bars represent the 1- and 2-LOD QTL interval. Solid rectangular bars represent significant QTLs, while blank bars represent putative QTLs. LG1, LG6 and LG7 were divided into subgroups '1A' and '1B', respectively, based on the association observed in the maximum likelihood mapping (MLM) due to insufficient linkage to complete the map using regression mapping (RM).

4.3.2.1 QTLs associated with yield components and morphological traits in the F_2, F_3 and F_4 segregating generations

Significant QTL for NS (LOD: 3.87, 19.1% of the PVE), NP (LOD: 3.34, 16.9% of the PVE) and putative QTL for PW (LOD: 2.10, 11.0% of the PVE) under well-watered conditions and putative QTL for IL (LOD: 2.11, 11.0% of the PVE) under drought-stressed conditions in the F_3 segregating population was co-located on LG2 (85.95 cM, nearest marker: 4181165 and 27636104) with overlapping confidence intervals (Table 4-3). Significant QTL for NS (LOD: 3.87, 21.9% of the PVE), NDP (LOD: 3.85, 21.8% of the PVE), SW (LOD: 4.19, 23.5% of the PVE), PW (LOD: 3.5, 19.9% of the PVE) and putative QTL for NP (LOD: 2.16, 12.8% of the PVE) and HI (LOD: 1.77, 11.3% of the PVE) under well-watered conditions in the F_4 segregating population were co-located on LG4 (3.29 cM, nearest marker: 4181663 and 4175954) with overlapping confidence intervals (Table 4-3). In addition to the co-located QTL on LG4, significant QTL for NDP and PW under well-watered conditions in the F_4 segregating population were also mapped on LG6A (LOD: 2.75, 16.1% of the PVE) and LG5 (LOD: 3.03, 17.6% of the PVE), respectively (Table 4-3).

Significant QTL for NP (LOD: 2.91) under drought-stressed conditions in the F_4 segregating population was observed to have mapped on LG11 (38.03 cM, nearest marker: 2764162 and 4182072), explaining 16.0% of the phenotypic variation (Table 4-3). However, putative QTL for NP (LOD: 2.16) was detected on LG4 (3.29 cM, nearest marker: 4181663) under well-watered conditions in the F_4 generation, explaining 12.8% of the phenotypic variation. Eight QTL loci were found to have overlapping confidence intervals for yield-related and morphological traits, which included 4181165 and 27636104 (85.95 cM) on LG2 (NS, NP and PW under well-watered conditions and IL under drought-stressed conditions in the F_3 generation), 4182352 (100.03 cM) on LG2 (DTF under well-watered conditions in the F_2

generation, P/I under drought-stressed conditions in the F_3 and F_4 generations), 4183509 (87.10 cM) on LG3 (SDW and PH under drought-stressed conditions in the F_4 generation), 4175954 and 4181663 (3.29 cM) on LG4 (NS, NP, NDP, SW, PW and HI under well-watered conditions in the F_4 generation), 4175814 (35.38 cM) on LG7A (NS, SW and PW under drought-stressed conditions in the F_3 generation), 4178651 (32.66 cM) on LG10 (SDW and NL under drought-stressed conditions in the F_3 generation), 4181438-1 (43.76 cM) on LG10 (SDW under well-watered conditions and PH under drought-stressed conditions in the F_3 generation), and 2764162 (38.03 cM) on LG11 (NS, NP, NL, PL and IL under well-watered conditions in the F_2 generation and NP under drought-stressed conditions in the F_4 generation) (Table 4-3 and 4-4).

Table 4-3 QTLs for yield components under well-watered conditions in the F_2 segregating population and under drought-stressed and well-watered conditions in the F_3 and F_4 segregating populations derived from S19-3 × DodR.

Traits	Treatment	Generation	GW LOD	IM LOD	Linkage group	Position (cM)	Nearest marker	PVE%	Additive effect	KW value	Significant levels
Shoot dry weight (SDW)	WW	F2	3.40	1.70	5	40.12	4180783	40.50	–14.16	5.71	*
	WW	F3	2.80	3.05	10	43.76	24383815, 4181438-1	15.50	–2.10	13.99	*****
	DS	F3	2.80	1.92	10	32.66	4178651	10.10	–1.42	7.95	**
	WW	F4	2.70	1.04	5	113.26	4179158	6.20	2.36	4.36	ns
	DS	F4	2.80	2.40	3	87.10	4183509	12.90	–1.62	9.96	***
Number of seeds per plant (NS)	WW	F2	2.80	2.15	11	38.03	2764162	11.10	–12.59	9.16	**
	WW	F3	2.70	3.87	2	85.95	4181165, 27636104	19.10	7.66	16.13	******
	DS	F3	2.80	2.04	7A	35.38	4175814	10.70	–3.78	9.99	***
	WW	F4	2.80	3.87	4	3.29	4181663, 4175954	21.90	–7.84	12.41	****
	DS	F4	2.80	1.40	5	0.00	4176576	8.10	–3.86	4.04	ns
Number of pods per plant (NP)	WW	F2	2.80	3.47	11	38.03	2764162	17.30	–18.04	13.94	*****
	WW	F3	2.90	3.34	2	85.95	4181165, 27636104	16.90	7.93	14.42	*****
	DS	F3	2.80	1.49	8	0.00	4178576	8.00	4.57	9.25	***
	WW	F4	2.70	2.16	4	3.29	4181663	12.80	–7.02	7.01	**
	DS	F4	2.80	2.91	11	38.03	2764162, 4176309	16.00	–7.36	7.15	**
Number of double-seeded pods per plant (NDP)	WW	F2	2.20	1.14	3	53.36	4183075	6.10	–0.86	11.75	****
	WW	F3	2.60	2.18	7A	29.74	4184098	11.40	–0.20	10.37	***
	DS	F3	2.20	1.42	7B	0.68	4182115, 4178408	7.50	–0.20	7.17	**
	WW	F4	2.60	3.85	4	3.29	4181663, 4175954	21.80	–0.23	18.92	*******
				2.75	6A	43.37	4178271	16.10	–0.22	23.84	*******

	DS	F4	1.30	0.87	1A	85.03	4182601	5.10	–0.21	6.15	**
Seed weight per plant	WW	F2	2.80	2.10	2	148.59	4183573	9.40	3.84	8.37	**
(SW)	WW	F3	2.90	2.18	6A	5.99	4178051	11.30	–1.89	8.55	**
	DS	F3	2.80	1.99	7A	35.38	4175814	10.40	–1.37	8.19	**
	WW	F4	2.70	4.19	4	3.29	4181663, 4175954	23.50	–2.04	13.19	****
	DS	F4	2.70	1.48	5	0.00	4176576	8.60	–0.94	4.79	*
Pod weight per plant	WW	F3	2.90	2.10	2	85.95	4181165	11.00	2.43	8.24	**
(PW)	DS	F3	2.80	1.93	7A	35.38	4175814	10.10	–1.63	8.09	**
	WW	F4	2.80	3.50	4	3.29	4181663, 4175954	19.90	–2.54	10.87	****
				3.03	5	30.51	42010841	17.60	–2.61	16.57	******
	DS	F4	2.70	1.29	11	38.03	2764162	7.70	–1.32	3.83	ns
100-seed weight	WW	F2	2.80	2.18	2	148.59	4183573	11.30	3.22	6.55	**
(100SW)	WW	F3	2.90	1.87	6A	5.99	4178051	9.90	–2.63	9.23	***
	DS	F3	2.70	1.24	11	26.63	4181067	6.70	–2.54	5.74	*
	WW	F4	2.80	1.69	5	30.51	42010841	10.40	–2.59	9.95	***
	DS	F4	2.80	2.45	2	71.63	4179802	13.80	–3.40	8.93	**
Harvest index (HI)	WW	F2	3.30	2.82	8	55.74	4181906	17.90	0.11	8.30	**
	WW	F3	2.80	3.33	5	13.99	4183046, 4181791	16.80	0.04	10.68	****
	DS	F3	2.80	1.23	8	63.86	4183359	6.60	–0.03	5.71	*
	WW	F4	2.70	1.77	4	3.29	4181663	11.30	–0.05	5.76	*
	DS	F4	2.80	1.35	8	5.04	4182383	7.80	0.04	6.10	**
Shelling percentage	WW	F3	2.60	0.87	3	0.00	4183000	4.70	0.01	1.28	ns
(SP)	DS	F3	2.80	1.93	9	30.66	37313543	11.50	0.06	8.15	**
	WW	F4	2.90	2.64	3	17.13	4184228	17.80	–0.06	9.87	***
	DS	F4	2.90	1.86	5	14.00	4183046	11.20	–0.06	9.13	**

*Note: WW Well-watered, DS Drought-stressed, GW LOD Genome-Wide logarithm of odds, IM LOD Interval mapping logarithm of odds, PVE phenotypic variation explanation, KW Non-parametric Kruskal-Wallis test, significant level * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.005$, ***** $p < 0.001$, ****** $p < 0.0005$, ******* $p < 0.0001$, ns not significant.*

Table 4-4 QTLs for morphological traits under well-watered conditions in the F_2 segregating population and under drought-stressed and well-watered conditions F_3 and F_4 segregating populations derived from S19-3 × DodR.

Traits	Treatment	Generation	GW LOD	IM LOD	Linkage group	Position (cM)	Nearest marker	PVE %	Additive effect	KW value	Significant levels
Days to flowering (DTF)	WW	F2	2.80	1.95	2	100.03	4182352	10.10	–1.32	9.01	**
	WW	F3	2.80	1.63	1A	24.74	4181231	8.60	–0.60	8.14	**
	DS	F3	2.80	1.80	6A	0.00	4182879	9.50	–0.68	7.43	**
	WW	F4	2.90	2.34	6B	5.51	4181907	12.20	–1.00	9.51	***
	DS	F4	2.80	2.52	9	26.20	4182850	12.20	–1.12	8.96	**
Number of leaves per plant (NL)	WW	F2	2.70	4.20	11	38.03	2764162, 4176309	20.60	–38.58	17.33	******
	WW	F3	2.80	1.77	1B	0.00	4181815	9.40	–4.83	9.44	***
	DS	F3	2.80	1.88	10	32.66	4178651	9.90	–4.88	8.23	**
	WW	F4	2.30	1.19	11	0.61	4184109	6.90	18.93	8.45	**
	DS	F4	2.80	2.43	7A	75.43	4180470	11.20	6.03	10.19	***
Plant height (PH)	WW	F2	2.80	1.83	5	102.19	4182450	9.50	–2.04	5.08	*
	WW	F3	2.80	1.22	6A	11.24	4181739	6.60	–1.10	5.86	*
	DS	F3	2.80	1.99	10	37.09	4181438-1	10.40	–1.53	9.67	***
	WW	F4	2.80	1.62	10	210.83	24383815	9.30	–1.45	8.65	**
	DS	F4	2.90	2.41	3	87.10	4183509	12.90	–1.25	10.76	****
Petiole length (PL)	WW	F2	2.80	2.46	11	38.03	2764162	12.60	–1.39	12.13	****
	WW	F3	2.70	2.26	6A	10.36	4181745	11.80	–0.93	9.35	***
	DS	F3	2.80	1.85	3	156.88	4181117	9.70	–0.94	9.67	***
	WW	F4	2.80	1.06	3	16.57	4182872	6.20	0.59	4.21	ns
	DS	F4	2.80	1.39	8	0.00	4178576	7.70	–0.61	5.47	*
Internode length (IL)	WW	F2	2.80	2.20	11	38.03	2764162	11.40	–0.43	13.41	****
	WW	F3	2.90	2.18	11	1.01	4181329, 24385209	11.40	–0.22	12.15	****
	DS	F3	2.90	2.11	2	87.45	27636104	11.00	–0.22	8.26	**
	WW	F4	2.80	1.45	5	0.00	4178576	8.40	–0.20	6.06	**
	DS	F4	2.70	2.38	8	0.00	4178576	12.80	–0.22	12.83	****

Petiole internode ratio (P/I)	WW	F2	2.80	1.79	5	14.05	27641212	9.30	1.06	7.08	**
	WW	F3	2.70	1.81	10	2.53	24384187, 37313638	9.60	0.58	10.39	***
	DS	F3	2.90	2.17	2	100.03	4182352	11.60	0.95	9.50	**
	WW	F4	2.80	1.33	1A	36.72	4184152	7.90	0.74	5.26	*
	DS	F4	2.80	1.94	2	100.03	4182352	10.70	0.92	8.66	**

*Note: WW Well-watered, DS Drought-stressed, GW LOD Genome-Wide logarithm of odds, IM LOD Interval mapping logarithm of odds, PVE phenotypic variation explanation, KW Non-parametric Kruskal-Wallis test, significant level * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.005$, ***** $p < 0.001$, ****** $p < 0.0005$, ******* $p < 0.0001$, ns not significant.*

4.3.2.2 QTLs associated with physiological traits in the F_3 and F_4 segregating generations

Two significant QTLs associated with CCI (LOD: 3.24, 38.03 cM, nearest marker: 42010841) and F_V/F_M (LOD: 3.01, nearest marker: 4176835) under drought-stressed conditions in the F_4 and F_3 segregating population mapped on LG5, explaining 16.5% and 15.4% of the phenotypic variation, respectively (Tables 4-5). Two QTL loci were found to have overlapping confidence intervals for yield-related, morphological and physiological traits, which included 42010841 (30.51 cM) on LG5 (PW under well-watered conditions and CCI under drought-stressed conditions in the F_4 generation) and 4182450 (102.19 cM) on LG5 (PH under well-watered conditions in the F_2 generation and F_V/F_M under well-watered conditions in the F_4 generation).

Table 4-5 QTLs for *leaf chlorophyll content index* (CCI), *quantum yield PSII photochemistry* (F_V/F_M) and *leaf relative water content* (RWC) under well-watered (WW) and drought-stressed (DS) conditions in the F_3 and F_4 segregating populations derived from S19-3 × DodR.

Traits	Treatment	Generation	GW LOD	IM LOD	Linkage group	Position (cM)	Nearest marker	PVE%	Additive effect	KW value	Significant levels
Leaf chlorophyll content index (CCI)	WW	F3	2.80	1.81	9	70.03	4181610	9.10	1.20	9.27	***
	DS	F3	2.90	1.56	2	66.18	4183031	8.30	–1.03	9.02	**
	WW	F4	2.90	1.10	5	71.63	4179802	5.70	–1.18	8.03	**
	DS	F4	3.00	3.24	5	30.51	42010841	16.50	–2.17	11.82	****
Quantum yield PSII photochemistry (F_V/F_M)	WW	F3	2.80	2.19	4	111.14	4182453	11.40	0.01	9.08	**
	DS	F3	2.80	3.01	5	58.41	4176835	15.40	–0.01	12.08	****
	WW	F4	2.90	2.04	5	102.19	4182450	26.10	–0.02	8.73	**
	DS	F4	3.00	1.49	9	58.36	4182089	19.30	0.02	7.03	**
Leaf relative water content (RWC)	WW	F3	2.80	2.21	3	11.94	24346055	11.50	1.38	8.15	**
	DS	F3	2.80	1.98	3	0.00	4183000	10.40	1.19	13.29	****
	WW	F4	2.80	1.97	6A	11.24	4181739	26.10	–1.27	6.96	**
	DS	F4	3.00	1.54	9	21.17	4181240	20.50	1.58	7.65	**

*Note: WW Well-watered, DS Drought-stressed, GW LOD Genome-Wide logarithm of odds, IM LOD Interval mapping logarithm of odds, PVE phenotypic variation explanation, KW Non-parametric Kruskal-Wallis test, significant level * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.005$.*

4.3.2.3 QTLs associated with seed size in the F_4 segregating population

Putative QTL for PER (LOD: 1.81, 51.00 cM, nearest marker: 4178265) was mapped on LG6A, explaining 13% of the PVE (Tables 4-6). Putative QTLs associated with WID (LOD: 1.55, 40.12 cM, nearest marker: 4176835) and LEN (LOD: 1.87, 44.53 cM, nearest marker: 4183179) under drought-stressed conditions were mapped on LG5, explaining 10.4% and 12.2% of the phenotypic variation, respectively (Tables 4-6).

Table 4-6 QTLs for seed size under well-watered (WW) and drought-stressed (DS) conditions in the F_4 segregating populations derived from S19-3 × DodR.

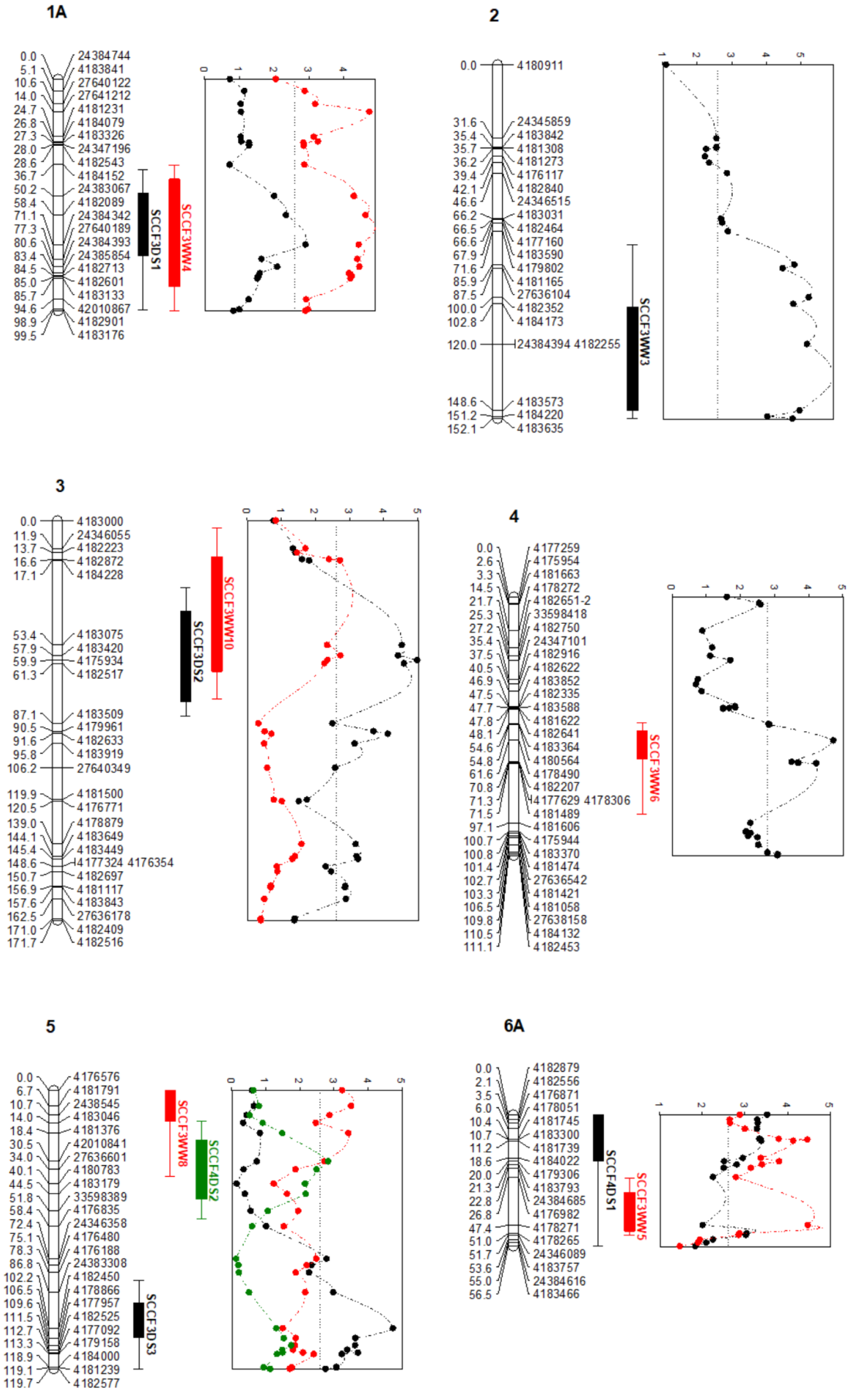
Traits	Treatment	GW LOD	IM LOD	Linkage group	Position (cM)	Nearest marker	PVE %	Additive effect	KW value	Significant levels
Seed perimeter (PER)	WW	2.80	1.81	6A	51.00	4178265	13.00	–1.86	10.80	***
	DS	2.80	0.96	7A	35.38	4175814	6.60	–1.69	3.50	ns
Seed width-length ratio (WLR)	WW	2.90	2.24	1B	1.85	4182837	15.80	–0.02	9.05	**
	DS	2.80	0.95	11	0.00	24346244	6.50	–0.02	5.26	*
Seed width (WID)	WW	1.90	1.12	11	26.63	4181067	9.40	0.47	5.06	*
	DS	2.50	1.55	5	40.12	4180783	10.40	0.68	4.81	*
Seed length (LEN)	WW	2.10	1.20	1A	71.15	24384342	8.70	0.56	6.69	**
	DS	2.70	1.87	5	44.53	4183179	12.20	1.09	5.27	*

*Note: WW Well-watered, DS Drought-stressed, GW LOD Genome-Wide logarithm of odds, IM LOD Interval mapping logarithm of odds, PVE phenotypic variation explanation, KW Non-parametric Kruskal-Wallis test, significant level * $p < 0.1$, ** $p < 0.05$, ns not significant.*

4.3.3 QTLs associated with seed coat colour in harvested seeds from the F_3 and F_4 generations

Multiple significant QTLs associated with SCC under well-watered and drought-stressed conditions in the F_3 and F_4 segregating generations were detected (Fig. 4-3). A total of 10 significant QTLs (LOD > 2.6) associated with SCC were mapped on LG1A, LG2, LG3, LG4, LG5, LG6A, LG7A, LG7B, LG8 and LG11 under well-watered conditions in the F_3 segregating generation, while 5 significant QTLs (LOD > 2.7) were mapped on LG1A, LG3, LG5, LG7A and LG7B under drought-stressed conditions in the F_3 segregating generation (Table 4-7). Two significant QTLs (LOD > 2.6) associated with SCC were mapped on LG5 and LG6A under drought-stressed conditions in the F_4 segregating generation (Table 4-7).

Four QTL loci were found to have overlapping confidence intervals for SCC, which included 27641212 (14.05 cM) under well-watered conditions and 24384342 (71.15 cM) under drought-stressed conditions on LG1A, 4183420 (57.89 cM) under well-watered conditions and 4175934 (59.95 cM) under drought-stressed conditions on LG3, 37320527 (0.00 cM) under well-watered and drought-stressed conditions on LG7A, and 4182115 (0.68 cM) under well-watered conditions and 27640313 (10.95 cM) under drought-stressed conditions on LG7B in the F_3 generation (Table 4-7, Figure 4-3).


1A
0.0 24384744
5.1 4183841
10.6 27640122
14.0 27641212
24.7 4181231
26.8 4184079
27.3 4183326
28.0 24347196
28.6 4182543
36.7 4184152
50.2 24383067
58.4 4182089
71.1 24384342
77.3 27640189
80.6 24384393
83.4 24385854
84.5 4182713
85.0 4182601
85.7 4183133
94.6 42010867
98.9 4182901
99.5 4183176
SCCF3DS1
SCCF3WW4
2
0.0 4180911
31.6 24345859
35.4 4183842
35.7 4181308
36.2 4181273
39.4 4176117
42.1 4182840
46.6 24346515
66.2 4183031
66.5 4182464
66.6 4177160
67.9 4183590
71.6 4179802
85.9 4181165
87.5 27636104
100.0 4182352
102.8 4184173
120.0 24384394 4182255
148.6 4183573
151.2 4184220
152.1 4183635
SCCF3WW3
3
0.0 4183000
11.9 24346055
13.7 4182223
16.6 4182872
17.1 4184228
53.4 4183075
57.9 4183420
59.9 4175934
61.3 4182517
87.1 4183509
90.5 4179961
91.6 4182633
95.8 4183919
106.2 27640349
119.9 4181500
120.5 4176771
139.0 4178879
144.1 4183649
145.4 4183449
148.6 4177324 4176354
150.7 4182697
156.9 4181117
157.6 4183843
162.5 27636178
171.0 4182409
171.7 4182516
SCCF3DS2
SCCF3WW10
4
0.0 4177259
2.6 4175954
3.3 4181663
14.5 4178272
21.7 4182651-2
25.3 33598418
27.2 4182750
35.4 24347101
37.5 4182916
40.5 4182622
46.9 4183852
47.5 4182335
47.7 4183588
47.8 4181622
48.1 4182641
54.6 4183364
54.8 4180564
61.6 4178490
70.8 4182207
71.3 4177629 4178306
71.5 4181489
97.1 4181606
100.7 4175944
100.8 4183370
101.4 4181474
102.7 27636542
103.3 4181421
106.5 4181058
109.8 27638158
110.5 4184132
111.1 4182453
SCCF3WW6
5
0.0 4176576
6.7 4181791
10.7 2438545
14.0 4183046
18.4 4181376
30.5 42010841
34.0 27636601
40.1 4180783
44.5 4183179
51.8 33598389
58.4 4176835
72.4 24346358
75.1 4176480
78.3 4176188
86.8 24383308
102.2 4182450
106.5 4178866
109.6 4177957
111.5 4182525
112.7 4177092
113.3 4179158
118.9 4184000
119.1 4181239
119.7 4182577
SCCF3DS3
SCCF3WW8
SCCF4DS2
6A
0.0 4182879
2.1 4182556
3.5 4176871
6.0 4178051
10.4 4181745
10.7 4183300
11.2 4181739
18.6 4184022
20.0 4179306
21.3 4183793
22.8 24384685
26.8 4176982
47.4 4178271
51.0 4178265
51.7 24346089
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55.0 24384616
56.5 4183466
SCCF4DS1
SCCF3WW5

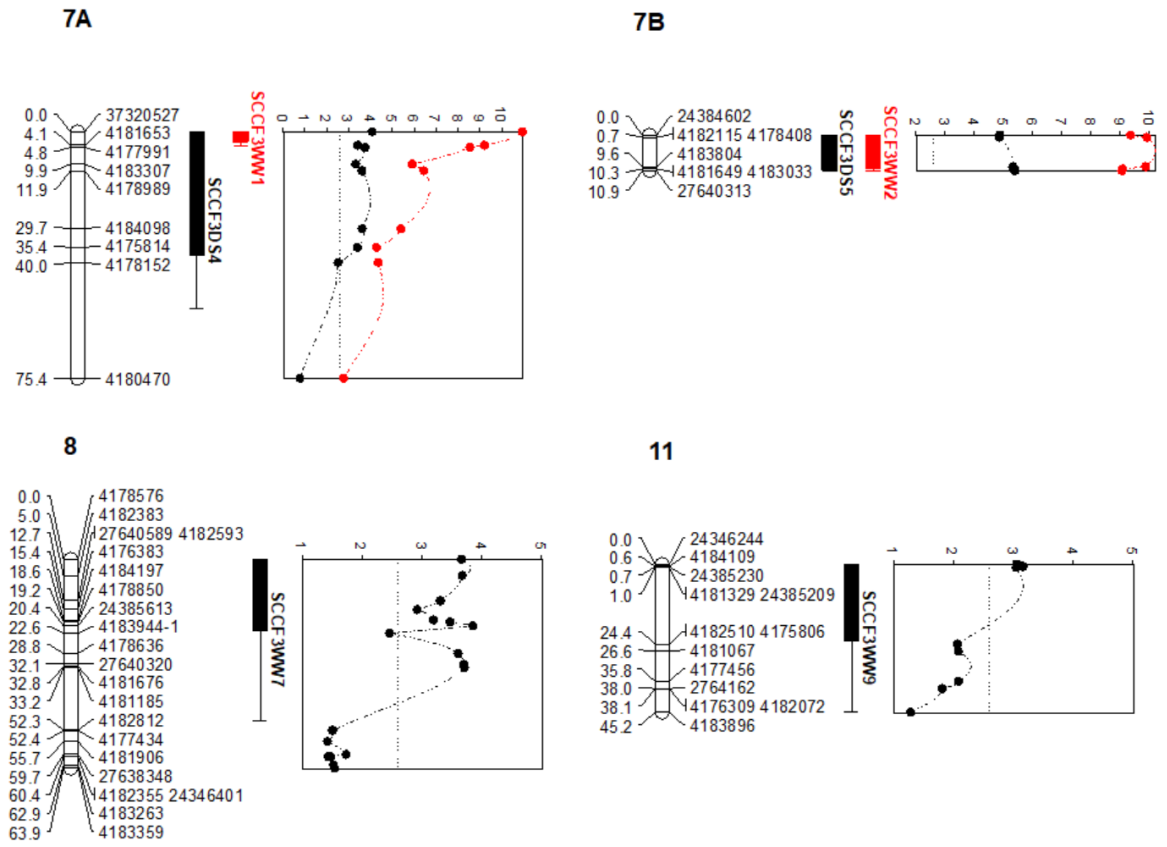

Figure 4-3 Map position of the quantitative trait loci (QTL) under well-watered (WW) and drought-stressed (DS) in the F_3 and F_4 segregating populations developed from S19-3 × DodR. Right: positions of markers (cM); left: name of the markers. Rectangular bars represent the 1- and 2-LOD QTL interval. Solid rectangular bars represent significant QTLs, while blank bars represent putative QTLs. LG1, LG6 and LG7 were divided into subgroups '1A' and '1B', respectively, based on the association observed in the maximum likelihood mapping (MLM) due to insufficient linkage to complete the map using regression mapping (RM).

Table 4-7 QTLs for seed coat colour under well-watered (WW) and drought-stressed (DS) conditions in the F_3 and F_4 segregating populations derived from S19-3 × DodR.

Generation	Treatment	PT LOD	Linkage group	position	Nearest marker	IM LOD	PVE%	Additive effect	KW value	Significant levels
F3	WW	2.60	7A	0.00	37320527	10.94	45.90	–14.15	35.79	*******
			7B	0.68	4182115, 4178408	9.91	42.70	–13.41	32.67	*******
			2	100.03	4182352	5.21	25.40	–10.63	18.47	*******
			1A	14.05	27641212	4.74	23.40	–10.39	19.11	*******
			6A	47.37	4178271	4.47	22.20	–10.25	13.78	******
			4	71.52	4181489	4.22	21.10	–10.12	15.04	******
			8	20.40	24385613	3.85	19.40	–9.49	13.94	******
			5	6.71	4181791	3.50	17.80	–8.96	10.57	****
			11	0.61	4184109	3.16	16.30	–8.68	13.74	******
			3	57.89	4183420	2.73	14.20	–8.13	11.19	*****
F3	DS	2.70	7B	10.95	27640313, 4181649	5.39	27.20	–13.43	19.51	*******
			3	59.95	4175934	4.95	25.30	–13.67	18.18	*******
			5	102.19	4182450	4.73	24.30	–13.35	15.33	*******
			7A	0.00	37320527	4.08	21.40	–12.16	14.39	******
			1A	71.15	24384342	2.90	15.70	–10.62	10.47	****
F4	WW	2.70	4	48.11	4182641	1.65	8.80	3.15	7.79	***
F4	DS	2.60	6A	0.00	4182879	3.52	19.00	–5.71	12.96	******
			5	30.51	42010841	2.83	15.60	–5.30	12.89	******

Note: WW Well-watered, DS Drought-stressed, GW LOD Genome-Wide logarithm of odds, IM LOD Interval mapping logarithm of odds, PVE phenotypic variation explanation, KW Non-parametric Kruskal-Wallis test, significant level *** $p < 0.01$, **** $p < 0.005$, ***** $p < 0.001$, ****** $p < 0.0005$, ******* $p < 0.0001$.

4.4 Discussion

Several molecular and genetic studies (Redjeki et al. 2013; Fadah et al. 2017; Chai et al. 2017) as well as physiological studies (Basu et al 2007; Jørgensen et al. 2010; Vurayai et al. 2011; Chai et al. 2016; Muhammad et al. 2016) have been focused on understanding the complexity of drought resistance in bambara groundnut. However, the inheritance and genetic architecture of quantitative traits for drought resistance in bambara groundnut are still not well understood. For the first time we identified and compared the QTLs under drought-stressed and well-watered conditions in the F_3 and F_4 segregating populations derived from S19-3 × DodR.

In the present study, significant QTLs were mapped to approximately the same position on LG4 (3.29 cM) for *number of seeds per plant, number of double-seeded pods per plant, seed weight per plant* and *pod weight per plant* with PVE ranged from 19.9–23.5% and putative QTL for *number of pods per plant* and *harvest index* were mapped on the same location on LG4 (3.29 cM) with PVE ranged from 11.3–12.8% under well-watered conditions in the F_4 segregating population. Such pleiotropism has also observed in other species, such as soybean, in which QTLs associated with *days to flowering, days to maturity, plant height, number of nodes on main stem, lodging* and *plot yield* mapped to the same chromosomal regions (Zhang et al. 2004). Chai et al. (2017) reported that QTLs controlling *pod number per plant, seed number per plant, internode length* and *peduncle length,* were centred around the same marker in an F_5 segregating population of bambara groundnut, Tiga Nicuru × DipC.

QTLs for *number of seeds per plant*, *number of pods per plant* and *pod weight per plant* under well-watered conditions and QTL associated with *internode length* under drought-stressed conditions in the F_3 segregating population were co-located on LG2 (85.95 cM) with

overlapping confidence intervals. The clustered QTL on the same loci could correspond to a single gene controlling yield and growth habit in bambara groundnut (Chai et al. 2017).

Multiple significant QTLs for *number of double-seeded pods per plant* under well-watered conditions in the F_4 segregating population were mapped on LG4 (3.29 cM) and LG6A (43.37 cM), explained 21.80% (LOD 3.85) and 16.10% (LOD 2.75) of the phenotypic variation, respectively, suggesting the inheritance of double-seeded pods was controlled by a major QTL and few minor QTLs. Similar results were also observed for *pod weight per plant* under well-watered conditions in the F_4 segregating population mapped on LG4 (3.29 cM) and LG5 (30.51 cM), explained 19.9% (LOD 3.5) and 17.6% (LOD 3.03) phenotypic variation, respectively, suggesting the inheritance of pod yield could probably be controlled by few QTLs with minor effect. QTLs identified under well-watered conditions could reflect the intrinsic genetic mechanisms underlying yield-related and morphological traits which vary between the parental lines, although there are also clear differences observed among individual lines and the interaction between genotypes and environment factors for these traits, clearly exists, as shown by difference in QTL between treatments.

Takono et al. (2016) reported that F_4 and F_3 populations would be almost as useful as RIL populations for QTL mapping. Bradshaw et al. (1998) estimated the accuracy of QTL detection in two different population size in interspecific crosses of monkeyflower (*Mimulusspp*.), 12 QTLs of relatively large effect were detected in the smaller population (n = 93), while 27 QTLs including 11 of the same QTLs were detected in the larger population (n = 465). Although the small population size (n = 86) in the present study is one of the limiting factors that could have affected the power of QTL detection, the estimated QTLs with PVE of ≥20% could be considered as major QTLs to control these traits, including *number of seeds per plant, number*

of double-seeded pods per plant, seed weight per plant, number of leaves per plant F_V/F_M and *relative water content*.

In the case of *seed coat colour*, significant QTLs were identified under both water regimes in the F_3 generation, suggesting multiple genes control this trait. Multiple consensus QTLs of these significant QTLs were clustered on LG1A, LG3, LG7A and LG7B. In bambara groundnut, dark-coloured seeds had better seedling emergence than light-coloured seeds under drought stress conditions due to the tannins present in dark-coloured seeds which are polyphenols and act as antioxidants under stress conditions (Chibarabada et al. 2015). Herniter et al. (2018, 2019) identified four candidate genes (*Vigun05g039500, Vigun07g110700, Vigun09g139900* and *Vigun10g163900*) involved in flavonoid biosynthesis pathway in cowpea. The future study could focus on identifying the candidate genes related to *seed coat colour* in response to drought stress in bambara groundnut. Candidate genes involved in flavonoid biosynthesis pathway in plants have been reported in Arabidopsis (Wang et al. 2020) and cowpea (Herniter et al. 2018, 2019), which could be used as reference genes to identify potential genes in the anthocyanin biosynthesis in bambara groundnut.

The genetic linkage map obtained in the present study could be used for the identification of molecular markers linked to important agronomic traits and syntenic regions in other closely related species such as cowpea. Integrating genetic linkage maps from different crosses or using a larger mapping population size will facilitate the development of fine and high marker density maps. Together with a fully assembled and annotated genome of bambara groundnut, the task of identifying markers associated with target traits and the function of candidate genes associated with specific traits will become a reality. The identified markers associated with target traits will be useful in breeding selection to accelerate bambara groundnut improvement

through MAS breeding. The development of DArT sequencing technology and the emergence of powerful genome editing techniques will further contribute to molecular breeding progress in bambara groundnut.

4.5 Conclusion

The present genetic linkage map covered 1,040.92 cM across 11 linkage groups with an average interval distance of 5.23 cM among 228 DArTseq markers in the F_2 segregating population from S19-3 × DodR. Significant and putative QTLs for yield-related, morphological and physiological traits under drought-stressed and well-watered conditions in the F_3 and F_4 segregating generations were identified. QTLs associated with *number of seeds per plant*, *number of pods per plant, pod weight per plant* under well-watered conditions and *internode length* under drought-stressed conditions in the F_3 generation were co-located on LG2 with overlapping confidence intervals, while *number of seeds per plant*, *number of pods per plant, number of double-seeded pods per plant, seed weight per plant, pod weight per plant and harvest index* under well-watered conditions in the F_4 generation were co-located on LG4 with overlapping confidence intervals. QTLs identified under well-watered conditions would reflect the intrinsic genetic mechanisms underlying yield-related and morphological traits. Multiple significant QTLs for *number of double-seeded pods per plant* and *pod weight per plant* were observed, suggesting inheritance of double-seeded pods and pod yield was controlled by many genes. A decrease in PVE under drought-stressed conditions compared to well-watered conditions, suggesting the traits identified under well-watered conditions were unable to fully express their potential trait values under drought conditions. Several QTLs with≥20% of the PVE were identified as major QTLs to control these traits, including *number of seeds per plant, number of double-seeded pods per plant, seed weight per plant, number of leaves per plant* F_V/F_M and *relative water content.* The major QTLs identified in this study are essential to

support the development of improved varieties of bambara groundnut in molecular-enabled breeding programmes.

Chapter 5 General discussions and conclusions

5.1 Issues and challenges for bambara groundnut

As an underutilised legume crop, bambara groundnut has the potential to contribute to global food security and nutrition as a climate-smart crop (Cleasby et al. 2016; Massawe et al. 2016; Atoyebi et al. 2017; Halimi et al. 2019; Mayes et al. 2019). However, bambara groundnut is still mainly grown by subsistence farmers in Africa and has received limited support and funding from National and International agencies (Massawe et al. 2005; Oyeyinka et al. 2015; Mayes et al. 2019).

Around 5000 accessions of bambara groundnut mainly collected from African countries are held by international or regional seed banks (Begemann and Engels 1997; Muhammad et al. 2020). A lack of improved, high yielding and climate-resilient varieties is one of the main factors limiting wider cultivation and utilization of bambara groundnut. In this regard, past research has reported on the variation among landraces or genotypes (Amadou et al. 2001; Massawe et al. 2003), ideotype selection under abiotic stress conditions (Jørgensen et al. 2010; Mabhaudhi et al. 2013; Chai et al. 2016; Nautiyal et al. 2017) and nutrition value (Minka and Bruneteau 2000; Suwanprasert et al. 2006; Okpuzor et al. 2010; Halimi et al. 2020) of bambara groundnut. Genotypes displaying drought response capabilities have been identified under drought stress conditions, which provide a breeding resource pool for variety improvement and ideotypes development (Jørgensen et al. 2010; Mabhaudhi et al. 2013; Nautiyal et al. 2017). There were no concerted breeding programmes in bambara groundnut until recently perhaps due to limited funding dedicated to underutilised crops and also a lack of research interest from breeders and the scientific community more generally. More recently, breeding activities have emerged with the development of structured populations of bambara groundnut for genetic

analysis and target individual lines selection (e.g. drought resistant varieties) (Chai et al. 2016; Gao et al. 2020; Kendabie et al. 2020). The present study utilised the genetic variation haboured within bambara groundnut landraces to develop breeding lines and structured populations in search of new and improved varieties and for genetic analysis and agronomic trait dissection.

As stated in the previous chapters, artificial hybridization approaches have been establishment and mastered by a few bambara groundnut scientists (Massawe et al. 2003; Suwanprasert et al. 2006; Kendabie et al. 2015). These approaches were used in the present study to enhance the genetic potential of bambara groundnut genotypes through cross-breeding, with the ultimate goal of developing improved varieties with adaptive features, reasonable yield and good nutritional value.

5.2 Landraces as important resources for crop breeding

Landraces are important resources in breeding as they harbour genetic variations necessary not only for the development of improved crop varieties with key adaptive features, but also to maintain functional ecosystems. One of the objectives of the present study was to investigate the phenotypic traits variations among twelve genotypes collected from different geographical locations and to select suitable parental material for breeding purposes. The variations observed in these genotypes provide resources for bambara groundnut ideotype development with favourable traits, for example., for high yield (high *harvest index, 100-seed weight*), early maturing (*days to flowering* or short life cycle).

Genotypes with a balanced development of vegetative growth and yield accumulation are critical breeding resources for improved varieties selection in breeding programmes. The identified morphological traits, i.e., *number of leaves per plant, petiole length, internode length, petiole internode ratio* and *plant height* and the correlations of these traits with yield-related

traits, i.e., *100-seed weight, harvest index* and *shelling percentage* could form a basis for the selection of parental lines to meet specific breeding objectives. For example, DodR, Gresik and LunT are recommended as high yield (high *100-seed weight* and *harvest index*) genotypes compare to other genotypes. Tiga Nirucu, Getso and DodR are recommended as early *days to flowering* genotypes, while Gresik and Ankapa4 are recommended as late flowering time genotypes. Of the parental materials investigated in the present study, Tiga Nicuru and S19-3 have also been recommended as drought-tolerant genotypes with short life cycle and superior root length distribution (Jørgensen et al. 2010; Mateva et al. 2020).

5.3 Exploiting the power of structured populations and selection of breeding lines

Two F_2 segregating populations (S19-3 × DodR and IITA × Tiga Nicuru) of bambara groundnut were developed to identify phenotypic variations in the segregating populations and obtain structured populations and breeding lines for genetic analysis and trait dissection (Chapter 2). High variability and transgressive segregation were observed in the two F_2 bi-parental segregating populations, which provided an opportunity for superior individual lines selection for further development into improved varieties and for genetic analysis. Individual lines (6, 38, 44, 48, 50 in the F_2 segregating population IITA-686 × Tiga Nicuru and 51, 73, 86, 108, 111 in the F_2 segregating population S19-3 × DodR) with combined desirable traits, such as earlier flowering, higher *harvest index, 100-seed weight*, shorter *plant height* and *internode length* than average of the population or their parents are recommended as superior lines for further field investigation to develop improved varieties.

Harvest index positively correlated with *100-seed weight,* negatively correlated with *plant height* and *internode length* in the F_2 segregating population, which suggests that genotypes with short height or *internode length* have potential to develop varieties with high yield.

However, the crop final yield is affected by multiple factors including genotype, environment and interaction between genetic and environmental factors (Karikari et al. 2004; Ntundu et al. 2006; Chai et al. 2016). *Plant height* and *internode length* could be used as surrogates for potential high yield genotypes selection.

In addition to developing breeding lines and advancing these for field investigation and multi-locational trials, the two F_2 populations will be advanced into recombinant inbred lines (RILs) to serve as powerful tools not only for the analysis of complex traits (e.g. *harvest index* and *100-seed weight*) but also for genetic mapping and marker-trait association analysis.

5.4 Drought stress impacts yield components and morphological traits

Having developed the two F_2 populations, one of the populations (S19-3 × DodR) was advanced into F_3 and F_4 populations and used for drought studies to understand the effect of drought stress on yield components, morphological and physiological traits and to select potential breeding lines with drought-tolerant capabilities for further analysis and variety of development. The significant differences observed among individual lines ($p < 0.05$) and the interaction between treatment and individual lines for yield-related and physiological traits, would suggest that individual lines in the segregating populations could be selected for superior performance under multiple environmental conditions (Zhao et al. 2016). Individual lines with higher *chlorophyll content index* and F_V/F_M under both water regimes (e.g. 14, 33, 36, 39, 43, 50, 63 and 64 in the F_3 segregating population and 3, 4, 5, 12 and 23 in the F_4 segregating population) are recommended as superior lines for genetic analysis and variety development (Chapter 3). The present study also identified individual lines with higher *leaf relative water content* (e.g. 6, 12, 20, 21 and 37) than their population mean or parental lines under drought-stressed conditions in the F_3 and F_4 segregating generations (Chapter 3).

The present study also showed *harvest index* and *100-seed weight* to be positively correlated with yield components in both the F_3 and F_4 segregating populations, which implies that *harvest index* and *100-seed weight* could be used as surrogates for selection of superior lines with drought resistance ability in bambara groundnut breeding programme (see also Chai et al. 2016). Individual lines with higher *harvest index* and *100-seed weight* (e.g., 11, 20, 26, 33, 35, 49, 50, 51, 65, 80, 82, 87, 90, 92, 93, 99, 105 and 108 in the F_3 segregating population and 10, 32, 53, 58, 62 and 86 in the F_4 segregating population) than population mean or parental lines are recommended as superior lines for genetic analysis and variety development (Chapter 3).

When subjected to water deficit, plants maintain high water status by stomatal closure, which reduces transpiration, CO_2 accumulation and net photosynthesis (Turner et al. 2001; Chaves and Oliveira 2004; Kavar et al. 2008). *Stomatal conductance, photosynthesis rate, transpiration rate* and *intracellular CO_2* were significantly reduced by drought but LWUE increased significantly. Individual lines with higher LWUE (e.g. 10, 12, 21, 28 and 33) and higher gs (e.g. 7, 11, 14, 19, 20, 30 and 32) are recommended as potential drought resistant lines for genetic analysis and variety development in breeding programmes (Chapter 3). The results of the phenotypic traits analysis in the F_2 population and the drought studies in both the F_3 and F_4 populations paved the way for genetic linkage mapping and QTL analysis as the foundations for marker-trait analysis and candidate gene identification.

5.5 Genotyping-by-sequencing (GBS) mapping of quantitative traits (QTLs) and qualitative traits

Having developed the two F_2 populations (see section 5.3 above), one of the populations (S19-3 × DodR) was subjected to GBS using the DArTseq platform. The genetic linkage map obtained covered 1,040.92 cM across 11 linkage groups and was constructed using 228

DArTseq markers. Mapping quantitative and qualitative loci to a location on the chromosomes of bambara groundnut have been reported in the segregating populations, which included DipC × and VSSP11 (Basu et al. 2007), Tiga Nicuru × DipC (Ahmad et al. 2016; Chai et al. 2017; Ho et al. 2017) and IITA686 × Ankpa4 (Ho et al. 2017). These genetic linkage maps are critical to identify QTLs that responsible for phenotypic variation in bambara groundnut breeding programme (Chai et al. 2017). The genetic linkage map constructed in the present study was used to identify QTLs associated with yield components, morphological and physiological traits under well-watered and drought-stressed conditions in bambara groundnut, which will provide critical insights into how genetic features control these traits in bambara groundnut in response to drought stress.

As shown in Chapter 4, significant QTLs for *shoot dry weight* were mapped on LG10 accounting for 15.5% of the PVE under well-watered conditions and a putative QTL for the same trait mapped on LG10 with reduced PVE (10.10%) under drought-stressed conditions in the F_3 segregating population. Significant QTLs associated with *number of seeds per plant, number of double-seeded pod per plant, seed weight per plant* and *pod weight per plant* were mapped on LG4 (nearest marker: 4181663 and 4175954) with overlapping confidence intervals and explaining 21.9%, 21.8%, 23.5% and 19.9% of the PVE, respectively, under well-watered conditions in the F_4 population, which could be considered as major QTL involved in the control of these traits. Seven QTL loci that were found to be consensus QTLs for yield-related and morphological traits across LG2, LG3, LG4, LG7A and LG10. The significant ($p < 0.05$) reduction observed in yield-related and morphological traits and a decrease in PVE under drought-stressed conditions compared to well-watered conditions, suggesting the traits identified under well-watered conditions were unable to fully express their potential trait values under drought conditions.

Multiple significant QTLs associated with seed coat colour were identified under well-watered and drought-stressed conditions, suggesting these traits are likely highly interacted with environmental stress and controlled by multiple genes. The first genome sequence of bambara groundnut has been assembled with 513 Mb in size and predicted 31,707 protein-coding genes (Chang et al. 2018). However, the current bambara groundnut genome information and QTL mapping does not afford adequate resolution to identify genes. High density genetic linkage maps and QTL detection are very useful tools to identify genomic regions that may be responsible for target traits for MAS breeding of bambara groundnut (Chai et al. 2017). Once the function of candidate genes is validated, breeders are able to design molecular markers for target varieties selection among large genotypes or populations and apply genome editing techniques to speed up breeding progress in bambara groundnut.

5.6 Implication of the present study

The present research clears the path for the development of structured populations and breeding lines for genetic analysis, trait dissection and selection of new improved varieties in bambara groundnut. The results of these investigations can be summarized into several main points.

1- The variations observed within twelve genotypes selected from landraces and segregating populations of bambara groundnut, S19-3 × DodR and IITA-686 × Tiga Nicuru, offer unique opportunities for crop improvement through utilisation of these breeding resources for variety development with favourable traits. The present study has also furthered our understanding of the correlations between yield components, morphological and physiological traits and the impact of drought stress on these traits (Chapter 2).

2- Investigation of the effect of drought stress on yield-related, morphological and physiological traits in the F_3 and F_4 segregating populations of S19-3 × DodR. The present study has also furthered our understanding of the variation of traits in segregating populations of bambara groundnut and the correlation between yield-related and morphological traits, and the impact of drought stress on these traits (Chapter 3).

3- Evaluation of the effect of drought stress on phostosynthetic parameters before drought stress was imposed, during drought stress period and after irrigation was resumed in a subset of the F_4 segregating populations of S19-3 × DodR (Chapter 3).

4- The recommended individual lines with desirable traits were identified in the F_2, F_3, and F_4 segregating populations of S19-3 × DodR. These are great resources for genetic analysis and variety of development in breeding programmes (Chapter 3).

5- Construction of the first genetic linkage map of S19-3 × DodR in bambara groudnut (Chapter 4). The genetic linkage map could be used for the identification of molecular markers linked to important agronomic traits and syntenic regions in other closely related species such as cowpea.

6- Significant and putative QTLs for yield-related, morphological and physiological traits under drought-stressed and well-watered conditions in the F_3 and F_4 segregating generations were identified. The identification of quantitative and qualitative trait loci under well-watered and drought-stressed conditions is essential to support the development of improved varieties of bambara groundnut in molecular-enabled breeding programmes (Chapter 4).

7- Multiple significant QTLs associated with seed coat colour were identified under drought-stressed and well-watered conditions in the F_3 and F_4 segregating generations. The significant QTLs observed under drought-stressed conditions are helpful to identify the candidate genes related to *seed coat colour* in response to drought stress in bambara groundnut. (Chapter 4).

5.7 Future work

Based on the results obtained from the present study, the following future work can be proposed briefly.

1- Replicated trials across multi-locations and different seasons are needed to further study the recommended lines to estimate environmental effects and genetic variation and to validate the present results of Chapter 2 and Chapter 3.

2- Further development of selected lines into RILs (It is now at F_6 generation of bambara groudnut derived from S19-3 × DodR) for various traits which can be used for marker-trait association analysis, QTL analysis and high-density mapping in the crop improvement program of bambara groundnut.

3- Further validation of consensus markers and significant QTLs associated with various traits is required in different populations, across locations and seasons. Integrating genetic linkage maps from different crosses or using a larger mapping population size will facilitate the development of fine and high marker density maps. Used together with a fully assembled and annotated genome of bambara groundnut, the task of identifying markers associated with target traits and the function of candidate genes associated with specific traits will become a reality. The validated markers associated with target traits and candidate genes will be useful in breeding selection to accelerate bambara groundnut improvement through MAS breeding. The

development of DArT sequencing technology and the emergence of powerful genome editing techniques will further contribute to molecular breeding progress in bambara groundnut.

4- More sequencing data, especially transcriptome sequence of bambara groundnut could be used to determine drought response mechanisms, biomarker detection, gene expression analysis and gene ontology classification in bambara groundnut. The transcriptome sequence could be used to develop synteny of bambara groundnut to close relative legume sequences to predict the gene expression mechanism in bambara groundnut.

5- Nitrogen fixation activity, nutritional quality and hard-to-cook phenomenon in bambara groundnut deserve further attention to fully exploit the potential of bambara groundnut to contribute to food security and nutrition and agricultural system resilience more generally.

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Appendix

Appendix 1 Characterization of the traits of Bambara groundnut used in this study.

No	Traits	Description
1	Days to emergence	Number of days from sowing to the appearance of first true leaf on the soil surface.
2	Days to flowering	Recorded from seedling emergence to the appearance of the first flower(s).
3	Leaf no./plant	Recorded at harvest as total leaf no/plant in the field experiment.
4	Plant height (cm)	Recorded at harvest and measured from the ground level (at the base of the plant) to the tip of the highest point, including the terminal leaflet.
5	Peduncle length (cm)	Average length of five peduncles per plant, measured at harvest.
6	Internode length (mm)	Average length of fourth internodes measured for five longest stems per plant at harvest.
7	Pod no./plant	Counted at harvest. Number of pods with more than one seed was also determined.
8	Pod weight (g/plant)	Weight of dried pods (at 12% moisture content) was recorded after maintaining the harvest pods for three weeks at 37^{o}C.
9	Seed no./plant	counted after removing the shells of all pods.
10	Seed weight (g/plant)	Weight of dried seed (at 12% moisture content).
11	Biomass dry weight (g/plant)	The dry weight of all organic materials produced by the plant was measured.
12	Days to podding onset	Number of days from seedling emergence to the discovery of the first pod(s) (at least 0.5 cm long).

13	Growth habit	Recorded 10 weeks after sowing for all individual plants, based on the 4th petiole (P)/4th internode (I) length ratio (P/I) as measured in descriptors; Bunch type (P/I = > 9), Semi-bunch type (P/I = 7 – 9) and Spreading type (open) (P/I = < 7).
14	Seed colour/pattern – Testa with pure colour, without eye pattern around hilum	1 Cream 2 Grey 3 Light red 4 Dark red 5 Light brownish red 6 Dark brown 7 Dark purple 8 Black
15	Seed colour/pattern – Testa with pure colour with an eye pattern around hilum	1 Cream testa with black butterfly-like eye 2 Cream testa with dark red butterfly-like eye 3 Cream testa with grey butterfly-like eye 4 Cream testa with black triangular eye 5 Cream testa with brown triangular eye 6 Cream with grey triangular eye 7 Cream testa with black irregular eye 8 Cream testa with grey double thick lines on both sides of the eye 9 Cream testa with brown circular eye 10 Light brown testa with grey butterfly-like eye 11 Light brownish red testa with dark brown triangular eye 12 Grey testa with black triangular eye
16	Seed colour/pattern - Testa with mixed colour, with or without eye pattern around hilum	1 Black small dotted spots on brown background without eye 2 Dark brown small dotted spots on cream background without eye 3 Black and grey mottles on cream background without eye 4 Black and brown mottles on cream background with grey butterfly-like eye 5 Black marbled spots on cream background with grey butterfly-like eye 6 Dark brown marbled spots on cream background with grey butterfly-like eye 7 Black rhomboid spots on cream background on the micropylar end with grey butterfly-like eye 8 Dark brown rhomboid spots on cream background on the micropylar end with grey butterfly-like eye 9 Black rhomboid spots on cream background on both micropylar and non-micropylar ends with grey butterfly-like eye 10 Dark brown rhomboid spots on cream background on both micropylar and non-micropylar ends with grey butterfly-like eye 11 Black stripes on cream background with black butterflylike eye

		12 Black stripes on cream background with black irregular eye 13 Brown stripes on cream background with brown butterfly-like eye 14 Brown stripes on cream background with grey butterflylike eye 15 Brown stripes on cream background with brown irregular eye 16 Cream rhomboid on black background on both sides of the hilum with grey triangular eye 17 Cream rhomboid on dark brown background on both sides of the hilum with grey triangular eye 18 Black Holstein on cream background 19 Dark brown Holstein on cream background

Appendix 2 Testa colour of parental lines, F_2 and F_3 seeds derived from S19-3 × DodR and IITA-686 × Tiga Nicuru.

Appendix 3 Potential lines (cross IITA-686 × Tiga Nicuru) with superior performance than population mean for advancement based on *days to flowering, harvest index and 100-seed weight, plant height* and *internode length*.

Line	*Days to flowering*	*Plant height* (cm)	*Petiole length* (cm)	*Internode length* (cm)	*Number of leaves per plant*	*Petiole internode ratio*	*100-seed weight* (g)	*Harvest index*
Line-6	33.00	25.50	19.50	1.95	82.00	10.00	39.18	0.42
Line-38	31.00	29.00	21.50	1.80	98.00	11.94	30.10	0.28
Line-44	33.00	24.00	19.17	1.95	86.00	9.83	29.78	0.31
Line-48	33.00	26.00	21.50	2.45	116.00	8.78	31.43	0.33
Line-50	33.00	27.00	21.33	1.55	122.00	13.76	31.75	0.35
Population mean	34.98	26.18	19.72	2.03	84.80	10.95	21.85	0.24
IITA-686 (Female parent) mean	32.50	28.15	14.79	2.09	266.50	7.15	24.68	0.38
Tiga Nicuru (Male parent) mean	32.33	22.71	12.52	0.96	77.67	13.36	24.58	0.21

Appendix 4 Potential lines (cross S19-3 × DodR) with superior performance than population mean for advancement based on *days to flowering, harvest index and 100-seed weight, plant height* and *internode length.*

Line	*Days to flowering*	*Plant height* (cm)	*Petiole length* (cm)	*Internode length* (cm)	*Number of leaves per plant*	*Petiole internode ratio*	*100-seed weight* (g)	*Harvest index*
Line-51	36.00	37.00	16.90	3.45	77.00	4.90	30.60	0.44
Line-73	36.00	31.50	17.50	2.75	111.00	6.36	37.86	0.47
Line-86	33.00	27.00	15.50	2.10	82.00	7.38	35.26	0.47
Line-108	36.00	35.00	14.25	2.45	85.00	5.82	35.47	0.49
Line-111	36.00	36.00	17.45	2.40	108.00	7.27	29.21	0.47
Population mean	36.41	28.34	16.20	2.48	77.67	28.34	28.72	0.33
S19-3 (Female parent) mean	33.40	27.33	17.44	2.46	84.80	27.33	27.47	0.47
DodR (Male parent) mean	32.10	34.35	21.60	4.04	266.50	34.35	45.49	0.28

Appendix 5 Recommended individual lines with high *chlorophyll content index* (CCI) and *quantum yield PSII photochemistry* (F_V/F_M) under drought-stressed and well-watered conditions in the F_3 segregating population.

Lines	F_V/F_M		CCI	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Line-14	0.68	0.67	47.45	46.85
Line-33	0.67	0.67	46.70	48.58
Line-36	0.68	0.65	46.10	47.57
Line-39	0.67	0.68	46.63	45.17
Line-43	0.68	0.66	47.02	47.07
Line-50	0.67	0.65	45.15	45.47
Line-63	0.69	0.66	48.73	49.50
Line-64	0.66	0.67	46.66	44.52
Population mean	0.65	0.64	44.50	43.75
S19-3 (maternal line)	0.67	0.64	40.37	43.97
DodR (paternal line)	0.67	0.64	45.23	46.13

Appendix 6 Recommended individual lines with high *chlorophyll content index* (CCI) and *quantum yield PSII photochemistry* (F_V/F_M) under drought-stressed and well-watered conditions in the F_4 segregating population.

Lines	F_V/F_M		CCI	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Line-3	0.65	0.68	41.04	40.82
Line-4	0.65	0.64	40.52	40.92
Line-5	0.65	0.66	39.47	36.40
Line-12	0.69	0.61	39.22	37.14
Line-23	0.69	0.66	39.53	32.30
Population mean	0.65	0.64	37.78	35.98
S19-3 (maternal line)	0.64	0.64	36.37	39.22
DodR (paternal line)	0.62	0.59	37.46	34.72

Appendix 7 Recommended individual lines with high relative water content (RWC) under drought-stressed and well-watered conditions in the F_3 and F_4 segregating population.

Lines	RWC (F_3 segregating population)		RWC (F_4 segregating population)	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Line-6	79.54	76.45	82.47	82.59
Line-12	78.65	76.80	81.25	81.74
Line-20	79.78	76.25	81.38	81.93
Line-21	77.76	74.80	81.14	81.95
Line-37	77.67	75.97	82.77	82.33
Population mean	77.38	74.66	81.06	80.11
S19-3 (maternal line)	76.62	76.94	80.11	79.42
DodR (paternal line)	74.78	77.45	81.95	82.61

Appendix 8 Recommended individual lines with high *harvest index* and *100-seed weight* in the F_3 segregating population.

Lines	*100-seed weight (g)*		*Harvest index*	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Line-11	36.87	30.55	0.37	0.38
Line-20	30.47	39.33	0.39	0.45
Line-26	37.05	31.42	0.41	0.35
Line-33	31.54	31.65	0.41	0.36
Line-35	30.36	30.53	0.43	0.37
Line-49	37.54	29.44	0.48	0.36
Line-50	36.23	37.45	0.42	0.37
Line-51	30.33	38.00	0.37	0.40
Line-65	34.21	32.62	0.44	0.41
Line-80	30.61	29.58	0.40	0.40
Line-82	33.36	36.52	0.42	0.44
Line-87	35.65	32.66	0.39	0.38
Line-90	31.29	30.53	0.47	0.42
Line-92	37.45	35.41	0.37	0.37
Line-93	37.40	39.58	0.39	0.37
Line-99	31.38	30.19	0.42	0.38
Line-105	32.67	35.42	0.38	0.44
Line-108	34.89	30.97	0.40	0.37
Population mean	29.24	28.87	0.36	0.34
S19-3 (maternal line)	19.44	22.69	0.30	0.36
DodR (paternal line)	30.83	32.65	0.26	0.33

Appendix 9 Recommended individual lines with high *harvest index* and *100-seed weight* under drought-stressed and well-watered conditions in the F_4 segregating population.

Lines	*100-seed weight* (g)		*Harvest index*	
	Well-watered	Drought-stressed	Well-watered	Drought-stressed
Line-10	20.93	22.14	0.17	0.19
Line-32	20.54	19.54	0.23	0.24
Line-53	21.80	23.17	0.26	0.23
Line-58	26.54	24.22	0.33	0.55
Line-62	20.05	22.02	0.23	0.36
Line-86	23.84	25.38	0.39	0.39
Population mean	19.59	18.79	0.16	0.19
S19-3 (maternal line)	12.73	15.70	0.12	0.19
DodR (paternal line)	24.79	13.22	0.16	0.09

Appendix 10 Recommended individual lines with high leaf water use efficiency (LWUE) under drought-stressed and well-watered conditions in the F_4 segregating population.

Group	Lines	LWUE (μmol mol^{-1})		*gs* (mol m^{-2} s^{-1})	
		Well-watered	Drought-stressed	Well-watered	Drought-stressed
1	Line-10	93.67	158.58	0.34	0.17
3	Line-21	104.69	136.09	0.17	0.18
3	Line-33	103.21	136.57	0.21	0.19
4	Line-12	90.25	143.52	0.35	0.23
4	Line-28	128.00	154.30	0.12	0.19
Population mean		88.33	129.58	0.37	0.25
S19-3 (maternal line)		97.69	114.5	0.27	0.32
DodR (paternal line)		103.62	108.92	0.29	0.12

Appendix 11 Recommended individual lines with high *gs* under drought-stressed and well-watered conditions in the F_4 segregating population.

Group	Lines	*gs* (mol m^{-2} s^{-1})		LWUE (μmol mol^{-1})	
		Well-watered	Drought-stressed	Well-watered	Drought-stressed
1	Line-7	0.78	0.38	46.50	112.13
1	Line-30	0.47	0.44	62.37	118.50
2	Line-14	0.38	0.36	78.45	109.30
3	Line-19	0.50	0.38	66.44	120.86
3	Line-20	0.38	0.28	104.30	118.31
3	Line-32	0.38	0.32	92.54	98.59
4	Line-11	0.44	0.28	124.47	127.05
Population mean		0.37	0.25	88.33	129.58
S19-3 (maternal line)		0.27	0.32	97.69	114.5
DodR (paternal line)		0.29	0.12	103.62	108.92

Appendix 12 Sequences of DArTseq markers in each linkage groups and their SNP positions.

Linkage group	Position	SNP marker name	SNP	PICRef	PICSnp	Trimmed Sequence 5' -> 3'
1A	0.00	24384744	32:G>T	0.45	0.46	TGCAGTCAAGGGCTTTATTTAGTGTTCCTGAAGCAGTTGGGAGTATTGAGGGAGAGAGTTTTCGCAAAT
1A	5.08	4183841	29:A>G	0.32	0.46	TGCAGTATAATAACTGAAATAAGTCCGCTATTATCAATGGCAGATGGCGGAATATGGTGGAGGAAAAAA
1A	10.61	27640122	39:C>T	0.19	0.11	TGCAGAGGGAAATGTAATTTATATGAGGAGCGGATAAGGCGGAGAATATCGCGAACCAATGAGAGTAAA
1A	14.05	27641212	15:A>G	0.08	0.17	TGCAGGGTGATTCATATGAAGGTTTTTTTGGAGAAGATGGATCAGAATCAATCAGAACTCTTGGAACAC
1A	24.74	4181231	50:T>C	0.48	0.33	TGCAGAAACTAGGAGGCGCCTTGCTGTGTATTGTTTGAAGGTTTGTCTTCTGGTTCCTTTGTCATAGGA
1A	26.83	4184079	38:A>T	0.33	0.47	TGCAGTGTATGTTATCCACTAATGCTTTTTTTGTTTCAACAGGTATGTTGATCACAGTTCCTCATTCTC
1A	27.32	4183326	25:T>G	0.32	0.47	TGCAGACCAGTACGTCAACCATGCTTACAAAACACACATTACCCTTGTTGAAGCTTTCAGAAACATGCA
1A	27.97	24347196	33:G>A	0.31	0.46	TGCAGGCTGCTTCTAATGGGAAATATATTGATTGGTGTTTGGAAATGCTTGTGAAACATTTTGTGCCCC
1A	28.61	4182543	18:T>G	0.46	0.30	TGCAGTAAGCAAAGCTAGTACACACGAAGTACTTTTTGGGGTCGGTGTCATCAATGTTCAGCTTACAGA
1A	36.72	4184152	33:G>A	0.21	0.42	TGCAGAAACTATGCACAAACAAATCCATCAATGGTGAAGGGAGAGCTGAACTAGGATGTCGATGAAAAG
1A	50.23	24383067	25:C>T	0.05	0.14	TGCAGAAAAAAGAGCATAATGACTGCGACCCTTTTTTTTTGTGTGGGGTTGTCTTTGTGTCCGATGTCT
1A	58.36	4182089	68:T>G	0.50	0.45	TGCAGCCATTACAGGATTTCTTGACCAGTTAGTGAATATGAGTCTCCTCAGTCCAGAATTTGTACTCTT
1A	71.15	24384342	17:T>C	0.49	0.37	TGCAGTTTTCAGTCTCGTCCCGAATATGTGTTAGTTTAC
1A	77.31	27640189	45:G>C	0.12	0.19	TGCAGATGTATTGATTATAAAGTCCACTTTGATAATACAGGAGCCGTCAAACTATCGGTACCCCATCAA
1A	80.63	24384393	51:A>C	0.22	0.14	TGCAGTTTTATCACCAAATTAGGATAAACTCTTATTCCTTTGATGCCCTCAAAGTATGTCTATGCTTTT

1A	83.42	24385854	33:C>A	0.15	0.07	TGCAGCCTATGATTGGTCTTTGTCTTGAAATTCCACCGAGCCTAAATCGAATTTTGAACATTCTGCTTT
1A	84.54	4182713	41:A>G	0.39	0.49	TGCAGCAATTGCTGAGAATGCCCAGCAACCCTCTGTATCAAAAAACCAATAACTTAGATAAAGAAACTA
1A	85.03	4182601	5:T>C	0.34	0.48	TGCAGTGAACTGAAACTGTAAAATGCAAAAAGAGACCATTGTAGTGGCATGTAACTTAC
1A	85.66	4183133	12:C>T	0.47	0.49	TGCAGCCCAATTCATACATTGCAAAAATTATCCTATAACACTGGGCTGCTTTCACCACTTTCATCTCTC
1A	94.63	42010867	5:T>C	0.05	0.13	TGCAGTTTGCTATTGTTTGGAGTAGTTGCATTGCATGATACAAATCATCATCATGATTGAACTGGGTGA
1A	98.88	4182901	34:A>C	0.37	0.49	TGCAGACTCACTTTTTACCCCTTTCAAATTTCTCAAGGTCTTTTTGATTGTCTTCTCCGACTTATTTCT
1A	99.53	4183176	20:A>G	0.37	0.49	TGCAGTGCTGGTCCTGGAAGAGAATTATTTTTTGCACTATCCTTACATGTTAGTTGAACTATCCTTTTT
1B	0.00	4181815	68:T>G	0.50	0.43	TGCAGTATTACGCAGCTCAAAGCTTCATAATTATATATTATAAATAATCTATGATTTTGTTGAGCTAAT
1B	1.85	4182837	26:T>C	0.49	0.38	TGCAGATTAGAACATCTCTGAGTTTATTATTTGTTTGTCGGAGTTCATCATATGTAGATGAAGAATTGG
1B	3.68	27638434	12:A>G	0.50	0.43	TGCAGTTGCCAAATCCATGAACTCTTGCTCACATCGCTCTTTCCCTCCCACGTGTTGAGTCATCATCAT
1B	4.90	4182716	27:T>C	0.50	0.43	TGCAGTTGCCAAGTCCATGAACTCTTGTTCACATCGCTCTTTCCCTCCCACGTGTTGAGTCATCATCAT
2	0.00	4180911	61:G>A	0.49	0.40	TGCAGATACATAGACAATATGGAAAATTATATCTTGACAACAGAGCACCACTTGACATTCAGTGAAAGT
2	31.58	24345859	55:G>T	0.18	0.39	TGCAGAACCAGCTTCCAATGAAAAAAAGTACAGAAAACTCGATTCGTTCCACCTAGGGAACTCAGATAT
2	35.39	4183842	38:T>G	0.50	0.42	TGCAGCTGTGTCAGCATAACATGCTGTTTCTATGACTGTTTTTCATATCCAACTGGTGTTTCTTCTTTG
2	35.69	4181308	52:G>T	0.42	0.50	TGCAGAAAGAATAGCAAGCATTGGACAAACAACAATTATTTCTATGGATAGAGATATTTAC
2	36.15	4181273	39:A>G	0.36	0.49	TGCAGTAGCATTCTATGGAGTTCCTTCTCCTCAGCTTGCAGACCCTGCCCAAGCCAAGGCTCCTGTTCA

2	39.43	4176117	19:C>T	0.37	0.49	TGCAGCAAAGGCAGAGCATCCTGAGTGGGATCTGCCAGATAATGCTGGGGAATCGAACGATATACCGGA
2	42.07	4182840	62:T>C	0.17	0.24	TGCAGGAACAGCTCACTCTGGTTCCAAAGTTCCACATCGTACATAGGACTGAAGTAAACAGGTTCAGAC
2	46.61	24346515	47:G>C	0.39	0.18	TGCAGCGGGTGAATCCACTGTGTTTTACTATAAGATGTGAGCATTTCGGACTCATTCTCTGCTACAGGG
2	66.18	4183031	29:C>T	0.32	0.47	TGCAGGCACATTGAGGATGTTTTGAATTTCTGACAGTAGGTGATATTGATATTGTTATACTGTGCATGA
2	66.46	4182464	55:T>C	0.32	0.47	TGCAGATGCGGGGTACTATTCGTCCGGGTCACAAGTGGGATGTAATGGTAAAATATCTGTTGCTTTTCT
2	66.60	4177160	63:T>A	0.32	0.47	TGCAGGATAGAGTGGCCACTTGGCTTTGGTTCGGGAAATGGGGTAAGTAAGGAATCAAAGAGCTCTAAA
2	67.91	4183590	6:A>G	0.32	0.47	TGCAGTAGCAAAGGTACTTTTCTAATAGTTAGAATTCAGATTATTAC
2	71.63	4179802	23:C>T	0.45	0.48	TGCAGGTTACACAAACAAGTTACCTACAAATGCTACTAACATGACGCTAATCTATCACATTTTAC
2	85.95	4181165	62:G>A	0.36	0.41	TGCAGTTTTGCGTAATATATTATCAGCCCTATCTTATATATGCATTGGAAAACACGGATGGTGTGTATA
2	87.45	27636104	41:G>A	0.30	0.24	TGCAGAACGTGAAGTCAGTTTTTGATGCTGCTATCAAGGTGGTCGTCAAGCCTCCGCAAAAACAAGAGA
2	100.03	4182352	58:T>A	0.19	0.40	TGCAGCCTGGCCCTCTTGGCAGGCTAAACAAACGACAGTCAGTCACACATAAAACACATCATGTTCTAT
2	102.80	4184173	68:A>C	0.50	0.41	TGCAGATAAAAGACAAAAAAATAAACAAGGTTAGGCAAAATGATCAAGCATAAAACATTACCAGGATTA
2	119.99	24384394	30:T>C	0.44	0.50	TGCAGAAGGGGAATGAATCCTCTTAGTCAGTTCTTTCATGTGTTAGGGCCATGTGAAGAGAGAGAGACA
2	120.02	4182255	25:G>A	0.50	0.44	TGCAGTTTGGAAGGACATTTTCTTTGTCATTATTTGAATAACAAATTAC
2	148.59	4183573	38:T>C	0.17	0.24	TGCAGCATCCTCATCAGTCCTGCAAAGGAAAAATTATGTGTTAGATGAACACAGCAACATTGTATTATA
2	151.20	4184220	44:G>C	0.30	0.47	TGCAGCATATCAATATGCAATGCTTGATATTGAGTTGTAGCTGAGAGATCAACTAAATCTTCATCGATA

2	152.07	4183635	40:C>A	0.30	0.46	TGCAGGTTCAAACTGATATTGTCATTTGTTTGGGTGCTGACA AATTGTGTACCATTAGATGGGTTTGTT
3	0.00	4183000	54:G>C	0.50	0.41	TGCAGAGAGGTTTGGGACGGTGAAGGAATGAATGGAGCAG ACGCAGAAACCCTAGTTGTCATTCCGCAA
3	11.94	24346055	11:G>C	0.37	0.16	TGCAGAGTCAAGTCAAAAAAGGTTAC
3	13.72	4182223	39:A>T	0.50	0.46	TGCAGACGAGAAGGTTTTCTCCTTTGTCAAAATGGAAGAAG TTGGCAAACTGGCTTCATTAC
3	16.57	4183872	65:T>C	0.35	0.50	TGCAGTGCAGAGTGGAAGGAATGTGTTTCAGTGTACAGCTC CTAGTTAGCAATATATGTACATGGAACA
3	17.13	4184228	51:G>A	0.36	0.41	TGCAGTTCATGAGACCGAATAAAACATGTAAGGCAAAGCA GATTGCCACAGGCAAGTTAC
3	53.36	4183075	48:G>C	0.41	0.50	TGCAGTGCAACCTTACGTTGCTGTCATCAAACCCTAGTTTTT CTGTCTGGTTGCTTATTGTGTTTTTCT
3	57.89	4183420	66:A>G	0.32	0.45	TGCAGTTGATGTTCCCTTATCCATCATAGTTTGGATTTTGTA GGCATAAATGTTCTTTGTGGAATTAGA
3	59.95	4175934	16:G>A	0.20	0.28	TGCAGAGTGCTCTCACGTCTTTCACTTCCCATGCATAGCAGC CCACGTCAAGAAACACCGAAGCCACAC
3	61.26	4182517	46:A>G	0.40	0.49	TGCAGGATGCTGATAAAAACCTTGGGAGAACCATAACACAA AAGCCAACTATTGTAGTTGGAGAGCACA
3	87.10	4183509	33:G>C	0.31	0.36	TGCAGCATGCACAGTTGCACCTTCATTTTCTACGGAAGGAT ACACCTGCACAGGTTCACATCGACGAAA
3	90.51	4179961	13:G>A	0.20	0.27	TGCAGCTGGACAAGTATGAAAACAACCCTGAACCCTGATGG AAGAGGATGATGTTTAC
3	91.65	4182633	30:G>A	0.17	0.24	TGCAGGGGGTTTAGTTTGCTTTCTTGACGTGTATGGCCAAAC TTTATATGTTTGTAACCCATTGACTCA
3	95.85	4183919	10:C>T	0.17	0.24	TGCAGTAAAACAATGACTAAATCTTATCATACAACTATGAG CACATCCACAAGCAAAATTCTTCTTACA
3	106.22	27640349	62:T>G	0.15	0.22	TGCAGCTGACATTTGGTCTGATGTTCTTCAAAGGTTTTCTCA CGGCGACAAATTTCGTATAGTAGATCT
3	119.91	4181500	67:T>C	0.47	0.49	TGCAGCAGCCATTGCTCGCCTCCTACATTATCAGACTGTTGA TTCTGCTGTAACTGATTGTATGAATTG
3	120.54	4176771	67:T>A	0.47	0.49	TGCAGCTGCAATTGACTGAGCTCTAGAAACTCCAGCTGGGC ATGCTGACCAAGCTTGTCTGAGCAGGTG

3	138.77	4178879	51:C>A	0.44	0.46	TGCAGGACCTCTTGAGACATTGAGTACACGCTTCTGTAAGTAACATCTGAACACAAGTACAAACGTGTA
3	143.85	4183649	56:C>G	0.21	0.13	TGCAGAGAACCTTCCCTTTCACTCTTCTCTTTCTTCACCTTTCATCTCATGTTTTCCTTTCATCATCAA
3	145.13	4183449	29:A>G	0.40	0.48	TGCAGTCATGAAAAGCTCCCTGAAGTTGGATGGTGCGGGATGAGTTTGATAAAAACTAAACCATTACAG
3	148.35	4177324	38:C>T	0.35	0.48	TGCAGCAGGAAGCGATGTGCCACTTCTTGATGTAATTGCAAAACCATATGTCATACACAAATAATCAAA
3	148.40	4176354	15:T>G	0.41	0.48	TGCAGTTTTTGTTGCTGTGTGTGTGGACTAGAAGACTGATGGAGAATATCGAGTGAAGTTGTAAAGGAT
3	150.46	4182697	35:C>G	0.40	0.49	TGCAGTTTGAGCCTGAACAGCAACAGGCATGGAAACAGCAAGAATGAGAGTGGCAACCATGGCCACAAC
3	156.66	4181117	42:A>G	0.16	0.22	TGCAGAACCATGGAAGTATATATTGAGTAGAAATGCATATAAATGCTATTGGGAATTTAGATTTACAGA
3	157.36	4183843	33:T>A	0.16	0.22	TGCAGTTGCTCCTGCTGTGAGGACCACTCTATCTGGGTCAAATTTTGCTCTTCCTCCTCTTATTTGTTC
3	162.31	27636178	15:G>A	0.08	0.15	TGCAGACCCAGGCCCGGAAAATTGGCGGGGCCAGGGCGTTTGGAAACAGCTTCTATTACGGCCCACTCG
3	170.98	4182409	15:C>A	0.48	0.36	TGCAGAGTAAATTTCCTGGAAAATACCGCAATGTGGATTGCGCCTGCATAAAACTTTTGAAGATGGAAT
3	171.67	4182516	22:T>C	0.28	0.45	TGCAGGTGAACATTATTTCTGATTCCTTTTTTATTTCAGCTTTTTTCTTTAC
4	0.00	4177259	8:A>G	0.47	0.34	TGCAGTAAATAAGTGGAAATAAACACAAGCGAAGTGTTACTGTATACAATAAAAGGTGTTACTTTTGTA
4	2.64	4175954	24:G>C	0.45	0.47	TGCAGTAACTTCAGTGTAGTAAGCGACCGATGCAGATTCAGATGTCATTGCCAGTTTGTTGTCATATCT
4	3.29	4181663	45:C>T	0.44	0.47	TGCAGTGTATTTTCTTTTTTTGAAGGTTAGATATTTTGTCTATTTCATGATATAGGTCTTTTATTCTTC
4	14.52	4178272	17:C>T	0.29	0.35	TGCAGTAATGTAAAGATCTGTGAAGAAAGTTTTTAC
4	21.74	4182651-2	18:T>C	0.35	0.48	TGCAGCGGCTTCAACTTTTCCCTCTCGAGAAAATGCTGAGTCTATTATGGGTTTAGGGCATACCTCAAC
4	25.32	33598418	17:A>G	0.19	0.40	TGCAGAGGAAGAAGAAGAGAGGGAAAAAGCTGCACAGGAAGAAGAAAAGAAAATGCCAGAAAAAACTCA

4	27.20	4182750	33:A>G	0.18	0.25	TGCAGTGCCATTGACCAATGCCAAACCCTCTTTAGGTTGCAACTCAAAGAAACCTCCATCAATTCCAGC
4	35.41	24347101	12:C>G	0.19	0.39	TGCAGCAGAATTCCACGTGTCCTGTTTGTCGAATATCGTTGCGCGAATTTCCAGATAGAAAGTGGTTAC
4	37.54	4182916	23:C>T	0.47	0.42	TGCAGCTGTTTGACGAATATGAACAAGTGTATATATATTATTTGGTTGCCCACAACTCTCTCTATATGT
4	40.48	4182622	52:A>G	0.41	0.48	TGCAGCTGGTATATAATTTTTTCTTTGTTATTTTTGGAATATCGGATTCTTATGAAGCTTATTGACAT
4	46.91	4183852	21:A>G	0.31	0.46	TGCAGAGAAACAAGCTGCATTAGCTAAAATGCGTCAAGAGAAAGCTCAGTCTCTTGGTGAAGAACCTGA
4	47.49	4182335	20:G>A	0.31	0.46	TGCAGAAGGCATTTTCTTCAGCGCTAGCTGAGCTGCATCTTTAC
4	47.66	4183588	39:A>G	0.46	0.33	TGCAGCGTGTTTAGAATGGTTATGCTCTTCGGGTTGTAGATGTTTGTGATTTGTTGTGATATGCGAATG
4	47.80	4181622	67:G>A	0.31	0.47	TGCAGAGAGTGCTTGAAGAAAGTTCCCCTGTTCTTGTTGCAGCTAGACTTGATGCAGAAGAAAGACGGA
4	48.11	4182641	48:G>A	0.33	0.47	TGCAGGGACATCTCATCCTTATTGCCACGTGACGAGTTTTATCTTTTTGTGTTTAC
4	54.63	4183364	22:G>A	0.30	0.36	TGCAGCACTTGTCGCAGCCATAGCTTCTGTCATCAGGACTTCGCATTCCACCACCACTGGTACCAGAAA
4	54.79	4180564	13:A>G	0.31	0.37	TGCAGAGGAAAAGAGAAGATATTGCATTCATGCATATGTTTCACGTTTCGTCTTTCGATTATTCCTCCA
4	61.60	4178490	25:G>A	0.24	0.44	TGCAGTAACATCTTCATAATAGCCTGCAATTAC
4	70.81	4182207	19:T>C	0.47	0.42	TGCAGCTTGTGGTTGTACGTGCTGACTTGAATGGCCACTTGAAGGCCTTAC
4	71.31	4177629	25:A>G	0.34	0.50	TGCAGTGCAGTATGCTAAATTTAGCATAGTTTTTTTACTCACCAATACCCTATATGTTGGACTGAATCT
4	71.32	4178306	15:C>A	0.42	0.47	TGCAGCTGACTATTCCAGAACATCTTAC
4	71.52	4181489	55:A>C	0.47	0.42	TGCAGTATGTACGCTCTATCCTGTTTGTATTATGAAAATATTGTCTTGAGTTCACAAAAGGGATTACCT
4	97.06	4181606	63:G>A	0.36	0.49	TGCAGTGTGGAACAACTTTTCATCTGCACTTTGACTCTCTCTTTCATTCCTCTCTGTTTCTTCGTTCTA

4	100.72	4175944	41:C>G	0.17	0.23	TGCAGAGTCTCGAAAAACCACTCCCATTGCAGTTCCAACTTCGTCGCGTACACAAGCAGAAAAATTGGC
4	100.83	4183370	40:G>A	0.43	0.47	TGCAGAAGCCATCCAGAAGAGGAGCTTGCAGGCCTGTAGAGCTCTGCAACATTATTCAGTGACGTGCTA
4	101.42	4181474	10:C>T	0.39	0.49	TGCAGAGTATCGAATTACATCGATTTAGTCCTGAATTGAGATTAC
4	102.67	27636542	19:T>A	0.32	0.27	TGCAGCCTGCACCAGAAACTTATGAACTTTTTGATGACATAATATTACTTTCAGAAGGTGAGATTGTTT
4	103.26	4181421	48:C>G	0.26	0.32	TGCAGCTCTAGCATCTAGACCAGTTGTTGGTTCATCCATAAAAATAATCGAAGGATTTGCAACCAATTC
4	106.52	4181058	65:C>T	0.09	0.15	TGCAGGAGCAAAAAGCTGTGCTGGAAAATGAAATTCTGCGCAAACAGGTGAGAACCAAATCTGTCCAAG
4	109.84	27638158	10:C>T	0.49	0.41	TGCAGCATTTCTTTATTTGACATTGTTGTCCTTCAAGTCTCTCTTCTCTCTCTCTCTGAACTCTCTT
4	110.51	4184132	53:A>G	0.45	0.44	TGCAGGCTGTTCCTGATCACGGTTGCTCGGAAATTCTGTCGAAGCATTTGCTGAGATGCAAGAGTCTTG
4	111.14	4182453	53:C>G	0.45	0.45	TGCAGATGTCAGAATACTAATGTAGGGTCATGTGATGGTTGCCTTTGTTTATTCAACTTGTGATTCTTC
5	0.00	4176576	19:A>T	0.05	0.13	TGCAGCATAATTCTTCCAAATATATTTTTTTTCACATGATATAAGCTGGGCTAGTTCGTTTTATGAAAG
5	6.71	4181791	9:T>A	0.27	0.44	TGCAGCAAATGAGAGAATTCTTATTCCCAGCGCCCAGAGCTTGAGCCTGACCTCAATTTGCCCATTTGA
5	10.66	2438545	38:T>G	0.01	0.01	TGCAGATTCCGAGTCCATGACGGAAAGTGACGCCGGATTCGTGAAACGGATCTCGCCGGAGTCCGCGAG
5	14.00	4183046	39:G>T	0.18	0.25	TGCAGGTAAGATATACATAAAGGGTAAATTTTCGCTAGAGTTTGTATTTCAGTCTCTGGTTCATGTGTT
5	18.35	4181376	44:A>C	0.39	0.49	TGCAGGACGAGAAAAAATTTGATGTGATAATCGTTTATGGACTTAGTTAC
5	30.51	42010841	13:C>T	0.06	0.14	TGCAGTTCGACTTCGAGTGCCGGAGCAAAATCCGCGTTTTAGTTTAC
5	33.96	27636601	16:A>T	0.42	0.21	TGCAGCTAGTTTGATAATTGCCTAATAATGGAAAACTCAATAATAGATTCTGCTACCATTTTAGAGTTT

5	40.12	4180783	12:A>C	0.33	0.38	TGCAGGAAAAAAAACTTATTGACCGTGCTGAAAACAAAAAGGAAATGACAATCTAAGATGAGAAAAAAA
5	44.53	4183179	45:A>G	0.41	0.48	TGCAGAAGCTTTATATTCAACTTCACATGAGGAGTGTGCCACAACACTTTGCTTCTTTGAACACCATTA
5	51.82	33598389	59:C>T	0.17	0.38	TGCAGGATGCACCAATTCAAAGTCCACTCAAGGGTCTTGGTTAGTATTACACGATTCTGCGTTCCACTA
5	58.41	4176835	10:G>A	0.42	0.50	TGCAGTGTGCGCTTTGAGTAATTTGCAATTAC
5	72.37	24346358	32:T>C	0.43	0.50	TGCAGCCCCAACAACATCCTATCAAATGCACATAGAAGAATTTTATTAC
5	75.12	4176480	22:A>G	0.38	0.49	TGCAGTAAATAGGACAGTTATTATAGTAGTTGGCAGTTTTCTTAC
5	78.34	4176188	5:T>C	0.18	0.40	TGCAGTTCTATGAAAACTTTCAGTGCATTCAAAAAAATAGATCATCCGATAAAGTGGTATTATTCTGAA
5	86.85	24383308	5:T>A	0.32	0.50	TGCAGTGTGTGATTTAGCCTGTGCACCCGAGAAAAAGATTGACCACCATAGTGGAACAATCACCGAGTC
5	102.19	4182450	27:T>C	0.37	0.50	TGCAGCAACATGAACTCACAAGTCAACTCAATTTGCAACCAATAGCTTAC
5	106.49	4178866	29:C>G	0.42	0.48	TGCAGTGCACGTTGGTCCACCATTCTTCTCCTGTGGATCCTAACATCTCTACCCTTATTCTTTGATTCT
5	109.57	4177957	44:G>C	0.42	0.46	TGCAGAAATTACGAAGGGTGGCTCAGGTACCAACTGCAAGAATGGAAATTCAAATTTCTGGTCATAAAC
5	111.52	4182525	33:G>A	0.50	0.43	TGCAGACAACAAAAACAAGTTATTTTTACTAATGTAAAAACTTGAAACAAAACATTTCATCTCATTATG
5	112.71	4177092	62:C>T	0.42	0.50	TGCAGCATCCATGTCCATCCATCTATCAAAGTTCAATTTAGTGTTACGTTCACTGACTCAGCCGAAAGC
5	113.26	4179158	55:G>T	0.50	0.43	TGCAGTTATATGAAGGTGATGGTTACTTCCATGAAAGAGATCGGTTGGATATGAAGGCTTCTCATCTGA
5	118.91	4184000	33:T>C	0.31	0.36	TGCAGAGGCTTGATGATTGCTTTACCATTGTAGTTGACATGAAACTTTGCCTTTGCCAAGAGCCCCTAG
5	119.07	4181239	10:C>G	0.34	0.38	TGCAGTTTTCCAATCATGGCTCTCTTCAGCAACATGGATTTGCAAGGAACAGGTTTTGGACATTAGATC
5	119.72	4182577	49:T>C	0.31	0.36	TGCAGGCATCCTAGTATTAGCTGTCATGGTATGTCTGCAAGTTTCAAAATATTTACCTATGACCATAAT

6A	0.00	4182879	36:G>C	0.34	0.50	TGCAGTAAATTATAATAAATATAGAAAATGTTTATGGCGATGGTATACTTCTACAACTTTGAATATAAA
6A	2.12	4182556	15:A>G	0.40	0.49	TGCAGTACAGGAAGTAACGTGATGTAAGTATAATTTCTGGATAGTAAACAAAGTAGACGATTATCAGTG
6A	3.47	4176871	25:T>G	0.41	0.45	TGCAGTTATGTTTTTACTGAACTTTTTAGAGTGTTATTGCTAAATACTTTTTTTAC
6A	5.99	4178051	40:G>T	0.50	0.46	TGCAGTTACGGTTCATCATTTTCCTACAATGTTTACCTTTGCATACAAATAAATGTACTCTGTTGTCGA
6A	10.36	4181745	9:T>C	0.44	0.48	TGCAGATGCTGATGCCATATCGCTGAAATGTTGGGATCAGGTAATTTGACAAAACCCAGTTTCATATGT
6A	10.66	4183300	11:T>C	0.23	0.42	TGCAGTTGCAATATTCACGAAGAAATCATCTGGGAGGACAAAACGGGGAACTTTAGATGACTTCCTGTC
6A	11.24	4181739	58:C>T	0.24	0.42	TGCAGTTTCTCCAAAGATGTGTTAGGAAGCCAAGAATGAAATCTCGAAGGATCAGAATCTTGATCAGGA
6A	18.61	4184022	63:C>T	0.35	0.48	TGCAGTGAAGGAAACACCTGCAACAAGTGCAGCCACCACAGAACAAGATTCAGACGTATCCTTCAGCCA
6A	19.98	4179306	16:A>G	0.25	0.44	TGCAGGTGATATGTGGAAAGCAATGGTCTTTCCATGAACATATGCATTTGATTAGCTTTATTTTTCATA
6A	21.26	4183793	38:A>C	0.43	0.47	TGCAGGAACAAACGAAGTTCAAAGTACAGACAAAAGACAAAGCAAATCATCTCCATCCCCAAAACACAG
6A	22.82	24384685	39:A>T	0.39	0.35	TGCAGGGTGTCCTCTACCCTCTCAGATTGGAGTTCCCCTACCGGGAAACTTGATTGGGGTGTCAACGGA
6A	26.77	4176982	7:A>T	0.24	0.43	TGCAGTCATGCAGATATATAGATATTGCTCATTTCACTTCCCGTTTTTCTTTAGCGCTAAAGACAAAGC
6A	47.37	4178271	30:C>G	0.46	0.38	TGCAGAGCAGGGCAATTGAGTGAAGCTGAGCATATCATTCGAACTATGCCATTCCATATTGATGATGTT
6A	51.00	4178265	6:T>G	0.21	0.43	TGCAGTTGTATATTGGATCCTTCCTTGTAGATCACATGATCATCTTAC
6A	51.66	24346089	5:T>C	0.47	0.32	TGCAGTCTCGGATACATCTCTCATCTTCTTTAC
6A	53.60	4183757	44:G>T	0.44	0.50	TGCAGGATTTTTCCTTATCTGCATAGAAGCGTCAAATAGTAAGAGTTCTTTATGATTGAGAGAGCCAAA
6A	55.00	24384616	58:T>G	0.14	0.07	TGCAGCTCCAGTCAAAGGATGCAACACATATGTTACCTGCTTGTGCCTTCCACTCACATTCTACACAAA

6A	56.53	4183466	50:A>C	0.49	0.47	TGCAGTGTTGGAGAAGAAATCCAAGAGCAAGCTTCATTTTC TAGAAAAGCAAAACTTAC
6B	0.00	4182897	66:C>T	0.29	0.37	TGCAGGGGCTATGAACCAGTAGATTTTGTATTCGGTGATGG ATGGAAAGTTGAATCAGTGAGTATCCTT
6B	0.91	4178211	11:A>G	0.42	0.47	TGCAGAGACGAAAATTTGGAAGAAGGACGTGAATCAATTC ACAACATTGCACACTGTTAC
6B	1.69	4181151	49:T>C	0.47	0.42	TGCAGAAACTATTTATGATTTTGACCAAAAGGGTTCCGAAG AAATAATGTCATAGGCGGAAAAACTGTG
6B	5.51	4181907	58:C>T	0.12	0.19	TGCAGATTTTGTTTTATGGTTCGGAATAAAACATTGCGATTT TGATTGATTTGTTGTACATAAGATATA
6B	8.11	24345939	15:T>A	0.41	0.21	TGCAGTTGATGAGTTTTTCTCTTTTTGTAGGCGGGTCCAGGG ACGATTGCAGAAGCCCAGATCAGAGGC
7A	0.00	37320527	33:A>G	0.06	0.15	TGCAGGATTTTTGTGCATTGAATCAGAATTTTTAGTTCTGCA CCTGTCATATGGATCTTGATAGATTTA
7A	4.14	4181653	28:G>C	0.48	0.34	TGCAGAAGAATGAAACCCAGGAATTTCAGAAACAATCATG GTGGGGAAATTAC
7A	4.77	4177991	18:A>G	0.49	0.50	TGCAGCTGCATTGATGTGACCATATCCAGATTTTGATGTCAG CATGGCAGAGACCTTAC
7A	9.94	4183307	38:A>T	0.26	0.31	TGCAGACCTCAAGCCAAAGATCCCAACGGTGAAACTCAAGA ACAATGTGTTATGGACCATATATCAAAA
7A	11.94	4178989	6:C>A	0.32	0.37	TGCAGTCAAAATTCCACCACTCAATCAATAAAAAAAAATAC AATCCAACTGACTTAGTTCCCAATAAAA
7A	29.74	4184098	55:A>G	0.29	0.34	TGCAGAGAGAAATGACTTTGATTGCTTCATAAATGCTATGT GGAGTTGGCTAAATATCAGTGATATTAC
7A	35.38	4175814	10:G>A	0.25	0.43	TGCAGTGTTTGGAAAATGATGGTCTTTTTCTGCTAGATGCTT CTTCCTCAGAAACCTTCGGAGGAAGGT
7A	39.98	4178152	11:T>C	0.22	0.40	TGCAGATATTTTGTAATACGTGGTCAAAAGTTGCAGATTAT ATTCTAGAACTTCTCTTTTGATATAGCT
7A	75.43	4180470	65:G>A	0.50	0.41	TGCAGAATCAGAAACAGAAATCTCTAAAAACGCCAAATTAG GGCACGAAAATGCAATGCAAGCCAGCAT
7B	0.00	24384602	31:G>A	0.11	0.19	TGCAGCGTGGAATTTACCACGCATATATGCAGCACACACAT TCTCGTGCACCATCTGTTACAGCTCCTC

7B	0.68	4182115	35:T>C	0.14	0.22	TGCAGGCCGCAAAATTGACAAGAATATATAGTTAGTTCGATGTCACTTCAATTGGTGACAGTACCGGCA
7B	0.70	4178408	8:T>A	0.11	0.20	TGCAGGCTTAGGATCCTTATTTCAATATTGTGGTTTCTGAGTGTTGTTTCTGCTAAAGTTAC
7B	9.63	4183804	20:A>G	0.42	0.47	TGCAGATACCAGCTGGGCCTATATTTAGTACAGTTTAC
7B	10.26	4181649	46:T>A	0.22	0.42	TGCAGTATCTCAACATAGGTGAATCAGTATTTACTATAATATTTATTCAGTATTTTAC
7B	10.27	4183033	29:A>G	0.22	0.42	TGCAGAAGAATGGCAGAAAAGGAATCTCAATGATGAAATTAGTAACTCTTTCTCTATGCAAACTAATTT
7B	10.95	27640313	15:C>G	0.42	0.23	TGCAGCGCGAGTCTTCACGTATTTCCCCCCCGAAGACGGTGGTTCCGAGCTTCTACGACAAACCGGAGC
8	0.00	4178576	67:G>A	0.25	0.31	TGCAGCTACCAATGGTACTCTCAATCCGCTTTTCACATTTTATCATCAACAAGATAACTTGATCATGGC
8	5.04	4182383	62:A>C	0.42	0.50	TGCAGCAGTAGGCCTTCCCTCGTTTATGTGGTTCTCAAAAGATTGAAGACCACGGTCAATATATCCTGC
8	12.68	27640589	20:T>C	0.32	0.45	TGCAGTGGTGCTCTAGGTGATATAGATGGCTCTTCACAATAATCCAGCTCATTCTGCAAGAGGAATGCA
8	12.71	4182593	60:A>C	0.28	0.45	TGCAGACCAGCTGTACAATAAAAGACAATATAAACTTGGTTATGGTTCTAAATGAAATATATTTGGCTT
8	15.37	4176383	56:T>C	0.41	0.50	TGCAGAATGTGGCCCACGTTGCACAGGTAGATGTTCAAATACGCAATACAAGAAACTGTCGATGTTTGG
8	18.64	4184197	57:A>G	0.13	0.20	TGCAGTAGTAGAGAAGGAAAGCAGTGGAAAAATTGAAGAAACATTATGTCATTTACTATGTTGTATGTT
8	19.22	4178850	5:T>C	0.11	0.18	TGCAGTTGATAGCATGGTACTCAAGACAATCCAATTCATTGTCTACTTTGCATTCAACAATCCTCACTC
8	20.40	24385613	20:A>G	0.50	0.41	TGCAGAAAAACGCGTGGTGGAGGTGAGATTTCTTAC
8	22.57	4183944-1	15:C>T	0.23	0.17	TGCAGCACAAATCATCATTTTCAACAATATCTCATTTAC
8	28.83	4178636	5:G>A	0.16	0.25	TGCAGGTGTGGGAATCAGCATCCAAAGCAGTGAGGGATGAAGAGGAAATAAAACAGAAATTATGTGAAG
8	32.12	27640320	36:G>A	0.26	0.17	TGCAGCGGCGGAGGTGGCGGTGGTTTCGACGGCGCGGATGGAGTTCAAGAGGCGGCTACGGTGGTCCAC
8	32.81	4181676	59:T>C	0.13	0.21	TGCAGAAATCAATTGTTACAGTATCATATGTACCAAAAAGAAACATGCAATTATAAGCTTTCACAACAT

8	33.17	4181185	49:T>A	0.13	0.21	TGCAGCAATGTTGACACAAAAAGATGAAAAGTAGCACACT ACCTCTCACTATTATGCTATTTTAGATTT
8	52.32	4182812	33:T>C	0.37	0.49	TGCAGCCCACACATCTACCCCTGGACCATACTGTTTGGTACC AAATAATAGTTCAGGTGCTCTATACCA
8	52.38	4177434	53:G>C	0.37	0.48	TGCAGATTTTGCAAAGTGAGTGAACATGATGAACACGCCCC AGATAAAATCATGCGAAAAAATCGAAAC
8	55.74	4181906	43:A>C	0.45	0.49	TGCAGTTTCCTTCTTCCTTCCTTTTTCTCATCTTTGTCCACAT ATTTCATTTTCACTGCTGTTTTTTAC
8	59.75	27638348	6:C>T	0.49	0.38	TGCAGTCCCTTTTCTCAACAGAGAAGGCTTCATGCTGCATAG AAGATGGGTTTTGTCGCCGGTGGCAAG
8	60.37	4182355	50:C>G	0.38	0.49	TGCAGAAAACAGTTCAGCAACGTTATTGTGCTGTCTCTTCTG CAATCTTCCATGAATTCATACCAAAAC
8	60.37	24346401	41:A>G	0.38	0.49	TGCAGAAGAAGCAGATTCTCGAGAGGGCGCAGGCTGAGAT TACCAAAATTCAGGAAGGTGATGAGGAGG
8	62.91	4183263	33:A>T	0.29	0.45	TGCAGGAATCAAACATAGGTGTAGATAAGTAAGAACACTAT AAATTTGGTCTAATGATTTATTTTAGAA
8	63.86	4183359	57:G>A	0.27	0.45	TGCAGATGTTGCTGCCAAGGCATATGAGCTGGGTTTGTGTTC ATTATCACAACTCTCGTGCTTTGTTCT
9	0.00	4181595	52:T>C	0.21	0.27	TGCAGGGTTCCCAATCATATTTGAGATTGGTGACATTGGCAT GGAGAGCCTCTTGAACCTCTTTTCTAT
9	4.56	4176923	11:G>C	0.31	0.37	TGCAGCCATCAGAATAAAGAAGATCCCTATCACGTTGACGA GGGAATTCAATTCAATAGGCATATTTCT
9	14.50	4181894	10:A>G	0.30	0.25	TGCAGCAGCGACAGGCCCACCAGTCTTGGCAGCATTTTGAA ATGTTGCAAATGCTGCTGGTGCAAAAGC
9	21.17	4181240	26:T>C	0.31	0.36	TGCAGAAGTGACAGCGTACCTGATTTTGATGAAGCTGTTCA AGGATCTGGTTGGTCACTCAAATACCAT
9	26.20	4182850	24:C>T	0.31	0.46	TGCAGTGACAAGGGAGAGAAACATCATTTTCATGCCTCTGG TATTTGGAAAGCATCAAACATCGGACAA
9	30.66	37313543	13:C>T	0.05	0.13	TGCAGTGAAAAGTCCTTCATTATCAGTGCAAACAAACTCAG TTTCAAAAACAAACAACTGTCTCGCTAA
9	44.81	4181385	52:C>T	0.30	0.43	TGCAGATATAAGGGAAGCAGCAAAAGCTGCTACTTCTGATG AAACTTTCACTCCAAACACTGCTTTTTC
9	48.65	4176822	11:A>T	0.28	0.46	TGCAGGAAAAGAAGGAAAAGTCCACTTTTATTAC

9	52.42	4182120	16:G>A	0.38	0.49	TGCAGTGATTTTCCTAGTTATGAATATAACAACTAAAAAACTCAACTTAC
9	66.76	4176229	18:G>A	0.25	0.45	TGCAGTCACCAAATCAAAGCCATAACACATCAAAATGCATACAAGTTTTCAATTTAC
9	70.03	4181610	29:A>G	0.32	0.37	TGCAGTATAGAGGATAGGTGAATTCCCACACTATAAAAAATATAATGGATAAATTTTATATAATTACAG
9	76.12	4177869	10:A>C	0.46	0.49	TGCAGAAAGAAACTGAGGGGGTGGAGAAAGCAATTACCCATGGTAGAAATCACCAATTTGAGCCCATAG
9	78.06	4182822	22:T>A	0.49	0.47	TGCAGCCACTAAATTTTCAAAATTGGCAATATTACCTTTATAAAAGTGGATTCCAGATTTTTATTTTGG
10	0.00	4183651	7:C>T	0.11	0.19	TGCAGCACCCCAATTTTTCTTCTTGCAGCACCCCCAATTTTTCCATAATTTCACAAATATTTTTTACAG
10	0.51	4183674	23:C>T	0.27	0.34	TGCAGCTTAGTCAAAATCATTTTCCTCTCTCTCCGCTCAATCTTCTCCAAGCACCTTCTCCGCTCAAGC
10	0.59	4182563	9:T>G	0.26	0.33	TGCAGGGTATCATATGGGGATGGTATTGGCGTGGTTTCACAGCAACTTTATACTGTCCTAACCAGATTA
10	2.53	37313638	29:A>G	0.07	0.15	TGCAGGAGGCTGAATGAAGAAAGGTCAACATCATTTCTGTGCCATCATTTAC
10	2.70	24384187	36:T>A	0.07	0.15	TGCAGAATAATTACGCTAATTACCTCCTAAGTAGATTAGTAATGGGATGTGTGATATTAC
10	18.58	24346652	16:C>T	0.47	0.49	TGCAGCTTGGTTTTGACTCTGTGCACTTCGCTAGGATTGATTATCAAGACAGAGAAAAACGCATAGTTG
10	32.66	4178651	27:T>C	0.11	0.38	TGCAGGAAGAAACAGCTAAACGATAAATGAGTTAC
10	37.10	4181438-1	27:T>C	0.12	0.37	TGCAGTAATGTCATGTGGCGTTCCCGATTTATTATTTAC
10	39.76	4181479	31:A>G	0.20	0.22	TGCAGCCCACCTTTTCTACTTACACCTCTAAATTAGAAGTTTTATCCCTCAGTCAAAGTCAAAACCATA
10	42.02	27640107	25:G>A	0.15	0.22	TGCAGAGCAGTAAGAGAGGTAAAAAGAACTCGTCCCAAAATGCATTTCTCGTGAGGTATTTCAAAATTT
10	43.76	24383815	25:G>A	0.13	0.20	TGCAGTAATATAATTTTTTCACCTCGTTCCTCTGTGATAGCCCTTGATCCTTTCTCCACCTGAGTTGTC
11	0.00	24346244	51:G>A	0.11	0.19	TGCAGCATCTCTTCCCAAAATTGTTCCACTATATGCTGCTGGTGCTACATCGATTCCACCATTATCATC

11	0.61	4184109	65:G>A	0.09	0.16	TGCAGATAATAGAGAAACTGAAGGAAAAGGTGTTGTATGTG GTTGGTAATGACTGTAAAGCCTAGGGAA
11	0.71	24385230	54:A>T	0.07	0.15	TGCAGCAGATAAACGTCCTCTTCTTAGAGAACCTCTCTCAG ATACAAGCCTTATAGCTAAGTCCCACCC
11	1.01	4181329	8:C>A	0.10	0.16	TGCAGAACCAATTATTCAATGATTATGTATTATTCTGAAATG ACTTATGGGTTTCCTTTTGAAGAAAGC
11	1.03	24385209	40:A>G	0.40	0.50	TGCAGAAGATGGTGGTTTCACGCGACGGCAAGTGGCTCGCA TCGTTTACGCATGACGGGAGGCTTTTAG
11	24.37	4182510	67:A>G	0.36	0.49	TGCAGCAAATTTCAATAATTTTGACCACATTGGTAAGGCAC AACGTAACAGCCCATCGAGGAGCAGCAC
11	24.42	4175806	15:G>A	0.48	0.36	TGCAGATTTCTTTATGTGTTTCAATTCAACTTCATGTTTTATA TTTCAACTTTTATCAATACAAAACAA
11	26.63	4181067	19:G>A	0.49	0.37	TGCAGTTAGATAAATCTGCGCATATTTTGTTAC
11	35.83	4177456	25:T>C	0.43	0.47	TGCAGAATTATATCCAATTCGTGGATTGTTTAC
11	38.03	2764162	37:T>C	0.33	0.50	TGCAGTTTTGAGCAGTTCTGCATTAC
11	38.06	4176309	42:A>T	0.44	0.45	TGCAGCTTCGGCCATAAAATTCTGCACCACATTCATCACTTG ATTTACTTGATTACCACGCATACTAAT
11	38.06	4182072	59:A>C	0.47	0.46	TGCAGGAGCCTTCTCCCTTCCCCCTGGATTACAAACAACAA AGTAACAAACTTTCAAATACAAAACAGC
11	45.18	4183896	66:C>T	0.44	0.47	TGCAGAGGAAGCTATCCAAACAAATGATTGGAGGTTGCCTG AGGAGGATGGTTGCGGATGCTTGGTCGA

Note: PICRef The polymorphism information content (PIC) for the reference allele row, PICSnp The polymorphism information content (PIC) for the SNP allele row, Trimmed sequence Same as the full sequence,but wtih removed adapters in short marker tags.