Root morphology for growth and physiology of drought and heat tolerance in chickpea (*Cicer arietinum* L., Fabaceae)

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Declaration

I, Grace Emmanuella Adoteye, declare that this dissertation is submitted in fulfilment of the requirements for the award of MSc degree, in the Department of Biological Science at the University of Cape Town. I acknowledge my knowledge of the meaning of plagiarism, and declare that all work in this document, except for that which is properly acknowledged, is my own. This document has not been submitted in whole, or in part, for the award of any degree at any other academic institution.

Signature:  Date: 16/02/2025

For Ebenezer and Ezerri.

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Abstract

Chickpea (*Cicer arietinum* L.) is an important legume crop valued for its nutritional quality and affordability, yet its production is increasingly threatened by drought and heat stress, particularly in arid and semi-arid regions where it is predominantly cultivated during the post-rainy season. Climate change is expected to intensify the frequency and severity of these stresses, underscoring the need to understand adaptive mechanisms that enhance stress resilience. While research on the individual effects of drought and heat stress in chickpea is relatively well established, their combined impact remains poorly understood. Moreover, most studies have focused on above-ground traits, leaving critical root system adaptations largely unexplored. This study therefore aimed to characterise chickpea root morphology and its responses to drought and heat stress - individually and in combination - as well as to low-nutrient stress and the combined effects of low nutrients and drought. Four genotypes were used, namely: ACC#7, ICCV 00108, ICCV 07113, and ACC#8. These genotypes differ in their stress responses, with ICCV 07113 and ACC#8 known to be heat- and drought-susceptible, ACC#7 and ICCV 00108 exhibiting drought tolerance, and ACC#7 additionally showing heat tolerance. Three complementary experiments were conducted.

In Chapter Two, a controlled environment study examined the physiological responses and root trait adaptations of three genotypes (ICCV 07113, ACC#8, and ICCV 00108) under individual and combined drought and heat stress. Drought promoted the development of deeper and more extensive root systems, whereas heat, alone or combined with drought, significantly suppressed root growth. The combined stress resulted in the most pronounced reductions in both root development and physiological performance. Among the three genotypes, ACC#8 exhibited superior stress tolerance both individual and combined stresses by maintaining pigment stability and biomass, while ICCV 07113 was the most sensitive.

Root system research in natural field conditions has long been considered the 'step-child of science' due to its laborious and time-consuming methods. To help bridge this knowledge gap, Chapter Three assessed root performance of four genotypes (ACC#7, ICCV 07113, ACC#8, and ICCV 00108) under contrasting field environments differing in temperature and rainfall. High-temperature conditions reduced biomass and most root traits, yet genotypes with greater root length, surface area, and volume exhibited enhanced physiological functioning, highlighting the role of robust root systems in mitigating heat-related declines in photosynthetic activity. ICCV 07113 possessed the most extensive root system and highest physiological resilience under field heat stress, whereas ACC#8 showed the weakest performance, demonstrating genotype-by-environment interactions and differing rankings between field and controlled conditions.

Plants growing in nutrient-rich soils typically develop a low root-to-shoot ratio, increasing their vulnerability to drought at later growth stages. Recognizing the importance of a higher root-to-shoot ratio in enhancing drought resilience, Chapter Four investigated whether plants that develop a larger root system under low-nutrient conditions gain an adaptive advantage when subsequently exposed to drought. Two genotypes (ICCV 07113 and ICCV 00108) were evaluated, although genotypic differences were minimal. Plants grown under low-nutrient conditions developed higher root-to-shoot ratios and greater root length, surface area, and volume. When later subjected to drought, these plants outperformed drought-stressed plants originating from nutrient-rich soils, exhibiting greater water retention and improved yield, although biomass, plant water status (stomatal conductance and relative water content), and absolute yield remained lower than in non-stressed controls.

Taken together, the findings from the three data chapters advance our understanding of plant responses to climate-induced stress and provide a foundation for breeding programmes to

enhance chickpea resilience to abiotic stress by integrating root traits, chlorophyll stability, and water conservation as key selection criteria.

Key words: chickpea root system; Fabaceae; climate change; abiotic stress; drought stress; heat stress; combined drought–heat stress; low-nutrient adaptation; root architectural traits; drought and heat tolerance; physiological resilience.

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List of Abbreviations

asl - above sea level

C_i - intracellular CO_2 concentration

E - rate of transpiration

g_s, - leaf stomatal conductance

P_n - net photosynthetic rate

PPFD - photon flux density

ppm - parts/ million

PSII - Photosystem II

RH - relative humidity

ROS - Reactive oxygen species

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

RWC - Relative water content

RSA – Root system architecture

RL – Root length

SA – Root surface area

RV – Root volume

RAD – Root average diameter

RLR – Root length ratio

RMR – Root mass ratio

RF – Root fineness

RTD – Root tissue density

SRL – Specific root length

Chapter 1

General Introduction

1.1 Chickpea

Cicer arietinum L., commonly known as chickpea or garbanzo bean, is the world's third most important pulse crop, with an annual production of 15 million tonnes, accounting for 15.42% of total global pulse production (Ajay et al., 2024; Merga & Haji, 2019). Chickpea belongs to the genus *Cicer*, from the family Fabaceae (POWO, 2025). Globally, two distinct types of chickpea – Desi and Kabuli – are mainly cultivated for human consumption. The Desi chickpea dominates global production (80-85%) and is mainly cultivated in South Asia, Australia, and East Africa (Eker et al., 2022). They are characterised by their smaller seeds and darker seed coat colours (brown or black). In contrast, the Kabuli chickpea has larger seeds with lighter seed coat colours (varying from cream to beige) and is usually cultivated in the Mediterranean basin and East Asia. The Desi variety has a thicker seed coat comprising 14% of the total seed weight whereas the Kabuli variety has a thin seed coat which makes up only 5% of its total seed weight (Wood et al., 2011). Of the two varieties, the Kabuli is known to be high yielding; however, the Desi is more tolerant to drought stress (Waqas et al., 2019).

1.1.1 Chickpea production

Chickpea is known to have originated from present-day south-eastern Turkey but is currently being cultivated in about 57 countries across all the continents with 85% of chickpea produced globally from Asia (Merga and Haji, 2019; Rani et al., 2020). In 2023, India, the world's single largest producer of chickpea accounted for 74% (12.27 million tonnes) of total global chickpea produced with Australia, Turkey, and the Russian Federation coming in as secondary producers (FAOSTAT, 2024). Ethiopia is the leading chickpea producer in Africa and the fifth-largest producer in the world with an annual production of 451,309 tonnes (FAOSTAT, 2024).

Although countries like Tanzania, Sudan, Kenya, and Malawi also contribute significantly to the crop's production in Africa, their production in addition to that of Ethiopia accounts for less than 4% of the global output (FAOSTAT, 2024). Chickpea production in Sub-Saharan Africa (SSA) constitutes approximately 88.6% of the continent's total production volume yet generates less than USD $100 million annually, accounting for less than 9% of the estimated USD $1.1 billion in global chickpea revenue. (Fikre et al., 2020).

Although originally a cool-season crop from temperate regions, chickpea cultivation has expanded into tropical and subtropical areas (Mohammed et al., 2017). In Mediterranean regions, chickpeas are typically sown during the winter months, capitalizing on seasonal rainfall, whereas in South Asia and parts of the Mediterranean, which are responsible for 90% of the global chickpea output, they are grown in the post-rainy season (Berger et al., 2004; Gaur et al., 2019). Optimal conditions for chickpea reproductive development occur when temperatures range from 20°C to 28°C (Devi et al., 2023). However, in warmer regions, the crop often experiences temperatures exceeding 30°C during critical growth stages, making it vulnerable to heat stress. Additionally, reliance on residual soil moisture in post-rainy season cultivation leaves the crop susceptible to terminal drought stress, further limiting productivity (Awasthi et al., 2017). Despite these challenges, chickpea remains a cost-effective crop compared to many other field crops, as it is typically grown towards the end of the cropping season, requiring minimal soil management and experiencing reduced weed pressure (Fikre, 2020). Beyond its economic advantages, chickpea contributes to sustainable agriculture by enhancing soil fertility through nitrogen fixation, facilitated by its symbiotic relationship with soil-borne *Rhizobium* bacteria (Waqas et al., 2019).

1.1.2 Consumption and nutritional value

Known as the 'poor man's meat', chickpeas are highly nutritious, containing 20% - 25% protein content, 48.2% - 67.6% carbohydrates and 6% fat (Das et al., 2020). They are also rich in dietary fibre (12g/100g), essential minerals like potassium, phosphorus, magnesium, calcium, sodium, and manganese, as well as vitamins A and K; and their low glycaemic index makes them a highly nutritious and healthy dietary choice (Rehm et al., 2023; Wallace et al., 2016). They are an important source of diet consumed across many regions of the world in various forms, including snacks, salads, hummus, and curry blends (Fikre, 2020). Their nutritional value and affordability have made them a critical component of global food security, particularly in addressing protein deficiencies in the diets of populations in India and SSA (Ajay et al., 2024). Malunga et al. (2014) highlighted the versatility of chickpeas by using them as a primary source of carbohydrates and protein in designing a chickpea-based follow-on formula. This formula meets WHO/FAO standards for complementary foods and adheres to EU regulations for follow-on formulas, requiring only minimal supplementation with oils, minerals, and vitamins.

1.1.3 Demand in South Africa

In South Africa, like many countries, chickpea is becoming one of the most consumed pulses whose demand is projected to continually increase with time. However, there is no commercial production of chickpea in the country, and as a result, it is imported from India, Canada, and Australia to meet its demand (Tiisetso and Sipho, 2018). Research carried out on the possible production of chickpea in South Africa indicates the crop can be cultivated in North-Eastern (NE) South Africa and performs better in the winter seasons of the region (Thangwana and Ogola, 2012). However, the identified regions are known to be semi-arid (Adeola et al., 2017), and like all other arid and semi-arid regions of the world, they are predicted to experience an increase in temperatures and a decrease in rainfall due to global warming (Vadez et al., 2011).

1.2 Abiotic stress and climate change

Climate change has become one of the most significant global challenges of the 21st century, with profound implications for agricultural productivity and food security. The Intergovernmental Panel on Climate Change (IPCC) has projected a continual rise in global temperatures accompanied by more frequent and intense extreme weather events, including droughts and heatwaves, in coming decades (IPCC, 2021). Among its impacts, the increasing frequency and intensity of abiotic stresses such as drought, heat, salinity, and flooding are particularly concerning, as they adversely affect plant growth and yield.

Drought and heat stress are two predominant abiotic stresses projected to intensify in severity and frequency as global temperatures rise and precipitation patterns become more erratic (IPCC, 2021; Trenberth et al., 2014). These stressors threaten the sustainability of agricultural systems, especially in regions that are already vulnerable to water scarcity and high temperatures, such as SSA (Lombe et al., 2024). Understanding how plants respond to these stresses individually and in combination is critical for developing climate-resilient crop varieties and ensuring food security under increasingly unpredictable environmental conditions.

1.2.1 Drought stress

Depending on the conditions leading up to a situation of drought, Wilhite and Glantz (1985) classified drought into four distinct categories: meteorological drought which is caused by dry weather conditions in a particular area due to erratic precipitation patterns or low precipitation levels; hydrological drought which occurs when there is low water supply in surface and groundwater levels, usually encountered after a long period of meteorological drought; agricultural drought which is associated with a decline in soil moisture having adverse effects on plant growth and development; and socio-economic drought relating to the inability to meet

the demand for some economic goods and services such as water, hydroelectric power, food grains, and fish, due to weather-related deficit in water availability.

Drought is a major limitation to the field of agriculture in that it often causes more yield losses than other stresses in agriculture (Seleiman et al., 2021). The phenomenon has in the past decade cost up to USD $30B of losses in global crop production (Gupta et al., 2020) through its negative impact on plant growth, physiology, and reproduction, of which chickpea is no exception (Rani et al., 2020). Globally, the effect of drought stress on chickpea has been estimated to cause about 45-50% yield reduction (Muruiki et al., 2018; Shah et al., 2020). Drought conditions inhibit photosynthesis as stomatal conductance is reduced, response widely reported across crops such as chickpea, wheat, and maize. For example, drought-tolerant chickpea genotypes ICCV 10 and JG 11 maintain higher stomatal conductance compared to sensitive genotypes (Kashiwagi et al., 2015), while drought-sensitive wheat cultivar 'Shiraz' shows rapid stomatal closure leading to severe photosynthetic decline (Farooq et al., 2009). The limited water availability also leads to a decline in the leaf relative water content (RWC) resulting in the loss of turgor pressure and shrinkage of cells in the leaves. When the drought is prolonged, it can lead to cellular dehydration and irreversible damage to the cell structures as the structural support, provided by the turgor pressure is compromised (Moore et al., 2008). In cases of extreme drought stress, reactive oxygen species (ROS) accumulate in the leaves, as demonstrated in rice cultivar IR64 and maize hybrid ZD958, both of which show sharp increases in H_2O_2 and superoxide radicals under water deficit (Guo et al., 2024; Wang et al., 2022). This causes oxidative damage to the photosynthetic apparatus, including the degradation of chlorophyll pigments and inhibition of photosystem (PSII) activity, further compromising photosynthetic performance, and hence reducing energy production (Zahoor et al., 2022). This leads to stunted plant growth, resulting in decreased biomass accumulation and yield losses in crops (Farooq et al., 2009). During the reproductive phase of a plant's growth cycle, drought

stress causes severe damage to crop yield more than it does compared to the vegetative state due to its influence on morphological and physiological processes in this phase (Wach and Skowron, 2022). By shortening the reproductive period, drought stress leads to flower or seed abortion, reducing yield, while also decreasing floral size and producing lower-quality nectar (Farooq et al., 2017). In addition to its effect on plants, drought stress compounds the effect of other abiotic and biotic stresses to which plants might be subjected (Cruz de Carvalho, 2008).

1.2.2 Heat stress

Heat stress generally arises when temperatures exceed a crop's optimal threshold for growth and development, leading to adverse effects on physiological, biochemical, and reproductive processes (Wahid et al., 2007). The severity of these impacts depends on the timing, intensity, and duration of high temperatures. For seed yield, heat stress is most impactful when it coincides with critical growth stages such as flowering, pollination, and grain filling, causing significant yield losses in many crops, including chickpea (Devasirvatham et al., 2010). Chickpea, which is commonly grown in semi-arid regions, is highly vulnerable to high temperatures during reproductive stages, with temperatures exceeding 30°C during flowering or pod formation causing drastic yield reductions – sometimes as high as 50% – primarily due to reduced pollen viability, flower drop, and pod abortion (Devasirvatham et al., 2012).

Heat stress negatively affects several physiological processes, particularly photosynthesis, which is highly sensitive to elevated temperatures. One of the primary effects is the decline in chlorophyll content, leading to a reduction in light absorption and energy conversion efficiency, as shown in heat-sensitive chickpea genotype ICC 10685, wheat cv. Cadenza, and soybean line PI 548313, all of which exhibit 20–40% pigment reduction under temperatures exceeding 35°C (Zhao et al., 2020). In addition, high temperatures cause instability in the photosynthetic apparatus, particularly in PSII, which experiences protein denaturation and

damage to the D1 protein, a core component essential for electron transport (Mathur et al., 2014). Furthermore, heat stress reduces the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the key enzyme responsible for carbon fixation in the Calvin cycle, thereby lowering the overall rate of CO_2 assimilation and disrupting carbohydrate production (Nosaka and Nosaka, 2017). Prolonged heat exposure also accelerates transpiration rates, leading to increased water loss and heightened vulnerability to drought conditions (Hu et al., 2020). Additionally, heat stress induces oxidative stress through excessive accumulation of ROS, such as superoxide radicals and hydrogen peroxide, which cause lipid peroxidation, protein oxidation, and damage to cellular membranes (Zhao et al., 2020).

During the reproductive stage, heat stress is particularly detrimental, as it interferes with key biochemical pathways responsible for flower development, pollination, and seed setting. High temperatures disrupt the synthesis of key proteins such as heat shock proteins (HSPs), which are critical for maintaining cellular homeostasis and protecting reproductive tissues from thermal damage (Gaur et al., 2019). In chickpea, heat stress also affects hormonal balance, reducing the levels of auxins and gibberellins, which are essential for pollen tube elongation and ovule development. As a result, pollen viability is significantly reduced, leading to lower fertilization success. Additionally, stigma and ovule abnormalities, along with impaired pollen germination, further decrease reproductive efficiency, ultimately leading to reduced pod set and grain yield (Devasirvatham et al., 2012). The shortening of the crop cycle under high temperatures exacerbates these effects by reducing the time available for grain filling, thereby lowering seed weight and quality. These ultimately lead to significant reductions in yield, making heat stress a critical challenge for sustainable chickpea production. Heat stress also amplifies the negative impacts of other abiotic stresses like drought, further exacerbating its overall impact on crops (Mittler, 2006).

1.2.3 Adaptive responses to drought and heat stress

Plants, being sessile organisms, have evolved to survive drought conditions with diverse biochemical, morphological, and physiological adaptations (Cruz de Carvalho, 2008). They adopt one of three strategies to cope with drought stress: escape, avoidance, or tolerance. Drought escape occurs when a plant rapidly completes its life cycle, ensuring reproduction before water scarcity becomes severe. Drought avoidance involves maintaining relatively higher tissue water content despite low soil moisture by minimizing water loss or enhancing water uptake (Basu et al., 2016; Gupta et al., 2020). Meanwhile, drought tolerance enables plants to survive under low tissue water content through multiple adaptive mechanisms, including increasing osmolyte concentrations, enhancing osmotic adjustment, and promoting water movement into cells to sustain metabolic activity (Min et al., 2020). The extent and effectiveness of these responses vary among plant species and genotypes, as well as with the duration and severity of drought stress and the specific organ or cell type affected (Barnabás et al., 2008).

To minimize water loss, one of the plant's initial responses is stomatal closure, which limits transpiration and helps conserve internal water (Manzi et al., 2015). However, this response causes a reduction in CO_2 fixation, thereby affecting key metabolic processes such as photosynthesis, carbon metabolism, and the synthesis of metabolites, including osmo-protectants and antioxidants (Nakashima et al., 2009; Thalmann and Santelia, 2017). Additionally, drought stress disrupts nitrogen assimilation and lipid metabolism, affecting amino acid biosynthesis and membrane stability (Araújo et al., 2011; Obata and Fernie, 2012). The limitation of intercellular CO_2 concentration in the leaves coupled with continued photosynthetic light reactions, disrupts the photosynthetic electron transport chain, leading to reduced molecular oxygen and the accumulation of ROS, which can cause severe oxidative damage (Basu et al., 2016; Cruz de Carvalho, 2008).

To further mitigate water loss, plants undergo morphological adaptations such as leaf shedding and reduced leaf number and size. Some species also develop sclerophylly, a trait that hardens leaves to prevent permanent damage from wilting, allowing them to regain full functionality once water availability is restored (De Micco and Aronne, 2012). To enhance water uptake and maintain cell turgor, plants regulate their water potential through the accumulation of osmolytes in dividing cells; a process known as osmotic adjustment (Sanders and Arndt, 2012). These osmolytes range from organic/compatible solutes such as sugars, amino acids, proline, and glycine betaine to inorganic ions mainly Na^+, K^+, $Ca^{2+,}$ and Cl^-. This adaptive response delays metabolic damage and leaf senescence while improving assimilate transport. Additionally, the compatible solutes protect plant cells from ROS-induced damage by detoxifying the accumulated ROS, stabilizing enzymes, and maintaining membrane integrity (Chen & Jiang, 2010). This shows that drought-induced osmolyte accumulation is not universal across all plant species or genotypes. While many species accumulate proline under water deficit, others show minimal or even suppressed proline accumulation. For instance, certain rice cultivars (e.g., IR52) and wheat genotypes ('Chinese Spring') exhibit limited proline increases under drought (Singh et al., 2015), whereas pearl millet lines have been reported to actively repress proline synthesis under prolonged water deficit (Reddy et al., 2017). Similarly, some chickpea genotypes such as ICCV 10 show moderate proline accumulation, while highly tolerant lines like ICC 4958 exhibit disproportionately low proline despite strong drought tolerance (Kashiwagi et al., 2015). These contrasting patterns reinforce that proline accumulation alone is not a universal indicator of drought tolerance.

Plants exposed to heat stress also develop a range of physiological, biochemical, morphological, and molecular strategies to mitigate damage and maintain productivity under high-temperature conditions (Upadhyaya et al., 2011). One of the primary physiological responses to heat stress is increased transpiration, which facilitates evaporative cooling to lower

leaf surface temperature. However, excessive transpiration can lead to significant water loss, making water use efficiency (WUE) a critical adaptive trait. Heat-tolerant plants often maintain higher WUE by optimizing stomatal conductance to balance transpiration and CO_2 uptake, thereby maintaining higher WUE and reducing the adverse effects of prolonged heat exposure (Vadez et al., 2012). At the biochemical level, plants employ multiple defence mechanisms to counteract heat-induced cellular damage. One of the key protective strategies is the accumulation of HSPs, which prevent the degradation of essential photosynthetic enzymes such as RuBisCO and protect photosystem II from heat-induced denaturation (Gupta et al., 2010; Wahid et al., 2007). Additionally, plants enhance their antioxidant defence system to mitigate oxidative stress caused by excessive production of ROS under high temperatures. Enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase also play pivotal roles in scavenging ROS and protecting cellular structures (Wahid et al, 2007). Furthermore, osmolytes like proline, glycine betaine, and sugars, aid in osmotic adjustment, stabilizing cellular membranes, and protecting proteins and enzymes from heat-induced denaturation (Hossain et al., 2018). It is however important to note that proline accumulation under heat stress is highly variable. Several studies report strong heat-induced proline increases in crops such as tomato and soybean (Djanaguiraman et al., 2018), whereas others show that heat can suppress proline biosynthesis. For example, in barley cultivar ‘Golden Promise’ and in chickpea genotype ICC 10685, heat stress led to reduced proline accumulation despite elevated ROS (Wahid et al., 2007). Some genotypes preferentially accumulate soluble sugars rather than proline under high temperatures, reflecting species-specific metabolic prioritisation. Such contrasting findings demonstrate that proline cannot be assumed to increase under all heat stress conditions.

Plants adapt to heat stress by modifying leaf characteristics to reduce heat absorption and water loss. Reduced leaf size, altered leaf orientation, and increased surface reflectance due to wax

deposition have been observed as effective strategies for minimizing heat-induced damage (Camejo et al., 2005). These structural modifications help lower leaf temperature by reducing direct solar radiation absorption.

1.2.3.1 Adaptations in the plant root system

In addition to aboveground adaptations, the root system plays a crucial role in plant adaptation to drought and heat stress. As the primary interface between the plant and its belowground environment, roots undergo structural and functional modifications under environmental stress conditions to optimize resource acquisition (Guo et al., 2024). Root system plasticity is strongly correlated with plant tolerance and susceptibility to stress, as plants modify root traits to enhance survival and maintain physiological functions (Yang et al., 2022).

One of the primary responses of plant roots to drought stress is an increase in root depth and length to access deeper soil moisture. Many drought-tolerant species, including chickpea, exhibit enhanced root elongation under water-deficit conditions, enabling them to reach subsurface water reserves (Varshney et al., 2021). Deeper rooting enhances water uptake efficiency and helps sustain transpiration and photosynthesis during prolonged dry periods (Kashiwagi et al., 2006). Additionally, the root-to-shoot ratio often increases under drought conditions, as plants allocate more resources to root development in order to optimize water absorption. Root diameter and branching patterns also change in response to drought stress. Plants often develop thinner roots with increased lateral root proliferation to maximize soil exploration and enhance water acquisition (Lynch, 2013). Fine-root proliferation under drought has been documented in soybean cultivar PI 416937 and lentil genotypes ILL 6002 and ILL 5888 (Singh et al., 2019). However, under severe drought conditions, some plants exhibit a shift towards thicker roots with reduced lateral branching, which helps minimize water loss and maintain structural integrity (Comas et al., 2013). In chickpeas, drought-resistant

genotypes have been observed to exhibit a balance between deep rooting and fine root proliferation, ensuring both access to deep water and efficient water uptake from upper soil layers (Kashiwagi et al., 2006). A well-developed root system also plays a key role in regulating transpiration, as increased root biomass enables greater water uptake, maintaining plant hydration and reducing the need for stomatal closure, which in turn supports continuous transpiration and cooling (Zhou et al., 2020).

Heat stress poses significant challenges to root system development, often reducing root biomass and limiting root system architecture (RSA) modifications, particularly in cool-season crops such as chickpea (Giri et al., 2017; Mishra et al., 2023). Unlike drought stress, which often promotes deeper rooting, excessive soil temperatures can impair root growth and function, thereby restricting water and nutrient uptake. These effects negatively impact overall plant performance, as damaged roots reduce transpiration efficiency and photosynthetic capacity. Despite these challenges, some heat-tolerant plants exhibit modifications in root growth patterns to maintain plant hydration by facilitating efficient water uptake, which helps sustain transpiration and cooling mechanisms, preventing damage from excessive soil temperatures (Kalra et al., 2024). For instance, heat-tolerant chickpea (*Cicer arietinum*) genotypes often maintain high root length density in deeper soil profiles to access cooler subsoil water (Kashiwagi et al., 2006). Similarly, tolerant maize (*Zea mays*) lines may adopt a steeper root growth angle to avoid excessive surface temperatures (Lynch, 2013). These architectural adjustments sustain transpiration and cooling mechanisms, preventing damage from excessive soil temperatures (Kalra et al., 2024).

Root traits such as increased length, higher root density, and enhanced surface area significantly improve water acquisition, particularly in heat-stressed environments where rapid soil drying occurs. In response to heat stress, plants with deeper root systems can access moisture from lower soil layers, mitigating the effects of surface-level desiccation (Zhang et

al., 2025; Zhou et al., 2020). Additionally, an extensive root surface area increases the contact points with soil particles, facilitating water uptake even under limited soil moisture conditions (Comas et al., 2013; Kalra et al., 2024). Another key adaptation to heat stress is the modification of root cortical structures. Increased cortical aerenchyma formation reduces metabolic costs and improves root penetration in compacted soils, enabling plants to access water more efficiently under high temperatures (Henry et al., 2012). This structural adjustment helps plants balance energy allocation between root development and other physiological processes crucial for survival.

Given the pivotal role of these traits in maintaining plant resilience under challenging environments, their relevance extends beyond physiology and directly informs breeding strategies. These root system adaptations also hold significant practical value for crop improvement, particularly within breeding programmes aimed at enhancing resistance to drought and heat stress. Root traits such as deeper rooting, increased root length density, balanced fine-root proliferation, and modified cortical structures are increasingly recognized as heritable characteristics that can be strategically targeted during selection (Lynch, 2015). Modern breeding initiatives prioritize these belowground traits because of their direct influence on water-use efficiency, nutrient uptake, and overall plant resilience under combined stress environments (Varshney et al., 2021). Integration of root architecture phenotyping into breeding pipelines, supported by high-throughput root imaging technologies and genomic tools, enhances the identification of superior genotypes with more stress-adaptive RSA (Kashiwagi et al., 2006). Such efforts contribute to the development of cultivars capable of sustaining yield stability under predicted climate-change conditions, underscoring the practical significance of root system plasticity in modern breeding strategies.

1.3 The combination of drought and heat stress

Under field conditions in nature, most abiotic stresses are found to occur concurrently such that plants are usually exposed to various combinations of abiotic stresses simultaneously (Makonya et al., 2020; Vescio et al., 2021). Drought and heat stress for instance, often co-occur in nature and can induce each other (Handayani, 2020). High-temperature conditions often stimulate drought stress as soil moisture is rapidly depleted due to increased evaporation and transpiration rates, resulting in a combination of heat and drought stress, if the soil water table is not replenished at the same rate at which water is lost (Handayani, 2020; Ullah et al., 2020; Vescio et al., 2021). The concurrent occurrences of drought and heat stress, referred to as compound events, have been documented worldwide, leading to devastating effects on ecosystems, agriculture, and livelihoods. For example, during the summers of 2003, 2010, and 2018, Europe experienced widespread heatwaves coupled with severe soil moisture deficits, which caused extensive damage to crop and forest ecosystems (Buras et al., 2020; Ciais et al., 2005; Li, 2024). Similarly, in Africa, compounded drought and heat stress are increasingly prevalent due to climate change, with profound implications for food security and water availability. The Horn of Africa, for instance, has endured persistent droughts accompanied by extreme heat in recent decades, severely impacting agricultural production and pastoralist livelihoods (Mwangi et al., 2021). In Southern Africa, the 2015-2016 El Niño event brought one of the most severe droughts in recorded history, coinciding with elevated temperatures that devastated maize yields and led to food insecurity for millions (Baudoin et al., 2017). South Africa, specifically, experienced unprecedented dry and hot conditions during this period, which significantly reduced water levels in reservoirs such as the Vaal Dam and exacerbated the challenges faced by both commercial and subsistence farmers (Archer et al., 2017).

Plant responses to combined heat and drought stress cannot be directly extrapolated from their responses to individual stressors, as their interaction often amplifies negative effects, leading

to more severe physiological and biochemical disruptions (Li, 2024; Raja et al., 2020). Under simultaneous heat and drought stress, plants experience exacerbated water deficits due to higher evaporative demand, coupled with impaired root function, reduced photosynthesis, and accelerated oxidative damage (Lamaoui et al., 2018; Mittler, 2006). Moreover, the combination of these stressors induces distinct metabolic pathways that are not activated under isolated stress conditions. For example, combined stress may enhance the accumulation of osmo-protectants such as proline and glycine betaine at higher levels than in single stress conditions, contributing to cellular osmotic adjustment and membrane stability (Gupta et al., 2016). Additionally, cross-talk between HSPs and ROS-scavenging enzymes intensifies under combined stress, suggesting a unique interplay between heat and drought stress response mechanisms (Mittler and Blumwald, 2015). Importantly, combined heat and drought do not always stimulate greater proline accumulation than individual stresses. In wheat, several tolerant genotypes (e.g., 'Kundan') show decreased proline under combined stress compared to drought alone, while sensitive genotypes exhibit strong accumulation (Cairns et al., 2013). Chickpea displays similar variability: genotypes such as ICC 4567 increase proline under combined stress, whereas ICCV 92944 shows suppressed accumulation (Devasirvatham et al., 2012). Some maize hybrids also preferentially accumulate sugars rather than proline under the dual stress (Zhao et al., 2020). These inconsistent responses further emphasise that the metabolic signature of combined stress differs fundamentally from individual drought or heat stress.

Furthermore, research indicates that tolerance to combined stressors is not necessarily linked to tolerance for individual stresses, as resilience to either heat or drought alone does not guarantee survival under their combined effects (Cairns et al., 2013; Hamidou et al., 2013). For instance, in wheat, genotype Kundan is tolerant to drought but collapses under combined heat–drought, whereas HD 2967 performs relatively better under the dual stress (Cairns et al., 2013).

To explain, while drought-tolerant genotypes for example often rely on stomatal closure to limit water loss, this strategy can be detrimental under heat stress, where maintaining transpiration is crucial for cooling. This trade-off necessitates adaptive responses such as enhanced root-to-shoot signalling via abscisic acid and cytokinin regulation to balance water conservation with evaporative cooling (Tardieu, 2016). Additionally, plants under combined stress conditions of high heat and high light exhibit changes in lipid metabolism to preserve membrane integrity, a crucial adaptation for maintaining cellular homeostasis under extreme environmental fluctuations (Balfagón et al., 2019). To mitigate the impacts of concurrent drought and heat stress, plants employ integrated adaptive strategies, including deeper rooting for sustained water uptake, increased antioxidant enzyme activity to counteract oxidative damage, and the accumulation of osmo-protectants such as proline and glycine betaine, which help maintain cellular water balance and stabilize proteins under stress conditions (Ohama et al., 2017). Additionally, plants regulate stress-responsive gene expression through signalling molecules like abscisic acid, which modulates stomatal conductance and water-use efficiency, ensuring a balance between water conservation and cooling through transpiration. Understanding these mechanisms is crucial for breeding climate-resilient crops capable of withstanding multiple environmental stressors.

1.4 Problem Statement

Numerous studies have extensively examined the effects of drought and heat stress on crop growth and yield, as well as the adaptive strategies plants employ to mitigate these stresses (Mittler, 2006). However, most research has focused on these stressors in isolation, despite increasing evidence that they frequently co-occur in nature. As climate change intensifies, the simultaneous occurrence of drought and heat stress is expected to become more prevalent, necessitating a deeper understanding of plant responses to these combined stressors

(Zandalinas et al., 2017). While recent studies have begun addressing this interaction, the focus has largely remained on aboveground plant traits, particularly leaf physiology and canopy responses, with limited attention given to the root system. Given that roots play a crucial role in water and nutrient uptake and stress adaptation, their exclusion represents a significant gap in the current understanding of plant response mechanisms. Furthermore, most investigations into root responses to combined drought and heat stress have been conducted in cereals and model crops under controlled environments. This bias exists partly because the fibrous root systems of cereals are generally more amenable to high-throughput phenotyping than the deep, taproot-dominant systems of legumes, which are more difficult to excavate intact and are prone to structural damage during sampling (Wasson et al., 2012; Trachsel et al., 2011). Additionally, unlike cereals, chickpea root studies must account for trade-offs between root growth and nodule development for nitrogen fixation, a biological complexity that complicates stress-response analysis (Kashiwagi et al., 2015). This leaves leguminous crops like chickpea understudied despite their importance in sustainable agriculture and nutritional security.

While controlled environment studies are valuable for understanding plant physiological responses, field-based studies are equally critical, as they capture the complex interactions between root traits and variable environmental factors such as soil composition and temperature fluctuations. The lack of field-based studies on chickpea root morphology under drought and heat stress limits the ability to identify traits that contribute to stress tolerance in real-world agricultural settings (Vadez et al., 2013). Additionally, modern crop production systems, which often rely on high fertilizer inputs to maximize yield, may inadvertently suppress the development of extensive root systems, reducing plant resilience under drought conditions. The relationship between root morphology, nutrient availability, and drought stress adaptation remains poorly understood, particularly in chickpea, where an extensive root system may provide a significant advantage under combined drought and heat stress.

1.5 General aim and objectives

This study, therefore, aims to characterize chickpea root morphology and assess its responses to drought and heat stress – both individually and in combination – as well as to low-nutrient stress and the combined effects of low nutrients and drought, thereby addressing a crucial gap in current research and contributing to the development of more resilient legume crops for future agricultural systems. It was hypothesized that chickpea plants that develop a more extensive and efficient root system would grow better under both individual and combined abiotic stress conditions.

The main objectives of this study are:

1. To characterize root morphology for drought, heat, and combined stress tolerance in chickpea genotypes and assess their contribution to water uptake and conservation.
2. To evaluate root responses of chickpea plants to temperature differences in field conditions and determine if identified root traits align with those observed under heat stress in controlled environments.
3. To determine whether a high root to shoot ratio and extensive root system under low nutrient adaptation is beneficial for plant growth and yield under drought stress.

Chapter 2

Root morphology for individual and combined drought and heat tolerance in chickpea (*Cicer arietinum* L.)

2.1 Introduction

Chickpea is a major global pulse crop, cultivated mainly in the Mediterranean and semi-arid regions, with an annual production of ~18.1 million tonnes from ~14.8 million hectares (FAO, 2022). As a cool-season crop, chickpea is winter-sown during in-season rainfall in some parts of the Mediterranean; whereas in South Asia and other Mediterranean areas, it is grown in the post-rainy spring season, relying on residual soil moisture with minimal to no rain during the growing season (Berger et al., 2004; Gaur et al., 2019). However, both growth seasons expose the chickpea plants to terminal drought, which is often accompanied by an increase in temperatures, both of which have negative impacts on the crop's productivity (Benali et al., 2023) causing yield losses ranging from 40% - 70% (Gaur et al., 2019), depending on the severity of the stress and the plant genotype (Rani et al., 2020).

Drought and heat stress each negatively impacts plant growth and development by limiting water availability, reducing photosynthetic efficiency, and disrupting metabolic processes, ultimately leading to reduced biomass accumulation and yield losses (Basu et al., 2016; Mittler, 2006). When these stressors occur simultaneously, their effects are often amplified, causing accelerated senescence, reproductive failure, and severe reductions in productivity due to increased oxidative stress, impaired nutrient uptake, and disruptions in hormonal balance (Cairns et al., 2013; Hamidou et al., 2013). The combined impact of drought and heat stress alters physiological and biochemical pathways differently than either stress alone, necessitating complex adaptive responses to sustain growth and reproduction (Raja et al., 2020). However, studies examining these adaptive responses under simultaneous drought and heat stress remain

limited. Research findings indicate that a strong antioxidant defence system, which minimizes oxidative damage by scavenging ROS, is a key trait associated with tolerance to combined drought and heat stress conditions (Awasthi et al., 2017; Hussain et al., 2019). Additionally, root system modifications, particularly an increase in primary root length, have been identified as crucial for improving water uptake and sustaining physiological functions under concurrent drought and heat stress (Vescio et al., 2021). These findings suggest that both biochemical and morphological adaptations play a critical role in determining plant resilience when exposed to multiple environmental stressors, highlighting the need for further studies to refine breeding strategies for enhanced stress tolerance.

In studies carried out on chickpea plants under the combined stress, the focus has been on the aerial plant parts and reporting on the plant's oxidative metabolism, nutritional quality and yield responses (Awasthi et al., 2017; Mohamed et al., 2023; Yadav et al., 2022). However, none so far have investigated the link between these responses and the root morphological traits of chickpea under combined drought and heat stress conditions. Considering the role of the root system in the absorption of water and nutrients in the plant, both of which are required for maintenance of physiological processes, and in alleviating negative impact of the individual stresses, it is therefore important to investigate the potential benefits that possessing a better root system might confer to plants under the combined heat-drought stress conditions, in order to understand the impact of such stress conditions on plant growth and yield.

Hence, the objectives of this chapter were to determine

1. the effect of individual and combined heat and drought stress on root morphology and transpiration
2. whether the root morphology contributes to individual and combined heat and drought tolerance in tolerant genotypes.

It was hypothesized that the drought tolerant genotype will have the adaptive root system for increased water uptake and conservation under drought and combined drought and heat stress conditions.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

The study was investigated with a pot experiment in a glasshouse at the University of Cape Town, South Africa (33°57.353' S, 18°27.727' E; see attached picture- appendix picture 2.1) from August to December 2022. Four Desi chickpea genotypes, namely ACC#7, ACC#8, ICCV 07113 and ICCV 00108, selected for their different characteristics (Table 2.1), were sourced from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT-Kenya) and used for this study. The experimental design consisted of a factorial combination of the four genotypes and four treatments (non-stressed, drought only, heat only, combined drought and heat), each with six replicates laid out in a completely randomised design.

Table 2.1. Characteristics of the Desi chickpea genotypes used in this study.

Genotype	**Known information**
ACC#7	Heat tolerant Drought tolerant
ICCV 07113	Heat susceptible Drought susceptible High yielding
ACC#8	Heat susceptible Drought susceptible
ICCV 00108	Drought tolerant

A total of 96 pots, each measuring 24.5 × 18 × 20 cm (top diameter, base, and height respectively), were used. The pots were filled with a mixture of 4.5 kg of commercial silica sand and 500 g of 70/30 coco-perlite (Hortishop-Hydroponics and Organics, South Africa). The substrate was fertilized with 6 g pot^{-1} of Multicote (4) 15–3–12 + Mg + Me (Haifa Chemicals, South Africa) and 1 g pot^{-1} of gypsum ($CaSO_4 \cdot 2H_2O$), following the nutrient formulation and substrate composition guidelines used in Makonya (2020). Based on the fertiliser composition (15% N, 3% P_2O_5, 12% K_2O) and a total substrate mass of 5.001 kg per pot, this fertiliser rate corresponds to approximately 180 ppm N (0.018%), 15.7 ppm P (0.00157%), and 119.5 ppm K (0.01195%) in the growth substrate. Elemental P and K were derived from P_2O_5 and K_2O using standard conversion factors, whereas nitrogen required no conversion as it is already expressed in elemental form on the fertiliser label.

In each pot, three chickpea seeds were sown at 1 cm depth, and 14 days after germination the seedlings were thinned to one plant per pot. All pots were watered to 75% field capacity (FC) until the commencement of the stress treatments. The FC of the soil for chickpeas was pre-determined from a preliminary experiment. At this stage, genotype ACC#7 was excluded from the study due to its poor germination rate (<25%), which prevented the establishment of a minimum of three biological replicates per treatment. Including this genotype would have resulted in $n < 3$ (i.e., $n = 0$ or 1) across treatments, thereby compromising statistical reliability and introducing bias in genotype comparisons. Its exclusion ensured that only genotypes with adequate replication were compared, thereby maintaining the integrity of the experimental design.

At the sight of flowering in each genotype, all pots (of the three remaining genotypes) were moved to and acclimatised for 48 hours in growth chambers set to 65% relative humidity (RH), 400 ppm CO_2 concentration, 400 µmol m^{-2} s^{-1} photon flux density (PPFD), 20°C/ 15°C day/ night temperature with a 12 h photoperiod until 50% flowering was recorded. After this period,

two temperature levels were set in two separate chambers, a control chamber with day/ night temperatures of 20°C/ 15°C and a heat stress chamber with day/ night temperatures of 35°C/ 30°C. All other conditions remained as they were during the acclimatization period in the growth chambers.

Twelve pots per genotype were moved to each of the two growth chambers, making 36 pots per chamber. Six pots per genotype in each chamber were water-stressed by keeping the FC at 25% (determined from a preliminary experiment) throughout the stress period, with the remaining six receiving the normal watering treatment of 75% FC. This resulted in four varying levels of stress treatments: non-stressed/ control (placed in the 20°C chamber and watered to 75% FC); drought only (placed in the 20°C chamber and watered to 25% FC), heat only (placed in the 35°C chamber and watered to 75% FC); and combined drought and heat (D+H, placed in the 35°C chamber and watered to 25% FC). Prior to the imposition of treatments, the plants that were allocated to the 25% FC treatment for both chambers (i.e. drought only and combined drought and heat stress) were not watered for three days while acclimatizing to enable the FC to drop from the initial 75% FC to 25% FC.

The treatments were imposed for eight days; after which data on stomatal conductance (g_s), transpiration (*E*), leaf gaseous exchange, RWC and chlorophyll content were collected. Three plants from each treatment per genotype were harvested for destructive data sampling including shoot and root biomass, root morphology, proline concentration and nutrient content analysis. The remaining plants were then returned to the glasshouse, at which point they were all rewatered at 75% FC to allow for recovery and a second dose of fertilizer (i.e. 6 g of Multicoat 4* 15-3-12+Mg+Me) was applied. Grain yield was determined at physiological maturity. Throughout the life cycle of the plants, all pots were moved around weekly within the glasshouse to minimize microclimate effects on the growth of the plants.

2.2.2 Biomass

Plant biomass was determined at the end of the eight-day stress period. Three plants per treatment and genotype were harvested and samples were taken for RWC, proline and chlorophyll concentrations, and root morphological analysis (details of sampling and analysis provided below).

The remaining biomass was separated into leaves, stems, and roots, oven-dried at 60°C for 72 h and the dry weight was recorded. From the organ measurements, the root: shoot (R/S) was calculated as:

$$R/S = \frac{root\ dry\ weight}{shoot\ dry\ weight} \text{----------(i)}$$

2.2.3 Chlorophyll and carotenoid concentrations

Chlorophyll and carotenoid concentrations were measured according to Lichtenthaler's (1987) 95% ethanol method. Three leaflets from the third fully expanded leaf of each plant were harvested and the leaflets were inserted in a vial containing 10 mL of 95% ethanol and left in the dark for 24 h to auto-extract. The leaflets were then removed from the vial and their surface area was determined using the STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Quebec, Canada). The total chlorophyll, chlorophyll a and b, and carotenoid absorbances were determined spectrophotometrically (Thermo Helios Epsilon spectrophotometer; Thermo Scientific, MA, USA) by reading the wavelengths of the resulting solutions at 664 nm, 648 nm, and 470 nm and their concentrations (expressed as µg cm^{-2}) calculated using the following equations:

$$C_a = 13.36\ (Abs_{664}) - 5.19\ (Abs_{648}) \text{ ----------(ii)}$$

$$C_b = 27.43\ (Abs_{648}) - 8.12\ (Abs_{664}) \text{ ----------(iii)}$$

$$C_{(x+c)} = [\ 1000\ (Abs_{470}) - 2.13\ (C_a) - 97.64\ (C_b)]\ /\ 209 \text{---------(iv)}$$

[Where C_a, C_b, and $C_{(x+c)}$ are chlorophyll a, chlorophyll b and carotenoid concentrations respectively].

2.2.4 Leaf relative water content

The leaf RWC, measured to determine the moisture-holding capacity of the plants under the various treatments, was determined at the end of the stress treatment, using the Barrs and Weatherly (1962) method. Three leaflets from three fully matured leaves were excised, placed in petri dishes in a cooler box and subsequently weighed for fresh weight (FW). Thereafter, the leaflets were floated for 3 h in distilled water at room temperature to obtain the turgid weight (TW) and oven-dried at 80°C for 24 h to obtain the dry weight (DW). Relative water content was then calculated using the equation:

$$\text{RWC} = \frac{FW - DW}{TW - DW} \times 100 \text{----------(v)}$$

2.2.5 Proline concentrations

Using the Bates et al. (1973) method, 0.5 g of fresh leaf samples was homogenized in 10 mL of 3% sulfosalicylic acid using a mortar and pestle on ice. The homogenate was centrifuged at 11,500 × *g* for 15 min, after which 2 mL of the supernatant was mixed with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin reagent and incubated for 1 h in a boiling water bath. The reaction was then terminated in an ice bath and 4 mL of toluene was added to the tube. The mixture was vortexed for 20 sec and left on the bench for 5 min to separate into the organic and water phases. The organic phase which was the chromophore containing toluene was then extracted and the absorbance read at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight (FW) basis as:

$$\text{Proline content (µmol g}^{-1}\text{ FW)} = \frac{\left[\frac{(\mu g\ proline\ mL^{-1} \times mL\ toluene)}{115.13\ \mu g\ \mu mol^{-1}}\right]}{\left[\frac{(g\ sample)}{5}\right]} \text{ ---------- (vi)}$$

[Where 115.13 µg mol^{-1} is the molecular weight of proline]

2.2.6 Root morphology

At harvest after the stress treatments, all adhering substrate on the root system was gently removed by washing under running tap water with roots placed in a sieve (pore size 2mm) to ensure any broken pieces were not lost. Approximately 15% of the total root biomass (Vandamme et al., 2013) was collected and stored in a 10% ethanol solution at 4°C for root morphological measurement. The root morphological parameters including total root length (RL; m), root surface area (SA; m^2), root average diameter (RAD; mm), and total root volume (RV; cm^3) were measured with a STD4800 scanner and WinRHIZO version 2013a (Regent Instruments, Quebec, Canada). After the analysis, the root samples were oven-dried at 60°C for 72 h to obtain the dry weight. The results of the analysed root parameters were then expressed as whole-root results by multiplying with the relevant conversion factors (i.e. using dry weights of the scanned root sample and that of the whole root system harvested) to 100% of the root. Based on the derived results, the root functional traits, specific root length (SRL), root mass ratio (RMR), root tissue density (RTD), and root fineness (RF) were calculated for each root as:

$$R/S = \frac{root\ biomass}{shoot\ biomass} \quad \text{---------- (vii)}$$

$$SRL\ (m\ g^{-1}) = \frac{root\ length}{root\ dry\ weight} \quad \text{---------- (viii)}$$

$$RMR\ (g\ g^{-1}) = \frac{root\ dry\ weight}{whole\ plant\ dry\ weight} \quad \text{---------- (ix)}$$

$$RTD\ (g\ cm^{-3}) = \frac{root\ dry\ weight}{root\ volume} \quad \text{---------- (x)}$$

$$RF\ (m\ cm^{-3}) = \frac{root\ length}{root\ volume} \quad \text{---------- (xi)}$$

The root length ratio (RLR) was then further related to the other functional traits (RMR, RF and RTD) using the Ryser and Lambers' (1995) equation below:

$$\text{RLR} = RMR \times \frac{RF}{RTD} \quad \text{---------- (xii)}$$

2.2.7 Leaf Nutrient Content Analysis

Leaf samples used in the determination of leaf biomass were milled using the Retsch Mixer Mill MM 200 (Fisher Scientific UK Ltd, England) for the nutrient analyses. Milled samples were weighed to 5.00 ± 0.01 µg on a Sartorius MC5 microbalance (Sartorius AG, Göttingen, Germany). Nitrogen concentration was analysed using a FlashEA 1112 series elemental analyser (Thermo Electron Corporation, Milan, Italy). Analyses of N, P, K, S, Ca, Mg, Fe, B, Cu, Mn, Na and Zn concentrations were done at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenburg (Stellenbosch, South Africa) using the dry ashing method (ALASA, 1998).

2.2.8 Stomatal conductance and transpiration

Stomatal conductance and transpiration rate were measured using a portable photosynthesis system (LI-6400XT, LI-COR Biosciences, Lincoln, NE, USA). The infrared gas analyzer (IRGA) sensor head was set to a reference CO_2 concentration of 400 ppm, a flow rate of 400 µmol s^{-1}, and a photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1}. Measurements were conducted on the youngest fully expanded leaf at the end of the stress period, between 1000 h and 1200 h. The selected leaf was carefully placed in the 2 cm^2 cuvette of the sensor head, ensuring full coverage with no visible gaps between leaflets. Readings were recorded every 10 seconds for three minutes, with the final six values averaged, as they were considered stable.

2.2.9 Grain yield and components

At physiological maturity, all pods were removed from the plants and air-dried to approximately 12% moisture content. The total number and weight of the pods were recorded before threshing to obtain the seed number and weight. Shelling percentage was then calculated as the ratio of seed weight to pod weight, expressed as a percentage, using the formula:

$$\text{Shelling Percentage} = \left(\frac{Seed\ weight}{Pod\ weight}\right) \times 100 \text{----------(xiii)}$$

2.2.10 Statistical analysis

All datasets were analysed by two-way ANOVA to compare the effect of water treatments and temperature levels for each genotype using Statistica. Where the results showed significant differences ($p < 0.05$), the Duncan's Multiple Range Test was used to separate the mean values.

2.3 Results

2.3.1 Plant Biomass

The effect of genotype on plant biomass was significant for leaf, stem, shoot and total plant biomass with no significant differences recorded between genotypes on root biomass and R/S. Where significant differences were recorded, genotype ACC#8 consistently accumulated the highest plant biomass, with genotypes ICCV 07113 and ICCV 00108 similarly accumulating a lower biomass (Table 2.2).

At the end of the stress treatments, all plant biomasses were significantly reduced relative to the control. The effect of heat treatment was similar to drought treatment on all organs/parameters except on leaf biomass where the values were lower than all the treatments. Biomass was lower in all organs under the combined drought and heat stress treatment compared to the drought-only treatment. The R/S as a measure of the allocation of biomass to aboveground and belowground systems in the plants was found to be significantly different across treatments (Table 2.2; see appendix picture 2.2). Of all treatments relative to the control, plants under the drought-only treatments shared similar values to the control, with the combined-stressed group recording the lowest R/S values. For all biomass parameters measured, the interaction between genotype and treatment was not significant.

Table: 2.2: Genotypic response to drought and heat stress treatments on biomass of chickpea at flowering growth stage. Data are means ± SE (n = 3 per treatment per genotype) followed by different lowercase letters in each column indicating significant differences by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	Leaf biomass (g)	Stem biomass (g)	Shoot biomass (g)	Root biomass (g)	Total Biomass (g)	Root: Shoot ratio
Genotype						
ICCV 07113	3.41 ± 0.45^{ab}	4.98 ± 0.47^{a}	8.39 ± 0.91^{a}	2.00 ± 0.30	10.39 ± 1.19^{a}	0.23 ± 0.02
ACC#8	3.68 ± 0.47^{b}	6.76 ± 0.56^{b}	10.44 ± 1.01^{b}	2.36 ± 0.41	12.79 ± 1.34^{b}	0.22 ± 0.03
ICCV 00108	2.80 ± 0.17^{a}	5.51 ± 0.49^{a}	8.30 ± 0.62^{a}	1.90 ± 0.43	10.20 ± 1.03^{a}	0.21 ± 0.03
Treatment						
Control	4.80 ± 0.46^{c}	7.57 ± 0.67^{c}	12.37 ± 1.00^{c}	3.60 ± 0.45^{c}	15.00 ± 1.31^{c}	0.29 ± 0.03^{c}
Drought	3.52 ± 0.32^{b}	5.98 ± 0.52^{b}	9.50 ± 0.76^{b}	2.32 ± 0.20^{b}	11.82 ± 0.91^{b}	0.25 ± 0.02^{bc}
Heat	2.69 ± 0.28^{a}	5.04 ± 0.39^{ab}	7.73 ± 0.62^{ab}	1.69 ± 0.18^{b}	9.42 ± 0.72^{ab}	0.23 ± 0.03^{b}
Drought + Heat	2.17 ± 0.16^{a}	4.41 ± 0.34^{a}	6.58 ± 0.47^{a}	0.72 ± 0.11^{a}	7.30 ± 0.50^{a}	0.11 ± 0.02^{a}
F ratio						
Genotype	3.688*	5.678**	4.463*	0.952^{ns}	3.750^{ns}	0.557^{ns}
Treatment	17.780**	9.610***	14.580***	18.073***	18.637***	13.824***
Genotype*Treatment	2.284^{ns}	0.788^{ns}	1.236^{ns}	0.384^{ns}	0.724^{ns}	2.259^{ns}

2.3.2 Transpiration and Stomatal Conductance

Relative to the control, g_s (Figure 2.1A) and *E* (Figure 2.1B) remained unchanged with the drought-only treatment. On the other hand, the heat-only treatment and combined-stress treatment recorded similar values of g_s and *E* that were lower compared to both the control and drought-only treatment. There were no significant genotypic and genotype and treatment interaction on both g_s and *E*.

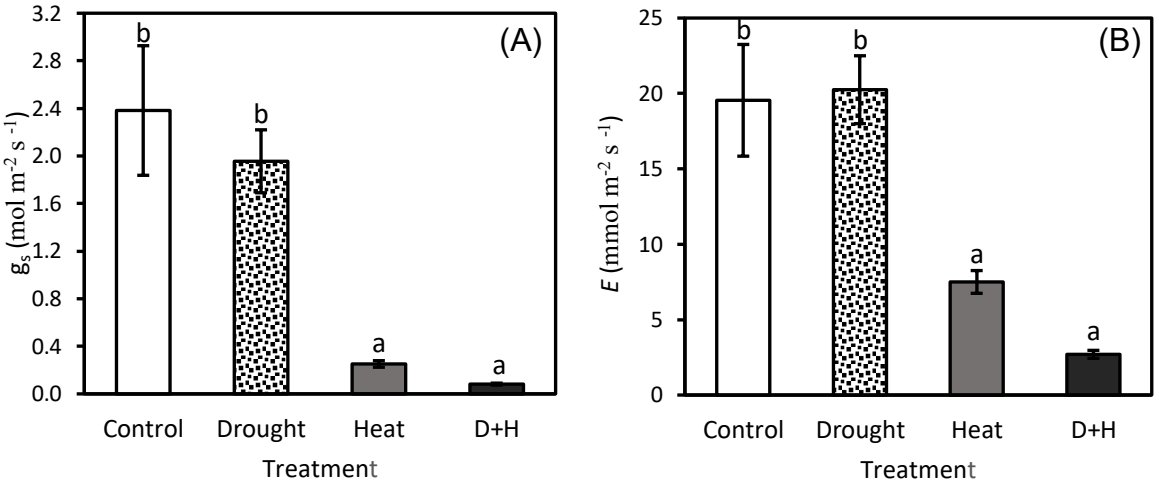

Figure 2.1: Treatment effect on stomatal conductance (A) and transpiration rate (B) of chickpea at the end of the stress period, D+H denotes combined drought and heat stress. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

2.3.3 Chlorophyll and carotenoid concentrations

Genotype and environment did not have an interactive effect on both chlorophyll a concentration and chlorophyll a/b ratio. For chlorophyll a concentration, the drought only treatment did not cause any impact as plants under this group recorded similar values relative to the control. On the other hand, both the heat only and the combined-stress treatments reduced chlorophyll a concentration with a higher reduction observed in the combined-stress group (Figure 2.2B). Conversely, chlorophyll a/b ratio in the combined-stress group increased in comparison to the control while the single stress treatments, drought only and heat only, shared similarities with the control (Figure 2.2D).

Genotypic differences in chlorophyll a concentration, indicated genotypes ACC#8 and ICCV 00108 had similar concentrations, whereas genotype ICCV 07113 recorded a lower chlorophyll a concentration- significantly different from the former two genotypes (Figure 2.2A). Conversely, genotype ICCV 07113 exhibited the highest chlorophyll a/b ratio while the lowest ratio was recorded in ICCV 00108 (Figure 2.2C).

Significant interactions between genotypes and stress treatments were recorded for carotenoid concentrations measured at the end of the stress period (Figure 2.2E). Both single and combined stress treatments scored lower values than the control for all genotypes except genotype ACC#8 where lower values were only recorded for the combined stress treatments with both drought only and heat only treatments sharing similar values to the control (Figure 2.2E).

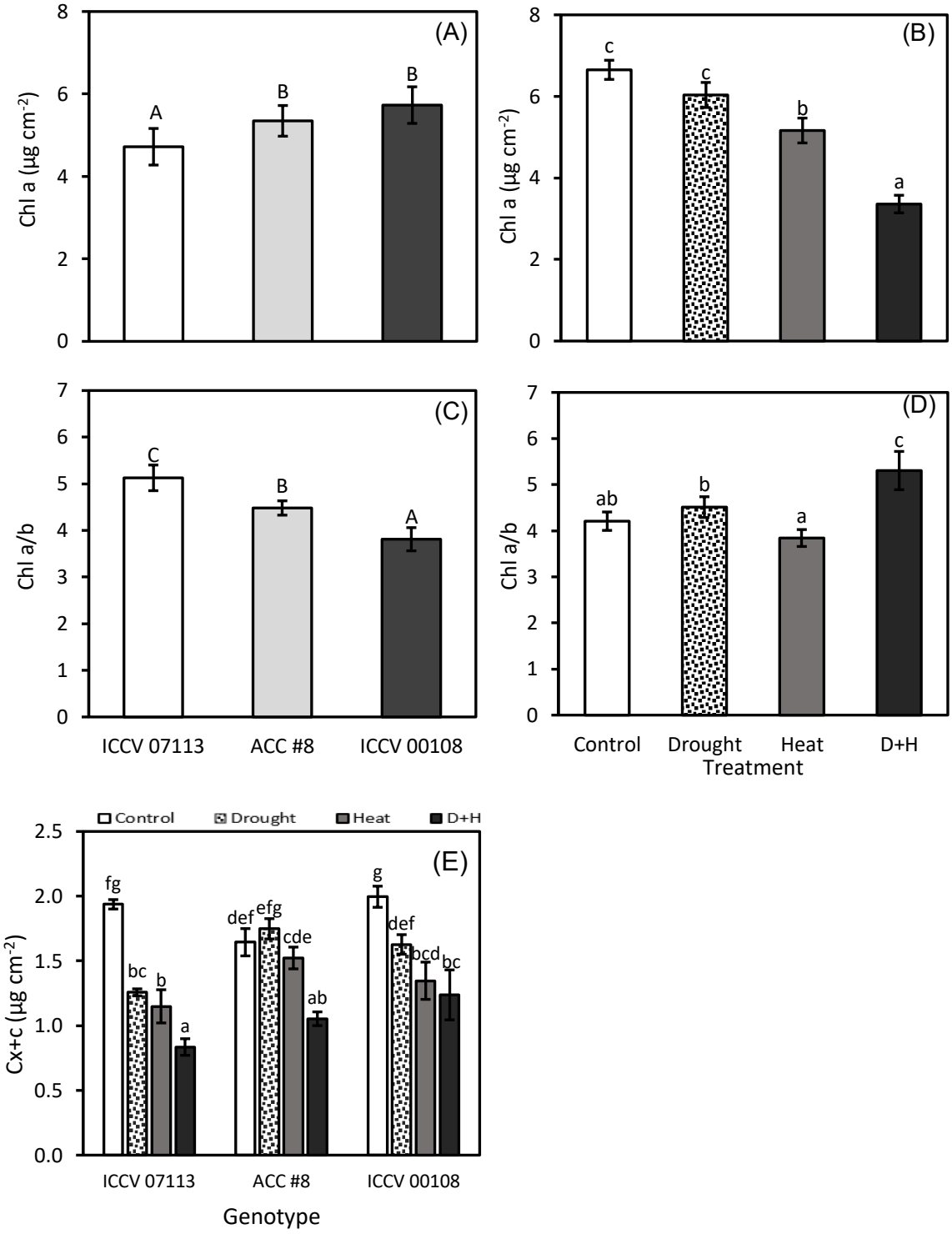

Figure 2.2: Effect of genotype on chlorophyll a (A) and chlorophyll a/b (B) of chickpea plants after the 8-day stress period. Treatment effect on chlorophyll a (B) and chlorophyll a/b (D); and the interactive effect of genotype and treatment on carotenoid concentration (E) of chickpea plants after the 8-day stress period. Measurements were taken at the end of the stress period. D+H denotes combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different uppercase and lowercase letters indicate significant differences between genotypes and treatments respectively, by Duncan's Multiple Range Test ($p < 0.05$). There was no significant interaction between genotype and treatments on chlorophyll a and chlorophyll a/b. Sample size: n = 3 per treatment per genotype.

2.3.4 Relative water content

The interaction between genotype and treatment had significant effects on RWC (Figure 2.3A). For all genotypes (except ACC#8), lower RWC than the control was recorded across stress treatments, whereas genotype ACC#8 recorded lower values for only the combined stress treatment. Of the three genotypes, ICCV 07113 retained the lowest RWC compared to the other two genotypes for corresponding treatments except heat stress with ICCV00108 that was similar (Figure 2.3).

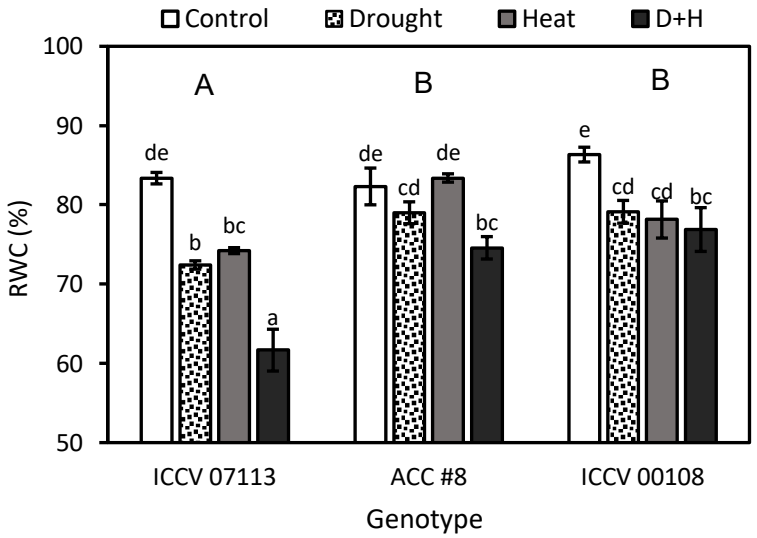

Figure 2.3: Interactive effect of genotype and treatment on relative water content of chickpea plants at the end of the stress treatment. D+H denotes combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different lowercase letters indicate significant differences between genotypes and stress treatments respectively by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

2.3.5 Proline concentration

Proline concentrations recorded significant differences between genotypes and across treatments. Within genotypes, ICCV 07113 expressed higher proline concentrations compared to the other two genotypes which were similar to each other (Figure 2.4A). Relative to the control, proline concentration within treatments was higher in both drought-only and the combined-stress treatments, with the heat-only treatment sharing similarities with all

treatments (Figure 2.4B). The interaction between genotype and treatment was however not significant for proline concentration.

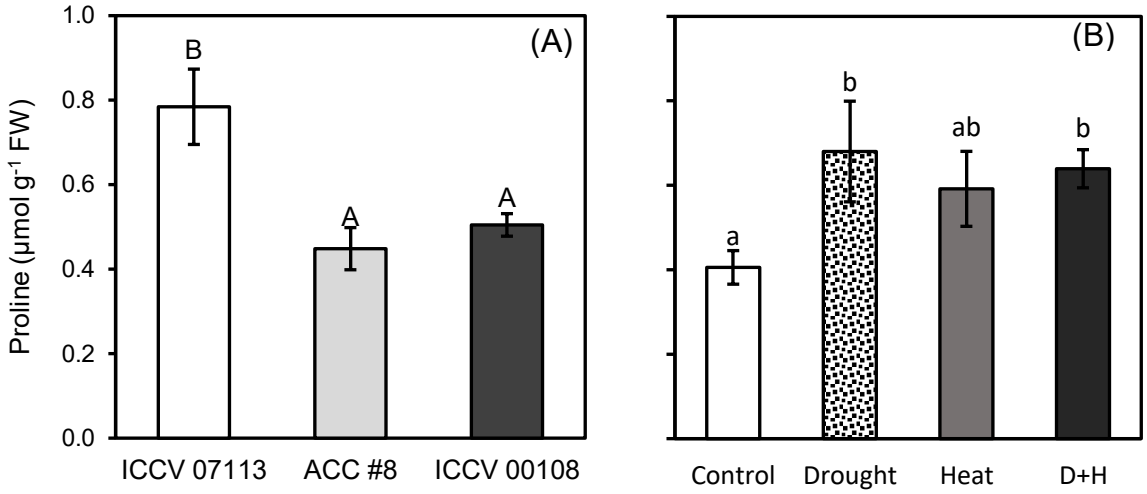

Figure 2.4: Effect of genotype (A) and treatment (B) on proline concentration of chickpea plants after the 8-day stress period. D+H denotes combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different uppercase and lowercase letters indicate significant differences between genotypes and treatments respectively, by Duncan's Multiple Range Test ($p < 0.05$). There was no significant interaction between genotype and treatments. Sample size: n = 3 per treatment per genotype.

2.3.6 Root morphology

Of all the root traits measured, stress treatments caused a variety of significant responses. The RL (Figure 2.5A) and RMR (Figure 2.5E) were found to share a pattern in results variation. In these two root traits, the drought-only group shared similar values as the control whereas the combined stress group had the greatest decline. On the other hand, an opposite trend of this was found to be true for RF (Figure 2.5F) and RTD (Figure 2.5I). For both traits, although the drought only group also shared similarities with the control, the combined drought and heat stress group resulted in values higher than all treatments. The root SA (Figure 2.5B), RAD (Figure 2.5C) and RV (Figure 2.5D), recorded a decline relative to the control across all stress treatments with the lowest values recorded in the combined-stress group. Compared to the

control, stress treatments showed no significant differences in SRL (Figure 2.5H) and RLR (Figure 2.5G), except for an increase observed in the combined-stress group for SRL and in the drought-only group for RLR.

Genotypic differences in the root traits were significant for RL (Figure 2.6A), RTD (Figure 2.6B), RF (Figure 2.6C) and RLR (Figure 2.6D). For RL, genotypes ICCV 007113 and ACC#8 shared similar values, both of which were higher than those of ICCV 00108 (Figure 2.6A). In contrast, RTD was highest in ICCV 00108, while ICCV 007113 and ACC#8 recorded similar but lower values (Figure 2.6B). RF values were higher in ICCV 00108 than ACC#8, with ICCV 07113 sharing similarities to both. In terms of RLR, ICCV 07113 had the highest values, leaving the other two genotypes with lower values that were similar (Figure 2.6BD).

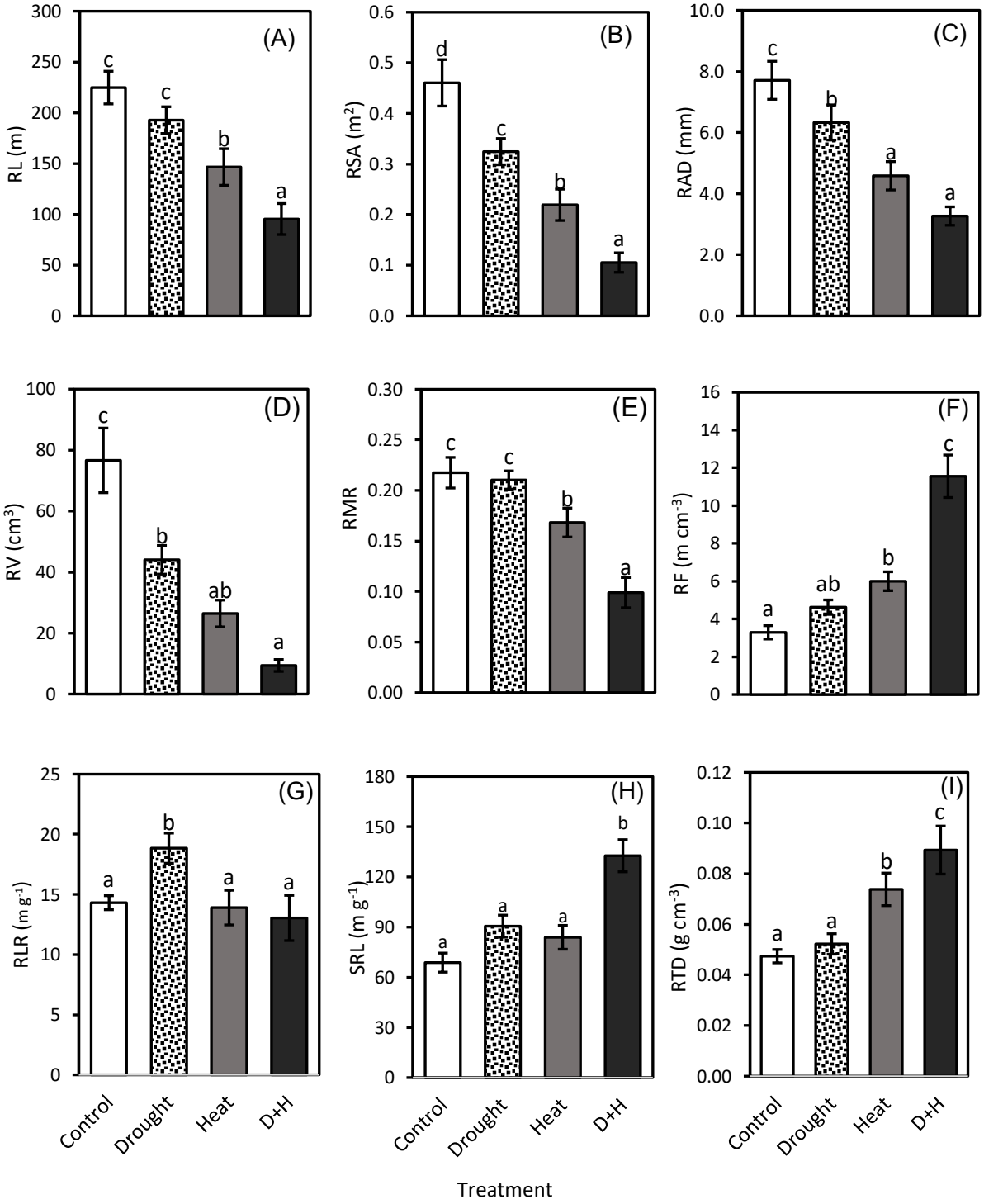

Figure 2.5: Effect of treatments on total root length (A), root surface area (B), root average diameter (C), root volume (D), root mass ratio (E), root fineness (F), root length ratio (G), specific root length (H) and root tissue density (I) of chickpea at the end of the stress treatment. D+H denotes combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

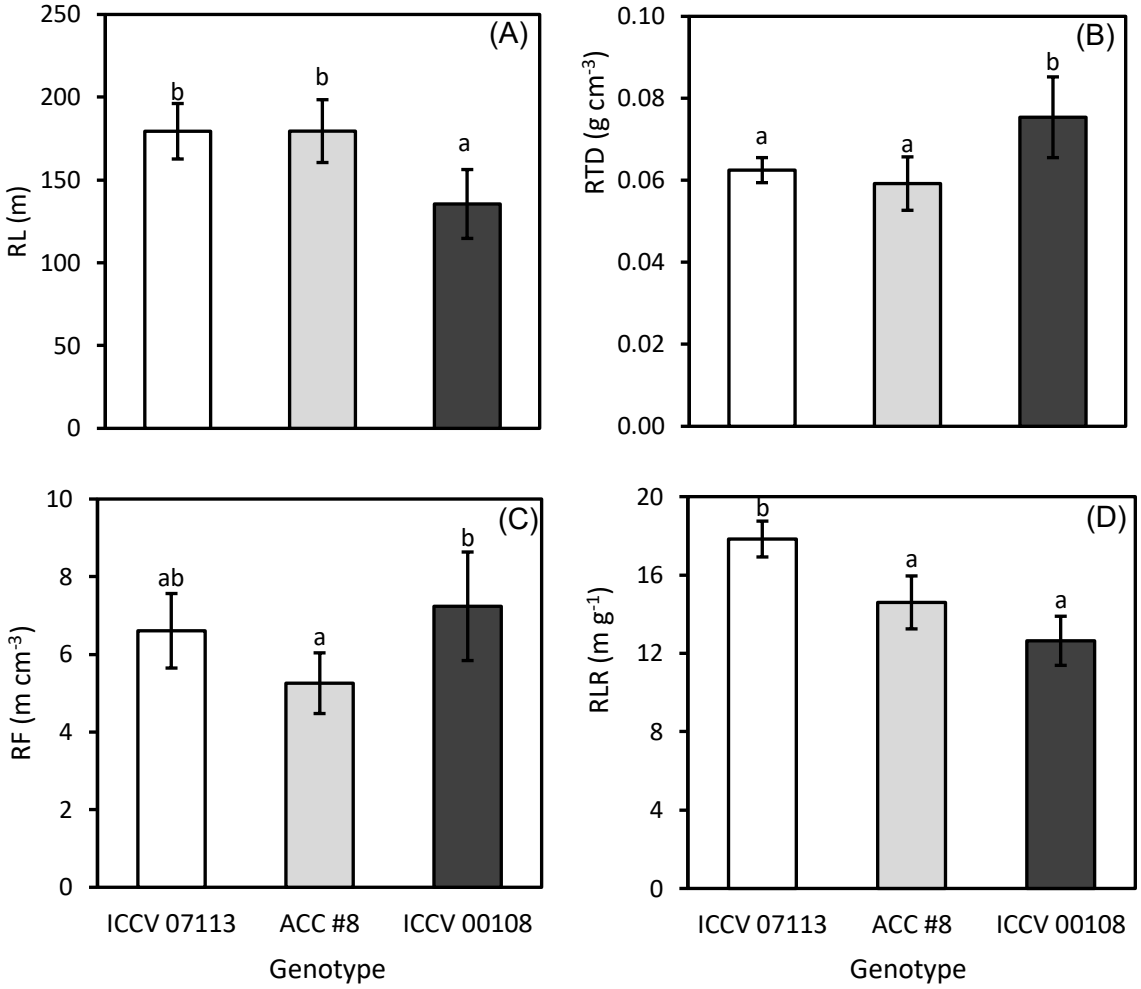

Figure 2.6: Genotypic effect on root length (A), root tissue density (B), root fineness (C) and root length ratio (D) of chickpea at the end of the stress treatment. D+H denotes plants under combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different letters indicate significant differences between genotypes by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

2.3.7 Nutrient content

Of the 12 nutrients analysed in the leaf, N and Al showed no significant differences between genotypes, across treatments and in the interaction of the two factors. The effect of genotype was however significant on Ca, Zn and Mn. Calcium concentration was highest in ICCV 07113, while Mn was highest in ICCV 00108. In both cases, the other two genotypes had lower but similar values (Table 2.3). For Zn, however, ICCV 07113 had the highest concentration and ICCV 00108 the lowest with ACC#8 being similar to both genotypes (Table 2.3).

The effect of treatment on nutrient concentrations showed a varying trend, with P, K, Na, and B being highest under the combined-stress treatment, followed by the heat stress treatment. Both treatments had higher concentrations than the control and drought treatments, which exhibited similar values (Table 2.3). On the other hand, Fe and Zn concentrations were lower across all stress treatments compared to the control. Copper and Mn concentrations were reduced under drought stress, while the heat and combined-stress treatments remained similar to the control. A similar pattern was observed for Mg, where the drought treatment had lower concentrations, the heat treatment was comparable to the control, and the combined-stress treatment recorded the highest values. Meanwhile, Ca concentration decreased under both drought and heat stress, while the combined-stress treatment remained similar to the control (Table 2.3).

Table 2.3: Nutrient content of chickpea genotypes under the four different treatments at flowering growth stage. Data are means ± SE (n = 3 per treatment per genotype) followed by different capital letters and lowercase letters in the rows indicating significant differences between genotypes and treatments respectively, by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	Nitrogen %	Phosphorus %	Potassium %	Calcium %	Magnesium %	Sodium mg/kg	Iron mg/kg	Copper mg/kg	Zinc mg/kg	Manganese mg/kg	Boron mg/kg
Genotype											
ICCV 07113	4.36 ± 0.15	0.59 ± 0.05	2.8 ± 0.27	0.82 ± 0.05^{B}	0.49 ± 0.03	427 ± 82	154 ± 21	2.2 ± 0.17	41.2 ± 3.1^{B}	105 ± 7^{A}	132 ± 15
ACC#8	4.49 ± 0.23	0.53 ± 0.03	2.4 ± 0.21	0.71 ± 0.03^{A}	0.46 ± 0.02	470 ± 101	150 ± 11	1.8 ± 0.12	36.9 ± 1.9^{AB}	104 ± 8^{A}	122 ± 9
ICCV 00108	4.23 ± 0.19	0.53 ± 0.04	2.7 ± 0.21	0.63 ± 0.03^{A}	0.47 ± 0.01	570 ± 122	140 ± 18	2.2 ± 0.23	32.4 ± 1.4^{A}	81 ± 5^{B}	122 ± 8
Treatment											
Control	4.49 ± 0.23	0.47 ± 0.03^{a}	2.2 ± 0.13^{a}	0.76 ± 0.03^{b}	0.47 ± 0.01^{b}	229 ± 30^{a}	222 ± 24^{b}	2.2 ± 0.29^{b}	43.8 ± 3.5^{c}	105 ± 6^{b}	97 ± 5^{a}
Drought	3.92 ± 0.20	0.45 ± 0.02^{a}	1.9 ± 0.04^{a}	0.63 ± 0.03^{a}	0.38 ± 0.01^{a}	260 ± 23^{a}	121 ± 6^{a}	1.5 ± 0.08^{a}	34.2 ± 2.1^{ab}	78 ± 5^{a}	95 ± 5^{a}
Heat	4.60 ± 0.19	0.57 ± 0.04^{b}	2.9 ± 0.13^{b}	0.67 ± 0.04^{a}	0.49 ± 0.02^{b}	503 ± 58^{b}	119 ± 8^{a}	2.4 ± 0.12^{b}	38.1 ± 2.4^{b}	101 ± 11^{b}	147 ± 6^{b}
Drought + Heat	4.43 ± 0.20	0.72 ± 0.02^{c}	3.7 ± 0.14^{c}	0.83 ± 0.06^{b}	0.55 ± 0.02^{c}	964 ± 109^{c}	129 ± 7^{a}	2.2 ± 0.17^{b}	31.3 ± 1.0^{a}	103 ± 8^{b}	164 ± 11^{c}
F ratio											
Genotype	0.637^{ns}	1.544^{ns}	2.293^{ns}	11.516^{***}	1.79^{ns}	2.097^{ns}	0.317^{ns}	2.385^{ns}	7.535^{**}	5.571^{*}	1.668^{ns}
Treatment	2.469^{ns}	17.647^{***}	57.105^{***}	8.035	41.491^{***}	33.296^{***}	11.651^{***}	6.157^{**}	8.638^{***}	3.656^{*}	46.732^{***}
Genotype*Treatment	2.049^{ns}	1.225^{ns}	1.526^{ns}	2.208^{ns}	4.349^{**}	1.7497^{ns}	0.553^{ns}	2.419^{ns}	2.824^{*}	1.454^{ns}	5.867^{***}

2.3.8 Grain yield

Pod number (Figure 2.7A), seed number (Figure 2.7B) and seed weight (Figure 2.7C) were all significantly reduced by the stress treatments relative to the control. In terms of seed number and seed weight, plants under the three stress treatments shared similar values that were similarly lower than the control (Figure 2.7B, C). Pod numbers were lowest under heat and combined-stress treatments compared to the control, while drought-treated plants had intermediate values, sharing similarities with both the control and other stress treatments (Figure 2.7A).

Pod weight was the only grain yield parameter that was significantly affected by the interaction of genotype and treatment (Figure 2.8B). Across all genotypes except ACC#8, pod weight for the stress treatments were similar but significantly lower than the control plants. In genotype ACC#8, however, pod weight for the drought and heat groups were similar to the control whereas the combined treatment was similar to the other treatment, but lower than the control (Figure 2.8B).

The effect of genotype as a main factor was only found to be significant for the shelling percentage where genotypes ACC#8 and ICCV 00108 recorded higher and similar values (~75%) in comparison to genotype ICCV 07113 which had a shelling percentage of ~50% (Figure 2.8A).

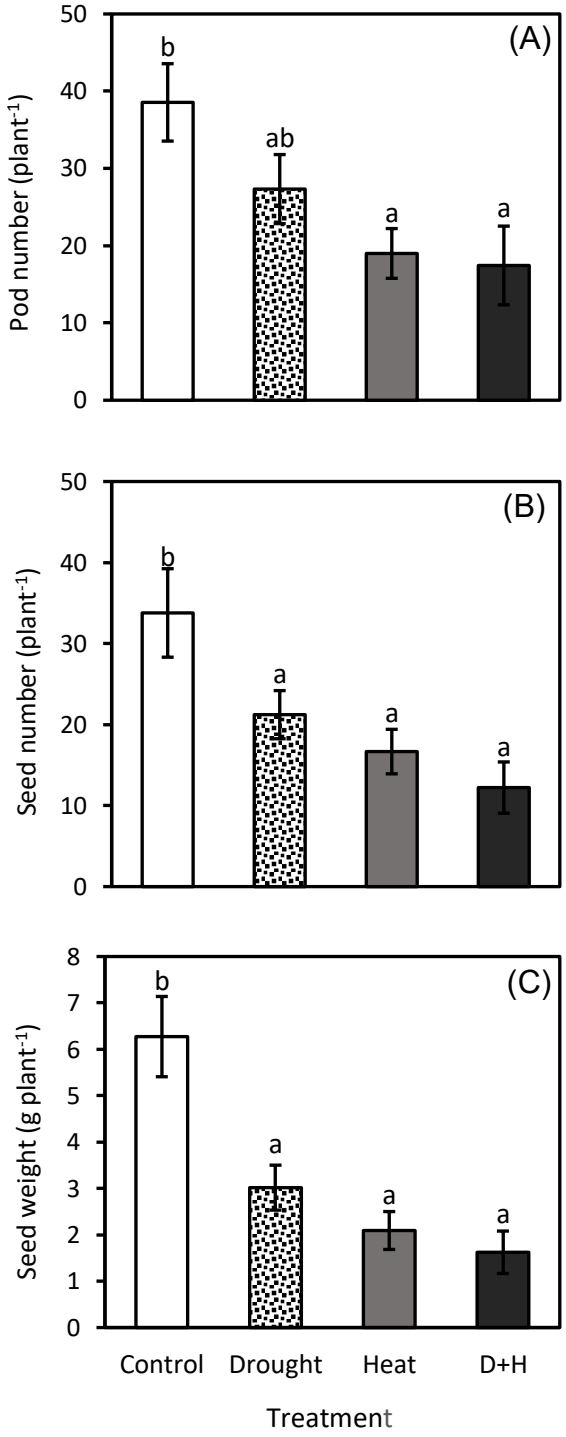

Figure 2.7: Effect of treatments on pod number (A), seed number (B) and seed weight (C) of chickpea at harvesting. D+H denotes combined drought and heat stress treatment. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

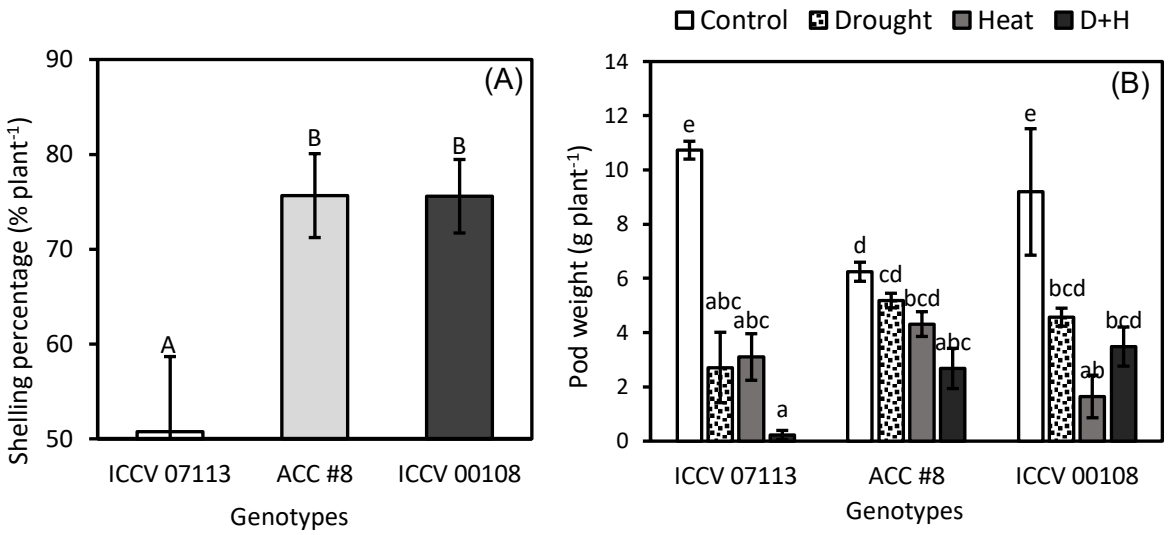

Figure 2.8: Effect of genotype on shelling percentage (A) and the interactive effect of genotype and treatment on pod weight of chickpea plants at harvesting. D+H denotes combined drought and heat stress treatments. Vertical lines on bars denote standard error. Different uppercase and lowercase letters indicate significant differences between genotypes and stress treatments respectively by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per treatment per genotype.

2.4 Discussion

2.4.1 *Impact of Stress Treatments*

In this study, three chickpea genotypes were exposed to individual drought and heat stress as well as their combined stress to assess root morphological responses and their impact on transpiration and plant growth. Results after eight days of stress showed that the combined drought and heat stress had the greatest detrimental effect on biomass accumulation, followed by heat stress alone, while the drought only stress exhibited a comparatively milder effect. Stress-induced biomass reductions observed in the current study align with findings from field and controlled environment experiments, where drought (Makonya et al., 2020; Muriuki et al., 2020) and heat stress (Devasirvatham et al., 2012) have consistently resulted in decreased biomass in chickpea, with the most pronounced declines occurring under combined stress conditions (Awasthi et al., 2014; Awasthi et al., 2017).

Biomass accumulation, closely associated with a plant's nutritional status and yield, serves as a critical agricultural indicator of crop growth and productivity (Li et al., 2020). Photosynthetic capacity, often evaluated through chlorophyll concentration, offers valuable insights into photosynthetic function under stress (Sehgal et al., 2017; Raja et al., 2020). In this study, the observed disparities in biomass reduction among treatments were likely linked to varying degrees of chlorophyll degradation and corresponding impairment of photosynthesis. Previous studies on crops such as cotton (Zafar et al., 2023) and tomato (Raja et al., 2020) have similarly reported chlorophyll reductions under individual and combined drought and heat stress, with photooxidation identified as a major driver of chlorophyll degradation. Consistent with these findings, this study observed significant reductions in chlorophyll concentration under heat stress, which is expected given that high temperatures often accelerate chlorophyll degradation (Fan et al., 2024). The combined effects of heat and drought stress were more pronounced, indicating a synergistic impact that exacerbated chlorophyll reduction beyond the effects of either stressor alone. This synergism underscores the compounded challenges these environmental stressors pose to chickpea growth and productivity. Interestingly, this study found no significant treatment effect on chlorophyll concentration in the drought-only group, deviating from the general expectation that drought stress typically reduces chlorophyll levels. This unexpected result aligns with observations by Hussain et al. (2019), who reported a similar outcome in maize, suggesting species-specific acclimation mechanisms that preserve chlorophyll under mild drought.

Root traits are critical determinants of plant resilience under environmental stress, as root systems optimize water and nutrient acquisition (Comas et al., 2013). The R/S ratio, in particular, is an indicator of a plant's resource allocation strategy, balancing belowground investment for resource uptake with aboveground structures essential for photosynthesis and growth. Under drought stress, studies (Blum, 2011; Wang et al., 2006) commonly report an

adaptive increase in the R/S ratio, reflecting a shift in resource allocation to prioritize root development for enhanced access to limited soil moisture and nutrients, often through the production of longer and finer roots. In this study, however, the R/S ratio and total root length of drought-stressed plants did not differ from the control. Instead, specific adaptive root traits, including an increase in RLR and a decrease in RAD, were enhanced under drought stress. An increase in RLR enhances a plant's capacity to explore a larger soil volume for residual moisture, improving water uptake efficiency during drought. Conversely, reductions in RAD reflect a shift towards finer, more absorptive roots, which increases radial conductivity and facilitates sustained water uptake when soil moisture is limited. Together, these functional advantages help maintain transpiration and carbon assimilation, supporting biomass accumulation under drought conditions.

The RLR is considered a more robust indicator of rooting depth and resource acquisition than [absolute] root length, because it integrates biomass allocation to the root system (RMR) and the pattern of biomass investment (RTD and RF; Ryser and Lambers, 1995). Therefore, an increase in RLR under drought conditions signifies improved adaptation for accessing deeper soil layers or more soil volume, thereby improving subsoil water capture. This finding aligns with research on maize (Vescio et al., 2020) and sugar beets (Romano et al., 2013), where higher RLR was positively correlated with better drought tolerance and growth performance. Additionally, the observed decrease in ARD under drought conditions suggests enhanced root fineness, which facilitates increased soil exploration and reduced hydraulic resistance, further supporting the plant's capacity to maintain water uptake under drought stress (Fan et al., 2024). In contrast, heat stress has been shown to often reduce root growth due to the root system's heightened sensitivity to high temperatures compared to shoots, primarily as a result of increased metabolic costs and potential cellular damage (Heckathorn et al., 2013; Mishra et al., 2023; Wahid et al., 2007). This was reflected in this study, where heat stress significantly

reduced the biomass allocated to underground resources, negatively impacting root traits including the R/S ratio. The observed differential responses of the root system to drought and heat stress align with Mohamed (2023), who demonstrated under field conditions that chickpea plants are more sensitive to heat stress during reproductive growth compared to drought stress.

When drought and heat stresses were combined, negative effects on root biomass and morphology were exacerbated, indicating a compounding impact on root development. Although adaptive root strategies, such as increases in specific root length (SRL), root tissue density (RTD), and root fineness (RF), were evident under combined stress, SRL and RF typically improve absorptive efficiency by producing longer, finer roots capable of accessing water from small soil pores, while increased RTD can enhance structural stability in compacted or heat-affected soils. In theory, these traits could help sustain water acquisition and support reproductive growth under simultaneous stressors; however, in this study their potential benefits were overridden by severe limitations in photosynthetic activity and impaired water transport. This explains the substantial reductions in pod number, seed set, and seed weight observed under combined drought and heat stress. Additionally, these root adaptations carry trade-offs, finer roots may be more fragile, and higher RTD may limit elongation, potentially constraining the plant's ability to sustain rapid soil exploration under extreme conditions (Ryser, 1996; Comas et al., 2013).

Plants growing under water-deficit conditions employ several adaptive mechanisms to mitigate stress. One of the earliest is stomatal closure, which conserves water but limits CO_2 uptake (Ahluwalia et al., 2021). Drought-tolerant plants often maintain deeper or more efficient root systems that allow continued access to subsoil water, minimizing the extent of stomatal closure and thereby maintaining CO_2 availability for photosynthesis (Kumar et al., 2012). In this study, plants in the drought-only treatment were able to maintain stomatal conductance and transpiration, likely due to their functionally intact root systems. By contrast, plants under

combined drought and heat stress exhibited significantly reduced stomatal conductance and transpiration rates, reflecting impaired root development and reduced access to water. Similarly, plants under heat-only stress also showed reduced stomatal conductance and transpiration rates, even in the presence of adequate soil moisture. Generally, high temperature conditions are known to cause an increase in stomatal conductance and transpiration rates as an adaptive trait to cool high leaf temperatures and prevent oxidative stress and damage to the photosynthetic apparatus (Ahluwalia et al., 2021). The anomaly observed in this study may therefore be likely due to heat-induced root dysfunction and increased vulnerability of roots with high RTD and RF to physical and thermal damage.

The reduced CO_2 assimilation resulting from stomatal closure often disrupts the photosynthetic electron transport chain, leading to an accumulation of ROS (Taiz & Zeiger, 2015). The generation of ROS can cause cellular damage, but plants have evolved mechanisms to mitigate these effects. One such adaptation is the accumulation of osmoprotectants, such as proline, which scavenges ROS, protects cellular structures, and enhances stress tolerance (Alagoz et al., 2022; Zafar et al., 2023). Hence, proline levels are frequently used as a marker to assess the intensity of stress on plants. Interestingly, under heat stress conditions, plants tend to accumulate soluble sugars as osmoprotectants instead of proline (Ahluwalia et al., 2021).

Nutrient accumulation is also sensitive to environmental stress. Soil water availability enhances nutrient solubility and transport while drought reduces these processes (Fahad et al., 2017). Similar to previous studies (Rouphael et al., 2017), drought in this study resulted in lower concentrations of most nutrients. However, heat and combined stress treatments showed elevated nutrient concentrations, contradicting earlier research (Lobell & Gourdji, 2012; Hasanuzzaman et al., 2013). The observed increases in this study may be attributed to the ‘concentration effect.’ According to Jarrell and Beverly (1981), environmental stressors that impede dry matter accumulation often lead to an imbalance between nutrient uptake and plant

growth. When the rate of dry matter production decreases faster than the rate of nutrient absorption, nutrients become concentrated in plant tissues. Thus, elevated nutrient levels reflect reduced growth rather than improved nutrient uptake.

Yield, being the ultimate indicator of crop productivity, is profoundly influenced by abiotic stress. Stress treatments disrupt resource allocation and developmental stability, often resulting in yield penalties. In this study, pod number, seed number, seed weight, and pod weight were reduced under drought, heat, and combined stress treatments, consistent with the vulnerability of chickpea reproductive processes during flowering, pod setting, and seed filling (Awasthi et al., 2014; Devasirvatham et al., 2010). The consistent decline in seed number and seed weight across stress treatments suggests that these stressors disrupt resource allocation and synthesis of metabolites, likely by impairing photosynthetic activity or limiting the translocation of assimilates to developing reproductive structures during these critical phases. Pod weight, as a parameter closely linked to assimilate partitioning and reproductive sink strength, was also significantly reduced across all stress treatments, further indicating disrupted carbon allocation. The similarity in reductions among the three stress treatments suggests a universal impairment in the plant's ability to allocate resources to pod development under stress conditions. This aligns with existing research showing that both heat and drought stress negatively affect carbohydrate assimilation and transport during pod filling, leading to smaller and lighter pods (Awasthi et al., 2017; Zafar et al., 2023).

Our findings further reveal that the combination of heat and drought stress exacerbates the negative impacts on all yield parameters, with reductions often more severe than those observed under individual stress treatments. This compounding effect may be attributed to the simultaneous disruption of multiple physiological processes, including photosynthesis, nutrient uptake, and water transport, which are critical for supporting reproductive growth.

2.4.2 Genotypic Responses to Stress Treatments

The genotypes evaluated in this study exhibited varying degrees of sensitivity and adaptive responses to abiotic stress conditions, providing insights into their potential for tolerance breeding programs. Despite the absence of significant differences in root biomass and the R/S ratio, differences in physiological and biochemical traits provided evidence of genotype-specific responses. While root biomass accumulation did not exhibit meaningful variation across genotypes, suggesting its limited utility for identifying tolerant genotypes in this study, ACC#8 demonstrated a distinct advantage in aboveground growth, achieving higher total biomass (Table 2.2) compared to the other genotypes. This ability to sustain growth under adverse conditions can be attributed to its capacity to maintain relatively high RWC, which is essential for mitigating water deficit and heat stress effects. In contrast, ICCV 07113 emerged as the most sensitive genotype, with consistently lower RWC values across all treatments. These findings align with prior research (Awasthi et al., 2014) suggesting that maintaining water status under stress is crucial for sustaining plant growth and productivity.

Photosynthetic pigments revealed genotype-specific stress responses where Chl a content was significantly lower in ICCV 07113, accompanied by a higher Chl a/b ratio compared to the other genotypes. This increase in the Chl a/b ratio reflects a stress-induced degradation of Chl b, a component of the light-harvesting complexes in photosystem II, which is particularly sensitive to oxidative damage under heat and drought stress (Lichtenthaler et al., 1988; Yordanov et al., 2000). While this adjustment may help optimize light capture and reduce photodamage under stress, the reduced efficiency of light harvesting could contribute to the poorer performance of ICCV 07113 under stressful environments. Carotenoid content followed a similar trend, with ICCV 07113 and ICCV 00108 recording reductions under all treatments relative to the control, whereas ACC#8 experienced declines only under combined stress. Carotenoids, essential for photoprotection and antioxidant activity (Demmig-Adams & Adams

III, 2006), may have played a critical role in mitigating oxidative stress in ACC#8, supporting its ability to maintain higher RWC and biomass accumulation under stress conditions.

Conversely, the elevated proline levels observed in ICCV 07113, while indicative of a robust stress response, suggest that this genotype experienced more severe oxidative stress and accumulated larger amounts of ROS compared to the others (Hayat et al., 2012). This imbalance, coupled with reduced carotenoid levels and altered pigment composition, underscores ICCV 07113's heightened sensitivity to stress. In contrast, ACC#8's superior pigment stability and efficient water-use strategy likely contributed to its resilience, further highlighting its potential as a stress-tolerant genotype.

2.5 Conclusion

This study highlights the profound effects of individual and combined drought and heat stress on chickpea growth, physiology, and yield, with combined stress presenting the most significant challenges. The findings highlight the pivotal role of root morphological traits, chlorophyll stability, and physiological adaptations in mediating plant responses to abiotic stress. While drought stress primarily influenced root traits and water uptake efficiency, heat stress exerted a greater impact on aboveground growth and chlorophyll degradation. The combined stress exacerbated these effects, underscoring the synergistic challenges posed by concurrent environmental stressors. Genotypic variations further revealed differential adaptive strategies, with ACC#8 demonstrating superior tolerance through efficient water-use strategies and pigment stability, while ICCV 07113 emerged as the most sensitive genotype, reflecting its limited stress tolerance.

These insights provide a foundation for breeding programs aimed at enhancing chickpea resilience to abiotic stress, emphasizing the importance of integrating root morphological traits, chlorophyll stability, and water conservation as selection criteria. Moreover, the study

contributes to a broader understanding of plant responses to climate-induced stress, highlighting the necessity for targeted interventions to sustain crop productivity under increasingly variable environmental conditions.

Chapter 3

Effect of heat stress on root architecture, photosynthesis, and nutrient composition of chickpea (*Cicer arietinum* L.) under field conditions

3.1 Introduction

Greenhouse gas (GHG) emissions have significantly increased in recent decades, resulting in global warming and consequent climate change (Cassia et al., 2018). This change has led to a rise in extreme weather events, particularly heat stress, which poses a major constraint on global crop productivity. The IPCC predicts that global temperatures will rise by at least 2 to 5°C by the end of this century if GHG emissions are not curtailed (IPCC, 2021). For every 1°C rise in average temperatures, experimental data and crop models indicate reduced global yields of major food crops: maize by 7.4%, wheat by 6.0%, rice by 3.2%, and soybean by 3.1% (Zhao et al., 2017). This decline in crop production, combined with the anticipated growth in the global population, presents significant challenges to food security and threatens to exacerbate malnutrition, particularly among vulnerable populations (Battisti and Naylor, 2009).

Chickpea is a highly nutritious crop, with a high protein content of 20 to 26%, fat (5 – 6%) and carbohydrate (61 – 68%), making it a valuable dietary component (Benali et al., 2023). As a cool-season crop, chickpea performs optimally when its reproductive stage coincides with temperatures ranging from 20°C to 28°C (Devi et al., 2023). However, in warmer chickpea-growing regions, the crop frequently experiences temperatures above 30°C during crucial late-stage growth phases, increasing its susceptibility to heat stress. Research by Kalra et al. (2008) has shown that even a minor increase of 1°C above optimal growth temperatures can lead to significant yield losses, ranging from 53 to 301 kg/ha. Given its economic importance, numerous studies (Devasirvatham et al., 2015; Devi et al., 2022; Makonya et al., 2019) have investigated chickpea's responses and tolerance to heat stress to mitigate the impact of rising global temperatures on its productivity. Heat stress affects chickpea’s physiological and

biochemical processes in various ways. The elevated temperatures reduce chlorophyll content, impair stomatal conductance, and decrease net photosynthetic rates, leading to lower biomass accumulation and yield (Devasirvatham et al., 2015; Devi et al., 2022; Makonya et al., 2019). Beyond these, heat stress also alters chickpea's root architecture by reducing root length, surface area, and volume, which in turn disrupts the crop's ability to uptake water and nutrients, adversely affecting its physiological processes (Kashiwagi et al., 2006).

Transpiration plays a critical role in regulating plant temperature and maintaining physiological processes. Under heat stress conditions, plants experience increased water demand due to higher transpiration rates, which serves as a cooling mechanism. Roots, being the primary organ responsible for water uptake, are therefore important in meeting these demands (Guo et al., 2024). A healthy and well-developed root system can access deeper soil moisture reserves, ensuring continuous water supply even under high evapotranspiration conditions (Tuberosa and Salvi, 2006). This is particularly important as efficient water uptake through an extensive root system can alleviate the adverse effects of heat stress, maintaining photosynthetic activity and overall plant health (Guo et al., 2024). In crops such as rice (Sailaja et al., 2014), oilseed rape (Wu et al., 2020), heat-tolerant varieties have been found to exhibit robust root systems that explore a larger soil volume, enhancing water and nutrient uptake. These plants are therefore better equipped to maintain higher transpiration rates, which help in cooling the plant and sustaining photosynthesis under elevated temperatures. Moreover, the root system's ability to increase hydraulic conductivity under heat stress conditions, facilitates the continuous movement of water across cell membranes, replenishing water lost during transpiration and maintaining cellular integrity (Maurel et al., 2008).

Considering the role of the root system in maintaining balance of the overall plant system under these conditions, it is quite surprising that few studies on chickpea have considered its response and adaptability to heat stress. This gap in research is partly due to the specialized tools and

labour-intensive methodologies required to study root systems. As such, the few studies that have reported on roots under heat stress conditions have been carried out as pot studies (Kumari et al., 2018). While this method facilitates easier handling and minimizes damage, it also restricts the growing environment and isolates each root system, making them less representative of natural field conditions (Asadullah et al., 2024). As a result, although pot studies provide valuable insights into root responses under heat stress, they do not fully capture the complexities of field environments, emphasizing the necessity of open-field screening for a more comprehensive understanding (Bhattarai et al., 2021). Furthermore, in several studies that identified heat tolerant chickpea genotypes, the root traits were not considered, neglecting their contribution in promoting heat tolerance.

The objectives of this chapter therefore were to (1) examine the root response of chickpea plants to temperature differences under field conditions and (2) assess how root responses contribute to heat tolerance in tolerant chickpea genotypes. It was hypothesized that chickpea genotypes that develop an extensive root system will perform better physiologically and yield maximally at the warmer site in Nkomazi.

3.2 Methodology

3.2.1 Study sites

The field experiment was conducted in northeastern South Africa during the winter of 2023, identified by Thangwana and Ogola (2012) as the ideal growing season for chickpea in the region. Two sites, Thohoyandou and Nkomazi, located in the Limpopo and Mpumalanga provinces respectively, and lying 338.45 km apart (in a straight line), were chosen for this study (see Table 3.1 for site coordinates and elevation). The sites Thohoyandou and Nkomazi are characterised by different soils (Table 3.1), mean annual temperatures of 16.0 – 28.6°C and 17.9 – 30.5°C (Figure 3.1), and an annual rainfall of 817.6 mm and 955.2 mm (Figure 3.1),

respectively. Rainfall in the region predominantly falls in the summer season with little to no rain in the winter season, leaving crops grown in the two sites during winter to depend on residual soil moisture and supplementary irrigation for growth.

3.2.2 Plant material and experimental design

Four Desi chickpea genotypes (ACC#7, ACC#8, ICCV 07113 and ICCV 00108), chosen due to their different characteristics (see Table 2.1 above), were sourced from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT- Kenya) and used for this study.

In both sites, treatments were arranged in a randomized complete block design and replicated three times with experimental units (plots) measuring 1.2 m × 0.8 m. Spacing between the experimental units and blocks was 0.5 m and 1.0 m respectively. Each plot consisted of four rows with an inter-row spacing of 0.4 m and an intra-row spacing of 0.1 m. At planting, the fields were fertilized using 20 kg N ha^{-1} of limestone ammonium nitrate (LAN 28%) as nitrogen and 60 kg P ha^{-1} of superphosphate fertilizer (20.3% P) as phosphorus. Seeds were sown manually with all plots uniformly watered to promote even germination and crop establishment. Supplemental irrigation was supplied whenever necessary, and the experimental plots were kept weed-free throughout the growing seasons. At the initiation of budding, a net cover was provided to deter monkey and bird herbivory.

3.2.3 Climate data and soil analysis

Average daily weather data (i.e. rainfall, maximum and minimum temperatures) was collected for each site via the Agricultural Research Council's Climate Monitoring Services (ARC-ISCW, Pretoria, South Africa), from stations ranging 12.5 km – 30.6 km away from the experimental sites (Figure 3.1).

Pre-sowing soil analysis was carried out at the start of the experiment. Soil samples from three replicates on each site were collected using an auger at a 20 – 30 cm depth, air-dried and then sieved through a 2 mm mesh to remove any debris available. The concentration of total nitrogen (N), phosphorus (P), and available phosphorus (Bray II P) was then determined at the analytical laboratory Bemlab (Pty) Ltd. (Somerset West, South Africa). Analyses of soil pH, boron (B), carbon (C), calcium (Ca), copper (Cu), potassium (K), manganese (Mn), sodium (Na), sulphur (S) and zinc (Zn) concentrations were also carried out at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenburg, South Africa following standard soil analysis methods (Non-Affiliated Soil Analysis Work Committee 1990).

3.2.4 Chlorophyll concentration

Chlorophyll and carotenoid concentrations were measured according to Lichtenthaler's (1987) 95% ethanol method. Please see details in Chapter 2 section 2.2.3.

3.2.5 Plant biomass and root morphology

At physiological maturity, a random row at the middle of each plot was selected and three adjacent plants within the selected row were harvested for whole plant biomass. A boundary of 0.2 m from the stem base of each plant was demarcated and a spade was used to excavate the soil containing the plant at a depth of 0.25 m. The root system with adhering soil was excised from the whole plant biomass, placed in a self-sealing bag stored in a cooler box and transported to the laboratory. The remaining shoot system was separated into leaves and stems, oven-dried at 60°C for 72 h and the dry weight was recorded. Based on these measurements, the R/S was then calculated as the ratio of root dry weight to shoot dry weight. At the laboratory, measurements for root morphological traits were carried out as outlined in Chapter 2, section 2.2.6 (please see above).

3.2.6 Leaf gaseous exchange

Gas exchange parameters including P_n, C_i, g_s and E were measured using a portable photosynthesis system, LI-6400XT (LI-COR Biosciences, Lincoln, NE, USA). At each site, measurements were taken at 50% flowering growth stage, on a sunny day between 0900 and 1200. Three plants within the inner rows were randomly selected and the youngest fully expanded leaf was used in taking the measurements. To take measurements, air temperature in the head sensor was set to ambient conditions with other parameters set according to protocol outline in Chapter 2, section 2.2.8.

3.2.7 Nutrient content

Leaf nutrient content was determined using leaf samples used in the determination of leaf biomass, following the protocol outlined in Chapter 2, section 2.2.7.

3.2.8 Statistical analysis

Soil analysis data was analysed by one-way analysis of variance (ANOVA) with the remaining data sets subjected to a two-way ANOVA to compare the effects of genotype and environments and their interaction. The Duncan's Multiple Range Test was used to separate mean values that were significantly different ($p < 0.05$). All statistical analyses were carried out using R (R Core Team, 2024).

3.3 Results

3.3.1 Climate and soil of study sites

A statistical comparison of the growing-season climate (April–June 2023) showed clear differences between the two sites. Nkomazi recorded higher maximum and minimum temperatures than Thohoyandou, with mean values of 29.7°C and 16.0°C, respectively, compared to 28.2°C and 13.9°C in Thohoyandou. This reflects increases of 1.5°C (5.3%) in maximum temperature and 2.1°C (15.1%) in minimum temperature at Nkomazi. Rainfall differences were even more pronounced: Thohoyandou received an average of 0.63 mm, whereas Nkomazi recorded 16.93 mm, representing a 16.30 mm absolute difference and an approximately 2,500% higher rainfall in Nkomazi during the same period. These statistical comparisons highlight the markedly hotter and wetter conditions at Nkomazi during the chickpea growing season.

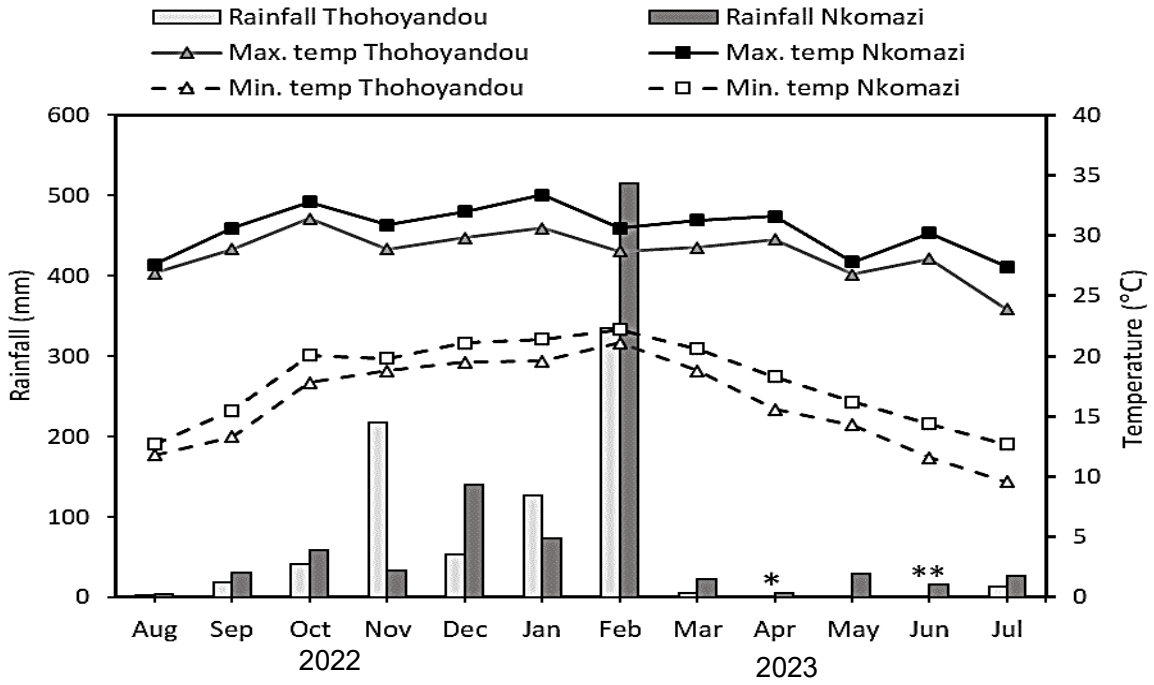

Figure 3.1: Annual recording of total rainfall and average minimum and maximum monthly temperatures at the two sites: Thohoyandou and Nkomazi. The single asterisk indicates the planting period, and the double asterisk indicates the data flowering stage/ data collection period.

Of all the chemical analyses done on the collected soil samples, soil pH, Total P, K, S, Na and Zn were not significantly different between the two sites (Table 3.1). Thohoyandou recorded higher levels of all the nutrients that showed significant differences except Available P and B which were higher in Nkomazi. Soils from both sites were found to be acidic (Table 3.1).

Table 3.1: Chemical analyses of soil collected at the two sites: Thohoyandou and Nkomazi. Data is mean values ± SE ((n = 3 per plot), followed by letters indicating significant differences between sites by Duncan's Multiple Range Test as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns = not significant.

Variable	Thohoyandou	Nkomazi	*F*-ratio
Coordinates	22°58.816' S, 30°26.292'	25°45.924' S, 31°47.163' E	---
Elevation	578 m asl	239 m asl	---
Soil type	Clay	Clay loam	---
pH	5.75 ± 0.05	5.97 ± 0.088	3.271^{ns}
C (%)	1.64 ± 0.19^{b}	0.8 ± 0.04^{a}	19.209*
N (%)	0.113 ± 0.003^{b}	0.087 ± 0.003^{a}	32**
Total P (mg kg^{-1})	151.3 ± 2.3	135.3 ± 14.1	1.254^{ns}
Bray II P (mg kg^{-1})	4.33 ± 0.52^{a}	58.3 ± 12.93^{b}	17.393*
K (mg kg^{-1})	133.7 ± 13.4	225.7 ± 50.7	3.073^{ns}
S (mg kg^{-1})	2.3 ± 0.06	3.4 ± 0.44	6.259^{ns}
Ca (cmol kg^{-1})	6.58 ± 0.37^{b}	4.99 ± 0.35^{a}	9.703*
Mg (cmol kg^{-1})	3.45 ± 0.15^{b}	2.907 ± 0.08^{a}	10.332*
B (mg kg^{-1})	0.133 ± 0.003^{a}	0.33 ± 0.044^{b}	20.238*
Cu (mg kg^{-1})	32.59 ± 0.43^{b}	10.74 ± 2.25^{a}	90.688***
Mn (mg kg^{-1})	809.9 ± 44.9^{b}	268.5 ± 10.7^{a}	137.25***
Na (mg kg^{-1})	21.3 ± 1.5	62.33 ± 17.05	5.739^{ns}
Zn (mg kg^{-1})	5.17 ± 0.24	6.02 ± 1.33	0.398^{ns}

3.3.2 Plant Biomass

Plants grown in the relatively cooler site, Thohoyandou, accumulated more biomass in the leaves (Figure 3.2A), shoot (Figure 3.2B) and root systems (Figure 3.2C) translating to an overall higher total biomass (Figure 3.2D) than those in the warmer Nkomazi site. The biomass for the stem did not show any significant differences (see appendix table 3.1). The interactive effect of genotype and environment was insignificant for all plant biomass parameters. Genotypic differences were also statistically insignificant (see appendix table 3.2).

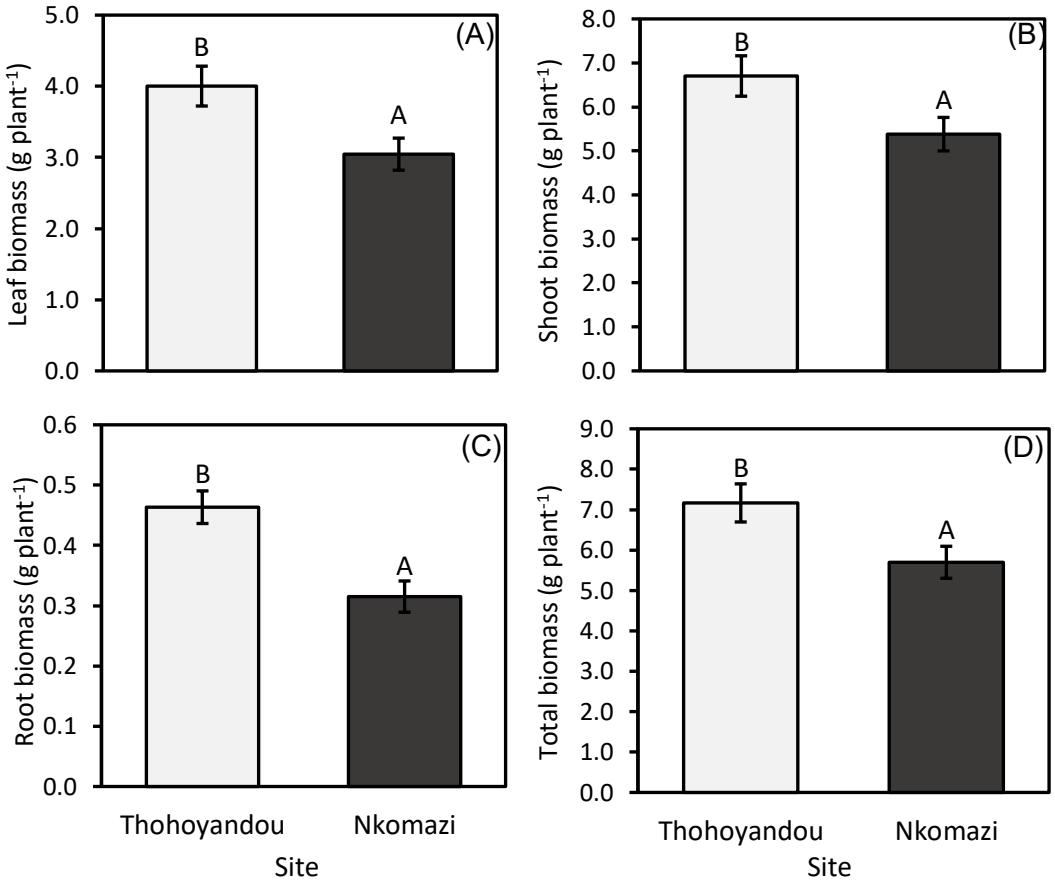

Figure 3.2: The effect of environment on leaf (A), shoot (B), root (C) and total biomass (D) of chickpea plants at 50% flowering. Data are mean values and vertical lines on bars denote standard error. Letters indicate significant differences between sites and genotypes respectively by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per plot per genotype.

3.3.3 Chlorophyll concentration

The interaction of environment and genotype for chlorophyll a, chlorophyll b and total chlorophyll concentrations were significant. At the relatively cooler site, Thohoyandou, genotypes ACC#7 and ICCV 07113 recorded a higher chlorophyll a concentration compared to the other genotypes (Figure 3.3). Despite the differences in chlorophyll a concentration between genotypes in Thohoyandou, the values for chlorophyll a concentration recorded in Nkomazi were similar for all genotypes. In terms of chlorophyll b and total chlorophyll concentrations however, ACC#7 had the highest concentrations in Thohoyandou, followed by ICCV 07113 which scored intermediate values sharing similarities with both ACC#7 and the other two genotypes. The differences recorded within genotypes in Thohoyandou however did not reflect in genotypic variation in Nkomazi as they all shared similarities.

3.3.4 Gaseous exchange

There were no significant genotype and environment interactions for all the measured gaseous exchange parameters. Between environments, the net photosynthesis (Figure 3.4A) and transpiration rate (Figure 3.4G) were higher in the warmer site, Nkomazi, compared to Thohoyandou whereas a higher intercellular CO_2 concentration was recorded for Thohoyandou (Figure 3.4E). There were no significant differences in the stomatal conductance between sites (Figure 3.4B).

Between genotypes, P_n, g_s, and *E* showed significant differences. Genotype ICCV 07113 was similar to ACC#7 but higher than ACC#8 and ICCV 00108 for P_n (Figure 3.4B) and g_s (Figure 3.4D). For *E*, genotype ICCV 07113 was higher only to ACC#8 (Figure 3.4H).

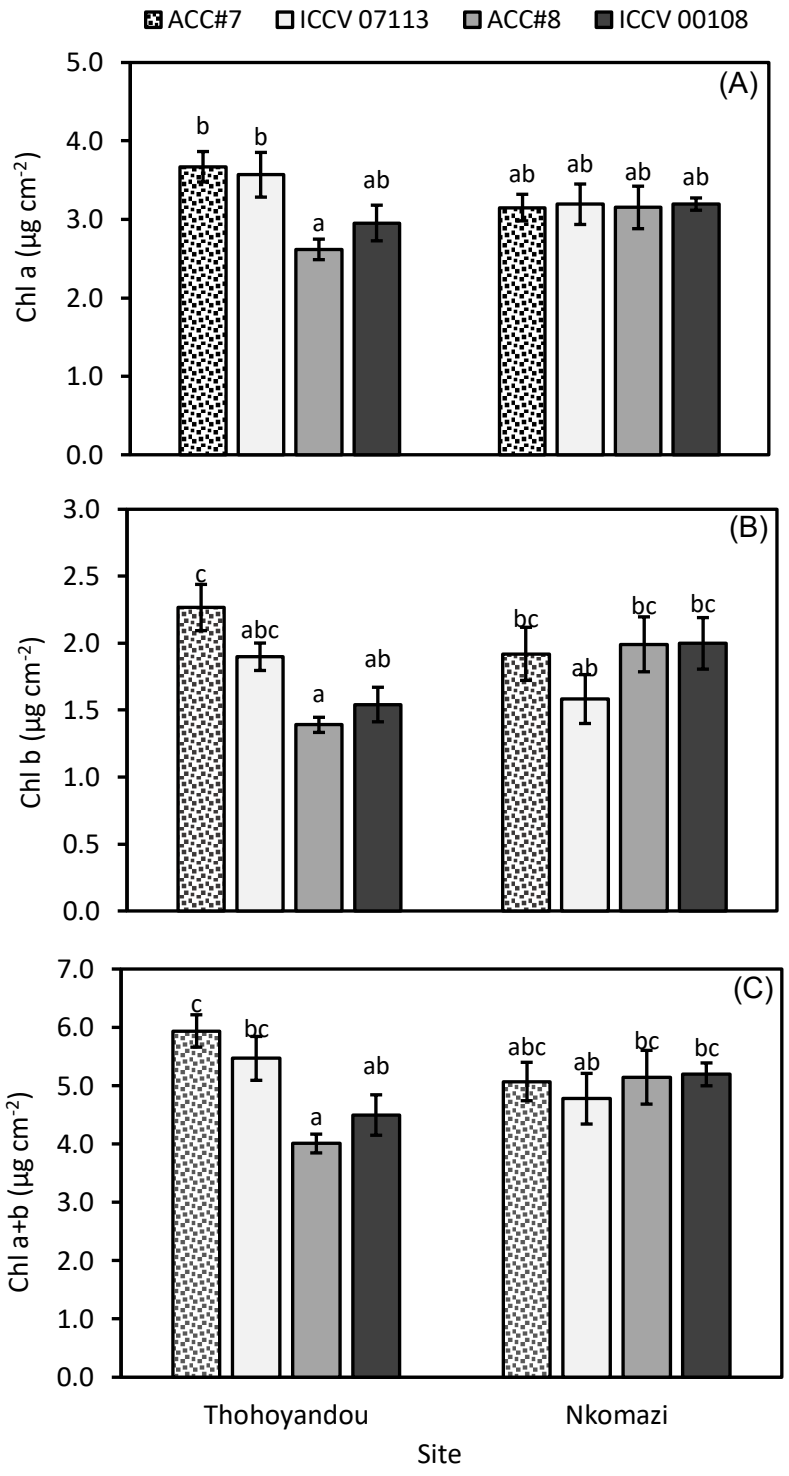

Figure 3.3: Interactive effect of environment and genotype on chlorophyll a (A), chlorophyll b (B) and total chlorophyll (C) concentrations of chickpea at 50% flowering. Data is mean values ± SE. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test (P<0.05). No significant differences were recorded for chlorophyll concentrations between sites. Sample size: n = 3 per plot per genotype.

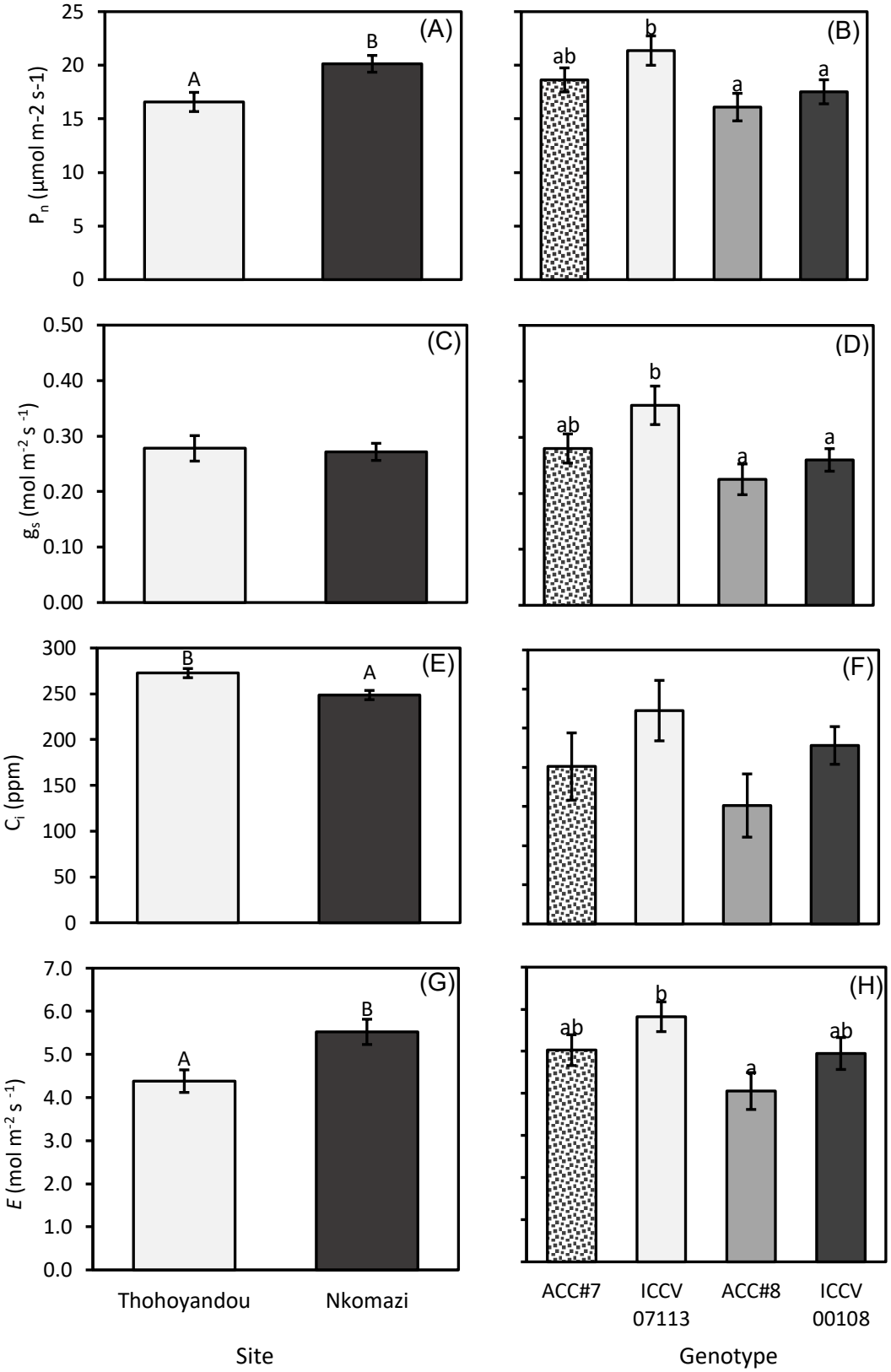

Figure 3.4: Environment (site) and genotype effect on P_n (A and B), g_s (C and D), C_i (E and F) and *E* (G and H) of chickpea. Measurements were taken at 50% flowering. g_s and C_i had no environmental and genotypic differences respectively. Data is mean values and vertical lines on bars denote standard error. Uppercase and lowercase letters indicate significant differences between sites and genotypes respectively by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 3 per plot per genotype.

3.3.5 Nutrient content

Of all the nutrients assessed, Cu and Mg showed no significant differences between sites, genotypes nor the interaction thereof. Environmental effects on nutrient composition in the leaves caused plants grown in the Nkomazi site to have higher levels of N, P, K, Na, Zn and B while Thohoyandou had higher levels of Ca, Fe, Mn and Al (Table 3.2). The effect of genotype was, however, insignificant for the majority of nutrients except N, Mn, and B. Nitrogen concentrations were higher in ACC#7 and ACC#8 compared to the remaining two genotypes (Table 3.2). For Manganese and boron, ICCV 07113 and ACC#7 recorded the highest values respectively relative to the other genotypes (Table 3.2).

The interactive effect of genotype and environment was significant in Ca and Mn (Table 3.3). For Mn, genotypes in Nkomazi had relatively lower concentrations compared to those in Thohoyandou whereas ICCV07113 and ICCV 00108 had higher concentrations than ACC#7 and ACC#8 at Thohoyandou. In both the two sites, Genotype ICCV 00108 interestingly recorded the highest concentration of Ca in Thohoyandou and the lowest in Nkomazi (Table 3.3.) Furthermore, ACC#7, and ICCV 07113 recorded significantly higher Ca concentrations in Thohoyandou than in Nkomazi, while genotype ACC#8 recorded similar values for both sites (Table 3.3).

Table 3.2: Analysis of variance (ANOVA) for nutrient content of chickpea plants collected at 50% flowering from the two sites: Thohoyandou and Nkomazi. Data is mean ± SE (n = 3 per plot per genotype) followed by capital letters and lowercase letters in the rows indicating significant differences between sites and genotypes respectively, by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	Nitrogen %	Phosphorus %	Potassium %	Calcium %	Sodium mg/kg	Iron mg/kg	Zinc mg/kg	Manganese mg/kg	Boron mg/kg	Aluminium mg/kg
Site										
Thohoyandou	3.86 ± 0.1^{A}	0.318 ± 0.007^{A}	2.07 ± 0.07^{A}	1.34 ± 0.02^{B}	200 ± 9.1^{A}	2107 ± 127^{B}	26.4 ± 0.6^{A}	150 ± 4.5^{B}	26 ± 0.9^{A}	1434 ± 78^{B}
Nkomazi	4.70 ± 0.1^{B}	0.6 ± 0.023^{B}	2.97 ± 0.09^{B}	1.16 ± 0.02^{A}	529 ± 26.5^{B}	648 ± 37^{A}	39.8 ± 3.7^{B}	93 ± 3.4^{A}	34 ± 0.8^{B}	764 ± 50^{A}
Genotype										
ACC#7	4.60 ± 0.19^{b}	0.498 ± 0.053	2.48 ± 0.13	1.28 ± 0.03	372 ± 50.3	1375 ± 219	37.9 ± 6.1	116 ± 6.8^{a}	33 ± 1.4^{b}	1139 ± 132
ICCV 07113	3.82 ± 0.16^{a}	0.412 ± 0.042	2.47 ± 0.17	1.25 ± 0.04	299 ± 40.5	1695 ± 285	32 ± 3.5	148 ± 10.4^{b}	29 ± 1.5^{a}	1256 ± 158
ACC#8	4.53 ± 0.1^{b}	0.435 ± 0.034	2.58 ± 0.16	1.21 ± 0.03	362 ± 50.4	1238 ± 174	30.3 ± 2.0	113 ± 6.7^{a}	27 ± 1.5^{a}	1020 ± 88
ICCV 00108	3.89 ± 0.15^{a}	0.429 ± 0.032	2.37 ± 0.17	1.29 ± 0.05	351 ± 48.3	1543 ± 258	29.5 ± 1.2	127 ± 11.0^{a}	29 ± 1.5^{a}	1138 ± 139
F-ratio										
Site	44.563^{***}	154.914^{***}	67.66^{***}	42.151^{***}	133.2019^{***}	95.5151^{***}	16.8701^{***}	103.83^{***}	44.143^{***}	43.995^{***}
Genotype	8.622^{***}	1.968^{ns}	0.898^{ns}	1.579^{ns}	0.0992^{ns}	0.5533^{ns}	1.5383^{ns}	3.186^{*}	4.292^{**}	0.3074^{ns}
Site*Genotype	1.984^{ns}	0.777^{ns}	1.274^{ns}	4.46^{**}	0.5388^{ns}	1.1192^{ns}	2.148^{ns}	2.927^{ns}	1.269^{ns}	1.2692^{ns}

Table 3.3: Analysis of variance for nutrient content of chickpea plants collected at 50% flowering from the two sites: Thohoyandou and Nkomazi. Data is means ± SE (n = 3plot per genotype) followed by capital letters and lowercase letters in the rows indicating significant differences between sites and genotypes respectively, by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

Site	Genotype	Calcium %	Manganese mg/kg
Thohoyandou	ACC#7	1.36 ± 0.04^{de}	137 ± 6.7^{b}
	ICCV 07113	1.31 ± 0.04^{cd}	167 ± 9.7^{c}
	ACC#8	1.25 ± 0.05^{bcd}	134 ± 7.5^{b}
	ICCV 00108	1.45 ± 0.03^{e}	164 ± 6.9^{c}
Nkomazi	ACC#7	1.19 ± 0.03^{abc}	95 ± 6.2^{a}
	ICCV 07113	1.12 ± 0.03^{ab}	106 ± 2.5^{a}
	ACC#8	1.18 ± 0.04^{abc}	93 ± 5.5^{a}
	ICCV 00108	1.10 ± 0.05^{a}	85 ± 8.1^{a}
F-ratio	Site	42.15^{***}	103.83^{***}
	Genotype	1.58^{ns}	3.19^{*}
	Site*Genotype	4.46^{**}	2.93^{*}

3.3.6 Root morphology

Chickpea plants growing in the relatively cooler Thohoyandou site recorded significantly higher root volume, larger surface area, and longer but thinner roots, resulting in higher SRL, RLR and RF values compared to those growing in the warmer Nkomazi site (Table 3.4). The effect of genotype on root traits showed a consistent trend of genotypes ICCV 07113 and ACC#8 recording the highest and lowest values respectively for all root parameters except root average diameter, root tissue density and root fineness (Table 3.4; Figure 3.5). For root fineness, ICCV 07113 shared similar values with the genotype, ICCV 00108. Similarly, genotype ACC#8, also shared similar values with genotype ACC#7, which were lower than the values for ICCV07113 and ICCV00108 (Table 3.4). In terms of root average diameter, however, a trend opposite to that of root fineness was observed with both genotypes ICCV 07113 and ICCV 00108 recording similar but a smaller root diameter than ACC#7

and ACC#8 that were also similar. Root mass ratio showed no significant differences between sites, within genotypes nor in the interaction between sites and genotype (see appendix table 3.3).

Table 3.4: Analysis of variance (ANOVA) for morphological root traits of chickpea plants collected at 50% flowering from the two sites: Thohoyandou and Nkomazi. Data is means ± SE (n = 3 per plot per genotype) followed by capital letters and lowercase letters in the rows indicating significant differences between sites and genotypes respectively, by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	RL (m)	RSA (cm^2)	RAD (mm)	RV (cm^3)	SRL (m g^{-1})	RTD (g cm^{-3})	RLR	RF (m cm^{-3})
Site								
Thohoyandou	9.27 ± 0.68^{B}	213.3 ± 15.6^{B}	0.739 ± 0.014^{A}	3.96 ± 0.31^{B}	20.02 ± 1.16^{B}	0.130 ± 0.006	1.39 ± 0.11^{B}	2.42 ± 0.09^{B}
Nkomazi	3.20 ± 0.25^{A}	93.9 ± 8.0^{A}	0.925 ± 0.023^{B}	2.22 ± 0.21^{A}	11.11 ± 0.87^{A}	0.147 ± 0.005	0.66 ± 0.09^{A}	1.57 ± 0.09^{A}
Genotype								
ACC#7	6.02 ± 0.94^{b}	162.9 ± 23.8^{b}	0.898 ± 0.033^{b}	3.56 ± 0.49^{b}	14.53 ± 1.64^{ab}	0.129 ± 0.009^{a}	0.86 ± 0.11^{ab}	1.69 ± 0.11^{a}
ICCV 07113	9.16 ± 1.39^{c}	212.9 ± 30.0^{b}	0.768 ± 0.035^{a}	4.00 ± 0.54^{b}	20.91 ± 2.55^{c}	0.122 ± 0.010^{a}	1.74 ± 0.24^{c}	2.30 ± 0.17^{b}
ACC#8	3.99 ± 0.56^{a}	103.5 ± 13.6^{a}	0.842 ± 0.028^{b}	2.18 ± 0.29^{a}	12.20 ± 0.76^{a}	0.158 ± 0.007^{b}	0.68 ± 0.08^{a}	1.90 ± 0.12^{a}
ICCV 00108	7.69 ± 1.13^{bc}	172.7 ± 21.9^{b}	0.769 ± 0.033^{a}	3.17 ± 0.36^{ab}	17.72 ± 1.89^{bc}	0.139 ± 0.006^{ab}	1.15 ± 0.16^{b}	2.34 ± 0.19^{b}
F ratio								
Site	77.44^{***}	45.15^{***}	51.74^{***}	19.47^{**}	34.79^{**}	2.87^{ns}	24.62^{***}	50.53^{***}
Genotype	6.86^{***}	4.51^{**}	5.31^{**}	3.35^{*}	5.39^{*}	4.12^{*}	9.68^{***}	6.77^{***}
Site*Genotype	3.80^{*}	2.09^{ns}	0.41^{ns}	1.26^{ns}	2.18^{ns}	2.77*	1.51^{ns}	1.89^{ns}

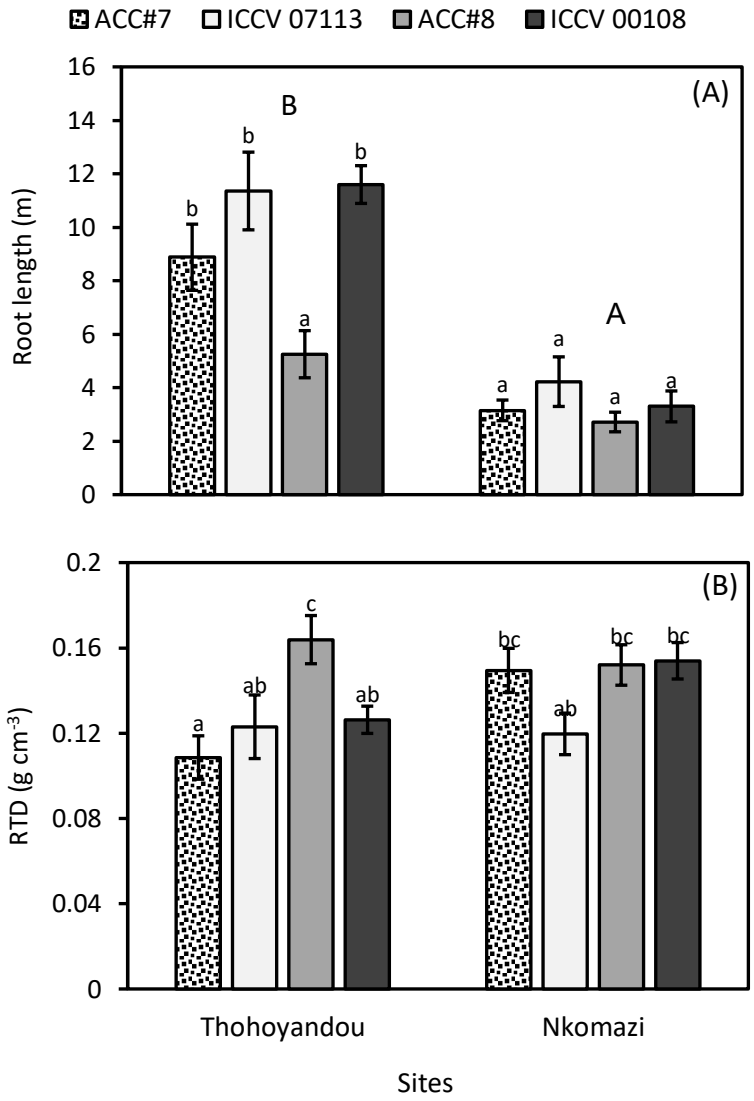

Figure 3.5: Interactive effect of environment and genotype on root length (A) and root tissue density (B) of chickpea at 50% flowering. The interaction between environment and genotype for the remaining root morphological traits was not significant. Data is mean values ± SE. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test $p < 0.05$). Sample size: n = 3 per plot per genotype.

3.4 Discussion

3.4.1 Environmental impact

In this study, four chickpea genotypes were cultivated across two open-field sites with varying environmental conditions to assess the effects of heat stress on growth and development. Research (e.g., Devasirvatham et al., 2015; Devi et al., 2023; Giri et al, 2017; Makonya et al., 2019) indicates that environmental variations often elicit diverse responses in chickpea plants, with cooler temperatures (20°C – 28°C) being more favourable for chickpea production compared to warmer temperatures (>28°C). It is therefore not surprising that chickpea grown in the Thohoyandou site, where temperatures were lower, exhibited greater biomass compared to those grown in Nkomazi, where temperatures were 2°C higher and exceeded the optimal maximum temperature of 28°C for chickpea. According to the IPCC (2021) report, a 2°C increase in atmospheric temperature often exceeds the critical tolerance threshold of agricultural systems, leading to significant negative impacts, such as reduced crop yields.

Under field conditions, elevated temperatures often induce soil drought, inherently causing stomatal closure and limiting the plant's ability to lower its leaf temperature through transpiration (MacAlister, 2020). Although this mechanism helps maintain plant water status, it adversely affects photosynthesis by restricting CO_2 uptake into the leaf (Basu et al., 2016). Studies on tomato (*Solanum lycopersicum L.;* Giri et al., 2017), water spinach (*Ipomoea aquatica* Forsk; Wang et al., 2023), and chickpea (Zeitelhofer et al., 2022), have reported lower photosynthetic rates under heat stress conditions due to reduced stomatal activity and chlorophyll degradation. In this study, however, despite the heat stress conditions in Nkomazi, stomatal conductance and chlorophyll concentration remained unchanged, recording similar values to those of the cooler Thohoyandou site. These results may be attributed to the higher rainfall in addition to the supplementary irrigation in Nkomazi, ensuring an abundance of soil moisture. As a result, higher transpiration and photosynthetic rates were recorded in this site.

The increased rate of photosynthesis in Nkomazi, however, did not translate to increased biomass accumulation. Under optimal conditions, higher photosynthetic rates generally correlate with higher biomass production (Silva et al., 2023). However, when exposed to suboptimal conditions such as heat stress, plants alter their physiological processes to maintain cellular function and survival, including the activation of pathways to produce heat shock proteins and other protective molecules, which require additional energy (Larkindale and Knight, 2002). This leads to accelerated respiration rates, diverting photosynthetic energy from growth and biomass accumulation to maintenance and stress responses. When heat stress is prolonged, this heightened energy expenditure leads to reduced plant growth and yield, as more resources are allocated to coping mechanisms rather than biomass production (Teskey et al., 2015). The lack of correlation between photosynthetic rates and biomass accumulation in Nkomazi therefore suggests that the observed temperature increase possibly placed the chickpea plants in this site under heat stress, causing them to expend much of their acquired energy on stress coping mechanisms. The possible increase in respiration rates of chickpea plants grown in Nkomazi may also be a contributing factor to the higher photosynthetic rates recorded in this site.

Previous studies (Giri et al., 2017; Heckathorn et al., 2013) have reported decreased shoot and root growth under heat stress conditions, with the root system being the most affected, resulting in a lower root-to-shoot ratio. In this study, although both root and shoot biomass were reduced in plants grown at the warmer Nkomazi site, root-to-shoot ratio was not affected which suggests that both systems were equally affected. However, the limited root growth affected the RSA of this plant group, resulting in lower values for primary (e.g., root length, root volume, and root surface area) and secondary root traits (e.g., specific root length, root tissue density, and root length ratio). Generally, a less-developed root system under stress conditions is often associated with thinner root structures (either absolute- i.e. smaller root diameter or relative i.e.

high SRL) as these traits are less expensive to produce but increases the volume of soil exploited per unit biomass invested in the root system, allowing for more efficient absorption of water and nutrients from the soil (Ostonen et al., 2007). The lack of development for these adaptive traits further supports the notion that the plants at Nkomazi were severely affected by the elevated temperatures. Heat stress affects cell differentiation and elongation (i.e. growth) and the roots are more susceptible to this than the shoot. Comparably, plants at Thohoyandou were able to establish a well-developed morphology (high root volume, large surface area and long and thin roots for efficient uptake of water and nutrients and plant growth.

The comparative analysis of soil nutrients between Thohoyandou and Nkomazi reveals significant differences that likely influenced the variations in chickpea biomass and nutrient concentrations observed at the two sites. Thohoyandou's soil, enriched with higher levels of C, N, Ca, Mg, Cu, and Mn, provided a more nutrient-rich environment compared to Nkomazi, where P and B were more prominent. Despite equal pre-sowing application of N and P across both sites, Thohoyandou maintained higher baseline nutrient levels, indicating the inherent fertility of this site. This elevated soil fertility likely supported more robust vegetative growth, as reflected in the higher plant biomass recorded in Thohoyandou. The relationship between soil fertility and plant growth is well-established, with increased nutrient availability driving enhanced metabolic processes and biomass accumulation (Lopez et al., 2023). However, the substantial biomass increase in Thohoyandou may have led to a "dilution effect," where nutrient content per unit biomass decreased as plant growth outpaced nutrient uptake (Jarrell and Beverly, 1981). This effect could account for the reduced nutrient concentrations in the plants from Thohoyandou, despite the higher overall soil nutrient levels. Conversely, in Nkomazi, the smaller plant biomass likely resulted in higher concentrations of nutrients within the plant tissues. Under environmental stress conditions, nutrient availability is crucial for enhancing a plant’s ability to cope with stress and sustain growth. The elevated levels of these

nutrients in plants at Nkomazi may have played a key role in sustaining certain physiological processes in the plants at this site. For example, higher levels of N, a key component of chlorophyll (Fathi, 2022), likely supported chlorophyll production and maintenance despite the heat stress conditions, contributing to the high photosynthetic rates recorded. Similarly, increased K levels, known for their role in stomatal regulation and osmoregulation (Sardans and Peñuelas, 2021), would have been particularly important in Nkomazi, where transpiration rates are high. Potassium aided in maintaining cellular turgor and sustaining photosynthetic efficiency by preventing stomatal closure, allowing for continuous gas exchange.

3.4.2 Genotypic effect

The chickpea genotypes selected for this study possess varying sensitivity to abiotic stress including heat stress. In this study, while the growth and performance of certain genotypes aligned with their known sensitivity levels, discrepancies were observed in others. For example, genotypes ACC#7 and ACC#8, previously identified as heat-tolerant and heat-susceptible, respectively (Makonya et al., 2019), performed in line with expectations, with the tolerant genotype displaying high performance and the susceptible genotype showing lower performance. On the other hand, genotype ICCV 07113, previously classified as heat-susceptible (Kaloki, 2012), performed better than anticipated, contradicting earlier findings. A brief contrast is also noted between its performance in this study and its behaviour under glasshouse conditions, which may reflect genotype × microclimate interactions. This study, therefore, confirms the importance of assessing the growth, physiological and seed yield of germplasm before being recommended for production in specific areas.

Of the four chickpea genotypes studied, ICCV 07113 alongside ACC#7, developed well-adapted root systems characterized by longer, more voluminous, and thinner root – traits that facilitate deeper soil penetration for enhanced water and nutrient uptake under heat stress conditions across both sites. This root adaptation possibly contributed to higher stomatal

conductance and increased transpiration rates, allowing for more effective leaf temperature regulation in these genotypes compared to others. Consistent with findings from a similar study on chickpea under open-field conditions (Devasirvatham et al., 2015), the ability of a plant to sustain transpiration under heat stress not only cools leaf temperatures but also prevents chlorophyll degradation, thereby maintaining photosynthetic efficiency. This possibly explains the lower chlorophyll concentrations and reduced photosynthetic rates observed in the poorly performing genotype ACC#8, which exhibited minimal root architectural development and the lowest transpiration rates.

Despite the differing physiological performances among the genotypes in this study, these differences did not translate into variations in biomass yield, as all genotypes exhibited similar shoot and root biomass across both sites. This suggests that differences in physiological parameters do not always correlate with plant biomass. This is because there are many other processes that affect plant biomass production and seed yield such as respiration (Amthor et al., 2019). Therefore, identifying genotypes for biomass production using physiological parameters should be used with caution.

3.5 Conclusion

The findings of this study provide important insights into the complex interactions between genotype performance, environmental conditions, and soil nutrient availability in chickpea cultivation. The results indicate that while soil fertility and environmental factors like temperature significantly influence plant biomass, they do not necessarily correlate with overall biomass productivity, particularly under stress conditions. The cooler Thohoyandou site, with its nutrient-rich soil, supported greater biomass accumulation, which may have contributed to a dilution effect, resulting in lower nutrient concentrations within the plant tissues. In contrast, the Nkomazi site, characterized by higher temperatures and lower soil fertility, exhibited

smaller biomass but higher nutrient concentrations and photosynthetic rates, likely due to the stress-induced physiological adjustments. Furthermore, the performance of the chickpea genotypes under varying stress conditions highlighted the importance of root architecture and physiological traits in coping with environmental stressors. The superior performance of genotypes with well-developed root systems underscores the value of root traits in breeding programs aimed at enhancing heat tolerance in chickpeas. However, the lack of correlation between biomass and productivity across the genotypes suggests that traditional measures of plant performance, such as biomass yield, may not fully capture a genotype's resilience and productivity potential under stress conditions. This study emphasizes the need for a more holistic approach in evaluating chickpea genotypes, taking into account both physiological and morphological traits to better predict performance under variable and challenging environmental conditions.

Chapter 4

Low-nutrient-induced extensive root system for tolerance in chickpea (*Cicer arietinum* L.) under drought conditions

4.1 Introduction

Drought stress is one of the major limiting factors to the field of agriculture in that it causes yield losses annually (Nasir and Toth, 2022). In the past decade, drought stress has caused up to USD $30B of losses in global crop production (Gupta et al., 2020) through its negative impact on plant growth, physiology, and reproduction (Rani et al., 2020). This abiotic stress triggers a series of physiological responses in plants, which adversely affects their growth and productivity. Drought stress significantly affects various physiological processes in plants, primarily by reducing water availability, which in turn disrupts normal cellular functions. A decline in soil moisture lowers plant water potential, reducing stomatal conductance as plants close their stomata to minimize water loss (Zahoor et al., 2022). While this response helps conserve water, it also limits CO_2 uptake, leading to a decline in photosynthetic rates and, consequently, reduced biomass accumulation (Farooq et al., 2009). Additionally, decreased transpiration rates under drought conditions impair the uptake and transport of essential nutrients, negatively impacting plant metabolism and growth (Hu and Schmidhalter, 2005). The restricted water supply also affects energy distribution during photosynthesis, disrupting electron transport chains and ATP synthesis, further exacerbating growth inhibition (Chaves et al., 2009). Moreover, these stress-induced disruptions often lead to an overproduction of ROS, causing oxidative damage to cellular structures, proteins, and lipids, ultimately compromising plant health and survival (Mittler, 2002).

Some plants have however evolved adaptive mechanisms to cope with drought stress and enhance their survival under water-limited conditions. These plants exhibit a myriad of morphological adaptations in their root systems to cope with water scarcity, as roots play a

pivotal role in water uptake and nutrient acquisition, making them essential for plant growth and survival, particularly under drought-stress conditions (Kalra et al., 2024). Among the various root traits crucial for plant survival under water-limited conditions, the increased root-to-shoot ratio stands out as a key determinant of drought resilience (Zhao et al., 2018). A higher root-to-shoot ratio allows plants to allocate more resources to root growth, enabling greater soil exploration for water uptake (Lynch, 2013). However, other root traits such as root volume, surface area, root length, and root thickness also play crucial roles in stress adaptation. An extensive root system with increased root length and higher root density enhances water uptake efficiency, especially under drought conditions where deep water extraction is necessary for survival (Comas et al., 2013; Kalra et al., 2024). Additionally, finer roots with smaller diameters improve soil penetration and increase the surface area available for water and nutrient absorption, further enhancing the plant's ability to sustain growth during stress periods (Zhou et al., 2019). The combination of these root adaptations ultimately enhances a plant's ability to withstand prolonged periods of drought, by optimizing water uptake and nutrient acquisition, thereby improving overall resilience under water-limited conditions.

In legumes, increases in root:shoot ratio under low-nutrient or drought conditions are widely recognised as classic expressions of phenotypic plasticity. This plasticity enables plants to reallocate biomass belowground to enhance water and nutrient foraging when resources become limiting. Comparative studies in chickpea, common bean, cowpea and soybean have shown that both nutrient deficiency and water scarcity commonly trigger proportional increases in root biomass relative to shoot biomass (Srinivasarao et al., 2006; Poorter et al., 2012; Kunert et al., 2016). Such plastic adjustments are considered adaptive because they enhance soil exploration, water uptake and resource acquisition, thereby supporting legume survival under combined nutrient and moisture limitations.

Despite its critical role in plant survival under drought, root morphology has often been overlooked in favour of aboveground traits such as leaf physiology and canopy architecture. Root systems are essential not only for water uptake but also for nutrient acquisition, particularly in low-fertility soils where extensive root development enhances resource capture. However, in modern crop production systems, high seed yield is frequently achieved through fertilization, which ensures nutrient availability but inadvertently reduces selection pressure for root traits that promote deeper soil exploration (Harris, 1992; Lopez et al., 2023). High soil fertility is often associated with a lower root-to-shoot ratio, as plants allocate more biomass to shoot growth rather than developing extensive root structures. While this strategy may be effective in well-fertilized environments, it poses a significant challenge under drought conditions. Over-fertilization in agricultural systems could, therefore, hinder the natural selection of drought-adaptive root traits, potentially reducing crop resilience in water-limited environments. Given the increasing frequency of drought events due to climate change, there is a pressing need to reconsider breeding and management strategies to enhance root traits that support both water and nutrient acquisition under stress conditions.

This study therefore aimed to determine whether plants with high root/shoot ratio and other root adaptive traits to drought stress such as high root volume, larger surface area, longer and thinner roots will have better physiological processes, growth and seed yield than those that lack them. The extensive and adaptive root traits were obtained by growing the plants in low nutrient conditions. This enabled us to determine whether the adaptive root traits acquired under low nutrient availability will indeed confer an advantage under drought conditions. The objective of this chapter was to determine the beneficial effect of low-nutrient induced extensive root systems on plant growth and seed yield under drought. It was hypothesized that an extensive root system under low nutrient confers growth advantage under drought stress.

4.2 Materials and Methods

4.2.1 Plant materials and growth conditions

The determination of the effect of low nutrient availability on drought stress in plants was investigated with a pot experiment in a glasshouse at the University of Cape Town, South Africa (33°57.353' S, 18°27.727' E) from March to July 2023. A total of 80 plastic pots each measuring 24.5 × 18 × 20 cm for the top diameter, bottom diameter, and height respectively, were used. The pots were filled with a mixture of 4.5 kg of commercial silica sand, 500 g of 70/30 coco-perlites (Hortishop- Hydroponics and Organics, South Africa) and 1 g of gypsum ($CaSO_4$. $2H_2O$). At the point of fertilisation, the pots were divided into two equal groups, with one group receiving the recommended rate of 6 g pot^{-1} of Multicote (4*) 15–3–12 + Mg + Me (Haifa Chemicals, South Africa) and the other group receiving 2 g pot^{-1} of the same fertiliser to create growth media with different nutrient levels. Based on the fertiliser composition (15% N, 3% P_2O_5, 12% K_2O) and the substrate mass (5.001 kg pot^{-1}), these application rates corresponded to approximately 180 ppm N (0.018%), 15.7 ppm P (0.00157%) and 119.5 ppm K (0.01195%) for the recommended treatment, and 60 ppm N (0.006%), 5.2 ppm P (0.00052%) and 39.8 ppm K (0.00398%) for the low-nutrient treatment. Elemental P and K were calculated from P_2O_5 and K_2O using standard conversion factors, while nitrogen required no conversion as it is reported directly in elemental form on fertiliser labels.

Two Desi chickpea genotypes, namely, drought tolerant ICCV 00108 and drought susceptible ICCV 07113 were used for this experiment with each genotype being allocated 20 pots of the two growth media. In each pot, three chickpea seeds were sown at 1 cm depth, and seedlings thinned to one plant per pot 21 days after sowing. All pots were watered to 75% field capacity (FC) until the commencement of the drought stress treatment. The recommended FC of the soil for chickpeas was pre-determined from a previous potted glasshouse experiment. At 50% flowering, four plants from each group were harvested and data on chlorophyll content, leaf

relative water content, stomatal content, shoot and root biomass, and root morphology was collected to assess the effect of the different nutrient levels on the plant's vegetative growth.

The remaining plants from each of the two groupings per genotype were then randomly divided into two sub-groups creating four groups of eight plants per genotype. The groups represented the four treatments to be imposed on the plants namely, control (grown in recommended nutrient levels; watered to 75% FC), drought-only (grown in recommended nutrient levels; watering withheld), low-nutrient (grown in low nutrient levels, watered to 75% FC) and combined drought and nutrient stress (grown in low nutrients and watering withheld; Figure 4.1). The treatments were imposed for 20 days, during which data on stomatal conductance, leaf relative water content and chlorophyll content were collected on four plants per treatment for each genotype.

The 20-day duration was possible in this chapter [in comparison to Chapter 2] because no heat-related treatments were applied; preliminary trials for Chapter 2 showed that plants exposed to heat, and especially the combined drought and heat treatment, could not tolerate stress beyond eight days without losing leaf colour, wilting, or showing severe decline. Therefore, a longer stress duration was not feasible in that experiment in order to prevent plant mortality and ensure measurable physiological data.

At the end of the treatment, data on the above-listed parameters were collected and the four plants were harvested for destructive data sampling including shoot and root biomass, root morphology, proline, and nutrient content analysis. The remaining four plants were rewatered to 75% FC across all treatments and left for recovery to determine the grain yield. Throughout the life cycle of the chickpea plants, all pots were moved around weekly within the glasshouse to minimize microclimate effects on plant growth.

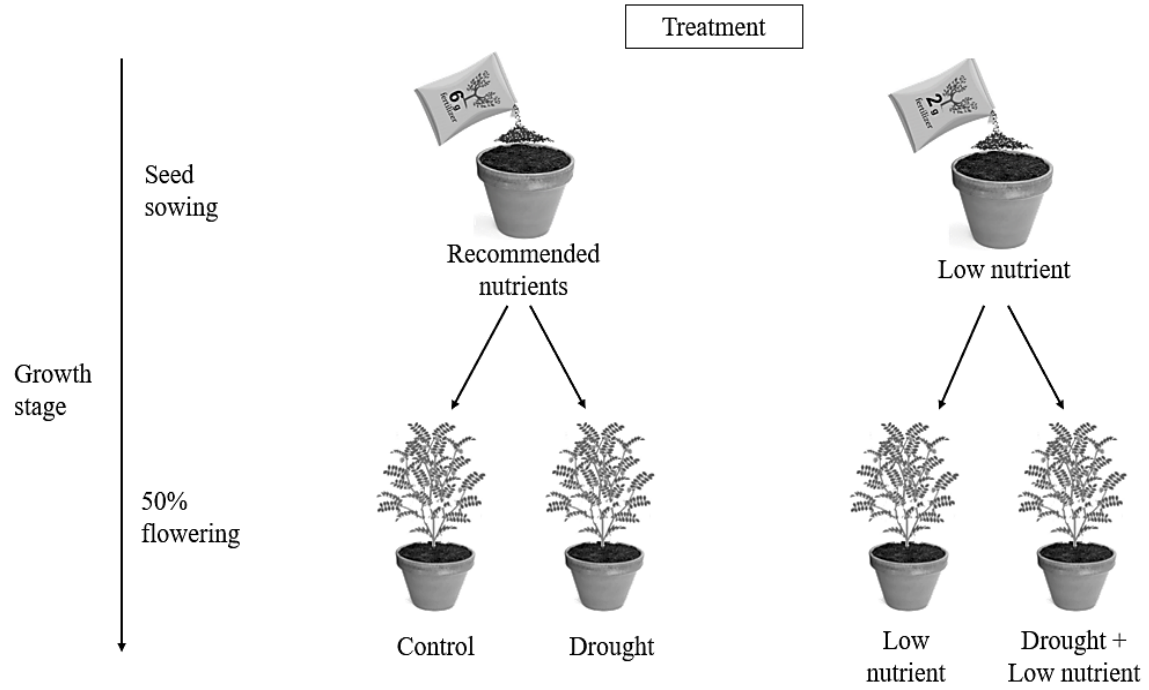

Figure 4.1: Experimental design for the determination of the effect of low-nutrient availability on plant growth under drought stress. Control (plants growing in recommended nutrient soils and watered to 75% FC throughout the growth period); Drought (plants growing in recommended nutrient soils but watering withheld for 20 days at 50% flowering); Low nutrients (plants growing in low nutrient soils and watered to 75% FC throughout the growth period); Drought + Low nutrient (plants growing in low nutrients and watering withheld for 20 days at 50% flowering).

4.2.2 Biomass

Four plants per treatment and genotype were harvested and samples were taken for leaf relative water content, proline and chlorophyll concentrations, and roof morphological analysis. The remaining plant parts were used in determining plant biomass parameters. See details in section 2.2.1.

4.2.3 Leaf relative water content

The leaf RWC, measured to determine the moisture-holding capacity of the plants under the various treatments, was determined using the Barrs and Weatherly (1962) method. Detailed description outlined in Chapter 2, section 2.2.4.

4.2.4 Stomatal conductance

Stomatal conductance was measured at the start and end of the stress period and every fourth day in between using the Leaf Porometer Model SC-1 (Decagon Devices Inc., USA). The youngest fully matured leaf of the plants was used for the measurements which were all taken between 0900 and 1100.

4.2.5 Proline concentrations

Proline concentration was determined using the Bates et al. (1973) method. Please see Chapter 2, section 2.2.5 for detailed description.

4.2.6 Chlorophyll and carotenoid concentrations

Chlorophyll and carotenoid concentrations were measured according to Lichtenthaler's (1987) 95% ethanol method. Please see detailed protocol outlined in Chapter 2 section 2.2.3.

4.2.7 Root morphology

At both harvest times (i.e. at 50% flowering and at the end of the stress period), root morphological traits were measured using protocol outlined in Chapter 2, section 2.2.6.

4.2.8 Leaf nutrient content analysis

Leaf nutrient content was determined using leaf samples used in the determination of leaf biomass, following the protocol outlined in Chapter 2, section 2.2.7.

4.2.9 Grain yield and components

At harvest maturity, all pods were removed from the plants and air-dried to about 12% moisture content. The pod number and weight were then determined, and the pods were threshed to obtain the seed number and weight.

4.2.10 Statistical analysis

The experiment was arranged in a complete randomised design with four replicates per treatment. Repeated measures ANOVA was used to test the effects of genotype and stress treatment on g_s and RWC across time. The remaining measured parameters were analysed by two-way ANOVA to compare the effect of genotype and stress treatments and their interactions. Where the results showed significant differences ($P<0.05$), the Duncan's Multiple Range Test was used to separate the mean values.

4.3. Results

4.3.1 Plant biomass and root morphology at 50% flowering

Prior to the commencement of drought stress treatments, the data indicated no significant difference between the two genotypes in all the parameters measured (Table 4.1) nor the interaction of genotype and treatment, except for root biomass (Figure 4.2). The plants from the two nutrient levels did not show significant differences in leaf and stem biomass, with the plants growing in soils containing the recommended nutrient levels (i.e. control) had values averaging approximately twice that of those growing in soils containing low nutrient levels (Table 4.1). The root system however, recorded values for the root biomass, root length, root surface area, root average diameter, root volume, root mass ratio and root length ratio significantly higher in low-nutrient plants than in the control plants (Table 4.1). This translated

to plants under the low-nutrient treatment having a higher R/S ratio compared to the control plants (Table 4.1).

In terms of the interaction between the genotypes and treatment, the root biomass of the drought tolerant genotype (ICCV 00108) was affected by the treatment with low soil nutrients causing an increase in root biomass (Figure 4.2). On the other hand, root biomass for drought susceptible genotype (ICCV 07113) remained unchanged by the low-nutrient soil conditions (Figure 4.2). However, the values recorded for genotype ICCV 07113 shared similarities to both the control and low-nutrient plant groups under genotype ICCV 00108 (Figure 4.2).

Table 4.1: Biomass and root morphological data of the two chickpea genotypes growing under two nutrient mediums recorded prior to the commencement of drought stress treatment. Data are means ± SE (n = 4 per treatment per genotype) followed by lowercase letters in each column indicates significant differences between treatments by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	Leaf biomass (g plant^{-1})	Stem biomass (g plant^{-1})	Root biomass (g plant^{-1})	Root: shoot ratio	Root length (m plant^{-1})	Root surface area (m^2 plant^{-1})	Root average diameter (mm plant^{-1})	Root volume (cm^3 plant^{-1})	Root mass ratio	Root length ratio
Genotype										
ICCV 07113	0.34 ± 0.06	0.33 ± 0.05	0.44 ± 0.07	0.84 ± 0.28	44.2 ± 8.0	0.08 ± 0.02	4.00 ± 0.45	11.0 ± 2.3	0.39 ± 0.06	41.1 ± 6.8
ICCV 00108	0.36 ± 0.13	0.30 ± 0.09	0.44 ± 0.10	1.11 ± 0.29	54.4 ± 13.1	0.09 ± 0.02	4.45 ± 0.56	12.2 ± 2.8	0.43 ± 0.09	55.3 ± 11.9
Treatment										
Control	0.48 ± 0.12	0.41 ± 0.09	0.30 ± 0.07^{a}	0.48 ± 0.15^{a}	31.0 ± 6.2^{a}	0.05± 0.01^{a}	3.34 ± 0.35^{a}	7.1 ± 1.6^{a}	0.28 ± 0.06^{a}	32.2 ± 7.8^{a}
Low nutrients	0.22 ± 0.03	0.23 ± 0.02	0.57 ± 0.08^{b}	1.46 ± 0.28^{b}	67.6 ± 10.5^{b}	0.12± 0.02^{b}	5.11 ± 0.43^{b}	16.0 ± 2.2^{b}	0.55 ± 0.06^{b}	64.3 ± 8.3^{b}
F ratio										
Genotype	0.032^{ns}	0.125^{ns}	0.000^{ns}	0.723^{ns}	0.871^{ns}	0.489^{ns}	0.632^{ns}	0.189^{ns}	0.234^{ns}	1.845^{ns}
Treatment	3.964^{ns}	3.518^{ns}	9.280*	9.843**	11.338**	11.682**	9.925**	10.778**	10.258**	9.374**
Genotype*Treatment	0.466^{ns}	0.125^{ns}	5.356*	1.841^{ns}	4.676^{ns}	3.789^{ns}	1.019^{ns}	2.540^{ns}	3.297^{ns}	2.755^{ns}

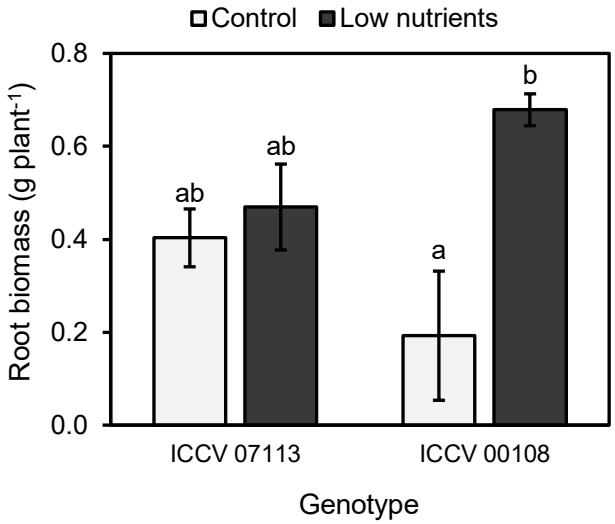

Figure 4.2: Interactive effect of genotype and nutrient treatment on root biomass of chickpea plants at 50% flowering, prior to the commencement of drought stress treatment. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 4 per genotype per treatment.

4.3.2 Plant biomass after drought stress treatment

At the end of the drought stress treatments, total biomass values for all treatments were similar but significantly (P<0.001) lower than the control plants (Figure 4.3E). However, there were treatment variations in the allocation of the biomass to the various organs. When compared to the control plants, low-nutrient and combined-stressed plants recorded greater reductions in leaf biomass (P<0.001; Figure 4.3A). In terms of the stem and shoot biomass however, the value of the combined drought and low nutrient treatment was similar to the low nutrient but lower than the drought treatment (Figure 4.3B, C). Conversely, root biomass (P<0.001) in the drought treatment was lower than the control and the low-nutrient and combined-stressed plants (Figure 4.3D; see appendix figure 4.1). The discrepancy in the allocation of biomass to the different organs led to varying R/S ratios (P<0.001), with plants growing in low-nutrient soil and combined-stress treatments having similar and higher R/S ratio compared to the control and drought stressed plants (Figure 4.3F).

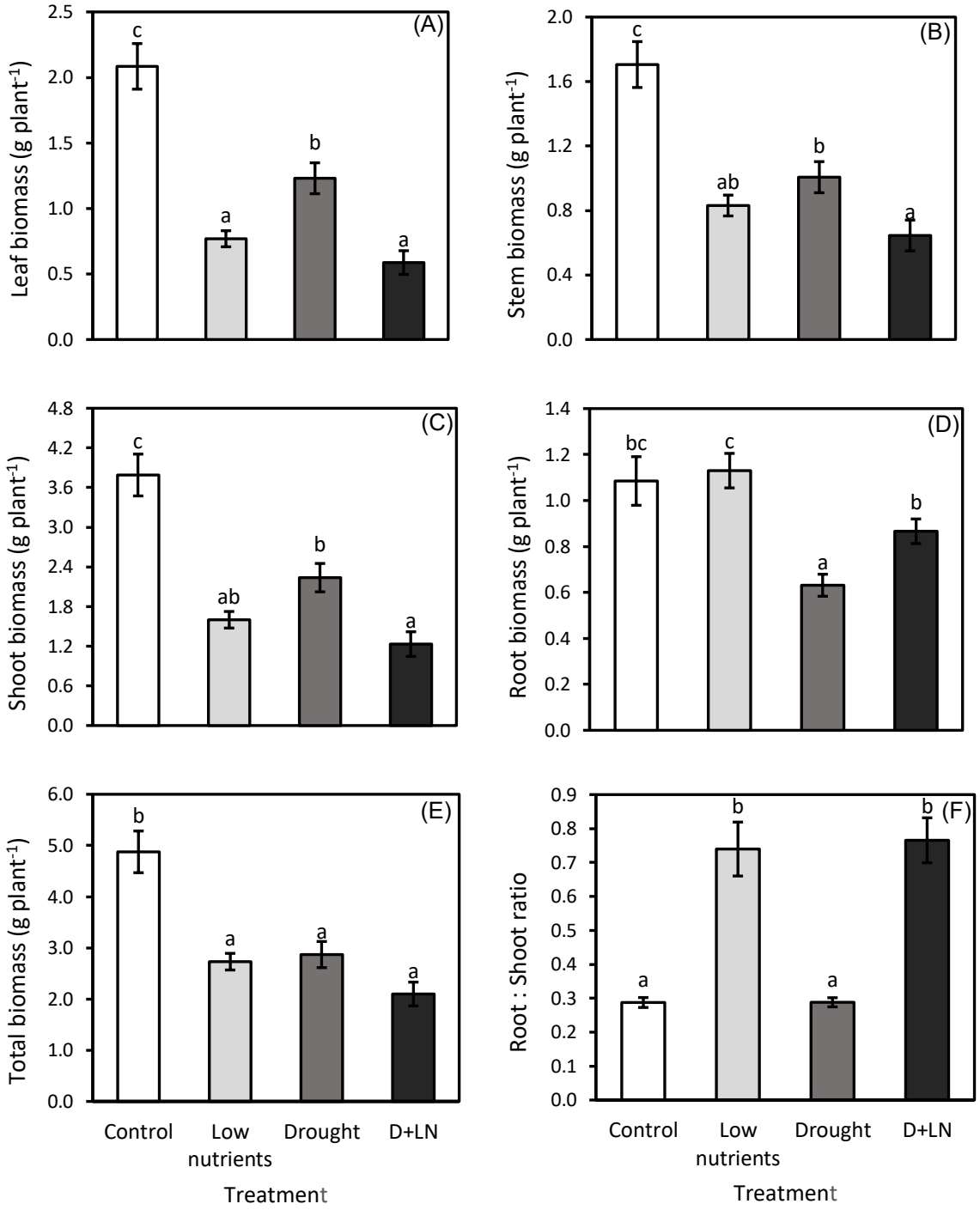

Figure 4.3: Treatment effect on leaf- (A), stem- (B), shoot- (C), root- (D), total biomass (E), and root: shoot ratio (F) of chickpea. LN (Low nutrients only plants) and D+LN (combined-stress plants). Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 4 per genotype per treatment.

4.3.3 Relative water content and stomatal conductance

The stomatal conductance of plants under drought and combined-stress treatments were similar throughout the stress period, except on day eight when the combined-stress treatment recorded a significantly higher value ($P<0.005$) than the drought-stressed treatment. However, both stress treatments had lower stomatal conductance compared to the control and low-nutrient treatments (Figure 4.4A). Similarly, the RWC under drought and combined drought and low nutrient plants decreased ($P<0.001$) over time relative to the control and low nutrient plants (Figure 4.4B). Within the first four days, both drought and D+LN maintained high RWC levels similar to the control and low nutrient plants. However, by the eighth day, the RWC of the drought-only plants sharply declined relative to the control and the other treatments until the 20th day when the plants were harvested. The combined stress treatment, on the other hand, maintained a RWC similar to the control until the 20th day when it was lower. Notably, the RWC of the combined stress was higher than the drought-only stress from day eight until harvesting on day 20 (Figure 4.4B). In terms of genotypic differences, RWC showed a gradual decline over time in both genotypes (Figure 4.4C). However, genotype ICCV 07113 recorded significantly higher RWC values compared to ICCV 00108 during the first eight days of withholding water, with the two genotypes recording significantly similar values during the rest of the stress treatment period.

Stomatal conductance showed no genotypic differences, and neither was the interaction of genotype and treatment for both RWC and g_s.

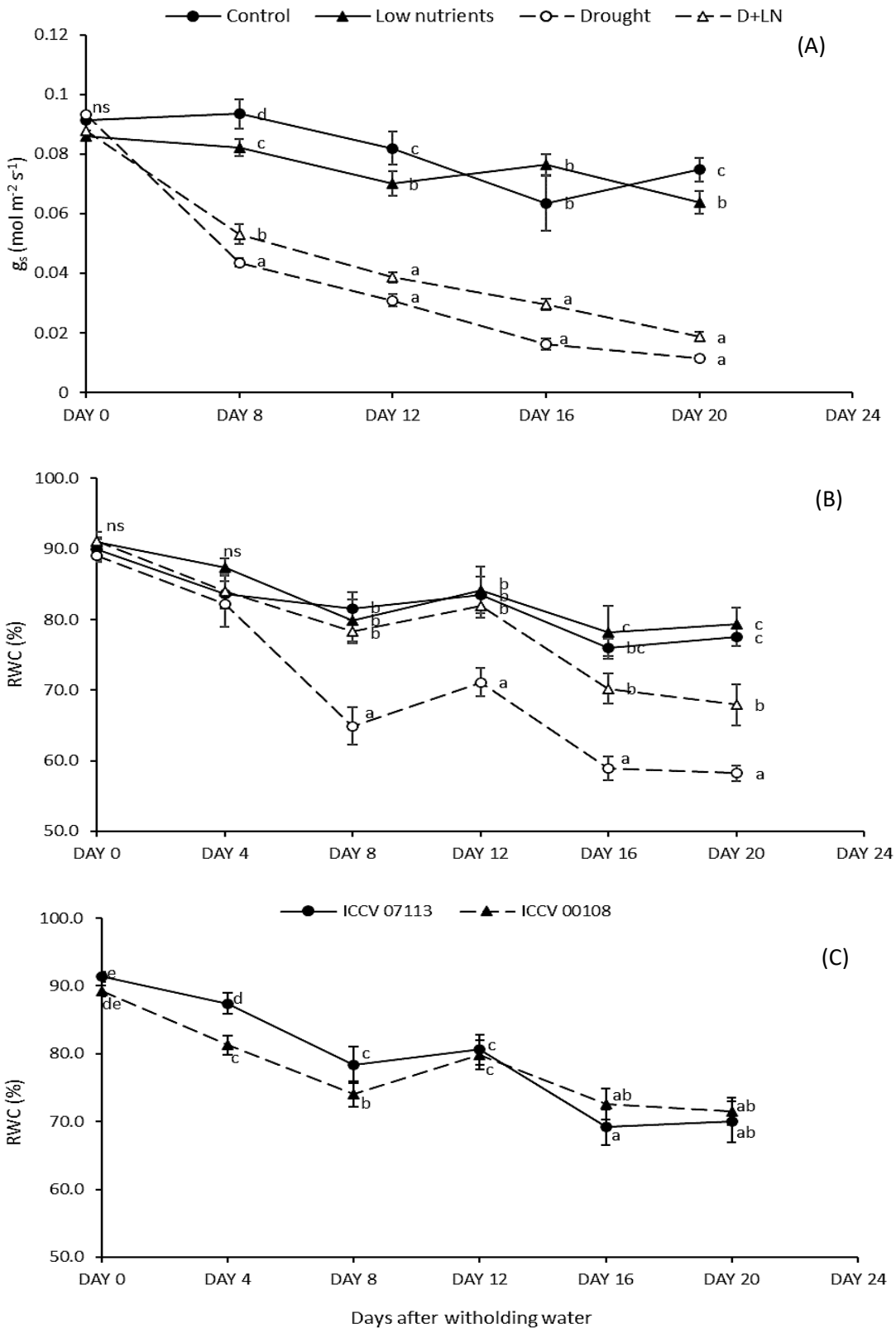

Figure 4.4: Effect of stress treatment (A and B) and genotype (C) on stomatal conductance (A) and RWC (B and C) of chickpea over 20 days of drought stress. D+LN denotes combined-stress plants. Data are mean values ± SE (n=4). Different letters indicate significant differences between treatments on each day by Duncan's Multiple Range Test ($P<0.05$). Sample size: n = 4 per genotype per treatment.

4.3.4 Chlorophyll concentrations

There were no significant differences between genotypes and in the interactive effect between genotype and treatment for all chlorophyll parameters measured. Stress treatments caused a similar response pattern in the concentrations of chlorophyll a and total chlorophyll. For chlorophyll a and total chlorophyll, the single stress treatments (i.e. low-nutrient and drought stresses) caused a decrease in concentrations whereas the combined stress treatment recorded values that were similar to the control (Figure 4.5 A and C). In chlorophyll b however, drought stress caused a decline in concentration (~30%) relative to the control whereas low-nutrient and combined stresses showed similar values to the control (Figure 4.5B). Relative to the control, the chlorophyll a/b ratio was higher in the drought stressed plants, with the other stress treatments sharing similarities with the control (Figure 4.5D).

4.3.5 Carotenoid and proline concentration

At the end of the stress period, concentration of carotenoid varied in the leaf depending on the stress treatment ($p<0.0002$). The low nutrient and drought stress treatments had similar values of carotenoids that were lower than the controls, whereas the value of the drought stress was lower than the combined stress (Figure 4.5E). Proline concentration, on the other hand, had both low-nutrient and combined stress plants recording low values similar to the control. In contrast, drought-stressed plants had a high ($P<0.001$) accumulation of proline, as much as 15x in the control (Figure 4.5F).

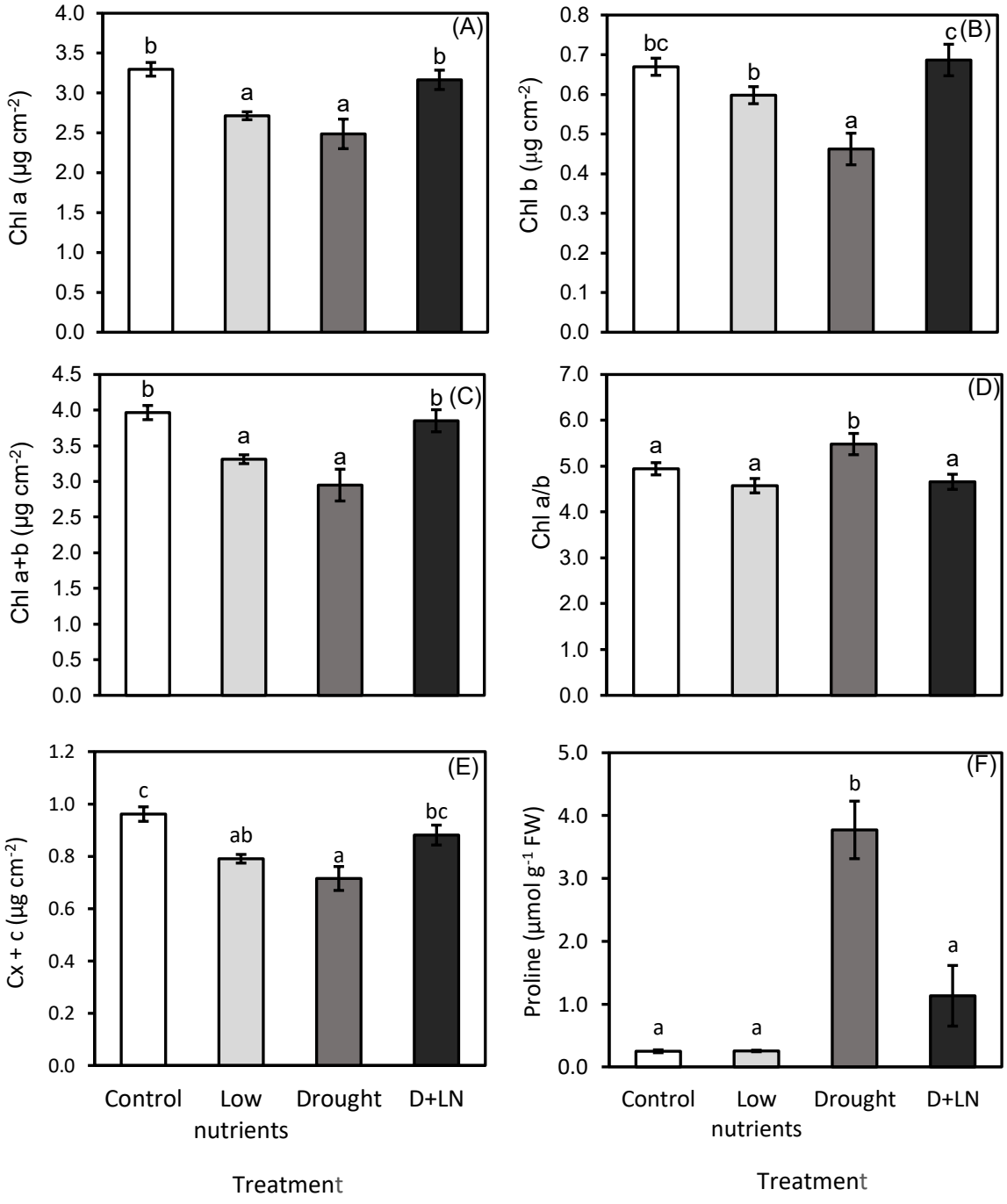

Figure 4.5: Effect of low nutrients and withholding watering on chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), chlorophyll a:b ratio (D), carotenoids (E) and proline content (F) of chickpea at the end of the stress treatment. LN denotes Low nutrients only plants and D+LN denotes combined-stress plants. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 4 per genotype per treatment.

4.3.6 Root morphology after drought stress treatment

All root morphological traits showed significant treatment differences with genotype and the interaction between genotype and treatment not recording significant differences. Out of the nine measured root morphological traits, root average diameter, specific root length and root tissue density, did not show any significant treatment differences ($p > 0.05$; see in appendix table 4.1), leaving six root traits that were found to have been significantly modified by the stress treatments in comparison to the control (Figure 4.6A – F).

Root length, root surface area and root volume were all reduced significantly by water deficit in both drought-only and combined-stress treatments with the lowest values recorded under drought-only treatment. Plants under low nutrient stress, on the other hand, recorded similar root length, root surface area and root volume values to the control (Figure 4.6A – C). The RMR in the drought-only treatment was similar to the control. However, plants in the low-nutrients and the combined-stress treatments recorded higher RMR values, almost twice as high as the control (Figure 4.6E).

The root length ratio (RLR) was increased by the stress in all three treatments except the drought only treatment that shared similarities with the control. Between the three treatments however, the combined-stress treatment recorded similar RLR values with the low nutrient plants but higher than the droughted plants (Figure 4.6F). Compared to the control, root fineness, on the other hand, was not affected by the combined-stress treatment but was increased in plants under drought stress and decreased in those under low nutrient stress (Figure 4.6D).

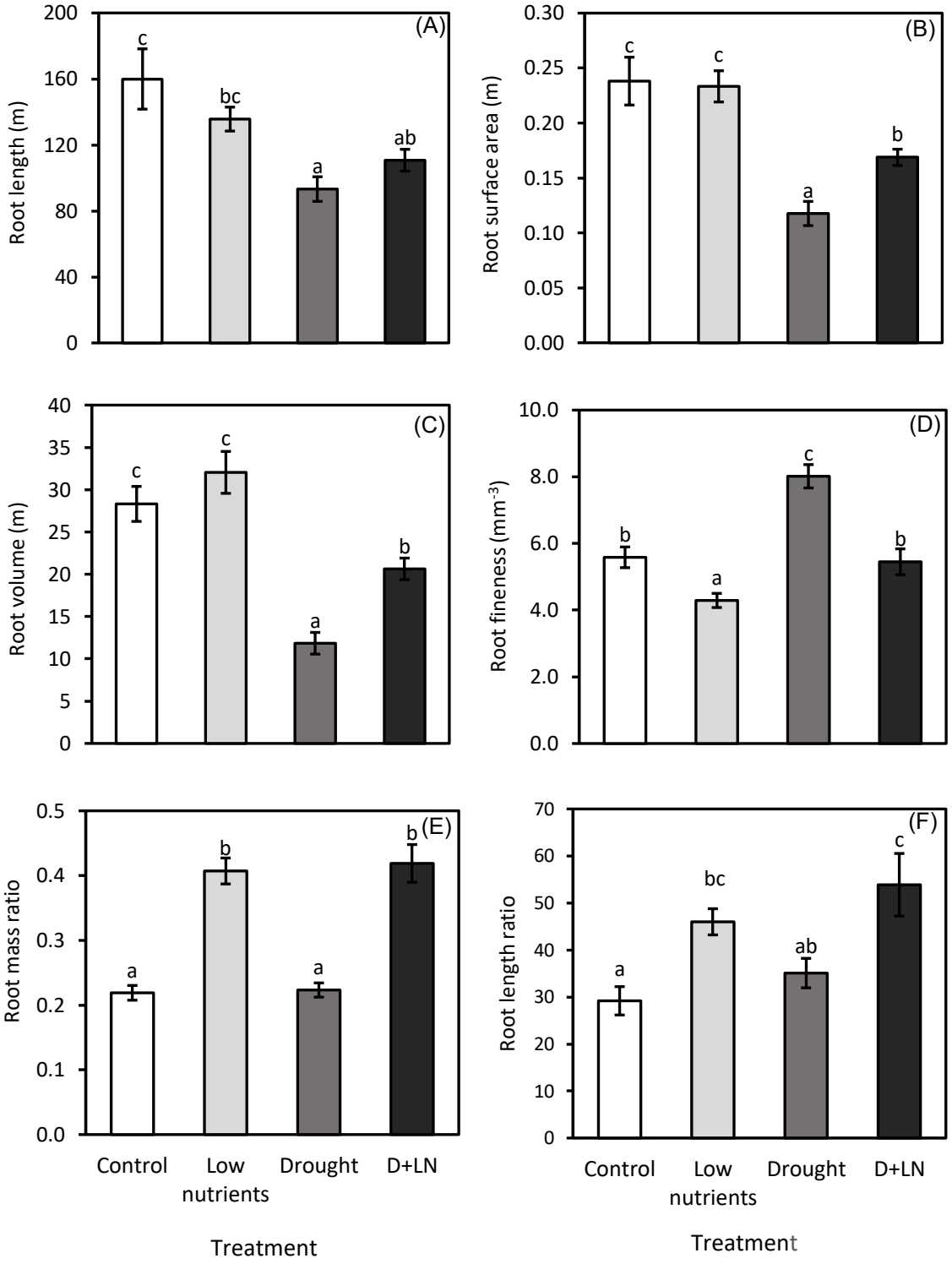

Figure 4.6: Effect of low nutrients and withholding watering on total root length (A), total root surface area (B), total root volume (C), root fineness (D), root mass ratio (E) and root length ratio (F) of chickpea at flowering growth stage. D+LN denotes combined-stress plants. Vertical lines on bars denote standard error. Different letters indicate significant differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 4 per genotype per treatment.

4.3.7 Nutrient content

There were no significant differences between genotypes and in the interactive effect between genotype and treatment for all leaf nutrient content measured (Table 4.2). Of the five macronutrients and five micronutrients measured, the effect of low nutrient supply and withholding watering was insignificant only in Mg and B concentrations.

For N and P, the combined-stressed plants recorded the lowest concentrations when compared to the control with the drought stress recording similar values as the control in nitrogen concentrations (Table 4.2). Potassium on the other hand, was reduced in the drought and combined-stressed plants. Ca, Fe, Cu, Zn and Mn shared a similar trend where plants under low-nutrient stress recorded increased concentrations compared to the control, but the concentration of these elements were unaltered in the drought and combined stress plants (Table 4.2).

Table 4.2: Nutrient content of chickpea at flowering growth stage. Data are means ± SE (n = 4 per treatment per genotype) followed by lowercase letters in a column indicate significant differences between treatments by Duncan's Multiple Range Test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns = not significant.

	Nitrogen %	Phosphorus %	Potassium %	Calcium %	Iron mg/kg	Copper mg/kg	Zinc mg/kg	Manganese mg/kg
Genotype								
ICCV 00108	3.94 ± 0.18	0.32 ± 0.03	2.21 ± 0.19	1.30 ± 0.11	184.5 ± 20.63	3.50 ± 0.38	73.0 ± 8.37	112.9 ± 12.55
ICCV 07113	3.94 ± 0.18	0.32 ± 0.02	2.29 ± 0.15	1.38 ± 0.05	234.5 ± 38.26	2.95 ± 0.20	71.8 ± 4.79	131.6 ± 13.78
Treatment								
Control	4.68 ± 0.19^{c}	0.42 ± 0.03^{c}	2.54 ± 0.18^{b}	1.12 ± 0.03^{a}	151.4 ± 14.45^{a}	2.48 ± 0.15^{a}	59.6 ± 2.80^{ab}	93.1 ± 7.43^{a}
Low nutrients	3.65 ± 0.12^{b}	0.29 ± 0.01^{b}	2.93 ± 0.16^{b}	1.64 ± 0.09^{b}	285.5 ± 59.40^{b}	4.59 ± 0.31^{b}	94.7 ± 3.45^{c}	162.8 ± 20.09^{b}
Drought	4.29 ± 0.12^{c}	0.35 ± 0.01^{b}	1.82 ± 0.07^{a}	1.23 ± 0.03^{a}	144.2 ± 16.10^{a}	2.52 ± 0.21^{a}	54.1 ± 4.23^{a}	88.3 ± 3.83^{a}
D and LN	3.14 ± 0.10^{a}	0.22 ± 0.03^{a}	1.71 ± 0.22^{a}	1.38 ± 0.19^{ab}	256.9 ± 47.10^{ab}	3.31 ± 0.49^{a}	81.2 ± 14.40^{bc}	144.9 ± 21.68^{ab}
F ratio								
Genotype	0.001^{ns}	0.0620^{ns}	0.184^{ns}	0.441^{ns}	1.535^{ns}	3.104^{ns}	0.022^{ns}	1.339^{ns}
Treatment	21.736***	14.760***	10.864***	3.999*	3.212*	10.208***	5.241**	5.344**
Genotype*Treatment	0.304^{ns}	0.921^{ns}	0.450^{ns}	0.204^{ns}	0.411^{ns}	0.904^{ns}	0.374^{ns}	0.035^{ns}

4.3.8 Grain yield

There were significant differences between treatments for all grain yield parameters (pod number, <0.000; pod weight, <0.000; seed number, <0.000; and seed weight, P<0.000) with genotype and the interaction of genotype and treatment recording no differences. All the stress conditions decreased plant yields (Figures 4.7A – D). However, although all three stress treatments reduced yield in comparison to the control, the yield of plants under the combination of both drought and low nutrient stress decreased the yield only by ~50%, whereas the yield under the drought treatment was reduced by ~80%.

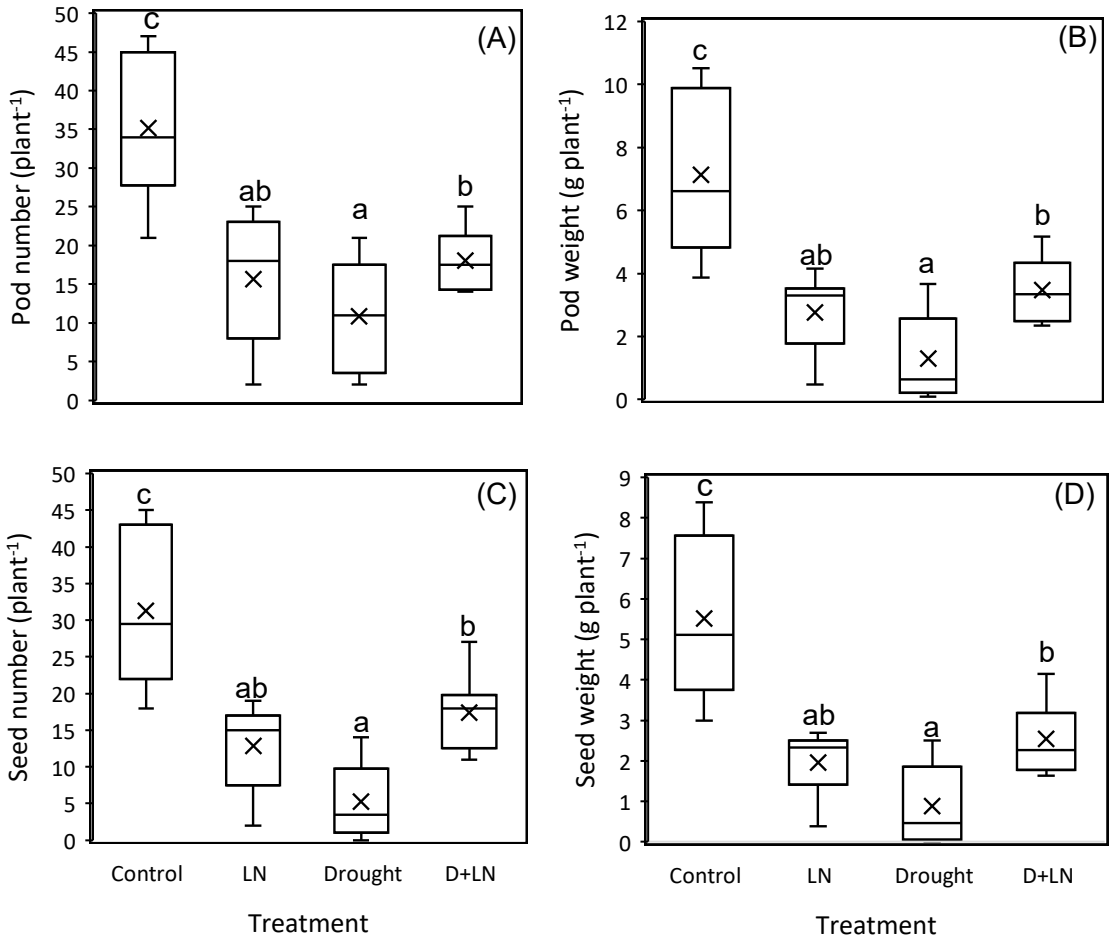

Figure 4.7: Effect of low nutrients and withholding watering treatments on pod number (A), pod weight (B), seed number (C) and seed weight (D) of chickpea at harvesting. LN denotes low nutrients and D+LN denotes combined-stress. The horizontal line and the 'x' in the middle of the box is the median value and mean marker respectively of the dataset for each treatment. The lower and upper boundaries indicate the 25th and 75th percentiles, respectively. The end of the whiskers shows the largest and smallest values observed. Lowercase letters indicate differences between treatments by Duncan's Multiple Range Test ($p < 0.05$). Sample size: n = 4 per genotype per treatment.

4.4 Discussion

Prior to the commencement of the drought stress treatment in this study, plants growing in soils containing low nutrient concentrations recorded a (~50%) reduction in plant shoot biomass. Lower nutrient concentrations in soils have been found to decrease shoot biomass in plants such as chickpea (Gülüt and Özdemir, 2021; Srinivasarao et al., 2006), sorghum (*Sorghum bicolor;* Zhao et al., 2005) and castor bean plants (*Ricinus communis*; Reddy and Matcha, 2010) growing under N and P deficiencies. On the other hand, nutrient deficiency was found to cause an increase in R/S ratios; that is, plants growing in the low-nutrient soils invested more resources belowground to the root system causing them to have a higher root biomass than those growing in fertile soils. The extensive root system developed in the low-nutrient plant group at this phase is in line with the literature (Gülüt and Özdemir, 2021; Reddy and Matcha, 2010; Srinivasarao et al. 2006) as decreasing nutrient levels are known to increase root development as an adaptive feature of plants growing in such environments. The increase in root length, root volume, root surface area, root mass ratio and root length ratio is therefore an attempt of plants to expand their belowground organs in order to gain access to larger soil volumes and possibly reach nutrient-rich pockets in the soil to increase their nutrient uptake.

Plants growing under the combined stresses of drought and low nutrients had higher R/S ratios than those under the single drought treatment. This meant that although water deficit caused a reduction in root length, surface area and volume, the low nutrient soil conditions of the combined-stress treatment counteracted the effect of drought stress to a degree, providing the combined-stress plants with a better-developed root system compared to the drought-only plants. Despite acquiring this adaptive feature, the prolific development of an extensive root system, a non-photosynthetic tissue, relative to the photosynthetic system (leaves) is a costly trade-off that reduces shoot biomass as more energy is diverted from the production of new photosynthetic tissues and used in maintaining the root systems (MacAlister., 2020). Thus,

although water limitation caused a reduction in leaf biomass in both plant groups, it was more pronounced in the combined-stressed plants.

According to Kumar et al. (2012), deep and well-developed root systems in chickpea plants are however effective for maintaining plant moisture due to the extraction of water by the deep roots from higher soil depths. Plants under the combined-stress treatment were therefore able to maintain a relatively higher leaf water content due to their prolific root systems. The evidence of this can further be seen in the g_s where the combined-stress plants had a relatively higher g_s throughout the 20 days of withholding water, indicating a lesser degree of stomatal closure compared to the drought-only group.

The sharp decline in gs and RWC for the drought-only plants by day eight suggests that CO_2 fixation may have been limited early and throughout the drought treatment. Reduced water and CO_2 availability are known to decrease photosynthetic rates, often due to imbalances in the photosynthetic electron transport chain (Taiz et al., 2015). Such imbalances can promote the formation of reactive oxygen species (ROS), which, when accumulated beyond the plant's antioxidant capacity, have the potential to disrupt cellular structures (Mittler, 2002). Although ROS were not directly measured in this study, the significant increase in proline in the drought-only group is consistent with patterns commonly observed in plants experiencing water limitation. Proline accumulation is widely recognised as part of a multifaceted stress-response system, functioning not only as an osmoprotectant but also as an osmolyte supporting osmotic adjustment, a stabiliser of proteins and membranes, and a readily mobilisable reservoir of carbon and nitrogen for post-stress recovery (Chen & Jiang, 2010). The elevated proline levels observed here therefore likely reflect the drought-related physiological strain experienced by the drought-only plants, consistent with findings in chickpea (Mafakheri et al., 2010) and rice cultivars (Nasrin et al., 2020).

The reduction of chlorophyll a, chlorophyll b and total chlorophyll concentrations in the low single stressed plants (i.e. low nutrient-only or drought-only groups) are expected as nutrient deficiency and water deficit are known to be associated with a reduction in chlorophyll concentrations. Previous studies carried out by Mafakheri et al. (2010) and Zhao et al. (2005) on drought-stressed chickpea plants and nutrient-deficit sorghum plants, respectively, both recorded reduced chlorophyll concentrations under the various stresses. Worth noting however in this study, is the lack of treatment effect on chlorophyll concentrations in the combined-stressed plant group as they recorded similar values as the control. This observed similarity, although unexpected, can be attributed to the similar carotenoid values recorded between the combined-stress and the control groups. Under stress conditions, carotenoids are known to offer photoprotection to chlorophyll molecules by dissipating excess excitation energy into heat via the xanthophyll cycle (Choudhury and Behera, 2001). This reduces the energy load on chlorophylls and minimizes photo-oxidative stress, thus stabilizing and preserving chlorophyll concentrations in the chloroplast (Demmig-Adams & Adams III, 2006). The increase of chlorophyll a/b ratio in the drought-only group is similar to the report of Saglam et al. (2014), and it indicates that plants under this stress group produced chlorophyll with fewer light-harvesting proteins, likely as a means to mitigate the stress of absorbing high amounts of light energy and essentially protecting the chloroplasts relative to the combined low nutrient and drought stress.

Grain yield was reduced by both water-deficit and low-nutrient stress treatments. However, the lowest reduction rate (~50%) was surprisingly recorded in the combined-stress plants relative to ~80% reduction from drought stress. True to our hypothesis, possessing a prolific root system gave an advantage to plants under the combined-stress treatment relative to only drought stress as they were able to maintain a relatively higher plant water status throughout the drought stress period.

The varying responses of the leaf's nutritional content to the various stresses were particularly interesting. The reduction in leaf N concentration of the plants under low nutrient stress can be correlated to the pre-existing nutrient deficiency present in their soil systems. This concurs with a previous study conducted by Song et al. (2019), who reported lower N concentrations with decreasing N treatments in *Pistacia chinensis.* On the other hand, the use of K by the plant's system under limited water conditions to mitigate the negative impact of drought stress (Fang et al., 2022) can be attributed to the reduced concentrations recorded in plant groups under water-deficit conditions. Additionally, the increase in the elements Ca, Fe, Zn, and Mn in plants under low-nutrient-soils could be a result of their lower shoot biomass. When plants experience increased shoot biomass accumulation due to conditions such as an increase in soil fertility, without a proportional increase in its nutrient uptake or concentrations, the nutrient content per unit of plant biomass decreases and this process is termed 'nutrient dilution' (Jarrell and Beverly, 1981).

From this study, both abiotic stresses, low-soil-nutrients and drought stress, negatively impacted plant growth and yield. Despite the two abiotic stresses sharing similar detrimental impacts on chickpea plant growth (i.e. reduced shoot biomass, reduced chlorophyll and carotenoid concentrations, and decreased plant grain yield), drought stress exerted a more severe effect on plant systems compared to low-soil-nutrients. Surprisingly, plants subjected to the combined stresses performed better than those under only one stressor. Interestingly, in agricultural practice, high nutrient application is typically used to maximise seed yield, often resulting in low root-to-shoot ratios. However, as demonstrated in this study, plants grown under such nutrient-rich conditions struggled under drought. These findings suggest that imposing low-nutrient conditions during early-generation screening could help identify genotypes that inherently invest more in root development when resources are limited, a trait that may confer improved drought tolerance. Such low-nutrient screening approaches are also

practical and cost-effective, making them suitable for early selection stages where large numbers of breeding lines must be evaluated. Therefore, breeding plants that can develop an extensive root system across varying soil fertility conditions could help alleviate the impact of drought in a changing climate.

4.5 Conclusion

Compared with plants under high nutrient soils, plants growing in low-nutrient soils had higher R/S ratios which translated to a well-developed root system, confirming the theory that R/S ratios increase with decreasing nutrient availability. The prolific root system developed by the plants exposed to low nutrient conditions was found to ameliorate the effects of drought stress when irrigation was withheld for an extensive period. The plants under the combined-stress consistently outperformed their drought stress counterpart growing in high nutrient soil in all physiological and morphological traits measured but for the aboveground biomass. Relative to the control plants, the reduction in grain yield was less pronounced in the combined-stress plants, yielding only ~50% losses compared to the ~80% losses recorded for the drought-only group. Therefore, the need to breed for crops that would develop extensive root systems in good soil fertile conditions, especially in the face of climate change is essential.

Chapter 5

Synthesis, conclusions and recommendations

Chickpea is a nutrient-rich crop vital for food security, but its production is significantly hampered by drought and heat stress, causing global yield losses of up to 50% and 30%, respectively (Awasthi et al., 2014). Rising greenhouse gas emissions are expected to intensify heatwaves and rainfall variability, compounding stress conditions for chickpea cultivation (IPCC, 2021). While research has explored drought and heat stress individually, their combined effects remain poorly understood, particularly in belowground systems. This research characterized root morphological traits associated with drought and heat tolerance in chickpea genotypes and their influence on physiological processes under individual and combined drought and heat stresses.

Across the three experimental chapters, a consistent pattern emerged: root traits played a central role in determining the degree of tolerance expressed by chickpea plants under different stress environments. The controlled environment study (Chapter Two) demonstrated the fundamental mechanisms of root and physiological responses to drought, heat, and their combination, while the field study (Chapter Three) highlighted how these mechanisms manifest under more complex and heterogeneous conditions. Chapter Four further explored whether low-nutrient-induced root proliferation confers a functional advantage under drought stress. Collectively, these studies showed that environments characterised by water limitation and high temperatures consistently selected for enhanced root development, and that genotypes capable of maintaining or improving critical root traits tended to perform better under such stresses. To provide a clear overview of how individual genotypes performed across the different experimental conditions, a summary table is presented below to allow quick cross-chapter reference.

Table 5.1: Summary of chickpea genotype performance across experimental chapters. The table provides a cross-chapter comparison of genotype performance under controlled stress, field environments, and low-nutrient-induced drought conditions.

Study / Conditions	**ACC#8**	**ICCV 07113**	**ICCV 00108**	**ACC#7**
Chapter 2 – Controlled Experiment (Drought, Heat, Combined)	Best performer; high biomass; stable pigments; maintained RWC except in combined stress; moderate root response.	Most sensitive; lowest RWC; low chlorophyll a; high Chl a/b; highest proline; high RL & RLR but poor performance.	Intermediate; highest RTD & RF; lowest RL; stable pigments; moderate RWC & biomass.	Excluded (<25% germination).
Chapter 3 – Field Study (Cool vs Hot Sites)	Weakest root traits; lower P_n & g_s; biomass reduced at hot site.	Strongest field performer; highest SA, volume, SRL, RLR; high chlorophyll; more tolerant in field.	Intermediate; variable nutrient trends; moderate root traits.	High chlorophyll at cool site; intermediate physiology; low root traits.
Chapter 4 – Low Nutrients + Drought Stress	Not included.	Weak LN-induced rooting; higher early RWC; stable chlorophyll under LN+Drought.	Strong LN-induced rooting; ↑ RL, SA, volume; high RMR; more drought-sensitive early.	Not included.

It was hypothesized that the development of an extensive root system would enhance plant growth under heat and drought stress. The findings generally supported this hypothesis: tolerant genotypes maintained or developed traits such as greater root length, surface area and volume, all of which contributed to improved water uptake and physiological regulation under stress. Although the specific traits differed depending on stress type, the overarching pattern was that chickpea relies on different combinations of root traits, rather than a single trait, to cope with drought versus heat. For example, drought tolerance was more strongly associated with below-ground biomass allocation patterns (e.g., high RMR, high RLR), while heat tolerance relied more on root structural efficiency and stability (e.g., RTD, SRL, RF). This confirms the notion that adaptation to drought and heat involves distinct, stress-specific root strategies rather than a uniform response.

Interestingly, plants subjected to combined drought and heat stress in this study (Chapter Two) exhibited enhanced root traits (SRL, RF, and RTD) similar to those of heat-stressed plants rather than drought-stressed plants. While drought and heat stress can elicit both shared and unique adaptive responses (Pandey et al., 2015; Awasthi et al., 2013), literature suggests that one stressor may sometimes suppress the adaptive mechanisms induced by the other due to antagonistic interactions (Sato et al., 2024). In this case, heat stress may have inhibited key drought-adaptive root traits, resulting in root characteristics that more closely resembled those of heat-stressed plants. Although this pattern was evident in the belowground system, physiological processes measured in aerial plant parts showed an additive response under combined stress, with significantly reduced values across measured parameters, resulting in severe growth limitations. Despite the heightened physiological strain on the combined stress treatment compared to individual stresses, grain yield did not follow this pattern. Instead, all stress treatments produced similar grain yields, suggesting that factors beyond these physiological responses influenced final yield outcomes. This suggests that factors beyond immediate physiological responses, such as improved assimilate remobilization (Farooq et al., 2017), enhanced root plasticity or shifts in water-use efficiency (Zaman-Allah et al., 2011), may have played a role in mitigating further yield losses. These results emphasise the complexity of plant responses under combined stress, where belowground and aboveground systems may not always follow the same adaptive trajectory.

In the field of agriculture, a long-standing debate concerns the reliability of controlled experiments and the extent to which their findings can be extrapolated to field conditions. The basis of this has been that while controlled environments allow for precise manipulation of variables, they do not fully capture the complexity of actual field conditions, leading to potential discrepancies between the knowledge gained by these studies and the knowledge required to develop plants and crops with enhanced tolerance to field conditions (Plessis, 2023;

Mittler, 2006). The comparison of Chapters Two and Three offered valuable insights into this issue. Although heat stress consistently impaired root development across both studies, the adaptive root traits expressed differed slightly between environments. In the controlled environment study, plants primarily developed RTD and RF, which contributed to maintaining root function under heat stress. In contrast, field-grown plants exhibited these same traits plus additional adaptations: SRL and RLR. This indicates that field conditions impose a broader set of selective pressures, revealing additional layers of root plasticity that may not manifest in controlled growth chambers. The development of SRL and RLR under field conditions suggests that these traits may be crucial for improving water uptake efficiency and nutrient acquisition in more complex and heterogeneous soil environments. The greater diversity of adaptive traits in the field study also underscores the limitations of controlled experiments in fully capturing plant responses to heat stress. While the controlled environment study confirmed the fundamental role of RTD and RF, the field study demonstrated that additional root modifications occur when plants are exposed to natural fluctuations in moisture, temperature, soil structure, and biotic interactions. Together, these findings reinforce the value of complementing controlled environment screening with field-based evaluations when identifying traits for breeding programmes, as combined approaches offer a more holistic understanding of plant resilience under agricultural conditions.

While general stress trends were consistent between the two studies, genotypic responses varied. In the controlled environment study, ACC#8 exhibited higher tolerance to stress conditions, whereas ICCV 07113 was identified as susceptible. However, the roles were reversed in the field study: ACC#8 displayed heightened sensitivity to heat stress, while ICCV 07113 performed comparatively better. This variation in response may be linked to disparities in the severity and duration of stress exposure under the controlled and field conditions (Wahid et al., 2007). In the controlled environment study, whereas heat stress was imposed a uniform

and abrupt manner, plants under the field conditions were exposed to more dynamic and prolonged stress fluctuations, which may have influenced their physiological responses differently. Nonetheless, this observation raises a critical question about the nature of plant stress responses, specifically, whether the physiological changes observed in experimental conditions represent heritable adaptive traits or context-dependent acclimation responses. While adaptation involves genetic modifications that confer stress tolerance across generations, acclimation consists of short-term physiological adjustments that may not be consistently expressed across different environments. Notably, both genotypes (ACC#8 and ICCV 07113) have been previously classified as susceptible to drought and heat stress (Makonya et al., 2020; ICRISAT- Kenya), suggesting that their observed shifts in tolerance reflect an acclimation response influenced by the contrasting environmental conditions in controlled and field studies rather than inherent genetic tolerance.

To determine whether the stress responses observed in this study represent true adaptive traits or short-term acclimation, future research should extend beyond single-season evaluations and incorporate multi-environment trials across contrasting temperature and rainfall regimes. In addition, evaluating root traits within early-generation breeding materials, such as F_1/F_2 mapping populations or recombinant inbred lines (RILs), under combined drought and heat stress would help identify heritable root characteristics associated with tolerance. Also, incorporating detailed physiological measurements with molecular analyses, including QTL mapping or genome-wide association studies (GWAS), would allow the identification of genomic regions controlling key root traits such as SRL, RLR, RTD and RF. Finally, long-term studies examining trait stability, plasticity and yield performance across generations would also help distinguish acclimation responses from genetically fixed adaptations. Such targeted investigations would provide breeders with robust trait-based markers for selecting chickpea genotypes with improved resilience to drought and heat stress.

Chapter Four further contributed to the synthesis by demonstrating that nutrient availability itself shapes root adaptive responses. Low-nutrient conditions consistently induced extensive and highly proliferated root systems, characterised by increased root biomass allocation, longer roots, and larger surface area, which later conferred a functional advantage when drought stress was imposed. Although the combined low-nutrient and drought treatment performed better than drought alone despite lower overall yields, demonstrating that prior investment in root proliferation can buffer the effects of subsequent water limitation. This introduces an important additional dimension to the study: root traits are not only shaped by immediate stress but can also be primed by earlier environmental conditions, which has implications for early-generation screening and breeding in resource-limited systems. Integrating the role of nutrient availability therefore adds a third dimension to the drought–heat framework explored in Chapters Two and Three and reinforces the central role of root plasticity in chickpea stress tolerance.

Overall, this research demonstrates that chickpea resilience to drought and heat stress is strongly dependent on the development, maintenance and strategic deployment of key root traits, many of which operate differently under distinct environmental constraints. By integrating evidence from controlled environments, field conditions and low-nutrient systems, the study reveals that no single root trait defines tolerance; rather, tolerance emerges from a coordinated suite of morphological adjustments that enhance water uptake, resource acquisition and physiological stability. The findings not only advance understanding of the belowground mechanisms underpinning chickpea stress resilience but also provide a clear framework for incorporating root traits into breeding programmes. As climate change intensifies the frequency and severity of drought and heat events, breeding strategies that deliberately target these functional root traits, supported by multi-environment testing and

early-generation root screening, will be essential for developing chickpea varieties capable of sustaining productivity in future agricultural landscapes.

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Appendix

Appendix Table 3.1: Environmental response of all four chickpea genotypes on stem biomass. Data are means ± SE (n = 3 plants per plot per genotype).

Site	Stem biomass (g)
Thohoyandou	3.99 ± 0.41
Nkomazi	3.06 ± 0.38

Appendix Table 3.2: Genotypic response to site differences on plant biomass. Data are means ± SE (n = 3 plants per plot per genotype).

Genotype	Leaf biomass (g)	Stem biomass (g)	Shoot biomass (g)	Root biomass (g)	Total biomass (g)
ACC#7	3.99 ± 0.41	2.76 ± 0.31	6.76 ± 0.68	0.40 ± 0.04	7.17 ± 0.71
ICCV 07113	3.06 ± 0.38	2.24 ± 0.29	5.31 ± 0.62	0.45± 0.05	5.76 ± 0.66
ACC#8	3.24 ± 0.34	2.44 ± 0.23	5.68 ± 0.56	0.32 ± 0.04	6.0 ± 0.59
ICCV 00108	3.84 ± 0.37	2.60 ± 0.23	6.45 ± 0.60	0.41 ± 0.04	6.97 ± 0.61

Appendix Table 3.2: Genotypic and site responses on RMR. Data are means ± SE (n = 3 plants per plot per genotype).

	RMR
Site	
Thohoyandou	0.07 ± 0.004
Nkomazi	0.05 ± 0.005
Genotype	
ACC#7	0.06 ± 0.004
ICCV 07113	0.08 ± 0.006
ACC#8	0.06 ± 0.005
ICCV 00108	0.07 ± 0.008

Appendix Table 4.1: Treatment response of the two chickpea genotypes on nutrient content of chickpea at flowering growth stage. Data are means ± SE (n = 4 plants per treatment per genotype).

Genotype	Root average diameter (mm)	SRL (m g^{-1})	RTD
Control	4.96 ± 0.54	135 ± 17	0.04 ± 0.003
Low nutrients	5.23 ± 0.31	113 ± 6	0.04 ± 0.003
Drought	4.01 ± 0.51	156 ± 10	0.05 ± 0.053
D and LN	4.64 ± 0.59	127 ± 10	0.04 ± 0.043

Appendix picture 2.1: Chickpea plants grown under controlled glasshouse conditions at the University of Cape Town.
(A) Two-week-old seedlings showing early vegetative establishment.
(B) Plants at the onset of flowering, displaying advanced vegetative growth and canopy development.

Appendix picture 2.2: Effect of treatment on root morphology (genotype: ICCV 00108). Non-stressed treatment (A), drought-only treatment (B), heat-only treatment (C) and combined drought and heat treatment (D).

Appendix picture 4.1: Effect of treatment on root morphology (genotype: ICCV 00108). Non-stressed treatment (A), low-nutrient treatment (B), drought-only treatment (C) and combined low-nutrient and drought treatment (D).