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Genetic Architecture of Salt and Drought Tolerance in Cowpea

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Genetic Architecture of Salt and Drought Tolerance in Cowpea

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid and nutrient-dense legume species. It provides affordable source of protein to human. Cowpea cultivation is prevalent in Africa, Asia, the western and southern U.S., and Central and South America. However, earlier reports have shown that drought and salt stress can be devastating to cowpea production. The objectives of this study were to screen for salt and drought tolerance in cowpea and to identify molecular markers associated with these traits. Simple methodologies to screen for drought (Chapter 2) and salt tolerance were developed (Chapter 3). Results suggested that: 1) a total of 14, 18, 5, 5, and 35 SNPs were associated with plant growth habit change due to drought stress, drought tolerance index for maturity, flowering time, 100-seed weight, and grain yield respectively in a MAGIC cowpea population, the network-guided approach revealed clear interactions between the loci associated with the drought tolerance traits, and GS accuracy varied from low to moderate for this population, 2) a total of 7, 2, 18, 18, 3, 2, 5, 1, and 23 SNPs were associated with various traits evaluated for salt tolerance in a MAGIC cowpea population, some of these SNPs were in the vicinity of potassium channel and biomolecule transporters, and significant epistatic interactions were found 3) a large variation of salt tolerance and drought tolerance was found in the panel involving 331 cowpea genotypes which were genotyped with 14,465,516 SNPs obtained from whole-genome resequencing, 4) tolerance to salt and drought-related traits seemed to be associated with the geographical origins of the cowpea genotypes, 5) a significant GWAS peak defined by a cluster of 196 significant SNPs and mapped on a 210-kb region of chromosome 5 was identified to be a good locus candidate for tolerance to trifoliate leaf chlorosis under drought stress in cowpea and harbored hormone-induced genes, and 6) a strong candidate locus for tolerance to leaf score injury under salt stress and defined by a cluster of

1,400 significant SNPs on chromosome 3 was identified and this region harbored a potassium channel gene. The results from this study could contribute to a better understanding of salt and drought tolerance in cowpea. The salt- and drought-tolerant genotypes could be used as parents in cowpea breeding programs.

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Dedication

This dissertation is dedicated to:

- My dad: Second Modeste Velombola
- My mom: Francie Razanamala
- My sister: Francia Second Ravelombola

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Chapter 1. Introduction

Cowpea

Cowpea [*Vigna unguiculata* (L.) Walp] is a diploid legume species (2n=2x=22). Cowpea belongs to the family *Fabaceae* (Verdcourt, 1970). The center of origin for cowpea has puzzled scientists. Some reports evidenced that cowpea was first domesticated in Africa (Richard, 1851). Vaillancourt and Weeden (1992) suggested Nigeria to be a center of domestication for cowpea. High similarity was identified between the chloroplast DNA from wild cowpea genotypes originated Nigeria and those currently cultivated. In addition, Ba et al. (2004) stated that cowpea was domesticated during the Neolithic age by African farmers. However, another investigation claimed that either Ethiopia or southern Africa could be the center of origin (Carvalho et al., 2017). The claim on cowpea being originated from India is also under investigation. Cowpea is widely grown in Africa, Asia, southern Europe, the southern and western U.S., Central and South America, the Middle East, and Oceania (Perrino et al., 1993). Cowpea is grown on over 14 million hectares globally and is considered a legume of economic importance (Singh et al., 2003). More than 9 million hectares of cowpea lands are planted in Central and West Africa (Agbicodo et al., 2009).

Annual cowpea production is estimated to be 5.4 million tons of dry seeds globally. Of which, Africa accounts for 70% of the production (Olufajo, 2012). Nigeria is the leading world cowpea producer (Singh et al., 2003). Significant cowpea production can also be found in Brazil, the U.S., and some countries in Asia (http://www.fao.org/inpho/content/compend/text/ch32/ch32.htm). Average seed yield of cowpea varies between countries. The highest cowpea seed yield is recorded in the U.S. Langyintuo et al. (2003) reported that cowpea seed yield averages (t/ha) were 1.950, 0.110, 0.244, 0.777, 0.284,

0.635, 0.341, 0.663, 0.331, 0.500, 0.489, and 0.827 for the U.S., Nigeria, Niger, Mali, Burkina Faso, Togo, Benin, Senegal, Ghana, Mauritania, Côte d'Ivoire, Chad, and Cameroon, respectively.

Cowpea production has multiple purposes. Cowpea consumption is an affordable way to be provided with proteins having better digestibility; cowpea leaves can be used as fodder for livestock feed. In addition, incorporating cowpea in the diet is health-promoting since it is a nutrient-dense crop. Weng et al. (2017) found that seed protein content was in the range of 21.0%-26.7% from a total of 240 cowpea genotypes. One hundred grams (g) of cowpea seed provides 323.4 kcal consisting of 24.5 g of protein, 51.4 g of carbohydrates, 2.2 g of lipid 16.6 g of insoluble fiber, 2.7 g of soluble fiber, and 2.6 g of ash (Frota et al., 2008). Fatty acid analysis in cowpea seed revealed 29.4% of saturated fatty acids and 70.7% of unsaturated fatty acids. Cowpea seed mineral compounds were (in mg per 100-g seed) 6.8 iron, 4.1 zinc, 1.5 manganese, 510.0 phosphorus, and 1430.0 potassium (Frota et al., 2008). Cowpea seeds are rich in antioxidants. Moreira-Araújo et al. (2017) estimated the cowpea phenolic compound gallic acid ranges from 45.4 to 9.4 mg/100g in cowpea. Average estimates of catechin, epicatechin, ferulic acid, chlorogenic acid, and caffeic acid were (in mg per 100-g seed), 5.7-6.5, 2.9-8.7, 11.1-13.8, 2.4-0.6, and 24.8-30.8, respectively.

Genetic diversity

Cowpea is a highly genetically diverse crop. The worldwide cowpea germplasm consists of approximately 27,600 accessions (Hall, 2012). Of these accessions, 14,000 can be found at the International Institute of Tropical Agriculture (IITA); 8,000 are maintained by the United States Department of Agriculture (USDA; 5,000 are kept at the University of California, Riverside (UC Riverside); and 600 Mediterranean and African cowpea landraces are preserved at The Istituto di

Genetica Vegetale in Bari, Italy. Wild cowpea relatives are held at the Botanical Research Institute in Pretoria, South Africa (Hall, 2012). In the U.S., the University of Arkansas, Fayetteville, accounts for most of the public cowpea breeding lines nationally (Dr. Ainong Shi, personal communication).

Investigations toward sequencing the cowpea genome

Progress has been made toward cowpea genome sequencing. Timko et al. (2008) analyzed the gene-rich regions and hypomethylated spots within the cowpea genome using methylation filtration. A total of 250000 gene-space sequence reads (GSRs) were obtained, of which, 41,260 were annotated. Of the annotated GSRs, 19,789 were unique. A total of 5,888 GSRs corresponded to transcription factors. The sequences are available at http://harvest.ucr.edu and the physical map can be found at http://phymap.ucdavis.edu/cowpea/. Sakai et al. (2016) established for the first Vigna server (http://viggs.dna.affrc.go.jp) based on the azuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi) genome, which is still of assistance for cowpea scientists.

In 2016, Dr. Timothy J. Close from UC Riverside received a 1.6 million US dollar-grant to sequence the cowpea genome (https://ucrtoday.ucr.edu/35843). The establishment of a draft genome for cowpea is underway. The most recent information on the cowpea genome has been provided by Lonardi et al. (2019) and is available on the phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html). A cowpea sequence information of 519.4 Mb is organized within 11 pseudomolecules and 722 scaffolds. 518.8 Mb of data sequence are located in 765 contigs. N50 (L50) for scaffolds was 16.4 Mb, whereas that of contigs was 10.9 Mb (https://phytozome.jgi.doe.gov/pz/portal.html). A total of 29,773 loci harboring 42,287 transcripts were identified. Of which, 12,514 could be alternative splicing-derived (https://phytozome.jgi.doe.gov/pz/portal.html).

Drought stress in cowpea

Significance

Drought stress, due to the insufficient soil moisture, can impair plant growth and development (Blum and Ebercon, 1981). Previous reports have demonstrated that drought occurring at early vegetative growth significantly reduced cowpea yield (Ajayi et al., 2018). Even though cowpea is one of the most drought-tolerant legumes, cowpea plants cannot withstand a long period of drought (Agbicodo et al., 2009), which frequently occurs in areas where cowpea is grown. In addition, drought stress can impair the biological nitrogen fixation of cowpea plants (Elowad et al., 1987). However, breeding for drought tolerance in cowpea remains less advanced compared to other legumes (Specht et al., 2001).

Screening for drought tolerance in cowpea

Screening for drought tolerance has been a challenging task for cowpea breeders. Identifying a simple and reliable parameter for drought tolerance evaluation has long been one of the major objectives of drought tolerance phenotyping in cowpea. Matsui and Singh (2003) suggested that root characteristics were worth considering when phenotyping for drought tolerance. However, Kumar et al. (2008) stated that leaf water content was a reliable parameter for drought tolerance evaluation in cowpea. Screening methodology is also an important aspect to take into account when phenotyping for drought tolerance. Ogbonnaya et al. (2003) evaluated four cowpea genotypes with contrasting response to drought stress. Hydroponic, pot, and field screening approaches were used for drought phenotyping. Results showed low correlation coefficients of the drought-tolerant parameters among the three methodologies. Verbree et al. (2015) used a “Shallow box” approach to evaluate drought tolerance of 40 cultivars and breeding lines from UC Riverside. Drought stress was imposed until most of the plants were completely

dead. Highly drought-tolerant genotypes were IT99K-241-2 and TX2028-1-3-1, whereas highly drought-susceptible genotypes consisted of Bambey 21 and TVu-7778.

Cowpea drought tolerance is commonly evaluated at seedling stage since doing so was practical. Labuschagne et al. (2008) evaluated drought tolerance of 20 African cowpea accessions, measuring stomatal-related parameters and cell membrane stability under drought stress. Bastos et al. (2011) phenotyped 20 cowpea accessions for drought tolerance at the seedling stage. Parameters for drought-stress phenotyping were leaf area index, chlorophyll content, and yield components. The genotypes, BRS-Paraguaçu, Pingo-de-ouro-1-2 and Pingo-de-ouro-2, were drought-tolerant, whereas Santo Inácio and Tracuateua-192 performed the least.

Screening drought tolerance within a population panel of significant size has allowed cowpea breeders to increase the diversity of genotypes being drought-tolerant. A total of 1,288 cowpea accessions from the International Institute of Tropical Agriculture (IITA) was evaluated for drought tolerance in fields (Fatokun et al., 2012). Plant greenness, flowering time, and grain yield were used for drought tolerance phenotyping. Of the 1,288 cowpea genotypes, 142 were highly-drought tolerant. Drought tolerance phenotyping was also conducted using bi-parental mapping populations (Muchero et al., 2013) and an association mapping panel (Wu et al., 2015). Sousa et al. (2015) investigated the drought tolerance of 219 cowpea progenies derived from a recurrent selection program. Water supply was limited to 205 mm, which was one-half less than cowpea plants' requirement. Of the 219 cowpea genotypes, 10 were found to be drought-tolerant.

A study conducted by Belko et al. (2014) demonstrated that maturity time was correlated to drought tolerance in cowpea. A total of 30 early and 30 medium-maturing cowpea genotypes was evaluated for drought tolerance. Data on drought tolerance index and grain yield were collected. Results suggested that medium-maturing cowpea genotypes were more drought-

tolerant than the early ones. Drought-tolerant genotypes were IT85F-3139, IT93K-693-2, IT97K-499-39, IT93K-503-1, IT96D-610, IT97K-207-15, KVx-61-1, KVx-403, KVx-421-25, and Mouride.

Both physiological and agronomic traits can be used to assess drought tolerance in cowpea. Bahadur et al. (2017) investigated leaf water content, photosynthesis, stomatal conductance, transpiration rate, and quantum yield of PSII photochemistry of 29 cowpea genotypes under drought stress. Results showed that the genotypes EC-30590, EC-37988, EC-390241, EC-15296, EC-472283, and Gomti performed well under drought stress. Ajayi et al. (2018) evaluated drought tolerance of 10 cowpea accessions at seedling stage. Plants were drought-stressed for 21 days. After that time, plants were rewatered. Cowpea drought tolerance was evaluating using agronomic straits such as visual rating, wilting percentage, plant height, number of leaves, terminal leaflet length, terminal leaflet with, stem circumference, stomatal conductance and resistance, and recovery rate after plant rewatering. TVu-241, TVu-207, TVu-235, and TVu-199 were identified as drought-tolerant genotypes, whereas TVu-218 and IT98K-555-1 were highly drought-susceptible.

Factors associated with the mechanism of drought tolerance in cowpea

Drought tolerance in cowpea consisted of complex mechanisms (Agbicodo et al., 2009). Tolerance to limited water supply can be associated to morphological, biochemical, and physiological changes (Carvalho et al., 2017). Cowpea root architecture plays a substantial role in drought tolerance (Matsui and Singh, 2003). Slabbert et al. (2004) associated cowpea drought tolerance with the increase in biochemical compounds such as abscisic acid, proline, carotenoid, and oxidases. Cowpea adapting to drought stress exhibited an increase in osomoprotectants (amino acids, sugars, and quaternary amine) (Khan et al., 2015). In addition, cowpea drought

tolerance has been attributed to water leaf status, relative turgidity, vapor pressure deficit, chlorophyll stability, and photosynthesis activity (Mitra, 2001).

Genes associated with cowpea drought tolerance have been investigated via cDNA isolation. These genes encoded for proteins involved in various physiological pathways for drought stress adaptation. Most of these geneses were hormone-induced genes. To date, cowpea drought-tolerant genes consisted of *CPRD8* (Iuchi et al., 1996), *CPRD14* (Iuchi et al., 1996), *CPRD22* (Iuchi et al., 1996), *CPRD12* (Iuchi et al., 1996), *CPRD 46* (Iuchi et al., 1996), *VuNCED1* (Iuchi et al., 2000), *VuABA1* (Iuchi et al., 2000), *VuPLD1* (Maarouf et al., 1999), *VuPAP-α* (Marcel et al., 2000), *VuPAP-β* (Marcel et al., 2000), *VuPAT1* (Matos et al., 2001), *VuC1* (Diop et al., 2004), *dtGR* (Contour-Ansel et al., 2006), *cGR* (Contour-Ansel et al., 2006), *VucAPX* (D'Arcy-Lameta et al., 2005), *VupAPX* (D'Arcy-Lameta et al., 2005), *VusAPX* (D'Arcy-Lameta et al., 2005), *VutAPX* (D'Arcy-Lameta et al., 2005), *GST* families (Gazendam and Oelofse, 2009), *PR-1* (Gazendam and Oelofse, 2009), *VuNSR4* (Silva et al., 2012), *VuNSR10* (Silva et al., 2012), *VuNSR44* (Silva et al., 2012), *VuNSR47* (Silva et al., 2012), and *VuNSR49* (Silva et al., 2012). MircoRNAs were shown to have a positive regulatory role in conferring drought tolerance in cowpea (Barrera-Figueroa and Gao, 2011; Shui et al., 2013).

Epigenetic control of drought stress

Tricker et al. (2013) showed that cytosine methylation assisted Arabidopsis with adaptation to drought conditions, and DNA methylation was heritable. Granot et al. (2009) stated that modifications occurring on the N-terminal tail of histone H3 conferred drought tolerance in shrub (*Zygophyllum dumosum* Boiss.). To the best of our knowledge, studies on drought tolerance at the epigenetic level have not yet been carried out in cowpea.

Salt stress in cowpea

Significance

Salinity is one of the major factors constraining crop production worldwide. Salinity-related issues were estimated to be 12 billion U.S. dollars per year (Läuchli and Lüttge, 2002). Factors such as rock weathering and seawater can increase soil salinity in crop lands (Omami and Hammes, 2006). Poor quality water from irrigation could also increase soil salinity problems (Rengasamy et al., 2006). Cowpea is widely grown in semi-arid tropics (Mishra et al., 2015). Effects of salinity are detrimental to crop growth and development in those areas (Zhang et al., 2012). Salinity has been shown to be yield-reducing for cowpea (Dutta and Bera, 2014). In addition, Aragão et al. (2016) demonstrated that high Na+ concentration in soils could inhibit the uptake of important elements such as $NO3^-$, which resulted in nutrient deficiency in cowpea plants.

Screening for salt tolerance in cowpea

Phenotyping salt tolerance provides cowpea breeders with information on the degree of salt tolerance of the genotypes found in the germplasm. The information resulted from the phenotyping could be used as a screening tool in plant breeding. Selecting for salt-tolerant cowpea genotypes has been carried out at both germination and seedling stages. Murillo-Amador et al. (2000) evaluated a total of 25 cowpea genotypes at germination stage. NaCl concentrations for cowpea salt tolerance screening were 0, 85, and 170 mM NaCl. Overall, a significant decrease in seed germination was found upon imposition of salt stress. The 25 cowpea genotypes were divided into three groups according to their responses to salt tolerance. A later study conducted by Murillo-Amador et al. (2002) reported that ion concentrations in leaves played a substantial role for cowpea salt tolerance phenotyping at seedling stage. A total

of 25 genotypes were evaluated for salt tolerance at seedling stage. Results revealed that the cowpea genotypes Sonorense, CB3, CB27, Cuarenteño, CB46, Paceño, and IT82D-889 exhibited lower Na^+ content in leaves, thus being salt-tolerant. The 25 cowpea genotypes were evaluated for salt tolerance at germination stage. These findings suggested that salt tolerance at germination was not necessarily related to salt tolerance at seedling stage.

Wilson et al. (2006) stated that cowpea could be used as a cover crop in the western part of the U.S.; however, the growing threat imposed by salinity in these areas would prohibit growers from using cowpea as cover crops. To tackle this issue, a total of 12 U.S. cowpea cultivars including CB5', 'CB27', 'CB46', 'IT89KD-288', 'IT93K-503-1', 'Iron Clay', 'Speckled Purple Hall', 'UCR 134', 'UCR 671', 'UCR 730', '8517', and '7964' were screened for salt tolerance.

Results suggested that leaf area and leaf dry weight were correlated to salt tolerance. Wilson et al. (2006) found that the most salt-tolerant cultivar was 'UCR 134', whereas the most affected by salt stress was 'UCR 671'. Almeida et al. (2012) evaluated the vigor of 10 cowpea genotypes (CE-09, CE-11, CE-31, CE-67, CE-70, CE-88, CE-104, CE-182, CE-250, and CE-551) under an increasing NaCl concentration (0, 25, 50, and 75 mM NaCl). Salt phenotyping was performed at seedling stage. The genotypes CE-9, CE-551, and CE-182 were found to be highly salt-tolerant. Ashebir et al. (2013) evaluated salt tolerance of cowpea at seedling stage using higher NaCl concentrations (0, 50, 100, and 200 mM). Results showed that salt stress unfavorably impacted root and shoot length, and root and shoot weight. Effects of salt stress on cowpea were most severe at 200 mM NaCl. The top cowpea performers were 210856, 211557, and Asebot.

A more detailed phenotyping of cowpea salt tolerance was suggested by Mini et al. (2015). A total of 23 cowpea genotypes was evaluated for salt tolerance. Cowpea plant materials were CPD121, PGCP6, KBC5, CoVu702, PGCP5, GC3, NBC5, GC0817, PGCP12, DC15, GC521, KBC2, ACM002, CP16, CO(CP)7, VBN1, VBN2, VCP09-001, IVT-VCP-09-013, VCP-09-016, VCP-09-030, VCP-09-019, and VCP-09-035.

Chlorophyll content, carbohydrate content, proline content, soluble protein, Na+ and K+ contents, salt tolerance index for shoot and root length, and shoot and root biomass were used for salt tolerance evaluation. Mini et al. (2015) stated that salt tolerance in cowpea was highly correlated with K^+/Na^+ ratio in leaf, soluble protein, and chlorophyll content. Salt-tolerant genotypes were KBC2, IVT-VCP-09-013, VBN1, VBN2, CO (CP) 7, VCP-09-001, DC15, PGCP5, and VCP-09-030.Sá et al. (2017) reported on salt tolerance of 19 cowpea genotypes subjected to salt tolerance at both germination and seedling stages. Parameters for salt tolerance screening involved germination speed index, shoot and root length, and fresh and dry shoot biomass accumulation. Results showed that the genotypes 6-MNC02-689F-2-8, 10-MNCO2-675F-4-10, 12-MNCO3-737F-5-9, 16-MNCO2-677F-2, 18-BRS-Pajeti, and 19-Paulistinha were salt-tolerant, whereas 11-MNCO2-675F-9-5, 13-BRS-Tumucumaque, 15-MNCO3-736F-7, and 17-BR17-Gurgueia were salt-sensitive.

In efforts to increasing the variability of salt-tolerant cowpea genotypes for salt tolerance, Ravelombola et al. (2017a) evaluated 151 cowpea genotypes at germination stage. Results revealed that PI582422, 09–529, PI293584, and PI582570 showed higher salt tolerance compared to other genotypes at germination stage.

Complex mechanisms for salt tolerance in cowpea

Salt tolerance in cowpea consists of interdependent complex mechanisms. Significant progress has been made toward understanding cowpea salt tolerance at seedling stage. Despite of these efforts, important pathways leading to salt tolerance in cowpea have remained unexplored.

Proteomic reports relevant to salt tolerance in cowpea

De novo synthesized proteins under salt stress are critical in contributing toward salt tolerance in cowpea. A proteomic study conducted by Sousa et al. (2004) showed that proteins encoded by LEA family genes are *de novo* synthesized under salt stress in cowpea (Fig. 1.1). These proteins were demonstrated to protect leaf cells from being dehydrated under salt stress. A total of nine *de novo* synthesized proteins were found in cowpea stems under salt-stressed conditions, which can contribute to plant tolerance to stress (Sousa et al., 2004). Proteins related to photosynthesis and energy metabolism played an important role in helping cowpea plants to cope with salt stress. In salt-tolerant cowpea genotypes, de Abreu et al. (2014) found an increase in rubisco activase, ribulose-5-phosphate kinase (Ru5PK) (EC 2.7.1.19), glycine decarboxylase (EC 1.4.4.2), and oxygen-evolving enhancer (OEE) protein 2 (Fig. 1.1), whereas a significant decrease in OEE protein 1, Mn-stabilizing protein-II, carbonic anhydrase (EC 4.2.1.1) and Rubisco (EC 4.1.1.39) was identified in the susceptible genotypes. Most of these proteins are involved in the Calvin cycle to capture CO_2. The failure to properly process atmospheric CO_2 under salt stress will result in reduced plant growth and plant death in cowpea as previously described (Mini et al., 2015; Praxedes et al., 2010; W. Ravelombola et al., 2017b) (Fig. 1.1).

A significant increase in proline and other soluble proteins production was associated with cowpea salt tolerance. Salt-tolerant cowpea plants exhibited higher protein and amino acids content compared to the sensitive ones under salt stress (Cavalcanti et al., 2004; Maia et al.,

2013; Mini et al., 2015; Praxedes et al., 2009) (Fig. 1.1). Proline contributes to osmotic adjustment under salt stress (Mini et al., 2015). Moreover, upon removal of salt stress, the accumulated proteins are used to help with plant recovery in salt-tolerant genotypes (Mini et al., 2015). Salt-sensitive cowpea genotypes fail to accumulate proteins under salt stress, which resulted in a loss of recovery ability upon salt stress removal (Cavalcanti et al., 2004).

Roles of oxidases in salt tolerance in cowpea

Previous investigations evidenced the role of oxidases in assisting cowpea plants with withstanding salt stress. Maia et al. (2010) demonstrated that difference in cowpea responses to salt tolerance was attributed to the amount of superoxide dismutase, ascorbate peroxidase, and phenol peroxidase produced under salt stress (Fig. 1.1). These findings were supported by (El-Mashad and Mohamed, 2012).

Brassinolides were significantly increased in salt-tolerant cowpea genotypes under salt stress. El-Mashad and Mohamed (2012) stated that cowpea brassinolides promoted the activity of α-esterase, β-esterase, polyphenol oxidase, peroxidase, acid phosphatase, and superoxide dismutase SOD, ascorbic acid, tocopherol, and glutathione, which help cowpea cope with salt stress (Fig. 1.1). However, Cavalcanti et al. (2004) reported that oxidases such as superoxide dismutase, catalase, and peroxidases fail to protect cowpea leaf cell structure from being damage by oxidative, which was triggered by Na^+ in leaves. A later study conducted by Praxedes et al. (2014) demonstrated that ascorbate peroxidase, glutathione reductase, and guaiacol peroxidase did not confer salt tolerance in cowpea.

Despite of these contrasting finding, catalase and superoxide dismutase played an important in protecting cowpea from intensive lipid peroxidation under salt stress (Praxedes et al., 2014). Mini et al. (2015) supported that peroxidases contribute to hardening cell wall under

stress, which resulted in a reduced plant growth but less susceptible to oxidative damage in cowpea (Fig. 1.1). Alternative oxidases called Aox proteins were found to help cowpea to withstand salt tolerance (Costa et al., 2007). Aox proteins are encoded by *VuAox2b* genes in cowpea. Costa et al. (2007) reported that overexpression of *VuAox2b* in cowpea not only contributes to salt stress but also limits the effects of limited water supply (Fig. 1.1).

Involvement of carbohydrates in salt tolerance in cowpea

The importance of carbohydrates to salt tolerance in the cowpea literature have been conflicting. Salt-tolerant cowpea plants exhibited higher carbohydrate contents that the salt-sensitive ones when salt stress was applied (Mini et al., 2015). The accumulation of carbohydrates could contribute to cowpea survival through various physiological pathways within plants (Fig. 1.1). However, Praxedes et al. (2011) stated that there was a poor correlation between carbohydrate accumulation and salt tolerance in cowpea. Therefore, further investigations are needed to unravel the possible roles of carbohydrates on salt tolerance in cowpea.

Genetic mechanism of salt tolerance in cowpea

Antiporter Na^+/H^+-associated genes were one of the most investigated genes affecting tolerance of crops to salt stress. In soybean (*Glycine max* L.), Qi et al. (2014) identified an antiporter Na^+/H^+ *GmCHX1* conferring salt tolerance. *GmCHX1* was located on chromosome 3 in soybean. It was also co-localized with previously identified major salt-tolerant-associated QTLs (Qi et al., 2014). *GmCHX1* limited Na^+ uptake from roots. In addition, *GmCHX1* was highly expressed in soybean leaves. In cowpea, prior to plant establishment, cowpea ribonuclease in cotyledons was shown to significantly increase tolerance of newly emerged plants to salt stress

(Gomes-Filho et al., 2008) (Fig. 1.1). Cowpea ribonucleases were also associated with seed germination salt tolerance in cowpea (Gomes-Filho et al., 2008).

To the best of our knowledge, mechanisms of tolerance to Cl^- have not been investigated at the gene level in cowpea. Therefore, we will focus on mechanisms of Na^+ tolerance. Mishra et al. (2015) described a candidate cowpea Na^+/H^+ antiporter gene, *VuNHX1*, which can affect salt tolerance in cowpea. *VuNHX1* transcript was 1,981 bp with an open reading frame of 1,629 bp. A BLAST between *VuNHX1* against the soybean genome using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that *VuNHX1* sequence was 91% identical (E-value=0.0) to *GmCHX1*, which was the Na+/H+ antiporter gene described in soybean, suggesting that *VuNHX1* could confer salt tolerance in cowpea. Mishra et al. (2015) found that *VuNHX1* was highly expressed in cowpea leaves and roots (Fig. 1.1). This finding was in agreement with reports of Praxedes et al. (2010) and Mini et al. (2015) who stated that cowpea salt tolerance was highly correlated with Na^+ concentration in leaves and roots. Salt-tolerant plants had the ability to prevent Na^+ from being taken up at the root level (Fig. 1.1).

Cowpea plants which were sensitive to salt stress failed to stop the excessive Na+ concentration within the rhizosphere from entering the plant system, which resulted in cowpea leaves being highly saturated with Na^+ (Praxedes et al., 2010) (Fig. 1.1). The excessive Na+ within leaf cells engendered osmotic stress leading to stomatal closure, which caused a significant restriction of CO_2 uptake (Cavalcanti et al., 2004) (Fig. 1.1). In addition, the high leaf Na^+ concentrations triggered intensive oxidative damage. This led to lipid superoxidation resulting in cell membrane and constituent damage, thus destruction of cell membrane integrity (Cavalcanti et al., 2004; Praxedes et al., 2010; Praxedes et al., 2014) (Fig. 1.1). de Abreu et al. (2014) showed a significant decrease in proteins involved in photosynthesis and energy

metabolism in cowpea such as OEE protein 1, Mn-stabilizing protein-II, carbonic anhydrase (EC 4.2.1.1) and rubisco (EC 4.1.1.39) in salt-sensitive cowpea after salt stress (Fig. 1.1). A dramatic decrease in chlorophyll content was also reported in salt-sensitive cowpea genotypes (Mini et al., 2015; Praxedes et al., 2010). As a result, photosynthetic activity and physiological pathways were significantly impaired, leading to plant death (Fig. 1.1).

VuNHX1 in salt-tolerant cowpea plants is translated into a protein containing a conserved amiloride binding site (Mishra et al., 2015). The amiloride binding domain has been shown to inhibit Na+ channels (Xing et al., 2011) (Fig. 1.1). Since *VuNHX1* is highly expressed in both salt-stressed cowpea roots and leaves (Mishra et al., 2015), the transport of Na^+ to the upper part of the cowpea plants is limited. Praxedes et al. (2010) stated that salt-tolerant cowpea plants had a lower Na^+ content in leaves, which resulted in less oxidative damage occurring in leaves. Mini et al. (2015) reported that salt-tolerant cowpea plants had higher K^+/Na^+ ratio in leaves, suggesting an enhanced K^+ transport and an inhibited Na^+ transport. Imamura et al. (2008) stated that *VuCIPK1* in cowpea was activated through phosphorylation under salt stress in cowpea. *VuCIPK1* encodes for calcineurin B-like protein-interacting protein kinase, which is involved in K+ transport in cowpea (Fig. 1.1). A lower Na+ content in cowpea leaves resulted in a less damaged cell membrane structure and cell constituent (Cavalcanti et al., 2004), which lead to salt-tolerant cowpea plants exhibiting higher chlorophyll content than the susceptible ones (Praxedes et al., 2010; Mishra et al., 2015). The net photosynthetic activity has been demonstrated to be less impaired in salt-tolerant cowpea genotypes (Praxedes et al., 2010) (Fig. 1.1).

The role of Na^+/H^+ antiporter genes in conferring salt tolerance in cowpea was further investigated by Mishra et al. (2014). A mungbean (*Vigna radiata* (L.) R. Wilczek) Na^+/H^+

antiporter gene, *VrNHX1*, was isolated and used to transform cowpea plants. Successfully transformed cowpea plants showed K^+/Na^+ ratio, greater Na^+ in roots, lower lipid peroxidation, hydrogen peroxide, and oxygen radical. In addition, transgenic cowpea plants exhibited higher water, proline, ascorbate, and chlorophyll contents compared to the non-transgenic ones under salt stress (Mishra et al., 2014). Cowpea salt tolerance involves complex mechanisms. However, more research is required in order to find the most prominent mechanisms that will help breeders be provided with markers for major salt-tolerant genes in cowpea.

Epigenetic regulations of salt tolerance

Previous reports evidenced that salt tolerance was heavily epigenetically controlled in plants. Wang et al. (2016) showed that epigenetics significantly contributed to salt tolerance in upland cotton (*Gossypium hirsutum* L.). DNA-methylation occurred across the genome of salt-stressed cotton plants. Results showed that some genes were hypermethylated, whereas the others were hypomethylated, leading to a change in expression of salt tolerance-related genes in cotton. In wheat (*Triticum aestivum* L.), Kumar et al. (2017) stated that methylation occurring on cytosine of high-affinity K^+ transporter (HKT) genes was key to confer salt tolerance. Methylation was used as epigenetic mark for salt tolerance in wheat. Bharti et al. (2015) reported that demylathylation of the promoter and the coding region of *AtROS1* gene (involved in flavonoid biosynthetic pathway) in transgenic tobacco provided salt tolerance. Golldack et al. (2011) reported that DNA methylation promoted expression of salt stress-induced in plants. In soybean (*Glycine max* L.), Song et al. (2012) found change in chromatin structure enhanced salt tolerance.

Despite of the critical role of epigenetics in salt tolerance, epigenetic-related mechanisms triggering salt tolerance in cowpea remain unexplored. Understanding the control of salt

tolerance at epigenetic level in cowpea will provide new insights to salt tolerance, and will have applications to modern cowpea breeding programs.

Single nucleotide polymorphism (SNP) markers

Single nucleotide polymorphism (SNP) refers to a single variation in nucleotides between DNA sequences or fragments resulting in polymorphism among individuals (Batley and Edwards, 2007). SNPs have been frequently used in efforts toward unraveling the genetic control of important traits in various organisms since SNP markers are cost effective (Seeb et al., 2011). In crop genetics, SNP discovery has significantly contributed to genome mapping and gene isolation research (Varshney et al., 2009).

Regarding cowpea genetics and breeding, SNPs have been commonly used to perform genome-wide association studies (GWAS) (Shi et al., 2016) and quantitative trait loci (QTL) analysis-related studies (Lucas et al., 2013). Muchero et al. (2009a) established a 1,536-SNP GoldenGate genotyping platform for cowpea. A cowpea 60K-SNP chip is also available for SNP genotyping (Close et al., 2015). The current advance in sequencing technology (next generation sequencing) has allowed the discovery of high density SNPs across crop genomes.

Next generation sequencing (NGS)

Next generation sequencing (NGS) technology has provided scientists with a cost and effective method of DNA sequencing. Current NGS plaforms are Roche 454®, Illumina®, SoliD®, HeliScope®, Ion Torrent®, PacBio®, and Oxford® nanopore (Glenn, 2011). Platforms differ in terms of read length and cost per million bases (Rhoads and Au, 2015). The cowpea accession IT97K-499-35 was whole genome shotgun-sequenced with a sequencing coverage of

62X using Illumina HiSeq series (Muñoz-Amatriaín et al., 2017). Genotyping-by-sequencing (GBS) and whole genome resequencing (WGRS) are currently among the most common approaches for genome-wide SNP genotyping.

Genotyping-by-sequencing (GBS) and whole genome resequencing (WGRS)

Genotyping-by-sequencing (GBS) is a sequencing approach aiming at providing a reduced representation of the genome, thus cost-effective. GBS technology was first described by Elshire et al. (2011). Briefly, a restriction enzyme digests DNA fragments, which lead to a mixture of sticky-ended restriction fragments. A barcode adaptor along with a common adaptor is ligated to each fragment end. Fragments with both adaptors will be further processed for *in situ* PCR and sequencing in order to generate reads. However, GBS can generate a significant amount of missing data.

Whole genome resequencing (WGRS) has become more popular since the cost per million bases for DNA sequencing has significantly decreased. Thanks to the relatively recent published draft and complete genomes of various crops, whole genome resequencing has been possible. This approach allows the discovery of a large number of SNPs across the genome. Increasing sequencing coverage can substantially decrease the issues caused by sequencing error. The discovery of high density markers has permitted the establishment of a more accurate marker-trait association study (Thudi et al., 2016).

Genome-wide association study (GWAS) in cowpea

Linkage disequilibrium (LD)

Genome-wide association studies (GWAS) have been widely adopted in plant genetic research. GWAS refers to a genetic mapping strategy based upon linkage disequilibrium (LD), or nonrandom association of alleles at different loci as described by (Nordborg and Tavaré, 2002). GWAS provides a higher resolution mapping through the establishment of a detailed recombination events at a kilobase level within the genome (Nordborg and Tavaré, 2002). Inbreeding, relatively small effective population size, low recombination rate, admixture with a population, and selection process increase LD, whereas factors such as outcrossing, and high recombination and mutation rate can result in a decrease in LD (Gupta et al., 2005). GWAS is also performed using regression analysis (Remington et al., 2001).

LD calculation is complex and achieved by using statistics. Some LD calculations commonly used in the literature for alleles at two loci are the following.

(1): Disequilibrium coefficient (Weir, 1979)

$D_{AB}=p_{AB}-p_Ap_B$

where D_{AB} is the disequilibrium coefficient, p_{AB} is the frequency of the AB haplotype, and p_A and p_B are the frequency of alleles A and B, respectively.

(2): Normalized disequilibrium coefficient (Weir, 1979)

$D'_{AB}=D_{AB}/\max(-p_Ap_B,-p_ap_b)$ if $D_{AB}<0$

$D'_{AB}=D_{AB}/\min(p_ap_B,p_Ap_b)$ if $D_{AB}>0$

where A and B are two loci with alleles A/a and B/b, respectively, D'_{AB} is the normalized disequilibrium coefficient, D_{AB} is the disequilibrium coefficient as previously described,

frequencies of alleles A/a are denoted p_A and p_a, respectively, frequencies of alleles B/b are denoted p_B and p_b, respectively.

(3): Squared Pearson's correlation coefficient between two loci (Hill and Robertson, 1968)

$r^2 = D^2_{AB}/(p_A p_a P_B p_b)$

where D^2_{AB} refers to the square of the disequilibrium coefficient, and p_A, p_a, P_B, and p_b are the allelic frequency.

(4): Square of the difference in proportions (Kaplan and Weir, 1992)

$d^2 = [(p_a p_B/p_A)-(p_A p_B/p_a)]^2$

(1), (2), and (3) are symmetric LD measurements, whereas (4) is not since allele order matters (Nordborg and Tavaré, 2002).

GWAS workflow

An overall workflow of a GWAS-based approach is shown in Fig. 1.2. Briefly, an association mapping panel consists of a set of individuals with supposedly distantly linked genetic background. Phenotypic data on the association panel is collected. Phenotyping is carried out based upon appropriate experimental designs if doing so is possible

Genotyping is conducted across all individuals within the mapping population. Recently, SNPs are popular in providing high-throughput genotyping. SNP genotyping is achieved by using either genotyping-by-sequencing (GBS), double digest RADseq (ddRADseq), SNP chip, or whole genome resequencing (WGRS). A 60K SNP Illumina Infinium BeadChip is available for cowpea (Close et al., 2015). GWAS is conducted using in-built statistical models in TASSEL 5, GAPIT, FarmCPU, BLINK or PLINK.

Agronomic traits

Qin et al. (2016) reported three SNP markers (C35063613_1497, Scaffold81493_886, and Scaffold84620_6785) associated with seed coat color in cowpea (Table 1.1). A total of 339 cowpea accessions were genotyped using 1049 SNPs postulated from GBS. For pod length, a total of 72 significant SNPs were found (Xu et al., 2017). These SNPs were located on chromosomes 1,2,3,4,5,9,10, and 11. The association panel consisted of 299 cowpea plant materials which were genotyped using 30211 SNPs. Glycosyl transferase was reported as candidate gene involved in pod length in cowpea (Xu et al., 2017) (Table 1.1). Studies on the genetics of root architecture have been reported by Burridge et al. (2017) using GWAS. SNPs, 4749_1972, 11851_914, 2326_226, 14604_737, and 1004_587, were found to be highly associated with adventious root angle, basal root angle, root tissues angle, median root width, and root density, respectively. SNP markers associated with cowpea stem diameter were 13772_1075, 5084_519, 4836_807, 139_439, 8969_1386, and 11138_624, which were identified on chromosomes 1, 3, 6, and 7 (Burridge et al., 2017). Ravelombola et al. (2017b) reported three SNPs, C35063613_1497, Scaffold81493_886, and Scaffold84620_6785, associated with seed germination in cowpea. A total of 10 SNPs were identified to be highly associated with plant growth habit in a cowpea panel accession consisting of 487 genotypes (Ravelombola et al., 2017c), which were genotyped with 1,031 SNPs from GBS.

Abiotic stress

Studies on the genetics of drought tolerance in cowpea were undertaken. Muchero et al. (2013) evaluated the drought tolerance of 383 cowpea genotypes using GWAS under field conditions. The experiments were conducted in the U.S., Burkina Faso, Nigeria, and Senegal. A total of 13 SNPs (1_0029, 1_0589, 1_0067, 1_0206, 1_0888, 1_0049, 1_0108, 1_1150, 1_0279,

1_0983, 1_0140, 1_0759, and 1_1405) (Table 1.1) were found to be associated with drought tolerance in cowpea. Xu et al. (2015) investigated 95 cowpea genotypes for tolerance to soil drought. The association panel was genotyped using a 1,536-SNP assay. A total of 39 drought tolerant-significant SNPs were identified. SNP markers were located on chromosomes 2,3,4,5,6,7,8,9,10, and 11.

Ravelombola et al. (2017d) reported 10 SNPs associated with low phosphorus conditions and rock phosphate response in a panel 357 cowpea genotypes. The association panel was genotyped with 1,018 SNPs from GBS. The genetic control of salt tolerance in cowpea was investigated by Ravelombola et al. (2017b). SNPs, Scaffold87490_622, Scaffold87490_630, and C35017374_128, were associated with salt tolerance at germination stage, whereas Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 were reported to be associated with salt tolerance at seedling stage. A total of 116 and 155 cowpea genotypes were used for salt tolerance research at germination and seedling stage, respectively (Table 1.1).

Biotic stress

Providing cowpea breeders with molecular markers associated with disease resistance is critical in speeding up the process of releasing new disease-resistant cowpea cultivars. Bhattarai et al. (2017) reported SNPs (C35069548_1883, scaffold65342_6794, scaffold66293_6549, scaffold95805_2175, C350 81948_540, and scaffold17319_4417) associated with cowpea mosaic virus in a panel of 333 cowpea genotypes. A total of 1,033 SNPs were used for the GWAS analysis.

Significant SNPs associated with cowpea bacterial blight (CoBB) due to *Xanthomonas axonopodis* pv. *vignicola* (Xav) were reported by Shi et al. (2016). CoBB-resistant SNPs were

C35025883_1166, C35046071_1260, C35083564_3310, C35084634_455, scaffold89853_3955, scaffold92472_1355, scaffold96328_3387, and scaffold96765_4430. The plant materials consisted of 249 cowpea genotypes. Genotyping was achieved using 1,031 SNPs obtained from GBS. SNP LOD values were in the range of 1.4 to 12.4 using EcMLM of GAPIT.

Wu et al. (2015) reported 18 SNP markers (1_0075, 1_1111, 1_1147, 1_0251, 1_0895, 1_0691, 1_0897, 1_0298, 1_0410, 1_0857, 1_0981, 1_1369, 1_0691, 1_0330, 1_1062, 1_0629, 1_0318, and 1_1504) through GWAS for resistance to fusarium wilt resistance, which is caused by *Fusarium oxysporum* f. sp. *Tracheiphilum* (Table 1.1). SNP marker 1_0410 had an LOD of 24.5, suggesting a major QTL affecting fusarium wilt resistance in cowpea. This major QTL was located on linkage group 8.

A GWAS analysis of aphid (*Aphis craccivora* C.L.Koch) resistance was conducted by Qin et al. (2017). A total of 338 cowpea materials were phenotyped for aphid resistance and GWAS was performed using GBS. Two SNP markers, C35011941_894 and Scaffold30061_3363, were found to be highly associated with aphid resistance in the 338 accessions.

Seed antioxidant content

Seed antioxidant content was evaluated in a set of 339 cowpea genotypes, and GWAS on this compound was conducted using 1,047 SNPs postulated from GBS as described by Qin et al. (2016). SNP markers, Scaffold7139_14363 and Scaffold29110_4657, were reported to be associated with seed antioxidant content in cowpea (Table 1.1).

GWAS using a MAGIC population for cowpea

The first Multiparent Advanced Generation Inter-Cross (MAGIC) cowpea population was developed by Huynh et al. (2017) to advance trait pyramiding in cowpea. A total of eight parents, SuVita 2, CB27, IT93K-503-1, IT89KD-288, IT84S-2049, IT82E-18, IT00K-1263, and

IT84S-2246, each having one or more of the aforementioned traits (abiotic and biotic stress tolerance) were used to develop the MAGIC cowpea population. The F_1 population was verified using the Kompetitive Allele-Specific PCR (KASP) cowpea assay (LGC Genomics Ltd., Hoddesdon, UK) based on the 1,536 SNP Illumina Golden Assay established by (Muchero et al., 2009a). True F_1 lines were further processed to generate 305 $F_{8:10}$ MAGIC cowpea RILs.

The MAGIC cowpea population was phenotyped for different traits in the summers of 2015 and 2016. Results suggested a major QTL (LOD=7.8) for flowering time exaplaining 30% of the variation in the phenotype was attributed to the detected QTL (Huynh et al., 2017). In addition, more recombination events were identified within the MAGIC population compared to other traditional bi-parental populations. Crossovers likely occurred at an average of 1.43 cM/Mb within the MAGIC cowpea genome.

Higher recombination rate was found in the vicinity of the telomeric distal regions of the chromosomes. The highest recombination rate was identified on chromosome 3 (1.76 cM/Mb), whereas the lowest one was on chromosome 10 (0.88 cM/Mb). The high recombination rate detected in the magic MAGIC cowpea population increases the likelihood of QTL identification as described by Huynh et al. (2017). However, this MAGIC population could lack salt-tolerant traits since the founder parents to establish the population were not phenotyped for salt tolerance. Moreover, no reports on salt tolerance were established for this MAGIC population.

QTL mapping in cowpea

QTL studies on cowpea have been conducted in efforts to understand the genetics governing traits of interest such as yield, seed size, pod characteristics, leaf morphology,

photoperiod sensitivity, phenology, disease resistance, nematode resistance, insect resistance, and abiotic-related stress resistance such as drought and heat.

Agronomic traits

QTLs associated with agronomic traits were reported in cowpea. Fatokun et al. (1992) identified two QTLs, *pO103* and *pA816,* associated with 100-seed weight. These QTLs were located on linkage groups 2 and 5, respectively. A total of 58 F_2 lines derived from the cross between TVNI 963 and IT2246-4 (Improved cultivar) were used for QTL analysis. Those lines were genotyped using 84 RFLPs (Table 1.2). A study conducted by Andargie et al. (2014a) showed 7 QTLs *qsw1, qsw2.1, qsw2.2, qsw3.1, qsw3.2, qsw7,* and *qsw10* for 100-seed weight. These QTLs were located on chromosomes 1, 2, 3, 7, and 10 respectively. Andargie et al. (2014a) suggested that cowpea seed germination is quantitatively inherited and reported that seed germination was controlled by a QTL (LOD=3.3) explaining 12.9% of the variation in the phenotype.

Pottorff et al. (2012a) identified a major QTL (LOD=30.9-33.8, KW p-values<0.0001) (Table 1.2) controlling leaf shape in cowpea, which accounted for 34.7 % of the variation in the phenotypic data. A candidate gene, EZA1/SWINGER, was suggested to affect leaf morphology in cowpea (Pottorff et al., 2012a). The mapping population consisted of 122 RIL lines (F_{10}) from the cross between Sanzi (sub-globose leaf shape) and Vita 7 (hastate leaf shape). The lines were genotyped using 416 SNPs. The genetics of cowpea floral scent compounds were investigated by Andargie et al. (2014b). A cross between 524B (domesticated) and 219-01 (wild type) was established to generate 159 RIL lines (F_7). QTL analysis was conducted based on 202 SSRs. A total of 64 QTLs found on chromosomes 1, 2, 4, 5, 6, 7, 8, and 10 were reported.

Four QTLs *Dro-7*, *Dro-8*, *Dro-1*, and *Dro-3*, controlling biomass yield under drought stress were mapped on chromosomes 6, 10, 7, 1, and 2, respectively; six grain yield-QTL*s*, *Dro-7*, *Dro-10*, *Dro-8*, *Dro-1*, *Dro-3*, and *Dro-4*, found on chromosomes 6, 7, 1, and 2, respectively, were reported from a cross between CB46 (drought-tolerant) X IT93K-503-1 (drought-susceptible) (Muchero et al., 2013). Kruskal-Wallis (KW) model was used to perform QTL analysis. KW p-values varied from 0.0005 to 0.05 (Table 1.2).

Flowering time was controlled by a QTL (LOD=3.1) located on chromosome 1, with an R-square value of 18.5% (Andargie et al., 2014a), which were not in agreement with the results found by Huynh et al. (2017). In fact, Huynh et al. (2017) reported a major QTL on chromosome 9 for days to flowering. The ovule number of cowpea flowers was suggested to be controlled by two QTLs, *qon1* (LOD=3.9, R-square=11.6%) and *qon3* (LOD=3.0, R-square=10.6%), both mapped on chromosome 1. Pod features in cowpea were reported to be affected by QTLs. An $F_{2:3}$ population consisting of 188 individuals and genotyped with 23 SSRs was developed to perform a QTL analysis for pod cellulose, hemicellulose, lignin, and twistiness (Suanum et al., 2016). Two major QTLs, *qCel1.1* (LOD=15.9, R-square=31.6%) and *qCel7.1* (LOD=8.1, R-square=15.5%) (Table 1.2), located on chromosomes 1 and 7, respectively, were found to impact pod cellulose. A candidate gene, cellulose synthase, was described to be located within *qCel1*.1 region. Pod hemicellulose was controlled by a major QTL (LOD=25.6, R-square=61.1%) *qHem7.1* (Table 1.2). A major QTL, *qLig7.1* (LOD=20.0, R-square=47.8) was found to affect pod lignin in cowpea.

Pod twistiness was controlled by one QTL *qLig7.1* (LOD=9.0, R-square=28.4%) (Table 1.2). All QTLs associated with pod hemicellulose, lignin, and twistiness were located on linkage group 7 of the $F_{2:3}$ population derived from the cross between JP81610 (yardlong bean) and

TVnu457 (wild cowpea). Overall, these findings were consistent with the results from a backcross BC_1F_1 (JP81610) population except for two additional QTLs located on chromosomes 2 and 4 for pod cellulose and hemicellulose, respectively. *MYB* gene families were found to be candidate genes for pod hemicellulose and lignin in cowpea, whereas no candidate genes were reported for the QTL controlling pod twistiness (Suanum et al., 2016) (Table 1.2). Xu et al. (2017) reported one major QTL affecting pod length on chromosome 3. Glycosyl transferase was suggested as a candidate gene for pod length.

Abiotic stress

Previous reports showed that drought tolerance in cowpea was controlled by QTLs. A total of 10 QTLs were found to affect drought tolerance in a RIL (F_8) population involving 127 individuals derived from a cross between CB46 (drought-susceptible) and IT93K-503-1 (drought-resistant), which were genotyped using 306 AFLPs (Muchero et al., 2009b). These QTLs were termed *Dro-1* (KW p-value=0.0001, LOD=6.0, R-square=24.2%), *Dro-2* (KW p-value=0.005, LOD=2.0, R-square=7.1%), *Dro-3* (KW p-value=0.0005, LOD=2.4, R-square=9.3%), *Dro-4* (KW p-value=0.0001, LOD=5.9, R-square=19.6%), *Dro-5* (KW p-value=0.001, LOD=3.1, R-square=10.8%), *Dro-6* (KW p-value=0.005, LOD=2.2, R-square=5.6%), *Dro-7* (KW p-value=0.0001, LOD=6.1, R-square=20.2%), *Dro-8* (KW p-value=0.0001, LOD=3.7, R-square=13.0%), *Dro-9* (KW p-value=0.0001, LOD=3.7, R-square=12.5%), and *Dro-10* (KW p-value=0.0001, LOD=4.0, R-square=15.2%) (Table 1.2), which were mapped on chromosomes 1, 1, 2, 3, 5, 6, 6, 7, 9, and 10, respectively (Muchero et al., 2009b).

Heat tolerance is a major abiotic stress which has unfavorably impacted cowpea production. A cross between IT82E-18 (heat-susceptible) X CB27 (heat-tolerant) was performed

to establish a RIL (F8) population in efforts to finding QTLs associated with heat tolerance in cowpea (Lucas et al., 2013). A total of five QTLs, *Cht–1* (LOD=5.1, R-square=18.1%), *Cht–2* (LOD=5.7, R-square=17.1%), *Cht–3* (LOD=5.4, R-square=16.2%), *Cht–4* (LOD=4.5, R-square=16.0%), and *Cht–5* (LOD=3.7. R-square=11.5%) (Table 1.2), were identified to be associated with heat tolerance in cowpea. These QTLs were found on chromosomes 5, 7, 6, 10, and 3, respectively. Lucas et al. (2013) suggested heat shock family protein, hydroxyproline-rich glycoprotein family, heat shock transcription factor, late embryogenesis abundant hydroxyproline-rich glycoprotein family, and proline transporter as candidate genes for heat tolerance in cowpea based on the reported QTLs.

Pottorff et al. (2014) found a major QTL affecting tolerance heat-induced seed coat browning (HBS) in cowpea. The mapping population was derived from a cross between IT93K-503-1 (Hbs positive) and CB46 (hbs negative). The QTL explained up to 77.3% of the variation in the phenotypic data. Ethylene forming enzymes (EFE) and ACC synthase 1 were suggested as candidate genes for tolerance heat-induced seed discoloration in cowpea (Pottorff et al., 2014).

Biotic stress

The genetics underlying resistance of cowpea to pathogens such as bacteria and nematodes, and insects were investigated using QTL-based approach. Muchero et al. (2011) identified a QTL (LOD=5.8, R-square=40.0%) affecting resistance to *Macrophomina phaseolina* (Tassi) Goid. in cowpea. QTL mapping was performed on 108 RILs ($F_{2:3}$) derived from CB46 (Macrophomina-susceptible) X IT93K-503-1 (Macrophomina-resistant), which were genotyped using 26 SNPs and 9 AFLPs. A candidate gene, pectin esterase inhibitor, was identified to confer resistance to *M. phaseolina*. Pottorff et al. (2012) reported a QTL on chromosome 1 for resistance to *Fusarium oxysporum* f.sp. *tracheiphilum* Race 3 in cowpea. The mapping

population involved 90 RIL lines (F_{10}), which were obtained from the cross between 24-125B-1 (susceptible) X CB27 (resistant). A total of 339 SNP markers were used for QTL analysis. Leucine-rich repeat serine/threonine protein kinases were candidate genes for resistance to *F. oxysporum* in cowpea.

Studies revealed that resistance to bacterial blight (CoBB) due to *Xanthomonas axonopodis* pv. vignicola (Xav.) were controlled by three QTLs, *CoBB-1*, *CoBB-2*, and *CoBB-3* in cowpea (Agbicodo et al., 2010). QTL analysis was achieved using Kruskal-Wallis and Multiple-QTL Model Mapping (MQM). KW p-values were 0.001, 0.0001, and 0.001 for *CoBB-1*, *CoBB-2*, and *CoBB-3*, respectively, LODs were 3.0 (*CoBB-1*), 3.4 (*CoBB-2*), and 2.3 (*CoBB-3*), and R-square values were 15.8% (*CoBB-1*), 22.1% (*CoBB-2*), and 9.7% (*CoBB-3*) (Table 1.2). These QTLs were found on chromosomes 3, 5, and 9. Candidate genes associated with these QTLs were extracellular dermal glycoprotein, acetyl esterase family protein, and ribosomal protein fibronectin (Agbicodo et al., 2010). A study conducted by Dinesh et al. (2016) reported three QTLs *qtlblb-1* (LOD=2.6, R-square=30.6%), *qtlblb-2* (LOD=2.6, R-square=10.8%), and *qtlblb-3* (LOD=3.0, R-square=10.6%) associated with bacterial leaf blight in cowpea. QTL *qtlblb-1* was found on chromosome 8, whereas both *qtlblb-2* and *qtlblb-3* were mapped on chromosome 11. E3 ubiquitin protein ligase RIN2-like mRNA, a positive regulator of the protein involved in the resistance to *Pseudomonas syringae* (Kawasaki et al., 2005), was the candidate gene for resistance to bacterial leaf blight in cowpea (Dinesh et al., 2016).

A major QTL *qCLScc9.1* (LOD=83.8, R-square=89.3%) (Table 1.2) located on chromosome 9 controlling resistance to cercospora leaf spot disease caused by *Cercospora canescens* Ellis & G. Martin was identified in F_2 and $F_{2:3}$ mapping populations derived from the cross between CSR12906 (susceptible) and IT90K-59-120 (resistant) (Duangsong et al., 2016).

The population was genotyped using SSRs. Inclusive composite interval mapping (ICIM) was used as statistical model for conducting QTL mapping.

Two mapping populations 24-125B-1 (susceptible) X CB27 (resistant) and UCR 779 (susceptible) X IT84S-2049 (resistant) showed one major QTL, *QRk-vu11.1* (LOD=60.8, R-square=83.1%) (Huynh et al., 2016), for resistance to root-knot nematodes caused by *Meloidogyne incognita* Kofoid & White and *Meloidogyne javanica* Treub. The two populations were genotyped using the 1536 SNP Illumina Golden Assay developed by (Muchero et al., 2009a). Experiments were conducted over 3 years, and the QTL was consistent was over years (Table 1.2).

Cowpea resistance to aphids and thrips was elucidated using a QTL approach. Resistance to flower bud thrips *Megalurothrips sjostedti* (Thysanoptera: Thripidae) was controlled by 5 QTLs *Fth1* (LOD=3.0, R-square=13.9%), *Fth2* (LOD=3.0, R-square=8.3%), *Fth5* (LOD=2.0, R-square=9.9%), *Fth4* (LOD=2.0, R-square=6.9%), and *Fth3* (LOD=2.0, R-square=7.4%) (Table 1.2) located on chromosomes 3, 2, 1, 7, and 6, respectively (Omo-Ikerodah et al., 2008). The mapping population involving 245 RIL lines (F_{10}) was derived from a cross between VITA7 (Thrips-susceptible) and Sanzi (Thrips-resistant). A total of 134 AFLPs and 5 SSRs was used for QTL mapping. Resistance to *Thrips tabaci* and *Frankliniella schultzei* (Thysanoptera: Thripidae) was suggested to be controlled by three QTLs *Thr-1* (KW p-value= 0.005, LOD=2.6, and R-square=9.1%), *Thr-2* (KW p-value=0.0001, LOD=5.7, and R-square=19.3%), and *Thr-3* (KW p-value=0.001, LOD=0.001, and R-square=14.1%), located on chromosomes 5, 5, and 7 respectively (Muchero et al., 2010). Aphid resistance-related QTLs were described by Huynh et al. (2015). Two QTLs located on chromosomes 1 (*QAc-vu1.1* with LOD=3.6 and R-square=7.8%) and 7 (*QAc-vu7.1* with LOD=17.1 and R-square=62.7%) (Table 1.2) were

identified. Resistance to aphid was suggested to be conferred by the candidate gene UDP-Glycosyl transferase.

Genomic selection (GS)

Relatively recently, predictive breeding has become more frequent in modern breeding programs. Since the cost of DNA sequencing has significantly decreased and conducting phenotyping could be challenging, predicting phenotypes of interest using marker data is a cost-effective way to advance plant breeding. Genomic selection is defined as the process of estimating breeding values of individuals within a population by utilizing marker data, thereby increasing genetic gain per unit of time (Beaulieu et al., 2014; Heffner et al., 2009).

Statistical models

The basic model is given by $y_i=g(X_i) + e_i$ (Honarvar and Rostami, 2013) where y_i is the genomic estimated breeding value, $g(X_i)$ is the genotype vector, and e_i is random error.

The following models have been widely used in genomic selection-related studies.

(1): Ridge-regression best linear unbiased predictor (rr-BLUP) (Meuwissen et al., 2001)

$y= \mu + g + \varepsilon$ with $g=\sum_j x_{ij}\beta_j$ $g \sim N(0,K\sigma^2_g)$ and $\varepsilon \sim N(0,I\sigma^2_e)$

where y is the vector phenotype, μ is the population mean, g is the vector of genetic values, K is the additive relationship matrix obtained from the marker data, σ^2_g is the genetic variance, and σ^2_e is the error variance.

The regression coefficients can be solved using $\hat{\beta}=(X^TX + I\lambda)^{-1}X^Ty$ where λ is a constant.

(2) Least absolute shrinkage and selection operator (LASSO) (Tibshirani, 1996)

LASSO is solved using the set of β_j satisfying $\min\left\{\sum_i (y_i - \sum_j x_{ij}\beta_j)^2\right\}$ constrained by $\sum_j |\beta_j| \leq t$

with $t \geq 0$, i=1,2,….,m denotes the individuals, j=1,2,….,m refers to the markers, y_i is the phenotype of the i^{th} genotype ($yi=\sum_j x_{ij}\beta_j + e_i$, where e_i is a random error), x_{ij} is the genotype of the i^{th} individual at the j^{th} marker, β_j is the effect due to allele substitution for the j^{th} marker.

(3): Bayes A and Bayes B methods (Meuwissen et al., 2001)

Additive marker effects are modeled as $a_j=\sum_j m_{ai}w_{ij}I_{ai}$ for i=1,….,n individuals and j=1,…..,m markers, I_{ai} is an indicator variable with $I_a \sim Bin(n,\pi)$ where $\pi=1$ for Bayes A and determined for Bayes B, w_{ij} are elements in the matrix vector, m_{ai} are marker effects with $m_{ai}|\sigma^2_{mai} \sim N(0, \sigma^2_{mai})$ and $\sigma^2_{mai} \sim X^{-2}(\nu_{ma}, s^2_{ma})$, the marginal prior distribution of additive markers is described by $m_{ai}|\nu_{ma}, s^2_{ma} \sim t(0, \nu_{ma}, s^2_{ma})$.

Dominant marker effects are described as $d_j=\sum_j m_{ai}s_{ij}I_{di}$ for i=1,….,n individuals and j=1,…..,m markers, I_{di} is an indicator variable with $I_d \sim Bin(n,\pi)$ where $\pi=1$ for Bayes A and determined for Bayes B, s_{ij} are elements in the matrix vector, m_{di} are marker effects with $m_{di}|\sigma^2_{mdi} \sim N(0, \sigma^2_{mdi})$ and $\sigma^2_{mdi} \sim X^{-2}(\nu_{md}, s^2_{md})$, the marginal prior distribution of dominant marker effects is $m_{di}|\nu_{md}, s^2_{md} \sim t(0, \nu_{md}, s^2_{md})$.

Genomic selection research

Significant genetic gain has been obtained via genomic selection in animal breeding (Tribout et al., 2012).To date, genomic selection remains limited for cowpea. Genomic selection-related research has been investigated in crops such as wheat (Battenfield et al., 2016), maize (Shikha et al., 2017), rice (Onogi et al., 2016), and soybean (Xavier et al., 2016). Previous studies reported the accuracy of genomic selection prediction trough cross-validation approach (Dawson et al., 2013; Michel et al., 2016). The size of the training population is critical in genomic selection (Xavier et al., 2016).

Rationale and significance

Salt and drought stress can cause significant cowpea yield losses (Ajayi et al., 2018; Dutta and Bera, 2014), which can threaten the livelihood of farmers who depend on cowpea cultivation. Cowpea has better drought tolerance ability than other legumes (Agbicodo et al., 2009). Understanding the genetic aspects of tolerance to these stresses will enhance cowpea breeding programs aiming at releasing salt and drought-tolerant cowpea cultivars. However, drought tolerance in cowpea is understudied compared to other legumes (Fig. 1.3) (Table 1.3). Genome-wide association study (GWAS) can help in generating robust outcomes in efforts towards understanding the genetics of drought and salt tolerance in cowpea, thus contributing to a more enhanced cowpea breeding. Genome-wide association study (GWAS) allows for high resolution mapping. Generally, efficiency of GWAS can be improved by using a large number of markers. Therefore, a whole genome resequencing-based GWAS could contribute in generating more robust data. Thanks to the high mapping resolution, identification of candidate genes associated with salt and drought tolerance is attainable, which is critical in modern plant breeding programs.

Objectives

The objectives of this research were to:

- Evaluate drought tolerance in cowpea
- Evaluate salt tolerance in cowpea
- Conduct a GWAS for drought tolerance in cowpea using a whole genome resequencing approach
- Identify SNP markers and candidate gene(s) for drought tolerance

- Conduct a GWAS for salt tolerance in cowpea using a whole genome resequencing approach
- Identify SNP markers and candidate gene(s) for salt tolerance
- Conduct GWAS and GS for drought and salt tolerance in a MAGIC cowpea population

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Tables

Table 1.1. GWAS-related studies in cowpea. Markers associated with agronomic traits, abiotic stress (drought tolerance, low phosphorus conditions, and salt tolerance) tolerance, resistance to biotic stress (disease and insect), and chemical compounds are reported.

Traits		Significant Markers	LOD range/p-value range	R-square (%) range	LG	Size	Genotyping strategy	Markers	Statistical model	Candidate gene	References
Agronomic traits	Pod length	72 SNPs	4.8E-5-1E-5	4.6-7.6	1,2,3,4,5,9,10, and 11	299	50K SNP Chip	30211 SNPs	MLM (Q + K)	Glycosyl transferase	Xu et al. (2017)
	Adventious root angle	4749_1972	3.9	NA	6	189	1536-SNP assay	1091SNPs	MLM (Q + K)	NA	Burridge et al. (2017)
	Basal root angle	11851_914	3.1	NA	10						
	Root tissue angle	2326_226	3.9	NA	3						
	Mediam root width	14604_737	4.5	NA	8						
	Root density	1004_587	4.0	NA	5						
	Stem diameter	13772_1075, 5084_519, 4836_807, 139_439, 8969_1386, and 11138_624	2.2-4.3	NA	1, 3, 6, and 7						
	Seed coat color	C35063613_1497, Scaffold81493_886, and Scaffold84620_6785		NA		339	GBS	1047 SNPs			Qin et al. (2016)
	Seed germination	C35042053_245, Scaffold27032_5665, and Scaffold94454_419	2.2-2.5	8.4-9.6	NA	116	GBS	1049SNPs	SMR	NA	Ravelombola et al. (2017b)
			2.3-2.8	9.2-11.0					GLM(Q)		
			2.2-2.6	9.5-10.3					MLM(Q+K)		
			2.3-2.9	NA					FarmCPU		

Table 1.1 (Cont.)

Traits		Significant Markers	LOD range/ p-value range	R-square (%) range	LG	Size	Genotyping strategy	Markers	Statistical model	Candidate gene	References
	Growth habit	C35060651_729, C35061339_799, C35062457_1855, C35072764_1384, C35080248_2355, Scaffold2771_4351, Scaffold29522_3213, Scaffold35913_2678, Scaffold53560_188, and Scaffold58098_4297	2.4-7.1	2.6-6.8	NA	487	GBS	1031SNPs	SMR	NA	Ravelombola et al. (2017c)
			2.0-6.9	2.1-6.6					GLM(Q)		
			1.5-3.9	1.2-3.9					GLM(PCA)		
			1.9-3.2	1.4-3.0					MLM(Q+K)		
			1.1-3.2	1.3-3.1					MLM(PCA+K)		
			1.3-6.6	NA					FarmCPU		
Abiotic stress	Drought tolerance	39 SNPs	>3	NA	2,3,4,5,6,7,8,9,10, and 11	95 (*Vigna unguiculata* ssp. *sesquipedalis*)	1536-SNP assay	1127 SNPs	GLM (Q) and MLM (Q + K)	B47-specificallyregulated SAUR-like gene, ethylene biosynthesis/response GO terms, Aquaporins (AQPs)	Xu et al. (2015)
		1_0029, 1_0589,1_0067, 1_0206, 1_0888, 1_0049, 1_0108, 1_1150, 1_0279, 1_0983, 1_0140, 1_0759, and 1_1405	7.71E-05-9.40E-03	0.8-7	1,2,3,4,,5,7, and 10	383	1536-SNP assay	1080 SNPs	MLM		Muchero et al. (2013)

Table 1.1 (Cont.)

Traits	Significant Markers	LOD range/p-value range	R-square (%) range	LG	Size	Genotyping strategy	Markers	Statistical model	Candidate gene	References
Abiotic stress	Low phosphorus efficiency	C35006753_110, C35028233_482, C35072764_1384, C35084634_455, Scaffold21750_4938, Scaffold26894_5408, Scaffold41885_14420, Scaffold45170_4650, Scaffold50732_679, and Scaffold88448_741	2.6-6.9	3.5-8.9	NA	357	GBS	1018 SNPs	SMR	NA	Ravelombola et al. (2017a)
			2.5-6.9	3.3-8.1					GLM(Q)		
			1.6-6.2	2.0-7.5					GLM(PCA)		
			1.3-3.8	1.8-5.1					MLM(Q+K)		
			1.3-3.3	1.8-4.9					MLM(PCA+K)		
			1.1-5.4	NA					FarmCPU		
	Rock phosphate response	C35028233_482, C35058535_121, Scaffold26894_5408, Scaffold45170_4650, Scaffold51609_507, Scaffold53730_7339, Scaffold74389_5733, and Scaffold87916_4921	2.2-5.6	3.0-7.1					SMR		
			1.6-3.5	2.1-4.9					GLM(Q)		
			1.9-4.4	2.4-5.4					GLM(PCA)		
			2.2-5.5	3.1-7.0					MLM(Q+K)		
			1.2-3.3	1.7-4.5					MLM(PCA+K)		
			1.3-6.0	NA					FarmCPU		
	Germination stage salt tolerance	Scaffold87490_622, Scaffold87490_630, and C35017374_128	2.3-2.8	8.8-15.2	NA	116	GBS	1049 SNPs	SMR	NA	Ravelombola et al. (2017b)
			2.4-2.8	8.4-15.0					GLM(Q)		
			1..9-2.6	9.2-14.1					MLM(Q+K)		
			2.0-3.2	NA					FarmCPU		

Table 1.1 (Cont.)

Traits	Significant Markers	LOD range/p-value range	R-square (%) range	LG	Size	Genotyping strategy	Markers	Statistical model	Candidate gene	References
	Seedling stage salt tolerance	Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089	2.0-3.4	4.8-13.4		155		SMR		
			2.1-3.3	5.3-13.1				GLM(Q)		
			1.6-3.2	4.2-12.3				MLM(Q+K)		
			1.6-4.1	NA				FarmCPU		
Disease resistance	Cowpea mosaic virus (CPMV)	C35069548_1883, scaffold65342_6794, scaffold66293_6549, scaffold95805_2175, C350 81948_540, and scaffold17319_4417	3.3-12.3	5.0-16.6	NA	333	GBS	1033 SNPs	SMR	NA	Bhattarai et al. (2017)
			3.0-5.6	3.8-9.5				GLM (PCA)		
			3.1-8.8	3.9-10.9				GLM (Q)		
			0.0-5.1	0.0-10.3				MLM (PCA + K)		
			0.0-5.1	0.0-10.3				MLM (Q + K)		
	Cowpea bacterial blight (*Xanthomonas axonopodis* pv. *vignicola* (Xav))	C35025883_1166, C35046071_1260, C35083564_3310, C35084634_455, scaffold89853_3955, scaffold92472_1355, scaffold96328_3387, andscaffold96765_4430	2.41E-16-0.008	4.4-31.3	NA	249	GBS	1031 SNPs	SMR	NA	Shi et al. (2016)
			1.4E-10-0.12	1.7-15.3				GLM (PCA)		
			2.1E-10-0.097	1.6-15.8				GLM (Q)		
			1.4E-05-0.99	0-12.4				MLM (PCA + K)		
			2.4E-05-0.99	0-12.9				MLM (Q + K)		

Table 1.1 (Cont.)

Traits	Significant Markers	LOD range/p-value range	R-square (%) range	LG	Size	Genotyping strategy	Markers	Statistical model	Candidate gene	References
			0.3-4.0	NA				SMR from QGene		
			1.4-12.4	1.9-16.7				EcMLM from Gapit		
	Fusarium wilt resistance (*Fusarium oxysporum* f. sp. *Tracheiphilum*)	1_0075, 1_1111, 1_1147, 1_0251, 1_0895, 1_0691, 1_0897, 1_0298, 1_0410, 1_0857, 1_0981, 1_1369, 1_0691, 1_0330, 1_1062, 1_0629, 1_0318, and 1_1504	2.4-24.5	2.0-4.4	1,3,4,5,6,8,10, and 11	95 (*Vigna unguiculata* ssp. *sesquipedalis*)	1536-SNP assay	1127 SNPs	MLM (Q + K)	NA	Xu et al. (2015)
Insect resistance	Aphid (*Aphis craccivora* C.L.Koch) resistance	C35011941_894 and Scaf-fold30061_3363	>2.5	NA	NA	338	GBS	1047 SNPs	MLM	NA	Qin et al. (2017)
Chemical compounds	Seed antioxidant	Scaffold7139_14363 and Scaffold29110_4657	>2.5	NA	NA	339	GBS	1047 SNPs	NA	NA	Qin et al. (2016)

Table 1.2. Previously reported major and minor QLTs associated with agronomic traits, disease resistance, nematode resistance, insect resistance, and tolerance to abiotic stress in cowpea. Closest markers associated with the QTL peak and molecular marker type for QTL analysis are provided. Candidate genes associated with QTLs are reported if available.

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Agronomic traits	Pod cellulose	*qCel1.1*	1	15.9	31.6	140.7	–	SSR	23	JP81610 (yardlong bean) X TVnu457 (wild cowpea)	*Vigan.01G359600.01* (Cellulose synthase)	Suanum et al. (2016)
		qCel7.1	7	8.1	15.5	15.0	cp05517				*Vigan.07G046100.01 (MYB)*	
	Pod hemicellulose	*qHem7.1*	7	25.6	61.1	15.0	–				*Vigan.07G046100.01 (MYB)*	
	Pod lignin	*qLig7.1*	7	20.0	47.8	15.0	–				*Vigan.07G046100.01 (MYB)*	
	Pod twistiness	*qLig7.1*	7	9.0	28.4	28.4	cp05517				–	
	Pod cellulose	*qCel1.1*	1	20.2	30.0	129.3	–	SSR	23	(JP81610 X TVnu457) X JP81610	*Vigan.01G359600.01* (Cellulose synthase)	
		qCel2.1	2	5.3	5.3	34.7	cp03873				–	
		qCel7.1	7	40.5	40.5	10.0	VR2094				*Vigan.07G046100.01 (MYB)*	
	Pod hemicellulose	*qHem4.1*	4	3.0	2.4	26.9	cp03825					
		qHem7.1	7	49.1	75.2	9.0	VR2094				*Vigan.07G046100.01 (MYB)*	
	Pod lignin	*qLig7.1*	7	37.3	67.2	8.0	VR2094				*Vigan.07G046100.01 (MYB)*	
	Pod twistiness	*qPdt1.1*	1	7.9	9.4	130.5	cp08288				–	
		qPdt7.1	7	25.8	40.8	12.6	–				–	

Table 1.2 (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Agronomic traits	Thickness of pod fiber	*qpft5*	5	3.4	10.8	49.6	SSR6790 (49.6)	SSR	202	524B (domesticated) X 219-01 (wilt type)	–	Andargie et al. (2014)
	Pod length	*Qpl.zaas-3*	3	18.7	45.7	–	–	SNP	7988	ZN016 (medium-pod long) X Zhijiang28 (long-pods)	glycosyl transferase	Xu et al. (2017)
		Qpl.zaas-5	5	6.3	13.7	–	–					
	Seed germination	*qsdg1*	1	3.3	12.9	208.9	SSR6243 (208.9)					
	Seed coat permeability	*qsdp2*	2	3.9	12.2	19.8	SSR6705 (19.8)					
		qsdp10.1	10	3.2	10.3	36.0	SSR6919 (36.0)					
		qsdp10.2	10	3.2	9.9	36.0	SSR6919 (36.0)					
	100-seed weight	*qsw1*	1	3.3	19.1	8.6	SSR7117 (8.6)	SSR	202	524B (domesticated) X 219-01 (wilt type)	–	Andargie et al. (2014)
		qsw2.1	2	3.1	8.9	31.2	SSR6314 (21.2)					
		qsw2.2	2	3.8	13.8	19.8	SSR6705 (19.8)					
		qsw3.1	3	3.2	10.1	16.5	SSR6701 (16.5)					
		qsw3.2	3	4.1	13.3	15.7	SSR6924-2 (15.7)					
		qsw7	7	4.8	15.6	32.0	SSR7027-2 (32.0)					

Table 1.2 (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
		pA816	5	3.0	–	–	pA816					
		qsw10	10	3.3	9.2	36.0	SSR6919 (36.0)	SSR	202	524B (domesticated) X 219-01 (wilt type)	–	Andargie et al. (2014)
		Dro-7	6	KW p-value=0.005								
		Dro-10	10	KW p-value=0.05								
		Dro-8	7	KW p-value=0.0005								
	Grain yield	*Dro-1*	1	KW p-value=0.05								
Agronomic traits		*Dro-3*	2	KW p-value=0.001	–		–	SNP	1536	CB46 (drought-tolerant) X IT93K-503-1 (drought-susceptible)	–	Muchero et al. (2013)
		Dro-4	2	KW p-value=0.05								
		Dro-7	6	KW p-value=0.0005								
	Biomass yield	*Dro-8*	7	KW p-value=0.005								
		Dro-1	1	KW p-value=0.001								
		Dro-3	2	KW p-value=0.05								
	Days to flowering	*qdf1*	1	3.1	18.5	31.5	SSR6188 (31.5)	SSR	202	524B (domesticated) X 219-01 (wilt type)	–	Andargie et al. (2014)
	Ovule number	*qon1*	1	3.9	11.6	162.3	SSR7041 (167.2)					

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Agronomic traits	Leaf morphology (greenhouse)	*Hls*	4	33.8	74.7	34.7	1_0992 (34.7)	SNP	416	Sanzi (sub-globose leaf shape) X Vita 7 (hastate leaf shape)	EZA1/ SWINGER	Pottorff et al. (2012)
			4	KW p-value=0.0001	–	–	1_0992 (34.7)					
	Leaf morphology (field)		4	30.9	71.5	34.7	1_0992 (34.7)					
			4	KW p-value=0.0001	–	–	1_0992 (34.7)					
	Floral scent compounds	*64 QTLS*	1,2,4,5,6,7,8, and 10	2.7-4.7	–	–	–	SSR	202	524B (domesticated) X 219-01 (wild type)	–	Andargie et al. (2013)
Disease	Bacterial leaf blight	*qtlblb-1*	8	2.6	30.6	74.6	VuMt397	SSR and CISP	96 (79 SSRs and 17 CISPs)	C-152 (susceptible) X V-16 (resistant)	E3 ubiquitin protein ligase RIN2-like mRNA	Dinesh et al. (2016)
		qtlblb-2	11	2.6	10.8	15.0	VuMt338					
		qtlblb-3	11	3.0	10.6	89.1	VuMt338					
	Cowpea bacterial blight (CoBB) caused by *Xanthomonas axonopodis* pv. vignicola (Xav.)	*CoBB-1*	3	KW p-value=0.001	–	111.6	1_0853	SNP	282	Tvu7778 (susceptible) X Danila (resistant)	extracellular dermal glycoprotein, acetylesterase family protein, and ribosomal protein fibronectin	Agbicodo et al. (2010)
		CoBB-2	5	KW p-value=0.0001	–	16.8	1_0037					
		CoBB-3	9	KW p-value=0.001	–	78.6	1_1202					

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)		QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Disease	Cowpea bacterial blight (CoBB) caused by *Xanthomonas axonopodis* pv. vignicola (Xav.)	*CoBB-1*	3	3.0	15.8	111.6	1_0853						
		CoBB-2	5	3.4	22.1	16.8	1_0037						
		CoBB-3	9	2.3	9.7	78.6	1_1202 (78.6)						
	Cercospora leaf spot disease caused by *Cercospora canescens* Ellis & G. Martin 60 days after planting	*qCLScc9.1*	9	26.8	48.4	39.0	CEDG070 (38.5)						
	Cercospora leaf spot disease caused by *Cercospora canescens* Ellis & G. Martin 70 days after planting	*qCLScc9.1*	9	40.3	63.2	39.0	CEDG070 (38.5)	SSR	33	CSR12906 (susceptible) X IT90K-59-120 (resistant)	–		Duangsong et al. (2016)
	Cercospora leaf spot disease caused by *Cercospora canescens* Ellis & G. Martin 60 days after planting	*qCLScc9.1*	9	67.6	86.9	39.0	CEDG070 (38.5)						

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Disease	Cercospora leaf spot disease caused by *Cercospora canescens* Ellis & G. Martin 70 days after planting	*qCLScc9.1*	9	83.8	89.3	39.0	CEDG070 (38.5)					
	Cercospora leaf spot disease caused by *Pseudocercospora cruenta* (Sacc.) 60 days after planting	*qCLScc9.1*	9	10.7	25.4	39.0	CEDG070 (38.5)					
	Cercospora leaf spot disease caused by *Pseudocercospora cruenta* (Sacc.) 60 days after planting	*qCLScc9.1*	9	13.4	30.4	39.0	CEDG070 (38.5)					
	Fusarium oxysporum f.sp. *tracheiphilum* Race 3	*Fot3-1*	1	3.1	18.4	49.4	1_1107 (49.4)	SNP	339	24-125B-1 (susceptible) X CB27 (resistant)	leucine-rich repeat serine/threonine protein kinases	Pottorff et al. (2012)
				KW p-value=0.0005	–	49.4	1_1107 (49.4)					
	Macrophomina phaseolina (Tassi) Goid. resistance	*Mac-2*	3	5.8	40.0	9.3	1_0853(9.3)	AFLP and SNP	35(26 SNPs and 9 AFLPS)	CB46 (Macrophomina-susceptible) X IT93K-503-1 (Macrophomina-resistant)	Pectin esterase inhibitor	Muchero et al. (2011)
		Mac-2	3	KW p-value=0.0001	–	9.3	1_0853(9.3)	AFLP and SNP	35(26 SNPs and 9 AFLPS)			

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)		QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Nematodes	Resistance to root-knot nematodes: egg masses of *Meloidogyne incognita* Kofoid & White	*QRk-vu11.1*	11	27.0	72.9	16.0	1_0414 (16.6)						
	Resistance to root-knot nematodes: gall score for *Meloidogyne incognita* Kofoid & White	*QRk-vu11.1*	11	16.0	70.9	14.0	1_0757 (15.4)		1536	24-125B-1 (susceptible) X CB27 (resistant)			
	Resistance to root-knot nematodes: gall score of *Meloidogyne javanica* Treub (field 2008)	*QRk-vu11.1*	11	10.2	59.2	15.0	1_0757 (15.4)	SNP				–	Huynh et al. (2016)
	Resistance to root-knot nematodes: gall score of *Meloidogyne javanica* Treub (field 2012)	*QRk-vu11.1*	11	8.2	52.4	13.0	1_0757 (15.4)						
	Resistance to root-knot nematodes: gall score *Meloidogyne incognita* Kofoid &White (field 2010)	*QRk-vu11.1*	11	60.8	83.1	19.0	1_0414 (20.8)		323	UCR 779 (susceptible) X IT84S-2049 (resistant)			

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Nematodes	Resistance to root-knot nematodes: gall score *Meloidogyne incognita* Kofoid & White (field 2010)	*QRk-vu11.1*	11	29.3	64.5	14.0	1_0757 (14.5)		323	UCR 779 (susceptible) X IT93K-503-1 (resistant)		
Insects	Aphid *Aphis craccivora* Koch	*QAc-vu1.1*	1	3.6	7.8	17.6	1_0357 (17.6)	SNP	1536	CB27(Aphid-susceptible) X IT97K-556-6 (Aphid-resistant)	UDPGlycosyltransferases	Huynh et al. (2015)
		QAc-vu7.1	7	17.1	62.7	22.0	1_0391 (22.2)					
	Thrips tabaci and *Frankliniella schultzei* (Thysanoptera: Thripidae)	*Thr-1*	5	KW p-value=0.005	–	28.4	ACC-CAT7	AFLP	306 AFLPs	CB46 (Thrips-susceptible) X IT93K-503-1 (Thrips-resistant)	–	Muchero et al. (2009)
		Thr-2	5	KW p-value=0.0001	–	53.4	ACG-CTC5					
		Thr-3	7	KW p-value=0.001	–	35.6	AGG-CAT1					
		Thr-1	5	2.6	9.1	28.4	ACC-CAT7					
		Thr-2	5	5.7	19.3	53.4	ACG-CTC5					
		Thr-3	7	4.1	14.1	35.6	AGG-CAT1					

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
	Flower bud thrips *Megalurothrips sjostedti* (Thysanoptera: Thripidae)	*Fth1*	3	3.0	13.9	32.0	ACTCAA376 (13.9)	AFLP and SSR	139 (134 AFLPs and 5 SSRs)	VITA7 (Thrips-susceptible) X Sanzi (Thrips-resistant)	–	Omo-Ikerodah et al. (2007)
		Fth2	2	3.0	8.3	18.4	ACGCTT2 (8.3)					
Insects	Flower bud thrips *Megalurothrips sjostedti* (Thysanoptera: Thripidae)	*Fth4*	7	2.0	6.9	11.9	AACCTA120 (6.9)					
		Fth3	6	2.0	7.4	12.6	AACCAA155 (NA)					
Abiotic stress	Drought tolerance	*Dro-1*	1	KW p-value=0.0001	–	76.6	–	AFLP	306	CB46 (drought-susceptible) X IT93K-503-1 (drought-resistant)	–	Muchero et al. (2009)
		Dro-2	1	KW p-value=0.005	–	99.1	–					
		Dro-3	2	KW p-value=0.0005	–	97.7	–					
		Dro-4	3	KW p-value=0.0001	–	68.5	–					
		Dro-5	5	KW p-value=0.001	–	64.9	–					
		Dro-6	6	KW p-value=0.005	–	22.7	–					
		Dro-7	6	KW p-value=0.0001	–	64.0	–					
		Dro-8	7	KW p-value=0.0001	–	40.5	–					
		Dro-9	9	KW p-value=0.0001	–	29.9	–					

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
		Dro-10	10	KW p-value=0.0001	–	27.6	–					
		Dro-1	1	6.0	24.2	76.6	–					
Abiotic stress	Drought tolerance	*Dro-2*	1	2.0	7.1	99.1	–					
		Dro-3	2	2.4	9.3	97.7	–					
		Dro-4	3	5.9	19.6	68.5	–					
		Dro-5	5	3.1	10.8	64.9	–					
		Dro-6	6	2.2	5.6	22.7	–					
		Dro-7	6	6.1	20.2	64.0	–					
		Dro-8	7	3.7	13.0	40.5	–					
		Dro-9	9	3.7	12.5	29.9	–					
		Dro-10	10	4.0	15.2	27.6	–					
	Heat tolerance	*Cht–1*	5	5.1	18.1	–	–	SNP	1536	IT82E-18 (heat-susceptible) X CB27 (heat-tolerant)	heat shock family protein, hydroxyproline-rich glycoprotein family	Lucas et al. (2013)
		Cht–2	7	5.7	17.1	–	–				NA	
		Cht–3	6	5.4	16.2	–	–				heat shock family protein, hydroxyproline-rich glycoprotein family	

Table 1.2. (Cont.)

Traits		QTL	LG	LOD/Kruskal-Wallis p-value	R-square (%)	QTL peak (cM)	Closest marker	Marker	Number of markers	Parents	Candidate gene(s)	References
Abiotic stress	Heat tolerance	*Cht–4*	10	4.5	16.0	–	–				heat shock family protein, heat shock transcription factor, late embryogenesis abundant hydroxyproline-rich glycoprotein family heat	
		Cht–5	3	3.7	11.5	–	–				shock protein family, heat shock transcription factor, proline transporter	
	Heat-induced browning (*Hbs*)	*Hbs-1*	8	KW p-value=0.0001	–	60.5	1_0032 (60.5)	SNP	1536	IT93K-503-1 (*Hbs* positive) X CB46 (*hbs* negative)	ethylene forming enzymes (EFE)	Pottorff et al. (2014)
		Hbs-2	3	KW p-value=0.0001	–	50.8	1_1343 (50.8)				NA	
		Hbs-1	8	30.2	77.3	60.5	1_0032 (60.5)				ethylene forming enzymes (EFE)	
		Hbs-2	3	2.8	12.3	50.8	1_1343 (50.8)				NA	
		Hbs-1	9	KW p-value=0.0001	–	49.5	1_0032 (49.5)				ethylene forming enzymes (EFE)	
		Hbs-3	3	KW p-value=0.0001	–	17.8	1_1534 (17.8)			IT84S-2246 (*Hbs* positive) X TVu 14676 (*hbs* negative)	ACC synthase 1	
		Hbs-1	9	12.1	28.3	49.5	1_0032 (49.5)				ethylene forming enzymes (EFE)	
		Hbs-3	3	2.0	6.8	17.8	1_1534 (17.8)				ACC synthase 1	

Table 1.3. Number of academic-related materials (peer-reviewed only) whose titles included both drought tolerance research and some of the most economically important grown legumes worldwide.

Year	Journal article				Conference proceeding			
	Soybean[x]	Common bean	Chickpea	Cowpea	Soybean	Common bean	Chickpea	Cowpea
1996-1998[y]	22[z]	11	2	3	1	0	0	0
1999-2004	38	11	4	16	0	0	0	0
2005-2010	65	21	41	23	3	0	0	1
2011-2016	170	65	79	23	3	0	1	1
Total	295	108	126	65	7	0	1	2

z Data were obtained from the online library website (http://libraries.uark.edu/) of the University of Arkansas, Fayetteville USA
y Materials were searched from January 1st until December 31st for each year.
x Globally important legumes as defined by Singh et al. (2003) in Genetic and Genomic Resources of Grain Legume Improvement.

Figures

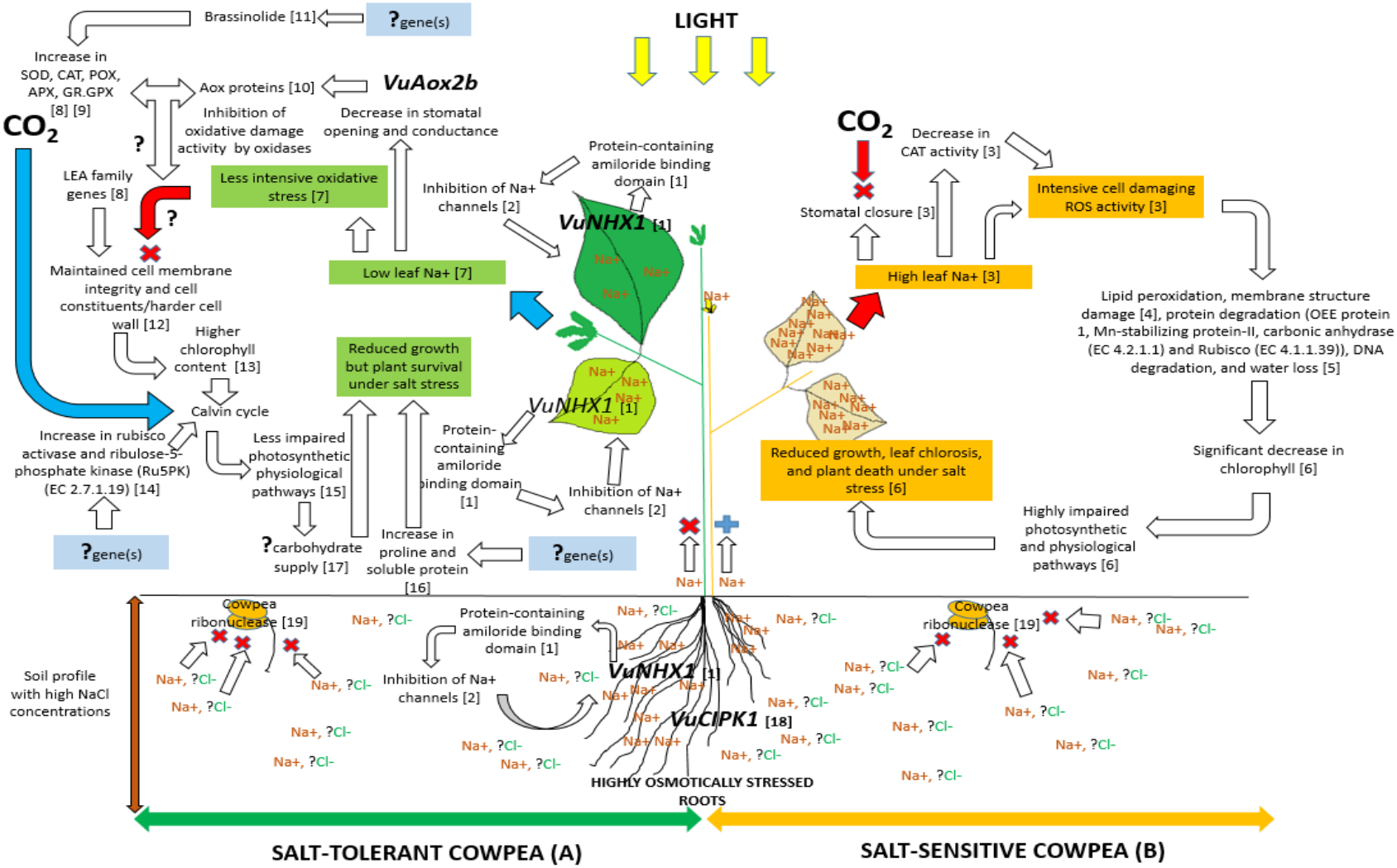

Fig. 1.1. Complex machinery mechanism of salt tolerance in cowpea plants.

(A) Resistant scenario: High expression of *VuNHX1* in roots and leaves [1] (Mishra et al., 2005). *VuNHX1* is translated into a protein containing an amiloride binding domain [1] (Mishra et al., 2005), which can inhibit Na+ channels [2] (Xing et al., 2011). Salt-tolerant cowpea cultivars have been proven to have less accumulation in Na+ in leaves [7] (Praxedes et al., 2010; Mini et al., 2015), which limit the occurrence of oxidative stress. LEA family genes are highly expressed in salt-tolerant cowpea genotypes under salt stress. LEA proteins prevent cowpea leaf cells from being dehydrated upon salt stress conditions [8] (Sousa et al., 2003). Sal-tolerant cowpea genotypes showed higher increase in SOD, CAT, POX, APX, GR, and GPX compared to the salt-sensitive ones [8] (Maia et al., 2010; Mini et al., 2015). However, this increase in oxidases is not necessarily correlated to salt-tolerance in cowpea [9] (Cavalcanti et al., 2004). An alternative oxidation pathway has been shown to confer salt-tolerance in cowpea trough expression of *VuAox2b* [10] (Costa et al., 2007). Cowpea brassinolide has been shown to help cowpea plants to cope with salt stress by increasing antioxidant contents in leaves [11]. Cowea POX has been shown to maintain cell-wall structure under salt-stress [12] (Cavalcanti et al., 2004; Mini et al., 2015). Higher chlorophyll content in leaf confers salt tolerance in cowpea [13]. Salt-tolerant cowpea plants showed increase in rubisco activase and ribulose-5-phosphate kinase (Ru5PK) (EC 2.7.1.19) [14]. Sal-tolerance has been associated with net photosynthetic activity under stress [15] (Praxedes et al., 2010). Increase in proline and synthesized de novo proteins assist in coping with salt tolerance in cowpea [16] (de Abreu et al., 2014; Maia et al., 2013; Mini et al., 2015). Correlation between increase in carbohydrate under salt stress remains and salt tolerance remains unclear [17] (Praxedes et al., 2014b; Mini et al., 2015). *VuCIPK1* is a cowpea gene contributing indirectly to salt tolerance by improving K+ uptake [18] (Imamura et al., 2008). Cowpea ribonuclease in the cowpea cotyledons contributes to salt tolerance at early plant establishment. **(B) Susceptible scenario**: Failure from preventing the high soil Na+ concentration to being uptaken by roots will result in toxic Na+ in the upper part of the plants. High Na+ concentration in leaf will trigger intensive oxidative damage and impairs the catalase activity, which is essential in scavenging relative oxygen species (ROS) [3] (Cavalcanti et al., 2004; Praxedes et al., 2014). The high Na+ leads also to stomatal closure, which can limit CO2 uptake [3] (Mini et al., 2015). Intensive relative oxygen species activity result in lipid superoxidation, which damages cell membrane structure afterwards [4] (Calvacanti et al., 2004; Mini et al., 2015). Chlorophyll content, and photosynthetic and physiological-related proteins are significantly impaired [5] [6] (Praxedes et al., 2009, de Abreu et al., 2004). Due to the loss in carbon, plant growth is reduced, which can lead to plant death [6] (Mini et al., 2015, Ravelombola et al., 2017).

SOD: superoxide dismutase, CAT: catalase, POX: peroxidase, APX: ascorbate peroxidase, GR: glutathione reductase, GPX: guaiacol peroxidase, and Aox: alternative oxidase proteins.

? Unknown mechanisms or conflicting conclusions in the cowpea literature.

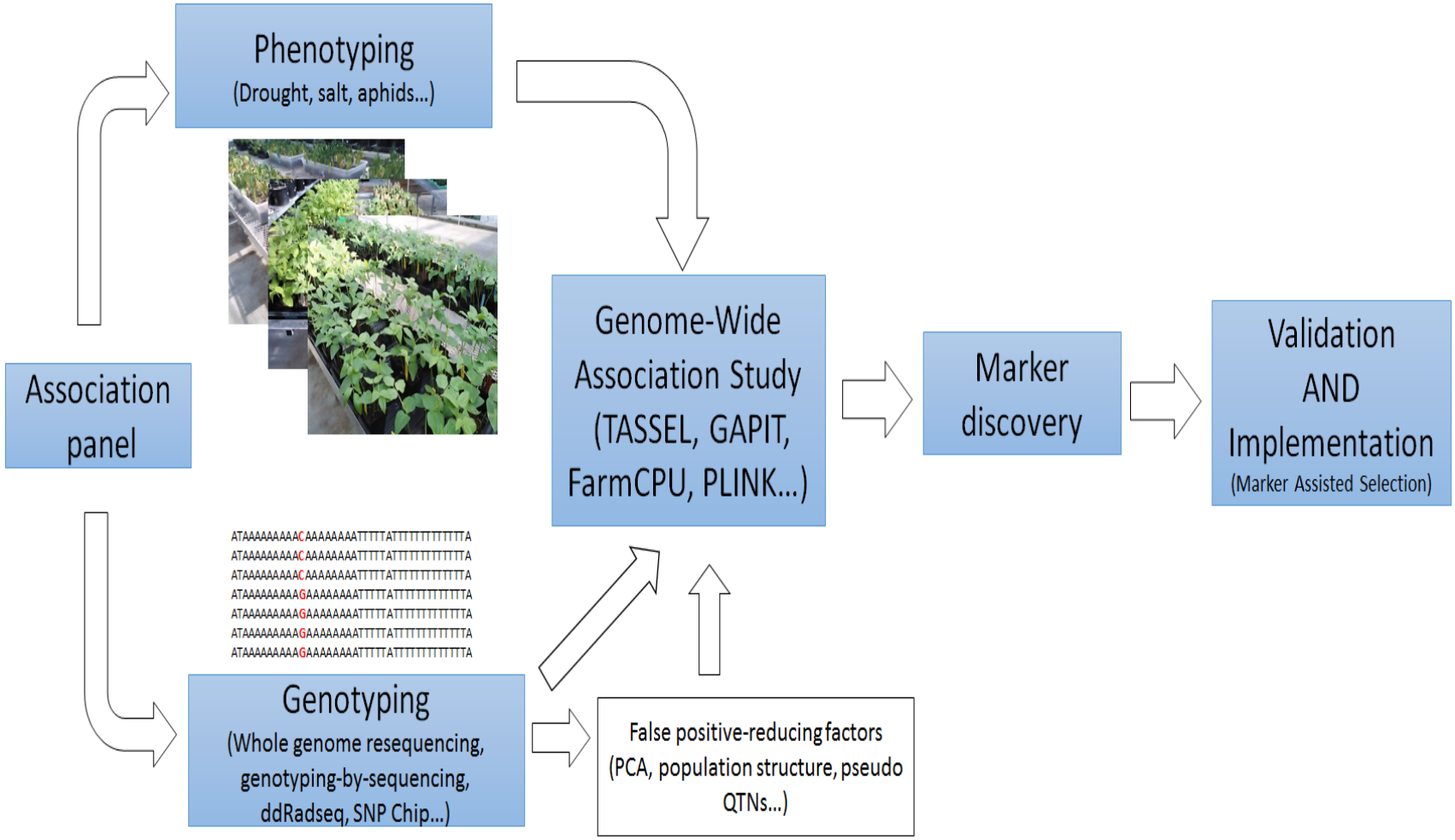

Fig. 1.2. Genome-wide association study workflow. The association mapping is phenotyped and genotyped. Phenotypic and genotypic data are merged for GWAS study in order to identify significant markers associated with the trait. Upon marker discovery, validation is required prior to its implementation in a breeding program or its use in gene cloning-related studies.

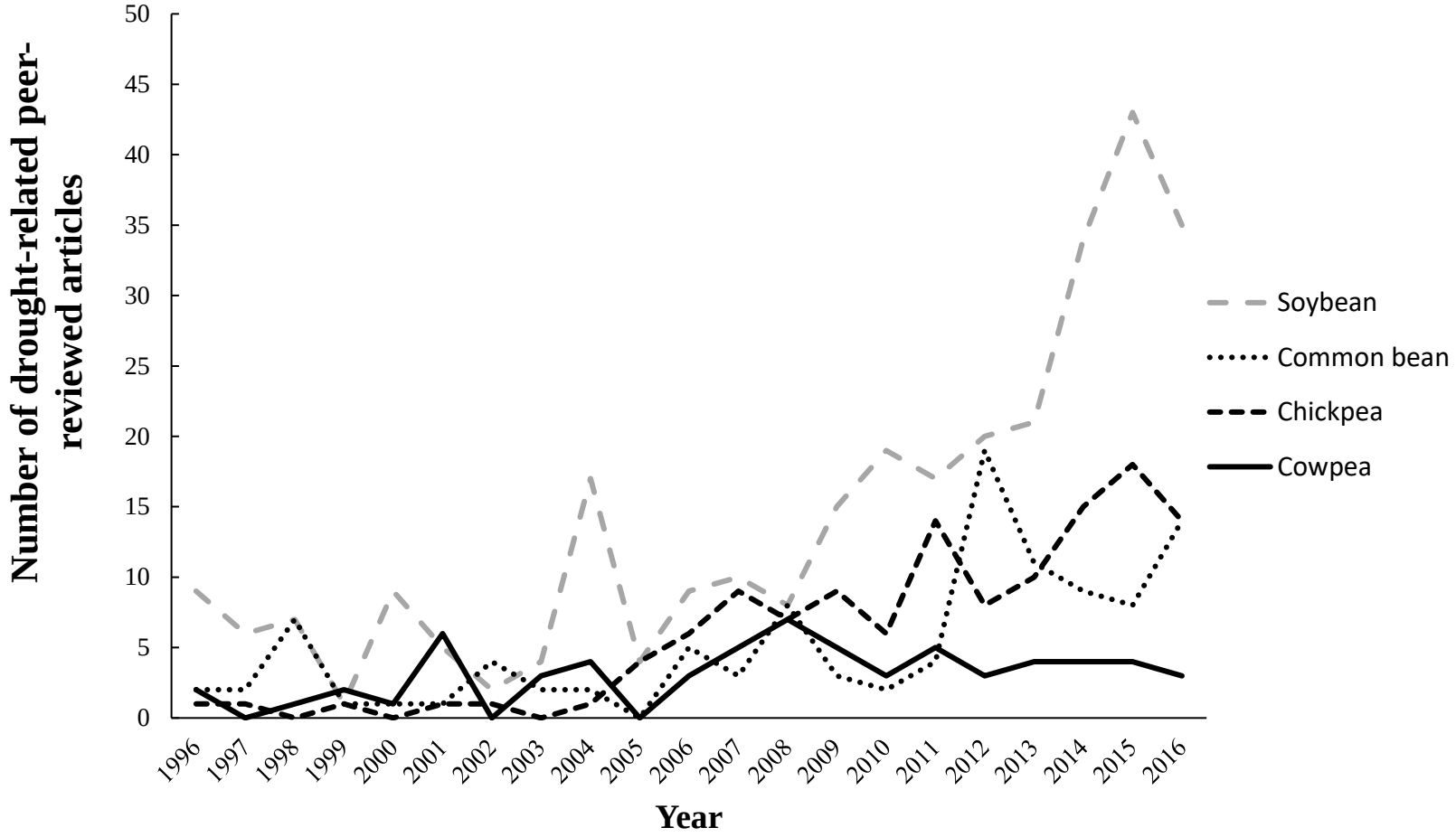

Fig. 1.3. Number of scholarly materials pertaining to drought research (Data were obtained from the online library: http://libraries.uark.edu/ of the University of Arkansas, Fayetteville USA.

Chapter 2. Investigation on Various Above-Ground Traits to Identify Drought Tolerance in Cowpea Seedlings

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Abstract

Impacts of drought stress on crop production can significantly impair farmer's revenue, hence adversely impacting the gross national product growth. For cowpea [*Vigna unguiculata* (L.) Walp.], which is a legume of economic importance, effects of drought at early vegetative growth could lead to substantial yield losses. However, little has been done with respect to breeding for cowpea cultivars withstanding drought at early vegetative growth. In addition, previous investigations have been focusing on how plant morphology and root architecture can confer drought tolerance in cowpea, which is not sufficient in efforts to unraveling unknown drought tolerance-related genetic mechanisms, potentially of great importance in breeding, and not pertaining to neither plant morphology nor root architecture. Therefore, the objective of this study was to evaluate above-ground drought-related traits of cowpea genotypes at seedling stage. A total of 30 cowpea genotypes were greenhouse-grown within boxes and the experimental design was completely randomized design with three replicates. Drought stress was imposed for 28 days. Data on a total of 17 above-ground related traits were collected. Results showed that: 1) a large variation in these traits was found among the genotypes; 2) more trifoliate wilt/chlorosis tolerance but more unifoliate wilt/chlorosis susceptible were observed; 3) delayed senescence was related to the ability of maintaining a balanced chlorophyll content in both unifoliate and trifoliate leaves; and 4) the genotypes PI293469, PI349674, and PI293568 were found to be slow-wilting and drought-tolerant. These results could contribute to advancing breeding programs for drought tolerance in cowpea.

Introduction

Drought stress has been constraining agricultural production in various ways, which increasingly threatens food availability globally. Drought has been described as the effects of a sustained lack of soil moisture required for plants to properly grow and provide sufficient crop yields (Blum and Ebercon, 1981). Long period of drought conditions adversely impacts plant growth development and extreme cases result in plant death (Golldack et al., 2014). As a result, drought stress can significantly impair the economy (Ishiyaku and Yilwa, 2009). In the U.S., Rosine and Bull (1989) reported that crop losses due to drought stress unfavorably affected the gross national product growth. Therefore, crop scientists have been working on developing strategies to address the concerns imposed by drought stress on agriculture.

Breeding for drought-tolerant cultivars is one the most cost-effective ways to cope with the effects of insufficient water supplies on crops. Research aiming at identifying drought-tolerant cultivars has been recently of interest since doing so is critical toward delivering substantial information to plant breeders (Dhanapal et al., 2015; Ajayi et al., 2018). For crops which are rain-dependent, the lack of rainfall occurring at early vegetative growth could be insidious for further development. Predicting water shortage due to insufficient rainfall is still challenging despite of the advances in technology (Ajayi et al., 2018), leading to serious concerns pertaining to effectively planning agricultural activities.

The U.S. National Drought Center at the University of Nebraska stated that little has been done to help farmers being well prepared with drought stress (Wu and Wilhite, 2004). Cultivars which can tolerate limited water supplies at early vegetative growth could be an affordable solution to overcome drought conditions. Reports showed that impacts of drought on crops such as cowpea have been acute in tropical and sub-tropical regions (Carvalho et al. 2017).

Cowpea [*Vigna unguiculata* (L.) Walp.], 2n=2x=22, is one the most economically important legumes widely grown in sub-Saharan Africa (Singh et al., 2003). Cowpea is a good source of protein for human consumption (Weng et al., 2017). Cowpea provides micronutrients such Iron and Zin, which are essential to human's diet (Frota et al., 2008). Cowpea is also a health-promoting food due to the significant amount of antioxidants found in cowpea seeds (Moreira-Araújo et al., 2017). In addition to being part of human's diet, cowpea is also used as feed for livestock.

Cowpea is one of the most-drought legumes (Agbicodo et al., 2009). However, drought conditions occurring at early season could be detrimental to cowpea production (Muchero et al., 2009). Significant industry dealing with cowpea cultivation has been noticed in the Southern and Western part of the U.S. since cowpea is an economically profitable crop to grow (Okiror et al., 2008). Evidence of drought conditions has been reported in these areas (Escalante et al., 2016), which could limit cowpea production. However, little has been done towards advancing breeding programs for drought tolerance in cowpea compared to other legumes (Specht et al., 2001).

Since drought tolerance consists of complex mechanisms, identifying traits for reliably assessing drought tolerance could be challenging in cowpea (Verbree et al., 2015). Providing growers with crops that better withstand drought conditions require effective and strong breeding programs through the establishment of better phenotyping and screening approach. Fatokun et al. (2012) conducted a field experiment to evaluate drought tolerance in cowpea. However, possible heterogeneity due to uncontrolled factors such as temperature and water transmission within soils could significantly affect field results.

Seedling stage is one of the most sensitive stages to drought stress in cowpea (Agbicodo et al., 2009). Phenotyping drought tolerance at seedling stage in a controlled condition could

contribute towards advancing breeding programs for drought tolerance in cowpea. In addition, little has been done regarding screening drought tolerance in cowpea by limiting adaptation due to plant morphology and root architecture, which can contribute to finding unexplored genetic mechanisms underlying drought tolerance. To date, cowpea cultivars that have been proven to be drought-tolerant at seedling stage remain limited. Therefore, the objective of this study was to assess the effects of drought on above-ground traits in cowpea, and to identify drought-tolerant cowpea genotypes based on those traits at seedling stage.

Materials and Methods

Plant materials

A total of 30 cowpea genotypes were used in this study, and they originated from 14 countries (Australia, Botswana, Brazil, Ghana, India, Iran, Kenya, Mexico, Nigeria, Paraguay, Saudi Arabia, Tanzania, Trinidad and Tobago, and the United States) (Table 2.1). Of the 30 cowpea genotypes, 3 were advanced breeding lines developed by the University of Arkansas, Fayetteville, AR. The remaining was plant introductions (PIs) from the United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea accessions, which was provided by the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. Seeds were increased at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville, AR, during the summer 2016.

Growth conditions and drought stress

Evaluation of drought tolerance was conducted in the greenhouse of Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville, AR. Greenhouse day/night temperatures were maintained at 26℃/21℃, and daylight length was 14 hours (Fig. 2.1).

Screening methodology was similar to those adopted by Singh et al. (1999) and Verbree et al. (2015) with slight modifications. Cowpea planting was conducted in the Sterilite polypropylene boxes (Sterilite Corporation, Townsend, MA) with dimensions 88.6 cm X 42.2-cm X 15.6 cm, previously filled with Sunshine® Mix #1 Natural & Organic (Agawan, MA) up to 10.5 cm high. Two days before planting, each box was irrigated with 12 L of tap water so that field capacity was attained at sowing time.

Within each box, a total of ten 7.5 cm-spaced rows were designed across the box length. Each cowpea genotype was planted within each row. A total of 6 uniform and vigor plants were kept at each row when the first trifoliate leaf began to expand. One week after plant emergence from soil medium, fertilizers consisting of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI) were applied. Each row was irrigated with 150 mL of tap water every three days until the first trifoliate leaf was fully developed. Drought stress was imposed by stopping water irrigation when the first trifoliate was completely expanded, and pursued until some genotypes were completely dead, indicating susceptibility to drought stress. Soil moisture measure within boxes was recorded using HH2 Moisture Meter (Cambridge, England) every 3 days.

The experiment was conducted using a completely randomized design (CRD) with three replicates per genotype and six plants in each replicate. Treatments were the 30 cowpea genotypes for evaluation of drought tolerance. The treatment was assumed to have fixed effect. Experimental unit was each row where genotypes were planted as fixed effect as well in the study.

Measurements

Above-ground related traits

Traits involving plant greenness, stem diameter, lodged plants, wilted plants, plants exhibiting necrotic stems, plants showing dead growing points, percentage of dead plants, and recovery rate after rewatering were recorded. Plant greenness was assessed using a 1-5 scale (1= Plants were completely green, 2= Plants began losing greenness, 3=Signs of chlorosis and necrosis were visible, 4= Chlorosis and necrosis was severe, and 5= Plants were completely dead) (Fig. 2.2). Data on plant greenness was recorded on a per plant basis in 4 weeks after first imposing drought stress. At that time, some genotypes were completely dead (Fig. 2.3). If the average plant greenness scores was lower than the population average at 4 weeks of drought stress, the genotype was considered slow wilting; otherwise, it would be a fast-wilting one (Fig. 2.3). When the first signs of wilting appeared, stem diameter was recorded at 1cm above the soil medium using a digital caliper. Data on percentage of dead plants, lodged plants, wilted plants, plants exhibiting necrotic stems, and plants showing dead growing points were collected on a per row basis at 4 weeks after the last watering. Recovery rate after rewatering for each genotype was evaluated on per row basis as well.

Leaf-related parameters

Leaf-related traits have been used to identify drought tolerance in cowpea (Verbree et al., 2015). Unifoliate leaf length and width were measured before drought stressing the cowpea plants. When some genotypes were completely dead whereas others remained green, the number of plants showing unifoliate leaf wilt and chlorosis and trifoliate wilt and chlorosis was counted on a per row basis.

In vivo chlorophyll measurement

Chlorophyll was measured using SPAD-502 Plus (Spectrum Technologies, Inc., Plainfield, IL). Chlorophyll on trifoliate leaves and unifoliate leaves was measured separately since tolerance to trifoliate leaf wilting/ chlorosis and unifoliate leaf wilting/ chlorosis are two different mechanisms of drought tolerance in cowpea as described by Verbree et al. (2015). Measurements were conducted weekly after drought stress was applied. Data on chlorophyll content were taken from all plants. On each leaf, measurements were done three times at different positions to avoid edge effect. Average between the three measurements was recorded. In addition, ratio between the chlorophyll contents from trifoliate leaves and unifoliate leaves, respectively, was calculated.

Data analysis

Analysis of variance (ANOVA) was performed using PROC MIXED of SAS® 9.4. Mean separation was done using a protected least significant difference procedure (protected LSD) at α=0.05 in SAS® 9.4. Analysis of chlorophyll content was achieved through ANOVA using time as a repeated measure since observations over time were from the same experimental unit, thus could not be assumed independent. ANOVA involving time series required the identification of the appropriate covariance matrix prior to the analysis (Littell et al., 2000). Covariance matrix used for ANOVA with repeated measures was that of corresponding to the lowest Bias-Corrected Small Sample Akaike Information Criterion (AICC) as described by Littell et al. (2000).

Types of covariance structure from which the selection were done were unstructured, independence with equal variance, first order autoregressive, Toeplitz, Toeplitz with 2 bands, Toeplitz with 3 bands, heterogeneous independence, and heterogeneous first autoregressive

(Littell et al., 2000). The values of AICC for each covariance structure were calculated through SAS® 9.4 using the options 'type=un', 'type=vc', 'type=ar(1)', 'type=toep', 'type=toep(2)', 'type=toep(3)', 'type=un(1)', and 'type=arh(1)', respectively.

The statistical model for ANOVA with repeated measures for a completely randomized design was the following.

$$Y_{ijk} = \mu + G_i + \eta_{k(i)} + D_j + GD_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} represented the chlorophyll content of the i^{th} genotype (i=1, 2,…., 30) at the j^{th} week (j=1, 2, 3) of drought stress and on the k^{th} replicates (k=1, 2, 3), μ was the overall mean, G_i was the effect of the i^{th} genotype (fixed effect) on the mean response, $\eta_{k(i)}$ were independent error terms associated with the genotypes where $\eta_{k(i)} \sim N(0, \sigma^2_\eta)$, D_j was the effect of j^{th} week on the mean response, GD_{ij} denoted the interaction effect between the i^{th} genotype and the j^{th} week on the mean response, and ε_{ijk} was the error term associated with the interaction effect whose covariance matrix structure depended on the AICC value.

Pearson's correlation coefficients between trait values were calculated using JMP Genomics ®7 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics were generated using the 'Tabulate" options of JMP Genomics ®7 (SAS Institute Inc., Cary, NC, USA). Combined violin and boxplots were drawn using the packages 'ggplot2', 'labeling' and 'gridExtra' of R 3.3.0. Network path analysis between traits evaluated for drought tolerance and heatmap for chlorophyll content were drawn using the packages 'network' and 'gplots', respectively, of R 3.3.0 as well.

Results

Soil moisture content

Soil moisture content within the Sterilite polypropylene boxes where cowpea was grown significantly dropped from an average of 55 % to 22 % at 7 days of salt stress (Fig. 2.4). At 14 days, average soil moisture content was close to 10%, and in that time, the plant wilting was observed. The decreasing moisture in soil triggered drought stress in cowpea plants. The sustained insufficiency in soil moisture over time (Fig. 2.4) induced severe drought conditions, which is critical for drought tolerance evaluating in cowpea at seedling stage. Some cowpea genotypes were not able to withstand a long period of drought conditions as shown in Fig. 2.1.

Above-ground related traits

Some cowpea genotypes were completely dead at 28 days after drought stress. Plant greenness score at 28 days after drought stress varied from 1.42 to 4.47, with an average of 3.69 and a standard deviation of 0.58 (Table 2.2). A significant variation in plant greenness score was identified among the 30 cowpea genotypes evaluated for tolerance to drought stress (F value=7.31, p-value<0.0001) (Table 2.3). Mean separation analysis revealed data PI293469 (1.42), PI349674 (2.83), and PI293568 (2.89) (Table 2.2) had the lowest overall plant greenness score, indicating significant delayed senescence to cope with drought condition in those genotypes, thus tolerant to drought stress based on plant greenness score. PI582573 (4.47), PI582665 (4.33), PI229734 (4.33), PI255774 (4.33), PI666260 (4.28), and PI666260 (4.13) (Table 2.2) had the highest overall plant greenhouse score, suggesting that these genotypes failed to delay leaf senescence under drought stress, hence these genotypes were drought-susceptible based on plant greenness score. Since the population mean for plant greenness score was 3.69, for this experiment, those having a plant greenness score lower than the population mean was

considered slow-wilting; otherwise, they were fast-wilting. Slow-wilting genotypes were 09-1090, 09-655, PI293469, PI293568, PI311119, PI582340, PI582366, PI582402, PI582551, PI582697, and PI583209 (Table 2.2).

Stem diameter was recorded at first sign of plant wilting. Stem diameter was in the range of 2.45 mm to 3.69 mm, with an average of 2.96 mm and a standard deviation of 0.28 mm. ANOVA revealed a statistically significant difference in stem diameter among the cowpea genotypes (F value=3.52, p-value<0.0001) (Table 2.3). The genotypes having the largest stem diameter at the first sign of wilting were PI293469 (3.69 mm), PI582402 (3.62 mm), and 09-714 (3.48 mm), whereas those having the shortest stem diameter were PI180014 (2.69 mm), PI582366 (2.67 mm), PI582573 (2.67 mm), PI339563 (2.66 mm), PI582512 (2.62 mm), and PI582812 (2.45 mm) (Table 2.2).

The percentage of dead plants per genotype was recorded at 28 days after drought stress. At that time, some genotypes were completely dead. The percentage of dead plants per genotype varied from 0 to 100%, with an average of 54.26% and a standard deviation of 25.98%. Statistical analysis showed that the significant differences were observed in percentage of dead plants among the cowpea genotypes (F-value=29.86, p-value<0.0001) (Table 2.3). The genotypes having the lowest percentage of dead plants were PI293469 (0), PI293568 (8.33%), PI349674 (8.44%), and PI582402 (9.70%), indicating that the four genotypes were drought-tolerant, whereas accessions showing the highest percentage of dead plants were PI582665 (91.67%), PI255774 (91.67%), PI582573 (93.89%), PI229734 (97.22%), and PI666260 (100%.00) (Table 2.2). Slow-wilting genotypes had a percentage of dead plants lower than 50 % on average, whereas that of fast-wilting genotypes was greater than 50 % (Fig. 2.5B).

A large variation in percentage of lodged plants was found among the cowpea genotypes. Percentage of lodged plants varied from 0 to 100%, with an average of 44.28% and a standard deviation of 26.74%. The percentage of lodged plants was statistically significantly different among the genotypes (F-value=21.06, p-value=<0.0001) (Table 2.3). On average, less than 10% of plants were lodging under drought stress for the genotypes PI582340 (0), PI293469 (0), PI339610 (8.33%), PI293568 (8.33%), and PI349674 (9.11%), whereas percentage of lodged plants was greater 90% for the genotypes PI229734 (92.22%), PI582573 (93.56%), and PI666260 (100) (Table 2.2), suggesting that these genotypes were highly-susceptible to drought stress. Percentage of lodged plants in the fast-wilting genotypes was higher than in the slow-wilting ones (Fig. 2.2D).

Most of the cowpea genotypes presented wilting signs under severe drought conditions (Table 2.2) (Fig. 2.1). ANOVA revealed significant different in percentage of wilted plants among the cowpea genotypes evaluated from drought tolerance at seedling stage (F-value=20.57. p-value<0.0001) (Table 2.3).

Significant differences in proportion of plants with necrotic stems were identified (F-value= 15.17, p-value<0.0001) (Table 2.3). The percentage of plants showing necrotic stems ranged from 8.33% to 100%, with an average of 55.37%, and a standard deviation of 27.32%. Few plants were affected by stem necrosis for the genotypes PI293469 (8.33%), PI349674 (10.17%), and PI293568 (16.67%), indicating that the four genotypes were tolerant to stem necrosis under drought conditions. The genotypes PI339563 (86.90%), PI582812 (87.50%), PI582573 (87.78%), PI582468 (90), PI339610 (91.67%), and PI229734 (100) were highly susceptible to stem necrosis under drought stress (Table 2.2). Both distributions and average

percentage of plants with necrotic stem were different between fast-wilting and slow-wilting genotypes (Fig. 2.5B).

The percentage of plants with dead growing points was in the range of 0 and 100%, with an average of 56.76% and a standard deviation of 27.24%. There was a significant difference in percentage of plants with dead growing points among the cowpea genotypes (F-value=18.63, p-value<0.0001) (Table 2.3). Growing point of the genotypes PI349674 (0), PI293568 (0), and PI293469 (0) were free of damage, suggesting that these genotypes were highly tolerant to growing point death under extreme drought conditions. Significant amount of dead growing points was recorded for the genotypes PI582468 (80), PI582812 (87.50%), PI582573 (89.61%), PI582665 (91.67%), PI255774 (91.67%), PI229734 (93.44%), and PI666260 (100%) (Table 2.2). Distributions of dead growing points were bimodal for both fast-wilting and slow-wilting genotypes, and slow-wilting genotypes had a lower percentage of plants showing dead growing points (Fig. 2.6B).

Cowpea plants drought-stressed in 28 days were re-watered. Recovery in plant greenness was noticed in some genotypes, whereas damage caused by drought conditions was not reversible in other genotypes. Number of recovered plants was counted in one week after re-watering. Percentage of recovered plants varied from 0 to 100, with an average of 30.92% and a standard deviation of 24.38%. Recovery rate was significantly different among the cowpea genotypes (F-value=26.32, p-value<0.0001) (Table 2.3). The genotypes PI293469, 09-655, PI582402, and PI349674 (Table 2.2) had a good capability of recovering from a prolonged period of extreme drought conditions at seedling stage upon re-watering, whereas the genotypes 09-1090, PI180014, PI229734, PI255774, PI339563, PI339610, PI582340, PI582428, PI582468, PI582530, PI582573, PI582665, PI583209, and PI666260 were not capable of recovering.

Discrepancy in distributions and recovery rate were identified between fast-wilting and slow-wilting genotypes (Fig. 2.5C).

Leaf-related parameters under drought stress

Measurements on unifoliate leaves

Unifoliate leaf length and width were measured prior to drought stressing the cowpea plants. Results showed that unifoliate leaf length ranged between 6.78 cm and 11.22 cm, with an average of 9.44 cm and a standard deviation of 0.88 cm (Table 2.4). Unifoliate leaf length was significantly different among the cowpea genotypes (F-value=5.72, p-value<0.0001) (Table 2.5). The lowest unifoliate leaf length was recorded for PI180014 (8.81 cm), PI582340 (8.80 cm), PI582697 (8.72 cm), PI582512 (8.70 cm), PI293568 (8.67 cm), PI255774 (7.28 cm), and PI582812 (6.78 cm), PI582402 (11.22 cm), 09-714 (10.76 cm), PI582665 (10.43 cm), PI293469 (10.22 cm), and PI582368 (10.18 cm) had the highest unifoliate leaf length.

Unifoliate leaf width was in the range of 4.37 cm and 8.50 cm, with an average of 6.29 cm and a standard deviation of 0.87 cm. ANOVA showed significant differences in unifoliate leaf width among the cowpea genotypes (F-value= 7.30, p-value<0.0001) (Table 2.5). Genotypes with the largest unifoliate leaves were PI582402 (8.50 cm), PI293469 (7.85 cm), PI582468 (7.54 cm), 09-1090 (7.44 cm), and PI339563 (7.32 cm). Those with the narrowest unifoliate leaves were PI255774 (5.17 cm), PI180014 (5.11 cm), and PI582812 (4.37 cm). Both unifoliate leaf length and width were nearly normally distributed and almost similar for fast-wilting and slow-wilting genotypes (Fig. 2.6 C-D).

Tolerance to unifoliate leaf wilting and chlorosis under drought stress

Unifoliate leaf wilting and chlorosis have been frequently used as criteria for drought tolerance evaluation in cowpea seedlings. Data on unifoliate leaf wilting and chlorosis were

collected at 28 days after drought stress. The percentage of plants having wilted unifoliate leaves varied from 22.22% to 100%, with an average of 77.97% and a standard deviation of 19.28% (Table 2.4). Data on unifoliate leaf wilt was skewed to the lower percentage for both fast-wilting and slow-wilting genotypes with higher percentage of wilting in fast-wilting genotypes (Fig. 2.7A). Unifoliate leaf wilting was significantly different among the cowpea genotypes (F-value=15.19, p-value<0.0001) (Table 2.5). Relatively lower percentage of plants showing wilted unifoliate leaves was identified for the genotypes PI349674 (40), PI293568 (33.33%), and PI293469 (22.22%), indicating that these genotypes were moderately tolerant to unifoliate leaf wilting under drought stress. However, all plants (100%) exhibited wilted unifoliate leaves for the four genotypes PI229734, PI582573, PI582812, and PI666260 (Table 2.5), suggesting that these genotypes were highly tolerant to unifoliate leaf wilting when drought-stressed.

A large variation in tolerance to unifoliate leaf chlorosis was identified among the cowpea genotypes evaluated for drought tolerance. The percentage of plants showing chlorotic unifoliate leaves ranged between 5.56% and 100%, with an average of 75.48% and a standard deviation of 26.22%. Unifoliate leaf chlorosis was skewed to lower percentage for the fast-wilting genotypes, whereas it was bimodal for the slow-wilting genotypes with a lower percentage compared to the fast-wilting genotypes (Fig. 2.7B). Significant differences in unifoliate leaf chlorosis was identified (F-value=16.14, p-value<0.0001) (Table 2.5). The lowest percentage of plants with chlorotic unifoliate leaves was recorded for the genotypes PI293568 (22.22%), PI349674 (14.39%), and PI293469 (5.56%) (Table 2.4), indicating that these genotypes were tolerant to unifoliate leaf chlorosis under drought stress. The genotypes highly susceptible (100%) to unifoliate leaf chlorosis were PI180014, PI229734, PI255774, PI582368, PI582530, PI582551, PI582573, and PI582812 (Table 2.4).

Tolerance to trifoliate leaf wilting and chlorosis under drought stress

The percentage of plants with wilted trifoliate leaf at 28 days after drought stress varied from 0 to 60.28%, with an average of 29.75% and a standard deviation of 13.90%. Distribution of trifoliate leaf wilt was bimodal for the fast-wilting genotypes, whereas it was skewed to higher percentage for the slow-wilting genotypes (Fig. 2.7C). The percentage of plants presenting chlorotic trifoliate leaves varied from 0 to 31.67%, with an average of 10.47% and a standard deviation of 6.09%. These results suggested that cowpea plants were more tolerant to trifoliate leaf chlorosis than trifoliate leaf wilting. Significant differences in both trifoliate leaf wilting (F-value=11.02, p-value<0.0001) and trifoliate leaf chlorosis (F-value=12.42, p-value<0.0001) were identified among the cowpea genotypes. The genotypes PI582551 (11.11%), PI349674 (9.17%), and PI293469 (0) were tolerant to trifoliate leaf wilting when drought-stressed, whereas the genotypes PI229734, PI255774, PI582468, and PI582573 were severely affected by trifoliate leaf wilting under drought conditions (Table 2.4). Most of the genotypes evaluated for drought tolerance were tolerant trifoliate leaf chlorosis expect for 09-714 (31.67%), PI229734 (25.94%), PI255774 (25.00%), PI582573 (24.81%), PI582368 (24.45%), PI582512 (20.89%), PI583209 (16.17%), and PI582812 (13.89%).

Chlorophyll contents under drought stress

Covariance matrix identification for repeated measure analysis

Estimates of -2 Res Log Likelihood, Akaike Information Criterion, Bias-corrected Small Sample Akaike Information Criterion, and Bayesian Information Criterion were calculated for a total of 8 types of covariance matrix (Unstructured, independence with equal variance, first order autoregressive, Toeplitz, Toeplitz with 2 bands, Toeplitz with 3 bands, heterogeneous independence, and heterogeneous first order autoregressive). For the traits involving chlorophyll

(SPAD values) in unifoliate leaves, chlorophyll (SPAD values) in trifoliate leaves, and ratio between chlorophyll content in trifoliate and unifoliate leaves, the lowest estimates were found using an unstructured covariance matrix type except for Bayesian Information Criterion for trifoliate leaf chlorophyll (Table 2.7). Therefore, ANOVA involving time series analysis for chlorophyll contents was conducted based on an unstructured covariance matrix type.

Time by genotype effect on chlorophyll content under drought stress

Extensive leaf damage was identified at 28 days after drought stress, which made chlorophyll measurement difficult at that time. Therefore, data on chlorophyll content was collected at 7 days, 14 days, and 21 days after drought stress, respectively. Unifoliate leaf and trifoliate leaf chlorophyll was near normally distributed (Fig. 2.8A-B). ANOVA with repeated measure analysis revealed significant genotype-by-time effects on the mean response of unifoliate leaf chlorophyll (F-value=5.69, p-value<0.0001), trifoliate leaf chlorophyll (F-value=4.40, p-value<0.0001), and ratio between chlorophyll content in unifoliate leaves and trifoliate leaves (F-value=9.81, p-value<0.0001) (Table 2.8). Overall, chlorophyll in unifoliate leaves decayed over time with the lowest average recorded at 21 days after drought stress (Fig. 2.8A), whereas that of trifoliate leaves slightly increased at 14 days after drought stress, and decreased at 21 days after drought stress as shown in Fig. 2.8B.

Ratio between chlorophyll content in trifoliate leaves and unifoliate leaves was calculated and used as an indicator to assess the discrepancy in chlorophyll content between the different leaf types of drought-stressed cowpea plants at seedling stage. Results indicated a ratio close to 1 at 7 days after drought stress, suggesting that nutrients were likely evenly distributed within plant shoot. Ratio increased with a value which gradually deviated from 1 (Fig. 2.8C), indicating a

mobilization of nutrients to the upper part of the plants when soil moisture became more and more insufficient.

A more detailed view of the average chlorophyll in unifoliate and trifoliate leaves on a per genotype basis was shown using a heatmap (Fig. 2.9). Overall, the cowpea genotypes were clustered into three groups based on the average chlorophyll over the period of drought stress (Fig. 2.9). Cluster 1 (middle section of the heatmap) consisted of genotypes with an overall increased in chlorophyll at 14 days after drought stress and a less severe decrease in chlorophyll content at 21 days after drought stress. PI349674, PI293469, and PI293568 had the highest average chlorophyll content at 21 days after drought stress, suggesting that these genotypes were drought-tolerant. Cluster 2 (upper section of the heatmap) included genotypes with a decrease in average chlorophyll content over time, whereas cluster 3 (lower section of the heatmap) involved genotypes with a rapid decrease in average chlorophyll content, resulting in plant death for some of the genotypes at 21 days after drought stress.

Correlation between traits and network analysis

High correlation coefficients (|r| greater than 0.65) (Table 2.9) were found between, percentage of dead plants and recovery rate (r= -0.70), percentage of dead plants and lodged plants (r= 0.73), percentage of dead plants and those showing necrotic stems (r= 0.69), percentage of dead plants and those with dead growing points (r= 0.87), percentage of dead plants and plant greenness score (r= 0.73), percentage of dead plants and tolerance to unifoliate leaf chlorosis (r= 0.71). In addition, results revealed high correlations between unifoliate leaf chlorosis and unifoliate leaf wilt under drought stress (r= 0.73), unifoliate leaf chlorosis and chlorophyll content (r= -0.72). Network between these highly correlated traits was established and shown in Fig. 2.10. Interestingly, low correlations were found between unifoliate leaf size

and tolerance to drought in cowpea seedlings (Fig. 2.10). Similar results were found between stem diameter and tolerance to drought tolerance. Plant death under drought conditions was lowly correlated with both trifoliate leaf wilt and chlorosis.

Discussion

Drought has been shown to be an increasing threat to crop production worldwide (Cairns et al., 2013; Upadhyaya, 2005; Upadhyaya et al., 2017). Being provided with crops which are more resilient to drought conditions is an affordable strategy to cope with the impacts of drought stress. Therefore, breeding for drought-tolerant crops could alleviate the effects of drought tolerance in agriculture. Drought occurring at early vegetative has been demonstrated to be extremely damaging to cowpea production (Agbicodo et al., 2009). However, less progress has been made toward breeding and releasing drought-tolerant cowpea cultivars which would better withstand drought stress at early season. The need of a robust, fast, and cost-effective phenotyping strategy would significantly assist cowpea breeders in advancing their programs for drought tolerance.

In this report, a large variation in different traits evaluated for drought was found among the cowpea genotypes. A total of 17 above-ground traits was evaluated under drought stress. Network analysis between these traits was established and indicated that failure to tolerate unifoliate leaf wiling/chlorosis and stem necrosis and to maintain plant greenness phenomenon lead to significant plant death in cowpea genotypes, which resulted in a low recovery rate when water supplies were re-established. Overall, most of the genotypes were more tolerant to trifoliate leaf wilting/chlorosis than unifoliate leaf wilting/chlorosis, which was in agreement with the results provided by Verbree et al. (2015). The mechanism of drought tolerance

occurring at leaf level during seedling stage is an important criterion in determining drought tolerance type in cowpea. Mai-Kodomi et al. (1999) described two types of drought tolerance in cowpea. Type I drought-tolerant cowpea has the ability to delay senescence in both trifoliate and unifoliate, whereas type II is more tolerant to trifoliate wilt/chlorosis but more susceptible to unifoliate wilt/chlorosis. Our results suggested that most of genotypes were type II drought-tolerant. The genotype PI293469 (Fig 2.3 and Fig. 2.9) was considered type I drought-tolerant.

Delayed senescence phenomenon was assessed by evaluating plant greenness and taking measurement on chlorophyll (SPAD data) in both trifoliate and unifoliate leaves in drought-stressed cowpea. Our results indicated an overall increase in chlorophyll content in trifoliate leaf at 14 days after drought stress. This could be explained by a transport of nutrients to the upper shoot part at 14 days after drought stress. Our data indicated that PI293469, PI349674, and PI293568 proved to successfully maintain this mechanism even at 21 days after drought stress. An attempt to unraveling the mechanisms of drought tolerance in legumes such as chickpea (*Cicer arietinum* L.) was conducted by Li et al. (2018). Candidate genes such as auxin efflux carrier protein (PIN3), p-glycoprotein, and nodulin MtN21/EamA-like transporter were identified to probably confer drought tolerance in chickpea. Auxin efflux carrier protein (PIN3) was reported to enhance cell-to-cell auxin transport, which is critical in maintaining plant growth (Zourelidou et al., 2014). In *Arabidopsis*, Remy et al. (2013) showed that these auxin transporters were further enhanced by a superfamily of transporters regulating potassium and proton movement between plant cells. In maize (*Zea mays* L.), Yue et al. (2015) reported high expression of auxin transporter-related genes under drought stress. With an enhanced auxin transport, drought-tolerant crops had better ability of mobilizing nutrients to younger plant tissues for surviving (Remy et al., 2013; Yue et al., 2015), which could explain the increase in

chlorophyll content in trifoliate leaves of cowpea plants at 14 days after drought stress as reported in this current investigation. However, further research is required in order to provide scientific evidence of the genetics of drought tolerance in cowpea.

Research aiming at identifying the most suitable plant morphology and root architecture for enhancing drought tolerance has been extensively investigated in cowpea (Ajayi et al., 2018; Bastos et al., 2011; Burridge et al.; 2017). In this study, the effects of plant architecture on enhancing drought tolerance were limited by growing cowpea within sterility polypropylene boxes, which explained the absence of path analysis between leaf size and drought tolerance. The type I drought-tolerant cowpea, PI293469 had the largest stem dimeter (p-value<0.0001) at first sign of wilting despite of limiting adaptation of cowpea due to plant morphology. This suggested that this genotype could have the ability to better store carbohydrate in stems under drought conditions, which could contribute to its tolerance to drought conditions. Similar results were reported to by Singh et al. (1999) and Verbree et al. (2015) claiming tolerance to drought was moderately to stem diameter in cowpea seedlings. This current investigation provides valuable insights to drought tolerance in cowpea in addition to identifying drought-tolerant cowpea genotypes.

Conclusions

A total of 30 cowpea genotypes were evaluated for drought tolerance at seedling stage by using a method that limited the effects of plant morphology to confer drought tolerance. A total of 17 above-ground traits of drought-stressed plants was assessed and analyzed. A network analysis between these traits was established. Based on the path analysis, the cowpea genotypes PI293469, PI349674, and PI293568 were found to be drought-tolerant, whereas PI229734,

PI582573, PI255774, PI582468, PI582368, and PI666260 were identified to be drought-susceptible. These results could contribute to advancing breeding programs for drought tolerance in cowpea.

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Tables

Table 2.1. Cowpea accessions (30 genotypes) used for drought tolerance evaluation at seedling stage.

Accessions	Seed color[z]	Country of origin	Plant name
09-1090[y]	Pink eye	USA	09-1090
09-655[y]	Brown eye	USA	09-655
09-714[y]	Pink eye	USA	09-714
PI180014	Tan	India	Cholan
PI190191	Tan	Mexico	TVu1557
PI229734	Black eye	Iran	Chesh Boldoli Lubi
PI255774	Cream	Nigeria	TVu2428
PI293469	Tan	USA	Brown Crowder
PI293568	Tan	NA	Six Weeks Georgia
PI311119	Red	Mexico	Tvu1799
PI339563	Tan	Australia	C2-576
PI339610	Grey	Tanzania	TVu1972
PI349674	Black	Australia	Aloomba
PI582340	Grey	Paraguay	UCR 86
PI582353	Black eye	Saudi Arabia	UCR 155
PI582366	Red	India	UCR 191
PI582368	Black holstein	India	UCR 193
PI582402	Tan	Brazil	Pitiuba
PI582428	Black eye	Trinidad and Tobago	Laura B
PI582468	Brown holstein	NA	UCR 347
PI582512	Brown eye	Nigeria	UCR 430
PI582530	Grey	Ghana	Sambrizie
PI582551	Black eye	Botswana	UCR 1004
PI582573	Brown eye	Kenya	KVu23
PI582665	Grey	Botswana	UCR 1016
PI582697	Tan	Botswana	UCR 1176
PI582812	Brown holstein	Botswana	UCR 794
PI583209	Tan	Nigeria	TVu2503
PI663011	Brown eye	USA	Louisiana Purchase
PI666260	Pink eye	USA	Corona

[z]Seed color was established using the cowpea seed color classification found at https://npgsweb.ars-grin.gov/gringlobal/descriptors.aspx?

Table 2.2. Above-ground related parameters for phenotyping drought tolerance at seedling stage in cowpea.

Accessions	Wilting[z]	Plant greenness scores[y]			Stem diameter[x]			Dead plants (%)[w]			Recovery rate after rewatering(%)[w]		
		LSMeans[v]		SD[u]	LSMeans		SD	LSMeans		SD	LSMeans		SD
09-1090	5	3.47	efghi	0.06	2.89	defgh	0.23	58.33	cdefg	25.00	0.00	g	0.00
09-655	5	3.41	fghij	0.09	2.96	defgh	0.15	36.56	jk	3.34	81.72	b	1.67
09-714	0	3.78	bcdefgh	0.41	3.48	abc	0.49	55.19	defgh	5.01	77.59	b	2.51
PI180014	0	3.82	bcdefg	0.34	2.69	ghi	0.18	36.56	jk	3.34	0.00	g	0.00
PI190191	0	3.70	cdefgh	0.26	2.82	defghi	0.07	50.56	efghi	10.00	33.33	de	5.78
PI229734	0	4.33	ab	0.58	2.97	defgh	0.16	97.22	a	4.81	0.00	g	0.00
PI255774	0	4.33	ab	0.58	2.97	defgh	0.20	91.67	a	8.34	0.00	g	0.00
PI293469	5	1.42	k	0.16	3.69	a	0.21	0.00	l	0.00	100.00	a	0.00
PI293568	5	2.89	ij	0.35	2.76	fghi	0.05	8.33	l	8.34	58.33	c	25.00
PI311119	5	3.67	cdefgh	0.42	3.15	cdef	0.06	46.78	fghijk	5.87	25.67	def	4.91
PI339563	0	3.78	bcdefgh	0.19	2.66	ghi	0.20	42.06	hijk	8.36	0.00	g	0.00
PI339610	0	3.94	abcdef	0.10	2.82	defghi	0.04	58.33	cdefg	8.34	0.00	g	0.00
PI349674	5	2.83	j	0.29	3.07	cdefg	0.15	8.44	l	0.84	80.44	b	9.83
PI582340	5	3.62	defgh	0.20	3.16	cdef	0.41	45.15	ghijk	5.01	0.00	g	0.00
PI582353	0	3.83	bcdefg	0.70	2.82	defghi	0.26	54.11	defgh	6.26	29.00	de	8.41
PI582366	5	3.56	defgh	0.14	2.67	ghi	0.18	50.00	efghij	10.00	30.00	de	30.00
PI582368	0	3.78	bcdefgh	0.35	2.91	defgh	0.71	66.67	bcd	16.67	16.67	efg	0.00
PI582402	5	3.29	ghij	0.18	3.62	ab	0.19	9.70	l	10.01	81.72	b	1.67
PI582428	0	3.80	bcdefg	0.53	2.91	defgh	0.05	66.83	bcd	5.92	0.00	g	0.00
PI582468	0	4.11	abcd	0.67	3.00	defgh	0.43	70.00	bc	10.00	0.00	g	0.00
PI582512	0	4.03	abcde	0.45	2.62	hi	0.06	73.44	b	5.68	19.51	ef	2.86
PI582530	0	4.03	abcde	0.55	2.75	fghi	0.02	66.67	bcd	0.00	0.00	g	0.00
PI582551	5	3.39	fghij	0.35	3.20	bcde	0.30	33.33	k	0.00	41.67	cd	8.34
PI582573	0	4.47	a	0.32	2.67	ghi	0.02	93.89	a	5.36	0.00	g	0.00
PI582665	0	4.33	ab	0.58	2.79	efghi	0.17	91.67	a	8.34	0.00	g	0.00
PI582697	5	3.18	hij	0.02	3.23	bcd	0.40	40.00	ijk	0.00	10.00	fg	10.00
PI582812	0	3.81	bcdefg	0.17	2.45	i	0.35	62.50	bcde	12.50	37.50	d	37.50
PI583209	5	3.58	defgh	0.33	3.17	cdef	0.29	60.33	bcdef	0.34	0.00	g	0.00
PI663011	0	4.13	abcd	0.35	2.91	defgh	0.11	53.61	defghi	5.68	17.40	ef	6.00
PI666260	0	4.28	abc	0.25	2.85	defghi	0.31	100.00	a	0.00	0.00	g	0.00

Accessions	Lodged plants (%)			Wilted plants (%)			Necrotic stems (%)			Dead growing points (%)		
	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
09-1090	66.67	bcd	16.67	100.00	a	0.00	25.00	klm	8.33	50.00	ijkl	16.67
09-655	26.87	hijk	6.67	100.00	a	0.00	18.28	lm	1.67	36.56	lm	3.34
09-714	56.85	cde	17.54	100.00	a	0.00	56.85	efghi	17.54	55.19	hijk	5.01
PI180014	36.56	fghij	3.34	100.00	a	0.00	46.26	ghij	13.35	45.15	jkl	5.01
PI190191	50.44	cdef	0.20	100.00	a	0.00	62.11	defgh	7.71	56.44	ghijk	10.44
PI229734	92.22	a	6.94	100.00	a	0.00	100.00	a	0.00	93.44	ab	11.36
PI255774	41.67	efghi	25.00	100.00	a	0.00	83.33	abc	16.67	91.67	abc	8.34
PI293469	0.00	m	0.00	66.67	b	16.67	8.33	m	8.34	0.00	n	0.00
PI293568	8.33	lm	8.34	100.00	a	0.00	16.67	lm	0.00	0.00	n	0.00
PI311119	42.50	efgh	2.78	100.00	a	0.00	44.17	hijk	4.73	47.00	ijkl	5.63
PI339563	57.94	cde	8.36	100.00	a	0.00	86.90	ab	12.54	73.81	cdefg	25.08

Table 2.2. (Cont.)

Accessions	Lodged plants (%)			Wilted plants (%)			Necrotic stems (%)			Dead growing points (%)		
	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
PI339610	8.33	lm	8.34	100.00	a	0.00	91.67	ab	8.34	75.00	cdef	25.00
PI349674	9.11	lm	0.84	67.11	b	5.58	10.17	m	1.69	0.00	n	0.00
PI582340	0.00	m	0.00	100.00	a	0.00	35.45	jkl	15.02	45.15	jkl	5.01
PI582353	33.29	ghijk	6.41	100.00	a	0.00	57.22	efghi	5.68	61.94	efghij	7.44
PI582366	50.00	defg	10.00	100.00	a	0.00	20.00	lm	20.00	40.00	klm	0.00
PI582368	58.33	cde	25.00	100.00	a	0.00	33.33	jkl	16.67	58.33	fghij	25.00
PI582402	18.28	kl	1.67	100.00	a	0.00	18.28	lm	1.67	26.87	m	6.67
PI582428	65.33	cd	4.84	100.00	a	0.00	73.78	bcde	10.78	63.44	efghi	5.68
PI582468	50.00	defg	10.00	100.00	a	0.00	90.00	ab	10.00	80.00	bcde	0.00
PI582512	23.17	jkl	7.29	100.00	a	0.00	67.06	cdef	11.21	75.28	cdef	4.84
PI582530	41.67	efghi	8.34	100.00	a	0.00	58.33	efghi	8.34	58.33	fghij	8.34
PI582551	25.00	ijkl	8.33	100.00	a	0.00	41.67	ijk	8.34	25.00	m	8.33
PI582573	93.56	a	11.16	100.00	a	0.00	87.78	ab	10.72	89.61	abc	9.06
PI582665	83.33	ab	16.67	100.00	a	0.00	83.33	abc	16.67	91.67	abc	8.34
PI582697	30.00	hijk	10.00	100.00	a	0.00	80.00	bcd	0.00	60.00	fghij	20.00
PI582812	50.00	defg	0.00	100.00	a	0.00	87.50	ab	12.50	87.50	abcd	12.50
PI583209	67.17	bc	5.63	100.00	a	0.00	61.93	defgh	2.93	70.28	defgh	9.92
PI663011	41.83	efgh	3.37	100.00	a	0.00	51.17	fghij	10.17	45.11	jkl	4.84
PI666260	100.00	a	0.00	100.00	a	0.00	64.44	cdefg	33.56	100.00	a	0.00

[z]If overall plant greenness score was lower than 3.5, the genotype was considered as slow-wilting, thus highly drought-tolerant.

[y]Overall plant greenness score was lower than 3.5.

[x]Stem diameter was measured when plans showed first signs of wilting.

[w]Percentage of dead, lodged, wilted, plants, and those showing necrotic stems and dead growing points were evaluated 28 days after upholding water.

[w]Percentage of plants recovering from severe drought conditions was evaluated one week after rewatering.

[v]Means followed by the same letter are not significantly different at α=0.05.

[u]Standard deviation.

Table 2.3. ANOVA table for overall plant greenness, stem diameter, dead, lodged, and wilted plants, and plants showing necrotic stems and dead growing points in 28 days of drought stress, and recovery rate after rewatering plants over one week.

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Prob > F
Plant_Greenness	Accession	29	30.45	1.05	7.31	<.0001
	Residual	60	8.62	0.14		
Stem_diameter_(mm)	Accession	29	7.20	0.25	3.52	<.0001
	Residual	60	4.23	0.07		
Dead_plants(%)	Accession	29	60761.70	2095.23	29.86	<.0001
	Residual	60	4210.82	70.18		
Recovery(%)	Accession	29	86051.09	2967.28	26.32	<.0001
	Residual	60	6763.45	112.72		
Lodged_plants(%)	Accession	29	64347.98	2218.90	21.06	<.0001
	Residual	60	6321.87	105.36		
Wilted_plants(%)	Accession	29	6140.04	211.73	20.57	<.0001
	Residual	60	617.65	10.29		
Necrotic_stem(%)	Accession	29	67171.51	2316.26	15.71	<.0001
	Residual	60	8845.14	147.42		
Dead_growing_point(%)	Accession	29	66772.39	2302.50	18.63	<.0001
	Residual	60	7413.64	123.56		

Table 2.4. Leaf-related traits for cowpea seedling under drought stress.

Accessions	Unifoliate_length(cm)[z]			Unifoliate_width(cm)[y]			Unifoliate_wilt(%)[y]			Unifoliate_chlorosis(%)[y]			Trifoliate_leaf_wilt(%)[y]			Trifoliate_leaf_ chlorosis(%)[y]		
	LSMeans[x]		SD[w]	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
09-1090	9.73	bcdefg	0.33	7.44	bcd	0.34	77.78	abcd	19.24	72.22	cdef	9.62	44.45	abc	38.49	0.00	e	0.00
09-655	9.99	bcd	0.53	5.59	jklmn	0.47	75.56	bcd	21.43	75.56	cde	21.43	40.00	abc	52.92	0.00	e	0.00
09-714	10.76	ab	0.75	5.68	ijklmn	0.17	73.33	bcd	23.09	85.00	abcd	13.23	38.33	abcd	37.53	31.67	a	16.07
PI180014	8.81	efgh	0.33	5.11	no	0.54	88.89	abc	19.24	100.00	a	0.00	27.78	abcd	25.46	0.00	e	0.00
PI190191	8.92	efgh	0.22	5.57	jklmn	0.29	84.55	abc	4.02	85.58	abcd	8.34	33.78	abcd	5.82	0.00	e	0.00
PI229734	9.50	cdefgh	0.36	5.51	klmn	0.36	100.00	a	0.00	100.00	a	0.00	60.28	a	9.75	25.94	ab	6.08
PI255774	7.28	i	1.25	5.17	mno	1.99	88.89	abc	19.24	100.00	a	0.00	55.56	ab	9.62	25.00	ab	8.33
PI293469	10.22	abcd	0.48	7.85	ab	0.31	22.22	f	9.62	5.56	i	9.62	0.00	d	0.00	0.00	e	0.00
PI293568	8.67	h	0.34	6.44	efghij	0.27	33.33	ef	0.00	22.22	hi	19.24	22.22	abcd	25.46	0.00	e	0.00
PI311119	9.41	cdefgh	0.35	6.70	cdefg	0.46	75.61	bcd	5.09	77.50	bcd	7.05	25.33	abcd	5.17	0.00	e	0.00
PI339563	9.83	bcde	0.58	7.32	bcde	0.14	72.22	bcd	25.46	55.56	fg	9.62	22.22	abcd	19.24	0.00	e	0.00
PI339610	9.69	cdefgh	0.50	6.58	defghi	0.25	83.33	abc	16.67	88.89	abc	9.62	16.67	bcd	16.67	0.00	e	0.00
PI349674	9.62	cdefgh	0.51	6.64	cdefgh	0.45	40.00	ef	6.54	14.39	i	3.63	9.17	cd	5.53	0.00	e	0.00
PI582340	8.80	efgh	0.48	6.04	ghijklm	0.67	72.22	bcd	9.62	57.78	efg	22.69	42.22	abc	36.72	0.00	e	0.00
PI582353	9.17	defgh	0.27	6.70	cdefg	0.50	80.72	abc	10.50	77.50	bcd	6.10	23.72	abcd	5.88	0.00	e	0.00
PI582366	9.26	defgh	0.48	6.27	fghijkl	0.09	55.56	de	13.88	67.78	def	13.47	30.00	abcd	26.46	0.00	e	0.00
PI582368	10.18	abcd	2.00	6.19	fghijkl	1.45	86.67	abc	23.09	100.00	a	0.00	36.67	abcd	32.15	24.45	abc	13.47
PI582402	11.22	a	0.39	8.50	a	0.07	83.33	abc	0.00	40.00	gh	17.32	18.89	bcd	20.09	0.00	e	0.00
PI582428	9.70	bcdefgh	0.21	5.46	lmn	0.24	54.56	de	20.38	83.78	abcd	7.68	28.50	abcd	7.17	0.00	e	0.00
PI582468	9.72	bcdefgh	0.41	7.54	bc	0.40	94.44	ab	9.62	94.44	ab	9.62	53.33	ab	50.33	0.00	e	0.00
PI582512	8.70	gh	0.53	5.73	hijklmn	0.45	87.78	abc	2.34	85.11	abcd	5.17	29.50	abcd	10.21	20.89	bcd	4.94
PI582530	9.16	defgh	0.38	6.32	fghijkl	0.45	88.89	abc	9.62	100.00	a	0.00	27.78	abcd	25.46	0.00	e	0.00
PI582551	10.05	bcd	0.25	5.53	jklmn	0.14	83.33	abc	16.67	100.00	a	0.00	11.11	cd	19.24	0.00	e	0.00
PI582573	9.37	cdefgh	0.26	5.93	ghijklmn	0.25	100.00	a	0.00	100.00	a	0.00	52.44	ab	6.04	24.81	ab	4.57
PI582665	10.43	abc	1.41	6.98	bcdef	0.43	94.44	ab	9.62	88.89	abc	19.24	27.78	abcd	25.46	0.00	e	0.00

Table 2.4. (Cont.)

Accessions	Unifoliate_length(cm)[z]			Unifoliate_width(cm)[y]			Unifoliate_wilt(%)[y]			Unifoliate_chlorosis(%)[y]			Trifoliate_leaf_wilt(%)[y]			Trifoliate_leaf_chlorosis(%)[y]		
	LSMeans[x]		SD[w]	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
PI582697	8.72	fgh	0.79	6.27	fghijkl	0.17	66.67	cd	16.67	44.44	g	19.25	17.78	bcd	16.78	0.00	e	0.00
PI582812	6.78	i	0.09	4.37	o	0.10	100.00	a	0.00	100.00	a	0.00	22.22	abcd	25.46	13.89	d	12.73
PI583209	9.78	bcdef	0.50	6.40	efghijk	0.10	88.67	abc	5.49	69.94	def	9.42	31.44	abcd	7.00	16.17	cd	8.30
PI663011	9.69	cdefgh	0.36	6.12	fghijkl	0.27	86.23	abc	5.46	83.35	abcd	6.90	26.61	abcd	6.31	0.00	e	0.00
PI666260	10.06	bcd	0.42	6.84	cdefg	0.62	100.00	a	0.00	88.89	abc	19.24	16.67	bcd	28.87	0.00	e	0.00

[z]Unifoliate length and width were measured on the last day of watering.

[y]Percentage of plants showing unifoliate leaf wilt/chlorosis and trifoliate leaf wilt/chlorosis 21 days after imposing drought stress.

[x]Means followed by the same letter are not significantly different at α=0.05.

[w]Standard deviation.

Table 2.5. ANOVA table for unifoliate leaf length and width measured on the last day of watering, percentage of plants showing wilted, chlorotic, and necrotic unifoliate and trifoliate leaves 21 days after drought stress.

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Prob > F
Unifoliate leaf length	Accession	29	70.45	2.43	5.72	<.0001
	Residual	60	25.48	0.42		
Unifoliate leaf width	Accession	29	68.26	2.35	7.30	<.0001
	Residual	60	19.34	0.32		
Unifoliate leaf wilt	Accession	29	33458.30	1153.73	15.19	<.0001
	Residual	60	13328.96	222.15		
Unifoliate leaf chlorosis	Accession	29	61873.74	2133.58	16.14	<.0001
	Residual	60	7932.64	132.21		
Trifoliate leaf wilt	Accession	29	17385.45	599.50	11.02	<.0001
	Residual	60	5215.63	86.93		
Trifoliate leaf chlorosis	Accession	29	9872.01	340.41	12.42	<.0001
	Residual	60	1644.89	27.41		

Table 2.6. Chlorophyll (SPAD values) content over time under drought stress.

Accessions	7 days of drought stress									14 days of drought stress								
	SPAD_Unifoliate(%)[z]			**SPAD_Trifoliate(%)**			**SPAD_Tri/Uni(%)[y]**			**SPAD_Unifoliate(%)**			**SPAD_Trifoliate(%)**			**SPAD_Tri/Uni(%)**		
	LSMeans[x]		SD[w]	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
09-1090	53.05	bcdefg	2.99	52.78	abc	9.77	0.99	abcdef	0.14	34.22	abcdef	15.83	54.13	bcde	1.95	1.80	cde	0.69
09-655	48.83	gh	3.91	50.75	abc	7.75	1.04	abcde	0.19	31.59	bcdef	13.96	51.81	de	5.84	1.85	cde	0.74
09-714	54.42	abcde	2.79	46.38	abcd	13.74	0.86	defg	0.28	29.67	bcdef	18.05	55.71	bcde	5.58	2.26	cde	0.97
PI180014	45.07	h	1.54	53.09	abc	11.81	1.18	a	0.25	23.61	ef	14.95	55.73	bcde	1.27	2.92	cde	1.33
PI190191	51.21	efg	1.01	50.78	abc	1.46	0.99	abcdef	0.02	50.33	abcd	1.92	57.62	bcd	0.78	1.14	e	0.03
PI229734	51.87	defg	1.10	51.30	abc	2.62	0.99	abcdef	0.05	48.64	abcd	0.88	56.22	bcde	1.66	1.15	e	0.03
PI255774	56.96	abc	0.25	57.63	a	2.85	1.01	abcde	0.05	31.49	bcdef	21.47	56.60	bcde	1.27	2.33	cde	1.21
PI293469	52.15	defg	1.63	38.68	d	7.49	0.74	g	0.14	41.71	abcdef	6.17	55.28	bcde	3.65	1.34	de	0.10
PI293568	53.96	bcdef	1.94	53.68	abc	9.91	1.00	abcde	0.23	38.61	abcdef	7.92	58.20	bc	4.87	1.54	cde	0.30
PI311119	54.41	abcde	2.04	50.36	abcd	3.22	0.93	cdefg	0.10	50.80	abc	2.93	56.55	bcde	1.02	1.12	e	0.08
PI339563	51.69	defg	0.34	55.98	abc	3.03	1.08	abc	0.07	28.67	def	16.47	56.76	bcde	4.47	2.32	cde	0.90
PI339610	54.35	bcde	1.73	57.52	a	10.20	1.06	abcd	0.16	32.61	abcdef	18.01	64.62	a	2.97	2.33	cde	0.96
PI349674	53.51	bcdef	2.14	50.65	abc	2.17	0.95	bcdefg	0.08	51.41	ab	1.16	56.94	bcde	2.89	1.11	e	0.04
PI582340	52.93	bcdefg	3.32	44.89	bcd	16.06	0.84	efg	0.26	36.92	abcdef	13.06	58.79	abc	7.43	1.70	cde	0.51
PI582353	51.63	defg	1.98	47.76	abcd	1.30	0.93	cdefg	0.04	44.73	abcde	3.13	50.88	e	1.34	1.14	e	0.07
PI582366	57.24	ab	2.73	44.38	cd	12.79	0.78	fg	0.24	32.63	abcdef	12.99	53.05	cde	3.24	1.77	cde	0.56
PI582368	49.70	fg	4.39	53.12	abc	8.22	1.07	abcd	0.17	23.54	ef	19.81	54.70	bcde	4.72	3.32	abc	1.79
PI582402	50.55	efg	0.76	46.62	abcd	8.17	0.92	cdefg	0.16	37.54	abcdef	9.21	56.38	bcde	5.88	1.54	cde	0.21
PI582428	54.49	abcde	2.79	53.82	abc	1.97	0.99	abcdef	0.02	54.04	a	2.45	59.52	ab	0.89	1.11	e	0.05
PI582468	50.54	efg	2.64	57.37	a	4.75	1.13	abc	0.08	25.27	ef	16.77	59.08	abc	0.63	3.07	bcd	1.74
PI582512	52.50	cdefg	2.38	51.26	abc	2.45	0.98	abcdef	0.01	50.15	abcd	2.53	57.71	bcd	2.01	1.15	e	0.03
PI582530	55.78	abcd	6.27	55.09	abc	2.26	0.99	abcdef	0.09	21.54	f	23.03	59.24	abc	4.91	5.09	a	3.34
PI582551	58.94	a	3.32	54.41	abc	5.21	0.92	cdefg	0.06	35.74	abcdef	14.70	58.10	bc	2.97	1.78	cde	0.56
PI582573	49.62	fg	1.72	56.39	ab	2.91	1.14	abc	0.02	48.84	abcd	1.42	58.84	abc	1.46	1.21	e	0.06
PI582665	51.47	defg	1.46	54.24	abc	6.23	1.05	abcde	0.14	28.66	def	18.63	58.19	bc	6.40	2.47	cde	1.00

Table 2.6 (Cont.)

Accessions	7 days of drought stress									14 days of drought stress								
	SPAD_Unifoliate(%)[z]			SPAD_Trifoliate(%)			SPAD_Tri/Uni(%)[y]			SPAD_Unifoliate(%)			SPAD_Trifoliate(%)			SPAD_Tri/Uni(%)		
	LSMeans[x]		SD[w]	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD	LSMeans		SD
PI582697	44.84	h	2.81	52.22	abc	6.69	1.16	ab	0.08	29.41	cdef	8.64	58.31	bc	5.48	2.06	cde	0.45
PI582812	48.71	gh	2.18	47.03	abcd	1.05	0.97	abcdef	0.04	22.65	f	24.06	58.56	abc	4.74	4.80	ab	3.21
PI583209	53.11	bcdefg	1.51	54.90	abc	2.03	1.03	abcde	0.01	48.79	abcd	3.25	59.24	abc	0.67	1.22	e	0.09
PI663011	50.93	efg	1.14	54.18	abc	1.77	1.07	abcd	0.04	48.24	abcd	3.18	57.79	bcd	1.99	1.20	e	0.12
PI666260	53.18	bcdefg	6.50	52.67	abc	5.35	0.99	abcdef	0.02	29.06	cdef	17.42	58.63	abc	5.21	2.39	cde	0.94

Accessions	21 days of drought stress								
	SPAD_Unifoliate(%)			SPAD_Trifoliate(%)			SPAD_Tri/Uni(%)		
	LSMeans		SD	LSMeans		SD	LSMeans		SD
09-1090	26.49	cdefg	2.45	48.67	bcde	2.19	1.85	jklmno	0.24
09-655	23.52	fghi	2.08	48.15	bcde	8.20	2.04	hijklm	0.30
09-714	19.71	jklm	0.93	49.33	bcde	3.58	2.50	efgh	0.16
PI180014	14.64	opq	0.79	50.22	bcd	3.93	3.43	c	0.16
PI190191	28.94	c	2.08	46.38	cde	2.79	1.61	lmno	0.11
PI229734	17.16	mno	1.89	33.46	hi	2.16	1.97	hijklmn	0.29
PI255774	19.60	jklm	2.49	44.32	def	3.05	2.28	fghij	0.32
PI293469	39.20	a	1.82	53.45	ab	3.44	1.36	o	0.03
PI293568	33.13	b	2.80	51.74	abc	6.03	1.58	lmno	0.31
PI311119	25.54	cdefgh	1.23	45.95	cde	3.05	1.80	jklmno	0.11
PI339563	18.17	lmno	1.81	53.19	ab	2.31	2.94	cde	0.18

Table 2.6 (Cont.)

Accessions	21 days of drought stress								
	SPAD_Unifoliate(%)			**SPAD_Trifoliate(%)**			**SPAD_Tri/Uni(%)**		
	LSMeans		SD	LSMeans		SD	LSMeans		SD
PI339610	21.48	ijkl	1.28	52.60	ab	4.59	2.44	efghi	0.08
PI349674	34.81	b	3.25	50.28	bcd	0.95	1.45	no	0.11
PI582340	28.04	cd	3.41	52.60	ab	5.20	1.91	ijklmno	0.39
PI582353	23.86	efghi	2.25	49.37	bcde	1.04	2.08	ghijklm	0.17
PI582366	26.94	cdef	3.22	39.67	fg	1.97	1.49	no	0.23
PI582368	12.75	pqr	1.67	37.27	gh	5.23	2.93	cde	0.23
PI582402	29.24	c	5.28	43.90	ef	1.18	1.54	mno	0.30
PI582428	28.04	cd	1.96	48.11	bcde	2.46	1.73	klmno	0.20
PI582468	15.62	nop	3.34	32.07	hi	8.23	2.07	ghijklm	0.40
PI582512	22.97	ghij	1.42	44.34	def	2.07	1.94	ijklmn	0.19
PI582530	8.98	s	1.28	48.65	bcde	3.64	5.48	a	0.71
PI582551	27.47	cde	2.37	52.61	ab	2.48	1.92	ijklmn	0.12
PI582573	11.78	qrs	0.70	30.38	i	1.94	2.59	defg	0.31
PI582665	18.08	lmno	0.35	56.62	a	3.41	3.13	cd	0.19
PI582697	25.20	defghi	1.42	53.45	ab	1.91	2.13	ghijkl	0.12
PI582812	10.38	rs	2.82	48.00	bcde	0.82	4.83	b	1.15
PI583209	21.99	hijk	1.51	48.26	bcde	2.24	2.21	ghijk	0.25
PI663011	24.75	defghi	2.55	48.66	bcde	1.22	1.99	hijklmn	0.26
PI666260	18.72	klmn	0.52	52.83	ab	4.00	2.83	def	0.29

[z]SPAD chlorophyll values for unifoliate and trifoliate leaves were measured the 14th, 21th, and 28th day of drought stress.

[y]Ratio between SPAD values for first triofoliate leaves and unifoliate leaves.

[x]Means followed by the same letter are not significantly different at α=0.05.

Table 2.7. Model selection criteria for identifying the best covariance matrix structure under which ANOVA involving time series was performed.

Covariance matrix structures	Fits Statistics	Chlorophyll (SPAD values) in unifoliate leaves	Chlorophyll (SPAD values) in trifoliate leaves	Ratio between chlorophyll content in trifoliate and unifoliate leaves
Unstructured	-2 Res Log Likelihood[z]	1137.5	1147.9	232.5
	AIC[y]	1149.5	1159.9	244.5
	AICC[x]	1149.9	1160.4	245
	BIC[x]	1164.5	1174.9	259.5
Independence with equal variance	-2 Res Log Likelihood	1357.9	1199.4	470.9
	AIC	1359.9	1201.4	472.9
	AICC	1359.9	1201.4	472.9
	BIC	1362.4	1203.9	475.4
First order autoregressive	-2 Res Log Likelihood	1357.8	1194.9	462.6
	AIC	1361.8	1198.9	466.6
	AICC	1361.9	1199	466.7
	BIC	1366.8	1203.9	471.6
Toeplitz	-2 Res Log Likelihood	1357.5	1190.4	462.3
	AIC	1363.5	1196.4	468.3
	AICC	1363.6	1196.5	468.4
	BIC	1371	1203.9	475.8
Toeplitz with 2 bands	-2 Res Log Likelihood	1357.8	1196.3	462.6
	AIC	1361.8	1200.3	466.6
	AICC	1361.8	1200.4	466.7
	BIC	1366.8	1205.3	471.6
Toeplitz with 3 bands	-2 Res Log Likelihood	1357.5	1190.4	462.3
	AIC	1363.5	1196.4	468.3
	AICC	1363.6	1196.5	468.4
	BIC	1371	1203.9	475.8
Heterogeneous independence	-2 Res Log Likelihood	1142	1163.4	251.3
	AIC	1150	1169.4	257.3
	AICC	1150.2	1169.6	257.4
	BIC	1160	1176.9	264.8
Heterogeneous first order autoregressive	-2 Res Log Likelihood	1142	1155.8	244.5
	AIC	1150	1163.8	252.5
	AICC	1150.2	1164.1	252.7
	BIC	1160	1173.8	262.5

[z]Maximization of the likelihood function $L(\Theta|y_1,\ldots,y_n)$.

[y]Akaike Information Criterion.

[x]Bias-corrected small sample Akaike Information Criterion, BIC: Bayesian Information Criterion.

Table 2.8. ANOVA (Type 3 Tests of Fixed Effects) involving time series analysis under unstructured covariance matrix model for chlorophyll (SPAD) contents in unifoliate leaves, trifoliate leaves, and ratio between chlorophyll content (SPAD) in unifoliate leaves and trifoliate leaves.

Parameters	Effect	Num DF	Den DF	F Value	Pr > F
Chlorophyll content in unifoliate leaves	Accessions	29	60	4.37	<.0001
	Time	2	60	2679.47	<.0001
	Accessions*Time	58	60	5.69	<.0001
Chlorophyll content in trifoliate leaves	Accessions	29	60	3.97	<.0001
	Time	2	60	251.02	<.0001
	Accessions*Time	58	60	4.4	<.0001
Ratio of chlorophyll content between trifoliate and unifoliate leaves	Accessions	29	60	6.23	<.0001
	Time	2	60	650.25	<.0001
	Accessions*Time	58	60	9.81	<.0001

Table 2.9. Correlation between traits evaluated under drought tolerance among 30 cowpea genotypes.

	Plant_Greenness	Stem_Diameter	Dead_plants	Recovery	Lodged_plants	Wilted_plants	Necrotic_Stem	Dead_growing_point	Unifoliate_leaf_length
Plant_Greenness	1.00								
Stem_Diameter	-0.47	1.00							
Dead_plants	0.73	-0.37	1.00						
Recovery	-0.61	0.42	-0.70	1.00					
Lodged_plants	0.57	-0.29	0.73	-0.47	1.00				
Wilted_plants	0.60	-0.32	0.48	-0.52	0.36	1.00			
Necrotic_Stem	0.60	-0.31	0.69	-0.60	0.50	0.39	1.00		
Dead_growing_point	0.71	-0.34	0.87	-0.66	0.67	0.50	0.81	1.00	
Unifoliate_leaf_length	-0.10	0.49	-0.17	0.23	0.10	-0.12	-0.27	-0.21	1.00
Unifoliate_leaf_width	-0.27	0.39	-0.29	0.15	-0.12	-0.24	-0.29	-0.24	0.63
Unifoliate_leaf_Wilt	0.67	-0.28	0.59	-0.47	0.49	0.51	0.51	0.63	-0.10
Unifoliate_leaf_chlorosis	0.71	-0.35	0.71	-0.55	0.51	0.60	0.55	0.65	-0.14
Trifoliate_leaf_wilt	0.30	0.03	0.36	-0.28	0.24	0.27	0.22	0.30	-0.05
Trifoliate_leaf_chlorosis	0.28	0.04	0.45	-0.09	0.33	0.14	0.32	0.37	-0.03
Chlorophyll_unifoliate_leaf	-0.63	0.41	-0.64	0.53	-0.52	-0.49	-0.57	-0.68	0.18
Chlorophyll_trifoliate_leaf	-0.34	0.17	-0.35	0.15	-0.34	-0.17	-0.20	-0.26	0.04
Ratio_Trifoliate_UnifoliateChlorophyll	0.31	-0.31	0.33	-0.32	0.23	0.25	0.34	0.39	-0.24

	Unifoliate_leaf_width	Unifoliate_leaf_Wilt	Unifoliate_leaf_chlorosis	Trifoliate_leaf_wilt	Trifoliate_leaf_chlorosis	Chlorophyll_unifoliate_leaf	Chlorophyll_trifoliate_leaf	Ratio_Trifoliate_UnifoliateChlorophyll
Plant_Greenness								
Stem_Diameter								
Dead_plants								
Recovery								
Lodged_plants								

Table 2.9 (Cont.)

	Unifoliate_leaf_width	Unifoliate_leaf_Wilt	Unifoliate_leaf_chlorosis	Trifoliate_leaf_wilt	Trifoliate_leaf_chlorosis	Chlorophyll_unifoliate_leaf	Chlorophyll_trifoliate_leaf	Ratio_Trifoliate_UnifoliateChlorophyll
Wilted_plants								
Necrotic_Stem								
Dead_growing_point								
Unifoliate_leaf_length								
Unifoliate_leaf_width	1.00							
Unifoliate_leaf_Wilt	-0.26	1.00						
Unifoliate_leaf_chlorosis	-0.45	0.73	1.00					
Trifoliate_leaf_wilt	-0.14	0.26	0.31	1.00				
Trifoliate_leaf_chlorosis	-0.28	0.23	0.33	0.37	1.00			
Chlorophyll_unifoliate_leaf	0.29	-0.66	-0.72	-0.25	-0.39	1.00		
Chlorophyll_trifoliate_leaf	0.10	-0.30	-0.32	-0.27	-0.42	0.34	1.00	
Ratio_Trifoliate_UnifoliateChlorophyll	-0.24	0.41	0.45	0.02	0.12	-0.79	0.12	1.00

Figures

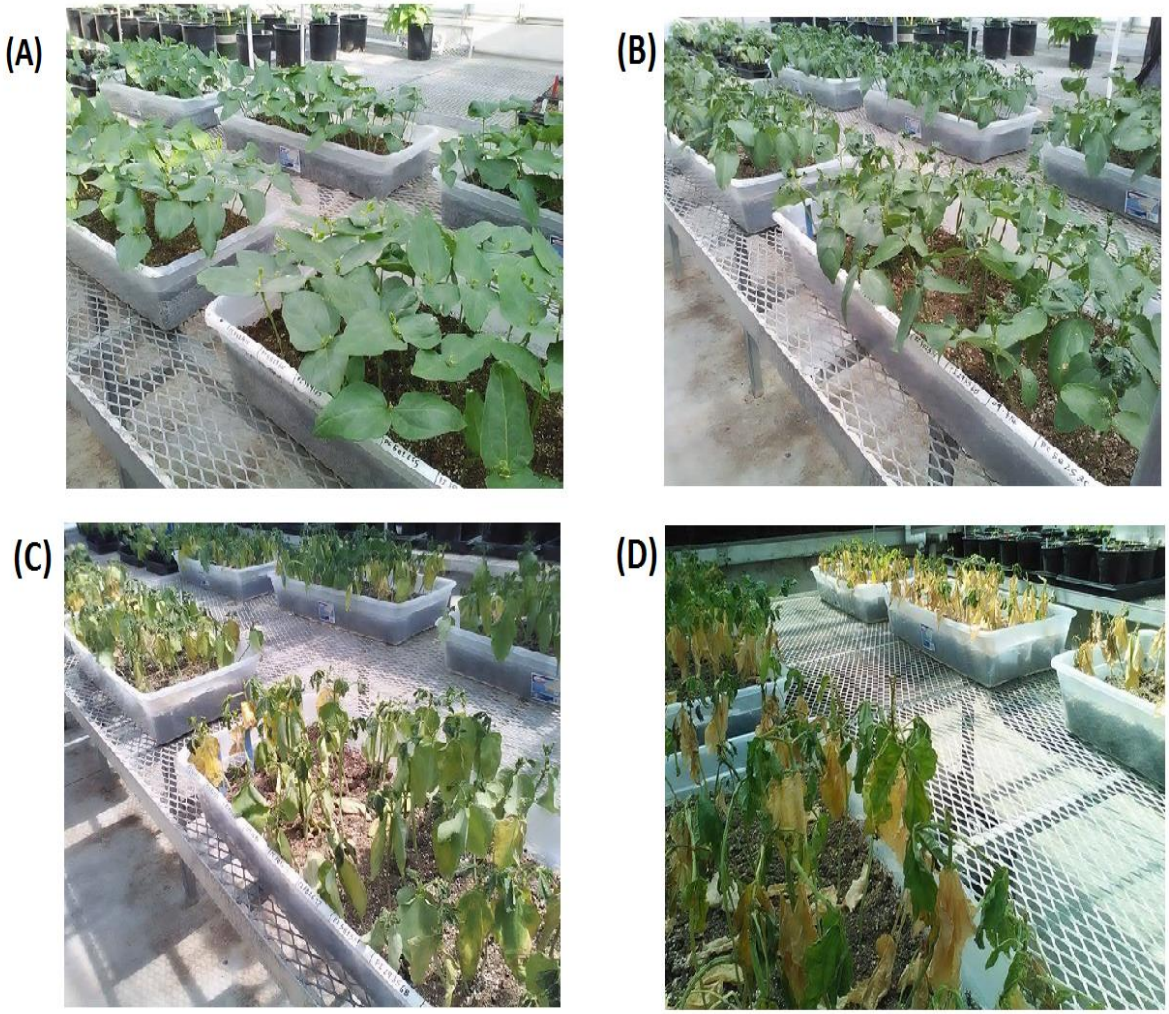

Fig. 2.1. Greenhouse phenotyping experiments for drought tolerance at seedling stage in cowpea: (A) drought stress was imposed for 7 days, (B) for 14 days, (C) for 21 days, and (D) for 28 days (Photo: Dr. Ainong Shi).

Fig. 2.2. Overall-plant greenness assessed on a 1-5 scale: 1= Plants were completely green, 2= Plants began losing greenness, 3=Signs of chlorosis and necrosis were visible, 4= Chlorosis and necrosis was severe, and 5= Plants were completely dead (Photo: Dr. Ainong Shi).

Fig. 2.3. Slow-wilting (green) and fast-wilting (yellow) cowpea genotypes 28 days of drought stress (Photo: Dr. Ainong Shi).

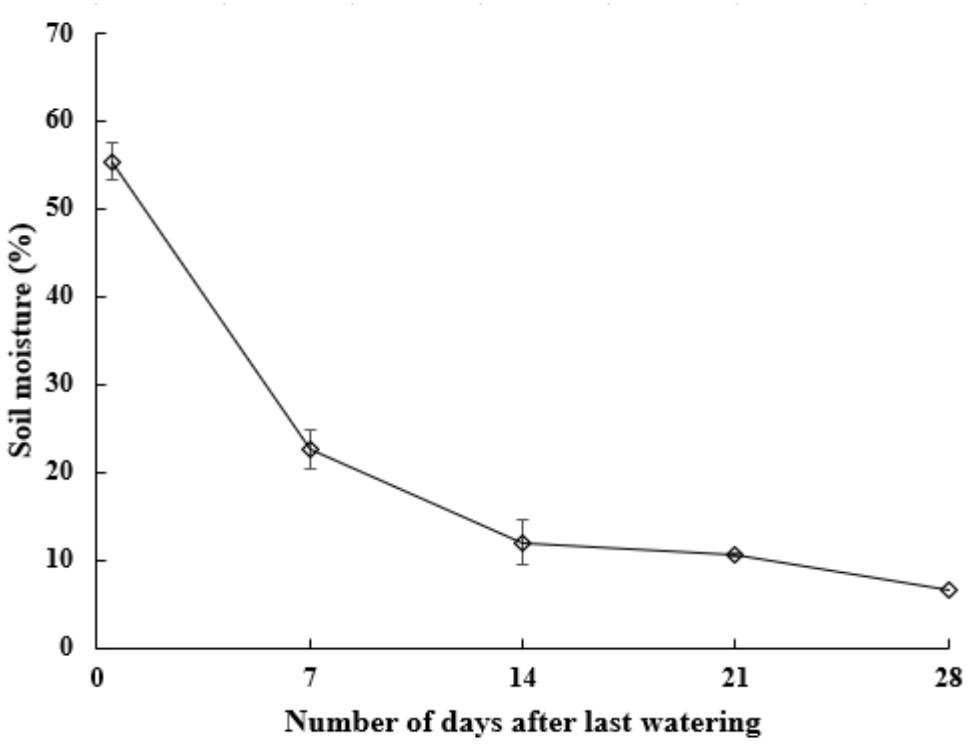

Fig. 2.4. Soil moisture content over time during drought stress.

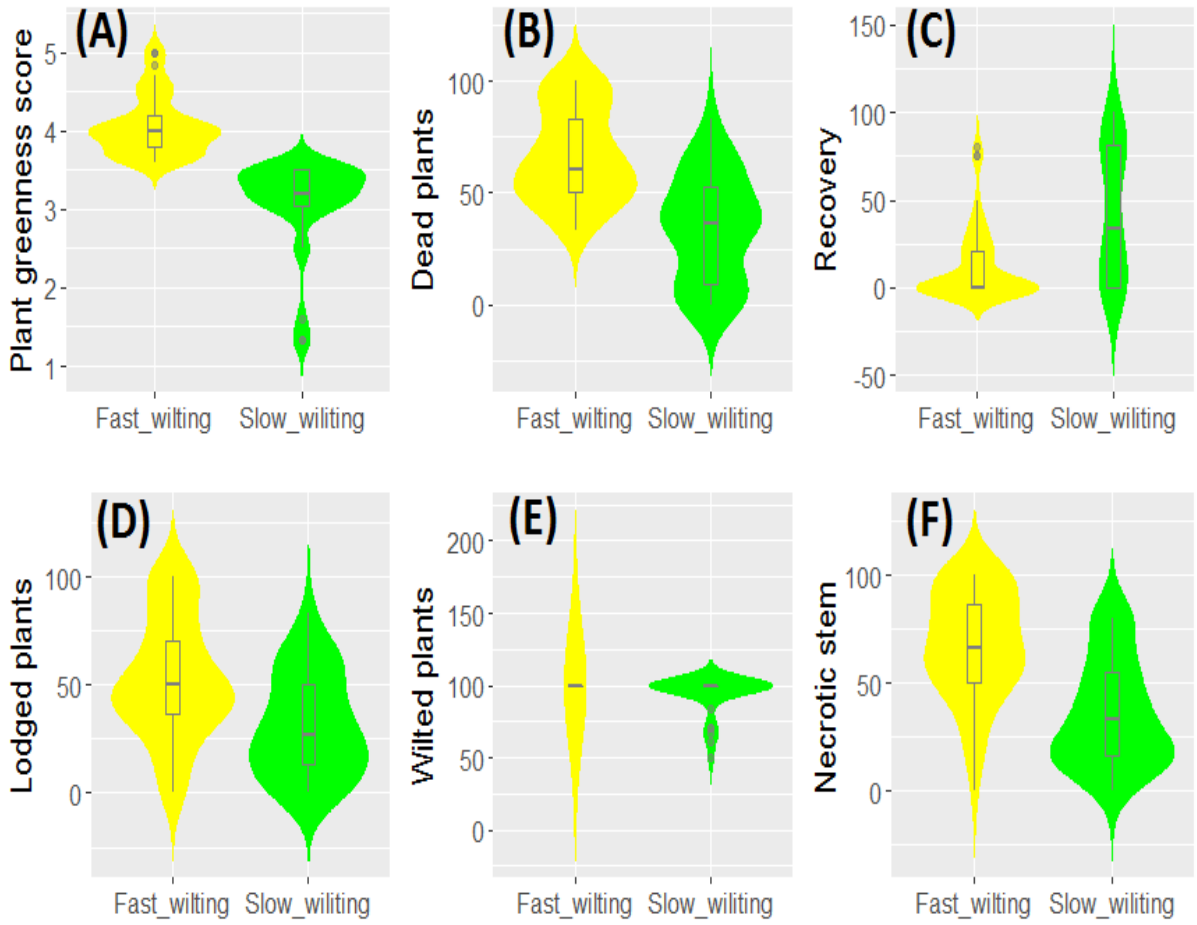

Fig. 2.5. Combined violin and boxplots of the values related to above-ground traits of cowpea under drought stress for 28 days: (A) plant greenness scores, (B) percentage of dead plants, (C) recovery rate after rewatering, (D) percentage of lodged plants, (E) percentage of plants showing wilting sign, and (F) percentage of plants exhibiting necrotic stems.

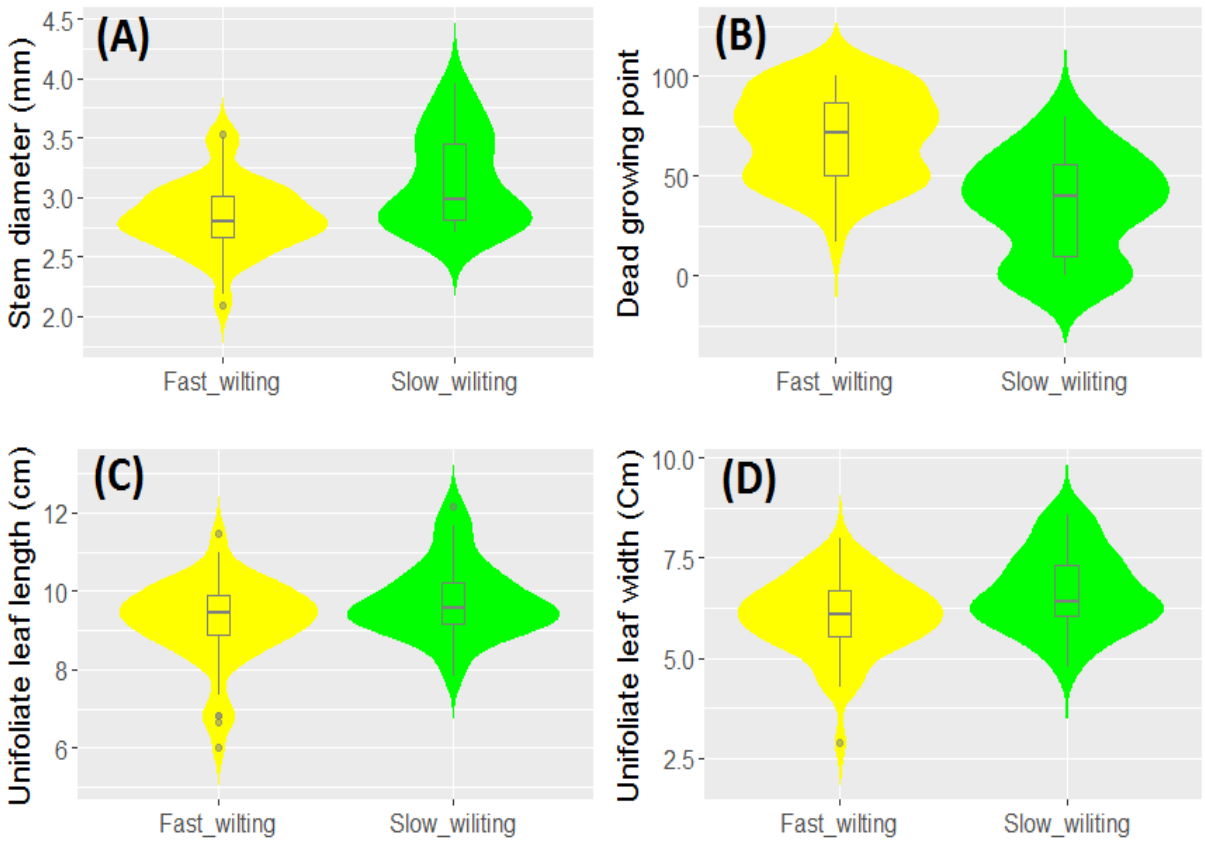

Fig. 2.6. Combined violin and boxplots for (A) stem diameter (mm) recorded at first sign of wilting, (B) percentage of plants showing dead growing point, (C) unifoliate leaf length, and (D) unifoliate leaf width. Percentage of plants having dead growing points was recorded at 28 days of drought stress. Stem diameter was measured at first sign of plant wilting. Unifoliate leaf length and width were recorded before imposing drought stress on cowpea plants.

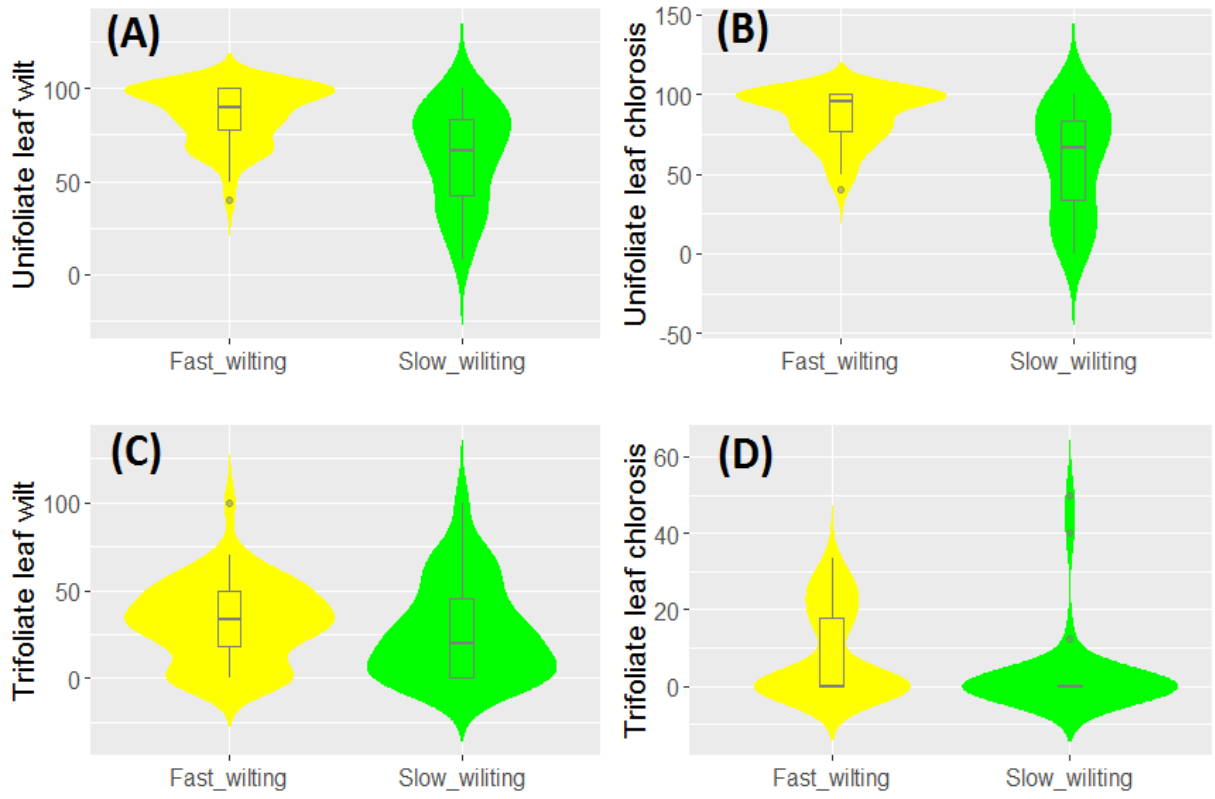

Fig. 2.7. Percentage of plants showing signs of (A) wilting on unifoliate leaves, (B) chlorosis on unifoliate leaves, (C) wilting on trifoliate leaves, and (D) chlorosis on trifoliate leaf. Data were recorded at 28 days of drought stress.

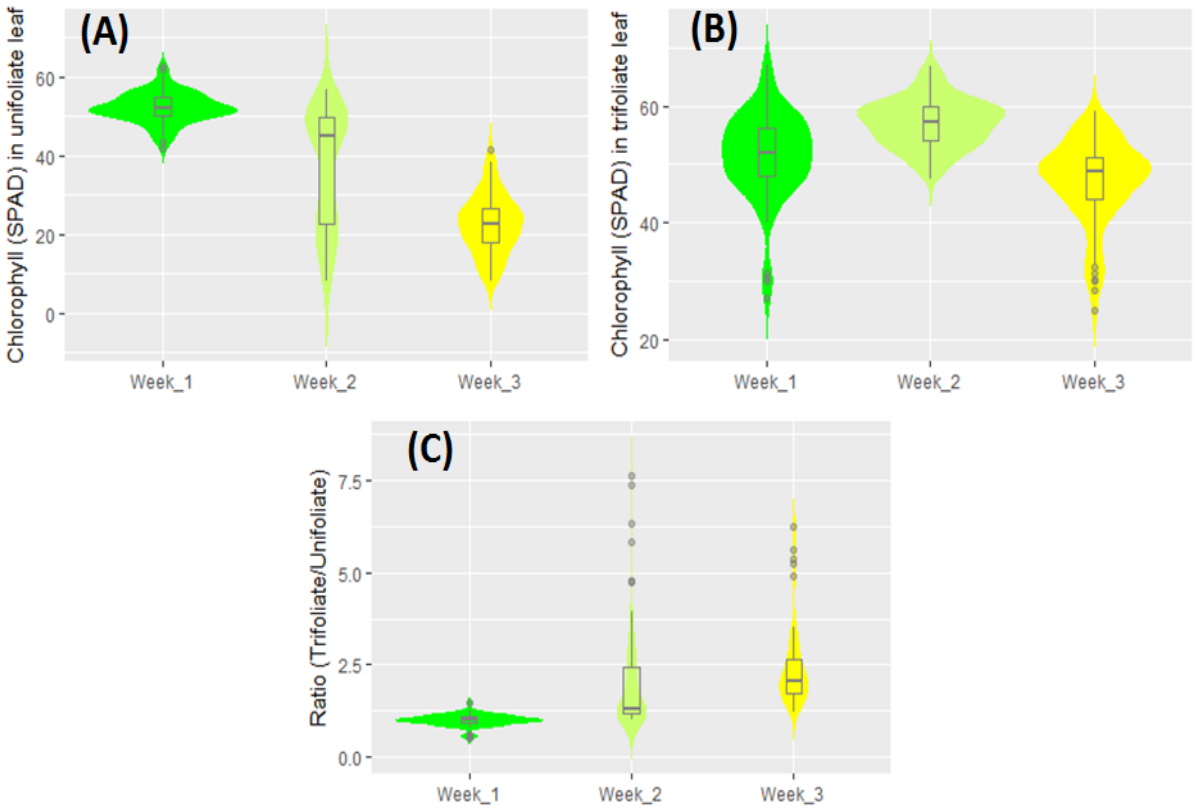

Fig. 2.8. Chlorophyll (SPAD values) in (A) unifoliate leaves and (B) trifoliate leaves over time. Ratio (C) between chlorophyll in unifoliate leaves and trifoliate leaves, respectively. Week1, week2, and week3 corresponded to 7 days, 14 days, and 21 days of drought stress.

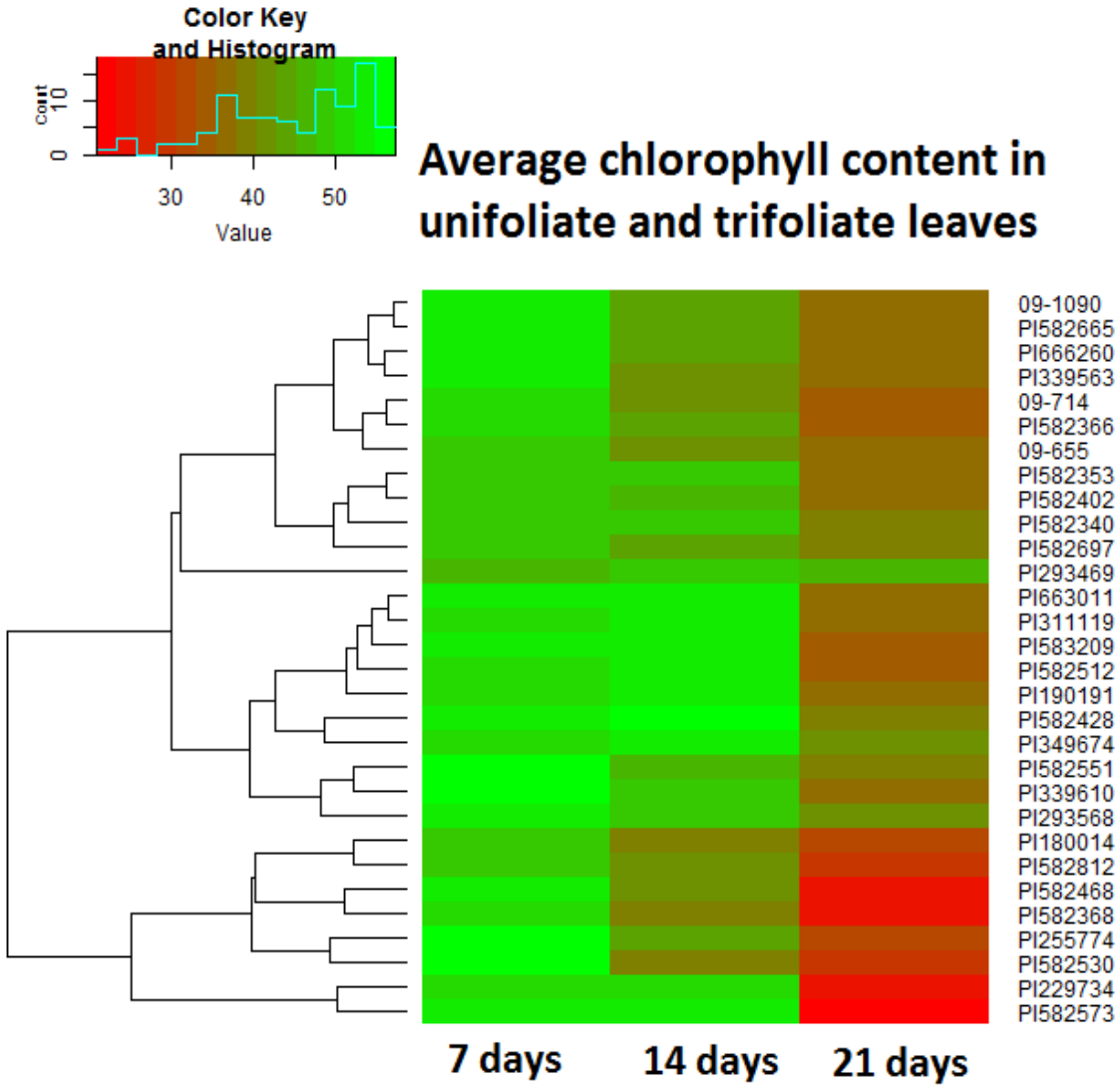

Fig. 2.9. Heatmap of the average chlorophyll content (SPAD) in unifoliate and trifoliate leaves at 7 days, 14 days, and 21 days of drought stress, respectively. Green indicated high chlorophyll content, whereas red indicated low chlorophyll content.

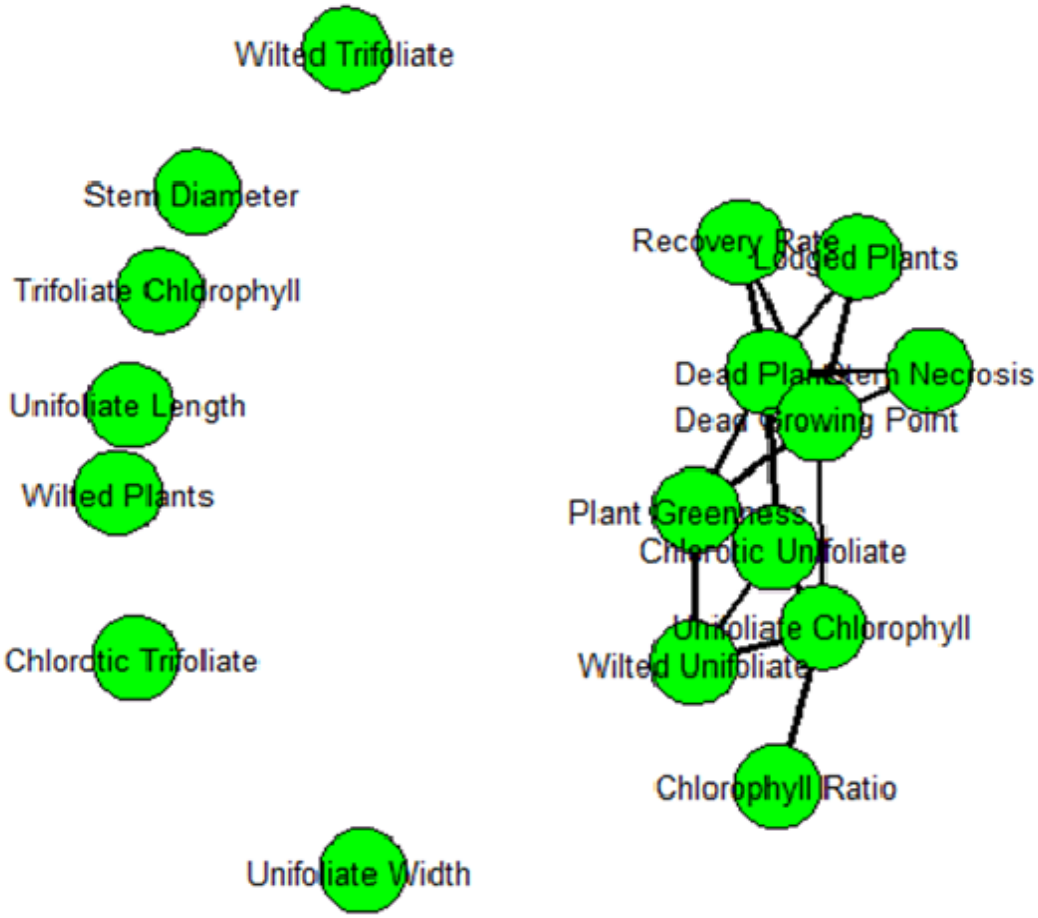

Fig. 2.10. Network analysis between traits evaluated under drought stress in cowpea. Path was shown using solid lines if Person's coefficient value between trait values was greater than 0.65.

Chapter 3. A Simple and Cost-effective Approach for Salt Tolerance Evaluation in Cowpea (*Vigna unguiculata*) Seedlings

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Abstract

Little has been done with respect to breeding for salt-tolerant cowpea cultivars despite of salt stress being a growing threat to cowpea production. Seedling stage is one the most susceptible stages to salt stress in cowpea. Establishing a streamlined methodology for rapidly screening a large number of genotypes will significantly contribute toward enhancing cowpea breeding for salt tolerance. Therefore, the objective of this study was to establish and validate a simple approach for salt tolerance evaluation in cowpea seedlings. A total of 30 genotypes including two controls (PI582468, a salt-tolerant genotype, and PI255774, a salt-sensitive genotype) were greenhouse-grown under 0 mM and 200 mM NaCl. A total of 14 above-ground traits were evaluated. Results revealed: 1) significant differences in average number of dead plants per pot, leaf injury scores, relative salt tolerance for chlorophyll, plant height, and leaf and stem biomass among the 30 genotypes, 2) all PI255774 plants were completely dead, whereas those of PI582438 were fully green after two weeks of salt stress, which validated this methodology, 3) relative salt tolerance for chlorophyll content was highly correlated with number of dead plants and leaf injury scores, 4) relative salt tolerance for leaf biomass was moderately correlated with number of dead plants and leaf injury scores, and 5) relative salt tolerance in plant height was poorly correlated with number of dead plants and leaf injury scores Therefore, less number of dead plants per pot, high chlorophyll content, and less leaf injury scores were good criteria for salt tolerance evaluation in cowpea. This study provided a simple methodology and suggested straightforward criteria to evaluate salt tolerance at seedling stage in cowpea.

Introduction

Cowpea [*Vigna unguicalata* (L.) Walp.] is a diploid legume species (2n=2x=22) widely grown in Africa, Asia, the Middle East, southern Europe, southern and western U.S., and Central and South America. Worldwide cowpea production is estimated to be 5.4 million tons of cowpea grain annually and Africa is the leading producer (Olufajo, 2012). Cowpea is cultivated on more than 14 million hectares (Singh et al., 2003). It provides good quality nutrition to human consumption (Frota et al., 2008). In addition, cowpea can contribute toward protecting soils from being eroded due to the fact that it is an excellent cover crop. In the western part of the U.S., a growing interest in using cowpea as a cover crop has been noticed since cowpea can tolerate drought conditions (Agbicodo et al., 2009). However, increasing concerns due to salinity in this part of the country can limit the use of cowpea as a cover crop (Wilson et al., 2006). In semi-arid regions where cowpea cultivation is predominant, the low rainfall frequency could lead to salt compounds not properly being leached out, hence accumulated within soils and exacerbated salinity-related issues (Zhang et al., 2012).

Salinity is one of the major limiting factors that have been constraining agricultural production globally (Allakhverdiev et al., 2000). In croplands, salinity is due to an undesirable increase in the concentration of cations such as K^+, Mg^{2+}, Ca^{2+}, and Na^+, and anions such as NO_3^-, HCO_3^-, SO_4^{2-}, and Cl^- according to Wallender and Tanji (2011). Salinity due to sodium chloride (NaCl) has been predominant (Ayers and Westcot, 1985), hence tolerance to this type of salt was reported in this current investigation. The estimate of cropland areas facing salinity was over 19.6 million hectares in the U.S. (Shannon, 1997). Costs related to concern imposed by salinity on agriculture were 12 billion U.S. dollars (Läuchli and Lüttge, 2002). Multiple factors

such as rock weathering, deforestation, poor quality of irrigation water, and inadequate fertilization practices can worsen salinity on cultivated lands (Omami and Hammes, 2006).

Studies have shown that salt stress can cause serious concerns to cowpea production. Cowpea germination has been shown to be unfavorably affected by salt stress (Zahedi et al., 2012). Salt-stressed cowpea plants exhibited a reduced plant growth and vigor (Mini et al., 2015). Salt stress can impair plant physiology, photosynthesis, and absolutely important functions such as cell extension and division (Maas and Hoffman, 1977). These aforementioned factors could lead to a significant cowpea yield reduction (Dutta and Bera, 2014). Breeding for cowpea salt-tolerant cultivars is one of the most affordable solutions to tackle these issues. However, few studies have focused on addressing salt stress in cowpea in efforts to adequately providing breeders with critical information on the tolerance of cowpea genotypes to salinity.

Phenotyping is a substantial process in screening genotypes for a particular trait of interest. It is usually a labor-intensive, time-consuming, and a costly task to undertake for plant breeders. The increasing needs for accurate and less expensive phenomics requires the establishment of a fast and cost-effective methodology. To the best of our knowledge, there is no reported methodology on salt tolerance phenotyping in cowpea. Salt phenotyping can be carried in fields. However, the uncontrolled factors such as differences in soil fertility, temperature, and transpiration could increase the unexplained part of the variation in salt tolerance among cowpea genotypes, thus leading to biased conclusions (Pathan et al., 2007). Hydroponic system has long been considered the ideal approach for salt tolerance phenotyping in crops. However, this requires adequate facilities and specialized skills (An et al., 2001), which could significantly increase the phenotyping cost. Since cowpea is predominantly cultivated in developing countries, a methodology that can be applied in these areas where funds and facilities are very limited

would be most helpful. In addition, the screening methodology should allow for a rapid and accurate salt tolerance phenotyping of a large number of genotypes to be efficient. Seedling stage is one of the most vulnerable stages to salt stress in cowpea (Win and Oo, 2015). Suggesting a strategy that can help cowpea breeders select for salt-tolerant genotype at this stage is therefore important and can also assist with at least narrowing down the number of genotypes for salt tolerance screening at a later stage. Therefore, the objective of this study was to establish an approach that can be easily applied for salt tolerance phenotyping for cowpea at seedling stage.

Materials and Methods

Plant materials

A total of 30 cowpea accessions originating from 13 countries was used in this study (Table 3.1). These genotypes were plant introductions (PI) from the United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea germplasm accessions. Cowpea seeds were obtained from the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. Seeds were increased in the summer of 2017 at the University of Arkansas, Fayetteville AR. Of the 30 cowpea genotypes, PI582468 (salt-tolerant) and PI255774 (salt-susceptible) (Ravelombola et al., 2017), were used as control to validate the methodology. At the end of the experiment, the two extreme genotypes from the remaining 28 along with the aforementioned controls were independently repeated from the current investigation to further validate the results.

Growth conditions and experiment design

The experiment was conducted in the greenhouse of Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville, AR (Fig. 3.1). Temperatures in the

greenhouse were 26°C/21°C (day/night) and day light length was 14 hours. Cowpea plants were established in pots previously filled up with 100 g Sunshine® Natural & Organic (Agawam, MA).

Holes were designed at the bottom of each pot, and paper was placed at the bottom of each pot as well to prevent soil medium from leaking during irrigation. In each pot, 6 to 8 seeds were sown. When cowpea plants emerged, 4 vigor and uniform plants were kept. One week after plant emergence, plants were fertilized with an application of a solution of 50 mL of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI) in each pot, and the same fertilizer was weekly applied to all pots until the end of the experiment.

Each genotype was planted in 6 pots. Of which, 3 pots were salt-treated, whereas the remaining 3 pots were irrigated with deionized water. Pots were placed on rectangular plastic trays to facilitate the irrigation. Salt (NaCl) treatment began when the first trifoliate leaf began to expand (V1 stage) (Fehr et al., 1971). Salt concentration was 200 mM NaCl as described previously (Abeer et al., 2015; Ashebir et al., 2013; Paul et al., 2011, Ravelombola et al., 2017). We conducted a preliminary test involving only the two accessions used as control under the aforementioned NaCl concentration and used the current screening methodology, and found that all plants from the salt-tolerant genotype (PI582468) were fully green and that of from the salt-susceptible genotype (PI255774) were completely dead after 14 days of salt stress (Fig. 3.2). Salt concentration was obtained by dissolving a total of 11.7 g of sodium chloride powder of Science Company® (Lakewood, CO) in one liter of deionized water.

Irrigation was performed by supplying either deionized water or salt solution to the plastic trays described above. Irrigation was achieved such that pots were soaked with solution up to two third of pot height. The solution was kept within the plastic trays for 2 hours every day.

The treatment was conducted until the susceptible check (PI255774) was completely dead. This irrigation strategy was key since it assisted cowpea roots with being permanently exposed to salt ions, which could lead to salt stress. In addition, doing so could limit within pot variation due to the differences in soil-root transmission if the rhizosphere was not completely soaked with solution. This irrigation approach has been proven to be efficient in salt tolerance screening in other crops (Ledesma et al., 2016).

The experiment design was completely randomized design (CRD) with three replications per genotype. Factor involved the set of 30 genotypes evaluated for salt tolerance. Genotypes were assumed to have fixed effects.

Measurements

Measurements were taken when the susceptible check was completely dead. Leaf injury was assessed based on a 1-7 scale (Fig. 3.3) (1=healthy plants, 2=first sign of leaf chlorosis, 3=expansion of chlorosis on leaf surface, 4= totally chlorotic leaf, 5=first sign of necrosis, 6=expansion of necrosis on leaf surface, and 7=completely dead plants).

Number of dead plants per pots was counted. Plant height (from the bottom part to growing point) for both non-stressed and salt-stressed plants was measured on per plant basis. Relative salt tolerance (RST) for plant height, described as the ratio between plant height under stress and non-stress conditions, was computed (Saad et al., 2014). Data on fresh leaf biomass under non-stress and stress conditions were collected and relative salt tolerance (RST) for fresh leaf biomass was calculated. Fresh stem biomass under non-stress and stress conditions along with the relative salt tolerance (RST) for fresh stem biomass were assessed as well. Leaf chlorophyll was measured using a chlorophyll SPAD-502 Plus (Spectrum Technologies Inc.,

Plainfield, IL) for non-stressed and salt-stressed plants and relative salt tolerance (RST) for chlorophyll content was computed.

Data analysis

Data were analyzed using PROC MIXED of SAS® v.9.4 (SAS Institute Inc., Cary, NC). Mean separation was done using a protected least square difference (LSD) procedure at $\alpha = 0.05$. LSD procedure was described as $LSD = t_{\alpha/2} \sqrt{2MSError/n}$ where $t_{\alpha/2}$ was a critical value from the t-table with df(SSError)= Number of observations-Number of genotypes, and n= number of replications. Person's correlation coefficients and descriptive statistics were computed using JMP Genomics ®7 (SAS Institute Inc., Cary, NC, USA). Graphs and path analysis were established using the packages 'MASS' and 'Network' of R® 3.1.1.

Results

Number of dead plants

The average number of dead plants per pot was evaluated for each genotyped at 14 days of salt stress. At that time, all plants from the susceptible check, PI255774, were completely dead, whereas those from the tolerant check, PI582468, were fully green (Fig. 3.1). The number of dead plants varied from 0.00 to 4.00 dead plants per pot, with an average of 3.18 dead plants per pot and a standard deviation of 1.20. Distribution of number of dead plants per pot was left-skewed (Fig. 3.4). ANOVA revealed significant differences in number of dead plants among the 30 genotypes (F-value=18.50, p-value<0.0001) (Table 3.2). The genotypes having less than 2 dead plants per pot were PI582468 (0.00), PI349674 (0.00), PI582812 (1.00), PI293469 (1.33), and PI190191 (1.67) (Table 3.3). All plants from the genotypes PI664517, PI664515, PI582852,

PI582573, PI582551, PI582428, PI582402, PI354860, PI354835, PI293586, PI291140, PI255774, and PI229734 were completely or almost dead at 14 days of salt stress.

To further validate the results, the two checks (PI582468 and PI255774) along with PI349674, having all plants being fully green at 14 days of salt stress, and PI582573, showing severe chlorosis at that time, were independently repeated from the previous trial. The results from the repeated experiment were consistent with the previous one as shown in Fig. 3.5. The tolerant control was fully green, whereas the susceptible check was completely dead. In addition, none of the plants from the genotypes PI349674 were dead, whereas those of PI582573 were chlorotic (Fig. 3.5), indicating that this current methodology could provide replicability of salt tolerance or salt susceptibility over time, hence stable and useful for investigating potential major genes affecting salt tolerance in cowpea.

Leaf injury score

Leaf injury was scored based on a 1-7 scale depending on leaf greenness and chlorosis. Leaf injury scores were in the range of 1.33 to 7.00, with an average of 5.66 and a standard deviation of 1.52, indicating a large variation of leaf injury score among the genotypes. Distribution of leaf injury scores was left-skewed (Fig. 3.4). A significant difference in leaf injury scores was found among the 30 genotypes (F-value=30.58, p-value<0.0001) (Table 3.2). Leaf score injury for tolerant control was 1.33, whereas the susceptible scored 7.00, suggesting that this methodology permitted a clear distinction between the two controls. In addition to the tolerant check, PI349674 (1.67), PI582812 (3.33), and PI190191 (3.50) scored the least (Table 3.3), suggesting that these genotypes were salt-tolerant.

Highest leaf injury score was recorded for the genotypes PI291140 (6.50), PI582368 (6.50), PI582863 (6.50), PI293586 (6.60), PI354865 (6.67), PI664515 (6.67), PI292898 (6.77),

PI664517 (6.83), PI582428 (7.00), PI582573 (7.00), PI582852 (7.00) (Table 3.3), which suggested that these genotypes were susceptible to salt stress. Leaf scoring was consistent in the repeated trials involving the controls along with PI349674 and PI582573, indicating that the methodology was stable.

Chlorophyll (SPAD)

Chlorophyll (SPAD) was assessed in both non-salt-treated and salt-stressed cowpea plants, and relative salt tolerance for chlorophyll content (SPAD) was calculated. Chlorophyll content of plants without salt stress was higher than those under salt stress at 14 days of salt stress, indicating that salt stress significantly affected leaf chlorophyll (Fig. 3.6). Distributions of chlorophyll content in leaves of salt-stressed and non-stress plants, and relative salt tolerance were approximately normally distributed (Fig. 3.6). For the salt-stressed plants, chlorophyll content varied from 2.00 to 26.07, with a mean of 13.07 and a standard deviation of 5.53, at 14 days of salt stress. Significant difference in chlorophyll content was found (F-value=9.27, p-value<0.0001) (Table 3.2). Chlorophyll content (SPAD) of the tolerant check (PI582468) was 26.07, whereas that of the susceptible check was 5.83 (Table 3.3). The well performing genotypes under salt stress in addition to the tolerant check were PI349674 (24.10), PI582812 (21.60), PI293469 (19.43), PI664524 (18.70), and PI190191 (18.43) (Table 3.3), indicating that these genotypes were tolerant to salt stress. The least performers in terms of chlorophyll content besides the susceptible check were PI354835 (9.90), PI293586 (9.77), PI582368 (9.73), PI664517 (9.17), PI292898 (8.67), PI582852 (8.47), PI582573 (4.30), and PI582428 (2.00) (Table 3.3).

Relative salt tolerance for chlorophyll content was the ratio between chlorophyll content of salt-stressed and non-stressed plants. The higher the relative salt tolerance was, the more salt-

tolerant the genotype was. Relative salt tolerance for chlorophyll content ranged from 0.08 to 0.97, with an average of 0.47 and a standard deviation of 0.19. Significant differences in relative salt tolerance among the genotypes were found (F-value=7.62, p-value<0.0001) (Table 3.2). The tolerant check had a relative salt tolerance value of 0.97, whereas the susceptible check had a relative salt value of 0.21. The most salt-tolerant genotypes based on relative salt tolerance for chlorophyll content in addition to the tolerant check were PI349674 (0.75), PI293469 (0.75), PI664524 (0.67), and PI582812 (0.67) (Table 3.3). Those having the lowest relative salt tolerance value besides the susceptible check were PI292898 (0.34), PI664515 (0.33), PI664517 (0.33), PI582852 (0.31), PI582573 (0.16), and PI582428 (0.08) (Table 3.3).

Plant height

Plant height of salt-stressed and non-stressed plants was measured at 14 days of salt stress when the susceptible check was completely dead. Salt stress significantly reduced plant height (Fig. 3.1). Plant height of non-stressed plants varied from 10.43 to 20.00 cm, with an average of 14.70 cm and a standard deviation of 2.70 cm. That of stressed plants ranged between 5.87 to 11.80 cm, with a mean of 8.18 cm and a standard deviation of 1.42 cm. Plant height under both conditions was approximately normally distributed (Fig. 3.7).

Significant differences in plant height without salt-stress (F-value=27.19, p-value<0.0001) and under salt stress (F-value=11.08, p-value<0.0001) (Table 3.2) were identified. Under salt treatment, the tallest genotypes were PI664524 (11.80 cm), PI582353 (10.50 cm), PI582551 (10.30 cm), PI664517 (9.73 cm), PI354865 (9.60 cm), PI582352 (9.57 cm), and PI293469 (9.47 cm), whereas the shortest ones were PI354860 (6.93 cm), PI582428 (6.83 cm), PI354832 (6.80 cm), PI582573 (6.00 cm), PI582366 (5.93 cm), and PI582812 (5.87 cm) (Table 3.3).

Relative salt tolerance was the ratio between plant height under salt stress conditions and plant height without salt stress. Relative salt tolerance for plant height varied from 0.37 to 0.70, with an average of 0.59 and a standard deviation of 0.07. Relative salt tolerance was significantly different among the genotypes (F-value=4.01, p-value<0.0001) (Table 3.2). Interestingly, relative salt tolerance for PI582468 (0.49) (tolerant control) was less the PI255774 (0.59) (susceptible control), suggesting that relative salt tolerance for plant height could not be accurately evaluated using the current methodology.

Fresh leaf biomass weight

Leaf biomass was measured when the susceptible check was completely dead. Distribution leaf biomass of plants without salt stress was approximately normally distributed, whereas that of salt-stressed plants was right-skewed (Fig. 3.8).

Under non-stress conditions, average leaf biomass per plant ranged from 1.51 g to 4.69 g, with an average of 2.55 g and a standard deviation of 0.67 g. Under salt treatment, leaf biomass varied between 0.15 and 1.39 g, with an average of 0.77 g and a standard deviation of 0.40g. In addition, correlation analysis showed week correlation (r=0.15) between leaf biomass under salt stress and non-stress conditions, indicating that the observed variation in leaf biomass under salt stress among the genotypes was more likely to be associated with a genetic response specific to the genotype rather than being correlated with an adaptation due to plant morphology. ANOVA showed significant differences in leaf biomass under salt stress among the genotypes (F-value=0.47, p-value<0.0001) (Table 3.2).

Genotypes having the heaviest leaf biomass under salt conditions were PI664524 (1.50 g), PI582551 (1.39 g), PI349674 (1.38 g), PI582352 (1.30 g), PI293469 (1.25 g), and PI582468 (1.18 g) (Table 3.4). Those having the lightest leaf biomass under salt stress were PI582368

(0.36 g), PI354865 (0.35 g), PI582428 (0.30 g), PI664515 (0.24 g), PI582573 (0.24 g), PI229734 (0.24 g), and PI255774 (0.15 g) (Table 3.4). Relative salt tolerance for leaf biomass had a right-skewed distribution (Fig. 3.8). Leaf biomass relative salt tolerance varied from 0.05 to 0.71, with a mean of 0.32 and a standard deviation of 0.18. Relative salt tolerance for leaf biomass was statistically significantly different among the genotypes (F-value=5.64, p-value<0.0001). Genotypes having the highest relative salt tolerance for leaf biomass were PI293469 (0.71), PI582551 (0.65), PI349674 (0.60), PI354864 (0.54), and PI354860 (0.51) (Table 3.4). The lowest relative salt tolerance for leaf biomass was recorded for PI229734 (0.10), PI582428 (0.09), PI255774 (0.08), and PI664515 (0.05) (Table 3.4).

Fresh stem biomass weight

Fresh stem biomass of plants under salt stress and without salt treatment was recorded on a per plant basis at 14 days of salt stress. At that time, the susceptible check was completely dead. Stem biomass of salt-treated plants was lower than plants without being salt-treated (Fig. 3.9). Stem biomass was nearly normally distributed for plants without salt stress, whereas distribution was right-skewed for stem biomass of salt-stressed plants (Fig. 3.9). Stem biomass per plant varied from 0.86 to 2.53 g, with an average of 1.64 g and a standard deviation of 0.46 under non-stress conditions. Under salt treatment, stem biomass was in the range of 0.36 and 1.19 g, with a mean of 0.71 g and a standard deviation of 0.25. Stem biomass was significantly different among the genotypes under salt stress (F-value=16.88, p-value<0.0001) and without salt stress (F-value=15.36, p-value<0.0001) (Table 3.2).

Relative salt tolerance for stem biomass varied from 0.18 to 0.68, with a mean of 0.45 and a standard deviation of 0.13. Values of relative salt tolerance were approximately normally distributed (Fig. 3.9). Relative salt tolerance for stem biomass was significantly different among

the genotypes (F-value=5.13, p-value<0.0001) (Table 3.2). Genotypes having the highest relative salt tolerance for stem biomass were PI582551 (0.68), PI354865 (0.68), PI293586 (0.64), PI354835 (0.61), PI582368 (0.59), PI664524 (0.59), and PI354860 (0.58) (Table 3.4). Lowest relative salt tolerance was recorded for PI582468 (0.38), PI349674 (0.37), PI229734 (0.35), PI582366 (0.34), PI582812 (0.30), PI291140 (0.25), PI582428 (0.22), and PI664515 (0.18) (Table 3.4). Similar to plant height, none of the two controls were grouped into these extreme genotypes, indicating that stem biomass was not a good indicator for salt tolerance under this methodology.

Network analysis between traits and correlation analysis

Network analysis revealed existing pathways between number of dead plants, leaf injury scores, relative salt tolerance for chlorophyll content, chlorophyll content under salt stress, relative salt tolerance for leaf biomass, and leaf biomass under salt stress (Fig. 3.10). Pearson's correlation coefficients between number of dead plants and leaf injury score, number of dead plant and chlorophyll content under salt stress, and number of dead plants and relative salt tolerance for chlorophyll were 0.91, -0.81, and -0.77 (Table 3.5), respectively, indicating that salt stress caused sever leaf chlorosis, which resulted in leaf tissue damage and reduction in leaf matter, thus plant death. Another pathway defined by plant height under salt stress, plant height without salt stress, stem biomass under salt stress, and leaf biomass under salt stress was identified (Fig. 3.10).

All parameters within the second network were related to non-stressed plants except for stem biomass and plant height, suggesting that phenotypic values obtained using these parameters were likely associated with plant morphology rather that response to salt tolerance. Since the second pathway was independent from the first one (Fig. 3.10), there was almost no

correlation between the network defined by number of dead plants, leaf injury scores, relative salt tolerance for chlorophyll content, chlorophyll content under salt stress, relative salt tolerance for leaf biomass, and leaf biomass under salt stress, and that of plant height under salt stress, plant height without salt stress, stem biomass under salt stress, and leaf biomass under salt stress.

Discussion

Salt stress has been increasingly threating crop production globally (Flowers, 2004). Salinity affects more than 830 million hectares of croplands worldwide (Chaitanya et al., 2014). Shannon (1997) estimated a total of 1 to 60 metric tons of salt compound being annually added to cultivated areas, which has made salinity a growing concern to agriculture. The effects of salinity has been found to be more severe is semi-arid regions where cowpea is widely grown (Zhang et al., 2012). Providing farmers with genotypes which better tolerate salt conditions would be the most affordable way to limit the negative effects of salinity on crop production. Establishing a straightforward phenotyping strategy to select for salt-tolerant genotype will significantly help cowpea breeders to do so.

Since cowpea cultivation is predominant in developing countries where there is a limited access to funding opportunities and facilities to set up hydroponic system to screen for salt tolerance in cowpea at seedling stage, providing cowpea scientists working in these areas with an easy-to-implement and cost-effective approach would help in enhancing breeding programs aiming at releasing salt-tolerant cowpea cultivars. In this current investigation, we developed a rapid screening methodology that can be followed and used by cowpea breeders when phenotyping for salt tolerance.

This research has been conducted in a controlled condition in order to limit potential effects of uncontrolled factors such as differences in soil fertility, transpiration, and root-soil transmission that commonly occurred in field phenotyping (Pathan et al., 2007).Therefore, cowpea breeders can rapidly replicate promising investigations identifying good genotypes prior to conducting a field phenotyping with a fewer number of genotypes to screen, which could significantly limit the unexplained variation due to field conditions as previously stated. In addition, we have established easy-to-track phenotyping traits such as leaf score injury and leaf biomass for assessing salt tolerance, which does not require substantial costs to record, hence can be easily scaled up.

The current methodology has been validated by the use of two checks, PI582468 (salt-tolerant) and PI255774 (salt-sensitive), as previously reported (Ravelombola et al., 2017). Substantial discrepancy in above-ground traits between these genotypes was found even at 10 days of salt stress, suggesting that this approach can help differentiate a salt-tolerant genotype from a salt-sensitive one.

Replicating is a critical part of applied sciences and data from investigations that fail to be replicated cannot be used for further experiments in general. Therefore, to further validate our results, the two checks along with the two contrasting genotypes were repeated. Similar results from the previous screening were obtained in the replicated trial. The two salt-tolerant genotypes were fully green, whereas the two sensitive ones were almost dead at 11 days of salt stress as shown in Fig. 3.4, which further validated the methodology and the data from this investigation.

A total of 14 above-ground parameters was evaluated in this study. Mini et al. (2015) reported a high correlation between accumulation of salt ions and chlorophyll content in leaves of salt-stressed cowpea plants. Therefore, we suggested that chlorophyll content is a good

indicator of salt tolerance in cowpea. Since analyzing ion contents within salt-stressed cowpea leaves and roots could be expensive, measuring chlorophyll content could give a good approximation of salt tolerance. In fact, our results suggested that the two controls (PI582468 and PI255774) were significantly contrasting in terms of chlorophyll content under salt stress. In addition, Murillo-Amador et al. (2002) reported that ion exchange mechanisms payed an imported role in conferring salt tolerance in cowpea. Praxedes et al. (2010) stated that salt-sensitive cowpea plants were not able to limit the uptake of $Na+$ and Cl^- under salt stress, which substantially lowered the chlorophyll content in the salt-sensitive genotypes as reported in this investigation.

A scoring-based scale for salt leaf injury (1=green plant and 7=completely dead plant) was established to help cowpea scientists quantify the stay-green phenomenon under a prolonged period of salt stress. Establishing a straightforward scoring for salt injury has been proved to allow for a rapid screening for salt tolerance in other crops such as soybean (*Glycine max* (L.) Merr.) (Ledesma et al., 2016). In addition, path analysis from this investigation revealed significant correlations between number of dead plants, leaf injury scores, chlorophyll content of salt-stressed cowpea plants, and leaf biomass of salt stressed cowpea plants. Therefore, leaf injury score could be also used as a good indicator for salt tolerance in cowpea.

The current methodology also allowed for clear distinction between the salt-tolerant genotype from the salt-sensitive one based on fresh leaf biomass weight under salt stress. El-Mashad and Mohamed (2012) reported that cowpea plants which were able to keep cell constituents from being extensively damaged by oxidative reaction occurring in leaf cells under salt stress were likely to withstand the stress, whereas those failing to prevent extensive lipid peroxidation occurring in leaves were highly susceptible to salt stress (Cavalcanti et al., 2004).

This research aimed at providing a streamlined protocol for salt tolerance phenotyping, which will have practical applications for cowpea breeding. The cowpea genotypes used as controls in this investigation can be freely accessed through the USDA GRIN website (https://npgsweb.ars-grin.gov/gringlobal/search.aspx) and available for orders, and can be used for further references when selecting for salt-tolerant genotypes. Since the controls were freely available to everyone, we can expect that the present protocol can be used by other cowpea scientists contributing towards unraveling the genetics of salt tolerance in cowpea.

Conclusions

Phenotyping is one of the most challenging tasks in plant breeding. Being provided with a fast and accurate phenotyping strategy will allow for enhanced salt tolerance phenomics-related investigations, which is common in modern breeding. In this study, we developed a simple and cost-effective salt tolerance methodology in cowpea, which is not yet available despite of being important, to the best of our knowledge.

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Tables

Table 3.1. List of 30 cowpea accessions including two accessions (PI255774 and PI582468) used as control.

Accession	Plant name[x]	Origin[y]
PI190191	TVu 1557	Mexico
PI229734	CHESH BOLBOLI LUBI	Iran
PI255774†	TVu 2428	Nigeria
PI291140	NEGRO	Australia
PI292898	TVu 1890	Hungary
PI293469	BROWN CROWDER	United States
PI293586	WILT RESISTANT BLACKEYE	NA[z]
PI349674	ALOOMBA	Australia
PI354832	P 1350	India
PI354835	P 1353	India
PI354860	P 1387	India
PI354864	P 1392	India
PI354865	P 1393	India
PI582352	UCR 154	Saudi Arabia
PI582353	UCR 155	Saudi Arabia
PI582366	UCR 191	India
PI582368	UCR 193	India
PI582402	PITIUBA	Brazil
PI582428	LAURA B	Trinidad and Tobago
PI582468†	UCR 347	NA§
PI582551	UCR 1004	Botswana
PI582573	KVu 23	Kenya
PI582697	UCR 1176	Botswana
PI582812	UCR 794	Botswana
PI582852	UCR 935	Botswana
PI582863	UCR 1017	Botswana
PI583232	UCR 3317	Senegal
PI664515	Bettergreen	United States
PI664517	Bettergro Blackeye	United States
PI664524	Green Dixie Blackeye	United States

[x]PI255774 is a salt-sensitive genotype, whereas PI582468 is a salt-tolerant one as previously reported (Ravelombola et al., 2017). These genotypes were used to validate the methodology.

[y]Plant name and country of origin were based on the information found at https://npgsweb.ars-grin.gov/gringlobal/descriptors.aspx?

[z]Information was not available.

Table 3.2. ANOVA table for traits evaluated for salt tolerance phenotyping at seedling stage.

Phenotype[a]	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Dead plants	Accession	29	125.16	4.32	18.50	<.0001
	Error	60	14.00	0.23		
Leaf injury score	Accession	29	201.61	6.95	30.58	<.0001
	Error	60	13.64	0.23		
Chlorophyll_NonStress	Accession	29	786.68	27.13	4.81	<.0001
	Error	60	338.59	5.64		
Chlorophyll_Stress	Accession	29	2659.46	91.71	9.27	<.0001
	Error	60	593.45	9.89		
Chlorophyll_RST[b]	Accession	29	3.15	0.11	7.62	<.0001
	Error	60	0.86	0.01		
Height_NonStress	Accession	29	636.48	21.95	27.19	<.0001
	Error	60	48.43	0.81		
Height_Stress	Accession	29	174.36	6.01	11.08	<.0001
	Error	60	32.56	0.54		
Height_RST	Accession	29	0.39	0.01	4.01	<.0001
	Error	60	0.20	0.003		
LeafBiomass_NonStress	Accession	29	38.92	1.34	11.99	<.0001
	Error	60	6.71	0.11		
LeafBiomass_Stress	Accession	29	13.60	0.47	8.74	<.0001
	Error	60	3.22	0.05		
LeafBiomass_RST	Accession	29	2.79	0.10	5.64	<.0001
	Error	60	1.02	0.02		
StemBiomass_NonStress	Accession	29	18.58	0.64	15.36	<.0001
	Error	60	2.50	0.04		
StemBiomass_Stress	Accession	29	5.36	0.18	16.88	<.0001
	Error	60	0.66	0.01		
StemBiomass_RST	Accession	29	1.42	0.05	5.13	<.0001
	Error	60	0.57	0.01		

[a]Phenotypes were collected at 14 days of salt stress on a per plant basis. The susceptible check was completely dead at 14 days of salt stress.
[b]RST (Relative Salt Tolerance) was the ratio between the phenotypic values under salt stress and without salt stress.

Table 3.3. LS Means of average number of dead plants per pot, leaf injury scores, chlorophyll content under non-salt conditions, chlorophyll content under salt stress, relative salt tolerance for chlorophyll content, plant height under non-salt conditions, plant height under salt stress, and relative salt tolerance for plant height.

	Average number of dead plants per pot			Leaf injury score			Chlorophyll_Non_Stress (SPAD value)			Chlorophyll_Stress (SPAD value)		
Accession	**Mean**	**SD[1]**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
PI190191	1.67	0.58	d[3]	3.50	0.50	ij	29.37	0.64	cde	18.43	2.30	cde
PI229734	4.00	0.00	a	6.43	0.31	abcd	26.37	3.30	defgh	10.17	0.76	hijkl
PI255774	4.00	0.00	a	7.00	0.00	a	27.90	0.17	defg	5.83	0.49	lmn
PI291140	4.00	0.00	a	6.50	0.50	abc	26.87	0.78	defgh	10.83	3.24	hijkl
PI292898	3.67	0.58	ab	6.77	0.40	abc	25.50	2.78	efgh	8.67	1.93	jklm
PI293469	1.33	0.58	d	4.17	1.04	hi	25.93	2.46	defgh	19.43	5.72	bcd
PI293586	4.00	0.00	a	6.60	0.36	abc	24.93	0.81	fgh	9.77	1.27	ijkl
PI349674	0.00	0.00	e	1.67	0.35	k	32.50	3.92	bc	24.10	2.26	ab
PI354832	3.67	0.58	ab	5.60	0.36	efg	27.27	2.72	defg	11.37	3.07	ghijk
PI354835	4.00	0.00	a	6.43	0.12	abcd	26.63	1.56	defgh	9.90	0.50	ijkl
PI354860	4.00	0.00	a	6.00	0.00	cdefg	27.07	3.35	defg	11.60	2.29	ghijk
PI354864	3.67	0.58	ab	6.17	0.76	bcdef	28.13	0.87	def	15.20	6.66	defgh
PI354865	3.67	0.58	ab	6.67	0.58	abc	25.60	4.51	defgh	10.80	4.57	hijkl
PI582352	2.67	0.58	c	4.50	0.50	h	27.50	1.73	defg	16.37	1.31	defg
PI582353	3.00	1.00	bc	4.50	0.50	h	28.20	2.79	def	17.43	4.51	cdef
PI582366	3.67	0.58	ab	6.00	0.00	cdefg	34.07	0.81	ab	12.30	1.76	fghijk
PI582368	3.67	0.58	ab	6.50	0.87	abc	23.17	1.00	h	9.73	4.97	ijkl
PI582402	4.00	0.00	a	6.33	0.58	abcde	24.73	0.71	fgh	12.33	2.74	fghijk
PI582428	4.00	0.00	a	7.00	0.00	a	25.73	4.64	defgh	2.00	0.82	n
PI582468	0.00	0.00	e	1.33	0.29	k	27.07	4.01	defg	26.07	0.92	a
PI582551	4.00	0.00	a	5.67	0.29	defg	29.40	1.71	cd	16.40	3.02	defg
PI582573	4.00	0.00	a	7.00	0.00	a	25.13	2.21	fgh	4.30	2.76	mn
PI582697	3.33	0.58	abc	6.33	0.29	abcde	26.73	1.50	defgh	10.23	1.93	hijkl
PI582812	1.00	0.00	d	3.33	0.29	j	32.40	0.79	bc	21.60	2.42	abc
PI582852	4.00	0.00	a	7.00	0.00	a	27.20	0.26	defg	8.47	3.31	klm
PI582863	3.00	1.00	bc	6.50	0.87	abc	25.03	2.21	fgh	13.70	4.85	efghij
PI583232	2.67	0.58	c	5.50	0.50	fg	24.20	3.39	gh	14.83	3.93	defghi
PI664515	4.00	0.00	a	6.67	0.58	abc	36.97	1.17	a	12.30	1.56	fghijk
PI664517	4.00	0.00	a	6.83	0.29	ab	27.50	1.51	defg	9.17	3.96	jklm
PI664524	2.67	1.15	c	5.33	0.58	g	27.87	2.00	defg	18.70	1.90	cde

	Chlorophyll_RST[2]			Height_Non_Stress (cm)			Height_Stress (cm)			Height_RST		
Accession	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
PI190191	0.63	0.08	bcde	14.27	1.36	defg	7.33	0.76	ijk	0.51	0.01	ijk
PI229734	0.39	0.04	ghijk	13.53	1.55	fgh	7.87	0.47	hijk	0.59	0.04	bcdefghij
PI255774	0.21	0.02	klm	13.57	0.55	fgh	7.97	0.45	ghijk	0.59	0.01	bcdefghij
PI291140	0.40	0.11	ghijk	12.03	1.07	ij	7.47	0.60	hijk	0.62	0.07	abcdef
PI292898	0.34	0.05	ijkl	13.87	0.72	efgh	7.53	0.55	hijk	0.54	0.02	defghijk
PI293469	0.75	0.18	bc	15.53	0.91	bcd	9.47	0.60	bcde	0.61	0.05	abcdefgh
PI293586	0.39	0.04	ghijk	19.37	0.35	a	9.13	0.81	cdefg	0.47	0.05	k
PI349674	0.75	0.14	b	16.37	1.72	bc	8.63	0.55	defgh	0.53	0.07	fghijk
PI354832	0.42	0.10	fghij	13.73	1.54	fgh	6.80	0.61	klm	0.50	0.09	jk

Table 3.3. (Cont.)

	Chlorophyll_RST[2]			Height_Non_Stress (cm)			Height_Stress (cm)			Height_RST		
Accession	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
PI354835	0.37	0.02	ghijk	16.03	0.38	bc	8.60	0.40	defgh	0.54	0.02	efghijk
PI354860	0.44	0.12	efghij	12.87	1.00	ghi	6.93	0.74	jklm	0.54	0.07	defghijk
PI354864	0.53	0.22	defghi	10.87	0.55	jk	7.13	0.83	ijkl	0.66	0.11	ab
PI354865	0.41	0.12	fghij	13.60	0.79	fgh	9.60	0.46	bcd	0.70	0.02	a
PI582352	0.60	0.08	bcdef	18.57	0.93	a	9.57	0.38	bcd	0.52	0.03	hijk
PI582353	0.62	0.18	bcde	20.00	0.36	a	10.50	0.95	b	0.53	0.06	ghijk
PI582366	0.36	0.05	hijk	10.57	0.47	jk	5.93	0.49	lm	0.56	0.07	cdefghijk
PI582368	0.42	0.20	fghij	12.67	0.95	hi	8.27	0.31	efghi	0.65	0.03	abc
PI582402	0.50	0.11	defghij	16.50	0.20	b	9.27	0.60	cdef	0.56	0.03	cdefghijk
PI582428	0.08	0.04	m	18.70	1.20	a	6.83	0.74	klm	0.37	0.05	l
PI582468	0.97	0.12	a	15.67	0.74	bcd	7.67	1.36	hijk	0.49	0.07	k
PI582551	0.56	0.08	cdefg	16.37	0.83	bc	10.30	0.82	bc	0.63	0.04	abcde
PI582573	0.16	0.10	lm	10.57	0.31	jk	6.00	0.70	lm	0.57	0.08	bcdefghijk
PI582697	0.38	0.08	ghijk	13.57	0.57	fgh	8.10	0.95	fghij	0.60	0.08	bcdefghi
PI582812	0.67	0.09	bcd	10.43	0.85	k	5.87	0.75	m	0.57	0.10	bcdefghijk
PI582852	0.31	0.12	jkl	14.97	0.70	cdef	7.83	1.40	hijk	0.52	0.08	ghijk
PI582863	0.55	0.18	defgh	12.73	1.16	hi	7.83	0.80	hijk	0.62	0.03	abcdefg
PI583232	0.62	0.22	bcde	16.43	0.95	bc	8.20	0.82	fghi	0.50	0.07	jk
PI664515	0.33	0.04	jkl	13.17	0.25	ghi	7.27	0.23	ijk	0.55	0.01	defghijk
PI664517	0.33	0.12	jkl	15.30	0.20	bcde	9.73	1.10	bcd	0.63	0.07	abcd
PI664524	0.67	0.09	bcd	19.27	0.91	a	11.80	0.10	a	0.61	0.04	abcdefg

[1]SD represents the standard deviation.
[2]RST (Relative Salt Tolerance) was the ratio between the phenotypic values under salt stress and without salt stress.
[3]Means followed by the same letter are not significantly different at α=0.05.

Table 3.4. LS Means of leaf biomass under non-salt conditions, leaf biomass under salt stress, relative salt tolerance for leaf biomass, stem biomass under non-salt conditions, stem biomass under salt stress, and relative salt tolerance for stem biomass.

	Leaf biomass_Non_Stress (g)			Leaf biomass_Stress (g)			Leaf biomass_RST			Stem biomass_Non_Stress (g)			Stem biomass_Stress (g)			Stem biomass_RST (g)		
Accession	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
PI190191	2.33	0.24	ghijk	1.04	0.31	bcdefg	0.45	0.17	bcde	1.54	0.20	ijkl	0.66	0.10	ghijk	0.43	0.03	l
PI229734	2.28	0.11	hijkl	0.24	0.09	no	0.10	0.04	hi	1.51	0.02	ijkl	0.53	0.04	ijklm	0.35	0.03	kl
PI255774	1.77	0.16	lm	0.15	0.03	o	0.08	0.03	hi	1.29	0.34	klm	0.52	0.14	jklmn	0.40	0.06	jkl
PI291140	2.49	0.23	fghij	0.83	0.13	efghij	0.34	0.04	cdefg	1.49	0.11	jkl	0.38	0.03	mn	0.25	0.03	ijkl
PI292898	2.63	0.18	efghi	0.56	0.12	ijklmn	0.22	0.06	fghi	1.40	0.28	jklm	0.76	0.15	fg	0.55	0.11	hijk
PI293469	1.78	0.11	lm	1.25	0.40	abcd	0.71	0.22	a	1.31	0.05	klm	0.66	0.04	ghijk	0.51	0.05	ghijk
PI293586	2.53	0.32	fghij	0.84	0.32	efghij	0.35	0.16	cdefg	1.89	0.21	efgh	1.19	0.08	ab	0.64	0.10	ghijk
PI349674	2.33	0.35	ghijk	1.38	0.25	ab	0.60	0.13	ab	1.97	0.10	defg	0.73	0.07	fgh	0.37	0.04	ghijk
PI354832	3.37	0.54	bc	0.88	0.20	defghij	0.26	0.04	efghi	1.84	0.21	fghi	0.71	0.08	fgh	0.40	0.08	ghij
PI354835	1.51	0.36	m	0.51	0.06	jklmno	0.35	0.09	cdefg	1.24	0.19	lm	0.74	0.09	fg	0.61	0.18	fghij
PI354860	2.11	0.30	ijkl	1.09	0.37	bcdef	0.51	0.13	abc	1.24	0.25	lm	0.69	0.07	ghij	0.58	0.18	fghij
PI354864	2.57	0.91	fghij	1.10	0.53	bcdef	0.54	0.46	abc	1.38	0.48	jklm	0.62	0.08	ghijkl	0.50	0.20	fghij
PI354865	2.48	0.26	fghij	0.35	0.11	lmno	0.15	0.06	ghi	1.60	0.20	hijk	1.07	0.13	bc	0.68	0.13	fghi
PI582352	2.83	0.15	cdefg	1.30	0.07	abc	0.46	0.02	bcde	2.28	0.12	abcd	0.88	0.12	def	0.39	0.07	efghi
PI582353	3.26	0.26	bcd	0.95	0.27	cdefgh	0.29	0.07	defgh	2.47	0.27	ab	1.19	0.28	ab	0.48	0.11	efghi
PI582366	2.15	0.26	ijkl	0.40	0.04	klmno	0.18	0.02	fghi	1.10	0.20	mn	0.36	0.07	n	0.34	0.15	defghi
PI582368	2.18	0.15	ijkl	0.36	0.04	lmno	0.17	0.01	ghi	1.11	0.01	mn	0.66	0.10	ghijk	0.59	0.09	defghi
PI582402	2.93	0.47	cdef	0.74	0.09	fghijk	0.25	0.03	efghi	1.66	0.07	ghij	0.73	0.07	fgh	0.44	0.04	defghi
PI582428	3.21	0.42	bcd	0.30	0.02	mno	0.09	0.01	hi	2.49	0.08	ab	0.57	0.05	hijkl	0.22	0.02	cdefgh
PI582468	3.15	0.40	bcde	1.18	0.33	abcde	0.38	0.11	cdef	2.03	0.09	cdef	0.77	0.14	efg	0.38	0.08	bcdefgh
PI582551	2.17	0.16	ijkl	1.39	0.44	ab	0.65	0.24	ab	1.50	0.24	jkl	0.99	0.02	cd	0.68	0.13	bcdefgh
PI582573	1.87	0.17	klm	0.24	0.13	no	0.12	0.07	hi	0.86	0.13	n	0.37	0.07	mn	0.44	0.13	bcdefg
PI582697	1.54	0.14	m	0.69	0.08	ghijkl	0.45	0.07	bcde	1.30	0.25	klm	0.60	0.03	ghijkl	0.47	0.09	abcdef
PI582812	2.05	0.41	jklm	0.93	0.18	cdefghi	0.47	0.15	bcd	1.22	0.16	lm	0.36	0.06	n	0.30	0.05	abcde

Table 3.4. (Cont.)

	Leaf biomass_Non_Stress (g)			Leaf biomass_Stress (g)			Leaf biomass_RST			Stem biomass_Non_Stress (g)			Stem biomass_Stress (g)			Stem biomass_RST (g)		
Accession	**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**	
PI582852	2.34	0.23	ghijk	0.53	0.20	jklmn	0.22	0.07	fghi	1.60	0.31	hijk	0.70	0.23	ghi	0.44	0.10	abcd
PI582863	2.62	0.13	efghi	0.63	0.31	hijklm	0.24	0.11	fghi	1.23	0.20	lm	0.51	0.02	klmn	0.42	0.08	abcd
PI583232	2.73	0.50	defgh	0.91	0.16	defghi	0.34	0.08	cdefg	1.54	0.13	ijkl	0.64	0.08	ghijk	0.41	0.06	abc
PI664515	4.69	0.09	a	0.24	0.04	no	0.05	0.01	i	2.53	0.16	a	0.46	0.04	lmn	0.18	0.03	ab
PI664517	3.50	0.30	b	0.59	0.23	hijklmn	0.17	0.05	ghi	2.36	0.16	abc	0.94	0.13	cde	0.40	0.08	a
PI664524	3.19	0.39	bcd	1.50	0.16	a	0.48	0.09	bcd	2.19	0.17	bcde	1.28	0.03	a	0.59	0.05	a

Table 3.5. Pearson's correlation coefficients between trait values used for phenotyping salt tolerance at seedling stage in cowpea.

	Dead	Leaf_injury	Chlorophyll_ NonStress	Chlorophyll_Stress	Chlorophyll_ RST	Height NonStress	Height_Stress
Dead	1.00						
Leaf_injury	0.91	1.00					
Chlorophyll_ NonStress	-0.22	-0.28	1.00				
Chlorophyll_ Stress	-0.81	-0.85	0.32	1.00			
Chlorophyll_ RST	-0.77	-0.79	0.07	0.96	1.00		
Height_NonStress	-0.10	-0.18	-0.18	0.13	0.19	1.00	
Height_Stress	-0.04	-0.08	-0.22	0.22	0.30	0.66	1.00
Height_RST	0.11	0.17	-0.09	0.07	0.10	-0.43	0.38
LeafBiomass_ NonStress	0.04	-0.02	0.20	0.10	0.08	0.29	0.13
LeafBiomass_ Stress	-0.52	-0.61	0.05	0.68	0.69	0.32	0.37
LeafBiomass_ RST	-0.46	-0.50	0.07	0.56	0.54	0.13	0.23
StemBiomass_ NonStress	-0.11	-0.19	0.19	0.17	0.14	0.66	0.38
StemBiomass_ Stress	-0.03	-0.10	-0.20	0.19	0.25	0.69	0.81
StemBiomass_ RST	0.14	0.12	-0.37	-0.03	0.05	0.15	0.48

	Height_RST	LeafBiomass_ NonStress	LeafBiomass_Stress	LeafBiomass_RST	StemBiomass_ NonStress	StemBiomass_Stress	StemBiomass_RST
Dead							
Leaf_injury							
Chlorophyll_ NonStress							
Chlorophyll_ Stress							
Chlorophyll_ RST							
Height_NonStress							
Height_Stress							
Height_RST	1.00						
LeafBiomass_ NonStress	-0.21	1.00					
LeafBiomass_ Stress	0.02	0.00	1.00				
LeafBiomass_ RST	0.10	-0.36	0.89	1.00			
StemBiomass_ NonStress	-0.35	0.79	0.15	-0.16	1.00		
StemBiomass_ Stress	0.11	0.19	0.39	0.21	0.45	1.00	
StemBiomass_ RST	0.40	-0.42	0.24	0.36	-0.37	0.62	1.00

Figures

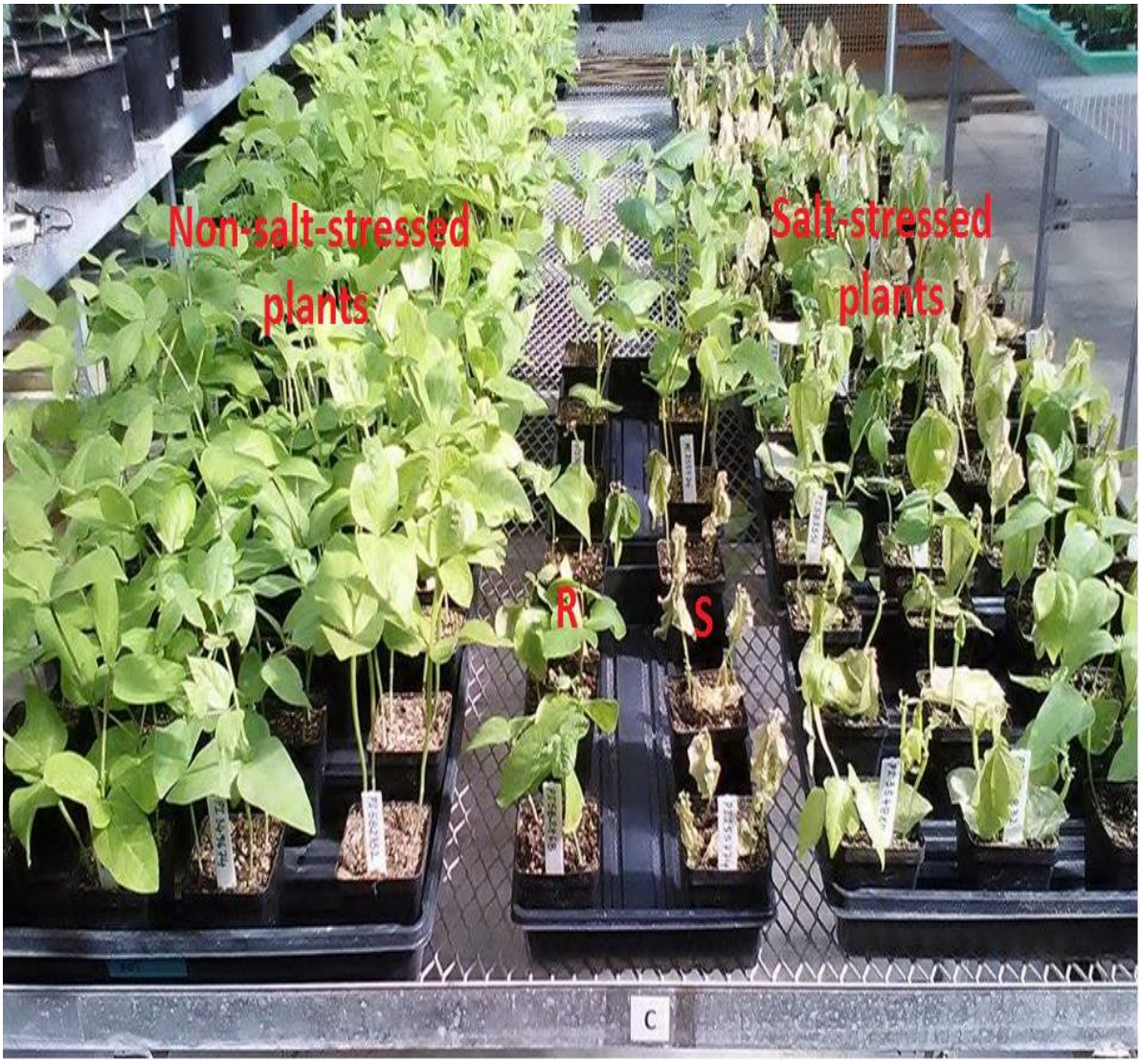

Fig. 3.1. Phenotyping of salt tolerance in cowpea at seedling stage 14 days of salt stress. (R) Salt-tolerant genotype, PI582468, and (S) salt-sensitive genotype, PI255774 used as controls. Salt treatment was conducted by irrigating each plastic pot from the bottom.

Fig. 3.2. Differences in above ground traits between salt-tolerant and salt-sensitive genotypes 14 days of salt stress (R: salt-resistant and S: salt-sensitive).

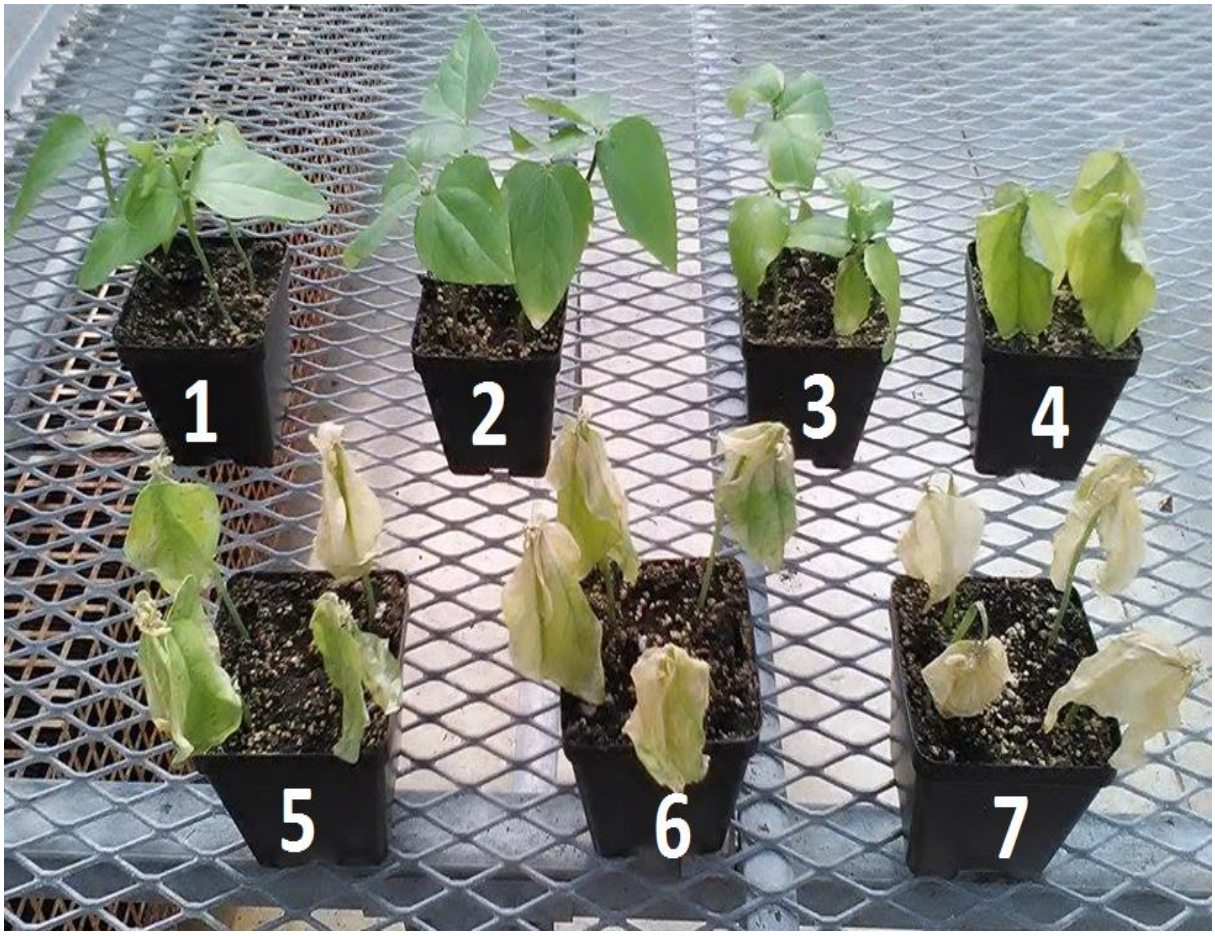

Fig. 3.3. Foliar injury due to salt stress: 1=healthy plants, 2=first sign of leaf chlorosis, 3=expansion of chlorosis on leaf surface, 4= totally chlorotic leaf, 5=first sign of necrosis, 6=expansion of necrosis on leaf surface, and 7=completely dead plants.

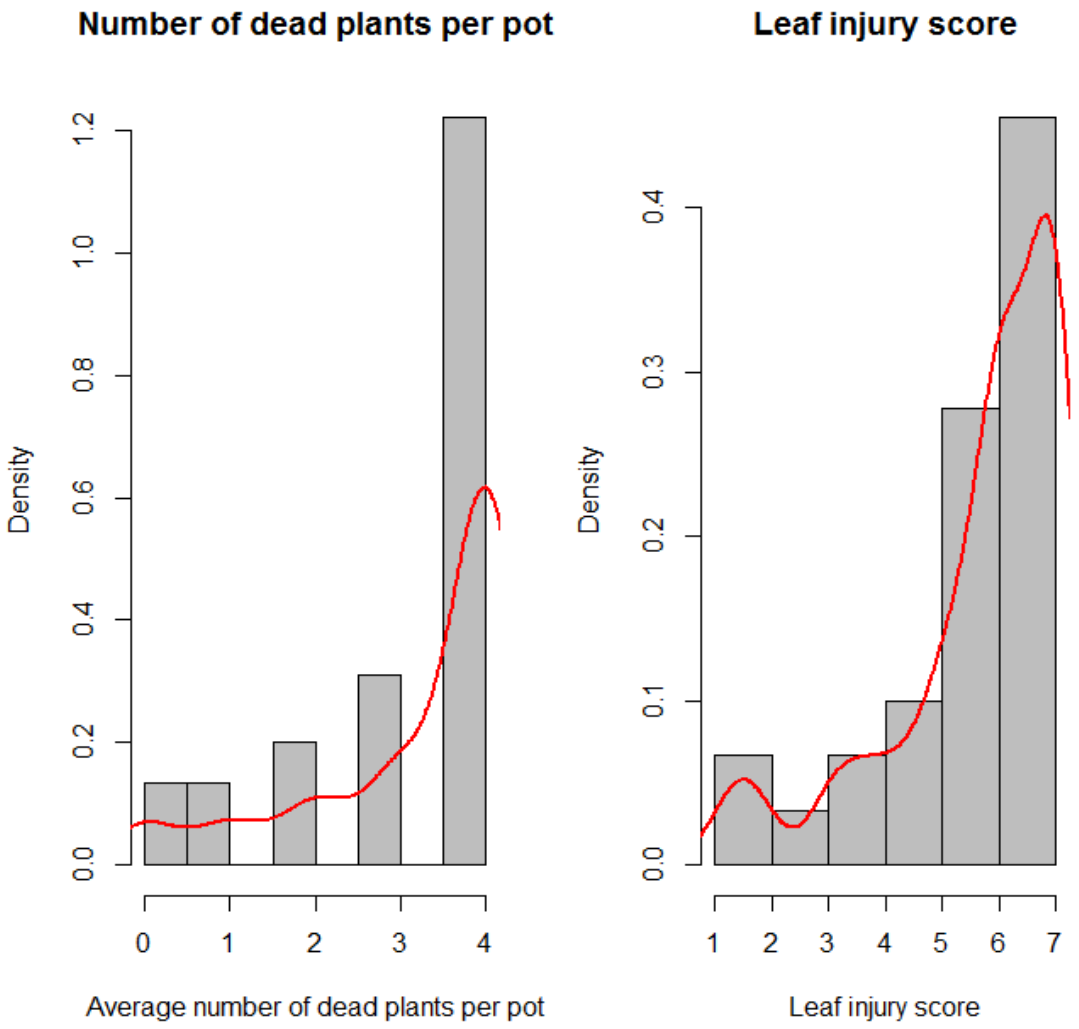

Fig. 3.4. Distributions of the average number of dead plants per pot and leaf injury score.

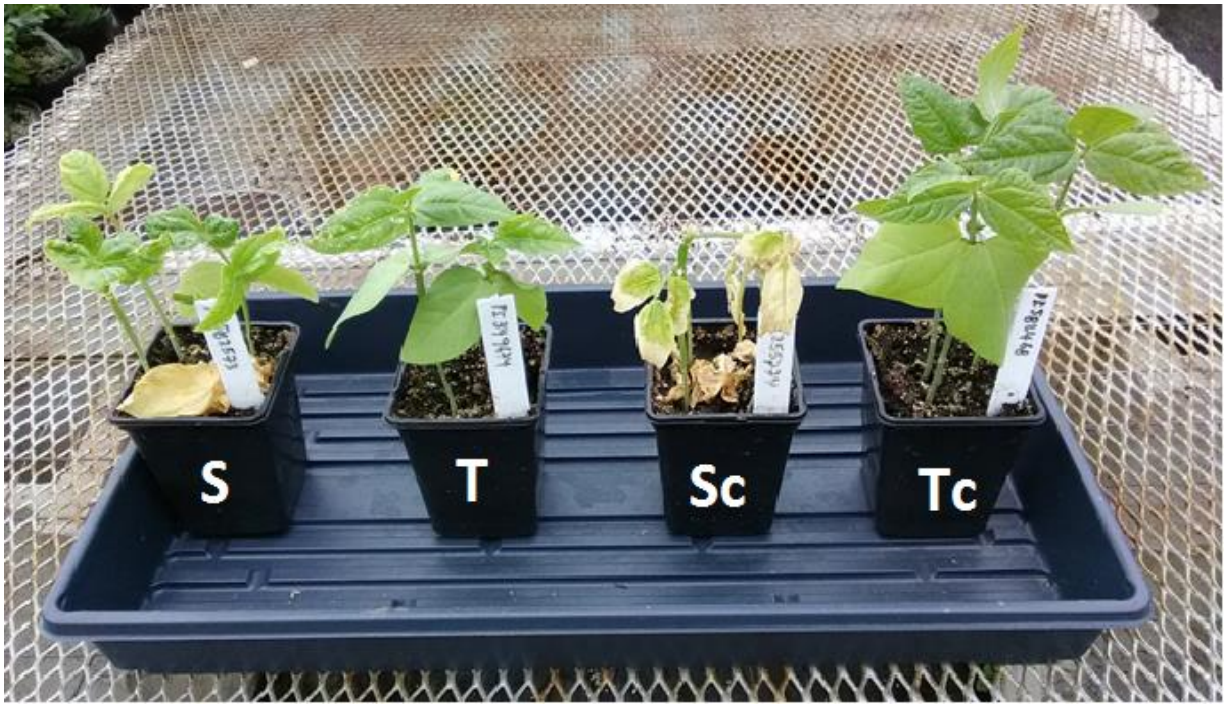

Fig. 3.5. Independent replicated trial involving the tolerant check (Tc: PI582468), the susceptible check (Sc: PI255774), and one of the salt-tolerant genotypes (T: PI349674) and salt-susceptible ones (S: PI582573) as identified in the previous experiment. The results from the independent replicated trials showed that the current methodology was stable.

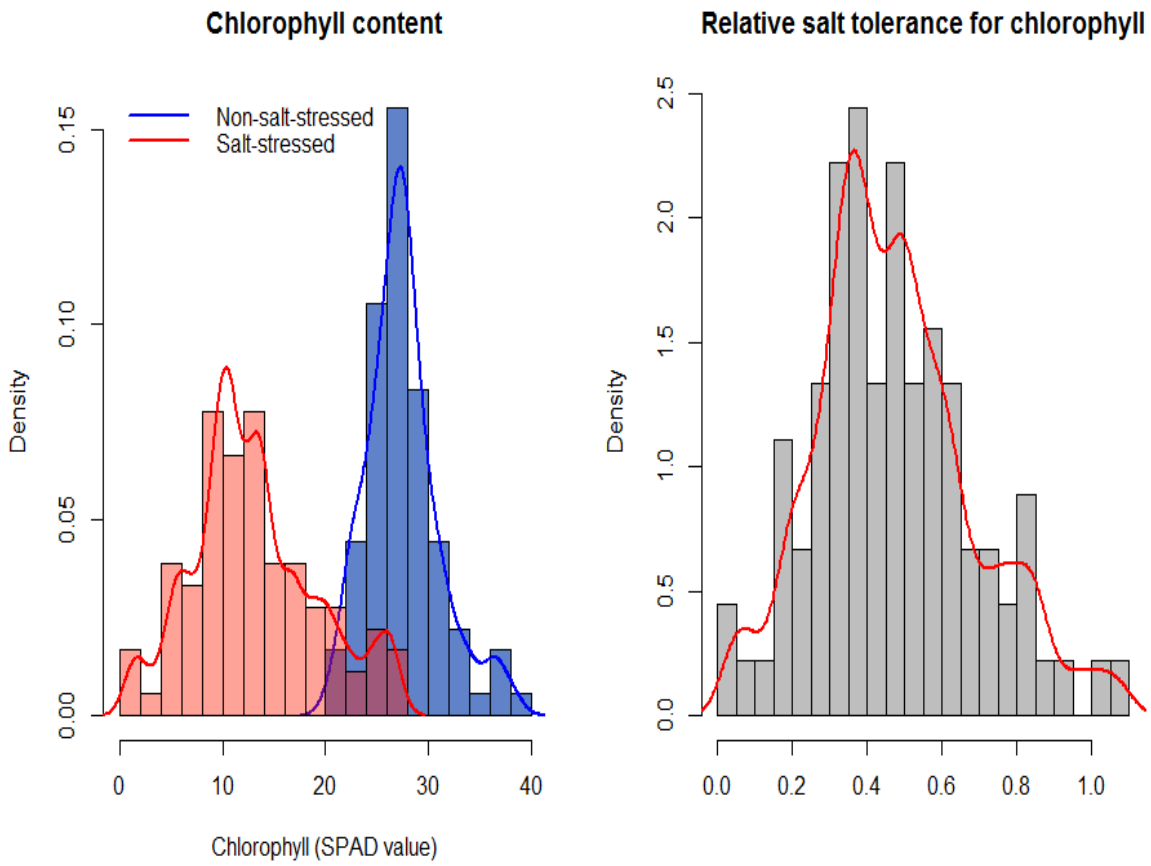

Fig. 3.6. Distributions of chlorophyll content of non-salt-stressed and salt-stressed plants, and relative salt tolerance for chlorophyll content.

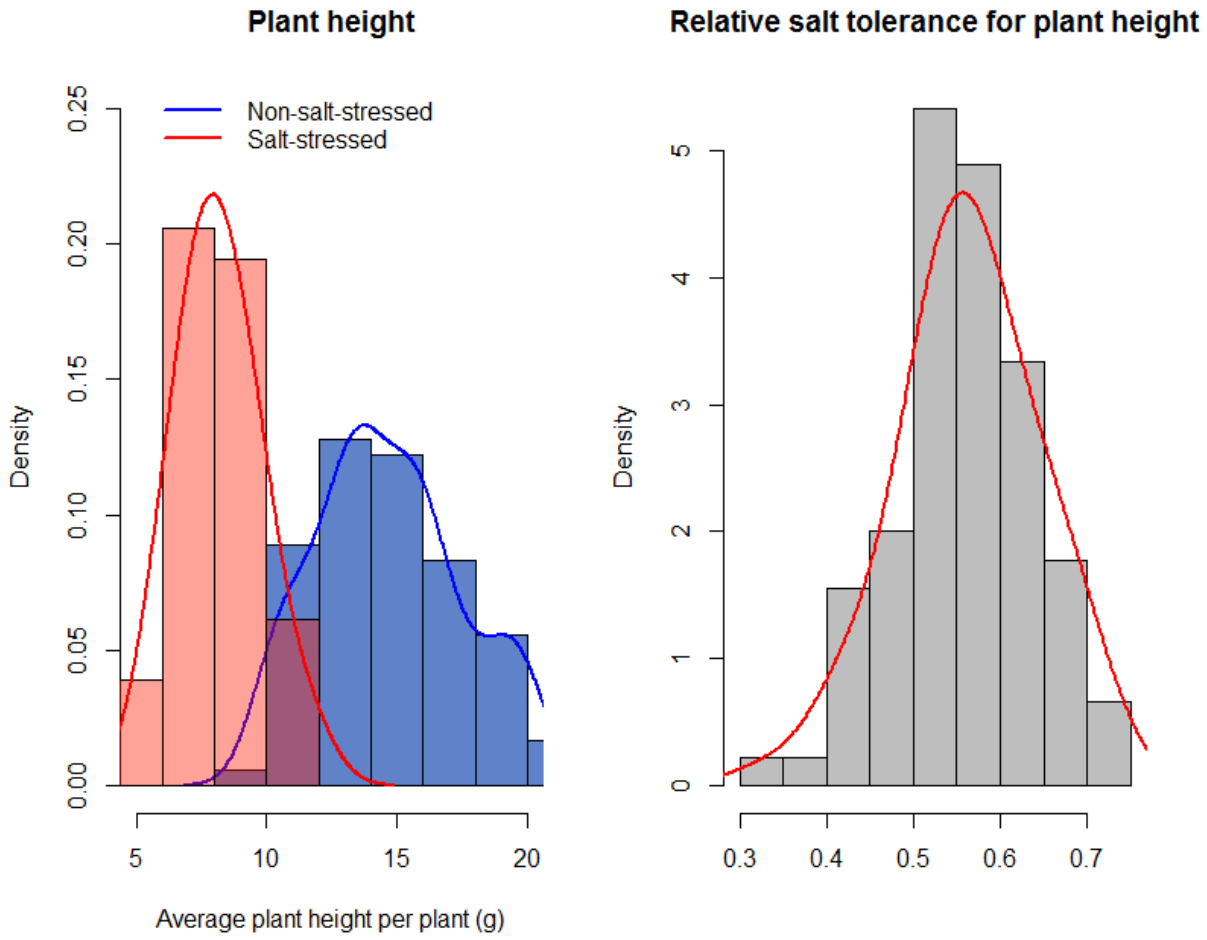

Fig. 3.7. Distributions of plant height of non-salt-stressed and salt-stressed plants, and relative salt tolerance for plant height.

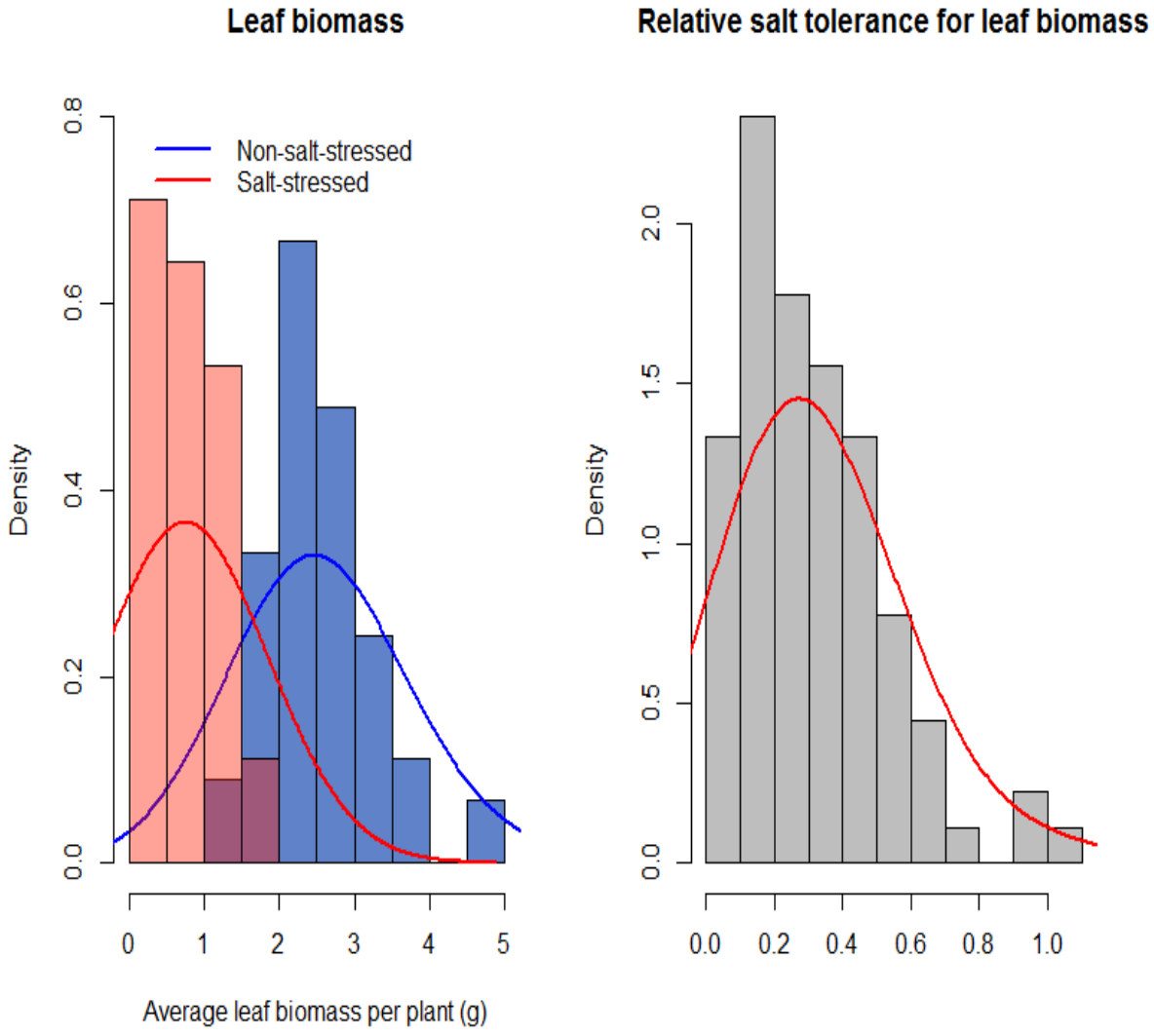

Fig. 3.8. Distributions of leaf biomass of non-salt-stressed and salt-stressed plants, and relative salt tolerance for leaf biomass.

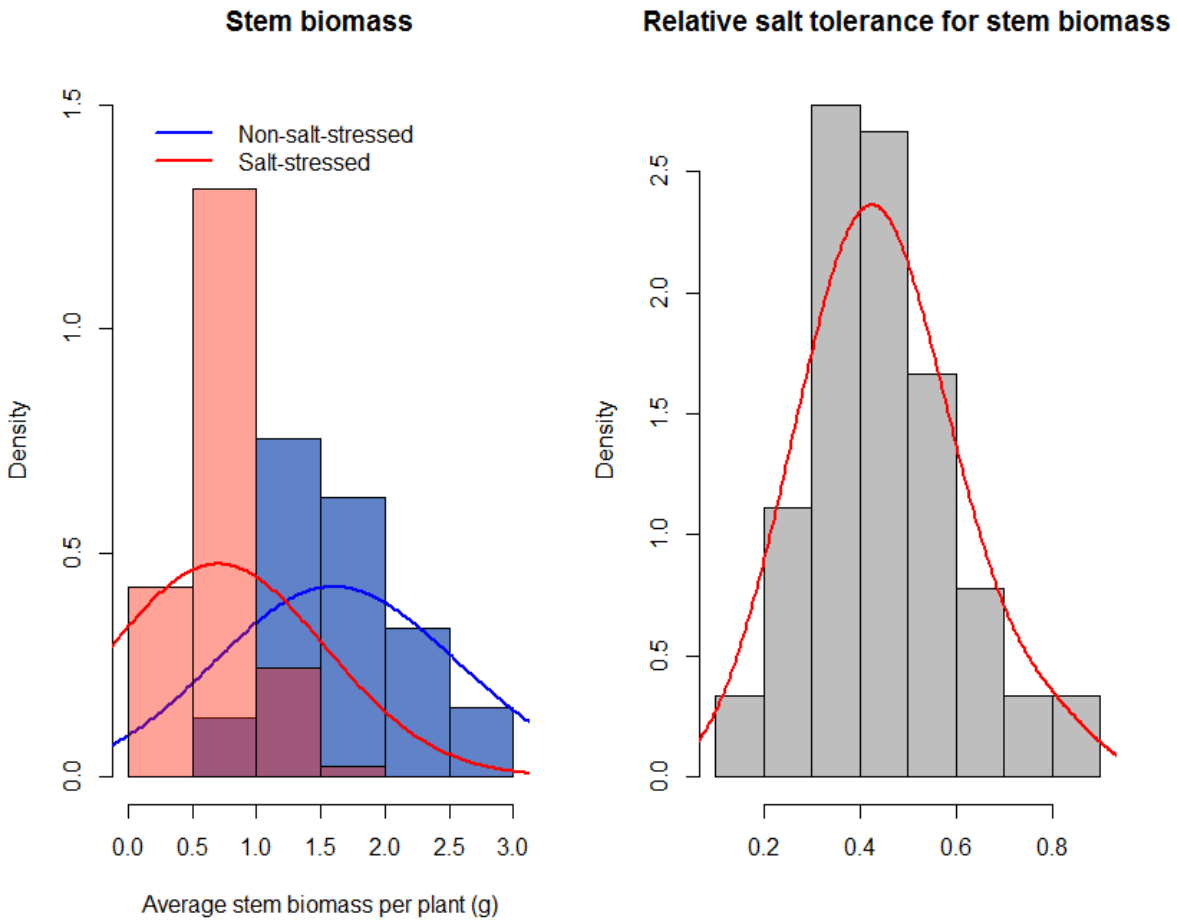

Fig. 3.9. Distributions of stem biomass of non-salt-stressed and salt-stressed plants, and relative salt tolerance for stem biomass.

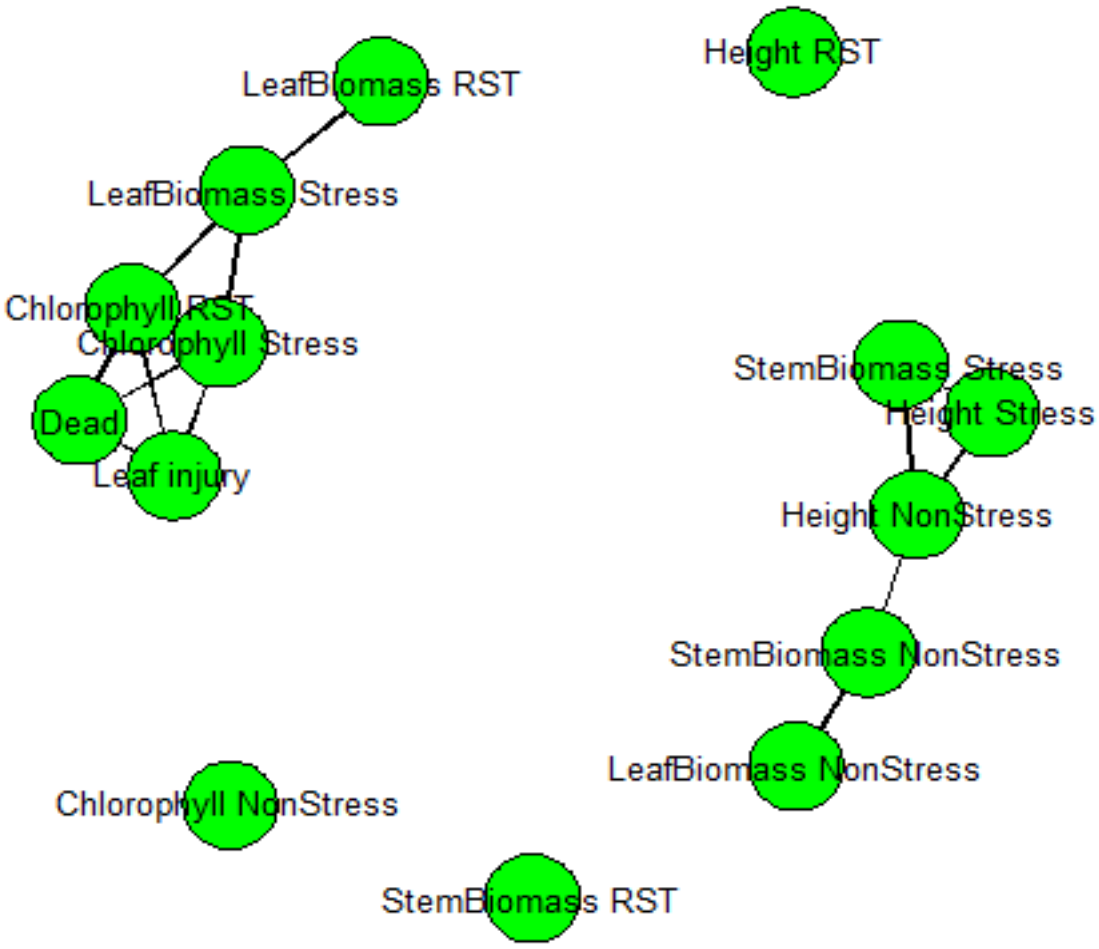

Fig. 3.10. Network analysis between traits evaluated under salt stress and non-salt conditions. Pathways were shown using solid lines when absolute value of Pearson's correlations was greater than 0.65.

Chapter 4. Loci Discovery, Network-Guided Approach, and Genomic Prediction for Drought Tolerance Index in a Multi-Parent Advanced Generation Inter-Cross (MAGIC) Cowpea Population

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Abstract

Cowpea is a nutrient-dense legume that significantly contributes to the population's diet in sub-Saharan Africa and other regions of the world. Improving cowpea cultivars to be more resilient to abiotic stress such as drought would be of great importance. The use of a MAGIC population has been shown to be efficient in increasing the frequency of rare alleles that could be associated with important agricultural traits. In addition, drought tolerance index has been reported to be a reliable parameter for assessing crop tolerance to water deficit conditions. Therefore, the objectives of this study were to evaluate the drought tolerance index for plant growth habit, plant maturity, flowering time, 100-seed weight, and grain yield in a MAGIC cowpea population, to conduct GWAS and identify single nucleotide polymorphism (SNP) markers associated with the drought tolerance indices, to investigate the potential relationship existing between the significant loci associated with the drought tolerance indices, and to conduct genomic selection (GS). The MAGIC population consisted of a total of 305 cowpea genotypes that were developed and phenotyped by the UC Riverside's team. The results indicated that: 1) a large variation in drought tolerance indices existed among the cowpea genotypes, 2) a total of 14, 18, 5, 5, and 35 SNPs were associated with plant growth habit change due to drought stress, drought tolerance index for maturity, flowering time, 100-seed weight, and grain yield respectively, 3) the network-guided approach revealed clear interactions between the loci associated with the drought tolerance traits, and 4) GS accuracy varied from low to moderate. The results from this study will have practical applications in cowpea breeding programs through marker-assisted selection (MAS) and genomic selection (GS). To the best of our knowledge, this is the first study identifying loci associated with the aforementioned drought tolerance indices using a MAGIC population in cowpea.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid legume (2n=2x=22) grown for its relatively high amount of seed protein (Weng et al. 2017). Cowpea cultivation is prevalent in Asia, Oceania, the Middle East, southern Europe, Africa, southern USA, and Central and South America (Perrino et al. 1993). Cowpea has also been shown to be nutrient-dense. Cowpea seeds consisted on average of 6.8 iron, 4.1 zinc, 1.5 manganese, 510.0 phosphorus, and 1430.0 potassium, in mg per 100-g seed (Frota et al. 2008). Cowpea consumption has been demonstrated to be health-promoting due to the high amount of antioxidant compounds found in cowpea seeds (Moreira-Araújo et al. 2017; Qin et al. 2016). In addition to being consumed for its good nutritional values, cowpea leaves can provide good quality feed for livestock and cowpea plants can be used as cover crops (Wison et al. 2006). Cowpea is grown on more than 11 million hectares worldwide and over 70% of the worldwide cowpea production has been provided by Africa with Nigeria being the top producer (Singh et al. 2003). Among the developed countries, the United States has the greatest potential for exporting cowpea with the highest average cowpea yield per hectare (Agbicodo et al. 2009).

Cowpea cultivation is usually rain-dependent and water shortage during cowpea developmental and growth stages could be detrimental to cowpea production (Fatokun et al. 2012). Evidence of the negative effects of drought stress on cowpea has been reported in areas where cowpea is cultivated (Burridge et al. 2017; Carvalho et al. 2017). Even though cowpea is one of the most drought-tolerant legumes, some cultivars with desirable agronomic traits were found to be sensitive to water deficit conditions (Verbree et al. 2015). Therefore, cowpea breeding program aiming at improving drought tolerance is still required. Breeding for drought tolerance requires a good understanding of the genetic mechanism conferring drought tolerance.

With an estimated genome size of 620 Mb (Timko et al. 2008), cowpea could be used as an excellent model crop for drought tolerance-related studies in legume research. The relatively small genome size of cowpea would allow for a rapid and efficient identification of genes contributing to drought tolerance. Drought tolerance in cowpea is a complex mechanism and involves sophisticated interactions between genes (Carvalho et al. 2017). Therefore, identifying genes for drought tolerance would be critical. However, incorporating the genetic finding into breeding programs for improving drought tolerance of the existing cowpea elite culticars would be time consuming. This could be addressed by performing drought tolerance research on a Multi-Parent Advanced Generation Inter-Cross (MAGIC) population derived from parents having drought tolerance and any other desirable agronomic traits.

Investigation into the genetic architecture governing traits of interest using MAGIC populations has recently received significant consideration. MAGIC populations provide both greater diversity and a balanced allele frequency, which is critical for efficiently conducting genetic-related studies (Huang et al. 2015). MAGIC populations were first developed to dissect the genetic architecture of important traits in animals and results were promising (Ram et al. 2014). For plants, MAGIC populations have been established for *Arabidopsis thaliana* (Kover et al. 2009), wheat (Huang et al. 2012), rice (Bandillo et al. 2013), and chickpea (Gaur et al. 2012). The genetics of yield and tolerance to abiotic stress such as drought have been successfully investigated in a MAGIC rice population (Bandillo et al. 2013). Investigating the genetics of drought tolerance on a MAGIC cowpea population could be also achieved. The first MAGIC cowpea population was developed by Huynh et al. (2018) from the University of California, Riverside.

This first MAGIC cowpea population was phenotyped under both full irrigation and restricted irrigation water regimes at UCR-CES (California) and CVARS (California). The MAGIC population was genotyped using a total of 51,128 SNPs postulated from the Illumina Cowpea Consortium Array (Muñoz-Amatriaín et al. 2017). Markers associated with drought tolerance and agronomic traits such as flowering time, growth habit, and maturity were investigated based upon QTL analysis. Genetic maps, recombination frequency analysis, and significant QTLs related to the aforementioned traits were established for the MAGIC cowpea population (B. Huynh et al. 2018). This study was complemented using a genome-wide association study (GWAS) approach as reported by Olatoye et al. (2019). GWAS provides a greater mapping resolution over QTL mapping and efficiently permits the discovery of new genes (Price 2006; Hamblin et al. 2011). However, the drought tolerance index trait, which is the relative change of the trait values due to drought stress (Ravelombola et al. 2018; Saad et al. 2014), was not investigated in this MAGIC cowpea population. Investigating the genetic architecture of the drought tolerance indices could lead to the discovery of new significant loci associated with drought tolerance in cowpea. In addition, the analysis can be further enhanced using genomic selection. Predictive breeding involving genomic selection has become more and more popular since it is cost-effective and provides breeders with a rapid genetic gain per unit of time (Hayes et al. 2009). Genomic selection has been reported to be highly efficient in investigating the genetic architecture of complex trait such as drought tolerance (Heffner et al. 2009). Therefore, the objectives of this study were to conduct a GWAS and GS for the drought tolerance indices, to identify SNP markers associated with drought tolerance indices, and to estimate the GS accuracy in predicting drought tolerance indices in a MAGIC cowpea population.

Materials and Methods

MAGIC population development and genotyping

The MAGIC cowpea population was derived from crosses between eight different cowpea parents (IT89KD-288, IT84S-2049, CB27, IT82E-18, SuViTa_2, IT00K-1263, IT84S-2246, and IT93K-503-1) (Huynh et al. 2018). The eight parents consisted of cultivars and breeding lines from Burkina Faso, Nigeria, and the United States. The parents were genetically diverse and details on population development were described previously (Huynh et al. 2018). IT93K-503-1 was an advanced drought-tolerant line developed by IITA, Nigeria (Muchero et al. 2009). The remaining parents harbored a combination of important agronomic traits such as resistance to *Striga*, fungi, bacteria, viruses, foliar thrips, root-knot nematode, and heat stress (Ehlers et al. 2000; Huynh et al. 2016; Lucas et al. 2012; Muchero et al. 2009; Muchero et al. 2011; Ouédraogo et al. 2002; Pottorff et al. 2014). The first crosses were done in early 2011. The resulting MAGIC population consisted of a total of 305 $F_{8:10}$ RIL lines.

The 305 RIL lines along with the parents were genotyped using of total of 51,128 SNPs form the Illumina Cowpea Consortium Array (Muñoz-Amatriaín et al. 2017). After SNP filtering, a total of 32,059 high-quality SNPs were retained (missing data <10%, heterozygosity <10%, and minor allele frequency >5%).

Growing conditions and phenotyping

Phenotypic data and filed phenotyping were conducted by Huynh et al. (2018) at CVARS. Data on plant growth habit, flowering date, maturity date, grain yield, and 100-seed weight were recorded under both full and restricted irrigation. A total of 12 seeds were planted for each MAGIC RIL line along with the 8 parents. Plantation areas were irrigated to field capacity before planting and restricted water regime was achieved by withholding water on the

2-week old cowpea plants (Huynh et al. 2018). Flowering date corresponded to the time where 50% of plants within a row had flowers. Plant growth habit was rated based on a 1 to 6-scale (1: acute erect, 2: erect, 3: semi-erect, 4: indeterminate, 5: semi-prostrate, and 6: semi-prostrate). Maturity date was recorded when over 95% of pods within a row were dry. Grain yield and 100-seed weight were recorded upon harvest as described by Huynh et al. (2018).

In order to assess the effects of restricted irrigation on the aforementioned agronomic traits, drought stress tolerance index was computed and defined as following (Saad et al. 2014) and change in plant growth habit was quantified using a binary approach(1: no change in plant growth habit between full irrigation and restricted irrigation and 9: otherwise).

$$\text{Tolerance index} = 100 * (Y_{\text{restricticed irrigation}}/Y_{\text{full irrigation}})$$

where $Y_{\text{restricticed irrigation}}$ represented flowering time, maturity, grain yield, and 100-seed weight under restricted irrigation and $Y_{\text{full irrigation}}$ referred to flowering time, maturity, grain yield, and 100-seed weight under full irrigation treatment. Data were visualized using the 'MASS' package of R® v.3.6.1 (R Developlment Core Team 2011).

Pearson's correlation coefficients between the quantitatively evaluated traits were calculated using R® v.3.6.1 and the association between the qualitative trait (change in growth habit) and the quantitatively evaluated traits was investigated through a univariate logistic regression, which was run in R® v.3.6.1 as well. The logistic regression model was the following.

$$\log[\pi/(1-\pi)] = \beta_0 + \beta_i X_i$$

where π was the probability of success of an event from the conditional binomial distribution $Y|N \sim Bin(N, \pi)$ with Y being the number of genotypes having change in plant growth habit under drought stress and N being the total number of genotypes, β_0 was the intercept, β_i was the effect

of the i^{th} covariate on the binomial response, X_i denoted the i^{th} covariate corresponding to each trait i={1: tolerance index for plant maturity, 2: tolerance for flowering time, 3: tolerance index for 100-seed weight, and 4: tolerance index for grain yield}.

Genome-wide association study (GWAS)

A Bayesian Information and Linkage Disequilibrium Iteratively Nested Keyway (BLINK) model was used to conduct GWAS. BLINK was run using in R® v.3.6.1 using the package 'BLINK' (Huang et al. 2019). Previous studies have shown that BLINK allowed for efficiently discovering SNPs highly associated with traits of interest over other models (Huang et al. 2019). SNPs with an LOD greater than 3 were declared significant (Kaler et al. 2017).

BLINK was a modified and improved version of Fixed and Random Model Circulating Probability Unification (FarmCPU). FarmCPU iteratively run both a fixed effect model (FEM) and a random effect model (REM). A major assumption when running FarmCPU was the even distribution of markers within the genome, which could be easily violated. In BLINK, this assumption was relaxed by using the information from a linkage disequilibrium (LD) analysis. The REM part of FarmCPU was replaced by a second FEM in BLINK, making the running time shorter. The two FEM models used in BLINK were the following

$$\text{FEM (1): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ik}b_k + M_{ij}d_j + e_i$$

$$\text{FEM (2): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ij}b_j + e_i$$

with y_i being the phenotypic data from the i^{th} sample; M_{i1},$M_{i2}b_2$, …, M_{ik} the genotypes of k pseudo QTNs, which were initially empty and with effects b_1, b_2, …, b_k, respectively; M_{ij} being the j^{th} genetic marker of the i^{th} sample; and e_i being the residual having a distribution with mean zero and a variance σ^2_e. In this study, we focused on the SNPs associated with the tolerance index trait. However, we re-ran the traits investigated by Huynh et al. (2018) and Olatoye et al. (2019) using BLINK and the SNPs identified for these traits were analyzed in the network

analysis section. LD heatmaps were established in R® v.3.6.1 using the package 'LDheatmap' (Shin et al. 2019).

Candidate gene(s) discovery

Significant SNPs were used for candidate gene(s) discovery. The 40-kb region harboring the significant SNP was considered for candidate gene search using the Phytozome 12 database (https://phytozome.jgi.doe.gov/) based on the SNP density. Functional annotation pertaining to candidate gene(s) was investigated using the Phytozome 12 database as well.

Association network

A network-guided association analysis was conducted to investigate the significant loci that were associated with two or more traits. The algorithm used for constructing the network was similar to that of established by Fang et al. (2017) with slight modifications. The nodes in the network corresponded to the traits and the significant SNPs associated with each trait. The traits investigated by Huynh et al. (2018) and Olatoye et al. (2019) were represented by solid circles, whereas the tolerance index traits were visualized by solid diamonds. The SNPs associated with each trait were denoted using solid dark grey circles. The size of each trait node was fixed, whereas the size of each SNP node was proportional to its LOD value that was obtained from GWAS. The bigger the SNP node was, the higher its LOD was. The edge of the network was represented using solid dark lines linking the SNP and trait nodes. The attribute of the edge between a pair of SNPs was proportional to the pairwise LD r^2 between the two SNPs, which was estimated using PLINK (Purcell et al. 2007). The attribute of the edge between a SNP node and a trait node was fixed. No edges were used between trait nodes. The network was designed using Cytoscape v. 3.7.2 (Otasek et al. 2019). A network was established when a SNP was associated with two or more traits, which was easily identified using a GWAS approach. In

addition, a network could be also constructed when two different SNPs were associated with two different traits, but these two SNPs were in high LD. This could not be detected with GWAS. Finally, a network was also defined when two SNPs in high LD were associated to one trait, which could be considered as epistasis (Fang et al. 2017).

Genomic selection (GS)

Genomic selection was carried out using all 32,059 high-quality SNPs. Genomic estimated breeding values (GEBVs) were estimated using a ridge regression best linear unbiased predictor model (rrBLUP) (Meuwissen et al. 2001). The rrBLUP model was $y=WG\beta + \varepsilon$ where y was the vector phenotype, β indicated the marker effect with $\beta \sim N(0, I\sigma^2_\beta)$, W corresponded to the incidence matrix relating the genotype to the phenotype, G denoted the genetic matrix, and ε was the random error. The solution for the model was $\hat{\beta}=(Z^TZ + I\lambda)^{-1}Z^Ty$ with Z=WG. The ridge parameter used in this study was $\lambda=\sigma^2_e/\sigma^2_\beta$. The parameter σ^2_e denoted the residual variance and σ^2_β the marker effect variance. rrBLUP was conducted in R® v.3.6.1 using the package 'rrBLUP' (Endelman 2011).

Genomic estimated breeding values (GEBVs) were estimated using a training population randomly chosen from the MAGIC population (Shikha et al. 2017). Since the genotypes with missing data could impact the results, they were removed prior to conducting genomic selection, leaving with a total of 249 cowpea genotypes for the analysis. Genomic selection was conducted using a two-, three-, four-, five-, six-, seven-, and eight-fold cross validation corresponding to a training/testing set of 125/124, 166/83, 186/63, 199/50, 207/42, 213/36, and 217/32, respectively. The training and testing sets were two disjoint groups. The training population was used to fit the model and the testing population was used to assess the accuracy of the model. A total of 100 replications were used for each cross-validation level. Genomic selection accuracy corresponded

to the Pearson's correlation coefficient between the GEBVs and the observed phenotypic values in the testing set (Shikha et al. 2017).

Results

Phenotyping

To quantify the relative change in maturity due to drought stress, tolerance index was evaluated. A tolerance index greater than 100 for plant maturity indicated that restricted irrigation made plant maturity longer, whereas a tolerance index lower than 100 suggested plant maturity being shorter due to water deficit. A large variation in tolerance index for maturity was identified among the RILs. Tolerance index was nearly normally distributed (Fig. 4.1A). Tolerance index ranged between 69.19 and 142.01, with an average of 104.74 and a standard deviation of 15.60.

Tolerance index for flowering time varied from 78.41 to 126.67, with an average of 97.48 and a standard deviation of 5.35. Tolerance index for flowering time was also approximately normally distributed (Fig. 4.1B). Tolerance index for 100-seed weight was approximately normally distributed (Fig. 4.1C) and ranged between 59.56 and 210.11, with an average of 113.09 and a standard deviation of 17.54.

Unlike the aforementioned parameters investigated in this study, tolerance index for grain yield was right-skewed as shown in Fig. 4.1D. Tolerance index ranged between 4.95 and 754.39, with an average of 41.89 and a standard deviation of 53.34, indicating that yield was negatively impacted by restricted irrigation. Plant growth habit under both full and restricted irrigations were recorded. A total of 154 RILs had a change in plant growth habit due to drought stress. Overall, the change pattern was semi erect and inderminate towards acute erect and erect.

Pearson's correlation coefficients between the different tolerance indices were calculated. Overall, correlation coefficients between traits were low. A moderate and positive Pearson's correlation coefficient was found between tolerance index for grain yield and tolerance index for 100-seed weight (r=0.33). A low Pearson's correlation coefficient was found between tolerance index for maturity and tolerance index for flowering time (r=0.17). The lowest Pearson's correlation coefficient was found between tolerance index for flowering time and tolerance index for 100-seed weight (r=0.01).

A univariate logistic regression model was used to assess the relationship between change in growth habit due to drought stress and the previously assessed tolerance indices. The univariate logistic regression model was used to fit the change in growth habit to each tolerance index trait, where the growth habit was a binomial response and each tolerance index was a continuous predictor variable. The univariate model showed that all tolerance indices except for tolerance index for grain yield were insignificant. The estimate of the effects of tolerance index for plant maturity, tolerance index for grain yield, tolerance index for 100-seed weight, and tolerance index for flowering time on the change of growth habit due to drought stress were -0.009 (Z-value=-1.170, p-value=0.142), 0.013 (Z-value=2.207, p-value=0.03), 0.006 (Z-value=0.851, p-value=0.395), and -0.019 (Z-value=-0.775, p-value=0.438), respectively. These results indicate that there is a significant association between tolerance index for grain yield and change in growth habit to drought stress.

Genome-wide association study (GWAS)

GWAS was conducted to identify SNP markers associated with growth habit change, tolerance indices for maturity, flowering time, 100-seed weight, and grain yield. A total of 14 SNP markers were found to be associated with tolerance index to plant growth habit change

(Table 4.1) (Fig. 4.2A). Of which, eight were mapped on a 10.1-Mb region of chromosome 8, indicating a strong likelihood of significant loci associated with plant growth habit change under drought stress in this genomic region. The top five SNPs associated with plant growth habit change under drought stress were 2_26924 (LOD= 4.06, MAF= 17.67%), 2_01300 (LOD= 3.88, MAF= 17.27%), 2_10658 (LOD= 3.88, MAF= 17.27%), 2_54501 (LOD= 3.88, MAF= 17.27%), and 2_45332 (LOD= 3.88, MAF= 17.27%) (Table 4.1), which were all located on chromosome 8. The LD analysis around the most significant SNP showed low pairwise LD values between SNPs (Fig. 4.3A).

The results indicated a total of 18 SNPs associated with tolerance index for maturity (Table 4.1) (Fig. 4.2B). Of which, 14 were found on a 584-Kb region of chromosome 8. A small portion of this region overlapped with the 10.1-Mb region found for plant growth habit change under drought stress. The remaining SNPs were located on chromosomes 2 and 7. The top 5 SNPs with the highest LOD value were 2_21981 (LOD= 5.68, MAF= 20.08%), 2_40337 (LOD= 4.27, MAF= 28.34%), 2_14976 (LOD= 4.23, MAF= 28.92%), 2_14158 (LOD= 3.63, MAF= 33.33%), and 2_51274 (LOD= 3.54, MAF= 13.65%) (Table 4.1). The region in the vicinity of the SNP with the highest LOD value indicated a moderate LD (Fig. 4.3B). In addition, no SNPs located within the 30-kb region flanking the most significant SNP, 2_21981, had an LOD greater than the declared threshold (3) (Fig. 4.3B).

The discrepancy in change in flowering time between full irrigation and restricted irrigation was also assessed using tolerance index for flowering time. However, no SNPs exceeding the LOD threshold (3) were found. We only reported the top 5 SNPs, 2_06470 (LOD= 2.84, MAF= 12.45%), 2_52919 (LOD= 2.84, MAF= 12.45%), 2_06137 (LOD= 2.84, MAF= 12.45%), 2_27706 (LOD= 2.83, MAF= 19.68%), and 1_0946 (LOD= 2.83, MAF= 11.65%) that

the GWAS analysis suggested for tolerance index for flowering time (Table 4.1) (Fig. 4.2C). One of these SNPs were located on chromosome 8 (Fig. 4.2C). However, this SNP was not located within the significantly associated loci identified for plant growth habit change and tolerance index for plant maturity. The region harboring the most significant SNP, 2_06470, had a high LD (Fig. 4.3C).

The results did not show any SNPs having an LOD greater than the threshold (3) for tolerance index for 100-seed weight under restricted irrigation. We just reported the top 5 SNPs having the highest LOD values (Table 4.1). These SNPs were 2_11122 (LOD= 2.95, MAF= 11.34%), 2_03731 (LOD= 2.89, MAF= 10.84%), 2_14932 (LOD= 2.89, MAF= 10.84%), 2_34365 (LOD= 2.89, MAF= 10.84%), and 2_07882 (LOD= 2.89, MAF= 10.84%). These SNPs were all found on chromosome 4 (Fig. 4.2D). Among all traits evaluated in this study, tolerance index for grain yield had the highest number of significant SNPs. Our data suggested indicated a total of 35 SNPs associated with tolerance index for grain yield (Table 4.2) (Fig. 4.2E). Of which, 26 were mapped on a 566.5-Kb region of chromosome 6, seven on a 2.5-Mb region of chromosome 7, and two on a 703-Kb region of chromosome 8 (Table 4.2). These regions could harbor significant loci associated with tolerance index for grain yield under drought stress in cowpea. The top five SNPs with the highest LOD value were 2_25334 (LOD= 3.51, MAF= 8.23%), 2_51818 (LOD= 3.38, MAF= 12.85%), 2_31565 (LOD= 3.35, MAF= 9.64%), 2_19053 (LOD= 3.35, MAF= 9.64%), and 2_33474 (LOD= 3.35, MAF= 9.64%). The LD heatmap shown in Fig. 4.3E revealed an independent LD block, which contained the most significant SNP associated tolerance index for grain under drought stress. This LD pattern was not identified for traits such as change in plant growth habit, tolerance index for maturity, flowering time, and 100-

seed weight. In addition, there is lack of overlap between the significant SNPs across different traits, indicating that drought stress is a complex mechanism.

Candidate genes

A total of nine candidate genes were found for growth habit change under drought stress (Table 4.1). These candidate genes consisted of *Vigun08g076600.1*, *Vigun08g077200.1*, *Vigun08g077800.1*, *Vigun08g080000.1*, *Vigun08g082400.1*, *Vigun08g082500.1*, *Vigun08g069700.1*, *Vigun10g104700.1*, *Vigun10g106600.1* that encode for aldehyde dehydrogenase family, organic solute transporter, multi-copper oxidase, TLC ATP/ADP transporter, membrane protein involved in ER to Golgi transport, cytochrome P450, and SNARE protein GS28, respectively (Table 4.1). Out of the 18 SNPs found to be associated with tolerance index for maturity, 15 had annotated genes in their vicinity. A significant cluster of patatin-like phospholipase was found and encoded by *Vigun08g022000.1*, *Vigun08g022100.1*, *Vigun08g021900.1*, and *Vigun08g022200.1* (Table 4.1). The genes found close to the top five SNPs associated with tolerance index for maturity were *Vigun08g020700.1*, *Vigun08g023500.1*, *Vigun08g023400.1*, and *Vigun08g023300.1*. The annotated gene *Vigun08g020700.1* encodes for a kinase. Both *Vigun08g023500.1* and *Vigun08g023400.1* encode for EF hands and *Vigun08g023300.1* encodes for a phosphatidate phosphatase. An annotated gene encoding for a leucine rich repeat was also found.

A total of seven annotated genes were found in the vicinity of the five significant SNPs associated with tolerance index for flowering time (Table 4.1). The SNP 1_0946 was mapped within a cluster of aspartyl proteases. The other candidate genes consisting of *Vigun03g417300.1*, *Vigun03g417700.1*, *Vigun08g220500.1*, and *Vigun08g220700.1* encode for importin alpha, Myb-like DNA-binding domain, 5'-AMP-activated protein kinase beta subunit,

and PPR repeat. No functional annotation was found for *Vigun08g220600.1* (Table 4.1). The results indicated two or more annotated genes in the vicinity of the significant SNPs associated with tolerance index for 100-seed weight (Table 4.1). Out of the 5 SNPs associated with tolerance index for 100-seed weight, 4 were mapped within a large cluster of cytochrome P450 and histone-modifying enzymes such as lysine-specific histone demethylase 1 homolog 1.

GWAS suggested a total of 35 SNPs associated with tolerance index for grain yield under drought stress (Table 4.2). Of which, only three were not mapped in the vicinity of an annotated gene. The loci associated with tolerance index for grain yield was rich in biomolecule transporters such as transmembrane amino acid transporter protein, organic solute transporter Ostalpha, organic solute transporter, nucleoside transporter, organic anion transporter polypeptide (OATP) family, inositol transporter 4-related, and sodium-dependent phosphate transporters. Oxidoreductases such as quinone oxidoreductase PIG3 and pyridine nucleotide-disulphide oxidoreductase were also found to be prevalent (Table 4.2). Epigenetic-related proteins such as lysine-specific histone demethylase 1 homolog 1, JMJC domain-containing histone demethylation protein, and demethylmenaquinone methyltransferase were also identified. A MYB transcription-related factor was also found for tolerance index for grain yield.

Network-guided GWAS

An association network was established in order to investigate the possible interactions existing between loci which were found to be significantly associated to each tolerance index trait in the MAGIC cowpea population evaluated in this study under drought stress. In addition, significantly associated loci for traits reported by Huynh et al. (2018) and Olatoye et al. (2019) were also incorporated into the network. The network was designed to be an extension of the

GWAS analysis in such a way that the SNPs in high LD (Linkage disequilibrium) with the SNP having the highest LOD value for each trait were used to perform the analysis.

The network-guided GWAS indicated 12 independent subnetworks as shown in Fig. 4.4. The solid diamonds on Fig. 4.4 showed the tolerance index trait, whereas the solid circles indicated to traits investigated by Huynh et al. (2018) and Olatoye et al. (2019). The solid dark grey circles surrounding each trait corresponded to the SNPs. These results provided a clear visualization of the genetic architecture affecting each trait and suggested that some traits were likely to be correlated at the genetic level, whereas other traits were more genetically independent from the others. Traits such as tolerance index for plant maturity (T2), tolerance index for flowering time (T3), and tolerance index for 100-seed weight (T6) had independent significant loci (Fig. 4.4), suggesting that these traits could have independent drought tolerance mechanism and should be investigated separately when studying drought tolerance in cowpea.

The network-guided GWAS revealed interacting loci for change in growth habit and tolerance index for grain as shown by the solid blue and red diamonds, respectively, in the upper right-corner of Fig. 4.4. The two interacting loci were highlighted using the empty red circles. This result suggested that tolerance index for grain yield and change in growth habit had common significantly associated loci. Interestingly, this network existing between loci affecting tolerance index for grain yield and change in growth habit was not identified via GWAS alone, indicating that a network analysis could complement GWAS to provide additional information to investigate the genetics of drought tolerance in cowpea.

The network analysis revealed common loci between traits, which were identified using GWAS. These findings showed that GWAS and network analysis could be used to validate each other. In addition, the network analysis displayed epistatic loci for each trait evaluated in this

study. Significant epistatic loci, shown by the interactions between SNPs within each trait, were found for tolerance index for grain yield, change in growth habit, and tolerance index for plant maturity (Fig. 4.4).

Genomic selection

Genomic selection was conducted using a ridge regression best linear unbiased predictor model (rrBLUP) for change in plant growth habit due to a restricted irrigation, tolerance index for plant maturity, tolerance index for flowering time, tolerance index for 100-seed weight, and tolerance index for grain yield. The accuracy of genomic selection was evaluated under different cross-validation folds. Overall, genomic selection was low for almost all traits. At each cross-validation fold, variation in genomic selection accuracy was identified between each tolerance index trait (Fig. 4.5). Genomic selection accuracy for change in growth habit was highest regardless of the training population size. The average genomic selection accuracy for change in growth habit was 0.18, 0.21, 0.19, 0.21, 0.19, 0.21, and 0.19 at 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, and 8-fold cross validation, respectively. Genomic selection accuracy for tolerance index for 100-seed weight was second highest at 2-fold (0.12), 3-fold (0.12), 5-fold (0.13), 6-fold (0.12), and 7-fold (0.15) cross validation (Fig. 4.5). The increase in training population size seemed to be more favorable to improving the genomic selection accuracy of tolerance for 100-seed weight than enhancing the genomic selection accuracy for tolerance index for grain yield. The lowest genomic selection accuracy was recorded for tolerance index for flowering time (2-fold: 0.05, 3-fold: 0.07, 4-fold: 0.07, 5-fold: 0.08, 6-fold: 0.08, 7-fold: 0.08, and 8-fold: 0.08) and for tolerance index for grain yield (2-fold: 0.05, 3-fold: 0.05, 4-fold: 0.05, 6-fold: 0.08, 7-fold: 0.08, and 8-fold: 0.08) (Fig. 4.5).

Discussion

Change in plant growth habit, tolerance index for plant maturity, tolerance index for flowering time, tolerance index for 100-seed weight, and tolerance index for grain yield were evaluated to quantify the relative tolerance to drought stress of the MAGIC cowpea population used for this study. Tolerance index has been used for efficiently assessing plant stress tolerance in previous studies (Ravelombola et al. 2018; Saad et al. 2014). Our results indicated a large variation in tolerance index trait among the cowpea genotypes evaluated in this study, suggesting that this population is genetically diverse and could be used to enhance drought tolerance in a cowpea breeding program. However, the Pearson's correlation coefficients analysis between the tolerance index traits were low, indicating that drought tolerance mechanism between the tolerance index traits could be independent. These results were in line with previously reported studies on the possible independent mechanisms affecting drought tolerance in cowpea (Singh et al. 1999; Verbree et al. 2015). The logistic regression model of change in plant growth habit on tolerance index for grain yield was significant, which suggested an association between these two traits. This funding was critical since it established a link between growth habit and tolerance to grain yield reduction due to drought stress in cowpea. Additional studies will be required to investigate the pathways that could lead to the association between plant growth habit and tolerance to the decrease in grain yield under restricted irrigation in cowpea.

Genome-wide association study (GWAS) was conducted to identify SNP markers associated with the tolerance index traits. The number of significant SNPs varied between the tolerance index traits. As expected, tolerance index for grain yield had the highest number of SNP markers, indicating that a large number of loci could contribute to maintaining high yield in cowpea genotypes subjected to restricted water supplies. These results were in agreement with

previous investigations reporting grain yield being a polygenic trait (Assefa et al. 2019; Diers et al. 2018). The MAGIC cowpea population used in this study was first investigated by Huynh et al. (2018) and Olatoye et al. (2019). They conducted GWAS for flowering time, plant maturity, plant growth habit, 100-seed weight, and grain yield under full irrigation and restricted irrigation, respectively. In this study, we improve their analysis by assessing the drought tolerance of each individual within the cowpea MAGIC population using the tolerance index formula (Ravelombola et al. 2018; Saad et al. 2014). The GWAS was re-analyzed based on tolerance indices. Results indicated the discovery of new loci affecting the tolerance index traits. These loci were not identified by Huynh et al. (2018) and Olatoye et al. (2019). Therefore, our findings complement the approach conducted by Huynh et al. (2018) and Olatoye et al. (2019) to investigate drought tolerance in the MAGIC cowpea population. In addition, we integrated the reported loci identified by Huynh et al. (2018) and Olatoye et al. (2019) into a network that displayed the newly discovered loci for tolerance index. The network analysis suggested a clear independency between the different loci, which supported our previous claim on the independency of drought tolerance mechanism affecting different traits in cowpea. Olatoye et al. (2019) investigated the epistatic interactions between loci affecting the traits evaluated by Huynh et al. (2018). These interactions were found using a network-guided approach as shown in Fig. 4.4, which suggests that the algorithm we used to establish the network analysis was valid. One of the significant findings from this current study was the discovery of two loci affecting both change in plant growth habit and tolerance index for grain yield (Fig. 4.4). These loci were rich in transmembrane amino acid transporters and MYB-transcription factors. The role of biomolecule transporters in regulating plant response to water deficit conditions has been well-documented. Jarzyniak and Jasiński (2014) stated that the transmembrane transporters

significantly affect stomatal and cuticular activities during drought stress in plant. These biomolecules could also affect root responses under water deficit conditions. MYB-transcription factors have been shown to assist plant with withstanding drought stress. The expression of MYB-transcription factors have been correlated with the capability of plants to survive under drought conditions (Butt et al. 2017; Tang et al. 2019; Stracke et al. 2001). These findings showed that the approach we used for investigating the genetic architecture of drought tolerance in this MAGIC cowpea population could efficiently target candidate genes that are relevant to drought tolerance. Genomic selection for change in growth habit, drought tolerance index for flowering time, plant maturity, 100-seed weight, and grain yield was conducted using a ridge regression best linear unbiased predictor model. Genomic selection has been proven to be effective when dealing with complex traits such as drought tolerance (Heffner et al. 2009; Ravelombola et al. 2019). In this study, genomic selection accuracy varied from low to moderate. This could be attributed to the complexity of the drought tolerance traits. Olatoye et al. (2019) evaluated the prediction accuracy of flowering time, maturity date, and seed size under full irrigation and restricted irrigation, respectively, from the data generated by Huynh et al. (2018) and using the same MAGIC population reported in this current work. The prediction accuracy was higher for flowering time, maturity date, and seed size under full irrigation and restricted irrigation, respectively. This could be explained by the fact that these traits were more heritable than their respective drought tolerance indices, which were calculated based on the ratio of the trait values from restricted irrigation and full irrigation, respectively. Even though the genomic selection accuracy varied from low to moderate, it can still supplement the phenotypic selection and would increase the genetic gain by at least 10% (Lozada et al. 2019).

Conclusions

In this study, a large variation in drought tolerance indices for plant growth habit, flowering time, plant maturity, 100-seed weight, and grain yield was found within the MAGIC cowpea population. New loci associated with these drought tolerance traits were identified and a network-guided strategy assisted with the discovery of overlapping significant loci associated with the drought tolerance indices. In addition, genomic selection accuracy varied from low to moderate. The results from this investigation will contribute to a better understanding of the genetic architecture governing drought tolerance in cowpea and could be used in cowpea breeding programs through marker-assisted selection (MAS) and genomic selection (GS).

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Tables

Table 4.1. Significant SNPs associated with growth habit change, tolerance indices for plant maturity, flowering time, and 100-seed weight with their respective LOD (-$\log_{10}$(p_value)) value, MAF (minor allele frequency), annotated gene found within a 40-kb genomic region flanking the significant SNP, and functional annotation corresponding to the candidate gene.

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
Growth habit change	2_40797	8	10549370	3.06	12.05	NA[a]	NA
	2_42112	8	10601329	3.06	12.05	NA	NA
	2_42607	8	11012105	3.41	31.33	*Vigun08g069700.1*	NA
	2_26924	8	13771284	4.06	17.67	*Vigun08g076600.1*	Aldehyde dehydrogenase family
	2_01300	8	14264077	3.88	17.27	*Vigun08g077200.1*	Organic solute transporter
	2_10658	8	15346859	3.88	17.27	*Vigun08g077800.1*	Multi-copper oxidase
	2_54501	8	16564006	3.88	17.27	*Vigun08g080000.1*	TLC ATP/ADP transporter
	2_45332	8	16871228	3.88	17.27	NA	NA
	2_06275	8	17354751	3.88	17.27	*Vigun08g082400.1*, *Vigun08g082500.1*	Membrane protein involved in ER to Golgi transport, NA
	2_43529	8	20159451	3.64	17.67	NA	NA
	2_40435	8	20618849	3.64	17.67	NA	NA
	2_50806	10	29754489	3.49	12.20	NA	NA
	2_26782	10	30148065	3.38	13.25	*Vigun10g104700.1*	Cytochrome P450
	2_38918	10	30517553	3.25	13.31	*Vigun10g106600.1*	SNARE protein GS28
Tolerance index for maturity	2_16403	2	32138108	3.13	42.17	*Vigun02g180500.1*	Beta-1,3-N-acetylglucosaminyltransferase
	2_45148	2	32146045	3.13	42.17	*Vigun02g180600.1*, *Vigun02g180700.1*, *Vigun02g180500.1*	Aldose 1-epimerase, Leucine Rich Repeat, Beta-1,3-N-acetylglucosaminyltransferase
	2_55009	7	14098180	3.54	13.65	NA	NA
	2_51274	7	14976910	3.54	13.65	NA	NA
	2_21981	8	1801037	5.68	20.08	*Vigun08g020700.1*	Kinase-like
	2_10862	8	1929122	3.20	33.33	*Vigun08g022000.1*, *Vigun08g022100.1*, *Vigun08g021900.1*	Patatin-like phospholipase, Patatin-like phospholipase, Patatin-like phospholipase
	2_10861	8	1929370	3.20	33.33	*Vigun08g022000.1*, *Vigun08g022100.1*, *Vigun08g021900.1*	Patatin-like phospholipase, Patatin-like phospholipase, Patatin-like phospholipase

Table 4.1. (Cont.)

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	*Gene_ID*	Functional_annotation
	1_0806	8	1950113	3.00	32.93	*Vigun08g022300.1*, *Vigun08g022400.1*, *Vigun08g022500.1*, *Vigun08g022200.1*	Eukaryotic translation initiation factor 3 -related, Carboxylesterase family, NA, Patatin-like phospholipase
	2_21676	8	1965506	3.00	32.93	*Vigun08g022800.1* , *Vigun08g022900.1*, *Vigun08g022700.1*, *Vigun08g022600.1*	NA, Origin recognition complex subunit 2, NA, NA
	2_21804	8	1970485	3.00	32.93	*Vigun08g022900.1*, *Vigun08g022800.1*	Origin recognition complex subunit 2, NA
	2_23871	8	1980059	3.00	32.93	*Vigun08g023000.1*, *Vigun08g023100.1*, *Vigun08g022900.1*	Protein phosphatase 2C, NA, Origin recognition complex subunit 2
	2_23870	8	1980643	3.00	32.93	*Vigun08g023000.1*, *Vigun08g023100.1*	Protein phosphatase 2C, NA
	2_44136	8	1985249	3.00	32.93	*Vigun08g023000.1*, *Vigun08g023100.1*	Protein phosphatase 2C, NA
	2_14976	8	2006627	4.23	28.92	*Vigun08g023300.1*, *Vigun08g023200.1*	Phosphatidate phosphatase, BRI1 kinase inhibitor 1
	2_40337	8	2013873	4.27	28.34	*Vigun08g023500.1*, *Vigun08g023400.1*, *Vigun08g023300.1*	EF hand, EF hand, Phosphatidate phosphatase
	2_14158	8	2338417	3.63	33.33	*Vigun08g026400.1*	Proteinaceous RNAse P 1-chloroplastic/mitochondrial
	2_16735	8	2361920	3.47	32.93	*Vigun08g026700.1*	Aminotransferase class I and II
	2_41533	8	2384266	3.34	33.20	NA	NA
Tolerance index for flowering time[b]	2_06470	3	62407410	2.84	12.45	*Vigun03g417300.1*	Importin alpha
	2_52919	3	62409665	2.84	12.45	*Vigun03g417300.1*	Importin alpha
	2_06137	3	62434051	2.84	12.45	*Vigun03g417700.1*	Myb-like DNA-binding domain
	1_0946	3	63722355	2.83	11.65	*Vigun03g433200.1*, *Vigun03g433300.1*	Aspartyl proteases , Aspartyl proteases

Table 4.1. (Cont.)

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	*Gene_ID*	Functional_annotation
Tolerance index for flowering time[b]	2_27706	8	37928961	2.83	19.68	*Vigun08g220500.1*, *Vigun08g220600.1*, *Vigun08g220700.1*	5'-AMP-activated protein kinase beta subunit, NA, PPR repeat
Tolerance index for 100-seed weight[b]	2_11122	4	1483784	2.95	11.34	*Vigun04g019600.1*, *Vigun04g019700.1*, *Vigun04g019500.1*	Cytochrome P450, Cytochrome P450, NA
	2_03731	4	1523145	2.89	10.84	*Vigun04g019900.1*, *Vigun04g020000.1*	Cytochrome P450, Cytochrome P450
	2_14932	4	1548833	2.89	10.84	*Vigun04g020400.1*, *Vigun04g020500.1*, *Vigun04g020300.1*, *Vigun04g020200.1*	Lysine-specific histone demethylase 1 homolog 1, Serine/threonine-protein phosphatase PP2A 65 kda regulatory subunit, Aspartyl proteases, Cytochrome P450
	2_34365	4	1549730	2.89	10.84	*Vigun04g020400.1*, *Vigun04g020500.1*, *Vigun04g020300.1*, *Vigun04g020200.1*	Lysine-specific histone demethylase 1 homolog 1, Serine/threonine-protein phosphatase PP2A 65 kda regulatory subunit, Aspartyl proteases, Cytochrome P450
	2_07882	4	1556026	2.89	10.84	*Vigun04g020500.1*, *Vigun04g020600.1*, *Vigun04g020400.1*	Serine/threonine-protein phosphatase PP2A 65 kda regulatory subunit, Aluminium activated malate transporter, Lysine-specific histone demethylase 1 homolog 1

[a]NA indicates no information was available.

[b]No SNPs having an LOD value greater than the chosen threshold (3) were found so that the top 5 SNPs with the highest LOD value are presented.

Table 4.2. Significant SNPs associated with tolerance index for grain yield with their respective LOD (-log10(p_value)) value, MAF (minor allele frequency), annotated gene found within a 40-kb genomic region flanking the significant SNP, and functional annotation corresponding to the candidate gene.

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
Tolerance index for grain yield	2_31564	6	32057972	3.35	9.64	*Vigun06g206600.1*	NA[a]
	2_31565	6	32058239	3.35	9.64	*Vigun06g206600.1*, *Vigun06g206700.1*	NA, Transmembrane amino acid transporter protein
	2_30808	6	32061499	3.35	9.64	*Vigun06g206700.1*, *Vigun06g206600.1*	Transmembrane amino acid transporter protein, NA
	2_19053	6	32061827	3.35	9.64	*Vigun06g206700.1*, *Vigun06g206600.1*	Transmembrane amino acid transporter protein, NA
	2_33474	6	32070478	3.35	9.64	*Vigun06g206800.1* , *Vigun06g206900.1*, *Vigun06g207000.1*	Endonuclease 1, Ribosomal proteins L26 eukaryotic, Quinone oxidoreductase PIG3
	2_28131	6	32077832	3.35	9.64	*Vigun06g207000.1*, *Vigun06g207100.1*, *Vigun06g206900.1*, *Vigun06g206800.1*	Quinone oxidoreductase PIG3, Organic solute transporter Ostalpha, Ribosomal proteins L26 eukaryotic, Endonuclease 1
	2_28570	6	32088910	3.09	9.80	*Vigun06g207300.1*, *Vigun06g207400.1*, *Vigun06g207200.1*, *Vigun06g207100.1*	NA, NA, NA, Organic solute transporter
	2_10632	6	32089786	3.35	9.64	*Vigun06g207300.1*, *Vigun06g207200.1*	T28P6.11 protein, NA
	2_13247	6	32107028	3.35	9.64	*Vigun06g207600.1*, *Vigun06g207700.1*	Syntaxin, Zinc finger CW-type coiled-coil domain protein 3
	2_18126	6	32147410	3.35	9.64	*Vigun06g208000.1*	NA
	2_14728	6	32165112	3.35	9.64	*Vigun06g208300.1*, *Vigun06g208200.1*	NA, Ribonucleoprotein
	2_02004	6	32184138	3.35	9.64	*Vigun06g208400.1*	Pyridine nucleotide-disulphide oxidoreductase
	2_25332	6	32186496	3.35	9.64	*Vigun06g208400.1*	Pyridine nucleotide-disulphide oxidoreductase
	2_33745	6	32186893	3.35	9.64	*Vigun06g208400.1*, *Vigun06g208500.1*	Pyridine nucleotide-disulphide oxidoreductase, Armadillo/beta-catenin-like repeat-containing protein

Table 4.2. (Cont.)

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	*Gene_ID*	Functional_annotation
	2_25331	6	32188321	3.35	9.64	*Vigun06g208400.1, Vigun06g208500.1*	Pyridine nucleotide-disulphide oxidoreductase, Armadillo/beta-catenin-like repeat-containing protein
	2_25334	6	32189396	3.51	8.23	*Vigun06g208500.1, Vigun06g208400.1*	Beta catenin-related armadillo repeat-containing, Pyridine nucleotide-disulphide oxidoreductase
	2_25333	6	32189710	3.35	9.64	NA	NA
	2_30533	6	32204324	3.35	9.64	*Vigun06g208800.1, Vigun06g208700.1, Vigun06g208600.1*	Inositol monophosphatase, Wound-induced protein, Vesicle-associated protein 4-2-related
	2_31969	6	32234310	3.35	9.64	*Vigun06g209000.1, Vigun06g209100.1, Vigun06g208900.1*	Chaperone-activity of BC1 complex CABC1 -related, JMJC domain-containing histone demethylation protein, MYB transcription related
Tolerance index for grain yield	2_32622	6	32239677	3.35	9.64	*Vigun06g209100.1, Vigun06g209000.1*	JMJC domain-containing histone demethylation protein, Chaperone-activity of BC1 complex CABC1 -related
	2_50666	6	32250975	3.11	9.92	*Vigun06g209200.1, Vigun06g209300.1, Vigun06g209100.1*	Nucleoside transporter, Nucleoside transporter, JMJC domain-containing histone demethylation protein
	2_21574	6	32454860	3.05	9.64	*Vigun06g212000.1, Vigun06g212100.1, Vigun06g212200.1, Vigun06g211900.1*	NA, NA, NA, NPH3 family
	2_29076	6	32461137	3.05	9.64	*Vigun06g212200.1, Vigun06g212300.1, Vigun06g212100.1, Vigun06g212000.1*	NA, Demethylmenaquinone methyltransferase, NA, NA
	1_0823	6	32612013	3.05	9.64	*Vigun06g214900.1, Vigun06g214800.1, Vigun06g214700.1*	Methionine sulfoxide reductase, NA, Organic Anion Transporter Polypeptide (OATP) family
	2_15103	6	32612013	3.05	9.64	*Vigun06g214900.1, Vigun06g214800.1*	Methionine sulfoxide reductase, NA

Table 4.2. (Cont.)

Traits	SNP	Chromosome	Position (bp)	LOD	MAF(%)	*Gene_ID*	Functional_annotation
Tolerance index for grain yield	2_01303	9	4760699	3.24	13.25	*Vigun09g048900.1*, *Vigun09g048800.1*	Inositol transporter 4-related, PPR repeat
	2_51818	9	4789752	3.38	12.85	NA	NA
	2_35898	9	4877591	3.16	13.65	*Vigun09g049500.1*, *Vigun09g049600.1*	PPR repeat, F-box and WD40 domain protein
	2_23949	9	5346101	3.26	14.46	*Vigun09g053500.1*, *Vigun09g053600.1*, *Vigun09g053400.1*	Protein phosphatase 2C, Sodium-dependent phosphate transporters, small subunit ribosomal protein S11e
	2_23950	9	5347304	3.26	14.46	*Vigun09g053500.1*, *Vigun09g053600.1*, *Vigun09g053700.1*, *Vigun09g053400.1*	Protein phosphatase 2C, Sodium-dependent phosphate transporters, NA, small subunit ribosomal protein S11e
	2_11952	9	5364438	3.26	14.46	*Vigun09g053800.1*, *Vigun09g053700.1*	Ring finger domain, NA
	2_34102	9	7298753	3.08	9.79	*Vigun09g068400.1*, *Vigun09g068300.1*	Alpha/beta hydrolase family, GPI biosynthesis protein family Pig-F

Figures

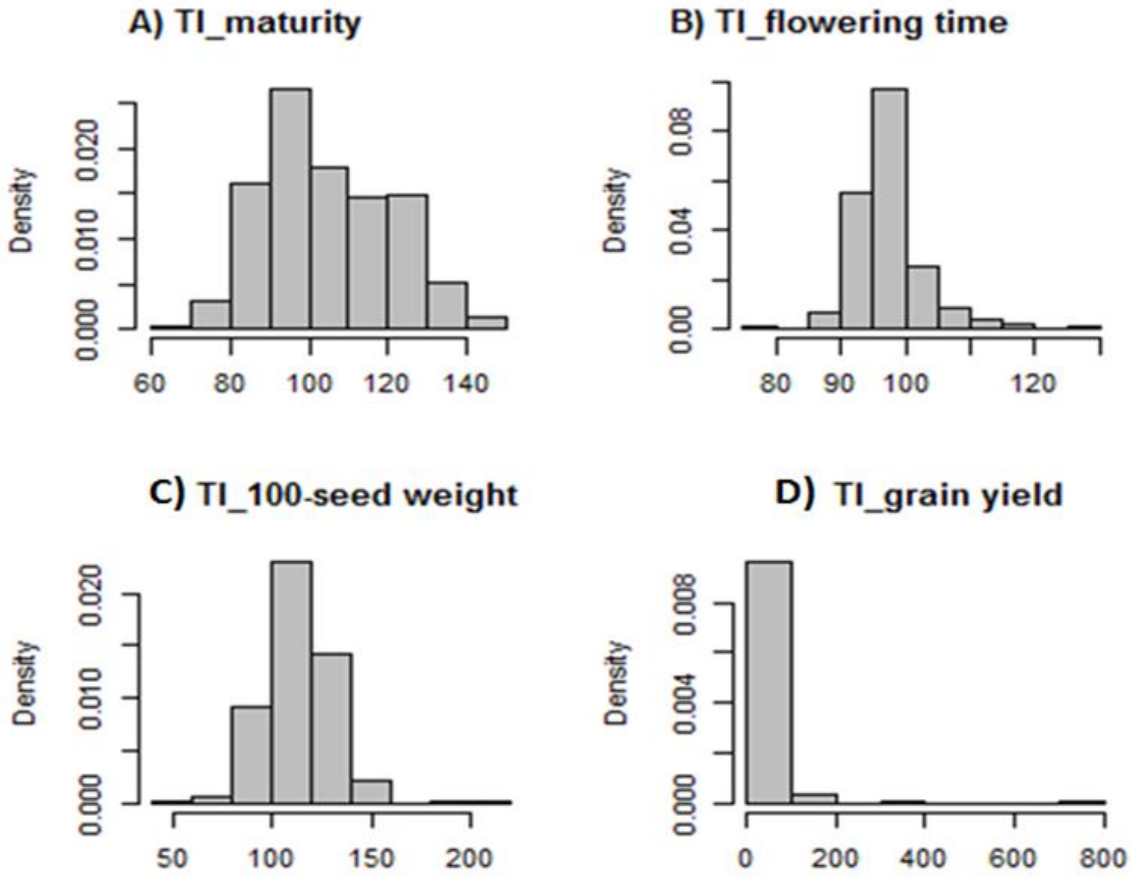

Fig. 4.1. Distribution of drought tolerance index for A) maturity, B) flowering time, C) 100-seed weight, and D) grain yield.

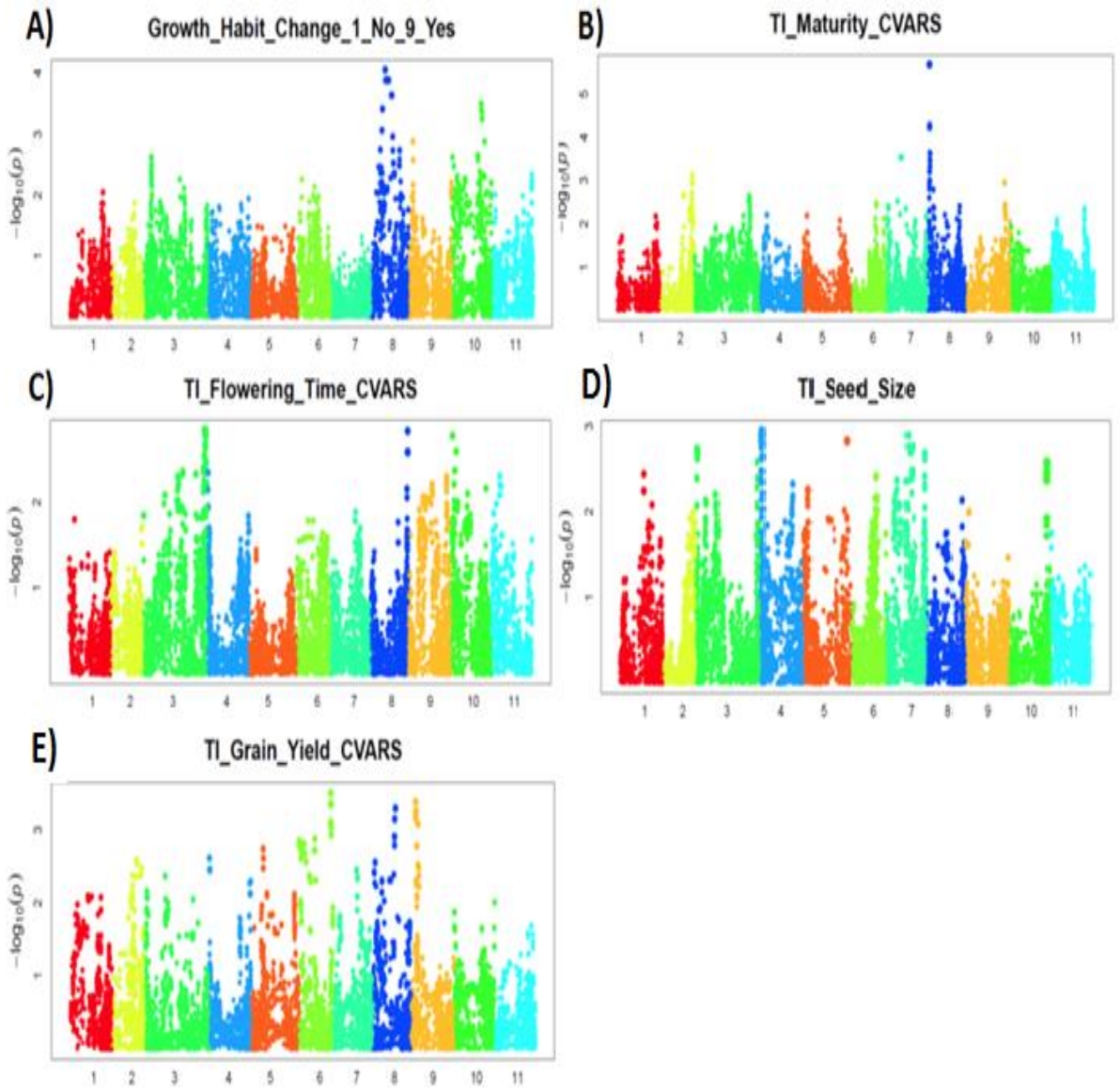

Fig. 4.2. Manhattan plots showing the LOD (-$\log_{10}$(p_value)) for each SNP used to conduct GWAS. The y-axis each of Manhattan plot represents the LOD (-$\log_{10}$(p_value)) and the x-axis displays the chromosome number. Color coding on each Manhattan plot was chromosome-wise. A) Manhattan plot for change in growth habit, B) Manhattan plot for tolerance index for maturity, C) Manhattan plot for tolerance index for flowering time, D) Manhattan plot for tolerance index for seed size, and E) Manhattan plot for tolerance index for grain yield.

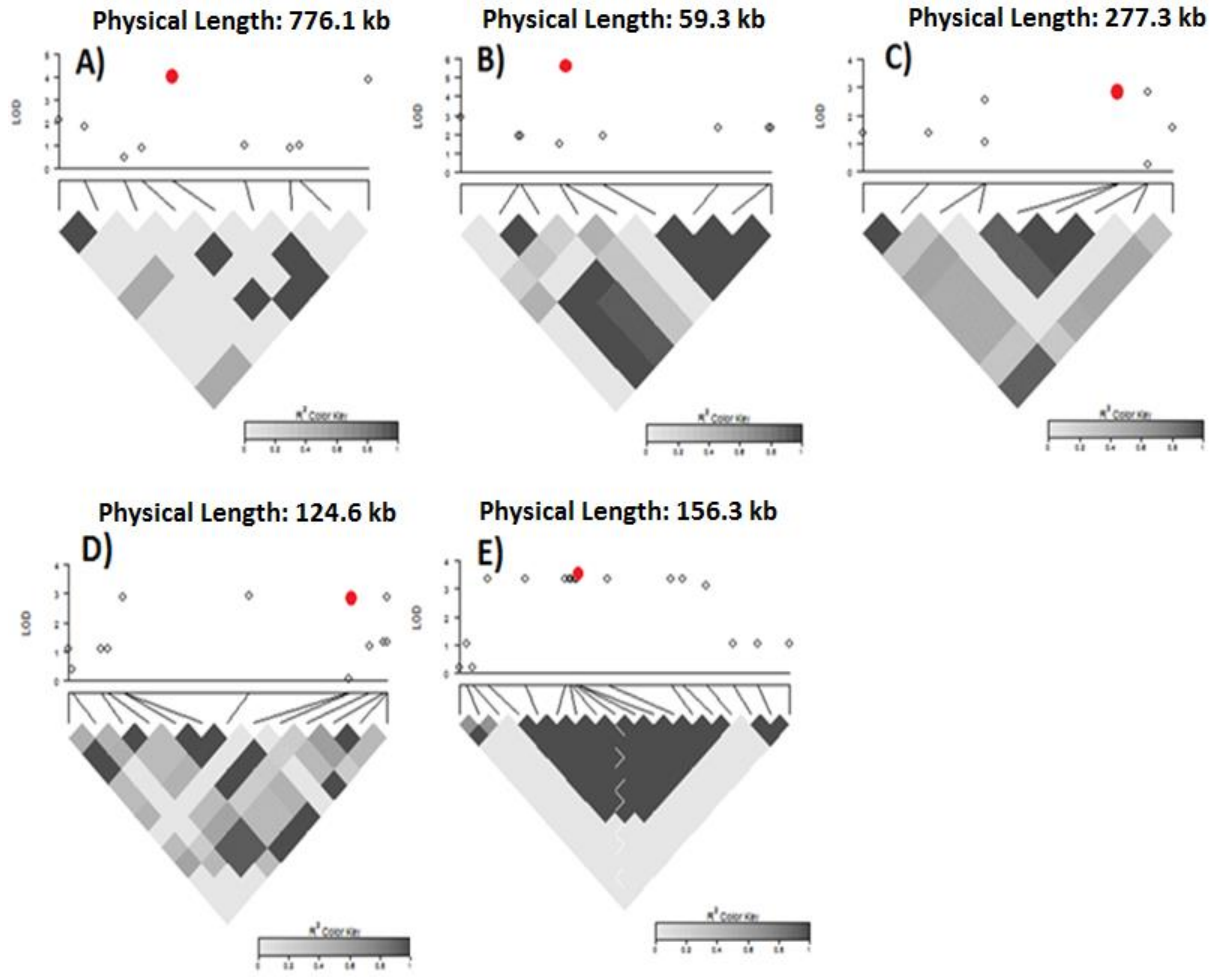

Fig. 4.3. Local Manhattan plots and linkage disequilibrium (LD) heatmaps around the most significant SNP for each trait, which is shown by the red dots. For each graph, the y-axis of the local Manhattan represents the LOD ($-\log_{10}$(p_value)) of the corresponding SNP. The x-axis of the local Manhattan shows the physical distance (kb) between two adjacent SNPs. Below each local Manhattan plot is displayed the LD heatmap. Color coding within the LD heatmap ranges from white to black and the parameter for estimating pairwise LD was R square. The white color within the LD heatmap corresponds to an R-square value of 0, whereas the black color corresponds to an R-square value of 1. A) Local Manhattan plot and LD heatmap on a 776.1-kb region of chromosome 8 harboring the SNP 2_26924 associated with change in growth habit, B) Local Manhattan plot and LD heatmap on a 59.3-kb region of chromosome harboring the SNP 2_21981 associated with tolerance index for maturity, C) Local Manhattan plot and LD heatmap on a 227.3-kb region of chromosome 3 harboring the SNP 2_06470 associated with tolerance index for flowering time, D) Local Manhattan plot and LD heatmap on a 124.6-kb region of chromosome 4 harboring the SNP 2_11122 associated with tolerance index for seed weight, and E) Local Manhattan plot and LD heatmap on a 156.3-kb region of chromosome 6 harboring the SNP 2_25334 associated with tolerance index for yield.

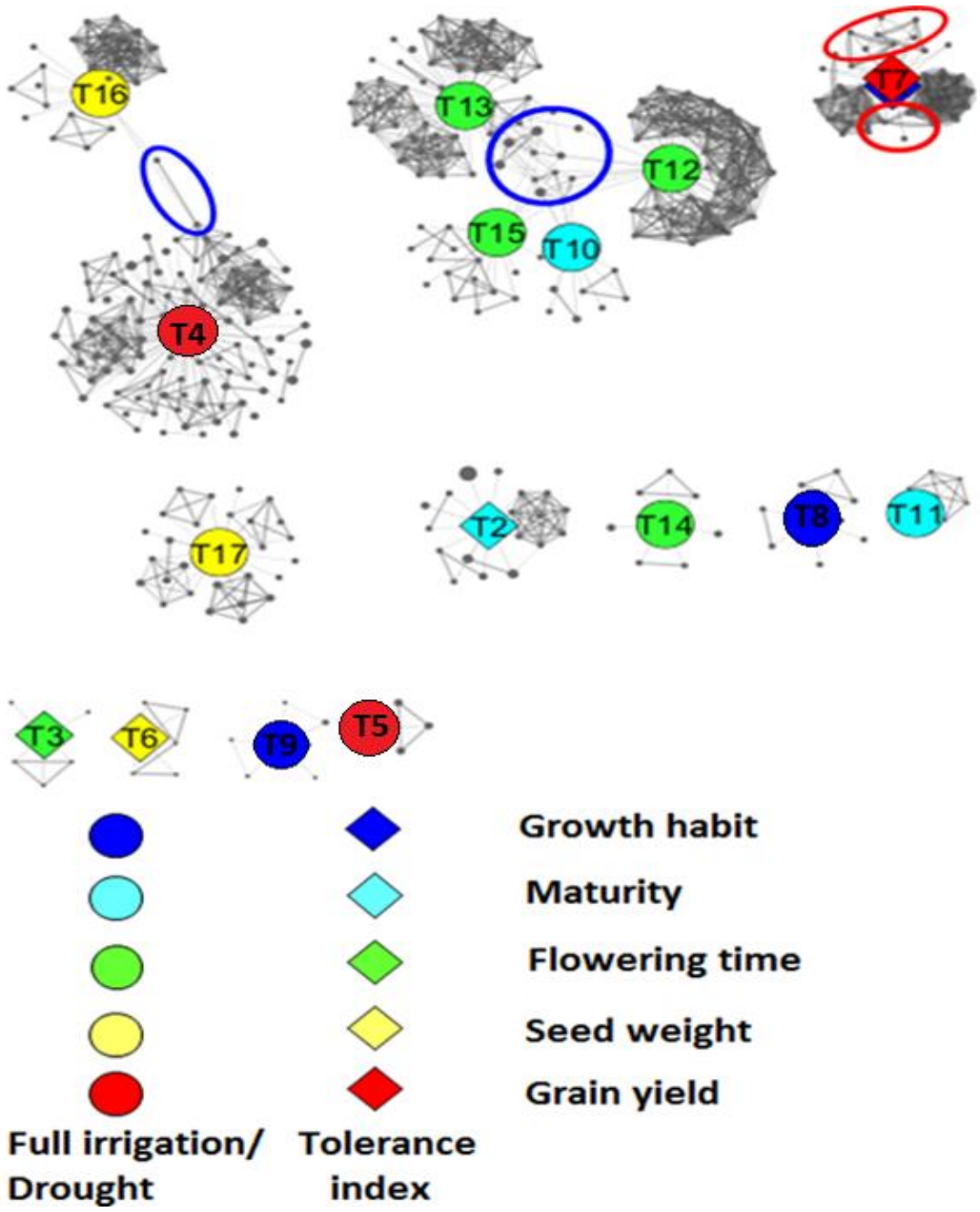

Fig. 4.4. Association networks displaying the tolerance indices of growth habit, maturity, flowering time, seed weight, and grain yield under drought stress in a MAGIC cowpea population. The solid circles represent the traits evaluated under full irrigation and drought stress conditions. The solid diamonds correspond to the tolerance indices for different traits under drought stress. The solid dark grey circles show the significant SNPs associated with each trait. The size of each SNP node is proportional to its LOD value. Edges between nodes are represented by solid black lines. Edges with similar size are used to link each trait node to each SNP node. Edges with different size are used to link different SNP nodes. The link power of the

edge between each SNP node was the R-square linkage disequilibrium (LD) value between the two SNPs. The empty red circles represent the significant loci associated with the tolerance index trait values. The empty blue circles display the epistatic loci reported by Olatoye et al. (2019). The legend corresponding to each trait node was the following: T1 = Tolerance index for growth habit change, T2 = tolerance index for plant maturity, T3 = tolerance index for flowering time, T4 = grain yield under full irrigation, T5 = grain yield under drought stress, T6 = tolerance index for 100-seed weight, T7 = tolerance index for grain yield, T8 = growth habit under full irrigation, T9 = growth habit under drought stress, T10 = maturity under full irrigation, T11 = maturity under drought stress, T12 = flowering time under full irrigation, T13 = flowering time under drought stress, T14 = flowering time under full irrigation at UCR, T15 = flowering time under drought stress at UCR, T16 = seed weight under full irrigation, and T17 = seed weight under drought stress. Tolerance index for flowering time at UCR was not calculated since the experiments were conducted under two different seasons at this location.

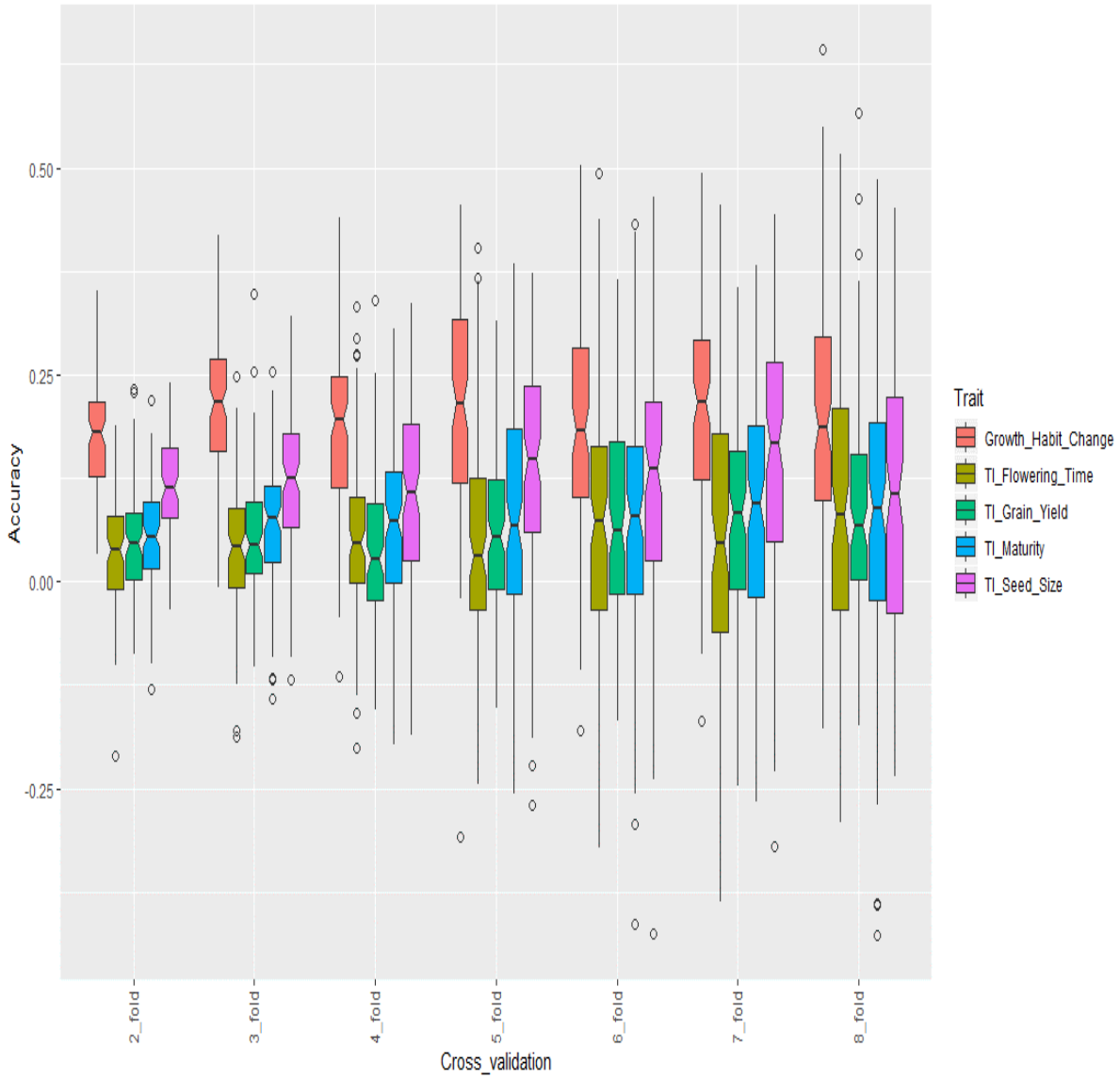

Fig. 4.5. Genomic selection accuracy using a ridge regression best linear unbiased predictor model (rrBLUP) for change in plant growth habit, tolerance index for flowering time, grain yield, plant maturity, and 100-seed weight. Genomic selection was conducted using a 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, and 8-fold cross validation. The y-axis of the figure represents the accuracy of genomic selection at each cross-validation fold for each trait.

Appendices

Table S4.1. List of cowpea genotypes with the corresponding phenotypic data on growth habit change, tolerance index for maturity, tolerance index for flowering time, tolerance index for 100-seed weight, and tolerance index for grain yield. The genotypes with missing data were removed from the list.

Table S4.2. Significant SNPs associated with growth habit at CVARS under full irrigation, growth habit at CVARS under restricted irrigation, maturity at CVARS under full irrigation, maturity at CVARS under restricted irrigation, flowering time at CVARS under full irrigation, flowering time at CVARS under restricted irrigation, flowering at UCR under full irrigation, flowering time at UCR under restricted irrigation, 100-seed weight at CVARS under full irrigation, 100-seed weight at CVARS under restricted irrigation, grain yield at CVARS under full irrigation, and grain yield at CVARS under restricted irrigation.

Chapter 5. Genetic Architecture of Salt Tolerance in a Multi-Parent Advanced Generation Inter-Cross (MAGIC) Cowpea Population

Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid legume species that has multiple uses. It provides good quality protein for humans and can also be used as supplement to fodder for livestock. Previous reports have shown that soil salinity is a growing threat to cowpea production, thus salt-tolerant cowpea cultivars need to be developed. Therefore, the objectives of this study were to evaluate salt tolerance in a Multi-Parent Advanced Generation Inter-Cross (MAGIC) cowpea population, to conduct a genome-wide association study (GWAS) for salt tolerance, to identify single nucleotide polymorphism (SNP) markers associated with salt tolerance, and to perform genomic selection (GS) for salt tolerance. A total of 234 MAGIC lines along with their eight founders were evaluated for salt tolerance under greenhouse conditions. GWAS was conducted using a total of 32,047 filtered SNPs. A large variation in traits evaluated for salt tolerance was identified among the MAGIC lines were found. A total of 7, 2, 18, 18, 3, 2, 5, 1 and 23 SNPs were associated with number of dead plants, salt injury score, leaf SPAD chlorophyll under salt treatment, relative tolerance index for leaf SPAD chlorophyll, fresh leaf biomass under salt treatment, relative tolerance index for fresh leaf biomass, relative tolerance index for fresh stem biomass, relative tolerance index for the total above-ground fresh biomass, and relative tolerance index for plant height, respectively, with overlapping SNP markers between traits. Candidate genes encoding for proteins involved in ion transport such as Na^+/Ca^{2+} K^+ independent exchanger and H^+/oligopeptide symporter were identified were found. Epistatic interactions were identified. GS accuracy varied from low to

moderate. These results will have direct applications in breeding programs aiming at improving salt tolerance in cowpea through marker-assisted selection and genomic selection. To the best of our knowledge, this study was one of the earliest reports using a MAGIC population to investigate the genetic architecture of salt tolerance in cowpea.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid legume crop ($2n=2x=22$) that is widely grown in various regions such as Africa, Central and South America, Asia, the Middle East, southern Europe, Oceania, and the western and southern United States (Perrino et al. 1993). The annual worldwide cowpea production is estimated to be 5.4 million tons of cowpea seed with Nigeria being the top producer (Olufajo 2012). Cowpea is grown on a total of 11 million hectares of croplands (Bahadur et al. 2017). Cowpea is a legume that has a multipurpose use. It provides an excellent and affordable source of protein to human (Weng et al. 2017). Cowpea seeds contain nutrients that are necessary to human's heath. One hundred g of cowpea seed has on average, in mg, 6.8 iron, 4.1 zinc, 1.5 manganese, 510.0 phosphorus, and 1430.0 potassium (Frota et al. 2008). The significant amount of antioxidant compounds within cowpea seeds provides additional nutritional value that would be of interest when incorporated into the diet (Moreira-Araújo et al. 2017; Qin et al. 2016).

In addition to significantly contributing to enhancing the human's diet, cowpea leaves could be used to supplement low quality feed for livestock. This practice is prevalent in sub-Sahara Africa (Olufajo 2012). Cowpea also provides effective ecosystem services by limiting soil erosion. In fact, with its excellent root architecture, cowpea can be used as cover crop. The

use of cowpea as cover crop has attracted considerable attention in recent years (Wilson et al. 2006).

Despite being a rich-protein crop, enhancing feed nutritional quality for livestock, and contributing to the ecosystem services, cowpea cultivation can be substantially limited by stresses such as soil salinity. Salinity has been reported to increasingly affecting agricultural production worldwide and contributing to an annual loss of 12 billion US dollars (Allakhverdiev et al. 2000; Läuchli and Lüttge 2002). Soil salinity has resulted from the accumulation of cations consisting of K^+, Mg^{2+}, Ca^{2+}, and Na^+ and anions such as NO_3^-, HCO_3^-, SO_4^{2-}, and Cl^-within the soil profile (Wallender and Tanji 2011). Soil salinity affects more than 19.6 million of croplands in the U.S. and areas facing salinity-related issues have increased (Shannon 1997). Cowpea cultivation is common in semi-arid areas since cowpea has a better capability to withstand a limited water condition (Karapanos et al. 2017). However, earlier reports suggested that the limited rainfall occurring in semi-arid areas significantly contributed to the salt-related compounds not being effectively leached out from the soil profile, which can exacerbate the effects of salinity on cowpea grown in semi-arid regions (Chinnusamy et al. 2005).

Salinity is also increased by the use of poor-quality irrigation water. In the U.S., cowpea cultivation is prevalent in the southern regions (Agbicodo et al. 2009). However, irrigation from groundwater in the southern U.S. accounts for more than 66 % of the water source used for agricultural activities and can contain up to 1639 mg of Cl^- per L of water (Kresse and Clark 2008; Zeng et al. 2017). A sodium chloride (NaCl) concentration greater than 90 mM, releasing around 526 mg/L of Cl^- , could significant reduce cowpea yield (Düzdemir et al. 2009). Therefore, salinity could limit cowpea production in southern U.S.

Significant cowpea production can also be found in western U.S. in addition to the increasing interest in the use of cowpea as cover crop in this part of the country (Wilson et al. 2006). However, the Coachella Valley of California has been increasingly impacted by salinity, which will limit cowpea cultivation expansion in western U.S. (Bower et al. 1969; Wilson et al. 2006). Salinity can also be increased by the overuse of fertilizers or natural factors such as rock weathering (Omami and Hammes 2006).

Salinity affects most of development and growth stages of cowpea with germination and seedling stages being the most sensitive stages (Dong et al. 2019; Waltram Ravelombola et al. 2017). Salinity can completely suppress cowpea germination and lead to plant death in cowpea seedlings (Ravelombola et al. 2017). In addition, high salt ion concentrations will result in significant height, biomass, and chlorophyll reduction in cowpea, causing serious physiological impairment within cowpea plants (Dong et al. 2019). Breeding for salt-tolerant cowpea cultivars would be one of the most affordable ways to limit the negative effects of salinity on cowpea cultivation. Significant efforts towards investigating salt tolerance in cowpea have been conducted in relatively recent years.

Salt tolerance at germination stage of a total of 151 diverse cowpea genotypes have been reported (Ravelombola et al. 2017). This study was complemented by Dong et al. (2019) who have identified promising cowpea genotypes that better withstand salt stress at seedling stage. Molecular markers have substantially assisted plant breeders with rapidly developing cultivars (Xu and Crouch 2008). Our previous article reported the first molecular markers associated with salt tolerance in cowpea (Ravelombola et al. 2017). Three SNP markers, Scaffold87490_622, Scaffold87490_630, and C35017374_128, were found to be associated with salt tolerance at both seedling stage and germination stages, and a total of 7 SNPs

Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 were reported to be seedling stage-specific in cowpea (Ravelombola et al. 2017). The aforementioned research was carried out on an association panel consisting of diverse cowpea germplasm but having a limited population size, which reduces the likelihood of finding rare alleles that potentially affect salt tolerance. This can be addressed by conducting a genome-wide association analysis (GWAS) on a multi-parent advanced generation inter-cross (MAGIC) population. The development of a MAGIC population can increase the frequency of rare alleles while providing a significant recombination between the chromosomal sections (Bandillo et al. 2013; Gaur et al. 2012; B. E. Huang et al. 2012; Kover et al. 2009).

The first MAGIC cowpea population was developed by Huynh et al. (2018). The founders were parents having desirable agronomic traits such as high yield, drought tolerance, resistance to diseases and insects (Huynh et al. 2018). However, salt tolerance was not investigated for this MAGIC population despite of salinity being an increasing threat to cowpea production worldwide. In addition, genomic selection has recently attracted significant scientific attention since it can contribute to achieving a faster genetic gain per unit of time in plant breeding (Meuwissen et al. 2001).

Previous investigations showed that genomic selection was efficient in breeding for complex agricultural traits (Bao et al. 2014). However, genomic selection-related research for salt tolerance in cowpea remains very limited despite of its usefulness in advancing cowpea breeding program aiming at improving salt tolerance. Therefore, the objectives of this study were to evaluate the salt tolerance among the MAGIC lines, to identify genotypes that are salt-

tolerant, to conduct GWAS and identify SNP markers associated with salt tolerance in this MAGIC population, and to assess the accuracy of GS for salt tolerance.

Materials and methods

Population development and genotyping

The MAGIC population was established using a total of 8 founders (IT89KD-288, IT84S-2049, CB27, IT82E-18, SuViTa_2, IT00K-1263, IT84S-2246, and IT93K-503-1) by Huynh et al. (2018) and the first crosses were conducted in 2011. The eight parents were cultivars and breeding lines from Burkina Faso, Nigeria, and the United States. A full description of the details regarding population development was previously reported (Huynh et al. 2018). IT93K-503-1 was a drought-tolerant breeding line that was established by the scientists from IITA, Nigeria (Muchero et al. 2009b). The other founders were proven to have desirable traits such as resistance to *Striga*, fungi, bacteria, viruses, foliar thrips, root-knot nematode, and heat stress (Ehlers et al. 2000; Huynh et al. 2016; Lucas et al. 2012; Muchero et al. 2009; Muchero et al. 2011; Ouédraogo et al. 2002; Pottorff et al. 2014). A total of 305 $F_{8:10}$ RIL lines were obtained from the University of California, Riverside, with 10 seeds each. Seeds were hand-planted using a 5-foot long row for each line and established at the research station of the University of Arkansas, Fayetteville during the summer of 2018. Some lines were not able to flower due to photoperiodism under the Arkansas climate. At harvest, a total of 234 lines were harvested. Seeds from each row were harvested separately from the other rows, but bulk-harvested within each row. Therefore, we investigated a total of 234 $F_{8:11}$ RIL lines along with their eight parents for the salt tolerance evaluation.

The MAGIC population and the founders were genotyped using a total of 51,128 SNPs obtained from the Illumina Cowpea Consortium Array (Muñoz-Amatriaín et al. 2017). An extensive study on the genetic diversity analysis of this population was previously reported (Huynh et al. 2018). After SNP quality check, a total of 32,047 SNPs were used for further analysis (missing data<10%, heterozygosity<10%, and minor allele frequency>5%).

Growth conditions and experiment design

Salt tolerance evaluation was conducted using a previously described methodology (Ravelombola et al. 2019). The experiment was carried out in the greenhouse of Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville where the average temperature was 26 °C/21 °C (day/light) and the daylight length was 14 h (Fig. 5.1). Cowpea seeds were sown in pots previously filled up with 100 g Sunshine Natural & Organic (Agawam, MA). Holes were placed at the bottom of each pot to prevent waterlogging, which could lead to plant root asphyxia. In addition, paper towels were placed at the bottom of each pot to prevent soil from leaking during irrigation. In each pot, a total of eight seeds were sown and thinned to a total of four vigorous and uniform plants at one week after emergence. Plants were fertilized weekly by applying a solution of 50 mL of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI) to each pot.

The experiment was run two times (used as a blocking variable) with two replications at each time due to limited number of seeds and space constraints. Therefore, each MAGIC line was replicated 4 times. Pots containing cowpea plants were placed on rectangular plastic trays to make the irrigation process more convenient. For each genotype, two pots were used as control by using deionized water during irrigation and two other pots were assigned to the salt

treatment. The two pots assigned to each treatment category (deionized water/salt treatment) corresponded to the two replications within each run.

Salt treatment (NaCl) started when the first trifoliate leaf began to expand (V1 stage) (Fehr et al. 1971). Salt concentration was 200 mM NaCl as previously suggested (Abeer et al. 2015; Ashebir et al. 2013; Paul et al. 2011; Ravelombola et al. 2017). Irrigation was conducted by supplying to each tray containing a total of 12 pots with either deionized water or salt solution. Irrigation was achieved such a way that two-third of pot height was soaked with the treatment solution. In addition to being less labor-intensive, this strategy has been demonstrated. In order to validated the experiments, one salt-tolerant cowpea genotype ('09-529') and one salt-susceptible cowpea genotype (PI255774) were used as controls (Dong et al. 2019; Ravelombola et al. 2017). The top 10 most salt-tolerant and 10 most salt-susceptible genotypes were repeated at the end of the experiments. The experiment design was a randomized complete block design (RCBD) using time as a blocking variable.

Measurements

In vivo chlorophyll measurement

Leaf chlorophyll was measured using a SPAD-502 Plus (Spectrum Technologies, Inc., Plainfield, IL). Measurements were achieved at one day prior to salt treatment and when the susceptible controls were completely dead, which was about 14 days after the first salt stress. For each plant, chlorophyll measurement was conducted three times on both trifoliate and unifoliate leaves, respectively, and the average read was recorded and analyzed. Measurements were done on three different positions on the leaf surface in order to limit the edge effect (Dong et al. 2019; Ravelombola et al. 2017). Data were taken from all plants under salt stress and non-salt stress conditions.

Plant height and above-ground fresh biomass

Plant height of the cowpea seedlings was recorded one day before the salt treatment began and when the susceptible controls were dead, indicative of the end of plant growth in the susceptible genotypes (Ravelombola et al. 2017). Plant height under salt stress and non-salt stress conditions was also recorded on a per plant basis. Data on both fresh leaf and fresh stem biomass from each plant were also taken. The above-ground fresh biomass corresponded to the sum of fresh leaf biomass and fresh stem biomass.

Leaf injury score

Leaf injury score has been successfully used as a reliable parameter for screening salt tolerance at seedling stage in cowpea (Ravelombola et al. 2017). It has been shown to be highly correlated with Na^+ and Cl^- contents in leaves (Ledesma et al. 2016), and can accurately assess salt tolerance/susceptibility when leaf ion extraction is financially expensive (Ledesma et al. 2016; Ravelombola et al. 2017). Leaf injury score was evaluated using a previously established scale (1 = healthy plants, 2 = sign of leaf chlorosis, 3 = expansion of chlorosis on leaf surface, 4 = totally chlorotic leaf, 5 = first sign of necrosis, 6 = expansion of necrosis on leaf surface, and 7 = completely dead plants) (Ravelombola et al. 2017). Leaf injury scoring was conducted when the susceptible controls were completely dead.

Phenotypic data analysis

Relative tolerance index (RTI) for chlorophyll, plant height, fresh leaf biomass, fresh stem biomass, and total fresh above-ground biomass were used to assess the impact of salt stress relative to the non-salt stress condition. RTI was calculated as following (Ravelombola et al. 2017; Saad et al. 2014).

- RTI_chlorophyll (RTI_C) = (Y_{c_S}/Y_{c_NS}) X 100

- RTI_plant_height (RTI_H) = (Y_{h_S}/Y_{h_NC}) X 100
- RTI_fresh_leaf_biomass (RTI_FL) = (Y_{l_S}/Y_{l_NS}) X 100
- RTI_fresh_stem_biomass (RTI_FS) = (Y_{s_S}/Y_{s_NS}) X 100
- RTI_total_above_fresh_ground_biomass (RTI_FB) = (Y_{b_S}/Y_{b_NS}) X 100

with Y_{c_S} being the chlorophyll content under salt stress, Y_{c_NS} the chlorophyll content under non-salt stress, Y_{h_S} the plant height under salt stress, Y_{h_NC} the plant height under non salt stress, Y_{l_S} the fresh leaf biomass under salt stress, Y_{l_NS} the fresh leaf biomass under non-salt stress, Y_{s_S} the fresh stem biomass under salt stress, Y_{s_NS} the fresh stem biomass under non-salt stress, Y_{b_S} the total fresh above ground biomass under salt stress, and Y_{b_NS} the total fresh above ground biomass under non-salt stress.

Data distribution was visualized using the MASS package of R® 3.6.1. Pearson's correlation coefficients were calculated using JMP Genomics 9 (SAS Institute Inc., Cary, NC). The analysis of variance (ANOVA) was done using PROC MIXED of SAS® 9.4 (SAS Institute Inc., Cary, NC). Mean separation was conducted using a protected least significant difference (LSD) procedure at $\alpha=0.05$. LSD procedure was defined as $LSD=t_{\alpha/2}\sqrt{2MSError/n}$, with $t_{\alpha/2}$ being the critical value from the t-table and having a degree of freedom [df(SSError)] corresponding to the difference between the number of observations and the number of replications, and n being the number of replications. The statistical model for conducting ANOVA was the following.

$$Y_{i(j)k} = \mu + T_j + G_k + R_{i(j)} + TG_{jk} + \varepsilon_{i(j)k} \text{ where } i=1,2,\ j=1,2,\text{ and } k=1\ldots231$$

with μ being the overall mean, $Y_{i(j)k}$ being the response from the k^{th} genotype (G_k) (fixed effect) at the i^{th} replication ($R_{i(j)}$), which was nested under the j^{th} run (block) (T_j)(fixed effect), and TG_{jk} being the interaction effect between the k^{th} genotype (G_k) and the j^{th} run (block) (T_j).

The broad sense heritability (*H*) was estimated using the following formula (Holland 2003).

$$H = \sigma^2_G / [\sigma^2_G + ((\sigma^2_{GXR})/n_b) + ((\sigma^2_e)/(n_b*n_r))]$$

with σ^2_G being the total genetic variance, σ^2_{GXR} being the Genotype X Run variance, σ^2_e being the residual variance, n_b being the number of runs, and n_r being the number of replications. The estimates for σ^2_G and σ^2_{GXR} were [EMS(G)-EMS(GXB)]/ n_b*n_r and [EMS(GXB)-Var(Residual)]/n_r. EMS(G), EMS(GXB), and Var(Residual) were obtained from the ANOVA table. Person's correlation coefficients between the average number of dead plants per pot, average leaf injury score, fresh leaf biomass under salt stress, SPAD chlorophyll content under salt stress, RTI_C, RTI_H, RTI_FL, RTI_FS, and RTI_FB were calculated using R® v.3.6.1. A chord diagram was used in order to better visualize the pairwise correlation between traits. Chord diagram was established in R® v.3.6.1 using the package 'circlize' (Gu and Gu 2019).

Genotyping and SNP filtering

The MAGIC population was genotyped using a total of 51,128 SNPs the Illumina Cowpea Consortium Array (Muñoz-Amatriaín et al. 2017) and obtained from Huynh et al. (2018). A total of 32,047 SNPs were used to conduct GWAS after SNP filtering (missing data <10%, heterozygosity <10%, and minor allele frequency >5%).

Genome-wide association study (GWAS)

GWAS was conducted using a Bayesian Information and Linkage Disequilibrium Iteratively Nested Keyway (BLINK) model and run in R® 3.6.1 using the package 'BLINK' (Huang et al. 2019). BLINK has been demonstrated to have an enhanced statistical power and to be more efficient compared to previously developed models (Huang et al. 2019). LOD threshold was set to 3 (Kaler et al. 2017). The BLINK model was built upon the Fixed and

Random Model Circulating Probability Unification (FarmCPU) model. In FarmCPU, markers are assumed to be evenly distributed across the genome. However, such assumption could be easily violated. BLINK relaxed this assumption by incorporating the LD information. The random effect model (REM) part in FarmCPU, which was computationally heavy, was replaced by a second fixed effect model (FEM) in BLINK. Therefore, the two FEM models in BLINK were defined as following.

$$\text{FEM (1): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ik}b_k + M_{ij}d_j + e_i$$

$$\text{FEM (2): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ij}b_j + e_i$$

with y_i being the vector phenotype, M_{i1},$M_{i2}b_2$, …, M_{ik} the genotypes of k pseudo QTNs that were initially empty and with effects b_1, b_2, …, b_k, respectively, M_{ij} being the j^{th} genetic marker of the i^{th} sample, and e_i being the residual having a distribution with mean zero and a variance σ^2_e. LD heatmaps were generated using the package 'LDheatmap' in R® 3.6.1 (Shin et al. 2019). Overlapping SNP markers between different traits were visualized using a Venn diagram that was established using the online software program accessible at http://jvenn.toulouse.inra.fr/app/example.html.

Candidate gene(s) discovery

A 40-kb genomic region harboring a significant SNP was used for candidate gene in the Phytozome 12 database (https://phytozome.jgi.doe.gov/). Candidate genes with functional annotations relevant to abiotic stresses were considered.

Epistatic interaction modelling

Pairwise epistatic interaction analysis (SNP X SNP interaction) was conducted using PLINK v1.07 (Purcell et al. 2007). The command line for conducting epistasis analysis in PLINK was 'plink --file mydata --epistasis'. The interaction effect of two SNPs was estimated using the following model (Purcell et al. 2007).

$$E[Y|Snp_i, Snp_j] = \beta_0 + \beta_i Snp_i + \beta_j Snp_j + \beta_{ij} (Snp_i \text{ X } Snp_j)$$

with $E[Y|Snp_i, Snp_j]$ being the vector of expected values for the response given the SNP data, β_0 being the intercept, β_i being the main effect for the Snp_i, β_j being the main effect for the Snp_j, and β_{ij} being the interaction effect (epistasis) between Snp_i and Snp_j. The parameter of interest in the above model was β_{ij} and the test to be conducted was H0: $\beta_{ij}=0$. Choosing a minimum p-value for declaring a significant interaction effect can inflate the Type 1 error rate (Wu et al. 2013). However, the current approach using various techniques for identifying a significant threshold while reducing the bias in estimating β_{ij} and limiting the Type 1 error rate could be still extremely computationally intensive. Therefore, we used an arbitrary threshold (p-value $\leq 10^{-6}$) in this study given the number of possible pairwise interactions and for practical reasons during the data visualization process, and while being biologically reasonable. Pairwise epistatic interaction was visualized using the package 'circlize' and run in R® 3.6.1 (Gu and Gu 2019).

Genomic selection

Genomic estimated breeding values (GEBVs) were estimated using a ridge regression best linear unbiased predictor model (rrBLUP) (Meuwissen et al. 2001). The rrBLUP model was defined as $y=WG\beta + \varepsilon$ with y being the vector phenotype, β being the marker effect with $\beta \sim N(0, I\sigma^2_\beta)$, W being the incidence matrix relating the genotype to the phenotype, G being the genetic matrix, and ε being the random error. The solution for the equation was $\hat{\beta}=(Z^TZ + I\lambda)^{-1}Z^Ty$ with $Z=WG$. The ridge parameter was defined as $\lambda=\sigma^2_e/\sigma^2_\beta$ with σ^2_e being the residual variance and σ^2_β being the marker effect variance. rrBLUP was conducted in R® v.3.6.1 using the package 'rrBLUP' (Endelman 2011).

Model fitting was conducted using a training dataset with various size (50, 100, 150, and 200). Marker effects were estimated by fitting the model 100 times and randomly selecting the training set at each replication. In addition, the effect of the number of markers on the accuracy of genomic selection was done by randomly 20% (6,409 SNPs), 40% (12,819 SNPs), 60% (19,228 SNPs), 80% (25,638 SNPs), and 100% (32,047 SNPs) of the filtered SNPs at each replication. The accuracy of genomic selection was assessed by computing the Pearson's correlation coefficient between GEBVS and the observed phenotype in the testing set (Shikha et al. 2017).

Results

Phenotypic data

The average number of dead plants per pot varied from 0.0 to 3.0, with an average of 1.0 and a standard deviation of 0.7 (Table S5.1). The distribution of the average number of dead plants per pot was right-skewed (Fig 5.2A). A significant difference in the average number of dead plants per pot was found among the genotypes (F-value=15.3, p-value<0.0001) and the genotype X block interaction effect was also significant (F-value=6.0, p-value<0.0001) (Table 5.1), which was expected. Despite the significant genotype X block interaction effect, the main factor genotype was still analyzed since analyzing salt tolerance between genotypes was the main purpose of the phenotypic evaluation in this study. Of the 242 genotypes evaluated for salt tolerance, 45 did not have any dead plants across four replications. In addition, a variation in the average number of dead plants per pot was identified as shown in Fig. 5.2A. Interestingly, none of the cowpea parents were among the top 45 with plant death. The cowpea parents that were least affected by salt stress in terms of plant death were

IT00K_1263 and IT84S_2049 with an average of one dead plant per pot for each. The genotypes with the highest average number of dead plants per pot with 4 plants were MAGIC194 (2.5), MAGIC048 (2.8), IT89KD_288 (3.0), MAGIC074 (3.0), and MAGIC092 (3.0) (Table 5.2). The broad sense heritability for the average number of dead plants per pot was 74.2%.

Leaf injury score was approximately normally distributed (Fig 5.2B) and ranged between 0.5 and 6.5 based on a 1-7 scale, with an average of 3.6 and a standard deviation of 1.1 (Table S5.1). A significant genotype effect on leaf injury (F-value=13.2, p-value<0.0001) and genotype X block interaction effect (F-value= 5.4, p-value<0.0001) were also identified (Table 5.1). The genotypes with the lowest leaf injury score were MAGIC208 (0.5), MAGIC027 (1.3), MAGIC040 (1.3), MAGIC062 (1.3), and MAGIC236 (1.3) (Table 5.2), which were the most tolerant genotypes in terms of leaf injury score. The genotypes with the highest leaf injury score were MAGIC259 (6.0), MAGIC298 (6.0), MAGIC194 (6.0), MAGIC048 (6.3), MAGIC092 (6.5) (Table 5.2), which were the most susceptible genotypes in terms of leaf injury score. None of the MAGIC parents were among the most tolerant and the most susceptible groups. The parent that was the most tolerant to salt stress in terms of leaf injury score were IT00K_1263 (3.3), whereas the one that was the most susceptible was IT89KD_288 (5.8) (Fig. 5.2B). The broad sense heritability (*H*) for leaf injury score under salt stress was 72.6%.

The distribution of leaf SPAD chlorophyll under salt treatment showed a nearly normal distribution as shown in Fig. 5.2C. The average leaf SPAD chlorophyll under salt treatment was 27.9 and with a standard deviation of 7.8 and varied from 7.1 to 51.5 (Table S5.1). Leaf SPAD chlorophyll was significantly different among the MAGIC lines (F-value=45.2, p-

value<0.0001) (Table 5.1). The genotype X block interaction effect was also significant (F-value=15.4, p-value<0.0001) (Table 5.1). The lines with the highest leaf SPAD chlorophyll under salt stress were MAGIC208 (51.5), MAGIC008 (47.2), MAGIC027 (46.0), MAGIC311 (45.3), and MAGIC236 (44.0), whereas those with the lowest leaf SPAD chlorophyll under salt stress were MAGIC122 (12.0), MAGIC110 (11.4), MAGIC194 (10.2), MAGIC048 (8.5), and, MAGIC092 (7.1) (Table 5.2). None of the parents were listed among the top performers and the least performing ones in terms of leaf SPAD chlorophyll under salt stress. The MAGIC parent with the highest leaf SPAD chlorophyll under salt treatment was IT84S_2246 (13.6), which was the most susceptible parent in terms of leaf SPAD chlorophyll under salt treatment. The MAGIC parent with the highest leaf SPAD chlorophyll under salt stress was IT93K_503_1 (21.9) (Table 5.2). The broad sense heritability (*H*) for leaf SPAD chlorophyll under salt treatment was 78.9%.

Relative tolerance index for leaf SPAD chlorophyll (RTI_C) showed a nearly normal distribution (Fig. 5.2D). RTI_C varied from 23.6% to 108.1%, with an average of 71.3% and a standard deviation of 17.3% (Table S5.1). RTI_C was significantly different among genotypes (F-value=26.8, p-value<0.0001) and genotype X block interaction effect was also significant (F-value=14.0, p-value<0.0001) (Table 5.1). The top 5 genotypes with the highest RTI_C were MAGIC119 (108.1%), MAGIC311 (107.8%), MAGIC343 (105.8%), MAGIC008 (104.5%), and MAGIC236 (104.0%) (Table 5.2). Their RTI_C was greater than 100%, indicating that they were highly salt-tolerant based on RTI_C and the leaf SPAD chlorophyll content under salt stress was greater than that of under non-salt stress. The lines with the lowest RTI_C were MAGIC194 (32.2%), MAGIC074 (30.8%), MAGIC110 (28.9%), MAGIC048 (27.4%), and MAGIC092 (23.7%) (Table 5.2), suggesting that these genotypes were the most susceptible to

salt stress based on RTI_C in this population. The MAGIC parent with the highest RTI_C was IT84S_2049 (68.8%), whereas the one with the lowest RTI_C was IT84S_2246 (42.7%). The broad sense heritability (*H*) for RTI_C was 63.6%.

Fresh leaf biomass under salt stress is also a good phenotype for assessing salt tolerance in cowpea at seedling stage. In this study, fresh leaf biomass of cowpea plants under salt treatment was approximately normally distributed (Fig. 5.2E). Fresh leaf biomass ranged between 0.5 g to 4.2g, with an average of 2.1 g and a standard deviation of 0.7 g (Table S5.2). Under salt stress, a significant difference in fresh leaf biomass was observed among the genotypes (F-value=11.9, p-value<0.0001), and the genotype X block interaction was also significant (F-value=6.4, p-value<0.0001) (Table 5.1). The genotypes with the highest fresh leaf biomass under salt stress were MAGIC208 (4.2 g), MAGIC336 (3.8 g), MAGIC271 (3.8 g), MAGIC187 (3.8 g), and MAGIC027 (3.8 g), whereas those with the lowest fresh leaf biomass under salt stress were Suvita_2 (0.7 g), MAGIC073 (0.6 g), MAGIC048 (0.6 g), MAGIC092 (0.5 g), and IT84S_2246 (0.5 g) (Table 5.2). Two of the parents were listed among the least performing in terms fresh leaf biomass under salt stress. The MAGIC parent with the highest fresh leaf biomass under salt treatment was IT93K_503_1 (1.9 g). The broad sense (*H*) heritability for fresh leaf biomass of cowpea plants grown under salt treatment was 61.3%.

The relative tolerance index for fresh leaf biomass (RTI_FL) varied from 10.0% to 93.2%, with an average of 56.8% and a standard deviation of 13.9% (Table S5.2). RTI_FL was normally distributed as shown in Fig. 5.2F. ANOVA indicated a significant effect of genotypes on RTI_FL (F-value=5.4, p-value<0.0001) (Table 5.1). The genotype X block interaction effect was also significant (F-value=2.6, p-value<0.0001) (Table 5.1). The genotypes with the highest RTI_FL were MAGIC177 (93.2%), MAGIC264 (93.1%), MAGIC188 (92.5%),

MAGIC265 (90.6%), and MAGIC201 (88.0%), which were the most tolerance in terms of relative tolerance index for fresh leaf biomass (Table 5.2). The genotypes that were most susceptible to salt stress in terms of RTI_FL were MAGIC207 (26.2%), MAGIC110 (21.2%), IT84S_2246 (17.9%), MAGIC130 (17.4%), and MAGIC073 (10.0%) (Table 5.2). The MAGIC parent with the highest RTI_FL was Suvita_2 (59.0%). RTI_FL values for the MAGIC parents were scattered across the distribution of RTI_FL for this population (Fig. 5.2F). The broad sense heritability (*H*) for RTI-FL was 64.1%.

Relative tolerance index for fresh stem biomass (RTI_FS) was normally distributed (Fig. 5.2G). RTI_FS varied from 23.0% to 89.9%, with an average of 54.7% and a standard deviation of 12.7% (Table S5.2). A significant difference in terms of RTI_FS was found among the cowpea genotypes investigated for salt tolerance in this study (F-value=4.3, p-value<0.0001) (Table 5.1). The genotype X block interaction effect was also significant (F-value=2.3, p-value<0.0001) (Table 5.1). The top performing MAGIC genotypes in terms of RTI_FS were MAGIC181 (89.9%), MAGIC270 (88.7%), MAGIC343 (88.5%), MAGIC271 (87.2%), and MAGIC238 (86.6%), and the MAGIC lines that were the least performing in terms of RTI_FS were MAGIC073 (28.0%), MAGIC119 (27.7%), MAGIC089 (27.5%), MAGIC130 (24.6%), and MAGIC207 (23.0%) (Table 5.2). The MAGIC parent with the highest RTI_FS was IT89KD_288 (77.5%), whereas the one with the lowest RTI_FS was IT82E_18 (40.6%) (Table S5.2). The broad sense heritability (*H*) for RTI_FS was 59.9%.

Relative tolerance index for total above-drought fresh biomass (RTI_FB) was normally distributed as shown in Fig. 5.2H. RTI_FB ranged between 9.6% and 47.9%, with an average of 35.5% and a standard deviation of 7.6% (Table S5.3). Results indicated that there was a significant difference in RTI_FB between the MAGIC lines (F-value=6.5, p-value<0.0001)

(Table 5.1). A significant effect of genotype X block interaction was also identified (F-value=3.4, p-value<0.0001) (Table 5.1). The MAGIC lines that were the most tolerant to salt stress in terms of RTI_FB were MAGIC188 (47.9%), MAGIC187 (47.0%), MAGIC282 (46.8%), MAGIC242 (46.6%), and MAGIC199 (46.5%), whereas those that were the most susceptible to salt stress based on RTI_FB were MAGIC146 (13.8%), MAGIC259 (13.2%), MAGIC134 (12.6%), MAGIC148 (12.4%), and MAGIC130 (9.6%) (Table 5.2). The parent with the highest RTI_FB was Suvita_2 (41.3%), whereas the one with the lowest RTI_FB was IT82E_18 (14.0%). The broad sense heritability (*H*) for RTI_FB was 61.5%.

The distribution of relative tolerance index for plant height (RTI_H) was approximately normal (Fig. 5.2I). RTI_H varied from 54.6% to 89.5%, with an average of 73.1% and a standard deviation of 6.0% (Table S5.3). A significant difference in RTI_H was identified among the genotypes (F-value=6.9, p-value<0.0001), and the genotype X block interaction effect was also significant (F-value=3.1, p-value<0.0001) (Table 5.1). The MAGIC lines with the highest RTI_H were MAGIC199 (89.5%), MAGIC117 (87.3%), MAGIC280 (86.9%), MAGIC138 (86.5%), and MAGIC077 (85.8%) (Table 5.2), thus the most tolerant to salt stress based on RTI_H. The ones that were the most susceptible to salt stress in terms of RTI_H were MAGIC030 (58.8%), MAGIC072 (58.4%), MAGIC206 (57.5%), MAGIC153 (56.7%), and MAGIC074 (54.6%) (Table 5.2). The parent with the highest RTI_H was IT93K_503_1 (79.8%), whereas the one with the lowest RTI_H was IT84S_2246 (59.2%). The broad sense heritability (*H*) for RTI_H was 67.2%.

Correlation analysis

The average number of dead plants per pot was strongly correlated with salt injury score (r=0.9). In addition, a high and negative correlation was found between the average

number of dead plants per pot and the leaf SPAD chlorophyll under salt stress (r=-0.8), and between the average number of dead plants per pot and the relative tolerance index for leaf SPAD chlorophyll (r=-0.8) (Table 5.3). A high and negative correlation was also identified between the average number of dead plants per pot and fresh leaf biomass under salt stress (r=-0.6). However, the average number of dead plants per pot was weakly correlated with the relative tolerance index for fresh stem biomass (r=-0.20), relative tolerance index for total above-ground fresh biomass (r=-0.30), and relative tolerance index for plant height (r=-0.10) (Table 5.3). The relative tolerance index for leaf SPAD chlorophyll was moderately correlated with fresh leaf biomass under salt stress (r=0.50), but the relative tolerance index for leaf SPAD chlorophyll was not correlated with the relative tolerance index for plant height (r=-0.10), indicating that the mechanism for tolerance to plant height reduction and leaf SPAD chlorophyll reduction under salt stress could be different. The trait having the highest correlation with relative tolerance index for plant height was fresh stem biomass (r=0.40) (Table 5.3).

The pairwise relationship that was based on the Person's correlation coefficient for the traits evaluated under salt stress was visualized used a chord diagram (Fig. 5.3). The thicker the link between traits was, the lower the Person's correlation coefficient was. The traits with the thickest link end were the average number of dead plants per pot, leaf injury score, leaf SPAD chlorophyll under salt stress, and relative tolerance index for leaf SPAD chlorophyll (Fig. 5.3), suggesting the possibility of common pathway(s) for salt tolerance mechanism for these traits. The traits with the thinnest link end were relative tolerance index for fresh stem and plant height, indicating that the mechanism for salt tolerance could be independent from the other traits evaluated in this study.

Genome-wide association study and candidate gene identification

A total of seven significant SNPs were identified to be associated with the average number of dead plants per pot (Table 5.4). Of which, three SNPs were located on chromosome 3 and four SNPs on chromosome 7 (Fig. 5.4). The 3 SNPs on chromosome 3 were located within a 48-kb region. These SNPs were 2_26528 (LOD=4.1, MAF=35.1%), 2_05819 (LOD=4.1, MAF=35.1%), and 2_28348 (LOD=3.7, MAF=35.7%). The significant SNPs on chromosome 7 were 2_25790 (LOD=4.1, MAF=13.6%), 2_07660 (LOD=3.7, MAF=11.6%), 2_02219 (LOD=3.7, MAF=11.6%), and 2_02220 (LOD=3.7, MAF=11.6%). The SNPs 2_07660, 2_02219, and 2_02220 were located within a 15-kb region of chromosome 7. One annotated gene was identified within the 20-kb region harboring each significant SNP. The annotated genes found within or in the vicinity of each SNP location encoded for a homeobox associated leucine zipper, xyloglucan:xyloglucosyl transferase, RNA helicase, leucine rich repeat, calcium-dependent protein kinase 32, typa-like translation elongation factor SVR3-related, and raffinose synthase/seed imbibition protein Sip1 (Table 5.4).

A total of two SNPs were found to be significantly associated with leaf injury score. The SNPs were 2_13484 (LOD=3.6, MAF=29.3%) and 2_13485 (LOD=3.6, MAF=29.3%), and located at 25,524,675 bp and 25,525,542 bp on chromosome 1, respectively (Fig. 5.4). The annotated gene found in the vicinity of these SNPs was *Vigun01g093100.1*, which encodes for a Na^+/Ca^{2+} K^+ independent exchanger (Table 5.4).

A strong candidate locus defined by a 4.2-Mb region of chromosome 3 was associated with the leaf SPAD chlorophyll under salt stress (Fig. 5.4). This locus was defined by a total of 18 significant SNPs (Table 5.4). Of the 18 SNPs, 2_33024 (LOD=4.2, MAF=49.6%), 2_26528 (LOD=4.1, MAF=35.1%), 2_05819 (LOD=4.1, MAF=35.1%), 2_28348 (LOD=4.0,

MAF=35.7%), 2_02054 (LOD=3.9, MAF=48.8%), and 2_29692 (LOD=3.9, MAF=48.8%) had the highest LOD values. At least one annotated gene was identified in the vicinity of each significant SNP except for the SNPs 2_46677 and 2_47326. The candidate genes encode for various proteins such as mitochondrial folate transporter/carrier, auxilin/cyclin g-associated kinase-related, clathrin coat assembly protein, phytoene dehydrogenase, retinaldehyde binding protein-related, succinate dehydrogenase flavoprotein subunit, protein Da1-related, cysteine-rich secretory protein family, vacuolar protein sorting-associated protein VPS13, alpha/beta hydrolase fold, and xyloglucan:xyloglucosyl transferase (Table 5.4).

GWAS for relative tolerance index for leaf SPAD chlorophyll (RTI_C) identified 17 significant SNPs (Table 5.4). These SNPs were the ones that were associated with leaf SPAD chlorophyll under salt stress and were located within the 4.2-Mb genomic region of chromosome 3 (Fig. 5.4), suggesting a high likelihood of QTL(s) affecting salt tolerance based on leaf SPAD chlorophyll in this genomic region. For fresh leaf biomass under salt stress, no any SNP was above the declared threshold (LOD $\geq$ 3.5). The top three SNPs with the highest LOD for fresh leaf biomass were 2_27478 (LOD=3.0, MAF=48.8%), 2_28348 (LOD=3.3, MAF=35.7%), and 2_50921 (LOD=3.1, MAF=24.3%). The SNPs 2_27478 and 2_28348 were within the candidate region associated with both leaf SPAD chlorophyll content and RTI_C, indicating that there could be a common pathway for salt tolerance based on fresh leaf biomass under salt stress, leaf SPAD chlorophyll under salt stress, and RTI_C.

Two SNPs were found to be significantly associated with the relative tolerance index for fresh leaf biomass (RTI_FL) (Fig. 5.4). The two SNPs were also identified to be associated with fresh leaf biomass under salt stress, RTI_C, and leaf SPAD chlorophyll under salt stress (Table 5.4). Results showed that no any SNP was above the chosen LOD threshold (3.5) for

relative tolerance index for fresh stem biomass (RTI_FS), so we only reported the ones with the highest LOD. The SNPs with the highest LOD were 2_20734 (LOD=3.4, MAF=10.3%), 2_13286 (LOD=3.4, MAF=10.3%), 2_13285 (LOD=3.4, MAF=10.3%), 2_44170 (LOD=3.4, MAF=10.3%), and 2_47221 (LOD=3.4, MAF=10.3%). These SNPs were located with a 50.6-kb region of chromosome 4. A total of six annotated genes were found in the vicinity of these SNPs. These genes encode for a glycosyltransferase 8 domain-containing protein, ccr4-not transcription complex related, H+/oligopeptide symporter, and zinc finger FYVE domain containing protein.

One SNP, 2_33574, was significantly associated with the relative tolerance index for total above-ground fresh biomass (RTI_FB) (Fig. 5.4). This SNP was located at 579544 Mb on chromosome 5 (Table 5.4). The annotated genes found in the vicinity of this SNP were *Vigun05g006800.1*, *Vigun05g006700.1*, *Vigun05g006600.1*, and *Vigun05g006500.1*. No functional annotations were found for *Vigun05g006600.1*. Functional annotations for *Vigun05g006800.1*, *Vigun05g006700.1*, and *Vigun05g006500.1* were Mannose-6-phosphate isomerase, alpha/beta hydrolase fold-containing protein, and neoxanthin biosynthesis, respectively. GWAS suggested a strong candidate locus associated with relative tolerance index for plant height (RTI_H) (Fig. 5.4). This genomic region harbored a total of 23 significant SNPs and were mapped on a 3.4-Mb region of chromosome 3 (Table 5.4). The significant SNPs with the highest LOD were 2_26489 (LOD=4.2, MAF=28.1%), 1_0247 (LOD=4.2, MAF=28.1%), 2_04756 (LOD=4.2, MAF=28.1%), 2_34159 (LOD=4.2, MAF=28.9%), 2_34562 (LOD=4.2, MAF=29.0%), 2_00955 (LOD=4.2, MAF=28.9%), 2_52154 (LOD=4.2, MAF=28.9%), 2_15515 (LOD=4.2, MAF=28.9%), 2_06057 (LOD=4.2, MAF=28.9%), 2_03596 (LOD=4.2, MAF=28.9%), and 2_45312 (LOD=4.2, MAF=28.9%). A

total of 27 annotated genes were identified in the vicinity of the significant SNPs associated with RTI_H (Table 5.4). Functional annotations associated with the candidate genes were O-methyltransferase-related, protein transport protein SEC23, peptidyl-prolyl cis-trans isomerase, cystatin-C, phospholipases, dolichol-phosphate mannosyltransferase, IQ-domain 9 protein, mutt-nudix-related, magnesium chelatase subunit I, ionotropic glutamate receptor, apoptosis inhibitor 5, peroxidase 19, triacylglycerol degradation, cytochrome P450, microfibril-associated protein, suberin monomers biosynthesis, homoserine dehydrogenase, and beta-galactosidase 9 (Table 5.4).

Overlapping SNPs between traits

Overlapping SNP markers were identified between the traits evaluated for salt tolerance in this MAGIC cowpea population. Out of the SNP markers associated with the average number of dead plants per pot, three SNPs were found to be associated with both leaf SPAD chlorophyll under salt treatment (S_Chloro) and relative tolerance index for leaf SPAD chlorophyll (RTI_C) (Fig. 5.5), indicating that there could be a common pathway for salt tolerance based on the two traits. A total of 14 significant SNPs were overlapping between S_Chloro and RTI_C (Fig. 5.5). Interestingly, none of the significant SNP markers associated with relative tolerance index for plant height (RTI_H) overlapped with any SNP markers associated with other traits (Fig. 5.5), suggesting that the mechanism for salt tolerance based on RTI_H could be independent. Using a Venn diagram with more than 5 sets would be difficult to visualize, so the Venn diagram (Fig. 5.5) did not include the data for leaf injury score (Score), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for the total above-ground fresh biomass (RTI_FB), fresh leaf biomass under salt stress (S_Leaf), and relative tolerance index for fresh leaf biomass (RTI_FL). None of the SNP makers

associated with Score overlapped with any SNP makers associated with other traits (Table 5.4). Similar results were found for RTI_FS and RTI_FB. One SNP associated with S_Leaf, 2_28348, overlapped with RTI_FL, S_Chloro, Dead, and RTI_C (Table 5.4). The SNP 2_27478, associated with S_Leaf, was also associated with RTI_C, S_Chloro, and RTI_FL (Table 5.4). These results indicated that there could be a common pathway for salt tolerance between S_Leaf, RTI_FL, S_Chloro, Dead, and RTI_C.

Epistatic interaction analysis

A total of 513,489,081 possible pairwise interactions were tested using PLINK v1.07 for each trait. Of which, a total of 949, 264, 161, 272, 413, 269, 1323, 395, and 341 pairwise interactions for the average number of dead plants per pot, leaf injury score, leaf SPAD chlorophyll under salt treatment (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (RTI_C), fresh leaf biomass under salt treatment (S_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index for plant height (RTI_H), respectively, were significant based on our chosen threshold (p-value $\leq 10^{-6}$).

All pairwise epistatic interactions found for the average number of dead plants per pot were between chromosomes with chromosomes 9 and 11 having the highest number of significant epistasis (Fig 5.6A). However, these epistasis-rich regions had SNPs with low LOD values. The genomic region of chromosome 7 that harbored some of the significant SNP markers associated with the average number of dead plants per pot was in epistasis with some SNPs found at the beginning of chromosome 8 (Fig. 5.6A). No significant interaction was identified between SNPs located within the two candidate loci, one on chromosome 3 and one

on chromosome 7, associated with the average number of dead plants per pot. Similar results were found for leaf injury score where no epistatic interactions were identified between the significant SNPs associated this trait (Fig. 5.6B). The chromosomes with the highest number of epistatic interactions were chromosome 3 and chromosome 8 (Fig. 5.6B). Interestingly, most of significant epistatic interactions for leaf injury score appeared to be located towards both ends of the chromosome as shown in Fig. 5.6B. The epistasis analysis results for S_Chloro were particular since the significant SNP markers associated with this trait, which were located on chromosome 3, were in epistatic interaction with SNPs located on chromosomes 2, 8, and 11 (Fig. 5.6C).

Results indicated a within-chromosome epistatic interaction (chromosome 4) for RTI_C (Fig. 5.6D). The pattern of epistasis for RTI_C was very similar to that of S_Chloro (Fig. 5.6C and 6D), which was expected since these traits were highly correlated. In addition, the interactions between SNPs of chromosomes 6 and 8 that were found for S_Chloro were identified for the average number of dead plants per pot (Fig. 5.6A and 5.6D). The chromosomes with the highest number of significant epistasis for S_Leaf were 3 and 4 (Fig. 5.6E). None of the significant SNP markers associated with S_Leaf were in epistasis with any SNPs. For RTI_FL, chromosomes 6 and 7 had the highest number of significant epistatic interactions and a within-chromosome epistasis was found on chromosome 7 (Fig. 5.7A). The significant SNP markers associated with RTI_FL and found on chromosome 3 were not in epistatic interaction with any other SNPs (Fig. 5.7A).

Epistatic interactions were also identified for RTI_FS. The chromosomes with the highest epistatic interaction were 1, 6, and 11 (Fig. 5.7B). The significant SNPs associated with RTI_FS and located on chromosome 4 were in epistatic interaction with some low LOD SNPs

of chromosome 7 (Fig. 5.7B). However, the other candidate locus containing significant SNPs and located on chromosome 7 was not in epistatic interaction with any genomic regions. RTI_FB had the highest number of significant epistatic interactions among all traits evaluated for salt tolerance in this study. The chromosomes with the highest number of significant epistatic interactions for RTI_FB were 3, 6, and 10 (Fig. 5.7C). The significant SNP markers associated with RTI_FB and mapped on chromosome 5 were in epistatic interaction with some low LOD SNPs of chromosome 6. No within-chromosome epistatic interactions were identified for RTI_FB. The significant SNP markers associated with RTI_H and located on chromosome 3 were not in epistatic interaction with any SNPs as shown in Fig. 5.7D. One within-chromosome epistatic interaction was found on chromosome 7.

Genomic selection

The accuracy of genomic selection was assessed for average number of dead plants per pot (Dead), leaf injury score (Score), leaf SPAD chlorophyll under salt treatment (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (RTI_C), fresh leaf biomass under salt stress (S_Leaf), relative tolerance index for fresh leaf biomass (RTI_FS), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index for plant height (RTI_H) (Table 5.5). Overall, genomic selection accuracy did not increase with the size of training set except for S_Leaf and RTI_H (Fig. 5.8E and 5.8I), which was unexpected. In addition, no clear correlation was found between the increase in the number of SNPs and the accuracy of genomic selection. For traits such as RTI_FS, the increase in the number of SNPs did not result in the decrease of genomic selection accuracy when a larger training data set was used to fit the model (Fig. 5.8G). Overall, RTI_FS and RTI_H had the highest selection accuracy, whereas RTI_FS and RTI_FB

had the lowest one (Table 5.5). Genomic selection was more accurate using a larger training dataset for traits such as S_Leaf and RTI_H (Fig. 5.8E and 8I). However, better accuracy was found using a smaller training dataset for traits such as RTI_C and RTI_FB (Fig. 5.8D and 5.8H).

Discussion

A total of 234 MAGIC lines along with their eight parent founders were evaluated for salt tolerance in this study. Results showed a large variation in the traits evaluated under salt stress among the MAGIC lines. The degree of tolerance to salt stress was also different among the eight founders, suggesting that this MAGIC population was an adequate population for salt tolerance phenotyping. To the best of our knowledge, this study was one of the earliest reports investigating salt tolerance based on a MAGIC population in cowpea. In addition, the population size for this study was larger than the previous reports investigating cowpea salt tolerance (Dong et al. 2019; Ravelombola et al. 2017).

GWAS identified significant SNP markers associated with various traits evaluated under salt stress in this MAGIC cowpea population. GWAS has been successfully to identify SNP markers associated with important traits in cowpea (Burridge et al. 2017; Qin et al. 2016; Shi et al. 2016; Xu et al. 2017). The earliest SNPs found to be associated with salt tolerance in cowpea were Scaffold87490_622, Scaffold87490_630, C35017374_128, Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 (Ravelombola et al. 2017). These SNPs were identified by conducting GWAS based on a total of 155 cowpea genotypes and 1,049 SNPs that were postulated from genotyping-by-sequencing. The present investigation has improved

this study by carrying out GWAS based on a larger panel and using a large number of SNPs. However, the first reported SNP markers for salt tolerance in cowpea did not have chromosome information since the cowpea genome was not published at the time when the study was investigated. Therefore, we could not assess whether the first salt-tolerant SNP markers overlapped with the SNPs identified with in this investigation. In addition, most of the SNP markers identified in this study were within or in the vicinity of annotated genes whose functional annotations involved salt tolerance mechanisms, which provides robustness to our results.

Various candidate genes encoding for protein having functions that could be relevant to salt tolerance mechanism have been identified. Our results identified a relationship between $Na^+/Ca2^+$ K^+ independent exchanger and salt tolerance in cowpea. The involvement of $Na^+/Ca2^+$ K^+ independent exchanger in salt tolerance has been well described in other species such as tomato and soybean (Assaha et al. 2017). Therefore, the SNP marker found in the vicinity of this gene could be reliably used for screening salt tolerance in cowpea since it is highly conserved across species, thus stable. H+/oligopeptide symporter has been shown to be associated with Cl- dynamic under salt stress in soybean (Teakle and Tyerman 2010). These results suggested that a common salt tolerance mechanism pathway could exist between soybean and cowpea. Calcium-dependent protein kinases have also been identified to be associated with salt tolerance based on our data. Gao et al. (2018) showed that calcium-dependent protein kinases are important in regulating responses to salt stress in cotton. These proteins play a role in stress signaling. Gao et al. (2018) found that transcripts encoding for calcium-dependent protein kinases were induced at early stage of salt stress in cotton. These findings suggested that similar salt tolerance mechanism could exist in cowpea. Candidate gene

search suggested the involvement of vacuolar proteins in salt tolerance in cowpea. Kim and Bassham (2011) demonstrated that vacuolar proteins are critical in maintaining the trans-Golgi network (TGN) during salt tolerance. The direct involvement of vacuolar proteins in salt tolerance supports the claim regarding the true association of the SNP marker with salt tolerance in cowpea. *Vigun03g290600.1* has been reported to encode for xyloglucan:xyloglucosyl transferase. Cho et al. (2006) showed that xyloglucan:xyloglucosyl transferase was induced upon salt stress in *Arabidopsis thaliana*. Cho et al. (2006) suggested that xyloglucan:xyloglucosyl transferase might play a role in cell growth during salt stress. However, the exact involvement of xyloglucan:xyloglucosyl transferase during salt stress is not fully understood. Despite of the possible relationship existing between functional annotations of the candidate genes identified in this study and salt tolerance mechanism, further studies including transcriptomic analysis would be required to increase the reliability of the results.

Genomic selection has become more and more popular in recent years. Genomic selection has signficant impacts in modern breeding and has been shown to be efficient when dealing with complex traits (Meuwissen et al. 2001). Previous studies have demonstrated that genomic selection can signficiantly increase the genetic gain per unit of time (Duhnen et al. 2017; Michel et al. 2016; Poland et al. 2012; Spindel et al. 2015). The accuracy of genomic selection is highly critical in a breeding program. Studies aiming at evaluating genomic selection accuracy remain very limited in cowpea. The earliest investigation on genomic selection for cowpea has been reported by Olatoye et al. (2019) who investigated the accuracy of genomic selection for flowering time, maturity date, and grain yield under drought stress. Olatoye et al. (2019) found a medium selection accuracy (0.2-0.6) for these traits. To the best of our knowledge, this is the first report aiming at evaluating the accuracy of genomic selection

for salt tolerance in cowpea. Unexpectedely, the accuracy of genomic selection was low for all traits and the study failed to identify clear relationship between selection accuracy and number of SNPs used for estimating the GEBVs and the size of training population. In addition, we have expected that traits with a higher heritability could have a higher genomic selection, which was not the case. Therefore, further investigations including additional model testing are required prior to drawing robust conclusions in the accuracy of genomic selection for salt tolerance in cowpea. We think that it is still early to establish a final conclusion on the feasability of genomic selection for selecting salt tolerance in cowpea. However, even with a low prediction accuracy, Lozada et al. (2019) reported that genomic selection can still be used to complement phenotypic selection.

Soil salinity has been shown to be a growing threat to agriculture worldwide (Allakhverdiev et al. 2000). Cowpea can be significantly impaired by soil salinity (Wilson et al. 2006). This investigation reported the variation of salt tolerance in a MAGIC cowpea population. Salt-tolerant MAGIC lines were identified. This MAGIC population has been registered (Huynh et al. 2019). However, information on the tolerance to salt stress of this MAGIC cowpea population has not been reported despite of its negative impact on cowpea production. Therefore, our results can complement the information collected by Huynh et al. (2019) on this MAGIC population, which will further increase the usefulness of this population in cowpea breeding.

Conclusions

A large variation in salt tolerance among the cowpea MAGIC lines has been identified. The salt-tolerant lines could be used as parents in breeding for salt tolerance in cowpea. In

addition, a large number of significant SNP markers were found within or in the vicinity of genes that were directly involved in salt tolerance. Therefore, these SNPs can be used for screening salt tolerance in cowpea via marker-assisted selection (MAS) and genomic selection (GS) upon validation. However, additional studies are required to validate the candidate genes identified in this study and to improve the genomic selection accuracy for salt tolerance in cowpea, which will be useful in establishing a modern cowpea breeding program.

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Tables

Table 5.1. ANOVA table for the MAGIC population evaluated under salt tolerance. The evaluated traits were the average number of dead plants per pot (Dead_plants), leaf injury score (Salt_score), SPAD chlorophyll under salt treatment (S_Chloro), relative tolerance index for SPAD chlorophyll (RTI_C), fresh leaf biomass under salt treatment (S_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), relative tolerance index for fresh stem biomass (RTI_FS), and relative tolerance index for total above-ground fresh biomass.

Traits	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
Dead_plants	Genotype	241	514.6	2.1	482	15.3	<.0001
	Block	1	9.3	9.3	482	66.8	<.0001
	Rep(Block)	2	0.3	0.1	482	0.9	0.397
	Genotype*Block	241	201.4	0.8	482	6	<.0001
	Residual	482	67.2	0.1	-	-	-
Salt_score	Genotype	241	1166.2	4.8	482	13.2	<.0001
	Block	1	109.8	109.8	482	299.7	<.0001
	Rep(Block)	2	0.4	0.2	482	0.6	0.579
	Genotype*Block	241	475.7	2	482	5.4	<.0001
	Residual	482	176.6	0.4	-	-	-
S_Chloro	Genotype	241	19857	82.4	482	45.2	<.0001
	Block	1	327.5	327.5	482	179.7	<.0001
	Rep(Block)	2	7.8	3.9	482	2.1	0.283
	Genotype*Block	241	6771.9	28.1	482	15.4	<.0001
	Residual	482	878.4	1.8	-	-	-
RTI_C	Genotype	241	287943	1194.8	482	26.8	<.0001
	Block	1	29566	29566	482	662.1	<.0001
	Rep(Block)	2	194.5	97.3	482	2.2	0.312
	Genotype*Block	241	151075	626.9	482	14	<.0001
	Residual	482	21522	44.7	-	-	-
S_Leaf	Genotype	241	472.6	2	482	11.9	<.0001
	Block	1	159.5	159.5	482	970.1	<.0001
	Rep(Block)	2	0.9	0.5	482	2.9	0.071
	Genotype*Block	241	254.8	1.1	482	6.4	<.0001
	Residual	482	79.2	0.2	-	-	-
RTI_FL	Genotype	241	187356	777.4	482	5.4	<.0001
	Block	1	92398	92398	482	638.6	<.0001
	Rep(Block)	2	654.8	327.4	482	2.3	0.1052
	Genotype*Block	241	90725	376.5	482	2.6	<.0001
	Residual	482	69744	144.7	-	-	-
RTI_FS	Genotype	241	154541	641.2	482	4.3	<.0001
	Block	1	62106	62106	482	419	<.0001
	Rep(Block)	2	481.6	240.8	482	1.6	0.1981
	Genotype*Block	241	80826	335.4	482	2.3	<.0001
	Residual	482	71449	148.2	-	-	-

Table 5.1 (Cont.)

Traits	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
RTI_FB	Genotype	241	134866	559.6	482	6.5	<.0001
	Block	1	84129	84129	482	980.8	<.0001
	Rep(Block)	2	30.1	15	482	0.2	0.6109
	Genotype*Block	241	70323	291.8	482	3.4	<.0001
	Residual	482	41344	85.8	-	-	-
RTI_H	Genotype	241	34176	141.8	482	6.9	<.0001
	Block	1	8263.1	8263.1	482	401	<.0001
	Rep(Block)	2	114.7	57.3	482	2.8	0.0629
	Genotype*Block	241	15626	64.8	482	3.1	<.0001
	Residual	482	9931.3	20.6	-	-	-

Table 5.2. List of top 5 genotypes and 5 least performers for average number of dead plants per plot (DeadPlants), the leaf injury score under salt treatment (Score), SPAD chlorophyll under salt treatment (StressSPADChloro), relative tolerance index for SPAD chlorophyll (RTI_C), fresh leaf biomass under salt treatment (StressLeaf), relative tolerance index for fresh leaf biomass (RTI_FL), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index for plant height (RTI_H). Sd represents the standard deviation across 4 replications.

Plant_ID	DeadPlants	Sd	Plant_ID	Score	Sd	Plant_ID	StressSPADChloro	Sd
MAGIC001	0	0	MAGIC208	0.5	0.6	MAGIC092	7.1	5.1
MAGIC008	0	0	MAGIC027	1.3	1	MAGIC048	8.5	5.3
MAGIC009	0	0	MAGIC040	1.3	0.5	MAGIC194	10.2	6.9
MAGIC012	0	0	MAGIC062	1.3	0.5	MAGIC110	11.4	5.1
MAGIC027	0	0	MAGIC236	1.3	0.5	MAGIC122	12	5.6
MAGIC194	2.5	0.6	MAGIC259	6	0.8	MAGIC236	44	8.6
MAGIC048	2.8	0.5	MAGIC298	6	0.8	MAGIC311	45.3	2.8
IT89KD_288	3	0	MAGIC194	6	0	MAGIC027	46	4.6
MAGIC074	3	0	MAGIC048	6.3	0.5	MAGIC008	47.2	1.9
MAGIC092	3	0	MAGIC092	6.5	0.6	MAGIC208	51.5	6
Plant_ID	**RTI_C**	**Sd**	**Plant_ID**	**StressLeaf**	**Sd**	**Plant_ID**	**RTI_FL**	**Sd**
MAGIC092	23.6	16.9	IT84S_2246	0.5	0.1	MAGIC073	10	8
MAGIC048	27.4	16.1	MAGIC092	0.5	0.4	MAGIC130	17.4	10
MAGIC110	28.9	11.3	MAGIC048	0.6	0.4	IT84S_2246	17.9	4.8
MAGIC074	30.8	27.7	MAGIC073	0.6	0.4	MAGIC110	21.2	12
MAGIC194	32.2	20.5	Suvita_2	0.7	0.1	MAGIC207	26.2	14.1
MAGIC236	104	10.7	MAGIC027	3.8	0.6	MAGIC201	88	4.4
MAGIC008	104.5	8.2	MAGIC187	3.8	0.5	MAGIC265	90.6	4.1
MAGIC343	105.8	9.2	MAGIC271	3.8	1	MAGIC188	92.5	6.5
MAGIC311	107.8	10.9	MAGIC336	3.8	1.8	MAGIC264	93.1	5.4
MAGIC119	108.1	10	MAGIC208	4.2	0.7	MAGIC177	93.2	3.5
Plant_ID	**RTI_FS**	**Sd**	**Plant_ID**	**RTI_FB**	**Sd**	**Plant_ID**	**RTI_H**	**Sd**
MAGIC207	23	12.9	MAGIC130	9.6	12.2	MAGIC074	54.6	2.2
MAGIC130	24.6	11.4	MAGIC148	12.4	13.8	MAGIC153	56.7	2.4
MAGIC089	27.5	18.4	MAGIC134	12.6	13.7	MAGIC206	57.5	11.1
MAGIC119	27.7	5	MAGIC259	13.2	15.4	MAGIC072	58.4	1.4
MAGIC073	28	25.1	MAGIC146	13.8	15.6	MAGIC030	58.8	5.1
MAGIC238	86.6	7	MAGIC199	46.5	53.1	MAGIC077	85.8	10.6
MAGIC271	87.2	5.5	MAGIC242	46.6	53.6	MAGIC138	86.5	10.6
MAGIC343	88.5	5.5	MAGIC282	46.8	53.6	MAGIC280	86.9	9.1
MAGIC270	88.7	9.1	MAGIC187	47	53.6	MAGIC117	87.3	5.8
MAGIC181	89.9	4.1	MAGIC188	47.9	54.4	MAGIC199	89.5	6.3

Table 5.3. Persons' correlation coefficients for the traits evaluated for salt tolerance in a MAGIC population. Traits consisted of average number of dead plants per plot (DeadPlants), the leaf injury score under salt treatment (Score), SPAD chlorophyll under salt treatment (StressSPADChloro), relative tolerance index for SPAD chlorophyll (RTI_C), fresh leaf biomass under salt treatment (StressLeaf), relative tolerance index for fresh leaf biomass (RTI_FL), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index (RTI) for plant height (RTI_Height).

	DeadPlants	**Score**	**StressSPADChloro**	**RTI_C**	**StressLeaf**	**RTI_FL**	**RTI_FS**	**RTI_FB**	**RTI_H**
DeadPlants	1	-	-	-	-	-	-	-	-
Score	0.9	1	-	-	-	-	-	-	-
StressSPADChloro	-0.8	-0.9	1	-	-	-	-	-	-
RTI_C	-0.8	-0.8	0.9	1	-	-	-	-	-
StressLeaf	-0.6	-0.6	0.6	0.5	1	-	-	-	-
RTI_FL	-0.4	-0.4	0.4	0.4	0.7	1	-	-	-
RTI_FS	-0.2	-0.2	0.3	0.3	0.5	0.6	1	-	-
RTI_FB	-0.3	-0.2	0.3	0.3	0.5	0.6	0.6	1	-
RTI_H	-0.1	-0.1	0.2	0.1	0.2	0.3	0.4	0.3	1

Table 5.4. List of SNPs significantly associated with the traits evaluated under drought tolerance in a MAGIC cowpea population, chromosome and physical position (bp) of each SNP, LOD (-log10(p-value)), minor allele frequency MAF (%), annotated genes found within the 20-kb region flanking each significant SNP, and functional annotations for each gene ID. LOD threshold was greater or equal to 3.5. If no SNPs were above the threshold, the top 3 SNPs with the highest LOD were listed in below table. The BLINK model does not compute R_square, so no R_square information is provided.

Traits	SNP	Chr	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
DeadPlants	2_26528	3	47346498	4.1	35.1	*Vigun03g290500.1*	Homeobox associated leucine zipper
	2_05819	3	47359021	4.1	35.1	*Vigun03g290600.1*	Xyloglucan:xyloglucosyl transferase
	2_28348	3	47394698	3.7	35.7	*Vigun03g290800.1*	NA
	2_25790	7	1969327	4.1	13.6	*Vigun07g023000.1*	RNA helicase
						Vigun07g023100.1	Leucine Rich repeat
	2_07660	7	3048839	3.7	11.6	*Vigun07g032400.1*	Calcium-dependent protein kinase 32
						Vigun07g032300.1	Typa-like translation elongation factor SVR3-related
	2_02219	7	3062497	3.7	11.6	*Vigun07g032500.1*	Raffinose synthase or seed imbibition protein Sip1
	2_02220	7	3063296	3.7	11.6	*Vigun07g032500.1*	Raffinose synthase or seed imbibition protein Sip1
Score	2_13484	1	25524675	3.6	29.3	*Vigun01g093100.1*	Na+/Ca2+ K+ independent exchanger
	2_13485	1	25525542	3.6	29.3	*Vigun01g093100.1*	Na+/Ca2+ K+ independent exchanger
S_Chloro	2_14317	3	43217726	3.7	38.0	*Vigun03g263100.1*	Mitochondrial folate transporter/carrier
	2_33024	3	43218173	4.2	49.6	*Vigun03g263100.1*	Mitochondrial folate transporter/carrier
	2_45043	3	43435268	3.6	38.4	*Vigun03g264700.1*	NA
	2_15070	3	43489540	3.6	38.8	*Vigun03g265200.1*	Auxilin/cyclin G-associated kinase-related
	2_02054	3	43739483	3.9	48.8	*Vigun03g267000.1*	Clathrin coat assembly protein
						Vigun03g267100.1	Lysine methyltransferase
						Vigun03g266900.1	Phytoene dehydrogenase
	2_29692	3	43757044	3.9	48.8	*Vigun03g267200.1*	Retinaldehyde binding protein-related

Table 5.4 (Cont.)

Traits	SNP	Chr	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
S_Chloro	2_07148	3	43786460	3.7	49.2	*Vigun03g267400.1*	Succinate dehydrogenase flavoprotein subunit
	2_46677	3	44031642	3.6	48.8	NA	NA
	2_47326	3	44089702	3.6	48.8	NA	NA
	2_31683	3	44242654	3.6	48.8	*Vigun03g269900.1*	Protein DA1-related
	2_51323	3	44389344	3.7	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein family
						Vigun03g270300.1	NA
	2_20981	3	44394170	3.7	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein family
						Vigun03g270300.1	NA
	2_20980	3	44394695	3.7	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein family
						Vigun03g270300.1	NA
	2_51556	3	44395302	3.7	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein family
						Vigun03g270300.1	NA
	2_27478	3	44562081	3.7	48.8	*Vigun03g271300.1*	Vacuolar protein sorting-associated protein VPS13
	2_26528	3	47346498	4.1	35.1	*Vigun03g263000.1*	Alpha/beta hydrolase fold
	2_05819	3	47359021	4.1	35.1	*Vigun03g290600.1*	Xyloglucan:xyloglucosyl transferase
	2_28348	3	47394698	4.0	35.7	*Vigun03g290800.1*	NA
RTI_C	2_14317	3	43217726	3.5	38.0	*Vigun03g263100.1*	Mitochondrial folate transporter/carrier
	2_33024	3	43218173	4.6	49.6	*Vigun03g263100.1*	Mitochondrial folate transporter/carrier
						Vigun03g263000.1	Alpha/beta hydrolase fold-containing protein
	2_15070	3	43489540	3.6	38.8	*Vigun03g265200.1*	Auxilin/cyclin G-associated kinase-related
	2_02054	3	43739483	4.1	48.8	*Vigun03g267000.1*	Clathrin coat assembly protein
						Vigun03g267100.1	Lysine methyltransferase
						Vigun03g266900.1	Phytoene dehydrogenase
	2_29692	3	43757044	4.1	48.8	*Vigun03g267200.1*	Retinaldehyde binding protein-related
	2_07148	3	43786460	3.8	49.2	*Vigun03g267400.1*	Succinate dehydrogenase flavoprotein subunit
	2_46677	3	44031642	3.9	48.8	NA	NA
	2_47326	3	44089702	3.9	48.8	NA	NA

Table 5.4. (Cont.)

Traits	SNP	Chr	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
RTI_C	2_31683	3	44242654	3.9	48.8	*Vigun03g269900.1*	Protein DA1-related
	2_51323	3	44389344	4.0	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein-related
						Vigun03g270300.1	NA
	2_20981	3	44394170	4.0	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein-related
						Vigun03g270300.1	NA
	2_20980	3	44394695	4.0	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein-related
						Vigun03g270300.1	NA
	2_51556	3	44395302	4.0	49.6	*Vigun03g270400.1*	Cysteine-rich secretory protein-related
						Vigun03g270300.1	NA
	2_27478	3	44562081	3.9	48.8	*Vigun03g271300.1*	Vacuolar protein sorting-associated protein VPS13
	2_26528	3	47346498	3.9	35.1	*Vigun03g290500.1*	Homeobox-leucine zipper protein HAT9
	2_05819	3	47359021	3.9	35.1	*Vigun03g290600.1*	Xyloglucan:xyloglucosyl transferase
	2_28348	3	47394698	3.8	35.7	*Vigun03g290800.1*	NA
S_Leaf	2_27478	3	44562081	3.0	48.8	*Vigun03g271300.1*	Na+/Ca2+ K+ independent exchanger
	2_28348	3	47394698	3.3	35.7	*Vigun03g290800.1*	NA
	2_50921	7	16162316	3.1	24.3	NA	NA
RTI_FL	2_27478	3	44562081	3.9	48.8	*Vigun03g271300.1*	Vacuolar protein sorting-associated protein VPS13
	2_28348	3	47394698	4.0	35.7	*Vigun03g290800.1*	NA
RTI_FS	2_20734	4	40193498	3.4	10.3	*Vigun04g178400.1*	Glycosyltransferase 8 domain-containing protein
						Vigun04g178500.1	NA
						Vigun04g178300.1	CCR4-not transcription complex related
	2_13286	4	40198028	3.4	10.3	*Vigun04g178400.1*	Glycosyltransferase 8 domain-containing protein
						Vigun04g178500.1	NA
	2_13285	4	40198314	3.4	10.3	*Vigun04g178400.1*	Glycosyltransferase 8 domain-containing protein
						Vigun04g178500.1	NA
	2_44170	4	40238551	3.4	10.3	*Vigun04g178900.1*	H+/oligopeptide symporter

Table 5.4. (Cont.)

Traits	SNP	Chr	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
	2_47221	4	40244092	3.4	10.3	*Vigun04g178800.1*	NA
						Vigun04g178900.1	H+/oligopeptide symporter
						Vigun04g179000.1	Zinc finger FYVE domain containing protein
RTI_FB	2_33574	5	579544	3.6	7.0	*Vigun05g006800.1*	Mannose-6-phosphate isomerase
						Vigun05g006700.1	Alpha/beta hydrolase fold-containing protein
						Vigun05g006600.1	NA
						Vigun05g006500.1	Neoxanthin biosynthesis
RTI_H	2_26489	3	20639699	4.2	28.1	*Vigun03g171400.1*	O-methyltransferase-related
	1_0247	3	20639954	4.2	28.1	*Vigun03g171400.1*	O-methyltransferase-related
	2_04756	3	20640004	4.2	28.1	*Vigun03g171400.1*	O-methyltransferase-related
	2_34159	3	21168375	4.2	28.9	*Vigun03g172900.1*	Protein transport protein SEC23
						Vigun03g173100.1	Peptidyl-prolyl cis-trans isomerase
	2_34562	3	21184999	4.2	29.0	*Vigun03g173200.1*	Cystatin-C
	2_00955	3	21195566	4.2	28.9	*Vigun03g173300.1*	Phospholipases
	2_52154	3	21311445	4.2	28.9	*Vigun03g173800.1*	Dolichol-phosphate mannosyltransferase
	2_15515	3	21332934	4.2	28.9	*Vigun03g174100.1*	IQ-domain 9 protein
						Vigun03g174000.1	Mutt-nudix-related
						Vigun03g173900.1	Magnesium chelatase subunit I
	2_06057	3	21415465	4.2	28.9	*Vigun03g174200.1*	Ionotropic glutamate receptor
						Vigun03g174300.1	Apoptosis inhibitor 5
	2_03596	3	21479991	4.2	28.9	*Vigun03g174500.1*	NA
						Vigun03g174400.1	Peroxidase 19
	2_45312	3	21500420	4.2	28.9	*Vigun03g174600.1*	Triacylglycerol degradation
	2_39953	3	21742682	3.8	28.9	NA	NA
	2_30884	3	21777011	3.8	28.9	*Vigun03g175900.1*	Cytochrome P450
	2_37604	3	21810301	3.8	28.9	NA	NA
	2_32781	3	21841991	3.8	28.9	*Vigun03g176100.1*	Microfibril-associated protein
	2_25800	3	21872524	3.8	28.9	*Vigun03g176300.1*	Suberin monomers biosynthesis

Table 5.4. (Cont.)

Traits	SNP	Chr	Position (bp)	LOD	MAF(%)	Gene_ID	Functional_annotation
	2_14391	3	21913428	3.8	28.9	*Vigun03g176400.1*	Homoserine dehydrogenase
	2_14392	3	21914412	3.8	28.9	*Vigun03g176400.1*	Homoserine dehydrogenase
	2_54159	3	22010385	3.8	28.9	*Vigun03g177000.1*	Beta-galactosidase 9
						Vigun03g177100.1	Beta-galactosidase 9
	2_52111	3	22014192	3.8	28.9	*Vigun03g177000.1*	Beta-galactosidase 9
						Vigun03g177100.1	Beta-galactosidase 9
	2_47286	3	22025277	3.8	28.9	*Vigun03g177100.1*	Beta-galactosidase 9
	2_49598	3	23926152	3.8	28.9	NA	NA
	2_15529	3	24031773	3.8	28.9	*Vigun03g184300.1*	NA

Table 5.5. Genomic selection accuracy of traits evaluated under salt stress in a MAGIC cowpea population. Genomic selection accuracy was obtained by computing the Person's correlation coefficient between the genomic estimated breeding values (GEBVs) of the testing data set and the phenotypic value. Genomic selection model was fitted using various sizes of training data set and SNP numbers. Evaluated traits were average number of dead plants per pot (Dead), leaf injury score (Score), leaf SPAD chlorophyll under salt treatment (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (RTI_C), fresh leaf biomass under salt stress (S_Leaf), relative tolerance index for fresh leaf biomass (RTI_FS), relative tolerance index for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index for plant height (RTI_H).

Training_Data	SNP		Dead	Score	S_Chloro	RTI_C	S_Leaf	RTI_FL	RTI_FS	RTI_FB	RTI_H
50	6409	Min	0.02	0.05	0.06	0.03	0.05	-0.02	-0.01	0	0.1
		Max	0.27	0.34	0.32	0.34	0.33	0.34	0.24	0.26	0.41
		Mean	0.17	0.2	0.17	0.18	0.22	0.19	0.13	0.13	0.24
		Sd	0.06	0.05	0.05	0.05	0.06	0.07	0.06	0.06	0.06
	12819	Min	0.02	0.05	0.07	0.08	-0.01	0	-0.03	0.03	0.09
		Max	0.31	0.31	0.29	0.32	0.34	0.35	0.27	0.3	0.39
		Mean	0.17	0.2	0.17	0.19	0.22	0.2	0.14	0.15	0.25
		Sd	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.05	0.06
	19228	Min	0.01	0.07	0.04	0.08	-0.01	0.01	0	-0.01	0.08
		Max	0.33	0.31	0.32	0.35	0.35	0.37	0.3	0.28	0.39
		Mean	0.18	0.2	0.17	0.19	0.22	0.2	0.14	0.16	0.25
		Sd	0.06	0.05	0.05	0.05	0.07	0.07	0.06	0.06	0.06
	25638	Min	0.02	0.05	0.02	0.03	0.07	0.06	0.02	-0.04	0.09
		Max	0.29	0.34	0.3	0.32	0.36	0.34	0.24	0.28	0.38
		Mean	0.17	0.2	0.18	0.19	0.23	0.2	0.15	0.15	0.25
		Sd	0.06	0.05	0.05	0.06	0.06	0.06	0.05	0.06	0.06
	32047	Min	-0.02	0.02	0.03	0.05	0.08	0	0.02	0.06	0.06
		Max	0.3	0.32	0.28	0.3	0.34	0.31	0.26	0.27	0.36
		Mean	0.16	0.19	0.17	0.18	0.21	0.19	0.14	0.16	0.24
		Sd	0.07	0.06	0.06	0.06	0.06	0.07	0.05	0.05	0.06
100	6409	Min	0.02	0.03	0	0.01	0.07	0.01	-0.03	-0.01	0.11
		Max	0.31	0.33	0.32	0.39	0.39	0.35	0.3	0.27	0.43
		Mean	0.17	0.21	0.16	0.18	0.23	0.21	0.11	0.14	0.26
		Sd	0.05	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07
	12819	Min	-0.03	0	-0.04	0.01	0.08	0.03	-0.05	0	0.08
		Max	0.27	0.31	0.29	0.31	0.34	0.34	0.28	0.27	0.42

Table 5.5 (Cont.)

Training_Data	SNP	Dead	Score	S_Chloro	RTI_C	S_Leaf	RTI_FL	RTI_FS	RTI_FB	RTI_H
		Mean	0.16	0.19	0.15	0.17	0.21	0.21	0.11	0.13	0.27
		Sd	0.06	0.06	0.07	0.06	0.06	0.06	0.07	0.06	0.06
	19228	Min	-0.08	-0.03	0	-0.01	0.01	0.04	-0.03	-0.03	0.06
		Max	0.28	0.32	0.3	0.31	0.37	0.32	0.24	0.28	0.41
		Mean	0.17	0.19	0.15	0.18	0.23	0.2	0.11	0.13	0.26
		Sd	0.06	0.07	0.07	0.07	0.07	0.06	0.06	0.06	0.06
	25638	Min	-0.11	0	-0.01	0	0.06	0.08	-0.05	-0.05	0.12
		Max	0.31	0.36	0.29	0.32	0.4	0.34	0.2	0.23	0.4
		Mean	0.15	0.19	0.15	0.17	0.24	0.21	0.11	0.13	0.26
		Sd	0.07	0.07	0.05	0.06	0.06	0.06	0.05	0.06	0.06
	32047	Min	-0.02	0	-0.03	-0.01	0.06	-0.05	-0.06	-0.02	0.1
		Max	0.29	0.32	0.31	0.33	0.34	0.32	0.21	0.25	0.4
		Mean	0.16	0.19	0.15	0.17	0.22	0.2	0.1	0.13	0.26
		Sd	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.06	0.06
150	6409	Min	0	0.03	-0.05	0.01	0.05	0.03	-0.04	-0.08	0.04
		Max	0.34	0.41	0.35	0.34	0.38	0.42	0.26	0.27	0.47
		Mean	0.16	0.2	0.14	0.17	0.23	0.22	0.09	0.11	0.27
		Sd	0.07	0.08	0.08	0.08	0.07	0.08	0.07	0.07	0.08
	12819	Min	-0.05	-0.05	-0.04	-0.03	0.06	0.02	-0.08	-0.05	0.04
		Max	0.35	0.4	0.34	0.35	0.39	0.39	0.24	0.27	0.44
		Mean	0.16	0.19	0.13	0.16	0.24	0.21	0.09	0.13	0.27
		Sd	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.06	0.07
	19228	Min	-0.05	-0.01	-0.02	-0.01	0.06	-0.01	-0.12	-0.13	0.07
		Max	0.3	0.45	0.31	0.36	0.43	0.41	0.27	0.31	0.45
		Mean	0.16	0.19	0.13	0.17	0.25	0.21	0.09	0.11	0.27
		Sd	0.07	0.08	0.07	0.08	0.08	0.09	0.08	0.08	0.07
	25638	Min	-0.09	-0.04	-0.01	-0.02	0.06	-0.01	-0.09	-0.05	0.08
		Max	0.31	0.37	0.39	0.36	0.5	0.4	0.27	0.39	0.5
		Mean	0.15	0.19	0.14	0.17	0.23	0.2	0.08	0.12	0.28
		Sd	0.08	0.07	0.08	0.08	0.07	0.07	0.07	0.07	0.08
	32047	Min	-0.02	0.01	-0.07	-0.04	0.05	0.04	-0.1	-0.09	0.1
		Max	0.34	0.38	0.32	0.36	0.46	0.43	0.24	0.31	0.46
		Mean	0.16	0.2	0.13	0.16	0.25	0.22	0.08	0.12	0.28
		Sd	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.07	0.07
200	6409	Min	-0.25	-0.14	-0.27	-0.19	-0.1	-0.13	-0.17	-0.22	-0.08
		Max	0.51	0.48	0.39	0.52	0.55	0.51	0.39	0.43	0.57
		Mean	0.13	0.18	0.1	0.12	0.27	0.23	0.08	0.1	0.29

Table 5.5 (Cont.)

Training_Data	SNP	Dead	Score	S_Chloro	RTI_C	S_Leaf	RTI_FL	RTI_FS	RTI_FB	RTI_H
200	12819	Sd	0.13	0.12	0.14	0.13	0.13	0.13	0.11	0.11	0.14
		Min	-0.22	-0.17	-0.38	-0.19	-0.06	-0.1	-0.2	-0.15	0.04
		Max	0.44	0.45	0.55	0.66	0.72	0.63	0.43	0.33	0.62
		Mean	0.14	0.17	0.1	0.14	0.25	0.23	0.08	0.12	0.31
		Sd	0.12	0.13	0.15	0.13	0.14	0.13	0.12	0.12	0.12
	19228	Min	-0.11	-0.1	-0.2	-0.13	-0.23	-0.1	-0.27	-0.16	0.02
		Max	0.47	0.49	0.49	0.54	0.61	0.52	0.36	0.42	0.59
		Mean	0.18	0.21	0.13	0.17	0.24	0.21	0.07	0.11	0.29
		Sd	0.12	0.13	0.15	0.14	0.14	0.13	0.11	0.12	0.13
	25638	Min	-0.2	-0.09	-0.24	-0.25	-0.06	-0.14	-0.21	-0.24	-0.08
		Max	0.55	0.52	0.42	0.48	0.54	0.72	0.47	0.46	0.57
		Mean	0.15	0.19	0.09	0.14	0.23	0.21	0.07	0.11	0.3
		Sd	0.14	0.13	0.14	0.14	0.13	0.16	0.14	0.13	0.13
	32047	Min	-0.24	-0.11	-0.21	-0.16	-0.06	-0.08	-0.19	-0.2	0.07
		Max	0.47	0.55	0.46	0.44	0.63	0.49	0.36	0.44	0.59
		Mean	0.13	0.17	0.09	0.13	0.24	0.22	0.05	0.07	0.31
		Sd	0.14	0.13	0.13	0.12	0.14	0.14	0.11	0.14	0.12

Figures

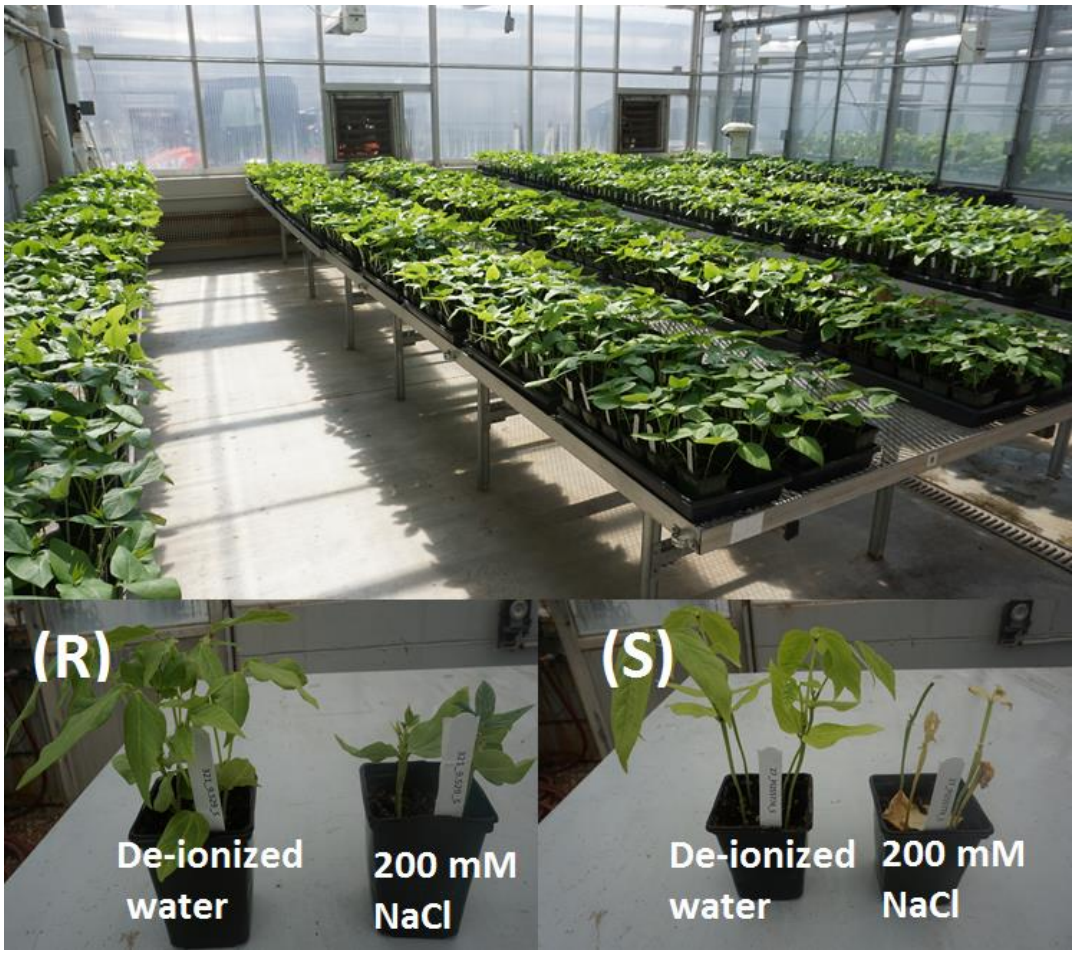

Fig. 5.1. Greenhouse experiment for salt tolerance evaluation on a MAGIC cowpea population. (R) indicates the resistant control, whereas (S) is the susceptible control.

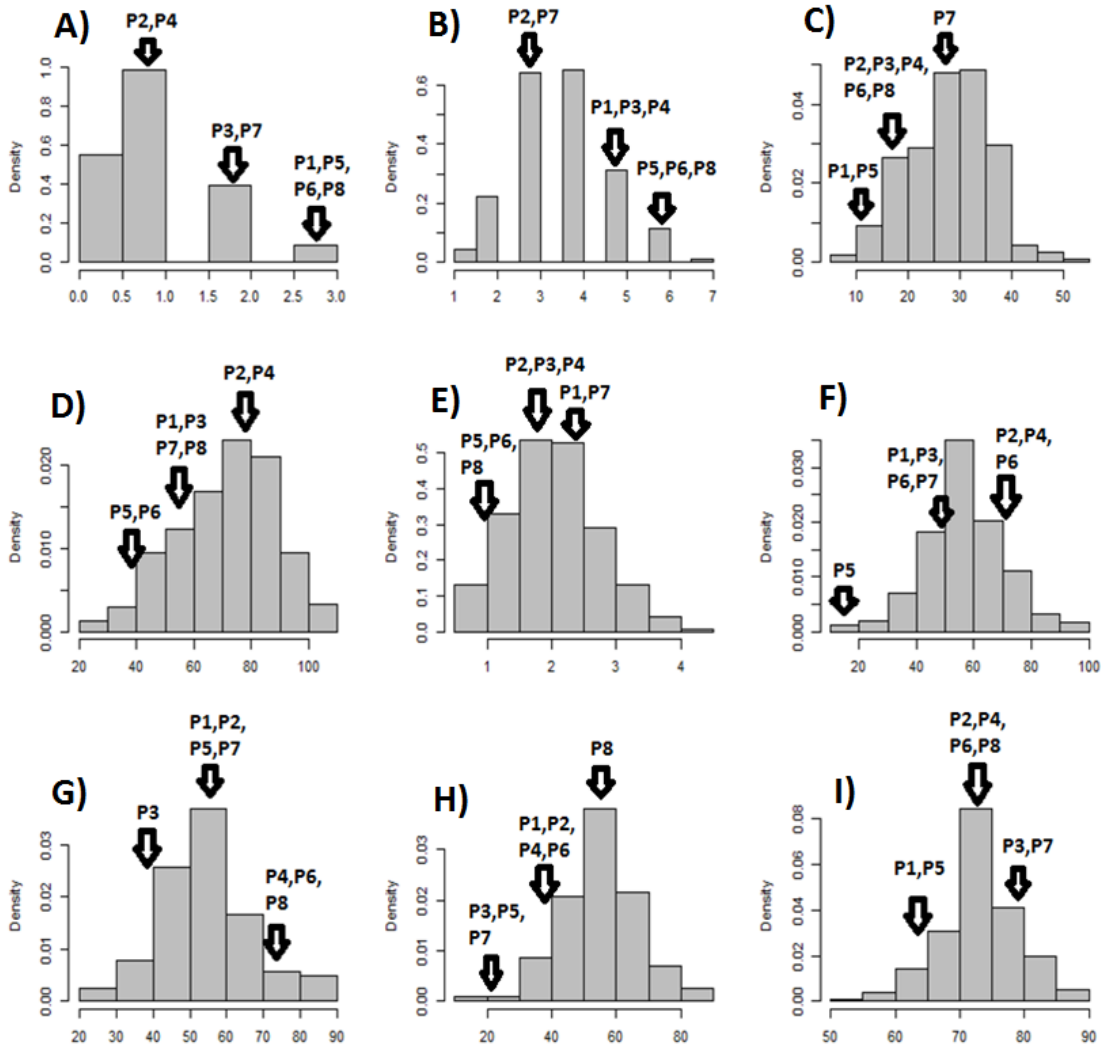

Fig. 5.2. Distribution of phenotypic values of traits evaluated under salt tolerance in a MAGIC cowpea population. A) Distribution of the average number of dead plants per pot. B) Distribution of leaf injury score. C) Distribution of SPAD chlorophyll of plants under salt stress. D) Relative tolerance index (RTI) for SPAD chlorophyll. E) Fresh leaf biomass of plants under salt stress. F) Relative tolerance index (RTI) for fresh leaf biomass. G) Relative tolerance index (RTI) for fresh stem biomass. H) Relative tolerance index (RTI) for total fresh above-ground biomass. I) Relative tolerance index (RTI) for plant height. The 8 founders were P1: CB27, P2: IT00K_1263, P3: IT82E_18, P4: IT84S_2049, P5: IT84S_2246, P6: IT89KD_288, P7: IT93K_503_1, and P8: Suvita_2.

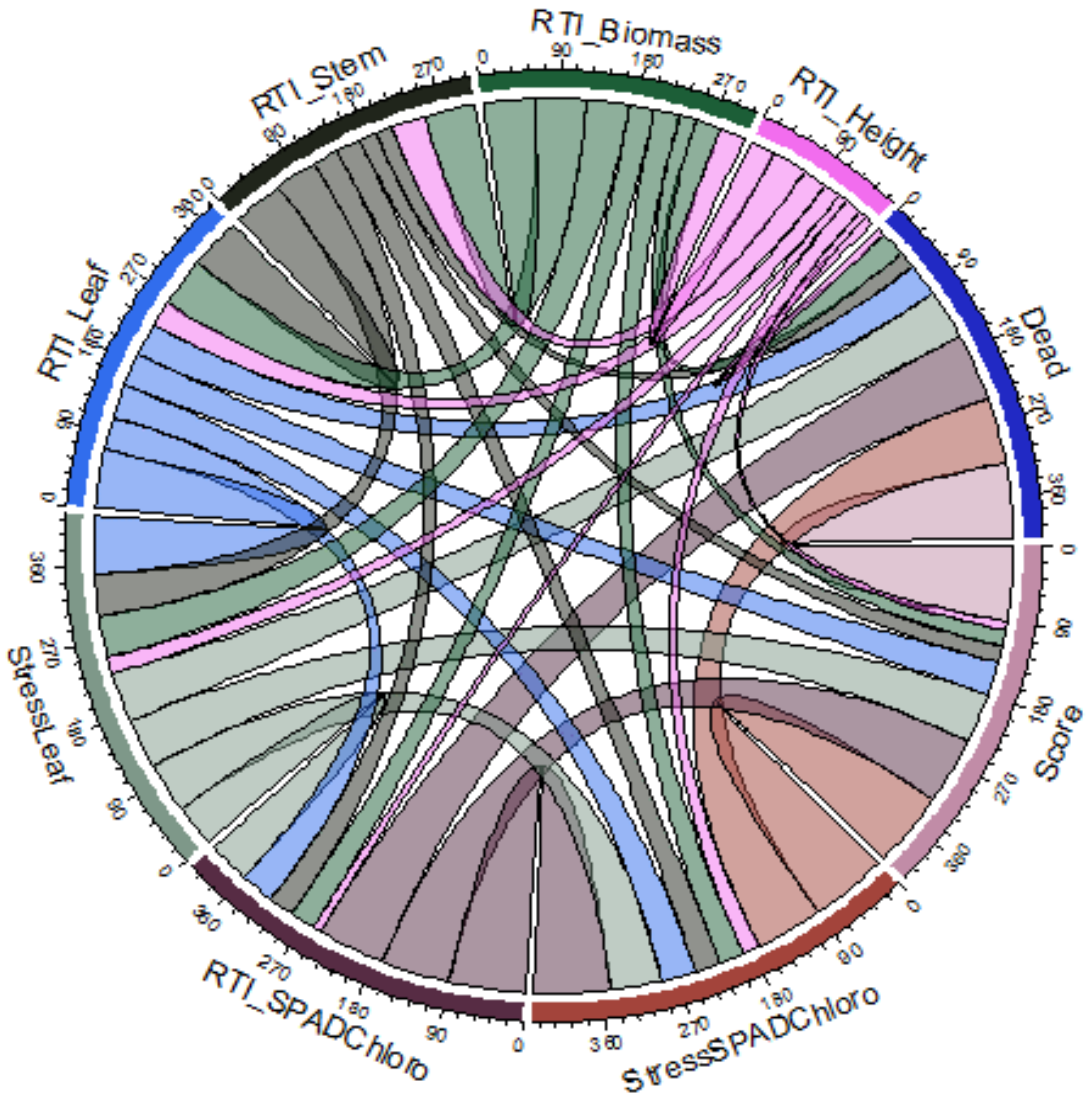

Fig. 5.3. Chord diagram showing the pairwise correlation between traits evaluated under salt tolerance in a MAGIC cowpea population. The legends outside the chord diagram correspond to the different traits (RTI_biomass= relative tolerance index for total above-ground fresh biomass, RTI_Height= relative tolerance index for plant height, Dead= average number of dead plants per pot, Score= leaf injury score, StressSPADChloro= leaf SPAD chlorophyll under salt stress, RTI_SPADChloro= relative tolerance index for leaf SPAD chlorophyll, StressLeaf= fresh leaf biomass under salt stress, RTI_Leaf= relative tolerance index for fresh leaf biomass, and RTI_Stem= relative tolerance index for fresh stem biomass). The width of the link between traits was proportional to the absolute value of the Pearson's correlation coefficient.

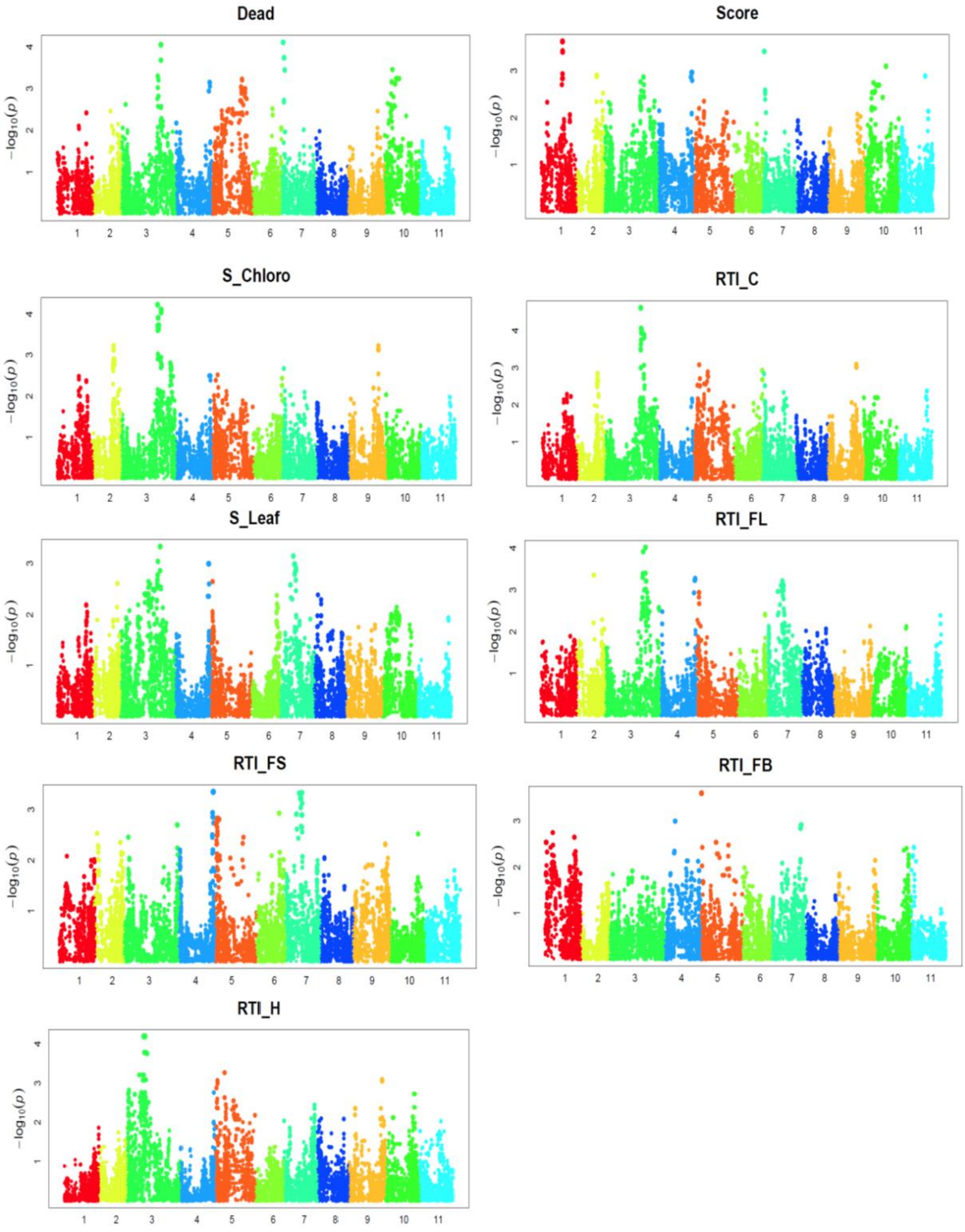

Fig. 5.4. Manhattan plots for genome-wide association study (GWAS) corresponding to the average number of dead plants per pot (Dead), leaf injury score (Score), leaf SPAD chlorophyll

under salt stress (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (RTI_C), fresh leaf biomass under salt stress (S_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), relative tolerance for fresh stem biomass (RTI_FS), relative tolerance index for total above-ground fresh biomass (RTI_FB), and relative tolerance index for plant height (RTI_H). For each Manhattan plot, the x-axis represents the chromosome number and the y-axis indicates the $-\log_{10}(p)$ where is the p-value corresponding to each SNP after running BLINK.

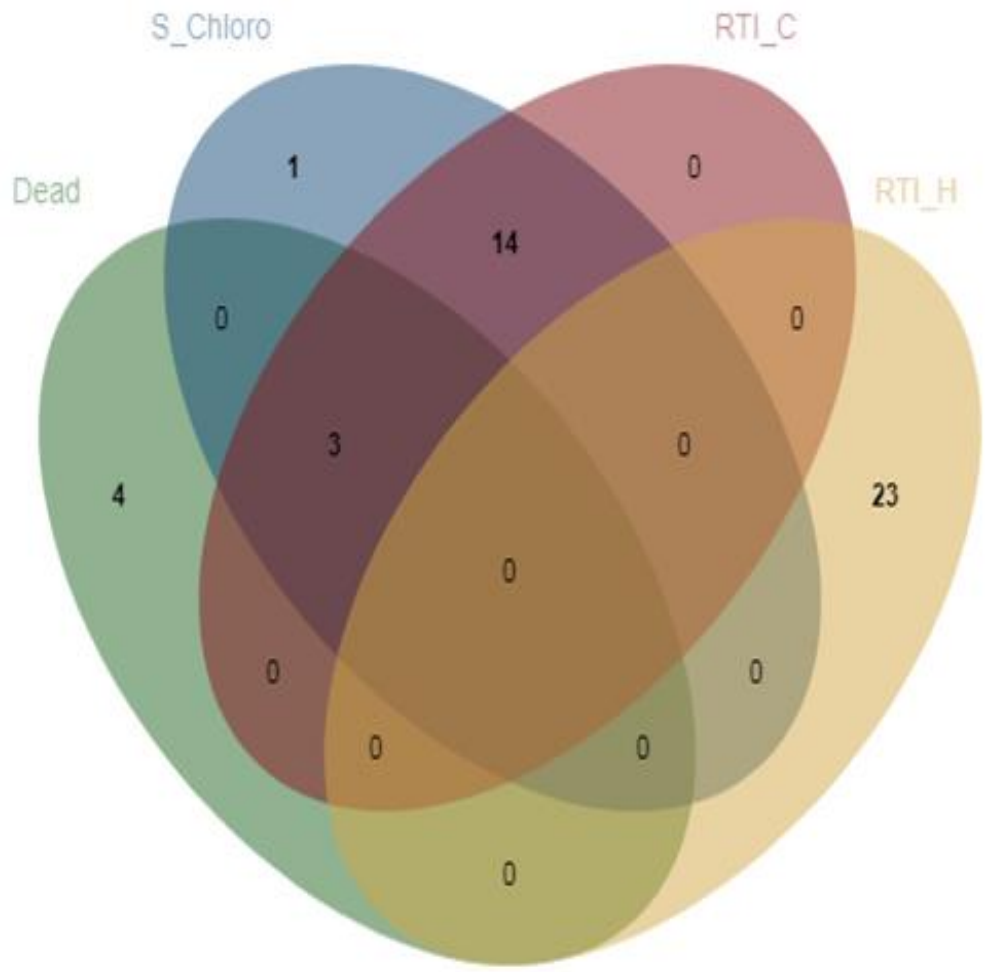

Fig. 5.5. Venn diagram showing the overlapping significant SNP markers between the average number of dead plants per pot (Dead), leaf SPAD chlorophyll under salt treatment (S_C), relative tolerance index for leaf SPAD chlorophyll (RTI_Chloro), and relative tolerance index for plant height (RTI_H). Venn diagrams were established using the online software program that is accessible at http://jvenn.toulouse.inra.fr/app/example.html.

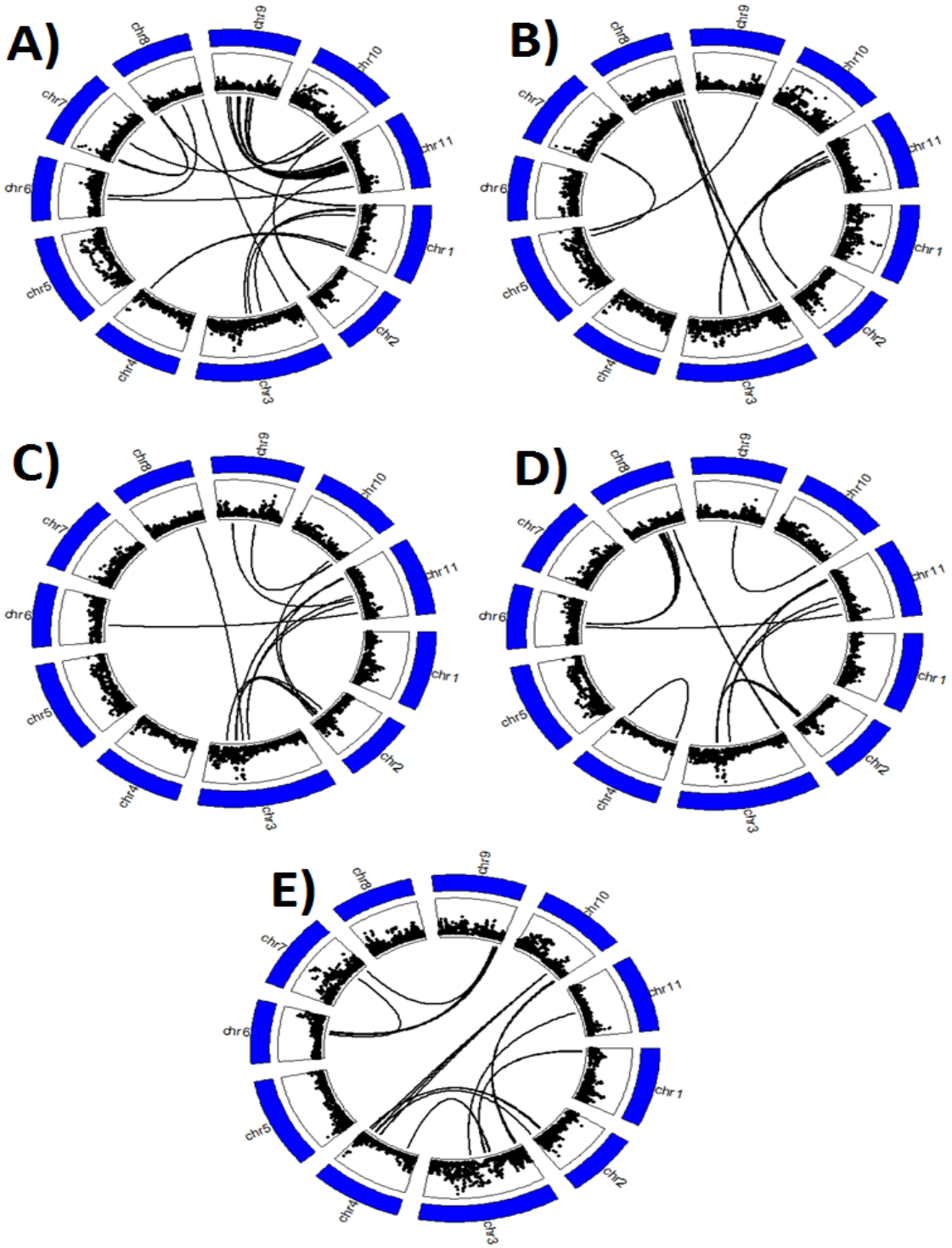

Fig. 5.6. Circos plots showing the significant pairwise epistatic interactions between SNPs. On each circos plot, the outermost layer represents the 11 chromosomes of cowpea and the length of each segment is proportional to the length of each chromosome. The innermost layer displays the SNPs used for conducting GWAS and each black dot represents one SNP. The

width of the innermost layer is proportional to the LOD values of each SNP. The further from the center the black dot is, the higher the LOD is. Links within each circos plot show a significant epistatic interaction between two SNPs. Since the resolution of the chromosomal length is in Mb (outermost layer), two closely located pairs of pairwise epistatic interactions can be cofounded in the above figure, so the number of links might not reflect the actual number of pairwise epistatic interactions. A) Average number of dead plants per pot, B) Salt injury score, C) leaf SPAD chlorophyll under salt treatment, D) relative tolerance index for leaf SPAD chlorophyll, and E) fresh leaf biomass under salt stress.

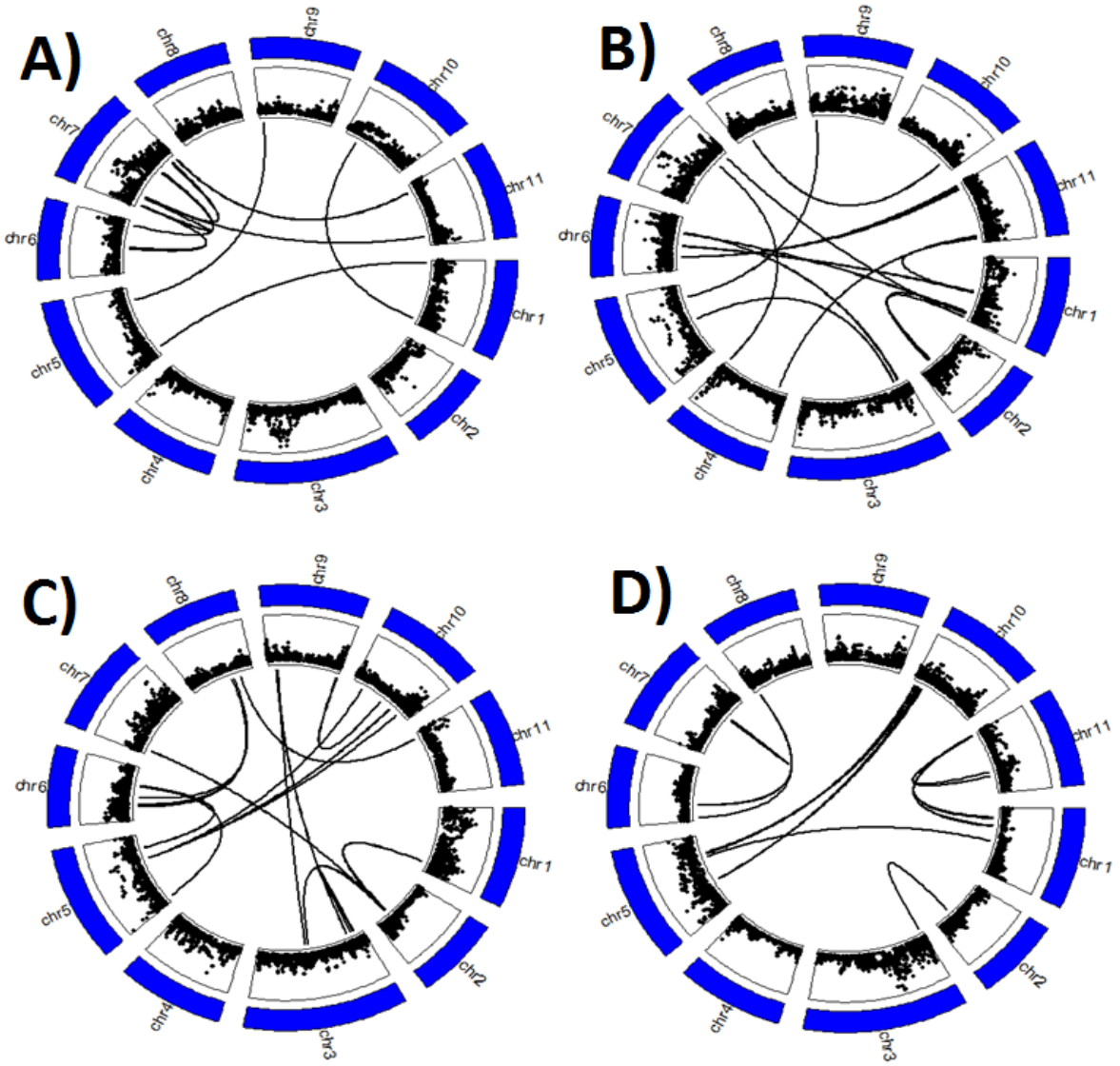

Fig. 5.7. Circos plots showing the significant pairwise epistatic interactions between SNPs. On each circos plot, the outermost layer represents the 11 chromosomes of cowpea and the length of each segment is proportional to the length of each chromosome. The innermost layer displays the SNPs used for conducting GWAS and each black dot represents one SNP. The width of the innermost layer is proportional to the LOD values of each SNP. The further from the center the black dot is, the higher the LOD is. Links within each circos plot show a significant epistatic interaction between two SNPs. Since the resolution of the chromosomal length is in Mb (outermost layer), two closely located pairs of pairwise epistatic interactions can be cofounded in the above figure, so the number of links might not reflect the actual number of pairwise epistatic interactions. A) Relative tolerance index for fresh leaf biomass, B) relative tolerance index for fresh stem biomass, C) relative tolerance index for total above-ground fresh biomass, and D) relative tolerance index for plant height.

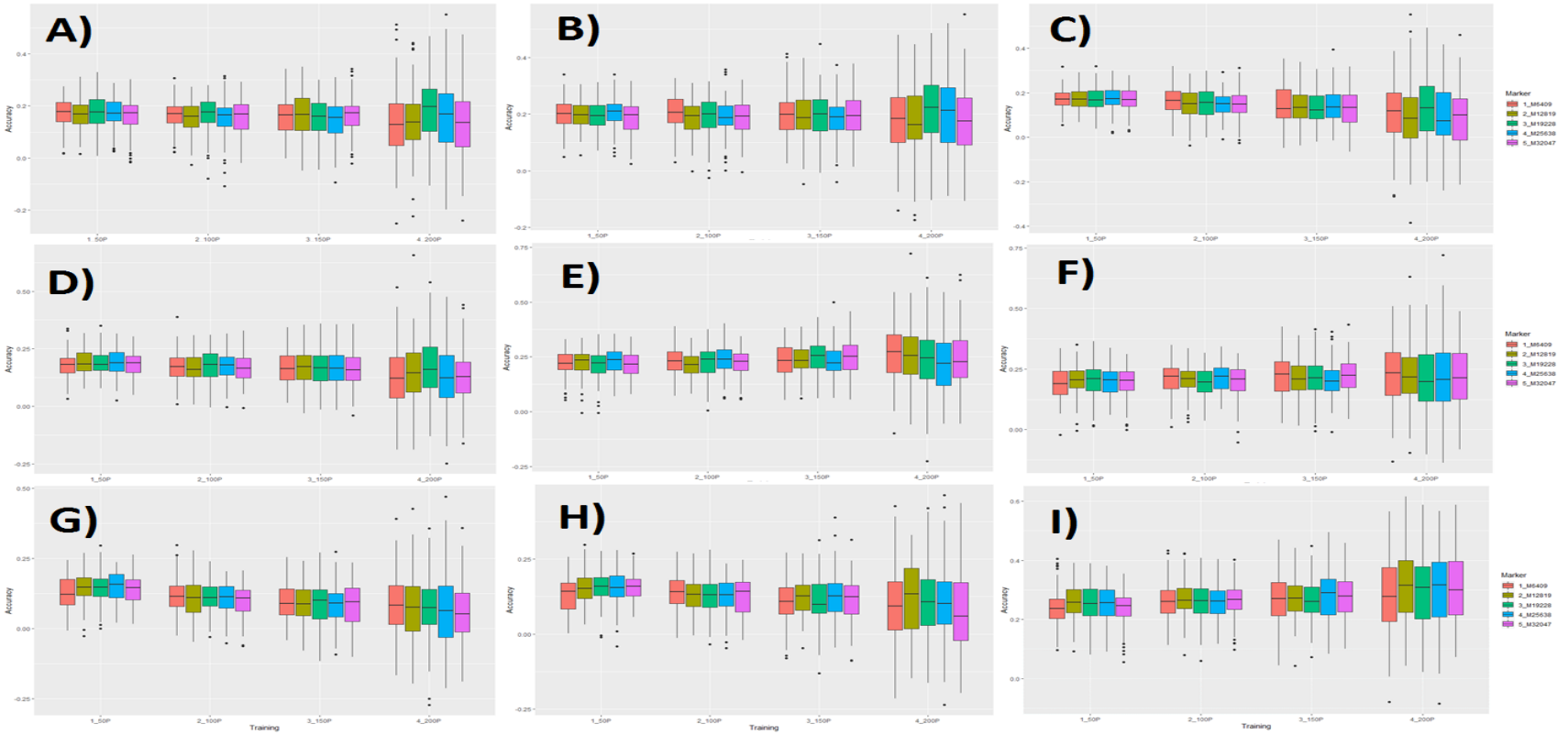

Fig. 5.8. Boxplots showing the accuracy of genomic selection for different traits evaluated under salt stress in a MAGIC cowpea population. The X-axis represented the size of training dataset (50, 100, 150, and 200). The Y-axis displayed the genomic selection accuracy. Boxplot color coding corresponded to the number of markers used during model fitting (6409 SNPs, 12819 SNPs, 19228 SNPs, 25638 SNPs, and 32047 SNPs). Traits consisted of A) average number of dead plants per pot (Dead), B) leaf injury score (Score), C) leaf SPAD chlorophyll under salt treatment (S_Chloro), D) relative tolerance index for leaf SPAD chlorophyll (RTI_C), E) fresh leaf biomass under salt stress (S_Leaf), F) relative tolerance index for fresh leaf biomass (RTI_FS), G) relative tolerance index for fresh stem biomass (RTI_FS), H) relative tolerance index for total above-ground fresh biomass (RTI_FB), and I) relative tolerance index for plant height (RTI_H).

Appendices

Table S5.1. List of the MAGIC lines evaluated for salt tolerance along with their 8 founders (top 8 genotypes on the list), average number of dead plants per plot, the leaf injury score under salt treatment, SPAD chlorophyll under no-salt treatment, SPAD chlorophyll under salt treatment, and relative tolerance index (RTI) for SPAD chlorophyll. Sd represents the standard deviation across 4 replications. RTI was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

Table S5.2. List of the MAGIC lines evaluated for salt tolerance along with their 8 founders (top 8 genotypes on the list), fresh leaf biomass under no-salt treatment, fresh leaf biomass under salt treatment, relative tolerance index (RTI) for fresh leaf biomass, fresh stem biomass under no-salt treatment, fresh stem under salt treatment, and relative tolerance index (RTI) for fresh stem biomass. Sd represents the standard deviation across 4 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

Table S5.3. List of the MAGIC lines evaluated for salt tolerance along with their 8 founders (top 8 genotypes on the list), total fresh above-ground biomass under no-salt treatment, total fresh above-ground biomass under salt treatment, relative tolerance index (RTI) for total fresh above-ground biomass, plant height under no-salt treatment, plant height under salt treatment, and relative tolerance index (RTI) for plant height. Sd represents the standard deviation across 4 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

Chapter 6. Evaluation of Cowpea for Drought Tolerance at Seedling Stage

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Ravelombola W., A. Shi, S. Chen, B. Mou, H. Xiong, Y. Yang , Q. Cui, and D. Samuel. 2020. Evaluation of cowpea for drought tolerance at seedling stage. Euphytica 216(8):1-9

Abstract

Cowpea is a health-promoting diploid legume species [*Vigna unguiculata* (L.) Walp., 2*n*=2*x*=22]. The annual cowpea production is 5.4 million tons of dry seed globally. Despite the fact that cowpea is one of the most drought-tolerant cowpea genotypes, some genotypes with excellent agronomic traits such as high yield under sufficient water supplies have been reported to be highly drought-susceptible, thus still requiring the need for breeding drought-tolerant cowpea genotypes. Therefore, the objectives of this study were to evaluate drought tolerance in cowpea at seedling stage and to identify drought-tolerant cowpea genotypes. In this study, a total of 331 cowpea genotypes were evaluated for drought tolerance at seedling stage. The experiment was conducted in a greenhouse and repeated 3 times. Drought tolerance phenotyping was conducted using a previously described methodology and a total of 11 traits were analyzed. The experiment was validated by the use of drought-tolerant and susceptible controls. Results showed that: 1) a large variation in the evaluated traits for drought tolerance was identified among the 331 cowpea genotypes, 2) a high correlation was found for traits such plant greenness score and tolerance to trifoliate leaf chlorosis under drought stress (r=0.8), whereas no linear correlation was found for traits such as tolerance to trifoliate leaf chlorosis and unifoliate leaf SPAD chlorophyll under non-drought stress (r=0.0), 3) a total of 21 genotypes were found to be drought-tolerant across different traits, and 4) country of origins could impact drought tolerance in cowpea. The top performing genotypes were repeated using an independent experiment to further validate the data. The results from this study would be of interest in breeding programs aiming at improving drought tolerance in cowpea.

Introduction

Cowpea is a diploid legume species [*Vigna unguiculata* (L.) Walp., 2*n*= 2*x*= 22], cultivated in various regions where climatic and edaphic are favorable for its production. Cowpea cultivation is prevalent in Africa, Asia, southern Europe, Oceania, and Central and Latin America. Cowpea is grown for its seeds that provide high quality protein to human consumption. In addition, cowpea seed contains nutrients that can ameliorate human's diet. Estimates of these nutrients were, in mg per 100-g seed, 6.8 mg of iron, 4.1 zinc, 1.5 manganese, 510.0 phosphorus, and 1430.0 potassium (Frota et al. 2008). Weng et al. (2017) reported that cowpea seeds contain on average 21.0-26.7% of protein. Frota et al. (2008) reported that cowpea seeds consisted of 2.2% lipid. Of which, 30% were saturated fatty acids and 70% were unsaturated fatty acids. In sub-Saharan Africa, cowpea is widely used as supplement to fodder for livestock. Cowpea leaves have been shown to enhance feed quality (Olufajo 2012). In addition to being part of the human's diet, cowpea can also be used as cover crop. In the United States, cowpea is known as southern pea or blackeye pea. A growing interest in processing cowpea into canned or frozen vegetables have been noticed in the U.S., which provides opportunities to cowpea growers to increase their production (Wilson et al. 2006).

Drought tolerance has been a growing threat to agriculture. Drought conditions can cause significant crop yield losses. Drought has been defined as being the results of lack of water supplies that are critical in maintaining proper plant growth and development and in providing reasonable crop yields (Blum and Ebercon 1981). Despite the fact that cowpea is one of the most drought-tolerant legumes, some cultivars that have excellent agronomic traits such as high yield under a normal water irrigation regime are still highly susceptible to drought stress (Ravelombola et al. 2018). The incorporation of drought-tolerant trait into these cultivars would

allow for their cultivation in areas where water deficit conditions are prevalent. Doing so will provide cowpea growers with additional production, which will make cowpea production more profitable (Okiror et al. 2008). Moreover, prediction of water shortage still remains challenging despite the significant progress being made in weather forecasting, which has resulted in a poor planning of agricultural activities. Choice of sowing date is one the critical activities that should be carefully taken into a consideration. However, an unpredicted rainfall shortage occurring few weeks after sowing could lead to severe drought conditions affecting plant seedling, thus leading to plant death (Ajayi et al. 2018). Being provided with genotypes that better withstand drought stress at seedling stage would be an efficient way to address the aforementioned constraints. However, the development of drought-tolerant cultivars requires a good phenotyping strategy and understanding of the genetics of drought tolerance, which has been reported to be a complex mechanism (Golldack et al. 2014).

Drought stress affects all developmental and growth stages of cowpea (Singh et al. 1999; Verbree et al. 2015). Seedling stage is one of the most critical stages to drought stress in cowpea (Agbicodo et al. 2009). Two types of drought tolerance have been described in cowpea. Type I drought-tolerant genotypes can maintain both unifoliate and trifoliate leaves fully green under drought conditions, whereas type II drought-tolerant genotypes can only delay senescence in trifoliate leaves (Mai-Kodomi et al. 1999). A total of 30 cowpea genotypes were tested for their types of drought tolerance and results suggested that type II drought tolerance were more prevalent (Ravelombola et al. 2018). In addition, traits such as leaf chlorosis and leaf SPAD chlorophyll have been demonstrated to be useful in assessing drought tolerance in cowpea (Ravelombola et al. 2018; Singh et al 1999; Verbree et al. 2015). However, little has been done regarding evaluating cowpea drought tolerance based on these traits and using a larger

population size. In addition, cowpea has a relatively small genome size (~620 Mb) (Lonardi et al. 2019), thus can be used as an excellent model crop to understand the genetics of drought tolerance in legumes. Therefore, the objectives of this study were to evaluate drought tolerance of cowpea at seedling stage and to identify drought-tolerant cowpea genotypes.

Materials and Methods

Plant materials

A total of 331 cowpea genotypes were evaluated for drought tolerance in this study (Tables S6.1-S6.2). Of which, 36 were breeding lines from the University of Arkansas, Fayetteville,

8 were obtained from the University of California, Riverside and were used to build the first cowpea multiparent advanced generation intercross (MAGIC) population (Huynh et al., 2018), 287 were Plant Introductions (PIs) from the U.S. Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea accessions. PIs were provided by the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. These cowpea genotypes originate from more than 32 countries. Seeds from each genotype were planted in the summer of 2018 at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville. At harvest, one plant was harvested for each genotype. Single-plant derived seeds were cleaned up and carefully sorted prior to conducting the drought tolerance experiment.

Growing conditions and experiment design

Drought tolerance evaluation has been conducted in the greenhouse at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville (Fig. 6.1). Greenhouse day/light temperatures were 26°C/21°C and daylight length was 14 hours. Drought

tolerance evaluation was carried using a previously described methodology (Ravelombola et al. 2018; Singh et al. 1999; Verbree et al. 2015). Sterilite propylene boxes (Sterilite corporation, Townsend, MA) with dimensions 88.6 X 42.2 X 15.6 cm were filled up with Sunshine® Mix #1 Natural & Organic (SunGro Horticulture, Agawan, MA) up to 10.5 cm high. Each box was irrigated with 12 L of tap water at 2 days before sowing to attain field capacity.

A total of 10 rows were established within each box and distance between each row was 7.5 cm. A total of 6 holes were designed within each row. Each genotype was planted within each row and a total of 2 seeds were sown within each hole. Plants were thinned to one plant per hole at emergence. Vigorous and uniform plants were kept. One week after plant emergence, fertilizers were applied by irrigating each row with a 150 mL solution of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI). Fertilizers were prepared by dissolving one tablespoon of Miracle-Gro into one gallon of tap water. Irrigation was conducted by watering each row with 150 mL tap water at 3-day interval until the first trifoliate was fully expanded. At this time, irrigation was stopped for one box, which was the drought-stressed box, whereas watering was pursued in another box, which was the well-watered treatment. In order to minimize the environmental effects within the greenhouse, each drought-stressed box was placed next to the well-watered one (Fig. 6.1). A total of 3 drought-tolerant genotypes (PI293469, PI349674, and PI293568) and 1 drought-susceptible genotype (PI255774) were used to validate the experiments (Ravelombola et al. 2018).

Due to space limitations, the experiment was conducted using 3 runs and each run was the replication. Therefore, the experiment was a randomized complete block design (RCBD) with 3 blocks (not a split-plot design since comparing drought and well-watered conditions was not the objective of this study). The experimental unit was one row where each genotype was

planted. The factor of interest was the set of 331 cowpea genotypes and each genotype corresponded to one treatment. Soil moisture within boxes was recorded using an HH2 Moisture Meter (Delta-T Devices, Cambridge, UK) every 3 days.

Data measurements

Plant greenness score and recovery rate

Plant greenness score and recovery rate have been previously shown to be accurate parameters for assessing drought tolerance at seedling stage in cowpea (Ravelombola et al. 2018). Plant greenness was recorded when the susceptible genotype was completely dead. Recovery rate corresponded to the number of plants that fully recovered after one week of rewatering. Plant greenness was assessed using a previously described scale (1 = plants were completely green, 2 = plants began losing greenness, 3 = signs of chlorosis and necrosis were visible, 4 = chlorosis and necrosis was severe, and 5 = plants were completely dead) (Ravelombola et al. 2018). Data on plant greenness under drought stress were recorded on a per plant basis.

Unifoliate and first trifoliate leaf chlorosis

Evaluating tolerance to unifoliate and first trifoliate leaf chlorosis has been shown to help in determining whether a genotype is type I drought-tolerant or type II drought-tolerant. Type I drought-tolerant cowpea genotypes showed tolerance to both unifoliate and first trifoliate leaf chlorosis, whereas those which were type II drought-tolerant were tolerant to trifoliate leaf chlorosis but susceptible to unifoliate leaf chlorosis (Verbree et al. 2015). For each genotype, the number of plants showing unifoliate chlorosis was evaluated at two different time points. The first one corresponded to the time when the susceptible control had more than 50 % of its unifoliate leaf being chlorotic. Unifoliate leaf chlorosis was assessed for the second time when

the susceptible control was completely dead. At this time, the number of plants having their first trifoliate leaves being chlorotic was also recorded.

In vivo chlorophyll for unifoliate and first trifoliate leaves

Leaf SPAD chlorophyll on both unifoliate and trifoliate leaves was an objective measurement of both plant greenness and tolerance to unifoliate/first trifoliate leaf chlorosis. Data on leaf SPAD chlorophyll were taken when the susceptible genotype was completely dead and were recorded using a SPAD-502 Plus (Spectrum Technologies, Inc., Plainfield, IL). For each plant, leaf SPAD chlorophyll was taken separately for the unifoliate leaves and trifoliate leaves. For each measurement, one unifoliate leaf was randomly chosen and measurements were taken at three different positions on the leaf surface in order to minimize the edge effect (Ravelombola et al. 2018). For the first trifoliate leaf, one measurement was conducted from each leaf and the average measurements from each first trifoliate leaf (first trifoliate leaves consisted of 3 leaves) was recorded.

Data analysis

ANOVA was conducted to analyze plant greenness score (Score), recovery rate (Recov), number of plants having chlorotic unifoliate leaves (Uni_1: when the susceptible genotype had more than 50 % of its unifoliate leaf being chlorotic, Unif: when the susceptible genotype was completely dead), number of plants having chlorotic trifoliate leaves (Tri), unifoliate leaf SPAD chlorophyll under drought stress (C_U_S), unifoliate leaf SPAD chlorophyll under non-drought stress (C_U_NS), relative tolerance index for unifoliate leaf SPAD chlorophyll (RTI_U= 100*(C_U_S/ C_U_NS)), trifoliate leaf SPAD chlorophyll under drought stress (C_T_S), trifoliate leaf SPAD chlorophyll under non-drought stress (C_T_NS), and relative tolerance index for trifoliate leaf SPAD chlorophyll (RTI_T=100*(C_T_S/ C_T_NS)).

ANOVA was run using PROC MIXED of SAS® 9.4 (SAS Institute Inc., Cary, NC). Mean separation analysis was done using a protected least significant difference (LSD) procedure at $\alpha=0.05$. LSD procedure was defined as $LSD=t\alpha/2\sqrt{2}MSError/n$, with $t\alpha/2$ being the critical value from the t-table and having a degree of freedom [df(SSError)] corresponding to the difference between the number of observations and the number of replications, and n being the number of replications. The statistical model for conducting ANOVA was the following.

$$Y_{ij} = \mu + B_i + G_j + \varepsilon_{ij} \text{ where } i=1,2,3, \text{ and } j=1\ldots.331$$

with μ being the overall mean, Y_{ij} being the response from the j^{th} genotype (G_j) (fixed effect) at the i^{th} block (B_i) (random effect), and ε_{ij} being the random error associated with the ij^{th} observation.

The effects of countries of origin on the different traits evaluated for drought tolerance were assessed using ANOVA. SAS® 9.4 (SAS Institute Inc., Cary, NC) was also used to conduct ANOVA via PROC MIXED. Country of origins was classified into 4 regions (Africa, America, Asia, Europe_The_MiddleEast). Groups could not be split further due to sample size limitation for some geographical areas. The statistical model for conducting ANOVA was the following.

$Y_{ij} = \mu + R_i + \varepsilon_{ij}$ where i=1,2,3,4 and j was the sample size within each geographical area with μ being the overall mean, Y_{ij} being the response from the i^{th} group (R_i) (fixed effect) and ε_{ij} being the random error associated with the ij^{th} observation.

Data distribution was visualized using the MASS package of R® 3.6.1. Pearson's correlation coefficients between the traits evaluated for drought tolerance were calculated using JMP Genomics 9 (SAS Institute Inc., Cary, NC). Cluster analysis was conducted using Ward'

method in JMP Genomics 9 (SAS Institute Inc., Cary, NC) (Sahu 2013). The broad sense heritability (*H*) was estimated using the following formula (Holland 2003).

$$H = \sigma^2_G / [\sigma^2_G + (\sigma^2_e/n_b)]$$

with σ^2_G being the total genetic variance, σ^2_e being the residual variance, and n_b being the number of blocks. The estimates for σ^2_G and σ^2_e were [EMS(G)- Var(Residual)]/ n_b and Var(Residual). EMS(G) and Var(Residual) were obtained from the ANOVA table.

Results

Plant greenness score

A large variation in plant greenness score was found among the 331 genotypes evaluated for drought tolerance. Plant greenness score varied from 1.7 to 5.0, with an average of 3.5 and a standard deviation of 0.6. Plant greenness score was approximately normally distributed as shown in Fig. 6.2A. Plant greenness was significantly different among the 331 cowpea genotypes (F-value=2.24, p-value<0.0001) (Table 6.1). The lower the plant greenness score was, the greener the plant was under drought score. The genotypes with the lowest plant greenness score were PI664524 (1.7), PI300173 (1.8), PI583550 (2.0), PI582575 (2.0), PI293476 (2.1), PI583251 (2.1), PI293568 (2.1), PI207527 (2.2), PI227829 (2.2), PI293469 (2.2), PI582469 (2.3), PI582697 (2.3), PI194211 (2.4), and PI221730 (2.4) (Table 6.2), indicating that these genotypes were drought-tolerant based on plant greenness score. The genotypes with the lowest plant greenness score were 'Early Acre' (4.6), PI582924 (4.6), PI582812 (4.6), PI527563 (4.6), PI582530 (4.6), PI406290 (4.7), PI229796 (4.8), PI583247 (4.9), and PI255774 (5.0) (Table 6.2), suggesting that these genotypes were susceptible to drought stress based on plant greenness

score. For all traits evaluated for drought tolerance, block effect was significant (p-values≤0.0059). The broad-sense heritability for plant greenness score was 78.8 %.

Recovery rate

The average number of fully recovered plants varied from 0.0 to 3.3, with an average of 0.3 and a standard deviation of 0.6. The distribution of the average number of fully-recovered plants was right-skewed (Fig. 6.2B). A $\log_2$ transformation was applied prior to conducting ANOVA. A significant genotype effect on the average number of fully recovered plants was identified (F-value=3.82, p-value<0.0001) (Table 6.2). The genotypes with the highest plants that were fully recovered after one week of rewatering were PI406293 (3.3), PI339587 (2.7), PI293582 (2.3), PI390421 (2.3), 09-481 (2.3), PI662992 (2.3), 09_1090 (2.3), PI664524 (2.0), PI75962 (2.0), PI339600 (2.0), 09-749 (2.0), PI608035 (2.0), PI610533 (2.0), 09-655 (2.0), and PI271256 (2.0) (Table 6.2), indicating that these genotypes have the ability to survive when water supplies become available after some time of drouth stress. However, a large number of genotypes did not recover. For example, the genotypes PI503326 (0), PI666251 (0), PI189374 (0), PI255774 (0), 'Epic Select.4' (0) (Table 6.2) fail to recover after rewatering. The broad-sense heritability for recovery rate was 73.8%.

Unifoliate leaf chlorosis 1 (Uni_1)

Tolerance to unifoliate chlorosis was first assessed when the susceptible control, PI255774, had more than 50% of its unifoliate leaves being chlorotic. The average number of plants having chlorotic unifoliate leaves (Uni_1) varied from 0.0 to 6.0, with an average of 2.5 and a standard deviation of 1.5. Uni_1 was approximately normally distributed (Fig. 6.2C). Uni_1 was significantly different among the 331 cowpea genotypes evaluated for drought tolerance (F-value=2.34, p-value<0.0001) (Table 6.1). The genotypes that were the most tolerant

to unifoliate chlorosis were PI152196 (0), PI152197 (0), PI167284 (0), PI180014 (0), PI190191 (0), PI194213 (0), PI582942 (0), PI583200 (0), PI583203 (0), PI583251 (0), PI583550 (0), PI662993 (0), PI292897 (0), Suvita_2 (0), IT84S_2246 (0), and PI75962 (0) (Table 6.2). The ones that were the most susceptible to unifoliate chlorosis were PI255774 (5.3), PI293545 (5.3), PI582354 (5.3), PI582468 (5.3), PI582541 (5.3), PI582727 (5.3), PI582850 (5.3), PI582926 (5.3), PI583247 (5.3), PI582815 (5.7), PI582810 (6.0), PI349674 (6.3) (Table 6.3). The broad-sense heritability for Uni_1 was 80.1%.

Unifoliate leaf chlorosis 2 (Uni_f)

Tolerance to unifoliate chlorosis was re-evaluated when the susceptible control, PI255774, was completely dead. The average number of plants having unifoliate chlorotic leaves (Uni_f) ranged between 2.0 and 6.0, with an average of 5.6 and a standard deviation of 0.6. The distribution of Uni_f was left-skewed (Fig. 6.2D). A $\log_2$ transformation was applied before running ANOVA. A significant difference in Uni_f was found among the cowpea genotypes (F-value=1.58, p-value<0.0001) (Table 6.1). The genotypes that were the most tolerant to unifoliate leaf chlorosis were PI664524 (2.0), PI582942 (3.0), PI598335 (3.0), PI293568 (3.3), PI194213 (3.7), PI583200 (3.7), PI583203 (3.7), PI583251 (3.7), PI292897 (3.7), PI583209 (3.7), and PI300173 (3.7) (Table 6.2). A large number of genotypes were susceptible to unifoliate leaf chlorosis. For example, the genotypes PI250416 (6.0), ‘Empire’ (6.0), ‘Empress’ (6.0), ‘Epic Select.4’ (6.0), and ‘Excel’ (6.0) (Table 6.2) were susceptible to unifoliate leaf chlorosis. The broad-sense heritability for Uni_f was 63.5%.

First trifoliate leaf chlorosis

A large variation in tolerance to first trifoliate leaf chlorosis was identified among the different cowpea genotypes evaluated for drought tolerance. The average number of plants

having chlorotic first trifoliate leaves (Tri) varied from 0.0 to 6.0, with an average of 4.5 and a standard deviation of 1.4. Tri was left-skewed distributed (Fig. 6.2E). A $\log_2$ transformation was done prior carrying out ANOVA. A significant difference in Tri among the 331 cowpea genotypes was identified (F-value=2.42, p-value<0.0001) (Table 6.1). The genotypes that were highly tolerant to first trifoliate leaf chlorosis were PI293476 (0), PI583550 (0), PI664524 (0.3), PI583251 (0.3), PI194211 (0.3), PI662993 (0.3), PI207527 (0.7), PI293568 (0.7), PI582575 (0.7), PI194213 (1.0), PI227827 (1.0), PI293470 (1.0), PI293582 (1.0), IT00K_1263 (1.0), PI194210 (1.0), and PI194209 (1.0) (Table 6.2). A large number of genotypes were susceptible to first trifoliate leaf chlorosis. For example, PI491193 (6.0), 'Early Scarlet' (6.0), 'Elegance' (6.0), 'Empress' (6.0), 'Epic Select.4' (6.0) (Table 6.2) were highly susceptible to first trifoliate leaf chlorosis.

Unifoliate leaf SPAD chlorophyll

Unifoliate leaf SPAD chlorophyll (C_U_NS) was evaluated for plants under non-drought stress conditions. A large variation in C_U_NS was identified among the cowpea genotypes. C_U_NS ranged between 18.5 and 54.5, with an average of 34.4 and a standard deviation of 4.2. C_U_NS was approximately normally distributed (Fig. 6.2F). A significant variation in C_U_NS was found among the 331 cowpea genotypes evaluated for drought tolerance (F-value=1.8, p-value<0.0001) (Table 6.1). The genotypes IT84S_2246 (54.5), IT93K_503_1 (53.8), PI582863 (46.6), IT89KD_288 (45.3), Suvita_2 (44.7) (Table 6.3) had the highest C_U_NS, whereas PI583202 (26.2), PI583513 (25.4), PI663148 (25.4), PI583551 (25.2), and PI583240 (18.5) (Table 6.3) had the lowest C_U_NS. The broad-sense heritability for C_U_NS was 70.5%.

A large variation in unifoliate leaf SPAD chlorophyll (C_U_S) was found among the 331 cowpea genotypes under drought stress. C_U_S varied from 5.1 to 53.7, with an average of 24.4

and a standard deviation of 7.3. The distribution of C_U_S was approximately normal (Fig. 6.2F). A large variation in C_U_S was identified among the cowpea genotypes (F-value=2.33, p-value<0.0001) (Table 6.1). The genotypes with the highest C_U_S were IT84S_2246 (53.7), IT93K_503_1 (48.0), PI583200 (47.0), Suvita_2 (44.4), and 'EpicSelect.4' (41.1) (Table 6.3), indicating that these genotypes were drought-tolerant based on unifoliate leaf SPAD chlorophyll under stress. The genotypes with the lowest C_U_S were PI582468 (10.1), PI293545 (9.2), PI582815 (7.7), PI582850 (7.2), and PI582810 (5.1) (Table 6.3), suggesting that these genotypes were susceptible to drought conditions based on unifoliate leaf SPAD chlorophyll. The broad-sense heritability for C_U_S was 79.9%.

Relative tolerance index for unifoliate leaf SPAD chlorophyll (RTI_C_U) was computed in order to assess the relative effect of drought stress on unifoliate leaf SPAD chlorophyll. A large variation in RTI_C_U was found among the 331 cowpea genotypes. RTI_C_U varied from 19.7 to 183.1, with a mean of 72.7 and a standard deviation of 20.7. RTI_C_U was approximately normally distributed (Fig. 6.2G). A significant difference in RTI_C_U was found among the cowpea genotypes (F-value=1.81, p-value<0.0001) (Table 6.1). The genotypes with the highest RTI_C_U were PI583240 (183.1), PI663148 (136.8), PI293500 (122.2), IT00K_1263 (118.4), and PI200867 (113.7) (Table 6.3), whereas those with the lowest RTI_C_U were PI293545 (27.1), AR_BE_1 (26.1), PI582850 (23.3), PI582815 (21.1), and PI582810 (19.7) (Table 6.3). The broad-sense heritability for RTI_C_U was 70.8%.

Trifoliate leaf SPAD chlorophyll

SPAD chlorophyll on the first trifoliate leaf (C_T_NS) was also analyzed for the plants under non-drought stress conditions. A large variation in C_T_NS was found among the cowpea genotypes evaluated for drought tolerance. C_T_NS ranged between 26.7 and 54.7, with an

average of 38.3 and a standard deviation of 4.2. The distribution of C_T_NS was approximately normal (Fig. 6.2H). The effect of the genotype on C_T_NS was significant (F-value=1.96, p-value<0.0001) (Table 6.1). The genotypes having the highest C_T_NS were IT84S_2246 (54.7), IT93K_503_1 (53.3), IT89KD_288 (51.9), PI582863 (50.9), and PI582789 (49.3) (Table 6.3), whereas those with the lowest C_T_NS were PI582566 (29.4), PI583274 (28.9), PI663011 (28.2), PI583551 (27.6), and PI583197 (26.7) (Table 6.3). The broad-sense heritability for C_T_NS was 74.2%.

Data on SPAD chlorophyll on the first trifoliate leaf (C_T_S) was also investigated. C_T_S varied from 22.0 to 57.7, with an average of 37.0 and a standard deviation of 5.0. C_T_S was approximately normally distributed (Fig. 6.2H). A large variation in C_T_S was identified among the 331 cowpea genotypes (F-value=686.13, p-value<0.0001) (Table 6.1). The genotypes with the highest C_T_S were IT84S_2246 (57.7), IT93K_503_1 (55.5), PI390421 (52.4), IT89KD_288 (50.3), and Suvita_2 (48.7) (Table 6.3), indicating that these genotypes had a good tolerance to trifoliate leaf chlorosis. The genotypes with the lowest C_T_S were PI582572 (25.3), PI582571 (24.6), PI582421 (24.3), PI582570 (24.1), and PI582567 (22.0) (Table 6.3), suggesting that these genotypes were susceptible to trifoliate leaf chlorosis under drought stress. The broad-sense heritability for C_T_S was 70.9%.

Relative tolerance index was calculated to assess the relative effect of drought stress on trifoliate leaf SPAD chlorophyll (RTI_C_T). A large variation in RTI_C_T was identified among the cowpea genotypes. RTI_C_T varied from 61.8 to 414.2, with an average of 98.3 and a standard deviation of 13.6. RTI_C_T was approximately normally distributed (Fig. 6.2I). A significant difference in RTI_C_T was found among the cowpea genotypes (F-value=1.24, p-value=0.0113) (Table 6.1). The genotypes with the highest RTI_C_T were PI583551 (141.2),

PI583550 (131.7), PI293584 (128.8), PI354860 (126.0), and PI354854 (125.9) (Table 6.3), indicating that these genotypes were drought-tolerant based on RTI_C_T. The genotypes PI582810 (71.2), PI582571 (68.6), PI582573 (68.4), PI582421 (63.6), and PI582567 (61.8) (Table 6.3) had the lowest RTI_C_T, suggesting that these genotypes were the most susceptible based on RTI_C_T. The broad-sense heritability for RTI_C_T was 41.7%.

Drought tolerance and geographical locations

The effect geographical locations on traits evaluated for drought tolerance were assessed. Results showed that geographical location differences were significant for traits such as plant greenness score (F-value=5.94, p-value=0.0005), recovery rate (F-value=4.09, p-value=0.0068), average number of plants having chlorotic unifoliate leaves when the susceptible control had more than 50% of its unifoliate leaves being chlorotic (F-value=11.39, p-value<0.0001), average number of plants having chlorotic first trifoliate leaves (F-value=9.7, p-value<0.0001), unifoliate leaf SPAD chlorophyll (F-value=4.65, p-value=0.0032), relative tolerance index for unifoliate leaf SPAD chlorophyll (F-value=7.33, p-value<0.0001), and relative tolerance index for trifoliate leaf SPAD chlorophyll (F-value=6.53, p-value=0.0002) (Table 6.4) (Fig. 6.3). Genotypes from America and Asia had the lowest plant greenness score, thus more drought-tolerant (Table 6.5). Interestingly, genotypes from Africa had the highest plant greenness score, which was not expected. Genotypes from America and Asia recovered the best after rewatering. Despite the fact that genotypes from America and Asia were equally recovered after rewatering, those from America had large variation in terms of recovery rate (Fig. 6.3B). Results suggested that genotypes from America and Asia had the highest unifoliate leaf SPAD chlorophyll, thus being more drought-tolerant based on this trait. However, genotypes from Europe and the Middle East had the lowest unifoliate leaf SPAD chlorophyll (Table 6.5). Relative tolerance index was the

highest for genotypes from Asia and America and was the lowest for those from Europe and the Middle East. In addition, genotypes from Africa, Europe, and the Middle East had more plants with unifoliate leaf chlorosis than those from America and Asia under drought stress (Table 6.5). Most of the genotypes from Africa were more susceptible to trifoliate leaf chlorosis than those from other regions under water deficit conditions. In addition, the genotypes from Asia were the best in terms relative tolerance index for trifoliate leaf SPAD chlorophyll, then followed by the genotype from Asia, and the genotypes from Africa, Europe, and the Middle East ranked last in terms of trifoliate leaf SPAD chlorophyll (Table 6.5).

No significant geographical location effects were identified for the average number of plants having chlorotic unifoliate leaves when the susceptible control was completely dead (F-value=0.78, p-value=0.5076), unifoliate leaf SPAD chlorophyll under non-drought stress (F-value=1.21, p-value=0.3039), trifoliate leaf SPAD chlorophyll under non-drought stress (F-value=2.28, p-value=0.078), and trifoliate leaf SPAD chlorophyll under drought stress (F-value=1.46, p-value=0.2241) (Table 6.5).

Correlation analysis and genotype ranking across traits

Correlation analysis between traits analyzed for drought tolerance was investigated. Plant greenness score was correlated highly correlated with tolerance to trifoliate leaf chlorosis (r=0.8), but was moderately correlated with unifoliate leaf chlorosis (r=0.4-0.5), unifoliate leaf SPAD chlorophyll under drought stress (r=-0.5), relative tolerance index for unifoliate leaf SPAD chlorophyll (r=-0.4), trifoliate leaf SPAD chlorophyll under drought stress (r=-0.4), and relative tolerance index for trifoliate leaf SPAD chlorophyll (r=-0.4) (Table 6.6). A high correlation was identified between unifoliate leaf SPAD chlorophyll under non-drought stress and trifoliate leaf SPAD chlorophyll under non-drought stress (r=0.7), unifoliate leaf SPAD chlorophyll under

drought stress and trifoliate leaf SPAD chlorophyll under drought stress (r=0.6), and trifoliate leaf SPAD chlorophyll and relative tolerance index for trifoliate leaf SPAD chlorophyll (r=0.6) (Table 6.6). However, trifoliate leaf SPAD chlorophyll under non-drought stress was not correlated with unifoliate leaf chlorosis under drought stress (r=0.0) and trifoliate leaf chlorosis under drought stress (r=0.0) (Table 6.6).

Genotype ranking across traits was analyzed in order to identify the genotypes that were drought-tolerant and drought-susceptible based on multiple trait. Genotypes were ranked for all traits (Table S6.3) and genotypes that overlapped between highly correlated traits were chosen. Highly correlated traits were score (overall greenness score), tri (average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead), and uni_1 (average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves). In fact, if some traits were highly correlated, ranking should be also consistent across traits. Therefore, the genotypes with the highest overall plant greenness and whose ranking was almost consistent across other highly correlated traits were PI664524, PI300173, PI583550, PI293476, PI583251, PI207527, PI227829, PI293469, PI194211, PI194213, PI291140, PI292892, IT84S_2246, PI194208, PI152197, PI354864, PI583209, PI598335, PI662993, and PI293500 (Table 6.7), indicating that these genotypes could be highly drought-tolerant. Of these genotypes, 9 were from America, 3, were from the Africa, and 1 from the Middle East. A similar approach was used to identify the most susceptible genotypes based on traits that were highly correlated. Results suggested that the genotypes PI255774, PI583247, PI582924, PI582530, PI582810, PI503326, PI582566, PI582468, ‘Early Scarlet’, and PI582850 were highly susceptible to drought stress (Table 6.7). A cluster analysis approach was used to further validate our results where the drought-tolerant

genotypes were successfully separated from the drought-susceptible ones (Fig. 6.4) (Fig. S6.1). The top 10 drought-tolerant genotypes and the susceptible control were repeated to further validate the results (Fig. 6.1D).

Discussion

Drought tolerance has resulted in significant crop yield losses worldwide (Cairns et al. 2013). The use of drought-tolerant crop cultivars could mitigate the effects of drought stress. Cultivar development requires an extensive phenotyping, which will contribute towards the identification of drought-tolerant lines. Drought stress occurring at seedling stage could be detrimental to cowpea production (Verbree et al. 2015). In this study, we have evaluated a total of 331 cowpea genotypes for their tolerance to drought stress at seedling stage. We found that the 3 genotypes that were reported to be drought-tolerant in our previous study (Ravelombola et al. 2018) ranked among the top 20 genotypes that were best performing in terms of plant greenness score in this current study, indicating that our experiments were robust. In addition, the 8 founders that were used to develop the first MAGIC cowpea population were included in the panel. Results showed that 2 founders, IT84S_2246 and IT00K_1263, were found to be highly drought-tolerant. Drought field phenotyping on this MAGIC cowpea population was conducted by Huynh et al. (2018), and results suggested that the 2 aforementioned founders were also drought-tolerant under filed conditions. However, Huynh et al. (2018) found a significant variation across locations and years when screening drought tolerance under field conditions. We suggest that the top genotypes that were proven to be drought-tolerant at seedling stage should be repeated under field conditions for future projects. The process of screening a large number of genotypes in a greenhouse setup and selecting the top ones for field screening would save a lot of

resources in a breeding program. Doing so will allow cowpea breeders to develop a large number of populations, each with significant size, and stack a significant number of alleles of interest. The macro greenhouse/field drought tolerance screening would be a powerful tool that could be used in plant breeding. This study is a first step towards establishing a macro greenhouse/field drought tolerance screening in cowpea.

Cowpea drought tolerance phenotyping using the 'wooden box' technique has been proven to be effective (Ravelombola et al. 2018; Verbree et al. 2015). Cowpea genotypes that are tolerant to unifoliate chlorosis and/or trifoliate chlorosis were well-differentiated using this technique (Fig. 6.1B). In addition to leaf chlorosis under drought stress, plant greenness score has also been used to assess drought tolerance in cowpea. Plant greenness score has been shown to help identify wilting status of cowpea plants under drought stress. Drought-tolerant genotypes were slow-wilting, whereas those that were more drought-susceptible were fast-wilting (Ravelombola et al. 2018; Verbree et al. 2015).

Drought tolerance has been reported to be a complex mechanism in crop (Golldack et al. 2014). Singh et al. (1999) suggested that drought tolerance should be investigated separately for different growth and developmental stages of cowpea, and each stage, different parameters such as tolerance to trifoliate leaf chlorosis or unifoliate leaf chlorosis should also be interpreted separately. We support the statement of Singh et al. (1999) since the Person's correlation coefficient between trifoliate leaf chlorosis and unifoliate leaf chlorosis was 0.4-0.5. In addition, the broad-sense heritability between traits was different, suggesting that the genetics mechanism underlying the different traits analyzed in this study could be different, especially for the traits that were not correlated at all. Mai-Kodomi et al. (1999) coined type I drought-tolerant cowpea the genotypes that have both unifoliate and trifoliate leaves fully green under drought stress, and

type II drought-tolerant the genotypes that were only able to delay senescence at the trifoliate leaf level. In this study, type II drought-tolerant genotypes were prevalent. In addition, we found that geographical locations could impact drought tolerance in cowpea. Similar results were identified for salt tolerant-related traits in cowpea (Ravelombola et al. 2017).

The drought-tolerant genotypes that were identified in this study could be used as parents to develop drought-tolerant cultivars. In addition, the drought-tolerant genotypes could be crossed with the susceptible ones to develop mapping populations for drought tolerance-related studies in cowpea, which is required for developing molecular markers that are used in marker-assisted selection (MAS).

Conclusions

In this study, a total of 331 cowpea genotypes were evaluated for drought tolerance at seedling stage and based on different traits. A large variation in the evaluated traits for drought tolerance was found among the 331 cowpea genotypes. A high correlation was found for traits such plant greenness score and tolerance to trifoliate leaf chlorosis under drought stress (r=0.8), whereas no linear correlation was found for traits such as trifoliate leaf chlorosis and unifoliate leaf SPAD chlorophyll under non-drought stress (r=0.0). The genotypes PI583550, PI583251, PI194213, IT84S_2246, PI152197, PI662993, PI664524, PI227829, PI293469, PI291140, PI292892, PI194208, PI354864, PI583209, PI300173, PI293476, PI207527, PI194211, PI582465, and PI293500 were found to be drought-tolerant across different traits. The results from this study could be used in breeding programs aiming at improving drought tolerance in cowpea.

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Tables

Table 6.1. ANOVA table for traits evaluated for drought tolerance in cowpea. Evaluated traits were score: overall greenness score, recov: average number of plants that fully recovered after one week of rewatering, uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves, uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, and tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead.

Traits	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
Score	Genotype	330	302.9	0.9	660	2.24	<.0001
	Block	2	5.7	2.9	660	6.92	0.0011
	Residual	660	272.1	0.4	.	.	.
Recov	Genotype	330	367.8	1.1	660	3.82	<.0001
	Block	2	10.6	5.3	660	18.19	<.0001
	Residual	660	193.4	0.3	-	-	-
Uni_1	Genotype	330	2157.8	6.5	660	2.34	<.0001
	Block	2	690.7	345.4	660	123.44	<.0001
	Residual	660	1849.4	2.8	-	-	-
Unif_f	Genotype	330	358.7	1.1	660	1.58	<.0001
	Block	2	7.1	3.6	660	5.18	0.0059
	Residual	660	456.4	0.7	-	-	-
Tri	Genotype	330	2070.7	6.3	660	2.42	<.0001
	Block	2	156.5	78.3	660	30.06	<.0001
	Residual	660	1721	2.6	-	-	-
C_U_NS	Genotype	330	17805	54	660	1.8	<.0001
	Block	2	61425	30712.5	660	1019.97	<.0001
	Residual	660	19904	30.2	-	-	-
C_U_S	Genotype	330	53313	161.6	660	2.33	<.0001
	Block	2	49997	24998.5	660	359.22	<.0001
	Residual	660	45999	69.7	-	-	-
RTI_C_U	Genotype	330	421558	1277.4	660	1.81	<.0001
	Block	2	326809	163404.5	660	230.9	<.0001
	Residual	660	467787	708.8	-	-	-

Table 6.1. (Cont.)

Traits	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
C_T_NS	Genotype	330	17322	52.5	660	1.96	<.0001
	Block	2	56500	28250	660	1049.96	<.0001
	Residual	660	17785	26.9	-	-	-
C_T_S	Genotype	330	24817	75.2	660	1.81	<.0001
	Block	2	57133	28566.5	660	686.13	<.0001
	Residual	660	27521	41.7	-	-	-
RTI_C_T	Genotype	330	182504	553	660	1.24	0.0113
	Block	2	90434	45217	660	100.97	<.0001
	Residual	660	295997	448.5	-	-	-

Table 6.2. List of cowpea genotypes along with their origin and traits evaluated under drought stress (Score: overall greenness score, Recov: average number of plants that fully recovered after one week of rewatering, Uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves, Uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, and Tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead). Sd represents the standard deviation (n=3). LSMeans followed by the same letter are not significantly different using a protected LSD at α=0.05.

Line_ID	Origin	Score		sd
PI664524	NA	1.7	w	0.7
PI300173	South Africa	1.8	vw	0.7
PI583550	NA	2	uvw	0.6
PI582575	NA	2	uvw	0.6
PI293476	United States	2.1	tuvw	0.7
PI583251	NA	2.1	tuvw	0.8
PI293568	United States	2.1	tuvw	1
PI207527	Afghanistan	2.2	stuvw	0.5
PI227829	Guatemala	2.2	stuvw	0.5
PI293469	United States	2.2	stuvw	1
PI582469	Philippines	2.3	rstuvw	0.7
PI582697	Botswana	2.3	rstuvw	1.3
PI194211	United States	2.4	qrstuvw	0.2
PI221730	South Africa	2.4	qrstuvw	0.7
EARLY_ACRE	United States	4.6	abcde	0.4
PI582924	Senegal	4.6	abcde	0.8
PI582812	Botswana	4.6	abcde	0.2
PI527563	Burundi	4.6	abcde	0.4
PI582530	NA	4.6	abcde	0.2
PI406290	Nigeria	4.7	abcd	0
PI229796	Iran	4.8	abc	0.2
PI583247	NA	4.9	ab	0.2
PI255774	Nigeria	5	a	0

Line_ID	Origin	Uni_1		sd
PI152196	Paraguay	0	l	0
PI152197	Paraguay	0	l	0
PI167284	Turkey	0	l	0
PI180014	India	0	l	0
PI190191	Mexico	0	l	0

Line_ID	Origin	Recov		sd
PI406293	Nigeria	3.3	a	2.5
PI339587	South Africa	2.7	ab	1.2
PI293582	NA	2.3	bc	1.5
PI390421	NA	2.3	bc	1.5
09_481	United States	2.3	bc	0.6
PI662992	NA	2.3	bc	2.1
09_1090	United States	2.3	bc	0.6
PI664524	NA	2	bcd	1
PI75962	NA	2	bcd	0
PI339600	South Africa	2	bcd	1.7
09_749	United States	2	bcd	0
PI608035	NA	2	bcd	1
PI610533	NA	2	bcd	2.6
09_655	United States	2	bcd	0
PI271256	India	2	bcd	2
PI503326	Turkey	0	h	0
PI666251	NA	0	h	0
PI189374	Nigeria	0	h	0
PI255774	Nigeria	0	h	0
EpicSelect.4	United States	0	h	0

Line_ID	Origin	Uni_f		sd
PI664524	NA	2	h	2
PI582942	Puerto Rico	3	gh	2.6
PI598335	NA	3	gh	1
PI293568	United States	3.3	fg	3.1
PI194213	United States	3.7	efg	1.2
PI583200	NA	3.7	efg	2.3
PI583203	NA	3.7	efg	2.1
PI583251	NA	3.7	efg	1.2

Table 6.2. (Cont.)

Line_ID	Origin	Uni_1		sd	Line_ID	Origin	Uni_f		
PI194213	United States	0	l	0	PI292897	Hungary	3.7	efg	2.1
PI582942	Puerto Rico	0	l	0	PI583209	NA	3.7	efg	2.5
PI583200	NA	0	l	0	PI300173	South Africa	3.7	efg	3.2
PI583203	NA	0	l	0	PI250416	Pakistan	6	a	0
PI583251	NA	0	l	0	EMPIRE	United States	6	a	0
PI583550	NA	0	l	0	EMPRESS	United States	6	a	0
PI662993	NA	0	l	0	EpicSelect.4	United States	6	a	0
PI292897	Hungary	0	l	0	EXCEL	United States	6	a	0
Suvita_2	Burkina Faso	0	l	0	**Line_ID**	**Origin**	**Tri**		**sd**
IT84S_2246	Nigeria	0	l	0	PI293476	United States	0	l	0
PI75962	NA	0	l	0	PI583550	NA	0	l	0
PI255774	Nigeria	5.3	abcd	1.2	PI664524	NA	0.3	kl	0.6
PI293545	NA	5.3	abcd	0.6	PI583251	NA	0.3	kl	0.6
PI582354	NA	5.3	abcd	0.6	PI194211	United States	0.3	kl	0.6
PI582468	NA	5.3	abcd	0.6	PI662993	NA	0.3	kl	0.6
PI582541	Mexico	5.3	abcd	1.2	PI207527	Afghanistan	0.3	kl	0.6
PI582727	Botswana	5.3	abcd	1.2	PI293568	United States	0.7	jkl	1.2
PI582850	Botswana	5.3	abcd	0.6	PI582575	NA	0.7	jkl	0.6
PI582926	NA	5.3	abcd	0.6	PI194213	United States	1	ijkl	1
PI583247	NA	5.3	abcd	1.2	PI227827	Guatemala	1	ijkl	1.7
PI582815	Botswana	5.7	abc	0.6	PI293470	United States	1	ijkl	1
PI582810	Botswana	6	ab	0	PI293582	NA	1	ijkl	1
PI349674	Australia	6	a	0	IT00K_1263	Nigeria	1	ijkl	1
-	-	-	-	-	PI194210	United States	1	ijkl	1.7
-	-	-	-	-	PI194209	United States	1	ijkl	1.7
-	-	-	-	-	PI491193	Turkey	6	a	0
-	-	-	-	-	EARLY_SCARLET	United States	6	a	0
-	-	-	-	-	ELEGANCE	United States	6	a	0
-	-	-	-	-	EMPRESS	United States	6	a	0
-	-	-	-	-	EpicSelect.4	United States	6	a	0

Table 6.3. List of cowpea genotypes found at the extreme tails of the distribution of the traits evaluated under drought stress (C_U_NS: unifoliate leaf SPAD chlorophyll under well-watered conditions, C_U_S: unifoliate leaf SPAD chlorophyll under drought stress, RTI_C_U: relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, C_T_NS: first trifoliate leaf SPAD chlorophyll under well-watered conditions, C_T_S: first trifoliate leaf SPAD chlorophyll under drought stress, and RTI_C_T: relative tolerance index for first trifoliate leaf SPAD chlorophyll under drought stress). Sd represents the standard deviation (n=3). Ratio presented in below table was the average of ratios from 3 replications and computing ratio using the big average for first trifoliate leaf SPAD chlorophyll and unifoliate leaf SPAD chlorophyll under drought stress form the below table will not correspond to the reported Ratio. Similar algorithm procedure is valid for all relative tolerance indices (RTI). LSMeans followed by the same letter are not significantly different using a protected LSD at α=0.05.

Line_ID	Origin	C_U_NS		sd	Line_ID	Origin	C_U_S		sd
IT84S_2246	Nigeria	54.5	a	13.7	IT84S_2246	Nigeria	53.7	a	14.8
IT93K_503_1	Nigeria	53.8	ab	19.7	IT93K_503_1	Nigeria	48	ab	10.6
PI582863	Botswana	46.6	abc	12	PI583200	NA	47	abc	10.9
IT89KD_288	Nigeria	45.3	bcd	8.4	Suvita_2	Burkina Faso	44.4	abcd	5.6
Suvita_2	Burkina Faso	44.7	cde	13.2	EpicSelect.4	United States	41.1	abcde	7.1
PI583202	NA	26.2	f2g2h2i2	8.6	PI582468	NA	10.1	y2z2a3b3c3	6.2
PI583513	Nigeria	25.4	g2h2i2	8.4	PI293545	NA	9.2	z2a3b3c3	4.7
PI663148	NA	25.4	g2h2i2	4.3	PI582815	Botswana	7.7	a3b3c3	5
PI583551	NA	25.2	h2i2	8.5	PI582850	Botswana	7.2	b3c3	3.6
PI583240	NA	18.5	i2	9.4	PI582810	Botswana	5.1	c3	4.4
Line_ID	**Origin**	**RTI_C_U**		**sd**	**Line_ID**	**Origin**	**C_T_NS**		**sd**
PI583240	NA	183.1	a	21.7	IT84S_2246	Nigeria	54.7	a	13.8
PI663148	NA	136.8	b	20.6	IT93K_503_1	Nigeria	53.3	ab	6
PI293500	United States	122.2	bc	17.4	IT89KD_288	Nigeria	51.9	abc	11.5
IT00K_1263	Nigeria	118.4	bcd	1.3	PI582863	Botswana	50.9	abcd	9.4
PI200867	Myanmar	113.7	bcde	18.6	PI582789	NA	49.3	abcde	6.3
PI293545	NA	27.1	n2o2p2q2r2	19.1	PI582566	NA	29.4	k2l2m2n2o2	12.8
AR_BE_1	United States	26.1	o2p2q2r2	6.1	PI583274	NA	28.9	l2m2n2o2	4.5
PI582850	Botswana	23.3	p2q2r2	14	PI663011	NA	28.2	m2n2o2	13
PI582815	Botswana	21.1	q2r2	15	PI583551	NA	27.6	n2o2	8.5
PI582810	Botswana	19.7	r2	19.9	PI583197	Senegal	26.7	o2	9.8
Line_ID	**Origin**	**C_T_S**		**sd**	**Line_ID**	**Origin**	**RTI_C_T**		**sd**
IT84S_2246	Nigeria	57.7	a	11.4	PI583551	NA	141.2	a	20.4
IT93K_503_1	Nigeria	55.5	ab	7.4	PI583550	NA	131.7	ab	20
PI390421	NA	52.4	abc	4.7	PI293584	NA	128.8	abc	14.6
IT89KD_288	Nigeria	50.3	abcd	1.5	PI354860	India	126	abcd	18.2
Suvita_2	Burkina Faso	48.7	abcde	3.5	PI354854	India	125.9	abcd	14.3

Table 6.3 (Cont.)

Line_ID	Origin	C_U_NS		sd	Line_ID	Origin	C_U_S		sd
PI582572	NA	25.3	i2j2k2l2m2	4.9	PI582810	Botswana	71.2	x1y1z1a2b2	24.3
PI582571	NA	24.6	j2k2l2m2	10.5	PI582571	NA	68.6	y1z1a2b2	15.8
PI582421	NA	24.3	k2l2m2	8.6	PI582573	Kenya	68.4	z1a2b2	11.1
PI582570	India	24.1	l2m2	10.8	PI582421	NA	63.6	a2b2	24.7
PI582567	NA	22	m2	5.7	PI582567	NA	61.8	b2	10.4

Table 6.4. ANOVA table for the geographical distributions of the cowpea genotypes. Evaluated traits were score: overall greenness score, recov: average number of plants that fully recovered after one week of rewatering, uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves, uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead, C_U_NS: unifoliate leaf SPAD chlorophyll under well-watered conditions, C_U_S: unifoliate leaf SPAD chlorophyll under drought stress, RTI_C_U: relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, C_T_NS: first trifoliate leaf SPAD chlorophyll under well-watered conditions, C_T_S: first trifoliate leaf SPAD chlorophyll under drought stress, and RTI_C_T: relative tolerance index for first trifoliate leaf SPAD chlorophyll under drought stress.

Traits	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
Score	Origin	3	9.9	3.3	674	5.94	0.0005
	Residual	674	373.3	0.6	.	.	.
Recov	Origin	3	6.8	2.3	674	4.09	0.0068
	Residual	674	371	0.6	.	.	.
Uni_1	Origin	3	149.8	49.9	674	11.39	<.0001
	Residual	674	2954.5	4.4	.	.	.
Uni_f	Origin	3	1.7	0.6	674	0.78	0.5076
	Residual	674	501.6	0.7	.	.	.
Tri	Origin	3	106.8	35.6	674	9.7	<.0001
	Residual	674	2473.9	3.7	.	.	.
C_U_NS	Origin	3	363.1	121	674	1.21	0.3039
	Residual	674	67225	99.7	.	.	.
C_U_S	Origin	3	1981.7	660.6	674	4.65	0.0032
	Residual	674	95836	142.2	.	.	.
RTI_C_U	Origin	3	22800	7600	674	7.33	<.0001
	Residual	674	698413	1036.2	.	.	.
C_T_NS	Origin	3	629.8	209.9	674	2.28	0.078
	Residual	674	62007	92	.	.	.
C_T_S	Origin	3	478.3	159.4	674	1.46	0.2241
	Residual	674	73567	109.1	.	.	.
RTI_C_T	Origin	3	10805	3601.7	674	6.53	0.0002
	Residual	674	371769	551.6	.	.	.

Table 6.5. LSMeans of traits evaluated for drought tolerance for each geographical area (origin). Evaluated traits were score: overall greenness score, recov: average number of plants that fully recovered after one week of rewatering, uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves, uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead, C_U_NS: unifoliate leaf SPAD chlorophyll under well-watered conditions, C_U_S: unifoliate leaf SPAD chlorophyll under drought stress, RTI_C_U: relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, C_T_NS: first trifoliate leaf SPAD chlorophyll under well-watered conditions, C_T_S: first trifoliate leaf SPAD chlorophyll under drought stress, and RTI_C_T: relative tolerance index for first trifoliate leaf SPAD chlorophyll under drought stress. Means followed by the same letter are not significantly different using a protected LSD at α=0.05.

Score				**C_U_S**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	3.6a	0.7	Asia	32	26.8a	9.5
Europe_Middle_East	17	3.4ab	0.8	America	77	25.5ab	11.3
Asia	32	3.4b	0.7	Africa	100	22.6bc	12.9
America	77	3.4b	0.8	Europe_Middle_East	17	22.2c	13
Recov				**RTI_C_U**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
America	77	0.4a	0.8	Asia	32	82.1a	28.3
Asia	32	0.3ab	0.8	America	77	74.8ab	32
Africa	100	0.3b	0.8	Europe_Middle_East	17	67.7bc	33
Europe_Middle_East	17	0.1b	0.3	Africa	100	66.1c	33.4
Uni_1				**C_T_NS**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	3a	2.1	Africa	100	39	9.6
Europe_Middle_East	17	3a	2.3	America	77	39	9.6
America	77	2.3b	2	Asia	32	36.9	9.7
Asia	32	1.8b	2	Europe_Middle_East	17	36.2	9
Uni_f				**C_T_S**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	5.7	0.8	Asia	32	38.7	9.5
Europe_Middle_East	17	5.6	1	America	77	37.7	9
Asia	32	5.6	0.7	Africa	100	37	11.8
America	77	5.6	1	Europe_Middle_East	17	35.2	10.1
Tri				**RTI_C_T**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	5a	1.6	Asia	32	107.3a	24.1

Table 6.5 (Cont.)

Tri				RTI_C_T			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Europe_Middle_East	17	4.4b	2.2	America	77	99.8b	24.1
America	77	4.2b	2.1	Europe_Middle_East	17	98.3bc	21.8
Asia	32	4.1b	2	Africa	100	95.3c	23
C_U_NS				-	-	-	-
Origin	**N**	**LSMeans**	**Sd**	-	-	-	-
America	77	35.3	10.3	-	-	-	-
Africa	100	34.2	10	-	-	-	-
Asia	32	33.5	9.4	-	-	-	-
Europe_Middle_East	17	33	9.5	-	-	-	-

Table 6.6. Pearson's correlation coefficients for traits evaluated for drought tolerance in cowpea. Evaluated traits were score: overall greenness score, recov: average number of plants that fully recovered after one week of rewatering, uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves, uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead, C_U_NS: unifoliate leaf SPAD chlorophyll under well-watered conditions, C_U_S: unifoliate leaf SPAD chlorophyll under drought stress, RTI_C_U: relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, C_T_NS: first trifoliate leaf SPAD chlorophyll under well-watered conditions, C_T_S: first trifoliate leaf SPAD chlorophyll under drought stress, and RTI_C_T: relative tolerance index for first trifoliate leaf SPAD chlorophyll under drought stress.

	Score	**Recov**	**Uni_1**	**Uni_f**	**Tri**	**C_U_NS**	**C_U_S**	**RTI_C_U**	**C_T_NS**	**C_T_S**	**RTI_C_T**
Score	1										
Recov	-0.2	1									
Uni_1	0.5	-0.1	1								
Uni_f	0.4	0	0.4	1							
Tri	0.8	-0.1	0.5	0.4	1						
C_U_NS	-0.2	0.2	-0.2	-0.1	-0.1	1					
C_U_S	-0.5	0.2	-0.8	-0.4	-0.5	0.4	1				
RTI_C_U	-0.4	0.1	-0.8	-0.3	-0.4	-0.1	0.8	1			
C_T_NS	-0.1	0.2	0	0	0	0.7	0.3	-0.1	1		
C_T_S	-0.4	0.2	-0.4	-0.1	-0.4	0.5	0.6	0.4	0.5	1	
RTI_C_T	-0.4	0.1	-0.4	-0.2	-0.4	-0.1	0.4	0.5	-0.4	0.6	1

Table 6.7. Ranking of genotypes across traits that were correlated (score: overall greenness score, tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead, and uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had more than 50% chlorotic unifoliate leaves).

Line_ID	Origin	Score	Tri	Uni_1	Tolerant (T)/Susceptibility (S)
PI664524	NA	1	3	17	T
PI300173	South Africa	2	20	39	T
PI583550	NA	4	2	5	T
PI293476	United States	5	1	40	T
PI583251	NA	6	4	6	T
PI207527	Afghanistan	8	7	41	T
PI227829	Guatemala	9	17	20	T
PI293469	United States	10	27	21	T
PI194211	United States	14	5	42	T
PI194213	United States	16	10	7	T
PI291140	Australia	23	91	22	T
PI292892	South Africa	24	115	23	T
IT84S_2246	Nigeria	27	46	2	T
PI194208	United States	28	33	24	T
PI152197	Paraguay	29	60	4	T
PI354864	India	32	28	18	T
PI583209	NA	36	79	25	T
PI598335	NA	37	58	44	T
PI662993	NA	38	6	8	T
PI293500	United States	39	18	36	T
PI255774	Nigeria	331	328	256	S
PI583247	NA	330	327	255	S
PI582924	Senegal	326	319	186	S
PI582530	NA	324	318	326	S
PI582810	Botswana	320	331	331	S
PI503326	Turkey	309	317	325	S
PI582566	NA	305	309	321	S
PI582468	NA	304	326	329	S
EARLY_SCARLET	United States	299	293	315	S
PI582850	Botswana	296	321	254	S

Figures

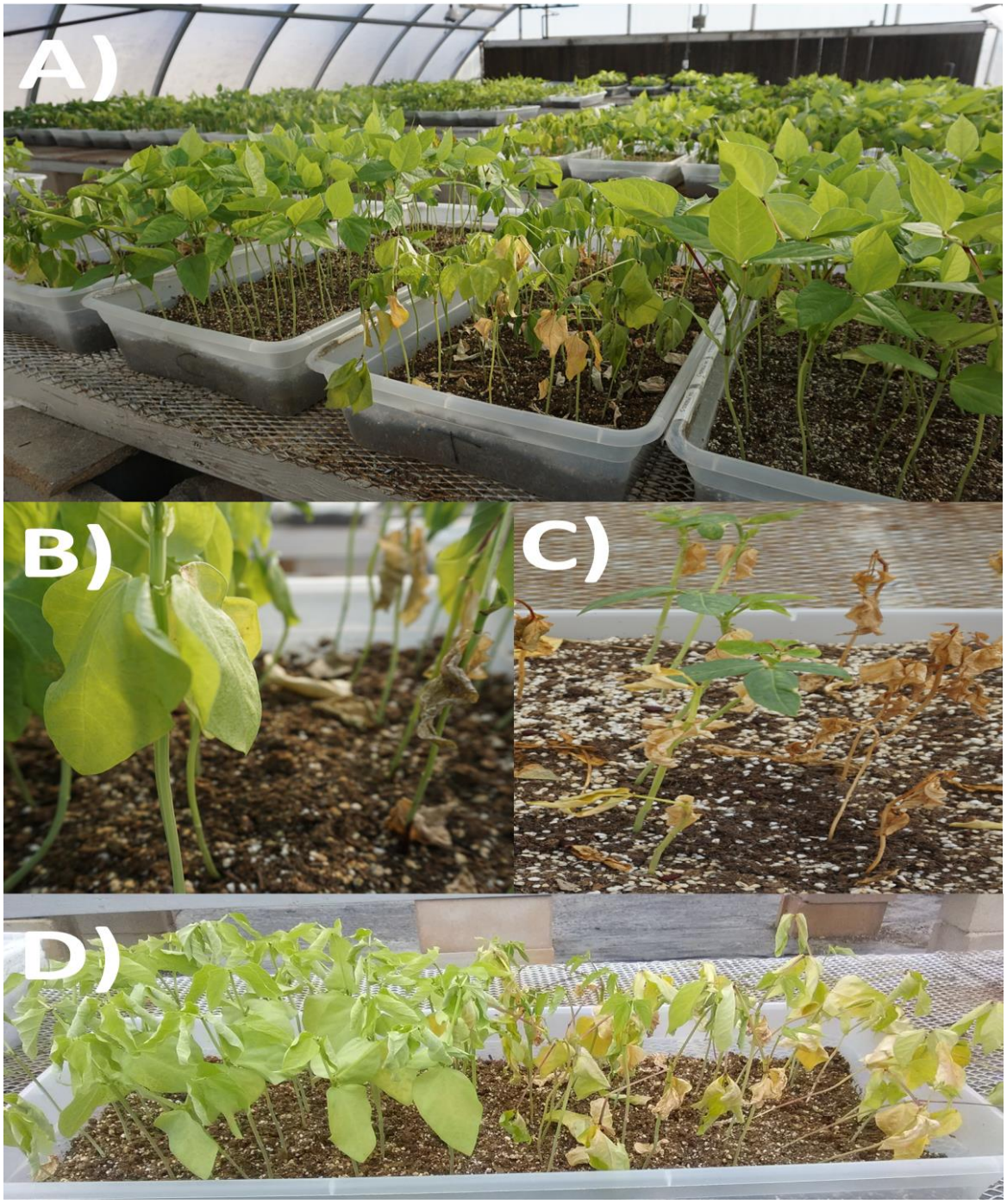

Fig. 6.1. Drought tolerance phenotyping. A) Overview of the greenhouse experiments, B) Discrepancy in slowing wilting between genotypes, C) Discrepancy in recovery rate between genotypes after rewatering, and D) Resistant and susceptible genotypes were repeated.

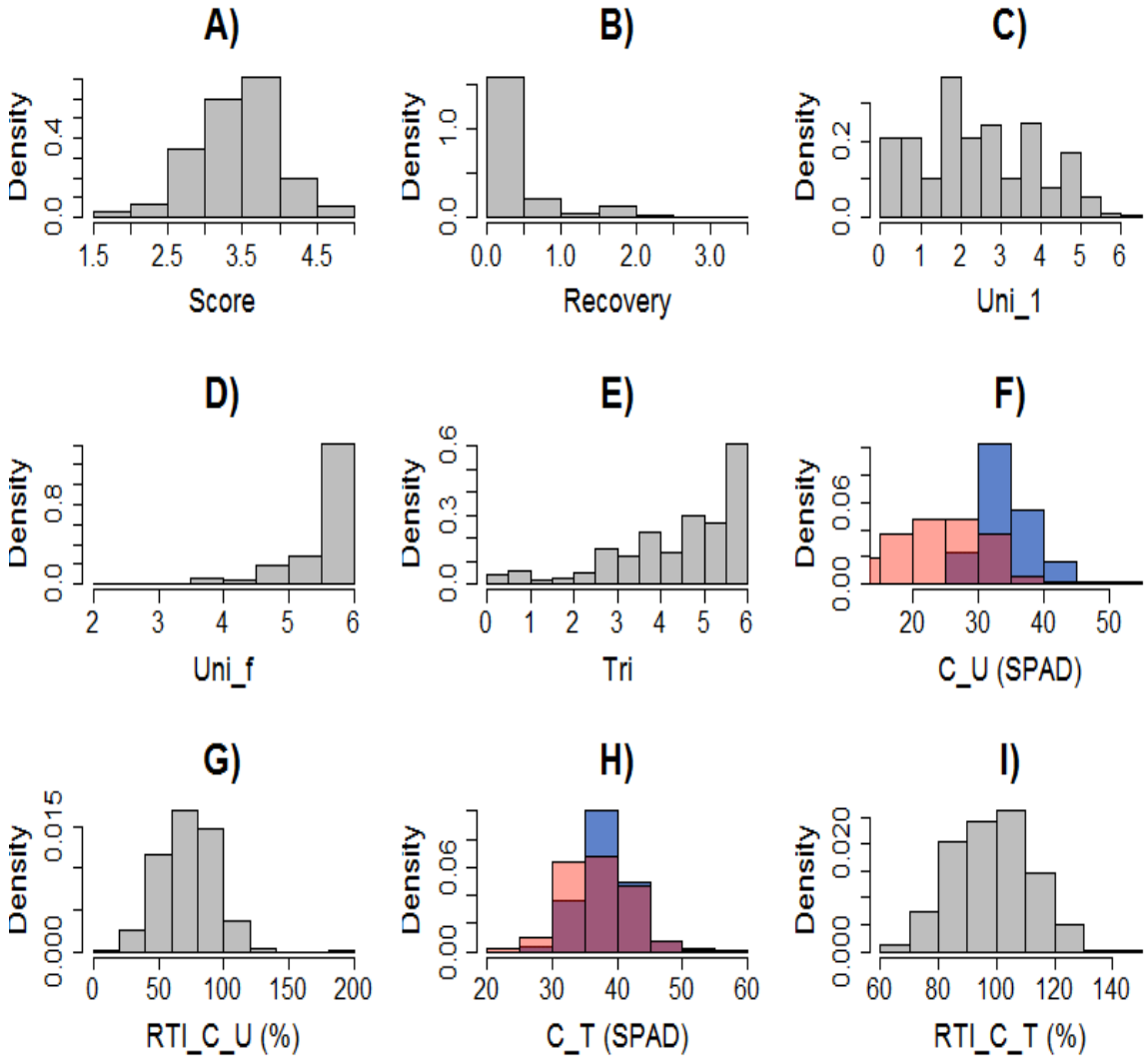

Fig. 6.2. Distributions of phenotypic trait values for drought tolerance in a total of 331 cowpea genotypes. For multicolor histograms, red histograms represented traits evaluated under drought stress, whereas blue histograms displayed traits evaluated under non-drought stress. A) Plant greenness score, B) Recovery rate, C) Average number of plants having chlorotic unifoliate leaves when more than half of the plants of the susceptible control have chlorotic unifoliate leaves, D) Average number of plants having chlorotic unifoliate leaves when the susceptible control was completely dead, E) Average number of plants having chlorotic trifoliate leaves, F) Unifoliate leaf SPAD chlorophyll under drought stress (red) and under non-drought stress (blue), G) Relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, H) Trifoliate leaf SPAD chlorophyll under drought stress (red) and under non-drought stress (blue), and I) Relative tolerance index for trifoliate leaf SPAD chlorophyll under drought stress.

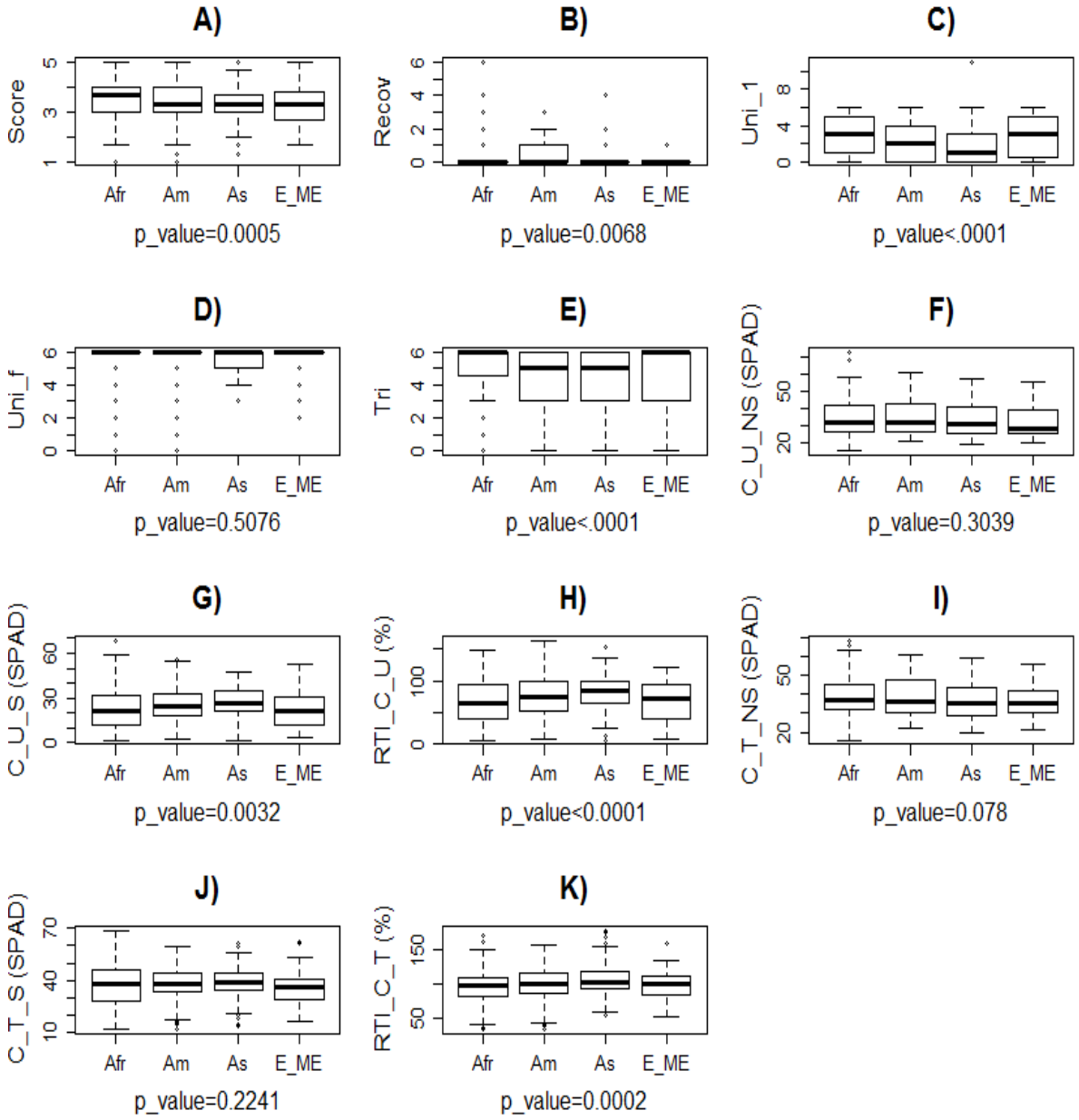

Fig. 6.3. Boxplots showing the variation of the traits evaluated for drought tolerance for each geographical area (origin). The x-axis represented the geographical where Afr=Africa (n=100), Am=America (n=77), As= Asia (n=32), and E_ME = Europe and the Middle East (n=17). Genotypes without information on the origin were not included in the analysis. Below each x-axis are shown the p-values obtained from the ANOVA. The y-axis displayed the different traits values. A) Plant greenness score, B) Recovery rate, C) Average number of plants having chlorotic unifoliate leaves when more than half of the plants of the susceptible control have chlorotic unifoliate leaves, D) Average number of plants having chlorotic unifoliate leaves when the susceptible control was completely dead, E) Average number of plants having chlorotic trifoliate leaves, F) Unifoliate leaf SPAD chlorophyll under non-drought stress, G) Unifoliate

leaf SPAD chlorophyll under drought stress, H) Relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, I) Trifoliate leaf SPAD chlorophyll under non-drought stress, J) Trifoliate leaf SPAD chlorophyll under drought stress, and I) Relative tolerance index for trifoliate leaf SPAD chlorophyll under drought stress.

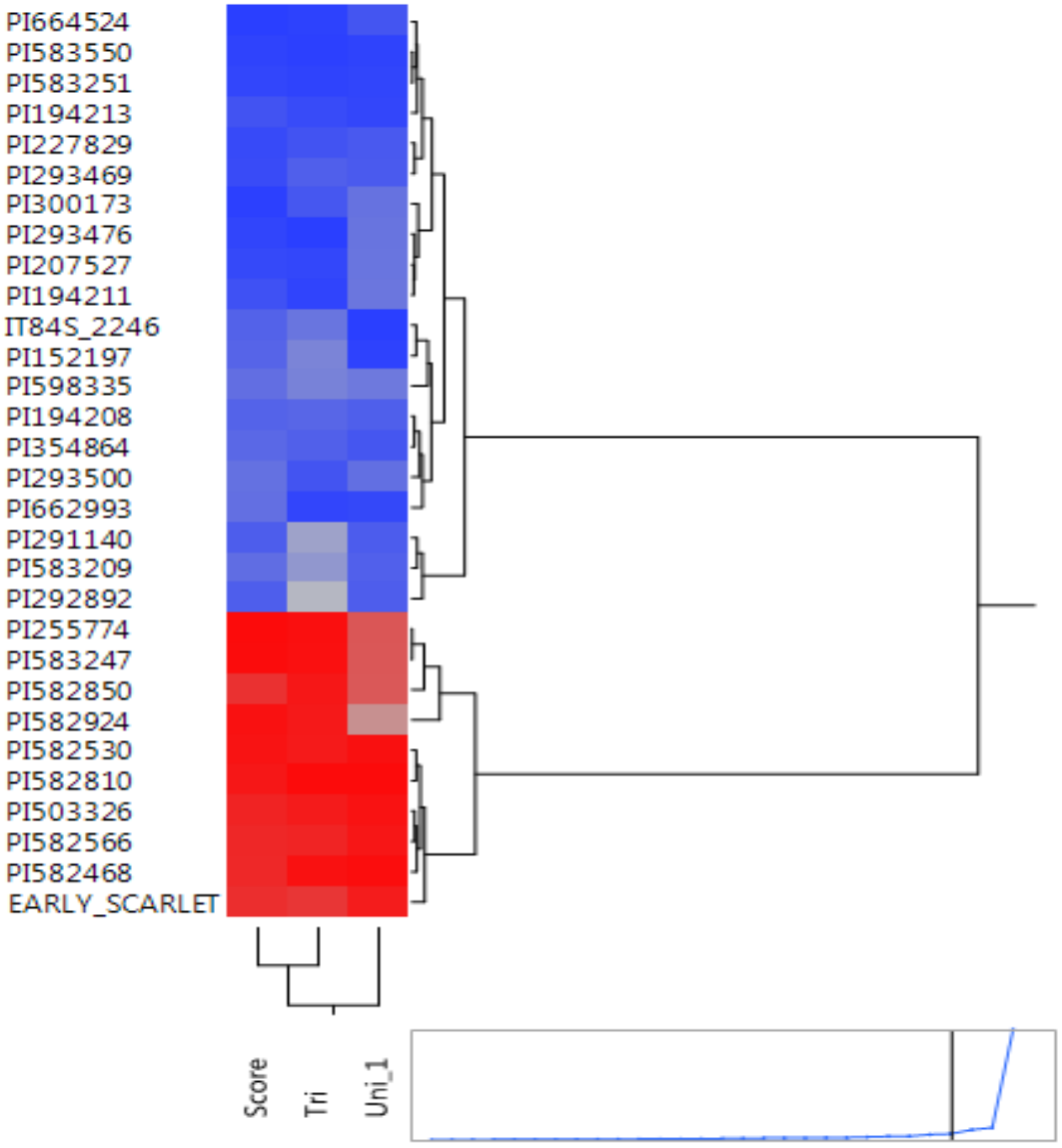

Fig. 6.4. Diversity of the drought-tolerant and drought-susceptible genotypes based on leaf injury score (Score), tolerance to trifoliate leaf chlorosis (Tri), and tolerance to unifoliate leaf chlorosis (Uni_1).

Appendices

Table S6.1. List of cowpea genotypes along with their origin and traits evaluated under drought stress (Uni_1: average number of plants with chlorotic unifoliate leaves when the susceptible control, PI255774, had its all unifoliate chlorotic, Uni_f: average number of plants with chlorotic unifoliate leaves when the susceptible control was completely dead, Tri: average number of plants with chlorotic first trifoliate leaves when the susceptible control was completely dead, Score: overall greenness score, and Recov: average number of plants that fully recovered after one week of rewatering). Sd represents the standard deviation (n=3).

Table S6.2. List of cowpea genotypes along with their origin and traits evaluated under drought stress (C_U_NS: unifoliate leaf SPAD chlorophyll under well-watered conditions, C_U_S: unifoliate leaf SPAD chlorophyll under drought stress, RTI_C_U: relative tolerance index for unifoliate leaf SPAD chlorophyll under drought stress, C_T_NS: first trifoliate leaf SPAD chlorophyll under well-watered conditions, C_T_S: first trifoliate leaf SPAD chlorophyll under drought stress, and RTI_C_T: relative tolerance index for first trifoliate leaf SPAD chlorophyll under drought stress). Sd represents the standard deviation (n=3). Ratio presented in below table was the average of ratios from 3 replications and computing ratio using the big average for first trifoliate leaf SPAD chlorophyll and unifoliate leaf SPAD chlorophyll under drought stress form the below table will not correspond to the reported Ratio. Similar algorithm procedure is valid for all relative tolerance indices (RTI).

Table S6.3. Ranking of each genotype for each trait.

Fig S6.1. Diversity of cowpea genotypes baes on drought tolerance-related traits

Chapter 7. Evaluation of Salt Tolerance in Cowpea at Seedling Stage

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Ravelombola. W., L, Dong, T.C. Barickman, H. Xiong, D. Samuel, G. Bhattarai, B. Zia, H. 2020. Alshaya, I. Alatawi, and A. Shi. Evaluation of Salt Tolerance in Cowepa at Seedling Stage (Submitted to Euphytica)

Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is a nutrient-dense diploid legume species (2*n*=2*x*=22) that provides protein to human. Its cultivation has provided farmers in various regions of the world with substantial income. However, cowpea production can be easily hampered by abiotic stresses such as soil salinity. In this study, we are aiming to screen 331 cowpea genotypes for their tolerance to salt stress, investigating potential correlations among various traits investigated for salt tolerance, and identifying salt-tolerant cowpea genotypes. The cowpea genotypes were screened in a greenhouse and were irrigated with deionized water (no salt treatment) and with a solution of 200 mM NaCl (salt treatment). The experiment was conducted using four runs and with two replications within each run, thus a total of eight replications for the whole experiment. Data on a total of 16 traits including leaf injury score, fresh leaf biomass, and plant height were recorded. Results demonstrated 1) a large variation in salt tolerance among the cowpea genotypes, 2) high correlation between traits such as leaf injury score, leaf SPAD chlorophyll, relative tolerance index for leaf SPAD chlorophyll, and fresh leaf biomass, but no correlation between leaf injury and relative tolerance index for plant height, 3) PI300173, 09-671, PI583209, PI582572, PI293545, PI339587, PI152195, PI582874, 09-529, PI583241, PI583550, PI293486, PI582823, PI293480, PI583237, 09-470, PI582474, PI582878, PI582864, PI583200, PI339603, and PI582469 were found to be salt-tolerant, and 4) country of origins could influence salt tolerance in cowpea. Salt-tolerant and salt-susceptible genotypes were repeated to further validate our results. The results could be used in cowpea breeding programs and allow for cowpea cultivation where soil salinity is predominant.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is diploid legume species (2*n*=2*x*=22). Cowpea is a protein-rich crop and provides an affordable source of protein. Cowpea cultivation is prevalent in Africa but can also be found in different regions of the world such as Asia, Oceania, southern Europe, the United States, and central and southern America (Perrino et al. 1993). The annual estimate for cowpea production is 5.4 million metric tons with Nigeria being the top producer (Singh et al. 2003). Fresh cowpea pods and seeds can be consumed as a vegetable, dried seeds are cooked and can be used to substitute soybean protein for people that are allergic to soybean protein, and the leaves can be used to supplement fodder for livestock (Karapanos et al. 2017; Nielsen et al. 1997).

Soil salinity has been a growing factor constraining crop production. Salinity has been reported to significantly reduce plant growth and lead to substantial crop yield losses (Allakhverdiev et al. 2000; Chinnusamy et al. 2005). These effects of soil salinity are severe in semi-arid areas (Zhang et al. 2012). In semi-arid regions, the low of occurrence of rainfall has resulted in the accumulation of salinity-related compound within soils. In fact, rainfall has significantly contributed to leaching out salt compounds within soils, which can reduce the threat imposed by soil salinity on crops (Karapanos et al. 2017). The increase in the concentration of Na^+, K^+, Mg^{2+}, Ca^{2+}, NO_3^-, HCO_3^-, SO_4^{2-}, and Cl^- has resulted in soil salinity (Wallender and Tanji 2011). Omami and Hammes (2006) reported that rock weathering, deforestation, poor quality of water used for irrigation, and overfertilization practices can rapidly increase soil salinity-related issues.

Cowpea cultivation is one of the most drought-tolerant legumes and its cultivation is prevalent in semi-arid regions (Karapanos et al. 2017). However, salinity can engender

significant concerns in these areas. In the U.S., salinity has affected over 19.6 million hectares of crop lands and cultivated areas facing salinity-related issues have increased (Shannon 1997). Soil salinity has caused serious concerns on cowpea production in the Coachella Valley of California where salinity has increased (Bower et al. 1969; Wilson et al. 2006). Climatic conditions of the southern U.S. are favorable to cowpea cultivation, which will provide cowpea growers with opportunities to expand their production. In southern U.S., more than 66% of the irrigation water used for crop production comes from groundwater (Kresse and Clark 2008). However, groundwater in southern U.S. can contain about 1639 mg of Cl^- per L of water (Kresse and Clark 2008; Zeng et al. 2017), which will limit cowpea production. In fact, Düzdemir et al. (2009) indicated that a sodium chloride (NaCl) concentration greater than 90 mM, potentially discharging about 526 mg/L of Cl^-, could be lethal to cowpea growth and development. Excess of salt ions within plants lead to plant death. Therefore, cowpea production will not be viable in near future in southern U.S.

Previous studies have been conducted to assess salt tolerance at seedling stage in cowpea. Ravelombola et al. (2017) evaluated a total of 155 cowpea genotypes under salt stress at both germination and seedling stages. A low correlation was found for salt tolerance between germination and seedling stages. Dong et al. (2019) evaluated another set consisting of 155 cowpea genotypes. Data such as reduction in plant height and leaf SPAD chlorophyll were used to asses salt tolerance and a large variation in salt tolerance was found among the 155 cowpea genotypes. Ayers and Westcot (1985) reported that salinity due to sodium chloride (NaCl) have been prevalent. Therefore, screening using NaCl will be of interest. Most of the genotypes previously used for salt tolerance evaluation in cowpea were from USDA and a large number of these genotypes were segregating. Improving the quality of the data for salt tolerance evaluation

is critical in breeding programs aiming at developing cowpea cultivars that are tolerant to salt stress. We selected one plant from each line and re-evaluated salt tolerance from seeds that were derived from single plants and added more genotypes and parameters for salt tolerance evaluation. Therefore, the objectives of this study were to evaluate salt tolerance in cowpea and to identify salt-tolerant cowpea genotypes.

Materials and methods

Plant materials

A total of 331 cowpea genotypes were evaluated for salt tolerance in this study (Tables S7.1-S7.2). Of which, 36 were breeding lines from the University of Arkansas, Fayetteville. Eight were obtained from the University of California, Riverside and were the founders of the first cowpea multiparent advanced generation intercross (MAGIC) population (Huynh et al. 2018). A total of 287 cowpea genotypes were Plant Introductions (PIs) from the U.S. Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea accessions, which were provided by the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. These cowpea genotypes were from more than 32 countries. Seeds from each genotype were planted in the summer of 2018 at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville. One plant from each line was harvested and seeds from each plant were cleaned. Uniform and non-misshaped seeds that were single plant derived were used for the experiment.

Growth conditions and experimental design

Salt tolerance evaluation was conducted in the greenhouse at Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville (Fig. 7.1). The average day/light

temperatures in the greenhouse were 26/21 °C and the average daylight length was 14 hours. Salt tolerance evaluation was conducted using a previously developed methodology (Ravelombola et al. 2019). Cowpea seeds were sown in pots previously filled up with 100 g Sunshine Natural & Organic (Agawam, MA). A total of eight seeds were sown per pot. One week after plant emergence, each pot was thinned to a total of four vigorous and uniform plants. Fertilizer was applied weekly by irrigating each pot with a solution of 50 mL of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI) that were obtained by dissolving one tablespoon on the fertilizers into one gallon of deionized water.

The experiment was arranged in a randomized complete block design with four blocks with four blocks and two replications within each block. The experiment was conducted using four runs and the run was used as a blocking variable. Within each run, two replications were used. Therefore, each genotype treatment was replicated eight times (4 runs X 2 replications/run). A total of 12 pots, within which cowpea plants were established, were placed on rectangular plastic trays. For each genotype, two pots were irrigated with deionized water and two other pots were salt-treated. Each pot corresponded to one replication within each run.

Salt treatment (NaCl) was initiated when the first trifoliate leaf began to expand (V1 stage) (Fehr et al. 1971). Salt treatment was conducted by applying a solution of 200 mM NaCl to each rectangular plastic tray (Abeer et al., 2015; Ashebir et al., 2013; Paul et al., 2011; Ravelombola et al. 2017). Irrigation was performed such a way that two-third of pot height was fully soaked with irrigation solution. The methodology we used for the screening was shown to be less labor-intensive and accurate (Ravelombola et al. 2019). The experiment was validated using a salt-tolerant genotype (’09-529’) and a salt-susceptible genotype (PI255774) (Dong et al. 2019; Ravelombola et al. 2019).

Measurements

In vivo chlorophyll measurements

Leaf SPAD chlorophyll was measured on both non-salt stress and salt stress conditions. Measurements were conducted using a SPAD-502 Plus (Spectrum Technologies, Inc., Plainfield, IL). Measurements were performed when the susceptible control was completely dead. Chlorophyll data were taken on a per plant basis. For each plant, one leaf was randomly chosen and measurements were conducted three times from different areas on the leaf surface in order to minimize the edge effect (Dong et al. 2019; Ravelombola et al. 2019). The average of the three measurements were recorded and analyzed.

Plant height and above-ground fresh biomass

Data on plant height were taken when the susceptible control was completely dead as previously described (Ravelombola et al. 2019). Plant height was recorded from each plant and the average plant height within each pot was used for the analysis. Data on plant height were recorded for both non-salt stress and salt-stress conditions. When the susceptible genotype was completely dead, fresh leaf biomass and fresh stem biomass were separately recorded as previously suggested (Ravelombola et al. 2019).

A positive correlation was found between fresh leaf biomass and leaf chlorosis under salt treatment, whereas almost no correlation was found between fresh stem biomass and leaf chlorosis (Ravelombola et al. 2019). Both fresh stem and leaf biomass were taken on a per plant biomass and the average from each pot were used for the analysis. The total fresh above-ground biomass, which was obtained by adding the fresh leaf biomass and to the fresh stem biomass, was also analyzed.

Leaf injury score

Leaf injury score has been demonstrated to be a reliable parameter for assessing salt tolerance in cowpea at seedling stage (Ravelombola et al. 2019). The most reliable parameters for assessing salt tolerance were Na^+/K^+ ratio and C^{l-} contents in plant roots and leaves. However, such chemical analysis could be expensive when a large number of genotypes was involved in the analysis. When budget is limited, leaf injury score can be used instead (Ledesma et al., 2016; Ravelombola et al., 2019). Leaf injury score was assessed using a 1-7 scale (1 = healthy plants, 2 = sign of leaf chlorosis, 3 = expansion of chlorosis on leaf surface, 4 = totally chlorotic leaf, 5 = first sign of necrosis, 6 = expansion of necrosis on leaf surface, and 7 = completely dead plants) (Ravelombola et al., 2017). Leaf score injury was recorded when the susceptible was completely dead.

Data analysis

ANOVA was conducted to analyze leaf injury score, leaf SPAD chlorophyll under salt treatment (S_Chloro), leaf SPAD chlorophyll under non-salt conditions (NS_Chloro), plant height under salt treatment (S_Height), plant height under non-salt treatment (NS_Height), fresh leaf biomass under salt treatment (S_Leaf), fresh leaf biomass under non-salt treatment (NS_Leaf), fresh stem biomass under salt treatment (S_Stem), fresh stem biomass under non-salt treatment (NS_Stem), total above-ground fresh biomass under salt treatment (S_Biomass), and total above-ground fresh biomass under non-salt treatment (NS_Biomass). Relative tolerance index (RTI) for leaf SPAD chlorophyll, plant height, fresh leaf biomass, fresh stem biomass, and total above-ground fresh biomass was calculated as following (Ravelombola et al., 2017; Saad et al., 2014).

- RTI_chlorophyll (RTI_C) = (Y_{c_S}/Y_{c_NS}) X 100

- RTI_plant_height (RTI_H) = (Y_{h_S}/Y_{h_NC}) X 100
- RTI_fresh_leaf_biomass (RTI_FL) = (Y_{l_S}/Y_{l_NS}) X 100
- RTI_fresh_stem_biomass (RTI_FS) = (Y_{s_S}/Y_{s_NS}) X 100
- RTI_total_above_fresh_ground_biomass (RTI_FB) = (Y_{b_S}/Y_{b_NS}) X 100

with Y_{c_S} being the chlorophyll content under salt stress, Y_{c_NS} the chlorophyll content under non-salt stress, Y_{h_S} the plant height under salt stress, Y_{h_NC} the plant height under non salt stress, Y_{l_S} the fresh leaf biomass under salt stress, Y_{l_NS} the fresh leaf biomass under non-salt stress, Y_{s_S} the fresh stem biomass under salt stress, Y_{s_NS} the fresh stem biomass under non-salt stress, Y_{b_S} the total fresh above ground biomass under salt stress, and Y_{b_NS} the total fresh above ground biomass under non-salt stress.

ANOVA was run using PROC MIXED of SAS® 9.4 (SAS Institute Inc., Cary, NC). Mean separation analysis was carried out using a protected least significant difference (LSD) procedure at $\alpha=0.05$. LSD procedure was defined as $LSD=t\alpha/2\sqrt{2MSError/n}$, with $t\alpha/2$ being the critical value from the t-table and having a degree of freedom [df(SSError)] corresponding to the difference between the number of observations and the number of replications, and n being the number of replications. The statistical model for conducting ANOVA was the following.

$$Y_{i(j)k} = \mu + T_j + G_k + R_{i(j)} + TG_{jk} + \varepsilon_{i(j)k} \text{ where } i=1,2,3,4\ j=1,2, \text{ and } k=1\ldots331$$

with μ being the overall mean, $Y_{i(j)k}$ being the response from the k^{th} genotype (G_k) (fixed effect) at the i^{th} replication ($R_{i(j)}$), which was nested under the j^{th} run (block) (T_j) (random effect), and TG_{jk} being the interaction effect between the k^{th} genotype (G_k) and the j^{th} run (block) (T_j).

The effects of country of origins on the different traits evaluated for salt tolerance were also analyzed using ANOVA, which was also conducted using PROC MIXED SAS® 9.4 (SAS Institute Inc., Cary, NC) and carried out using below statistical model. Country of origins was

grouped into 4 regions (Africa, America, Asia, Europe_The_MiddleEast). Increasing the groups into more than 4 regions would result in some groups having very few samples (<10) for the analysis.

$$Y_{ij} = \mu + R_i + \varepsilon_{ij}$$ where i=1,2,3,4, j was the sample size within each region

with μ being the overall mean, Y_{ij} being the response from the i^{th} region (R_i) (fixed effect) and ε_{ij} being the random error associated with the ij^{th} observation.

Data distribution was visualized using the MASS package of R® 3.6.1. Pearson's correlation coefficients between the traits evaluated for salt tolerance were calculated using JMP Genomics 9 (SAS Institute Inc., Cary, NC). Cluster analysis was conducted using JMP Genomics 9 (SAS Institute Inc., Cary, NC) (Sahu 2013). The broad sense heritability (*H*) was estimated using the following formula (Holland, 2003).

$$H = \sigma^2_G / [\sigma^2_G + ((\sigma^2_{GXR})/n_b) + ((\sigma^2_e)/(n_b * n_r))]$$

with σ^2_G being the total genetic variance, σ^2_{GXR} being the Genotype X Run variance, σ^2_e being the residual variance, n_b being the number of runs, and n_r being the number of replications. The estimates for σ^2_G and σ^2_{GXR} were [EMS(G)-EMS(GXB)]/ $n_b * n_r$ and [EMS(GXB)-Var(Residual)]/n_r. EMS(G), EMS(GXB), and Var(Residual) were obtained from the ANOVA table.

Results

Leaf injury score

Leaf injury score was one of the most accurate parameters for evaluating salt tolerance at seedling stage. Results indicated a large variation in leaf injury score among the 331 cowpea genotypes. Leaf injury score varied between 1.4 to 6.9, with an average of 4.0 and a standard

deviation of 1.0. Leaf injury score was normally distributed as shown in Fig. 7.2A. Genotypic differences in leaf injury score were identified (F-value=2.53, p-value<0.0001) (Table 7.1). The lower leaf injury score was, the more salt-tolerant the genotype was. The genotypes with the lowest leaf injury score were PI300173 (1.4), 09-671 (1.4), PI583209 (1.5), PI582572 (1.6), and PI293545 (1.8) (Table 7.2), indicating that these genotypes were salt-tolerant based on leaf injury score. The genotypes with the highest leaf injury were PI201498 (6.3), PI663011 (6.3), PI225922 (6.4), PI255774 (6.6), and PI582530 (6.9) (Table 7.2), suggesting that these genotypes were susceptible to salt stress. A significant genotype X block effect and a non-significant replication within block effect were found for all traits evaluated for salt tolerance in this study. The broad-sense heritability for leaf injury score was 64.6%.

Leaf SPAD chlorophyll

Leaf SPAD chlorophyll under salt stress (S_Chloro) has also been demonstrated to be a good indicator of salt tolerance. S_Chloro varied from 6.4 to 39.9, with an average of 21.9 and a standard deviation of 6.0. The distribution of S_Chloro was normal (Fig. 7.2B). Significant genotypic differences were identified among the 331 cowpea lines evaluated for salt tolerance (F-value=2.86, p-value<0.0001) (Table 7.1). The genotypes with the highest S_Chloro were PI300173 (39.9), PI152195 (37.8), PI583200 (37.4), 09-529 (37.1), and PI293545 (36.8) (Table 7.2), indicating that these genotypes contained high leaf SPAD chlorophyll content even under salt stress condition. The genotypes with the lowest S_Chloro were PI582530 (7.8), PI225922 (7.5), PI582984 (6.9), PI255774 (6.7), and PI663011 (6.4) (Table 7.2), suggesting that these genotypes contained low leaf SPAD chlorophyll contents under salt stress condition. The broad-sense heritability for S_Chloro was 66.2%.

A large variation in leaf SPAD chlorophyll was also identified under non-salt stress (NS_Chloro). Results indicated that NS_Chloro ranged between 26.0 and 44.8, with an average of 32.8 and a standard deviation of 2.4. NS_Chloro was normally distributed (Fig. 7.2B). A significant difference in NS_Chloro was found among the 331 cowpea genotypes (F-value=1.87, p-value<0.0001) (Table 7.1). The genotypes with the highest NS_Chloro were PI663101 (44.8), PI293588 (40.9), PI664515 (40.4), 09-749 (39.8), and IT89KD_288 (39.6) (Table 7.2), indicating these lines had high leaf SPAD chlorophyll content under normal condition. The genotypes with the lowest NS_Chloro were PI271256 (27.7), PI75962 (27.7), PI229551 (27.1), PI189374 (26.7), and IT84S_2049 (26.0) (Table 7.2), indicating these lines had low leaf SPAD chlorophyll content under normal condition. The broad-sense heritability for NS_Chloro was 57.2%.

Relative tolerance index was computed in order to assess the relative effect of salt stress on leaf SPAD chlorophyll (RTI_C). A higher RTI_C indicated a good tolerance to salt stress. A large variation of RTI_C was identified among the cowpea genotypes evaluated for salt tolerance. RTI_C varied from 16.7 to 121.0, with an average of 66.4 and a standard deviation of 17.9. RTI_C was normally distributed (Fig. 7.2C). Cowpea genotypes were significantly different in terms of RTI_C (F-value=2.38, p-value<0.0001) (Table 7.1). The genotypes with the highest RTI_C were PI582823 (121.0), PI293545 (114.6), 09-671 (113.6), PI300173 (113.5), PI152195 (112.0) (Table 7.2), suggesting that these genotypes were salt-tolerant based on RTI_C. The genotypes with the lowest RTI_C were PI225922 (22.3), PI582530 (21.3), PI663011 (19.1), PI582984 (18.3), PI255774 (16.7) (Table 7.2), indicating these lines were salt-sensitive. The broad-sense heritability for RTI_C was 62.0%.

Plant height

Results indicated a large variation in plant height under salt stress (S_Height). S_Height ranged between 9.9 cm and 20.7 cm, with an average of 14.6 cm and a standard deviation of 1.7 cm. S_Height was normally distributed (Fig. 7.2D). Significant genotypic differences were found in terms of S_Height (F-value=3.28, p-value<0.0001) (Table 7.1). The tallest genotypes under salt stress were PI582417 (20.7 cm), PI582354 (19.6 cm), PI582542 (19.2 cm), PI583201 (19.0 cm), and PI583204 (18.9 cm) (Table 7.2), whereas the shortest ones were PI300173 (11.2 cm), PI582812 (11.2 cm), PI582740 (11.2 cm), PI582850 (10.9 cm), and PI582823 (9.9 cm) (Table 7.2). The broad-sense heritability for S_Height was 70.0%.

A large variation in plant height under non-salt stress (NS_Height) was identified among the 331 cowpea genotypes involved in this study. NS_Height ranged between 15.3 cm to 28.4 cm, with an average of 21.4 cm and a standard deviation of 2.4 cm. NS_Height was normally distributed (Fig. 7.2D). The 331 cowpea genotypes were significantly different in terms of NS_Height (F-value=3.12, p-value<0.0001) (Table 7.1). The tallest genotypes under non-salt stress were PI582542 (28.4 cm), PI582417 (28.2 cm), PI582354 (27.8 cm), PI582541 (26.8 cm), and PI582420 (26.7 cm) (Table 7.2). The shortest genotypes under non-salt stress were PI582850 (16.4 cm), PI354883 (16.1 cm), ‘Empire’ (16.0 cm), PI339588 (15.8 cm), and 01-1781 (15.3 cm) (Table 7.2). The broad-sense heritability for NS_Height was 68.8%.

Results showed a large variation in relative tolerance index for plant height (RTI_H). RTI_H varied from 57.7 to 87.4, with an average of 70.3 and a standard deviation of 5.8. RTI_H was normally distributed (Fig. 7.2E). Genotypic differences in terms RTI_H were identified (F-value=1.67, p-value<0.0001) (Table 7.1). The genotypes with the highest RTI_H were PI666251 (87.4), ‘Encore’ (83.5), ‘Empire’ (82.9), IT93K_503_1 (82.2), and 09-393 (82.0) (Table 7.2),

indicating that these genotypes were salt-tolerant based on RTI_H. The genotypes with the lowest RTI_H were PI75962 (58.6), PI293476 (58.3), PI293500 (58.1), PI271256 (58.0), and PI229796 (57.7) (Table 7.2), suggesting that these genotypes were susceptible to salt stress based on RTI_H. The broad-sense heritability for RTI_H was 55.1%.

Fresh leaf biomass

Fresh leaf biomass under salt stress (S_Leaf) could also be used to assess salt tolerance at seedling stage in cowpea. S_Leaf varied from 0.2 g to 2.8 g, with an average of 1.4 g and a standard deviation of 0.5 g. S_Leaf was approximately normally distributed (Fig. 7.2F). S_Leaf was significantly different among the 331 cowpea genotypes evaluated for salt tolerance (F-value=2.38, p-value<0.0001) (Table 7.1). The genotypes with the highest S_Leaf were PI354762 (2.8 g), PI582465 (2.6 g), PI582878 (2.5 g), PI583205 (2.5 g), and 09-470 (2.5) (Table 7.2), indicating that these genotypes had high fresh leaf biomass even under salt stress condition. The genotypes with the lowest S_Leaf were PI582530 (0.4 g), PI225922 (0.4 g), PI367861 (0.4 g), PI503326 (0.4 g), and PI582428 (0.2 g) (Table 7.2), suggesting that these genotypes had low fresh leaf biomass under salt stress condition. The broad-sense heritability for S_Leaf was 65.3%.

A large variation in fresh leaf biomass under non-salt stress (NS_Leaf) was also identified among the 331 cowpea genotypes. NS_Leaf ranged from 1.4 g to 4.1 g, with an average of 2.7 g and a standard deviation of 0.5 g. The distribution of NS_Leaf was normal (Fig. 7.2F). Significant genotypic differences in terms of NS_Leaf were identified (F-value=2.28, p-value<0.0001) (Table 7.1). The genotypes with the highest NS_Leaf were PI666260 (4.1 g), PI582942 (4.0 g), PI578911 (4.0 g), PI608035 (4.0 g), and PI582924 (3.9 g) (Table 7.3), whereas those with the lowest NS_Leaf were PI610604 (1.6 g), PI582735 (1.6 g), PI367861 (1.6 g),

Suvita_2 (1.4 g), and PI339588 (1.4 g) (Table 7.3). The broad-sense heritability for NS_Leaf was 67.1%.

Relative tolerance for fresh leaf biomass (RTI_FL) varied from 8.4 to 86.4m with an average of 51.6 and a standard deviation of 14.2. RTI_FL was approximately normally distributed (Fig. 7.2G). A significant difference was found among the cowpea genotypes in terms of RTI_FL (F-value=1.82, p-value<0.0001) (Table 7.1). The genotypes with the highest RTI_FL were PI354762 (86.4), PI582980 (83.5), PI582850 (82.1), PI583241 (79.6), and PI293470 (77.9) (Table 7.3), indicating that these genotypes were salt-tolerant based on RTI_FL. The genotypes that performed the least in terms of RTI_FL were PI582530 (18.1), PI610520 (16.6), IT84S_2246 (16.3), PI503326 (15.1), and PI582428 (8.4) (Table 7.3), indicating that these genotypes were susceptible to salt based on RTI_FL. The broad-sense heritability for RTI_FL was 59.2%.

Fresh stem biomass

Resulted indicated a large variation in fresh stem biomass under salt stress (S_Stem). S_Stem ranged between 0.4 g and 2.2 g, with an average of 1.0 g and a standard deviation of 0.2 g. S_Stem distribution was normal (Fig. 7.2H). S_Stem was significantly different among the cowpea genotypes (F-value=2.2, p-value<0.0001) (Table 7.1). The genotypes with the highest S_Stem were IT89KD_288 (2.2 g), 09_175 (1.8 g), IT93K_503_1 (1.8 g), 09-470 (1.7 g), and 09-393 (1.6 g) (Table 7.3), whereas those with the lowest S_Stem were PI583247 (0.6 g), PI390421 (0.6 g), PI582681 (0.6 g), PI582984 (0.5 g), and PI293568 (0.4 g) (Table 7.3). The broad-sense heritability for S_Stem was 64.5%.

Fresh stem biomass under non-salt stress (NS_Stem) varied from 1.0 g to 3.6 g, with an average of 2.0 g and a standard deviation of 0.4 g. NS_Stem was normally distributed (Fig.

7.2H). Genotypic differences in terms of NS_Stem were found (F-value=2.32, p-value<0.0001) (Table 7.1). The genotypes with the highest NS_Stem were PI578911 (3.6 g), PI582924 (3.3 g), PI582354 (3.3 g), PI583186 (3.1 g), and PI167284 (3.1 g) (Table 7.3), whereas those with the lowest NS_Stem were PI75962 (1.2 g), 'Early Acre' (1.2 g), PI339588 (1.2 g), PI582735 (1.1 g), PI293568 (1.0 g) (Table 7.3). The broad-sense heritability for NS_Stem was 66.8%. Relative tolerance index for fresh stem biomass (RTI_FS) was the only parameter that was not significant different among the 331 cowpea genotypes (F-value=1.06, p-value=0.2642) (Table 7.1).

Total above-ground fresh biomass

A large variation in total above-ground fresh biomass under salt stress (S_Biomass) was identified. S_Biomass varied from 1.0 g to 4.2 g, with an average of 2.4 g and a standard deviation of 0.6 g. S_Biomass was normally distributed (Fig. 7.2J). S_Biomass was significantly different among the 331 cowpea genotypes evaluated for salt tolerance ((F-value=2.17, p-value<0.0001) (Table 7.1). The genotypes with the highest S_Biomass were 09-470 (4.2 g), 09-175 (4.0 g), PI354762 (3.9 g), 09-393 (3.9 g), and PI582878 (3.9 g) (Table 7.3), whereas those with the lowest S_Biomass were PI583247 (1.2 g), PI339588 (1.1 g), PI582681 (1.1 g), PI582428 (1.0 g), and PI582984 (1.0 g) (Table 7.3). The broad-sense heritability for S_Biomass was 63.0%.

Results indicated a large variation in total above-ground fresh biomass under non-salt stress (NS_Biomass). NS_Biomass ranged between 2.6 g and 7.6 g, with an average of 4.7 g and a standard deviation of 0.8 g. NS_Biomass was normally distributed (Fig. 7.2J). Genetypic differences were significant for NS_Biomass (F-value=2.23, p-value<0.0001) (Table 7.1). The genotypes with the highest NS_Biomass were PI578911 (7.6 g), PI582924 (7.2 g), PI608035 (7.1 g), PI592369 (7.0 g), and IT93K_503_1 (6.7 g) (Table 7.3), whereas those with the lowest

NS_Biomass were PI610604 (2.9 g), PI367861 (2.9 g), PI582735 (2.8 g), PI293568 (2.8 g), and PI339588 (2.6 g) (Table 7.3). The broad-sense heritability for NS_Biomass was 66.0%.

Relative tolerance index for total above-ground fresh biomass (RTI_FB) ranged between 18.9 and 77.3, with an average of 50.9 and a standard deviation of 10.4. RTI_FB was normally distributed (Fig. 7.2K). A significant difference was found in terms of RTI_FB among the 331 cowpea genotypes evaluated for salt tolerance. The genotypes that were top performers in terms of RTI_FB were PI354762 (77.3), PI582738 (74.8), PI582980 (72.7), PI311119 (72.1), and PI583241 (71.5) (Table 7.3), indicating that these genotypes were salt-tolerant based on relative tolerance index for total above-ground fresh biomass. The genotypes with the lowest RTI_FB were PI664515 (26.8), PI503326 (26.5), PI610520 (25.2), PI582984 (24.5), and PI582428 (18.9) (Table 7.3), suggesting that these genotypes were salt-susceptible in terms of RTI_FB. The broad-sense heritability for RTI_FB was 77.3%.

Salt tolerance and geographical locations

Salt tolerance between different geographical locations were compared. Results indicated that cowpea genotypes from Africa, America, Asia, Europe, and the Middle East were significantly different in terms of salt injury score (F-value=12.5, p-value<0.0001), leaf SPAD chlorophyll under salt treatment (F-value=16.7, p-value<0.0001), relative tolerance index for leaf SPAD chlorophyll (F-value=11.9, p-value<0.0001), plant height under non-salt stress (F-value=5.4, p-value=0.0011), relative tolerance index for plant height (F-value=12.4, p-value<0.0001), fresh leaf biomass under salt stress (F-value=10.3, p-value<0.0001), fresh leaf biomass under non-salt stress (F-value=9.5, p-value<0.0001), relative tolerance index for fresh leaf biomass (F-value=3.2, p-value=0.0213), fresh stem biomass under non-salt stress (F-value=3.1, p-value=0.0263), and total above-ground fresh biomass (F-value=6.6, p-

value=0.0002) (Table 7.4) (Fig. 7.3). Cowpea genotypes from America were the most salt-tolerant based on leaf score injury (3.7), whereas those from Europe and the Middle East were the most salt-susceptible (4.6) (Table 7.5). Similar results were found for leaf SPAD chlorophyll under salt stress where the genotypes from America had the highest leaf SPAD chlorophyll (23.7) and those from Europe and the Middle East had the lowest leaf SPAD chlorophyll (18.0) under salt stress (Table 7.5). In terms of relative tolerance index for leaf SPAD chlorophyll, cowpea genotypes from America performed the best, whereas those from Europe and the Middle East were the least performers (Table 7.5). Interestingly, cowpea genotypes from Europe and the Middle East were the tallest, whereas those America were the shortest under non-salt stress conditions. However, cowpea genotypes from America were the best in terms of relative tolerance index for plant height (77.4) and those from Asia, Europe, and the Middle East were the least performers based on relative tolerance index for plant height, thus being the most salt susceptible. These aforementioned results were also in agreement with fresh leaf biomass under salt stress where cowpea genotypes from America were the top performers (1.3 g) (Table 7.5). Cowpea genotypes from America were also the best in terms fresh leaf biomass under non-salt stress conditions. However, cowpea genotypes from America, Europe, the Middle East, and Asia were not significantly different in terms of relative tolerance index for fresh leaf biomass. In addition, results showed that cowpea genotypes from America had the highest fresh stem biomass under non salt-stress conditions. Cowpea genotypes from America were also significantly different from those that originated from Africa, Asia, Europe, and the Middle East in terms of total above-ground fresh biomass (Table 7.5).

No significant geographical location effects were found for traits such as leaf SPAD chlorophyll under non-salt stress conditions (F-value=2.2, p-value=0.0814), plant height under

salt stress (F-value=2.0, p-value=0.1127), fresh stem biomass under salt stress (F-value=1.8, p-value=0.1461), relative tolerance index for fresh stem biomass (F-value=1.6, p-value=0.1847), total above-ground fresh biomass under non-salt stress (F-value=2.2, p-value=0.0829), and relative tolerance index for total above-ground fresh biomass (F-value=2.0, p-value=0.1128) (Table 7.5).

Correlation analysis and genotype ranking across traits

Correlation analysis was conducted for the traits evaluated for salt tolerance. Leaf injury score was highly correlated with leaf SPAD chlorophyll under salt stress (r=-0.9), relative tolerance index for leaf SPAD chlorophyll (r=-0.8), fresh leaf biomass under salt stress (r=-0.6), relative tolerance index for fresh leaf biomass (r=-0.6), and relative tolerance index for total above-ground fresh biomass (r=-0.6) (Table 7.6). Leaf injury score was not correlated with plant height under salt stress (r=0.1), plant height under non-salt stress, (r=0.1), relative tolerance index for plant height (r=0.0), fresh stem biomass under salt stress (r=-0.1), fresh stem biomass under non-salt stress (r=-0.1), and relative tolerance index for fresh stem biomass (r=-0.2) (Table 7.6). Leaf SPAD chlorophyll under salt stress was highly correlated with relative tolerance index for leaf SPAD chlorophyll (r=0.9), fresh leaf biomass under salt stress (r=0.6), relative tolerance index for fresh leaf biomass (r=0.6) (Table 7.6). Leaf SPAD chlorophyll was moderately correlated with total above-ground fresh biomass (r=0.5) and relative tolerance index for total above-ground fresh biomass (r=0.5) (Table 7.6). Relative tolerance index for leaf SPAD chlorophyll was highly correlated with fresh leaf biomass under salt stress (r=0.6) and relative tolerance index for fresh leaf biomass (r=0.6) (Table 7.6). However, relative tolerance index for leaf SPAD chlorophyll was not correlated with plant height under salt stress (r=-0.1), plant height under non-salt stress (r=-0.1), relative tolerance index for plant height (r=0.0) (Table 7.6).

Genotype ranking across traits was conducted in order to identify which genotype ranked best for most of traits evaluated for salt tolerance (Table S7.3). Genotypes with ranking being consistent across highly correlated traits were further analyzed since it would be difficult to draw conclusions based on ranking from uncorrelated traits. A high correlation was found between leaf injury score, leaf SPAD chlorophyll under salt stress, and relative tolerance index for leaf SPAD chlorophyll. The top genotypes with the highest and almost consistent ranking across these traits were PI300173, 09-671, PI583209, PI582572, PI293545, PI339587, PI152195, PI582874, 09-529, PI583241, PI583550, PI293486, PI582823, PI293480, PI583237, 09-470, PI582474, PI582878, PI582864, PI583200, PI339603, and PI582469 (Table 7.7), indicating that these genotypes could be salt-tolerant. Of these genotypes, 6 were America and 5 were from Africa Similar approach was used to identify the salt-susceptible genotypes (Table 7.7). Results showed that cluster analysis successfully separated the salt-tolerant genotypes from the susceptible ones (Fig. 7.4) (Fig. S7.1). In addition, the experiments were repeated for the top 10 genotypes with the lowest leaf injury score (salt-tolerant) and the 10 least performing genotypes in terms of leaf injury score (salt-susceptible). Results showed that the leaf injury score for these genotypes were consistent.

Discussion

Soil salinity can be devastating to agricultural activities. Significant crop losses have been associated with soil salinity-related issues (Ghassemi et al. 1995; Reddy et al. 2017). In addition, concerns due to soil salinity keep increasing since more crop land areas are affected by soil salinity worldwide, thus making soil salinity being a growing threat to agriculture (Chinnusamy et al. 2005). Soil salinity is worsened by inappropriate agricultural practices such

as the excessive use of fertilizers and the application of poor irrigation water to plants have been highlighted to be strong driving factors leading to soil salinization (Omami and Hammes 2006). In addition, areas showing potential to cowpea production are facing rapidly increasing soil salinity-related issues in southern U.S. (Kresse and Clark 2008). In western U.S., soil salinity has also been shown to be a growing threat to cowpea production (Wilson et al. 2006). In addition, acute effects due to salinity were recorded in semi-arid regions, where cowpea cultivation is prevalent (Karapanos et al. 2017). Therefore, this study will significantly contribute towards developing salt-tolerant cowpea genotypes.

The cowpea seedling stage is one of the most susceptible stages to salt stress and being provided with salt-tolerant cowpea genotypes at this stage will assist with alleviating the effects of soil salinity (Dong et al. 2019). Screening for crop tolerance to salinity is challenging. Field screening for soil salinity tolerance in crops could result in significant bias due to uncontrolled factors such as temperature, soil fertility, and transpiration (Pathan and Lee 2007). Therefore, screening for salt tolerance should be conducted using a methodology that can minimize these uncontrolled factors. A simple methodology has been developed to screen cowpea for salt tolerance (Ravelombola et al. 2019). This methodology was used to evaluate salt tolerance in a cowpea panel consisting of 331 cowpea genotypes that were derived from a single plant. The resistant and susceptible controls had the same response as those previously described (Dong et al. 2019; Ravelombola et al. 2019).

Leaf injury score has been widely used for assessing salt tolerance and could be used when ion (Na^+, K^+, and Cl^-) extraction and analysis are expensive (Ledesma et al. 2016). A large variation in leaf injury score was found in this study. The genotypes with the highest leaf injury score were completely dead. This could be explained by the fact that these plants fail to limit salt

ions uptake, which lead to plant death (Zeng et al. 2017). In addition, chlorophyll content could be used as a good indicator of salt tolerance in cowpea (Dong et al. 2019). In this study, a high correlation was found between leaf injury score and leaf SPAD chlorophyll under salt treatment (r=-0.9), which was in agreement with a study conducted by Dong et al. (2019) for salt tolerance study in cowpea. Our results also indicated that no linear correlation was found between leaf injury score and relative tolerance index for plant height (r=0.0). Similar results were also found by Dong et al. (2019). These findings indicated that decrease in plant height due to salt stress could be affected by a genetic mechanism that is different from the one affecting leaf injury score and leaf SPAD chlorophyll. Results also indicated that country of origins of cowpea could affect salt tolerance, suggesting that country of origins should be considered when breeding for salt tolerance in cowpea. Salt tolerance mechanism is well-described in other crops such as soybean. The genetic mechanism underlying salt tolerance in soybean have been previously investigated and results identified strong loci affecting salt tolerance in soybean (Zeng et al. 2017). Most of the previously reported studies on crop salt tolerance have described biomolecular transporters to be associated with salt tolerance. For example, Qi et al. (2014) identified an ion transporter gene, *GmCHX1*, that contributes to salt tolerance in soybean. However, salt tolerance mechanism-related studies remain very limited in cowpea. Very few molecular markers have been reported to be associated with salt tolerance in cowpea and efforts are being made in order to identify strong QTL(s) associated with salt tolerance in cowpea (Ravelombola et al. 2017).

In addition to identifying salt-tolerant genotypes, this study could contribute towards understanding the genetic mechanism underlying salt tolerance in cowpea. The data could be used to conduct a genome-wide association study (GWAS) for salt tolerance in cowpea, which

will assist cowpea breeders with identifying molecular markers for rapidly screening salt tolerance, thus increasing the genetic gain per unit of time.

Conclusions

In this study, we evaluated salt tolerance in a total of 331 cowpea genotypes. Results indicated a large variation in salt tolerance among the cowpea genotypes. High correlation was found between traits such as leaf injury score, leaf SPAD chlorophyll under salt stress, relative tolerance index for leaf SPAD chlorophyll, and fresh leaf biomass under salt stress. However, leaf injury was not correlated with relative tolerance index for plant height. Geographical location differences were significant for traits such as leaf injury score, leaf SPAD chlorophyll under salt stress, relative tolerance index for leaf SPAD chlorophyll, relative tolerance index for plant height, fresh leaf biomass under salt stress, and relative tolerance index for fresh leaf biomass. PI300173, 09-671, PI583209, PI582572, PI293545, PI339587, PI152195, PI582874, 09-529, PI583241, PI583550, PI293486, PI582823, PI293480, PI583237, 09-470, PI582474, PI582878, PI582864, PI583200, PI339603, and PI582469 were found to be highly salt-tolerant based on different traits. The results from this study could be used in breeding programs aiming at improving tolerance of cowpea to salt stress.

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Tables

Table 7.1. ANOVA table for traits evaluated for salt tolerance in a total of 331 cowpea genotypes. Evaluated traits were salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), relative tolerance index for plant height (RTI_H), fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), relative tolerance index for total above-ground fresh biomass (RTI_FB).

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Score	Genotype	330	2815.15	8.53	2.53	<.0001
	Block	3	599.02	199.67	48.29	<.0001
	Genotype*Block	990	3340.78	3.37	9.53	<.0001
	Rep(Block)	4	4.44	1.11	3.13	0.0143
	Residual	1328	470.31	0.35	-	-
S_Chloro	Genotype	330	93660	283.82	2.86	<.0001
	Block	3	79933	26644	266.59	<.0001
	Genotype*Block	990	98171	99.16	28.85	<.0001
	Rep(Block)	4	16.29	4.07	1.18	0.3174
	Residual	1328	4565.27	3.44	-	-
NS_Chloro	Genotype	330	14558	44.12	1.87	<.0001
	Block	3	124280	41427	1759.84	<.0001
	Genotype*Block	990	23414	23.65	11.29	<.0001
	Rep(Block)	4	7.8	1.95	0.93	0.4465
	Residual	1328	2782.08	2.09	-	-
RTI_C	Genotype	330	848215	2570.35	2.38	<.0001
	Block	3	76052	25351	23.18	<.0001
	Genotype*Block	990	1068164	1078.95	26.62	<.0001
	Rep(Block)	4	214.85	53.71	1.32	0.26
	Residual	1328	53833	40.54	-	-
S_Height	Genotype	330	7900.45	23.94	3.28	<.0001
	Block	3	40756	13585	1573.93	<.0001
	Genotype*Block	990	7233.71	7.31	11.79	<.0001
	Rep(Block)	4	7.74	1.94	3.12	0.0145
	Residual	1328	822.77	0.62	-	-
NS_Height	Genotype	330	14682	44.49	3.12	<.0001
	Block	3	107699	35900	2541.37	<.0001

Table 7.1. (Cont.)

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Genotype*Block	990	14135	14.28	12.95	<.0001
NS_Height	Rep(Block)	4	3.72	0.93	0.84	0.499
	Residual	1328	1464.29	1.1	-	-
	Genotype	330	89469	271.12	1.67	<.0001
	Block	3	73216	24405	151.87	<.0001
RTI_H	Genotype*Block	990	161120	162.75	7.88	<.0001
	Rep(Block)	4	73.53	18.38	0.89	0.4706
	Residual	1328	27422	20.65	-	-
	Genotype	330	601.52	1.82	2.38	<.0001
	Block	3	325.52	108.51	172.71	<.0001
S_Leaf	Genotype*Block	990	758.08	0.77	4.81	<.0001
	Rep(Block)	4	0.08	0.02	0.13	0.2716
	Residual	1328	211.61	0.16	-	-
	Genotype	330	683.35	2.07	2.28	<.0001
	Block	3	498.93	166.31	142.14	<.0001
NS_Leaf	Genotype*Block	990	898.81	0.91	2.94	<.0001
	Rep(Block)	4	2.28	0.57	1.84	0.1186
	Residual	1328	410.58	0.31	-	-
	Genotype	330	529075	1603.26	1.82	<.0001
	Block	3	109151	36384	51.5	<.0001
RTI_FL	Genotype*Block	990	873418	882.24	4.54	<.0001
	Rep(Block)	4	68.99	17.25	0.09	0.486
	Residual	1328	258010	194.28	-	-
	Genotype	330	145.48	0.44	2.2	<.0001
	Block	3	150.49	50.16	265.27	<.0001
S_Stem	Genotype*Block	990	198.45	0.2	3.87	<.0001
	Rep(Block)	4	0.16	0.04	0.77	0.5421
	Residual	1328	68.85	0.05	-	-
	Genotype	330	449.62	1.36	2.32	<.0001
	Block	3	801.98	267.33	366.23	<.0001
NS_Stem	Genotype*Block	990	581.46	0.59	3.24	<.0001
	Rep(Block)	4	1.29	0.32	1.78	0.1306
	Residual	1328	240.77	0.18	-	-
	Genotype	330	207687	629.35	1.06	0.2642
RTI_FS	Block	3	7331.41	2443.8	4.08	0.0124
	Genotype*Block	990	589607	595.56	3.35	<.0001

Table 7.1. (Cont.)

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
RTI_FS	Rep(Block)	4	721.25	180.31	1.01	0.4003
	Residual	1328	236055	177.75	-	-
S_Biomass	Genotype	330	1048.01	3.18	2.17	<.0001
	Block	3	917.86	305.95	243.63	<.0001
	Genotype*Block	990	1447.72	1.46	4.95	<.0001
	Rep(Block)	4	0.35	0.09	0.29	0.3825
	Residual	1328	392.48	0.3	-	-
NS_Biomass	Genotype	330	1822.06	5.52	2.23	<.0001
	Block	3	2544.72	848.24	267.19	<.0001
	Genotype*Block	990	2447.12	2.47	3.21	<.0001
	Rep(Block)	4	5.89	1.47	1.9	0.1074
	Residual	1328	1023.73	0.77	-	-
RTI_FB	Genotype	330	286283	867.52	1.42	<.0001
	Block	3	31354	10451	20.3	<.0001
	Genotype*Block	990	605347	611.46	4.53	<.0001
	Rep(Block)	4	149.51	37.38	0.28	0.5934
	Residual	1328	179145	134.9	-	-

Table 7.2. LSMeans of the top 5 genotypes and 5 least performing genotypes for salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), and relative tolerance index for plant height (RTI_H). Sd represents the standard deviation across 8 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

PI_ID	Origin	Score	Sd	PI_ID	Origin	S_Chloro	Sd
PI300173	South Africa	1.4	0.7	PI300173	South Africa	39.9	8.7
09_671	United States	1.4	0.4	PI152195	Paraguay	37.8	10.1
PI583209	NA	1.5	0.8	PI583200	NA	37.4	11.5
PI582572	NA	1.6	0.7	09_529	United States	37.1	6
PI293545	NA	1.8	0.7	PI293545	NA	36.8	4.5
PI201498	Mexico	6.3	1.2	PI582530	NA	7.8	6.2
PI663011	NA	6.3	0.9	PI225922	Zambia	7.5	5.7
PI225922	Zambia	6.4	0.5	PI582984	Kenya	6.9	4.1
PI255774	Nigeria	6.6	0.5	PI255774	Nigeria	6.7	5.3
PI582530	NA	6.9	0.4	PI663011	NA	6.4	4.7
PI_ID	**Origin**	**NS_Chloro**	**Sd**	**PI_ID**	**Origin**	**RTI_C**	**Sd**
PI663101	NA	44.8	7.3	PI582823	Botswana	121	20.1
PI293588	NA	40.9	13.2	PI293545	NA	114.6	18.1
PI664515	NA	40.4	12	09_671	United States	113.6	11.6
09_749	United States	39.8	11.4	PI300173	South Africa	113.5	9.2
IT89KD_288	Nigeria	39.6	8.5	PI152195	Paraguay	112	8.2
PI271256	India	27.7	6.2	PI225922	Zambia	22.3	14.9
PI75962	NA	27.7	8.5	PI582530	NA	21.3	15.6
PI229551	Iran	27.1	9.5	PI663011	NA	19.1	14.6
PI189374	Nigeria	26.7	6.3	PI582984	Kenya	18.3	19.4
IT84S_2049	Nigeria	26	8.1	PI255774	Nigeria	16.7	16.9
PI_ID	**Origin**	**S_Height**	**Sd**	**PI_ID**	**Origin**	**NS_Height**	**Sd**
PI582417	Mexico	20.7	4.4	PI582542	NA	28.4	6.9
PI582354	NA	19.6	6.2	PI582417	Mexico	28.2	7.3
PI582542	NA	19.2	3.9	PI582354	NA	27.8	8.3
PI583201	Senegal	19	5.6	PI582541	Mexico	26.8	7.4
PI583204	NA	18.9	6.4	PI582420	NA	26.7	7.5
PI300173	South Africa	11.2	2.5	PI582850	Botswana	16.4	5.9
PI582812	Botswana	11.2	3.1	PI354883	India	16.1	4

Table 7.2 (Cont.)

PI_ID	Origin	S_Height	Sd
PI582740	Botswana	11.2	3.6
PI582850	Botswana	10.9	3.4
PI582823	Botswana	9.9	2.5

PI_ID	Origin	NS_Height	Sd
EMPIRE	United States	16	6.3
PI339588	South Africa	15.8	6.6
01_1781	United States	15.3	4.3

PI_ID	Origin	RTI_H	Sd
PI666251	NA	87.4	5.8
ENCORE	United States	83.5	8.9
EMPIRE	United States	82.9	10.9
IT93K_503_1	Nigeria	82.2	8.3
09_393	United States	82	12.2
PI75962	NA	58.6	12.4
PI293476	United States	58.3	9.3
PI293500	United States	58.1	6.8
PI271256	India	58	10.2
PI229796	Iran	57.7	5.6

Table 7.3. LSMeans of the top 5 genotypes and 5 least performing genotypes for fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), and relative tolerance index for total above-ground fresh biomass (RTI_FB). Sd represents the standard deviation across 8 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

PI_ID	Origin	S_FL	Sd	PI_ID	Origin	NS_FL	Sd
PI354762	India	2.8	0.4	PI666260	NA	4.1	1.9
PI582465	NA	2.6	0.3	PI582942	Puerto Rico	4	1.7
PI582878	Botswana	2.5	1.7	PI578911	China	4	0.6
PI583205	NA	2.5	0.8	PI608035	NA	4	0.8
09_470	United States	2.5	1.3	PI582924	Senegal	3.9	0.6
PI582530	NA	0.4	0.3	PI610604	NA	1.6	0.4
PI225922	Zambia	0.4	0.4	PI582735	Botswana	1.6	0.7
PI367861	India	0.4	0.4	PI367861	India	1.6	0.7
PI503326	Turkey	0.4	0.4	Suvita_2	Burkina Faso	1.4	0.8
PI582428	NA	0.2	0.2	PI339588	South Africa	1.4	0.4
PI_ID	**Origin**	**RTI_FL**	**Sd**	**PI_ID**	**Origin**	**S_FS**	**Sd**
PI354762	India	86.4	8.4	IT89KD_288	Nigeria	2.2	0.2
PI582980	Kenya	83.5	11.4	09_175	United States	1.9	1.1
PI582850	Botswana	82.1	12.4	IT93K_503_1	Nigeria	1.8	0.9
PI583241	NA	79.6	26.5	09_470	United States	1.7	1
PI293470	United States	77.9	14.7	09_393	United States	1.6	1.3
PI582530	NA	18.1	15.3	PI583247	NA	0.6	0.4
PI610520	NA	16.6	11.8	PI390421	NA	0.6	0.3
IT84S_2246	Nigeria	16.3	7.7	PI582681	Botswana	0.6	0.4
PI503326	Turkey	15.1	9.3	PI582984	Kenya	0.5	0.4
PI582428	NA	8.4	7.4	PI293568	United States	0.4	0.2
PI_ID	**Origin**	**NS_FS**	**Sd**	**PI_ID**	**Origin**	**RTI_FS**	**Sd**
PI578911	China	3.6	1.5	IT89KD_288	Nigeria	77.4	8.8
PI582924	Senegal	3.3	1.2	PI582738	Botswana	76.9	17.2
PI582354	NA	3.3	1.1	PI583196	NA	75.6	12.5
PI583186	NA	3.1	0.8	PI582932	Malawi	73.7	18.2

Table 7.3 (Cont.)

PI_ID	Origin	NS_FS	Sd
PI167284	Turkey	3.1	1.6
PI75962	NA	1.2	0.6
EARLY_ACRE	United States	1.2	0.6
PI339588	South Africa	1.2	0.6
PI582735	Botswana	1.1	0.4
PI293568	United States	1	0.5

PI_ID	Origin	S_FB	Sd
09_470	United States	4.2	2.3
09_175	United States	4	2.1
PI354762	India	3.9	0.5
09_393	United States	3.9	3.3
PI582878	Botswana	3.9	2.6
PI583247	NA	1.2	0.7
PI339588	South Africa	1.1	0.6
PI582681	Botswana	1.1	0.7
PI582428	NA	1	0.6
PI582984	Kenya	1	0.9

PI_ID	Origin	RTI_FB	Sd
PI354762	India	77.3	8.4
PI582738	Botswana	74.8	15.5
PI582980	Kenya	72.7	11.6
PI311119	Mexico	72.1	16.8
PI583241	NA	71.5	26
PI664515	NA	26.8	14.5
PI503326	Turkey	26.5	16.1
PI610520	NA	25.2	13.6
PI582984	Kenya	24.5	16.7
PI582428	NA	18.9	9.7

PI_ID	Origin	RTI_FS	Sd
Suvita_2	Burkina Faso	73.5	13
PI583247	NA	33.7	10.4
PI582428	NA	33.5	14.1
PI354854	India	33.2	12.6
PI582727	Botswana	32.4	6.4
PI582984	Kenya	26.2	7.2

PI_ID	Origin	NS_FB	Sd
PI578911	China	7.6	2
PI582924	Senegal	7.2	1.6
PI608035	NA	7.1	1.4
PI592369	NA	7	2.9
IT93K_503_1	Nigeria	6.7	1.4
PI610604	NA	2.9	1
PI367861	India	2.9	1.3
PI582735	Botswana	2.8	1.1
PI293568	United States	2.8	1.2
PI339588	South Africa	2.6	0.9

Table 7.4. LSMeans of traits evaluated for salt tolerance for each geographical area (origin). Evaluated traits were salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), relative tolerance index for plant height (RTI_H), fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), relative tolerance index for total above-ground fresh biomass (RTI_FB). LSMeans followed by the same letter are not significantly different using a protected LSD at α=0.05. Mean separation was conducted for traits for which ANOVA was significant. Genotypes without information on the origin were not included in the analysis.

Score				**S_Chloro**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Europe_Middle_East	17	4.6a	1.4	America	77	23.7a	10.9
Asia	32	4.1b	1.5	Asia	32	21b	8
Africa	100	4.1b	1.7	Africa	100	20.9b	10.1
America	77	3.7c	1.7	Europe_Middle_East	17	18c	7.9
NS_Chloro				**RTI_C**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	32.9	8.2	America	77	71.3a	29.3
America	77	32.8	7.8	Asia	32	66.3b	22.7
Asia	32	32.2	7.3	Africa	100	63.8bc	28.7
Europe_Middle_East	17	31.2	7.5	Europe_Middle_East	17	59c	24.9
S_Height				**NS_Height**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Europe_Middle_East	17	15.1	4.7	Europe_Middle_East	17	23a	7.9
America	77	14.3	4.4	Asia	32	21.1b	6.7
Africa	100	14.1	4.5	Africa	100	20.9b	7.3
Asia	32	14	4.2	America	77	20.3b	7
RTI_H				**S_Leaf**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
America	77	72.4a	11.3	America	77	1.5a	0.9
Africa	100	69.8b	11.9	Asia	32	1.3b	0.8
Asia	32	68.1bc	11.1	Africa	100	1.3b	0.9
Europe_Middle_East	17	67.8c	10.9	Europe_Middle_East	17	1.3b	0.7
NS_Leaf				**RTI_FL**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
America	77	2.8a	0.9	America	77	54a	26.4
Asia	32	2.6b	0.9	Europe_Middle_East	17	51.3ab	23.7
Africa	100	2.6b	1	Asia	32	50.9ab	25.9
Europe_Middle_East	17	2.5b	0.8	Africa	100	49.7b	25.8

Table 7.4 (Cont.)

S_Stem				NS_Stem			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
America	77	1	0.5	Europe_Middle_East	17	2.2a	1
Europe_Middle_East	17	1	0.4	Asia	32	2b	0.9
Africa	100	0.9	0.5	Africa	100	1.9b	0.9
Asia	32	0.9	0.4	America	77	1.9b	0.8
RTI_FS				**S_biomass**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
Africa	100	51.2	19.3	America	77	2.5a	1.4
America	77	50.9	21	Africa	100	2.2b	1.2
Asia	32	48.8	20	Europe_Middle_East	17	2.2b	1
Europe_Middle_East	17	48.2	18.2	Asia	32	2.2b	1.1
NS_Biomass				**RTI_FB**			
Origin	**N**	**LSMeans**	**Sd**	**Origin**	**N**	**LSMeans**	**Sd**
America	77	4.7	1.6	America	77	52	21.9
Europe_Middle_East	17	4.6	1.6	Africa	100	49.8	19.9
Asia	32	4.5	1.6	Asia	32	49.5	20.1
Africa	100	4.5	1.8	Europe_Middle_East	17	48.8	16.9

Table 7.5. ANOVA table for the geographical distributions of the cowpea genotypes. Evaluated traits were salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), relative tolerance index for plant height (RTI_H), fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), and relative tolerance index for total above-ground fresh biomass (RTI_FB). Genotypes without information on the origin were not included in the analysis.

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Score	Origin	3	102.92	34.31	12.51	<.0001
	Residual	1804	4948.97	2.74	-	-
S_Chloro	Origin	3	4968.92	1656.31	16.71	<.0001
	Residual	1804	178816	99.12	-	-
NS_Chloro	Origin	3	417.9	139.3	2.24	0.0814
	Residual	1804	111996	62.08	-	-
RTI_C	Origin	3	27679	9226.32	11.87	<.0001
	Residual	1804	1402772	777.59	-	-
S_Height	Origin	3	117.87	39.29	2	0.1127
	Residual	1804	35523	19.69	-	-
NS_Height	Origin	3	825.5	275.17	5.39	0.0011
	Residual	1804	92085	51.04	-	-
RTI_H	Origin	3	4934.06	1644.69	12.4	<.0001
	Residual	1804	239245	132.62	-	-
S_Leaf	Origin	3	22.49	7.5	10.28	<.0001
	Residual	1804	1316.12	0.73	-	-
NS_Leaf	Origin	3	25.82	8.61	9.5	<.0001
	Residual	1804	1633.88	0.91	-	-
RTI_FL	Origin	3	6505.44	2168.48	3.24	0.0213
	Residual	1804	1207508	669.35	-	-
S_Stem	Origin	3	1.22	0.41	1.8	0.1461
	Residual	1804	408.99	0.23	-	-
NS_Stem	Origin	3	7.08	2.36	3.09	0.0263
	Residual	1804	1380.22	0.77	-	-
RTI_FS	Origin	3	1916.89	638.96	1.61	0.1847
	Residual	1804	715267	396.49	-	-

Table 7.5. (Cont.)

Traits	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
S_Biomass	Origin	3	29.96	9.99	6.65	0.0002
	Residual	1804	2708.78	1.5	-	-
NS_Biomass	Origin	3	19.33	6.44	2.23	0.0829
	Residual	1804	5213.33	2.89	-	-
RTI_Biomass	Origin	3	2492.7	830.9	1.99	0.1128
	Residual	1804	751407	416.52	-	-

Table 7.6. Pearson's correlation coefficients for traits evaluated for salt tolerance. Evaluated traits were salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), relative tolerance index for plant height (RTI_H), fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), and relative tolerance index for total above-ground fresh biomass (RTI_FB).

Traits	Score	S_Chloro	NS_Chloro	RTI_C	S_Height	NS_Height	RTI_H	S_FL	NS_FL	RTI_FL	S_FS	NS_FS	RTI_FS	S_FB	NS_FB	RTI_FB
Score	1															
S_Chloro	-0.9	1														
NS_Chloro	-0.2	0.3	1													
RTI_C	-0.8	0.9	0.1	1												
S_Height	0.1	-0.1	0	-0.1	1											
NS_Height	0.1	-0.2	-0.2	-0.1	0.7	1										
RTI_H	0	0	0.1	0	0.4	-0.4	1									
S_FL	-0.6	0.6	0.1	0.6	0.1	0	0.1	1								
NS_FL	-0.2	0.2	0.1	0.2	0.1	0	0.2	0.6	1							
RTI_FL	-0.6	0.6	0.1	0.6	0	0	0	0.8	0.1	1						
S_FS	-0.1	0.1	0.1	0.1	0.4	0.3	0.2	0.5	0.5	0.3	1					
NS_FS	-0.1	0	0	0	0.4	0.4	-0.1	0.3	0.6	0	0.6	1				
RTI_FS	-0.2	0.2	0.1	0.2	0.2	0	0.2	0.3	0	0.4	0.6	-0.1	1			
S_FB	-0.5	0.5	0.1	0.5	0.2	0.1	0.2	0.9	0.6	0.7	0.7	0.5	0.4	1		
NS_FB	-0.2	0.2	0.1	0.1	0.3	0.2	0.1	0.5	0.9	0.1	0.6	0.9	-0.1	0.6	1	
RTI_FB	-0.6	0.5	0.1	0.5	0.1	0	0.1	0.8	0.1	0.9	0.4	0	0.7	0.7	0	1

Table 7.7. Ranking of genotypes across traits that were correlated (score: leaf injury score, S_Chloro: leaf SPAD chlorophyll under salt stress (S_Chloro), and RTI_C: relative tolerance for leaf SPAD chlorophyll).

PI_ID	Origin	Score	S_Chloro	RTI_C	Tolerant (T)/Susceptible (S)
PI300173	South Africa	1	1	4	T
09_671	United States	2	6	3	T
PI583209	NA	3	11	7	T
PI582572	NA	4	15	18	T
PI293545	NA	5	5	2	T
PI339587	South Africa	6	28	50	T
PI152195	Paraguay	7	2	5	T
PI582874	Botswana	8	14	15	T
09_529	United States	10	4	6	T
PI583241	NA	13	10	19	T
PI583550	NA	14	16	22	T
PI293486	United States	17	29	24	T
PI582823	Botswana	20	8	1	T
PI293480	United States	23	9	9	T
PI583237	NA	25	19	14	T
09_470	United States	26	13	10	T
PI582474	Botswana	27	23	55	T
PI582878	Botswana	28	26	53	T
PI582864	Botswana	32	18	46	T
PI583200	NA	34	3	8	T
PI339603	NA	37	12	31	T
PI582469	Philippines	39	36	39	T
PI582551	Botswana	303	320	323	S
PI666251	NA	304	321	314	S
PI582354	NA	308	315	309	S
PI293491	United States	311	318	311	S
PI503326	Turkey	313	319	320	S
PI582428	NA	316	305	313	S
PI527263	Zimbabwe	318	308	308	S
PI663059	NA	319	312	318	S
PI610520	NA	321	326	326	S
PI582984	Kenya	322	329	330	S
PI583247	NA	324	325	324	S
PI201498	Mexico	327	316	322	S
PI663011	NA	328	331	329	S

Table 7.7. (Cont.)

PI_ID	Origin	Score	S_Chloro	RTI_C	Tolerant (T)/Susceptible (S)
PI225922	Zambia	329	328	327	S
PI255774	Nigeria	330	330	331	S
PI582530	NA	331	327	328	S

Figures

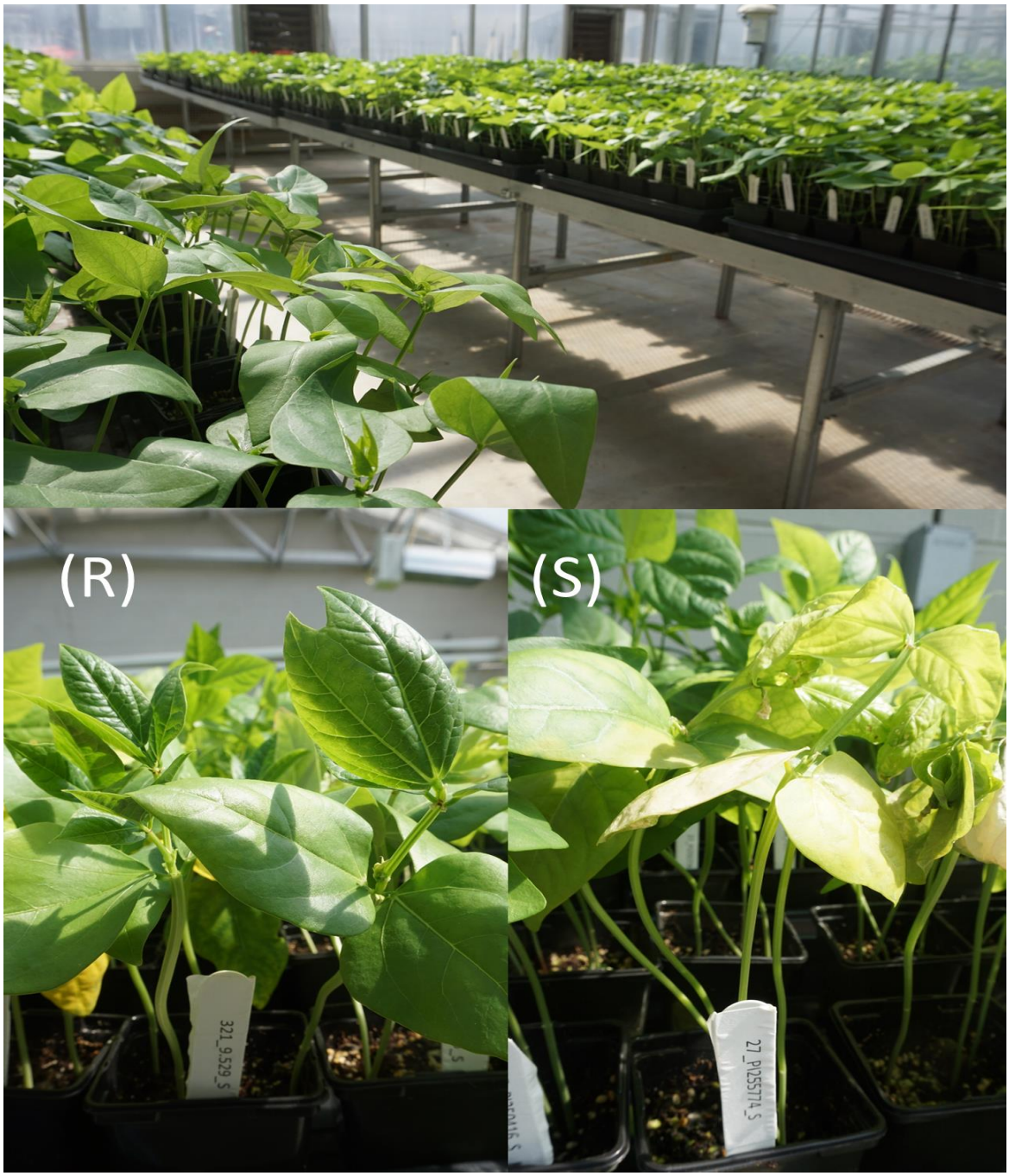

Fig. 7.1. Greenhouse experiment for salt tolerance in cowpea. (R) indicates the tolerant control, and (S) refers to the susceptible control.

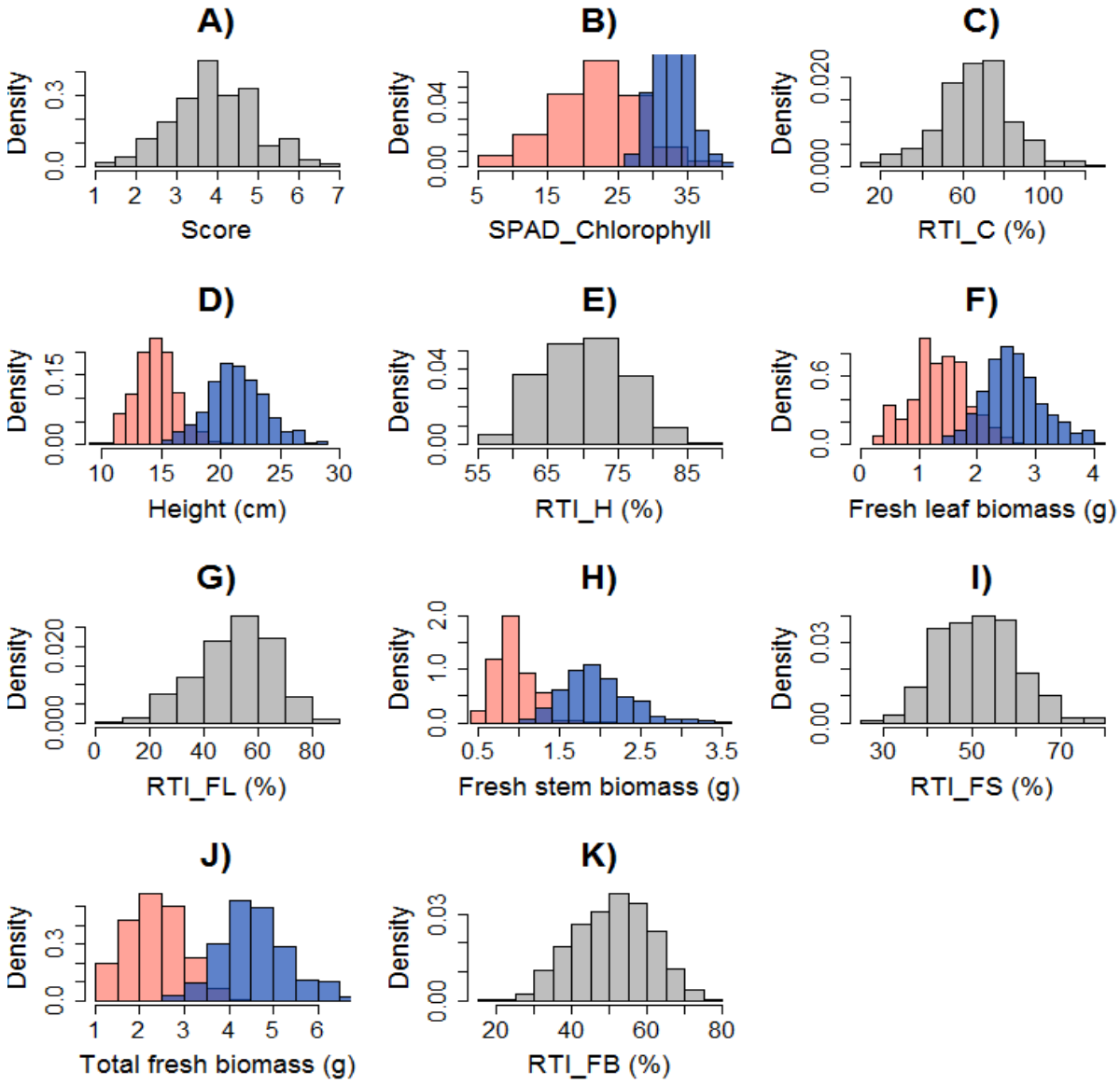

Fig. 7.2. Distributions of phenotypic trait values for salt tolerance in a total of 331 cowpea genotypes. For multicolor histograms, red histograms represented traits evaluated under salt stress, whereas blue histograms displayed traits evaluated under non-salt stress. A) Salt injury score, B) Leaf SPAD chlorophyll under salt stress (red) and under non-salt stress (blue), C) Relative tolerance index for leaf SPAD chlorophyll (RTI_C), D) Plant height under salt stress (red) and under non-salt stress (blue), E) Relative tolerance index for plant height (RTI_H), F) Fresh leaf biomass under salt stress (red) and under non-salt stress (blue), G) Relative tolerance index for fresh leaf biomass (RTI_FL), H) Fresh stem biomass under salt stress (red) and under non-salt stress (blue), I) Relative tolerance for fresh stem biomass (RTI_FS), J) Total above-ground fresh biomass under salt stress (red) and under non-salt stress (blue), and K) Relative tolerance index for total above-ground fresh biomass (RTI_FB).

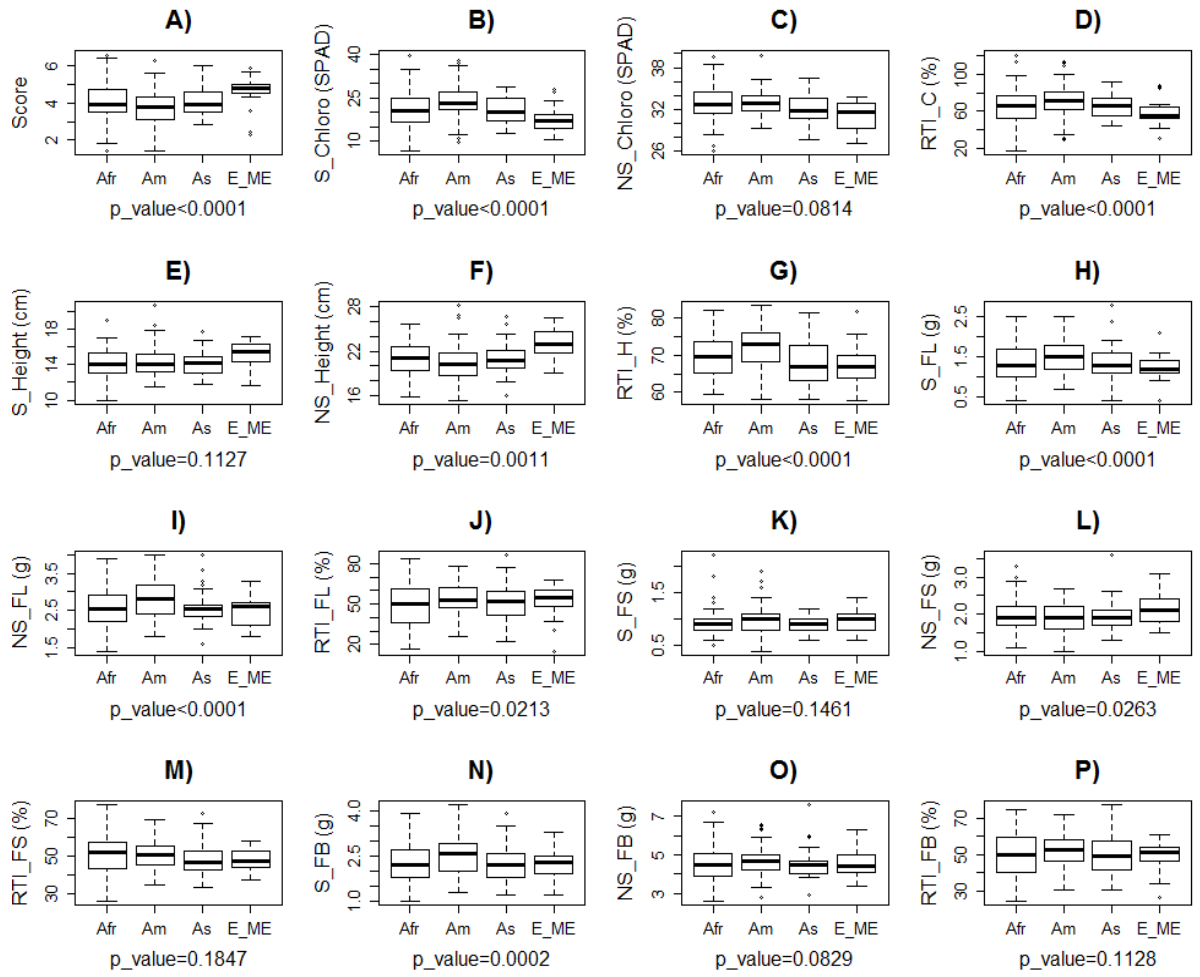

Fig. 7.3. Boxplots showing the variation of the traits evaluated for salt tolerance for each geographical area (origin). The x-axis represented the geographical where Afr=Africa (n=100), Am=America (n=77), As= Asia (n=32), and E_ME = Europe and the Middle East (n=17). Genotypes without information on the origin were not included in the analysis. Below each x-axis are shown the p-values obtained from the ANOVA. The y-axis displayed the different traits values. A) Salt injury score, B) Leaf SPAD chlorophyll under salt stress, C) Leaf SPAD chlorophyll under non-salt stress, D) Relative tolerance index for leaf SPAD chlorophyll (RTI_C), E) Plant height under salt stress, F) Plant height under non-salt stress, G) Relative tolerance index for plant height (RTI_H), H) Fresh leaf biomass under salt stress, I) Fresh leaf biomass under non-salt stress, J) Relative tolerance index for fresh leaf biomass (RTI_FL), K) Fresh stem biomass under salt stress, L) Fresh stem biomass under non-salt stress, M) Relative tolerance for fresh stem biomass (RTI_FS), N) Total above-ground fresh biomass under salt stress, O) Total above-ground fresh biomass under non-salt stress, P) Relative tolerance index for total above-ground fresh biomass (RTI_FB).

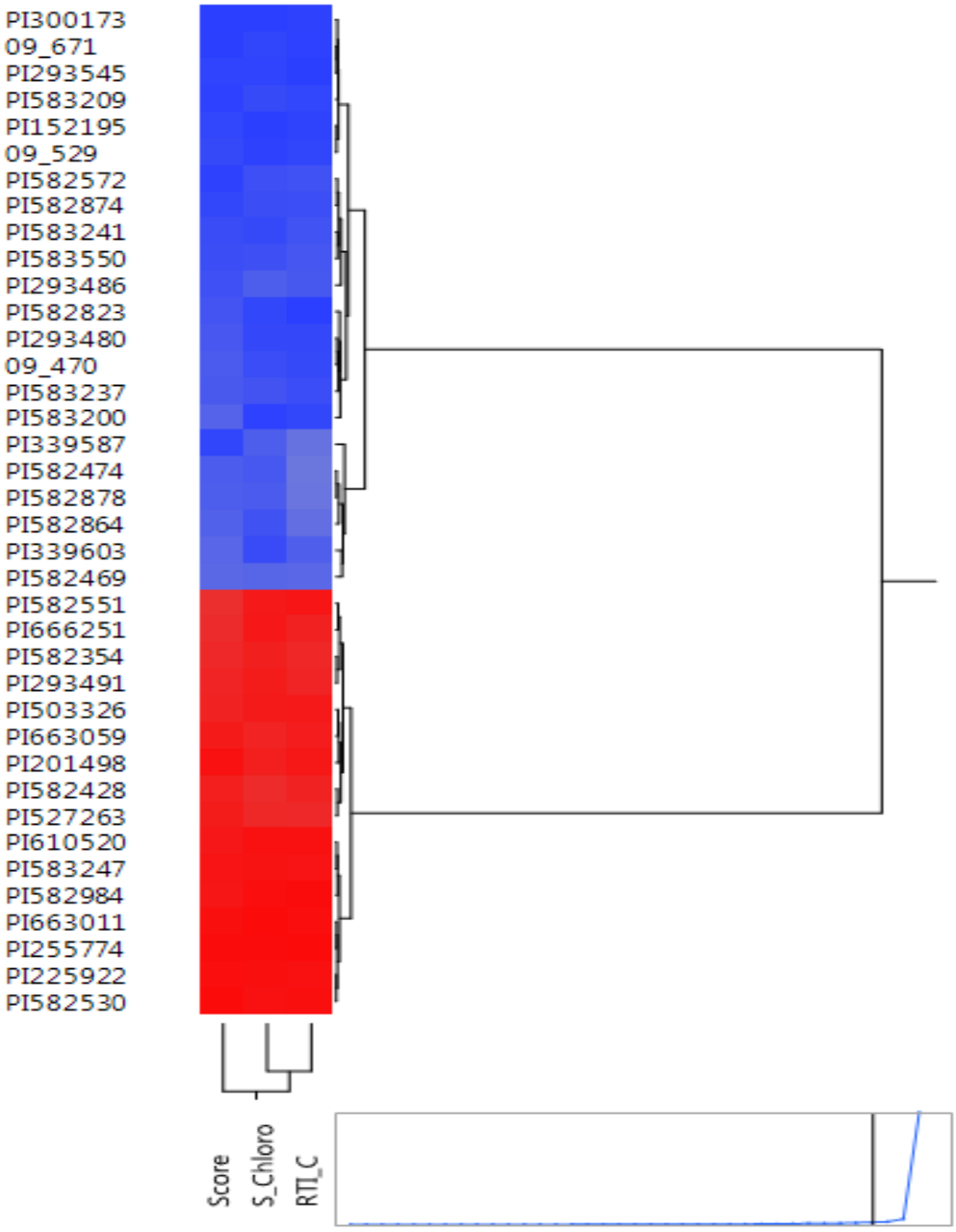

Fig. 7.4. Diversity of cowpea genotypes that were drought-tolerant based on leaf injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), and relative tolerance index for leaf SPAD chlorophyll (RTI_C).

Appendices

Table S7.1. List of 331 cowpea genotypes along with their country of origin. Cowpea genotypes were evaluated for salt injury score (Score), leaf SPAD chlorophyll under salt stress (S_Chloro), leaf SPAD chlorophyll under non-salt stress (NS_Chloro), relative tolerance for leaf SPAD chlorophyll (RTI_C), plant height under salt stress (S_Height), plant height under non-salt stress (NS_Height), and relative tolerance index for plant height (RTI_H). Sd represents the standard deviation across 8 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

Table S7.2. List of 331 cowpea genotypes along with their country of origin. Cowpea genotypes were evaluated for fresh leaf biomass under salt stress (S_Leaf), fresh leaf biomass under non-salt stress (NS_Leaf), relative tolerance index for fresh leaf biomass (RTI_FL), fresh stem biomass under salt stress (S_Stem), fresh stem biomass under non-salt stress (NS_Stem), relative tolerance index for fresh stem biomass (RTI_FS), total above-ground fresh biomass under salt stress (S_Biomass), total above-ground fresh biomass under non-salt stress (NS_Biomass), and relative tolerance index for total above-ground fresh biomass (RTI_FB). Sd represents the standard deviation across 8 replications. Relative tolerance index (RTI) was calculated as 100*(Phenotype_Stress/Phenotype_No_Stress). RTI was assessed for each replication and the RTI on the table was the average from each replication.

Table S7.3. Genotype ranking for each trait.

Fig. S7.1. Diversity of cowpea genotypes based on salt-related traits.

Chapter 8. Genome-Wide Association Study for Drought Tolerance in Cowpea (*Vigna unguiculata* (L.) Walp.) at Seedling Stage Using a Whole Genome Resequencing Approach

Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid legume species providing healthy nutrients for human consumption. Despite the fact that cowpea is one of more drought-tolerant legumes, some genotypes with a high yield under well-watered conditions have been shown to be susceptible to drought stress, thus requiring further improvement. The objectives of this study were to conduct a genome-wide association study (GWAS) to identify SNP markers, and to investigate candidate genes for drought tolerance in cowpea. A total of 331 cowpea genotypes were evaluated for drought tolerance. A total of 14,465,516 SNPs were obtained from a whole genome resequencing approach. After SNP filtering, 5,884,299 SNPs were used to conduct GWAS in 296 cowpea genotypes with high-quality SNP data using BLINK. From this study, a significant GWAS peak was observed with a cluster of 196 significant SNPs and is located at a 210-kb region of chromosome 5, which was identified as a the candidate locus for tolerance to trifoliate leaf chlorosis under drought stress in cowpea. This genomic region harbored the genes *Vigun05g006300.1* and *Vigun05g006500.1*, encoding for hormone-induced proteins. Another GWAS peak was found towards the end of chromosome 1 and it was a good candidate locus for tolerance to unifoliate leaf chlorosis under drought stress in cowpea. There were eight significant SNPs at this peak located at a 21-kb region of chromosome 1 and the gene *Vigun01g119000.1*, encoding for lysophosphatidic acid acyltransferase, was near the region. Two clusters > 500 SNPs located on chromosomes 8 and 10 were also found to be significantly associated with the tolerance to unifoliate leaf chlorosis under drought stress in cowpea. In addition, a total of 25

SNPs located on chromosomes 1, 3, 5, and 11were significantly associated with plant greenness under drought stress, and a total of 12 common SNPs were found between tolerance to trifoliate leaf chlorosis and plant greenness. These results could be used in cowpea breeding through marker-assisted selection (MAS). To the best of our knowledge, this is the first GWAS study using a whole genome resequencing data in cowpea.

Introduction

Breeding programs aiming at developing and releasing cultivars having the ability to better withstand drought conditions has been of interest over the last decades since the randomness of rainfall unfavorably impacts crop production. Severe drought conditions have been reported to lead to significant crop yield losses and plant death (Tester and Langridge 2010; Golldack et al. 2014). Drought related-issues are growing threats impairing legume production in tropical and sub-tropical areas (Carvalho et al. 2017). Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most widely grown legumes in these regions (Muñoz-Amatriaín et al. 2017).

Cowpea, (2n=2x=22), is a legume consumed for its protein. It belongs to the Family *Fabaceae* (Verdcourt 1970). Previous investigations showed that cowpea originated from Africa (Blackhurst and Miller 1980). In regions where cowpea is widely grown, limited access to water undermines cowpea production (Burridge et al. 2017). Cowpea cultivation is rain-dependent, and scarcity of water occurring at early vegetative growth is detrimental to cowpea production in spite of its high degree of drought tolerance over other crops (Fatokun et al. 2012). Therefore, improving drought tolerance of existing cowpea cultivars could address the increasing constraints imposed by drought conditions. In addition, with a relatively small genome size estimated to be 620 Mb (Timko et al. 2008) and a better ability to withstand drought (Contour-

ansel et al. 2006; Lucas et al. 2011), cowpea has been considered as a model crop for understanding drought mechanism in other crops (Carvalho et al. 2017).

Muchero et al. (2009) conducted a QTL mapping study for drought tolerance at seedling stage in 128 cowpea RILs derived from the cross between IT93K503-1 (drought tolerant) and CB46 (drought susceptible). A total of 306 amplified fragment length polymorphism (AFLP) markers were used. The results revealed 10 drought-related QTLs based recovery dry weight, visual rating of stem greenness and leaf senescence, and percent leaf damage under both greenhouse and field conditions. A later study by Muchero et al. (2011) suggested homology between seven previously reported drought QTLs and drought-related or abiotic stress-induced expressed sequence tags (EST) derived from cowpea or other plants. Since the number of QTLs reported by Muchero et al. (2009) was significantly large and the QTL resolution (22.7 cM to 76.6 cM) was poor, using such results for breeding purposes might be challenging.

Efforts toward effectively developing and improving crop drought-tolerant cultivars require knowledge pertaining to the genetic underlying such trait. Sequencing technologies have been tremendously improved recently, allowing scientists to perform whole genome (re)sequencing of crops for a reasonable cost even if only a reference genome is partially available. Further, gaps existing between model and crop species have been progressively filled over the last few years (Yao et al. 2016), which will speed up the discovery of genes controlling traits of agronomic interests. Whole genome (re)sequencing permits the discovery of a large set of SNPs which can be used for genome-wide association studies (GWAS) (Lee et al. 2015; Thudi et al. 2016). In regard to drought-related studies involving GWAS, previous reports have been proven to be promising at identifying molecular markers or regions of the genome associated with tolerance to drought. Varshney et al. (2012) evaluated a total of 223 barley

(*Hordeum vulgare* L.) accessions for drought and conducted a GWAS using 710 Dart markers, 61 SNPs, and 45 SSRs. In soybean [*Glycine max* (L.) Merr], Dhanapal et al. (2015) used the carbon isotope ratio ($\delta^{13}C$) as a surrogate for assessing water use efficiency in a soybean panel consisting of 373 genotypes. A total of 12,347 SNPs were used for GWAS; results showed that 39 SNPs were significantly associated with $\delta^{13}C$. In model plant such as *Arabidopsis,* Bac-Molenaar et al. (2015) evaluated 324 natural accessions of *Arabidopsis* and found six time-dependent QTLs for drought tolerance. Results showed that the earlier the flowering time was, the more likely to be drought tolerant the accession was. In rice (*Oryza sativa* L.), Pantalião et al. (2016) were able to identify 10 previously reported genes for drought tolerance using GWAS approach. A total of 175 rice accessions were analyzed and GWAS involved 150,325 SNPs. Zhang et al. (2015) phenotyped 140 canola (*Brassica napus* L.) accessions for drought tolerance; GWAS allowed the identification of 16 loci associated with drought. Kang et al. (2015) identified candidate genes for glutamate-cysteine ligase and aldehyde dehydrogenase associated with stomata density under drought conditions in *Medicago Truncatula* Gaertn. throught GWAS. In regard to common bean (*Phaseolus vulgaris* L.), traits consisting of wilting and leaf growth rate under drought conditions were evaluated in a panel of 96 genotypes, and GWAS revealed 27 significant SNPs associated with drought tolerance (Hoyos-Villegas 2015). Wang et al. (2016) conducted a marker-trait association involving 201 maize (*Zea mays* L.) inbred lines and using 41,101 SNPs. Results revealed 206 SNPs associated with drought-tolerance related traits with 115 candidate genes. Traits included final grain yield, total number of ears per plot, kernel number per row, plant height, anthesis-silking interval, days to anthesis, and days to silking.

QTL mapping in biparental crossings has also been used to identify genetic regions associated with drought tolerance. However, few genes have been identified from previously

identified QTLs (Price 2006). GWAS, a linkage disequilibrium-based approach, provides greater resolution, thus reliably allowing identification of specific region in the genome associated with traits (Hamblin et al. 2011). The use of SNPs (Fang et al. 2014) as molecular markers has been shown to be rewarding in the field of plant breeding. To our knowledge, there is not yet any report on GWAS for drought tolerance in cowpea in spite of the power of this technology in identifying genomic regions associated with traits of interest in agriculture and the potential of cowpea to be used a model crop for studying drought tolerance mechanism in plants. This study aimed to conduct a genome-wide analysis study for drought tolerance at seedling stage in cowpea, and to identify SNP markers and candidate genes for drought tolerance.

Materials and Methods

Plant materials and phenotyping

A total of 331 cowpea genotypes were evaluated for drought tolerance at seedling stage in this study. Of which, 36 were breeding lines from the University of Arkansas, Fayetteville, 8 were from the University of California, Riverside and were the founders of the first multiparent advanced generation intercross (MAGIC) population (Huynh et al. 2018), and 287 were Plant Introductions (PIs) from the U.S. Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea accessions. The PIs were obtained from the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. The cowpea genotypes were originally collected from than 32 countries and unknown sources. Seed increase was conducted in the summer of 2018 at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville. One plant from each genotype was harvested and developed to single plant-derived line. Cleaned and carefully sorted seeds were used for the experiments.

Cowpea drought tolerance evaluation was conducted in the greenhouse of Harry R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville, AR. Screening methodology was previously described (Ravelombola et al. 2018; Singh et al. 1999; Verbree et al. 2015). Sterilite polypropylene boxes (Sterilite Corporation, Townsend, MA) was used for drought phenotyping. Boxes were 88.6 cm-long, 42.2-cm wide, and 15.6 cm-high. Boxes were filled with Sunshine® Mix #1 Natural & Organic (Agawan, MA) up to 10.5 cm high. Soil medium within boxes was watered with 12 L of tap water two days before sowing so that field capacity was attained at planting time (Verbree et al. 2015).

A total of 10 rows were designed at each 7.5 cm through the box length. For each genotype, two cowpea seeds were sown in a 2-cm diameter hole across each row containing a total of 12 seeds. Cowpea plants were thinned to one plant per hole upon plant establishment so that six plants remain within each row. A solution of 150 mL Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI) were applied to each row at one week after plant emergence. Fertilizer solution was obtained by dissolving one tablespoon of Miracle-Gro into one gallon of tap water. Each row was irrigated with 150 mL tap water each three days and until the first trifoliate leaf was fully expanded. Plants were watered until the first trifoliate leaf was fully expanded and watering was stopped after this time in the drought-stressed box. Irrigation was still conducted in the well-watered box. The drought-stressed and well-watered boxes were placed next to each other in order to minimize the environmental effects within the greenhouse. A total of 3 drought-tolerant genotypes (PI293469, PI349674, and PI293568) and 1 drought-susceptible genotype (PI255774) were used to validate the experiments (Ravelombola et al. 2018). The experiments were conducted using 3 runs and each run was considered as a blocking variable. The experimental unit corresponded to each row within boxes. Soil moisture was assessed using an

HH2 Moisture Meter (Delta-T Devices, Cambridge, UK) every 3 days. Data measurements were previously described (Ravelombola et al. 2018).

Genotyping

DNA extraction, library preparation, and whole-genome resequencing

Young cowpea leaves were harvested from one plant and all seeds that were used for the experiments were form that plant. Genomic DNA was extracted from freeze-dried young cowpea leaves using the CTAB (hexadecyltrimethyl ammonium bromide) protocol (Kisha et al., 1997). Leaf samples were ground in Mixer Mill MM 400® (Haan, Germany). Samples were centrifuged at 13,000 rpm for 10 minutes after addition of DNA buffer. A solution of 1 ml of chloroform-isoamyl alcohol (24:1) was added to each sample to denature proteins. A solution of 1 ml of isopropanol allowed DNA to precipitate. Samples were stored at -20°C overnight. DNA pellets were washed by 70% and 90% ethanol. After ethanol washing, samples were air-dried. RNA was removed by adding 3 µl of RNAse to each sample. DNA was stored in a solution of 200 µl of 0.1X TE. The amount of DNA within each sample was quantified using a NanoDrop 200c spectrophotometer (Thermo SCIENTIFIC, Wilmington, DE). DNA was quality-checked on a 1%-agarose gel with ethidium bromide stain.

DNA sequencing was performed by Novogene (http://en.novogene.com/). Cleavage of DNA was done using Covaris S2® (Covaris, Inc., Woburn, MA). This generated a set of approximately 350-bp DNA fragments. DNA library consisted of sheared DNA fragments and NEBNext DNA Library Prep Reagent Set for Illumina (BioLabs, Inc., Ipswich, MA). DNA fragments were end-repaired. Poly-A tails were added to each fragment. Fragmented DNA was purified and subjected to *in situ* PCR amplification as described by van Dijk et al. (2014). Genomic DNA sequencing was achieved using Illumina HiSeq X Ten Series

(http://www.illumina.com/systems/hiseq-x-sequencing-system/system.html) with an average of 10X coverage. This study involved a total of more than 1.88 Tb of genomic information sequence.

SNP calling, mapping, and filtering

Short-reads were aligned to the cowpea reference genome (Lonardi et al. 2019). Alignment were done using SOAPaligner/soap2 (http://soap.genomics.org.cn/). Preliminary SNP calling was achieved using SOAPsnp v 1.05 (Li et al. 2009). Accessions having more than 20% missing SNP information were removed. Triallelic SNPs and those with more than 20% missing data were also not considered for GWAS. SNPs with more than 20% heterozygous calls were discarded from the analysis. The minor allele frequency (MAF) threshold was 5%. GWAS was conducted using filtered SNPs.

Population structure and genetic diversity analysis

STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to infer population structure. Population structure (K) analysis was conducted using an admixture-based model along with a correlated allele frequency one, which was independent for each run as described by Shi et al. (2016). For each estimated K value, 10 runs were conducted. Markov Chain Monte Carlo (MCMC) length of the burn-in period and the number of MCMC iterations after the burn-in period were 20000 and 50000, respectively. STRUCTURE Harvester (Earl and VonHoldt, 2011; http://taylor0.biology.ucla.edu/structureHarvester/) was used to select the appropriate K values. Screening for optimal K values was based on the formula established by Evanno et al. (2005). K value corresponding to the delta K peak was considered as optimal K. Cut-off probability for assigning an accession to a Q cluster was 0.55. Population structure was visualized using STRUCTURE PLOT using the option “Sort by Q” (Ramasamy et al., 2014). Since population

structure analysis is highly computationally intensive, a total of 60,000 (~10 % of the whole genome resequencing SNPs) were randomly chosen for the analysis.

Genetic diversity was performed using the Maximum Likelihood tree as statistical approach in MEGA 7 (Kumar et al., 2016). Phylogenetic tree was drawn using MEGA 7. The following parameters were considered as described previously (Shi et al., 2016; Xiong et al., 2016; Qin et al., 2017): Analysis: Phylogeny Reconstruction; Statistical method: Maximum Likelihood; Test of phylogeny: None; Substitutions type: Nucleotide; Model/Method: Tamura-Nei Model; Rates among sites: Gamma distributed with Invariant sites (G+I); No of Discrete Gamma Categories: 5; Gaps/Missing Data treatment; ML Heuristic Method: Nearest-Neighbor-Interchange (NNI); Initial Tree for ML: Make initial tree automatically (Default - NJ/BioNJ); Branch Swap Filter: Moderate; Number of threads: 1; Test of Phylogeny: None; No. of Bootstrap Replications: 500; Model/Method: General Time Reversible Model; Rates among Sites: Gamma distributed with invariant sites (G+1); Number of discrete gamma categories: 5; Gaps/Missing data treatment: use of all sites; ML Heuristic method: Subtree-Pruning-Regrafting-Extensive (SPR level 5); Initial tree for ML: Make initial tree automatically (Neighbor Joining); and Branch swap filter: Moderate.

Results including the Q groups from the population structure analysis were used in MEGA 7 for a combined genetic diversity analysis. Each Q cluster had different color by default in the STRUCTURE PLOTS. The sub-tree displaying each Q group in the phylogenetic tree, the shape of “Node/Subtree Marker”, and the “Branch Line” had the same color as shown in the STRUCTURE PLOTS.

Genome wide association study (GWAS) and genomic selection

GWAS was conducted using a Bayesian Information and Linkage Disequilibrium Iteratively Nested Keyway (BLINK) model (Huang et al. 2019). BLINK has been shown to have an improved statistical power and to be more efficient compared to previously models in reducing false positive discovery (Huang et al. 2019). SNP was declared to be significant when above the FDR-adjusted threshold and computed in R ($P < 3\ 10^{-8}$). BLINK model was derived from the Fixed and Random Model Circulating Probability Unification (FarmCPU) model. FarmCPU assumed markers being evenly distributed across the genome, which could be easily violated. Instead, BLINK used the LD information to relax this assumption. In addition, the heavy computational-related issue due to the random effect model (REM) was replace by a second fixed model (FEM) in BLINK. The two FEM models in BLINK were described below.

$$\text{FEM (1): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ik}b_k + M_{ij}d_j + e_i$$

$$\text{FEM (2): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ij}b_j + e_i$$

with y_i being the vector phenotype, M_{i1},$M_{i2}b_2$, …, M_{ik} the genotypes of k pseudo QTNs that were initially empty and with effects b_1, b_2, …, b_k, respectively, M_{ij} being the j^{th} genetic marker of the i^{th} sample, and e_i being the residual having a distribution with mean zero and a variance σ^2_e. Overlapping SNP markers between different traits were visualized using a Venn diagram that was designed using the online software program accessible at http://jvenn.toulouse.inra.fr/app/example.html.

Genomic selection was conducted using the rrBLUP model and run in R using the "rrBLUP" package. A 5-fold cross-validation study was used. A total of 100 replications were used. Genomic selection accuracy was assessed by computing the Person's correlation coefficient between the genomic estimated breeding values (GEBVs) and the phenotypic data.

Due to the extremely large number of SNPs, the SNPs with LOD > 4 were chosen to conduct GS. This threshold allowed for the SNP matrix size to be properly handled in R.

Candidate gene search and synteny analysis

Given the number of SNPs used in this study, the genome size of cowpea, and the average length of a gene within the cowpea genome, we looked at any annotated genes within 10-bk genomic region flanking a SNP using Phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html). Annotated genes having functional annotation relevant to plant physiology and/or tolerance to abiotic stress were considered. Functional annotations were also obtained from Phytozome v. 13 (https://phytozome.jgi.doe.gov/pz/portal.html). For the annotated genes with functional annotations addressing plant physiology and/or tolerance to abiotic stress, the coding sequences were extracted. The extracted sequences were used to conduct BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to investigate the amino acid sequence. The amino acid sequence was used to conduct protein homolog search in other legumes such as soybean, common bean, and *Medicago truncatula* Gaertn. Only hits with similarity greater than 90% were considered. The tertiary structure of the polypeptide/protein that was derived from the amino acid sequence was predicted using SWISS-MODEL (https://swissmodel.expasy.org/).

Results

Population structure and genetic diversity analysis

A peak delta K was found at K=2, indicating that the association panel had two subpopulations (Q1 and Q2). A relatively low level of admixture (Q1Q2) was also found. Q1

accounted for 49%. Q2 harbored 47% of the population. A combined analysis between population structure and genetic diversity is shown in Fig. S8.1.

First trifoliate leaf chlorosis under drought stress

Of the 5,884,299 SNPs used to conduct GWAS for tolerance to first trifoliate leaf chlorosis under drought stress in cowpea, a total of 1,047 SNPs were above the threshold (Table S8.1) (Figs. 8.1-8.3). Significant SNPs were located on chromosomes 1, 2, 3, 4, 5, 7, and 9. The number of significant SNPs was 2, 2, 1232, 610, 196, 2, 1, and 2 for the chromosomes 1, 2, 3, 4, 7, and 9, respectively. LOD values ($-\log_{10}$(p-value)) for the significant SNPs varied from 7.52 to 20.29. One of the most interesting findings from the study was the identification of four significant loci associated with tolerance to first trifoliate leaf chlorosis under drought stress. These loci were mapped at the start of chromosome 3, in the middle of chromosome 4, towards the end of chromosome 4, and at the beginning of chromosome 5.

The significant locus found on a 1.3-Mb region of chromosome 3 was defined by a total of 1149 SNPs (Table S8.1). This genomic region is gene-dense (Table 8.1). Functional annotations of the candidate genes found within regions showed proteins that were involved in hormone-induced response such as auxin and abscisic acid. This genomic region was also characterized by a significant cluster of biomolecule transporters (Fig. 8.1). Tertiary structure analysis of the proteins that were derived for the candidate genes were shown in Fig. 8.1. For example, a cluster of vacuolar iron transporters were mapped on a 30-kb genomic region and proteins derived from these transporters were slightly different from each other (Fig. 8.1). The SNPs that were found within or in the vicinity of these vacuolar iron transporters were Vu03_13295491, Vu03_13297714, Vu03_13302250 (Table 8.1). The candidate genes associated with the vacuolar iron transporters were *Vign03g135700.1*, *Vign03g135800.1*, and

Vign03g135900.1 (Table 8.1). The SNP that was found within the annotated gene associated with EamA-like transporter family/auxin-induced protein 5NG4, *Vigun03g136600.1*, was Vu03_13382599 (LOD= 9.59). In addition, an annotated gene, *Vigun03g137500.1*, encoding for an ABA responsive element binding was found in the vicinity of Vu03_13509429 (LOD= 10.25). Tolerance to trifoliate leaf chlorosis was assessed based on the level of leaf greenness. As expected, results identified a significant SNP, *Vu03_14815803* (LOD= 8.79), that was found on chromosome 3 and located within an annotated gene encoding for a chlorophyll a/b binding protein. In addition, a significant SNP, Vu03_36340055, was also mapped in the vicinity of an annotated gene encoding for ABC-2 type transporter family protein (Table 8.1). Other genomic regions of chromosome 3 also harbored significant SNPs associated with tolerance to trifoliate leaf chlorosis under drought stress in cowpea. However, these regions were less gene-dense and the annotated genes found within these regions had functional annotations that were less relevant to plant abiotic stress. Chromosome 4 had two significant loci defined by about 800-kb and 100-bk genomic regions, respectively (Fig. 8.2). The 800-bk genomic region harbored a total of 484 significant SNPs and the second one had 69 SNPs (Table S8.1). Of these SNPs, 19 were mapped within the structure of annotated genes that had functional annotations relevant to plant abiotic stress. These SNPs consisted of Vu04_26966450 (LOD= 8.37), Vu04_27157237 (LOD= 8.21), Vu04_27241963 (LOD= 8.3), Vu04_27298716 (LOD= 8.22), Vu04_27342140 (LOD= 8.56), Vu04_27505387 (LOD= 8.51), Vu04_27528973 (LOD= 8.1), Vu04_27714135 (LOD= 8.72), Vu04_27716250 (LOD= 8.35), Vu04_27778870 (LOD= 7.67), Vu04_27786623 (LOD= 9.08), Vu04_27797389 (LOD= 8.37), Vu04_27830859 (LOD= 7.81), Vu04_27913211 (LOD= 7.8), Vu04_27913980 (LOD= 8.06), Vu04_41785910 (LOD= 8.5), Vu04_41800041 (LOD= 7.67), Vu04_41826262 (LOD= 8.11), and Vu04_41832927 (LOD= 8.09) (Table 8.1). Two annotated

genes, *Vigun04g110600.1* and *Vigun04g110800.1*, having functional annotations that were directly relevant were found within the 800-kb locus associated with tolerance trifoliate leaf chlorosis. *Vigun04g110600.1* and *Vigun04g110800* encodes for no apical meristem protein (NAM) and a Myb-family protein. Structural analysis of these two proteins was investigated and visualized in Fig.8.2.

The most significant finding was the identification of a strong locus associated with tolerance to first trifoliate chlorosis on chromosome 5 (Fig. 8.3). The locus was defined by a 210-kb region and harbored a total of 196 significant SNPs (Table S8.1). In this region, LOD ($-\log_{10}$(p-value)) values varied from 7.52 to 20.29. SNPs with the highest LOD values were Vu05_539746 (LOD= 17.28), Vu05_539750 (LOD= 17.07), Vu05_539753 (LOD= 17.45), Vu05_539879 (LOD= 16.48), Vu05_539880 (LOD= 16.48), Vu05_539926 (LOD= 16.52), Vu05_540522 (LOD= 18.16), Vu05_540561 (LOD= 20.29), Vu05_541044 (LOD= 16.5), Vu05_541198 (LOD= 17.4), and Vu05_548993 (LOD= 17.18). Two SNPs, Vu05_540561 (LOD= 20.29) and Vu05_560665 (LOD= 14.25), were located within the structure of *Vigun05g006300.1* and *Vigun05g006500.1*, respectively. These annotated genes encode for an auxin-induced protein and a neoxanthin synthase involved in the abscisic acid biosynthesis. Chromosomes 7 and 8 also harbored significant SNPs associated with tolerance to trifoliate leaf chlorosis under drought stress in cowpea.

Unifoliate leaf chlorosis

Tolerance to unifoliate leaf chlorosis has also been described as mechanism to cope with water deficiency in cowpea. In this study, a total of 591 SNPs were found to be significantly associated with tolerance to unifoliate leaf chlorosis under drought stress (Table S8.2). A total of 8, 582, and 1 significant SNPs were found on chromosomes 1, 8, and 10, respectively (Figs. 8.4-

8.7). LOD ($-\log_{10}$(p-value)) values varied 7.52 to 14.45 for the significant SNPs. Results indicated three significant loci associated with tolerance to unifoliate leaf chlorosis. These loci were mapped on chromosomes 1 and 8 (Figs. 8.4-8.7).

The significant locus that was identified on chromosome 1 was defined by a total of 8 SNPs. These SNPs were mapped on a 27-kb region of chromosome 1 (Fig. 8.4). These SNPs were Vu01_29542433 (LOD= 9.98), Vu01_29544073 (LOD= 13.2), Vu01_29544191 (LOD= 14.45), Vu01_29544749 (LOD= 13.97), Vu01_29548480 (LOD= 12.43), Vu01_29549609 (LOD= 8.33), Vu01_29558145 (LOD= 8.72), and Vu01_29570238 (LOD =9.43) (Table 8.1). A total of 3 annotated genes were found within this region. Of the 3 annotated genes, *Vigun01g119000*.1 is the only one having a functional annotation. *Vigun01g119000*.1 encodes for lysophosphatidic acid acyltransferase (Fig. 8.4). The significant SNP that was closest to this annotated gene was Vu01_29544191 (LOD= 14.45).

A 42-kb region of chromosome 8 contained a total of 65 SNPs that were significantly associated with tolerance to unifoliate leaf chlorosis under drought stress in cowpea (Fig. 8.5). Of these SNPs, those with the highest LOD values were Vu08_4952393 (LOD= 9.83), Vu08_4946612 (LOD= 9.70), Vu08_4946618 (LOD= 9.70), Vu08_4945615 (LOD= 9.67), Vu08_4945627 (LOD= 9.61), Vu08_4946651 (LOD= 9.58), Vu08_4951347 (LOD= 9.51), Vu08_4951349 (LOD= 9.51), Vu08_4936939 (LOD= 9.40), Vu08_4946653 (LOD= 9.39), Vu08_4946682 (LOD= 9.39), Vu08_4946699 (LOD= 9.34), Vu08_4952509 (LOD= 9.34), Vu08_4952522 (LOD= 9.34), and Vu08_4952526 (LOD= 9.34). The significant locus defined by the 42-kb region of chromosome 8 harbored a cluster of three annotated genes encoding for a leucine-rich repeat (Table 8.1). The SNPs that were located in the vicinity or within the structure of these annotated genes were Vu08_4931701 (LOD= 8.32), Vu08_4945627 (LOD= 9.61), and

Vu08_4952526 (LOD= 10.59). The predicted tertiary structure of the protein derived from the 3 annotated genes was slightly different (Fig. 8.5).

The third significant locus associated with tolerance to unifoliate leaf chlorosis was mapped on a 184-kb region of chromosome 8 (Fig. 8.6). This region harbored a total of 517 significant SNPs. LOD (-log10(p-value)) values of the significant SNPs found in this region varied from 7.52 to 10.59. The SNPs with the highest LOD values were Vu08_26752606 (LOD= 10.59), Vu08_26852413 (LOD= 10.31), Vu08_26874709 (LOD= 10.27), Vu08_26898363 (LOD= 10.17), Vu08_26888097 (LOD= 10.11), Vu08_26877485 (LOD= 10.08), Vu08_26901689 (LOD= 10.04), Vu08_26878780 (LOD= 9.87), Vu08_26871649 (LOD= 9.83), Vu08_26871652 (LOD= 9.83), Vu08_26877438 (LOD= 9.78), Vu08_26874835 (LOD= 9.77), Vu08_26897604 (LOD= 9.77), and Vu08_26883655 (LOD= 9.75). The significant locus defined by the 184-kb region of chromosome 8 harbored 7 annotated genes with 6 having functional annotations. The SNPs Vu08_26752606 (LOD= 10.59), Vu08_26868733 (LOD= 8.83), Vu08_26877485 (LOD= 10.08), and Vu08_26901689 (LOD= 10.04) were found in the vicinity or within the structure of *Vigun08g107800.1*, *Vigun08g107900.1*, *Vigun08g108100.1*, and *Vigun08g108400.1* encoding for Ubiquitin carboxyl-terminal hydrolase, AT-hook DNA-binding family protein, Carbonic anhydrase, and DnaJ homolog subfamily, respectively. The predicted tertiary structure of these proteins is shown in Fig. 8.6. One significant SNP located on chromosome 10 was also found to be associated with tolerance to unifoliate leaf chlorosis under drought stress in cowpea (Fig. 8.7).

Plant greenness score

Plant greenness score was recorded in order to assess the degree of wilting due to drought stress in this study. Unlike tolerance to trifoliate leaf chlorosis and unifoliate leaf chlorosis under

drought conditions, a very few SNPs were identified to be associated with plant greenness score for the cowpea panel evaluated for drought tolerance at seedling stage. A total of 25 SNPs were identified and mapped on chromosomes 1, 3, 5, and 11 (Figs. 8.8-8.10). Chromosome 3 had the highest number of significant SNPs, whereas chromosome 1 had the lowest number of significant SNPs (Table S8.3).

The significant SNPs associated with plant greenness score under drought stress consisted of Vu01_10616486 (LOD= 8.13), Vu03_13509429 (LOD= 7.58), Vu03_14725410 (LOD= 7.56), Vu03_14725434 (LOD= 7.78), Vu03_14725437 (LOD= 7.78), Vu03_14725438 (LOD= 7.78), Vu03_14725450 (LOD= 7.69), Vu03_14730296 (LOD= 7.59), Vu03_14730297 (LOD= 7.59), Vu03_14735109 (LOD= 7.58), Vu03_15042787 (LOD= 7.73), Vu03_20084616 (LOD= 9.09), Vu03_24643282 (LOD= 9.19), Vu05_540561 (LOD= 8.32), Vu05_541044 (LOD= 8.62), Vu05_541198 (LOD= 8.84), Vu05_541677 (LOD= 7.95), Vu05_544287 (LOD= 8.12), Vu11_22285237 (LOD= 11.00), Vu11_22285238 (LOD= 11.00), Vu11_22285251 (LOD= 11.00), Vu11_22285317 (LOD= 11.24), Vu11_22285318 (LOD= 11.24), Vu11_22285324 (LOD= 9.77), and Vu11_22285327 (LOD= 10.48). On chromosome 1, the SNP that was located in the vicinity of an annotated gene, *Vigun01g054900.1*, was Vu01_10616486 (LOD= 8.13). This gene encodes for DCN1-like protein. The predicted tertiary structure of this protein is shown in Fig. 8.8. The genomic region harboring Vu01_10616486 contained also SNPs with relatively high LOD ($-\log_{10}$(p-value)) values as shown in Fig 8.8. However, these SNPs were just below the threshold that was chosen to declare significance in this study. The SNPs Vu03_13509429 (LOD= 7.58) and Vu03_14725438 (LOD= 7.78) were very close to the annotated genes *Vigun03g137600.1* and *Vigun03g144800.1*, respectively. The functional annotations of the proteins derived from these genes were P-loop containing nucleoside

triphosphate hydrolase superfamily protein and WRKY transcription factor, respectively. The predicted tertiary structure of these proteins is shown in Fig. 8.9. Interestingly, the significant locus found at the beginning of chromosome 5 overlapped with the locus associated with tolerance to first trifoliate leaf chlorosis under drought stress (Fig.8.10). One significant SNP associated with plant greenness score and mapped on chromosome 5 was just located at 1-kb of another SNP having the highest LOD value for tolerance to trifoliate leaf chlorosis. These results indicate that this genomic result could control both plant greenness score and tolerance to trifoliate leaf chlorosis under drought stress in cowpea. No annotated genes were found in the vicinity of the significant SNPs that were mapped on chromosome 11.

Protein homologs and gene ontology

Protein homolog search was conducted for the candidate genes with functional annotations that are relevant to plant abiotic stress. Search was conducted within the genomes of legumes such as soybean, common bean, and Medicago. Proteins with more than 90% with the query were only considered. Search was also conducted within the cowpea genome in order to investigate potential gene duplication within the cowpea genome. For the candidate genes associated with trifoliate leaf chlorosis, the number of homologs significantly varied across species (Table 8.2). On average, the soybean genome has a multiple copy of the same gene. The candidate genes *Vigun03g137500.1*, *Vigun03g135700.1*, and *Vigun04g110800.1* were unique within the cowpea genome. One or two copies of the candidate genes *Vigun05g006300.1*, *Vigun05g006500.1*, *Vigun03g136600.1*, and *Vigun04g110600.1* were identified within the cowpea genome (Table 8.2). The candidate genes *Vigun03g135800.1* and *Vigun03g135900.1* had more than four copies within the cowpea genome, 7 copies within the soybean genome, 5 copies within the common bean genome, and 4 copies within the Medicago genome.

Results for tolerance to unifoliate leaf chlorosis were interesting in a way that most of candidate genes were unique in the cowpea genome (Table 8.2). Candidate genes consisting of *Vigun08g046400.1*, *Vigun08g107800.1*, *Vigun08g108100.1*, *Vigun08g108400.1*, and *Vigun10g137100.1* were unique within the cowpea genome. In addition, no copy of *Vigun10g137100.1* was found within the genome of soybean, common bean, and Medicago. Overall, gene duplication of the candidate genes associated with tolerance to unifoliate leaf chlorosis seemed to be more significant within the common bean genome. Results for plant greenness score were also similar to that of tolerance to unifoliate leaf chlorosis under drought stress. In fact, *Vigun01g054900.1*, *Vigun03g137600.1*, *Vigun03g144800.1* were unique within the cowpea genome and only one copy was found for *Vigun05g006300.1*. No copies of *Vigun03g137600.1* were identified within the genome of common bean and Medicago. One copy of this gene was found within the soybean genome.

Overlapping SNPs and functional annotations

The number of overlapping SNPs between traits was visualized using a Venn diagram (Fig. 8.11A). The number of SNPs associated with tolerance to trifoliate leaf chlorosis, tolerance to unifoliate leaf chlorosis, and plant greenness score was 1047, 591, and 25, respectively. On the Veen diagram, SNPs associated with trifoliate leaf chlorosis, unifoliate leaf chlorosis, and plant greenness score were represented by solid green, blue, and pink circles, respectively (Fig. 8.11A). No overlapping SNPs were found between the 3 traits investigated for drought stress. However, a total of 12 SNPs overlapped between tolerance to trifoliate leaf chlorosis and plant greenness score in cowpea. No common SNPs were found between tolerance to unifoliate leaf chlorosis and plant greenness score. In addition, no overlapping SNPs were identified between

tolerance to trifoliate leaf chlorosis and tolerance to unifoliate leaf chlorosis under drought stress in cowpea, indicating that these two traits could have independent genetic mechanism.

Overlapping functions of candidate genes associated with different traits was also visualized using a Venn diagram (Fig. 8.11B). As expected from the results for overlapping SNPs, no candidate genes having common functions were found between tolerance to trifoliate leaf chlorosis, tolerance to unifoliate leaf chlorosis, and plant greenness score under drought stress in cowpea. However, overlapping functions were identified between tolerance to trifoliate leaf chlorosis and plant greenness. No overlapping functions were found between candidate genes associated with tolerance to unifoliate leaf chlorosis and plant greenness score, respectively, which was in agreement with the findings for overlapping SNPs.

Genomic selection

Overall, genomic selection accuracy was moderate for the tree traits evaluated for drought tolerance in this study. The average selection accuracy was 0.57, 0.52, and 0.47 for tolerance to first trifoliate leaf chlorosis, tolerance to unifoliate leaf chlorosis, and plant greenness score, respectively.

Discussion

To the best of our knowledge, this is the first report on GWAS for drought tolerance in cowpea using a whole genome resequencing data. A total of 14,465,516 SNPs were obtained from whole genome resequencing. Of which, 5,884,299 SNPs satisfied the filtering criteria and were further processed for GWAS. To date, this could be the largest amount of SNPs data that were used to conduct GWAS in cowpea. We have identified strong GWAS peaks that were associated with tolerance to trifoliate leaf chlorosis, tolerance to unifoliate leaf chlorosis, and

plant greenness under drought stress in cowpea at seedling stage. In addition to the individual GWAS peaks, a large number of significant SNPs were also identified and scattered across the cowpea genome, which could support earlier reports suggesting that drought tolerance is a complex mechanism (Golldack et al. 2014).

In this study, a total of 1047, 591, and 25 SNPs were identified to be associated with tolerance to trifoliate leaf chlorosis, tolerance to unifoliate leaf chlorosis, and plant greenness score under drought stress, respectively. Interestingly, no overlapping SNPs were found between the 3 traits. No common SNPs were identified between tolerance to trifoliate leaf chlorosis and tolerance to unifoliate leaf chlorosis. This could explain previous studies stating that there are two types of drought tolerance in cowpea and the mechanisms underlying these two types were independent (Singh et al. 1999). The type I drought-tolerant cowpea genotypes can delay senescence in both trifoliate and unifoliate leaves. However, the type II ones kept the trifoliate leaf green, but they were more susceptible to unifoliate leaf chlorosis. The strong GWAS peak on chromosome 5, which was associated with tolerance to trifoliate leaf chlorosis, was included in a significant drought-tolerant QTL region reported by Muchero et al. (2009). A QTL mapping study for drought tolerance in cowpea has been conducted by Muchero et al. (2009). The population was derived from cross between IT93K503-1 (drought tolerant) and CB46 (drought susceptible). Visual rating on leaf senescence under drought conditions was conducted. The QTL identified by Muchero et al. (2009) was within a 15-cM distance. Therefore, our results refined this QTL region.

Candidate genes involved in hormone biosynthesis pathways and membrane lipid degradation were also identified in this study. These genes were previously described as being directly involved in drought stress in cowpea. Iuchi et al. (1996) isolated 24 cDNA clones

pertaining to dehydration-induced genes from a highly drought-tolerant cowpea cultivar (IT84S-2246-4). Of the 24 cDNAs, nine were induced by water-deficit conditions. Five of them were characterized and known as cowpea clones responsive to dehydration (CRPD) genes (*CPRD8*, *CPRD12*, *CPRD14*, *CPRD22*, and *CPRD46*). Another CPRD gene (*CPRD86*) was studied later (Satoshi Iuchi et al. 2000). Two additional drought-tolerant genes, *VuNCED1* and *VuABA1*, were described and isolated from the aforementioned cultivar (Satoshi Iuchi et al. 2000). Investigations showed that *VuNCED1* encodes a 9-cis-epoxycarotenoid dioxygenase catalyzing a key step in the abscisic acid (ABA) biosynthesis (Schwartz et al. 1997; Satoshi Iuchi et al. 2000). *VuABA1* was demonstrated to encode a zeaxanthin epoxidase involved in another significant pathway for abscisic acid (ABA) biosynthesis (Satoshi Iuchi et al. 2000). Maarouf et al. (1999) described cowpea *VuPLD1* gene encoding a phospholipase D, which is stimulated by drought stress. Indeed, it is widely recognized that lipid metabolism is triggered upon degradation of membrane lipids under drought conditions (Paula and Thi 1993). Results revealed a highly expressed *VuPLD1* in a cowpea drought-susceptible cultivar to which drought stress was imposed; whereas its expression was unchanged in a drought-tolerant one (Maarouf et al. 1999).

A study by Marcel et al. (2000) described two cowpea cDNAs, *VuPAP-α* and *VuPAP- β*, encoding putative phosphatidate phosphatases (PAPs). Previous research showed that PAPs were significantly involved in the pathway related to membrane lipid degradation for plants under abiotic stresses or senescence (Sahsah et al. 1998). Study by Marcel et al. (2000) demonstrated that expression of *VuPAP-α* was stimulated for cowpea genotypes submitted to rehydration under a certain period of drought. However, expression of *VuPAP- β* was increased in air-desiccated leaves. Matos et al. (2001) isolated and characterized a cowpea *VuPAT1* (putative patatin-like) gene encoding for galactolipid acyl hydrolase whose expression was increased in a

cowpea cultivar susceptible to drought. Galactolipids, components of chloroplast membrane, were hydrolyzed in cowpea genotypes under drought stress (Carvalho et al. 2017). Diop et al. (2004) reported a cowpea *VuC1* gene encoding for cowpea cystatin, which is a leaf protease inhibitor regulating protein degradation and prevents leaf cells from oxidative damage under drought conditions (Cruz de Carvalho 2008). Study conducted by Contour-ansel et al. (2006) reported two cowpea genes, *dtGR* and *cGR*, encoding for dual-targeted glutathione reductase and cytosolic glutathione reductase, respectively. These are key enzymes involved in the detoxification of antioxidant metabolites under progressive drought conditions. Further cowpea antioxidant genes related to drought stress were isolated and characterized by D'Arcy-Lameta et al. (2005). These genes encode for cytolsolic ascorbate peroxidase (*VucAPX*), peroxisomal ascorbate peroxidase (*VupAPX*), stromatic ascorbate peroxidase (*VusAPX*), and thylakoidal ascorbate peroxidase (*VutAPX*). These enzymes are involved in detoxifying antioxidant species under drought stress in cowpea, which were similar to that of reported by Contour-ansel et al. (2006). Two additional abiotic-stress cowpea related genes, *GST* (glutathione-S-transferase) and *PR-1* (pathogenesis-related-protein-1), were described by Gazendam and Oelofse (2009). Research conducted by Silva et al. (2012) pointed out the effects of drought and heat on cowpea nodules. Results revealed that the genes *VuNSR4*, *VuNSR10*, *VuNSR44*, *VuNSR47*, and *VuNSR49*, encoding for digalactosildiacylglycerol synthase 1, kinase protein calcium dependent, CPRD12, CPRD8, and CPRD65, respectively, played a significant role in protecting cowpea nodules from drought and heat stresses. In addition to being regulated by proteins translated from genes, cowpea drought tolerance is also controlled by the effects of microRNAs (miRNAs). Barrera-Figueroa and Gao (2011) and Shui et al. (2013) reported 44 miRNAs which were associated to drought stress in cowpea. The number of genes that are involved in drought stress

tolerance in cowpea suggested the complexity of this trait (Carvalho et al. 2017). However, Verbree et al (2015) reported that a major gene could control drought tolerance in cowpea. In fact, crosses between TX2028-1-3-1 (drought-tolerant) and TVu-7778 (drought-susceptible), and TX2028-1-3-1 (drought-tolerant) and CB 46 (drought-susceptible) showed a segregation ratio 3:1 for unifoliate stay-green trait in F2 progenies. Therefore, further investigations are required to unravel more possible mechanisms of drought tolerance at the genetic level in cowpea.

This study has provided molecular markers associated with drought tolerance at seedling stage in cowpea. However, the significant SNP markers were not validated yet. Therefore, an additional study should be conducted in order to validate the SNP markers so that they can be reliably used for Marker-Assisted Selection (MAS). In addition, the results from this study contribute towards understanding the genetic architecture of drought tolerance in cowpea. The functional annotations of the annotated genes found within of in the vicinity of the location of the significant SNPs provided substantial hints on potential drought tolerance mechanism. These candidate genes will be validated in further projects. Despite the large amount of data generated in this study, one major limitation was related to the fact that the screening was conducted at seedling stage and under greenhouse conditions. To date, we do not have enough information whether these results can be replicated at reproductive stage and under field conditions. Further investigations are required to address this constraint.

Conclusions

Whole genome resequencing provided a total of 14,465,516 SNPs. GWAS was conducted using a total of 5,884,299 filtered SNPs. A total of 1047, 591, and 25 SNPs were found to be associated with tolerance to trifoliate leaf chlorosis, tolerance to unifoliate leaf

chlorosis, and plant greenness score under drought stress, respectively. A strong candidate locus was mapped on a 210-kb of chromosome 5 and associated with tolerance to tolerance to trifoliate leaf chlorosis. This region harbored hormone-induced genes. A strong GWAS peak was also identified for tolerance to unifoliate leaf chlorosis. A total of 12 overlapping SNPs were found for tolerance to trifoliate leaf chlorosis and plant greenness score under drought score in cowpea. These results could be used in cowpea breeding through Marker-Assisted Selection (MAS). To the best of our knowledge, this is the first report on cowpea GWAS for drought tolerance using a whole genome resequencing data.

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Tables

Table 8.1. List of significant SNPs close to candidate genes and associated with tolerance to trifoliate leaf chlorosis and unifoliate leaf chlorosis, and plant greenness under drought tolerance in cowpea.

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
Trifoliate leaf chlorosis	Vu01_10616309	10616309	1	6.54E-09	8.18	*Vign01g055000.1*	Mn plant transporter
	Vu01_25892353	25892353	1	3.74E-09	8.43	*Vign01g09400.1*	NADH-cytochrome b5 reductase
	Vu02_24537084	24537084	2	1.12E-08	7.95	*Vign02090500.1*	Retrotransposon
	Vu03_10803196	10803196	3	2.98E-08	7.53	*Vign03g116500.1*	PB1 domain containing protein
	Vu03_12666912	12666912	3	2.04E-08	7.69	*Vigun03g130400.1*	Protein phosphatase 2C
	Vu03_12897768	12897768	3	1.55E-08	7.81	*Vign03g132300.1*	Pumilio-family RNA binding repeat
	Vu03_13212508	13212508	3	2.99E-08	7.52	*Vign03g135100.1*	ATP-dependent DNA helicase 2 subunit 2
	Vu03_13274473	13274473	3	1.89E-08	7.72	*Vign03g135400.1*	RNA Methylase-related
	Vu03_13295491	13295491	3	5.40E-10	9.27	*Vign03g135700.1*	Vacuolar iron transporter
	Vu03_13297714	13297714	3	5.68E-12	11.25	*Vign03g135800.1*	Vacuolar iron transporter
	Vu03_13302250	13302250	3	1.84E-11	10.74	*Vign03g135900.1*	Vacuolar iron transporter
	Vu03_13352276	13352276	3	1.16E-09	8.93	*Vigun03g136300.1*	EamA-like transporter family/Glucose-6-phosphate/phosphate and phosphoenolpyruvate/phosphate antiporter
	Vu03_13361294	13361294	3	5.04E-10	9.30	*Vigun03g136400.1*	EamA-like transporter family/Glucose-6-phosphate/phosphate and phosphoenolpyruvate/phosphate antiporter

Table 8.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu03_13376628	13376628	3	1.96E-10	9.71	*Vigun03g136500.1*	EamA-like transporter family/Glucose-6-phosphate/phosphate and phosphoenolpyruvate/phosphate antiporter
	Vu03_13382599	13382599	3	2.55E-10	9.59	*Vigun03g136600.1*	EamA-like transporter family/auxin-induced protein 5NG4
	Vu03_13509429	13509429	3	5.66E-11	10.25	*Vigun03g137500.1*	ABA responsive element binding factor
	Vu03_14318570	14318570	3	9.08E-10	9.04	*Vigun03g142100.1*	Tetrahydroberberine oxidase
	Vu03_14743049	14743049	3	5.21E-09	8.28	*Vigun03g144800.1*	WRKY TRANSCRIPTION FACTOR 28-RELATED
Trifoliate leaf chlorosis	Vu03_14763808	14763808	3	2.88E-08	7.54	*Vigun03g144900.1*	O-acetyltransferase family protein
	Vu03_14806565	14806565	3	2.32E-09	8.63	*Vigun03g145200.1*	Starch synthase
	Vu03_14815803	14815803	3	1.63E-09	8.79	*Vigun03g145400.1*	Chlorophyll a/b binding protein
	Vu03_15222570	15222570	3	2.69E-08	7.57	*Vigun03g148300.1*	3-oxoacyl-synthase
	Vu03_36340055	36340055	3	2.93E-09	8.53	*Vigun03g218100.1*	ABC-2 type transporter family protein
	Vu03_58980712	58980712	3	3.74E-09	8.43	*Vigun03g384600.1*	H+-transporting ATPase
	Vu04_26966450	26966450	4	4.24E-09	8.37	*Vigun04g109300.1*	CemA-like proton extrusion protein-related
	Vu04_27157237	27157237	4	6.15E-09	8.21	*Vigun04g109500.1*	Protein FLOWERING LOCUS T (FT)
	Vu04_27241963	27241963	4	4.99E-09	8.30	*Vigun04g109600.1*	TatD DNase family protein
	Vu04_27298716	27298716	4	5.99E-09	8.22	*Vigun04g109700.1*	Aquaporin-like superfamily protein

Table 8.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu04_27342140	27342140	4	2.73E-09	8.56	*Vigun04g109800.1*	Nucleoside-triphosphatase
	Vu04_27505387	27505387	4	3.11E-09	8.51	*Vigun04g110000.1*	rRNA-processing protein FCF
	Vu04_27528973	27528973	4	7.92E-09	8.10	*Vigun04g110100.1*	Ubiquinone (electron-transporting coenzyme) biosynthesis protein
	Vu04_27714135	27714135	4	1.91E-09	8.72	*Vigun04g110600.1*	No apical meristem (NAM) protein
	Vu04_27716250	27716250	4	4.48E-09	8.35	*Vigun04g110700.1*	Tryptophan/tyrosine permease family
	Vu04_27778870	27778870	4	2.16E-08	7.67	*Vigun04g110800.1*	Myb family transcription factor-related
	Vu04_27786623	27786623	4	8.27E-10	9.08	*Vigun04g110900.1*	Pyruvate kinase
	Vu04_27797389	27797389	4	4.32E-09	8.37	*Vigun04g111000.1*	Zinc finger protein
	Vu04_27830859	27830859	4	1.57E-08	7.81	*Vigun04g111100.1*	CCR4-NOT transcription factor
	Vu04_27913211	27913211	4	1.59E-08	7.80	*Vigun04g111200.1*	Glucan endo-1,3-beta-D-glucosidase
	Vu04_27913980	27913980	4	8.65E-09	8.06	*Vigun04g111300.1*	GATA zinc finger
	Vu04_41785910	41785910	4	3.13E-09	8.50	*Vigun04g193600.1*	Protein kinase superfamily
	Vu04_41800041	41800041	4	2.14E-08	7.67	*Vigun04g193700.1*	NAD dependetn epimarase/dehydratase
	Vu04_41826262	41826262	4	7.69E-09	8.11	*Vigun04g193800.1*	Translation initiation factor 3 subunit G
	Vu04_41832927	41832927	4	8.14E-09	8.09	*Vigun04g194000.1*	Universal stress protein family
	Vu05_540561	540561	5	5.09E-21	20.29	*Vigun05g006300.1*	EamA-like transporter family/auxin-induced protein 5NG4

Table 8.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu05_560665	560665	5	5.63E-15	14.25	*Vigun05g006500.1*	Neoxanthin synthase/abscisic acid biosynthesis
	Vu07_23856082	23856082	7	5.11E-10	9.29	*Vigun07g129600.1*	Protein tyrosine kinase
	Vu07_24143183	24143183	7	7.91E-09	8.10	*Vigun07g131800.1*	Protoporphyrinogen oxidase/ chloroplast precursor
	Vu08_37171764	37171764	8	2.43E-08	7.61	*Vigun08g208700.1*	PPR repeat
Unifoliate leaf chlorosis	Vu01_29544191	29544191	1	3.51E-15	14.5	*Vigun01g119000.1*	Lysophosphatidic acid acyltransferase
	Vu08_4931701	4931701	8	4.83E-09	8.32	*Vigun08g046200.1*	Leucine-rich repeat
	Vu08_4945627	4945627	8	2.46E-10	9.61	*Vigun08g046400.1*	Leucine-rich repeat
	Vu08_4952526	4952526	8	4.61E-10	9.34	*Vigun08g046500.1*	Leucine-rich repeat
	Vu08_26752606	26752606	8	2.58E-11	10.6	*Vigun08g107800.1*	Ubiquitin carboxyl-terminal hydrolase
	Vu08_26868733	26868733	8	1.49E-09	8.83	*Vigun08g107900.1*	AT-hook DNA-binding family protein
	Vu08_26877485	26877485	8	8.31E-11	10.1	*Vigun08g108100.1*	Carbonic anhydrase
	Vu08_26901689	26901689	8	9.11E-11	10	*Vigun08g108400.1*	DnaJ homolog subfamily
	Vu10_35348050	35348050	10	2.58E-09	8.59	*Vigun10g137100.1*	Leucine-rich repeat
Plant greenness	Vu01_10616486	10616486	1	7.42E-09	8.13	*Vigun01g054900.1*	DCN1-like protein
	Vu03_13509429	13509429	3	2.63E-08	7.58	*Vigun03g137600.1*	P-loop containing nucleoside triphosphate hydrolase superfamily protein
	Vu03_14725438	14725438	3	1.65E-08	7.78	*Vigun03g144800.1*	WRKY transcription factor

Table 8.2. List of candidate genes having functional annotations that are relevant to plant abiotic stress. Protein homologs from each translated transcript was search in the cowpea (Vun), soybean (Gma), common bean (Pvu), and *Medicago truncatula* (Mtr) genomes. The number of protein homologs with similarity > 90% to that one from cowpea is reported.

Traits	Gene_ID	Functional_annotations	Vun	Gma	Pvu	Mtr
Trifoliate leaf chlorosis	*Vigun05g006300.1*	EamA-like transporter family/auxin-induced protein 5NG4	1	3	4	1
	Vigun05g006500.1	Neoxanthin synthase/abscisic acid biosynthesis	1	3	3	3
	Vigun03g136600.1	EamA-like transporter family/auxin-induced protein 5NG4	2	5	3	3
	Vigun03g137500.1	ABA responsive element binding factor	0	1	1	0
	Vigun03g135700.1	Vacuolar iron transporter	0	1	1	0
	Vigun03g135800.1	Vacuolar iron transporter	4	9	4	8
	Vigun03g135900.1	Vacuolar iron transporter	6	7	7	4
	Vigun04g110600.1	No apical meristem (NAM) protein	1	4	2	0
	Vigun04g110800.1	Myb family transcription factor-related	0	2	1	1
Uniifoliate leaf chlorosis	*Vigun01g119000.1*	Lysophosphatidic acid acyltransferase	1	4	2	2
	Vigun08g046200.1	Leucine-rich repeat	2	2	6	0
	Vigun08g046400.1	Leucine-rich repeat	0	1	4	0
	Vigun08g046500.1	Leucine-rich repeat	1	2	4	0
	Vigun08g107800.1	Ubiquitin carboxyl-terminal hydrolase	0	0	1	0
	Vigun08g108100.1	Carbonic anhydrase/Carbonate dehydratase/	0	1	1	1
	Vigun08g108400.1	DnaJ homolog subfamily	0	2	1	1
	Vigun10g137100.1	Leucine-rich repeat	0	0	0	0
Plant greenness	*Vigun01g054900.1*	DCN1-like protein	0	2	1	1
	Vigun03g137600.1	P-loop containing nucleoside triphosphate hydrolase superfamily protein	0	1	0	0
	Vigun03g144800.1	WRKY transcription factor	0	2	1	0
	Vigun05g006300.1	EamA-like transporter family/auxin-induced protein 5NG4	1	3	3	1

Figures

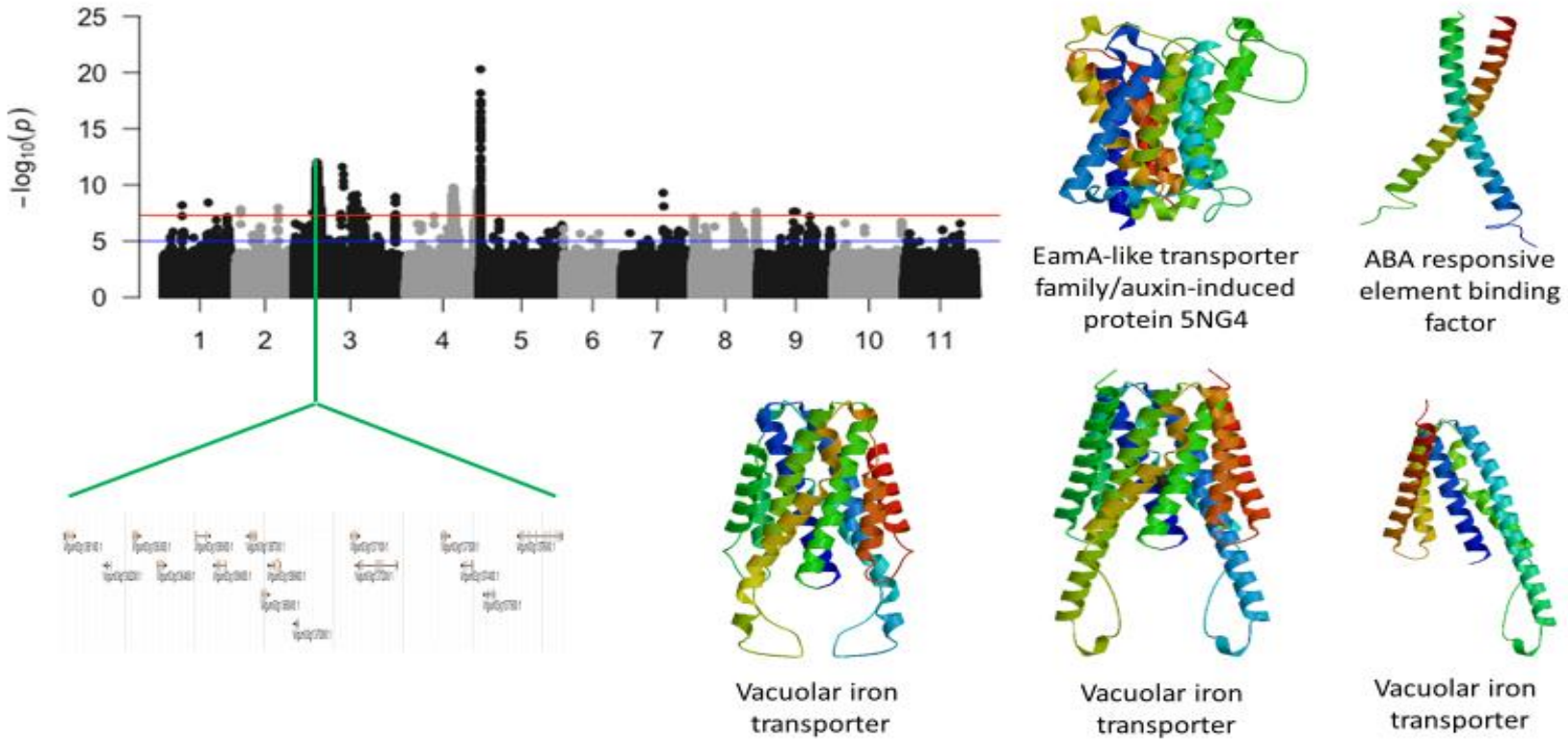


Fig. 8.1. Manhattan plot for chlorosis trifoliate leaf tolerance in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 2. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

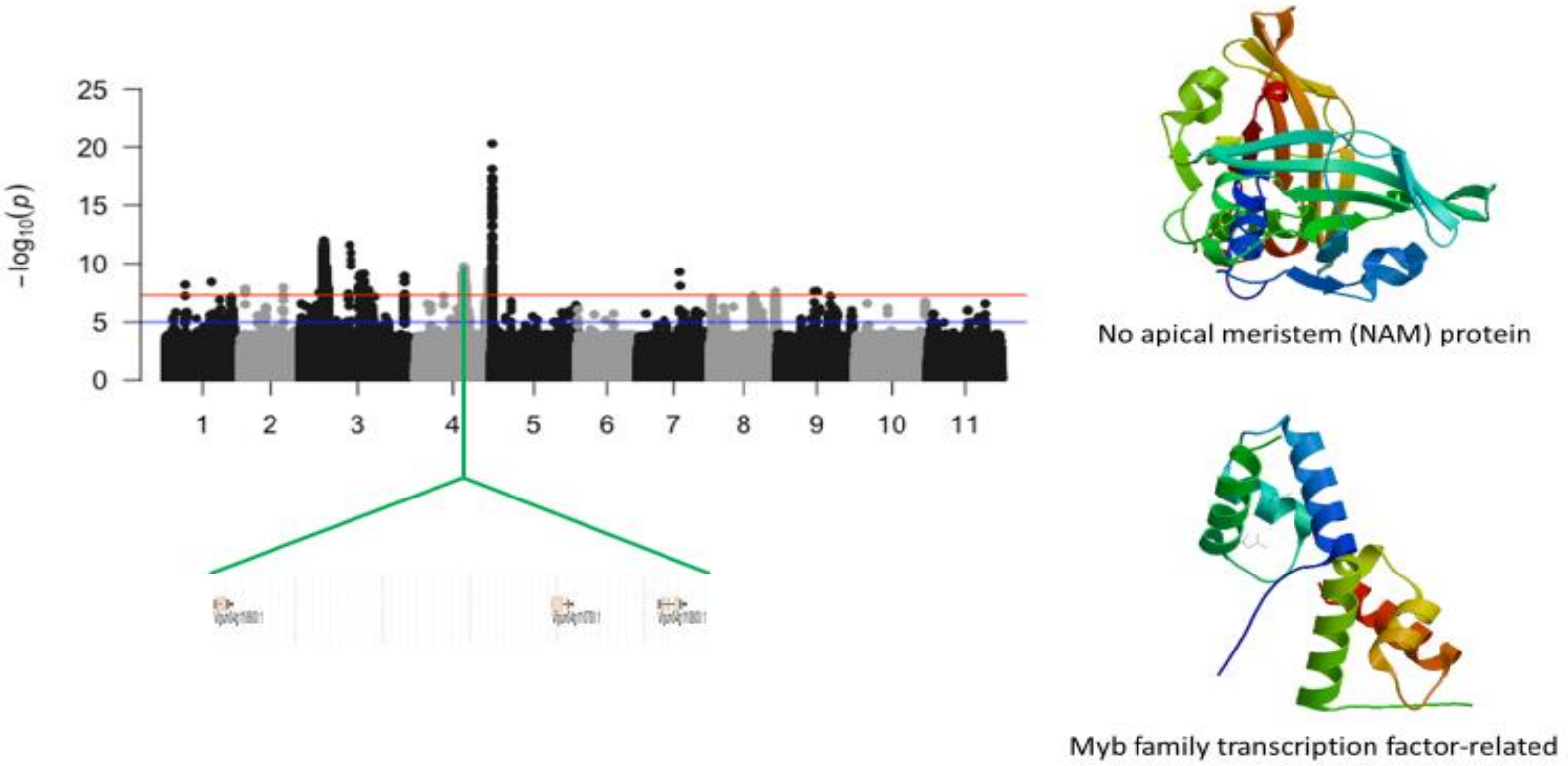


Fig. 8.2. Manhattan plot for chlorosis trifoliate leaf tolerance in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or $-\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 4. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

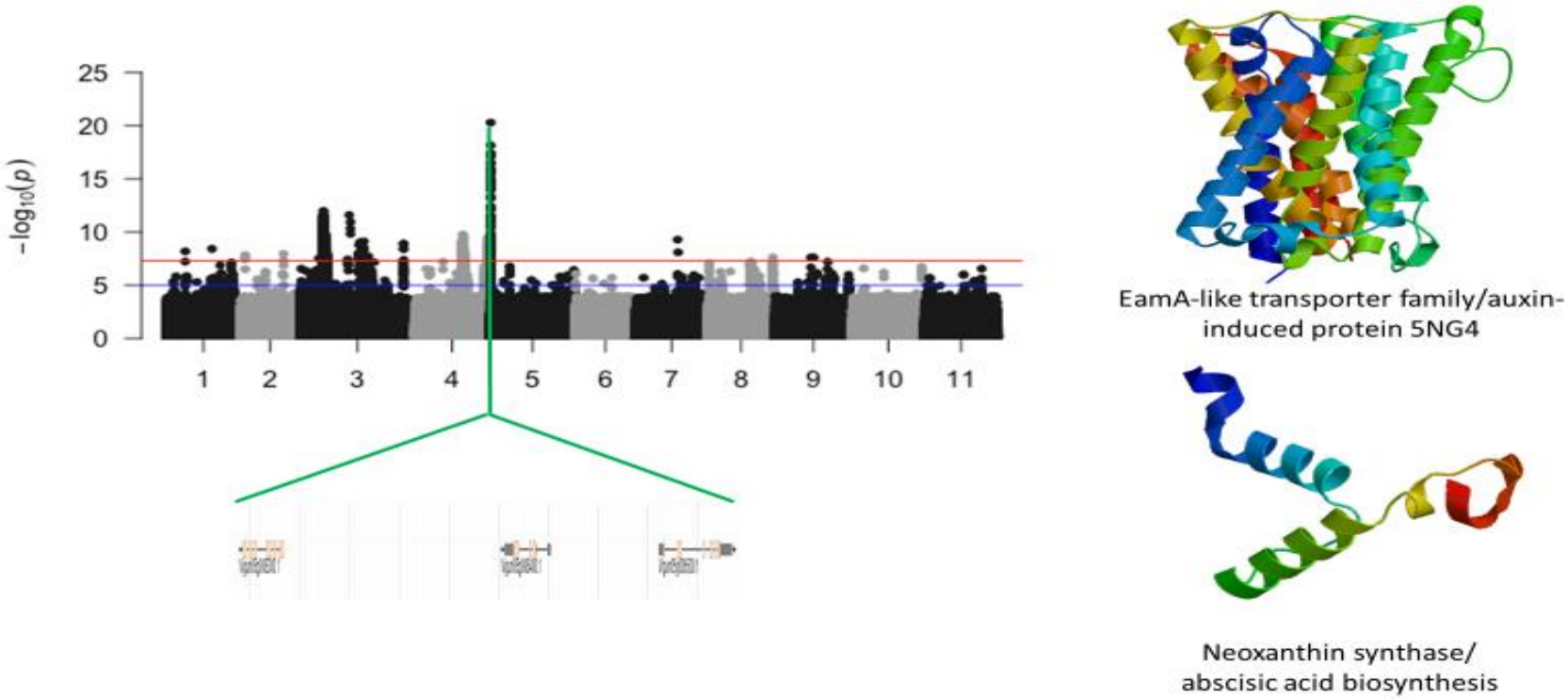


Fig. 8.3. Manhattan plot for chlorosis trifoliate leaf tolerance in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or $-\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 5. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

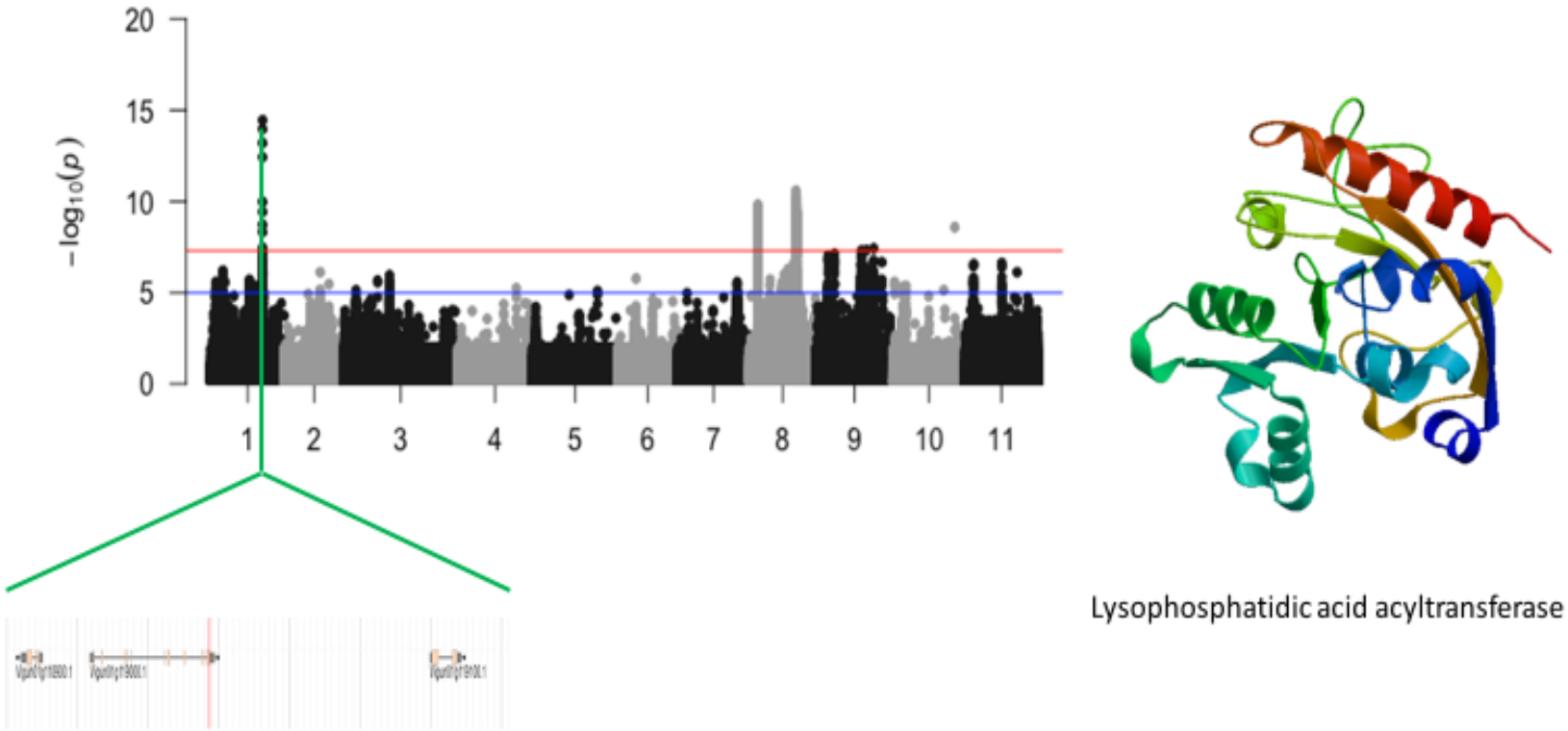


Fig. 8.4. Manhattan plot for tolerance to unifoliate leaf chlorosis under drought in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 1. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

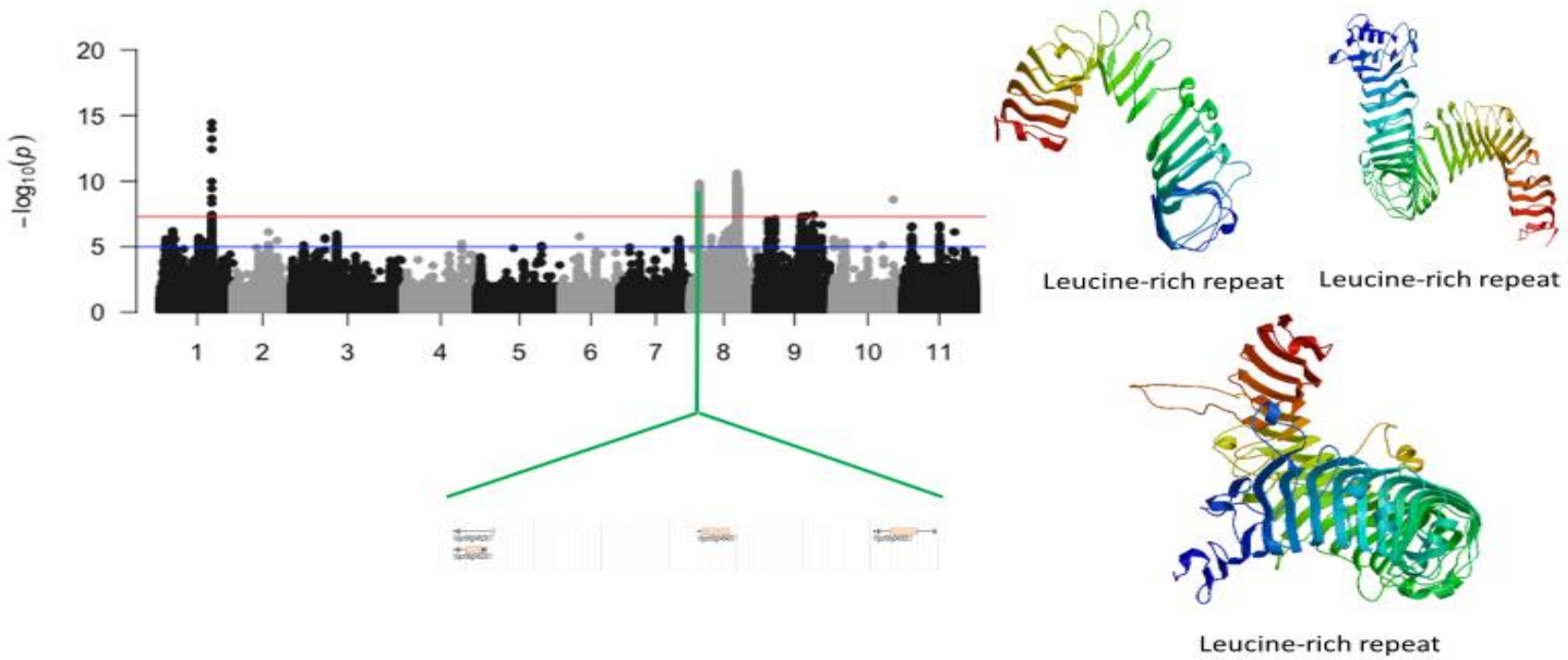


Fig. 8.5. Manhattan plot for tolerance to unifoliate leaf chlorosis under drought in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 8. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

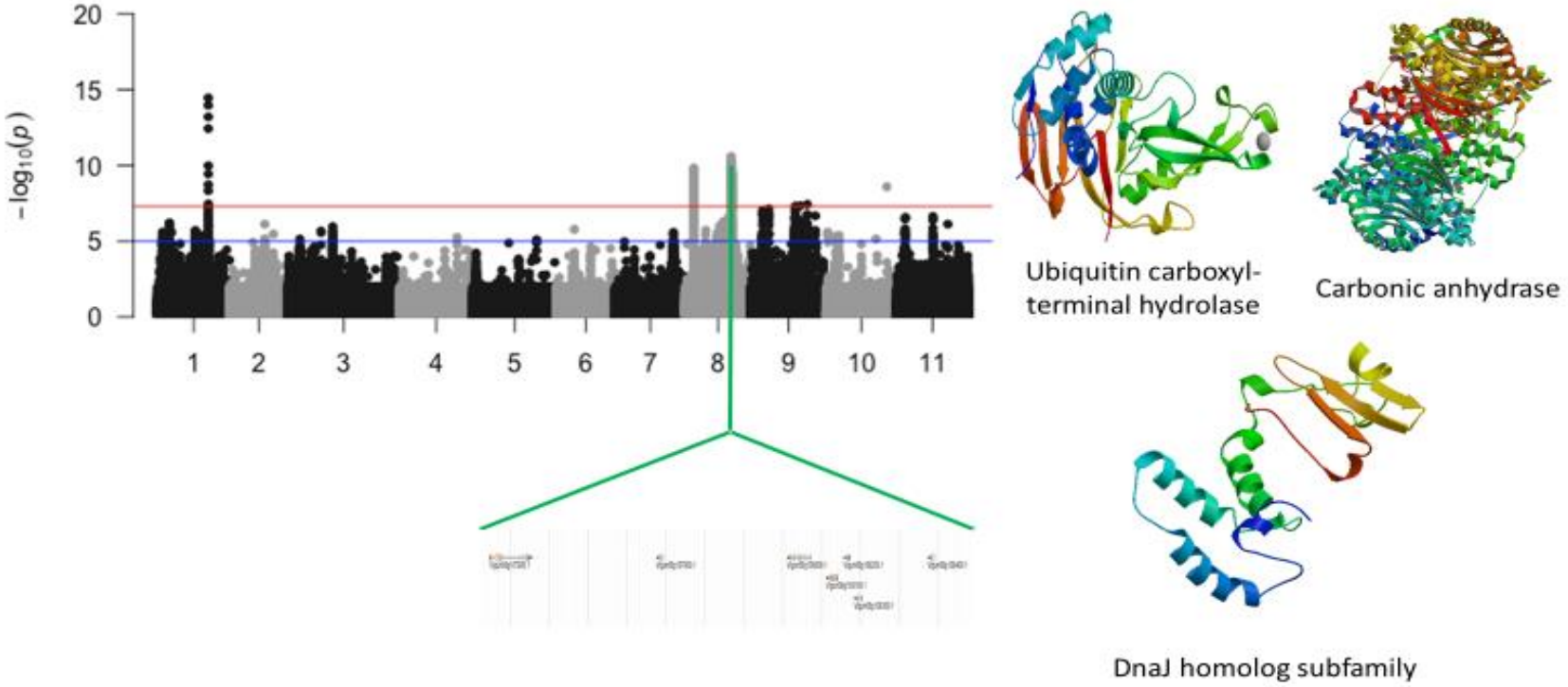


Fig. 8.6. Manhattan plot for tolerance to unifoliate leaf chlorosis under drought in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 8. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

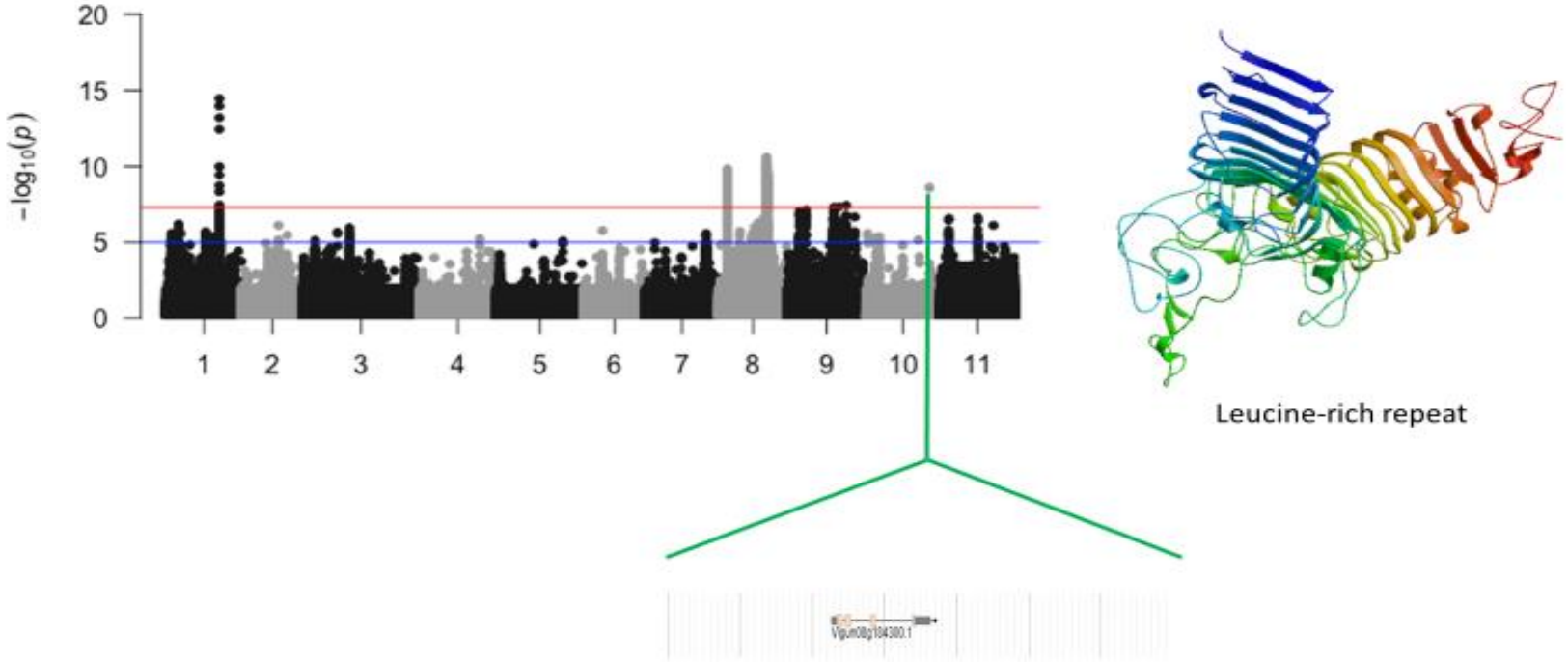


Fig. 8.7. Manhattan plot for tolerance to unifoliate leaf chlorosis under drought in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 10. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

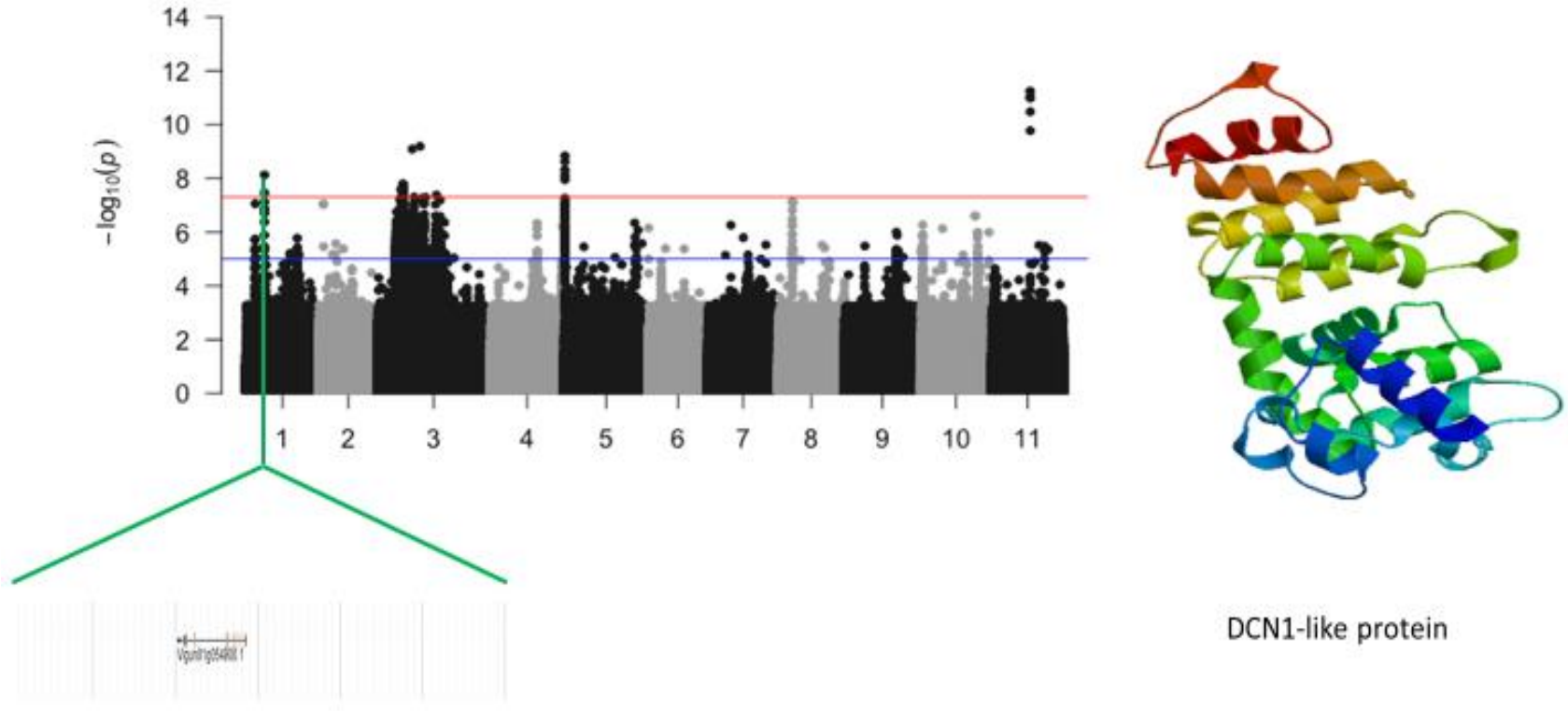


Fig. 8.8. Manhattan plot for plant greenness score under drought stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 1. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

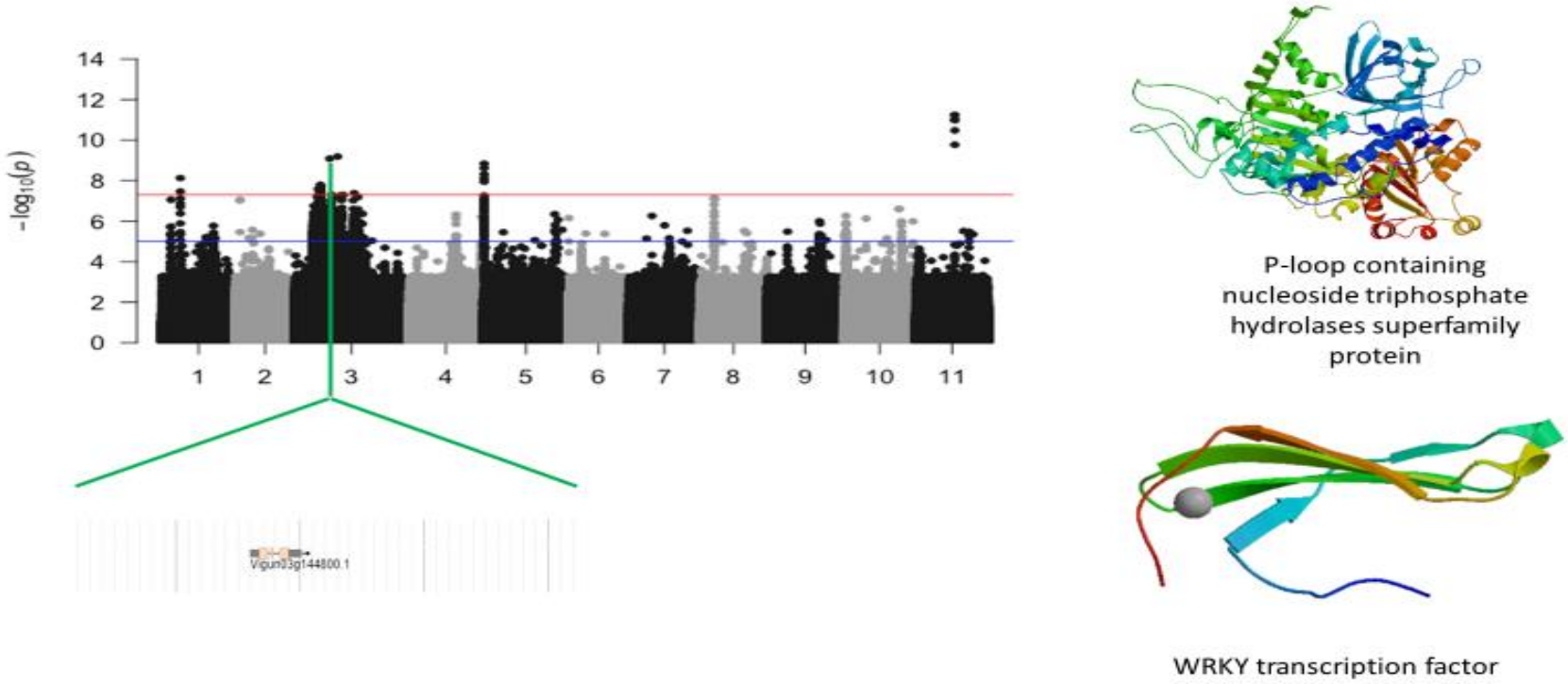
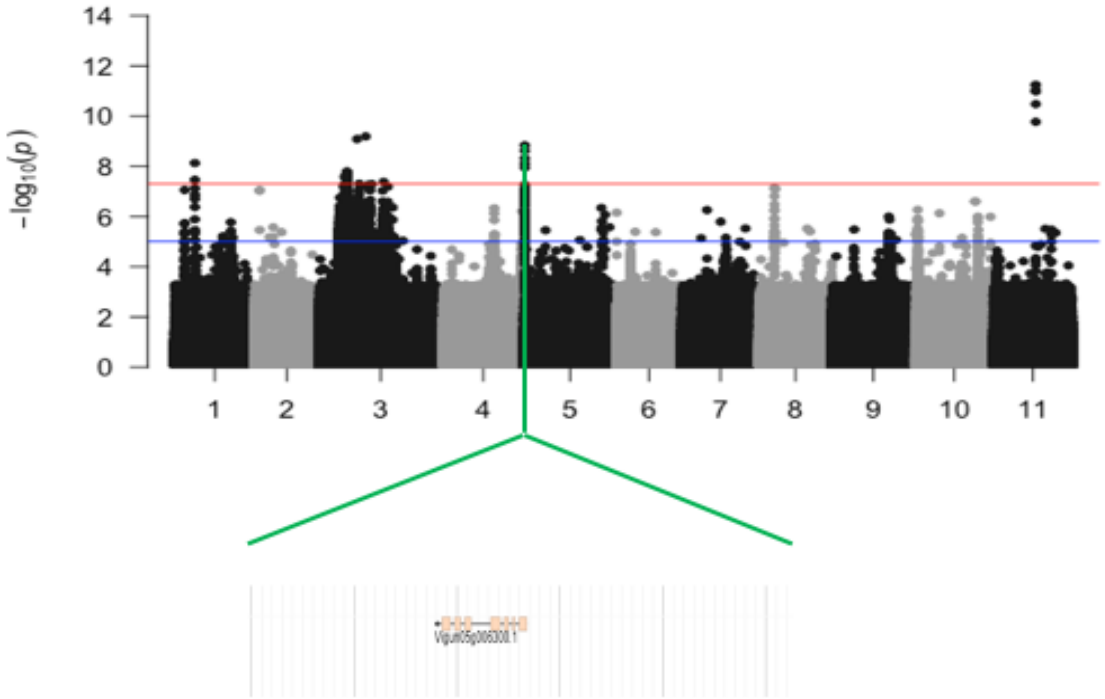
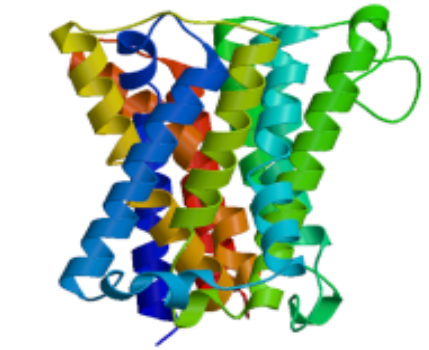


Fig. 8.9. Manhattan plot for plant greenness score under drought stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 3. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.





EamA-like transporter family/auxin-induced protein 5NG4

Fig. 8.10. Manhattan plot for plant greenness score under drought stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -log10 of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 5. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

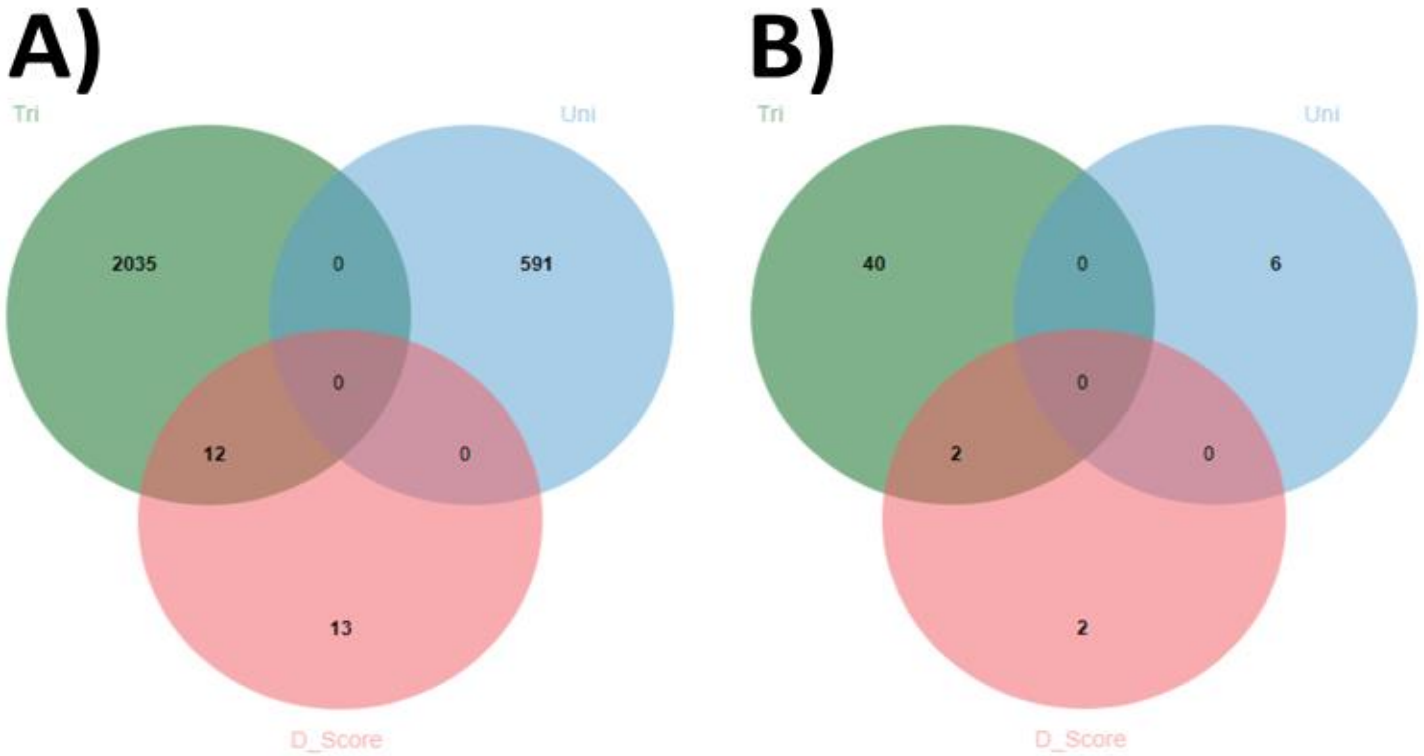


Fig. 8.11. A) Venn diagram showing the number of overlapping significant SNPs associated with tolerance to trifoliate leaf chlorosis (Tri), unifoliate leaf chlorosis (Uni), and plant greenness score (D_Score) under drought stress in cowpea. B) Venn diagram showing the number of unique functional annotations for candidate genes associated with tolerance to trifoliate leaf chlorosis (Tri), unifoliate leaf chlorosis (Uni), and plant greenness score (D_Score) under drought stress in cowpea. Venn diagrams were established using http://jvenn.toulouse.inra.fr/app/example.html.

Appendices

Table S8.1. List of significant SNPs associated with tolerance to trifoliate leaf chlorosis in cowpea.

Table S8.2. List of significant SNPs associated with tolerance to unifoliate leaf chlorosis under drought stress in cowpea.

Table S8.3. List of significant SNPs associated with overall plant greenness under drought stress in cowpea

Fig. S8.1. A) Origin of cowpea genotypes B) Combined genetic diversity and population structure analysis.

Chapter 9. Genome-Wide Association Study for Salt Tolerance in Cowpea (*Vigna unguiculata* (L.) Walp.) at Seedling Stage Using a Whole Genome Resequencing Approach

Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is a diploid nutrient-dense legume species. It provides affordable source of protein to human. Cowpea cultivation is prevalent in Africa, Asia, the western and southern U.S., and Central and South America. However, earlier reports have shown that salinity has been a growing threat to cowpea cultivation. The objectives of this study were to conduct a genome-wide association study (GWAS) to identify SNP markers, and to investigate candidate genes for salt tolerance in cowpea. A total of 331 cowpea genotypes were evaluated for salt tolerance. The cowpea panel was genotyped using a whole genome resequencing approach. A total of 14,465,516 SNPs were obtained and 5,884,299 SNPs were used after SNP filtering. GWAS was conducted on a total of 296 cowpea genotypes that were quality-checked. BLINK was used for conducting GWAS. From this study, a strong GWAS peak was observed on an 890-bk region of chromosome 2, where 56 significant SNPs were strongly associated with leaf SPAD chlorophyll under salt stress in cowpea. This genomic region harbored a significant cluster of NAD dependent epimerase/dehydratase genes such as *Vigun02g128900.1*, *Vigun02g129000.1*, *Vigun02g129100.1*, *Vigun02g129200.1*, and *Vigun02g129500.1*. The second and third GWAS peaks were observed to be strongly associated with relative tolerance index for chlorophyll and located on chromosomes 1 and 2. The peak on chromosome 1, consisted of a cluster of 10 significant SNPs, was located on a 5-kb region and was located in the vicinity of *Vigun01g086000.1*, encoding for a GATA transcription factor. The GWAS peak on chromosome 2, a cluster of 53 significant SNPs, was mapped on a 68-bk region

of chromosome 2. This region overlapped with the candidate locus for leaf SPAD chlorophyll under salt stress. The highest GWAS peak was identified on chromosome 3, which was associated with leaf score injury. This peak was defined by a 1-Mb region harboring a total of more than 1,400 SNPs. The GWAS peak corresponded to the SNP Vu03_14737814, which was within the structure of a potassium channel gene (*Vigun03g144700.1*). In addition, 19 SNPs overlapped between the 3 traits. The results from this study could be used in a marker-assisted selection (MAS) program for salt tolerance in cowpea. To the best of our knowledge, this is the first study in cowpea using a whole genome resequencing data.

Introduction

Abiotic stress has been limiting crop production globally. Crop breeders and geneticists have suggested a great deal of alternative to limit the negative impacts of abiotic stress-related issues on crops. Providing abiotic stress-tolerant cultivars will significantly help farmers better mitigate the problems caused by abiotic stress-damaging crops. One of the major abiotic stress constraining crop production is salt stress (Allakhverdiev et al., 2000). Reports have shown that salt stress is more detrimental to crops in semi-arid and arid areas (Zhang et al. 2012).

Cowpea (*Vigna unguiculata* (L.) Walp.), 2n=2x=22, is an important crop in the semi-arid regions of the sub-Sahara Africa and in some areas in the U.S. (Singh et al. 2003; Wilson et al. 2006). In addition to providing protein for human consumption, cowpea leaves are used for feeding the livestock (Itatat et al. 2013; Olufajo 2012). In semi-arid areas, salt compounds have been kept accumulating within soils since the rainfall has been not enough to contribute toward leaching them from crop lands (Zhang et al. 2012). Therefore, cowpea cultivation is under constant threat imposed by soil salinity.

Breeding for salt tolerance in cowpea will be more efficient if the salt tolerance mechanism can be unraveled at the genetic level. In this view, we conducted an association mapping for salt tolerance in cowpea. The study involved a total of 116 and 155 cowpea genotypes which were phenotyed for salt tolerance at germination and seedling stage, respectively (Ravelombola et al. 2017). However, due to the relatively small size of the association mapping and the relatively low number of markers being used, salt-tolerant SNP markers with a low LOD and R-square value were found. Therefore, the objective of this study was to conduct a genome-wide association study for salt tolerance in cowpea through a whole genome-resequencing approach and involving a significant association mapping panel, to identify SNP markers associated with salt tolerance, and to identify candidate genes for salt tolerance in cowpea.

Materials and Methods

Plant materials and phenotyping

A total of 331 cowpea genotypes were evaluated for salt tolerance. Of which, 36 were breeding lines from the University of Arkansas, Fayetteville, 8 were obtained from the University of California, Riverside and were the parents of the first cowpea multiparent advanced generation intercross (MAGIC) population (Huynh et al. 2018), 287 were Plant Introductions (PIs) from the U.S. Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) cowpea accessions and provided by the USDA Plant Genetic Resources Conservation Unit at Griffin, GA. The cowpea accessions were from more than 32 countries. Seeds were increased during the summer of 2018 at the Arkansas Agricultural Experiment Station of the University of Arkansas, Fayetteville. One plant from each plant were

harvested. Seeds from each plant were carefully sorted and cleaned prior to being used for the experiments.

Salt tolerance evaluation was conducted in the greenhouse of R. Rosen Alternative Pest Control of the University of Arkansas, Fayetteville. The average day/light temperatures in the greenhouse were 26 °C/21 °C and the average daylight length was 14 hours. Salt tolerance evaluation was done as previously described (Ravelombola et al. 2019). A total of 8 cowpea seeds were sown in pots filled with 100 g Sunshine Natural & Organic (Agawam, MA). Holes were established at the bottom of each pot to prevent water logging during irrigation. Paper towel was placed at the bottom of each pot to limit soil leaking during irrigation. At plant emergence, each pot was thinned to 4 plants per pot. Fertilizer was weekly applied by irrigating each pot with a solution of 50 mL of Miracle-Gro fertilizers (Scotts Miracle-Gro, Detroit, MI). The experiments were conducted using a randomized complete block design (RCBD) with 2 replications within each block. A total of 4 blocks were used. Each pot corresponded to one replication. For each genotype, one pot was subjected to salt treatment, whereas another one was irrigated with deionized water and used as a control. A total of 12 pots were established on a rectangular plastic tray to facilitate irrigation. Salt treatment (NaCl) was initiated when the firs trifoliate leaf began to expand (V1 stage) (Fehr et al. 1971). Salt treatment was achieved by irrigation a solution of 200 mM NaCl to each rectangular plastic tray (Abeer et al., 2015; Ashebir et al., 2013; Paul et al., 2011; Ravelombola et al. 2017). Two-third of pot height was fully soaked with either deionized water or salt solution during irrigation (Ravelombola et al. 2019). The experiment was validated using a salt-tolerant genotype (‘09-529’) and a salt-susceptible genotype (PI255774) (Dong et al. 2019; Ravelombola et al. 2019). Data measurements were previously described.

Genotyping

DNA extraction, library preparation, and whole-genome resequencing

Young cowpea leaves were harvested from one plant and all seeds used during the experiments were derived from that one plant. Genomic DNA was extracted from freeze-dried young cowpea leaves using the CTAB (hexadecyltrimethyl ammonium bromide) protocol (Kisha et al., 1997). Leaves were ground using a Mixer Mill MM 400® (Haan, Germany). DNA buffer was added to each sample. The mixture DNA buffer-sample was centrifuges at 13,000 rpm for 10 minutes. Proteins were denatured by adding a solution of 1 ml pf chloroform-isoamyl alcohol (24:1) to each sample. The addition of 1 ml pf isopropanol helped DNA precipitate. In order to optimize DNA precipitation, samples were stored at -20°C overnight. DNA pellets were washed using 70% and 90% ethanol. Washed DNA pellets were air dried. RNA was removed by adding 3 µl of RNAse to each sample. DNA was kept in a solution of 200 µl of 0.1X TE. DNA was quantity using a NanoDrop 200c spectrophotometer (Thermo SCIENTIFIC, Wilmington, DE) and quality-checked on a 1%-agarose gel with ethidium bromide stain.

DNA sequencing was conducted by Novogene (http://en.novogene.com/). DNA was cleaved in 350-pb fragments using Covaris S2® (Covaris, Inc., Woburn, MA). DNA library involved the sheared DNA fragments NEBNext DNA Library Prep Reagent Set for Illumina (BioLabs, Inc., Ipswich, MA). DNA fragments were end-repaired. Poly-A tails were added to each fragment. In situ PCR amplification was conducted as described by van Dijk et al. (2014). Sequecing was done using Illumina HiSeq X Ten Series (http://www.illumina.com/systems/hiseq-x-sequencing-system/system.html) with an average of 10X coverage.

SNP calling, mapping, and filtering

Reads were aligned to the cowpea reference genome (Lonardi et al. 2019) using SOAPaligner/soap2 (http://soap.genomics.org.cn/). SNP calling was conducted using SOAPsnp v 1.05 (Li et al. 2009). Accessions with more than 20% missing data were removed. Triallelic SNPs and those with more 20% missing data were also removed. SNPs with a heterozygosity greater than 20% were removed as well. The minor allele frequency (MAF) threshold was 5%. GWAS was conducted using filtered SNPs.

Genome-wide association study (GWAS) and genomic selection

GWAS was performed using Bayesian Information and Linkage Disequilibrium Iteratively Nested Keyway (BLINK) model (Huang et al. 2019). BLINK has been demonstrated to be statistically more powerful than the previously developed models (Huang et al. 2019). SNP was significant when above the FDR-adjusted threshold and computed in R ($P < 3\ 10^{-8}$). BLINK model was built upon the Fixed and Random Model Circulating Probability Unification (FarmCPU) model. In FarmCPU, markers were assumed to be evenly distributed across the genome, which was not necessarily true. BLINK used the LD information to relax this assumption. In addition, FarmCPU could be computationally intensive due the random model part of its algorithm. The random model was replaced by a fixed model in BLINK. The two fixed effect models in BLINK were described below.

$$\text{FEM (1): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ik}b_k + M_{ij}d_j + e_i$$

$$\text{FEM (2): } y_i = M_{i1}b_1 + M_{i2}b_2 + \ldots + M_{ij}b_j + e_i$$

with y_i being the vector phenotype, M_{i1},$M_{i2}b_2$, …, M_{ik} the genotypes of k pseudo QTNs that were initially empty and with effects b_1, b_2, …, b_k, respectively, M_{ij} being the j^{th} genetic marker of the i^{th} sample, and e_i being the residual having a distribution with mean zero and a variance σ^2_e. Overlapping SNP markers between different traits were visualized using a Venn diagram and

designed using the online software program accessible at http://jvenn.toulouse.inra.fr/app/example.html.

Genomic selection was conducted using the rrBLUP model and run using the "rrBLUP" package in R. A 5-fold cross-validation with 100 replications was done. Genomic selection accuracy was assessed by computing the Pearson's correlation coefficient between the GEBVs and phenotypic data. Due to the large number of SNPs that are involved in the study, we only use SNPs with LOD>4 for GS.

Candidate gene search and synteny analysis

By taking into account the number of SNPs involved in this study, the genome size of cowpea, and the average length of a gene within the cowpea genome, we investigated the annotated genes within 10-kb genomic region flanking a SNP using Phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html). We considered annotated genes that were involved in plant physiology and/or tolerance to abiotic stress. Functional annotations of each annotated gene were obtained using Phytozome v. 13 (https://phytozome.jgi.doe.gov/pz/portal.html). Coding sequences of the annotated genes relevant to plant physiology and/or tolerance to abiotic stress were extracted. The extracted sequences were used as query to perform BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to obtain the amino acid sequence. Protein homolog search in other legumes such as soybean, common bean, and *Medicago truncatula* Gaertn was done using the amino acid sequence. Only hits with similarity greater than 90% were considered. The tertiary structure of the amino acid sequence was predicted using SWISS-MODEL (https://swissmodel.expasy.org/).

Results

Leaf SPAD chlorophyll under salt stress

Results indicated that a total of 65 SNPs were significantly associated with leaf SPAD chlorophyll under salt stress in cowpea (Figs 9.1-9.2). These SNPs were located on chromosomes 1 and 2. Chromosome 1 harbored a total of 9 significant SNPs, whereas chromosome 2 had a total of 56 significant SNPs (Table S9.1). LOD ($-\log_{10}$(p-value)) values varied from 7.53 to 10.68. The first locus that was identified to be associated with leaf SPAD chlorophyll under salt stress was defined by a cluster of significant SNPs mapped on a 3-kb region of chromosome 1. The second locus that was found to be associated with leaf SPAD chlorophyll under salt stress was defined by a group of significant SNPs mapped on an 890-kb region of chromosome 2. The significant SNPs that were found on chromosome 1 were Vu01_24245081 (LOD= 7.57), Vu01_24246312 (LOD= 8.00), Vu01_24246319 (LOD= 8.00), Vu01_24246550 (LOD= 7.76), Vu01_24246587 (LOD= .94), Vu01_24246822 (LOD= 8.27), Vu01_24246905 (LOD= 8.08), Vu01_24246981 (LOD= 8.07), and Vu01_24248242 (LOD= 7.85) (Fig. 9.1). The SNP that was closest to an annotated gene, *Vigun01g086000.1*, was Vu01_24245081. *Vigun01g086000.1* encodes for GATA transcription factor whose predicted tertiary structure was shown in Fig. 9.1.

The second locus defined by an 890-kb region of chromosome 2 harbored 9 annotated genes. Within this region, the SNPs with the highest LOD ($-\log_{10}$(p-value)) were Vu02_28054154 (LOD= 10.68), Vu02_28050297 (LOD= 10.45), Vu02_28050011 (LOD= 10.26), Vu02_28050187 (LOD= 10.22), Vu02_28105724 (LOD= 10.05), Vu02_28105725 (LOD= 10.05), Vu02_28094085 (LOD= 9.71), Vu02_28084764 (LOD= 9.63), Vu02_28068945 (LOD= 9.61), Vu02_28054571 (LOD= 9.56), Vu02_28044965 (LOD= 9.43), Vu02_28064123

(LOD= 9.37), Vu02_28069038 (LOD= 9.33), Vu02_28067838 (LOD= 9.31), Vu02_28090457 (LOD= 9.25), Vu02_28064103 (LOD= 9.01), Vu02_28090387 (LOD= 8.93), and Vu02_28052297 (LOD= 8.91). The SNPs that were in the vicinity or within the structure of candidate genes were Vu02_28035590 (LOD= 8.33), Vu02_28044965 (LOD= 9.43), Vu02_28050297 (LOD= 10.45), Vu02_28054154 (LOD= 10.68), Vu02_28064103 (LOD= 9.01), Vu02_28068945 (LOD= 9.61), Vu02_28084764 (LOD= 9.63), Vu02_28090457 (LOD= 9.25), and Vu02_28105724 (LOD= 10.05) (Table 9.1). These SNPs were within or close to *Vigun02g128700.1*, *Vigun02g128800.1*, *Vigun02g128900.1*, *Vigun02g129000.1*, *Vigun02g129100.1*, *Vigun02g129200.1*, *Vigun02g129300.1*, *Vigun02g129400.1*, and *Vigun02g129500.1*. The candidate genes consisted of cluster of NAD dependent epimerase/dehydratase whose predicted tertiary structure is shown in Fig. 9.2.

Relative tolerance index for chlorophyll content

A total of 60 SNPs were found to be significantly associated with relative tolerance index for chlorophyll content in cowpea (Table S9.2). These SNPs were identified on chromosomes 1, 2,3,4,8,10, and 11 (Figs 9.3-9.6). The number of significant SNPs was 10, 21, 1, 1, 5, 20, and 2 on chromosomes 1, 2,3,4,8,10, and 11, respectively. LOD ($-\log_{10}$(p-value)) values ranged between 7.53 and 9.09. Three significant loci were found on chromosomes 1, 2, and 10. The significant SNPs that were mapped on chromosome 1 were Vu01_24245081 (LOD= 8.56), Vu01_24246312 (LOD= 8.56), Vu01_24246319 (LOD= 8.56), Vu01_24246550 (LOD= 8.26), Vu01_24246587 (LOD= 8.60), Vu01_24246822 (LOD= 8.95), Vu01_24246905 (LOD= 8.77), Vu01_24246981 (LOD= 8.64), Vu01_24248242 (LOD= 8.26), and Vu01_24249542 (LOD= 8.00). The SNP Vu01_24246822 was found within the structure of *Vigun01g086000.1*, which encoded for GATA transcription factor (Fig. 9.3).

An additional significant locus was found to be associated with relative tolerance index for chlorophyll. This locus was mapped on a 51-kb genomic region of chromosome 2 and defined by a total of 21 significant SNPs. This genomic region was gene-dense since a total of 7 annotated genes were identified in this locus (Fig. 9.4). The SNPs with the highest LOD values were within this region were Vu02_28094085 (LOD= 9.09), Vu02_28084764 (LOD= 8.53), Vu02_28105724 (LOD= 8.53), Vu02_28105725 (LOD= 8.33), Vu02_28075602 (LOD= 8.10), Vu02_28075604 (LOD= 8.10), Vu02_28112822 (LOD= 7.98), Vu02_28112832 (LOD= 7.98), Vu02_28071778 (LOD= 7.89), Vu02_28091358 (LOD= 7.78), Vu02_28111614 (LOD= 7.77), and Vu02_28108896 (LOD= 7.69). The following candidate genes consisting of *Vigun02g129000.1*, *Vigun02g129100.1*, *Vigun02g129200.1*, *Vigun02g129300.1*, and *Vigun02g129400.1*, were found close to the SNP location (Table 9.2). These candidate genes were a cluster of NAD dependent epimerase/dehydratase (Fig. 9.4).

The significant SNPs that were identified on chromosomes 3 and 4 were Vu03_10976477 (LOD= 7.58) and Vu04_41756724 (LOD= 7.98), respectively. The SNPs were in the vicinity of *Vigun03g118000.1* and *Vigun04g193500.1*, encoding for terpene synthase and phospholipid-transporting ATPase, respectively (Fig. 9.5). The significant SNPs that were located on chromosome 8 were Vu08_4118979 (LOD= 7.54), Vu08_7137752 (LOD= 7.58), Vu08_22719007 (LOD= 8.08), Vu08_22719008 (LOD= 8.08), and Vu08_22719042 (LOD= 7.58). However, no annotated genes were found in the vicinity of these SNPs. An 86-kb region of chromosome 10 could be also a good candidate locus for relative tolerance index for chlorophyll content under salt stress in cowpea. This region was defined by a total of 8 significant SNPs. These SNPs consisted of Vu10_29847718 (LOD= 7.55), Vu10_29848338 (LOD= 7.59), Vu10_29864524 (LOD= 7.78), Vu10_29864555 (LOD= 7.67), Vu10_29864829

(LOD= 7.78), Vu10_29865036 (LOD= 8.04), Vu10_29933934 (LOD= 7.63), and Vu10_29933946 (LOD= 7.63). In addition, this region harbored a cluster of cytochrome P450 (Fig. 9.6).

Leaf injury score under salt stress

A total of 1667 SNPs were found to be significantly associated with leaf injury score under salt stress in cowpea. These significant SNPs were located on chromosomes 1,2,3,4,5,3,10, and 11 (Figs. 9.7-9.10). The number was SNP was 18, 53, 1494, 84, 1, 3, 3, and 11 on chromosomes 1,2,3,4,5,3,10, and 11, respectively. LOD ($-\log_{10}$(p-value)) values varied from 7.52 to 13.63. The first significant locus associated with leaf injury score was a 140-kb region of chromosome 1. This genomic region contained the SNPs Vu01_24112868 (LOD= 8.33), Vu01_24245081 (LOD= 9.30), Vu01_24246312 (LOD= 9.44), Vu01_24246319 (LOD= 9.44), Vu01_24246550 (LOD= 9.23), Vu01_24246587 (LOD= 9.28), Vu01_24246822 (LOD= 9.64), Vu01_24246905 (LOD= 9.76), Vu01_24246981 (LOD= 9.58), Vu01_24248242 (LOD= 9.11), and Vu01_24249542 (LOD= 9.08). Two annotated genes, *Vigun01g085400.1* and *Vigun01g086000.1*, having functional annotations relevant to plant physiology were identified in this region (Fig. 9.7). An additional significant SNP, Vu01_25586428 (LOD= 7.69), mapped at more than 1 Mb of the 140-kb locus was located in the vicinity of *Vigun01g093400.1*, encoding for plasma-membrane choline transporter. A cluster of significant SNPs (Vu01_31228168 (LOD= 8.02), Vu01_31228899 (LOD= 7.77), Vu01_31228901 (LOD= 7.77), Vu01_31228974 (LOD= 7.59), Vu01_31228996 (LOD= 7.64), and Vu01_31229389 (LOD= 8.51)) located towards the end of chromosome 1 were also identified. However, no annotated genes were found in the vicinity of this cluster.

A group of 53 significant SNPs, mapped on a 68-kb region of chromosome 2, was also identified. The SNPs with the highest LOD values in this region were Vu02_28050011 (LOD= 9.53), Vu02_28054154 (LOD= 9.48), Vu02_28105724 (LOD= 9.09), Vu02_28105725 (LOD= 9.09), Vu02_28090457 (LOD= 8.94), Vu02_28050187 (LOD= 8.79), Vu02_28064123 (LOD= 8.46), Vu02_28090387 (LOD= 8.44), Vu02_28094085 (LOD= 8.37), Vu02_28084764 (LOD= 8.27), Vu02_28064103 (LOD= 8.26), Vu02_28050297 (LOD= 8.22), Vu02_28060786 (LOD= 8.20), Vu02_28091358 (LOD= 8.19), and Vu02_28068945 (LOD= 8.17). The 68-kb of chromosome 2 harbored a significant clusters of NAD dependent epimerase/dehydratase (Fig. 9.8).

Chromosome 3 harbored the most important significant locus associated with tolerance to leaf score injury under salt stress in cowpea (Fig. 9.9). This locus was a 1.5-Mb region of chromosome 3 and harbored more than 1,400 significant SNPs. The SNPs with the highest LOD values in this region were Vu03_14737814 (LOD= 13.63), Vu03_14726223 (LOD= 13.04), Vu03_14719792 (LOD= 13.01), Vu03_14737840 (LOD= 12.98), Vu03_14716271 (LOD= 12.94), Vu03_14714710 (LOD= 12.88), Vu03_14722481 (LOD= 12.87), Vu03_14722442 (LOD= 12.86), Vu03_14737848 (LOD= 12.65), Vu03_14725396 (LOD= 12.63), Vu03_14722398 (LOD= 12.58), Vu03_14734685 (LOD= 12.58), Vu03_14726150 (LOD= 12.54), and Vu03_14720653 (LOD= 12.52). Several annotated genes were found within the 1.5-Mb region of chromosome 3. The GWAS signal peak in this region was within the structure of a potassium channel (*Vigun03g144700.1*) (Fig. 9.9) (Table 9.1). In addition, biomolecule transporters (iron transporters, phosphate transporters…) such as *Vigun03g135800.1*, *Vigun03g135900.1*, *Vigun03g136000.1*, *Vigun03g136300.1*, and *Vigun03g136400.1* were found to be in the vicinity of the significant SNPs.

Significant GWAS peaks were also identified on chromosome 4. The SNPs that were closest to annotated genes were Vu04_1785520 (LOD= 8.55), Vu04_1801689 (LOD= 8.32), Vu04_1857562 (LOD= 8.14), Vu04_1876606 (LOD= 7.52), Vu04_1896799 (LOD= 8.49), Vu04_1916362 (LOD= 9.01), Vu04_2001620 (LOD= 8.23), Vu04_2535911 (LOD= 7.90), Vu04_5101729 (LOD= 7.78), Vu04_41757989 (LOD= 8.30), Vu04_41787263 (LOD= 7.95), Vu04_41800162 (LOD= 8.72), and Vu04_41850683 (LOD= 7.67). The annotated genes having functional annotations that were most relevant to tolerance to plant abiotic stress were *Vigun04g025900.1*, *Vigun04g031500.1*, and *Vigun04g054000.1*. These annotated genes encode for chlorophyllase, auxin efflux carrier family, and Myb-like DNA binding protein, respectively (Fig. 9.10). In addition, annotated genes involved in plant physiology were also identified. These genes consisted of *Vigun04g023800.1*, *Vigun04g193600.1*, *Vigun04g193700.1*, *Vigun04g194000.1*, and *Vigun04g194100.1*.

Protein homologs and gene ontology

Protein homolog search was investigated for the candidate genes with functional annotations that could be linked to tolerance to plant abiotic stress. In this study, search was carried out across the genomes of legumes such as soybean, common bean, and Medicago. Proteins that have similarity >90% with the query was taking into account. In order to estimate the number of copies of each candidate gene for cowpea, search was conducted within the cowpea genome. For the candidate genes associated with leaf SPAD chlorophyll under salt stress, multiple copies of *Vigun02g128900.1*, *Vigun02g129000.1*, and *Vigun02g129300.1* within the cowpea genome (Table 9.2). The candidate genes *Vigun01g086000.1*, *Vigun02g128700.1*, *Vigun02g129100.1*, *Vigun02g129200.1*, *Vigun02g129400.1*, and *Vigun02g129500*.1 had one to 3 copies within the cowpea genome. The number of protein homologs was highest within the

soybean genome, whereas it was lowest within the Medicago genome (Table 9.2). For the candidate genes associated with relative tolerance index for chlorophyll, a large number of copies of *Vigun10g104200.1*, *Vigun10g104300.1*, and *Vigun10g104400.1* were found within the cowpea genome. The candidate gene *Vigun04g193500.1* was unique within the cowpea genome. The candidate genes *Vigun01g086000.1*, *Vigun02g129000.1*, *Vigun02g129100.1*, *Vigun02g129200.1*, *Vigun02g129300.1*, *Vigun02g129400.1*, *Vigun03g118000.1*, and *Vigun10g093500.1* had 1 to 4 copies within the cowpea genome. Overall, the number of homologs between common bean and cowpea was very close. Among the 4 legume species compared in this study, the soybean genome had the largest number of copies. For the candidate genes associated with leaf injury score, the number of gene duplication is less significant compared to other traits. The candidate genes *Vigun01g086000.1*, *Vigun03g144700.1*, *Vigun04g025900.1*, and *Vigun04g193700.1* were unique within the cowpea genome. The candidate genes *Vigun04g193700.1*, *Vigun02g129000.1*, *Vigun02g129200.1*, *Vigun03g135800.1*, *Vigun03g136300.1*, *Vigun03g149400.1*, and *Vigun04g054000.1* had 1 to 4 copies within the cowpea genome. Vigun03g135800.1 seemed to be abundant within the soybean, common bean, and Medicago genomes. However, only one the common bean genome had a single copy of *Vigun01g086000.1*.

Overlapping SNPs and functional annotations

The number of overlapping SNPs between traits were visualized using a Venn diagram (Fig. 9.11A). On the Venn diagram, the significant SNPs associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf score injury was represented using solid green, blue, and pink circles, respectively (Fig. 9.11A). The number of SNPs

associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf score injury was 65, 60, and 1667, respectively.

A total of 19 SNPs overlapped between leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf score injury as shown in Fig. 9.11A, suggesting that there could be a common genetic mechanism controlling these traits. The number of common SNPs between leaf SPAD chlorophyll under salt stress and tolerance index for chlorophyll was 3. The number of overlapping SNPs between relative tolerance index for chlorophyll and leaf injury score was 4. The number of shared SNPs between leaf SPAD chlorophyll under salt stress and leaf injury score was 30. These results provided strong evidence on the interdependency between these traits at the genetic level.

Overlapping functional annotations between candidate genes associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf injury score were also visualized using a Venn diagram (Fig. 9.11B). Duplicated functional annotation names were removed and only the number of unique names were displayed on the Venn diagram. Color coding was similar to Fig. 9.11A. The 3 traits investigated for salt tolerance showed a common functional annotation, supporting the evidence on the potential common genetic mechanism controlling these traits (Fig. 9.11). In addition, a common functional annotation was identified for the candidate genes associated with leaf SPAD chlorophyll under salt stress and relative tolerance index for salt stress. No common functional annotation was found between for the candidate genes associated with leaf SPAD chlorophyll and leaf injury score under salt stress. Similar results were found the candidate genes associated with relative tolerance for chlorophyll and leaf injury score under salt stress.

Genomic selection

GS accuracy was assessed for leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll content, and leaf injury score under salt stress in this study. GS accuracy was 0.52, 0.43, and 0.67 for leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll content, and leaf injury score under salt stress, respectively.

Discussion

Whole genome resequencing has been more and more popular in plant genetic-related studies. It allows for the discovery for a large number of SNPs that can be used in GWAS. Thanks to the large number of SNPs, the likelihood of discovering good candidate genes is higher (Lee et al., 2015; Thudi et al., 2016). This study was one of the earliest reports in cowpea using a whole genome resequencing data to conduct GWAS for salt tolerance in cowpea. Whole genome resequencing provided a total of 14,465,516 SNPs. GWAS was conducted using a total of 5,884,299 filtered and high-quality SNPs.

In this study, a total of 65, 60, and 1667 SNPs were found to be significantly associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf score injury, respectively. The first reported molecular markers associated with salt tolerance in cowpea were Scaffold87490_622, Scaffold87490_630, C35017374_128, Scaffold93827_270, Scaffold68489_600, Scaffold87490_633, Scaffold87490_640, Scaffold82042_3387, C35069468_1916, and Scaffold93942_1089 (Ravelombola et al. 2017). These are SNP markers that were obtained from genotyping-by-sequencing. At the time this study was investigated, the cowpea genome was not yet available. These SNP markers do not have neither chromosome information nor physical position. In addition, the reads from genotyping-by-sequencing from

which the SNPs were obtained were not realigned yet with the new cowpea genome that is accessible at https://phytozome.jgi.doe.gov/pz/portal.html. Therefore, we could not pair yet our findings from this study with the first reported SNP markers associated with salt tolerance in cowpea.

One of the most interesting findings was the discovery of a strong GWAS signal that was mapped on a 1 Mb-region of chromosome 3, which was associated to tolerance to leaf score injury under salt stress. The peak of this signal corresponded to *Vigun03g144700.1*, which encodes for a potassium channel. This potassium channel has been described to be activated upon salt stress in cowpea in order to enhance the transport of K^+ under salt stress in cowpea (Mini et al. 2015). Previous investigations have showed that salt-tolerant cowpea had a higher K^+/Na^+ ratio in leaves (Cavalcanti et al., 2004; Maia et al., 2013; Mini et al., 2015; Praxedes et al., 2009). Therefore, the GWAS approach we used in this study has successfully targeted a gene that is involved in salt tolerance in cowpea. In addition, our previous research revealed a K+ channel protein being involved in salt tolerance in a MAGIC population, which is in agreement with the GWAS result in this study. Potassium channel proteins have also been well described for enhancing tolerance to salinity in other species . Assaha et al. (2017) showed that K+ channel-related genes were upregulated under salt stress in tomato and soybean.

Genes encoding for NAD dependent dehydratase have been also found in the vicinity of the significant SNPs associated with salt tolerance. These genes have been demonstrated to regulate stress in rice (Nan et al. 2020). A gene encoding for auxin efflux carrier was also found within the GWAS peaks. Korver et al. (2018) reported that auxin efflux proteins have a significant role in assisting Arabidopsis thaliana with regulating salt stress. The auxin efflux carriers regulate the variation in auxin flow during salt stress and are also involved in regulating

meristem size for plants under salt stress. Results also indicated the involvement of a chlorophyllase gene in salt tolerance. However, there is no report yet highlighting the role of chlorophyllase in salt tolerance. We would suggest that chlorophyllase is a salt-susceptible gene since it is involved in chlorophyll degradation (Harpaz-Saad et al. 2007). Genes involved in vacuolar iron transporters were also identified. Our previous investigation on salt tolerance identified these genes in a MAGIC population. These transporters are also involved in salt tolerance (Kim and Bassham 2011). In soybean, the Na^+/H^+ antiporter gene, *GmCHX1*, has been well described in conferring salt tolerance (Qi et al. 2014). A simple BLAST search showed that an orthologue of this gene can be found on chromosome 7 of cowpea. However, no strong GWAS peak was found on this chromosome. We could assume that this gene might be associated with a rare allele so that our GWAS approach failed to identify it.

A large number of molecular markers that are associated with cowpea salt tolerance have been identified in this study. A SNP validation is required prior to using these markers into a breeding program for Marker-Assisted Selection (MAS). The results from this investigation also contributed to a better understanding of the genetics of salt tolerance mechanism in cowpea. The candidate genes that were relevant to salt tolerance will be validated in further studies. Conducting the salt tolerance under greenhouse conditions could be a limitation for this study. However, to date, greenhouse phenotyping remains the most affordable and accurate way to evaluate salt tolerance since a lot of uncontrolled factors can occur during field screening. Therefore, repeating the experiments under field conditions could be a major challenge.

Conclusions

In this study, strong GWAS peaks associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and tolerance to leaf injury score under salt stress were identified. A total of 65, 60, and 1667 significant SNPs were found to be associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and tolerance to leaf injury score under salt stress, respectively. Leaf SPAD chlorophyll under salt stress was characterized by a strong candidate locus by an 890-kb region of chromosome 2. Two candidate loci were found to be associated with relative tolerance index for chlorophyll and mapped on chromosomes 1 and 2. A strong candidate locus defined by a 1-Mb region of chromosome 3 was associated with tolerance to leaf injury score under salt stress in cowpea. The results from this study could be used in cowpea breeding through Marker-Assisted Selection (MAS). To the best of our knowledge, this is the first report on cowpea GWAS using a whole genome resequencing data.

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Tables

Table 9.1. List of significant SNPs close to candidate genes and associated with leaf SPAD chlorophyll under salt stress, relative tolerance index for chlorophyll, and leaf injury score under salt stress in cowpea. SNP, CHR, BP, Pval, and LOD refers to SNP_ID, chromosome number, physical location (in bp), p-value, and -log10 of p-value (LOD), respectively. Gene_ID and functional annotations were obtained from Pythozome v.13.

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
Leaf SPAD chlorophyll under salt treatment	Vu01_24245081	1	24245081	2.70E-08	7.57	*Vigun01g086000.1*	GATA transcription factor
	Vu02_28035590	2	28035590	4.73E-09	8.33	*Vigun02g128700.1*	Inorganic phosphatase
	Vu02_28044965	2	28044965	3.71E-10	9.43	*Vigun02g128800.1*	Replication factor C
	Vu02_28050297	2	28050297	3.54E-11	10.5	*Vigun02g128900.1*	NAD dependent epimerase/dehydratase
	Vu02_28054154	2	28054154	2.09E-11	10.7	*Vigun02g129000.1*	NAD dependent epimerase/dehydratase
	Vu02_28064103	2	28064103	9.85E-10	9.01	*Vigun02g129100.1*	NAD dependent epimerase/dehydratase
	Vu02_28068945	2	28068945	2.47E-10	9.61	*Vigun02g129200.1*	NAD dependent epimerase/dehydratase
	Vu02_28084764	2	28084764	2.34E-10	9.63	*Vigun02g129300.1*	NAD dependent epimerase/dehydratase
	Vu02_28090457	2	28090457	5.66E-10	9.25	*Vigun02g129400.1*	NAD dependent epimerase/dehydratase
	Vu02_28105724	2	28105724	8.96E-11	10.1	*Vigun02g129500.1*	NAD dependent epimerase/dehydratase

Table 9.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
Relative tolerance index for chlorophyll	Vu01_24246822	1	24246822	1.14E-09	8.95	*Vigun01g086000.1*	GATA transcription factor
	Vu02_28061740	2	28061740	2.28E-08	7.64	*Vigun02g129000.1*	NAD dependent epimerase/dehydratase
	Vu02_28071778	2	28071778	1.30E-08	7.89	*Vigun02g129100.1*	NAD dependent epimerase/dehydratase
	Vu02_28084764	2	28084764	2.96E-09	8.53	*Vigun02g129200.1*	NAD dependent epimerase/dehydratase
	Vu02_28105725	2	28105725	4.70E-09	8.33	*Vigun02g129300.1*	NAD dependent epimerase/dehydratase
	Vu02_28112832	2	28112832	1.06E-08	7.98	*Vigun02g129400.1*	NAD dependent epimerase/dehydratase
	Vu03_10976477	3	10976477	2.64E-08	7.58	*Vigun03g118000.1*	Terpene synthase
	Vu04_41756724	4	41756724	1.05E-08	7.98	*Vigun04g193500.1*	Phospholipid-transporting ATPase-related
	Vu10_27003173	10	27003173	1.93E-08	7.72	*Vigun10g093500.1*	Xanthoxin dehydrogenase/Abscisic acid biosynthesis
	Vu10_29847718	10	29847718	2.79E-08	7.55	*Vigun10g104200.1*	Cytochrome P450
	Vu10_29864524	10	29864524	1.67E-08	7.78	*Vigun10g104300.1*	Cytochrome P450
	Vu10_29933934	10	29933934	2.37E-08	7.63	*Vigun10g104400.1*	Cytochrome P450

Table 9.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
Leaf injury score under salt stress	Vu01_24112868	1	24112868	4.68E-09	8.33	*Vigun01g085400.1*	No apical meristem (NAM) protein
	Vu01_24249542	1	24249542	8.23E-10	9.08	*Vigun01g086000.1*	GATA zinc finger
	Vu01_25586428	1	25586428	2.04E-08	7.69	*Vigun01g093400.1*	Plasma-membrane choline transporter
	Vu02_28050011	2	28050011	2.95E-10	9.53	*Vigun02g129000.1*	NAD dependent epimerase/dehydratase
	Vu02_28064123	2	28064123	3.44E-09	8.46	*Vigun02g129100.1*	NAD dependent epimerase/dehydratase
	Vu02_28090457	2	28090457	1.14E-09	8.94	*Vigun02g129200.1*	NAD dependent epimarase/dehydratase
	Vu02_28105725	2	28105725	8.07E-10	9.09	*Vigun02g129300.1*	NAD dependent epimarase/dehydratase
	Vu03_11383713	3	11383713	1.20E-09	8.92	*Vigun03g121600.1*	Malate dehydrogenase
	Vu03_13297388	3	13297388	9.20E-09	8.04	*Vigun03g135800.1*	Vacuolar iron transporter
	Vu03_13305589	3	13305589	6.45E-09	8.19	*Vigun03g135900.1*	Vacuolar iron transporter
	Vu03_13313938	3	13313938	8.57E-09	8.07	*Vigun03g136000.1*	Vacuolar iron transporter
	Vu03_13334160	3	13334160	4.32E-09	8.36	*Vigun03g136100.1*	Histidine decarboxylase
	Vu03_13357176	3	13357176	2.44E-09	8.61	*Vigun03g136300.1*	EamA-like transporter family/phosphate antiporter

Table 9.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu03_13363517	3	13363517	4.54E-09	8.34	*Vigun03g136400.1*	EamA-like transporter family/phosphate antiporter
	Vu03_13509429	3	13509429	1.37E-08	7.86	*Vigun03g137600.1*	tRNA-splicing endonuclease positive effector-related
	Vu03_14318570	3	14318570	3.03E-09	8.52	*Vigun03g142100.1*	Tetrahydroberberine oxidase
	Vu03_14369744	3	14369744	1.20E-08	7.92	*Vigun03g142200.1*	Tetrahydroberberine oxidase
	Vu03_14373278	3	14373278	1.75E-08	7.76	*Vigun03g142300.1*	Tetrahydroberberine oxidase
	Vu03_14737814	3	14737814	2.33E-14	13.6	*Vigun03g144700.1*	Potassium channel
	Vu03_14760979	3	14760979	8.65E-11	10.1	*Vigun03g144800.1*	WRKY transcription factor
	Vu03_15238396	3	15238396	1.78E-08	7.75	*Vigun03g148600.1*	Flavine reductase-related
	Vu03_15286489	3	15286489	2.79E-08	7.55	*Vigun03g148900.1*	CCR4-NOT transcription complex subunit
	Vu03_15308668	3	15308668	5.92E-09	8.23	*Vigun03g149000.1*	Eukaryotic cytochrome b561
	Vu03_15338189	3	15338189	5.92E-09	8.23	*Vigun03g149100.1*	DNA-directed RNA polymerase II subunit RPB7
	Vu03_15380199	3	15380199	2.32E-09	8.64	*Vigun03g149400.1*	Gibberellin 2-oxidase
	Vu03_16376823	3	16376823	1.95E-08	7.71	*Vigun03g154300.1*	Leucine-rich repeat protein

Table 9.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu03_26130498	3	26130498	9.70E-09	8.01	*Vigun03g190500.1*	Polysaccharide biosynthesis
	Vu04_1785520	4	1785520	2.83E-09	8.55	*Vigun04g023800.1*	Zinc finger protein-like protein
	Vu04_1801689	4	1801689	4.76E-09	8.32	*Vigun04g023900.1*	Core-2/I-Branching enzyme
	Vu04_1857562	4	1857562	7.27E-09	8.14	*Vigun04g024100.1*	Calmodulin binding protein
	Vu04_1876606	4	1876606	2.99E-08	7.52	*Vigun04g024200.1*	Protein kinase family
	Vu04_1896799	4	1896799	3.20E-09	8.49	*Vigun04g024700.1*	Protein tyrosin kinase
	Vu04_1916362	4	1916362	9.83E-10	9.01	*Vigun04g024900.1*	Protein tyrosin kinase
	Vu04_2001620	4	2001620	5.89E-09	8.23	*Vigun04g025900.1*	Chlorophyllase
	Vu04_2535911	4	2535911	1.26E-08	7.9	*Vigun04g031500.1*	Auxin efflux carrier family
	Vu04_5101729	4	5101729	1.65E-08	7.78	*Vigun04g054000.1*	Myb-like DNA-binding protein
	Vu04_41757989	4	41757989	5.05E-09	8.3	*Vigun04g193600.1*	Serine/threonine-protein kinase
	Vu04_41787263	4	41787263	1.13E-08	7.95	*Vigun04g193700.1*	NAD dependent epimerase/dehytrase
	Vu04_41800162	4	41800162	1.90E-09	8.72	*Vigun04g194000.1*	Universal stress protein family

Table 9.1 (Cont.)

Traits	SNP	CHR	BP	Pval	LOD	Gene_ID	Functional_annotation
	Vu04_41850683	4	41850683	2.13E-08	7.67	*Vigun04g194100.1*	3-hydroxyisobutyrate dehydrogenase-related
	Vu05_2631192	5	2631192	6.54E-09	8.18	*Vigun05g032800.1*	Transferase family protein
	Vu06_10043938	6	10043938	3.87E-10	9.41	*Vigun06g021500.1*	Coiled-coil regions of plant-specific actin-binding protein
	Vu06_30560091	6	30560091	2.52E-08	7.6	*Vigun06g186400.1*	Transcriptional repressor
	Vu11_1322049	11	1322049	1.02E-08	7.99	*Vigun11g010800.1*	Leucine-rich repeat
	Vu11_23659412	11	23659412	8.96E-10	9.05	*Vigun11g080000.1*	Serine/threonine-protein kinase

Table 9.2. List of candidate genes having functional annotations that are relevant to plant abiotic stress. Protein homologs from each translated transcript was search in the cowpea (Vun), soybean (Gma), common bean (Pvu), and *Medicago truncatula* (Mtr) genomes. The number of protein homologs with similarity > 90% to that one from cowpea is reported.

Traits	Gene_ID	Functional_annotations	Vun	Gma	Pvu	Mtr
Leaf SPAD chlorophyll under salt stress	*Vigun01g086000.1*	GATA transcription factor	1	4	2	1
	Vigun02g128700.1	Inorganic phosphatase	1	2	1	1
	Vigun02g128900.1	NAD dependent epimerase/dehydratase	5	12	5	3
	Vigun02g129000.1	NAD dependent epimerase/dehydratase	6	8	5	2
	Vigun02g129100.1	NAD dependent epimerase/dehydratase	2	9	4	3
	Vigun02g129200.1	NAD dependent epimerase/dehydratase	2	5	2	1
	Vigun02g129300.1	NAD dependent epimerase/dehydratase	4	5	2	1
	Vigun02g129400.1	NAD dependent epimerase/dehydratase	3	5	2	1
	Vigun02g129500.1	NAD dependent epimerase/dehydratase	3	5	2	0
Relative tolerance index for chlorophyll	*Vigun01g086000.1*	GATA transcription factor	1	2	2	1
	Vigun02g129000.1	NAD dependent epimerase/dehydratase	3	12	5	3
	Vigun02g129100.1	NAD dependent epimerase/dehydratase	2	9	4	3
	Vigun02g129200.1	NAD dependent epimerase/dehydratase	3	5	3	1
	Vigun02g129300.1	NAD dependent epimerase/dehydratase	3	5	2	1
	Vigun02g129400.1	NAD dependent epimerase/dehydratase	4	5	2	1
	Vigun03g118000.1	Terpene synthase	1	1	1	1
	Vigun04g193500.1	Phospholipid-transporting ATPase-related	0	2	1	0
	Vigun10g093500.1	Xanthoxin dehydrogenase/Abscisic acid biosynthesis	1	3	2	1
	Vigun10g104200.1	Cytochrome P450	9	8	3	4
	Vigun10g104300.1	Cytochrome P450	9	9	2	4
	Vigun10g104400.1	Cytochrome P450	9	7	3	3
Leaf injury score	*Vigun01g085400.1*	No apical meristem (NAM) protein	1	4	2	1
	Vigun01g086000.1	GATA zinc finger	0	0	1	0
	Vigun02g129000.1	NAD dependent epimerase/dehydratase	1	4	4	2
	Vigun02g129200.1	NAD dependent epimarase/dehydratase	3	5	3	1
	Vigun03g135800.1	Vacuolar iron transporter	3	7	3	4

Table 9.2 (Cont.)

Traits	Gene_ID	Functional_annotations	Vun	Gma	Pvu	Mtr
	Vigun03g136300.1	EamA-like transporter family/phosphate antiporter	4	5	4	3
	Vigun03g144700.1	Potassium channel/Ion Channel	0	2	1	0
	Vigun03g149400.1	gibberellin 2-oxidase	1	5	2	2
	Vigun04g025900.1	chlorophyllase	0	3	1	0
	Vigun04g054000.1	Myb-like DNA-binding protein	1	2	1	0
	Vigun04g193700.1	NAD dependent epimerase/dehytrase	0	1	1	1

Figures

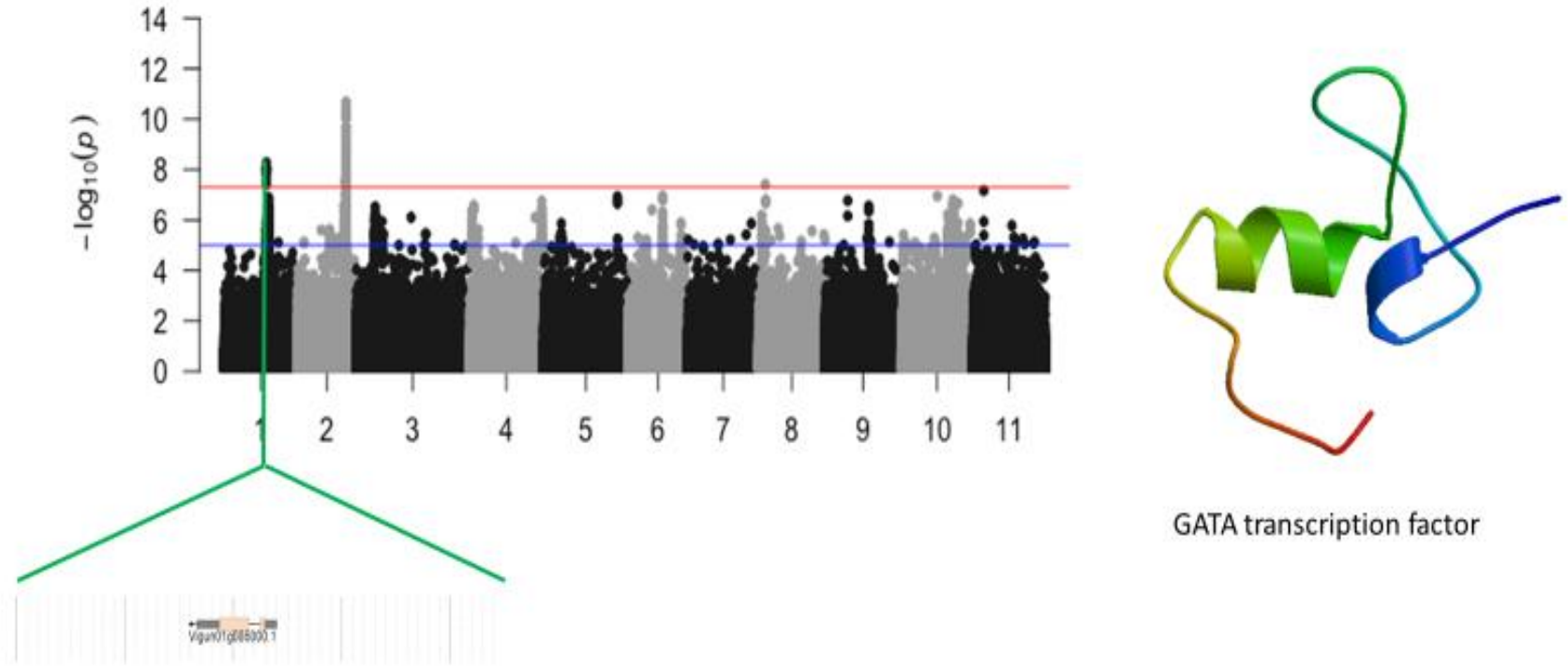

Fig. 9.1. Manhattan plot for leaf SPAD chlorophyll under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 1. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

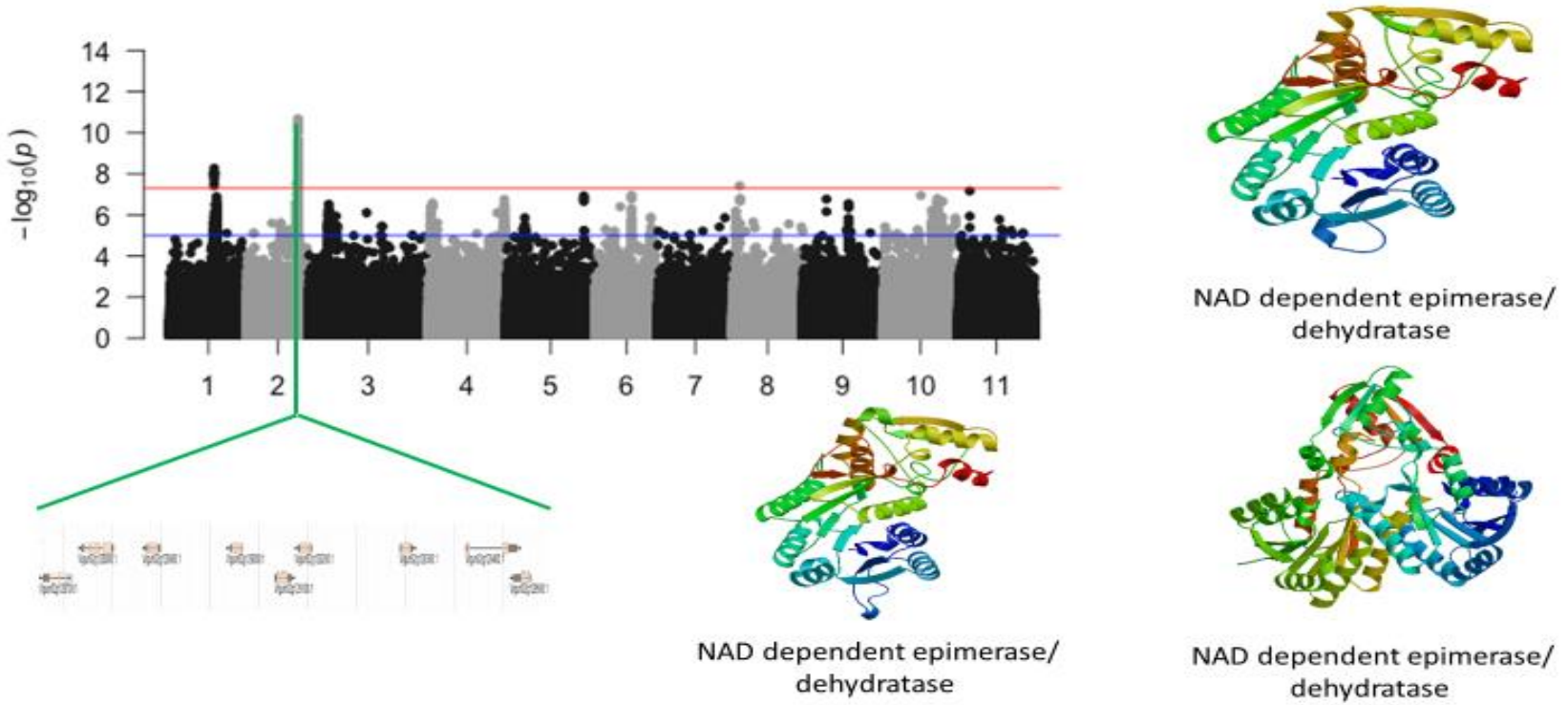

Fig. 9.2. Manhattan plot for leaf SPAD chlorophyll under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 2. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

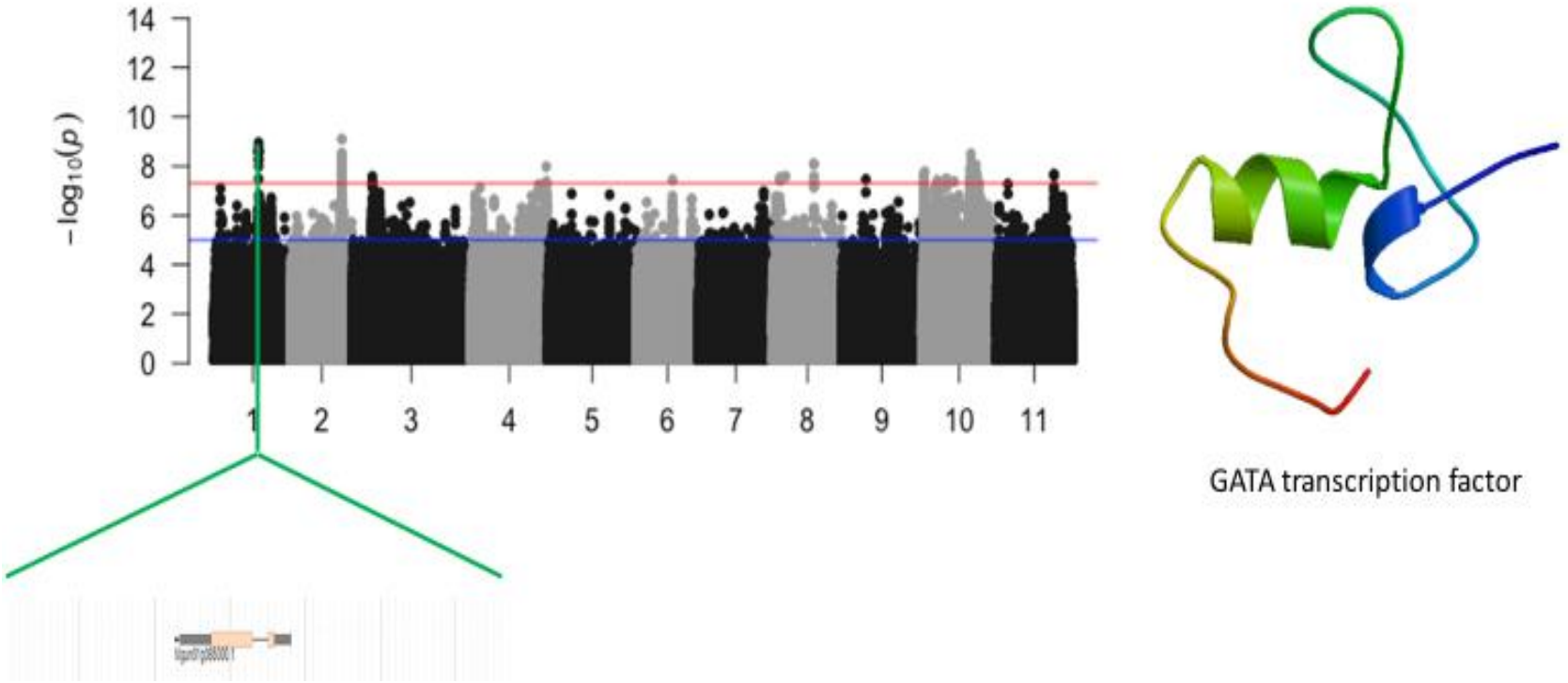

Fig. 9.3. Manhattan plot relative tolerance index for leaf SPAD chlorophyll for salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 1. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

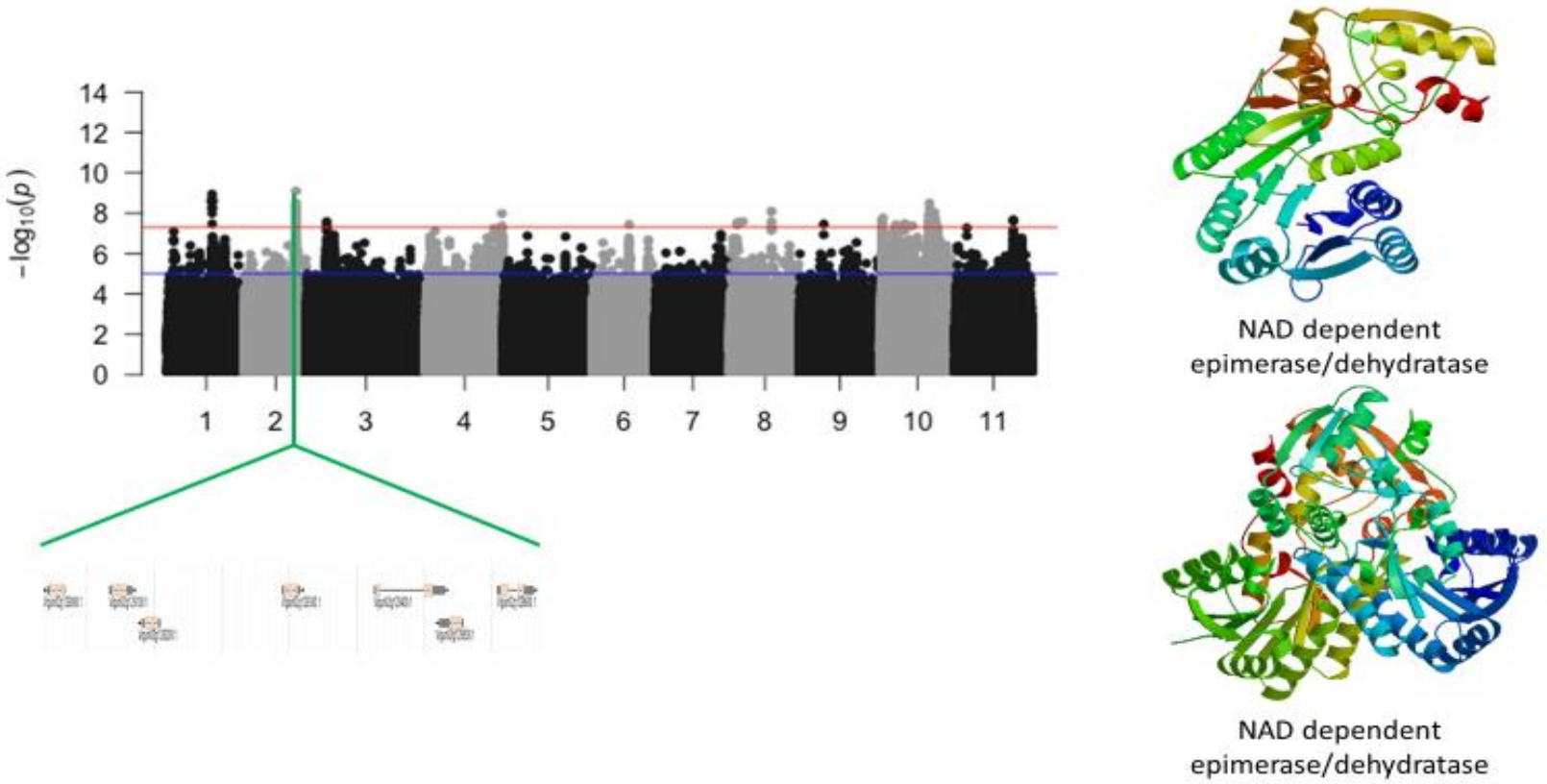

Fig. 9.4. Manhattan plot relative tolerance index for leaf SPAD chlorophyll for salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 2. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

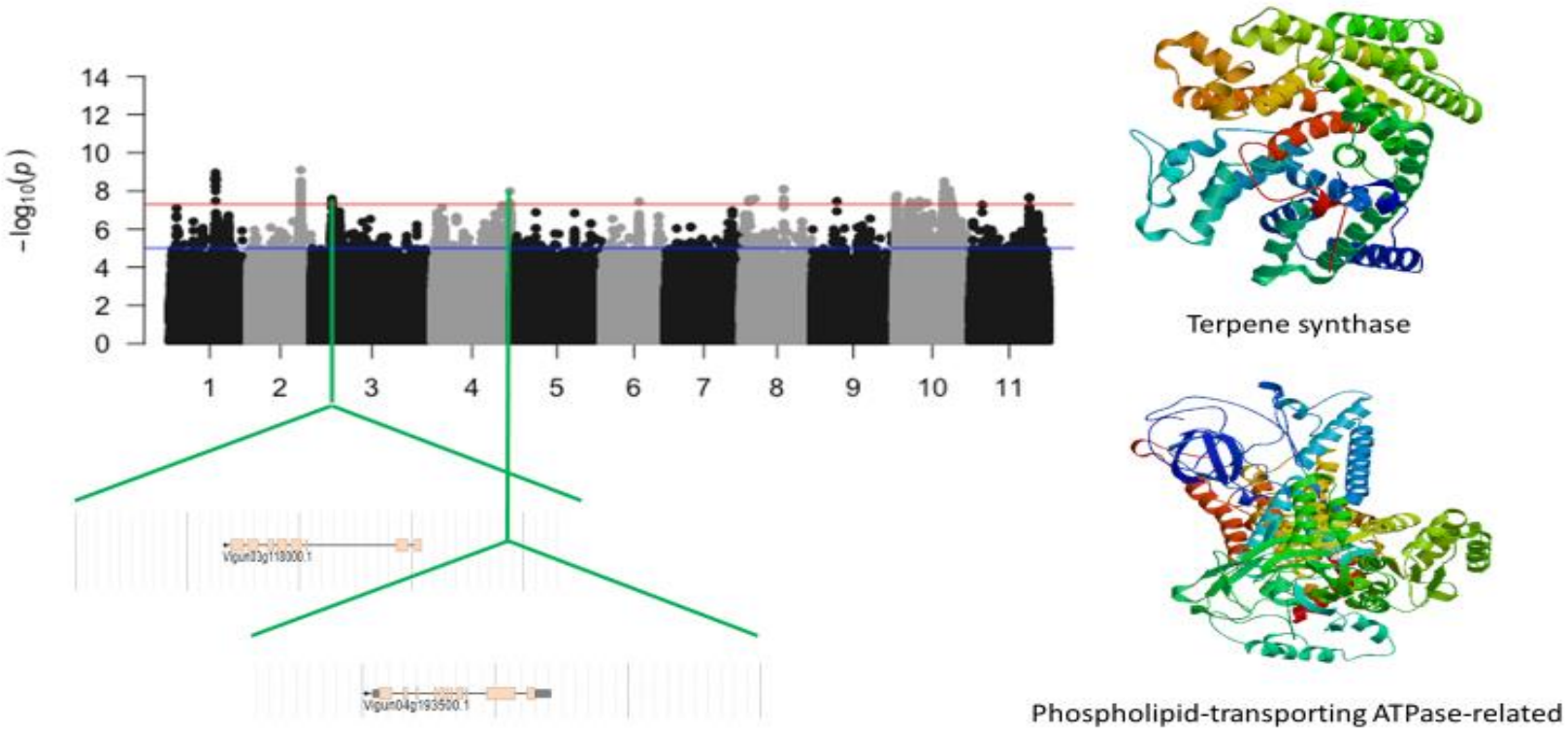

Fig. 9.5. Manhattan plot relative tolerance index for leaf SPAD chlorophyll for salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or $-\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosomes 3 and 4. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

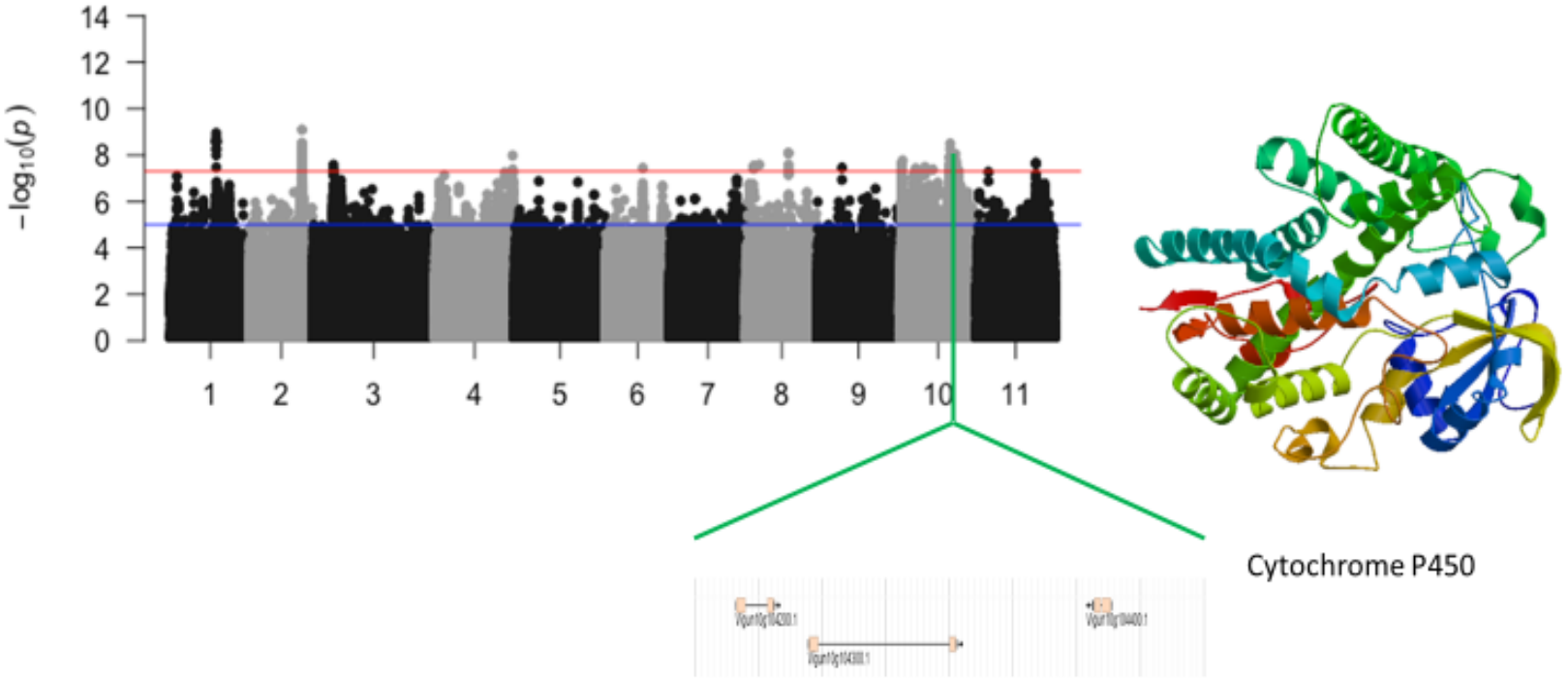

Fig. 9.6. Manhattan plot relative tolerance index for leaf SPAD chlorophyll for salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 8. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

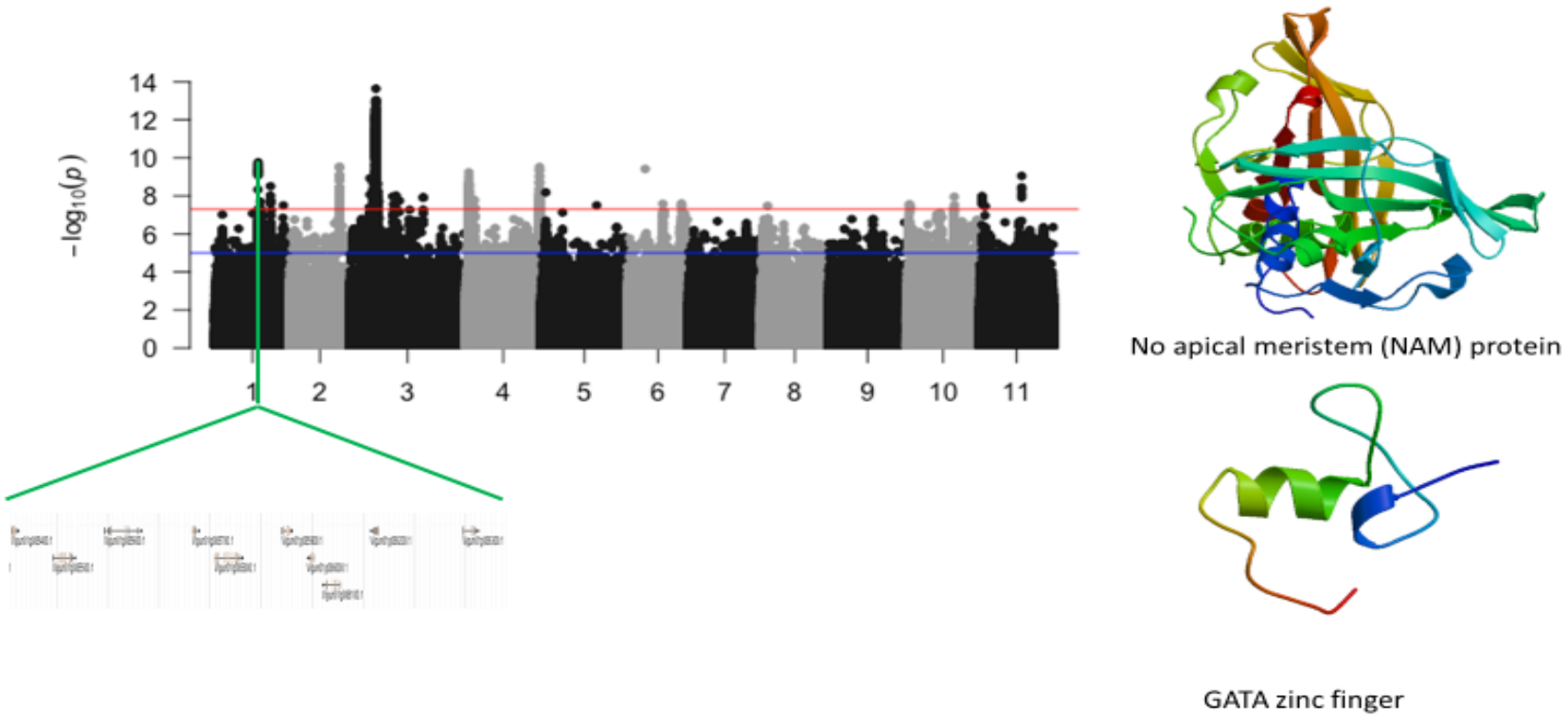

Fig. 9.7. Manhattan plot for tolerance to leaf injury score under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 1. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

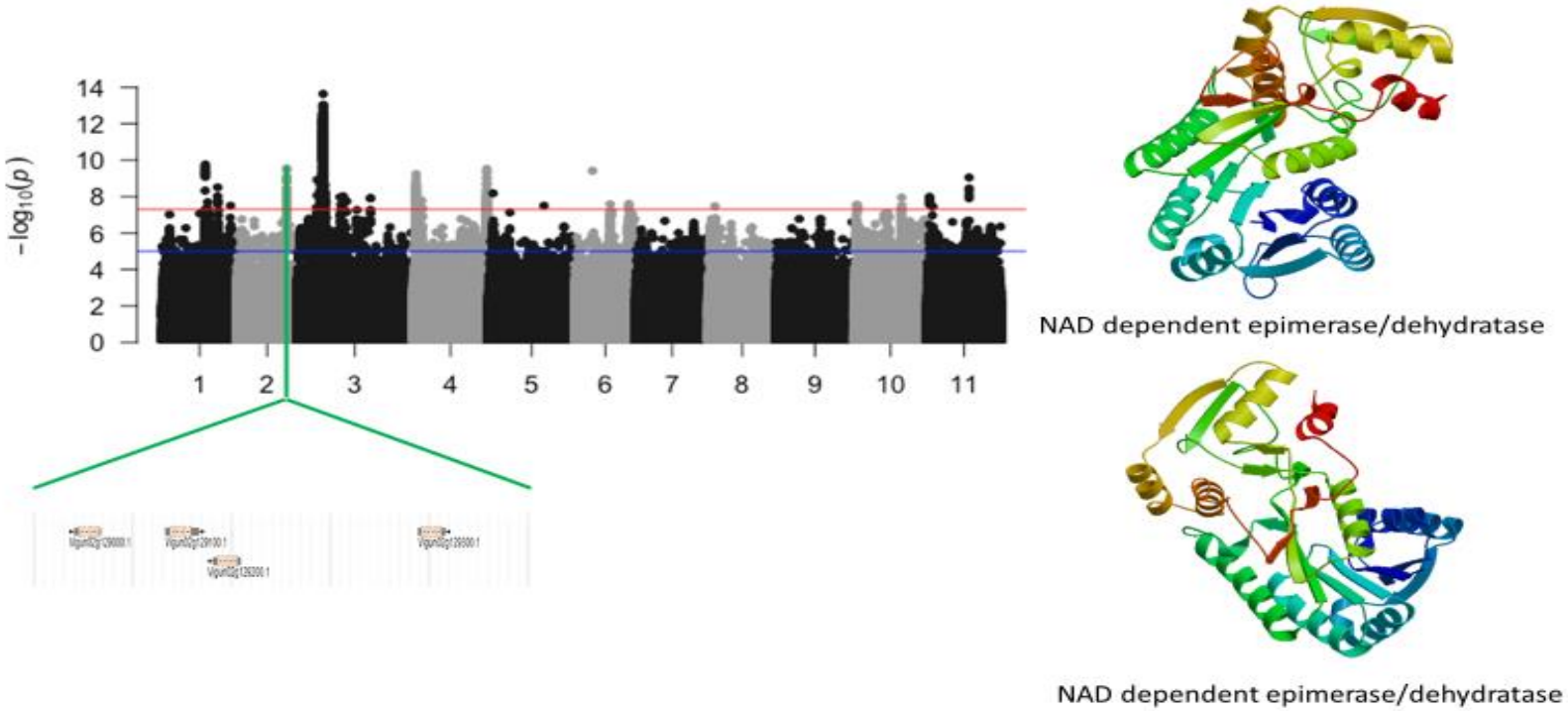

Fig. 9.8. Manhattan plot for tolerance to leaf injury score under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 2. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

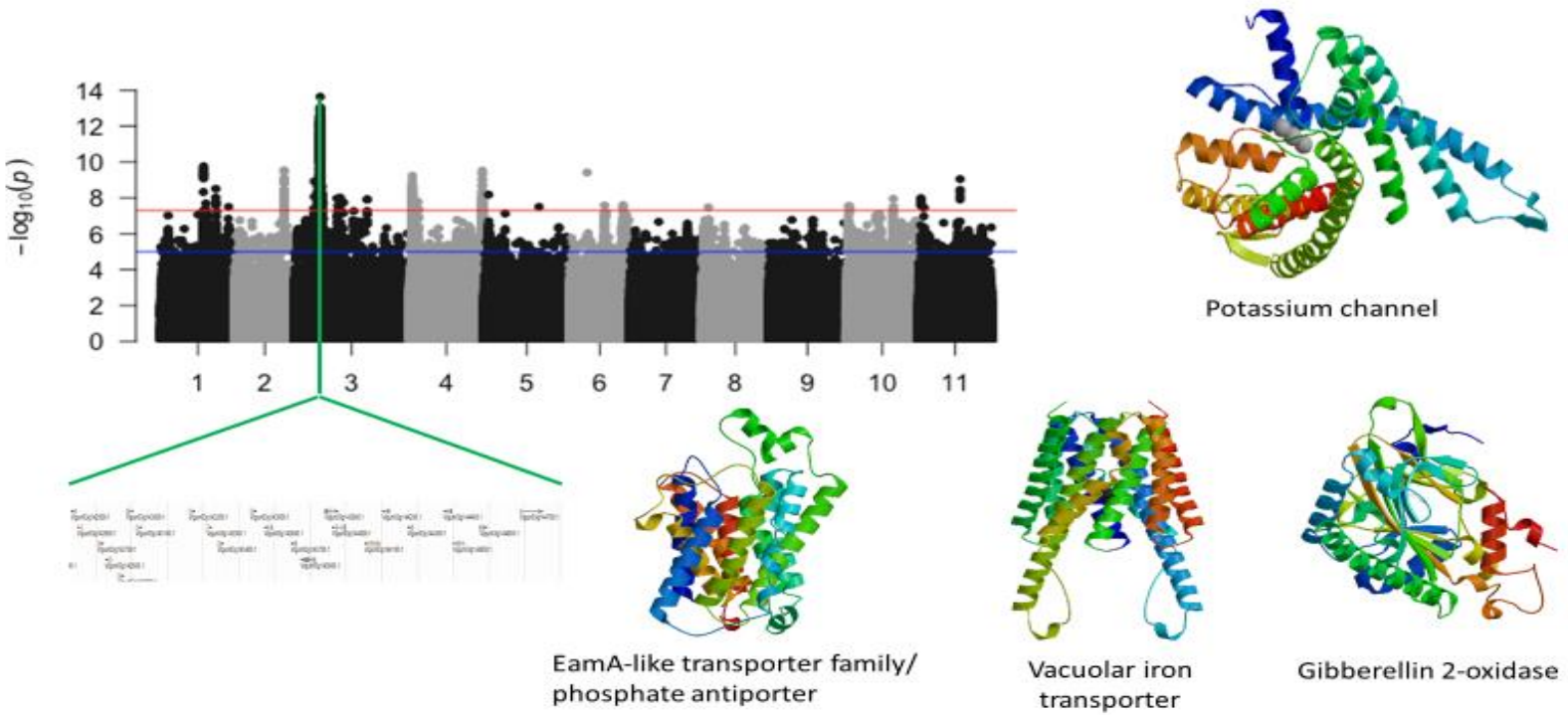

Fig. 9.9. Manhattan plot for tolerance to leaf injury score under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 3. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

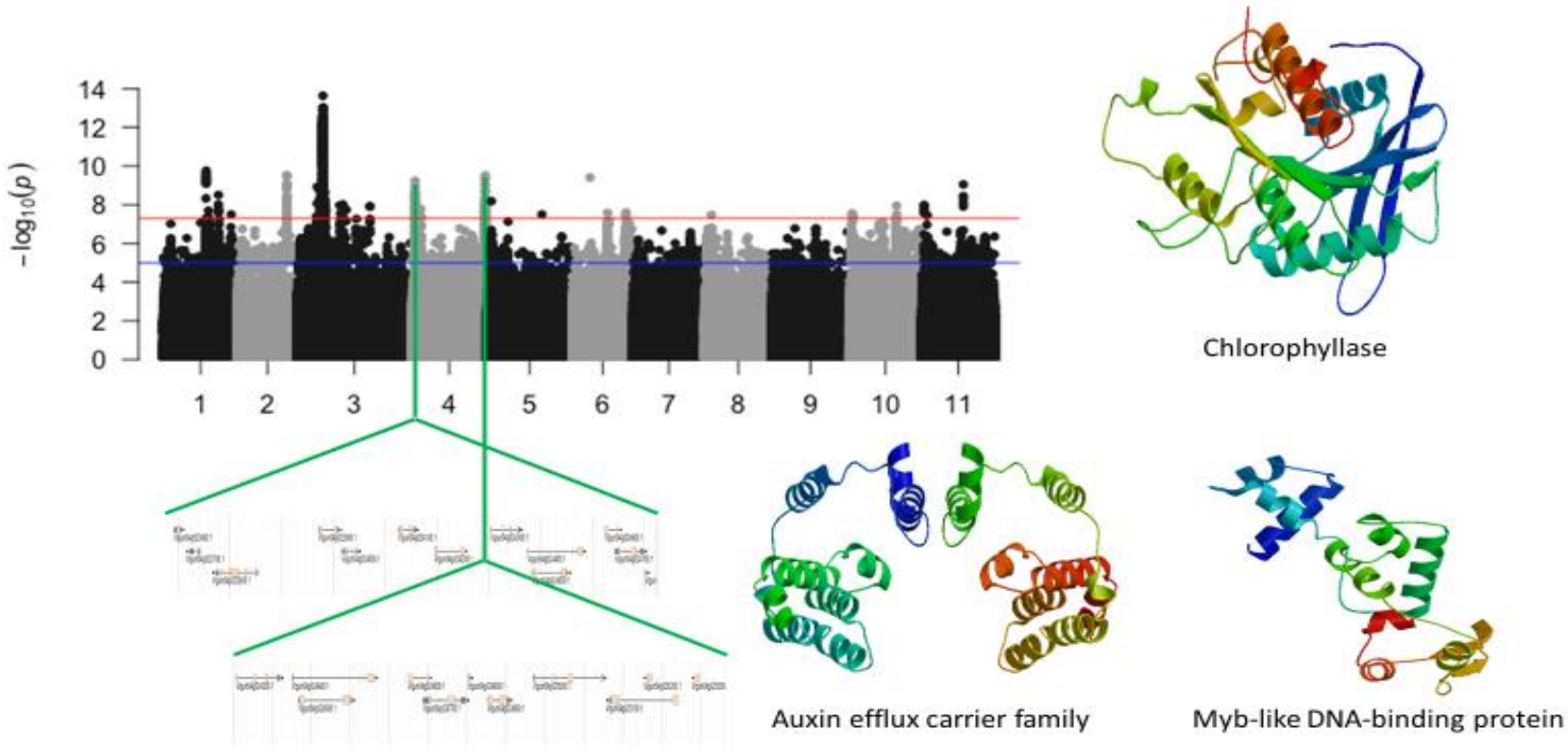

Fig. 9.10. Manhattan plot for tolerance to leaf injury score under salt stress in cowpea. The solid black and grey dots represent the SNPs. The x-axis is the chromosome number and the y-axis the LOD or -$\log_{10}$ of p-value. The horizontal red and blue bars are two different LOD thresholds. Below the Manhattan plot are gene IDs from phytozome v.13 (https://phytozome.jgi.doe.gov/pz/portal.html) corresponding to the significant locus on chromosome 4. Codifying sequences of the genes IDs whose functions were related to drought stress were extracted and converted to amino acid sequence using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary structures of the proteins/polypeptides derived from BLASTX were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) and presented on the left-hand side in the above figure.

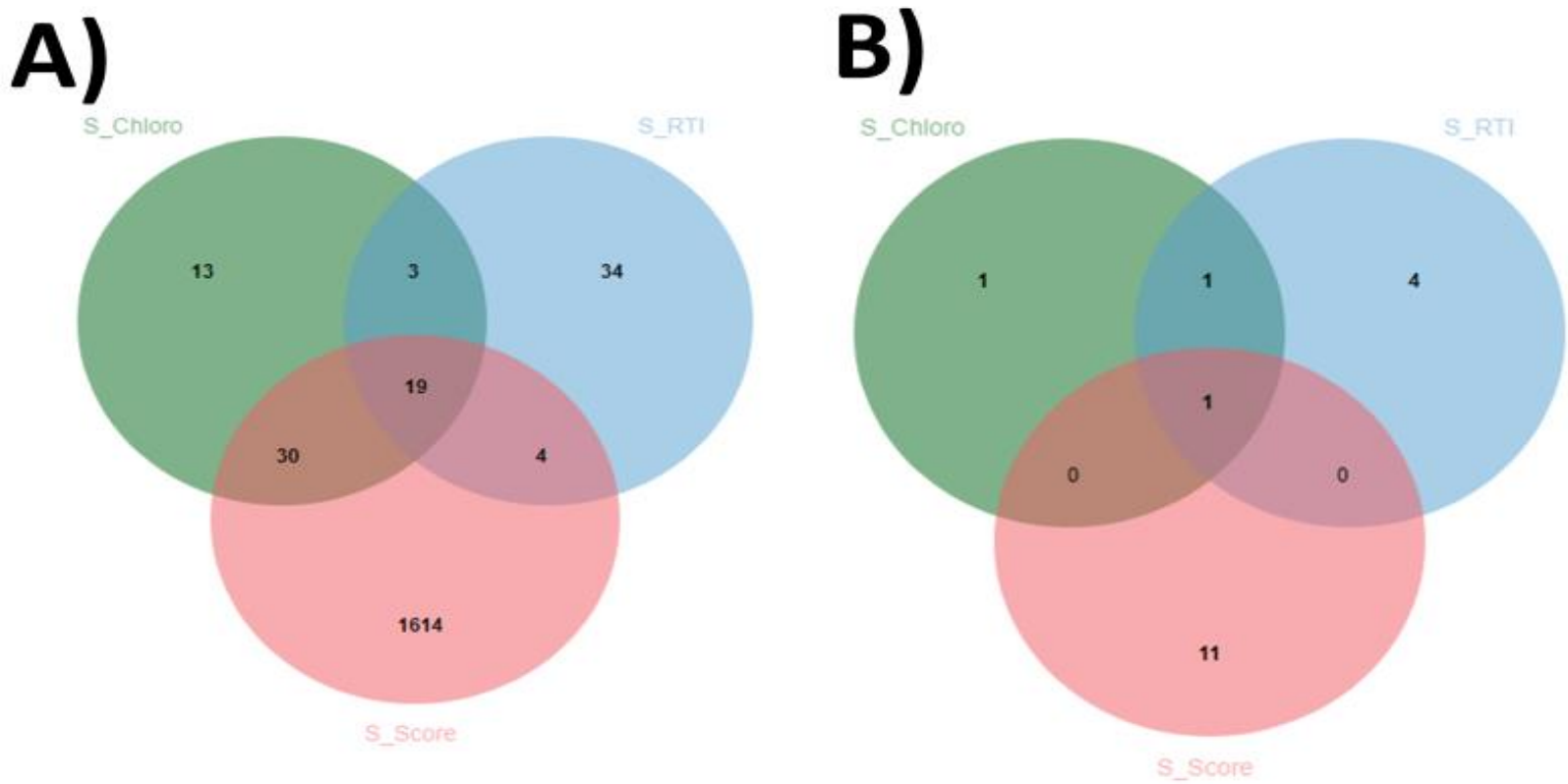

Fig. 9.11. A) Venn diagram showing the number of overlapping significant SNPs associated with leaf SPAD chlorophyll under salt stress (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (S_RTI), and leaf injury score under salt stress (S_Score) in cowpea. B) Venn diagram showing the number of unique functional annotations for candidate genes associated with leaf SPAD chlorophyll under salt stress (S_Chloro), relative tolerance index for leaf SPAD chlorophyll (S_RTI), and leaf injury score under salt stress (S_Score) in cowpea. Venn diagrams were established using http://jvenn.toulouse.inra.fr/app/example.html

Appendices

Table S9.1. List of significant SNPs associated with leaf SPAD chlorophyll under salt stress in cowpea.

Table S9.2. List of significant SNPs associated with relative tolerance index for leaf SPAD chlorophyll for salt stress in cowpea.

Table S9.3. List of significant SNPs associated with tolerance to leaf score injury in cowpea.

Ravelombola, W., A. Shi, B. Huynh, and P. Roberts. 2020. Loci discovery, network-guided approach, and genomic prediction for drought tolerance index in a multi-parent advanced generation inter-cross (MAGIC) cowpea population (Submitted to Frontiers in Plant Science)

Ravelombola. W., L, Dong, T.C. Barickman, H. Xiong, D. Olaoye, G. Bhattarai, B. Zia, H. 2020. Alshaya, I. Alatawi, and A. Shi. Evaluation of Salt Tolerance in Cowepa at Seedling Stage (Submitted to Euphytica).

Ravelombola, W., J. Qin, A. Shi, W. Lu, Y. Weng, H. Xiong, W. Yang, G. Bhattarai, S. Mahamane, W.A. Payne, J.C. Miller, Jr., D. Scheuring. 2017. Association mapping revealed SNP markers for adaptation to low phosphorus conditions and rock phosphate response in USDA cowpea germplasm. Euphytica 213:183

Ravelombola, W., J. Qin, A. Shi, Y. Weng, G. Bhattarai, L. Dong, J.B. Morris. 2017. A SNP-based association analysis for plant growth habit in worldwide cowpea (*Vigna unguiculata* (L.) Walp) germplasm. Euphytica 213:284

Ravelombola W, J. Qin, A. Shi, J.C. Miller, D.C. Scheuring, Y. Weng, G. Bhattarai, L. Dong, and W. Yang. 2018. Population structure analysis and association mapping for iron deficiency chlorosis in worldwide cowpea (*Vigna unguiculata* (L.) Walp) germplasm. Euphytica. 214(6):96

Ravelombola, W., J. Qin, A. Shi, L. Nice, Y. Bao, A. Lorenz, J. H. Orf, N. D. Young, and S. Chen. 2019. Genome-wide association study and genomic selection for soybean chlorophyll content associated with soybean cyst nematode. BMC Genomics 20(1):904

Ravelombola, W., J. Qin, A. Shi, L. Nice, Y. Bao, A. Lorenz, J. H. Orf, N. D. Young, and S. Chen. 2019. Genome-wide association study and genomic selection for tolerance of soybean biomass reduction under to soybean cyst nematode infestation. PloS One (Accepted)

Chapter 10. Conclusions

We have investigated the genetic architecture of salt and drought tolerance in cowpea using a large number of genotypes. These genotypes were screened for salt and drought tolerance using appropriate methodologies. A large variation of salt and drought tolerance has been found among the cowpea genotypes. The MAGIC population was genotyped using a 50-k SNP chip and the association panel was genotypes using a whole genome resequencing approach with 10 X coverage, which resulted in a total of more than 14 million SNPs. GWAS suggested SNP markers that were associated with salt and drought tolerance in cowpea. GS accuracy varied from low to moderated. Candidate genes associated with salt and drought tolerance in cowpea were reported. The results from this study could be used in cowpea breeding and genetics.