Thermo and drought tolerance markers and regulation of heat stress proteins for chickpea (*Cicer arietinum L.*; Fabaceae) production in NE South Africa

By

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I am now presenting the thesis for examination for the Degree of PhD.

The thesis contains one published paper and one submitted manuscript done with my supervisors John B.O. Ogola, A. Muthama Muasya, Olivier Crespo and Samson B.M. Chimphango, as well as other collaborators namely Sipho Maseko, Alex J. Valentine, Carl-Otto Ottosen and Eva Rosenqvist.

- Makonya GM, Ogola JBO, Muasya M, Crespo O, Maseko S, Valentine, AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2019. Chlorophyll fluorescence and carbohydrate concentration as field selection traits for heat tolerant chickpea (*Cicer arietinum* L.; Fabaceae) genotypes. Plant Physiology and Biochemistry.Volume 141, Pages 172-182.
- Makonya GM, Ogola JBO, Muasya M, Crespo O, Maseko S, Valentine, AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Intermittent moisture supply induces drought priming responses in some Chickpea (Cicer arietinum L.; Fabaceae) genotypes. Scientia Horticulturae (under review).

My supervisors have testified that I made substantial contributions to the conceptualisation and design of the papers and I independently ran the experiments and wrote the manuscripts, with their guidance in the form of comments and suggestions (see Appendix A)

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Dedication

To my late beloved mother, thank you for cultivating the zeal in me.

List of abbreviations

asl - above sea level

C_i - intracellular CO_2 concentration

DLI - daily light integral

E - rate of transpiration

F_q'/F_m' - operating quantum efficiency of Photosystem II photochemistry

$\mathrm{F_v/F_m}$ - maximal photochemical efficiency of photosystem II

$\mathrm{g_s}$, - Leaf stomatal conductance

IWUE - Intrinsic water use efficiency

LCMS - liquid chromatography mass spectrometry

PAGE - Polyacrylamide gel electrophoresis

PEP - Phospho-enoyl pyruvate

P_n - net photosynthetic rate

PPFD - photon flux density

ppm - parts/ million

PSII - Photosystem II

RH - relative humidity

R_n - night respiration

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RuBisCo - Ribulose-1,5-biphosphate carboxylase/oxygenase

RWC - Leaf relative water content

TCA - Trichloroacetic acid

T_{Max} - Maximum temperature

T_{Min} - Minimum temperature

VpdL - vapour pressure deficit

ABSTRACT

Chickpea (*Cicer arietinum*) is an important legume crop globally ranked third after dry bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum*). It constitutes 20% of the total global pulse production and around 95% of its production and consumption takes place in developing countries. Major constraints to chickpea production in sub Saharan Africa (SSA) have broadly been related to abiotic stresses, particularly drought and heat stresses, predicted to increase due to the global climatic changes. Due to the imperativeness of research for identifying heat tolerance markers for potential chickpea genotype selection, in chapter two of the thesis, the response of four chickpea genotypes to a natural temperature gradient in the field was assessed using chlorophyll fluorescence, non-structural carbohydrate, gas exchange and grain yield. Field experiments were carried out in two winter seasons at three locations with known differences in temperature in NE South Africa. Results showed two genotypes (Acc#3 and Acc#7) were tolerant to heat stress with an F_v/F_m of 0.83-0.85 at the warmer site, while the two sensitive genotypes (Acc#RR-2 and Acc#8) showed lower F_v/F_m of 0.78-0.80. Both chlorophyll fluorescence measurements: dark-adapted F_v/F_m and F_q'/F_m' (where $F_q' = F_m' – F$) measured at comparable high light levels correlated positively with grain yield. The two tolerant genotypes also showed higher photosynthetic rates, starch, sucrose and grain yield than the sensitive genotypes at the warmer site. However, these parameters were consistently higher at the cooler than at the warmer sites. It was concluded that genotypes Acc#RR-3 and Acc#7 are heat tolerant and chlorophyll fluorescence and leaf carbohydrates are suitable tools for selection of heat tolerant chickpea genotypes under field conditions. The coolest site of Polokwane showed favourable conditions for chickpea production. Heat and drought stresses are two abiotic factors that often occur simultaneously and are predicted to increase, consequently hampering plant growth. Response of different species to either stresses is well documented but information on the response of the same genotypes to both stresses in chickpea is limited. We aimed to determine whether previously noted heat stress tolerant genotype (Acc#7) is drought tolerant and the heat sensitive (Acc#8) is drought sensitive, and whether intermittent moisture supply at vegetative stage would induce priming effect to later drought at flowering. At vegetative stage, plants were divided into three groups, non-stressed (watered to 75% field capacity (FC), severe water stress (moisture-withholding for 14 days) and treated to 40% FC throughout the experiment (mild-stress), with recovery for the severely stressed plants after which they were stressed (double-stress) at flowering. Drought treatments at vegetative and flowering growth stages decreased physiological parameters and biomass accumulation in both genotypes except low water supply at 40% FC that decreased biomass in Acc#7 but not Acc#8. Double drought stress resulted in priming effect in Acc#7, having higher biomass, chlorophyll fluorescence, stomatal conductance, net photosynthesis, and relative water content in comparison to the introduction of stress only at flowering growth stage, as well as in comparison to Acc#8. These results showed that both Acc#7 and Acc#8 are sensitive to drought whereas after priming Acc#7 is better acclimated to drought than Acc#8 associated with osmotic adjustment on leaf relative water content (RWC) and higher capacity to protect photosynthetic activity, making Acc#7 potentially ideal for areas associated with intermittent drought spells. This observation, however, disapproved the hypothesis that Acc#7 is more drought tolerant than Acc#8 but is rather better acclimated than Acc#8, because of its superiority only in primed plants and not those stressed only at either vegetative or flowering stages. The findings emphasise the importance of matching chickpea physiological performance to expected rainfall amounts and distribution in drought prone areas during genotype selection. Chapter four of the thesis was an interrogative proteome analysis of the differences in the heat tolerant and sensitive chickpea (*Cicer arietinum* L.; Fabaceae) genotypes along a temperature gradient under field conditions which will help in identifying the molecular mechanisms involved in the crop's tolerance. Few studies have thus far combined chickpea physiological and proteome analysis to elucidate the changes in abundance and/or activity of relevant enzymes and expression of heat responsive proteins. In this study, analyses of chlorophyll concentrations, gas exchange, flavonoids and anthocyanin concentrations from a chamber experiment, as well as proteomic parameters from field studies in both the heat tolerant and sensitive genotypes are presented. The heat tolerant genotype Acc#7 maintained unaltered physiological performance at flowering growth stage when exposed to high (35/30°C) and moderate (30/25°C) heat stress, under climate chamber conditions compared to the two heat susceptible genotypes (Acc#RR-2 and Acc#8). Results from the proteomic studies showed an up-regulation in proteins related to protein synthesis (e.g. ribulose bisphosphate carboxylase/oxygenase activase), intracellular traffic (e.g. mitochondrial dicarboxylate/tricarboxylate transporter DTC), defence (e.g. HSP70) and transport (e.g. GTP-binding protein SAR1A-like) in heat tolerant Acc#7 compared to the susceptible Acc#8. Results from KEGG analyses support the involvement of probable sucrose-phosphate synthase and sucrose-phosphate phosphatase proteins in the starch and sucrose pathway, that were up-regulated in the heat tolerant genotype Acc#7. This result was in support of our earlier report where tolerant genotype Acc#7 had higher leaf starch and sucrose concentrations in comparison to the susceptible genotype Acc#8. The presence of these differentially regulated proteins including HSP70, ribulose bisphosphate carboxylase/oxygenase activase, plastocyanin and protoporphyrinogen oxidase shows their potential role in field grown chickpea tolerance to heat stress at flowering growth stage. In conclusion, chlorophyll fluorescence (both F_v/F_m and F_q'/F_m') and leaf carbohydrates were identified as selection markers that can potentially be used for chickpea phenotyping for heat stress under field conditions with the chlorophyll fluorescence parameters correlating positively with seed yield. Due to its higher biomass, chlorophyll fluorescence (F_v/F_m), stomatal conductance, net photosynthesis and RWC, heat tolerant genotype Acc#7 was identified to have better adaptive tolerance to drought stress after priming through exposure to intermittent dry spells than Acc#8. Furthermore, under controlled climate chamber conditions, Acc#7 consistently showed characteristics of tolerance to heat stress while Acc#RR-2 and Acc#8 were heat susceptible. Higher chlorophyll fluorescence, grain yield, chlorophyll concentrations, gas exchange, flavonoids and anthocyanin concentrations for Acc#7 compared to Acc#8 in the climate chamber was further validated by the higher up-regulation of proteins involved in protein synthesis, intracellular traffic, defence and transport in Acc#7 compared to Acc#8. The incorporation of proteomics in heat and drought stress studies will potentially help further the understanding of mechanisms by which the crop responds to these stresses.

Key words: chickpea; heat stress; intermittent drought; photochemical efficiency; leaf carbohydrates; thermo-tolerance; climate change; chlorophyll fluorescence; photosynthesis; priming; proteomics

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Chapter 1: Introduction and problem statement

1.1 Introduction

Chickpea (*Cicer arietinum* L.) is a vital legume crop globally ranked 3rd after dry bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum*) and constitutes 20% of the total global pulse production (FAOSTAT, 2018). The crop is a relatively cheap source of protein (23%), carbohydrates (40%), oil (6%) (Gil et al., 1996), and minerals (Mg, K, P, Fe, Zn, and Mn) (Ibrikci et al., 2003). The crop is generally grown under rainfed conditions, using either residual moisture in subtropical environments (dominated by summer rains), or rainfall in the Mediterranean environments (Yadav et al., 2006). It is believed to have originated in south east Turkey through to Iraq, in association with other crops like wheat and barley, 12 000 years ago (Abbo et al., 2003). Chickpea production spread into Sub Saharan Africa (SSA) through Ethiopia by the Iron Age (Abbo et al., 2003).

Domesticated chickpea and its wild relatives are in the genus *Cicer*, which is in the family Fabaceae within the tribe Cicereae (USDA, 2005). Domestication of chickpea came with its own changes that initially included loss of dormancy, larger plant sizes and variants with more erect habits: these traits form the basis of the current selection of desirable adaptive characteristics (Abbo et al., 2003). The domesticated chickpea crop is self-pollinated and pollination completes in the flower bud stage. It is an upright annual legume, 30 to 70 centimetres in height with primary, secondary and tertiary branching resembling a small bush (Monyo and Laxmipathi, 2014; Machado et al., 2004). There are two major types of chickpea under production globally (Monyo and Laxmipathi, 2014). The desi type genotypes have purple/pink coloured flowers and the grains are small, light to dark brown in colour, smooth or wrinkled and have a thick seed coat. In contrast, the kabuli chickpea types have white flowers, larger and whitish-cream coloured grains and have a thin seed coat (Bampidisa and Christodoulou, 2011).

Currently, chickpea is produced in over 40 countries across all continents but the major chickpea producing countries are India, Turkey, Pakistan, Iran, Mexico, Australia, Ethiopia, Myanmar, and Canada (Randhawa et al., 2014; Wubneh, 2016). It is grown on about 11 million hectares globally with 65% share mainly belonging to India (FAOSTAT, 2018; Muehlbauer and Sarker, 2017). Average global annual production of chickpea is about 12.1 million tonnes with 95% production and consumption occurring in developing countries (FAOSTAT, 2018; Muehlbauer and Sarker, 2017). In SSA, Ethiopia, Tanzania, Kenya and Malawi are major chickpea producers but the whole African continent contributes less than 4% of the global production (Toker and Yadav, 2010; Monyo and Laxmipathi, 2014). Most of the chickpea production (69%) in SSA, a mixture of desi and kabuli types, is concentrated in the near-equatorial East African region (Toker and Yadav, 2010; Monyo and Laxmipathi, 2014). Indeed, Ethiopia, Kenya, Malawi and Tanzania account for 52% of the total area and 76% of the total chickpea production in SSA with average yields of 1000 kg ha^{-1} compared to 769 kg ha^{-1} in the rest of SSA (Monyo and Laxmipathi, 2014).

Between the years 1961 and 2005, chickpea production in Africa rose by an average of 0.7% per year, associated with an annual increase of 0.5% in the area committed to chickpea production as well as a 0.2% yield increase per year (Toker and Yadav, 2010). The SSA region accounts for approximately 4% (398, 000 ha) of the global total area under chickpea production. A total of 36 countries in SSA are known to import chickpea mainly from Australia and India (Merga and Haji, 2019), Sudan being the highest importer accounts for 71% volume and 56% value, followed by South Africa, Mauritius, Malawi, Zambia, Zimbabwe, Niger, Madagascar, and Swaziland (Monyo and Laxmipathi, 2014). Due to this demand, chickpea production in SSA is projected to increase by 7.1% per annum, rising from 548,000 tonnes in 2010 to an estimated 1.125 million tonnes by 2020 (Kassie et al., 2009; Monyo and Varshney, 2016). Despite its high yield potential (4000 kg ha^{-1}), chickpea yields, at a global scale, are relatively low (910 kg ha^{-1}) due to a combination of biotic (diseases and insect pests) and abiotic (drought, heat and soil infertility) stresses (Awasthi et al., 2014).

In the SSA region, chickpea production is also constrained by management factors such as appropriate varieties, spacing and poor soil fertility (Monyo and Laxmipathi, 2014). Being a cool season crop, high temperatures during critical growth stages, like the reproductive period, can limit chickpea grain yield to a greater extent than warm season legumes (Devasirvatham et al., 2012a). Climate change is recognized as inevitable and one of the most complex challenges that humankind faces now and, in the future, with global simulation models predicting a 4 to 5°C increase in atmospheric temperatures by end of the century (Harris and Roach, 2016). This predicted rise in temperature, associated with changes in timing, magnitude and distribution of precipitation, are likely to increase drought and heat stress on crops as well as incidences of pests and diseases (Thorpe, 2005; Farooq et al., 2009; Mulwa et al., 2010). Winter crops, like chickpea, grown in subtropical regions where incidences of drought events and elevated temperatures are already relatively high, are likely to be affected significantly (Martin, 2015). There is overwhelming evidence that as climate has been changing, the amount of precipitation, intensity and frequency have also changed significantly (Trenberth, 2008). In addition, incidences of drought have markedly increased, especially in Africa, southwestern United States, Australia and in the Mediterranean region (Vadez et al., 2011). As temperatures rise, evaporation increases, land-surface dries and likely leads in increased incidence and severity of droughts (Leport et al., 1999). Climate simulations and empirical evidence concur and confirm that warmer climates, owing to increased water vapour, lead to more intense precipitation events even when the total annual precipitation is reduced slightly. (Trenberth, 2008). Therefore, a warmer climate has the potential to increase the risk of both drought (where there are no rains) and floods (where it would be raining in excess at different times and/or places). The ENSO cycle greatly affects the distribution and timing of floods and droughts, particularly in the tropics, hampering crop production (Vadez et al., 2011).

1.2. Drought stress

Three types of drought, i.e. meteorological, hydrological and agricultural drought, affect all forms of life directly or indirectly (Gan, 2004). Meteorological drought is regionally specific since atmospheric conditions resulting in precipitation deficiencies vary regionally and is defined on the degree of dryness and duration of the period (Wilhite, 2000). Hydrological drought is defined as the deficiency of water from the surface or sub-surface supplies, for example, from reservoirs, ground water and streams and conditions for this form of drought are built over extended time (Tallaksen et al., 2009). Agricultural drought, in contrast, links the various characteristics of either meteorological or hydrological drought to agricultural impacts focusing on precipitation shortages, differences between potential and actual evapotranspiration, soil water deficits, all of which affect plant growth and development (Wilhite, 2000). It is greatly influenced by changing climate and may arise collectively from insufficient rainfall, high temperatures and lower soil moisture during the growing season to sustain crop growth and yields (Vadez et al., 2011). Future climate projections show an increase in the intense rain events as well as reduced number of rain days leading to increased risk of drought (Vadez et al., 2011).

Drought in general, is one of the most important abiotic stresses which limit crop production in different parts of the world, with chickpea being no exception (Singh et al., 1987). Chickpea originated and is historically well adapted to the Mediterranean winter season conditions experiencing concurrently rain and relatively low temperatures. In Africa, where the majority of the chickpea production is grown in cool and dry winter and relies largely on residual moisture, incidences of drought stress are higher, including terminal droughts and mid-season dry spells during critical crop growth stages (Kumar and Abbo, 2001). Continued exposure to limited moisture conditions in the post-rainy season sowings may result in shortened life cycles and reduced dry matter production and water use efficiency (Singh et al., 1987).

Stomatal closure is one of the first responses to drought stress in plants, which ultimately results in declining stomatal conductance and hence conserve water (Blum et al., 1999). In water limited conditions, a growth retardant hormone abscisic acid, ABA, is produced in the roots, acting as a root-shoot signal eliciting stomatal closure and thus reducing stomatal conductance and transpiration (Cornic and Fresneau, 2002). Also, drought stress affects growth and yield of plants through its effects on membrane integrity, root depth and expansion, leaf area development, chlorophyll fluorescence, transpiration, proteins, and production of reactive oxygen species (Praba et al., 2009; Mafakheri et al., 2010; Wang et al., 2014a) but the effects of drought stress on grain yield is more severe during the flowering and reproductive stages (Barnabas et al., 2008; Leport et al., 2006; Leport et al., 1999). Drought stress reduces flower development time, resulting in flower abortion, small flowers with nectar of low quality and quantity, ultimately hindering embryo development due to lack of photosynthates (Leport et al., 1999). Drought stress typically causes oxidative damage to the photosynthesis apparatus by disrupting all its major components like stomatal control of CO_2 supply, reduction in carbon fixation and assimilate translocation (Zlatev and Lidon 2012), reduction in grain set and development and ultimately grain yield (Vadez et al., 2011).

Several shoot and root traits have also been noted to improve plant's resistance to drought (Araujo et al., 2015), with their contributions to superior plant growth dependent on the type of drought (early, intermittent and terminal), the agroecology in which the crop is grown, as well as genotype (Leport et al., 2006). Furthermore, plants tend to develop various physiological and biochemical responses, for adaptation, through different mechanisms such as drought escape, drought avoidance or drought tolerance (Praba et al., 2009). Through drought avoidance, plants can maintain relatively high tissue water content despite reduced moisture in the soil, through a variety of adaptive traits involving minimization of water loss (water savers) and optimized water uptake (water spenders) (Levitt, 1980). High leaf relative water content (RWC) has been associated with drought resistance, making it a valuable indicator of plant water status drought conditions (Abdallah et al., 2017), therefore making its maintenance through high leaf water status a key indicator for dehydration avoidance (Rahbarian et al., 2011). Through drought tolerance, plants tend to decrease their water potential by osmotic adjustments which increase osmolyte concentrations, while increasing water movement in the cells and tissues. Such adaptations delay metabolic damage and leaf senescence, and improve assimilate transport, thereby improving water absorption, stomatal conductance and CO_2 assimilation (Leport et al., 1999). Moreover, priming (pre-exposure to a moderate stress) could enhance the tolerance to subsequent stresses, a phenomenon known as stress memory (Valdés et al., 2013; Wang et al., 2014a; Abdallah et al., 2017).

Researchers in the SSA region have made inroads in trying to identify ways to mitigate drought risk and stabilize chickpea yields. Morpho-physiological root traits linked to drought tolerance in chickpea were noted in studies carried out in Kenya, where genotypes which had high root length density (RLD), root biomass, and total length did better under field drought conditions (Muriuki et al., 2016). In a study on identification of morpho-physiological characteristics associated with drought tolerance in selected chickpea germplasms in Nakuru and Baringo Counties of Kenya, drought tolerance index, biological mass, days to physiological maturity and days to 50% flowering were associated with drought tolerance due to their direct contribution to grain yield (Kirui and Njoka, 2013). In Ethiopia, eighteen drought tolerant genotypes were identified amongst a pool of different landraces, based on their drought tolerance indexes (Anbessa and Bejiga, 2002). Preliminary results from studies carried out in Limpopo Province of South Africa concluded that use of adapted chickpea genotypes and management practices like P fertilizer application, maximized water extraction from the soil and the efficiency with which the crop utilizes water (Ogola and Thangwana, 2013; Ogola et al., 2013); these may provide an option for improved productivity in most of Sub Saharan African countries.

However, more still needs to be done to address limitations to chickpea production brought about by drought stress. Root traits such as fibrous rooting system, root length, density and rooting depth have shown potential for use in chickpea genotype selection for terminal drought avoidance (Khan et al., 2010; Duc et al., 1994). Other research initiatives that can be exploited to address drought stress are low leaf conductance under limiting water conditions during the vegetative growth stages and low leaf expansion when plant growth is restricted under progressive exposure to stress. Adjustments to sowing time can influence critical crop growth stages such as flowering time and pod filling, and thus can reduce effects of drought during these growth stages (Mubvuma et al., 2015; Kumar and Abbo, 2001).

1.3 Heat Stress

With industrialization, natural environment deterioration and climate change, heat stress has become an increasingly important factor severely affecting global crop production (Harris and Roach, 2016; IPCC 2007). Crop productivity has been predicted to reduce with increases in temperatures (1°C to 2°C) at lower latitudes, especially in seasonal dry and tropical regions of the world (IPCC, 2007). High temperatures in various regions occur in combination with high solar irradiance, drought and strong winds, all which can aggravate plant injury (Hall, 1992; Wahid et al., 2007).

Chickpea has a relatively narrow genetic base, making development of heat stress tolerant cultivars a major challenge (Abbo et al., 2003). Being a cool season crop, high temperatures during critical growth stages like the reproductive period can limit chickpea grain yield compared to warm season legumes like soybean, pigeon pea and groundnut (Summerfield et al., 1984; Devasirvatham et al., 2012a). Crops in the subtropics normally experiencing cool temperatures (5°C to 10°C) during vegetative stages, tend to experience high temperatures beyond 30°C during the day over the reproductive development phase (Summerfield et al., 1984; Devasirvatham et al., 2012a).

Elevated temperatures adversely affect germination, photosynthesis, membrane stability, nutrient absorption, hormone activity, pod set, pod development, seed set, seed quality and quantity as well as protein synthesis (Wahid et al., 2007; Summerfield et al., 1984). At temperatures greater than 30°C, severe cellular injury and even death may occur within minutes, due to collapse of cell organization (Siddique et al., 1999). Similar elevated temperatures during seed filling stage accelerate leaf senescence, diminish seed set and seed weight, and ultimately reduce seed yield (Covell et al., 1986; Siddique et al., 1999). This has primarily been due to the diversion of resources, from reproductive growth, to heat shock response for maintenance of normal cell structure and function, as they cope with the heat stress, limiting availability of photosynthates for reproductive development (Siddique et al., 1999). Reduced photosynthetic rates and carbon assimilation, as well as high transpiration rates, tend to occur during high temperature stress, leading to reduced plant establishment and reduced carbon reserves (Singh et al., 1987; Mathur et al., 2011). Reduction in photosynthesis is attributed partly to thermal instability of Rubisco and inhibition of the electron transport chain and Photosystem II (Mathur et al., 2011; Brestic et al., 2012), primarily limiting photochemistry (Baker and Rosenqvist, 2004). This has been associated to the heat induced increase in thylakoid membrane fluidity and electron transport-dependent integrity of PSII (Prasad et al., 2008). Also, the heat stress induced damage and disruption of the integrity of thylakoid membranes causes photophosphorylation process to cease (Dias and Lidon, 2009). The inactivation of the PSII reaction centres after heat stress due to the damaged thylakoid membranes (composed of different types of lipids together with a significant amount of protein), has also been associated with the phase changes and ultimately the separation that the lipid components of the thylakoid membranes go through (Sharkey and Zhang, 2010). Moreover, inhibition of PSII activity after exposure to heat stress usually results in reduced chlorophyll biosynthesis due to the deactivation of various enzymes (Dutta et al., 2009).

Plants acclimate to elevated temperatures by developing appropriate morphological, physiological and biochemical characteristics (Wahid et al., 2007). For example, although heat stress leads to misfolding of newly synthesised proteins and the denaturation of existing ones, it induces accumulation of heat shock proteins (HSPs) that prevent protein degradation (Wahid et al., 2007). The mechanism of the pathway to the synthesis of HSPs is composed of sensing the temperature that is connected to the signal transfer to the heat transcription factors, where the activation of gene expression occurs by binding to the HSE, a specific recognition sequence located in the region of gene activator in DNA (Larkindale et al., 2005). Furthermore, increased carbohydrate (*e.g.* sucrose and glucose) availability during heat stress exposure represents a vital physiological trait associated with heat stress tolerance (Liu et al., 2011). Previous studies have also shown that high carbohydrate availability during periods of heat stress was an important physiological trait associated with thermotolerance (Liu and Huang, 2000). Reductions in carbon accumulation in plants could result from higher levels respiration compared to photosynthesis, subsequently increasing the rate of carbon consumption due to heat stress (Wahid et al., 2007). Indeed sugars, particularly sucrose are important metabolic signals in plants, aiding in regulation of plant development and response to stresses through carbon allocation and sugar signalling (Liu and Huang, 2000; Wahid et al., 2007). High cell wall and vacuolar invertases activities as well as increased sucrose import into young tomato fruit contributed to heat tolerance, through an elevated sink strength and sugar signalling activities (Li et al., 2012), hence their potential use as thermotolerance selection tools. Heat stress is known to disrupt sexual reproductive success, with the pollen being most sensitive, in several legume species including chickpea (Devasirvatham et al., 2012a), and common bean *Phaseolus vulgaris* L. (Monterroso and Wien, 1990). Exposure to heat stress during flowering leads to yield losses due to the reduction of pollen viability, pollen production per flower and pod set in chickpea, hence their use as potential heat tolerance selection candidates in chickpea (Devasirvatham et al., 2012a). Proteome analysis has also been found to be a useful tool in the investigation of mechanisms by which plants respond to abiotic stress (Singh and Jwa, 2013). Proteins related to electron transport chain, heat shock and glycolysis have a significant role in protecting plants from heat stress (Liu et al., 2014). However, not many studies have thus far combined physiological and proteome analyses in the quest to elucidate changes in abundance of relevant compounds that protect and enhance plant survival under stressful environmental conditions (Wang et al., 2015).

Thermotolerance research in chickpea has been receiving global attention in the recent past, with studies by Devasirvatham et al. (2012a) identifying genotype ICCV 92318 as a source of heat tolerance in the semi-arid environments. Moreover, ICRISAT-Nairobi has received 123 lines of heat tolerant nursery (61 desi and 62 kabuli), suppling the best lines of desi and kabuli to Kenya and Tanzania. Seventeen desi and seventeen kabuli genotypes were evaluated in Tanzania, with the superior genotypes of both desi and kabuli recommended for further evaluation (Rao et al., 2012). 'Feed the Future' has set up an innovation laboratory in Ethiopia for climate resilient chickpea, with one of their primary objectives being to characterize wild chickpea varieties from representative ranges of environments by systematic phenotyping, ultimately identifying and quantifying the contribution of useful alleles. Various researchers collaborated and genetically dissected drought and heat tolerance in chickpea through genome-wide and candidate gene-based association mapping approaches, resulting in the identification of 312 Marker trait associations (MTAs) for use, after validation, in molecular breeding for superior drought and heat tolerant chickpea varieties (Muehlbauer and Sarker, 2017).

While genetic improvement is centred upon development of cultivars which tolerate heat stress and produce an economic yield, modification of cultural practices like plant density, planting time as well as soil and irrigation management can also minimize stress effects. This can be achieved by synchronizing stress sensitive growth stages with the most favourable time within the season. The heat tolerance mechanism, like that of tolerance to drought, could be homeostasis to high temperature by maturing early before rises in temperatures. A thorough understanding of chickpea morphological (leaf coloration and degree of leaf chlorosis, necrosis and mottling), physiological (chlorophyll fluorescence, maximum quantum yield and gaseous exchange) and biochemical characteristics (carbon partitioning, accumulation of heat shock proteins and phenolics) in response to heat stress is integral for improved chickpea thermotolerance (Zhao et al., 2010; Wahid et al., 2007). For example, measurement of chlorophyll fluorescence ((F_v/F_m and F_q'/F_m') has been used successfully as a quantitative assessment of inhibition or damage to the electron transport system (Baker and Horton, 1988) due to excessive light in several crops including maize (Sinsawat et al., 2004). The chlorophyll fluorescence parameter (F_v/F_m) reflects the maximum quantum efficiency of PSII photochemistry in dark adapted leaves (Baker and Rosenqvist, 2004), with a decrease in F_v/F_m resulting in lowering of maximum quantum yield of photosynthesis (Ögren, 1988). The relationships between primary photosynthetic reactions and chlorophyll fluorescence (F_v/F_m) are important as they provide information on the plant's photosynthetic capability as well as its acclimation capacity under stressful environmental conditions (Lichtenthaler, 1988; Brestic et al., 2018).The use of chlorophyll fluorescence parameters (F_v/F_m and F_q'/F_m') are becoming a common tool in plant heat stress response studies with emphasis on PSII photochemistry since the technique is relatively rapid, sensitive, non-destructive and can show damage before visible stress symptoms appear (Wilson and Greaves, 1990). However, the field application of chlorophyll fluorescence measurements also poses some challenges due to the mostly high and varying light levels (Chaerle et al., 2007), hence the need to iscolate the measurements to days when there is no apparent cloud cover. Drought and heat stress are key stress factors with potential impact on chickpea yield, therefore future breeding attempts should aim to generate new knowledge acquired on the processes, determining plant development and its responses when exposed to these stresses.

1.4 Problem statement

Despite widespread production and use in some parts of Africa, chickpea and other legume crops have historically been considered ‘orphan’ crops in SSA (Mabhaudhi et al., 2019). Given the focus on cereal crops like maize and wheat in South African farming systems, legumes like chickpea have not been considered as high value and important crops (Woomer et al., 2014). This has resulted in lack of public and financial investment in initiating and advancing their production (Moran, 2013). Over the years, South Africa has been importing chickpea from India, Australia and Canada worth at least US$ 1.1 million annually (Monyo and Varshney, 2016).

Potential chickpea production in South Africa may be affected by environmental conditions (rainfall, temperature and soil types) and appropriate agronomic practices. For example, more than 50% of South Africa is semi-arid, receiving average annual rainfall of 464 mm (Kruger and Nxumalo, 2017). The summer rainy seasons in South Africa run from November to February and the winter seasons between May and August, with the summers predominated by the major crops like maize, sorghum and wheat. The summer and winter seasons are characterized by average mean daily maximum air temperatures of 25-35°C and 17-25°C, respectively (Du Plessis, 2009). Chickpea, being of Mediterranean origin, would be ideal for the winter season in NE South Africa, making use of residual soil moisture as well as temperatures that are suitable for its growth and development on land normally left uncultivated (Nieuwenhuis and Nieuwelink, 2005).

There are 1.3 million smallholder farming households in South Africa on about 14 million hectares of agricultural land which are marginalized into regions of poor productive land with little infrastructural support and water resources (Du Plessis, 2009). Chickpea being a nitrogen fixing legume (Nasr Esfahani et al., 2014), would be an ideal option for the poor resource farmers that form most of the local farming communities, helping in replenishing soil fertility as well as providing them with protein rich supplements for themselves and surplus for sale.

Evaluation of crop germplasms for suitability in Southern African conditions is a prerequisite, for which all future breeding work is based. Development of high yielding varieties with broad genetic base and wider adaptation could help increase the chickpea productivity. Genotypes with high yield potential have the capacity to immensely contribute to adoption of chickpea production in South African. Table 1.1 shows genotype evaluation studies for chickpea yield in NE South Africa and other SSA countries where production has been carried out over the years and indicate great potential with yields ranging between 0.5-3.9 tonne/ ha. However, further research with a wide range of genotypes, plant densities, seasons and different sites based on temperature and moisture supply can better advance the potential for chickpea production in NE South Africa. Use of management practices that aid in maximising water extraction and efficiency of water utilization provide an option for improved productivity in dry environments. Studies on responses of chickpea to different planting densities for improved yields have been carried out in Limpopo, NE South Africa (Thangwana and Ogola, 2012; Ogola and Thangwana, 2013), with the results showing a general increase in grain yields and water use efficiency with increase in plant populations (Table 1.2). These results are consistent with studies carried out elsewhere in the SSA region (Kibe and Kamithi, 2007; Kamithi et al., 2009). However, further research, with the inclusion of wide-range of cultivars, planting densities, seasons and test sites have been recommended before definite conclusions can be drawn.

Table 1.1: Genotypic variation in chickpea grain yield (t ha^{-1}) from studies carried out in Kenya, Ethiopia and South Africa

Kenya (Mallu, 2015)			
Long rain		Short rain	
Genotype	*Grain yield (tha^{-1})	Genotype	*Grain yield (tha^{-1})
ICC 9636	3.2	ICC 1052	1.6
ICCV 97165 (check)	2.8	ICC 4182	1.6
ICC 3325	2.8	ICC 7867	1.5
ICC 8522	1.6	ICC 9002	0.5
ICC 11944	1.5	ICC 11942	0.5
ICC 9862	1.5	ICC 791	0.4

Ethiopia (Tilahun et al., 2015)	
Mean yield (5 sites)	
Genotype	*Grain yield (tha^{-1})
DZ-2012-CK-0001	2.3
DZ-2012-CK-0013	2.6
Arerti (standard check)	2.4
DZ-2012-CK-0006	1.7
DZ-2012-CK-0011	1.8
DZ-10-4 (local check)	1.5

South Africa (Thangwana and Ogola, 2012)		
	*Grain yield (tha-1)	
	Summer	Winter
Genotype		
Kabuli type		
ICCV97314	2.1	2.7
ICCV92337	2.4	2.6
ICCV97306	-	3.9
Desi types		
ICCV88202	1.3	3.7
ICCV97031	1.1	3.8
ICCV201	1	3.3
ICCV37	1	3.1

*Data presented is of 3 of the highest and 3 of the lowest yielding genotypes

Table 1.2: Effect of planting densities on chickpea grain yield (tha^{-1}) carried out in Kenya, and South Africa

Plant populations (plants/ha)	Grain yield (tha^{-1})		Reference
	Long rains	Short rains	
74,074	1.3	1	
88,889	2.1	1.3	
111,111	2.7	1.7	
148,148	3.3	2	Kamithi et al., 2009
Plant populations (plants/ha)	Grain yield (tha^{-1})		
	Summer	Winter	
200,000	1	2.8	
250000	1.2	3.6	
330000	2.1	3.6	Thangwana and Ogola, 2012

Although plant performances to either of the individual drought and heat stresses are relatively well-known, their physiological responses in more complex environments with multiple abiotic stresses, occurring simultaneously, is fragmentary (Farooq et al., 2009, Zhou et al., 2017). This reality further necessitates the need for studies to identify genotypes that are tolerant to both heat and drought stress (Asensi-Fabado et al., 2013; Sapeta et al., 2013; Kumar et al., 2013, Randhawa et al., 2014; Awasthi et al., 2014). The two stresses (drought and heat) represent an excellent example of two different abiotic stress factors that often occur simultaneously, especially in the arid and semi-arid regions (Mittler, 2006; Rizhsky et al., 2004; Jedmowski et al., 2015). The increased risk of simultaneous occurrences of both stresses, due to the ever-changing climate conditions, implies that global agriculture will have to face their deleterious effects on chickpea (Awasthi et al., 2014) and other crop plants (Rizhsky et al., 2004; Rollins et al., 2013). This threat to the advancement of chickpea production, due to warming climates, is expected to be more severe in the tropics and subtropics, where temperatures are already quite high (Serdeczny et al., 2017).

Measurement of chlorophyll fluorescence (F_v/F_m) has however been used successfully to quantitate the inhibition or the damage to the electron transport system (Baker and Horton, 1988) in several crops like maize (Sinsawat et al., 2004). This has primarily been due to the chlorophyll fluorescence parameter's ability to reflect the maximum quantum yield of PS11 photochemistry in dark adapted leaves (Baker and Rosenqvist, 2004), with its decrease resulting in the lowering of the maximum quantum yield of photosynthesis (Ögren, 1988). So, the primary relationship between photosynthetic processes and chlorophyll fluorescence (F_v/F_m) are vital as they provide an insight on the plant's photosynthetic capabilities as well as its capacity to acclimate under environmentally stressful conditions (Lichtenthaler, 1988; Brestic et al., 2018). This, together with the reality that the technique is relatively rapid, sensitive, non-destructive and can show damage before visible stress symptoms appear, further strengthens its use as a phenotyping tool for germplasm screening under field conditions (Wilson and Greaves, 1990).

Most of the studies on heat and drought stress in chickpea have however been on different sets of genotypes being exposed to either of the stresses in isolation (Leport et al., 1999, Devasirvatham et al., 2012a, Kumar et al., 2013, Randhawa et al., 2014), thereby resulting in limited information on the responses of the same sets of genotypes to both heat and drought stress. On the other hand, plant exposure to intermittent drought over time has been noted to induce priming responses which alter a plant's subsequent stress response by the production of a faster and /or stronger reaction, providing enhanced protection benefits (shown through higher RWC, photosynthesis, F_v/F_m and biomass yield) (Walter et al., 2011; Abdallah et al., 2017). The reality that most drought stress studies in chickpea have focused on terminal drought stress (Leport et al., 1999; Mafakheri et al., 2010; Purushothaman et al., 2016), with little to no evidence in literature on chickpea studies specifically focusing on intermittent drought stress with recovery periods, makes its exploration very important. Despite reports from past studies showing that heat stress leads to increased expression of heat shock proteins (Baniwal et al., 2004; Wahid et al., 2007), several studies have also shown that heat stress exposure resulted in expressions of other proteins associated with energy, metabolism, photosynthesis, detoxification, transport, signal transduction as well as defensive systems against diseases (Rollins et al., 2013; Li et al., 2013). Fewer studies have also combined chickpea physiological and proteome analysis to explain the changes in the activities and relative abundance of some enzymes and the expression of heat responsive proteins after exposure to heat stress.

An understanding of the plant phenological modifications due to heat and drought stress and their interaction with genotypes becomes vital in chickpea field and glass house studies. This is importantly so, as they form the basis for germplasm selections. The effects of heat and drought stresses in isolation and the possible ways to deal with them during the vegetative and reproductive stages using morphological, physiological and yield data have been documented in several crops (Leport et al., 1999; Devasirvatham et al., 2012a; Kumar et al., 2013; Randhawa et al., 2014; Sharma et al., 2005). However, this study intended on identifying thermo and drought tolerant chickpea genotypes through an established use of desirable physiological phenotyping and biochemical tools under glasshouse and field conditions for maintained grain yields under changing climates and as resource pools for plant breeding. It was also hypothesized that chlorophyll fluoresce (F_v/F_m) is a thermotolerance trait in chickpea under field condition in NE South Africa, with tolerant genotypes maintaining their photochemical efficiency under heat stress. It was also hypothesized that the heat tolerant genotypes would be tolerant to drought stress and will upregulate heat shock proteins.

The broad objectives of the study were to:

1. Assess the physiological performance and identify traits for thermotolerance in four chickpea genotypes (Acc#RR-2, Acc#RR-3, Acc#7 and Acc#8) exposed to heat stress along a temperature gradient in NE South Africa

2. Assess if the identified heat tolerant genotypes were also drought tolerant

3. To identify the molecular mechanisms involved in chickpea thermotolerance using proteome analysis

Chapter 2: Chlorophyll fluorescence and carbohydrate concentration as field selection traits for heat tolerant chickpea genotypes

2.1 Introduction

Measurement of chlorophyll fluorescence has been used successfully as a quantitative assessment of inhibition or damage to the electron transport system (Baker and Horton, 1988) in several crops including maize (Sinsawat et al., 2004). The chlorophyll fluorescence parameter (F_v/F_m) reflects the maximum quantum efficiency of PSII photochemistry in dark adapted leaves (Baker and Rosenqvist, 2004), with a decrease in F_v/F_m resulting in lowering of maximum quantum yield of photosynthesis (Ögren, 1988). The relationships between primary photosynthetic reactions and chlorophyll fluorescence (F_v/F_m) are important as they provide information on the plant's photosynthetic capability as well as its acclimation capacity under stressful environmental conditions (Lichtenthaler, 1988; Brestic et al., 2018).The use of chlorophyll fluorescence is becoming a common tool in plant heat stress response studies with emphasis on PSII photochemistry since the technique is relatively rapid, sensitive, non-destructive and can show damage before visible stress symptoms appear (Wilson and Greaves, 1990). A group of other fluorescence parameters called the JIP-test that quantify the stepwise flow of energy through PSII using input data from fluorescent transient have, in some studies, shown a greater sensitivity to plant heat stress (Jiang et al., 2006; Brestic et al., 2012; Brestic and Zivcak, 2013). We, however, opted to use F_v/F_m test in this study because in a previous experiment (Sharma et al., 2012), it was noted that the F_v/F_m test had no genetic component of the variation in control conditions in climate chamber experiment, while the JIP-test parameters showed an increase in the genetic component in both the heat stress and the control plants.

Therefore, the validation of the relationships between measured chlorophyll fluorescence (F_v/F_m) and carbohydrate accumulation with plant agronomic performance will strengthen the use of these markers as phenotyping tools during germplasm screening under field conditions.

The objectives of this study were to determine (i) response of chlorophyll fluorescence of chickpea to a temperature gradient under field conditions, and (ii) the effect of heat stress on non-structural carbohydrates and gas exchange in four chickpea genotypes.With this study, we intend to establish the use of chlorophyll fluorescence and leaf carbohydrate concentrations for identifying thermotolerant genotypes with desirable agronomic traits under field conditions in southern Africa for the maintenance of higher grain yields under warming climates and as genetic resources for plant breeding.

2.2 Materials and methods

2.2.1 Study Sites

Field experiments were conducted in north eastern South Africa at three sites during the winter seasons of 2016 and 2017. The winter growing season traditionally falls between April and August (Thangwana and Ogola, 2012). The three sites included the University of Venda experimental farm in Thohoyandou (22°35’ S and 30°15’ E and 595 m asl), Vhugela River Queen farm in Louis Trichardt (23°02′ S; 29°54′ E and 495 m asl) and the University of Limpopo experimental farm in Polokwane (23°49’ S; 29°41’ E and 389 m asl). The straight-line distance between the two furthest sites (Venda and Polokwane) is 149 km. Automatic weather stations located approximately 100 m from the experimental plots recorded rainfall (mm), maximum and minimum air temperatures (°C), and relative humidity (%) each day during the experiments. The three sites are along a temperature gradient with average minimum and maximum winter temperatures of 12–24°C (Venda), 7–22°C (Louis Trichardt) and 4–20°C (Polokwane) characterized by different soils (Table 2.1), cumulative monthly rainfall and average maximum air temperatures for 2016 (Fig. 2.1a) and 2017 (Fig. 2.1b). The University of Venda and University of Limpopo experimental farms generally had the highest and lowest minimum and maximum air temperatures of the three sites throughout 2016 and 2017 respectively. The rainfall predominantly falls in the summer season and little to no rainfall in the winter season, with Venda receiving the highest cumulative rainfall and Polokwane the least rainfall of the three sites. Global radiation was measured daily and recalculated to daily light integral (DLI) across the environments. Pre-sowing analyses of soil physiochemical properties were carried out in all the three sites at the start of the study in 2016 (Table 2.1). Prior to analysis, soils were air-dried and sieved through a 1-mm mesh. The concentration of total N in the soil was measured using mass spectrometry at the Archaeology Department at the University of Cape Town. Samples were combusted in a Flash EA 1112 series elemental analyser (Thermo Electron) and the gases were passed to a Delta Plus XP isotope ratio mass spectrometer (Thermo Finnigan), via a Conflo III gas control unit (Thermo Finnigan). The P concentration in the extract was then determined via inductively coupled plasma optical emission spectrometry (Varian). Available P (Bray II P) was determined using the molybdenum blue method as per Bray and Kurtz (1945). Soil concentrations of C, Ca, Fe, K, Mg and Na were analysed at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenberg, Stellenbosch, South Africa (Non-Affiliated Soil Analysis Working Committee 1990).

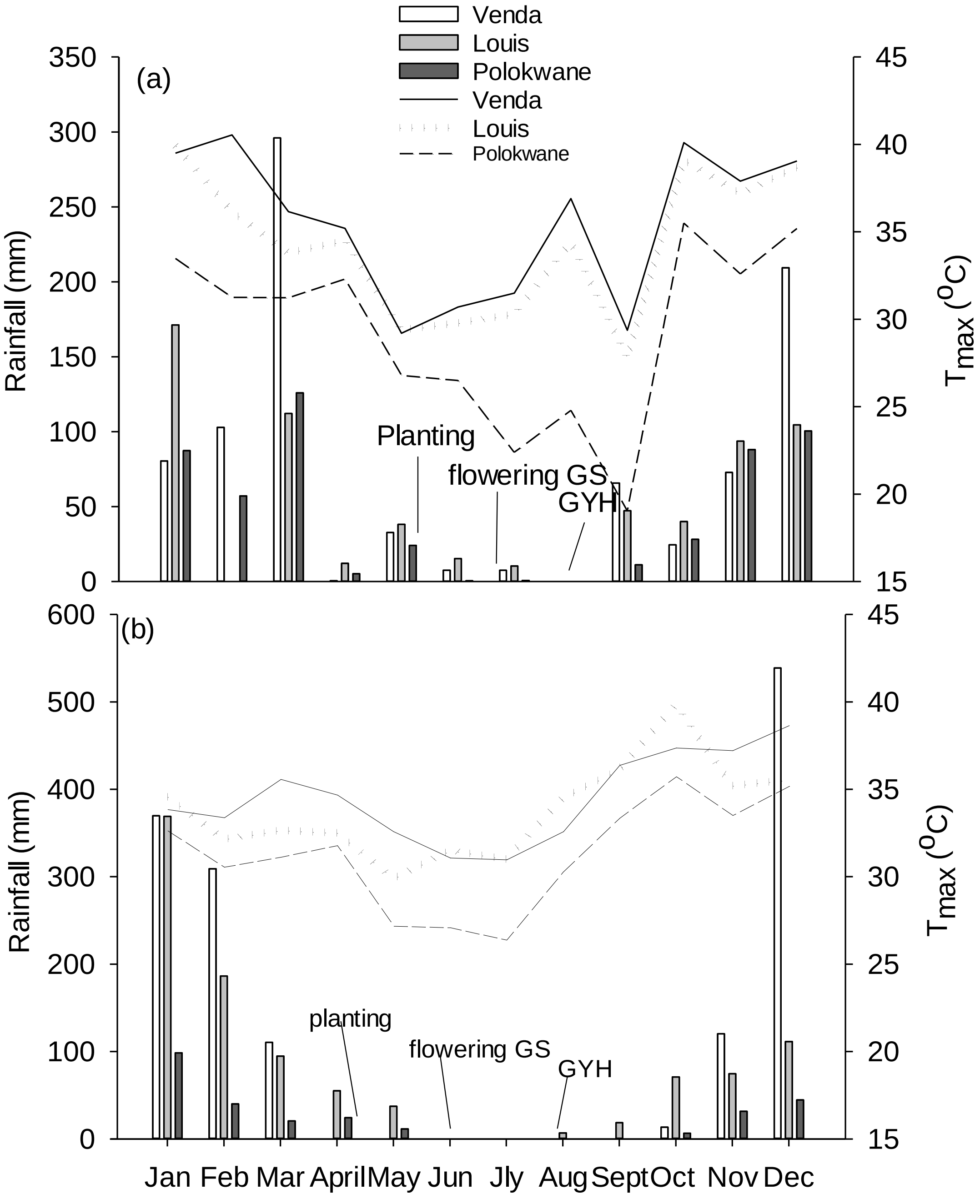

Figure 2.1: Summary of total rainfall and average minimum and maximum monthly temperatures at the three experimental sites for (a) 2016 and (b) 2017 cropping seasons. The experimental sites are Venda (University of Venda experimental farm, Thohoyandou), Louis (Vhugela River Queen Farm, Louis Trichardt) and Polokwane (University of Limpopo experimental farm, Polokwane). Flowering GS is flowering growth stage and GYH is grain yield harvesting. Rainfall is the monthly accumulated precipitation and T_{max} is the monthly mean daily maximum air temperature.

Table 2.1: Soil physiochemical analyses for the three experimental sites for 2016 cropping season. The sites are as in Fig. 2.1. Data is mean values ± se (n=8).

Site	Venda	Louis	Polokwane
Soil Type	*Clay*	*Sandy Loam*	*Sandy loam*
Carbon (%)	2.7^{7a}	1.42^{a}	0.32^{c}
pH	5.10^{b}	5.88^{a}	5.18^{b}
Total P (mg/kg)	209.3^{a}	154.2^{b}	74.3^{c}
Bray II P (mg/kg)	10.00^{c}	51.00^{a}	27.75^{b}
N (%)	0.08^{b}	0.12^{a}	0.05^{c}
K(mg/kg)	311.00^{ab}	368.50^{a}	146.75^{b}
Ca (cmol/kg)	7.34^{a}	4.93^{b}	2.48^{c}
Mg (cmol/kg)	2.46^{b}	3.34^{a}	3.65^{a}
Fe (mg/kg)	126.70^{b}	327.00^{a}	131.02^{b}

2.2.2 Plant material, management and experimental design

The experiments consisted of a factorial treatment combination of the three sites and four desi type chickpea (*Cicer arietinum* L.) genotypes (Acc#RR-2, Acc#RR-3, Acc#7 and Acc#8). Genotypes were selected based on their superior grain yield potentials from experiments previously carried out in N. E South Africa. At each site, the treatments were arranged in randomized complete block design and replicated four times. Each treatment consisted of a plot measuring 3.2 × 1.2 m with nine rows of chickpea. Spacing between plots and blocks was 0.5 m and 1 m, respectively. The plots were fertilized at planting by superphosphate fertilizer (20.3% P with 60 kg P ha^{-1}) and nitrogen (N) as limestone ammonium nitrate (LAN 28% N with 20 kg N ha^{-1}) (NTK, South Africa**).** The winter 2016 experiments were sown between the 7th to the 12th of May and the 2017 experiments were planted between the 14th and 19th of April. Field sowing was done manually at a spacing of 0.4 m inter-row and 0.1 m intra-row spacing. All the plots were watered uniformly after sowing to promote even germination, emergence and crop establishment. Supplemental irrigation was applied in all three experiments when necessary. Experimental plots were weeded throughout the growing seasons in all the three sites. A net was used to cover the plants at physiological maturity to deter monkeys and birds herbivory.

2.2.3 Chlorophyll fluorescence

Following the fluorescence nomenclature proposed by Baker and Rosenqvist (2004), leaf chlorophyll fluorescence values, including minimal fluorescence F_o, Maximum Fluorescence F_m, variable fluorescence F_v and the maximum photochemical efficiency of photosystem II F_v/F_m ($F_v = F_m - F_o$), were taken at the early reproductive stage on the youngest, fully expanded leaf using a PAM-2100 portable chlorophyll fluorometer (Walz, Eiffeltrich, Germany). Chlorophyll fluorescence readings were taken at night from 1900 hours onwards to enable prior dark adaptation (sun sets between 1730 hours and 1800 hours in winters of N.E. South Africa). Five plants from each of the sixteen plots were randomly selected from the four inner-most rows, clamped on using light-exclusion clips (Walz, Eiffeltrich, Germany) and readings recorded. The operating efficiency of PSII (F_q'/F_m') (where $F_q' = F_m' - F$) and leaf temperature readings were taken and recorded during the day from 0800 hours on five randomly selected plants from each of the sixteen plots from the four inner most rows with the fiber optics attached to leaf clip holder 2030-B (Walz, Eiffeltrich, Germany). Corresponding time and photosynthetic photon flux density (PPFD) values were recorded simultaneously.

2.2.4 Gas exchange

Gas exchange variables including net photosynthetic rate (P_n), rate of transpiration (E), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and night respiration (R_n) were measured at CO_2 concentration of 400 µmol mol^{-1} using a LI-6400 portable photosynthesis system infrared gas analyser (LiCor, Lincoln, NE, USA) with an automatic cuvette of up to 6 cm^2 leaf area. Measurements were taken when the crop had reached 50% flowering growth stage. The gas exchange measurements were taken on well-watered plants to avoid moisture stress between 0800 hours and 12 noon on a sunny day (average sunrise and sunset times in NE South Africa in winter are 0530 hours to 1800 hours respectively). Five plants from each of the 16 plots were randomly selected from four inner most rows. Readings were taken from the youngest, fully expanded leaves that were allowed to equilibrate to 20°C cuvette conditions and at PPFD of ca 1000 µmol m^{-2} s^{-1} for three minutes. The same procedure at PPFD of 0 µmol m^{-2} s^{-1}was repeated at night from 19 00 hours until 2300 hours for respiration measurements.

2.2.5 Non-structural carbohydrates

During the flowering stage, leaf samples were collected, and oven dried for 48 hours at 70°C. Dried samples were finely ground using a Hammer Mill (United Scientific Pty Ltd, Pretoria, South Africa) for analysis of the non-structural carbohydrates. A glucose stock solution (1.0 mg ml^{-1}) packed with the GAHK-20 kit was used to make standard solutions containing 0 to 5 mg ml $^{-1}$ glucose by diluting the stock solution with deionized water prior to analysis. Concentrations of fructose, glucose and starch were determined using an enzymatic method as described by Zhao et al. (2010). In this method, 70 mg of ground tissue was mixed with 2 ml 80% ethanol and heated at 80°C in a water bath for 15 minutes. The same sample was further extracted 2 more times and centrifuged for 10 minutes at 1811×g in an Eppendorf 5810 R Centrifuge (Hamburg, Germany).

Three supernatants from the same sample were then combined and brought to 6 ml final volume with 80% ethanol. Finely ground 60mg of activated charcoal was added into each tube and briefly shaken to mix contents. After being left to stand for 5 minutes, the tubes were centrifuged at 1811×g for 15 minutes to obtain clear extracts. Clear aliquots from these samples were transferred into fresh tubes and used for glucose, sucrose and fructose analyses. Glucose concentrations were determined using a glucose hexokinase (HK) assay reagent kit Sigma-Aldrich, Inc. (St Louis, MO, USA) on a microplate reader. Fructose, the second of the three assays was initiated by addition of phosphoglucose isomerase to each well with the glucose aliquots and resultant absorbance obtained as the sum of glucose and fructose. Addition of the invertase enzyme is the initial step in the determination of the final sucrose assay by obtaining the overall sum of glucose, fructose and sucrose equivalent concentrations as glucose. Subsequently, sucrose concentration was determined using the following equation:

$$Sucrose = \left[overall \sum of\ glucoseequivalents - (glucose + fructose)\right] \times 0.96$$

Where 0.96 accounts for a water molecule added during sucrose hydrolysis. Starch concentration was determined by measuring glucose in the aliquot of the supernatant after hydrolysis of starch in the sample residue remaining after extraction of the non-structural carbohydrates. Hydrolysis was done using amyloglusidase and the glucose released was measured as described above. All absorbances were obtained spectrophotometrically at 340 nm on a ThermoMultiskan Plate reader (ThermoScientific, USA). Starch concentration was then calculated according to glucose concentrations in the tissue residue multiplied by 0.9 to account for water loss when glucose units are linked in starch formation (Zhao et al., 2010).

2.2.6 Total biomass, grain yield and yield components

Whole plant biomass was quantified by harvesting three adjacent plants within randomly selected rows in each plot at the flowering stage (9-13 leaf stage). Plant samples were dried at 70°C for 48 hours and biomass weight recorded. Grain yield was determined at harvest maturity from 20 plants in the four inner most rows (five successive plants within a row) of the total nine rows in a plot. Pods were removed from the plants, threshed and seed air dried to 12% seed moisture and weighed to obtain total grain yield in kg ha^{-1}. Total pod number and 100 seed weight were also determined.

2.2.7 Statistical analysis

Data was analysed by two-way analysis of variance (ANOVA) to test for the significance of the different environments and the four genotypes on each measurable variable. The Tukey’s Honest Significant Difference (HSD) test was used to separate means that were significantly different ($P<0.05$).

2.3 Results

2.3.1 Chlorophyll fluorescence parameters

The daily light integral (DLI), which is the number of photons in the photosynthetic range integrated over the day (Poorter et al., 2016) were measured across all sites during the cropping seasons and were relatively comparable, ranging from 26.6 to 47.3 mol m^{-2} d^{-1} and 31.4 to 54.7 mol m^{-2} d^{-1} in 2016 and 2017 seasons respectively (data not shown). The interaction of the genotypes and environment for F_v/F_m was significant ($p<0.05$) for the plants grown in the 2017 season. At the cooler site of Polokwane, Acc#7 had higher ($p<0.05$) F_v/F_m (0.86) compared to the other three genotypes with no significant decline at Venda (Fig. 2.2). The F_v/F_m values for the other three genotypes were similar except that there was a significant decline from Polokwane to Venda for Acc#RR-2 whereas there was no decline in F_v/F_m for Acc#RR-3 and Acc#8. Noteworthy is that Acc#8 recorded lower F_v/F_m values than Acc#7 at all sites whereas the values for Acc#RR-3 were lower than Acc#7 only at Polokwane sites (Fig. 2.2). The values of F_q'/F_m' at the cooler site in Polokwane were greater ($p<0.05$) than those in Venda both in 2016 and 2017 seasons (Table 2.2). However, the F_q'/F_m' values at Louis Trichardt were similar to those in Venda in 2016 and to those in Polokwane in 2017 season. The PPFD at the time of data collection in 2016 were significantly higher ($p<0.001$) in Venda than in Polokwane which was similar to Louis Trichardt (Table 2.2). In 2017, however, the PPFD values on the day of data collections were similar across all environments. Genotypic differences in F_q'/F_m' were observed in both years where Acc#7 and Acc#RR-3 showed similar but significantly ($p<0.001$) higher values than Acc#RR-2 and Acc#8 which were also similar.

2.*3.2 Gas exchange parameters*

There was no genotype by environment interactions for all the measured gas exchange parameters in neither 2016 nor 2017 (Table 2.3). There were environmental differences ($p<0.05$) on net photosynthesis in 2016 with the cooler Polokwane site recording highest ($p<0.05$) P_n (8.5 µmol CO_2 m^{-2} s^{-1}) and Venda the lowest P_n (6.9 µmol CO_2 m^{-2} s^{-1}) (Table 2.3). In 2017, the warmer site of Venda showed higher ($p<0.001$) stomatal conductance g_s (0.39 mol H_2O m^{-2} s^{-1}), internal carbon dioxide concentration (C_i = 309 µmol CO_2 mol^{-1}) and leaf transpiration rate (E = 2.8 mmol H_2O m^{-2} s^{-1}) than the other two sites. Results for leaf temperatures varied between the years being higher ($p<0.05$) at the warmer Venda site than at Polokwane in 2016, while the highest leaf temperatures (24°C) were observed at Louis Trichardt site in 2017. The genotypic differences were significant

($p<0.01$) on P_n (Table 2.3) 2017 where genotype Acc#7 was similar to Acc#RR-3, but higher ($p<.01$) than the two similar Acc#RR-2 and Acc#8 genotypes.

The warmer Venda site showed higher ($p<0.01$) R_n values than the cooler Polokwane site in both years with Louis Trichardt values similar to Polokwane in 2016 and similar to Venda in 2017 (Fig. 2.3a and b). There were no genotypic differences in R_n in 2016 while in 2017, R_n in Acc#7 were similar to Acc#8 and Acc#RR-2, but higher ($p<0.01$) than Acc#RR-3.

2.3.3 Non-Structural carbohydrates

Genotype and environment did not have interactive effect on concentration of non-structural carbohydrate in leaves of plants grown in 2017. The concentration of starch in plants at the cooler Polokwane site was lower ($p<0.05$; Fig. 2.4a) than that at the warmer Venda site while that of sucrose ($p<0.001$; Fig. 2.4b) and glucose ($p<0.05$; Fig. 2.4c) were similar in the two sites. However, leaves of plants at Louis Trichardt recorded the highest ($p<0.05$) concentrations of starch and sucrose while that of glucose was the least relative to the other two sites (Figs. 2.4a, b, and c respectively). The concentration of starch also differed with genotypes where Acc#7 showed similar values to Acc#RR-3 and Acc#8, but higher ($p<0.05$; Fig. 2.4d) than Acc#RR-2. For the concentration of sucrose, Acc#7 showed similar values to Acc#RR-3 and Acc#8 but higher ($p<0.05$; Fig. 2.4e) than Acc#RR-2. The concentration of glucose in leaves of Acc#7 was similar to that in Acc#8 and Acc#RR-2 but significantly higher ($p<0.01$; Fig. 2.4f) than that of Acc#RR-3.

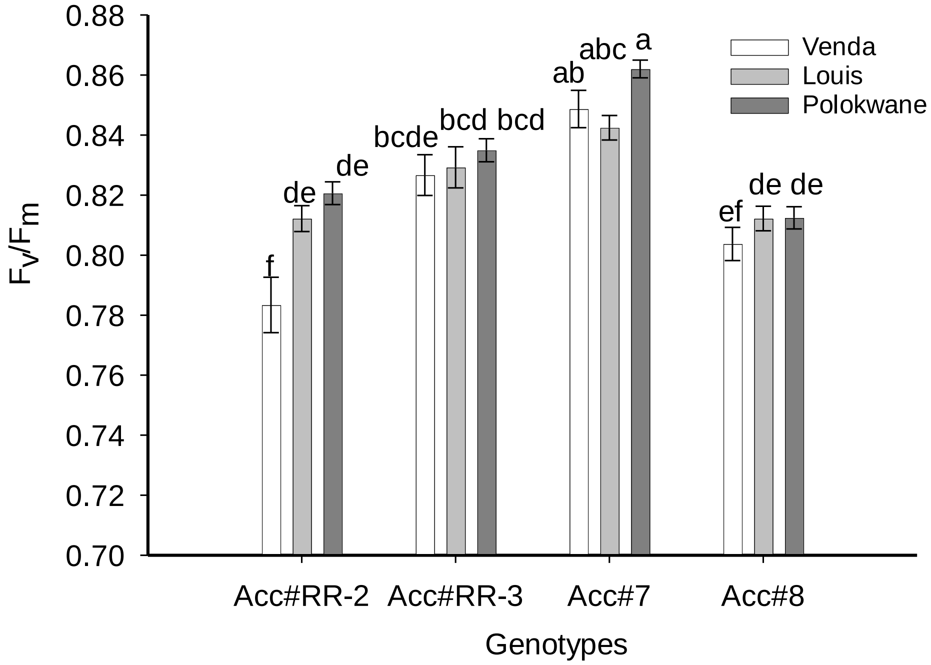

Figure 2.2: Interaction of (a) genotype and environment on maximum quantum yield of PSII (F_v/F_m) at 50% flowering stage of chickpea in 2017 cropping season at 50% flowering stage of chickpea treatment in a growth climate chamber. Data is mean values ± se (n=4). Different letters indicate significant differences between genotypes and sites by Tukey's honest significant difference *post hoc* test ($p<0.001$).

Table 2.2: Effect of environment (growth site) and genotypes on operating efficiency of PSII (F_q'/F_m') and average PPFD (µmol m^{-2} s^{-1}) at time of data collection of chickpea in 2016 and 2017 cropping seasons. The data is mean values ± se (n=16 for environment and n = 12 for genotype) with different letters showing significant difference as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant. The sites are as in Fig. 2.1.

Treatment	F_q'/F_m'		PPFD, µmol m^{-2} s^{-1}	
Site	*2016*	*2017*	*2016*	*2017*
Venda	0.66 ± 0.003^{b}	0.53 ± 0.007^{b}	1156.2 ± 25.9^{a}	1147.6 ± 28.0^{ns}
Louis	0.65 ± 0.006^{b}	0.57 ± 0.009^{a}	978.5 ± 45.3^{b}	1121.0 ± 35.9^{ns}
Polokwane	0.69 ± 0.005^{a}	0.59 ± 0.008^{a}	887.0 ± 17.8^{b}	1062.2 ± 20.5^{ns}
Genotypes				
Acc#RR-2	0.66 ± 0.007^{b}	0.53 ± 0.008^{b}	995.2 ± 55.2^{ns}	1041.3 ± 24.4^{ns}
Acc#RR-3	0.67 ± 0.007^{a}	0.59 ± 0.008^{a}	988.3 ± 56.1^{ns}	1136.3 ± 37.7^{ns}
Acc#7	0.67 ± 0.008^{a}	0.64 ± 0.009^{a}	1047.1 ± 42.6^{ns}	1160.1 ± 35.3^{ns}
Acc#8	0.65 ± 0.008^{b}	0.52 ± 0.008^{b}	998.4 ± 59.3^{ns}	1105.4 ± 30.1^{ns}
F probability				
Site	29.7***	16.0***	15.3***	2.5^{ns}
Genotype	9.1***	25.9***	0.4^{ns}	2.3^{ns}
Site*Genotype	2.2^{ns}	1.7^{ns}	0.3^{ns}	1.0^{ns}

Table 2.3: Effect of environment (growth site) and genotypes on gas exchange parameters at 50% flowering stage of chickpea in 2016 and 2017 cropping seasons measured at a PAR of 1000 µmol m^{-2} s^{-1}. The data is mean values ± se (n=16 for environment and n = 12 for genotype) with different letters showing significant difference as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant. The sites are as in Fig. 2.1. The reason for the Ci values to be over 400 is not known.

Treatments	Net photosynthetic rate (P_n),µmol m^{-2} s^{-1}		Stomatal conductance (g_s), mol m^{-2} s^{-1}		Intercellular CO_2concentration (C_i), ppm		Transpiration rate (E), mmol m^{-2} s^{-1}		Vpd$_{Leaf}$, kPa		T$_{Leaf}$, °C	
Site	*2016*	*2017*	*2016*	*2017*	*2016*	*2017*	*2016*	*2017*	*2016*	*2017*	*2016*	*2017*
Venda	6.9 ± 0.48^{b}	13.3± 0.59	0.29±0.07	0.39 ± 0.03^{a}	286.5±39.9	308.6 ± 6.31^{a}	2.1±0.23	2.8 ± 0.21^{a}	0.9±0.04	0.8 ± 0.01^{c}	20.8 ± 0.26^{a}	21.0 ± 0.12^{c}
Louis	-	12.4± 0.38	-	0.17 ± 0.01^{b}	-	234.7 ± 9.52^{c}	-	2.2 ± 0.12^{b}	-	1.3 ± 0.02^{a}	-	24.0 ± 0.18^{a}
Polokwane	8.5 ± 0.42^{a}	12.4± 0.39	0.25±0.02	0.21 ± 0.01^{b}	319.1±1.9	261.5 ± 5.84^{b}	2.4± 0.12	2.0 ± 0.10^{b}	0.9±0.02	0.9 ± 0.02^{b}	19.4 ± 0.15^{b}	22.0 ± 0.04^{b}
Genotypes												
Acc#RR-2	7.1±0.71	12.6 ± 0.42^{bc}	0.35±0.12	0.23±0.03	325.6±14.8	407.0±1.23	2.4±0.35	2.1± 0.22	0.9±0.06	1.0±0.07	19.9±0.48	22.1±0.41
Acc#RR-3	6.7±0.71	13.5 ± 0.60^{ab}	0.21±0.02	0.27±0.04	320.2±10.3	420.4±5.63	2.0±0.13	2.4± 0.22	1.0±0.03	1.0±0.06	20.2±0.41	22.1±0.31
Acc#7	8.2±0.71	14.2 ± 0.56^{a}	0.22±0.03	0.28±0.04	244.3±27.1	423.7±6.53	2.0±0.29	2.5± 0.18	0.9±0.02	1.0±0.06	20.2±0.43	22.0±0.35
Acc#8	8.9±0.48	11.7 ± 0.56^{c}	0.30±0.04	0.25±0.03	321.2±8.03	419.8±5.13	2.6±0.22	2.2± 0.17	0.9±0.04	1.0±0.07	20.3±0.41	21.9±0.33
F probability												
Site	7.6*	1.7^{ns}	0.4^{ns}	24.1 ***	0.7^{ns}	22.4***	1.8^{ns}	6.9**	3.2^{ns}	163.4***	16.0*	87.4***
Genotype	2.9^{ns}	5.1**	0.9^{ns}	0.7^{ns}	0.9^{ns}	0.7^{ns}	1.2^{ns}	1.1^{ns}	1.3^{ns}	0.6^{ns}	0.2^{ns}	0.4^{ns}
Site*Genotype	0.3^{ns}	2.2^{ns}	0.7^{ns}	0.2^{ns}	1.0^{ns}	0.2^{ns}	0.6^{ns}	0.2^{ns}	0.7^{ns}	0.6^{ns}	0.03^{ns}	0.3^{ns}

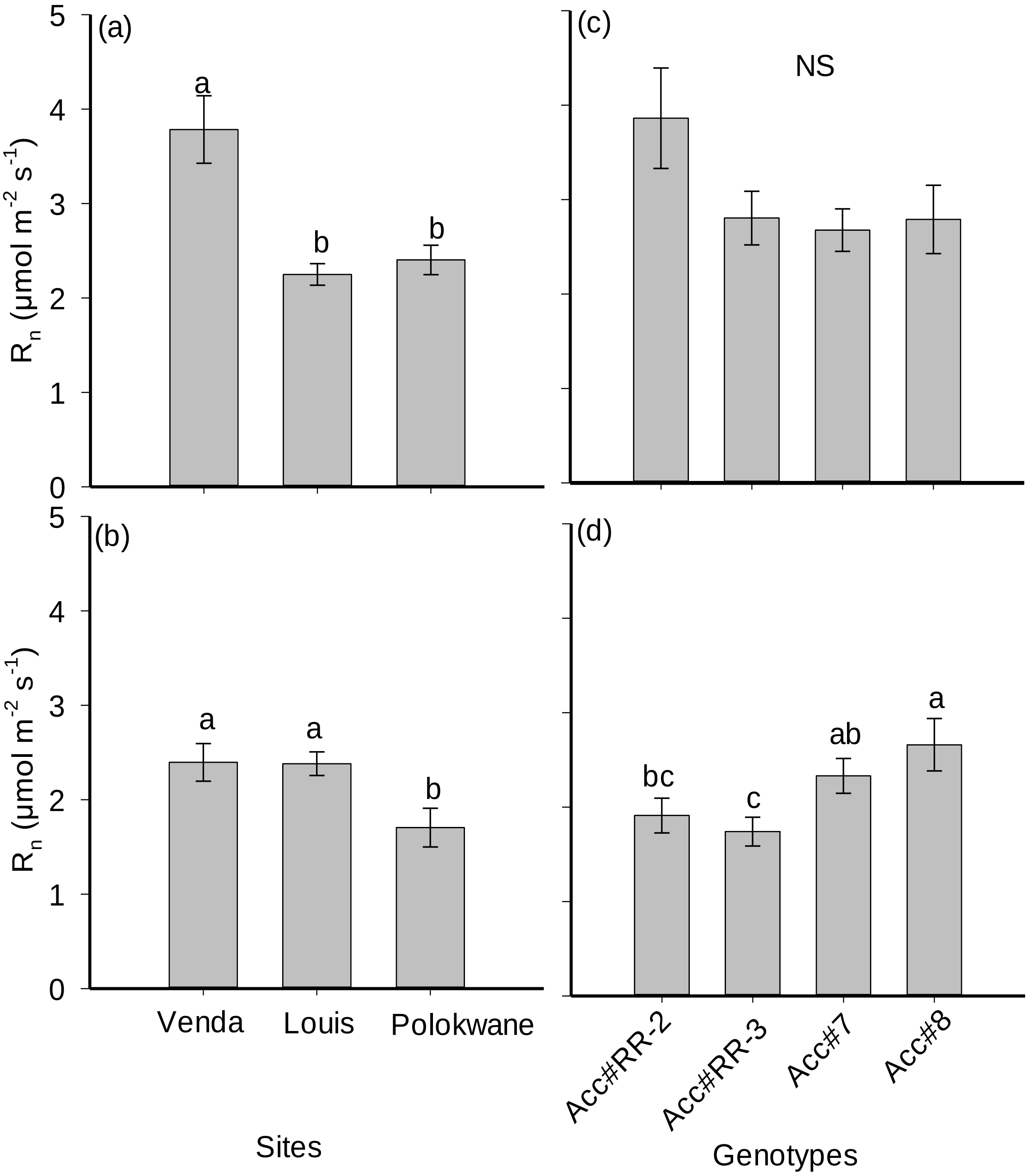

Figure 2.3: Effect of environment (a and b) and genotype (c and d) on respiration at 50% flowering stage of chickpea in 2016 (a and c) and 2017 (b and d) cropping season. Data is mean values of pooled sites or genotypes ± se (n=16 for environment and n = 12 for genotype). Different letters indicate significant differences between genotypes or site by Tukey's honest significant difference *post hoc* test ($p<0.001$) in respective figure; ns = not significant

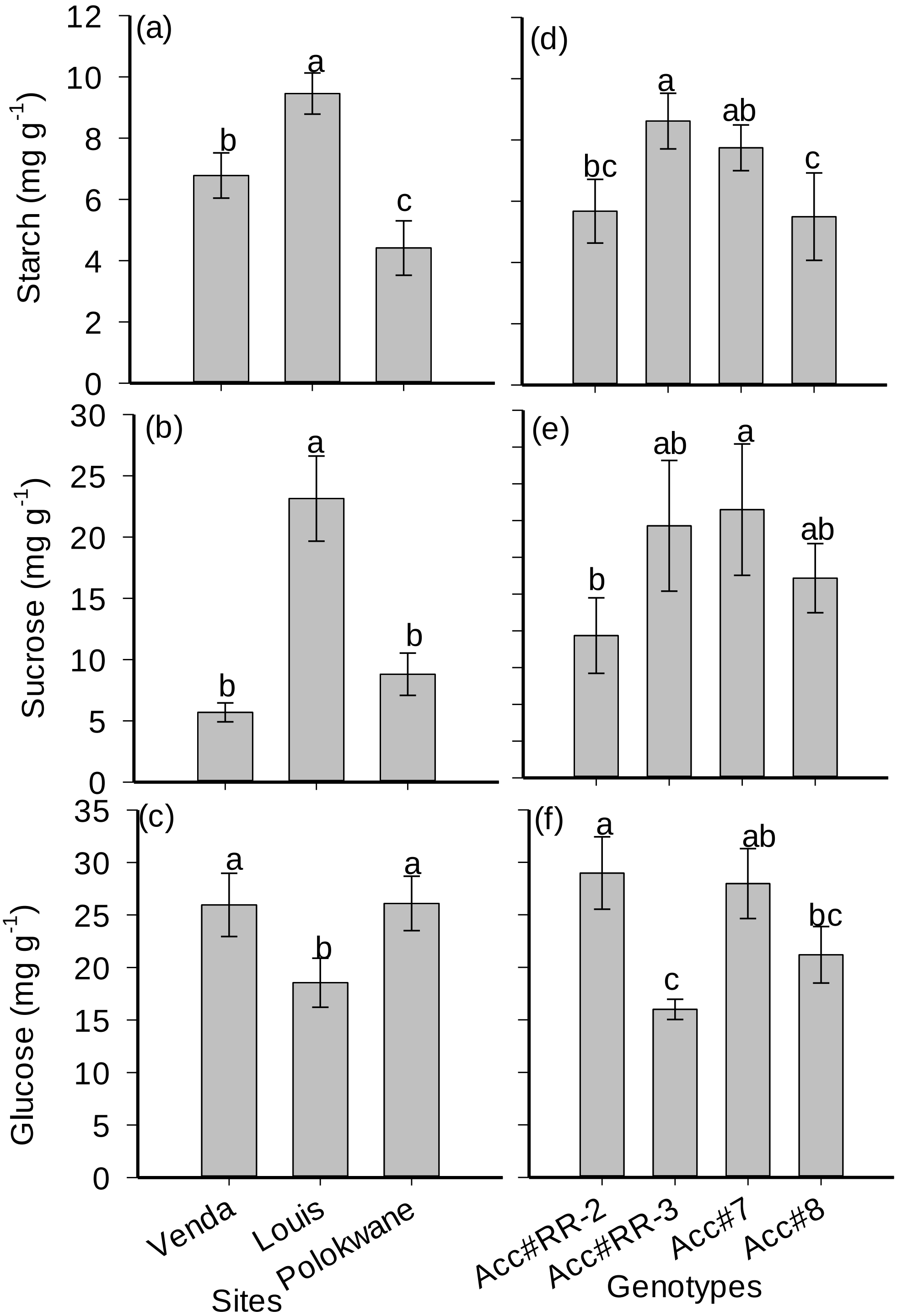

Figure 2.4: Effect of environment on starch (a), sucrose (b), glucose (c), and genotype on starch (d), sucrose (e), glucose (f) leaf concentrations at 50 % flowering stage of chickpea in 2017 cropping season. Data is mean values of pooled genotypes (a, c and d) or sites (b, e and f) ± se (n=16 for environment and n = 12 for genotype). Different letters indicate significant differences between genotypes and sites by Tukey's honest significant difference *post hoc* test ($p<0.001$).

2.3.4 Plant harvest and yield components

There was no interaction between genotypes and environment on yield and yield components of plants grown in neither 2016 nor 2017. The environmental effects showed that the cooler site of Polokwane recorded higher ($P<0.001$) grain yield than both Venda and Louis Trichardt 2017 (Table 2.4). However, grain yield in Polokwane was similar to that of Venda in 2016. Pod numbers per plant were significantly higher in Polokwane than in Venda and Louis Trichardt and in both years. The 100-seed weight was highest ($P <0.01$) at Polokwane relative to the other sites only in 2016. However, total biomass was significantly higher ($p<0.01$) at Venda than at Polokwane and Louis Trichardt with the two sites showing no differences. Genotypic differences were observed for grain yield and pod number in 2017 where Acc#7 recorded similar values with Acc#RR-3 and Acc#RR-2, but higher than Acc#8 (Table 2.4). Although Acc#7 recorded similar total biomass to all genotypes, total biomass for Acc#RR-3 was significantly higher ($p<0.05$) than Acc#8 (Table 2.4). We note that the low grain and pod number per plant at Louis Trichardt in 2016 were partly due to about 50% of the grain yield being destroyed by pod borer infestation.

It was interesting to see that there were significant and positive correlation of F_q'/F_m' (Fig. 2.5a) and F_v/F_m (Fig. 2.5b) with grain yield in 2017. Also, for leaf starch concentration, there was a significant correlation to the grain yield within each site, which was however negative at the warmer site of Venda (Fig. 2.5c).

Table 2.4: Effect environment and genotype on grain yield (plant^{-1}and ha^{-1}), pod yield plant^{-1}, 100 seed weight and total biomass yield (plant^{-1}and ha^{-1})of chickpea in 2016 and 2017 winter cropping seasons. Mean±se with different letters are significantly different as at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant. Venda-University of Venda experimental farm, Thohoyandou, Louis-Vhugela River Queen Farm, Louis Trichardt and Polokwane- University of Limpopo experimental farm, Polokwane

	Grain yield (g plant^{-1})		Grain yield (kg ha^{-1})		Pod number plant^{-1}		100 seed weight (g)		Total Biomass (g plant^{-1})	Total Biomass (kg ha^{-1})
Treatment	2016	2017	2016	2017	2016	2017	2016	2017	2017	2017
Site										
Venda	10.9±0.68^{a}	9.7±1.37^{c}	1948±122^{a}	1729±245^{c}	41±2.63^{b}	31±4.62^{c}	23.8±1.06^{b}	25.5±1.58	12.2±1.45^{a}	2170±258^{a}
Louis	4.0±0.61^{b§}	16.5±1.41^{b}	717±109$^{b\ \S}$	2942±252^{b}	18±3.14$^{c\ \S}$	47±4.57^{b}	20.7±1.37^{c}	25.8±0.94	8.4±0.71^{b}	1500±128^{b}
Polokwane	12.3±2.17^{a}	23.3±1.94^{a}	2204±388^{a}	4155±347^{a}	57±9.94^{a}	67±5.44^{a}	25.8±1.46^{a}	25.9±1.11	7.6±0.49^{b}	1369±88^{b}
Genotypes										
Acc#RR-2	8.1±2.20	16.1±2.07^{ab}	1438±391	2878±369^{ab}	28±7.23	47±6.34^{b}	19.7±2.13	25.9±1.01	10.1±0.70^{ab}	1796±124^{ab}
Acc#RR- 3	8.7±1.72	18.6±2.22^{a}	1550±302	3313±396^{a}	37±5.87	62±7.44^{a}	21.0±1.38	22.6±1.48	11.6±1.96^{a}	2072±350^{a}
Acc#7	9.2±1.77	19.5±3.18^{a}	1636±315	3482±569^{a}	41±7.67	52±7.02^{ab}	23.7±1.39	23.9±1.55	9.0±1.10^{ab}	1599±195^{ab}
Acc#8	7.8±1.59	11.8±1.58^{b}	1399±285	2096±281^{b}	31±7.58	31±4.65^{c}	25.7±1.09	27.7±1.34	7.0±0.43^{b}	1246±77^{b}
F probability										
Site	27.6***	21.8***	27.6***	21.8***	15***	20.5***	8.62**	1.5^{ns}	7.5**	7.5**
Genotype	0.8^{ns}	4.2*	0.8^{ns}	4.2*	2.2 ^{ns}	7.6***	2.7^{ns}	2.7^{ns}	3.6*	3.6*
Site * Genotype	0.4^{ns}	0.9^{ns}	0.4^{ns}	0.9^{ns}	0.1^{ns}	1.3^{ns}	2.07^{ns}	1.1^{ns}	0.8^{ns}	0.8^{ns}

§ - Infestation by pod borer in 2016 affected the pod number and grain yield

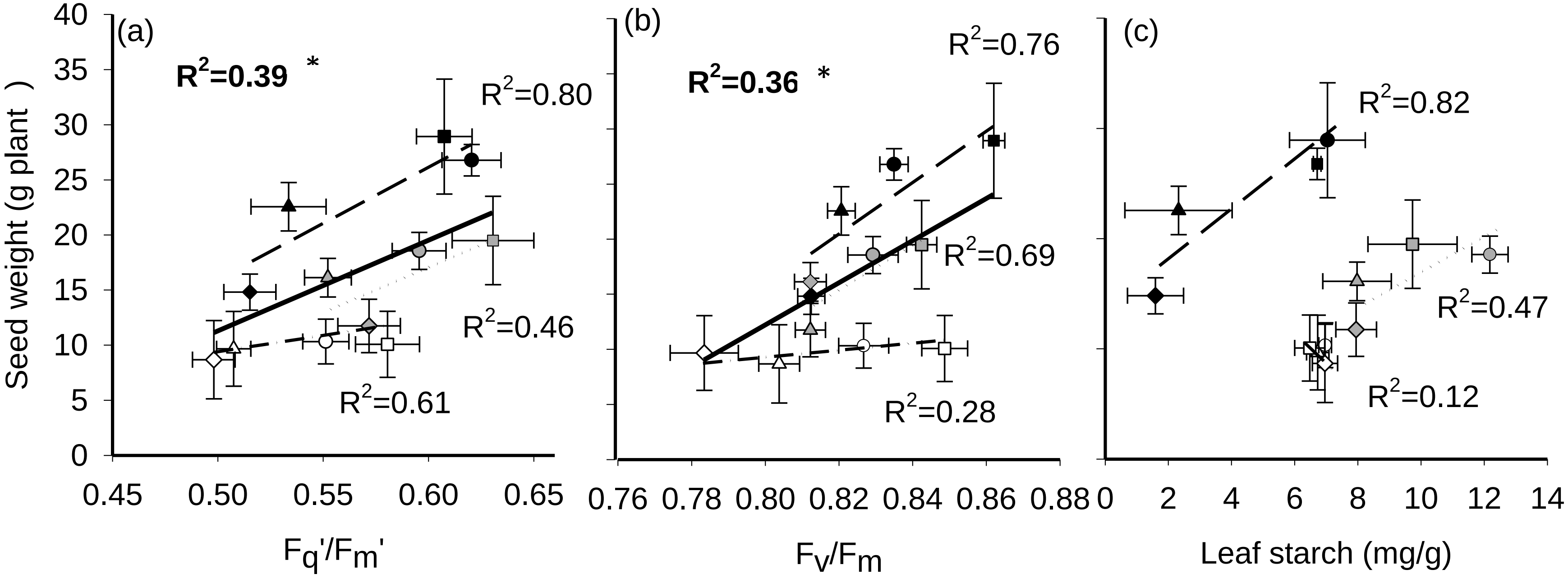

Figure 2.5: Correlation of chickpea grain yield (g plant^{-1}) and operating efficiency (F_q'/F_m') (a), F_v/F_m (b) and leaf starch concentrations (mg/g) (c) in 2017 cropping season. 20 g plant^{-1} corresponds to 3333 kg ha^{-1}. The data represents mean values ± se (n=4) where the symbols are triangles (Acc#RR-2), circles (Acc#RR-3), squares (Acc#7), and diamonds (Acc#8) and the closed symbols are from Polokwane, grey from Louis Trichardt and open symbols from Venda. Bold regression lines and R^2 values (where; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) represent combined regression across sites and accessions while the non-bold R^2 relate to respective sites. The flat lines for F_q'/F_m' and F_v/F_m for the Venda site are due to the scale for the y- axis (seed weight) and the non-presentation of the combined regression for leaf starch is due to the negative correlation for the Venda site.

2.4 Discussion

The maximum photochemical efficiency of PSII (F_v/F_m) measured on dark adapted leaves (Kumar et al., 2013) in combination with the operating efficiency of PS11 (F_q'/F_m') can be used as indicators of some environmental stresses and as screening tools for heat tolerance as shown in this study. The genotype Acc#7 was regarded as the most heat tolerant partly due to its high F_v/F_m at the cooler site of Polokwane with no significant decline in the warmer Venda site. On the other hand, genotype Acc#2 is regarded as the most heat sensitive as it was the only one to show a significant decline in F_v/F_m at the warmer site while Acc#3 is intermediate between Acc#7 and Acc#8 for F_v/F_m at both Polokwane and Venda and also did not decline with warmer temperature. The F_v/F_m for the sensitive Acc#RR-2 in Venda is lower than the published value of 0.832 (Demmig and Björkman, 1987) for non-stressed plants, hence a lower adaptive response to heat stress compared to the heat tolerant Acc#7 genotype. At the cooler Polokwane site with maximum temperatures of 27°C at flowering stage, the tolerant genotype Acc#7 clearly had the highest F_v/F_m compared to the other three genotypes, despite none of the genotypesbeing stressed, suggesting a more ideal site and temperatures for chickpea production. However, when grown at the warmer Venda site, characterised by maximum temperatures around 32°C at the flowering stage, Acc#7 and Acc#RR-3 were able to maintain a higher $F_v/\mathrm{F_m}$ than the heat sensitive genotype *Acc#RR-2.* Similarly, heat tolerant bean genotypes were found to maintain high F_v/F_m when exposed to heat stressful conditions (Petkova et al., 2007). In a study on 30 field grown chickpea genotypes, genotype Pusa 240 maintained a high F_v/F_m when exposed to temperatures above 30°C (Kumar et al., 2013). Contrasting reports have however reported that PSII inhibition does not occur until leaf temperatures are as high as 35°C to 42°C (Wise et al., 2004) and around 40°C (Al Khatib and Paulsen, 1999). Rubisco has been shown to deactivate at temperatures causing no harm to PS11 (Feller et al., 1998), with this deactivation being proposed as the primary constraint to photosynthesis in this temperature range (Crafts-Brandner and Salvucci, 2000). However, it would be difficult to make similar conclusions from short term heat treatments on the chronic heat stress applied in the current study.

Genotypes Acc#7 and Acc#RR-3 were also able to maintain higher operating efficiencies (F_q'/F_m') in both cropping seasons compared to the other two genotypes. The higher values of $F_q'/F_m'$for Acc#7 and Acc#RR-3 (between 0.58 and 0.67) show their superior operating efficiencies under heat stress probably due to maintenance of the PSII quinone electron acceptors partially more oxidised (Rosenqvist, 2001) than the heat sensitive Acc#RR-2 and Acc#8 genotypes. Similar observations were made in a study of cotton, where 17 of the 40 selected genotypes with operating efficiencies between 0.56 and 0.67 were concluded to be the most tolerant to heat stress (Wu, 2013). A decrease in the operating efficiency of PSII from the cooler site of Polokwane to the warmer site of Venda was observed in 2016, a result attributed to the higher PPFD at Venda during the time of measurements as a natural consequence of the shape of the light dependency of photosynthesis. Fluctuations of light irradiance in the field may occur over short time scales as well as from year to year, leading to varied photosynthetic assimilation (Petridis et al., 2018), similarly making field F_q'/F_m' data collection and use a challenge. We therefore recommend using F_q'/F_m'measurements in conjunction with F_v/F_m data. Noteworthy is that the observed values of up to 0.66 for F_q'/F_m' at about 1200 µmol m^{-2} s^{-1} are higher than those reported by Bilger et al. (1995) for pumpkin plants (about 0.45 at 1200 µmol m^{-2} s^{-1}) grown in summer in Temperate region in the Northern Hemisphere. There are several factors than can be attributed to the differences including species and location differences with the current study using chickpea genotypes that are acclimated to semi-arid conditions in Southern Africa.

Putting together results for from the F_v/F_m and F_q'/F_m'measurements, genotypes Acc#7 and Acc#RR-3 are showing heat tolerating characteristics in all sites. In a study of heat stress response by wheat, genotypic differences were also supported by superior photosynthetic performance of the genotypes (Sharma et al., 2015) as highlighted by the high operating efficiencies in the current study. The significant correlation of maximum photochemical efficiency and PSII operating efficiencies with grain yield seems valid because tolerant genotypes Acc#7 and Acc#RR-3 which both had higher grain yield also had higher operating efficiencies of PSII than the heat sensitive genotypes. The correlation of the two fluorescence parameters to grain yield was valid both within the sites and for the whole data set. Therefore, use of F_v/F_m as a tool for selection of field grown chickpeas continues to show potential, especially with the incorporation of F_q'/F_m' measured under high light conditions, which to the best of our knowledge has very few records in literature, if any, on chickpea before our study for the use of a modulated chlorophyll fluorimeter in the field. This is a vital finding of our study for it signifies the potential use of both quick and non-destructive parameters (Sharma et al., 2012) as selection tools for heat tolerant chickpea genotypes under field conditions.

In this study we found a significant difference in F_v/F_m in Acc#7 compared to the other three genotypes also in the coolest location in Polokwane. This raises the question if the found differences in heat tolerance amongst the four genotypes arises from a difference in ‘base line’ F_v/F_m in non-stressed plants or in the decline in F_v/F_m after heat stress, or in both. In previous studies of 41 wheat genotypes of as diverse origin as Sweden and Pakistan, no genetic component was found in the variation of the control values (Sharma et al., 2012). In 28 genotypes of tomato that are used as well performing cultivars in the field in Nepal, no significant difference was found in F_v/F_m in control conditions, while they showed pronounced differences after heat stress when screened in climate chambers (Poudyal et al., 2018). Two heat tolerant and two heat susceptible cultivars were subsequently grown in a well irrigated field experiment in Nepal, where they by coincidence were exposed to a natural heat wave. The heat tolerant group had considerably smaller loss of harvest yield and stayed greener than the heat susceptible group from a climate chamber screening (Poudyal et al., 2018). In both these investigations no significant difference was found in F_v/F_m in control conditions. In our study only one out of four genotypes had higher F_v/F_m in the 'control' conditions, while two genotypes were considered more heat tolerant. For that reason, we cannot challenge the previous conclusions that it is the decrease in F_v/F_m that distinguish the heat susceptible from the heat tolerant genotypes, rather than an intrinsic difference in F_v/F_m in un-stressed plants.

Genotypic and site differences on F_v/F_m and F_q'/F_m' were further supported by photosynthetic measurements, night respiration and carbohydrates in the leaf and grain yield. For example, similar to genotypic differences in F_v/F_m and F_q'/F_m', the tolerant genotypes Acc#7 and Acc#RR-3 had higher P_n than Acc#RR-2 and Acc#8. The ability of plants to sustain leaf gas exchange and CO_2 assimilation rates under heat stress is directly correlated with heat tolerance in snap bean (Kumar et al., 2005) and wheat (Yang et al., 2006). However, the correlation between P_n and grain yield was not universal across sites (data not shown). This is logical since growth is not directly dependent on photosynthesis but rather on the balance between photosynthesis, maintenance and growth respiration (Dewar et al., 1994). Since F_v/F_m reflects the activity in only parts of the photosynthetic apparatus, it is rather surprising to find a general correlation to the grain yield. Only further investigation will reveal if this relationship is universal. Plant respiration at night also varied with genotypes with Acc#8 having the highest respiration rate compared to the other three genotypes. Respiration has been noted to consume between 30% to 80% of the CO_2 taken up by photosynthesis per day (Atkin et al., 2005), increasing with increase in temperature (McCullough and Hunt, 1993). High R_n in peanut was associated with a potential increase in reactive oxygen species, leading to cell damage and decrease in pollen viability (Prasad et al., 1999). Low respiration has been associated with higher biomass accumulation in a *Lolium spp.* breeding programme in a temperate environment (Wilson and Jones, 1982). Therefore, the high respiration rates, lower operating efficiency and lower photosynthesis in our study may have ultimately led to significantly lower grain yield of Acc#8.

The higher leaf starch concentrations in genotype Acc#7 and Acc#RR-3 compared to Acc#8 also suggest tolerance to heat stress (Subrahmanyam and Rathore, 1995). Indeed, sugars, have been noted to be important metabolic signals in plants that aid in the regulation of plant development and response to stresses through carbon allocation and sugar signalling (Kaushal et al., 2013). Reduction in the starch accumulation in heat sensitive genotypes may be partly attributed to the limited activity of starch synthesising enzymes which ultimately leads to reduced sucrose availability to developing seeds (Snider et al., 2011). In a study where heat stress induced reproductive failure, a significant reduction in sucrose concentrations in leaves of heat intolerant chickpea genotypes was observed, with the tolerant genotypes having higher sucrose concentration which correlated with higher sucrose phosphate synthase and sucrose synthase (Kaushal et al., 2013). Availability of higher sucrose concentrations for the reproductive organs (Snider et al., 2011) may have been critical in their sustained function of Acc#7. Increased sucrose availability in genotype Acc#7 may have been due to increased Rubisco activity, as noted by the high photosynthetic rates, possibly resulting in reduced flower and pod abortions, which may have contributed to its superior pod numbers and grain yield compared to Acc#8. Despite the relatively lower sucrose and starch concentrations, Acc#RR-2 was able to maintain a higher leaf glucose concentration. Concentrations of starch, fructose and sucrose decreased with glucose remaining relatively higher in some heat intolerant varieties of crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.) and redtop (*Agrostis alba* L.) (Chatterton et al., 1987). The correlation of leaf starch to grain yield was valid both within the sites and for the whole data set, also making it a potential selection marker for heat tolerant chickpea genotypes in the field.

In our study, photosynthesis, grain yields and yield components followed a similar pattern being significantly higher at the cooler site of Polokwane compared to the warmer site of Venda. In soybean, heat stress (38/20°C) significantly reduced F_v/F_m (5%), photosynthesis (20%) and sucrose concentrations, 20% (Hasanuzzaman et al., 2013). A 2.2 and 3.1°C increase in atmospheric temperatures from the ambient caused a respective 18.8 and 37.5% reduction in the P_n of chickpea (Chakrabarti et al., 2013). However, in 2017 g_s, leaf transpiration, and C_i were highest at Venda and least at Polokwane, with leaf temperatures lower at Venda. These results contrasted with 2016 observations in this study and we attribute this to the relatively lower temperatures (22.6°C) on the day of data collection at the normally warmer site of Venda compared to the other two sites, which might have led to a higher stomatal opening, hence more internal CO_2 concentrations (Greer et al., 2012).

Interestingly, there were no differences in the leaf glucose and sucrose concentrations of plants grown in Venda and those grown in Polokwane, with a higher starch concentration in plants grown in Venda that lead to higher biomass accumulation at flowering. The lower grain yield of plants at Venda compared to Polokwane site, similar to the warmer treatments in the chamber experiment, might partly be due decreased carbohydrate export from leaves to reproductive organs of plants grown in warmer conditions (Plaut et al., 2004). Starch synthase has been identified as a major gene and protein that is reduced by heat stress and this consequently leads to reduced utilization of incoming carbohydrate, followed by a reduction in sugar transport to the developing grain (Keeling et al., 1993). In heat stress studies on potato, raised temperatures during tuber growth resulted in redirection of photosynthates to vegetative tissues at the expense of starch accumulation in growing tubers (Wolf et al., 1990). Furthermore, elevated temperatures during grain filling stages of chickpea have previously been reported to reduce grain yield as well as seed sizes, which may also lower grain yield (Ong, 1983). Reactive oxygen species (ROS) accumulate in male reproductive organs during prolonged heat stressful conditions, particularly in microspores / pollen grains, evidenced by protein and membrane degradation, potentially leading to male reproductive abortion (Sage et al., 2015). Heat stress has also been noted to result in severely reduced flower bud initiation, decreased flower number and size, leading to loss of flowers and young pods, ultimately lowering grain yields (Morrison et al., 2002). The impact of heat stress on pod characteristics like pod numbers ultimately result in reduction of overall seed yield (Krishnamurthy et al., 2011). Average maximum temperatures at grain filling stages of chickpea in Polokwane in 2016 and 2017 were 27.1°C and 27°C respectively while in Venda they were 31.5°C and 31.2°C respectively. Chickpea grain yields have been reported to reduce by 53-330 kg ha^{-1} for every 1°C increase in mean seasonal temperatures in India (Kalra et al., 2008). The higher pod numbers per plant and grain yield in Polokwane reflect the site's association with higher chickpea reproductive efficiency. Chickpea grain yields in similarly cooler South African environments as the Polokwane site, with mean winter maximum temperatures ranging between 15 and 24°C have average yield between 3000 and 4000 kg ha^{-1} (Thangwana and Ogola, 2012). This is consistent to prior research on chickpea showing that elevated temperatures above 30°C, like the Venda site at the critical growth stages between flowering and pod formation, adversely affected pod set (Devasirvatham et al., 2012a). Consequently, any potential increase in temperatures associated with climate change in cooler sites like Polokwane may adversely affect chickpea production of the area.

2.5 Conclusion

Overall, our results show that chlorophyll fluorescence parameters (F_v/F_m and F_q'/F_m') and carbohydrate concentrations for Acc#7and Acc#RR-3were unaffected by exposure to heat stress, showing their potential use as heat tolerance markers and genotype Acc#7 and Acc#RR-3 as heat tolerant genotypes. Heat stress in the field generally leads to reduced photosynthesis, F_q'/F_m', F_v/F_m as well as carbohydrate concentrations in leaves of heat sensitive genotypes. However, the heat tolerant genotype Acc#7 and Acc#RR-3 maintained unaltered physiological response at flowering stage as well as their grain yields after exposure to heat stressful conditions in the field environment. The observed relationships between measured chlorophyll fluorescence (both F_v/F_m and F_q'/F_m'), a relatively rapid and non-destructive method, with plant agronomic performance supports its use as a phenotyping tool during germplasm screening under field conditions. The site of Polokwane showed temperature range that is conducive to chickpea production.

Chapter 3: Intermittent moisture supply induces drought priming responses in some heat tolerant Chickpea (*Cicer arietinum* L.; Fabaceae) genotypes

3.1 Introduction

Chickpea (*Cicer arietinum* L.) is a vital legume crop globally ranked 3rd after dry bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum*) (FAOSTAT 2018). It is grown in different regions around the world, mainly in India, Pakistan, Australia and Ethiopia (Randhawa et al., 2014). The crop is generally grown under rainfed conditions, using either residual moisture in subtropical environments dominated by summer rains, or current rainfall in either winter or summer in Mediterranean environments (Yadav et al., 2006). Despite its high yield potential (4000 kg ha^{-1}), global chickpea yields are relatively low (910 kg ha^{-1}) due to a combination of biotic (e.g., pod borer- *Helicoverpa armigera*) and abiotic (e.g., drought and temperature) stresses (Awasthi et al., 2014). Intermittent and terminal drought are the two distinct kinds of droughts associated with limited rainfall (Xangsayasane et al., 2014). Intermittent drought is due to either climatic patterns of sporadic rainfall that cause intervals of drought and can occur at any time during the growing season (Schneider et al., 1997), or when farmers have options to irrigate but the supply is limited. In contrast, terminal drought occurs when plants suffer lack of water during later stages of reproductive growth e.g. flowering, pod set and pod filling (Leport et al., 1999). Being a cool season crop, high temperatures and/or unavailability of soil moisture during critical growth stages affects the physiological performance as well as grain yield of chickpea (Yadav et al., 2006, Devasirvatham et al., 2012, Randhawa et al., 2014).

Agricultural water deficit greatly impacted by changing climate, arises from both insufficient rainfall and soil moisture during the growing season to sustain crop growth and yields (Vadez et al., 2011). Future climate projections show an increase in the intense rain events as well as reduced number of rain days leading to increased risk of drought (Vadez et al., 2011). Stomatal closure is one of the first responses to drought stress, which ultimately results in declining stomatal conductance to conserve water (Blum et al., 1999). Also, drought stress affects growth and yield of plants through its effects on membrane integrity, root depth and expansion, leaf area development, chlorophyll fluorescence, transpiration, proteins and production of reactive oxygen species (Praba et al., 2009, Mafakheri et al., 2010, Wang et al., 2014), with the effects of drought stress on grain yield more severe during the reproductive growth stage compared with the vegetative growth stage (Barnabás et al., 2008).

Several shoot and root traits have been noted to improve plant’s tolerance to drought (Araujo et al., 2015) but the contribution of these traits to superior plant growth are dependent on the type of drought (early, intermittent and terminal), the agroecology in which the crop is grown, as well as genotype (Leport et al., 1999). Furthermore, plants tend to develop various physiological and biochemical responses for adaptation through different mechanisms such as drought escape, drought avoidance or drought tolerance (Praba et al., 2009). Through drought avoidance, plants might be able to maintain (relatively) higher tissue water content despite reduced moisture in the soil, which is achieved through a variety of adaptive traits involving minimization of water loss (water savers) and optimized water uptake (water spenders) (Levitt, 1980). Through drought tolerance, plants tend to decrease their water potential by osmotic adjustments which increase osmolyte concentrations, while increasing water movement in the cells and tissues. Such adaptations delay metabolic damage and leaf senescence and improve assimilate transport, thereby improving water absorption, stomatal conductance and CO_2 assimilation (Leport et al., 1999). Moreover, high leaf relative water content (RWC) has been associated with drought tolerance and this has been proposed as a valuable indicator of plant water status under drought conditions (Abdallah et al., 2017). Therefore, RWC is an important trait in drought studies, and maintenance of leaf water status is a key indicator for dehydration avoidance (Rahbarian et al., 2011).

Plant priming, referred to as stress memory, is a process by which an earlier exposure to abiotic stress (e.g. drought) may alter a plant's subsequent stress response by producing a faster and/or stronger reaction that may provide the benefits of enhanced protection (Walter et al., 2011, Abdallah et al. 2017). However, the time span between stress events, e.g. rehydration between two drought episodes, might be an important factor (Bruce et al. 2007), and there appears to be a mechanism of storing information from previous drought stress exposure (Walter et al., 2011, Wang et al., 2014). These phenomena have been ascribed to the changes in some key signalling processes, transcription factors and epigenetic modifications (Bruce et al. 2007). Retaining this information, the imprint memory of the stress, may be short or long term (Walter et al., 2011). For example, drought priming lead to enhanced tolerance to subsequent drought by improving grain weight (wheat, Abid et al., 2017), leaf photosynthesis (olive, Abdallah et al., 2017) and photo protection (*Arrhenatherum elatius,* Walter et al., 2011). Furthermore, it has been reported that drought priming confers to primed wheat plants the ability to retain water more efficiently than non-primed plants (Wang et al., 2014).

In field studies carried out along a temperature gradient in NE South Africa during the winter seasons of 2016 and 2017 that aimed at identifying thermotolerant traits, chickpea genotype Acc#7 appeared to be tolerant to heat stress with a maximum quantum efficiency of PSII, F_v/F_m of 0.85 and operating efficiency of PSII, F_q'/F_m' (where $F_q' = F_m' - F$) of 0.60 at the warmest site (PAR 1100 µmol m^{-2} s^{-1}, T_{max} 30.1-33.1°C), while two apparently sensitive genotypes, Acc#8 and Acc#RR-2, showed lower F_v/F_m of 0.78 and 0.80 respectively and F_q'/F_m' of 0.52 and 0.53, respectively (Chapter 2). Genotype Acc#7 also showed higher non-structural carbohydrates (starch and sucrose) concentrations, photosynthetic rates, and grain yield than the 'sensitive' genotypes at the warmest site, confirming the potential use of these parameters as heat tolerance markers, and genotype Acc#7 as a heat tolerant genotype. Since high air temperatures often occur concurrently with water shortages under field conditions (Comas et al., 2013), we irrigated the plants in the heat stress study to eliminate the confounding effect of water stress. Indeed, the negative effects of drought and heat stress on crop productivity, associated with climate change, are expected to be more severe in the tropics and sub-tropics where temperatures are already quite high (Serdeczny et al., 2017).

The two stresses (drought and heat) represent examples of two different abiotic stress factors that often occur simultaneously, especially in the arid and semi-arid regions (Mittler, 2006; Rizhsky et al., 2004; Jedmowski et al., 2015). The ever-changing climatic conditions worsened by the increased chances of simultaneous occurrences of heat and drought stresses will result in their combined deleterious effects on growth of chickpea (Awasthi et al., 2014) and other crop plants (Rizhsky et al., 2004; Rollins et al., 2013). However, most studies on the two abiotic stresses have looked at either of the stresses in isolation (Leport et al., 1999, Devasirvatham et al., 2012, Kumar et al., 2013, Randhawa et al., 2014) leading to limited information of single genotypic responses to both stresses (i.e. cross tolerance). In one of the rare studies looking at combination of both drought and heat stress, Awasthi et al. (2014) observed that heat tolerant chickpea genotypes were also tolerant to drought stress, and vice versa. Therefore, we hypothesise that chickpea genotype Acc#7, having shown evidence of heat tolerance, may also be drought tolerant.

Although a number of studies have been conducted on drought stress in chickpea, most of the research has focused on terminal drought (Leport et al., 1999, Mafakheri et al., 2010, Purushothaman et al. 2016), with little evidence in literature on studies specifically focusing on intermittent drought stress with recovery periods in-between. This is rather surprising considering that intermittent drought stress approximates natural conditions better than single stress treatments (Izanloo et al., 2008). We aimed to determine whether previously noted heat stress tolerant genotype (Acc#7) is drought tolerant and the heat sensitive (Acc#8) is drought sensitive, and whether intermittent moisture supply at the vegetative stage would induce priming effect to a later drought at flowering growth stage

3.2 Materials and Methods

3.2.1 Plant materials, growth conditions and watering treatments.

Two Desi chickpea (*Cicer arietinum* L.) genotypes, Acc#7 and Acc#8, were selected for this study based on the results from field experiments carried out in NE South Africa in 2016 and 2017 growing seasons where Acc#7 was deemed heat tolerant and Acc#8 heat susceptible (Chapter 2). A pot experiment was conducted from March to May 2018 in a glasshouse at the University of Cape Town, South Africa (33.955889S, 18.462111E) with an average temperature of 25°C during the growth period and mean relative humidity of 53.5%. The experiment consisted of a factorial combination of two genotypes (Acc#7 and Acc#8) and three watering regimes (75% FC as the control, 40% FC as mild stress and a water withholding treatment), replicated four times. A total of 144 pots, each measuring 22.5 cm and 16.5 cm top and bottom diameters respectively were filled with a mixture of 5 kg of commercial sand and promix organic (Hortishop and Hydroponics, South Africa) in a 1:1 ratio and fertilized with a Multicoat (4*) 15-3-12+Mg+Me (Haifa chemicals, South Africa; 6 g pot^{-1}) and gypsum ($CaSO_4$. $2H_2O$. 1g pot^{-1}). The soil water content at field capacity (FC) was pre-determined by filling the pre-weighed dry promix/sand mixes with water in the free draining pots, allowing them to drain for 48 hours and then taking weight measurements of the pots.

Three seeds were sown in each pot, which were later thinned to one seedling per pot. All pots were initially uniformly watered, to 75% field capacity (FC), from seed germination to vegetative growth stage. The watering treatments were imposed at vegetative stage (3-5 leaf; 21 days (d) after planting (DAP)) and at flowering growth stage (50% flowering; 45 DAP). The plants allocated to the 40% FC treatment (mild stress) were not watered for three days prior to imposition of treatments to allow for the FC to drop from the initial 75% FC to 40% FC. At the vegetative growth stage, 24 pots for each genotype were watered to 75% FC (control) 24 pots to 40 % FC (mild stress) and 24 had their watering withheld (severe water stress) for the 14 d period. Before watering every other day, pots were re-weighed and water added to maintain the 75% and 40% field capacity. During the 14 d of stress treatment impositions, data on gas exchange, stomatal conductance, soil moisture content, and chlorophyll fluorescence were collected on four plants (4 pots) from each treatment. At the end of the 14 d stress treatment, data on relative leaf water content and shoot biomass were collected on four plants from each treatment, with all the remaining plants rewatered to recovery (75% FC) (Fig. 1).

At the flowering growth stage, eight pots from the control plants and another eight from the group of plants whose watering had been withheld at the vegetative growth stage had their watering withheld for 8 d, with the remaining eight pots from the control and those whose watering had been withheld at vegetative growth stage watered to 75% FC. Plants watered to 40% FC from vegetative growth stage continued to receive the same water supply to maturity (Fig. 1). Similar data as collected during the stress period at vegetative growth stage were obtained on four plants from each treatment at the flowering growth stage, with the same plants subsequently harvested for determination of shoot biomass and root morphology. The 14 d and eight day water withdrawal periods at vegetative and flowering growth stages respectively, were determined in preliminary experiments to avoid plants reaching permanent wilting point. The treatments at flowering growth stage as shown in Fig. 1 were; NS-NS (control plants well-watered to 75% FC); S-NS (severely stressed at vegetative stage then well-watered to 75% FC at flowering growth stage); S-S was the priming treatment (severely stressed at both vegetative and flowering growth stages [double stress]); NS-S was the non-priming treatment (well-watered to 75% FC at vegetative stage and severely stressed at flowering growth stage); 40% FC (watered to 40% FC throughout the experiment[mild stress]).

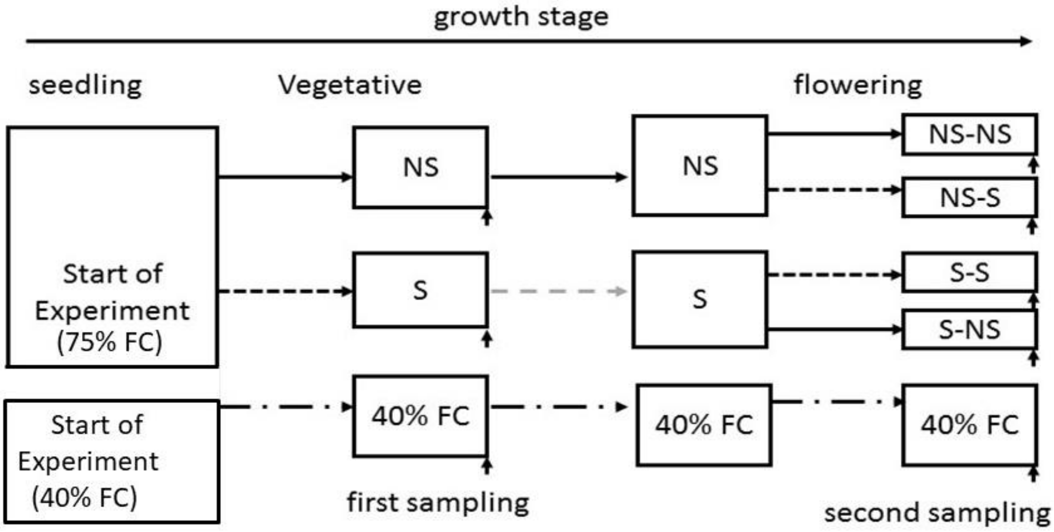

Figure 3.1: Experimental design used to determine the effect of progressive drought imposed by withholding water at vegetative (14 days) and flowering (8 days) stages and subsequent recovery (+7days under well-watered conditions). Stress and recovery periods are depicted by black and grey dashed arrows respectively, while continuous black arrows are for non- stressed plants and those watered to 40% field capacity (mild stress). **NS (**control plants well-watered to 75% FC at vegetative stage); **S (**severely stressed at vegetative stage); **NS-NS (**control plants well-watered to 75% FC at vegetative and at flowering growth stages); **NS-S** was the non-priming treatment **(**well-watered to 75% FC at vegetative stage and severely stressed at flowering growth stage); **S-S** was the priming treatment **(**severely stressed at both vegetative and flowering growth stages [double stress]); **S-NS (**severely stressed at vegetative stage then well-watered to 75% FC at flowering growth stage); **40% FC(**watered to 40% FC throughout the experiment [mild stress]). The small up arrows indicate time of sampling/ measurement.

3.2.2 Stomatal conductance and soil moisture content

Stomatal conductance (g_s) was measured daily, using a porometer (Model SC-1, Decagon Devices Inc., USA), during the stress periods at both the vegetative and flowering growth stages. Although the IRGA, used for the other gas exchange parameters, also measured leaf conductance, the ease of use of the porometer allowed for relatively quicker measurements *in situ* making it the preferred option for leaf conductance measurements. Measurements of g_s

were performed on clear sunny days between 0900 hours and 1200 noon. The uppermost fully expanded sunlit leaf was used on all four replicates per drought treatment and genotype during the stress period.

Soil moisture content was also measured daily between 0900 hours and 1200 noon, using a portable moisture meter (ML2X Moisture Meter, WET Sensor, Delta-T Devices, Cambridge, England), during the stress periods at vegetative and flowering growth stages on four replicates per treatment and genotype.

3.2.3 Shoot biomass and relative drought index (RDI)

Shoot biomass was determined by harvesting four plants per genotype and treatment at the end of the stress period at both the vegetative and flowering growth stages. Plants were separated into leaves, stems and roots and then oven dried at 70°C for 48 hours and biomass recorded.

Relative drought index (RDI) was determined at flowering stage according to Fischer et al. (1979) as follows:

$$\text{Relative drought Index} = (Y_S/Y_P)/\ (\overline{Y}_S/\overline{Y}_P)$$

In the above formula, Y_S, Y_P, $\overline{Y}_S$ and $\overline{Y}_P$ represent shoot biomass yield in drought stress treatments and non-stress treatments for each genotype, and shoot biomass yield mean in drought stress treatments and non-stress treatments for all genotypes, respectively.

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3.2.4 Relative water content

Leaf relative water content was estimated according to Henston et al. (1981). The leaf samples for determination of relative water content (RWC) were collected between 1100 hours and 1200 hours. Ten leaflets from one fully matured leaf for each four plants per treatment were excised, placed in plastic bags and their fresh weights subsequently recorded. The leaflets were floated in distilled water under low light conditions for 6 hours to measure their saturated weight. After recording fully turgid weight, leaves were then oven dried at 70°C for 48 hours and their dry weights measured. Relative water content was calculated using the following equation:

RWC = [(fresh weight) – (dry weight) / (turgid weight) – (dry weight)] × 100

3.2.5 Chlorophyll fluorescence

Following the fluorescence nomenclature proposed by Baker and Rosenqvist (2004), leaf chlorophyll fluorescence values, including minimal fluorescence F_o, maximum fluorescence F_m, variable fluorescence F_v and the maximum photochemical efficiency of photosystem II F_v/F_m ($F_v = F_m - F_o$), were collected daily during the stress periods at the vegetative and flowering growth stages on youngest fully expanded leaves using a PAM-2100 portable chlorophyll fluorometer (Walz, Eiffeltrich, Germany). F_v/F_m readings were taken on four plants per treatment after dark adaptation for 30 minutes using light exclusion clips (Walz, Eiffeltrich, Germany) attached to the adaxial surface of the youngest fully expanded leaf. The operating efficiency of PSII (F_q'/F_m') and leaf temperature readings were taken and recorded in light using the same equipment between 0900 hours and 12 00 noon on four plants per treatment, with the fiber optics attached to a leaf clip holder 2030-B (Walz, Eiffeltrich, Germany). Corresponding time and photosynthetic photon flux density (PPFD) values were recorded simultaneously. Only data for both chlorophyll fluorescence parameters during the stress periods at both vegetative and flowering stages where significant differences were observed are presented.

3.2.6 Gas exchange

Gas exchange variables including net photosynthetic rate (P_n), g_s, rate of transpiration (E) and intracellular CO_2 concentration (C_i) were measured at CO_2 concentration of 400 µmol mol^{-1} using a LI-6400 portable photosynthesis system infrared gas analyser (LiCor, Lincoln, NE, USA) with an automatic cuvette of up to 6 cm^2 leaf area. Instantaneous water use efficiency (IWUE) was also calculated by dividing the net photosynthetic rate (P_n) by rate of transpiration (E) for each data point. Data were collected on four plants daily during the stress period between 0900 hours and 12 noon at the vegetative and flowering growth stages. Readings were taken from the youngest, fully expanded leaves that were allowed to equilibrate to 20°C cuvette conditions, flow rate at 500 µmol s^{-1} and at PPFD of ca 1000 µmol m^{-2} s^{-1} for three minutes.

3.2.7 Assessment of root morphology using WinRHIZO

Roots were gently washed out and collected by soaking individual pots in water. An estimated 15% of the total root mass per individual pot (Van Damme et al. 2013) was collected during the destructive sampling of plants at flowering growth stage and stored in 10% ethanol solution at room temperature for root morphological analyses. Roots were stained with 2% (w/v) gentian violet for better visualisation on the scanner and stored in ethanol again before assessment. Total root length (m), mean root diameter (mm), and total surface area (m^2) were measured with a STD4800 scanner and WinRHIZO software version 2013a (Reagent Instruments, Quebec, Canada) and converted to whole-root results by multiplying by the relevant conversion factor of 6.6 to 100% of the root.

3.2.8 Statistical analysis

Two-way analysis of variance (ANOVA) was used to test for the significance of the different drought treatments and the two genotypes on each measured variable in Statistica 12 (StatSoft, Inc.). Means were compared by Tukey's Honestly Significant Difference (HSD) tests at the 5% probability level

3.3 Results

3.3.1 Stomatal conductance and soil moisture content

Drought stress during the 14-d period of water withholding at the vegetative stage decreased g_s from about 0.08 to 0.050 mmol $m^{-2}s^{-1}$ in both genotypes (Fig. 3.2A); Acc#7 had higher ($P< 0.05$) g_s than Acc#8 on 3 measurement dates during this period (Fig. 3.2A). The stressed plants had lower g_s compared to the control between the 6th and 14th d after watering (DAW), and the plants subjected to 40% FC water regime recorded lower g_s than the non-stressed plants between the 6th and 11th DAW (Fig. 3.2B). The pattern of soil moisture depletion in the pots during the 14-d period of water withdrawal was similar to the changes in g_s with withdrawal of water (Figs. 3.2C and D). The soil moisture decreased from about 18% to 14% (in Acc#7) and 12% (in Acc#8) within the period, and the water content in the pots of Acc#7 was higher ($P< 0.05$) than those of Acc#8 on six different days during the period of water withdrawal (Fig. 2C).

3.3.2 Shoot biomass

There were significant interactions between genotypes and drought treatments on shoot biomass at both vegetative ($p<0.001$) and flowering growth stages ($p<0.05$) (Fig. 3.3a). At the vegetative growth stage, withdrawing watering for a period of 14 d decreased biomass accumulation similarly to supplying water at 40% field capacity for Acc#7. For genotype Acc#8 in contrast, only the withdrawal of watering, but not watering at 40% FC, reduced biomass accumulation. At flowering growth stage (Fig. 3.3a), all drought treatments significantly reduced biomass accumulation in Acc#7 after withholding water for 8 d, with stressed plants only at flowering (non-priming treatment) being the most severe and no differences being observed between double stressed plants (priming treatment) and those watered to 40% FC for the same genotype.

On the other hand, shoot biomass accumulation in genotype Acc#8 reduced in all droughted treatments except 40% FC relative to the control (Fig. 3.3a). However, unlike Acc#8, genotype Acc#7 had a significantly higher shoot biomass in double stress treatment compared to plants that were stressed only at flowering.At flowering growth stage, the drought treatments differed in their relative tolerance index, with the primed plants for both genotypes significantly higher than then other drought treatments, which were similar (Supplementary Figure 1). However, no genotypic differences were observed the on relative tolerance index

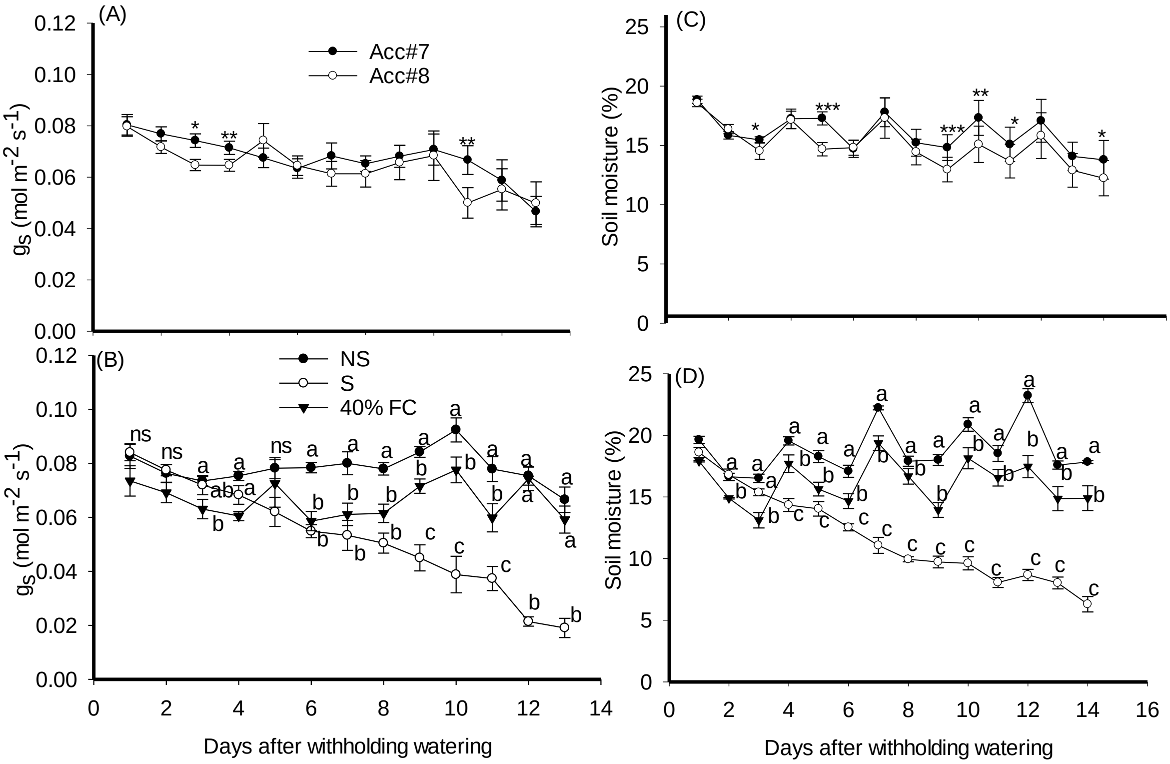


Figure 3.2: Effect of genotype (A and C) and drought treatments (B and D) on stomatal conductance, g_s (A and B) and soil moisture content (C and D) of chickpea at vegetative growth stage**.** The genotype and drought treatment interaction for both stomata conductance and soil moisture were not significant. **NS (**control plants well-watered to 75% FC at vegetative stage); **S (**severely stressed at vegetative stage); **NS-NS (**control plants well-watered to 75% FC at vegetative and at flowering growth stages); **NS-S** was the non-priming treatment **(**well-watered to 75% FC at vegetative stage and severely stressed at flowering growth stage); **S-S** was the priming treatment **(**severely stressed at both vegetative and flowering growth stages [double stress]); **S-NS (**severely stressed at vegetative stage then well-watered to 75% FC at flowering growth stage); **40% FC(**watered to 40% FC throughout the experiment [mild stress]). Data is mean values of pooled drought ± se (n=8) and pooled genotypes ± se (n=12). Different letters indicate significant differences between genotypes by Tukey's honest significant difference *post hoc* test ($p<0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant

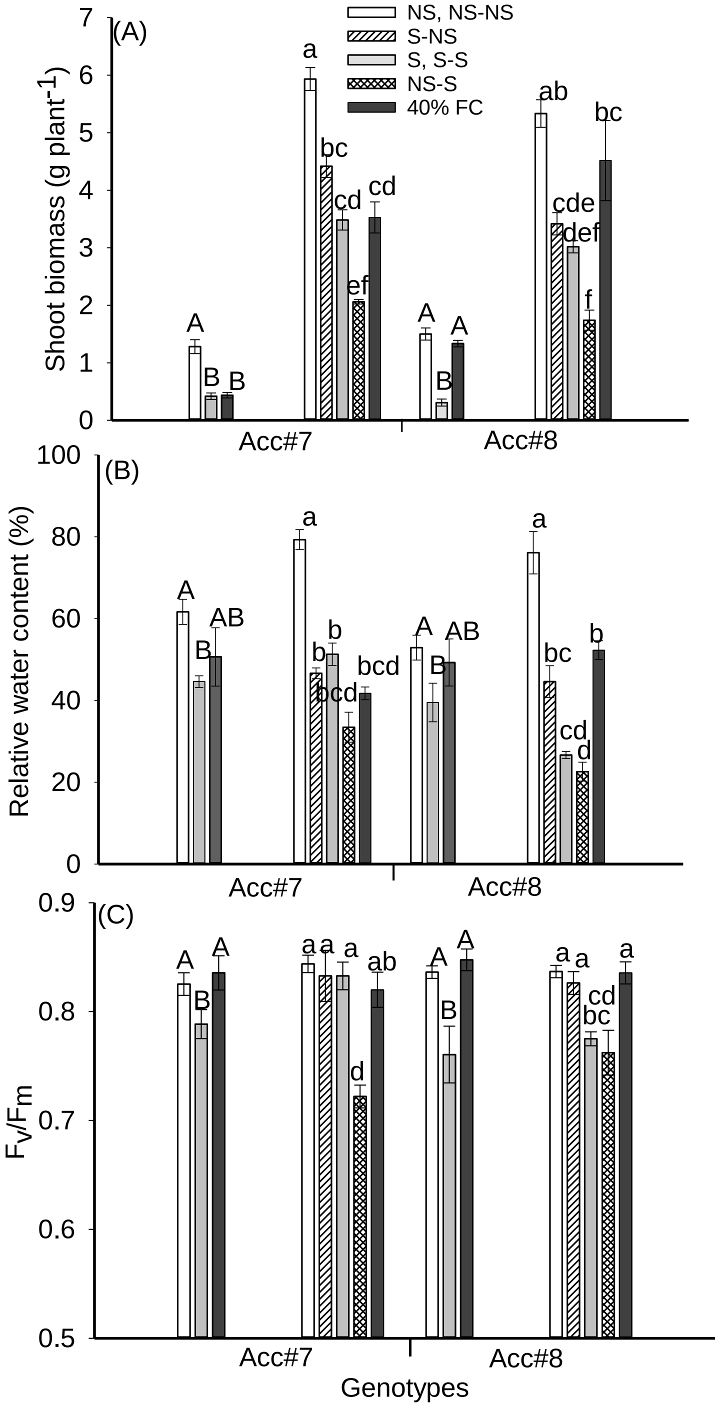


Figure 3.3: Interaction of genotypes and drought treatments at vegetative (Upper case letters) and flowering (lower case letters) growth stages of chickpea on shoot biomass (A), leaf relative water content (B) and Fv/Fm (C). Data presented was collected on the final day of moisture withholding. Treatment descriptions are as in Figure 3.1. Uppercase and lowercase letters indicate significant differences between genotypes and drought treatments regimes at vegetative and stage flowering stages respectively by Tukey's honest significant difference *post hoc* test ($P < 0.01$). The nomenclature "NS, NS-NS" on the figure represents non-stress treatment for vegetative growth stage (NS) and non-stress at both the vegetative and flowering growth stages (NS-NS), while "S, S-S" represents severe stress at vegetative stage (S) and severe stress at both the vegetative and flowering stages (S-S)

3.3.3 Relative water content

Relative to the control, the RWC of drought stressed plants was lower on the 14th DAW at vegetative stage but RWC in 40% FC treatments did not differ from either the stressed or the non-stressed treatments (Fig. 3.3b). At flowering growth stage, the interactive effect of genotypes and drought treatments on RWC was significant ($p<0.01$; Fig. 3.3b). While all the drought treatments decreased RWC compared to the control in both genotypes, the treatment reductions were similar for Acc#7, whereas in Acc#8, the effects were greater for double stress and those stressed at only vegetative stage compared to those stressed at only vegetative stage and 40% FC treatments (Fig. 3.3b).

3.3.4 Chlorophyll fluorescence parameters

At the vegetative growth stage, reduction in F_v/F_m was apparent at the 14th day of withholding moisture (Fig. 3.3c), with no differences observed on all other days during the stress period (data not shown). At the flowering stage, the genotype and drought treatment interaction on F_v/F_m was significant ($p<0.001$); Acc#8 plants exposed to double stress and those stressed only at flowering stage showed lower F_v/F_m compared to the control, but in Acc#7, F_v/F_m was reduced only in plants stressed at flowering stage (Fig. 3.3c). Plant exposure to stress at only vegetative stage and those receiving 40% FC did not alter F_v/F_m in both genotypes. Moisture withdrawal at vegetative stage reduced ($p<0.001$) F_q'/F_m' on the 14th DAW (Fig. 3.4a), with no differences observed on the corresponding PPFDs at time of measurements. Double stress and stress at only flowering stage reduced ($p<0.001$) F_q'/F_m' at fourth DAW relative to the control treatment, with no differences observed on the corresponding PPFDs at the time of data collection (Fig. 3.4 b). On the 8th day, no differences were also observed on the PPFDs at the time of data collection but in contrast, plants stress at only flowering stage lowered F_q'/F_m' compared to the control (Fig. 3.4c).

There was negative correlation ($R^2 = 0.72$) between F_q'/F_m' and PPFD at vegetative stage (Fig. 3.4a) but none at flowering stage (Figs. 3.4b and c). It was also interesting to note, from our results, that there were significant and positive correlations of F_v/F_m (Fig. 3.5a) and shoot biomass (Fig. 3.5b) with RWC.

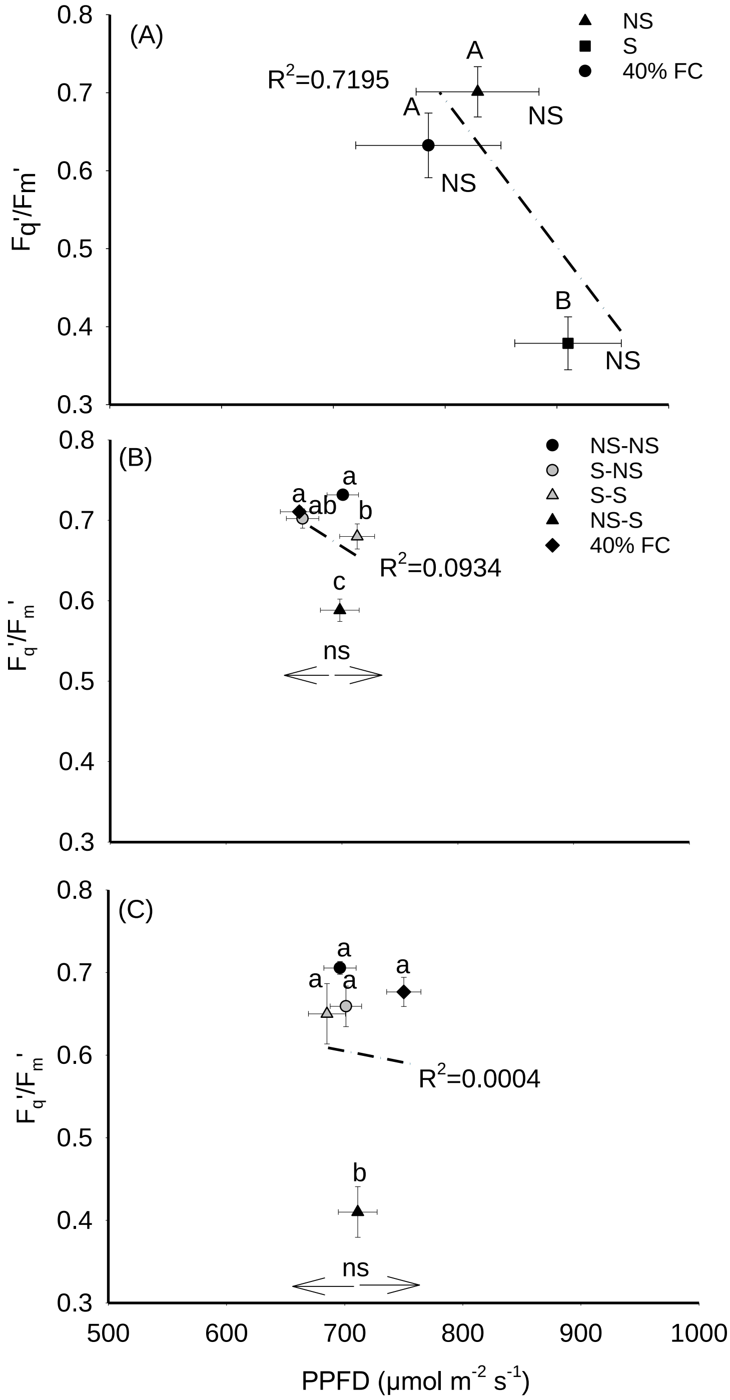


Figure 3.4: Correlation of PPFD (µmol m^{-2} s^{-1}) at time of data collection and operating efficiency of PSII (F_q'/F_m') of chickpea at vegetative stage (A) and on the 4th and 8th DAW at flowering stage (B and C respectively). Treatments are as in Figure 3.1. Data is mean values of pooled stress treatments and PPFDs ± se (n=8). Different letters indicate significant differences between stress treatments by Tukey's honest significant difference *post hoc* test ($p<0.001$), **ns = not** significant. Water withdrawal periods at vegetative and flowering stages were 14 and 8 days respectively

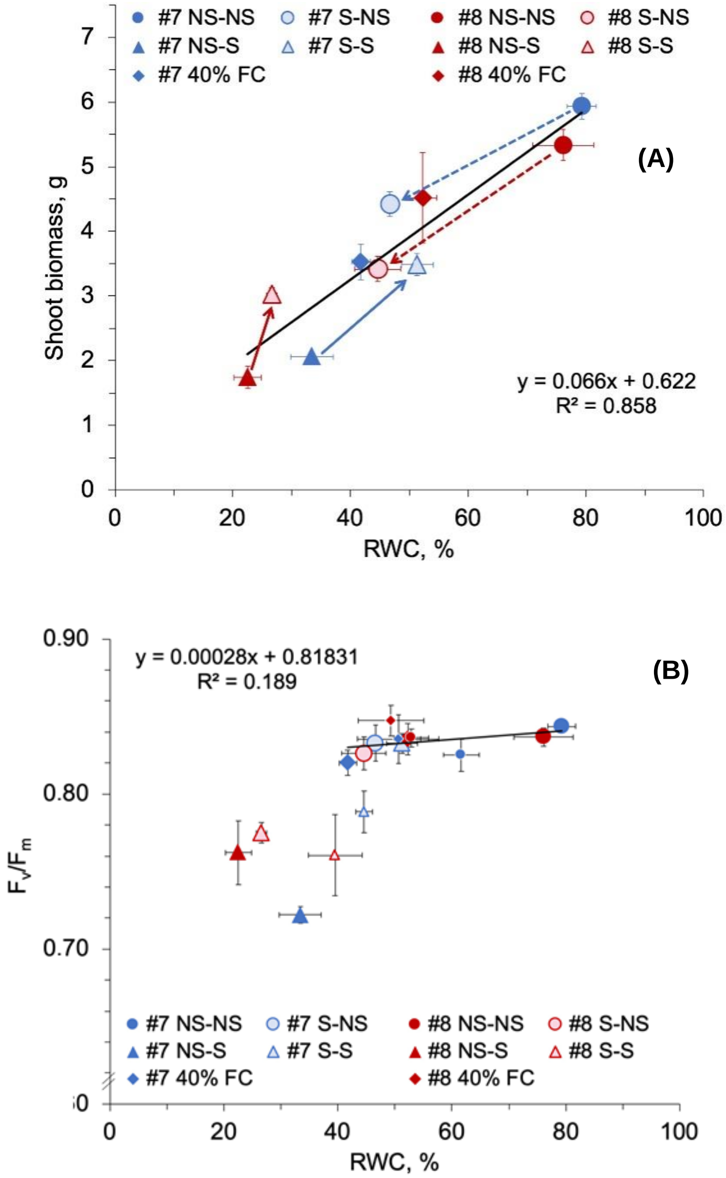


Figure 3.5: The effect of drought stress on the relationship between the leaf relative water content (RWC) and F_v/F_m (A) and RWC and the shoot biomass (B) at the end of each drought stress exposure in vegetative and flowering stage (A) and flowering stage only (B), respectively, where *small symbols* are after the vegetative treatments and the large symbols after the flowering stage treatments. The solid line in **A** is a trend line for pooled non-stressed and 40% FC treatments of both accessions, while the solid line in **B** is a trend line for all treatments and both accessions at the flowering stage. The dashed arrows indicate the effect that the vegetative drought stress have had after the flowering stage without stress, compared to the control non-stressed plants (NS-NS → S-NS) and the solid arrows the priming effect that the vegetative stress have had after the generative drought stress (NS-S → S-S). The data represents mean values of n = 4 +/- SEM when bigger than the symbol.

3.3.5 Gas exchange

A significant ($P < 0.05$) decrease in P_n in plants where watering was withheld relative to the control was observed on both days of data collection at the vegetative stage (Figs. 3.6a and b) but remained unaltered in plants receiving 40% FC. However, genotypic changes on photosynthesis at the vegetative stage with withdrawal of watering was only apparent on the 14th DAW, with Acc#7 being higher than Acc#8 (Fig 3.6b). At the flowering stage, the photosynthetic response to drought treatment by the two genotypes differed ($p<0.01$) on both days of data collection (Fig. 3.6a and Fig. 3.6b). All the drought treated plants, except those watered to 40% FC, had significantly lower P_n than the control treatment in both genotypes. However, at the fourth DAW (Fig. 3.6a), double stress and stress at only flowering stage decreased P_n by similar magnitude in Acc#8, whereas P_n in plants stressed only at flowering was lower than of the plants exposed to double stress treatment in Acc#7 (Fig. 3.6a). The pattern of plant response to the drought treatments changed at the 8th DAW, where drought stress only at flowering decreased ($P < 0.05$) P_n in Acc#7 relative to the control and the other treatments of the same genotype, but in Acc#8, it was decreased by both double stress and stressed only at flowering treatments (Fig. 3.6b).

No differences ($P > 0.05$) on the intercellular CO_2 concentrations were observed on both days of measurements at the vegetative stage (Figs. 3.6c and 3.6d). At the flowering stage, the intercellular CO_2 concentrations for all drought treated plants, except those watered to 40% FC, in both genotypes, were significantly ($p<0.0001$) reduced on the fourth DAW (Fig. 3.6d). However, on the eighth DAW, only plants stressed at flowering stage for both genotypes had significantly lower ($p<0.0001$) intercellular CO_2 concentrations relative to the controls (Fig. 3.6d)

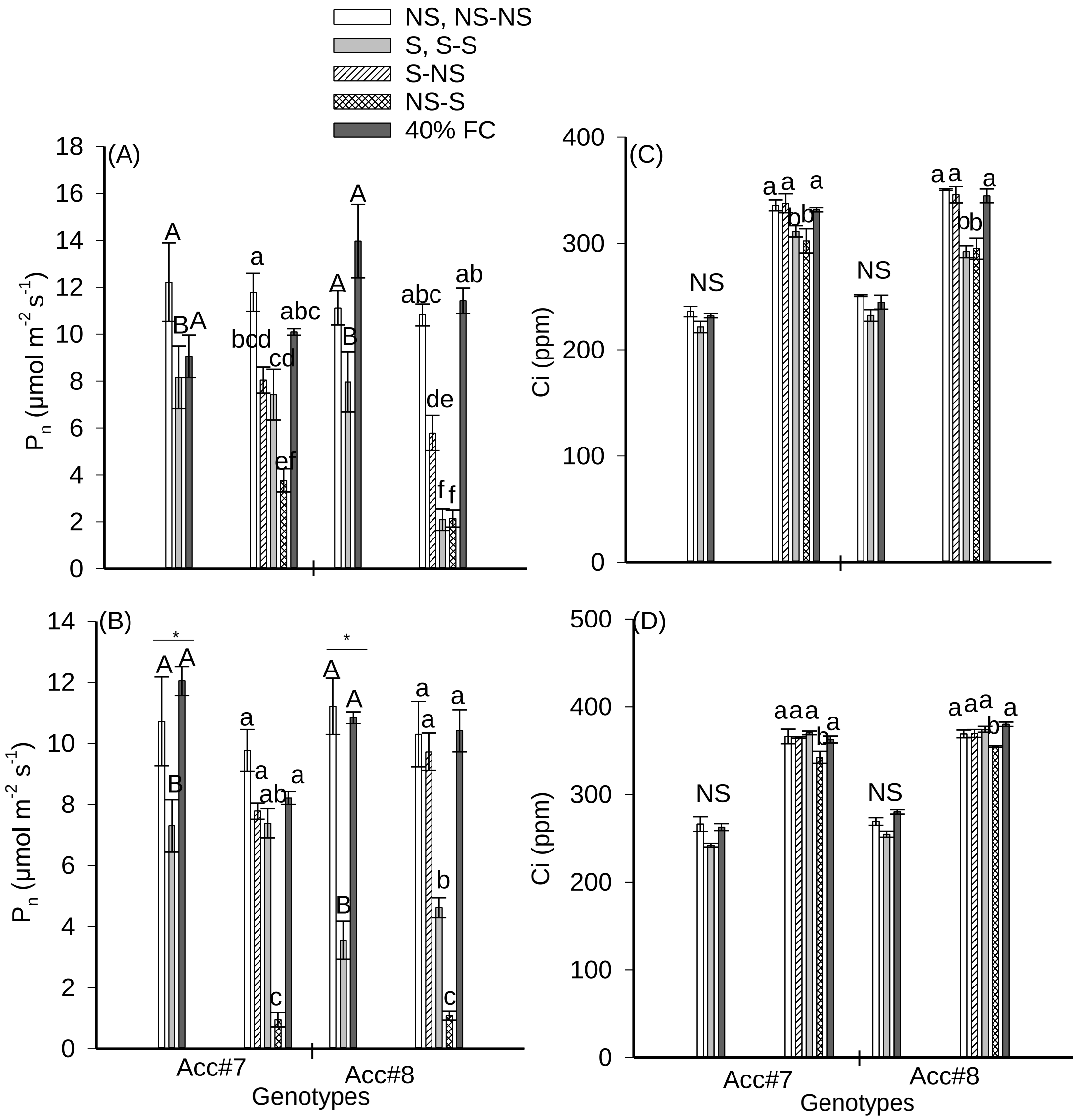


Figure 3.6: Interaction of genotype and drought treatments on P_n at flowering stage (lower case letters), as well as drought treatment and genotype effect on P_n at vegetative stage (upper case letters) (A and B) and effect of drought treatments on intercellular CO_2 concentration at vegetative and flowering stages (C and D). The genotype and drought treatment interaction on intercellular CO_2 concentration was not significant. Measurements were taken on the 8th DAW (vegetative) and 4th DAW (flowering) (A and C) and the 14th DAW (vegetative) and 8th DAW (flowering) (B and D). Treatment descriptions are as in Figure 3.1. Uppercase and lowercase letters indicate significant differences between genotypes and drought treatments regimes at vegetative and flowering stages respectively by Tukey's honest significant difference *post hoc* test ($P < 0.01$). Asterisk show genotypic differences (P<005) in P_n (B) at vegetative stage on the 14th DAW. The nomenclature "NS, NS-NS" on the figure represents non-stress treatment for vegetative growth stage (NS) and non-stress at both the vegetative and flowering growth stages (NS-NS), while "S, S-S" represents severe stress at vegetative stage (S) and severe stress at both the vegetative and flowering stages (S-S). Ci values seem too high in relation to the g_s (Fig. 3.7), probably due to in the inaccuracies of Ci calculations under drought.

At vegetative stage, *E* and g_s decreased (P <0.05) at both days in plants where watering was withheld relative to the control (Figs. 3.7a, b, d and e), but remained unaltered in plants receiving 40% FC (Figs. 3.7a, b and d), with the g_s for plants receiving 40% FC significantly lowered (Fig. 3.7e). Significant interaction between genotypes and drought treatments on *E* at the flowering growth stage was observed only on the 4th day after stopping watering (Fig. 3.7a). Double stress and stress only at flowering treatment significantly lowered *E* in Acc#8 relative to the control, with the double stress and stress only at flowering treatment in Acc#7 similar to stress at vegetative stage and to plants watered to 40% FC. Plants that were stressed only at vegetative stage and those receiving 40% FC water showed similar *E* values to the non-stressed plants. At the 8th DAW, plants subjected to water stress only at flowering stage as well as double stress plants decreased *E* relative to the control in both genotypes (Fig. 3.7d). A significant ($p<0.0001$) decrease in g_s for double stress and stress only at flowering treatment was observed on both days of measurements (Figs. 3.7a and d) relative to the control. Genotypic differences on g_s were also apparent on the eighth DAW, with Acc#7 higher that Acc#8 (Fig. 3.7e).

Plants whose watering was withheld had a significantly ($p<0.001$) higher instantaneous water use efficiency (IWUE) on both days of measurements at the vegetative stage, relative to the controls (Figs. 3.7c and d). At the flowering stage, the IWUEs of double stress and stress only at flowering treatments for both genotypes was significantly higher ($p<0.0001$) relative to the controls on the fourth DAW (Fig. 3.7c). In contrast, the result was only apparent for double stress plants on the eighth DAW, relative to the controls (Fig. 3.7f). Genotype Acc#7 had a significantly higher IWUE compared to Acc#8 on the eighth DAW (Fig. 3.7f).

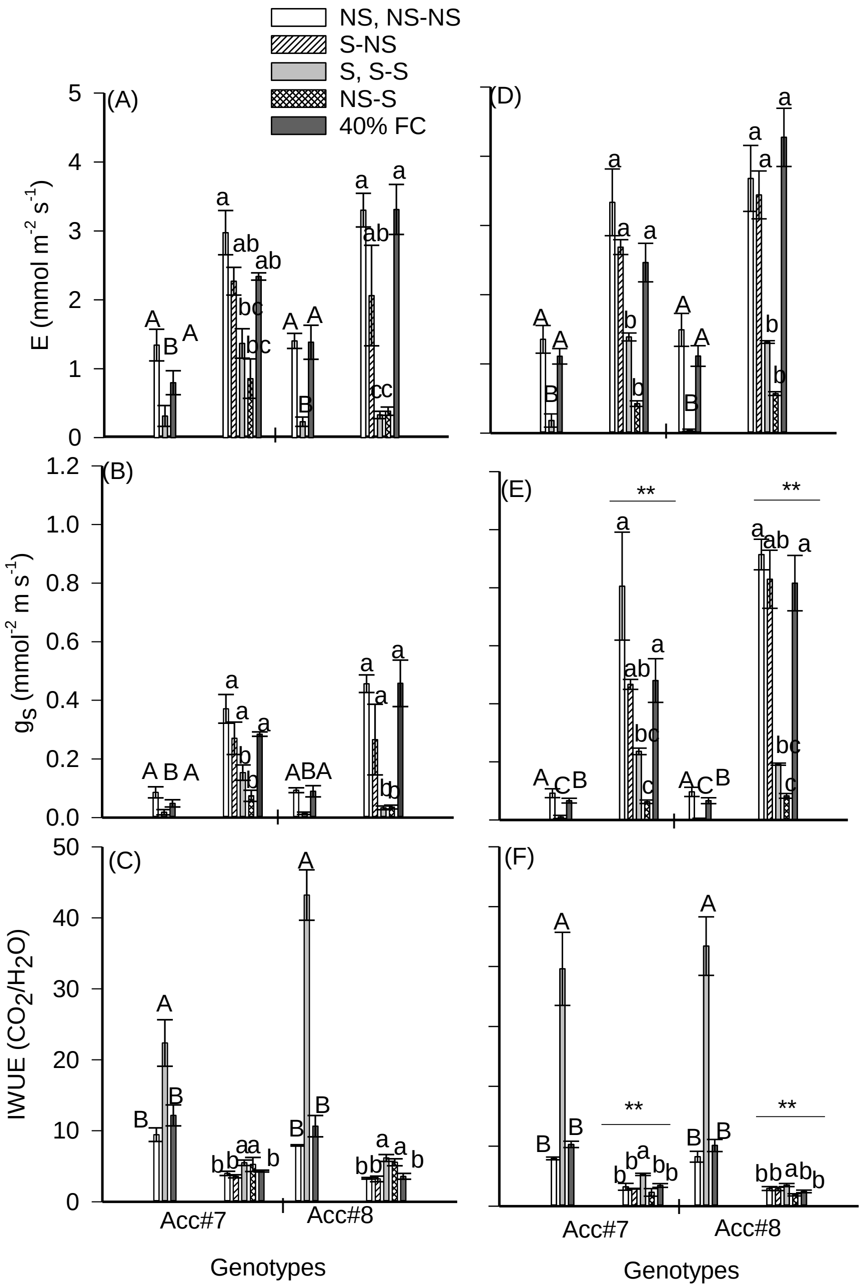


Figure 3.7: Effect of genotype and drought treatments at vegetative (upper case letters) and flowering (lower case letters) growth stages of chickpea on *E* (A and D), g_s (B and D) and IWUE (C and F). The genotype and drought treatment interaction on *E* (D), g_s and IWUE was not significant. Measurements were taken on the 8th DAW (vegetative) and 4th DAW (flowering) (A, B and C) and on the 14th DAW (vegetative) and 8th DAW (flowering) (D, E and F). Treatment descriptions are as in Figure 3.1. Uppercase and lowercase letters indicate significant differences between genotypes and drought treatments regimes at vegetative and flowering stages respectively by Tukey's honest significant difference *post hoc* test ($P < 0.01$). Asterisks show genotypic differences ($P<0.01$) in g_s (E) and IWUE (F) on the 8th DAW at flowering stage. The nomenclature "NS, NS-NS" on the figure represents non-stress treatment for vegetative growth stage (NS) and non-stress at both the vegetative and flowering growth stages (NS-NS), while "S, S-S" represents severe stress at vegetative stage (S) and severe stress at both the vegetative and flowering stages (S-S)

3.3.6 Root morphology

At flowering growth stage, all drought treatments in both genotypes had lower total root length relative to the control treatments (Fig. 3.8a). However, all root length did not differ among the drought treatments in Acc#8, but in Acc#7, plants stressed only at flowering stage showed lower ($P<0.001$) total root length compared to those stressed only at vegetative stage. Total root surface area was similarly reduced ($p<0.001$) in both genotypes by the drought treatments (Fig. 3.8b). There were significant genotypic differences on average root diameter with genotype Acc#7 having a significantly smaller root diameter than Acc#8 (Fig. 3.8c).

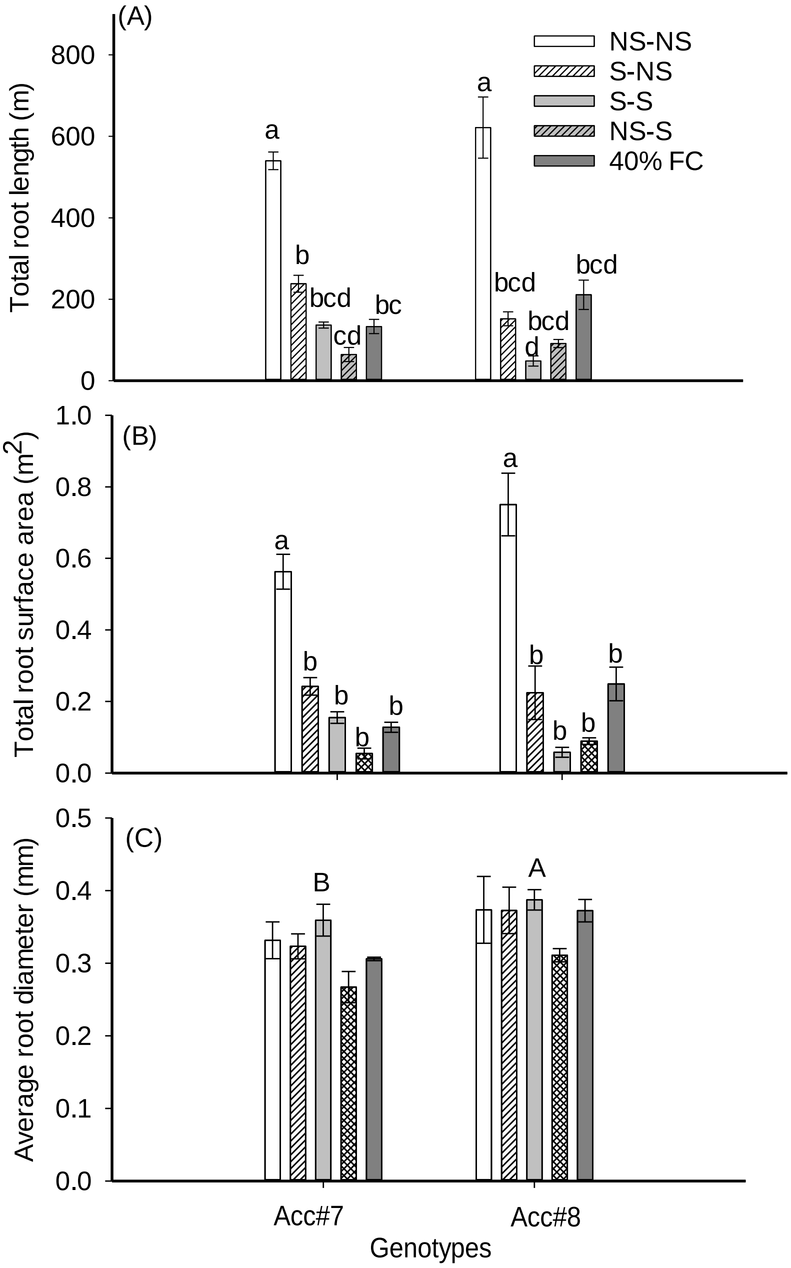


Figure 3.8: Effect of interaction of genotype and drought treatments on total root length (1), drought treatments on total root surface area (2) and genotypes on average root diameter (3) of chickpea at flowering growth stage. The genotype and drought treatment interactions on total root surface area and average root diameter were not significant. Treatments are as in Figure 3.1. Data is mean values of different genotypes under varying stress regimes ± se (n=4). Different letters indicate significant differences between genotypes and stress regimes by Tukey's honest significant difference *post hoc* test ($p<0.001$)

3.4 Discussion

In this study, chickpea plants from both the heat tolerant and heat susceptible genotypes were drought primed by double stress at both the vegetative and flowering growth stages, and subsequently compared to non-primed ones that were well irrigated control plants and stressed only at the flowering growth stage. Our results have shown that double drought stress resulted in effective priming in heat tolerant Acc#7 plants, with the genotype having higher biomass, F_v/F_m, g_s, P_n and RWC compared to when it was stressed only at flowering growth stage, as well as in comparison to the same treatment combinations for heat susceptible Acc#8. However, it should be noted that, compared to the controls, primed plants for both genotypes had lower accumulation of shoot biomass, RWC, E and P_n (for Acc#8), with the decrease in most of the parameters greater in Acc#8 than in Acc#7. The differences between the genotypes were observed only after drought priming, which was associated with the intermittent moisture supply. Therefore, these differences are likely to be adaptive differences rather than constitutive. Thus, the heat tolerant Acc#7 is better acclimated to drought than the heat susceptible Acc#8. Relative to constitutive responses, adaptive or inducive defences are considered less costly to the productivity of the plant because they are triggered only on demand (Hamilton et al., 2008). Constitutive responses are common in plants that have evolved in more stressful environments (Karban, 2011) and often correlate negatively with plant productivity due to competition for energy and metabolites for growth in the absence of the stress factor (Singhal et al., 2016).

The ability of primed Acc#7 to maintain a higher growth and photosynthetic rate can potentially be attributed to higher electron transport under drought stress (Wang et al., 2014). The observed higher F_v/F_m in the primed Acc#7 relative to Acc#8 was due to the genotype's ability to maintain higher cellular hydration under drought stressful conditions, avoiding photo inhibition and ultimately maintaining D1 protein associated with PSII (Abid et al., 2017). As a consequence of water stress induced oxidative stress, plants tend to deactivate the antennae of PSII resulting in the decline of their F_v/F_m and F_q'/F_m' ratios, generally leading to low carbon assimilation rates under such conditions (Yuan et al., 2016). However, tolerant plants tend to adaptively reduce their electron transport rates maintaining equilibrium with production of ATP and NADP, while maintaining higher photochemistry as shown by Acc#7 (Baker and Rosenqvist 2004). The general decline observed in F_v/F_m and F_q'/F_m' with exposure to drought is in contrast to Cornic and Fresneau (2002) and Banks (2018) who found that PSII was highly resistant to water shortages and thus cautioned against overreliance on these parameters as stress indicators in drought stress research, but rather to use them in conjunction with other supporting parameters. There was, also, a high dependency of F_q'/F_m' on the PPFD at vegetative stage, with the low operating efficiency observed in stressed plants associated with high PPFD at time of measurement, compounding the effect of the drought.

The reduction in P_n at flowering growth stage was also noted to be less pronounced (Figures 6a and 6b) than g_s (Figure 7b) in primed plants for Acc#7 in comparison to those stressed only at vegetative stage and those mildly stressed (40% FC). This result reflects a better protection of leaf metabolic apparatus in the primed plants for Acc#7 compared to the non-primed plants, pointing to a better stomatal adjustment of stomatal conductance as well as regulation of non-stomatal leaf activities (Bota et al., 2004). Previously, Rahbarian et al. (2011) observed higher RWC in the drought tolerant chickpea genotypes MCC392 and MCC877 after drought stress at vegetative and at flowering growth stages. Thus, the maintenance of high RWC and subsequently P_n in primed Acc#7 suggests better osmoregulation and greater capacity to protect the photosynthetic activity in response to a later drought stress (Blum, 2005; Wang et al., 2014). The water uptake was also most likely enhanced in Acc#7 with the observed smaller root diameter than that of Acc#8, which represents a selection directly related to resistance to low soil water potential, possibly emanating from thinner xylem elements in prevention of cavitation within the rooting system (Tucker et al., 2011). Drought tolerance after priming has also previously been observed in plants such as *Arabidopsis, Eucalyptus,* wheat and olive exposed to recurring drought stress, undergoing an acclimation process and ultimately becoming more successful in the responses to the follow-up drought exposure (Knight et al., 1998; Valdés et al., 2013, Wang et al., 2014, Abdallah et al., 2017). However, the relatively very low values of RWC (<40%) in primed plants and those stressed at flowering growth stage only for Acc#8 was surprising, but was similarly reported by Berger and Ludwig (2014) in *Lupinus luteus* and attributed to adaptive patterns that are likely to be species and habitat specific along Mediterranean rainfall gradients.

Future climate projections have also suggested increased probability that plants will encounter combinations of abiotic stresses associated with climate change (Pandey et al., 2017). Therefore, it is important to screen genotypes for multiple tolerance to abiotic stresses as explored in the study using chickpea genotype Acc#7 that was earlier observed to be heat tolerant due to its superior F_v/F_m, P_n, leaf carbohydrate concentrations and grain yield (Chapter 2). In the current study, heat tolerant Acc#7 was similarly sensitive to drought stress compared to heat sensitive Acc#8, due to the lack of differences on F_v/F_m, P_n, RWC shoot biomasses and ultimately RDI of the genotypes when drought stressed only at either vegetative or flowering growth stages. Although similarities between tolerance to drought and heat stress have been observed (Rang et al., 2011; Awasthi et al., 2014), our results support the notion that be used to select for tolerance to the different stresses. Studies have shown that photosynthetic efficiency, transpiration rates and PSII function decrease under both heat and drought stresses, consequently due to stress-induced stomatal closure which leads to reduced internal available CO_2 concentrations as well as also non-stomatal limitations such as decreased leaf expansion, leaf senescence and inhibition of the photosynthetic machinery's function through degradation of vital components of PSII, such as D1, D2 and CP47 (Zandalinas et al., 2016, Wahid et al., 2007, Aswathi et al., 2014). However, extended exposure to high temperatures has been noted to primarily trigger decrease in chlorophyll content, thylakoid grana disintegration, thermal instability of Rubisco and inhibition of the electron transport chain and Photosystem II (PSII), reduction of leaf carbohydrates and disruption of assimilate transport (Wahid et al., 2007, Kozłowska et al., 2007, Awasthi et al., 2014, Chapter 2). In contrast, excessive drought primarily results in closed stomata, low root length density and reduced relative leaf water content (Leport et al., 1999; Wahid et al., 2007), which leads to reduced root hydraulic conductivity hence preventing water losses from the plant to the dry soil. Reduced photosynthetic rates after drought exposure have also been directly linked to water use efficiency (WUE), with stomatal closure noted to result in increased WUE (Ruggiero et al., 2017), with the tool noted to be very critical in assessments of phenotypic variations within large drought stress tolerance breeding pools (Ellsworth and Cousins, 2016). Therefore, the lack of F_v/F_m and P_n differences between the genotypes used in the current study after exposure to drought stress, despite previously there being observed after exposure to heat stress (Chapter 2), as well as the noted differences in the mechanisms involved in tolerance to either of the stresses, further highlights that tolerance to either drought or heat stress does not automatically imply tolerance to the other or the combination of both (Jagadish et al., 2007, 2010). Ultimately, the generation of novel chickpea genotypes, displaying tolerance to both heat and drought stress, can only be a reality when studies exploring the underlying mechanisms associated with combined heat and drought stresses are carried out at various plant developmental stages, physiological cascades, and biochemical and molecular reactions occurring at the cellular, tissue, or whole plant level (Awasthi et al., 2014; Sehgal et al., 2017, Zhou et al., 2019).

Drought stress indirectly leads to reduction in CO_2 fixation, photosynthetic rates and finally lower assimilate production (Mafakheri et al., 2010). This is often dependent on the growth stage affected (vegetative or reproductive) (Serraj et al., 2004), more severely so at flowering growth stage (Barnabás et al., 2008). In the current study, severe water stress at vegetative stage only as well as at flowering growth stage only was detrimental to the RWC, stomatal conductance, F_v/F_m, P_n and shoot biomass for both genotypes relative to the control conditions. Several studies have shown that drought stress notably limits vegetative growth by reducing leaf water content in plant tissue (Sehgal et al., 2017; Awasthi et al., 2014), which may markedly be influenced inhibition of stomatal conductance/ transpiration (Blum et al., 1999), causing membrane damage (Awasthi et al., 2014), chlorophyll loss (Rahbarian et al., 2011) and reduced photosynthesis (Mafakheri et al., 2010), due to disruption of stomatal or non-stomatal associated mechanisms. At vegetative stage however, Acc#8 accumulated more shoot biomass than Acc#7 when both genotypes were exposed to mild water stress. This may have been due to Acc#8 being more efficient in its water use compared to Acc#7 under these conditions; as evidenced by its lower stomata conductance (Fig 2a) hence more available plant water. Some *Populus deltoids* and *Populus nigra* genotypes which had a lower stomata conductance after exposure to drought conditions were deemed water use efficient after they accumulated higher biomass (Monclus et al., 2006). However, at vegetative stage, Acc#7 was able to maintain a higher soil moisture content than Acc#8 while at the same time, recording higher stomatal conductance, a result seemingly contradictory. This may have been due to better partitioning of water use into the useful transpiration, rather than the wasteful escape from the soil surface by Acc#7, probably due to the shading effect of its rather “more bushy” growth habit at early development. Ogola et al. (2002) demonstrated that reduction in wasteful loss of water through reduction of evaporative escape, coupled with a simultaneous increase in transpiration led to an increase in water use efficiency of maize. In our study, genotype Acc#7 showed a higher WUE compared to Acc#8 at flowering growth stage.

3.5 Conclusion

In our study, genotype Acc#7 plants had higher shoot biomass, P_n, F_v/F_m, RWC and a smaller root diameter compared to Acc#8 probably due to the beneficial effects of priming, hence acquired drought tolerance. The combination of these factors showed that heat tolerant Acc#7 has better adaptive tolerance to drought after priming than heat sensitive Acc#8 associated with osmotic adjustment on leaf RWC, stomata regulation, water uptake and higher capacity to protect photosynthetic activity. This makes Acc#7 potentially ideal for areas associated with intermittent drought stress. We partially disapprove the hypothesis that heat tolerant Acc#7 is also more drought tolerant than heat sensitive Acc#8, but rather it is better acclimated to drought than Acc#8, because of its superiority only in primed plants and not those severely stressed only at either vegetative or flowering growth stages. This therefore implies that the physiological responses to drought and heat stresses are largely independent of one another and that generation of genotypes displaying cross tolerance through exposure to combined heat and drought are required. Genotype selection by matching chickpea crop physiological performance to the expected rainfall amount and distribution in drought prone areas is also notably essential.

Chapter 4: A physiological and proteomic study on the response to heat stress at flowering growth stage of different chickpea (*Cicer arietinum* L.; Fabaceae) genotypes

4.1. Introduction

Increased frequencies and duration of high temperatures continue to limit crop growth and development (Barnabas et al., 2008). Being a cool season crop, high temperatures during critical growth stages like the reproductive period can limit chickpea (*Cicer arietinum* L.) grain yield more than warm season legumes like soybean, pigeon pea and groundnut (Summerfield et al., 1984; Devasirvatham et al., 2012a). Crops in the subtropics normally experiencing cool temperatures (5°C to 10°C) during vegetative stages, tend to experience high temperatures beyond 30°C during the day over the reproductive development phase (Summerfield et al., 1984; Devasirvatham et al., 2012a). The heat stressful temperatures greater than 30°C may lead to membrane damage, protein denaturation, inactivation of enzymes in the mitochondria and chloroplasts, synthesis of new proteins and impaired carbon metabolism (Wahid et al., 2007). Heat stress also leads to loss of chlorophyll, disruption of the photosynthetic electron transport and a reduction in the activity of Rubisco in the heat sensitive photosynthetic apparatus (Sinsawat et al., 2004) as well as oxidative metabolism in plants through overproduction of reactive oxygen species (ROS) such as superoxide radicals (O_2^-) and hydroxyls (OH^-), leading to cell membrane peroxidation, protein oxidation and DNA damage (Chen et al., 2013).

However, plants may respond to environmental stress through physiological and biochemical changes aimed at restoration of cellular homeostasis which enables them to survive under such adverse conditions (Huber and Bauerle, 2016). For example, plants have evolved complex antioxidative detoxification systems, such as the biosynthesis of antioxidants like anthocyanins and flavonols, to counteract the injurious effects of overproduced ROS under heat stress (Harsh et al., 2016). Anthocyanin accumulation contributes to non-specific disease resistance, having a pivotal role in plants' responses to abiotic and biotic stresses (Castellarin et al., 2007) in crops like rice (Finocchiaro et al., 2010) and maize (Moreno et al., 2005) due to their antioxidant properties, hence their involvement in plant mechanisms of photoprotection (Azuma et al., 2012; Chen et al., 2013).

Heat stress leads to increased expression of several proteins with chaperone functions, especially members of the large family of heat-shock proteins which are classified into five distinct sub-families (HSP110, HSP90, HSP70, HSP60 and the small HSPs) according to their molecular weight (Baniwal et al., 2004). However, proteomic analyses have uncovered several proteins, other than HSPs, which are crucial for acclimation to heat as well as drought stress in several crops like rice, chickpea and barley (Pandey et al.,2017; Kosová et al., 2011; Singh and Jwa, 2013; Rollins et al., 2013; Parankusam et al., 2017). For example, Lee et al. (2007) identified 48 proteins, among them HSP70, HSP100, dnak-type molecular chaperone and Cpn60 in rice that was exposed to 12 to 24 hours of high temperature. Also, regulated proteins (99) associated with energy, metabolism, photosynthesis, detoxification and translation were differentially expressed in barley under heat stress (Rollins et al., 2013), and 81 over-expressed proteins involved in protein synthesis, storage, transport, signal transduction as well as defensive systems against diseases and heat stress were observed in alfalfa (Li et al., 2013). Therefore, the identification and introgression of heat stress tolerant proteins could be incorporated into modern biotechnology tools for improvement of heat stress tolerance in various crops including chickpea.

Presently, few studies have combined chickpea physiological and proteome analysis to elucidate the changes in abundance and/or activity of relevant enzymes and expression of heat responsive proteins and their associated metabolic roles. In this study, analysis of chlorophyll concentrations, gas exchange, concentrations of flavonoids and anthocyanin from controlled climate chamber experiment are presented to support the heat tolerance status of chickpea genotypes from a field study where samples for the proteomic analyses were obtained. In the field experiment, the genotypes were classified as heat tolerant (Acc#7 and Acc#RR-3) and heat susceptible (Acc#RR-2 and Acc#8) (Chapter 2) using physiological markers. Leaf samples of the tolerant and sensitive chickpea genotypes from the field study were subjected to a detailed Label free quantification proteome analysis to determine the expression of proteins involved in heat tolerance.

4.2 Materials and methods

4.2.1 Plant material, experimental design and management

The climate chamber experiment (experiment 1) was conducted once in September 2018 at the Department of Food Science, Aarhus University, Aarslev, Denmark (55.30N, 10.44E). The four Desi chickpea genotypes earlier used in the field experiment (experiment 2) were sown under greenhouse conditions. Genotypes were sown with a 12 hr photoperiod combination of natural and supplementary light, 65 ±15% air relative humidity, RH (%) and an average temperature of 25°C ± 1.5°C. Three seeds were placed one cm deep into 0.6 L truncated cone plastic pots (9 cm height, 11 cm in diameter across the top and 7.5 cm in diameter across the bottom) filled with a commercial peat-based potting substrate (Pindstrup Færdigblanding 2, PindstrupMosebrug A/S, Ryomgaard, Denmark). Seedlings were thinned to one plant per pot at the 4th leaf stage (approximately 14 days after sowing, DAS). When the first flower appeared, a total of 12 plants per genotype were initially moved to the control climate chamber (MB teknik, Brøndby, Denmark), with 11 hour photoperiod, photon flux density (PPFD) of 300 μmol m^{-2} s^{-1} (LED

FL300 Sunlight, Fionia Lighting, Søndersø, Denmark), RH set at 65%, and a CO_2 concentration of 400 ppm for a 24 hour acclimatisation. Three temperature regimes, including the control, were established in three separate chambers: a control chamber with day/night temperatures of 25/20°C, moderate heat stress of 30/25°C and high heat stress chamber with day/ night temperatures of 35/30°C. The three chambers were set at relative humidity of 65%, 70% and 80% respectively to maintain a constant VpdL across treatments. At 50% flowering, four replicates from each genotype in the control climate chamber were subjected to moderate heat stress at 30/25°C and high heat stress at 35/30°C for 3 days, with four replicates per genotype remaining in the control climate chamber. Plants in the 25/20°C and 30/25°C chambers were watered with a full nutrient solution twice a day, the plants in the 35/30°C were watered thrice daily to avoid moisture limitation. The day length was set from 0800 to 1900 hours in all chambers. Plants were returned to the control chamber after data collection for recovery until final grain yield determination.

Planting material, experimental design and, management for the field experiment (Experiment 2) is as described in section 2.2.1 to 2.2.2 but data on proteomic analysis in experiment 2 is presented.

4.2.2 Chlorophyll, flavanoid and anthocyanin concentrations

Chlorophyll, anthocyanin and epidermal flavanoid concentrations were measured simultaneously *in vivo* using a Dualex 4 Scientific (Dx4) device. (Dualex Scientific^{TM}, Force-A, Paris, France). The device was clamped on the youngest, upper most and fully expanded leaf and measurements taken on four plants per treatment between 0900 hours and 1000 hours after three days of prior exposure to heat stress (35/30°C and 30/25°C treatments, and the control). The indexes of the measurements are defined as described by Shen et al. (2017).

4.2.3 Gas exchange

Gas exchange parameters including net photosynthetic rate (P_n), rate of transpiration (E), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured at CO_2 concentration of 400 μmol mol^{-1} for experiment 1 using a portable open system infrared gas analyser (CIRAS-2, PP systems, Amesbury, MA, USA). Data were collected on the youngest fully expanded leaf after three days of prior exposure to heat stress in the 35/30°C and 30/25°C treatments, as well as the control treatments. The CIRAS cuvette and the plant were placed in a controlled climate cabinet (with corresponding temperature regimes) during the measurements. Each plant in the cuvette was given 5 minutes to achieve a steady state, after which the subsequent two minutes average gas exchange rates were recorded. A moist cloth was placed around the water vapour equilibrator of the gas analyser to increase the RH (%) of the air during measurements on the plants from the two heat stress treatments. The youngest fully expanded leaves were affixed in the 1.7 cm^2 leaf cuvette, with a flow rate of 200 mL min^{-1}, light intensity of 1000 μmol photons m^{-2} s^{-1} (controlled by an LED light unit) and a CO_2 concentration of 400 ppm. Measurements were taken between 09:00 and 12:00 and plants were kept hydrated throughout the measurements by maintaining a thin layer of water underneath the pots in order to avoid stomatal closure.

4.2.4 Chlorophyll fluorescence

F_v/F_m measurements were taken on four plants per genotype (in each temperature regime) using a Plant Efficiency Analyser, Handy PEA (Hansatech Instrument, King’s Lynn, UK) with excitation light energy of 3000 $\mu molm^{-2}$ s^{-1}. Data was collected on the youngest fully expanded leaf after three days of exposure to heat stress. Leaves were prior dark-adapted, using leaf dark clips, for 30 minutes (Hansatech Instrument). Measurements were taken at 16:00 to maximise on the light exposure, hence the stressful conditions on the adaxial leaf surface.

4.2.5 *Grain yield and yield components*

Number of pods plant^{-1} (Experiment 1) were determined at harvest maturity from four harvested plants per genotype and treatment, which were then dried to 12% moisture content and seed weight in grams plant^{-1} determined.

4.2.6 Chickpea physiological data analyses (Experiment 1)

For experiment 1, a two-way analysis of variance (ANOVA) was used to test the effect of temperature regimes and genotypes on the measured parameters. The Tukey's Honest Significant Difference (HSD) test was used to separate means that were significantly different ($p<0.05$).

4.2.7 Brief background on proteomic study (Experiment 2)

This section of the study aimed at determining the influence of heat stress on the proteome profile of two tolerant and one susceptible genotype (Chapter 2), (Acc#RR-3 and Acc#7; and Acc#8 respectively). The samples were collected from the coolest site (Polokwane), which is therefore considered as a reference point/ control site, as well as the warmest site (Venda) which is the site considered as heat stressful site.

4.2.8 Protein extraction

Leaf samples were collected randomly from four plants per plot, quickly snap frozen in liquid nitrogen and then stored on ice from the field and then transferred into a -80ºC freezer until further analysis. Total leaf protein was extracted from the four biological replicate samples for each treatment using a phenol extraction protocol (Isaacson et al., 2006). The recovered pellets from all samples were washed three times using ice cold methanol (4ºC) for 10 minutes at 5,000 g. The pellets were dried under vacuum, stored at -80ºC and eventually sent to the Centre for Proteomic and Genomic Research (Rondebosch, South Africa) for analysis.

4.2.9 Protein solubilisation and quantification

Protein samples were resuspended in 100mM triethylammonium bicarbonate (TEAB; Sigma T7408) 2% Sodium dodecyl sulfate (SDS; Sigma 71736), 5% SDS 50 mM TEAB and then placed at 95ºC for ten minutes. Samples were then clarified by centrifugation at 10 000 × *g* for ten minutes at room temperature. Quantification was performed using the QuantiPro BCA assay kit (Sigma QPBCA) according to the manufacturer's instructions.

4.2.10 On-bead HILIC digest

The HILIC beads (ReSyn Biosciences, HLC010) were aliquoted into a new tube and then washed with 250 µl wash buffer twice (15% ACN, 100 mM Ammonium acetate (Sigma 14267) pH 4.5) for one minute. The beads were then resuspended in loading buffer (30% ACN, 200 mM Ammonium acetate pH 4.5) to a concentration of 5 mg/ml. A total of 50 µg of protein from each sample was transferred to a protein LoBind plate (Merck, 0030504.100). Protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) which was added to a final concentration of 10 mM TCEP and incubated at 60˚C for one hour. Samples were cooled to room temperature and then alkylated with methylmethanethiosulphonate (MMTS; Sigma 208795), added to a final concentration of 10 mM MMTS and then incubated at room temperature for 15 minutes. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 beads:total protein. The plate was then incubated at room temperature on the shaker at 900RPM for 30 minutes for binding of protein to beads. After binding, the beads were washed four times with 500 µl of 95% ACN each time for one minute. For digestion Trypsin (Promega PRV5111), made up in 50 mM TEAB was added at a ratio of 1:10 total protein and the plate was incubated at 37˚C on the shaker for four hours. After digestion, the supernatant containing peptides was removed and dried down. Samples were then resuspended in LC loading buffer: 0.1% FA, 2.5% ACN.

4.2.11 LCM S analysis

LCMS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. Data was acquired using Xcalibur v4.1.31.9, Chromeleon v6.8 (SR13), Orbitrap MS v2.9 (build 2926) and Thermo Foundations 3.1 (SP4). Peptides were dissolved in 0.1% Formic Acid (Sigma 56302), 2% Acetonitrile (Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 9027905000, 300 µm × 5 mm × 5 µm). Samples were trapped onto the column and washed for 3 minutes before the valve was switched and peptides eluted onto the analytical column as described hereafter. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm). The solvent A system was employed; LC water (Burdick and Jackson BJLC365), 0.1% FA and solvent B: ACN, 0.1% FA.

The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 5 min, gradient change: 2–5% Solvent B, time change 40 min, gradient change 5–18% Solvent B, time change 10 min, gradient change 18–30% Solvent B, time change 2 min, gradient change 30–80% Solvent B. The gradient was then held at 80% solvent B for 10 minutes before returning it to 2% solvent B and conditioning the column for fifteen minutes. All data acquisition was obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523). The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV.

4.2.12 Proteomic data analysis (Experiment 2)

For experiment 2, label free quantification was conducted using Progenesis QI for Proteomics v2.0 (Non-linear Dynamics, UK). Raw data processing included peak picking, running alignment and normalisation (singly charged spectra were removed from the processing pipeline) and valid proteins containing at least two unique peptides were reported. Relative quantification was based on four biological replicates per condition using non-conflicting peptides. A protein with a fold change $\geqslant 2$ with a corresponding q-value <0.05 was considered regulated. Database interrogation was performed with Byonic Software v2.6.46 (Protein Metrics, USA) using a chickpea database sourced from Uniprot-KB and downloaded on 26/09/2018.

The Label free identified proteins were functionally annotated using the UniProt database (http://www.uniprot.org). The database was used to search for the Gene Ontology (GO) analysis using three key terms, i.e. Biological Processes, Molecular Processes and the Cellular Component as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. The conserved domains and family names of the identified proteins were identified to the reference canonical pathways of chickpea in the KEGG database (http://www.genome.jp/tools) (Kanehisa and Goto, 2000).

4.3 Results

4.3.1 Chlorophyll, flavanoids and anthocyanin concentrations

The Genotype × temperature regime interaction did not affect chlorophyll and flavonoid concentration, but the main effects of genotype and temperature regime were significant (Table 4.1). Plants grown in the high heat stress had significantly ($p<0.001$) the least chlorophyll concentrations, with those grown in the moderate heat stress intermediate. Also, Acc#8 (heat susceptible) had significantly ($p<0.05$) lower chlorophyll concentration compared with Acc#7 which was classified previously as heat tolerant (Table 4.1). Plants exposed to high heat stress had significantly ($p<0.05$) higher flavanoid concentrations compared with moderate heat stress and the control plants (Table 4.1). In contrast, anthocyanin concentration was significantly affected by the genotype × temperature regime interaction (Fig. 4.1); only Acc#7 (heat tolerant) and Acc#RR-2 had significantly higher anthocyanin concentrations in the high heat stress treatment compared to the heat susceptible Acc#8. Indeed, it is only the heat tolerant genotype (Acc#7) that exhibited significantly higher anthocyanin concentration at the high and moderate heat stress compared to the control (Fig. 4.1).

4.3.2 Chlorophyll fluorescence

The interaction between genotype and temperature regime affected ($p<0.05$) F_v/F_m (Fig. 4.2); Heat stress (high and moderate) did not lower F_v/F_m in the heat tolerant Acc#7 but a significant decline in F_v/F_m in the moderate heat stress (0.78) and high heat stress(0.74) relative to the control treatment (0.84), was observed in the heat susceptible Acc#8 and a decline observed only in the high heat stress for Acc#RR-2 and Acc#RR-3 (Fig. 4.2)

Table 4.1: Effect of temperature and genotype on leaf chlorophyll, flavonoids and leaf temperatures of chickpea at 50% flowering stage in a climate chamber experiment. Mean±se with different letters are significantly different as at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant.

Treatments	Chlorophyll	flavanoids
Temperature		
25/20°C (control)	41.0 ± 1.42^{a}	1.25 ± 0.04^{b}
30/ 25°C (moderate heat stress)	34.1 ± 0.74^{b}	1.24 ± 0.04^{b}
35/ 30°C (high stress)	29.3 ± 1.93^{c}	1.41 ± 0.04^{a}
Genotypes		
Acc#RR-2	33.9 ± 2.47^{ab}	1.31±0.06
Acc#RR-3	34.5 ± 2.60^{ab}	1.25±0.06
Acc#7	38.7 ± 1.16^{a}	1.33±0.04
Acc#8	32.2 ± 1.89^{b}	1.30±0.06
F probability		
Temperature	21.0***	5.1*
Genotypes	3.4*	0.5^{ns}
Temperature*Genotype	1.8^{ns}	1.6^{ns}

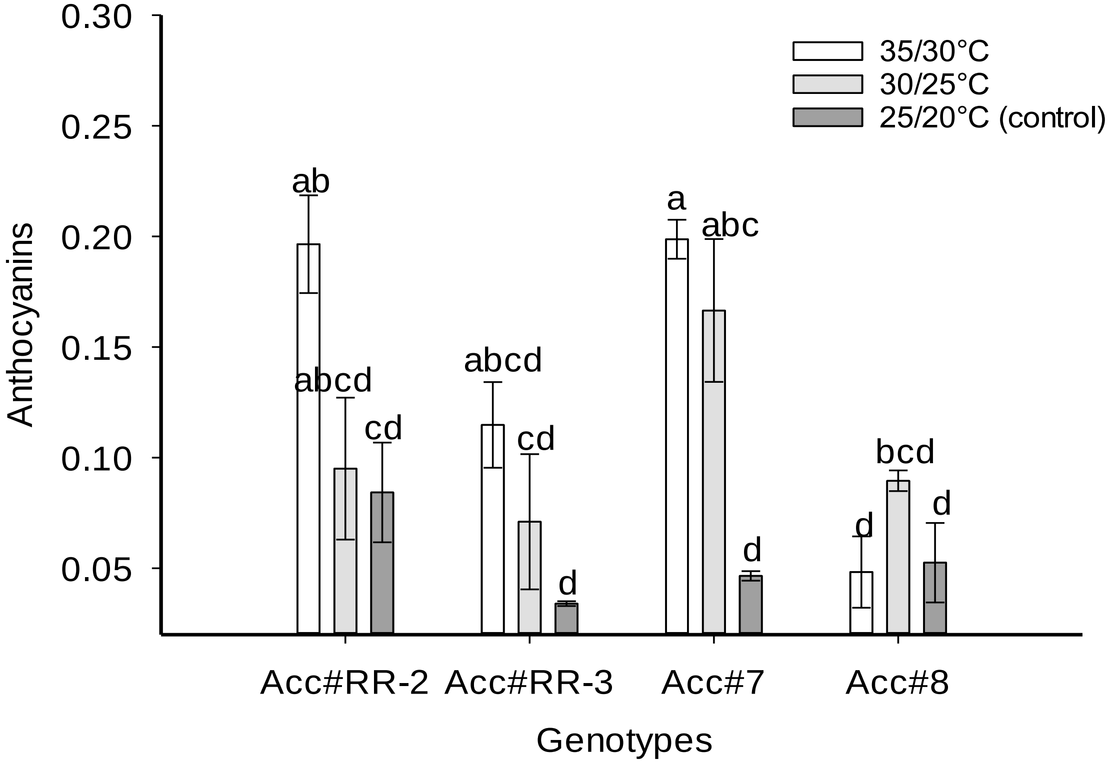


Figure 4.1: Interaction of genotype and temperature treatment on anthocyanin concentrations at 50% flowering stage of chickpea treatment in a growth climate chamber. Data is mean values ± se (n=4). Different letters indicate significant differences between genotypes and temperature treatments by Tukey's honest significant difference post hoc test ($p<0.001$).

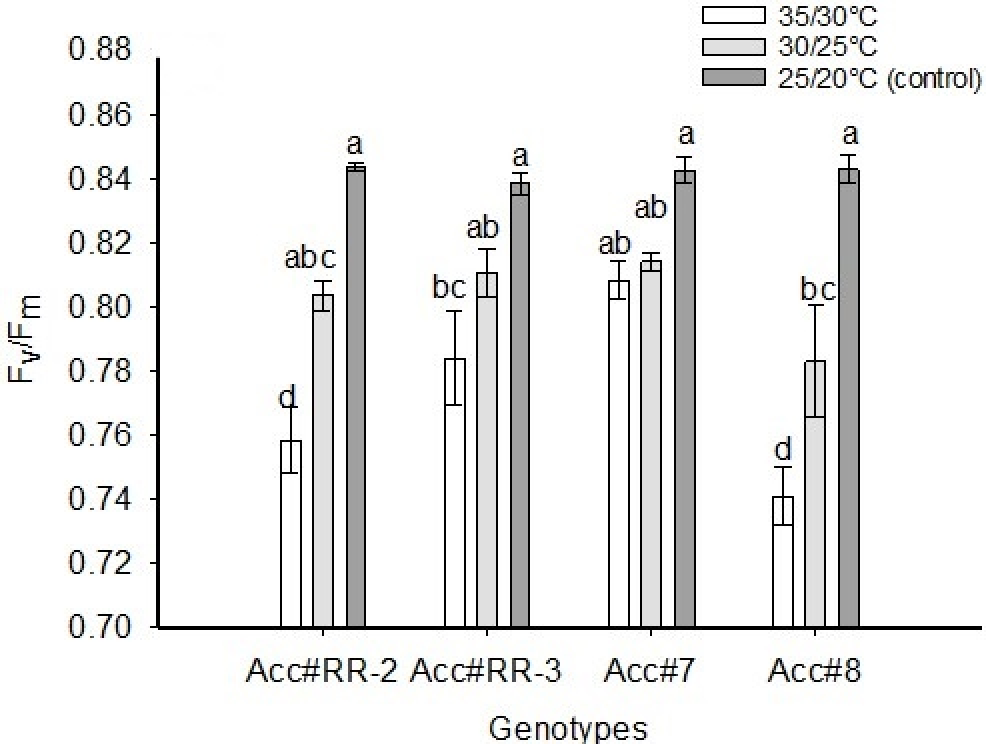


Figure 4.2: Interaction of genotype and temperature treatment on maximum quantum yield of PSII (F_v/F_m) at 50% flowering stage of chickpea in a growth climate chamber. Data is mean values ± se (n=4). Different letters indicate significant differences between genotypes and temperature treatments by Tukey's honest significant difference *post hoc* test ($p<0.005$).

4.3.3 Gas exchange parameters

The main effects of genotype (G) and temperature regime (T) but not the G × T interaction on net photosynthesis (P_n) was significant (Fig. 4.3a). The heat tolerant Acc#7 exhibited significantly higher P_n than Acc#RR-2 and the heat susceptible Acc#8, and both high and moderate heat stress decreased P_n across the genotypes (Fig. 4.3a). Both the high heat stress and moderate heat stress decreased the internal carbon dioxide concentrations (Ci) (Fig. 4.3b). There was a significant interaction ($p<0.05$) between genotype and temperature regime on leaf transpiration rates, *E* (Fig. 4.3c). Acc#3 plants subjected to high heat stress treatment had significantly higher *E* relative to the moderate heat stress and the control plants which were similar. Heat susceptible genotype Acc#RR-2 had significantly lower *E* than Acc#RR-3 in the high heat stress (Fig. 4.3c). Genotype had significant effect on stomatal conductance ($p<0.001$) (Fig.4.3d), with Acc#7 (heat tolerant) significantly higher than Acc#2 (heat susceptible).

4.3.4 Grain yield and yield components

Grain yield (g plant^{-1}) was significantly affected by the G × T interaction ($p<0.05$) as well as the main effects of G and T ($p<0.001$) (Table 4.2). There was a significant decline in the grain yield of all genotypes for both the moderate and high temperature chambers relative to their controls. However, Acc#8 and Acc#7 had the most and least severe decline in grain yield (94% and 65% respectively) in the high heat stress relative to their controls. Also, the heat tolerant Acc#7 had significantly higher grain yield (which was similar to the yield of Acc#8 at the control temperature regime) compared to the other 3 genotypes at the moderate heat stress. Though not different from Acc#RR-2 and Acc#RR-3, Acc#7 also had significantly higher grain yield plant^{-1} compared to Acc#8 in the high heat stress; indeed, the grain yield of Acc#7 subjected to high heat stress was similar to the grain yield of the other genotypes that were subjected to moderate stress (Table 4.2).

The G × T interaction on number of pods per plant was not significant but heat stress (both moderate and high) decreased ($P<0.001$) the number of pods per plant. Also, genotypic differences ($P<0.001$) were observed, with Acc#7 recording higher number of pods per plant compared to the other three genotypes which were similar (Table 4.2).

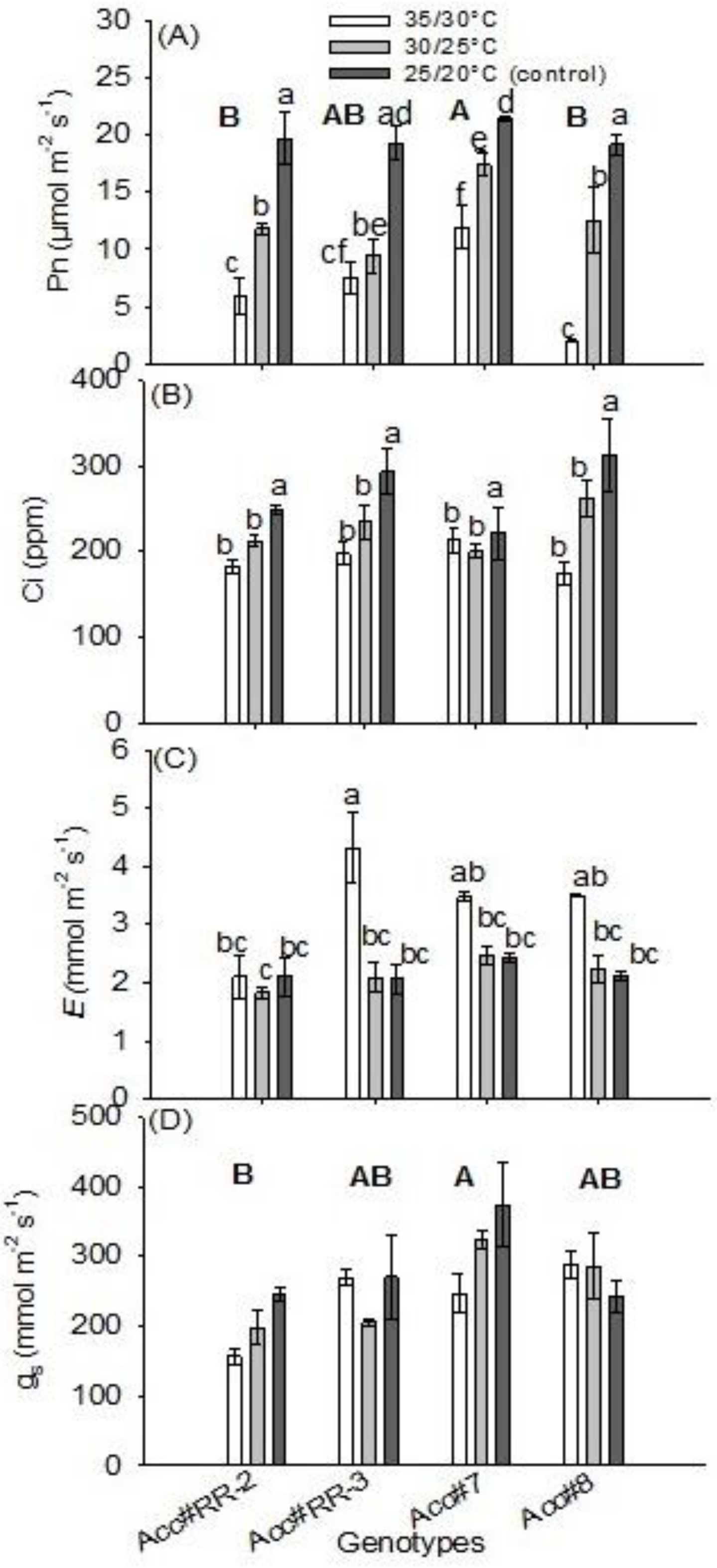


Figure 4.3: The effect of genotype and temperature treatment on (a) net photosynthesis, (b) internal carbon dioxide concentration, (c) leaf transpiration and (d) stomatal conductance at 50% flowering stage of chickpea in a growth climate chamber. Data is mean values ± se (n=4). Uppercase and lowercase letters indicate significant differences between genotypes and temperature treatments respectively by Tukey's honest significant difference post hoc test ($p<0.05$).

Table 4.2: Effect temperature and genotype on grain yield (plant^{-1}) and number of pods per plant of chickpea in a climate chamber experiment. Mean±se with different letters are significantly different as at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant

Treatment	Grain yield	pod number plant^{-1}
Temperature		
25/20°C (control)	6.8±0.29^{a}	19.1±0.98^{a}
30/ 25°C	2.3±0.33^{b}	10.8±1.10^{b}
35/ 30°C	1.2±0.22^{c}	4.1±0.64^{c}
Genotype		
Acc#RR-2	3.3±0.92^{b}	10.3±2.03^{b}
Acc#RR-3	3.3±0.79^{b}	10.1±1.60^{b}
Acc#7	4.6±0.62^{a}	15.2±2.33^{a}
Acc#8	2.6±0.75^{b}	9.7±2.08^{b}
Temperature*Genotype		
25/20°C (control)		
Acc#RR-2	7.4±0.75^{a}	18.3±1.4
Acc#RR-3	6.9±0.63^{a}	16.3±1.5
Acc#7	6.9±0.57^{a}	24.0±1.5
Acc#8	6.0±0.36^{ab}	17.8±1.1
30/25°C		
Acc#RR-2	1.6±0.26^{cd}	9.8±1.9
Acc#RR-3	1.9±0.14^{cd}	9.0±0.9
Acc#7	4.4±0.34^{b}	14.8±1.7
Acc#8	1.4±0.22^{cd}	9.8±1.5
35/30°C		
Acc#RR-2	0.9±0.16^{cd}	3.0±0.8
Acc#RR-3	1.1±0.17^{cd}	5.0±1.2
Acc#7	2.4±0.44^{c}	6.8±0.6
Acc#8	0.3±0.09^{d}	1.5±0.4
F probability		
Temperature	12.7***	8.9***
Genotype	219.3***	100.3***
Temperature*Genotype	3.0*	ns

There was a significant positive correlation between grain yield and F_v/F_m (Fig. 4.4a), and a significant negative relationship between grain yield and leaf anthocyanin (Fig. 4.4b) and leaf flavanoids (Fig. 4.4c) concentrations.

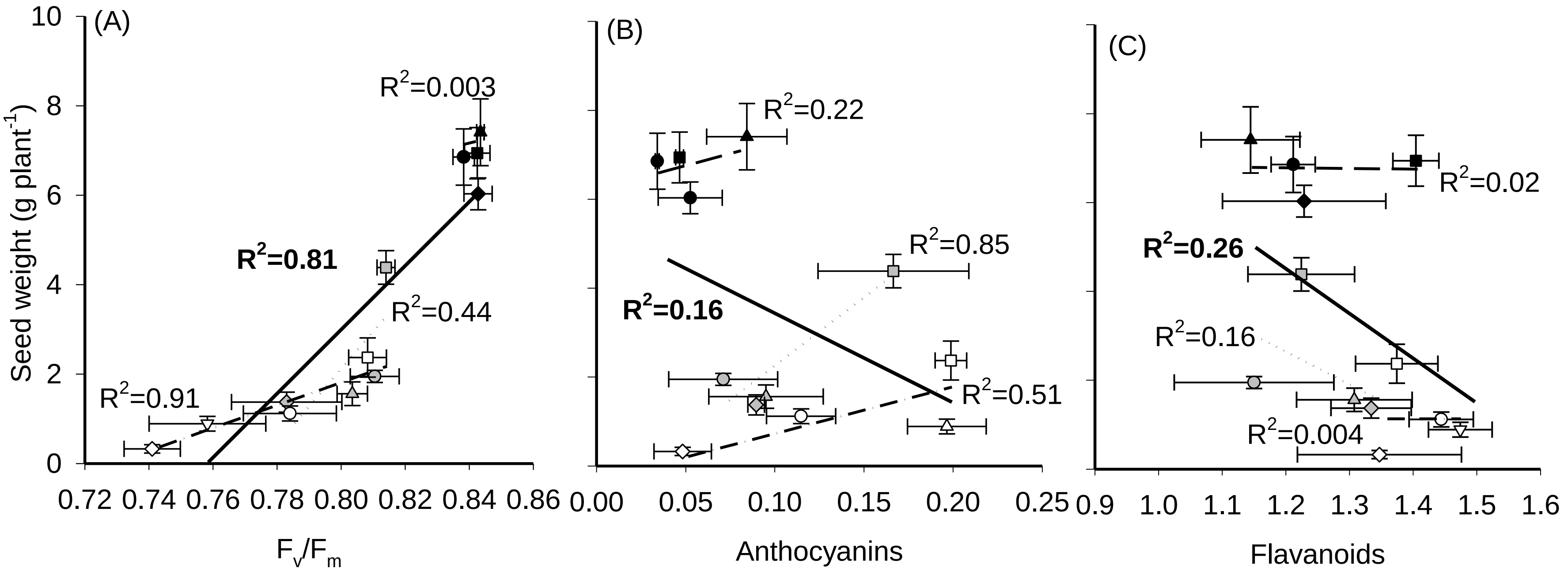


Figure 4.4: Correlation of chickpea grain yield (g plant^{-1}) and F_v/F_m ($P<0.001$) (A), leaf anthocyanins $P<0.05$) (B) and leaf flavanoids ($P<0.05$) (C) in a growth climate chamber. The data represent mean values ± se (n=3) where the symbols are triangles (Acc#RR-2), circles (Acc#RR-3), squares (Acc#7), and diamonds (Acc#8) and the closed symbols are for 25/ 20°C (control), grey for 30/ 25°Cand open symbols for 35/ 30°C. Bold regression lines and R^2 values represent combined regression across treatments and accessions while the non-bold R^2 relate to respective treatments.

4.3.5 Protein profile from field experiment 2 for the heat tolerant Acc#3 and Acc#7 and heat susceptible genotype Acc#8

A combined total 3287 unique proteins were identified in the three genotypes assessed (i.e. 1036, 1073 and 1178 for Acc#3, Acc#7 and Acc#8 respectively). Of the positively identified proteins, a combined 542 proteins were differentially expressed in response to heat stress in Acc#3 (129), Acc#7 (221) and Acc#8 (192). Amongst the differentially expressed proteins, 41, 103 and 94 were unique to Acc#3, Acc#7 and Acc#8 respectively, while 29, 39 and 9 proteins were common to Acc#3 and Acc#7, Acc#7 and Acc#8 and Acc#3 and Acc#8 respectively (Fig. 4.5). Fifty heat responsive proteins were common in all three genotypes.

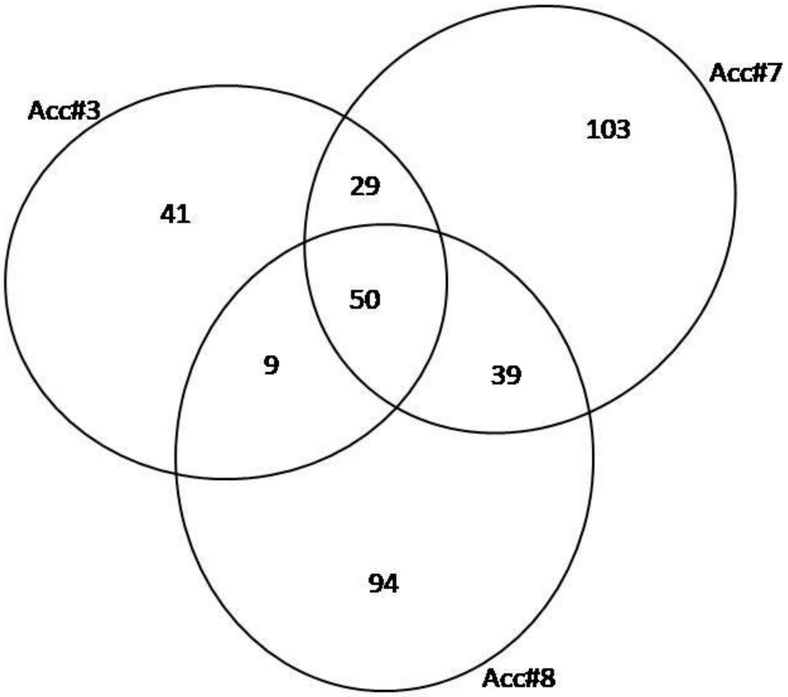


Figure 4.5: The identified relationship amongst the heat stress responsive proteins for Acc#RR-3, Acc#7 and Acc#8.

In order to determine whether the heat responsive proteins were either up-regulated or down-regulated, a comparison was done between the warmer (Venda) and the cooler (Polokwane) sites. For Acc#RR-3, 75 (58%) were up-regulated (highest mean condition in warmer Venda site compared to the cooler site of Polokwane), while 54 (42%) were down regulated (lowest mean condition in warmer Venda site compared to the cooler site of Polokwane). In the warmer site of Venda, 120 (54%) proteins were up-regulated for Acc#7, while 100 (46%) were down regulated. For Acc#8, 95 (49%) proteins were up-regulated in the warmer site of Venda while 97 (51%) were down regulated (up-regulated in the cooler site of Polokwane). The fold change ranged from 2 to 21.46 for up-regulated proteins in Acc#RR-3 and from -49.61 to -2 for the down regulated proteins. For Acc#7, the range for up-regulated proteins was from 2 to 48.56 while the down regulated protein ranged from -35.73 to -2.01. The range for up-regulated proteins for Acc#8 was from 2.01 to 27.8 and -61.61 to -2.0 for down regulated proteins. A summary list of the heat stress responsive proteins identified in the three genotypes and discussed in text is given in Table 4.3 as well as in detailed supplementary Tables 1 to 3.

All the heat stress responsive proteins for Acc#RR-3 (129), Acc#7 (221) and Acc#8 (192) were GO annotated and classified into molecular (Fig. 4.6), biological (Fig. 4.7) and cellular components (Fig. 4.8). Of the heat responsive proteins identified, 6%, 4% and 16% (Acc#RR-3, Acc#7 and Acc#8 respectively) had no predicted molecular functions (Fig. 4.6). Heat responsive proteins predicted to be involved in molecular functions comprised the largest proportion compared to the other two categories (Fig. 4.6 to 4.8). Proteins involved in binding activities that encompass, but not limited to, nucleotide, protein, nucleic acid, ion, ATP, GTP, heme and rRNA binding comprised 47%, 42% and 36% for Acc#RR-3, Acc#7 and Acc#8, respectively (Fig. 4.6 to 4.8). Also, there was relatively high activity predicted for structural constituent of ribosome (14%, 14% and 6%), oxidoreductase activity (8%, 6% and 9%), hydrolase activity, hydrolyzing O-glycosyl compounds (5%, 3% and 2%), catalytic activity (4%, 5% and 5%) and GTPase activity (2%, 4% and 1%) for Acc#RR-3, Acc#7 and Acc#8, respectively. Other relatively high activity observed included for protein phosphatase inhibitor, electron transfer, signalling receptor, protein disulfide oxidoreductase, peptidyl-prolyl cis-trans isomerise, carbonate dehydratase, peroxidise, chitinase, and sucrose synthase (Fig. 4.6).

The heat responsive proteins for the respective genotypes were also GO annotated across a wide range of biological functions. There were no predicted biological processes for 35%, 40% and 46% of the proteins identified for Acc#RR-3, Acc#7 and Acc#8 respectively (Fig. 4.6). Proteins predicted to be involved in translational processes constituted the largest proportions (14%, 15% and 7% for Acc#RR-3, Acc#7 and Acc#8, respectively) compared to other categories. The other highly represented proteins in this category were those involved in metabolic processes with 12%, 4% and 7%, biosynthesis processes with 5%, 3% and 2%, oxidation-reduction processes with 7%, 8% and 10%, transport with 6%, 5% and 7% and the cell redox homeostasis processes with 5%, 2% and 4% for Acc#RR-3, Acc#7 and Acc#8 respectively. The remaining proteins belonged to the other biological processes, albeit, in insignificant proportions (Fig. 4.7).

There were no cellular component predictions for 75%, 73% and 78% of the heat responsive proteins for Acc#RR-3, Acc#7 and Acc#8 respectively (Fig.). The most highly presented cell components included the ribosome with 13%, 11% and 7%, outer membrane with 3%, 2% and 4%, photosystem II with 2%, 1% and 1%, mitochondria with 0%, 2% and 2%, nucleus with 1%, 1% and 1%, as well as the chloroplast with 1.5%, 0.5% and 0.5% for Acc#RR-3, Acc#7 and Acc#8 respectively. In addition, there was a lower representation of the other cell components like photosystem II oxygen evolving complex, nascent polypeptide-associated complex, microtubule, thylakoid membrane, proteasome regulatory particle clathrin coat of trans-Golgi network vesicle, clathrin complex across the three genotypes (Fig. 4.8).

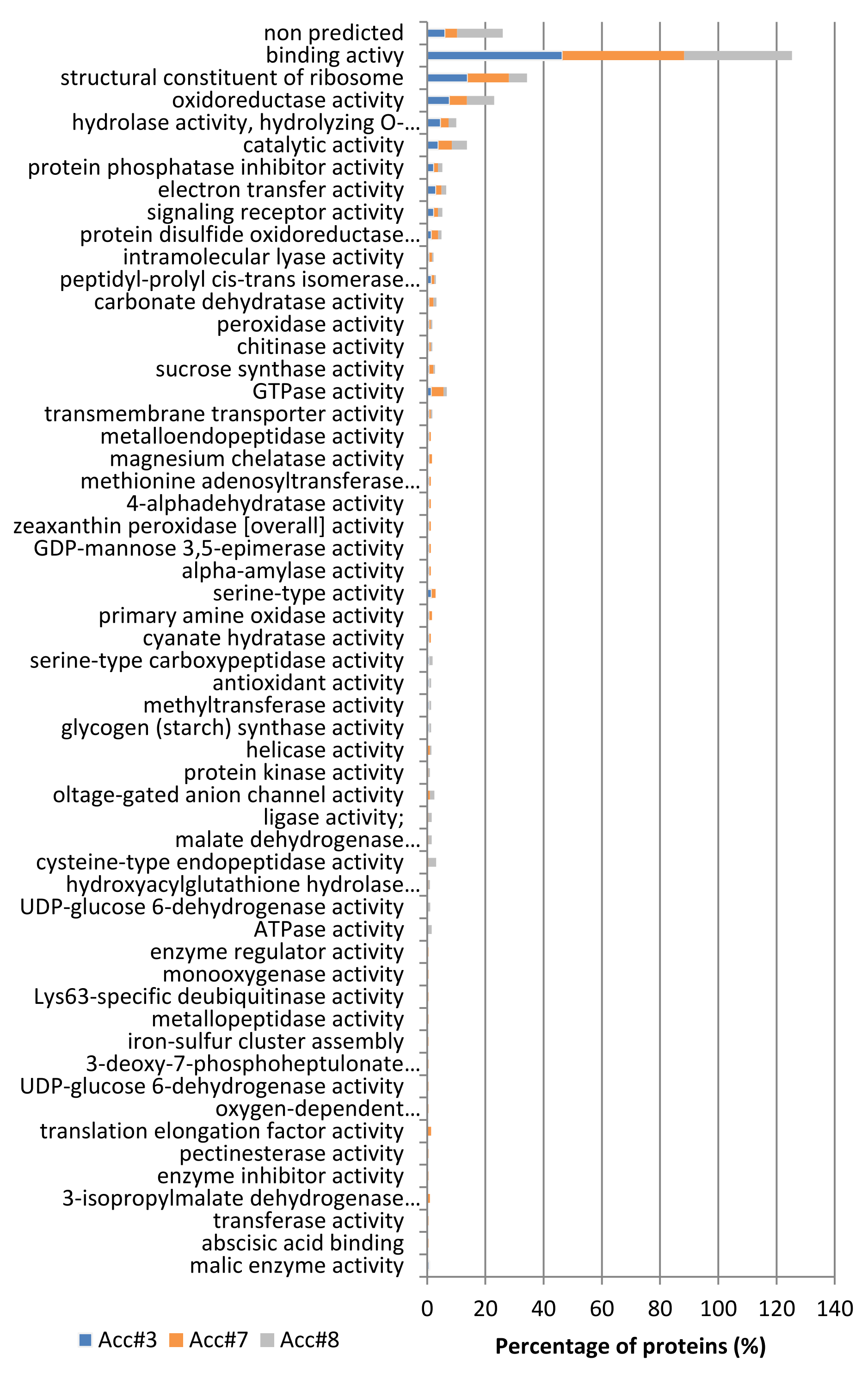


Figure 4.6: Molecular component predictions of the identified heat stress responsive total soluble protein based on GO annotation.

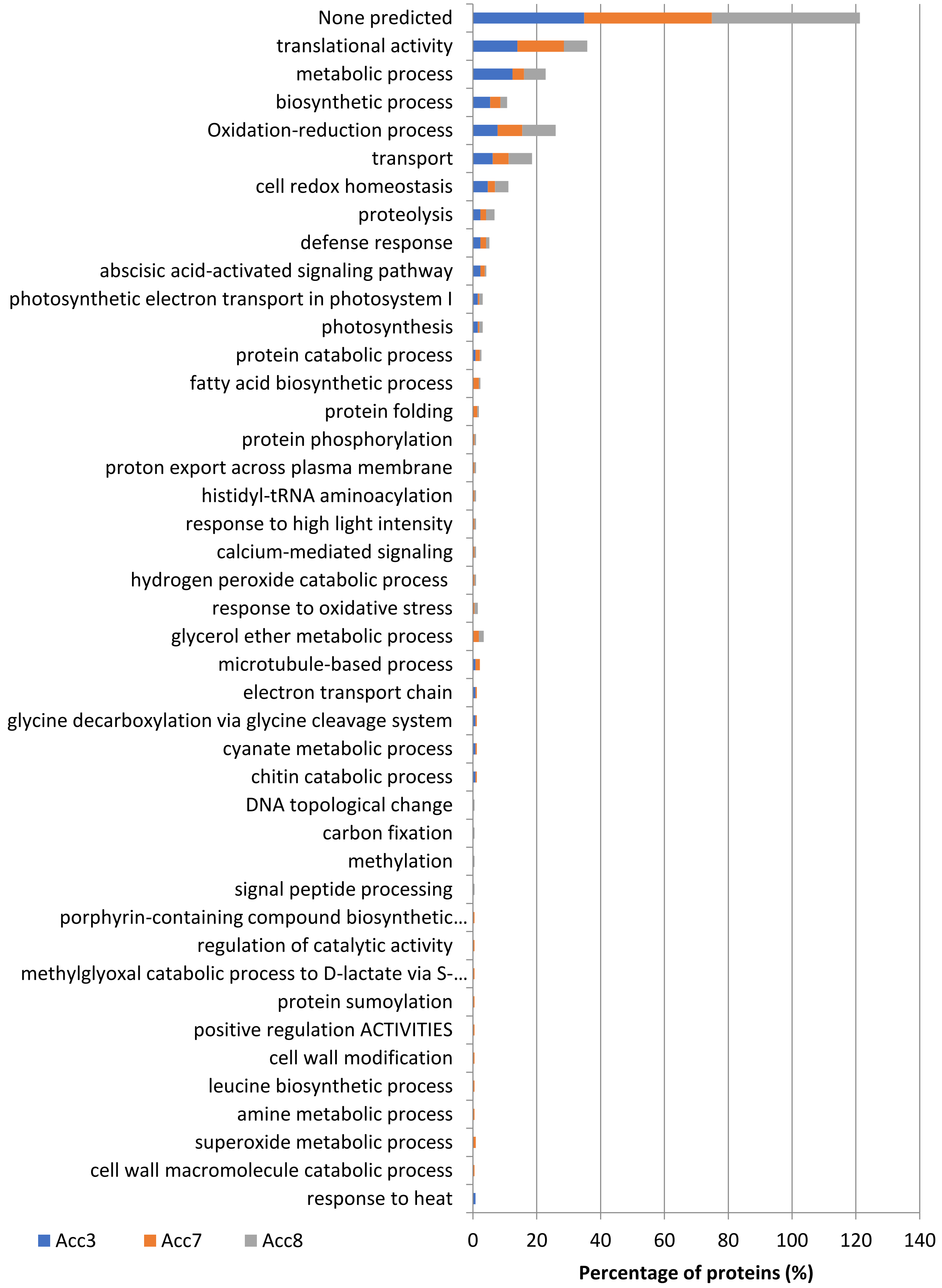


Figure 4.7: Biological component predictions of the identified heat stress responsive total soluble protein based on GO annotation.

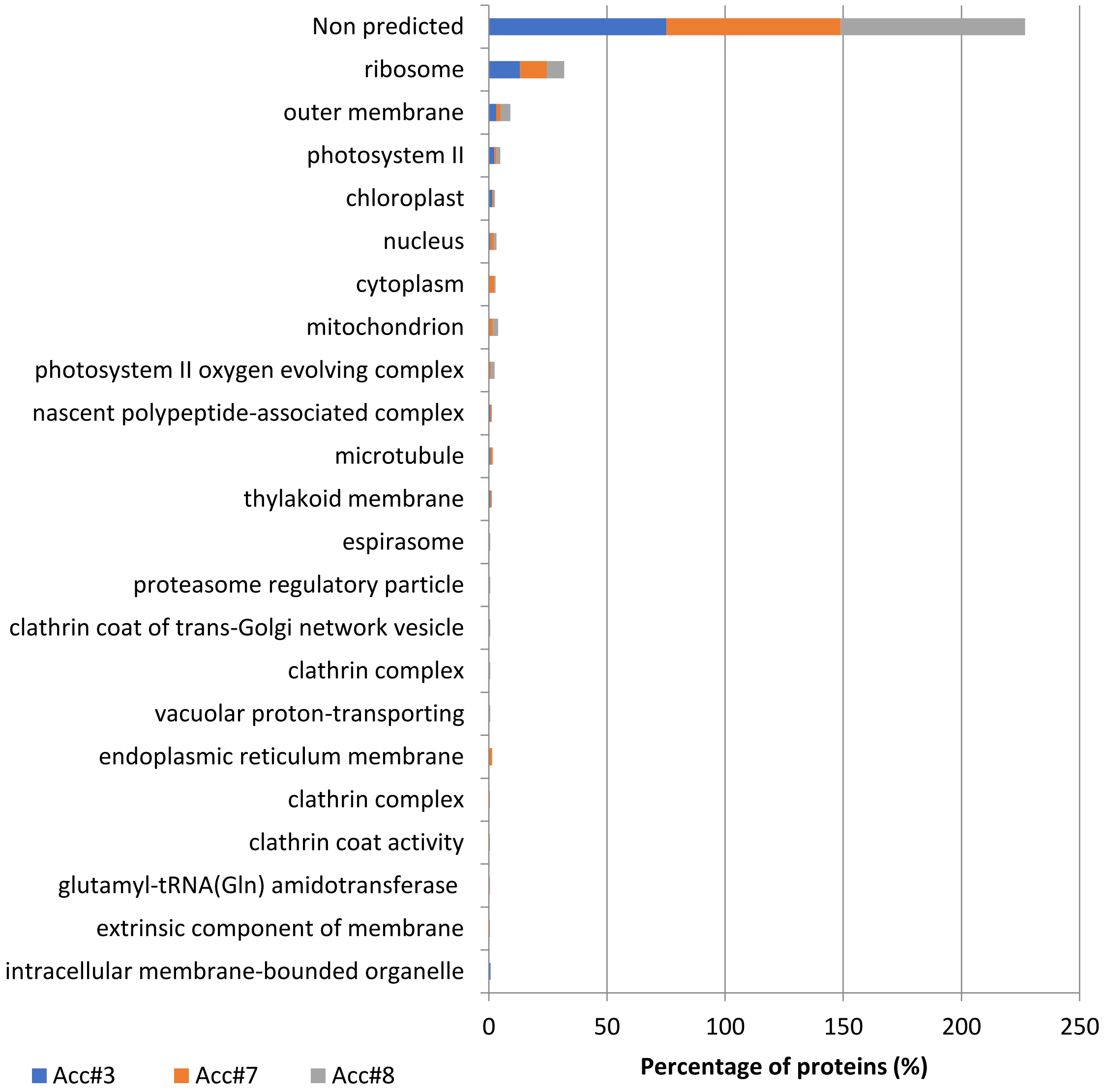


Figure 4.8: Cellular component predictions of the identified heat stress responsive total soluble protein based on GO annotation

4.3.7 Functional categories of differentially expressed heat responsive proteins

Based on the functional categories described by Bevan et al. (1998), the highest representation for differentially expressed proteins for Acc#RR-3 were those involved in metabolism (25%), protein synthesis (17%), disease and defence (17%), transporters (13%) and energy (10%) (Fig. 4.9). The highest representation for differentially expressed proteins for Acc#7 were those involved in metabolism (21%), protein synthesis (20%), disease and defence (19%), transporters (14%), and energy (6%) (Fig. 4.9). Metabolism (20%), disease and defence (18%), protein synthesis (13%), followed by transporters (9%) and energy (7%) had the highest representation for the differentially expressed heat responsive proteins for Acc#8 (Fig. 4.9).

Most of the characterised heat responsive proteins under the protein synthesis category were down regulated in the warmer site of Venda and up-regulated in Polokwane for Acc#RR-3, except for four that were up-regulated in Venda (Fig. 4.10). For Acc#7, 38 proteins involved in protein synthesis were up-regulated under heat stressful conditions in Venda and of this group, the greatest representation was from proteins that were structural constituents of the ribosomes. In contrast, only six proteins in the protein synthesis category were up-regulated when Acc#8 was grown in the warmer site of Venda (Fig. 4.10).

For Acc#RR-3, 19 proteins categorized under metabolism were up-regulated in the warmer site of Venda and down regulated in the cooler site of Polokwane. For Acc#7, 21 proteins involved in metabolism were up-regulated when the genotype was grown in Venda and down regulated when it was grown in cooler site of Polokwane. For the same metabolism category proteins, 23 of the total were up-regulated when Acc#8 was grown in warmer site of Venda and down regulated in cooler Polokwane (Fig. 4.10).

Twenty proteins categorized under disease and defence for Acc#RR-3 were up-regulated in Venda and down regulated in Polokwane. Seven proteins involved in disease/defence were up-regulated in Acc#7 when grown at the warmer Venda site and were down regulated when the genotype was grown at the cooler site of Polokwane. Seven proteins involved in defence were up-regulated when Acc#8 was grown in the warmer site of Venda (Fig. 4.10; Supplementary Tables 1 to 3).

Of the proteins involved in transport, 12 were up-regulated for Acc#RR-3 in the warmer site of Venda and down regulated in the cooler site of Polokwane. On the other hand, 21 were up-regulated in Acc#7 when it was grown in Venda and were down regulated in Polokwane. For Acc#8, six proteins involved in transport were up-regulated and down-regulated in Venda and Polokwane respectively (Fig. 4.10; Supplementary Tables 1 to 3).

Eleven proteins categorized under energy, were up-regulated for Acc#RR-3 at the warmer site of Venda and down-regulated in Polokwane, while four energy related proteins were up-regulated for Acc#7 when it was grown in the warmer site and down regulated at the cooler site. Three proteins were up-regulated at the warmer site of Venda and down-regulated in Polokwane for Acc#8 in the energy category (Fig. 4.10; Supplementary Tables 1 to 3).

We further performed the KEGG analyses of each of the heat responsive proteins to identify their involvement in the molecular, biological and cellular processes and the molecular pathways involved. From our results, enzymes sucrose synthase and sucrose-phosphate synthase involved in starch and sucrose pathway (Fig. 4.11) were identified to be associated with probable sucrose-phosphate synthase that was upregulated in tolerant Acc#7 and downregulated in susceptible Acc#8 at the warmer site of Venda (Table 4.3). Another enzyme, sucrose-phosphate phosphatase, also involved in the sucrose and starch pathway (Fig. 4.11), was uniquely expressed and upregulated in the tolerant Acc#7 when grown at the warmer site of Venda (Table 4.3).

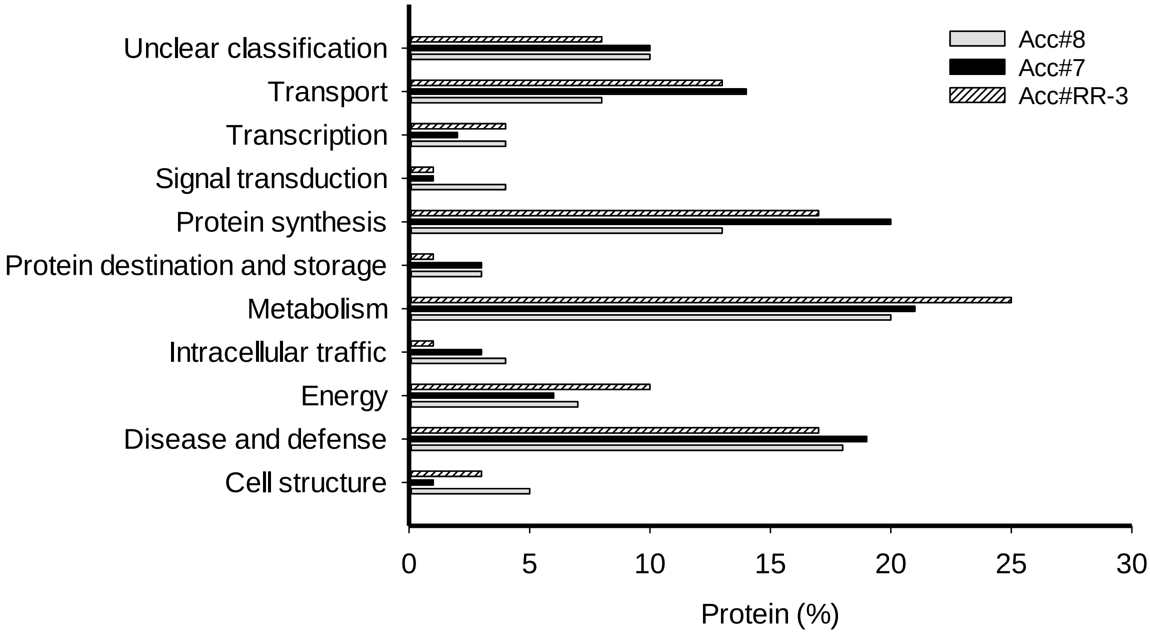


Figure 4.9: Functional classification of the differentially expressed heat stress responsive proteins for chickpea genotypes Acc#RR-3, Acc#7 and Acc#8

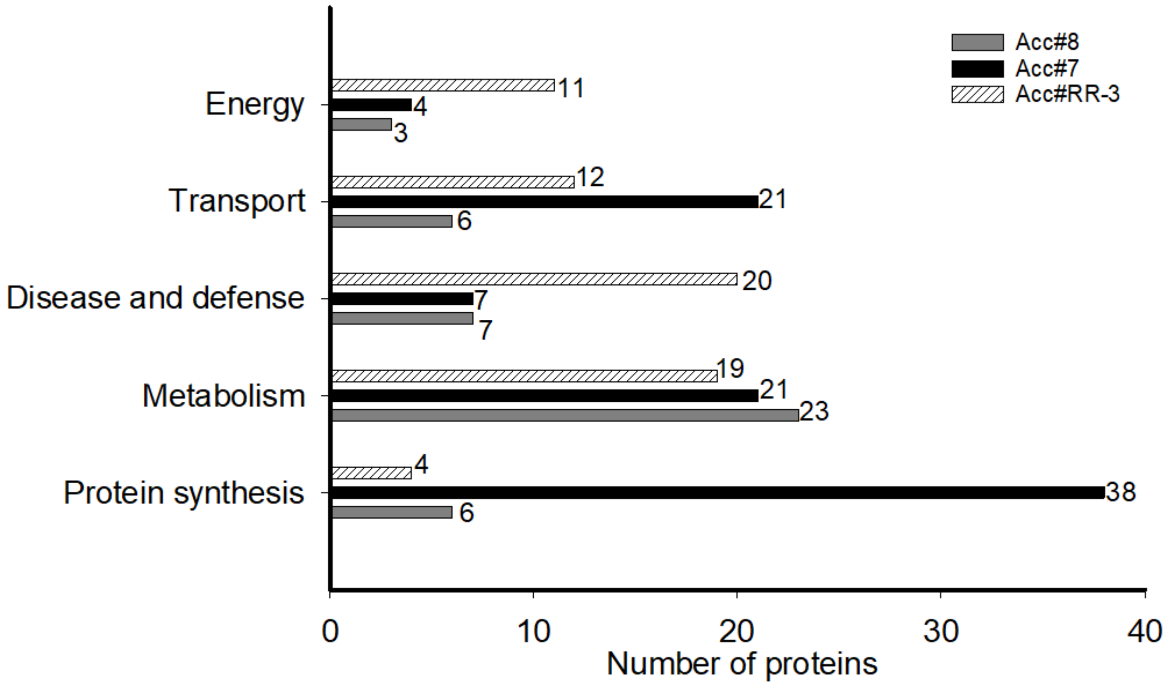


Figure 4.10: Functional classification of the upregulated heat stress responsive proteins for chickpea genotypes Acc#RR-3, Acc#7 and Acc#8 at the Venda site.

Figure 4.11: KEGG pathway image of starch and sucrose metabolism for chickpea under heat stress at flowering growth stage. Enzymes involved in sucrose and starch synthesis identified in Acc#7 and Acc#8 in the current study and depicted in circles were sucrose-phosphate phosphatase (EC:3.1.3.24), Sucrose-phosphate synthase (EC 2.4.1.14) and sucrose synthase (EC 2.4.1.13).

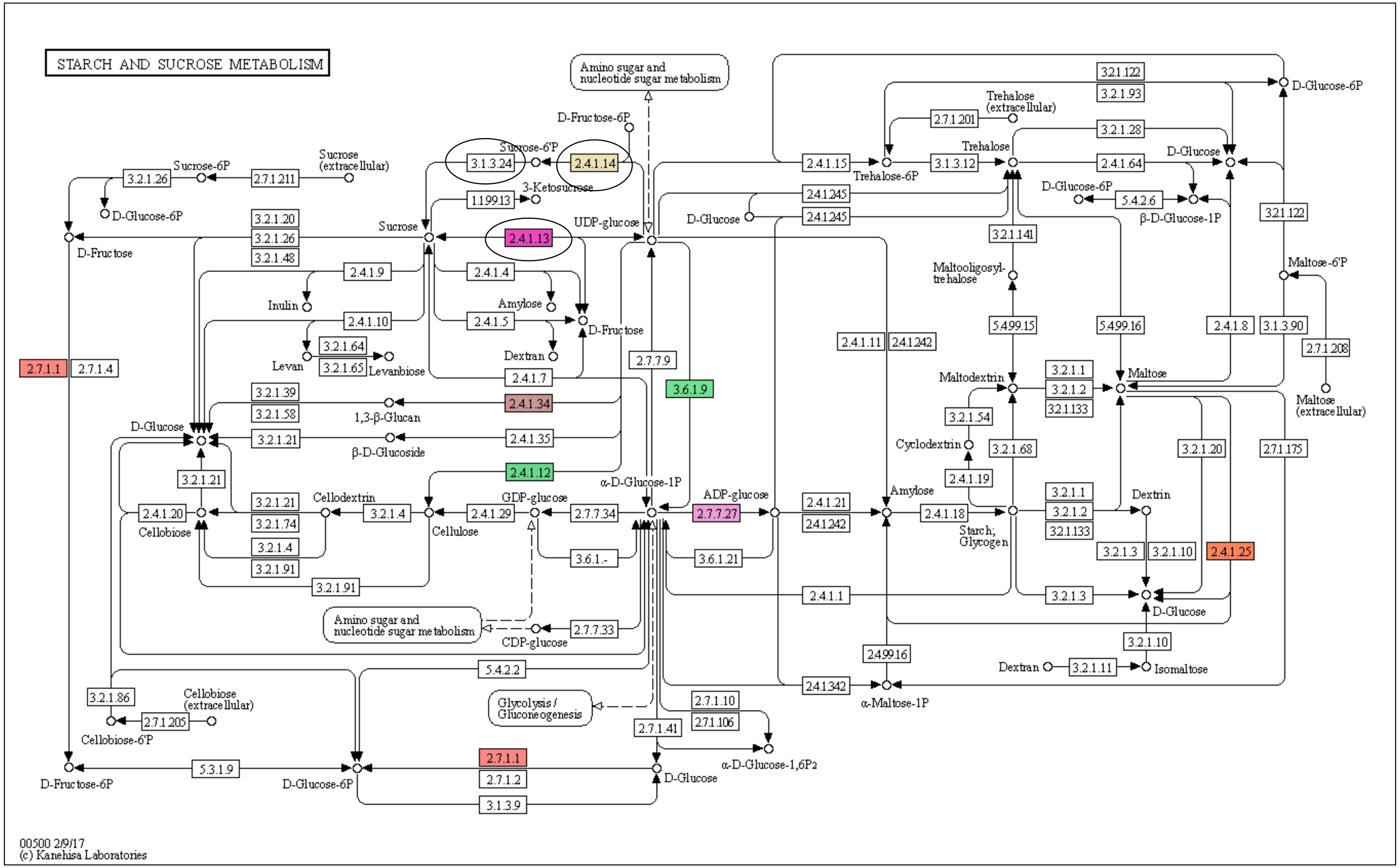


Table 4.3: Some of the heat stress responsive proteins identified from Acc#RR-3, Acc#7 and Acc#8 samples discussed in text. Identification was done using the Label free quantification and database searches. All protein identifications were from *Cicer arietinum*. The complete lists of differentially regulated proteins for Acc#RR-3, Acc#7 and Acc#8 can be found in Supplementary Tables 1 to 3

Accession	Protein Name	Score^C	q value	max fold	Function
Acc#RR-3					
tr|A0A1S2Y874	Plastocyanin	78.197	0.019	13.175	energy
tr|A0A1S2YPS5	Sucrose synthase	5.942	0.038	3.018	energy
tr|A0A1S2YPS0	peroxiredoxin Q, chloroplastic	235.481	0.007	2.344	metabolism
Genotype Acc#7					
tr|A0A1S2YQ32	dihydrolipoyl dehydrogenase 1, chloroplastic-like	13.998	0.048	2.99	energy
tr|A0A1S2XB11	ribulose bisphosphate carboxylase/oxygenase activase (Rubisco), chloroplastic	21.267	0.008	2.94	protein synthesis
tr|A0A1S2YPS0	peroxiredoxin Q, chloroplastic	235.481	0.007	2.344	metabolism
tr|A0A1S2Y115	sucrose-phosphatase 1-like	26.247	0.005	2.34	metabolism
tr|A0A1S2Y835	Catalase	160.915	0.007	2.315	disease and defence
tr|A0A1S2XWV1	Zeta-carotene desaturase	10.834	0.011	2.263	metabolism
tr|A0A1S2YUC7	serine/threonine-protein kinase STN8, chloroplastic-like	11.018	0.012	2.106	metabolism

tr|A0A1S2YQR3	probable sucrose-phosphate synthase	14.833	0.003	2.063	metabolism
tr|A0A1S2YMB2	heat shock 70 kDa protein (HSP70) 15-like isoform X1	55.18	0.048	2.025	Disease and defence
tr|A0A1S2YGU4	mitochondrial dicarboxylate/tricarboxylate transporter DTC	78.459	0.01	2.008	intracellular traffic
tr|A0A1S2XGQ1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2-like	8.905	0.005	-3.106	energy
tr|A0A1S3EGD4	Sucrose synthase	11.43	0.011	-3.314	metabolism
tr|A0A1S2Y874	Plastocyanin	83.8	0.003	-10.074	energy
Genotype Acc#8					
tr|A0A1S2Y874	Plastocyanin	103.708	0.011	10.568	energy
tr|A0A1S2YQR3	probable sucrose-phosphate synthase	16.153	0.014	-2.063	metabolism
tr|A0A1S2Y835	Catalase	195.318	0.034	-2.286	disease and defence
tr|A0A1S2YGU4	Mitochondrial dicarboxylate/tricarboxylate transporter DTC	94.699	0.018	-2.403	intracellular traffic
tr|A0A1S2XB11	ribulose bisphosphate carboxylase/oxygenase activase (Rubisco), chloroplastic	22.568	0.04	-2.439	protein synthesis
tr|A0A1S2XSJ5	26S proteasome regulatory subunit 4 homolog A	8.964	0.017	-2.484	protein synthesis

tr|A0A1S2YMB2	heat shock 70 kDa protein (HSP70)15-like isoform X1	50.18	0.048	-2.725	disease and defence

4.4 Discussion

Plant chlorophyll concentration is an important indicator of photosynthetic capacity and is generally correlated to thylakoid membrane stability under heat stress (Vijayalakshmi et al., 2010; Jiang and Huang, 2001). Genotype Acc#7, showing heat tolerating traits, was able to maintain higher chlorophyll concentrations compared to the less tolerant genotype Acc#8. Clearly, Acc#7 plants were better at staying green compared with the less tolerating Acc#8, which resultantly exhibited signs of early senescence. Delayed senescence or stay-green traits have been associated with heat and drought tolerance in crops like cowpea and wheat (Muchero et al., 2009; Sharma et al., 2015). However, high temperature stress generally led to decreased average chlorophyll concentrations (across genotypes) which suggests that high chlorophyll concentration can be used as a reliable indicator for PSII efficiency in chickpea after exposure to heat stressful conditions (Vijayalakshmi et al., 2010). The reduction in photosynthetic pigments under heat stress may be attributed to the inhibition of biosynthesis of, and changes in, ultrastructure of the chloroplast, especially the membrane, as well as photodegradation (Tewari and Tripathy, 1998; Reda and Mandoura, 2011). In the climate chamber study, the tolerant genotype Acc#7 showed superior rates of photosynthesis compared to the susceptible Acc#8. This trend was followed up by the genotype consistently showing a higher accumulation of anthocyanins compared to Acc#8 when the genotypes where exposed to high heat stress. Based on the chlorophyll concentrations, flavanol and anthocyanins concentrations and net photosynthesis, genotype Acc#7 maintained unaltered physiological performance at flowering growth stage when exposed to high and moderate heat stress, a result consistent with the superior physiological and grain yield performance of genotype Acc#7 compared to Acc#8 under field conditions (Chapter 2).

Genotype Acc#7 had higher grain yield and pod yield plant^{-1} than the sensitive Acc#8 when exposed to high heat stress in a climate chamber. This result was consistent with the result from the field study, where Acc#7 accumulated more grain and shoot biomass yields compared to Acc#8 after exposure to perennial heat stress (Chapter 2). Protein synthesis has been noted to account for a significant part of the energy required for plant growth (Penning de Vries, 1975; Amthor, 2000). In Acc#7, 38 proteins associated with protein synthesis were up-regulated under heat stressful conditions compared to the four and six in Acc#RR-3 and Acc#8 respectively (Fig. 8). When plants are exposed to heat treatments beyond optimum growing temperatures, protein synthesis rates decline, owing to a coordinate loss of most mRNAs, especially in non-tolerant species (Dubey, 1999). However, in this study, ribosomal proteins dominated the protein synthesis category with 2, 26 and 3 proteins being upregulated in genotypes Acc#RR-3, Acc#7 and Acc#8 respectively (Supplementary Tables 1 to 3). Despite the limitation in reports on the role of ribosomal genes in stress response (Moin et al., 2016), ribosomal proteins have been known to play an important role in escaping protein synthesis from being inhibited by heat stress (Kim and Jang, 2002; Duncan and Hershey, 1989) as well as improving cell growth and overall plant development (Chang et al., 2005; Giavalisco et al., 2005). In their study, Cheng et al. (2020) noted that 12 ribosomal proteins were significantly increased in mustard sprouts under heat stress. In this study, heat stressful conditions directly impacted 26S proteasome regulatory subunit 4 homolog A, one of the down-regulated proteins in susceptible genotype Acc#8 (Table 2), leading to reduction in cell proliferation and ultimately decreased cell division rates and growth (Reinheckel et al., 2000). Ji et al. (2017) showed that the overexpression of an Arabidopsis ribosomal protein gene in Sweet potato (*Ipomoea batatas* L) not only led to better antioxidant activity, but improved the crop’s photosynthetic performance and tolerance to both heat and cold temperatures, consequently improving its tuber and above ground biomass yields and storage ability under stressful conditions.

The heat tolerant genotype Acc#7 exhibited higher stomatal conductance, an important factor that modulates photosynthetic rates in plants, than Acc#RR-3 and the heat susceptible Acc#8. This result was also consistent with the superior photosynthetic performance of genotype Acc#7 compared to Acc#RR-3 and Acc#8. Eamus et al. (2008) have shown that the stomatal conductance of some *Eucalyptus haemastoma* leaves declined with leaf temperatures above 30–32°C, with considerable reductions at 40°C. In *Vitis vinifera cv. Semillon* leaves, Greer and Weston (2010) observed that after a four-day heat stress exposure, temperatures beyond 35°C caused sustained reductions in photosynthesis that was mainly (95%) attributed to reduction in g_s, suggesting that stoma of this plant was highly susceptible to heat stress. Peroxiredoxin Q chloroplastic was induced and upregulated in Acc#3 and Acc#7 after exposure to heat stress (Table 2). This protein is involved in redox homeostasis in the chloroplast (Rasouli et al., 2020) and their upregulation in Acc#RR-3 and Acc#7 shows their ability to maintain photosynthesis under heat stressful conditions. Stoma rely on the production of ATP in the guard cells for their movement and under heat stressful conditions, more energy is required for osmotic adjustment and dealing with elevated temperatures (Wahid et al., 2007; Tyerman et al., 2019). Therefore, it is not surprising that proteins associated ATP synthesis were upregulated in chickpea leaves under heat stress.

Rubisco plays an important role in the Calvin cycle and the up-regulation of ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic in tolerant Acc#7 and downregulation in susceptible genotype Acc#8 at the warmer site, suggests its superior thermostability (Rollins et al., 2013) in the tolerant genotype compared to the susceptible Acc#8. Rubisco activase functions in acclimation of photosynthetic CO_2 fixation (Law and Crafts-Brandner, 2001), increasing photosynthetic rates during high temperature stress and keeping the steady state of photosynthesis approached with increased light intensity (Yamori et al., 2012). The down-regulation of Rubisco activase might lead to short-term disruptions in

CO_2 assimilation under elevated temperatures and the plants may have a lower thermal stability. This observation clearly shows the consistency with the superior photosynthetic performance by the heat tolerant genotype Acc#7 compared to the susceptible Acc#8 (Fig. 2) as well as the tolerant genotype's superior grain yield (Chapter 2). On the other hand, Plastocyanin, which was up-regulated at the warmer site in the sensitive genotype Acc#8 (Table 2), has been shown to donate electrons to PSI thus increasing the total rate of energy transduction in photosynthetic membranes (Li et al., 2013). Indeed, plastocyanin along with other proteins-like sucrose synthase and NADH dehydrogenase [ubiquinone] 1 alpha sub complex subunit 2-like involved in plant respiration were down-regulated only in tolerant Acc#7 at the warmer Venda site (Table 2). The down-regulation of these proteins may contribute to the adaptation of Acc#7 to heat stress by lowering the respiratory energy consumption in this specific genotype (Rachmilevitch et al., 2006a, b).

Results from the KEGG analysis showed that enzymes sucrose synthase and sucrose-phosphate synthase involved in starch and sucrose pathway were associated with probable sucrose-phosphate synthase and sucrose-phosphate phosphatase that were upregulated in the tolerant genotype Acc#7 at the warmer Venda site. The protein sucrose-phosphate synthase is believed to play a major role in the control of the rate of partitioning of fixed carbon during photosynthesis between starch and sucrose (Stitt and Quick, 1989; Chen et al., 2005) with its activity significantly reduced in susceptible plants when exposed to heat stressful conditions (Kaushal et al., 2013), while the sucrose-phosphate phosphatase catalyses the final step in the sucrose biosynthesis pathway (Lunn and Rees, 1990). These findings are consistent with results from field studies carried out using the same genotypes, where heat tolerant genotype Acc#7 had a higher leaf starch and sucrose concentration compared to the susceptible Acc#8 after exposure to heat stressful conditions (Chapter 2).

Heat shock proteins (HSPs) are known as stress-induced proteins or stress proteins (Wahid et al., 2007) and high temperatures induce the synthesis of high (60-110kDa) and low (15-45kDa) molecular mass HSPs in plants (Miernyk, 1999). In this study, Acc#7 was the only genotype that exhibited the upregulation of heat shock 70 kDa protein 15-like isoform X1 in the warmer site of Venda with the same protein down-regulated in Acc#8 (Table 2). Similarly, Zhu et al. (2006) reported that the induction of HSP70 in transgenic soybean plants by introgressing HsfA1 enhanced tolerance to high temperature stress. There is evidence that HSPs play an important role of augmenting thermotolerance, and that some HSPs are causally involved in signal transduction during heat stress and with deduced function like chaperones, folding and unfolding of cellular proteins, and protection of functional sites from the adversity of heat stress (Vierling, 1991; Wahid et al., 2007). The heat tolerant genotype, Acc#7, had significantly higher anthocyanin concentrations compared to heat susceptible Acc#8, at high heat stress. In addition, all genotypes (except Acc#8) had higher anthocyanin in the high heat stress compared to the controls. For Acc#7, the anthocyanin levels increased significantly in response to high heat stress, which is in line with previous findings in wheat (Hosseinian et al., 2008), indicating the role of the antioxidant defence system in conferring heat stress tolerance. A significant and negative correlation being observed between grain yield and both flavonoid and anthocyanin concentrations (Fig. 4.4). This finding further highlights the role of antioxidants in heat tolerance of chickpea, as well as their incorporation in heat stress tolerance selections. Anthocyanins have been noted to reduce photoinhibition and chlorophyll bleaching in foliar tissues by masking the chlorophyll pigments, hence higher photosynthesis (Johnston et al., 2007). In addition to their role as UV screens, anthocyanins accumulation under heat stress serves to decrease leaf osmotic potential, resulting in increased water uptake, properties that enable the plant to respond quickly to changing environments (Wahid et al., 2007). Flavonoid concentrations were also higher under high heat stress compared to the control treatments. Flavonoids, which play a role in co-pigmentation with anthocyanins and are affected by increased sunlight and temperature exposure were elevated under high heat stress. These conditions usually promote strong enhancement in flavanoid concentrations and in the expression of flavonol biosynthesis genes (Spayd et al., 2002; Downey et al., 2004). However, no genotypic differences were noted on flavonoid concentration with exposure to heat stress. Temperature seems to have less of an effect on flavonol synthesis control, and under heat stress flavonols may not be affected (Spayd et al., 2002) or slightly reduced (Azuma et al., 2012).

Catalase, an antioxidant enzyme which participates in protecting cells against excess ROS was up-regulated in Acc#7 and down-regulated in the susceptible Acc#8 in the warmer site of Venda (Table 2). Dihydrolipoyl dehydrogenase 1, chloroplastic-like, an energy-categorized protein which was only identified and up-regulated in the heat tolerant Acc#7 at the warmer site, relative to the cooler site (Table 2). This protein is part of the glycine cleavage system responsible for the conversion of glycine to serine in the photorespiratory cycle (Bauwe and Kolukisaoglu, 2003). Its downregulation in a heat susceptible wheat cv. ‘1039’ and upregulation in the heat tolerant cv‘810’ was associated with the tolerant genotype’s better maintenance of photorespiration and alliviation of oxidative stress (Wang et al., 2015). The increased abundance of catalase and Dihydrolipoyl dehydrogenase 1, chloroplastic-like in Acc#7 observed in the current study is consistent with higher anthocyanins concentrations for the same genotype after exposure to high heat stress (Fig. 2).

In their study on heat responsive proteome changes that reveal molecular mechanisms underlying heat tolerance in chickpea, Parankusam et al. (2017) classified the proteins into three categories based on expression: 1) Proteins that were enhanced in abundance in the tolerant genotype but reduced in the sensitive genotype, 2) Proteins that were induced in the heat sensitive genotype but were repressed in the tolerant genotype and 3) Proteins that were specific to one genotype and not expressed in the others. In that study Parankusam et al. (2017) reported an upregulation of proteins like PAL 2, beta galactosidase, glucanase, sucrose synthase, WAT1 like transporter and Rubisco in the tolerant JG14 genotype and the same proteins were downregulated in the heat sensitive ICC16374 genotype. Similarly, in the current study, Rubisco was upregulated in the tolerant genotype Acc#7 compared to the sensitive genotype Acc#8. Furthermore, in the current study, several other proteins potentially vital in heat stress tolerance, were upregulated in the heat tolerant genotype and downregulated in the susceptible genotype e.g. heat shock 70 kDa protein 15-like isoform X1, Catalase, 26S proteasome regulatory subunit 4 homolog A. Parankusam et al. (2017) also noted proteins that were upregulated in the heat susceptible genotype and downregulated in the tolerant genotype like SAMS and monodehydroascorbate reductase. In the current study, the susceptible genotypes upregulated proteins like Plastocyanin, sucrose synthase and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2-like among others, which were however downregulated in the tolerant genotype Acc#7. Proteins specific to the heat tolerant JG14 chickpea genotype like 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase, cystathionine gamma-synthase, threonine synthase, P5CS cystathionine gamma-synthase, 1-aminocyclopropane-1-carboxylic acid oxidase and abscisate beta-glucosyltransferase, and flavoprotein were observed in the study by Parankusam et al. (2017). In the current study, tolerant genotype Acc#7 uniquely expressed proteins like Dihydrolipoyl dehydrogenase 1, chloroplastic-like, PSI reaction centre subunit proteins, protoporphyrinogen oxidase, Zeta-carotene desaturase and Serine/threonine-protein kinase after exposure to heat stress in the field (Table 2). Based on the results from these two studies, the expression, and up and down regulation of heat stress responsive proteins in chickpea varies due to among other factors, genotypic differences as well as the levels of heat stress. This is evidenced by the plants in the current study having been exposed to chronic heat stress in the field whereas the study by Parankusam et al. (2017) was a controlled experiment with short term stress induced through artificial light sources in growth chambers over an 8-day period.

4.5 Conclusions

The genotype Acc#7 maintained unaltered physiological performance at flowering growth stage when exposed to high and moderate heat stress, as well as superior grain yield under climate chamber conditions, consistent with the field observations (Chapter 2). The genotype was therefore considered heat tolerant compared to the two genotypes Acc#RR-2 and Acc#8 that were susceptible. The proteomic studies brought to the fore, the roles in heat stress tolerance played by proteins involved in plant growth, disease and defence, carbon partitioning and photosynthesis and transport, through their differential upregulation in heat tolerant Acc#7 compared to the susceptible Acc#8. This study suggests that the superior performance of Acc#7 is due to its ability to escape inhibition of protein synthesis by heat stress, as well as the genotype's upregulation of sucrose phosphate synthase, believed to play a major role in the control of the rate of partitioning of fixed carbon during photosynthesis. The unique upregulation of heat shock 70 as well as antioxidant enzymes by Acc#7 also potentially contributed to its cellular defence protection and eventual superiority compared to Acc#8. The comparative differences between the current field study and previous studies on chickpea shows the variation in the expression of heat stress responsive proteins due to differences in genotypes used and growing conditions. Nevertheless, there is need for further characterization of these candidate proteins identified in the current study as well as their specific roles in the tolerance to heat stress in chickpea for the increased rate of crop improvement initiatives.

Chapter 5: Overall Discussion, conclusions and recommendations

Chickpea is one of the most vital legume crops globally, constituting 20% of the total global pulse production (FAOSTAT, 2018). The crop is generally grown under rainfed conditions, using either residual moisture in subtropical environments (dominated by summer rains), or rainfall in the Mediterranean environments (Yadav et al., 2006). Despite its high yield potential (4000 kg ha^{-1}), chickpea yields at a global scale have been relatively low (910 kg ha^{-1}) due to a combination of biotic (diseases and insect pests) and abiotic (drought, heat and soil infertility) stresses (Awasthi et al., 2014). Climate change is inevitably a complex challenge to humankind currently and in the future (Harris and Roach, 2016). Predicted rises in temperatures, associated with changes in timing, magnitude and distribution of precipitation, are likely to increase drought and heat stress on crops (Thorpe, 2005; Farooq et al., 2009; Mulwa et al., 2010). Drought stress, which is a consequence of insufficient rainfall or soil moisture, could potentially decrease grain yield by 30 to 100% (Leport et al., 1999). Moreover, a warmer climate has the potential to increase the risk of both drought and heat stress. Therefore, a thorough understanding of chickpea physiological (chlorophyll fluorescence, relative water content and gaseous exchange) and biochemical characteristics (carbon partitioning, accumulation of heat shock proteins and phenolics) in response to heat and drought stress is integral for improved chickpea drought and thermotolerance (Zhao et al., 2010; Wahid et al., 2007).

Chickpea, having been historically considered an ‘orphan’ crop in SSA despite widespread production in other parts of Africa (Mabhaudhi et al., 2019), has not been regarded as an important crop in South African cropping systems (Woomer et al., 2014), as more attention has been given to cereal crops like wheat and maize. This is despite efforts that have been made to introduce chickpea to the dry environments of North East South Africa, with the results thus far showing huge potential (Thangwana and Ogola, 2012; Ogola and Thangwana, 2013; Ogola, 2015). Evaluation of crop genotypes for suitability in SSA conditions is, however, a prerequisite for which all future breeding is based. The reality that the effects of climate change (drought and high temperatures) are expected to be more severely felt in the tropics and subtropics, where temperatures are already high, threatens the advances in chickpea production in the region (Serdeczny et al., 2017).

Genotype Acc#7, registered a lower decline in F_v/F_m, P_n, and grain yield in comparison to the sensitive ones at 35/30 °C (chapter 4), relative to their controls, under controlled climate chamber conditions, a trend that was consistent with the performance of the genotypes under natural field conditions (chapter 2). There were also significant and positive correlations between grain yields and F_v/F_m, both in the climate chamber and the natural field conditions (chapter 2 and chapter 4). This is particularly an interesting consistency as plants grown under field conditions are often subjected to various stresses, which sometimes extend throughout their lifetimes, often causing discrepancies between controlled and field-based plant responses and phenotyping (Mittler, 2006; Zhou et al., 2017). For instance, Poudyal et al. (2019) noted that two tolerant tomato cultivars from a controlled chamber experiment had a considerably smaller harvest loss and stayed greener, maintaining higher F_v/F_m than the susceptible genotypes when grown under natural field conditions. The plants grown in the glasshouse and the climate chamber were dark adapted for 30 minutes using light exclusion leaf clips before F_v/F_m measurements were collected (chapters 3 and 4) and on the contrary, plants grown in the field (chapter 2) were dark adapted by taking measurements at 1900 hours, one hour after darkness. However, there was a consistency on the readings of F_v/F_m of control and stressed plants in the field and under controlled environments. Therefore, this consistency in the differences point to the convenience and reliability of dark adaptation by the use of light exclusion clips for 30 minutes during the day as well as the correlations observed (chapter 2), gives further credence to the potential use of F_v/F_m and P_n as phenotyping tools for heat tolerance, as well as linking the screening of chickpea in controlled environments to its production in the field.

The heat stress tolerant genotype Acc#7 (chapter 2 and chapter 4) was, however, noted to be better acclimated to drought stress (chapter 3) but not necessarily tolerant to drought stress. Although similarities between drought and heat stress have been observed (Rang et al. 2011), this result further brings to light the reality in the differences of the mechanisms implored by plants in their tolerance to either of the stresses. The result also highlights that tolerance to either drought or heat stress does not automatically imply tolerance to the other or the combination of both (Jagadish et al., 2007, 2010). This variation has also been attributed to differences in intensity and duration of the stresses (Wahid et al., 2007). Both heat and drought have been reported to decrease electron transport, degrade proteins and release magnesium and calcium ions from their protein-binding partners (Wahid et al., 2007; Zlatev and Lidon, 2012). However, extended exposure to high temperatures has also been noted to primarily trigger decrease in chlorophyll content, increased amylolytic activity, thylakoid grana disintegration and disruption of assimilate transport (Kozłowska et al., 2007). In contrast excessive drought primarily results in closed stomata, low root length density and reduced relative leaf water content (Leport et al., 1998; Wahid et al., 2007). Therefore, the generation of novel chickpea genotypes, displaying tolerance to both heat and drought stress, can only be a reality when studies exploring the underlying mechanisms associated with combined heat and drought stresses are carried out.

Simultaneous heat and drought stress initiates various processes like decreased rate of photosynthesis coupled with abnormal respiration, and closed stomata and high leaf temperatures; these effects may be synergistic, antagonistic or hypo-additive on plant development and yield (Wahid et al., 2007; Qaseem et al., 2019). This challenge/reality makes the identification of traits to be used for selection for tolerance to both stresses, whether individually or in combination, very difficult. For example, in the drought stress study (chapter 3), the chlorophyll fluorescence parameters (F_v/F_m and F_q'/F_m') did not show any variation amongst the genotypes on majority of days of drought stress exposure at vegetative and flowering growth stages, despite being identified as a suitable tool for selection and identification of heat tolerant chickpea genotypes (chapter 2). Under drought stress, stomatal closure imposes a limitation on photosynthesis by decreasing the availability of CO_2, while heat stress, in contrast, inhibits photosynthesis mainly through alterations in non-stomatal traits such as electron transport capacity and activity of Rubisco (Salvucci and Crafts-Brandner, 2004; Way and Oren, 2010). These differences in plants' individual stress responses have been attributed mainly to the differences in signalling pathways, which may interact and inhibit one another in cases of combined stress (Mittler, 2006; Rasmussen et al., 2013). For example, heat stressed plants may increase stomatal conductance in order to cool the leaves by transpiration, but if the heat stress was to simultaneously occur with drought, plants would not be able to open their stomata and therefore leaf temperature would increase by 2–5°C (Rizhsky et al., 2004), making the use of stomatal conductance in combined heat and drought stress studies not as reflective as during individual heat stress studies.

However, some studies have also noted that the combined effects of heat and drought stress were generally additive, suggesting a certain degree of independence between the mechanisms regulating the responses of plants to drought and heat stress (Wahid et al., 2007; Awasthi et al., 2014). This has resulted in the successful use of leaf chlorophyll content, leaf sucrose and starch concentrations, net photosynthesis, F_v/F_m, proteome analysis, osmolyte accumulation analysis, and quantitative trait loci (QTL) approach in studies for tolerance to individual drought and heat stress and their combination (Leport et al., 1998; Behboudian et al., 2001; Chen and Harmon 2006; McCann and Huang, 2007; Barnabas et al., 2008; Reynolds and Tuberosa, 2008; Awasthi et al., 2014; Sehgal et al., 2017; Zhou et al., 2017; Qaseem et al., 2019). The heat tolerant genotype Acc#7 used in the current study, which registered higher net photosynthesis in the field as well as under climate chamber conditions, also maintained higher net photosynthesis in primed plants after exposure to drought stress at vegetative and flowering stages (chapter 2, chapter 3 and chapter 4).

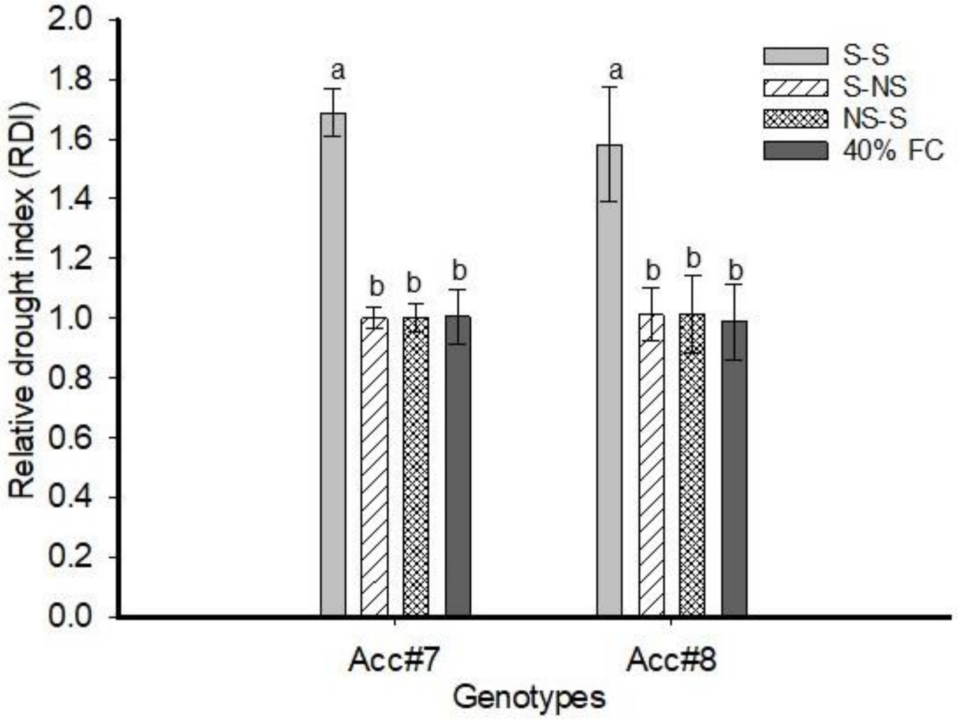
Chickpea is sensitive to high temperatures (>30°C) and drought stress (Shah and Paulsen, 2003), which often adversely affect plant growth and development, and grain biomass accumulation (Farooq et al., 2009; Awasthi et al., 2014). The interactions between heat and drought indicate that productivity of chickpea is potentially reduced considerably more by the combined stresses than the individual stresses alone (Hamidou et al., 2013; Awasthi et al., 2014) and therefore an understanding of the morphological, physiological and biochemical basis of combined heat and drought tolerance is a prerequisite for the selection of chickpea genotypes that are tolerant to drought and heat stress in follow up studies.

There is evidence that heat shock proteins (HSPs), a significant class of molecular chaperones, plays an important role in thermotolerance, and that some HSPs are causally involved in signal transduction during heat stress and with deduced function like chaperones, folding and unfolding of cellular proteins, and protection of functional sites from the adversity of heat stress (Vierling, 1991; Wahid et al., 2007). For example, HSP101 in maize and *Arabidopsis* (Hong and Vierling, 2001; Nieto-Sotelo et al., 2002), HSP90 in *Arabidopsis* (Ludwig-Muller et al., 2000), HSP 70 in tobacco and chickpea (Cho and Choi, 2009; Parankusam et al., 2017) and small HSPs in maize and bentgrass (Heckathorn et al., 1998; Wang et al., 2014b), have been shown to provide protection of the functional sites of the respective crops when exposed to heat stress. There is, however, evidence that other proteins have also been found to be important in tolerance to heat stress (Wahid et al., 2007; Huang and Xu, 2008). For example, He and Huang (2007) reported synthesis of several heat inducible proteins in the cytoplasm and membranes of Kentucky bluegrass (*Poa pratensis* L.). There seems to be extensive attention being given to HSPs, and lesser attention towards the other classes of heat stress inducible proteins in heat stress studies. In the current study, HSP 70 was the only heat shock protein identified and upregulated after exposure to heat stress in the leaves of the heat tolerant genotype Acc#7 yet 541 other heat stress inducible proteins were differentially expressed in response to heat stress in Acc#3 (129), Acc#7 (220) and Acc#8 (192) (chapter 4). Several studies in the past involving different crops and in the current study have revealed major roles played by the various heat inducible proteins (e.g. Rubisco, methyltransferase, Catalase, Dihydrolipoyl dehydrogenase 1, chloroplastic-like) related to major functional categories such as protein synthesis, disease and defence, energy and metabolism (Miernyk, 1999; Bauwe and Kolukisaoglu, 2003; Fatehi et al., 2012; Budak et al., 2013; Ghabooli et al., 2013; Gharechahi et al., 2014; Wang et al., 2015; Parankusam et al., 2017). Indeed, the heat stress inducible proteins also show organelle and tissue specific expression with deduced function like chaperones, folding and unfolding of cellular proteins, and protection of cellular and subcellular structures from adverse effects of dehydrative forces and heat stress (Wahid et al., 2007; Huang and Xu, 2008). Therefore, in addition to the importance of HSPs in plant heat stress responses, attention should be paid towards the other heat inducible proteins in the efforts to develop genotypes tolerant to heat stress. However, with the inevitability of combined heat and drought stress, synthesis patterns may differ in roots from shoots due to their differences in temperature and moisture sensitivities and their distinct functions (Huang and Xu, 2008). Therefore, follow up studies should focus also on exploring patterns of protein synthesis to elevated temperatures and drought stress in roots of chickpea, potentially providing insights into molecular mechanisms of the plant adaptation to high temperatures and drought in terms of root functionality.

Overall, past studies have demonstrated that measurement of chlorophyll fluorescence F_v/F_m, with the incorporation of F_q'/F_m' has been used successfully to quantitate inhibition or damage by heat stress to the electron transport system (Baker and Horton, 1988) in several crops, albeit mostly under controlled environments. This study showed that there is a strong argument on the identification and use of both chlorophyll fluorescence parameters and carbohydrate concentrations as suitable phenotyping tools for chickpea germplasm screening under field conditions in Southern Africa. Genotypes Acc#7 and Acc#RR-3 were resultantly identified as heat tolerant genotypes, while Acc#RR-2 and Acc#8 as heat susceptible. The identification of different phenotyping tools, as well as the heat tolerant and susceptible genotypes in this study will be foundational in their use as selection markers and control genotypes in the subsequent studies on germplasm selection for tolerance to heat stress in chickpea. The superior physiological performance by the tolerant genotype Acc#7 compared to Acc#8 under controlled conditions was further validated by the higher up-regulation of proteins involved in protein synthesis, intracellular traffic, defence and transport in Acc#7 compared to Acc#8. The heat tolerant genotype Acc#7 uniquely expressed proteins like Dihydrolipoyl dehydrogenase 1, chloroplastic-like, PSI reaction centre subunit proteins, protoporphyrinogen oxidase, Zeta-carotene desaturase, Serine/threonine-protein kinase and heat shock 70 kDa protein 15-like isoform X1 after exposure to heat stress, which were not reported by Parankusam et al. (2017) also on chickpea albeit under controlled environment. The identification of these heat responsive proteins provided an opportunity to identify molecular mechanisms involved in chickpea heat tolerance.

Most of the studies on heat and drought stress in chickpea and other crops have seen different sets of genotypes being exposed to either of the two stresses (Leport et al., 1999, Devasirvatham et al., 2012a, Kumar et al., 2013, Randhawa et al., 2014, chapter 2), with limited information on the responses of the same sets of genotypes to both drought and heat stress (i.e. cross tolerance). This study has demonstrated that the heat tolerant Acc#7 genotypes was susceptible to drought, just like Acc#8 implying that the physiological responses to drought and heat stresses are largely independent of one another and that generation of genotypes displaying cross tolerance through exposure to combined heat and drought are required. However, the respective contribution of double drought stressing to the priming effect in the heat tolerant and susceptible genotypes was demonstrated. This was at the backdrop that most studies have focused on terminal drought with little to no evidence in literature on chickpea studies specifically focusing on intermittent drought stress. From these results, attention should be given to matching chickpea physiological performances to expected rainfall amounts and distribution in drought prone areas during genotype selection. The characterization of chickpea production areas in NE South Africa using different soil and environmental variables showed that Polokwane area has favourable conditions for its production.

Supplementary Material



Supplementary Figure 1**:** Effect of drought treatments on Relative drought index (RDI) of chickpea at flowering growth stage. The genotype and drought treatment interaction on Relative drought index (RDI) was not significant. Treatments are as in Figure 3.1. Data is mean values of different genotypes under varying stress regimes ± se (n=4). Different letters indicate significant differences between genotypes and stress regimes by Tukey's honest significant difference *post hoc* test ($p<0.001$).

Supplementary Table 1: List of heat stress responsive proteins identified from Acc#RR-3 samples using the Label free quantification and database searches. All protein identifications were from *Cicer arietinum.*

Accession	Protein Name	Score^C	q value	max fold	Function
tr|A0A1S2XTR8	calvin cycle protein CP12-2, chloroplastic-like	3.649	0.014	21.455	transport
tr|A0A1S2YWP4	uncharacterized protein LOC101504873	7.909	0.012	19.432	unclear classification
tr|A0A1S2Y9R4	acidic mammalian chitinase-like	29.846	0.01	17.679	metabolism
tr|A0A1S2Z987	thaumatin-like protein 1b	14.65	0.014	16.449	disease and defence
tr|Q9ZP12	uncharacteristic protein LOC101488649	69.233	0.019	14.95	unclassified
tr|A0A1S2Y874	Plastocyanin	78.197	0.019	13.175	energy
tr|O81927	Thaumatin-like protein PR-5a	69.298	0.019	10.189	disease and defence
tr|A0A1S2YFV1	Ferredoxin	10.541	0.019	9.857	transport
tr|A0A1S2YQ46	ABA-responsive protein ABR18-like	10.832	0.009	9.526	metabolism
tr|A0A1S2Y090	keratin, type I cytoskeletal 10-like	17.723	0.019	9.521	cell structure
tr|A0A1S2YSZ0	Pathogenesis related protein	35.419	0.046	9.468	metabolism

tr|A0A1S2XGT2	class-10 pathogenesis-related protein 1-like	16.364	0.017	9.083	metabolism
tr|A0A1S2XDV0	ABA-responsive protein ABR17-like	36.526	0.01	8.773	metabolism
tr|A0A1S2Z702	Non-specific lipid-transfer protein	49.617	0.012	8.682	transport
tr|A0A1S2XDV2	Pathogenesis-related protein 10	68.625	0.012	8.594	metabolism
tr|A0A1S2XZ19	calvin cycle protein CP12-2, chloroplastic-like	32.22	0.043	7.471	transcription
tr|A0A1S2YLG2	Non-specific lipid-transfer protein	34.473	0.01	7.384	transport
tr|A0A1S2Y6U8	non-specific lipid-transfer protein 1-like	12.422	0.012	7.125	transport
sp|P36908	Acidic endochitinase	55.97	0.012	6.717	disease and defence
tr|A0A1S2Y9F3	far upstream element-binding protein 2-like	9.74	0.019	6.097	protein synthesis
tr|A0A1S2XBN8	Ferredoxin	10.419	0.027	5.602	energy
tr|A0A1S2Y2B7	1-aminocyclopropane-1-carboxylate oxidase homolog 4-like	23.632	0.037	5.432	metabolism

tr|A0A1S2YBP2	betaine aldehyde dehydrogenase 1, chloroplastic-like	19.245	0.014	5.428	disease and defence
tr|A0A1S2Y6K3	uncharacteristic protein LOC101488529	28.373	0.019	5.142	unclassified
tr|Q9ZNQ4	Superoxide dismutase [Cu-Zn]	32.378	0.009	5.111	disease and defence
sp|Q00016	Isoflavone reductase	19.085	0.024	4.58	metabolism
tr|O65757	Putative Pi starvation-induced protein	10.318	0.021	4.115	disease and defence
tr|Q700A6	Putative lipid transfer protein GPI-anchored	31.395	0.013	4.057	transport
tr|A0A1S2XG53	photosystem II 10 kDa polypeptide, chloroplastic	95.42	0.019	3.976	transport
tr|A0A1S2Y294	Chalcone-flavonone isomerase family protein	28.617	0.009	3.854	metabolism
tr|A0A1S2Y0R0	glutathione S-transferase L3-like	55.202	0.024	3.758	disease and defence
tr|A0A1S2XNK3	Non-specific lipid-transfer protein	12.14	0.019	3.564	transport
tr|A0A1S2Z038	probable carboxylesterase 18	2.926	0.033	3.449	metabolism
tr|A0A1S2Y5Z2	thylakoid lumenal 19 kDa protein, chloroplastic	4.815	0.02	3.398	transport

tr|A0A1S2XPQ3	vestitone reductase	12.038	0.038	3.366	disease and defence
tr|A0A1S2YQU2	methyl-CpG-binding domain-containing protein 11-like	19.125	0.027	3.342	cell structure
tr|A0A1S2YKP6	Superoxide dismutase [Cu-Zn]	150.992	0.038	3.3	energy
tr|A0A1S2YY37	Glycine cleavage system H protein	90.096	0.035	3.181	energy
tr|A0A1S2YE45	Malic enzyme	15.116	0.012	3.068	metabolism
tr|A0A1S2YPS5	Sucrose synthase	5.942	0.038	3.018	energy
tr|A0A1S2XFN2	persulfide dioxygenase ETHE1 homolog, mitochondrial	42.464	0.02	2.945	metabolism
tr|A0A1S2XSM5	glutathione S-transferase L3	21.011	0.014	2.814	disease and defence
tr|A0A1S2XJR8	Carbonic anhydrase	222.378	0.019	2.807	disease and defence
tr|A0A1S2Z2Z2	thioredoxin-like isoform X1	21.628	0.01	2.709	metabolism
tr|A0A1S2YCD8	ribosome-binding factor PSRP1, chloroplastic	24.237	0.049	2.648	protein synthesis
tr|A0A1S2XFX0	thioredoxin M4, chloroplastic	33.632	0.009	2.567	disease and defence

tr|A0A1S2YUR2	1-deoxy-D-xylulose 5-phosphate reductoisomerase, chloroplastic-like	309.179	0.011	2.509	disease and defence
tr|A0A1S2YQJ2	endo-1,31,4-beta-D-glucanase-like	48.107	0.043	2.494	energy
tr|A0A1S2YB16	1-aminocyclopropane-1-carboxylate oxidase	44.823	0.02	2.478	disease and defence
tr|A0A1S2YIQ0	uncharacterized protein LOC101494085	29.671	0.047	2.469	unclassified
tr|A0A1S2Y6T5	cinnamoyl-CoA reductase 1	86.45	0.028	2.45	disease and defence
tr|A0A1S2YE48	photosystem I reaction center subunit N, chloroplastic isoform X1	52.876	0.027	2.45	energy
tr|A0A1S2XJG9	Aldose 1-epimerase	11.694	0.03	2.367	disease and defence
tr|A0A1S2YDD8	thylakoid lumenal 15 kDa protein 1, chloroplastic	40.198	0.027	2.358	energy
tr|A0A1S2YKF7	probable 2-carboxy-D-arabinitol-1-phosphatase isoform X1	4.212	0.024	2.304	transport
tr|A0A1S2YDG7	thioredoxin-like	90.062	0.018	2.285	metabolism

tr|A0A1S2Y875	CO (2)-response secreted protease-like	81.132	0.044	2.272	Protein destination and storage
tr|A0A1S2XDT5	beta-D-xylosidase 1	51.676	0.033	2.269	energy
tr|A0A1S2YLN1	arginase 1, mitochondrial	31.222	0.038	2.263	energy
tr|A0A1S2Y2Y0	Cyanate hydratase	25.018	0.038	2.225	metabolism
tr|B5LMS5	Photosystem I iron-sulfur center	70.676	0.038	2.214	transport
tr|A0A1S2YPS0	peroxiredoxin Q, chloroplastic	194.359	0.027	2.195	disease and defence
tr|A0A1S2YD43	uncharacterized protein LOC101494289 isoform X1	8.159	0.019	2.173	unclassified
tr|A0A1S3E819	LOW QUALITY PROTEIN: cytochrome b5	14.02	0.013	2.172	unclear classification
tr|A0A1S3E3F5	uncharacterized protein LOC101510142	17.114	0.033	2.171	transport
tr|A0A1S2YKU0	CDGSH iron-sulfur domain-containing protein NEET	31.373	0.038	2.157	energy
tr|A0A1S2YD89	Starch synthase, chloroplastic/amyloplastic	17.382	0.027	2.107	metabolism
tr|A0A1S2YAR8	Amine oxidase	21.276	0.009	2.066	metabolism
tr|A0A1S2YUP6	Carboxypeptidase	19.123	0.039	2.059	Protein destination and storage

tr|A0A1S2YXU1	thylakoid lumenal protein At1g12250, chloroplastic	41.342	0.021	2.057	metabolism
tr|A0A1S2Y0V9	Peptidylprolyl isomerase	31.156	0.038	2.057	metabolism
tr|A0A1S2XEB9	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	2.709	0.038	2.04	disease and defence
tr|A0A1S2Y089	thioredoxin X, chloroplastic	30.058	0.046	2.04	disease and defence
tr|A0A1S2Y9J2	glycine-rich RNA-binding protein-like	26.212	0.02	2.034	protein synthesis
tr|A0A1S2YDG4	60S ribosomal protein L9	89.617	0.019	2.002	protein synthesis
tr|A0A1S2YBD7	protein HHL1, chloroplastic	19.227	0.019	-2.003	protein synthesis
tr|A0A1S2Z2Y7	50S ribosomal protein L6, chloroplastic	80.81	0.043	-2.055	protein synthesis
tr|A0A1S4ETK8	50S ribosomal protein L14, chloroplastic	26.091	0.02	-2.058	protein synthesis
tr|A0A1S2XSJ5	26S proteasome regulatory subunit 4 homolog A	12.35	0.048	-2.061	protein synthesis
tr|A0A1S2YZR4	60S ribosomal protein L17-2-like	20.206	0.028	-2.063	unclassified
tr|A0A1S2XMM1	40S ribosomal protein S4	52.004	0.01	-2.068	protein synthesis

tr|A0A1S2XWV0	uncharacterized protein LOC101503283	8.281	0.014	-2.07	unclassified
tr|A0A1S2YTZ6	60S ribosomal protein L27a-2	36.739	0.044	-2.071	unclassified
tr|A0A1S2YR41	calcium sensing receptor, chloroplastic	67.521	0.016	-2.074	disease and defence
tr|A0A1S2Y808	L-ascorbate oxidase homolog	10.484	0.019	-2.098	metabolism
tr|B5LMM7	30S ribosomal protein S2, chloroplastic	24.156	0.009	-2.121	protein synthesis
tr|A0A1S2YAQ0	DEAD-box ATP-dependent RNA helicase 56-like isoform X1	9.062	0.021	-2.122	transcription
tr|A0A1S3EA23	phytoene dehydrogenase, chloroplastic/chromoplastic	16.714	0.028	-2.141	metabolism
tr|A0A1S3E663	phosphomethylpyrimidine synthase, chloroplastic	5.287	0.03	-2.166	metabolism
tr|A0A1S2XKI0	protein TIC110, chloroplastic	24.39	0.043	-2.177	cell structure
tr|A0A1S2XWP5	60S ribosomal protein L18-like	22.971	0.044	-2.178	protein synthesis
tr|B5LMR4	50S ribosomal protein L23, chloroplastic	11.727	0.027	-2.187	protein synthesis

tr|A0A1S2Y534	40S ribosomal protein S15a-1	35.261	0.029	-2.254	protein synthesis
tr|A0A1S2Z515	uncharacterized protein LOC101496302	13.362	0.038	-2.281	unclassified
tr|A0A1S2YEX2	chaperone protein ClpC, chloroplastic	228.2	0.038	-2.298	metabolism
tr|A0A1S2Y710	50S ribosomal protein L17, chloroplastic	14.758	0.019	-2.32	protein synthesis
tr|A0A1S2Z5B3	50S ribosomal protein L4, chloroplastic	45.207	0.038	-2.323	protein synthesis
tr|A0A1S2YSD6	Zeaxanthin epoxidase, chloroplastic	7.775	0.01	-2.369	disease and defence
tr|A0A1S2YJW9	60S ribosomal protein L35	15.996	0.046	-2.386	protein synthesis
tr|A0A1S2YVU9	60S ribosomal protein L5	20.478	0.043	-2.426	protein synthesis
tr|A0A1S2YMU2	protein TOC75, chloroplastic	20.986	0.012	-2.54	transport
tr|A0A1S2XAS4	Obg-like ATPase 1	12.408	0.023	-2.566	transport
tr|A0A1S2XWK8	GTP-binding protein SAR1A-like	14.756	0.019	-2.575	signal transduction
tr|A0A1S2YPA1	Diacylglycerol kinase	9.024	0.019	-2.607	disease and defence

tr|A0A1S2XW15	probable 60S ribosomal protein L14	24.571	0.038	-2.62	protein synthesis
tr|B5LMQ8	30S ribosomal protein S8, chloroplastic	36.236	0.012	-2.622	protein synthesis
tr|A0A1S2XIQ1	50S ribosomal protein L22, chloroplastic-like	19.261	0.033	-2.759	protein synthesis
tr|A0A1S2XME4	60S ribosomal protein L9-like	39.943	0.017	-2.862	protein synthesis
tr|A0A1S2YCE5	nascent polypeptide-associated complex subunit alpha-like protein 2	14.631	0.039	-3.017	transcription
tr|A0A1S2YFW9	translocase of chloroplast 159, chloroplastic-like	39.855	0.011	-3.331	metabolism
tr|A0A1S2XUG0	GDP-mannose 3,5-epimerase 2	62.627	0.019	-3.616	metabolism
tr|A0A1S2XH51	uncharacterized protein LOC101502403	9.701	0.021	-3.688	energy
tr|A0A1S2YWG7	Annexin	9.432	0.049	-3.845	disease and defence
tr|A0A1S3ED54	DEAD-box ATP-dependent RNA helicase 30-like	46.498	0.046	-3.966	unclassified
tr|A0A1S2Z480	alpha-amylase 3, chloroplastic-like	44.999	0.01	-4.127	metabolism

tr|A0A1S2YQB7	uncharacterized protein LOC101493781	3.896	0.019	-4.331	disease and defence
tr|A0A1S2Z2C9	translation factor GUF1 homolog, chloroplastic	21.011	0.019	-4.49	transport
tr|A0A1S2YLJ9	Tubulin beta chain	80.091	0.046	-4.606	cell structure
tr|A0A1S2XGE6	trifunctional UDP-glucose 4,6-dehydratase/UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-L-rhamnose-reductase RHM1	30.144	0.024	-4.891	metabolism
tr|A0A1S2XTU9	S-adenosylmethionine synthase	50.936	0.012	-5.035	metabolism
tr|A0A1S2X9V3	Mg-protoporphyrin IX chelatase	55.876	0.025	-5.314	transport
tr|A0A1S2Y3V2	ATP-dependent zinc metalloprotease FTSH 11, chloroplastic/mitochondrial	3.649	0.038	-5.635	intracellular traffic
tr|A0A1S2Z4A6	putative bark agglutinin LECRPA3	14.723	0.019	-8.82	disease and defence
tr|A0A1S2YV42	Lipoxygenase	16.029	0.038	-10.312	metabolism
tr|A0A1S2XX37	DEAD-box ATP-dependent RNA helicase 3, chloroplastic isoform X1	13.846	0.023	-12.144	transcription

tr|A0A1S2XBY4	DNA-damage-repair/toleration protein DRT100	4.2	0.036	-12.159	disease and defence
tr|A0A1S2YV39	Lipoxygenase	56.7	0.031	-15.05	metabolism
tr|A0A1S2XYW4	early light-induced protein, chloroplastic-like	35.812	0.009	-29.986	metabolism
tr|A0A1S2YZK2	probable methyltransferase PMT21	4.221	0.038	-49.609	transcription

Supplementary Table 2: List of heat stress responsive proteins identified from Acc#7 samples using the Label free quantification and database searches. All protein identifications were from *Cicer arietinum.*

Accession	Protein Name	Score^C	q value	max fold	Function
tr|A0A1S2XYW4	early light-induced protein, chloroplastic-like	22.988	0	48.562	metabolism
tr|A0A1S2YV39	Lipoxygenase	23.301	0.001	25.858	metabolism
tr|A0A1S2Z4A6	putative bark agglutinin LECRPA3	13.253	0.008	7.352	disease and defence
tr|A0A1S3DZ63	cytochrome P450 CYP736A12-like	14.656	0.004	5.034	metabolism

tr|A0A1S3ECZ3	secoisolariciresinol dehydrogenase-like	32.873	0.018	4.861	energy
tr|A0A1S2XME4	60S ribosomal protein L9-like	30.392	0.003	4.617	protein synthesis
tr|A0A1S2Z2C9	translation factor GUF1 homolog, chloroplastic	27.808	0.001	4.553	transport
tr|A0A1S2YPD3	Plasma membrane ATPase	38.206	0.005	4.463	transport
tr|A0A1S2Y1Y6	uncharacterized protein LOC101513695	7.642	0.001	4.442	unclassified
tr|A0A1S2YNF6	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	2.924	0.008	4.325	protein destination and storage
tr|A0A1S2XYG3	ADP-ribosylation factor	29.148	0.005	4.279	intracellular traffic
tr|A0A1S2Y2D5	histidine--tRNA ligase	2.495	0.004	4.229	metabolism
tr|B5LMS1	NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic	14.145	0.015	4.198	disease and defence
tr|A0A1S2YFW9	translocase of chloroplast 159, chloroplastic-like	17.633	0.004	4.188	metabolism
tr|A0A1S2YQB7	uncharacterized protein LOC101493781	3.585	0.003	4.133	disease and defence
tr|A0A1S2XSL0	calcium-transporting ATPase 4, endoplasmic reticulum-type-like	11.504	0.008	4.066	transport

tr|A0A1S2YJ61	ADP,ATP carrier protein 3, mitochondrial	44.149	0.002	3.989	transport
tr|A0A1S2XRC1	small subunit processome component 20 homolog	4.303	0.045	3.969	cell structure
tr|A0A1S2Z480	alpha-amylase 3, chloroplastic-like	38.54	0.001	3.934	metabolism
tr|A0A1S2YMU2	protein TOC75, chloroplastic	40.836	0.003	3.847	transport
tr|A0A1S2XT74	uncharacterized protein LOC101505098	33.623	0.001	3.668	unclassified
tr|A0A1S2XW14	GTP-binding protein SAR1A-like	14.921	0.001	3.648	transport
tr|A0A1S2YCR8	40S ribosomal protein SA	55.069	0.029	3.592	protein synthesis
tr|A0A1S2XA10	CSC1-like protein ERD4	7.435	0.003	3.544	transport
tr|A0A1S2XV31	60S ribosomal protein L35-like	11.595	0.007	3.424	protein synthesis
tr|A0A1S2XUN8	40S ribosomal protein S23-like	8.666	0.014	3.365	protein synthesis
tr|A0A1S3EA23	phytoene dehydrogenase, chloroplastic/chromoplastic	15.841	0.004	3.355	metabolism
tr|A0A1S2YG51	Tubulin alpha chain	25.025	0.01	3.323	protein synthesis
tr|A0A1S2YH83	26S protease regulatory subunit 8 homolog A	12.278	0.002	3.317	protein synthesis

tr|B5LMQ8	30S ribosomal protein S8, chloroplastic	34.538	0.007	3.314	protein synthesis
tr|A0A1S2Y3C7	DEAD-box ATP-dependent RNA helicase 56-like isoform X1	14.065	0.004	3.25	transport
tr|A0A1S2YSD6	Zeaxanthin epoxidase, chloroplastic	7.793	0.002	3.244	disease and defence
tr|A0A1S2X999	nifU-like protein 2, chloroplastic	2.399	0.005	3.229	transport
tr|A0A1S2YX76	putative 12-oxophytodienoate reductase 11	50.78	0.032	3.195	protein synthesis
tr|A0A1S2YDG4	60S ribosomal protein L9	97.985	0.005	3.177	protein synthesis
tr|A0A1S2XFW1	NADPH-protochlorophyllide oxidoreductase	48.801	0.001	3.102	transport
tr|A0A1S2XWP5	60S ribosomal protein L18-like	23.844	0.007	3.091	protein synthesis
tr|A0A1S2X9G4	Phospho-2-dehydro-3-deoxyheptonate aldolase	8.951	0.004	3.078	metabolism
tr|A0A1S3EBL9	LOW QUALITY PROTEIN: 9-divinyl ether synthase-like	7.585	0.011	3.049	metabolism
tr|A0A1S2YQ32	dihydrolipoyl dehydrogenase 1, chloroplastic-like	13.998	0.048	2.99	energy

tr|A0A1S2YG09	40S ribosomal protein S15a-1-like	28.653	0.009	2.985	protein synthesis
tr|A0A1S2XB11	ribulose bisphosphate carboxylase/oxygenase activase (Rubisco), chloroplastic	21.267	0.008	2.94	protein synthesis
tr|A0A1S2YEX2	chaperone protein ClpC, chloroplastic	239.756	0.001	2.904	metabolism
tr|A0A1S2XN51	UDP-glucose 6-dehydrogenase	11.793	0.003	2.878	metabolism
tr|A0A1S2YY93	mitochondrial outer membrane protein porin 2	48.519	0.007	2.842	transport
tr|A0A1S2XL88	Clathrin heavy chain	18.634	0.002	2.837	protein synthesis
tr|A0A1S2XUM9	protein CURVATURE THYLAKOID 1A, chloroplastic	28.06	0.006	2.833	metabolism
tr|A0A1S2Y1I5	protein PROTON GRADIENT REGULATION 5, chloroplastic	22.018	0.001	2.816	energy
tr|A0A1S3EIC8	eukaryotic peptide chain release factor GTP-binding subunit-like isoform X2	7.526	0.022	2.785	energy
tr|A0A1S2X7K7	Tubulin beta chain	43.141	0.003	2.783	protein synthesis
tr|A0A1S2XYX6	Protoporphyrinogen oxidase	14.83	0.001	2.779	metabolism

tr|B5LMR4	50S ribosomal protein L23, chloroplastic	15.266	0.006	2.743	protein synthesis
tr|A0A1S2YMI5	uncharacterized protein LOC101497983 isoform X1	21.416	0.001	2.662	disease and defence
tr|A0A1S2YR41	calcium sensing receptor, chloroplastic	79.763	0.001	2.661	disease and defence
tr|A0A1S2XSJ5	26S proteasome regulatory subunit 4 homolog A	6.35	0.004	2.63	protein synthesis
tr|A0A1S2YV29	chaperonin CPN60-like 2, mitochondrial	9.697	0.025	2.619	transport
tr|A0A1S2XUG0	GDP-mannose 3,5-epimerase 2	58.139	0.002	2.615	metabolism
tr|A0A1S2YUI7	protein TRIGALACTOSYLDIACYLGLYCEROL 4, chloroplastic	9.515	0.008	2.603	unclear classification
tr|C3TS15	S-adenosylmethionine synthase	24.553	0.003	2.535	metabolism
tr|A0A1S2Z1R2	eukaryotic initiation factor 4A-15	41.695	0.001	2.518	transport
tr|A0A1S3EB28	magnesium protoporphyrin IX methyltransferase, chloroplastic	9.956	0.001	2.503	transcription
tr|A0A1S2Y9L5	uncharacterized protein LOC101504606	3.79	0.011	2.486	unclassified
tr|A0A1S2YZP5	26S proteasome non-ATPase regulatory subunit 13 homolog B	2.477	0.005	2.438	protein synthesis

tr|A0A1S2YHM9	3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplastic	3.23	0.002	2.432	metabolism
tr|A0A1S2Z5B3	50S ribosomal protein L4, chloroplastic	49.626	0.011	2.429	protein synthesis
tr|A0A1S2Y710	50S ribosomal protein L17, chloroplastic	15.004	0.008	2.421	protein synthesis
tr|A0A1S2Z2H7	magnesium-chelatase subunit ChlH, chloroplastic	24.35	0.015	2.411	disease and defence
tr|A0A1S2Z827	probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5, mitochondrial	13.793	0.008	2.373	transport
tr|A0A1S2YAB3	elongation factor Tu, chloroplastic	173.549	0.003	2.361	protein synthesis
tr|A0A1S2YRJ4	Plasma membrane ATPase	46.806	0.005	2.361	transport
tr|A0A1S2YNL1	glutamyl-tRNA reductase-binding protein, chloroplastic	12.358	0.002	2.359	transcription
tr|A0A1S2YPS0	peroxiredoxin Q, chloroplastic	235.481	0.007	2.344	metabolism
tr|A0A1S2Y115	sucrose-phosphatase 1-like	26.247	0.005	2.34	metabolism
tr|A0A1S2X9V3	Mg-protoporphyrin IX chelatase	39.747	0.009	2.338	transport
tr|A0A1S2Z945	26S proteasome non-ATPase regulatory subunit 14 homolog	12.201	0.008	2.335	protein synthesis

tr|A0A1S2YGS7	40S ribosomal protein S17-like	26.477	0.009	2.324	protein synthesis
tr|A0A1S2Z236	uncharacterized protein LOC101504453	3.223	0.043	2.317	protein destination and storage
tr|A0A1S2YCX7	Translation factor GUF1 homolog, chloroplastic	2.459	0.005	2.317	transport
tr|A0A1S2Y835	Catalase	160.915	0.007	2.315	disease and defence
tr|A0A1S2Y9S1	protein CutA, chloroplastic isoform X1	11.612	0.036	2.308	intracellular traffic
tr|A0A1S2Z0R7	fe-S cluster assembly factor HCF101, chloroplastic	18.387	0.008	2.295	metabolism
tr|A0A1S2Y808	L-ascorbate oxidase homolog	7.395	0.012	2.287	metabolism
tr|A0A1S2YCE5	nascent polypeptide-associated complex subunit alpha-like protein 2	17.062	0.006	2.286	transcription
tr|A0A1S2YJH9	60S ribosomal protein L11	32.008	0.01	2.267	protein synthesis
tr|A0A1S2XWV1	Zeta-carotene desaturase	10.834	0.011	2.263	metabolism
tr|A0A1S2XM48	cell division protein FtsZ homolog 1, chloroplastic	13.636	0.007	2.259	transport

tr|A0A1S2Y152	40S ribosomal protein S3-2-like	68.271	0.005	2.226	protein synthesis
tr|A0A1S2Z2Y7	50S ribosomal protein L6, chloroplastic	79.774	0.016	2.208	protein synthesis
tr|A0A1S2XKI0	protein TIC110, chloroplastic	28.537	0.002	2.202	cell structure
tr|A0A1S2XHH6	uncharacterized protein LOC101501957	15.055	0.008	2.194	unclassified
tr|B5LMM7	30S ribosomal protein S2, chloroplastic	28.226	0.002	2.189	protein synthesis
sp|O65731	40S ribosomal protein S5 (Fragment)	33.613	0.012	2.189	protein synthesis
tr|A0A1S2XMH6	60S ribosomal protein L35a-1	8.378	0.006	2.185	protein synthesis
tr|A0A1S2Y0H8	probable plastid-lipid-associated protein 12, chloroplastic isoform X1	10.982	0.005	2.164	transport
tr|A0A1S3E2C4	ATP-dependent RNA helicase 3, chloroplastic-like	47.499	0.001	2.162	transport
tr|A0A1S2YMA8	clustered mitochondria protein isoform X1	8.606	0.029	2.156	intracellular traffic
tr|A0A1S2XUE6	40S ribosomal protein S24	22.394	0.004	2.156	protein synthesis

tr|A0A1S2XV81	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	27.43	0.012	2.155	disease and defence
tr|A0A1S3E9T4	40S ribosomal protein S16-like	33.696	0.008	2.153	protein synthesis
tr|A0A1S2YEN0	26S proteasome non-ATPase regulatory subunit 1 homolog	15.123	0.02	2.152	protein synthesis
tr|B5LMR1	30S ribosomal protein S3, chloroplastic	46.399	0.003	2.136	protein synthesis
tr|A0A1S2XUW0	40S ribosomal protein S15-4	6.673	0.026	2.135	protein synthesis
tr|A0A1S2YSN4	GTP-binding protein ERG isoform X1	7.486	0.007	2.109	signal transduction
tr|A0A1S2YUC7	serine/threonine-protein kinase STN8, chloroplastic-like	11.018	0.012	2.106	metabolism
tr|A0A1S2XFR8	caffeic acid 3-O-methyltransferase	7.837	0.004	2.102	disease and defence
tr|A0A1S2Y357	60S ribosomal protein L24-like	14.781	0.027	2.102	protein synthesis
tr|A0A1S2YB52	calcium sensing receptor, chloroplastic isoform X2	23.114	0.008	2.097	signal transduction
tr|A0A1S2Z6J8	uncharacterized protein LOC101506186	38.366	0.007	2.092	unclassified

tr|A0A1S2XJZ3	outer plastidial membrane protein porin-like	46.743	0.01	2.089	transport
tr|A0A1S2YQR3	probable sucrose-phosphate synthase	14.833	0.003	2.063	metabolism
tr|A0A1S2XCX9	40S ribosomal protein S14	56.191	0.016	2.063	protein synthesis
tr|A0A1S2YX91	uncharacterized protein LOC101496924 isoform X1	28.346	0.009	2.056	unclassified
tr|A0A1S2YD12	calnexin homolog	27.832	0.021	2.046	protein destination and storage
tr|A0A1S2XHQ9	Elongation factor 1-alpha	125.216	0.008	2.027	protein synthesis
tr|A0A1S2YMB2	heat shock 70 kDa protein (HSP70) 15-like isoform X1	55.18	0.048	2.025	Disease and defence
tr|A0A1S2XKN6	T-complex protein 1 subunit theta-like	2.038	0.002	2.017	transport
tr|A0A1S2YGU4	mitochondrial dicarboxylate/tricarboxylate transporter DTC	78.459	0.01	2.008	intracellular traffic
tr|B5LML4	30S ribosomal protein S4, chloroplastic	39.426	0.008	2.008	protein synthesis
tr|A0A1S2YTM9	mitochondrial carnitine/acylcarnitine carrier-like protein	57.31	0.01	2.005	intracellular traffic

tr|A0A1S2YXA2	uncharacterized protein LOC101499642	17.099	0.021	2.004	disease and defence
tr|A0A1S4ETM2	30S ribosomal protein S15, chloroplastic	19.465	0.003	2.003	protein synthesis
tr|A0A1S2YDG7	thioredoxin-like	93.944	0.001	-2.008	metabolism
tr|A0A1S2YRM0	betaine aldehyde dehydrogenase 1, chloroplastic	50.3	0.015	-2.011	disease and defence
tr|A0A1S2YLN1	arginase 1, mitochondrial	30.796	0.004	-2.017	energy
tr|A0A1S3DZE5	Small ubiquitin-related modifier	2.833	0.012	-2.025	disease and defence
tr|A0A1S2YK76	alpha carbonic anhydrase 7-like	79.936	0.004	-2.028	energy
tr|A0A1S2Z038	probable carboxylesterase 18	6.765	0.017	-2.05	metabolism
tr|A0A1S2YHI8	Peptidylprolyl isomerase	40.549	0.002	-2.056	metabolism
tr|A0A1S2YPM6	threonine synthase 1, chloroplastic	24.353	0.007	-2.065	unclear classification
tr|A0A1S2Y2Y0	Cyanate hydratase	27.101	0.003	-2.081	metabolism
tr|A0A1S2Y6T5	cinnamoyl-CoA reductase 1	81.057	0.012	-2.088	disease and defence
tr|A0A1S2XB48	3-isopropylmalate dehydrogenase	50.315	0.004	-2.102	disease and defence

tr|A0A1S2Y089	thioredoxin X, chloroplastic	32.429	0.008	-2.118	disease and defence
tr|A0A1S2YE45	Malic enzyme	12.105	0.031	-2.14	metabolism
tr|A0A1S2XH89	Ferredoxin-thioredoxin reductase, catalytic chain	12.829	0.002	-2.177	metabolism
tr|A0A1S3E0N0	probable glutathione S-transferase	5.81	0.002	-2.178	disease and defence
tr|A0A1S2XJR8	Carbonic anhydrase	277.758	0.004	-2.202	disease and defence
tr|A0A1S2YPG7	macrophage migration inhibitory factor homolog	14.546	0.016	-2.22	disease and defence
tr|A0A1S2XFX0	thioredoxin M4, chloroplastic	41.722	0.001	-2.223	disease and defence
tr|A0A1S2YGC4	pectinesterase/pectinesterase inhibitor 51	12.327	0.006	-2.229	disease and defence
tr|A0A1S2YMH7	uncharacterized protein LOC101496020	16.605	0.006	-2.237	unclassified
tr|A0A1S2XJI4	60S acidic ribosomal protein P2-1-like	6.988	0.035	-2.254	protein synthesis
tr|A0A1S2YKJ9	Lactoylglutathione lyase	20.731	0.004	-2.26	disease and defence

tr|A0A1S2Y615	Glutamyl-tRNA(Gln) amidotransferase subunit A, chloroplastic/mitochondrial	29.087	0.018	-2.268	transcription
tr|A0A0X9LEN0	Glutathione s-transferase 1	11.978	0.008	-2.269	disease and defence
tr|A0A1S2YIB9	thioredoxin M3, chloroplastic	3.729	0.009	-2.271	disease and defence
tr|A0A1S2XHF9	uncharacterized protein At5g39570 isoform X1	23.842	0.003	-2.291	unclassified
tr|A0A1S2YQJ2	endo-1,31,4-beta-D-glucanase-like	51.528	0.005	-2.339	energy
tr|A0A1S2YUP6	Carboxypeptidase	28.548	0.01	-2.357	Protein destination and storage
sp|Q00016	Isoflavone reductase	15.886	0.009	-2.362	metabolism
tr|A0A1S2YRE0	Eukaryotic translation initiation factor 5A	6.009	0.007	-2.366	protein synthesis
tr|A0A1S2XNK3	Non-specific lipid-transfer protein	15.619	0.008	-2.402	transport
tr|A0A1S2Y9F3	far upstream element-binding protein 2-like	1.653	0.028	-2.408	protein synthesis
tr|A0A1S2XSM5	glutathione S-transferase L3	16.825	0.007	-2.495	disease and defence

tr|A0A1S2Y105	60S acidic ribosomal protein P2-4-like	10.156	0.021	-2.541	protein synthesis
tr|A0A1S2XK69	uncharacterized protein LOC101509461	15.078	0.012	-2.548	unclassified
tr|A0A1S3E278	NHL repeat-containing protein 2	26.752	0.002	-2.555	unclassified
tr|A0A1S3EET3	Acyl carrier protein	7.906	0.02	-2.566	protein synthesis
tr|A0A1S2YAR8	Amine oxidase	39.113	0.003	-2.586	metabolism
tr|A0A1S2YB16	1-aminocyclopropane-1-carboxylate oxidase	51.055	0.008	-2.616	disease and defence
tr|A0A1S2Y0A3	geraniol 8-hydroxylase-like	2.288	0.003	-2.623	metabolism
tr|A0A1S2XVA7	cathepsin B-like	3.898	0.021	-2.636	disease and defence
tr|A0A1S2YPL0	uncharacterized protein LOC101500266	17.392	0.001	-2.648	unclassified
tr|A0A1S2XBQ8	hydroxyacylglutathione hydrolase 2, mitochondrial-like isoform X1	11.659	0.02	-2.668	intracellular traffic
tr|A0A1S2XU67	alpha carbonic anhydrase 1, chloroplastic	11.541	0.002	-2.673	metabolism
tr|A0A1S3E7G7	uncharacterized protein LOC105852205	3.087	0.002	-2.697	unclassified
tr|A0A1S2Y9C4	uncharacterized protein LOC101501607	2.744	0.003	-2.782	unclassified
tr|A0A1S2Z2Z2	thioredoxin-like isoform X1	24.023	0.002	-2.832	metabolism
tr|A0A1S2XM46	cytochrome c oxidase subunit 6b-1-like	2.705	0.018	-2.854	transport

tr|A0A1S2YX62	Lactoylglutathione lyase	3.235	0.01	-2.884	metabolism
tr|A0A1S2XG53	photosystem II 10 kDa polypeptide, chloroplastic	87.861	0.011	-2.887	energy
tr|A0A1S2Y875	CO(2)-response secreted protease-like	111.781	0.004	-2.897	Protein destination and storage
tr|A0A1S2XWI5	uncharacterized protein LOC101515092	9.189	0.012	-2.932	unclassified
tr|A0A1S2Y5Z2	thylakoid lumenal 19 kDa protein, chloroplastic	9.438	0.003	-2.938	transport
tr|A0A1S2YEW6	Acyl carrier protein	11.768	0.016	-2.949	metabolism
tr|A0A1S2YKF7	probable 2-carboxy-D-arabinitol-1-phosphatase isoform X1	12.354	0.004	-2.962	transport
tr|A0A1S2YKM2	Carboxypeptidase	12.576	0.01	-2.969	Protein destination and storage
tr|O65757	Putative Pi starvation-induced protein	17.398	0.005	-2.991	disease and defence
tr|A0A1S2Y6K3	uncharacterized protein LOC101488529	31.155	0.015	-3.051	unclassified
tr|A0A1S2YAN1	uncharacterized protein LOC101493535 isoform X1	6.735	0.048	-3.056	unclassified

tr|Q7DLT8	CaM protein	17.228	0.005	-3.069	disease and defence
tr|A0A1S2XGQ1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2-like	8.905	0.005	-3.106	energy
tr|A0A1S2XVS3	R probable aldo-keto reductase 1	11.332	0.003	-3.12	disease and defence
tr|A0A1S2YDD8	thylakoid lumenal 15 kDa protein 1, chloroplastic	44.272	0.003	-3.125	energy
tr|A0A1S2Y2B7	1-aminocyclopropane-1-carboxylate oxidase homolog 4-like	29.568	0.008	-3.193	metabolism
tr|A0A1S2Y0R0	glutathione S-transferase L3-like	58.754	0.003	-3.3	disease and defence
tr|A0A1S3EGD4	Sucrose synthase	11.43	0.011	-3.314	metabolism
tr|A0A1S2XFN2	persulfide dioxygenase ETHE1 homolog, mitochondrial	38.96	0.004	-3.546	metabolism
tr|A0A1S2YI59	uncharacterized protein LOC101506653	18.208	0.021	-3.555	unclassified
tr|A0A1S2XNJ7	Non-specific lipid-transfer protein	13.113	0.003	-3.72	unclear classification
tr|A0A1S2Y294	Chalcone-flavonone isomerase family protein	27.091	0.001	-3.741	metabolism
tr|A0A1S2YY37	Glycine cleavage system H protein	104.469	0.004	-3.877	energy

tr|Q9ZNQ4	Superoxide dismutase [Cu-Zn]	38.857	0.003	-3.975	disease and defence
tr|A0A1S2Z521	Peroxidase	2.324	0.002	-4.09	disease and defence
tr|A0A1S2YLG2	Non-specific lipid-transfer protein	38.932	0.01	-4.579	transport
tr|A0A1S2YQU2	methyl-CpG-binding domain-containing protein 11-like	26.548	0.004	-4.71	cell structure
tr|A0A1S2YKP6	Superoxide dismutase [Cu-Zn]	228.48	0.007	-4.743	energy
tr|A0A1S2XDV0	ABA-responsive protein ABR17-like	33.697	0.004	-5.042	metabolism
tr|A0A1S2YFV1	Ferredoxin	13.503	0.005	-5.171	transport
tr|A0A1S2XBN8	Ferredoxin	10.874	0.004	-5.523	energy
tr|A0A1S2Z702	Non-specific lipid-transfer protein	53.946	0.001	-5.653	transport
sp|P36908	Acidic endochitinase	67.804	0.003	-6.026	disease and defence
tr|A0A1S2XZ19	calvin cycle protein CP12-2, chloroplastic-like	17.679	0.008	-6.15	transcription
tr|A0A1S2XPR0	vestitone reductase-like	9.369	0.001	-7.394	metabolism
tr|A0A1S2XA51	Acyl carrier protein	11.197	0.007	-7.477	intracellular traffic

tr|A0A1S2YWP4	uncharacterized protein LOC101504873	7.301	0.009	-7.507	unclear classification
tr|A0A1S2Y6U8	non-specific lipid-transfer protein 1-like	14.957	0.001	-7.668	transport
tr|A0A1S2Y9R4	acidic mammalian chitinase-like	37.887	0.001	-7.746	metabolism
tr|A0A1S2XDV2	Pathogenesis-related protein 10	59.895	0	-7.78	disease and defence
tr|A0A1S2XGT2	class-10 pathogenesis-related protein 1-like	21.038	0.002	-7.925	metabolism
tr|A0A1S2YSZ0	Pathogenesis related protein	34.624	0	-8.626	disease and defence
tr|Q700A6	Putative lipid transfer protein GPI-anchored	27.24	0.001	-8.631	transport
tr|A0A067XU00	UDP-glycosyltransferase 83A1	19.687	0.001	-9.042	metabolism
tr|A0A1S2Z987	thaumatin-like protein 1b	17.77	0.009	-9.769	disease and defence
tr|A0A1S2Y874	Plastocyanin	83.8	0.003	-10.074	energy
tr|A0A1S2YQ46	ABA-responsive protein ABR18-like	22.834	0.001	-10.192	metabolism
tr|A0A1S2Y090	keratin, type I cytoskeletal 10-like	42.207	0.006	-10.565	unclear classification

tr|A0A1S2XYW5	early light-induced protein, chloroplastic-like	33.53218	0.007859	6.68034	metabolism
tr|A0A1S2YV40	Lipoxygenase	33.5609	0.007853	-6.74391	metabolism

Supplementary Table 3: List of heat stress responsive proteins identified from Acc#8 samples using the Label free quantification and database searches. All protein identifications were from *Cicer arietinum*

Accession	Protein Name	Score^{C}	q value	max fold	Function
tr|A0A1S2XTR8	calvin cycle protein CP12-2, chloroplastic-like	21.104	0.014	27.773	transport
tr|A0A1S2XQS4	light-regulated protein	5.245	0.021	27.31	cell structure
tr|A0A1S2Y090	keratin, type I cytoskeletal 10-like	21.813	0.018	12.758	cell growth/development
tr|A0A1S2XD74	60S acidic ribosomal protein P2B-like	10.261	0.019	11.342	protein synthesis
tr|A0A1S2XA51	Acyl carrier protein	12.414	0.014	10.737	intracellular traffic
tr|A0A1S2Y874	Plastocyanin	103.708	0.011	10.568	energy

tr|A0A1S2XZ19	calvin cycle protein CP12-2, chloroplastic-like	48.286	0.035	9.097	transcription
tr|A0A1S2YB46	glycine-rich RNA-binding protein 2, mitochondrial-like	19.579	0.01	8.535	transcription
tr|A0A1S2Y9R4	acidic mammalian chitinase-like	18.732	0.025	7.131	metabolism
tr|A0A1S2XDV2	Pathogenesis-related protein 10	49.663	0.004	7.122	disease and defence
tr|A0A1S2XQH9	protein SQS1-like	12.841	0.04	6.795	transcription
tr|A0A1S2XHF9	uncharacterized protein At5g39570 isoform X1	27.896	0.009	6.719	unclassified
tr|Q9ZP12	Glucan endo-1,3-beta-d-glucosidase	17.569	0.021	6.631	metabolism
tr|A0A1S2Y6I8	uncharacterized protein LOC101509734	3.694	0.027	6.164	unclassified
tr|A0A1S2YFV1	Ferredoxin	16.17	0.017	6	energy
tr|A0A1S2YQ46	ABA-responsive protein ABR18-like	7.693	0.016	5.619	disease and defence
tr|A0A1S2Z702	Non-specific lipid-transfer protein	47.043	0.023	5.543	transport
tr|A0A1S2YSZ0	Pathogenesis related protein	29.206	0.001	5.066	disease and defence
tr|A0A1S2XPR0	vestitone reductase-like	4.963	0.008	4.996	metabolism
tr|A0A1S2YTZ2	albumin-2-like	4.995	0.022	4.904	unclear classification
tr|A0A1S2YY37	Glycine cleavage system H protein	113.588	0.015	4.659	energy
tr|Q700A6	Putative lipid transfer protein GPI-anchored	27.486	0.022	4.654	transport

tr|A0A1S2YNE3	glycine-rich RNA-binding, abscisic acid-inducible protein-like	25.751	0.014	4.305	protein synthesis
tr|A0A1S2Y5Z2	>tr|A0A1S2Y5Z2|A0A1S2Y5Z2_CICAR thylakoid lumenal 19 kDa protein, chloroplastic	15.564	0.011	3.924	transport
tr|A0A1S3EGJ5	LOW QUALITY PROTEIN: ribulose bisphosphate carboxylase large chain	39.407	0.018	3.89	metabolism
tr|A0A1S2XG53	photosystem II 10 kDa polypeptide, chloroplastic	130.849	0.017	3.865	energy
tr|A0A1S2Z2Z2	thioredoxin-like isoform X1	31.638	0.001	3.831	metabolism
tr|A0A1S2YG15	Acyl carrier protein	16.057	0.049	3.827	metabolism
tr|A0A1S2XWI5	uncharacterized protein LOC101515092	4.593	0.033	3.677	unclassified
tr|A0A1S2XYG5	thioredoxin Y1, chloroplastic-like	16.337	0.011	3.419	metabolism
tr|Q9ZNQ4	Superoxide dismutase [Cu-Zn]	26.724	0.016	3.404	metabolism
tr|A0A1S2YLG2	Non-specific lipid-transfer protein	36.033	0.034	3.344	metabolism
tr|A0A1S2Y294	Chalcone-flavonone isomerase family protein	20.344	0.009	3.336	metabolism
tr|A0A1S2YEH0	flocculation protein FLO11-like	5.204	0.011	3.261	cell structure
tr|A0A1S2Y7P0	DNA topoisomerase 2	2.584	0.047	3.223	metabolism
tr|A0A1S3E8Q6	lysosomal beta glucosidase-like	6.44	0.016	3.186	Protein destination and storage
tr|A0A1S2XQC0	thioredoxin F-type, chloroplastic-like	7.44	0.021	3.184	cell growth/development

tr|A0A1S2XDZ4	translationally-controlled tumor protein homolog	27.06	0.014	3.144	cell growth/development
tr|A0A1S3E0G2	calmodulin-like	23.443	0.026	3.142	signal transduction
tr|A0A1S2Y0X8	thylakoid lumenal 15.0 kDa protein 2, chloroplastic	15.08	0.003	3.118	cell structure
tr|A0A1S2XDV0	ABA-responsive protein ABR17-like	14.464	0.034	3.102	signal transduction
tr|A0A1S2XUH0	Glutathione synthetase	8.366	0.014	3.042	metabolism
tr|A0A1S2YDD8	thylakoid lumenal 15 kDa protein 1, chloroplastic	51.373	0.007	3.032	energy
tr|A0A1S2Z111	30S ribosomal protein 3, chloroplastic-like	18.823	0.034	3.003	protein synthesis
tr|A0A1S2XWM2	uncharacterized protein LOC101504472	6.882	0.031	2.989	unclassified
tr|A0A1S2XFN2	persulfide dioxygenase ETHE1 homolog, mitochondrial	24.981	0.024	2.957	metabolism
tr|A0A1S2XPA0	high mobility group B protein 1	5.614	0.016	2.943	intracellular traffic
tr|A0A1S2Z4L2	protein BOLA4, chloroplastic/mitochondrial	26.618	0.007	2.938	cell structure
tr|A0A1S2XAG9	protease Do-like 5, chloroplastic	2.683	0.012	2.93	cell structure
tr|A0A1S2XVX2	AT-hook motif nuclear-localized protein 14-like	15.346	0.045	2.925	cell structure
tr|A0A1S2XUQ1	33 kDa ribonucleoprotein, chloroplastic	23.773	0.04	2.886	transcription
tr|A0A1S2YCD8	ribosome-binding factor PSRP1, chloroplastic	40.931	0.009	2.786	metabolism

tr|A0A1S2YUP6	Carboxypeptidase	28.532	0.009	2.758	Protein destination and storage
tr|A0A1S2XG58	uncharacterized protein LOC101505718 isoform X2	6.723	0.008	2.751	unclassified
tr|A0A1S2Y089	thioredoxin X, chloroplastic	30.99	0.007	2.737	disease and defence
tr|A0A1S2YE45	Malic enzyme	12.388	0.042	2.721	metabolism
tr|A0A1S3E326	valine--tRNA ligase	2.549	0.026	2.689	transcription
tr|A0A1S2Y875	CO(2)-response secreted protease-like	95.928	0.013	2.623	Protein destination and storage
tr|A0A1S2YQJ2	endo-1,31,4-beta-D-glucanase-like	57.271	0.01	2.609	energy
tr|A0A1S2XU67	alpha carbonic anhydrase 1, chloroplastic	5.471	0.007	2.564	metabolism
tr|A0A1S2XTU0	uncharacterized protein OsI_027940-like	39.099	0.007	2.539	unclassified
tr|A0A1S2Z271	aminoacyl tRNA synthase complex-interacting multifunctional protein 1	8.057	0.012	2.526	transcription
tr|A0A1S2XH89	Ferredoxin-thioredoxin reductase, catalytic chain	8.117	0.016	2.522	metabolism
tr|A0A1S2XK69	protein CHLORORESPIRATORY REDUCTION 7, chloroplastic	14.026	0.009	2.495	energy
tr|A0A1S2YHI8	Peptidylprolyl isomerase	35.581	0.005	2.49	metabolism
tr|A0A1S2YKM2	Carboxypeptidase	12.413	0.02	2.466	Protein destination and storage
tr|A0A1S2YZ64	thiosulfate sulfurtransferase 16	26.714	0.015	2.457	protein synthesis

tr|A0A1S2YD43	uncharacterized protein LOC101494289 isoform X1	6.206	0.017	2.422	metabolism
tr|A0A1S2XVM5	uncharacterized protein LOC101512811	16.1	0.009	2.372	unclassified
tr|A0A1S2XSM5	glutathione S-transferase L3	29.331	0.013	2.353	disease and defence
tr|A0A1S2XM30	CBS domain-containing protein CBSX3, mitochondrial isoform X1	92.691	0.02	2.336	cell growth/development
tr|A0A1S2YMH7	uncharacterized protein LOC101496020	23.025	0.034	2.322	unclassified
sp|O49818	Lactoylglutathione lyase	12.177	0.045	2.291	metabolism
tr|A0A1S2XT07	Inosine-5'-monophosphate dehydrogenase	5.756	0.02	2.282	cell growth/development
tr|A0A1S2YKP6	Superoxide dismutase [Cu-Zn]	192.617	0.031	2.244	energy
tr|A0A1S2YRM0	betaine aldehyde dehydrogenase 1, chloroplastic	41.549	0.023	2.242	disease and defence
tr|A0A1S2YQU2	methyl-CpG-binding domain-containing protein 11-like	20.745	0.049	2.213	cell structure
tr|A0A1S2YXU1	thylakoid lumenal protein At1g12250, chloroplastic	65.825	0.006	2.21	metabolism
tr|A0A1S2XHY0	14 kDa zinc-binding protein	12.33	0.012	2.209	disease and defence
tr|A0A1S2YFZ3	40S ribosomal protein S12	25.111	0.018	2.203	protein synthesis
tr|A0A1S2Y9C4	uncharacterized protein LOC101501607	13.469	0.005	2.166	unclassified
tr|A0A1S2YIG4	10 kDa chaperonin	5.971	0.018	2.159	cell structure

tr|A0A1S2Z5B7	plasminogen activator inhibitor 1 RNA-binding protein-like	25.456	0.043	2.156	transcription
tr|A0A1S2YLN1	arginase 1, mitochondrial	27.799	0.043	2.154	transport
tr|A0A1S2YII8	14 kDa zinc-binding protein	14.917	0.023	2.143	signal transduction
tr|A0A1S2YC79	thylakoid lumenal 17.4 kDa protein, chloroplastic	66.541	0.007	2.094	cell structure
tr|A0A1S2XFX0	thioredoxin M4, chloroplastic	48.713	0.009	2.094	metabolism
tr|A0A1S2YC17	psbP domain-containing protein	45.044	0.014	2.051	transport
tr|A0A1S2XRG1	10 kDa chaperonin-like	11.354	0.044	2.028	Protein destination and storage
tr|A0A1S2YPL0	uncharacterized protein LOC101500266	17.805	0.011	2.018	unclassified
tr|A0A1S2YLZ5	leucine--tRNA ligase, cytoplasmic isoform X1	14.545	0.023	2.014	protein synthesis
tr|A0A1S2YQ71	peroxisomal fatty acid beta-oxidation multifunctional protein MFP2	19.255	0.025	-2.006	metabolism
tr|A0A1S2YXE2	uncharacterized protein LOC101488830	59.63	0.019	-2.016	metabolism
tr|A0A1S2YB16	1-aminocyclopropane-1-carboxylate oxidase	65.015	0.014	-2.054	disease and defence
tr|A0A1S2Y7N8	uncharacterized protein ycf39	45.502	0.013	-2.056	unclassified
tr|A0A1S2YQR3	probable sucrose-phosphate synthase	16.153	0.014	-2.063	metabolism
tr|A0A1S2XK74	2-methylene-furan-3-one reductase-like	58.899	0.048	-2.073	disease and defence

tr|A0A1S2XVW1	probable plastid-lipid-associated protein 6, chloroplastic	171.624	0.03	-2.074	metabolism
tr|A0A1S2YCA3	rhodanese-like domain-containing protein 14, chloroplastic	18.396	0.018	-2.075	disease and defence
tr|A0A1S2XAS4	Obg-like ATPase 1	23.122	0.024	-2.086	transport
tr|A0A1S2XLQ0	Mitochondrial fission 1 protein	19.059	0.043	-2.095	signal transduction
tr|A0A1S2YPG7	macrophage migration inhibitory factor homolog	12.744	0.036	-2.106	disease and defence
tr|A0A1S2YNI6	epimerase family protein SDR39U1 homolog, chloroplastic	29.928	0.04	-2.108	metabolism
tr|A0A1S2YQQ6	uncharacterized protein At2g34460, chloroplastic	48.02	0.022	-2.11	unclassified
tr|A0A1S3ECZ3	secoisolariciresinol dehydrogenase-like	24.504	0.034	-2.127	energy
tr|A0A1S2Z6J8	uncharacterized protein LOC101506186	32.18	0.017	-2.128	unclassified
tr|Q9ZRU2	uncharacterized protein LOC101508404	7.431	0.007	-2.132	unclassified
tr|A0A1S2YF30	mitochondrial-processing peptidase subunit alpha-like	36.977	0.048	-2.136	signal transduction
tr|A0A1S2YWE9	carotenoid 9,10(9',10')-cleavage dioxygenase 1	49.82	0.011	-2.144	metabolism
tr|B5LMR4	50S ribosomal protein L23, chloroplastic	12.676	0.021	-2.167	protein synthesis
tr|A0A1S2Z1R2	eukaryotic initiation factor 4A-15	67.209	0.047	-2.181	transport
tr|A0A0X9LEN0	Glutathione s-transferase 1	6.53	0.019	-2.197	disease and defence

tr|A0A1S2XJY4	ABC transporter B family member 26, chloroplastic isoform X1	12.222	0.044	-2.204	transport
tr|A0A1S2XT74	uncharacterized protein LOC101505098	33.727	0.031	-2.219	unclassified
tr|A0A1S2XMM1	40S ribosomal protein S4	70.453	0.031	-2.248	protein synthesis
tr|A0A1S2Y1I5	protein PROTON GRADIENT REGULATION 5, chloroplastic	21.979	0.011	-2.253	energy
tr|A0A1S2XKE7	cytochrome c1-2, heme protein, mitochondrial	24.206	0.019	-2.255	cell structure
tr|A0A1S2Z0M6	40S ribosomal protein S7	43.451	0.015	-2.255	protein synthesis
tr|A0A1S2YFB6	probable plastid-lipid-associated protein 7, chloroplastic	11.292	0.016	-2.275	protein synthesis
tr|A0A1S2Z854	uncharacterized protein LOC101497856	11.812	0.011	-2.276	unclassified
tr|A0A1S2XQN6	mitochondrial phosphate carrier protein 3, mitochondrial	24.087	0.012	-2.279	intracellular traffic
tr|A0A1S2Y835	Catalase	195.318	0.034	-2.286	disease and defence
tr|A0A1S2YSK4	V-type proton ATPase subunit H	5.662	0.012	-2.293	transport
tr|A0A1S2YTM9	mitochondrial carnitine/acylcarnitine carrier-like protein	50.67	0.011	-2.324	intracellular traffic
tr|A0A1S2Y710	50S ribosomal protein L17, chloroplastic	20.62	0.029	-2.326	protein synthesis
tr|A0A1S2YDH7	26S proteasome non-ATPase regulatory subunit 7 homolog A	9.649	0.034	-2.326	protein synthesis
tr|A0A1S2YKB0	pheophorbide a oxygenase, chloroplastic	7.139	0.045	-2.335	energy

tr|A0A1S2XV81	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	21.58	0.015	-2.35	disease and defence
tr|A0A1S2YG09	40S ribosomal protein S15a-1-like	28.059	0.016	-2.369	protein synthesis
tr|A0A1S2YGU4	mitochondrial dicarboxylate/tricarboxylate transporter DTC	94.699	0.018	-2.403	intracellular traffic
tr|A0A1S3E2C4	DEAD-box ATP-dependent RNA helicase 3, chloroplastic-like	45.082	0.012	-2.409	transport
tr|A0A1S2XME2	neutral ceramidase-like	2.639	0.013	-2.416	disease and defence
tr|A0A1S2Y0H8	probable plastid-lipid-associated protein 12, chloroplastic isoform X1	18.588	0.009	-2.431	transport
tr|A0A1S2XB11	ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	22.568	0.04	-2.439	protein synthesis
tr|A0A1S2Z665	geraniol 8-hydroxylase-like	10.486	0.018	-2.454	energy
tr|A0A1S2Y654	outer envelope pore protein 24, chloroplastic	11.92	0.018	-2.459	intracellular traffic
tr|A0A1S2YRJ4	Plasma membrane ATPase	60.167	0.006	-2.461	transport
tr|A0A1S2YFW9	translocase of chloroplast 159, chloroplastic-like	38.256	0.026	-2.476	metabolism
tr|A0A1S2Y966	Eukaryotic translation initiation factor 3 subunit B	11.725	0.038	-2.476	protein synthesis

tr|A0A1S2XSJ5	26S proteasome regulatory subunit 4 homolog A	8.964	0.017	-2.484	protein synthesis
tr|A0A1S2XR87	bifunctional protein FolD 4, chloroplastic	10.248	0.04	-2.487	protein synthesis
tr|A0A1S2Y6T5	cinnamoyl-CoA reductase 1	80.382	0.021	-2.489	disease and defence
tr|A0A1S2Z827	probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5, mitochondrial	15.846	0.02	-2.489	transport
tr|A0A1S2Z166	uncharacterized protein LOC101492619	5.667	0.033	-2.503	unclassified
tr|A0A1S3EAP9	external alternative NAD(P)H-ubiquinone oxidoreductase B2, mitochondrial-like	11.419	0.011	-2.544	disease and defence
tr|A0A1S2XHH6	uncharacterized protein LOC101501957	12.881	0.006	-2.546	unclassified
tr|A0A1S2YDG4	60S ribosomal protein L9	102.783	0.042	-2.555	protein synthesis
tr|O65757	Putative Pi sta	18.039	0.012	-2.571	disease and defence
tr|A0A1S2YJ62	prohibitin-1, mitochondrial	58.256	0.016	-2.58	intracellular traffic
tr|A0A1S2YLZ0	30S ribosomal protein S31, chloroplastic	17.091	0.018	-2.648	protein synthesis
tr|A0A1S2Y6M9	outer envelope pore protein 37, chloroplastic	4.721	0.043	-2.665	intracellular traffic
sp|O65751	40S ribosomal protein SA	81.904	0.026	-2.682	protein synthesis
tr|A0A1S2YNJ2	40S ribosomal protein S25-2-like	15.995	0.037	-2.692	protein synthesis
tr|A0A1S2Z192	thioredoxin-like protein Clot	5.965	0.025	-2.71	disease and defence

tr|A0A1S2YMB2	heat shock 70 kDa protein (HSP70)15-like isoform X1	50.18	0.048	-2.725	disease and defence
tr|A0A1S3EJE6	(+)-neomenthol dehydrogenase-like isoform X2	10.592	0.018	-2.73	Energy
tr|A0A1S3DY93	cytochrome b-c1 complex subunit 8-like	10.517	0.039	-2.762	protein synthesis
tr|A0A1S2YR41	calcium sensing receptor, chloroplastic	95.334	0.011	-2.772	disease and defence
tr|B5LMP8	ATP-dependent Clp protease proteolytic subunit	11.836	0.041	-2.79	protein destination and storage
tr|A0A1S2YZY1	probable mannitol dehydrogenase	13.832	0.045	-2.791	energy
tr|A0A1S2XWW1	PITH domain-containing protein At3g04780	4.487	0.015	-2.798	disease and defence
tr|A0A1S2Z480	alpha-amylase 3, chloroplastic-like	48.304	0.017	-2.828	metabolism
tr|A0A1S2XVH6	2-methyl-6-phytyl-1,4-hydroquinone methyltransferase, chloroplastic	24.49	0.023	-2.871	protein synthesis
tr|A0A1S3EBL9	LOW QUALITY PROTEIN: 9-divinyl ether synthase-like	4.505	0.045	-2.883	metabolism
tr|A0A1S2Y8W1	Clathrin heavy chain	26.501	0.018	-2.885	signal transduction
tr|A0A1S3EA23	phytoene dehydrogenase, chloroplastic/chromoplastic	2.892	0.011	-2.89	metabolism
tr|A0A1S2Z2C9	translation factor GUF1 homolog, chloroplastic	19.366	0.026	-2.958	transport
tr|A0A1S2XMD4	uncharacterized aarF domain-containing protein kinase At5g05200, chloroplastic	2.626	0.037	-3	signal transduction

tr|A0A1S2YZB9	60S ribosomal protein L4	98.19	0.007	-3.047	protein synthesis
tr|A0A1S2XME4	60S ribosomal protein L9-like	41.082	0.034	-3.088	protein synthesis
tr|A0A1S2YY93	mitochondrial outer membrane protein porin 2	20.59	0.011	-3.175	transport
tr|A0A1S2YAY9	uncharacterized aarF domain-containing protein kinase At1g79600, chloroplastic	5.227	0.007	-3.223	protein synthesis
tr|A0A1S2Z521	Peroxidase	5.798	0.029	-3.278	disease and defence
tr|A0A1S2YMU2	protein TOC75, chloroplastic	32.258	0.004	-3.45	transport
tr|A0A1S2YIQ0	uncharacterized protein LOC101494085	48.393	0.049	-3.457	disease and defence
tr|A0A1S2Z5P2	30S ribosomal protein 2, chloroplastic	28.996	0.036	-3.689	protein synthesis
tr|A0A1S2YJ61	ADP,ATP carrier protein 3, mitochondrial	50.197	0.008	-3.714	transport
tr|B5LMQ8	30S ribosomal protein S8, chloroplastic	38.059	0.008	-3.74	protein synthesis
tr|A0A1S2XN51	UDP-glucose 6-dehydrogenase	17.129	0.022	-3.749	metabolism
tr|A0A1S2YD89	Starch synthase, chloroplastic/amyloplastic	14.837	0.009	-3.906	metabolism
tr|A0A1S2XGE6	trifunctional UDP-glucose 4,6-dehydratase/UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-L-rhamnose-reductase RHM1	15.844	0.033	-3.965	metabolism
tr|A0A1S2YV39	Lipoxygenase	89.569	0.031	-3.976	metabolism
tr|A0A1S2Y0R0	glutathione S-transferase L3-like	57.926	0.006	-4.098	disease and defence

tr|A0A1S2XH77	serine protease SPPA, chloroplastic-like OS	11.186	0.011	-4.621	protein synthesis
tr|A0A1S2Z5K5	uncharacterized aarF domain-containing protein kinase At1g79600, chloroplastic-like	4.347	0.001	-4.793	protein synthesis
tr|A0A1S2Z4A6	putative bark agglutinin LECRPA3	3.583	0.04	-5.073	disease and defence
tr|A0A1S3DZ63	cytochrome P450 CYP736A12-like	28.403	0.016	-5.17	metabolism
tr|A0A1S2Y105	60S acidic ribosomal protein P2-4-like	12.342	0.03	-5.259	protein synthesis
tr|O81927	Thaumatin-like protein PR-5a	43.701	0.02	-7.688	disease and defence
tr|A0A1S2XGT2	class-10 pathogenesis-related protein 1-like	15.301	0.014	-7.837	disease and defence
sp|P36908	Acidic endochitinase	37.306	0.015	-9.848	disease and defence
tr|A0A1S2XYW4	early light-induced protein, chloroplastic-like	25.147	0.002	-61.61	metabolism

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18 November 2019

TO WHOM IT MAY CONCERN

Re: Contribution to co-authored publications

Madam, Sir,

I have had the pleasure to be involved through targeted engagements to the following publications, of which Givemore Munashe Makonya is the first author.

1. Makonya GM, Ogola JBO, Muasya M, Crespo O, Maseko S, Valentine, AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2019. Chlorophyll fluorescence and carbohydrate concentration as field selection traits for heat tolerant chickpea (*Cicer arietinum* L.; Fabaceae) genotypes. Plant Physiology and Biochemistry. Volume 141, Pages 172-182.
2. Makonya GM, Ogola JBO, Muasya M, Crespo O, Maseko S, Valentine, AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Intermittent moisture supply induces drought priming responses in some Chickpea (*Cicer arietinum* L.; Fabaceae) genotypes. Scientia Horticulturae (under review),

I would like to confirm that in both cases Mr Makonya was the lead researcher, and that the papers are directly reflecting Mr Makonya's research work, including conception, design, data collection, experiments and data analysis. He independently wrote the manuscripts, integrating comments and suggestions from the co-authors, and my particular involvement is one of a supervisor to a well capable PhD candidate.

Sincerely

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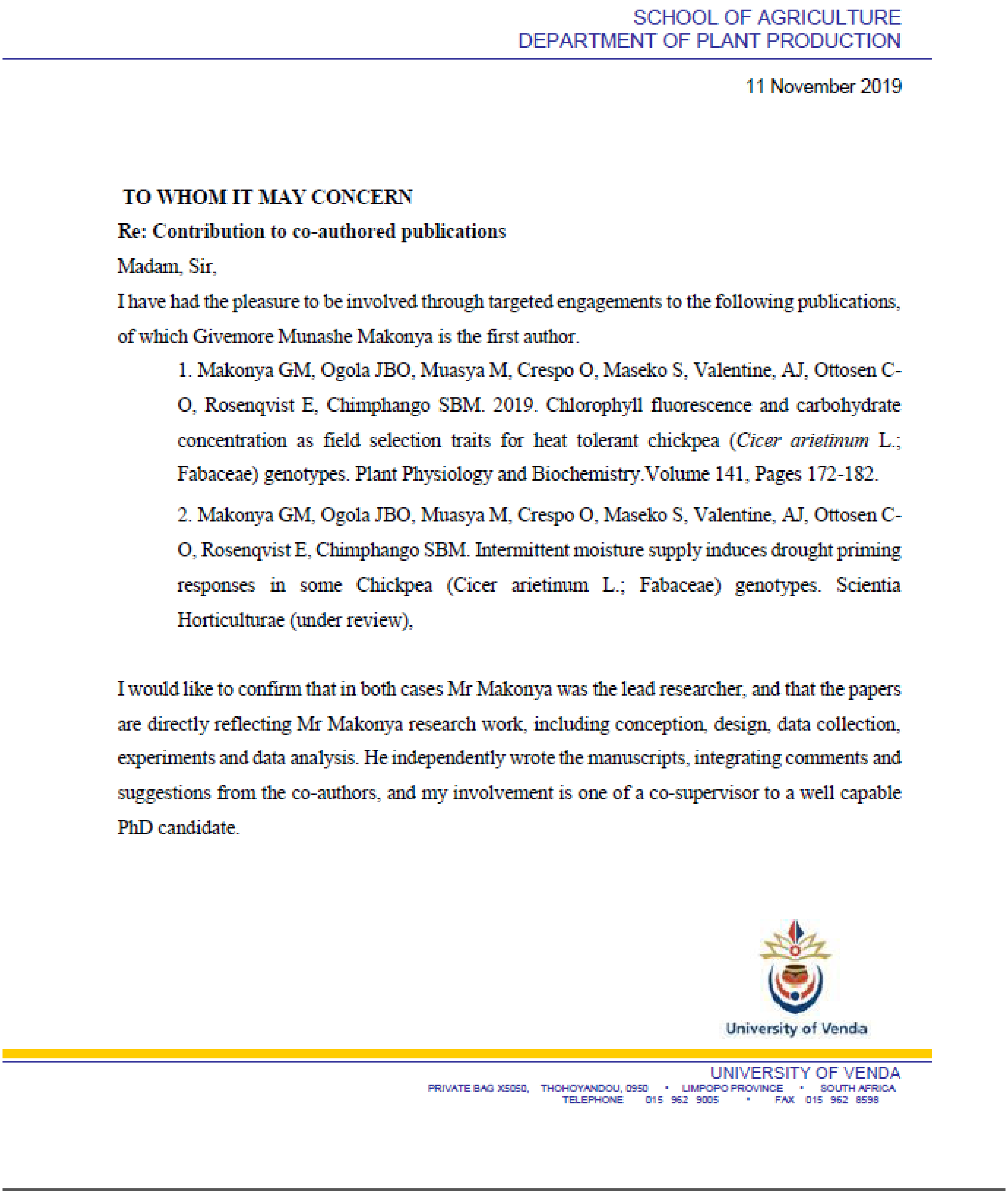
Samson BM Chimphango

Our Mission is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."



SCHOOL OF AGRICULTURE
DEPARTMENT OF PLANT PRODUCTION

11 November 2019

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University of Venda

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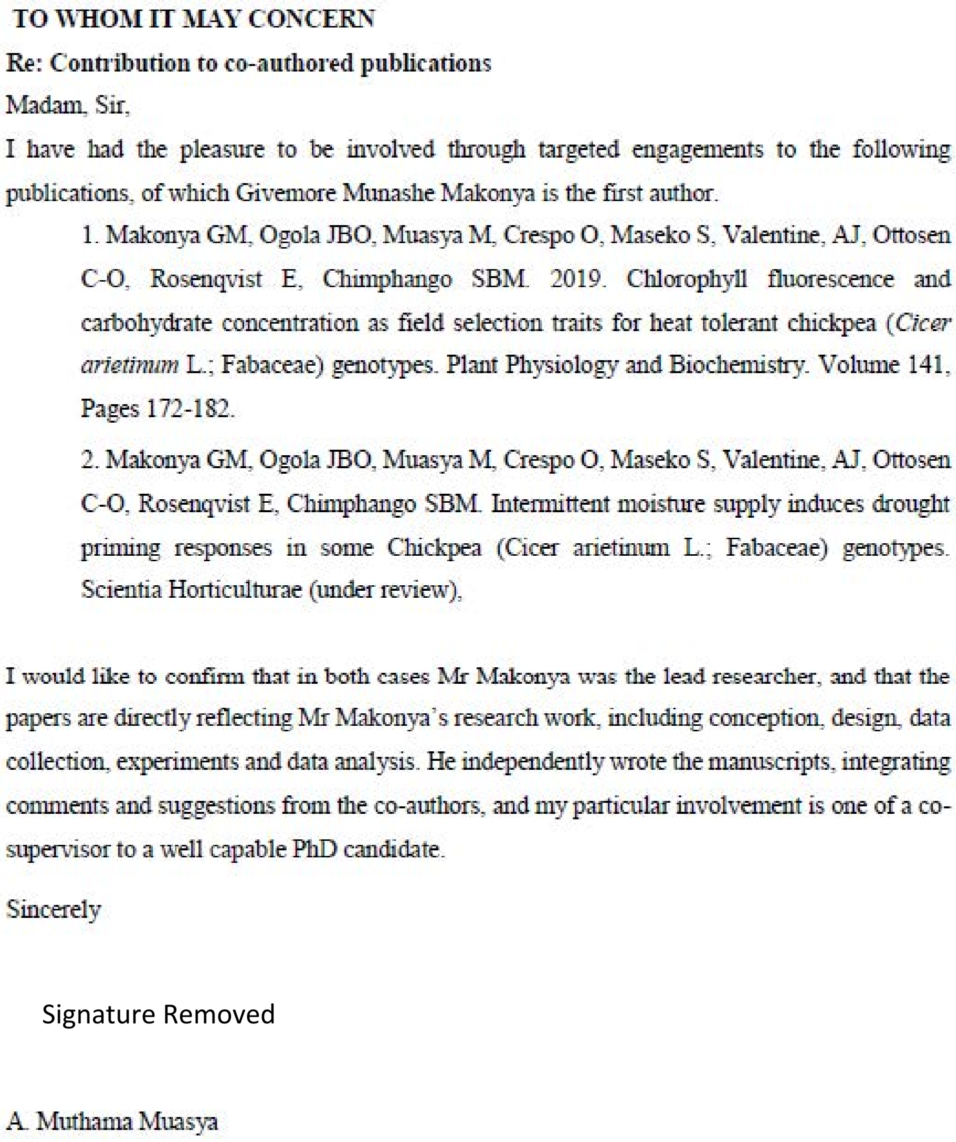
Prof. J.B.O. Ogola, Pr.Sci.Nat.



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Sincerely

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Cape Town, October 31, 2019

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Yours sincere'

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Olivier Crespo

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