Investigation Into Physiology and Genetic Infrastructure Regarding the Impact of Heat Stress on Commercially Cultivated Chickpea

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1. Abstract

This thesis is the culmination of a multi-faceted investigation into the impacts, physiological management, and genetic architecture of heat stress and tolerance in chickpea (*Cicer arietinum*). Previous research in stress tolerance of this crop has had a predominant focus on disease, with heat stress research increasing in the last 10 years. Such research has typically focused on stress escape via accelerated development, primarily in the reproductive phase when the plant is the most vulnerable. Twelve field trials were conducted across two Australian growing regions, Narrabri (New South Wales) and Kununurra (Western Australia), in which a delayed sowing approach was used to create differentiated heat treatments. These were then paired with glasshouse trials in which heat stress was induced at different developmental stages. These experiments were collectively done to identify differences in physiology and trait performance when heat is induced at flowering versus podding.

Using a growing degree day model, the 2019 field trials were assessed to quantify temperature regimes according to both exposure time and intensity. 2019 was chosen as a model year, as the growing seasons did not experience confounding weather patterns, such as extreme storm or drought. This modelling was performed in order to detail the impact of heat stress on plant physiology, as well as yield and seed size. This was used alongside a genotype main effect plus Genotype by Environment (GxE) interaction model to determine each genotypes "preference" for a specific environment and identified the phenotypic plasticity of yield under heat stress, along with the genetic stability of seed size under the same conditions. Following this, glasshouse trials of top performing genotypes were assessed alongside that of the field trials to identify trait correlations that may be further investigated to inform future GAB programs. The hope is that through the identification of trait correlations, such as the positive correlation with yield and height, there can be a greater understanding of the growth and developmental phenology that underlies yield. Through this, it is possible that GAB will be more successful, as yield is seen to be a greatly variable and a phenologically plastic trait. It was also observed that the degree of phenotypic trait variation varied greatly between locations and treatments, particularly

yield which showed a greater variation in Kununurra where nutrient and water was more available.

Finally, single nucleotide polymorphism (SNP) sequencing was completed for all genotypes and combined with data from the field trials to complete a genome-wide association study. 14 Quantitative Trait Loci (QTLs) were identified for 5 physiological traits under either commercially typical, or heat stressed conditions. These were aligned with genes and QTLs related to heat stress in chickpea identified in previous studies. This is the first study to identify genomic regions linked with final canopy closure in chickpea, therefore the 3 QTLs linked to final canopy closure are novel. In addition, a flowering QTL was identified that is likely linked to earliness of flowering under heat stress. This is due to its co-location with a known heat stress transcription factor (HSF) under heat stressed conditions. This HSF is known to be linked to acceleration of flower and shoot growth under heat stress, and the co-location suggests that the QTL may be involved in the up-regulation of this HSF.

This thesis provides a great deal of value to the chickpea breeding community. The research involved in its development has uncovered novel tools and information, along with building on the existing knowledge of previous academics.

2. Declaration by Author

I declare this thesis to be composed of my original work, containing no material previously published or written by another person except where appropriate reference has been made within the text. I have made clear any contribution by other authors to works included in the thesis and stated their proportion of assistance to its production. This thesis does not include any work submitted to qualify for the award of any other degree in any other institution.

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3. Publications During Candidature

Peer-reviewed Journal Articles

Jeffrey, C., Trethowan, R., & Kaiser, B. (2021). Chickpea tolerance to temperature stress: Status and opportunity for improvement. *Journal of Plant Physiology*, 267, 153555. Doi:https://doi.org/10.1016/j.jplph.2021.153555

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Conference Presentations

Jeffrey C, Ziems LA, Kaiser B, Trethowan R (2022), Investigation into physiological impacts of heat stress on Chickpea (Cicer arietinum), and genomic mapping for breeding advancements, poster presented to the 16th Australasian Plant Breeding Conference 2022

Jeffrey C, Kaiser B, Trethowan R (2020), Looking for Tough Chickpeas: Determining Mechanisms of Heat Tolerance, oral presentation to the Sydney Institute of Agriculture's Research Symposium 2020

4. Publications Included in this Thesis

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Incorporated as Chapter 1

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Brent Kaiser (Principal Advisor)	Edited the paper (15%)
Richard Trethowan (Co-Supervisor)	Edited the paper (15%) Designed experiments (50%)
Angela Pattison (Auxiliary Supervisor)	Designed experiments (30%) Review of Literature (20%) Edited the paper (10%)

5. Contributions By Others to the Thesis

In addition to those cited in publications contributions to the thesis by others include:

- Dr. Urmil Bansal: DNA extractions for genotyping
- Isobella Revell: advice for data collection and management
- Jack Daniel: management of Kununurra trials and advice on crop management
- Helena O'Dwyer: data collection and management in Kununurra trials

Statement of Parts of The Thesis Submitted to Qualify for The Award of Another Degree

None

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Keywords

Legumes, Abiotic Stress, Heat, Climate, Degree Days, Breeding, Legumes, Abiotic Stress, Pulses, Heat, Future Directions, Quantitative Trait Loci

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List of Abbreviations

ANOVA	Analysis of Variance
BAC	Bacterial Artificial Chromosome
CIAT	International Centre for Tropical Agriculture
CV	Coefficient of Variation
DarT	Diversity Arrays Technology
DC	Drought Control
FAO	Food and Agriculture Organization of the United Nations
GDD	Growing Degree Day
GGE	Genotype by Genotype x Environment
GWAS	Genome Wide Association Study
GxE	Genotype x Environment
HSF	Heat Shock Transcription Factor
HSP	Heat Shock Protein
HSW	Hundred Seed Weight
ICARDA	International Center for Agricultural Research in the Dry Areas
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IPCC	Intergovernmental Panel on Climate Change
MET	Multi Environment Trial
MTA	Multi Trait Analysis
NACRA	Northern Australia Crop Research Alliance
PCA	Principal Component Analysis
PIC	Polymorphism Information Content
QTL	Quantitative Trait Loci
SDD	Stress Degree Day
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
TOS	Time of Sowing
WC	Water Control

1. General Introduction

1.1 Background

Chickpea is a crucial source of protein, iron, and starch globally, particularly in countries with largely vegetarian populations (Abbo, Berger, & Turner, 2003). Chickpea is considered to be one of the first grain legumes domesticated (Lev-Yadun, Gopher, & Abbo, 2000), however has been referred to as the "orphan legume" due to the lack of progress in cultivar improvement since the 1980s. Within the last 5 years, technology advances have transformed chickpea from an orphan crop to a genomic resource enriched crop (Srivastava, Dixit, & Singh, 2017). Research into improving the genetic architecture of this crop has primarily focused on disease tolerance (Abbo, Shtienberg, Lichtenzveig, Lev-Yadun, & Gopher, 2003), so there is a considerable knowledge gap to fill in order to produce new heat tolerant genotypes.

In the last decade, there have been great advancements in mapping the chickpea genome (Jeffrey, Trethowan, & Kaiser, 2021). There have been a number of markers found for physiological traits linked to heat stress, such as seed size, flowering time, and plant height (Jagdish Kumar & Abbo, 2001; Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014). However, many of these studies have been performed on only a small subset of genotypes. There is a continued need for research into the location, structure, and function of genomic regions involved in the stress response. While the average chickpea chromosome is roughly 12 % the size of a wheat chromosome, the latest genomic map consists of only 3339 Single-Nucleotide Polymorphisms (SNPs) (Farahani, Maleki, Mehrabi, & Kanouni, 2019), compared to the 119,000 SNPs included in one of the wheat arrays (Cui et al., 2017). The most recent comprehensive cross-population genome-wide marker map was produced using SNPs in 2013 (Rajeev K Varshney, Song, Saxena, & Azam, 2013) with the previous iteration produced with microsatellite Simple Sequence Repeats (SSRs) in 2000 (Winter, Benko-Iseppon, Hüttel, & Ratnaparkhe, 2000). Because of this, there is inconsistency in the markers used in research that has occurred since 2000, making it difficult to understand the relative locations of known Marker Trait Associations (MTAs) and Quantitative Trait Loci (QTLs). In addition to these larger maps, there have also been several smaller maps made, based on more simple parentage crosses, which can be used to further investigate genomic regions of interest (S. Gupta, Kumar,

Verma, & Bharadwaj, 2015; Jha, Nayyar, Palakurthi, & Jha, 2021; Kale, Jaganathan, Ruperao, & Chen, 2015; Mallikarjuna, Samineni, Thudi, & Sajja, 2017; Paul, Samineni, Thudi, Sajja, & Rathore, 2018).

1.2 Hypothesis and Aims

Overall hypothesis:

Heat-stress is life stage dependent, new physiological and genomic discoveries will enhance targeted breeding efforts regarding heat tolerance.

Overall thesis aim:

Understand genotype and lifestage-specific impact of heat stress in a diverse population of chickpea cultivars and build on the existing genomic breeding tools via genome-mapping.

- Determine the impact of heat stress on various life stages of a diverse population of chickpeas.
- Identify GxE interactions regarding heat stress response.
- Understand trait plasticity in more depth to inform future breeding efforts.
- Identify key QTLs involved in heat stress response.

1.3 Significance of Research

The latest Global Nutrition Report states that in 2020, 1 in 9 people worldwide were hungry (UNICEF, 2020), highlighting the need to safeguard such a nutritionally rich crop to ensure future food security (Atlin, Cairns, & Das, 2017). Additionally, the latest NASA models tracking the progression of climate change have determined that its impacts on the global agricultural industry are occurring faster than predicted (Jägermeyr, Müller, Ruane, Elliott, & Balkovič, 2021). Heat stress can cause a significant, and sometimes total loss of yield (Sanjeev Kumar et al., 2013). Therefore, there is a pressing need to develop hardy and resilient chickpea genotypes.

Breeding new cultivars to improve and protect crop production is not a new (Blum, 1988), and the last 20 years have seen great improvements in pulse genetic gains (Rajeev K Varshney et al., 2019). The majority of existing research has focused on heat escape and reproductive development rather than investigating traits that allow plants to manage their own stress response and endure high temperature stress (Jeffrey et al., 2021). This research aims to examine the genetics of heat stress over a wider scope to gain further understanding of the issue and find new directions for future research.

1.4 Methodology

Using a delayed sowing model in two contrasting environments, a diverse population of 164 chickpea genotypes were grown and studied under commercial cropping conditions annually for a total of 3 years. The data from these trials was then used to select a subset of genotypes for controlled-environment study to examine plant development in greater detail to further the understanding of physiological impacts of heat stress. Additionally, a "model" year was selected to calculate the extent of heat stress required to cause an impact of the plants and identify any ameliorating factors. Finally, data collected from field trials was used alongside DArTseq sequencing data for each genotype to identify QTLs involved in the growth and development of these plants under their respective climates.

1.5 Thesis Outline

This thesis seeks to address the issue of heat stress on chickpea under commercial conditions through as wide a lens as possible. This is to ensure an extensive understanding of both the issue itself and the identification of a number of possible solutions to pursue. The goal is to study heat stress in chickpea through physiological, genetic, and climatological perspectives, though specific weight has been given to the capacity of genomic resources to manage and improve the current risks posed by climate change.

2. Chickpea Tolerance to Temperature Stress: Status and Opportunity for Improvement

2.1. Abstract

Chickpea is a globally important commercial crop and a key source of protein for vegetarian populations. Though chickpea was domesticated at least 3000 years ago, research into abiotic stress tolerance has been limited compared to cereal crops such as wheat. This review investigates the impacts of heat stress on chickpea, focusing on reproductive development. The fertilisation process is particularly sensitive to environmental stress, such as drought and heat that can reduce yields by up to 70%. Current research has largely focused on breeding cultivars that reach maturity faster to avoid stress rather than true thermotolerance and little is known of the impact of heat on cellular processes. This review suggests that there is ample variation within the chickpea gene pool for selective breeding to achieve improved abiotic stress tolerance. Rates of genetic progress will improve once key QTL are identified and the link between thermotolerance and pollen viability confirmed. Other benefits may arise from better understanding of heat shock proteins and molecular chaperones and their role in the protection of reproductive processes.

2.2. Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated true diploid that can be classified into two types: the desi, characterised by small purple flowers and small seeds, and the kabuli, known for its white flowers and large, pale seeds (Blum, 1988). Cultivated chickpeas are an excellent source of protein, beta-carotene, and minerals like P, Ca, Mg, Fe, and Zn, and they are a crucial source of protein and starch in countries with large vegetarian populations (Abbo, Berger, et al., 2003).

Globally, more than 2 billion people suffer from micronutrient deficiency, particularly anaemia, Zn, and vitamin A (Ritchie & Roser, 2017). It is thought that research, development, and adoption of new crop varieties with improved yield and nutrition will be an ameliorating factor, especially in South Asia and Sub-Saharan Africa (Atlin et al., 2017).

Chickpea is a rich source of protein, Fe and Zn where increased consumption as part of a balanced diet can alleviate malnutrition. Additionally, chickpea has several beneficial agronomic benefits, including adaptation to low-nutrient soils, tolerance of dry periods, readily grown on stored soil moisture, and efficient nitrogen extraction from soil, at 80% of total requirement (S. Sharma, Upadhyaya, Roorkiwal, Varshney, & Gowda, 2013). Thus inclusion of chickpea in the cropping system can reduce nitrogen inputs, as its symbiotic relationship with nitrogen-fixing bacteria within the root system increases the access of bioavailable nitrogen, decreasing the need for additional fertiliser, making it economical and beneficial to the environment (Jagdish Kumar & Abbo, 2001).

Chickpea seems unusual among other cool-season legumes, in that it is already relatively tolerant to warmer climates (Wery, Turc, & Lecoeur, 1993). However, while chickpea is adapted to cultivation in low-moisture systems and possesses some heat tolerance, it is believed that abiotic stresses, including drought and heat, cause over 70% of global yield losses (Rajeev K Varshney et al., 2019), and in extreme circumstances heat alone can cause a total loss of yield (Sanjeev Kumar et al., 2013). The International Centre for Tropical Agriculture (CIAT) believes that new cultivars could improve product quality and yield, directly benefitting small-scale farmers as well as larger industrial operations (Blum, 1988). Significant advances in genetic trait mapping over the past 15 years has lifted rates of genetic gain in several pulses, including chickpea (Liu, Gan, Warkentin, & McDonald, 2003; Rajeev K Varshney et al., 2019). Currently, most high temperature related research focuses on heat escape, including traits such as early flowering and maturity, rather than the physiological basis of heat tolerance (Jha et al., 2021).

Selective breeding for tolerance to stress has generally focussed on stages when the plant is producing flowers, pods, or in both reproductive stages, as this is when plants are most vulnerable, and thus yield losses are greatest (Charles & Harris, 1972; Zinn, Tunc-Ozdemir, & Harper, 2010). However, sexual reproduction studies are often difficult to quantify, as the processes involved are complex, short-lived and unpredictable, and predominately difficult

to observe and characterise, being hidden within the reproductive tissues of the plant (Zinn et al., 2010). As such, efforts need to be made to further this research as much as possible.

2.3. Domestication

Chickpea is thought to be one of the first grain legumes to be domesticated in the "old world" originating in the fertile crescent 10,000 years ago (Lev-Yadun et al., 2000). The genus Cicer, is classified into 3 gene pools based on the crossability of the 43 wild species (Blum, 1988), and the wild progenitor of the cultivated chickpea (*Cicer arietinum*) is thought to be *C. reticulatum,* a rare species reported from only 18 narrow pockets in South-eastern Turkey (Abbo, Berger, et al., 2003). It is thought that *C. reticulatum* was domesticated and cultivated in several locations, including Syria (7250-6750BC), Turkey (6700BC), and Jericho (8350-7370BC), and there is ample documentation of cultivation in Egypt and the Middle East from 3300 BC (Abbo, Berger, et al., 2003). It appears that the species migrated from the fertile crescent to India, followed by East Africa and Central Asia (Abbo, Berger, et al., 2003).

During domestication, chickpea overcame several genetic bottlenecks as farmers selected desirable traits for production and quality. The initial concern was disease susceptibility. This led to cropping on residual soil moisture to limit vegetative growth to avoid disease (Abbo, Berger, et al., 2003; Blum, 1988). Nutritional benefits were and continue to be largely unselected where the crop has evolved in accordance with the needs of production and reliability (Blum, 1988). Although chickpea evolution has progressed, Abbo (2003) has suggested that four key bottlenecks still remain:

1. Restricted distribution of the genetically diverse *C. reticulatum* compared to other Neolithic crops. The progenitor of the modern chickpea was restricted to South East Turkey. Therefore the original species gene pool was genetically limited compared to progenitor crops like wheat, which benefited from a much larger regional distribution (Abbo, Berger, et al., 2003).
2. Domestication: *C. reticulatum* demonstrated higher diversity compared to *C. arietinum* using isozyme (Labdi, Robertson, Singh, & Charrier, 1996) and microsatellite markers (Choumane, Winter, Weigand, & Kahl, 2000), suggesting that as *C. arietinum* evolved there was a loss of allelic diversity.

3. Current reliance on stored soil moisture rather than mid-season rainfall (Abbo, Berger, et al., 2003). It is assumed that when chickpea moved from a West Asian to Mediterranean environment prior to the bronze age (Jagdish Kumar & Abbo, 2001), the species was then bred for a spring sowing rather than its original autumn pattern in order to avoid damage by disease such as Aschochyta blight.
4. Creation of elite but fragile cultivars due to farming pressure (Tanksley & McCouch, 1997). It is thought that the varieties of *C. arietinum* currently cultivated contain only 20% of the genetic diversity held by its wild progenitors (Rajeev K Varshney et al., 2019).

2.4. Climate and Production

Chickpeas are a major global crop, making up ~17% of total pulse production according to official FAO records (FAOSTAT, 2023). While chickpea has been grown in over 50 countries worldwide (Blum, 1988), and over 15 million tonnes were produced in 2021 (FAOSTAT, 2023), the industry faces challenges, with slow production growth over the last 60-70 years and changes in food habits globally. Attempts have been made to systematically increase pulse production and productivity globally with little success. This challenge is becoming increasingly difficult with increasing urbanization and loss of cultivated land, rising temperatures and increased drought frequency and intensity (Jitendra Kumar, Choudhary, Solanki, & Pratap, 2011).

Human-driven climate change remains a key risk to the agricultural industry overall, with the global mean departure year (the year when the projected mean climate moves to a state permanently outside historical bounds) sitting at 2069 ± 18, if global emissions are stabilised, and 2047 ± 14 if not, (Mora, Frazier, Longman, Dacks, & Walton, 2013). In Australia, there has been an increase in both day and night temperatures, as well as an increase in the frequency of extreme heat events and the frequency and severity of drought conditions (CSIRO, 2018). In the latest Intergovernmental Panel on Climate Change (IPCC) climate report, it has been determined as “established fact” that there is an increased frequency and severity of weather and climate extremes since 1850, particularly for high temperature events (IPCC, 2021). There has been an uptick in research focus regarding heat stress in chickpea (Arriagada, Cacciuttolo, Cabeza, Carrasco, & Schwember, 2022; Karalija et

al., 2022). Thus to safeguard the crop, genetic diversity for heat tolerance must be packaged into varieties that also increase yield potential, end user quality, and agronomic fit to common cropping systems so that wide-spread adoption can be achieved (Atlin et al., 2017).

2.5. Room to Move with Genetics

Genetic diversity in chickpea was eroded by domestication, a genetically narrow progenitor with limited geographic distribution, and changes in seasonal production during domestication (Tanksley & McCouch, 1997). It is estimated that 80% of this diversity has been lost in modern cultivars (Rajeev K Varshney et al., 2019). While it would be expected that domestication and subsequent selective breeding would decrease genetic diversity, limited molecular marker studies have identified genomic regions linked to traits such as drought and heat tolerance in modern cultivars (Thudi, Upadhyaya, et al., 2014a; Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014; Rajeev K Varshney, Thudi, Nayak, & Gaur, 2014; Zinn et al., 2010). Early breeding efforts were often focused on disease and cold resistance (Tanksley & McCouch, 1997). Therefore, with the increased risk of heat stress through global climate change (CSIRO, 2018), it has been considered beneficial in the last two decades to increase focus into heat and drought tolerance (Devasirvatham, Tan, Gaur, & Trethowan, 2015; Maphosa, Richards, Norton, & Nguyen, 2020).

In tandem with advances in chickpea genomics, large scale molecular markers, transcriptomic resources and genome sequences have been developed (P. Jain, Ramgiry, & Singh, 1998; Rajeev K Varshney et al., 2013). These resources include: (1)> 3,000 SSR markers (Nayak, Zhu, Varghese, Datta, & Choi, 2010; Thudi et al., 2011); (2) 15360 DarT loci (Thudi et al., 2011); (3) > 3000 SNP markers (Gaur, Azam, Jeena, & Khan, 2012; Hiremath, Kumar, Penmetsa, Farmer, & Schlueter, 2012); (4) Genetic maps (Gaur et al., 2012; Gujaria, Kumar, Dauthal, & Dubey, 2011; Hiremath et al., 2012; T Millan, Winter, JŘngling, & Gil, 2010; Thudi et al., 2011); (5) Hundreds of QTLs for many tolerance traits such as salinity (Vadez et al., 2012), drought tolerance (Jaganathan, Thudi, Kale, Azam, & Roorkiwal, 2015; Kale et al., 2015; Rajeev K Varshney, Thudi, et al., 2014), Ascochyta blight (Anbessa, Taran, Warkentin, & Tullu, 2009), and fusarium wilt (Udupa & Baum, 2003); (6) The creation of several draft genome sequences (Sonal Gupta, Nawaz, Parween, & Roy, 2017; M. Jain,

Misra, Patel, Priya, & Jhanwar, 2013; Ruperao et al., 2014; Rajeev K Varshney et al., 2013); (7) Discovery and mapping of drought responsive genome regions (Jaganathan et al., 2015; Kale et al., 2015); (8) Development of a genome-wide physical map (Rajeev K Varshney, Mir, Bhatia, & Thudi, 2014); (9) Comprehensive characterisation of a chickpea reference set with > 16,000 markers, detection of 917 SSRs, and an allele frequency and mean heterozygosity of 0.21 and 0.04 respectively (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014) and (10) genetic map made of 1,291 markers on eight linkage groups by DarT, made of 46,270 BAC-end sequences (Thudi et al., 2011).

In the last decade, two bacterial artificial chromosome (BAC) libraries were constructed from high molecular weight DNA and these used to estimate a genome size of 738MB and identify 654 genes in a QTL hotspot that housed a subset of genes linked to drought avoidance, via enhanced transpiration efficiency (Rajeev K Varshney, Mir, et al., 2014). As of 2012, more than 10,000 SNP markers were available for accelerating genetic research in chickpea (Hiremath et al., 2012). More recently, Farahani utilised a high density SNP analysis method created by DarT with 1,291 marker loci (Roorkiwal, Rathore, Das, Singh, & Jain, 2016), for genetic diversity, population structure, and linkage disequilibrium analysis for 186 chickpea genotypes, made of 11% desi landraces and 89% advanced kabuli breeding lines (Farahani et al., 2019). Building on earlier work (R. Singh, Singhal, & Randhawa, 2008), 1152 SNPs were selected for diversity analysis from an initial dataset of 3339 SNPs, with 144 SNPs per linkage group on average (Farahani et al., 2019). Heterozygosity observed with these SNPs was less than 0.2 in 75% of genotypes, which was expected due to the self-pollinating nature of chickpeas (Farahani et al., 2019). Research on 320 elite desi and kabuli breeding lines showed polymorphism information content (PIC) ranging from 0.01 – 0.38 with a mean of 0.19 for DarT-SNPs, and a gene diversity of 0.01 – 0.5 for SilicoDArTs (Roorkiwal et al., 2016).

While these markers are available, they have little practical value without reliable and relevant phenotypes for heat tolerance and a fuller understanding of the physiological mechanisms of stress tolerance and avoidance. The exception being earliness in flowering; an adaptation for heat escape, which is relatively well understood (Toker & Canci, 2007). At least 3 loci affect flowering time in chickpea (Weller, Reid, Taylor, & Murfet, 1997). This

includes a major recessive gene labelled *efl-1,* for early flowering from a cross between an extra early (ICCV2) and medium (JG62) flowering variety which was responsible for a 3 week difference in flowering time between parents (Jagdish Kumar & Abbo, 2001). There have been SNPs found with significant associations with traits such as 100-seed weight, plant height, and pods per plant, and these can be used for molecular breeding to produce tolerant varieties (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014). Recently, 122 candidate gene domestication regions and 204 genes have been found that underwent selection during the domestication process, and these can be investigated to shed light on the mechanisms controlling physiological response to stress (Rajeev K Varshney et al., 2019).

More recently, a number of studies have been conducted regarding physiological impacts of heat stress on the offspring of a number of specific parental crosses. These studies provide valuable pilot information that can further inform species-wide genome discovery and enhance breeding efforts (Barmukh et al., 2021; Jha et al., 2021; Rezaei et al., 2019; Yadav, Juneja, & Kumar, 2021)

However, a few factors have limited genetic research in chickpea compared to other grain crops. Seed multiplication produces just 10-30 seeds per plant compared with up to 1000 for wheat, delaying cultivar release while seed producers multiply sufficient seed for market (K. B. Singh, 1997). Genotype by Environment (GxE) interactions control 70-80% of yield variation in chickpea (S Kumar & Ali, 2006) and indeterminate growth makes it more difficult to visually assess genetic variation compared to crops such as wheat (K. B. Singh, 1997). Therefore, further research and assessment into existing lines should be conducted to bring the crop into line with the advances in crops such as wheat.

2.6. Even Tough Crops Have Their Limits

The difference between potential and actual yield of cultivated crops is typically defined by the presence of stress. Dudal (1976) estimated that only 10% of the world's arable land can be considered free of stress. Of these stresses, temperature is most limiting to crop distribution and the interaction of temperature with photoperiod makes it difficult for plants to adapt to large changes in temperature (Blum, 1988). Temperature impacts

phenology, reproductive development, growth rate and yield (Blum, 1988). As climate change forces a redistribution of crop cultivation areas, improved understanding of temperature and photoperiod responses will help mitigate these effects (Berger et al., 2011). Climate change has many impacts on commercial crops, including but not limited to inappropriate phenology following permanent changes in climate (Miglietta, Tanasescu, & Marica, 1995). For example, Indian chickpea cultivars will flower much earlier when stressed (Berger et al., 2011), speeding their development and risking a reduction in yield (Boote, Allen, Prasad, Baker, & Gesch, 2005); growing season length will decline due to drought (Nelson, Berger, & Erskine, 2010), and in extreme cases, a change in geographic distribution may require a completely new phenology to that currently grown (Berger et al., 2011). Therefore, it may be difficult to identify suitable material for adaptation to new climates. However, chickpea is a robust species, and there is likely a cache of adaptation/tolerant genes residing in the genome of the *C. arietinum* progenitor (Abbo, Berger, et al., 2003).

2.6.1. Factors Associated with Heat Stress

As crops typically experience several stressors in the same season, such as drought, heat, salinity, alkalinity, etc., it is necessary to account for phenology and its role in the stress response so that specific physiological, biochemical and molecular characteristics can be identified and selected (Stoddard et al., 2006). Heat stress (HS) is affected by many factors, and different growth stages may have different temperature optima (Dwyer & Stewart, 1986). Growth and development follow sigmoidal temperature response curves, showing a plateau at the optimum temperature, where anything above this is considered a stress temperature (Blum, 1988). For chickpea, this optimum temperature is dependent on the physiological process considered and the plant structure or organ; however, it is generally considered that temperatures of 35/25 °C (Table 1.) (Sanjeev Kumar et al., 2013) cause damage. Important plant processes like nitrogen fixation and photosynthesis have an optimum of 22 °C (Dart, Islam, & Eaglesham, 1975; K. B. Singh, 1997) and are particularly vulnerable to high temperature.

Factors linked to heat stress include:

1. **Evapotranspiration.** According to Thornthwaite and Mather (1955), plant stress is essentially a function of the balance between supply and demand of water, and this is impacted by solar radiation, humidity, soil, and vegetation properties, which all impact the availability of water for transpiration. During drought, heat stress can be exacerbated as reduced transpiration leads to canopy heating by as much as 5-7 °C (Blum, 1988).
2. **Canopy climate.** The canopy is the site of reproductive organ exposure, and controlled by leaf temperature, which is ultimately controlled by leaf size, angle, surface properties, and wind velocity (Gates, 1968). Characteristics controlling canopy climate are predominately responsible for heat avoidance (Wery et al., 1993).
3. **Soil temperature** of the surface layer may act as a heat sink, depending on reflectance of sunlight by the soil (albedo) and water content (Blum, 1988). This can be exacerbated or mitigated by the level of canopy closure and sun penetration to the soil surface (Gates, 1968). Soil temperature will control factors like nitrogen fixation, leading to impacts on vegetative growth (Lewis, Noctor, Causton, & Foyer, 2000).
4. **Earliness (Early Maturity)** is considered the most important trait for resistance to drought and heat stress as it is an escape rather than a tolerance mechanism (Toker & Canci, 2007). However, earliness is risky if both drought and heat occur simultaneously (Turner, Wright, & Siddique, 2001). In Australian conditions, early maturing chickpeas have higher yields compared to late when water is limiting and temperature is optimal, but in climates with adequate water, late maturing genotypes have higher yield potential (Sabaghpour, Kumar, & Rao, 2003). Therefore, the success of earliness is dependent on water availability during reproduction and pod filling, and is a benefit only in low-water scenarios (Devasirvatham, Tan, Trethowan, Gaur, & Mallikarjuna, 2010), potentially due to a decreased transpiration potential (Mathur, Agrawal, & Jajoo, 2014).
5. **Growth vigour** is controlled by 2 genes with duplicate dominant epistasis; Gv1 and Gv2 (Sabaghpour et al., 2003). There is a positive correlation between growth vigour, 100-seed weight and leaf size (Narayanan, Saxena, & Sheldrake, 1981; Raje, 1992; Sabaghpour et al., 2003; Van der Maesen, 1987), as well as a positive correlation

between seedling vigour and early maturity (P. Gupta, Kumar, Mir, & Kumar, 2010; Sabaghpour et al., 2003; K. B. Singh, 1997). Productivity is likely dependent on efficient utilisation of soil moisture. As the majority of chickpea crops are grown on stored moisture (J Kumar & Van Rheenen, 2000), early growth vigour should reduce evaporative losses from bare soil surfaces, thus improving water use efficiencies (P. Jain et al., 1998).

2.7. Heat Stress in Chickpeas

It is interesting to note that chickpea defies the stereotype of other cool season legumes, being relatively tolerant to warmer climates (Wery et al., 1993). The prevailing theory is that strong selection pressure during the migration of the crop selected for earlier and more tolerant genotypes (Berger, Ali, Basu, & Chaudhary, 2006). As chickpea moved to Indian and African environments, strong photoperiod responses were maladaptive, and in selecting for a diminished response, the farmers of the bronze age may have inadvertently selected for some level of temperature tolerance (Jagdish Kumar & Abbo, 2001).

Temperature is a key determinant of crop growth (Summerfield, Virmani, Roberts, & Ellis, 1990). As temperatures rise, brief exposure has the potential to cause severe and irreparable damage. However, damage to the plant is not universal, as heat stress damage is a function of time, temperature and the process impacted. Heat stress causes an acceleration of growth and development which can cause significant reductions in yield, and this may lead to senescence rather than the physiological perturbations alone (Blum, 1988).

Heat tolerance is likely polygenic (H. D. Upadhyaya, Dronavalli, Gowda, & Singh, 2011), and plant adaptation mechanisms can be classified into three groups, escape, avoidance and tolerance (Wery et al., 1993). Escape, is controlled by early phenology (Toker & Canci, 2007), where flower initiation and plant maturation occur early and rapidly, so that plants mature prior to the onset of high temperatures (Toker & Canci, 2007). This is particularly relevant for Mediterranean and South Indian chickpea environments (Berger et al., 2011). Avoidance, refers to leaf reflectance, orientation and transpiration to avoid the direct impacts of the sun and the accumulated heat (Wery et al., 1993). This is an area that has not been

extensively addressed in chickpea. Tolerance, is linked to membrane stability, alteration of membrane lipid composition, and heat shock proteins (Blum, 1988).

Heat stress has been studied extensively for impacts on growth stages, morphology, phenology, physiology and agronomy in wheat (P. Sharma, Sharma, & Deswal, 2005), rice (Weerakoon, Maruyama, & Ohba, 2008) and cotton (Cottee, Tan, Bange, Cothren, & Campbell, 2010). We have information about the cardinal temperatures for a large number of processes in chickpea (Sanjeev Kumar et al., 2013; Shah et al., 2021), but this research is often based on small numbers of genotypes (Berger et al., 2011). Much of the research in chickpea focusses on heat escape through early flowering and maturity rather than tolerance (Dua, 2001) combined with physiology and transcriptomics rather than deeper metabolic processes (R. R. Kumar, Goswami, Sharma, Singh, & Gadpayle, 2012; Wang, Gan, Clarke, & McDonald, 2006). However, it has long been known that early flowering tends to reduce biomass accumulation and root system growth, which subsequently reduces yield potential and renders plants vulnerable to drought (Johansen et al., 1997). Plants experiencing heat stress are found to have higher chlorophyll content than un-stressed plants (Bhasker, Nandwal, Kumar, & Chand, 2018). It is also suggested within this thesis that heat stress can cause a remobilisation of carbohydrates, altering seed number and size, particularly when experienced during flowering (Chapter 4).

Nevertheless, some early flowing genotypes were observed to have equivalent yield but lower biomass than later flowering adapted materials, indicating a higher harvest index (Jagdish Kumar & Abbo, 2001).

2.7.1 Reproductive Processes

Heat stress during the reproductive phase in crops can lead to the following: (1) Impaired micro-sporogenesis and megasporogenesis (Porch & Jahn, 2001), (2) loss of pollen viability (Sanjeev Kumar et al., 2013), (3) poor pollen germination, and pollen tube growth (Sanjeev Kumar et al., 2013), (4) loss of stigma receptivity (Sanjeev Kumar et al., 2013), (5) loss of ovule function (Devasirvatham et al., 2010), (6) limited embryogenesis (Sun, Van Montagu,

& Verbruggen, 2002), (7) reduced ovule number (Devasirvatham, Gaur, Mallikarjuna, Raju, & Trethowan, 2013), and (8) increased ovule abortion (Devasirvatham et al., 2010) (Table 2.1).

The most In-depth phenological and biochemical study conducted on chickpea under heat stress conditions was that of Kumar et al (2013). The results are summarised below:

Table 2.1: The responses of different processes and characteristics to increasing temperatures of four commercially available chickpea cultivars (Sanjeev Kumar et al., 2013).

		30/20 °C	**35/25 °C**	**40/30 °C**	**45/35 °C**
Dry biomass (g/plant)	S	6.56-7.11	7-10% increase	13-18% decrease	38-41% decrease, greater chlorosis and necrosis of leaves
	T	6.46-6.96	6-14% increase	8-9% decrease	24-25% decrease
Pods/plant	S	20.4-20.8	7-15% increase	37-45% decrease and 28-37% fewer pods than tolerant	No pods
	T	19.4-21.5	14-15% increase	15-16% decrease	79-84% decrease, 3-4.5 pods plant^{-1}
Unfilled pods/plant	S	No unfilled pods		Unfilled pods increased, especially for sensitive	No pod set
	T				More unfilled pods
Seed dry mass (g/plant)	S	4.21-4.6	11-17% increase	54-56% decrease	No seeds
	T	4.32-4.98	4-16% increase	26-32% decrease	83-87% reduction
Harvest index	S	64.1-64.6	4-6% increase	35-41% decrease	No pods
	T	63.9-71.5	9-10% increase	19-25% decrease	77-83% decrease
Chlorophyll (mg/g FW)	S	4.86-5.31	No change	36-38%% decrease	50-52% decrease an 22-32% less chlorophyll than tolerant
	T	5.42-4.64		4-20% decrease	27-43% decrease
Membrane damage (%)	S	9.1-10.1	5-24% increase	180-190% increase	230-234% increase
	T	8.9-10.4	11-16% increase	87-88% increase	117-131% increase
Cellular oxidising ability	S	0.16-0.2	35-38% increase	50-106% increase	37-39% decrease
	T	0.16-0.18	28-44% increase	111-156% increase	29-32% decrease
Photochemical efficiency (Fv/Fm ratio)	S	1.4-1.5	0-14% increase	59-63% decrease	74-78% decrease
	T	1.3-1.5	-13-23% increase	25-39% decrease	55-65% decrease
Pollen viability %	S	90.6-91.3	7-9% decrease	10-11% decrease	67-73% decrease, 25-30% viability
	T	89.6-90.1	No change	8-10% decrease	53-62% decrease, 34-43% viability
Pollen germination %	S	88.1-90.2	4-7% decrease	36-43% decrease, 51-56% germination	77-80% decrease, 18-20% germination
	T	87.6-89.4	2-3% decrease	15-18% decrease, 71-76% germination	53-57% decrease, 38-42% germination

Pollen tube length (μm)	S	17	8-14% increase	39-51% decrease	No tube growth
	T	18	-3-15% increase	11-18% decrease	59-61% decrease
Stigma receptivity (esterase test, 1-5 scale)	S	4.2-4.4	2-4% increase	59-60% decrease	83-87% decrease
	T	4.1-4.6	-10-7% increase	24-43% decrease	65-66% decrease
Pod set %	S	86.1-88.2	1-2% increase	59-68% decrease,28-35% pod set	No pod set
	T	85.4-87.5	-3-2% increase	34-41% decrease, 51-56% pod set	73-79% decrease
Oxidative stress	S	Unaffected		- MDA content 29-32% higher - H2O2 18-21% higher	- MDA content higher by 40-49% - H2O2 almost twice the level than tolerant
	Both			Increased antioxidant levels	Significantly decreased antioxidant levels

Two heat "sensitive" (ICC14183, ICC5912) and "tolerant" (ICCV07110, ICCV92944) cultivars were chosen for testing. Temperature profiles are in degrees Celsius (°C) day/night, with 250μmol m^{-2} s^{-1} daylight, and 80% relative humidity, with frequent irrigation. For the sake of brevity, sensitive (S) and tolerant (T) genotype data are averaged within the table.

426

427 *2.7.1.1 Flowering*

428 Heat shortens the flowering period. This is considered a stress response in plants where
429 yield is proportional to the number of flowers per plant (Blum, 1988). Devasirvatham
430 determined that high temperature for 3 or more days initially reduced pollen fertility, and
431 after 7 days the stigma and ovule were also be impacted (Devasirvatham et al., 2010).
432 Temperature, daylength and genotype interactions are key determinants of flowering in
433 chickpea (Berger et al., 2011). Nayyar showed that reproductive structures are the most
434 sensitive and vulnerable to heat stress in chickpea, making these tissues useful indicators of
435 abiotic stress tolerance (Nayyar, Bains, & Kumar, 2005). Heat stress causes structural
436 abnormalities in anthers; producing small, shrunken, and empty pollen grains and in ovaries,
437 causing ovule abnormalities and decreased stigma receptivity (Devasirvatham et al., 2013;
438 Devasirvatham, Tan, Gaur, Raju, & Trethowan, 2012; Kaushal, Awasthi, Gupta, & Gaur, 2013;
439 Sanjeev Kumar et al., 2013). This was confirmed by the observation that pre-anthesis
440 temperature stress produced lower pod set compared to stress post-anthesis (Wang et al.,
441 2006). However, there is genetic variation in pollen fertility. It is generally believed that
442 35/20 °C day/night temperatures are critical temperatures that decrease pollen viability and
443 pod set in chickpea (Table 2.1.) (Devasirvatham et al., 2010; Sanjeev Kumar et al., 2013). The

effect of heat stress on pollen fertility starts at the third day post-stress, whereas the effects on ovule and ovary become clear after 7 days (Devasirvatham et al., 2013). Pollen viability is thought to be the main cause of sterility in chickpea under high temperature stress at anthesis (Devasirvatham et al., 2010), predominately due to reduced accumulation of carbohydrates and altered sink-source dynamics in pollen grains and stigmatic tissue (Kaushal et al., 2013). It has also been suggested that stomatal conductance plays an important role during heat stress, with stomatal conductance and leaf water content found to be lower in "heat-sensitive" genotypes compared to "tolerant" (Kaushal et al., 2013), in accordance with the previous definitions (Wery et al., 1993), the "tolerant" genotypes would be said to have improved heat avoidance capacity. Research has also found a positive correlation between high Fv/Fm ratios and heat tolerant plants (P. Kumar, Yadav, & Singh, 2020). Pollen developmental stages and heat stress have not been extensively studied in chickpea, however, heat stress can cause asynchrony between male and female organs in pulses (Zinn et al., 2010).

2.7.1.2 Podding

Podding is the final vulnerable period of the chickpea life cycle and seed yield per plant is determined by the following characteristics: number of flowers per plant, percentage pod set, number of seeds per pod, and seed size (Zaiter & Barakat, 1995). During heat stress the reproductive phase is accelerated, ultimately reducing the duration of growth and limiting the yield potential and value of the crop (Boote et al., 2005). This can be due to pod abortion (Warrag & Hall, 1983), or seed with small wrinkled endosperms that have lower market value (Davies, Turner, Siddique, Leport, & Plummer, 1999). The smaller endosperm size is likely due to reduced remobilisation of photosynthates and nutrients to the grain during stress, and more research is needed to address remobilisation under stress in legumes as a whole (Davies et al., 1999).

Chickpea has a high level of phenological plasticity (Liu et al., 2003). Plants can recover from short (10 d) periods of heat stress, producing new and healthy pods (Wang et al., 2006), but will still yield less. Chickpea exposed to 35/20 °C temperatures can show a 50% reduction in pod set compared to an optimum temperature of 28/16 °C (Devasirvatham et al., 2010). In a

study conducted in India, an increase in seasonal temperature of 1 °C reduced chickpea yield by 53/300 kg/ha (Kalra, Chakraborty, Sharma, Rai, & Jolly, 2008). Eight QTLs related to pod setting and filling under heat stress were identified (Paul et al., 2018).

Grain number and weight will both decrease at high temperatures (Devasirvatham et al., 2013; Summerfield, Hadley, Roberts, Minchin, & Rawsthorne, 1984; Wang et al., 2006). Another way to maintain yield under abiotic stress is to select cultivars with larger seeds. Davies et al (1999) showed that despite variation in drought response, larger-seeded chickpea genotypes maintained larger seeds when stressed compared to smaller-seeded cultivars, even though there was still a reduction in size. There can be, however, a negative correlation between seed size and seed number (Chapter 4).

2.7.1.3 Other Developmental Stages

There is little research available on the impacts of heat stress on seed germination in chickpea. However, the thermal range is said to be between 15-35℃, with a thermal optimum of 20℃, and a decrease in germination rate occurring above 30℃ (Sleimi, Bankaji, Touchan, & Corbineau, 2013). At extreme temperatures, above 42.5 °C, germination will decrease more significantly, and above 45 °C germination ceases (Ibrahim, 2011; K. B. Singh, 1997; N. Singh & Dhaliwal, 1972). Heat stress will also decrease biomass with 40/30℃ being the critical temperature (Sanjeev Kumar et al., 2013).

2.7.1.4 Heat Shock Proteins

All organisms respond to heat stress by inducing the synthesis of heat shock proteins (HSPs), which are evolutionarily conserved polypeptides. HSPs bind denatured proteins after stress and protect them from misfolding, premature clearance, and aggregation, thereby maintaining and restoring natural conformations (Bukau, Weissman, & Horwich, 2006; Prahlad & Morimoto, 2009). HSPs are controlled by heat transcription factors (HSFs) (Schöffl, Prändl, & Reindl, 1998), which together are key to the heat stress response in plants (Larkindale & Vierling, 2008; Scharf, Berberich, Ebersberger, & Nover, 2012). Accumulation of small HSPs is correlated with stress tolerance in many plants (Sun et al.,

2002). During plant reproduction, HSFs and HSPs become critically important to floral reproduction growth stages (Zhang et al., 2012). Under normal conditions, HSPs are only produced at specific stages of development, such as embryogenesis, germination, pollen development, and fruit maturation (Sun et al., 2002). Genome-wide expression analysis of HSFs in male tomato reproductive organs demonstrated that HSFs are closely managed in anther tissues during heat stress, suggesting that HSFs and HSPs are linked to how plants manage their heat-stress response (Giorno, Wolters-Arts, Mariani, & Rieu, 2013). HSFs and HSPs also occur during pollen formation without stress (Frank, Pressman, Ophir, Althan, & Shaked, 2009; Giorno, Wolters-Arts, Grillo, Scharf, & Vriezen, 2010; Kotak, Larkindale, Lee, von Koskull-Döring, & Vierling, 2007). During pollen development, HSPs may function as molecular chaperones for protein folding involved in meiosis, and tetrad formation (Bukau et al., 2006).

This understanding of HSFs and HSPs, and their genetic control, is difficult to use in selective breeding as they are difficult to identify and select. Nevertheless, they are probably more useful in transgenesis and can be targeted for introgression. Arabidopsis plants transformed with heat-shock factor *Athsf1* exhibited a low level of HSP expression in non-stressed plants, but these seedlings had elevated heat tolerance at temperatures that were lethal in non-transformed plants (Lee, Pokala, & Vierling, 1995). For chickpea the research is still new, but several recent studies have associated key proteins to thermotolerance. Twenty-two *HSFs* were identified in both desi and kabuli genomes for heat stress during podding and seedling growth (Chidambaranathan, Jagannadham, Satheesh, & Kohli, 2018), 482 heat-responsive proteins were correlated with heat response (Parankusam, Bhatnagar-Mathur, & Sharma, 2017), and five putative genes were identified encoding for HSPs or heat shock transcription factors contributing to thermotolerance (Agarwal, Garg, Kudapa, Doddamani, & Pazhamala, 2016).

2.7.1.5 Rhizobia and Symbiosis

Rhizobial bacteria, under symbiotic conditions, fix atmospheric nitrogen into ammonia from the safety of specialised structures called nodules. These nodules exist on the roots of

532 legume plants, and this relationship provides reduced nitrogen to the plant (Sindhu, Dahiya,
533 Gera, & Sindhu, 2020).

534 Inoculation of legumes is used in agriculture to ensure that planting soils have rhizobia of
535 sufficient number and compatibility (Atieno, Herrmann, Okalebo, & Lesueur, 2012; Catroux,
536 Hartmann, & Revellin, 2001; Deaker, Hartley, & Gemell, 2012). There is limited information
537 available regarding abiotic stress on rhizobia and their relationship with legumes, but it is
538 known that high soil temperature in tropical regions is a constraint for legume nitrogen
539 fixation (Sindhu et al., 2020). Root zone temperature impacts both rhizobial survival, and
540 also the symbiosis, via the exchange of molecular signals between partners, the adherence
541 of rhizobia to roots, root hair formation, and infection thread formation (Werner & Newton,
542 2005). High temperatures can also decrease nitrogenase and enzyme activity (Hungria &
543 Vargas, 2000) and cause reductions in growth rate, rate of colonisation, nodule number and
544 size, and delay nodulation (Gopalakrishnan, Sathya, Vijayabharathi, & Varshney, 2015). In
545 arid regions, the majority of root-nodulating rhizobia function well at 25-30 ℃, which is the
546 same for optimal nodule function in common bean (Alexandre & Oliveira, 2013). It has been
547 reported that the maximum temperature for chickpea rhizobial function is 40 ℃ for *M.*
548 *20icero* and *M. mediterraneum* (Nour, Cleyet-Marel, Normand, & Fernandez, 1995; Nour,
549 Fernandez, Normand, & Cleyet-Marel, 1994), but one more recent report describes the
550 range to be 30-35 ℃ (Maâtallah, Sanjuan, & Lluch, 2002). There is some evidence that
551 directed rhizobia inoculation may assist nodule formation and fixation at high temperatures.
552 The genotype ILC 482 was observed to grow and nodulate at prohibitive temperatures
553 (40/25 °C) following inoculation with a heat tolerant *Rhizobium leguminosarium* L. strain,
554 CP37A (Laurie & Stewart, 1993).

555 Nodulation and N_2 fixation involves many genes hosted by the rhizobia, known as *nod* and
556 *nif* symbiosis genes (Cooper, 2007). Rhizobia possess several genomic features that allow
557 them to survive temperature stress, including high numbers of goESL operons, multiple
558 rpoH copies, a large number of sHSPs, and regulatory mechanisms similar to gram positive
559 and negative bacteria (Alexandre & Oliveira, 2013). The need for plasticity is crucial for heat
560 tolerance in bacteria, which is permitted by alternative sigma factors, that allow bacteria to
561 rapidly redirect the RNA polymerase pool to the set of genes required for a certain condition
562 (Gruber & Gross, 2003). Rhizobial genomes have a large number of these alternative sigma

563 factors, including copies of rpoH, which encodes σ^{32}, the major sigma factor involved in heat
564 shock response (Barnett, Bittner, Toman, Oke, & Long, 2012). Some reports suggest that
565 *ClpB* has a key role in bacterial heat shock tolerance, as shown in *Ensifer meliloti* (Sauviac,
566 Philippe, Phok, & Bruand, 2007), *M. 21icero* (Brígido, Robledo, Menéndez, Mateos, &
567 Oliveira, 2012), and *M. loti* (Alexandre, Laranjo, & Oliveira, 2014). To improve the symbiotic
568 performance of chickpea rhizobium, the strain *Mesorhyzobium mediterraneum* UPM-Ca36^{T}
569 was transformed with extra copies of the *ClpB* gene, and the evaluation of growth under
570 abiotic stress showed that the expression of additional copies of the gene led to a higher
571 growth after heat shock (Paço, Brígido, Alexandre, Mateos, & Oliveira, 2016). The study
572 suggests the extra copies of the gene improve the symbiotic performance through an
573 increase in root hair curling and nodulation abilities, leading to more efficient nodules (Paço
574 et al., 2016). Analysis of *M. loti* MAFF303099 transcriptome identified 2186 protein-coding
575 genes that differentially expressed after heat shock, indicating that the transcript levels of
576 ~30% of the protein-coding genes were altered by the stress, including a larger number of
577 downregulated than upregulated genes (Alexandre et al., 2014).

578 At the cellular level, the key consequences of heat stress are protein denaturation and
579 aggregation (Richter, Haslbeck, & Buchner, 2010). After a sudden temperature increase,
580 there is an induction of the genes that encode for HSPs, and these are divided into
581 chaperones and proteases (da-Silva, Alexandre, Brígido, & Oliveira, 2017). Chaperones like
582 GroESL and DnaKJ repair misfolded proteins to allow them to resume their functional
583 confirmation (da-Silva et al., 2017), and are more highly induced in heat-tolerant rhizobia
584 strains compared to heat sensitive ones (Alexandre & Oliveira, 2011). Analyses of bacterial
585 transcriptomes and proteomes suggest that chaperone genes are strongly involved in the
586 symbiotic process (Sarmah et al., 2004), and more recently, it has been demonstrated that
587 the ClpB chaperone is involved in mesorhizobium-chickpea nodulation (Brígido et al., 2012).
588 The chaperone systems that are the most widely studied and found to be involved in the
589 heat stress response are DnaK-DnaJ and GroES-GroEL (Alexandre & Oliveira, 2013). In
590 chickpea rhizobia, a 60kDa protein thought to correspond to GroEL was found to be
591 overproduced when the bacteria was exposed to heat stress (Rodrigues, Laranjo, & Oliveira,
592 2006), and similar results have been found with other *Mesorhizobium* strains (Laranjo &
593 Oliveira, 2011). The ClpB chaperone, belonging to the HSP100 protein family, is also

considered important to heat shock responses, due to its function as an ATP-dependent disaggregase, able to disaggregate and activate aggregated proteins that accumulate under stress (Biter, Lee, Sung, & Tsai, 2012; Kedzierska & Matuszewska, 2001). Less studied chaperones, including the RNA chaperone Hfq, may also be key in rhizobia due to their assistance in helping RNA molecules attain their functional confirmation (Rajkowitsch, Chen, Stampfl, Semrad, & Waldsich, 2007).

2.7.1.6 Photosynthesis and Respiration

The plant's photosynthetic status at seed development will determine the extent of pod filling in chickpea (Awasthi, Kaushal, Vadez, Turner, & Berger, 2014). In chickpea, the rate of photosynthesis has a negative linear relationship with increasing temperature (Grace, 1988). The productivity peak occurs at 22°C, and decreases at 28°C with increased transpiration efficiency (K. B. Singh, 1997). This is caused by a reduction in the SPS and SS enzyme activity involved in sucrose synthesis, and tolerant chickpea genotypes are able to maintain higher sucrose contents and higher SS and SPS activities (Kaushal et al., 2013). Heat stress can also reduce carbohydrates in pollen grains and the generation of ATP in stigmatic tissue, reducing the available energy for pollen tube growth, and causing asynchrony between the maturity of male and female structures (Herrero, 2003).

Part of the photosynthesis story relates to transpiration, and high temperature affects leaf water status, stomatal conductance, and intercellular CO_2 concentration (Mathur et al., 2014). Temperature changes impact water vapour pressure density, plant hydraulic conductance, and decrease available water supply to the leaf surface (Matzner & Comstock, 2001; Yang et al., 2012). Stomatal conductance is higher in tolerant genotypes, likely resulting in better cooling potential under heat stress (Kaushal et al., 2013). The water status of leaves also decreased in heat stressed plants, which could be attributed to either increased water loss through attempts at cooling, or reduction in uptake from the roots (Morales, Rodríguez, Dell'Amico, Nicolas, & Torrecillas, 2003). Transpiration is an effective heat avoidance mechanism, and plants with small leaves, such as chickpea, are more likely to avoid heat stress by evacuating heat more quickly, with smaller air boundary resistance (Mathur et al., 2014). In well-hydrated plants, effective transpiration can cool the leaves 6-

10 °C (Mathur et al., 2014), but it has been suggested that a lower specific leaf area is beneficial, as it allows reduced water loss and greater carbon exchange, protecting the plant (Ramamoorthy, Lakshmanan, Upadhyaya, Vadez, & Varshney, 2016). Transpiration efficiency was shown to be a highly heritable (up to 70%), and 22 MTAs explained up to 35% of trait phenotypic variation (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014).

2.8 Conclusion and Future Directions

The overarching challenge for chickpea researchers is the need to accelerate genetic advance to safeguard the crop from the effects of climate change. However, the genetic improvement of chickpea lags other important grain crops. As the globe warms, farmers that rely on chickpea as a source of income become increasingly vulnerable, and while the knowledge gaps are not insurmountable, these must be addressed to safeguard the industry.

Most breeding for heat tolerance in this species has focused on earliness to escape heat stress. This is appropriate for current production systems as temperature shifts have so far been minimal (Mora et al., 2013). However, with the production environment rapidly changing, increased focus on true heat tolerance rather than avoidance is required. This can be achieved by melding high-throughput and accurate phenological, morphological, physiological and biochemical studies with the latest genomic technologies (Rajeev K Varshney et al., 2019). In this process, large numbers of genotypes must be phenotyped accurately with emphasis given to Multi Environment Trial (MET) data to account for G x E interactions (Jagdish Kumar & Abbo, 2001). The Kumar et al (2013) approach is sound, and this research could be used as a pilot for further study of hundreds of additional genotypes. Finally, future research and breeding should investigate heat tolerance and avoidance across the growing season including flowering time (Devasirvatham et al., 2010), pod-fill and nodulation (Dart et al., 1975), and photorespiration (Lewis et al., 2000). Many chickpea studies of heat stress have focused on physiological responses and/or transcriptomics (Kudapa, Azam, Sharpe, & Taran, 2014; R. R. Kumar et al., 2012; Wang et al., 2006). Since metabolic processes and related proteins are very sensitive to heat stress, proteomics could augment the breeding and selection process (Parankusam et al., 2017). Clearly there is a

655 berth of knowledge for genetic improvement of crops available and in use for other
656 agricultural products, and with the use of this knowledge and the available breeding and
657 genetic techniques, there will be great improvement seen in the yield stability and heat
658 tolerance of chickpea under heat stress.

659

660

3. A Growing Degree Day (GDD) Model Determines the Effect of Temperature Stress on Diverse Chickpea Genotypes

3.1 Abstract

Chickpeas are a globally crucial agricultural product, currently at risk due to human-induced climate change. There has been little research into the impact of heat stress on chickpea compared to other crops, but it is known that heat stress can cause up to 100% yield loss. This study utilises an existing calculation, known as Growing Degree Days (GDD), and expanded this formula for heat stress: Stress Degree Days (SDD), to examine the effects of high temperature stress on commercially important traits such as yield and seed size. Using a multi-environment trial, traits such as time to flowering, and seed size were observed in 148 chickpea cultivars across two sowing times within 1 year in two different Australian locations (Narrabri in New South Wales, and Kununurra in Western Australia). It was determined that there is a significant correlation between yield, GDD, and SDD at all locations, sowing times, and life stages of the crop. These metrics allowed greater differentiation between environments when compared to a count of the number of calendar days required for each cultivar to reach a set life stage (flowering and maturation), allowing more accurate investigation the impacts of high temperature stress. It was also determined that loss of yield and a decrease in seed size was significantly correlated with high GDD and SDD, though seed size had less environmental plasticity (variability) compared to yield, and therefore higher stability under stress. GDD and SDD were shown to be useful for predicting genotype adaptation to locations and seasons thus providing a basis for varietal recommendations. This information could also be used to breed environment specific cultivars and to understand trait plasticity.

3.2. Introduction

Chickpea (*Cicer arietinum*) is a crucial source of protein and starch in many countries (Abbo, Berger, et al., 2003), making up roughly 20% of global pulse production (FAO, 2020). Chickpea is adapted to low-nutrient, water-limited conditions, and is able to extract 80% of its nitrogen requirements from soil (S. Sharma et al., 2013). Nevertheless, abiotic stresses such as drought and heat cause ~70% of global yield losses (Rajeev K Varshney et al., 2019).

Human driven climate change remains a critical risk to global agriculture, with the global mean departure year (the year when the projected mean climate moves to a state permanently outside historical bounds) at 2069 ± 18, if global emissions are stabilised, and 2047 ± 14 if not (Mora et al., 2013). Previous research has demonstrated that high temperature stress can severely damage chickpea plants, with processes such as photochemical efficiency, pollen viability and germination, and pod set all negatively impacted (Sanjeev Kumar et al., 2013). Improved knowledge of the mechanisms of heat tolerance will assist the genetic improvement of this important crop and its continuation as an important food staple (Blum, 1988).

Growing Degree Days (GDD) is a unit of measurement that relates plant growth and development to air temperature. A degree day is a calculation of the cumulative temperature experienced by a plant at which growth is possible, calculated as GDD = ∑[Tmax + Tmin/2] – Tbase, where (Tmax+Tmin)/2 refers to the average daily temperature, and Tbase is the minimum temperature at which the crop can grow (Mavi & Tupper, 2004).

GDD can be more effective than season length for recognising physiological responses to temperature (Russelle, Wilhelm, Olson, & Power, 1984) and has been used to calculate yield potential in wheat, cowpea, grape, soybean, and tomato (Akyuz, Kandel, & Morlock, 2017; Bondade & Deshpande, 2021; Hall, 2000; Pathak & Stoddard, 2018; Raun et al., 2001). This calculation has also been used to model the growth and development of chickpea, with the earliest study published in 1996 (P. Singh & Virmani, 1996). Since then, there have been studies examining critical temperatures (Viola Devasirvatham et al., 2012; Sadras & Dreccer, 2015), and phenological variation across varied environments (Dreccer, Fainges, Whish, Ogbonnaya, & Sadras, 2018). These studies have been limited in scope due to their use of a small number of genotypes (Dreccer et al., 2018; Sadras & Dreccer, 2015; Verghis, McKenzie, & Hill, 1999), and data collected from simulations (Dreccer et al., 2018; Soltani & Sinclair, 2011). In order to strengthen this crop against the impacts of increasing temperatures, it is essential to understand trait plasticity and genetic control among a diverse population under commercial field conditions. This would vastly improve genetic selection and breeding efforts for future cultivars.

GDD is a useful metric with which to evaluate specific crop genotypes for individual farmers, based on their expectation of the season (Hall, 2000). Appropriate application could allow

farmers to continuously supply product to market, by choosing genotypes with different GDD requirements and sowing times at different locations (Bondade & Deshpande, 2021). Additionally, the ability to more effectively and accurately align season and harvest expectations could assist in management decisions such as scheduling labour, processing, and long term water management based on season length (Pathak & Stoddard, 2018). In this study, the calculation was modified to include Stress Degree Days (SDD), which refers to the cumulative temperature experienced by plants exposed to temperature stress. This stress is recorded here as a decrease in yield or seed size, as per existing literature (Davies et al., 1999; Sanjeev Kumar et al., 2013; Zinn et al., 2010). In this paper, stressful temperatures are considered to be above 30 ℃ as per existing literature (Sleimi et al., 2013).

This study uses GDD to evaluate 148 genotypes in two contrasting environments to select genotypes based on their performance and phenotypic plasticity. These were; a tropical environment that experiences consistent high temperatures but is otherwise "ideal" due non-limited water availability and light conditions, and a more variable, temperate environment considered more typical to the "Mediterranean" type for which Chickpea was domesticated (Abbo, Berger, et al., 2003). SDDs were calculated to understand the impact of cumulative heat-related stress on the crop to determine both genetic and environmental effects on the traits assessed. Better understanding the response of genotypes to varying climates provides a critical tool that farmers can use to improve and secure harvest outcomes.

3.3. Materials and Methods

3.3.1. Data Collection – Field Trials

Two Australian locations with varying climates were chosen to collect physiological data. The first was in Kununurra, Western Australia (15.7783° S, 128.7439° E), the second in Narrabri, New South Wales (30.2737° S, 149.7350° E). The recorded minima and maxima temperature for these locations are shown in Appendix 3.1, and these data were collected from stations managed by ozforcast.com.au and weather.agric.wa.gov.au, respectively. In summary, for the purpose of this trial, Kununurra is considered an environment in which the

752 trials experienced only high temperature stress, as the trials were flood irrigated and
753 regularly treated with nitrogen. Due to this, the Kununurra site could be considered an
754 “ideal” environment when studying heat stress, as other confounding stressors are absent.
755 Narrabri can be considered a more typical environment for chickpea production,
756 experiencing the milder mid-season temperatures, with the crop receiving rain and
757 supplementary irrigation as required.

758 Field experiments were conducted at these locations in 2019, at two sowing times. The first
759 time of sowing (TOS) was based on typical commercial growing times within each region,
760 and the second was delayed so that flowering would occur when average temperatures
761 were higher, thus generating heat stress from reproductive development onwards. In
762 Kununurra, this delay was 1 month, as the higher temperatures caused a shorter season
763 length. In Narrabri it was 2 months, due to the milder conditions. This resulted in a total of 4
764 experiments across 2 environments and 2 TOS

765 The 148 elite chickpea genotypes evaluated included 10 Desis and 138 Kabulis, originating
766 from Syria (129), India (10), and Australia (9) (Appendix 3.2.). Those genotypes not from
767 Australia were provided by The International Center for Agricultural Research in the Dry
768 Areas (ICARDA) and The International Crops Research Institute for the Semi-Arid Tropics
769 (ICRISAT) and were considered high yielding in hot environments. Australian genotypes
770 were chosen as comparison, being already used in Australian environments. Genotypes
771 were sown according to commercial standards (~169 seeds and ~381 seeds per plot in
772 Narrabri and Kununurra respectively) in randomised complete block designs of 2 replicates
773 for each TOS in plots of 8 m^2. Each trial was prepared and treated using standard agronomic
774 practice including fertilizer, herbicide and pesticide recommendations at each location
775 (Appendix 3). Plots were inspected daily to identify date of flowering and maturity.
776 Flowering is determined as the date at which ≥ 50% of plants have ≥ 1 flower, maturity is
777 determined as the date at which ≥ 50% of plants are ≥ 50% desiccated (brown). This was
778 recorded as the number of days from sowing to each stage of development. From these
779 developmental markers, growth was separated into two classifications, in which vegetative
780 is defined as the period between sowing and flowering, and reproductive as the period
781 between flowering and physiological maturity. According to standard commercial practices,
782 the growth season for Chickpea in Kununurra is typically shorter than in Narrabri (GRDC,

2016; Regan, 2015). All plots were harvested when the entire experiment attained harvest ripeness. Gross yield and 100 seed weight (HSW) was assessed in grams post-harvest. Gross yield data was collected by weighing the harvest of each plot, which was bagged and labelled accordingly. HSW data was collected by randomly sampling 100 seeds from each harvest bag (1 per plot) and taking their weight in grams. Weather data was collected using an on-site weather station which sampled every 15 minutes and was stored in online databases mentioned previously. This was collated into daily averages across the season. Across all trials, data can be categorised into plant physiological and climate data. Plant physiological data comprised number of days to flowering, and maturity, the weight of 100 seeds in grams, and gross plot yield in grams. Climate data refers to the temperature regimes at each location over the duration of the trial.

3.3.2. Data Analysis

3.3.2.1. Physiology and Environment

Preparation of the data, and other relevant statistical tests were completed using Genstat (VSN-International, 2022a). Genotypes and times of sowing were considered fixed effects at each location in each year, and rows and ranges within replications and times of sowing as random effects. Predicted means were calculated for further analysis. Correlations between the generated GDD and SDD data and the crop physiological traits, and their significance, were assessed using the Spearman's rank correlation coefficient.

GDD was calculated from sowing to each key life stage using the R package "cropgrowdays" (https://cran.r-project.org/package=cropgrowdays). The base temperature was set at 10 ℃, and a chosen thermal maximum at 30 ℃ in accordance with existing literature (Sleimi et al., 2013), to ensure the calculation only accounted for temperatures at which growth was optimal. SDD was calculated by repeating this script, with the thermal maximum set to 50 ℃, then subtracting the original GDD value ($\mathrm{SDD} = \mathrm{GDD}_{50} - \mathrm{GDD}_{30}$, Where GDD = ∑ [Tmax + Tmin/2] – Tbase, and subscripts refer to the selected thermal maximum).

50 ℃ was chosen as it was deemed higher than the recorded maximum temperature experienced in all experiments, thus isolating all days above 30 ℃ for the calculation.

Genotypes are labelled numerically for simplicity (Appendix 3.2.), and a summary of the temperature regimes recorded in each experiment is given in Appendix 3.1.

Superiority refers to a performance "score" for each genotype for the given trait, compared to the others in the population. The stability coefficient refers to variance among means in various environments (Lin & Binns, 1988; Nassar & Huehn, 1987). Superiority and static stability coefficients were also calculated using the Meta-Analysis suite found in Genstat by grouping data by genotype and TOS. One calculation was completed for each location, with Y-variate and Environment represented by the assessed trait and TOS respectively, and data was then sorted according to the ranks of each trait's performance superiority score (Table 3.1), and then according to the ranks of the Static Stability scores (Table 3.1). Those that were ranked 10^{th} or lower were selected to highlight in Table 3.1.

3.3.2.2. Genotypes

The Genotype by Genotype x Environment (GGE) biplots were constructed using the Genstat Meta-Analysis suite, with data grouped by both experiment and genotype. GGE biplots are multivariate Principal Component Analysis (PCA) biplots that represent genetic versus phenotypic plasticity of a particular trait among a range of genotypes and environments. PC refers to the principal component, with PC1 representing the maximum variance direction in the data. The origin of each individual graph represents a calculated "virtual" genotype with average performance or broad adaptation across environments, and genotypes that fall closer to a specific environmental vector perform better for that trait in that environment. The angle between environmental vectors indicates the degree of correlation between environments, with a smaller angle denoting greater similarity (Simon Harding, 2010).

Broad sense heritability was calculated using the equation $H^2 = (s_g^2)/[s_g^2 + (s_e^2/r)]$, in which s_g^2 represents the genetic variance, s_e^2 represents residual variance, and r represents the number of genotype replicates (Gitonga, Koning-Boucoiran, Verlinden, Dolstra, & Visser, 2014). The genetic and residual variance were calculated using a Linear Mixed Model in Genstat, in which the fixed and random models were experiment and genotype respectively, and the Y-variate was the assessed trait.

3.4. Results

3.4.1. Environment and Physiology

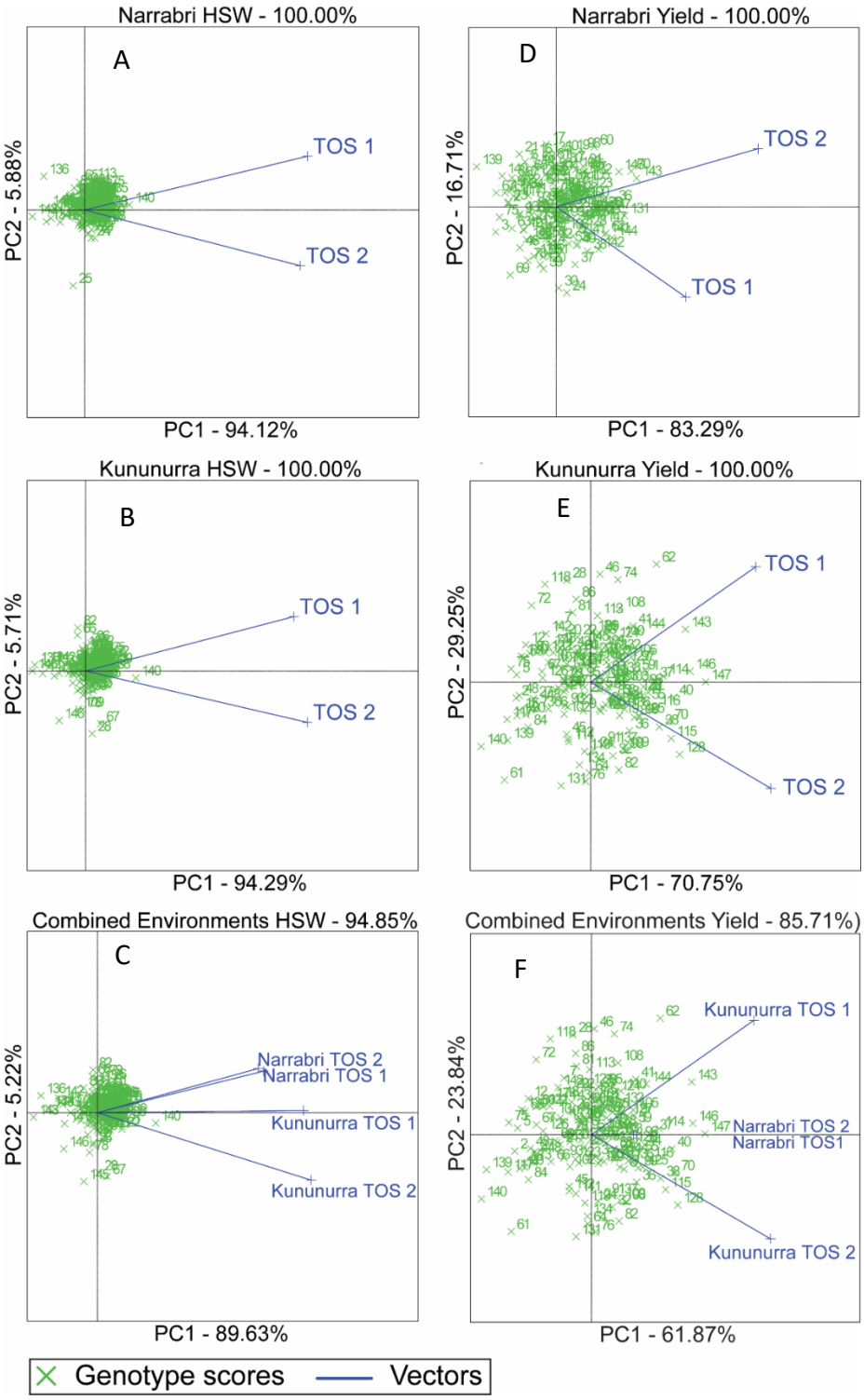


Figure 3.1. *Biplots comparing the GGE* ***effects across all genotypes, in each environment and combined across environments for grain yield and HSW***. Numbers refer to individual genotypes, whose official titles and countries of origin can be found in Appendix 3.2.

Table 3.1. *Superiority and Static stability coefficients for HSW and yield* depicting the 10 highest ranked genotypes for each trait and metric. Genotypes denoted with * have high ranking for the trait in both environments, and those denoted with ** have high rankings for both traits in that environment. Superscript D refers to genotypes that are Desi type, and superscript A refers to Australian genotypes.

		Superiority		Static Stability	
	Rank	Narrabri	Kununurra	Narrabri	Kununurra
HSW	1	* [A]139	* [A]139	38	89
	2	*1	*138	109	11
	3	*88	*88	34	**68
	4	60	22	10	71
	5	*75	*21	12	58
	6	*138	*75	122	[D] 141
	7	61	*53	**39	22
	8	127	137	82	*100
	9	*53	98	70	5
	10	*21	*1	*100	[D] 133
Yield	1	* [D] 142	[A] 146	24	97
	2	*70	* [A]145	30	127
	3	[A] 131	*142	69	77
	4	* [A]145	40	**39	92
	5	36	114	78	54
	6	47	*70	120	33
	7	[A]143	115	46	75
	8	76	128	88	67
	9	62	37	3	**68
	10	[D, A]140	116	116	[D, A]140

Table 3.2 *Summary of average number of days to reach each life stage (Age), average number of days with temperature suitable for growth (# Days), average growing degree days (GDD), average stress degree days (SDD), average yield, and average HSW* for ***each TOS and location.***

Trial	TOS	Flowering				Maturity				Yield (T/ha)	HSW (g)
		Age	# Days	GDD	SDD	Age	# Days	GDD	SDD		
Narrabri	1	85	62	182	0	134	111	511	13	2.23	35.16
	2	65	58	281	5	102	95	673	25	1.15	31.57
Kununurra	1	57	54	783	118	128	125	1561	216	3.51	36.95
	2	55	55	641	76	131	131	1591	246	3.3	39.04

Table 3.3 *Spearman's rank correlation between Growing Degree Days (GDD) and Stress Degree Days (SDD)* of two growth periods with grain yield and HSW at each location. * and ** denote significant ($p < 0.05$) and highly significant ($p < 0.001$) correlations respectively.

Location	Growth Period	Variate 1	Variate 2	Adjusted R^2
Kununurra	Vegetative	GDD	HSW	0.041
			Yield	*-0.18
		SDD	HSW	-0.082
			Yield	**-0.198
	Reproductive	GDD	HSW	0.015
			Yield	*0.183
		SDD	HSW	*0.164
			Yield	**0.187
Narrabri	Vegetative	GDD	HSW	**-0.29
			Yield	**-0.807
		SDD	HSW	**-0.333
			Yield	**-0.489
	Reproductive	GDD	HSW	*-0.182
			Yield	**-0.615
		SDD	HSW	**-0.306
			Yield	**-0.802

There was greater range and variability in the SDD and GDD data when compared to Grow Days (Table 3.2). This was particularly notable when considering the difference in grow days between locations for maturity, compared to their respective GDD and SDD values. The GDDs for Kununurra at maturity were triple those of Narrabri, and the SDDs tenfold, whereas the grow days (# Days) were 30% higher at most. Additionally, there was a greater proportional difference between flowering GDD at Narrabri (TOS2 was 35.3% larger than TOS1), than between TOS in Kununurra (TOS2 was 18.1% larger than TOS1). There was greater variation for age at flowering between TOS in Narrabri (23.5%) compared to Kununurra (3.5%), and both TOS in Kununurra reached flowering in a much shorter time compared to Narrabri (32.9% and 15.4% faster, respectively). However, the time between flowering and maturity was shorter for both TOS in Narrabri (49 and 37 days, respectively) compared to Kununurra (71 and 76 days, respectively).

A large difference in yield was observed between locations, with mean yield at Kununurra ($\dot{x}$ = 3.4 T/ha) 50% higher and HSW ($\dot{x}$ = 38.0 g) 12.1% higher than Narrabri. However, there was less variation between TOS in Kununurra for both yield (6%) and HSW (5.4%), than Narrabri (48.5% and 10.2% respectively). There was also less variation for HSW than Yield, (19.2% and 67.3% respectively) across all experiments.

GDD and SDD are predominately significantly correlated with HSW throughout the lifespan of the crop (Table 3.3). This correlation was mostly negative, particularly at Narrabri (R^2 = -0.333, -0.29, -0.182, $P < 0.001$, $P < 0.001$, $P = 0.002$), though the SDD value in Narrabri was consistently much lower than Kununurra (Table 3.2). However, there was a significant positive correlation between HSW and SDD observed during the reproductive stage in Kununurra ($R^2 = 0.164$, $P = 0.005$). There was no significant correlation between GDD and SDD for HSW in the vegetative period at Kununurra ($P = 0.479$, $P = 0.158$), nor was there for GDD during the reproductive period ($P = 0.802$).

From Table 3.3, it can be noted that both GDD and SDD are highly significantly correlated with Yield ($P < 0.05$): particularly in Narrabri ($P < 0.001$). This correlation is exclusively negative in the vegetative growth period (R^2 from -0.807 to -0.180) but positive in Kununurra during the reproductive period, with a stronger correlation between yield and SDD ($R^2 = 0.187$, $P < 0.001$) than GDD ($R^2 = 0.183$, $P = 0.002$).

3.4.2. Trait Plasticity

GGE effects of each environment on yield and HSW are given in Figure 3.1 A-F. HSW was more influenced by genotype than environment, evidenced by high PC1 scores (89.63 – 94.29%) and acute angles between each environmental vector. Genotypes that clustered towards the origin demonstrated a similar performance to the virtual genotype.

Yield was impacted more significantly by the environment in comparison to HSW. There are wider angles between environmental vectors, and lower PC1 scores (61.87 – 83.29%). Genotypes are spread further from each other, with some genotypes associated with specific environmental vectors.

Variance in yield at Narrabri was more strongly related to genotype (83.29%) than Kununurra (73.25%). In Kununurra, a larger number of genotypes aligned more closely with specific environmental vectors than Narrabri, demonstrating superior performance in this environment.

For HSW, 60% of top performers were common to both environments, and 10% were top performers or highly stable across traits in each environment (Table 3.1). For yield, 30% of top performers were common to both environments and 10% were highly stable (Table 3.1). In terms of consistent stability across traits, genotypes 39 and 68 had high stability for both HSW and yield in Narrabri and Kununurra (Table 3.1).

No genotypes were both high performers and highly stable in each environment.

Desi genotypes represent 6.76% of the genotypes assessed in these experiments but represented 30% of the genotypes ranked in the top 10 for yield (Table 3.1). Desi genotypes represented 20% of the genotypes ranked in the top 10 for HSW stability, but these genotypes were only considered highly ranked based on performance in Kununurra (Table 3.1).

3.4.3. Heritability

Table 3.4. broad sense heritability estimates (H2) across location TOS for HSW and gross yield.

Location	TOS	HSW H^2	Yield H^2
Narrabri	1	0.970	0.785
	2	0.968	0.956
Kununurra	1	0.950	0.395
	2	0.916	0.378

HSW had a higher heritability than yield overall (H^2 = 0.916 – 0.970 and 0.378 – 0.956, respectively). HSW had a more consistent heritability estimated across environments when compared to yield (3.6 % and 60.4 % variation, respectively). HSW had a more consistent heritability estimate in Narrabri compared to Kununurra (0.2% and 4.6% variation respectively), whereas yield showed consistently lower heritability in Narrabri compared to Kununurra (17.9% and 4.3% variation respectively). The experiment with the highest heritability across both traits was Narrabri TOS 2, and the lowest was Kununurra TOS 2.

3.5. Discussion

3.5.1. Climate and Physiology

This study showed that GDD and SDD provide greater variation in field data compared to a simple count of the number of days that were at suitable temperature for growth. Data for both geographical locations were vastly different for each calculation, however this was to be expected, considering the vast differences overall climate (Appendix 3.1), and the differing agronomy practices at each location, such as more extensive watering at Kununurra. However, this variation allowed detailed investigation of genotypic and environmental influences on key traits (Figure 3.1), due to the bespoke environment of each trial. This diversity of conditions highlights the need to develop location-specific cropping strategies and genotypes for farmers.

Both TOS in Kununurra reached flowering sooner than Narrabri, likely due to a larger accumulation of GDD in Kununurra over both TOS (Table 3.2). Additionally, yield in Kununurra was 50% higher on average than Narrabri (Table 3.2). This high GDD indicates a

consistently warm environment, with reduced vegetative development and earlier flowering. Furthermore, several studies have shown a correlation between early flowering and high yield (Sanjeev Kumar et al., 2013; Rubio, Moreno, Cubero, & Gil, 2004; Siddique, Loss, Thomson, & Saxena, 2003). Apart from higher GDD, Kununurra also had higher SDD across the season compared to Narrabri. As the SDD calculation refers to the stress experienced at this location, one may expect a decrease in yield in Kununurra compared to Narrabri. However, commercial irrigation standards in Kununurra are considerably more generous, thus plants had greater capacity for stress-management via transpiration cooling, compared to Narrabri, where trials were irrigated according to typical rainfed conditions for the region. Previous research on the impacts of temperature and water stress on chickpea, found that sites with high rainfall typically demonstrated high yield performance (Dreccer et al., 2018), and this was likely due to the plant's ability to reduce canopy temperatures by 6-10 ℃ (Mathur et al., 2014). Interestingly, HSW in TOS2 was also 5.4% larger than TOS1 in Kununurra (Table 3.2), likely due to a longer period between flowering and maturity. This longer grain filling period results in larger seeds (Davies et al., 1999; Zaiter & Barakat, 1995), as well as the consistent development of new seeds/pods due to the indeterminate nature of the species (K. B. Singh, 1997). There is a delicate balance here, as it has been shown that in Australian conditions, early maturing chickpeas with limited water have higher yields only when temperatures are optimal (Sabaghpour et al., 2003). In this study, SDD and GDD were both negatively correlated with yield throughout the season in Narrabri, particularly in TOS2 (Table 3.2, Table 3.3). Thus, it can be seen that even though SDD was lower than Kununurra, stress management is often reliant on resource availability (Mathur et al., 2014).

3.5.2. Trait Plasticity

3.5.2.1. Seed Size Stability

HSW showed less variation than yield across environments across TOS (Table 3.2). Multivariate analysis showed that HSW was more strongly influenced by genotype than the environment, evidenced by high PC1 scores (89.63 – 94.29%) and acute angles between each environmental vector (Figure 3.1, A-C). Stability coefficients for HSW showed that 60% of the top-ranked genotypes occurred in both locations, compared to 30% for yield, and

976 correlations between GDD and SDD for HSW were lower than observed for yield (Table 3.3).
977 Seed size was also found to be 10.15 % and 58.57 % more heritable than yield in Narrabri
978 and Kununurra respectively (Table 3.4). These data confirm that yield is an environmentally
979 plastic trait, whereas HSW is more genotypically fixed (Figure 3.1) and therefore stable.
980 Others reported that seed size in chickpea is controlled by two genes with dominance
981 epistasis (H. D. Upadhyaya, Kumar, Gowda, & Singh, 2006) and by additive effects (H.
982 Upadhyaya, Sharma, & Gowda, 2011) and molecular markers directly related to seed size
983 have also been identified (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal,
984 Nayak, Chaturvedi, Basu, Gangarao, et al., 2014). These key genetic loci are likely to be
985 present among the genotypes in this study, given that seed size was clearly under relatively
986 strong genetic control. Whereas yield is known to be significantly influenced by
987 environment and therefore less stable (Teresa Millan, Clarke, Siddique, Buhariwalla, & Gaur,
988 2006).

989

990 *3.5.2.2. Room for Enhancement with Yield Plasticity*

991 One of the benefits of environmental impacts on yield is the option to breed specifically
992 adapted cultivars once the degree of yield plasticity is known. Figure 3.1 demonstrates the
993 possibility of choosing genotypes specifically adapted to each environment type. Figure 3.1
994 D, E, and F show a greater spread of data points across the biplot, along with lower PC1
995 scores compared to those of A, B, and C. It is therefore possible to identify genotypes that
996 are better adapted to TOS2, where temperature stress is likely to be higher. This is
997 particularly evident in Kununurra (Figure 3.1, E), which could be a consequence of the
998 transpiration cooling mentioned previously (Mathur et al., 2014).

999 In Narrabri, variance in yield was more strongly related to genotype (83.29%) than
1000 Kununurra (73.25%) (Figure 3.1 D, E), which is also supported by the higher heritability of
1001 yield at Narrabri compared to Kununurra ($\dot{x}$ = 0.871 and 0.387 respectively) (Table 3.4).
1002 Several genotypes in plot E align more closely with one specific vector, demonstrating more
1003 variation in genotype performance at each TOS in this location (Figure 3.1). Clearly, some
1004 genotypes are better adapted to TOS2 than TOS1 for yield (and vice-versa). For example, in
1005 Narrabri, genotypes 38, 144, and 142 appear to have superior yield in TOS2, whereas

genotypes 70, 146, and 143 have higher yield in TOS1 (Figure 3.1 D). Similarly, in Kununurra, genotypes 38, 115, and 128 appear to have superior yield in TOS2, whereas genotypes 105, 143, and 144 were higher yielding in TOS1 (Figure 3.1 E). Understanding these relationships provides a basis for future chickpea improvement, as parents can be selected to either breed for broad adaption or specific adaptation to particular environment types.

3.5.3. Future Directions and Breeding

Australian genotypes represented 5 of the 10 highest yielding genotypes with highest HSW across environments HSW (Table 3.1). However, in terms of static stability, only one genotype was represented, ranking 10th in yield, and this was predominately observed only at Narrabri (Table 3.1). This suggests that the Australian genotypes are well adapted to this environment, although the average yield loss between Narrabri times of sowing was 32.34% (Table 3.2). It can therefore be concluded that the heat tolerance of Australian cultivars could be improved by utilising some of the international genotypes that ranked highly in static stability as parents (Table 3.1, Appendix 3.2).

There is clearly a trade-off between seed size and seed number with consequences for yield. However, this allows for tailoring of the product to several markets. Farmers will be driven by price. If gross margins are greater for high yield, then smaller seeded types will be produced. However, a premium price for large seed may change this equation. Unfortunately, no genotypes that ranked highly for both stability and yield. However, some genotypes had both HSW and yield in specific environments, suggesting these may be good choices as breeding stock. Due to the vast differences in environments both between and within locations, it would be unlikely that high performers for either trait would also be stable. However, stable genotypes (Table 3.1) may be a good choice for farmers in highly variable environments with unpredictable seasons.

3.6. Conclusion

The GDD metric calculation is a viable and highly effective tool for assessing the impact of temperature regimes on commercially cultivated chickpeas. With the addition of SDDs, it is

possible to further examine the effects of temperature stress on commercially important traits such as yield and seed size. As risks related to temperature stress increase globally, it is crucial to rapidly find new ways to model and improve genotypes to safeguard food production. This study demonstrated there is a significant correlation between heat stress and yield loss, as well as heat stress and a decrease in seed size. However, seed size was found to be impacted significantly less by environmental factors than yield and largely related to genotype. This research provides a basis for selecting chickpea genotypes adapted across environments and/or adapted to specific environment types including high temperatures.

4. An Investigation into The Impacts of Heat Stress on Chickpea, Using Field and Glasshouse-Based Experiments

4.1 Abstract

High temperature stress has the potential to be a serious limiting factor when considering crop success in chickpea. Heat stress is known to impact every developmental stage of the crop, with the reproductive flowering and podding stages seen to be most vulnerable. Even considering this, research regarding both the impacts and amelioration of heat stress in chickpea lags behind that of its sister crops, such as soybean and cowpea, particularly when attempting to identify physiological responses to such stress, and correlations between physiological traits under stress. With this in mind, a number of commercial-style field trials were carried out across two locations in Australia, to assess the impact of heat stress on a population of 147 chickpea genotypes, primarily Kabulis. Developmental ages and yield-based traits were examined throughout the life of the plants under commercially typical and heat-stressed field environments, with subsequent glasshouse trials conducted to study these traits in more detail. Through these experiments, this study describes the impacts of heat stress on the growth and development of chickpea throughout its growth stages, identifying trait correlations that aid our understanding of the plants stress management. Additionally, these correlations and associated trait data can be used as future breeding targets for the production of improved, reliable cultivars in a changing climate.

4.2 Introduction

Chickpeas are a globally important food crop, known to be a crucial source of protein and starch in many countries (Abbo, Berger, et al., 2003). With the latest Global Nutrition Report stating that in 2020 1 in 9 people worldwide are hungry (UNICEF, 2020), there is a need to safeguard such a nutritionally rich crop, to ensure future food security (Atlin et al., 2017). There are also agronomic benefits to the cultivation of chickpea, including the crops adaptation to nutrient-sparse soils, efficient soil-nitrogen deposition and extraction, and tolerance of dry periods making it a hardy and reliable option for farmers (S. Sharma et al., 2013). However, even a crop that is regularly grown in arid environments has limits, and it is known that heat stress can lead to yield losses of 10-15% per degree above optimum

temperature (H. D. Upadhyaya et al., 2011). Heat stress is known to impact a number of different chickpea growth and development processes, and in extreme cases will lead to a complete loss of yield (Sanjeev Kumar et al., 2013). The latest Intergovernmental Panel on Climate Change (IPCC) report has found that between 2021-2040 it is very likely that the global surface temperature will increase by 1.5 ℃ relative to 1850-1900, along with an increase in drought, aridity, and fire events (IPCC, 2021). With this knowledge, it is critical to understand the impacts of heat stress on chickpea in detail, to advance both genetic and agronomic practices to protect and improve the crop against this oncoming threat.

Heat stress is known to impact every developmental stage of the plant, with the reproductive phase being the most vulnerable to such stress resulting in yield decreases, as summarised in Jeffrey et al. (2021). In the reproductive phase, heat stress impacts pollen development, germination, and tube growth, as well as reducing ovule number and function (Devasirvatham et al., 2010; Sanjeev Kumar et al., 2013). Heat-stress is less studied for other stages, but it is thought that germination rate will decrease at temperatures above 30 ℃, with a cessation at 45℃ (Ibrahim, 2011), and biomass growth decreasing above 40 ℃ (Sanjeev Kumar et al., 2013).

Additionally, the adaptations possessed by plants to manage heat stress can be characterised into escape, avoidance, and tolerance (Wery et al., 1993). These present as: development prior to the onset of high temperatures (Toker & Canci, 2007), developing physiology that permits mitigation of stress (Wery et al., 1993), and using cell biochemistry and heat shock proteins to manage stress (Blum, 1988), respectively. Understanding the method of stress management used by each genotype is critical for informing breeding and agronomic choices, to ensure the correct genotypes are targeted for sustainable and effective improvement.

There have been several studies relating genomic regions to physiological traits, including SNPs related to seed weight and plant height (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014), a gene for early flowering (Jagdish Kumar & Abbo, 2001), and 204 genes that experienced selection pressure

during domestication (Rajeev K Varshney et al., 2019). However, there has been little research done to link stress responses with their relevant gene expression patterns, or to link phenological combinations to a plants capacity to withstand heat stress. This information is crucial to enhance our understanding regarding which traits are best to target when selecting suitable candidates for breeding. Additionally, it is thought that Genotype by Environment (GxE) interactions influence 70-80% of variation in yield for chickpea (S Kumar & Ali, 2006), which indicates that trait plasticity is as important to understand as is the genotype itself (Pierce, 2020). By understanding the plasticity of a key trait, breeders can choose whether or not it is worth targeting, as well as choosing cultivars that are more suited to specific environments, or those that will have stable performance in variable conditions (Jeffrey et al. in Prep).

This study aims to address the impact of heat stress on several key traits, in order to further understand their relationship to each other, environmental plasticity, and variation within a given population.

4.3 Materials and Methods

4.3.1 Data Collection

4.3.1.1 Field Trials

Field trials were conducted in two locations within Australia, under the typical agronomic management of commercial chickpea cropping in each region. These regions are Kununurra, Western Australia (15.7783° S, 128.7439° E), and Narrabri, New South Wales (30.2737° S, 149.7350° E). The recorded minima and maxima temperature for these locations were collated from stations managed by weather.agric.wa.gov.au and ozforcast.com.au, respectively and can be found in Appendix 4.1. These trials were carried out annually from 2018 to 2020, with two sowing times per location and year, representing the control and heat stress treatments. The control treatment was sown according to each region's commercial standard time, and the heat treatment's sowing delayed by 1 month in Kununurra, and 2 in Narrabri. This variation in sowing delay between the regions is due to the shorter season length in Kununurra, caused by consistently higher temperatures than those of Narrabri (Appendix 4.1). In total, there were 12 field trials, split by 3 years, two

locations, and 2 TOS. Trials were managed according to the agronomic norms of the region, and it should be noted that this varies. Kununurra trials were flood irrigated fortnightly and had regular nutrient applications throughout the season. Narrabri trials were either rainfed irrigated or simulated-rainfed, with rhizobial inoculation and pre-emergent applications at planting.

In total, 164 genotypes were examined, though this number varies for each year and location (Appendix 3.2). These genotypes were made up of 150 Kabuli lines, and 14 Desi lines, with 9 originating from Australia, and 155 provided by ICARDA and ICRISAT from India and Syria. Genotypes were sown according to commercial standards (~169 seeds and ~381 seeds per plot in Narrabri and Kununurra respectively) in randomised complete block designs of 2 replicates for each TOS in plots of 8 m^2. Plots were inspected as often as possible, ranging from every 1-3 days based on field conditions, and the number of days was recorded for each plot, from sowing to each key developmental stage, flowering, podding, and maturity. Flowering and podding dates were established by the day at least 50% of plants within a plot had a flower or pod respectively, and maturity as the day at least 50% of a plot was brown. Temperature regimes were collected using on-site weather stations, sampling every 15 minutes, and collated into daily average across the growing season (Appendix 4.1). When all plots had reached full desiccation, they were harvested using a plot header, and gross yield and HSW were collected for each plot.

4.3.1.2 Glasshouse Trials

Glasshouse trials were conducted in Sydney Australia, at the University of Sydney's Centre for Carbon, Water, and Food. Two trials were conducted, the first having heat stress induced at podding, and the second at flowering. Control glasshouses were maintained at temperatures between 25-28 ℃, deemed optimal by previous research (Devasirvatham et al., 2010), and treatment glasshouses were maintained at above 30 ℃, deemed the beginning of the critical temperature range according to Kumar et al (2013).

Seeds were planted in 7L plastic pots filled with Grange Premium potting mixture (www.grangegardenhealth.com.au/) , which was pasteurised prior to use. Each pot had 3 seeds planted, and germinated plants were thinned to 1 per pot after 2 weeks. Pots were ordered according to a randomised block design (VSN-International, 2022b), and plants were trellised to stakes to ensure minimum confounding by shading of their neighbours.

Water and temperature regimes are as below:

4.3.1.3 Podding Trial

For the podding trial, 20 genotypes were selected based on their stable seed size, flowering time, and yield performance under stress in the field trials (Appendix 4.3). The date of the first flower and pod for each plant was recorded, and heat stress (≥ 30 ℃ for day and night) was induced once 70% of plants had at least 1 pod (Mahajan, Sapra, Umesh, Singh, & Sharma, 2000). Once the glasshouse containing the stress treatment had at least 70% of all plants browning due to desiccation, watering was stopped for both treatments, and plants were left to fully dry for harvest.

4.3.1.4 Flowering Trial

For the flowering trial, a subset of 10 genotypes were selected based on the similarity of their flowering time in the podding trial, to ensure stress would be equally distributed according to development stage (Appendix 4.3). The date of the first flower and pod for each plant was recorded, and heat stress (≥ 30 ℃ for day and night) was induced once 70% of plants had at least 1 flower.

This trial also had duplicated controls, kept at 25-28 ℃, denoted as drought control (DC), and watering control (WC). In each control and treatment, there were 6 replicates per genotype. In DC, watering was halted at the same time as in the treatment, irrelevant of the desiccation state of the control plants, to standardise the amount of water given to each set of plants. In WC, watering continued until 70% of WC plants were browning, irrespective of the treatment plants, to examine any changes in physiology under optimal temperature and high-water availability. For the purposes of this study, results for the podding trial can only

be compared to those of the flowering trial with the exclusion of WC. This is to ensure that data is only compared when it has been gathered under a standardised watering schedule. Results that assess low and high-water availability and their impact on heat stress will only be compared within the bounds of the flowering trial.

At harvest, each plant had all pods removed, bagged, and counted, and the plant was cut at the base of the stem, and placed inside a labelled paper bag, along with its respective pods. These bags were then placed in a dehydrator at 40°C for 48 h, at which time plants were processed to record vegetative biomass weight, number and weight of pods, and number and weight of seeds. With this, HSW was calculated as a standardised measurement of average seed size, and seeds per 100 pods was calculated as a measure of the plants assimilate partitioning under stress.

4.3.2 Data Analysis

The traits measured for each trial type are as below:

Field Trials	Glasshouse Trials
Flowering	Yield (g)
Yield (g)	HSW (g)
HSW (g)	Vegetative Mass (g)
Height (cm)	Pod Filling (%)
	Seed number

All data analysis and preparation was performed using Genstat software (VSN-International, 2022a).

Physiological traits that were not immediately measurable were calculated as follows:

HSW	Vegetative Mass	Pod filling
$\frac{\text{Seed weight}}{\text{Number of seeds}} \text{ x } 100$	$\text{Total biomass (g)} - \text{Seed weight (g)}$	$\frac{\text{Number of seeds}}{\text{Number of pods}} \text{ x } 100$

Average trait performance was calculated using predicted means from a generalised linear regression model, and significance via Analysis of Variance (ANOVA). Trait correlations were calculated via Spearman's rank correlation coefficient test.

Significance between genotype, TOS, and each trait was calculated using ANOVA, with further output giving coefficient of variation values. The coefficient of variation (CV) refers to the ratio of the standard deviation to the mean, and shows the degree of dispersion within the data as a percentage (VSN-International, 2022b). In this study, low CV values across environments indicate that the trait shows little variation under environmental pressure, and so can be considered genetically stable (Hill & Mackay, 2004). Percent loss values were calculated between the treatment and control to indicate the difference between trait performance in an optimal versus high temperature environment ($\frac{TOS1-TOS2}{TOS1}$ x 100). For the Flowering trial, Percent loss values were calculated twice, to examine the impact of high temperature stress compared to a standardised watering control (Drought difference) and compared to a control where water is not limiting (Watering Difference).

4.4 Results

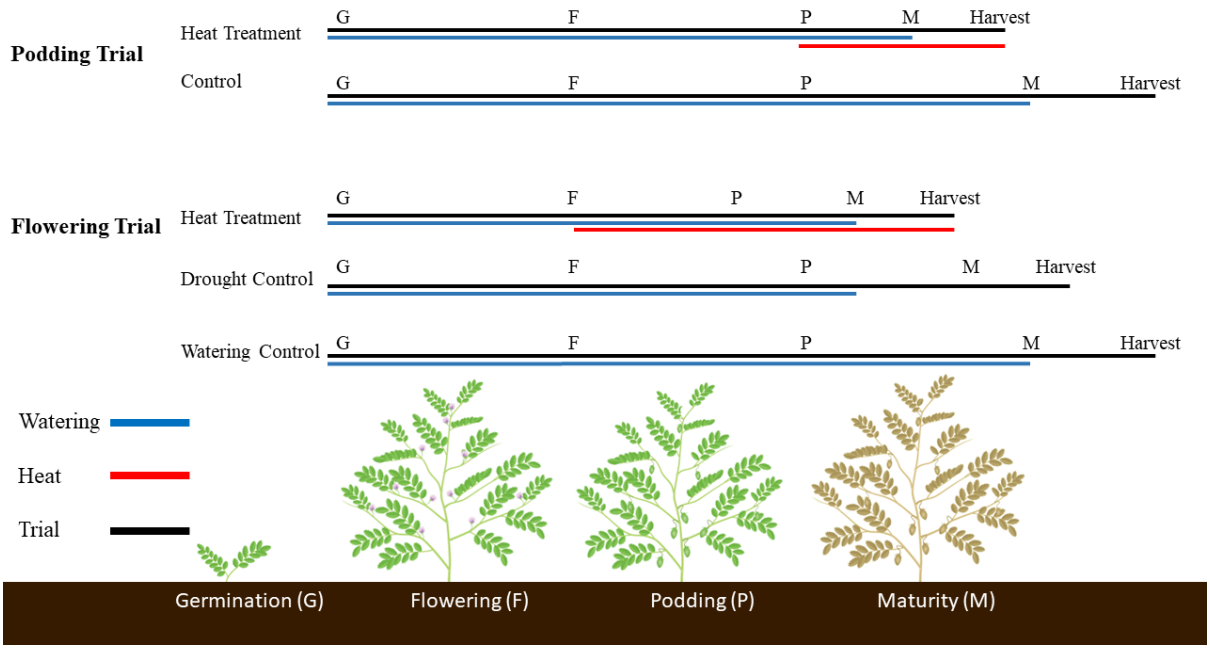


Figure 4.1: Visual description of heat and water regimes for glasshouse trials, based on developmental stages.

Heat stress has a significant and consistent impact on all measured traits across almost all trials. In the field trials, there was an average yield loss of 38.22 % in Narrabri between TOS1 and TOS2 (Table 4.1). However, in Kununurra, the difference between TOS was variable, with 2018 and 2019 seeing an average yield gain of 5.58 % and 8.61 % respectively, whereas 2020 experienced an average loss of 18.37 % (Table 4.1.). In comparison, there was a consistently lower variation between locations in terms of loss of seed size, with an average seed size loss of 1.86 % and 9.22 % for Kununurra and Narrabri, respectively (Table 4.1.). However, average TOS1 seed size was 11% higher in Narrabri than Kununurra, subsequently becoming 6.7 % lower in TOS2 (percent loss for both traits was highly significant for TOS in all trials ($P < 0.01$), except for 2019 Kununurra seed size ($P = 0.417$), and 2018 Kununurra yield ($P = 0.002$) (Table 4.1). Percent loss for both traits was highly significant for genotype across all trials ($P < 0.01$) (Table 4.1).

In the Podding glasshouse trial, high temperature stress had a highly significant impact on all measured traits ($P < 0.01$), except for HSW, which was merely significant ($P < 0.05$) (Table 2). The trait that underwent the largest highly significant impact was pod filling, which improved 31.04 % under the heat treatment compared to the control, and the trait with the smallest impact was vegetative mass, which improved 5.43 % under the heat treatment (Table 2). However, pod filling was the only trait in these trials that has no significance to genotype ($P = 0.071$) (Table 4.2). Yield and Vegetative mass saw a large decrease under temperature stress (32.97 % and 21.17 % respectively), whereas HSW and pod filling experienced an increase (5.43 % and 31.04 % respectively) (Table 4.2).

In the flowering glasshouse trial, percent loss values for all traits were highly significant between the treatment and both controls ($P < 0.01$), however HSW was the only trait that experienced a significant difference in relation to genotype ($P < 0.01$ for Drought difference and $P < 0.05$ for watering difference) (Table 4.3). On average, the WC demonstrated higher percent loss values compared to DC, most highly for yield (45.3 % higher) and least for pod

filling (9.8 % higher). Between the three trials (DC, WC, and Heat), WC had the highest values for all traits other than pod filling, where the heat treatment experienced a 33.45 % improvement (Table 4.3). Across all glasshouse trials, pod filling is the only trait that consistently experienced an improvement under heat stress, particularly in the flowering trial (40.67 % on average) with an average 35.86 % improvement under heat stress across all trials (Tables 2 and 3).

WC exhibited the best performance for all traits, with the exception of pod filling (Table 4.3). When compared to DC, WC demonstrated higher values in yield (45.3 %), HSW (15.7 %), vegetative mass (19.2 %), and seed number (35.0 %) (Table 4.3).

4.4.1 The Impact of Heat Stress

Table 4.1: Average performance for two commercially critical traits, yield and seed size, in each sowing time (treatment) for all sets of field trials, as well as the percentage loss of weight (g) between TOS1 and TOS2. Values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05). Subscript G also denotes Percent loss values that were highly significant (P < 0.01) for genotype.

	Location	Year	Time of Sowing 1	Time of Sowing 2	Percent Loss
Gross Yield (g)	Kununurra	2018	2017.52 ± 33.16	2130.02 ± 31.44	$^{*}{}_{G}$ -5.58
		2019	2657.23 ± 41.18	2885.95 ± 42.13	$^{**}{}_{G}$ -8.61
		2020	2555.48 ± 51.62	2086.10 ± 45.70	$^{**}{}_{G}$ 18.37
	Narrabri	2018	2170.87 ± 29.25	1416.38 ± 11.870	$^{**}{}_{G}$ 34.76
		2019	1732.80 ± 20.76	1166.50 ± 14.19	$^{**}{}_{G}$ 32.69
		2020	978.89 ± 12.88	516.73 ± 14.79	$^{**}{}_{G}$ 47.22

	Location	Year	Time of Sowing 1	Time of Sowing 2	Percent Loss
Weight of 100 Seeds (g)	Kununurra	2018	36.14 ± 0.45	34.21 ± 0.43	$^{**}{}_{G}$ 5.35
		2019	37.17 ± 0.47	39.53 ± 0.50	$_{G}$ -6.35
		2020	33.48 ± 0.38	31.28 ± 0.36	$^{**}{}_{G}$ 6.58
	Narrabri	2018	36.04 ± 0.48	34.22 ± 0.40	$^{**}{}_{G}$ 5.05
		2019	35.00 ± 0.33	31.52 ± 0.31	$^{**}{}_{G}$ 9.95
		2020	37.00 ± 0.35	32.32 ± 0.32	$^{**}{}_{G}$ 12.65

Table 4.2: Average performance for several physiological traits when exposed to an optimal and high-temperature environment. Data are shown for treatment for glasshouse trials

where heat exposure was initiated at the time of podding. Percent loss values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05). Subscript G also denotes Percent loss values that were highly significant (P < 0.01) for genotype.

Trait	Control	Heat	Percent Loss
Yield (g)	18.38 ± 0.80	12.32 ± 0.43	$^{**}{}_{G}$ 32.97
HSW (g)	28.92 ± 0.63	30.49 ± 0.58	$^{*}{}_{G}$ -5.43
Vegetative Mass (g)	46.52 ± 0.85	36.67 ± 0.75	$^{**}{}_{G}$ 21.17
Pod Filling (%)	74.42 ± 2.20	97.52 ± 1.61	**-31.04
Seed Number	66.94 ± 3.55	42.41 ± 1.86	$^{**}{}_{G}$36.64

Table 4.3: Average performance for several physiological traits when exposed to an optimal and high-temperature environment. Data are shown for treatment for glasshouse trials where heat exposure was initiated at the time of flowering. Difference in performance is shown between each treatment. Percent loss values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05). Subscript G also denotes Percent loss values that were highly significant (P < 0.01) for genotype.

	Drought Control	Water Control	Heat Treatment	Drought Percent Loss	Watering Percent Loss
Yield (g)	6.41 ± 0.23	11.71 ± 0.29	4.95 ± 0.27	**15.20	**31.44
HSW (g)	31.78 ± 0.69	37.70 ± 1.05	27.90 ± 0.78	$^{**}{}_{G}$ 22.78	$^{**}{}_{g}$ 57.73
Vegetative Mass (g)	12.24 ± 0.21	15.14 ± 0.29	10.38 ± 0.18	**12.21	**26.00
Pod Filling (%)	80.11 ± 1.60	88.80 ± 2.12	118.48 ± 3.52	**-47.90	**-33.43
Seed number	20.5 ± 0.83	31.53 ± 1.12	17.77 ± 1.00	$^{**}{}_{g}$13.3	$^{**}{}_{g}$43.66

Among all field trials, all traits show high significance for genotype, except for TOS1 yield in Kununurra 2020 (Table 4.6). CV varies by trait between each location, with the lowest average CV belonging to flowering in Narrabri and HSW in Kununurra (average CV = 2.87 and 4.75, respectively) and the highest belonging to yield in both locations (average CV = 18.17 and 24.85) (Table 4.6). Between locations, all traits have consistently lower CV values in Narrabri compared to Kununurra, with flowering at 65.2 %, yield at 26.9 %, HSW at 39.1 %, and height at 9.7 % lower average CV. Height has the lowest variation in CV values across all trials (SD = 1.93), and yield has the highest (SD = 7.66) (Table 4.6).

Between treatments for Narrabri there was an increase in genotypic trait variation under the heat treatment for all traits except height, which saw a 5.37 % decrease in variation

(Table 4.6). These increases were: (1) 37.74 % for flowering, (2) 14.31 % for yield, (3) 16.13 % for HSW (Table 4.6). In Kununurra, these variations were lower: (1) 18.58 % decrease for flowering, (2) 2.31 % decrease for yield, (3) 5.81 % increase for HSW, (3) 14.34 % increase for height (Table 4.6).

4.4.2 Trait Correlations

Table 4.4: *R2 correlation values between traits in all field trials,* grouped by year and location. Values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05). Table is colour coded according to correlation strength, with dark green representing strong ($R^2 > 0.7$), pale green representing moderate ($R^2 > 0.5$), and yellow representing weak ($R^2 < 0.5$).

Narrabri	2018		Yield	HSW	
		Flowering	0.448	0.109	
		Yield		0.25	
	2019		Yield	HSW	Height
		Flowering	0.504	0.367	0.628
		Yield		0.162	0.361
		HSW			0.216
	2020		Yield	HSW	Height
		Flowering	0.607	0.363	0.742
		Yield		0.313	0.565
		HSW			0.356
Kununurra	2018		Yield	HSW	
		Flowering	-0.260	0.142	
		Yield		-0.210	
	2019		Yield	HSW	Height
		Flowering	-0.111	0.136	0.170
		Yield		-0.071	0.057
		HSW			0.037
	2020		Yield	HSW	Height
		Flowering	-0.223	0.077	-0.047

		Yield	0.104	0.065
		HSW		0.145

Table 4.5: R2 correlation values between traits in both glasshouse trials. Values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05). Table is colour coded according to correlation strength, with dark green representing strong (R^2 > 0.7), pale green representing moderate (R^2 > 0.5), yellow representing weak (R^2 < 0.5), and orange representing moderate negative (R^2 < -0.5).

Flowering		Seed Number	Yield	HSW	Pod Filling (%)
	Veg Mass (g)	**0.316	**0.525	**0.584	**-0.515
	Seed Number		**0.894	*0.158	0.008
	Yield (g)			**0.521	*-0.193
	HSW (g)				**-0.422
Podding		Seed Number	Yield (g)	HSW (g)	Pod Filling (%)
	Veg Mass (g)	-0.037	-0.064	-0.121	**-0.541
	Seed Number		**0.926	**-0.462	*0.165
	Yield (g)			*-0.142	*0.161
	HSW (g)				-0.032

Between field trial locations, Narrabri is more consistent for highly significant correlations between traits, as well as strong and positive correlation values, particularly for yield (Table 4.4). The trait with the most consistent correlation for Narrabri is HSW (SD = 0.107, R^2 between 0.415 and 0.109), and the least consistent is height (R^2 between 0.742 and 0.216) (Table 4.4). For Kununurra, the most consistent is height (SD = 0.078, R^2 between 0.170 and -0.047), and the least is yield (SD = 0.144, R^2 between 0.104 and -0.260). All trait correlations in Narrabri are positive, with the weakest existing between HSW and flowering in 2018 (R^2 = 0.109), and the strongest between height and flowering in 2020 (R^2 = 0.742) (Table 4.4). Two thirds (6 of 9) of trait correlations in Kununurra were negative, with the strongest negative between yield and flowering in 2018 (R^2 = -0.26), and the strongest positive between height and flowering in 2019 (R^2 = 0.170) (Table 4.4).

In the glasshouse trials, there were more significant correlations between traits in the flowering trial compared to the podding trial, with the only correlation lacking significance

being between pod filling and number of seeds (R^2 = 0.008, P = 0.914) (Table 4.5). In the flowering trial, the trait with the highest consistent correlation was pod filling (SD = 0.235, R^2 between

0.008 and -0.515), and the lowest was vegetative mass (SD = 0.508, R^2 between 0.584 and -0.515) (Table 4.5). 70% of the trait correlations in the flowering trial were positive, and the strongest of these was between yield and seed number (R^2 = 0.894), followed by vegetative mass and HSW (R^2 = 0.584) (Table 4.5). The strongest negative exists between pod filling and vegetative mass (R^2 = -0.541) (Table 4.5). In the podding trial, the trait with the highest consistent correlation was HSW (SD = 0.227, R^2 between -0.032 and -0.462), and the lowest was seed number (SD = 0.519, R^2 between 0.926 and -0.462) (Table 4.5). 30% of the trait correlations in the podding trial were positive, with the following correlations that had positive correlations in the flowering trial now having negative: (1) seed number x vegetative mass, (2) yield x vegetative mass, (3) HSW x vegetative mass, (4) HSW x seed number, (5) HSW x yield, (6) pod filling x HSW (Table 4.5). The strongest positive correlation was between yield and seed number (R^2 = 0.926), followed by pod filling and seed number (R^2 = 0.165) (Table 4.5). The strongest negative correlation was between pod filling and vegetative mass (R^2 = -0.541), which is the same rank as in the flowering trial (Table 4.5).

4.4.3 Genotypic Significance and Variation

Table 4.6: Coefficient of variation values between genotype and trait for each field trial, grouped by Time of Sowing. Values labelled with ** are highly significant (P < 0.01), and with * are significant (P < 0.05).

		TOS	Flowering	Yield	HSW	Height
Narrabri	2018	1	** 1.6	** 16.3	** 4.2	
		2	** 7.2	** 10.4	** 4.6	
	2019	1	** 3.2	** 16.9	** 4.9	** 12.1
		2	** 1.9	** 12.8	** 4.3	** 12.9
	2020	1	** 1.8	** 17.1	** 3.9	** 9.5
		2	** 1.5	** 35.5	** 6.6	** 7.6
Kununurra	2018	1	** 6.0	** 24.1	** 5.1	
		2	** 8.7	** 20.2	** 5.0	
	2019	1	** 12.6	** 20.5	** 7.2	** 10.1

		2	** 10.7	* 22.8	** 9.1	* 13.5
	2020	1	** 8.2	30.8	** 10.4	* 11.4
		2	** 3.2	** 30.7	** 10.0	* 11.6

4.5 Discussion

4.5.1 Heat Stress – Impacts and Management

The impact of heat stress in chickpea has been well documented, however little is known regarding the mechanisms or development of tolerance within the crop (Jeffrey et al., 2021). There is ample research regarding heat escape, which involves early and rapid development (Berger et al., 2011; Toker & Canci, 2007). However, this method is highly reliant on water availability (Turner et al., 2001), and fails to take into account the potential this species has to endure and manage stress. There is evidence that chickpea plants are able to recover themselves from short (3 day) periods of temperature stress early in their development, and that this may prime them for improved tolerance to future heat stress events (Wang et al., 2006; Yadav et al., 2021). There is also evidence to suggest that the impact of heat stress can be mitigated by the increased availability of nutrients and water, with the ability to maintain yield by up to 80 % (H. D. Upadhyaya et al., 2011). This may explain the variation between trait performance between field trials in this study (Table 4.1). When looking at temperature alone, it could be assumed that the trials in Kununurra would show a reduction in yield and HSW when compared to Narrabri (Appendix 4.1). These trials experienced higher temperature throughout the growing period and experienced an average yield loss of 1.39 % between years (Table 4.1). It is possible that as the plants germinated under a high temperature they may have been primed to withstand further increase (Yadav et al., 2021). Additionally, as the standard agronomic practices of each region means that the Kununurra trials were irrigated and fertilised at much higher rates compared to Narrabri, these plants likely had a greater potential for stress management (H. D. Upadhyaya et al., 2011). As transpiration is a crucial part of cellular function, water availability is a key factor when considering stress management (Mathur et al., 2014), and it has been shown that while drought is a more impactful stressor than heat, the combination of both can be severe (Awasthi et al., 2014). This is reflected in the results using both WC and DC in the Flowering Glasshouse trial, demonstrating that increased water availability led

to an improvement in almost all traits, even with the plants held at optimal temperature (Table 4.3). Additionally, when examining trait stability across environments.

The developmental stage at which heat stress is applied can also cause a marked change in the way stress is managed, with multiple previous studies indicating the most vulnerable stage of development is flowering (Devasirvatham et al., 2010; Kaushal et al., 2013; Wang et al., 2006). However, while it is important to identify the most vulnerable life stage, it can also be beneficial to determine the way relationships between certain traits may change under stress. This is seen in the glasshouse, where the relationship between podding and vegetative mass is positive when stress is applied at flowering, but negative when it is applied at podding (Table 4.5). In Narrabri, trait correlations are exclusively positive with varying strength, however, in Kununurra the correlations associated with yield become either weaker, or negative (Table 4.4). This suggests that in Kununurra, genotypes that reached flowering sooner also had a higher yield, whereas in Narrabri, the genotypes with slower development yielded better. This may be due to these early genotypes experiencing heat stress slightly later in their development, when pod set had already begun, along with the fact that resource availability was higher in Kununurra compared to Narrabri, allowing these plants greater capacity to endure stress in the short term. Narrabri plants may have required a slowing in development in order to focus more energy towards stress management with the lesser amounts of nutrient and water available (Awasthi et al., 2014; Russelle et al., 1984). The glasshouse trials depict this trend in more detail, particularly when examining the traits that comprise seed yield, being seed number, pod filling, and HSW (Table 4.5). The variation in these trait relationships is likely due to a change in the use and partitioning of sucrose and starch based on the stress induction (Awasthi et al., 2014). The change in relationship between seed number and HSW is likely an example of this, as heat induced at flowering can lead a significant reduction in pollen viability and germination (Sanjeev Kumar et al., 2013), preventing the inception of new pods (Devasirvatham et al., 2013). When plants experience stress at podding, germination has already occurred, and there is already a higher demand for assimilates, so seeds will not grow large (Davies et al., 1999). More recent research has shown that heat stress impairs the photosynthetic pathway, reducing the supply and accumulation of carbohydrates to the developing seed (Awasthi et al., 2014). In the field, the relationship between yield and HSW is negative in

Kununurra, but positive in Narrabri, even though the yield in Kununurra is higher. This is potentially due an increased number of seeds in Kununurra with an inability to grow them to a larger size (Table 4.1). In contrast, the plants in Narrabri may have a lower seed number, but this would permit the allocation of more carbohydrate to each seed (Table 4.1). In the glasshouse, stress initiated at flowering caused a 22.78 % decrease in HSW ($P < 0.01$), whereas when it was initiated at podding, HSW actually increased 5.43 % ($P < 0.05$), though this was significantly dependant on genotype ($P < 0.01$) (Table 4.2, Table 4.3). This may be due to chickpea being an indeterminate plant, still producing flowers as pods are forming. The stress at podding would have caused abortion of these late-developed flowers, decreasing the number of seeds produced and thus enabling the existing seeds to reach a larger size. Additionally, there are several studies demonstrating a correlation between early flowering and high yield (Sanjeev Kumar et al., 2013; Rubio et al., 2004; Siddique et al., 2003), which may align with the suggestion of tolerance priming to explain the yield increase under high temperatures in Kununurra (Table 4.1).

As stated previously, much of the research into chickpea heat tolerance has focused on escape, but it is known that the plants have the capacity to manage stress through the provision of sufficient water (Mathur et al., 2014). One study has found that growing chickpea plants in the presence of Proline can increase heat tolerance via the protection of key enzymes, also reducing cellular injury (Kaushal, Gupta, Bhandhari, & Kumar, 2011). With a recent push in research devoted to the study of genome mapping for heat tolerance (Devasirvatham et al., 2015), there is space and demand for new discoveries and breeding directions involving traits other than rapid development.

4.5.2 Breeding – Capacity and Future Directions

When examining the impact of heat stress on key traits across a range of conditions, it becomes possible to assess their stability and thus suitability for breeding. In a previous study, it has been shown that HSW is both more heritable and genetically stable when compared to yield (Jeffrey et al, in Prep). This is further demonstrated within this study by the consistently low CV values for HSW across trials (Table 4.6). In contrast, the CV values for yield are much higher and more varied, suggesting a greater degree of plasticity in the

trait (Table 4.6). This is to be expected, as yield is comprised of several other traits (Zaiter & Barakat, 1995). Additionally, it can be noted that the average CV values for yield are 26.7 % lower in Narrabri compared to Kununurra (Table 4.6), likely due to the high water availability allowing for a range of stress management strategies, including avoidance and tolerance (Toker & Canci, 2007). Due to its high degree of phenotypic plasticity, breeding efforts for increased yield under heat stress will have minimal success, but by identifying stable traits with positive correlations to yield, it may yet be possible to find improvement. For example, height has relatively stable CV values both within and between environments (Table 4.6), and considering it has a consistently positive correlation with yield, this may be a suitable target.

There have been several studies investigating genomic regions that may be targeted for crop improvement under heat stress, including genes for flowering time (Ridge et al., 2017), and several heat stress transcription factors (Chidambaranathan et al., 2018). There have also been many studies locating QTLs for key traits, such as yield, pod filling, height, and seed size (Cho et al., 2002; Cobos et al., 2007; Lichtenzveig, Bonfil, Zhang, Shtienberg, & Abbo, 2006). Unfortunately, there is a large knowledge gap pertaining to biochemical and mechanistic responses to heat stress, but this is closing over time. Stomatal conductance has been found to be lower in heat sensitive genotypes (Kaushal et al., 2013), and a QTL hotspot has been located that is linked to enhanced transpiration efficiency under drought stress (Rajeev K Varshney, Thudi, et al., 2014). Recent studies have also found higher chlorophyll content in leaves under heat stress (Bhasker et al., 2018) and a positive correlation between Fv/Fm ratios and heat tolerance (P. Kumar et al., 2020), however it is unclear whether these traits are heritable. It is crucial that traits such as these are studied in more detail, and their capacity for genomic selection be determined, for the improvement of the crop.

4.6 Conclusion

By examining a range of traits under optimal and high temperature environments, across a range of locations, this study has demonstrated the impacts of heat stress on the growth, development, and yield capacity of chickpea. In particular, this study has demonstrated the environmental plasticity of chickpea plants when exposed to heat stress at different

developmental stages, with certain traits such as height showing a change in trait correlation consistency between locations in both field and glasshouse trials. In particular, the capacity for trait plasticity is shown to be highly beneficial in environments with high water availability. Within both field and glasshouse trials, plants demonstrated an extreme capability for managing and enduring heat stress when water availability was high, particularly when considering HSW and yield. Additionally, strong trait correlations have been identified among the trials that demonstrate a strong potential for further understanding of the ways in which these plants manage stress, as well as future breeding targets for the production of improved, reliable cultivars in a changing climate. Finally, recommendation is made to shift breeding focus to target traits that will permit plants to endure stressful conditions, rather than trying to outpace them.

5. A Genome-Wide Association Study to Investigate Heat Tolerance in Chickpea Identifies QTLs for Canopy-Closure and Early Flowering

5.1 Abstract

High temperature stress is a universal danger for agricultural products, particularly with the accelerating impacts of climate change. Chickpeas are a critical source of protein and starch for a large portion of the world's population and are known to be impacted by heat stress at every life stage. Previously known as the "Orphan Legume", there is little known regarding genetic linkages to either the effect or management of heat stress, and much previous research has focused on heat avoidance rather than tolerance. This study utilised a population of 147 chickpea genotypes, primarily Kabulis, across 12 field trials conducted in 2 locations and across 3 years. Physiology was examined, and data was used alongside DArT sequencing to perform a Genome Wide Association Study to connect physiology to genomic regions. This study has identified 14 QTLs related to yield, seed size, time to flowering, time to maturity, and final canopy closure. Among these, are the first QTLs ever identified for canopy closure in chickpea, along with a QTL that is likely linked to early flowering under heat stress. Additionally, several other QTLs serve to align with previous research in identifying QTL hotspots that can be targeted for selective breeding of several traits at once. The overall goal of this research is to identify new targets for genome assisted breeding in order to produce new genotypes that will be able to manage stress responses and endure heat stress, rather than use early phenology to escape it.

5.2 Introduction

Chickpea (*Cicer arietinum*) is a crucial source of protein and starch in many countries (Abbo, Berger, et al., 2003), making up roughly 20% of global pulse production (FAO, 2020). Chickpeas are a nutrient dense food, with high protein, iron, and starch contents, and are an agronomically strong crop, surviving on low nutrient soils through highly efficient nitrogen extraction via symbiosis with rhizobial bacteria (S. Sharma et al., 2013). Heat stress impacts every stage of plant growth and development (Jeffrey et al., 2021) and the latest

Intergovernmental Panel on Climate Change (IPCC) report has found that between 2021-2040 it is very likely that the global surface temperature will increase by 1.5 ℃ relative to 1850-1900 (IPCC, 2021). It has been shown that high temperatures can decrease potential yield of this crop by 10-15% for every degree increase beyond the minimum (H. D. Upadhyaya et al., 2011), with extreme heat events leading to a complete loss of yield (Sanjeev Kumar et al., 2013). With the increased urgency of mitigating the effect of climate change on global cropping systems, the last two decades have seen an increased focus on breeding for heat and drought tolerance in chickpea (Devasirvatham et al., 2015; Maphosa et al., 2020). However, up until the last 10 years, there have been limited studies linking genetic markers to relevant traits (Farahani et al., 2019; Thudi, Upadhyaya, et al., 2014b). As of 2012, more than 10,000 SNP markers were available for accelerating genetic research in chickpea (Hiremath et al., 2012), and there have been a number of studies utilising these to understand genetic diversity, linkage disequilibrium, and polymorphism information content (Farahani et al., 2019; Roorkiwal et al., 2016; Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014).

The availability of these markers, and improvement in the scientific community's understanding of the chickpea genome, there is now a need for a deeper understanding of the physiology of chickpea, and its alteration under heat stress. There have been markers found for traits that are known to be linked to a plant's performance under heat stress, such as flowering time, seed size, and plant height (Jagdish Kumar & Abbo, 2001; Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014). However, the extent of this research is limited, and many of these studies have been performed on only a small subset of genotypes, missing the full diversity of physiological responses possible in the crop.

One of the main issues in chickpea genomic research, is that its genome has been mapped much less extensively compared to other crops such as wheat (Alonge, Shumate, Puiu, Zimin, & Salzberg, 2020), even though the average chickpea chromosome size is roughly 12 % of a wheat chromosome (Rajeev K. Varshney, Thudi, & Muehlbauer, 2017). Additionally, the most recent genome-wide marker map was produced using Single-Nucleotide Polymorphisms (SNPs) in 2013 (Rajeev K Varshney et al., 2013) with the previous iteration produced with microsatellite SSRs in 2000 (Winter et al., 2000). Because of this, there is a

large deal of inconsistency in the markers used in research that has occurred since 2000, making it difficult to understand the locations of MTAs QTLs. All *Cicer* species are diploid, with 16 somatic chromosomes, but there is significant enough difference between chromosome length and chromosomal constrictions to be able to speciate *C. arietinum* from the wild progenitor *C. reticulatum* while still keeping a large degree of intraspecific variation in karyotype (Rajeev K. Varshney et al., 2017). Due to this speciation, early attempts to form heterotic hybrids have been mostly unsuccessful, due to post fertilisation abortion (Ahmad & Slinkard, 2004). As such, the primary direction for crop improvement via genomic technologies seems to be improvement of existing map resources, with the goal of developing enhanced genomic assisted breeding (GAB) programs (Pandey, Roorkiwal, Singh, Ramalingam, & Kudapa, 2016).

This study used 149 chickpea genotypes in 2 commercial growing regions in Australia to assess physiological traits under typical and heat-stressed conditions. With this data, a Genome-Wide Association Study (GWAS) was completed. The aim of this research is to identify key QTLs associated with traits related to or impacted by heat stress, in order to advance future breeding efforts and research into heat tolerance in this key crop. Additionally, this study aims to combine the findings of previous research to produce a chromosome map showing key genes and QTLs associated with heat stress and similar physiological traits. The hope is to create a simplified resource that depicts known genomic regions of interest, regardless of the marker type that was used to find them.

5.3 Materials and methods

5.3.1 Measured Traits and Data Curation

In total, data was collected across 3 years, 2 locations, and 2 sowing times, resulting in 12 field trials. 147 genotypes were examined, comprising 10 desi and 137 kabuli types. These genotypes originated from Australia (9), ICARDA (129), and ICRISAT (9), and are a mix of released cultivars and elite breeding lines (Appendix 4.1). Appendix 5.1 indicates the genetic relationship between individuals through a phylogenetic cluster dendrogram, visualised with Base R package (R Development Core Team, 2022) . For all 3 years and locations, the following traits were measured: Gross yield per plot (grams), HSW (grams), flowering time,

and maturity time. Canopy closure was also measured in the 2019 Narrabri trials using the "canopeo" app (Patrignani & Ochsner, 2015). For flowering and maturity time plots were inspected as regularly as possible, typically every 2-3 days, to determine the date at which 50 % of the plants in each plot had at least 1 flower and the date at which 50 % of each plot was desiccated and a brown colour. These dates were then used to calculate the number of days it took from sowing for the plants to reach flowering and maturity development stages respectively (Pundir, Reddy, & Mengesha, 1988).

Phenotypic data is summarised in Table 5.1. The predicted means were calculated using Genstat software (VSN-International, 2022b), and subsequent data was organised into QQ plots in Excel, grouped by location and year. This was done to determine the data's similarity to a normal distribution, to assess quality for GWAS analysis (Appendix 4.3).

5.3.2 DNA Extraction

Seeds were sprouted in petri dishes until large enough to produce sufficient leaves for extraction. 3-4 young leaves were collected and dried in an Eppendorf tube with silica gel for 2-3 days. Two ball bearings were added to each tube, and tissue was subsequently crushed with a lyser for 2min at 20 rpm. Ball bearings were then removed, and 800 µL of CTAB buffer added to each tube, with samples incubated at 65 ℃ for 30-40 min, followed by 5 min at room temperature. 600 µL Phenol-chloroform (24:1 ratio) was added to each tube, and samples were mixed for 2 min and then centrifuged at 10,000 rpm for 10 min. 600 µL supernatant was removed and placed in a 1.5 mL microcentrifuge tube, followed by 500 µL cold isopropanol. Samples were then chilled at -20 ℃ for 20 min, and then centrifuged at 10,000 rpm for 10 min. Supernatant was discarded, and remaining ethanol was removed through drying. The DNA pellet was washed via the addition of 500 µL washing to each tube, and the use of a centrifuge at 7,000 rpm for 10 min. Samples were then dried, and 100 µL TE (pH 8) and RNAse (1:100 ratio) was added to each tube, before incubation at 37 ℃ for 1-2 hours. DNA samples were diluted with double-distilled autoclaved water to 50 ng/µL and stored at -20 ℃ until use.

5.3.3 Field Trials

Field trials were conducted in Kununurra, Western Australia (15.7783° S, 128.7439° E), and Narrabri, New South Wales (30.2737° S, 149.7350° E), under the respective region's typical agronomic management. It should be noted that management practice between these sites was not identical, due to the research's aim to assess growth under typical commercial conditions. Trials in Kununurra experienced fortnightly flood-irrigation, with regular side-dressing throughout the season, whereas Narrabri trials were rainfed-simulation irrigated with rhizobial inoculation and pre-emergent applications at planting. Temperature was collected in hourly intervals for each location, and daily minima and maxima has been provided in Appendix 4. This data can be found in raw form through weather.agric.wa.gov.au (Kununurra site) and ozforcast.com.au (Narrabri site). Delayed sowing trials were carried out annually from 2018-2020 inclusive, with the first sowing (TOS1) occurring according to the regions' typical commercial season, and the second (TOS2) delayed by 1 month in Kununurra, and 2 in Narrabri. This variation in sowing delay between the regions is due to the shorter season length in Kununurra, caused by consistently higher temperatures than those of Narrabri (Appendix 4).

5.3.4 GWAS and Statistical Analysis

DNA samples were sent for DArT-SeqTM genotyping through Diversity Arrays, which returned 3,339 SNP markers. These markers were filtered for minor allele frequency < 0.5 %, removal of markers that provided information for > 20 % of lines, and removal of markers that were not present on the Cicer arietinum CDC Frontier genome v1.0 map used for GWAS (BioProject PRJNA175619) (Rajeev K Varshney et al., 2013). After filtering for duplication, minor allele frequency less than 0.05, and those that failed to provide information for greater than 20 % of the lines, 2,361 reported SNPs and 1,919 mapped positions were used for the analysis. In total, 83 GWAS (184 individuals from 3 countries, across 2 environments, 3 years, 2 TOS, and 11 traits) were used. From this, 56 significant MTA were identified using the threshold $-\log10p \geq 3$, leading to 14 QTLs identified across 4 chromosomes.

Population structure was analysed using PCA and phylogenetic cluster dendrogram to assess genetic relationships between individuals, with populations isolated for Desi and Kabuli type

chickpeas (Appendix 5.1, Figure 5.1). This was conducted using the “synbreed” package in R (V Wimmer, 2012), and visualised using ggplot2 in R (Wickham, 2016).

GWAS analysis and predicted allele effects were conducted using the “rrBLUP” package (Endelman, 2011). The analysis was aiming at identifying QTLs, which refers to an identifiable genomic region that can be associated with the presentation of a particular phenotype (Abiola et al., 2003). This analysis led to the detection of 14 QTLs that could be located on the CDC Frontier Version 1 genome assembly (Rajeev K Varshney et al., 2013). Visualisation of this map was completed using Mapchart 2.32 (Voorrips, 2002). Known genes and QTLs were located from existing literature, and their sequences identified using the NCBI Basic Local Alignment Search Tool (BLAST) (Sayers et al., 2022). GO terms were extracted, and these genes were added to the same map to determine validity of data and existence of novel QTLs.

5.4 Results

5.4.1 Suitability of Population Panel and Phenotypic Data.

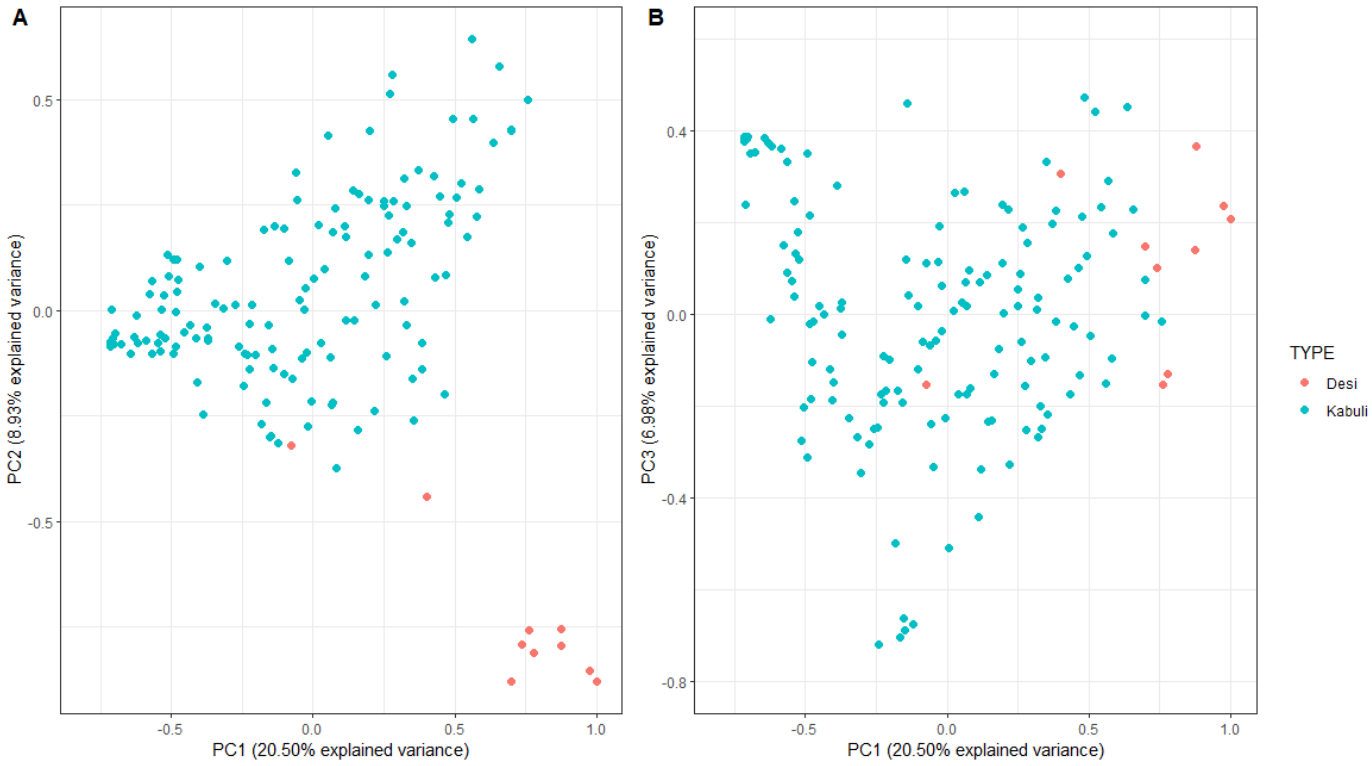
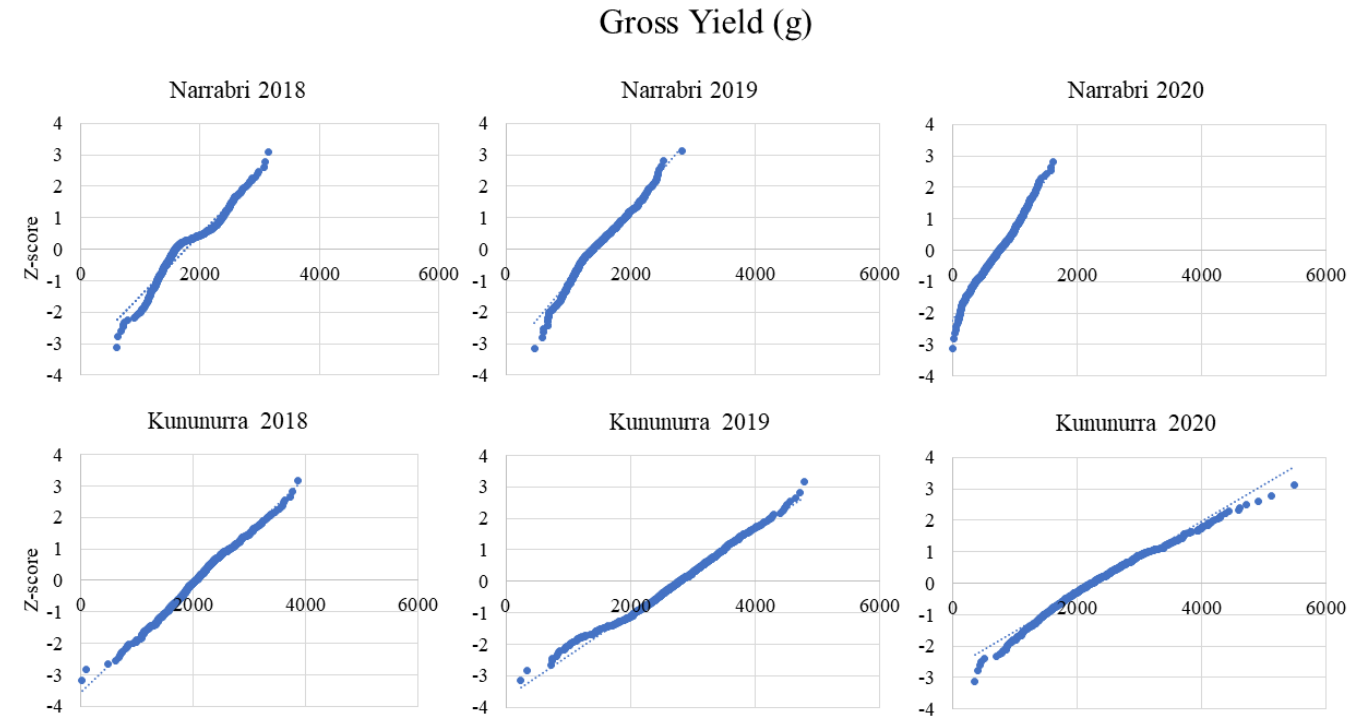
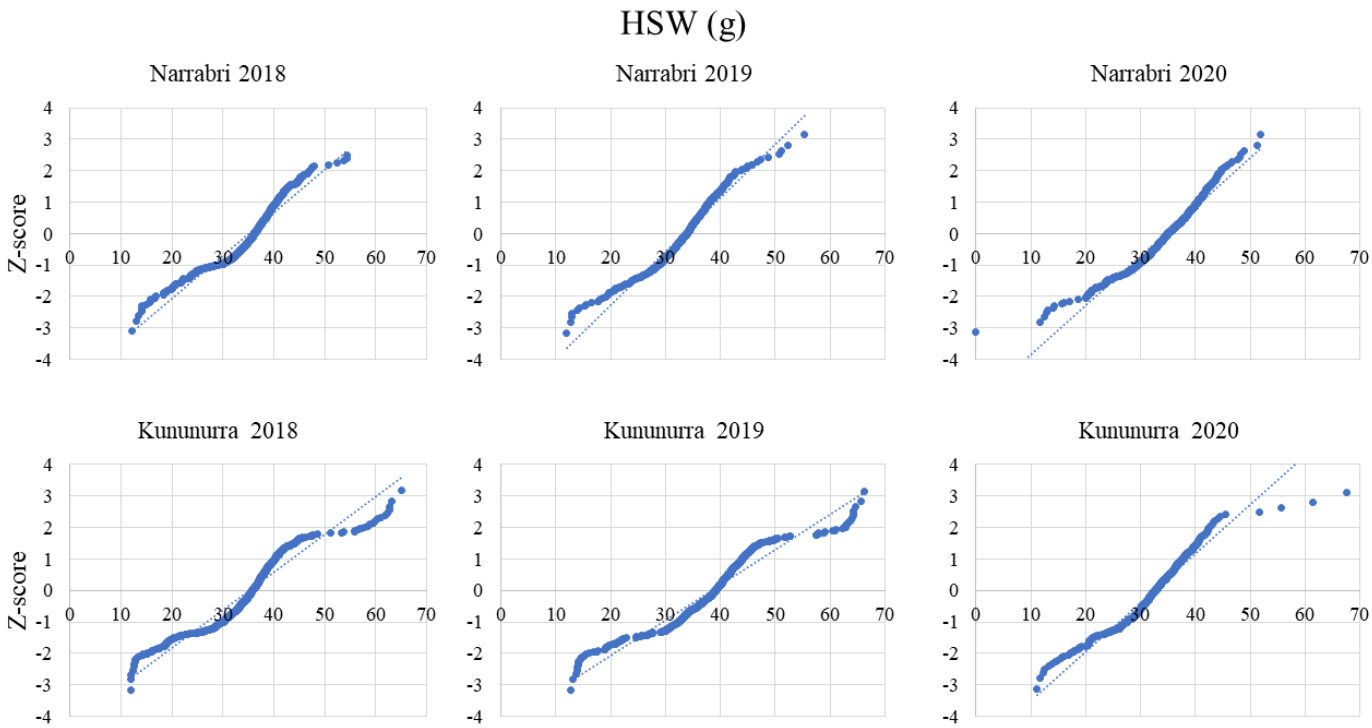


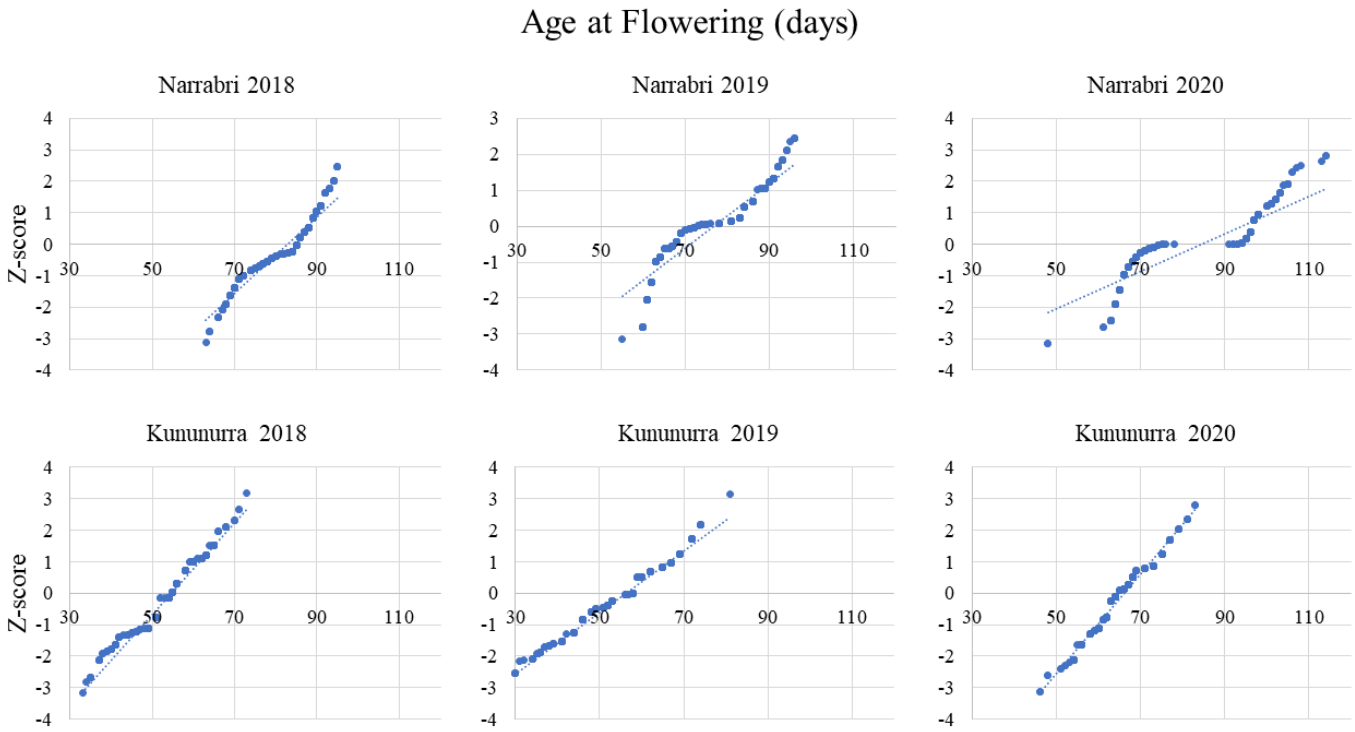
Figure 5.1: *Principal component analysis (PCA) differentiating the 147 desi and kabuli chickpea genotypes.* PC1 and PC2 refer to genetic variation within the population


Gross Yield (g)
Narrabri 2018
Narrabri 2019
Narrabri 2020
Kununurra 2018
Kununurra 2019
Kununurra 2020
Z-score
4
3
2
1
0
-1
-2
-3
-4
0
2000
4000
6000

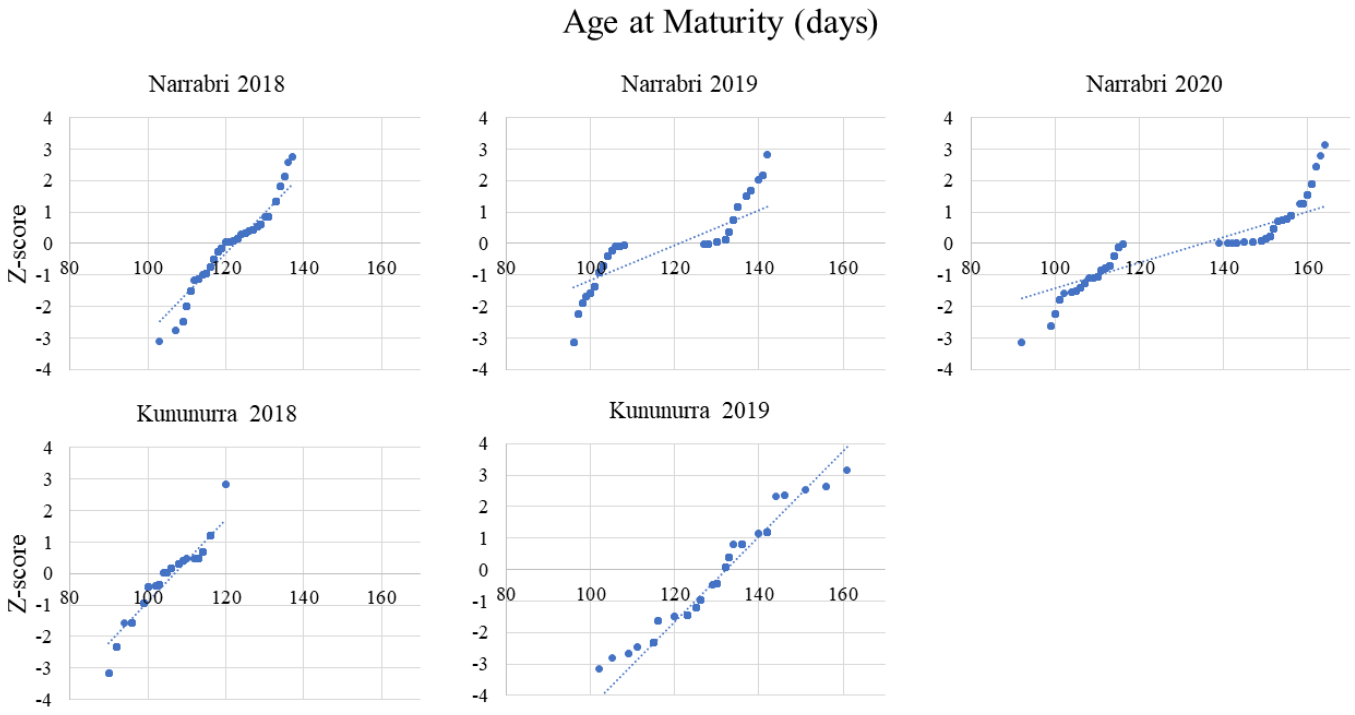
1659


HSW (g)
Narrabri 2018
Narrabri 2019
Narrabri 2020
Kununurra 2018
Kununurra 2019
Kununurra 2020
Z-score
4
3
2
1
0
-1
-2
-3
-4
0
10
20
30
40
50
60
70

1660


Age at Flowering (days)
Narrabri 2018
Narrabri 2019
Narrabri 2020
Kununurra 2018
Kununurra 2019
Kununurra 2020
Z-score

1661


Age at Maturity (days)
Narrabri 2018
Narrabri 2019
Narrabri 2020
Kununurra 2018
Kununurra 2019
Z-score

1662

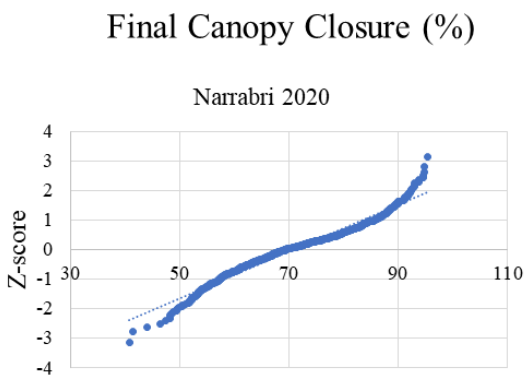


Figure 5.2: QQ plots depicting data distribution against the expected trend.

Principal component analysis (PCA) demonstrates that there is genetic separation between desi and chickpea type chickpeas, and there does not seem to be duplication of any genotypes. Even with the clustering of chickpea type, there is overlap enough for interbreeding among genotypes, and the population has suitable diversity to mine for QTLs. Along with this genetic variation, there is a strong relationship between environment and trait performance, demonstrated by the variation among all treatment levels (Year, TOS, Environment) and across all traits (Table 5.1), while phenotypic data remains within the limits of normal distribution (Figure 5.2).

5.4.2 Novel and Validated QTLs Identified by This Study

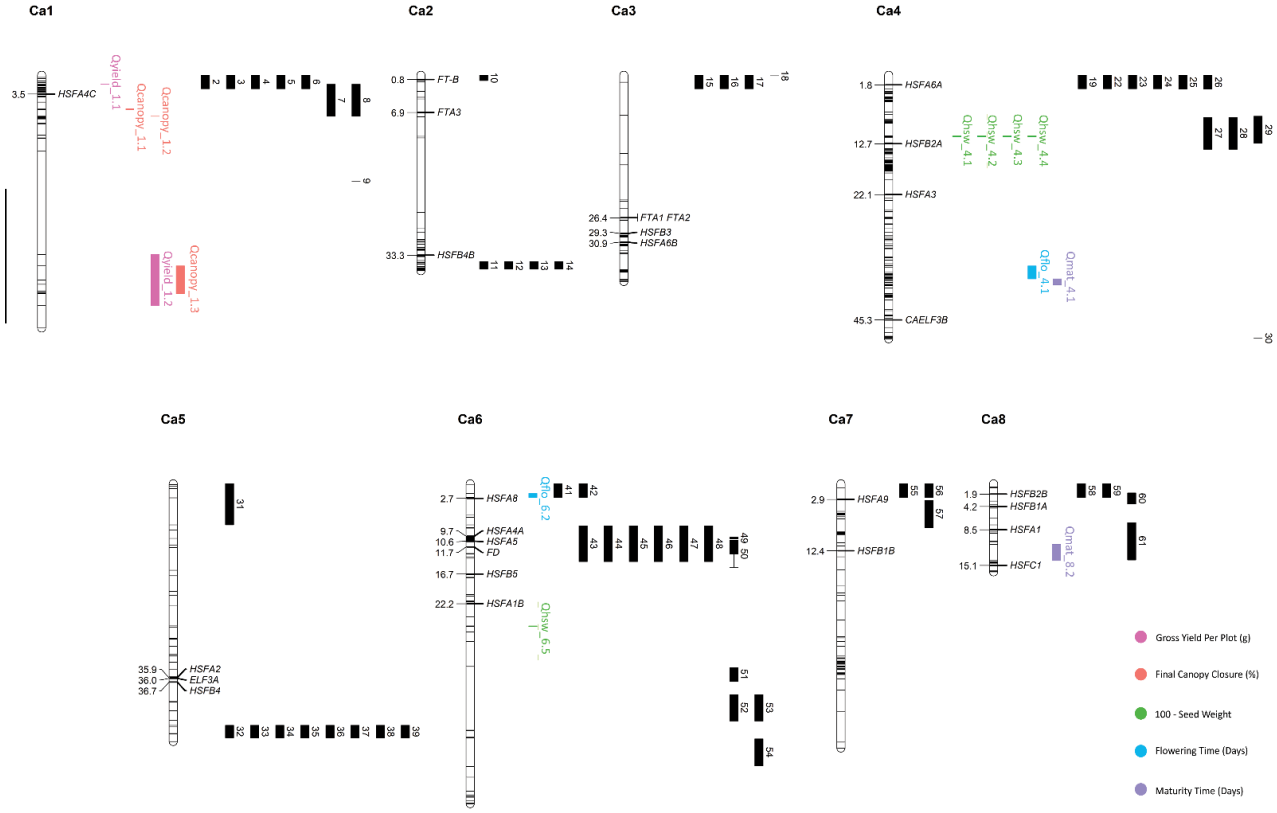


Figure 5.3: Physical map of QTLs identified in this study, along with the location of relevant and related gene and QTL findings from previous research. Chromosomes are represented as white vertical lines, with mapping markers denoted by black lines across the chromosomes. Genes are positioned according to Mb, and external QTLs are represented by black bands, numbered in order of chromosome and position, to the right of their relevant chromosome. QTLs identified by this study are represented by coloured bands, and labelled according to their linked trait. Positions are based on the CDC Frontier genome assembly (BioProject ID: PRJNA175619, NCBI Reference: NC_011163.1). A complete list of genes and QTLs, including their original position formats can be found in Supplemental Data Set 1. Colours denote putative roles in the following traits: Pink, total yield per plot (grams); Orange, final canopy closure (percentage); Green, 100-seed weight; Blue, days to 50 % plot flowering; Purple, days to 50 % maturation.

Table 5.1: Summary table of examined traits across trials.

	Kununurra	Narrabri

		Year	2018	2019	2020	2018	2019	2020
Gross Yield (g)	TOS 1	Mean	2015.72 ± 39.01	2704.67 ± 45.38	2568.22 ± 57.19	2179.44 ± 29.46	1724.68 ± 23.95	978.53 ± 15.36
		SD	496.45	564.9	693.29	353.51	293.23	186.79
	TOS 2	Mean	2133.07 ± 37.99	2917.73 ± 47.13	2073.07 ± 52.24	1404.42 ± 15.15	1165.58 ± 18.03	516.74 ± 18.02
		SD	483.43	586.68	633.27	187.35	220.04	219.14
HSW (g)	TOS 1	Mean	35.64 ± 0.57	36.51 ± 0.57	33.44 ± 0.49	37.21 ± 0.56	35.16 ± 0.45	37 ± 0.49
		SD	7.14	7.00	5.86	6.66	5.46	5.87
	TOS 2	Mean	33.78 ± 0.54	38.81 ± 0.6	31.24 ± 0.47	34.65 ± 0.5	31.57 ± 0.44	32.32 ± 0.43
		SD	6.78	7.38	5.60	6.10	5.30	5.14
Flowering Age (days)	TOS 1	Mean	52.13 ± 0.45	57.98 ± 0.76	67.45 ± 0.55	87.78 ± 0.25	84.91 ± 0.44	97.49 ± 0.28
		SD	5.64	9.36	6.63	2.99	5.32	3.32
	TOS 2	Mean	55.22 ± 0.49	52.49 ± 0.55	62.93 ± 0.25	78.18 ± 0.55	65.17 ± 0.28	67.23 ± 0.27
		SD	6.14	6.79	2.99	6.79	3.32	3.24
Maturity Age (days)	TOS 1	Mean	111.03 ± 0.37	127.55 ± 0.26		129.3 ± 0.28	133.62 ± 0.2	153.68 ± 0.31
		SD	4.65	3.24		3.30	2.45	3.68
	TOS 2	Mean	99.39 ± 0.25	133.39 ± 0.47		116.04 ± 0.25	102.34 ± 0.2	110.32 ± 0.35
		SD	3.16	5.82		3.09	2.39	4.21
Final Canopy Closure (%)	TOS 1	Mean						79.7 ± 0.67
		SD						8.09
	TOS 2	Mean						62.09 ± 0.45
		SD						5.41

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1692

1693 *Table 5.2: QTLs identified for a number of physiological traits across 147 genotypes in 12*

1694 *trials.*

Ch	QTL Name	Trait	Linked Trial	# of Associated Markers	Marker Effect	Left Location	Right Location
1	Qyield_1.1	Gross Yield Per Plot	Kun_2019_TOS1	2	-228.14	1.744678	1.744744
	Qcanopy_1.1	Canopy Closure	Na_2020_TOS1	2	-4.42	6.161699	6.383597
	Qcanopy_1.2	Canopy Closure	Na_2020_TOS2	2	0.91	7.630859	7.649141
	Qyield_1.2	Gross Yield Per Plot	Na_2018_TOS1	11	-376.00	33.185604	42.615142

	Qcanopy_1.3	Canopy Closure	Na_2020_TOS1	8	-9.61	35.323486	40.351158
4	Qhsw_4.1	Seed Size	Kun_2018_TOS2	5	-3.12	11.212337	11.441367
	Qhsw_4.2	Seed Size	Na_2019_TOS2	5	-2.39	11.212337	11.441367
	Qhsw_4.3	Seed Size	Na_2020_TOS1	5	-2.98	11.212337	11.441367
	Qhsw_4.4	Seed Size	Na_2020_TOS2	5	-3.00	11.212337	11.441367
	Qflo_4.1	Flowering Time	Na_2019_TOS1	3	-0.08	35.29603	37.725454
	Qmat_4.1	Maturity Time	Na_2020_TOS2	3	2.15	37.725454	38.784252
6	Qflo_6.2	Flowering Time	Kun_2020_TOS2	2	-1.41	1.829402	2.53797
	Qhsw_6.5	Seed Size	Kun_2018_TOS2	2	-3.91	26.349028	26.360262
8	Qmat_8.2	Maturity Time	Na_2019_TOS1	2	-1.07	11.150126	14.248237

Table 5.3: Genes identified by external literature for chickpea, related to heat stress.

Symbol	LG	Description	Gene ID	Reference
HSFA4C	Ca1	Heat Stress Transcription Factor A-4c	101515265	(Chidambaranathan et al., 2018)
HSFB4B	Ca2	Heat Stress Transcription Factor B-4b	101499841	
HSFA6B	Ca3	Heat Stress Transcription Factor A-6b	101499033	
HSFB3		Heat Stress Transcription Factor B-3	101502316	
HSFA3	Ca4	Heat Stress Transcription Factor A-3	101513159	
HSFA6A		Heat Stress Transcription Factor A-6a	101493622	
HSFB2A		Heat Stress Transcription Factor B-2a	101505110	
HSFA2	Ca5	Heat Stress Transcription Factor A2	101510605	
HSFB4		Heat Stress Transcription Factor B-4	101514043	
HSFA1B	Ca6	Heat Stress Transcription Factor A-1b	101514812	
HSFA4A		Heat Stress Transcription Factor A-4a	101489852	
HSFA5		Heat Stress Transcription Factor A-5	101494390	
HSFA8		Heat Stress Transcription Factor A-8	101489530	
HSFB5		Heat Stress Transcription Factor B-5	101504520	
HSFA9	Ca7	Heat Stree Transcription Factor A-9	101495918	
HSFB1B		Heat Stree Transcription Factor B-1b	101504213	
HSFA1	Ca8	Heat Stree Transcription Factor A-1	101492154	
HSFB1A		Heat Stress Transcription Factor B-1a	101494022	
HSFB2B		Heat Stress Transcription Factor B-2b	101502512	
HSFC1		Heat Stress Transcription Factor C-1	101489265	
FTA3	Ca2	Protein FLOWERING LOCUS T	101515383	(Ridge et al., 2017)
FT-B		Protein FLOWERING LOCUS T	101505276	
FTA1	Ca3	Protein FLOWERING LOCUS T	101497376	
FTA2		Protein FLOWERING LOCUS T-Like	101496618	
CAELF3B	Ca4	Protein EARLY FLOWERING 3b	101488316	
ELF3A	Ca5	Protein EARLY FLOWERING 3a	101489432	
FD	Ca6	Protein FLOWERING LOCUS D	101490188	

Table 5.4: QTLs identified by external literature for chickpea, related to traits measured in this study.

Related To	QTL_ID In This Map	LG	QTL Name	Reference
Yield	2	1	Q1-1	(Rehman, Malhotra, Bett, & Tar'an, 2011)
	7	1	Qgy01_1	(Paul et al., 2018)
	8	1	Qgy01_1	(Paul et al., 2018)
	21	4	Qtlyd	(Cobos et al., 2007)
	26	4	Q4-1	(Rehman et al., 2011)
	36	5	Qgy02_5	(Paul et al., 2018)
	37	5	Qgy02_5	(Paul et al., 2018)
	47	6	Qgy03_6	(Paul et al., 2018)
	48	6	Qgy03_6	(Paul et al., 2018)
	49	6	Casypp_NS6	(Jha et al., 2021)
Seed Size	20	4	Qtlsw1	(Cobos et al., 2007)
	24	4		(Cho et al., 2002)
	42	4		(Jamalabadi, Saidi, Karami, Kharkesh, & Talebi, 2013)
	57	7	Qph-2	(S. Gupta et al., 2015)
Seed Number/Plant	23	4		(Cho et al., 2002)
Pod Setting %	27	4	Q%Podset03_4	(Cobos et al., 2007; Paul et al., 2018)
	28	4	Q%Podset03_4	(Cobos et al., 2007; Paul et al., 2018)
	32	5	Q%Podset06_5	(Cobos et al., 2007; Paul et al., 2018)
	33	5	Q%Podset06_5	(Cobos et al., 2007; Paul et al., 2018)
	43	6	Q%Podset08_6	(Cobos et al., 2007; Paul et al., 2018)
	44	6	Q%Podset08_6	(Cobos et al., 2007; Paul et al., 2018)
Pod Filling	11	2	Qfpod01_2	(Cobos et al., 2007; Paul et al., 2018)
	12	2	Qfpod01_2	(Cobos et al., 2007; Paul et al., 2018)
	34	5	Qfpod02_5	(Cobos et al., 2007; Paul et al., 2018)
	35	5	Qfpod02_5	(Cobos et al., 2007; Paul et al., 2018)
	45	6	Qfpod03_6	(Cobos et al., 2007; Paul et al., 2018)
	46	6	Qfpod03_6	(Cobos et al., 2007; Paul et al., 2018)
Number Of Seeds	13	2	Qts01_2	(Cobos et al., 2007; Paul et al., 2018)
	14	2	Qts01_2	(Cobos et al., 2007; Paul et al., 2018)
	38	5	Qts02_5	(Cobos et al., 2007; Paul et al., 2018)
	39	5	Qts02_5	(Cobos et al., 2007; Paul et al., 2018)
Number Of Filled Pods	51	6	Cafp_NS6	(Jha et al., 2021)

Height	54	3	Qsw-2	(S. Gupta et al., 2015)
	6	1	Q1-1	(Rehman et al., 2011)
	25	4	Q4-3	
Early Flowering	22	4	QTLEF1	(Cobos et al., 2007)
Double Podding	41	6		(Cho et al., 2002)
Days To Podding	9	1	Cadpi_LS1	(Jha et al., 2021)
	53	6	Cadpi_LS6	
Days To Pod Filling	30	4	Cadpf_NS4	
	60	8	Cadpf_LS8	
Days To Maturity	4	1	Q1-1	(Rehman et al., 2011)
	16	3	Q3-1	
	55	7	Q7-1	
Days To Flowering	1	1	Qefl2-1	(Mallikarjuna et al., 2017)
	3	1	Q1-1	(Rehman et al., 2011)
	10	3	Qefl1-1	(Jamalabadi et al., 2013)
	18		Qefl3-2	
	15	3	Q3-1	(Rehman et al., 2011)
	19	4	Qefl1-2	(Mallikarjuna et al., 2017)
	29		Qefl2-3	
	31	3	Qefl3-1	
	40	6	Qefl4-1	
	50	1	QTL1	(Lichtenzveig et al., 2006)
	52	6	Cadfi_LS6	(Jha et al., 2021)
	58	8	Qefl2-4	(Mallikarjuna et al., 2017)
	59	8	Q8-2	(Rehman et al., 2011)
	61	8	Cadfi_LS8	(Jha et al., 2021)
Days From Flowering to Maturity	5	1	Q1-1	(Rehman et al., 2011)
	17	3	Q3-1	
	56	7	Q7-1	

This study identified a total of 14 QTLs, relating to gross yield, HSW, flowering age, maturity age, and final canopy closure. QTLs identified in this study exist on chromosomes 1 ,4, 6 and 8 (Table 5.2; Figure 5.2). Five of these QTLs, one for each trait; are novel, located in regions that have not previously been linked to traits of interest (Figure 5.2). For each of the measured traits, there is at least 1 QTL aligning with genetic region associated with previously described QTLs (Figure 5.2). QTLs for flowering are the most extensively located among external literature (Table 5.4), whereas for this study, the trait with the highest representation of QTLs was HSW (Figure 5.2, Table 5.2). Most QTLs located in this study are related to the Narrabri field trials (10 of 14) particularly TOS1 (6), conversely there are more

linked to the Kununurra TOS2 trials (3) than TOS1 (1) (Table 5.2). QTLs associated with yield were both detected at TOS1 trials, one at each location, whereas those for HSW span multiple trials (Table 5.2).

Several of the QTLs identified in this study have overlapping positions with QTLs and genes identified by previous research, such as the co-location of Qyield_1.1 and Q1-1 (Rehman et al., 2011). In total, there are 5 regions where QTLs in this study have overlapping positions with mapped QTLs and genes found in previous literature. These are as follows: (1) Qyield_1.1, overlapping with QTL IDs 1-6, linked to days to flowering, days to maturity, days from flowering to maturity, and height ((Mallikarjuna et al., 2017; Rehman et al., 2011), and HSFA4C (Chidambaranathan et al., 2018), (2) Qcanopy_1.1 and Qcanopy_1.2, overlapping with QTL IDs 7 and 8, linked to yield (Paul et al., 2018), (3) Qhsw_4.1-4.4, overlapping with QTL IDs 27-29, linked to pod setting and days to flowering (Cobos et al., 2007; Mallikarjuna et al., 2017), and HSFB2A (Chidambaranathan et al., 2018), (4) Qflo_6.2, overlapping with QTL IDs 41 and 42, linked to double podding and seed size (Cho et al., 2002; Jamalabadi et al., 2013), and HSFA8 (Chidambaranathan et al., 2018), (5) Qmat_8.2, overlapping with QTL ID 61 linked to days to flowering (Jha et al., 2021) (Figure 5.3, Table 5.2, Table 5.3, Table 5.4).

Based on the review of existing literature, this study has identified the first QTLs to be associated with final canopy closure in Chickpea, with Qcanopy_1.1, 1.2, and 1.3 identified on chromosome 1, and closely linked to yield-linked QTLs Qyield_1.1 and 1.2, and those discovered in previous literature designated QTL 7 and 8, also linked to yield (Paul et al., 2018) (Figure 5.3, Table 5.2). These QTLs are also found across both trials in which the trait was measured (Table 5.2).

5.5 Discussion

The QTLs identified in this study provide strong potential to both support and advance current research regarding the chickpea genome. The overlap of new and previously discovered QTLs on chromosomes 1, 4, 6 and 8 suggest the presence of highly valuable QTL hotspots, which could be targeted for marker-assisted breeding (Figure 5.2, Table 5.2, Table

5.4). This is particularly exciting, considering the range of traits these QTLs are associated with.

Due to the large number of trials on which these findings are based, it is also possible to make inference and prediction regarding the expression of each QTL, based on the presence of a stressor. This allows a deeper understanding of the plasticity of these traits, along with an improved understanding of the expression of these traits under particular conditions. The majority of the QTLs found in this study are linked to Narrabri field trials, particularly those of TOS1, and these are linked both to physiology and yield traits (Table 5.2). This may be related to the domestication of chickpeas to a Mediterranean environment, which is similar in climate to Narrabri (Jagdish Kumar & Abbo, 2001), and therefore a selection pressure for performance under these conditions. However, the location of Qflo_6.2, linked to flowering time in Kununurra TOS2 2020, may indicate the expression of a particular genomic region linked to flowering under heat stress, particularly as it does not co-occur with any of the other mapped QTLs for flowering time (Lichtenzveig et al., 2006) (Figure 5.2, Table 5.4). Qflo_6.2 overlaps with the mapped gene HSFA8, which is a HSF linked to shoot apical meristem and floral tissue development under heat stress (Chidambaranathan et al., 2018) (Figure 5.2, Table 5.4). This overlap could indicate that Qflo_6.2 is linked to a developmental acceleration under heat stress, particularly as early flowering, or “escape”, is a known physiological strategy for performance under heat stress (Jagdish Kumar & Abbo, 2001), and plant growth was accelerated under the higher temperatures in Kununurra. This is further suggested by the presence of the other QTL for flowering time, Qflo4.1, which was identified in Narrabri TOS1 2019, overlapping with Qmat_4.1 (Figure 5.2, Table 5.2). This suggests differential protein expression for this trait, depending on the environmental conditions experienced by the plant. In contrast, the QTLs identified for HSW predominately share an overlapping location on chromosome 4, though they are linked to different trials (Figure 5.2). This suggests that the expression of the genomic region responsible for seed size is not strongly impacted by environmental conditions, making this trait desirable for genomic selection in breeding (Pierce, 2020). This is supported by an investigation by Jeffrey et al. (In prep (Chapter 3)), which found HSW to be highly heritable, particularly when compared to yield. Additionally, the overlap of Qhsw_4.1-4.4 and the gene HSFB2A may indicate that under heat stress, plants divert energy away from new growth and into

existing structures, such as seeds. HSFB2A is an HSF that is upregulated under heat stress, linked to the development of all tissue types except those of shoots, and linked most heavily with the development of young pods (Chidambaranathan et al., 2018). Similarly, the QTLs associated with yield in this study were both linked to TOS1 trials, although different locations, suggesting that yield may be more heritable under stress-free conditions (Figure 5.2, Table 5.2).

5.5.1 Canopy Closure – A Recommendation for Future Research

This study appears to be the first to describe genomic regions associated with final canopy closure in chickpea. This is an exciting discovery both from the perspective of novel QTL discovery, and the potential to build on research that is focused on stress management. Much previous research has focused on heat escape, examining traits such as earliness in flowering and maturity (Berger et al., 2011). However, while breeding for heat escape can be beneficial, this is only a solution as long as seasons remain predictable, and temperature shifts minimal, which does not align with recent predictions (NASA, 2022). It has been known for some time that canopy climate is crucial for the maintenance of healthy plant tissues, particularly reproductive organs (Gates, 1968). The creation of shade via canopy closure has the potential to protect root systems, and their rhizobial symbionts, from overheating (Lewis et al., 2000). Additionally, as effective transpiration can cool leaves from 6-10 °C, canopy closure can create a cooler zone in which pods and other delicate tissues can grow with reduced heat stress (Mathur et al., 2014). This study has identified 3 novel QTLs linked to final canopy closure, which also show linkage with yield-linked QTLs found by both this and previous research (Jha et al., 2021; Paul et al., 2018). This doesn't just give the potential for the production of new chickpea genotypes with an ability to survive under high temperature, but the potential to create genotypes that can manage their own stress response and thrive through physiology alone, without the sacrifice of yield. Finally, Qyield_1.1, Qcanopy_1.1, and Qcanopy_1.2 overlap with a QTL for flowering date (Mallikarjuna et al., 2017), a QTL for yield, flowering, height, and maturity (Rehman et al., 2011), and a QTL for yield (Paul et al., 2018), along with gene HSFA4C, which is linked to a heat stress transcription factor (HSF), and expressed in the presence of a *fusarium* wilt infection (Chidambaranathan et al., 2018) (Figure 5.2, Table 5.2, Table 5.4). This overlap

suggests that these particular traits are linked, suggesting the potential of not only a QTL hotspot, but a powerhouse for GAB to vastly improve the performance of future chickpea genotypes (Pierce, 2020).

The discovery of QTLs linked to this trait creates the potential for further understanding of its expression, control, and breeding potential, and this could lead to the development of genotypes that can respond to and manage stress at a significantly more efficient and effective rate. Additionally, as transpiration efficiency has been found to have a heritability rate of up to 70 % (Thudi, Upadhyaya, Rathore, Gaur, Krishnamurthy, Roorkiwal, Nayak, Chaturvedi, Basu, Gangarao, et al., 2014), and stomatal conductance is seen to be higher in heat tolerance genotypes (Kaushal et al., 2013), breeding plans can be created that target all of these traits, enhancing the tools we currently have for breeding.

5.6 Conclusion

This study has used 12 multi environment trials to investigate genetic linkages between specific physiological traits in chickpea in order to gain an improved understanding regarding its management and the improvement of future genotypes. Through the identification of 14 QTLs related to these key traits, this research builds on previous discoveries of potential QTL hotspots, as well as locating novel QTLs for seed size, yield, flowering time, and maturity time. Within this collection is a QTL likely linked to earliness under heat stress (Qflo_6.2) that could be targeted in GAB programs for chickpeas that utilise a heat escape physiology. In addition, this study is the first to investigate potential genomic linkages to canopy closure in chickpea and has discovered the first QTLs related to this trait. The hope of this research is that this discovery will encourage further examination into breeding for true stress management and tolerance, rather than heat escape.

6. General Discussion

Heat stress is a universal danger when considering the scope of global agriculture, and grain legumes are no exception. In an ever-changing world, stable access to food is one of the most critical needs faced by the global population, and also one of the most threatened by climate change (IPCC, 2021). While chickpea is a staple food in many countries, its susceptibility to heat stress at all stages of development means that its reliability both as a source of sustenance and agricultural income is at great risk (Kumar et al., 2013). Labelled as the orphan legume until recently, research into heat stress in chickpea has only become prominent within the last decade, with vast knowledge gaps left to be filled (Srivastava, Dixit, & Singh, 2017).

With the latest developments in genomic breeding and mapping technologies, there is now a capacity to fill these gaps. This thesis aims to do so by investigating the impacts of heat stress through several lenses. While the overall aim of this research is to better understand the genetic underpinnings of development and yield-based traits, there has been a parallel goal to improve predictive growth tools and better understand physiological responses to heat stress. In this way, this research aims to provide a thorough and complex understanding of both how heat stress impacts this crop, and what potential there is to improve plant resilience and provide agency to growers in their ability to predict crop performance.

One of the overarching themes of this research has involved trait plasticity, namely that of seed size versus gross yield. Chapters 3 and 4 investigate this in detail, with the coefficient of variation, heritability, and principal component values all indicating that seed size has reliable performance across environments, and a considerable degree of phenological stability when compared to all other traits. Additionally, when examining the QTLs identified in this study, there is locational overlap for almost all QTLs related to seed size, irrelevant of the environment in which they occur. This suggests an area that shows little variation in its activation under varying conditions (Pierce, 2012). These findings support previous work, which has found two dominant genes involved in seed size, as well as several molecular

markers (Thudi et al., 2011; Upadhyaya, Dronavalli, Gowda, & Singh, 2011; Upadhyaya, Kumar, Gowda, & Singh, 2006).

In contrast, yield is known to be strongly influenced by environment (Millan et al., 2006), and this can be seen by the varying performance, heritability, and coefficient of variation values across trials (Tables 3.4, 4.1, and 4.6). While it can be said that selective breeding is less effective when targeting highly plastic traits (Gage et al., 2017; Pierce, 2012), this environmental variation in phenology can also be used as a tool. Figure 3.1 shows that the large spread of yield performance across environments can also be used to select specific genotypes based on the environment they are expected to grow in. Some genotypes were seen to have a higher yield under heat stress, while others showed consistent performance irrelevant of their stress level. This is a great benefit to GAB programs, as it allows selective breeding based on predicted environmental conditions, producing either a genotype that will have enhanced yield in a hotter climate, or one that will be predictable irrelevant of the temperature regime. Additionally, these high performers can be targeted for more detailed study, to attempt to identify the genomic regions responsible for their resilience.

Much of previous research regarding genomic regions associated with heat tolerance in chickpea has focused on reproductive traits such as time to flowering, along with those associated with yield, such as seed size and podding (Devasirvatham et al., 2013; Kumar et al., 2013; Paul et al., 2018). This thesis demonstrates a high potential for genomic discovery, with the identification of 3 novel QTLs linked to canopy closure in chickpea. Canopy closure is known to be involved in stress management, protecting developing pods from solar radiation while the plant creates a cooler environment through increased transpiration (Mathur, Agrawal, & Jajoo, 2014). Through further study of this trait and its genetic control in chickpea, there is the potential to develop new genotypes with a heightened ability to protect their reproductive tissues and continue to yield well under normally stressful conditions. Additionally, this same study has identified a QTL (Qflo_6.2) that shares a position with a known HSF involved in shoot and floral tissue development under heat stress (Chidambaranathan et al., 2018). This QTL was only identified under heat-stressed conditions, and its shared position with this HSF may indicate an involvement with early floral development to escape heat stress. Chickpea is a species that has not received the

same attention as other grain crops when it comes to genomic mapping. In chapter 5, 2,361 SNPs were used to conduct a GWAS into heat tolerance in chickpea, whereas a similar study in wheat was able to utilise 52,610 (Khan et al., 2022). This is especially remarkable as the average chickpea chromosome is 12 % of the size of that of wheat (Cui et al., 2017). With the discoveries made through this thesis' GWAS, it can be seen that there is great potential in gaining a more detailed understanding of the plant's physiology under stress conditions. In doing so, genomic maps with improved resolution can be developed, which will subsequently permit more trait-based QTL discovery. The discovery of novel QTLs related to canopy closure also suggests that there is great potential for research into other physiological traits that may be involved with the management of stress responses. Table 4.4 depicts a positive correlation between yield and height across almost all trials, and while this study did not identify any QTLs linked to height, this correlation is an example that there may be potential for improved yield performance by building an understanding of the traits that show positive correlation with it, along with their genomic linkages.

Along with new discovery, the research within this thesis has also given rise to a range of new questions, and potential for future research. As with much GAB-based research, there is recommendation for duplicate research that involves more climates, more genotypes, and more traits to be assessed. The lack of resolution for the chickpea genome compared to other crops requires addressing as soon as possible, and this requires the collection of dense and detailed data. Part of this process should involve a focus on traits that have not received a great deal of focus, such as canopy coverage, branching, leaf area, and processes involved in photosynthesis and transpiration such as the findings depicted in table 2.1 (Kumar et al., 2013). This will vastly improve future genotype development speed and success.

Finally, it should be noted that there exists a disparity between developed and developing countries regarding access to agricultural technologies and knowledge. This includes access to improved genotypes (Tadesse, Bishaw, & Assefa, 2019), and access to knowledge regarding agronomic strategies to mitigate the effects of stress (Abid, Scheffran, Schneider, & Elahi, 2019). In order to successfully implement research such as that found in this thesis, there is a need to improve the dissemination of seed, strategies, and their use.

This thesis serves to provide an improved understanding of the impacts of heat stress on commercially cultivated chickpea, its physiological and genetic infrastructure, and future directions that can be taken to further the work.

7. References

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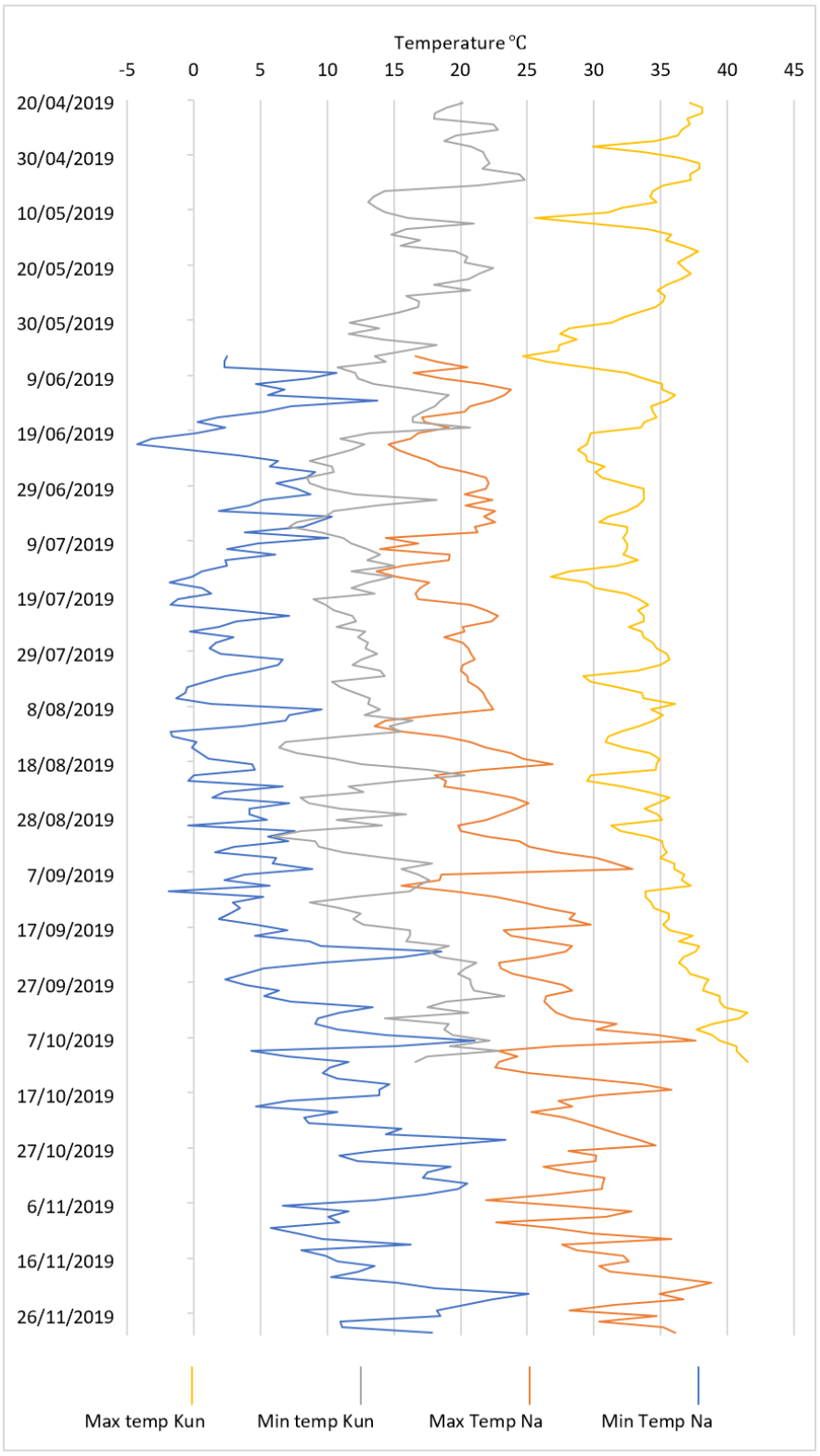
8. Appendix

Appendix 3.1. *Recorded season temperature maxima and minima for the trial in both Narrabri and Kununurra 2019,* collected from stations managed by ozforcast.com.au and weather.agric.wa.gov.au respectively.



2496 *Appendix 3.2. Genotypes used in this study, organised alphabetically and numerically, with*

2497 *their type and country of origin labelled.*

#	Genotype	Type	Origin
1	Almaz	Kabuli	Syria
2	FLIP 07-291C	Kabuli	Syria
3	FLIP 07-295C	Kabuli	Syria
4	FLIP 07-306C	Kabuli	Syria
5	FLIP 07-312C	Kabuli	Syria
6	FLIP 07-328C	Kabuli	Syria
7	FLIP 07-329C	Kabuli	Syria
8	FLIP 07-339C	Kabuli	Syria
9	FLIP 07-340C	Kabuli	Syria
10	FLIP 09- 84C	Kabuli	Syria
11	FLIP 09- 90C	Kabuli	Syria
12	FLIP 09-125C	Kabuli	Syria
13	FLIP 09-126C	Kabuli	Syria
14	FLIP 09-129C	Kabuli	Syria
15	FLIP 09-133C	Kabuli	Syria
16	FLIP 09-136C	Kabuli	Syria
17	FLIP 09-140C	Kabuli	Syria
18	FLIP 09-141C	Kabuli	Syria
19	FLIP 09-146C	Kabuli	Syria
20	FLIP 09-148C	Kabuli	Syria
21	FLIP 09-150C	Kabuli	Syria
22	FLIP 09-154C	Kabuli	Syria
23	FLIP 09-155C	Kabuli	Syria
24	FLIP 09-156C	Kabuli	Syria
25	FLIP 09-157C	Kabuli	Syria
26	FLIP 09-158C	Kabuli	Syria
27	FLIP 09-159C	Kabuli	Syria
28	FLIP 09-160C	Kabuli	Syria
29	FLIP 09-164C	Kabuli	Syria
30	FLIP 09-165C	Kabuli	Syria
31	FLIP 09-168C	Kabuli	Syria
32	FLIP 09-169C	Kabuli	Syria
33	FLIP 09-170C	Kabuli	Syria
34	FLIP 09-173C	Kabuli	Syria
35	FLIP 09-179C	Kabuli	Syria
36	FLIP 09-180C	Kabuli	Syria
37	FLIP 09-183C	Kabuli	Syria
38	FLIP 09-184C	Kabuli	Syria
39	FLIP 09-185C	Kabuli	Syria
40	FLIP 09-186C	Kabuli	Syria
41	FLIP 09-187C	Kabuli	Syria

#	Genotype	Type	Origin
42	FLIP 09-193C	Kabuli	Syria
43	FLIP 09-194C	Kabuli	Syria
44	FLIP 09-201C	Kabuli	Syria
45	FLIP 09-202C	Kabuli	Syria
46	FLIP 09-203C	Kabuli	Syria
47	FLIP 09-207C	Kabuli	Syria
48	FLIP 09-208C	Kabuli	Syria
49	FLIP 09-209C	Kabuli	Syria
50	FLIP 09-213C	Kabuli	Syria
51	FLIP 09-214C	Kabuli	Syria
52	FLIP 09-216C	Kabuli	Syria
53	FLIP 09-218C	Kabuli	Syria
54	FLIP 09-219C	Kabuli	Syria
55	FLIP 09-234C	Kabuli	Syria
56	FLIP 09-263C	Kabuli	Syria
57	FLIP 09-269C	Kabuli	Syria
58	FLIP 09-277C	Kabuli	Syria
59	FLIP 09-280C	Kabuli	Syria
60	FLIP 09-284C	Kabuli	Syria
61	FLIP 09-300C	Kabuli	Syria
62	FLIP 09-308C	Kabuli	Syria
63	FLIP 09-320C	Kabuli	Syria
64	FLIP 09-321C	Kabuli	Syria
65	FLIP 09-322C	Kabuli	Syria
66	FLIP 09-326C	Kabuli	Syria
67	FLIP 09-329C	Kabuli	Syria
68	FLIP 09-330C	Kabuli	Syria
69	FLIP 09-332C	Kabuli	Syria
70	FLIP 09-333C	Kabuli	Syria
71	FLIP 09-334C	Kabuli	Syria
72	FLIP 09-335C	Kabuli	Syria
73	FLIP 09-336C	Kabuli	Syria
74	FLIP 09-337C	Kabuli	Syria
75	FLIP 09-338C	Kabuli	Syria
76	FLIP 09-341C	Kabuli	Syria
77	FLIP 09-342C	Kabuli	Syria
78	FLIP 09-344C	Kabuli	Syria
79	FLIP 09-345C	Kabuli	Syria
80	FLIP 09-347C	Kabuli	Syria
81	FLIP 09-350C	Kabuli	Syria
82	FLIP 09-351C	Kabuli	Syria

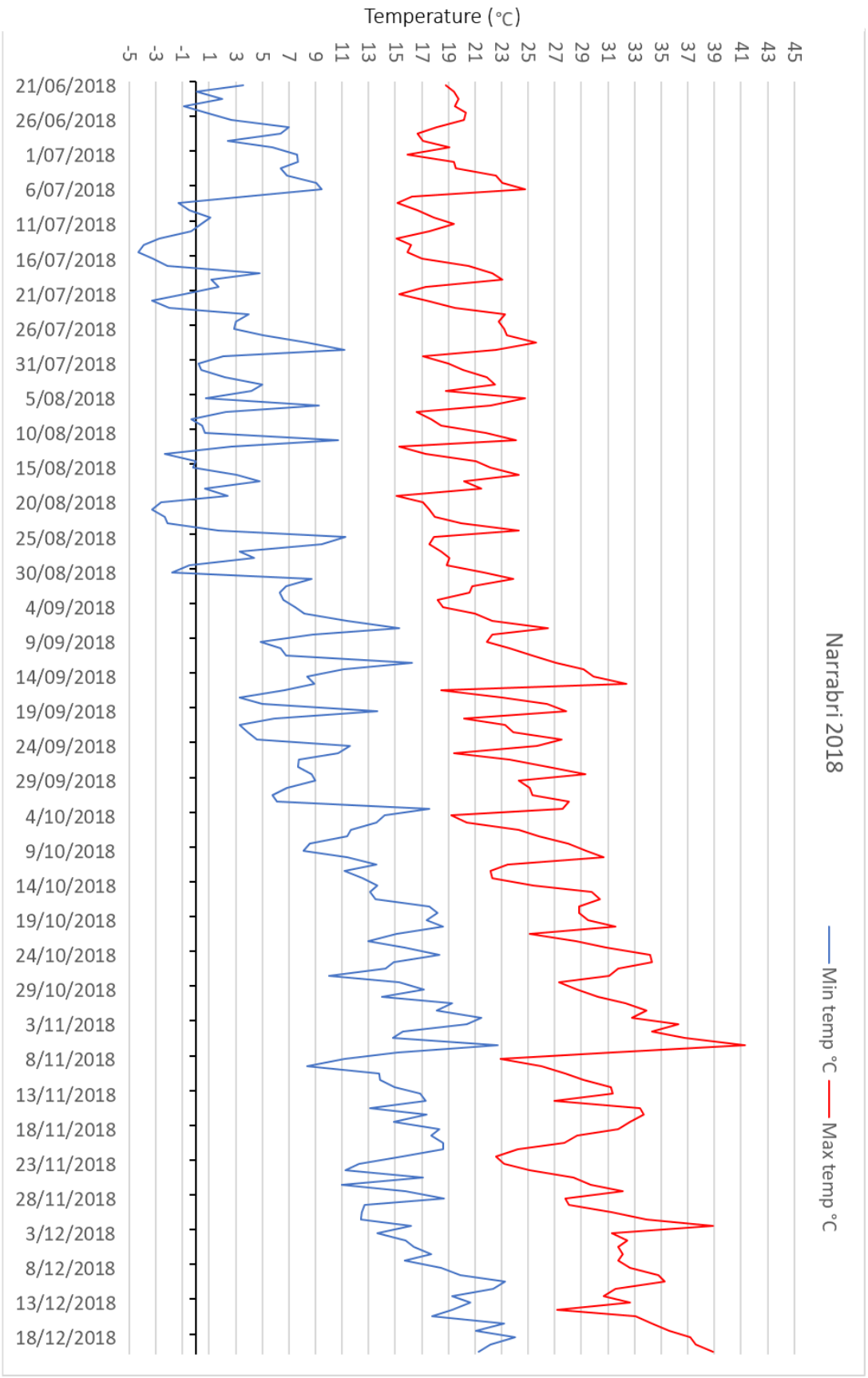
#	Genotype	Type	Origin
83	FLIP 09-352C	Kabuli	Syria
84	FLIP 09-353C	Kabuli	Syria
85	FLIP 09-354C	Kabuli	Syria
86	FLIP 09-355C	Kabuli	Syria
87	FLIP 09-356C	Kabuli	Syria
88	FLIP 09-358C	Kabuli	Syria
89	FLIP 09-359C	Kabuli	Syria
90	FLIP 09-361C	Kabuli	Syria
91	FLIP 09-362C	Kabuli	Syria
92	FLIP 09-363C	Kabuli	Syria
93	FLIP 09-366C	Kabuli	Syria
94	FLIP 09-367C	Kabuli	Syria
95	FLIP 09-370C	Kabuli	Syria
96	FLIP 09-371C	Kabuli	Syria
97	FLIP 09-372C	Kabuli	Syria
98	FLIP 09-374C	Kabuli	Syria
99	FLIP 09-377C	Kabuli	Syria
100	FLIP 09-378C	Kabuli	Syria
101	FLIP 09-386C	Kabuli	Syria
102	FLIP 09-387C	Kabuli	Syria
103	FLIP 09-388C	Kabuli	Syria
104	FLIP 09-389C	Kabuli	Syria
105	FLIP 09-391C	Kabuli	Syria
106	FLIP 09-392C	Kabuli	Syria
107	FLIP 09-394C	Kabuli	Syria
108	FLIP 09-396C	Kabuli	Syria
109	FLIP 09-397C	Kabuli	Syria
110	FLIP 09-399C	Kabuli	Syria
111	FLIP 09-400C	Kabuli	Syria
112	FLIP 09-401C	Kabuli	Syria
113	FLIP 09-403C	Kabuli	Syria
114	FLIP 09-404C	Kabuli	Syria
115	FLIP 09-405C	Kabuli	Syria

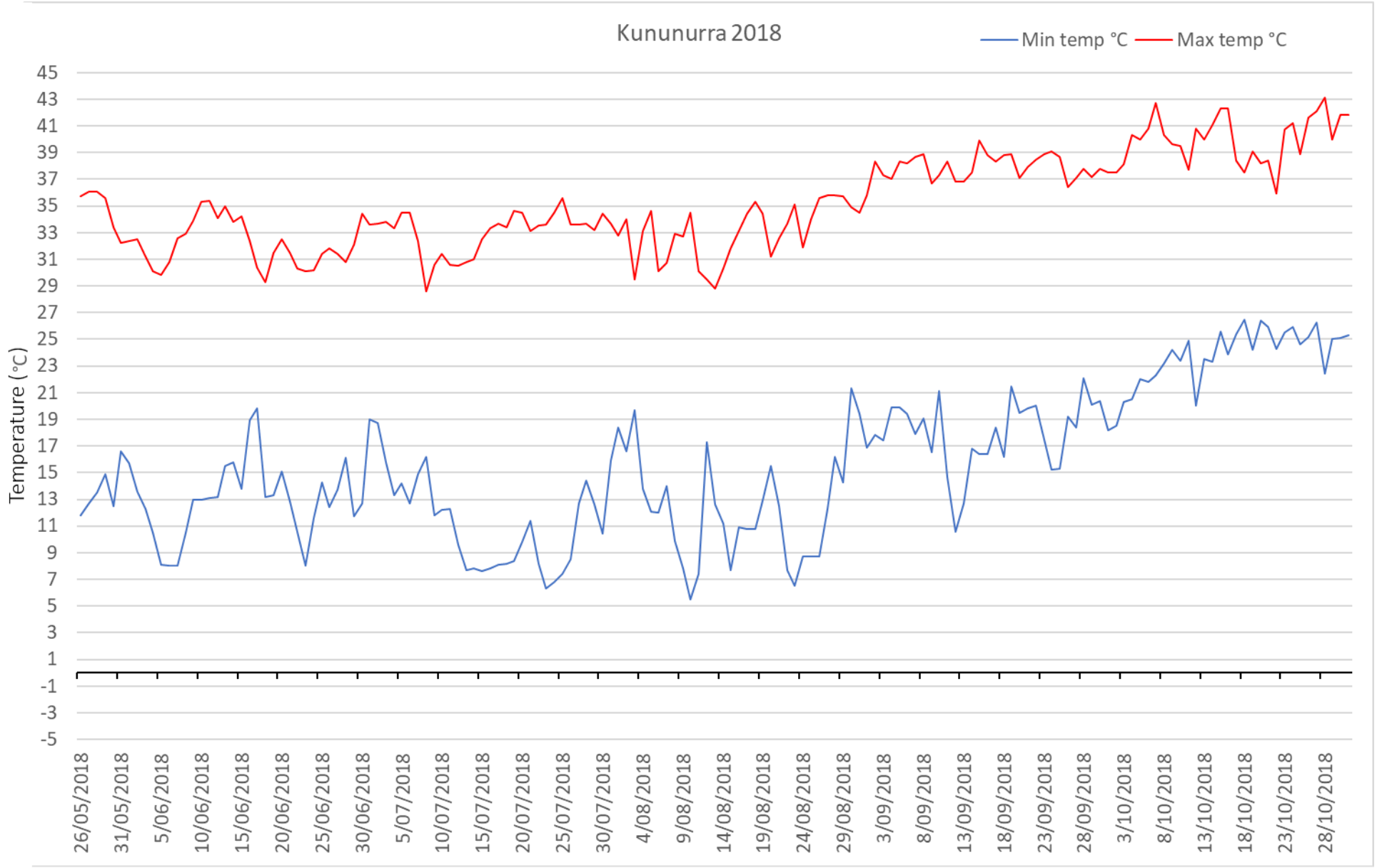
#	Genotype	Type	Origin
116	FLIP 09-408C	abuli	Syria
117	FLIP 09-409C	Kabuli	Syria
118	FLIP 09-411C	Kabuli	Syria
119	FLIP 09-412C	Kabuli	Syria
120	FLIP 09-416C	Kabuli	Syria
121	FLIP 09-423C	Kabuli	Syria
122	FLIP 09-425C	Kabuli	Syria
123	FLIP 09-430C	Kabuli	Syria
124	FLIP 09-437C	Kabuli	Syria
125	FLIP 09-438C	Kabuli	Syria
126	FLIP 2001-56C	Kabuli	Syria
127	FLIP 2003-127C	Kabuli	Syria
128	FLIP 94-62C	Kabuli	Syria
129	FLIP 97-210C	Kabuli	Syria
130	FLIP 97-230C	Kabuli	Syria
131	Genesis 090	Kabuli	Australia
132	Genesis Kalkee	Kabuli	Australia
133	ICC 06098	Desi	India
134	ICC 16796	Kabuli	India
135	ICCV 91216	Desi	India
136	ICCV 95224	Desi	India
137	ICCV 96329	Kabuli	India
138	IG131610C	Kabuli	India
139	Kimberly Large	Kabuli	Australia
140	Kyabra	Desi	Australia
141	NEC 02185C	Desi	India
142	PANT G-114C	Desi	India
143	PBA Monarch	Kabuli	Australia
144	PBA Pistol	Desi	Australia
145	PBA Seamer	Desi	Australia
146	PBA Slasher	Desi	Australia
147	S98477	Kabuli	India
148	ICC 01671	Kabuli	India

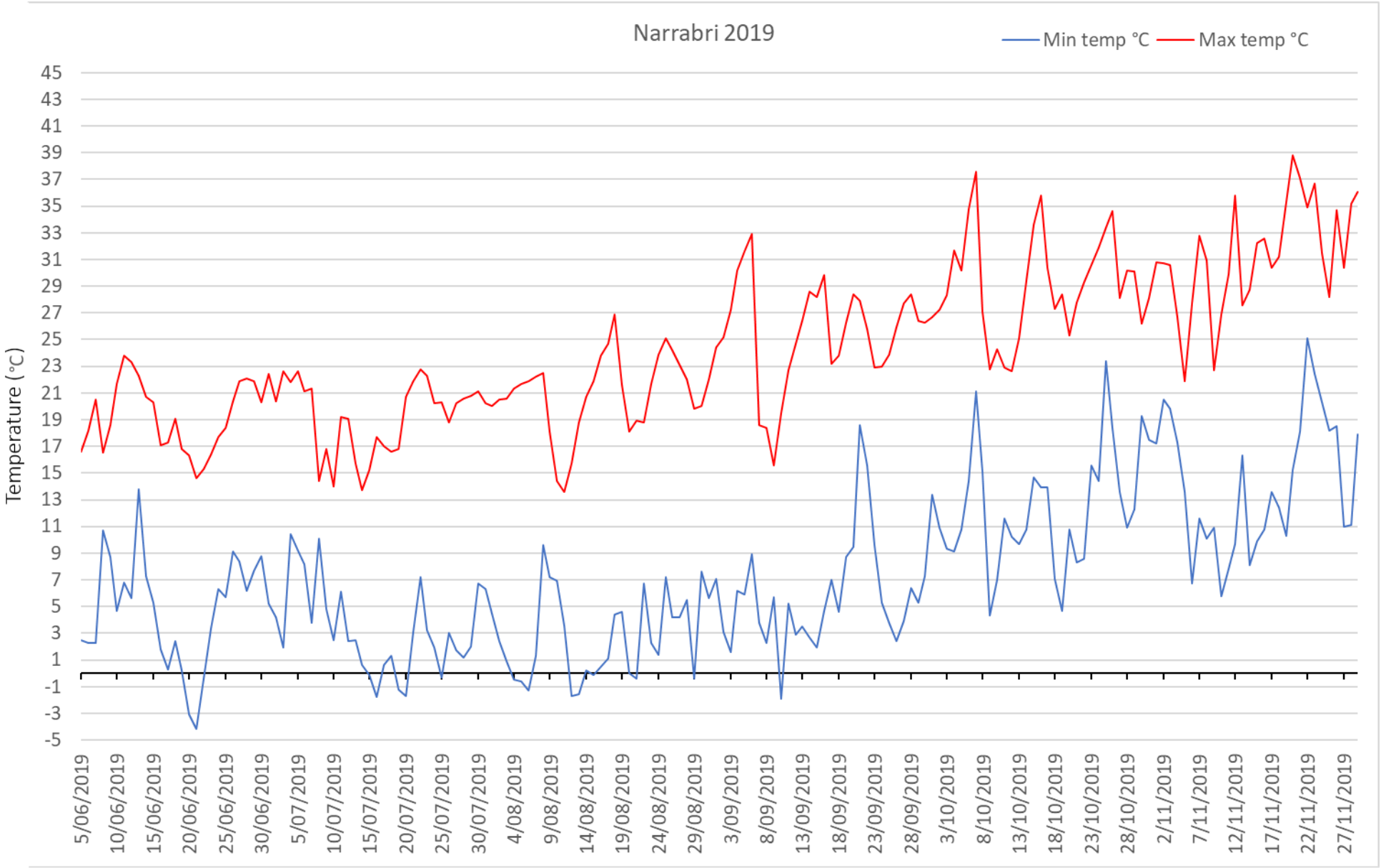
Appendix 3.3 *Pre-planting field preparations and continued field management.*

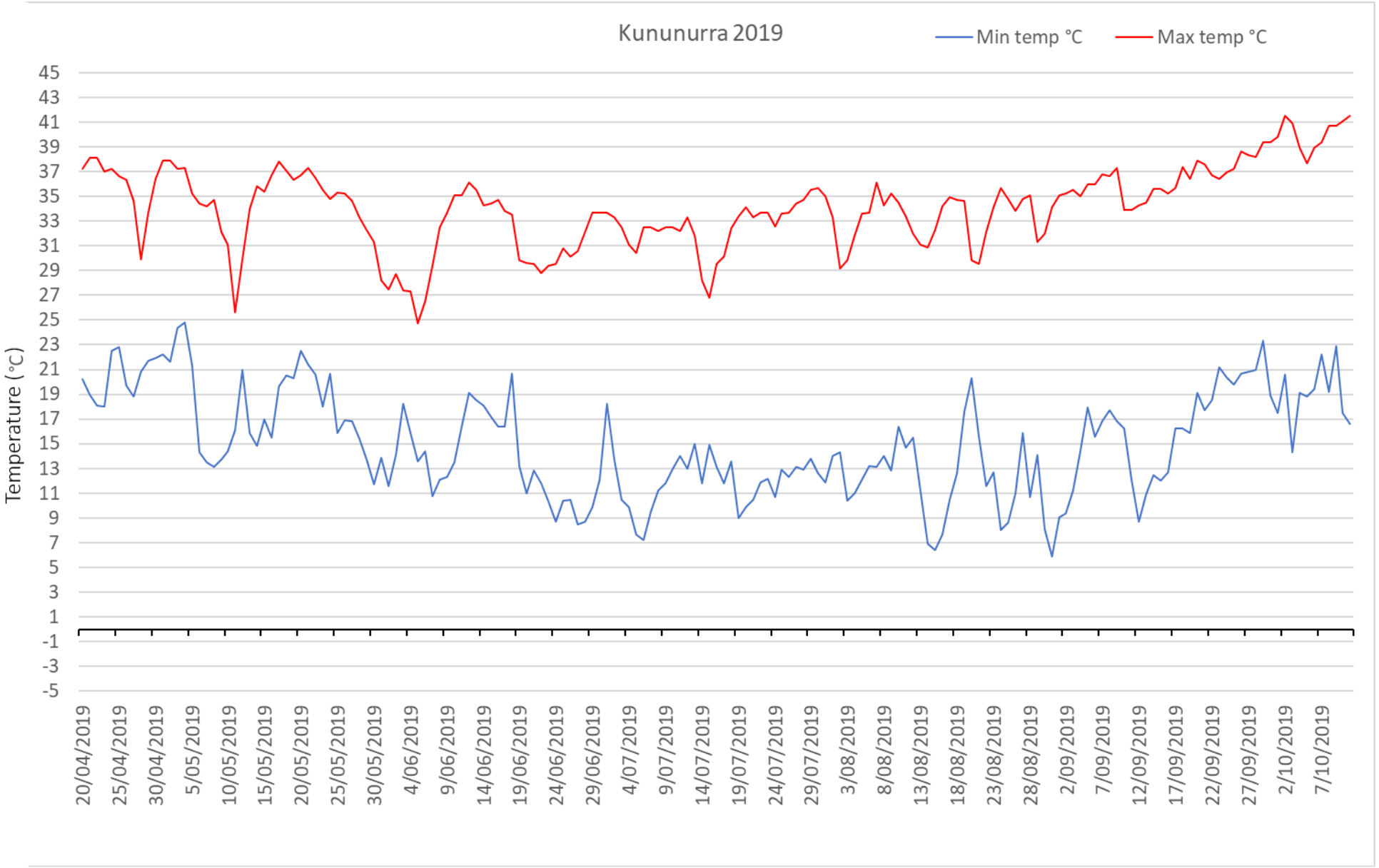
	Narrabri	Kununurra
Starter	Granulock Z Extra at 80kg/Ha	200 kg/ha DAP, 100 kg/ha SOP, 44 kg/ha Sulphur 90kg/ha, Zinc monosulphate 10 kg/ha
Inoculum	TagTeam® peat	None
sowing depth	9cm	9cm
Seed treatment	P-Pickle T	P-Pickle T

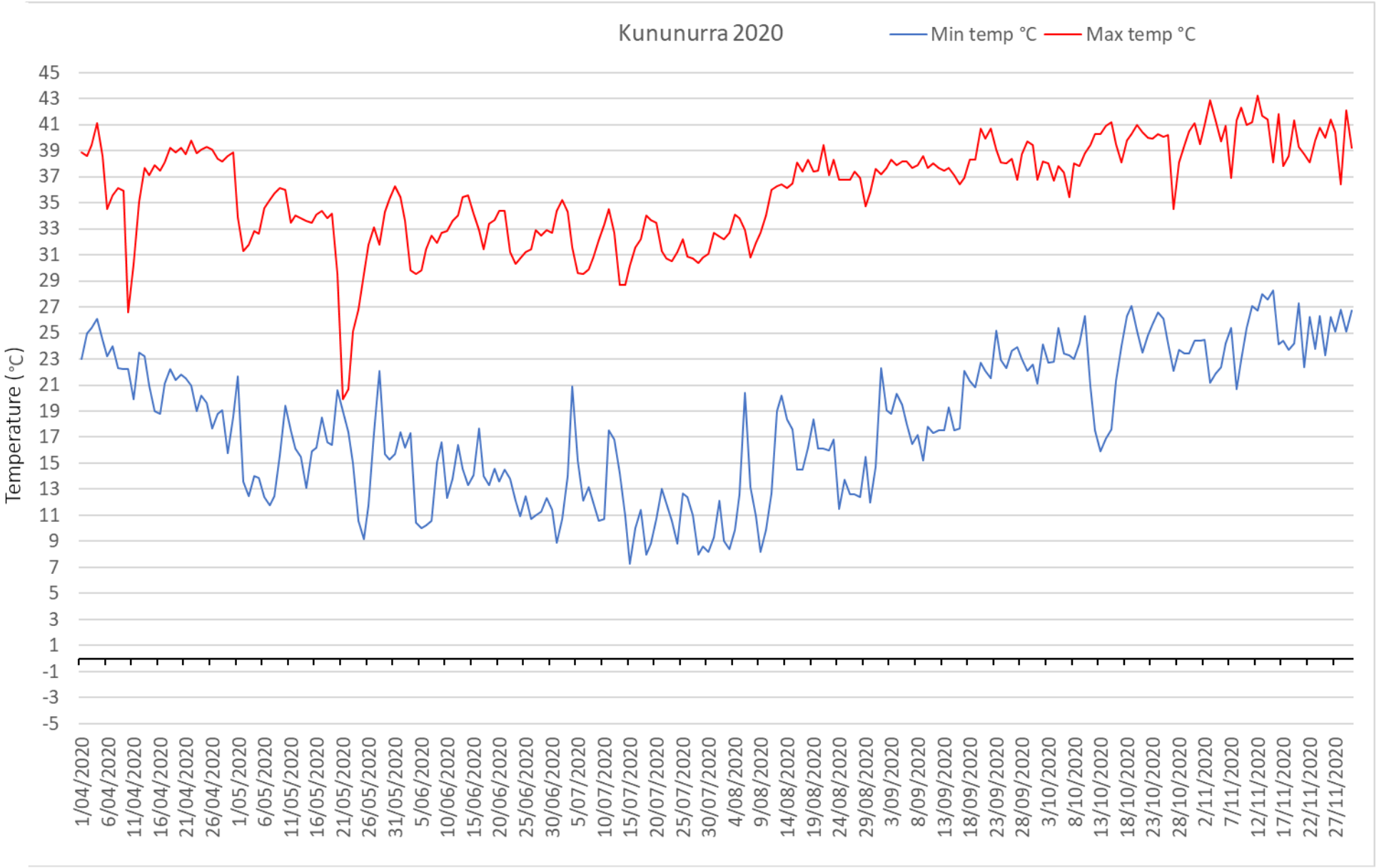
Appendix 4.1. *Weather data for each field trial location and year*

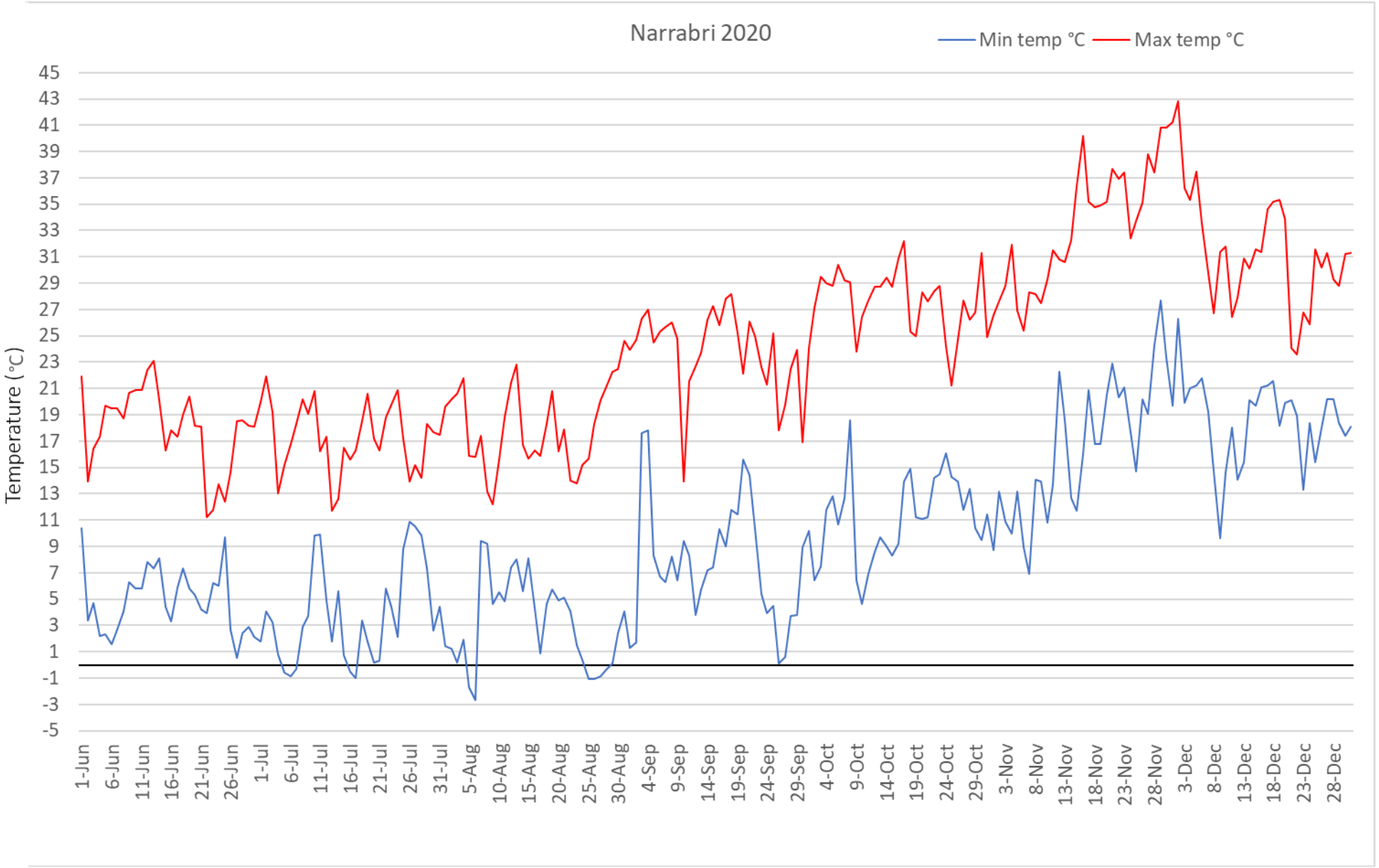



Kununurra 2018
Min temp °C
Max temp °C
Temperature (°C)
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
-1
-3
-5
26/05/2018
31/05/2018
5/06/2018
10/06/2018
15/06/2018
20/06/2018
25/06/2018
30/06/2018
5/07/2018
10/07/2018
15/07/2018
20/07/2018
25/07/2018
30/07/2018
4/08/2018
9/08/2018
14/08/2018
19/08/2018
24/08/2018
29/08/2018
3/09/2018
8/09/2018
13/09/2018
18/09/2018
23/09/2018
28/09/2018
3/10/2018
8/10/2018
13/10/2018
18/10/2018
23/10/2018
28/10/2018


Narrabri 2019
Min temp °C
Max temp °C
Temperature (°C)
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
-1
-3
-5
5/06/2019
10/06/2019
15/06/2019
20/06/2019
25/06/2019
30/06/2019
5/07/2019
10/07/2019
15/07/2019
20/07/2019
25/07/2019
30/07/2019
4/08/2019
9/08/2019
14/08/2019
19/08/2019
24/08/2019
29/08/2019
3/09/2019
8/09/2019
13/09/2019
18/09/2019
23/09/2019
28/09/2019
3/10/2019
8/10/2019
13/10/2019
18/10/2019
23/10/2019
28/10/2019
2/11/2019
7/11/2019
12/11/2019
17/11/2019
22/11/2019
27/11/2019


Kununurra 2019
Min temp °C
Max temp °C
Temperature (°C)
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
-1
-3
-5
20/04/2019
25/04/2019
30/04/2019
5/05/2019
10/05/2019
15/05/2019
20/05/2019
25/05/2019
30/05/2019
4/06/2019
9/06/2019
14/06/2019
19/06/2019
24/06/2019
29/06/2019
4/07/2019
9/07/2019
14/07/2019
19/07/2019
24/07/2019
29/07/2019
3/08/2019
8/08/2019
13/08/2019
18/08/2019
23/08/2019
28/08/2019
2/09/2019
7/09/2019
12/09/2019
17/09/2019
22/09/2019
27/09/2019
2/10/2019
7/10/2019


Kununurra 2020
Min temp °C
Max temp °C
Temperature (°C)
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
-1
-3
-5
1/04/2020
6/04/2020
11/04/2020
16/04/2020
21/04/2020
26/04/2020
1/05/2020
6/05/2020
11/05/2020
16/05/2020
21/05/2020
26/05/2020
31/05/2020
5/06/2020
10/06/2020
15/06/2020
20/06/2020
25/06/2020
30/06/2020
5/07/2020
10/07/2020
15/07/2020
20/07/2020
25/07/2020
30/07/2020
4/08/2020
9/08/2020
14/08/2020
19/08/2020
24/08/2020
29/08/2020
3/09/2020
8/09/2020
13/09/2020
18/09/2020
23/09/2020
28/09/2020
3/10/2020
8/10/2020
13/10/2020
18/10/2020
23/10/2020
28/10/2020
2/11/2020
7/11/2020
12/11/2020
17/11/2020
22/11/2020
27/11/2020


Narrabri 2020
Min temp °C
Max temp °C
Temperature (°C)
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
-1
-3
-5
1-Jun
6-Jun
11-Jun
16-Jun
21-Jun
26-Jun
1-Jul
6-Jul
11-Jul
16-Jul
21-Jul
26-Jul
31-Jul
5-Aug
10-Aug
15-Aug
20-Aug
25-Aug
30-Aug
4-Sep
9-Sep
14-Sep
19-Sep
24-Sep
29-Sep
4-Oct
9-Oct
14-Oct
19-Oct
24-Oct
29-Oct
3-Nov
8-Nov
13-Nov
18-Nov
23-Nov
28-Nov
3-Dec
8-Dec
13-Dec
18-Dec
23-Dec
28-Dec

Appendix 4.2 *Genotypes used in each year and location for field trials.*

Narrabri 2018	Narrabri 2019	Narrabri 2020	Kununurra 2018	Kununurra 2019	Kununurra 2020
Almaz	Almaz	Almaz	Almaz	Almaz	Almaz
FLIP 07-291C	FLIP 07-291C	FLIP 07-291C	FLIP 07-291C	FLIP 07-291C	FLIP 07-291C
FLIP 07-295C	FLIP 07-295C	FLIP 07-295C	FLIP 07-295C	FLIP 07-295C	FLIP 07-295C
FLIP 07-306C	FLIP 07-306C	FLIP 07-306C	FLIP 07-306C	FLIP 07-306C	FLIP 07-306C
FLIP 07-312C	FLIP 07-312C	FLIP 07-312C	FLIP 07-312C	FLIP 07-312C	FLIP 07-312C
FLIP 07-328C	FLIP 07-328C	FLIP 07-328C	FLIP 07-328C	FLIP 07-328C	FLIP 07-328C
FLIP 07-329C	FLIP 07-329C	FLIP 07-329C	FLIP 07-329C	FLIP 07-329C	FLIP 07-329C
FLIP 07-339C	FLIP 07-339C	FLIP 07-339C	FLIP 07-339C	FLIP 07-339C	FLIP 07-339C
FLIP 07-340C	FLIP 07-340C	FLIP 07-340C	FLIP 07-340C	FLIP 07-340C	FLIP 07-340C
			FLIP 09- 77C		
FLIP 09- 84C	FLIP 09- 84C		FLIP 09- 84C	FLIP 09- 84C	
FLIP 09- 90C	FLIP 09- 90C		FLIP 09- 90C	FLIP 09- 90C	
FLIP 09-125C	FLIP 09-125C	FLIP 09-125C	FLIP 09-125C	FLIP 09-125C	FLIP 09-125C
FLIP 09-126C	FLIP 09-126C	FLIP 09-126C	FLIP 09-126C	FLIP 09-126C	FLIP 09-126C
	FLIP 09-129C	FLIP 09-129C	FLIP 09-129C	FLIP 09-129C	FLIP 09-129C
FLIP 09-133C	FLIP 09-133C	FLIP 09-133C	FLIP 09-133C	FLIP 09-133C	FLIP 09-133C
FLIP 09-136C	FLIP 09-136C	FLIP 09-136C	FLIP 09-136C	FLIP 09-136C	FLIP 09-136C
FLIP 09-140C	FLIP 09-140C	FLIP 09-140C	FLIP 09-140C	FLIP 09-140C	FLIP 09-140C
FLIP 09-141C	FLIP 09-141C	FLIP 09-141C	FLIP 09-141C	FLIP 09-141C	FLIP 09-141C
FLIP 09-146C	FLIP 09-146C	FLIP 09-146C	FLIP 09-146C	FLIP 09-146C	FLIP 09-146C
FLIP 09-148C	FLIP 09-148C	FLIP 09-148C	FLIP 09-148C	FLIP 09-148C	FLIP 09-148C
FLIP 09-150C	FLIP 09-150C	FLIP 09-150C	FLIP 09-150C	FLIP 09-150C	FLIP 09-150C
			FLIP 09-152C		
FLIP 09-154C	FLIP 09-154C	FLIP 09-154C	FLIP 09-154C	FLIP 09-154C	FLIP 09-154C
FLIP 09-155C	FLIP 09-155C	FLIP 09-155C	FLIP 09-155C	FLIP 09-155C	FLIP 09-155C
FLIP 09-156C	FLIP 09-156C	FLIP 09-156C	FLIP 09-156C	FLIP 09-156C	FLIP 09-156C
FLIP 09-157C	FLIP 09-157C	FLIP 09-157C	FLIP 09-157C	FLIP 09-157C	FLIP 09-157C
FLIP 09-158C	FLIP 09-158C	FLIP 09-158C	FLIP 09-158C	FLIP 09-158C	FLIP 09-158C
FLIP 09-159C	FLIP 09-159C	FLIP 09-159C	FLIP 09-159C	FLIP 09-159C	FLIP 09-159C
FLIP 09-160C	FLIP 09-160C	FLIP 09-160C	FLIP 09-160C	FLIP 09-160C	FLIP 09-160C
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FLIP 09-165C	FLIP 09-165C	FLIP 09-165C	FLIP 09-165C	FLIP 09-165C	FLIP 09-165C
FLIP 09-168C	FLIP 09-168C	FLIP 09-168C	FLIP 09-168C	FLIP 09-168C	FLIP 09-168C
FLIP 09-169C	FLIP 09-169C	FLIP 09-169C	FLIP 09-169C	FLIP 09-169C	FLIP 09-169C
FLIP 09-170C	FLIP 09-170C	FLIP 09-170C	FLIP 09-170C	FLIP 09-170C	FLIP 09-170C
FLIP 09-173C	FLIP 09-173C	FLIP 09-173C	FLIP 09-173C	FLIP 09-173C	FLIP 09-173C
FLIP 09-179C	FLIP 09-179C	FLIP 09-179C	FLIP 09-179C	FLIP 09-179C	FLIP 09-179C
FLIP 09-180C	FLIP 09-180C	FLIP 09-180C	FLIP 09-180C	FLIP 09-180C	FLIP 09-180C
FLIP 09-183C	FLIP 09-183C	FLIP 09-183C	FLIP 09-183C	FLIP 09-183C	FLIP 09-183C
FLIP 09-184C	FLIP 09-184C	FLIP 09-184C	FLIP 09-184C	FLIP 09-184C	FLIP 09-184C
FLIP 09-185C	FLIP 09-185C	FLIP 09-185C	FLIP 09-185C	FLIP 09-185C	FLIP 09-185C

FLIP 09-186C	FLIP 09-186C	FLIP 09-186C	FLIP 09-186C	FLIP 09-186C	FLIP 09-186C
FLIP 09-187C	FLIP 09-187C	FLIP 09-187C	FLIP 09-187C	FLIP 09-187C	FLIP 09-187C
FLIP 09-193C	FLIP 09-193C	FLIP 09-193C	FLIP 09-193C	FLIP 09-193C	FLIP 09-193C
	FLIP 09-194C	FLIP 09-194C	FLIP 09-194C	FLIP 09-194C	FLIP 09-194C
FLIP 09-201C	FLIP 09-201C	FLIP 09-201C	FLIP 09-201C	FLIP 09-201C	FLIP 09-201C
FLIP 09-202C	FLIP 09-202C	FLIP 09-202C	FLIP 09-202C	FLIP 09-202C	FLIP 09-202C
FLIP 09-203C	FLIP 09-203C	FLIP 09-203C	FLIP 09-203C	FLIP 09-203C	FLIP 09-203C
FLIP 09-207C	FLIP 09-207C	FLIP 09-207C	FLIP 09-207C	FLIP 09-207C	FLIP 09-207C
FLIP 09-208C	FLIP 09-208C	FLIP 09-208C	FLIP 09-208C	FLIP 09-208C	FLIP 09-208C
FLIP 09-209C	FLIP 09-209C	FLIP 09-209C	FLIP 09-209C	FLIP 09-209C	FLIP 09-209C
FLIP 09-213C	FLIP 09-213C	FLIP 09-213C	FLIP 09-213C	FLIP 09-213C	FLIP 09-213C
FLIP 09-214C	FLIP 09-214C		FLIP 09-214C	FLIP 09-214C	
FLIP 09-216C	FLIP 09-216C	FLIP 09-216C	FLIP 09-216C	FLIP 09-216C	FLIP 09-216C
FLIP 09-218C	FLIP 09-218C	FLIP 09-218C	FLIP 09-218C	FLIP 09-218C	FLIP 09-218C
FLIP 09-219C	FLIP 09-219C	FLIP 09-219C	FLIP 09-219C	FLIP 09-219C	FLIP 09-219C
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FLIP 09-263C	FLIP 09-263C	FLIP 09-263C	FLIP 09-263C	FLIP 09-263C	FLIP 09-263C
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FLIP 09-280C	FLIP 09-280C	FLIP 09-280C	FLIP 09-280C	FLIP 09-280C	FLIP 09-280C
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FLIP 09-300C	FLIP 09-300C	FLIP 09-300C	FLIP 09-300C	FLIP 09-300C	FLIP 09-300C
FLIP 09-308C	FLIP 09-308C	FLIP 09-308C	FLIP 09-308C	FLIP 09-308C	FLIP 09-308C
FLIP 09-320C	FLIP 09-320C	FLIP 09-320C	FLIP 09-320C	FLIP 09-320C	FLIP 09-320C
FLIP 09-321C	FLIP 09-321C	FLIP 09-321C	FLIP 09-321C	FLIP 09-321C	FLIP 09-321C
	FLIP 09-322C	FLIP 09-322C	FLIP 09-322C	FLIP 09-322C	FLIP 09-322C
FLIP 09-325C			FLIP 09-325C		
FLIP 09-326C	FLIP 09-326C	FLIP 09-326C	FLIP 09-326C	FLIP 09-326C	FLIP 09-326C
FLIP 09-329C	FLIP 09-329C	FLIP 09-329C	FLIP 09-329C	FLIP 09-329C	FLIP 09-329C
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FLIP 09-335C	FLIP 09-335C	FLIP 09-335C	FLIP 09-335C	FLIP 09-335C	FLIP 09-335C
FLIP 09-336C	FLIP 09-336C	FLIP 09-336C	FLIP 09-336C	FLIP 09-336C	FLIP 09-336C
FLIP 09-337C	FLIP 09-337C	FLIP 09-337C	FLIP 09-337C	FLIP 09-337C	FLIP 09-337C
FLIP 09-338C	FLIP 09-338C	FLIP 09-338C	FLIP 09-338C	FLIP 09-338C	FLIP 09-338C
FLIP 09-341C	FLIP 09-341C	FLIP 09-341C	FLIP 09-341C	FLIP 09-341C	FLIP 09-341C
FLIP 09-342C	FLIP 09-342C	FLIP 09-342C	FLIP 09-342C	FLIP 09-342C	FLIP 09-342C
FLIP 09-344C	FLIP 09-344C	FLIP 09-344C	FLIP 09-344C	FLIP 09-344C	FLIP 09-344C
FLIP 09-345C	FLIP 09-345C	FLIP 09-345C	FLIP 09-345C	FLIP 09-345C	FLIP 09-345C
FLIP 09-347C	FLIP 09-347C	FLIP 09-347C	FLIP 09-347C	FLIP 09-347C	FLIP 09-347C
FLIP 09-350C	FLIP 09-350C	FLIP 09-350C	FLIP 09-350C	FLIP 09-350C	FLIP 09-350C
FLIP 09-351C	FLIP 09-351C	FLIP 09-351C	FLIP 09-351C	FLIP 09-351C	FLIP 09-351C

FLIP 09-352C	FLIP 09-352C	FLIP 09-352C	FLIP 09-352C	FLIP 09-352C	FLIP 09-352C
FLIP 09-353C	FLIP 09-353C	FLIP 09-353C	FLIP 09-353C	FLIP 09-353C	FLIP 09-353C
FLIP 09-354C	FLIP 09-354C	FLIP 09-354C	FLIP 09-354C	FLIP 09-354C	FLIP 09-354C
FLIP 09-355C	FLIP 09-355C	FLIP 09-355C	FLIP 09-355C	FLIP 09-355C	FLIP 09-355C
FLIP 09-356C	FLIP 09-356C	FLIP 09-356C	FLIP 09-356C	FLIP 09-356C	FLIP 09-356C
FLIP 09-358C	FLIP 09-358C	FLIP 09-358C	FLIP 09-358C	FLIP 09-358C	FLIP 09-358C
FLIP 09-359C	FLIP 09-359C	FLIP 09-359C	FLIP 09-359C	FLIP 09-359C	FLIP 09-359C
FLIP 09-361C	FLIP 09-361C	FLIP 09-361C	FLIP 09-361C	FLIP 09-361C	FLIP 09-361C
FLIP 09-362C	FLIP 09-362C	FLIP 09-362C	FLIP 09-362C	FLIP 09-362C	FLIP 09-362C
FLIP 09-363C	FLIP 09-363C	FLIP 09-363C	FLIP 09-363C	FLIP 09-363C	FLIP 09-363C
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FLIP 09-367C	FLIP 09-367C	FLIP 09-367C	FLIP 09-367C	FLIP 09-367C	FLIP 09-367C
FLIP 09-370C	FLIP 09-370C	FLIP 09-370C	FLIP 09-370C	FLIP 09-370C	FLIP 09-370C
FLIP 09-371C	FLIP 09-371C	FLIP 09-371C	FLIP 09-371C	FLIP 09-371C	FLIP 09-371C
FLIP 09-372C	FLIP 09-372C	FLIP 09-372C	FLIP 09-372C	FLIP 09-372C	FLIP 09-372C
FLIP 09-374C	FLIP 09-374C	FLIP 09-374C	FLIP 09-374C	FLIP 09-374C	FLIP 09-374C
FLIP 09-377C	FLIP 09-377C	FLIP 09-377C	FLIP 09-377C	FLIP 09-377C	FLIP 09-377C
FLIP 09-378C	FLIP 09-378C	FLIP 09-378C	FLIP 09-378C	FLIP 09-378C	FLIP 09-378C
FLIP 09-386C	FLIP 09-386C	FLIP 09-386C	FLIP 09-386C	FLIP 09-386C	FLIP 09-386C
FLIP 09-387C	FLIP 09-387C	FLIP 09-387C	FLIP 09-387C	FLIP 09-387C	FLIP 09-387C
FLIP 09-388C	FLIP 09-388C	FLIP 09-388C	FLIP 09-388C	FLIP 09-388C	FLIP 09-388C
FLIP 09-389C	FLIP 09-389C	FLIP 09-389C	FLIP 09-389C	FLIP 09-389C	FLIP 09-389C
FLIP 09-391C	FLIP 09-391C	FLIP 09-391C	FLIP 09-391C	FLIP 09-391C	FLIP 09-391C
FLIP 09-392C	FLIP 09-392C	FLIP 09-392C	FLIP 09-392C	FLIP 09-392C	FLIP 09-392C
FLIP 09-394C	FLIP 09-394C	FLIP 09-394C	FLIP 09-394C	FLIP 09-394C	FLIP 09-394C
			FLIP 09-395C		
	FLIP 09-396C	FLIP 09-396C	FLIP 09-396C	FLIP 09-396C	FLIP 09-396C
FLIP 09-397C	FLIP 09-397C	FLIP 09-397C	FLIP 09-397C	FLIP 09-397C	FLIP 09-397C
FLIP 09-399C	FLIP 09-399C	FLIP 09-399C	FLIP 09-399C	FLIP 09-399C	FLIP 09-399C
FLIP 09-400C	FLIP 09-400C	FLIP 09-400C	FLIP 09-400C	FLIP 09-400C	FLIP 09-400C
FLIP 09-401C	FLIP 09-401C	FLIP 09-401C	FLIP 09-401C	FLIP 09-401C	FLIP 09-401C
FLIP 09-403C	FLIP 09-403C	FLIP 09-403C	FLIP 09-403C	FLIP 09-403C	FLIP 09-403C
	FLIP 09-404C	FLIP 09-404C	FLIP 09-404C	FLIP 09-404C	FLIP 09-404C
FLIP 09-405C	FLIP 09-405C	FLIP 09-405C	FLIP 09-405C	FLIP 09-405C	FLIP 09-405C
FLIP 09-408C	FLIP 09-408C	FLIP 09-408C	FLIP 09-408C	FLIP 09-408C	FLIP 09-408C
	FLIP 09-409C	FLIP 09-409C	FLIP 09-409C	FLIP 09-409C	FLIP 09-409C
FLIP 09-411C	FLIP 09-411C	FLIP 09-411C	FLIP 09-411C	FLIP 09-411C	FLIP 09-411C
FLIP 09-412C	FLIP 09-412C	FLIP 09-412C	FLIP 09-412C	FLIP 09-412C	FLIP 09-412C
FLIP 09-416C	FLIP 09-416C	FLIP 09-416C	FLIP 09-416C	FLIP 09-416C	FLIP 09-416C
FLIP 09-423C	FLIP 09-423C	FLIP 09-423C	FLIP 09-423C	FLIP 09-423C	FLIP 09-423C
FLIP 09-425C	FLIP 09-425C	FLIP 09-425C	FLIP 09-425C	FLIP 09-425C	FLIP 09-425C
FLIP 09-430C	FLIP 09-430C	FLIP 09-430C	FLIP 09-430C	FLIP 09-430C	FLIP 09-430C
FLIP 09-433C			FLIP 09-433C		
FLIP 09-434C			FLIP 09-434C		

FLIP 09-437C	FLIP 09-437C	FLIP 09-437C	FLIP 09-437C	FLIP 09-437C	FLIP 09-437C
FLIP 09-438C	FLIP 09-438C	FLIP 09-438C	FLIP 09-438C	FLIP 09-438C	FLIP 09-438C
		FLIP 09-84			FLIP 09-84
		FLIP 09-90C			FLIP 09-90C
	FLIP 2001-56C	FLIP 2001-56C	FLIP 2001-56C	FLIP 2001-56C	FLIP 2001-56C
FLIP 2003-127C	FLIP 2003-127C	FLIP 2003-127C	FLIP 2003-127C	FLIP 2003-127C	FLIP 2003-127C
	FLIP 94-62C	FLIP 94-62C	FLIP 94-62C	FLIP 94-62C	FLIP 94-62C
FLIP 97-210C	FLIP 97-210C	FLIP 97-210C	FLIP 97-210C	FLIP 97-210C	FLIP 97-210C
FLIP 97-230C	FLIP 97-230C	FLIP 97-230C	FLIP 97-230C	FLIP 97-230C	FLIP 97-230C
GENESIS 079	GENESIS 079	GENESIS 079	GENESIS 079	GENESIS 079	GENESIS 079
Genesis 090	Genesis 090	Genesis 090	Genesis 090	Genesis 090	Genesis 090
Genesis Kalkee	Genesis Kalkee	Genesis Kalkee	Genesis Kalkee	Genesis Kalkee	Genesis Kalkee
ICC 01671	ICC 01671	ICC 01671	ICC 01671	ICC 01671	
ICC 06098	ICC 06098	ICC 06098	ICC 06098	ICC 06098	ICC 06098
ICC 11531			ICC 11531	ICC 11531	
ICC 14735			ICC 14735	ICC 14735	
			ICC 15888		
ICC 16796	ICC 16796	ICC 16796	ICC 16796	ICC 16796	ICC 16796
			ICCV 05314	ICCV 05314	
			ICCV 06302	ICCV 06302	
	ICCV 91216	ICCV 91216	ICCV 91216	ICCV 91216	ICCV 91216
ICCV 95224	ICCV 95224	ICCV 95224	ICCV 95224	ICCV 95224	ICCV 95224
	ICCV 96329	ICCV 96329	ICCV 96329	ICCV 96329	ICCV 96329
	IG 131610	IG 131610	IG 131610	IG 131610	IG 131610
ILC 01272			ILC 01272	ILC 01272	
Kimb. Large	Kimb. Large	Kimb. Large	Kimb. Large	Kimb. Large	Kimb. Large
Kyabra	Kyabra	Kyabra	Kyabra	Kyabra	Kyabra
NEC 02185C	NEC 02185C	NEC 02185C	NEC 02185C	NEC 02185C	NEC 02185C
PANT G-114C	PANT G-114C	PANT G-114C	PANT G-114C	PANT G-114C	PANT G-114C
PBA Hattrick					
PBA Monarch	PBA Monarch	PBA Monarch	PBA Monarch	PBA Monarch	PBA Monarch
PBA Pistol	PBA Pistol	PBA Pistol	PBA Pistol	PBA Pistol	PBA Pistol
PBA Seamer	PBA Seamer	PBA Seamer	PBA Seamer	PBA Seamer	PBA Seamer
PBA Slasher	PBA Slasher	PBA Slasher	PBA Slasher	PBA Slasher	PBA Slasher
	S98477	S98477	S98477	S98477	S98477

Appendix 4.3. *Genotypes selected for Glasshouse trials.*

Podding	Flowering
FLIP 09-164C	FLIP 09-164C
FLIP 09-169C	
FLIP 09-184C	
FLIP 09-186C	FLIP 09-186C

FLIP 09-201C	[redacted]
FLIP 09-209C	FLIP 09-209C
FLIP 09-218C	FLIP 09-218C
FLIP 09-234C	FLIP 09-234C
FLIP 09-263C	FLIP 09-263C
FLIP 09-333C	[redacted]
FLIP 09-336C	
FLIP 09-338C	
FLIP 09-362C	FLIP 09-362C
FLIP 09-363C	FLIP 09-363C
FLIP 09-399C	FLIP 09-399C
FLIP 09-438C	[redacted]
Genesis 090	
Kimberley Large	
Kyabra	
PBA Monarch	PBA Monarch