GENETIC AND PHYSIOLOGICAL STUDIES OF HEAT TOLERANCE IN COWPEA

A Dissertation

by

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important legume crop commonly used for grains and for fodder in some parts of the world. It is grown in over 65 countries worldwide. In the United States, it is primarily grown in the southern states, with major production areas in Texas and California. A systematic breeding program was initiated at Texas A&M University to identify genetic variability for heat tolerance in cowpea germplasm, and the variability was utilized to develop a RIL (recombinant inbred line) mapping population by crossing a heat-tolerant (GEC) and a heat-susceptible (IT98K-476-8) parent. The RILs were planted in three field environments – College Station in 2014, and Corpus Christi and Weslaco, Texas in 2015, and in a hot greenhouse to screen for heat tolerance, days to flowering, plant height, and other agronomic traits. The RILs were also genotyped using SNPs markers, and QTLs (quantitative trait loci) were mapped for the phenotypic traits measured.

Significant phenotypic variability was identified in cowpea germplasm. Both the selected parents utilized to develop RILs were significantly different for all the measured traits, and transgressive segregation was detected in the RIL population. A genetic linkage map was constructed having 11 linkage groups using genotypic data, and one significant QTL was detected on linkage group 3 (LOD of 2.78 and explained 7.66% of variation) for heat-tolerance visual ratings in Corpus Christi, and another on linkage group 10 (LOD of 3.86 and explained 10.64% of variation) for ratings in the greenhouse.

For seed weight per plant (SWT), we have detected two QTLs, one on linkage group 3 (LOD of 7.86 and explained 17.05% of variation) and another on 10 (LOD of 5.07 and explained 11.37% of variation). For number of pods per plant (PODN), three QTLs were detected, one on linkage group 3 (LOD of 11.43 and explained 22.93% of variation) and two on linkage group 10 (first – LOD of 3.34 and explained 5.93% of variation; second – LOD of 4.04 and explained 7.62% of variation) using BLUPs (best linear unbiased predictions).

DEDICATION

I would like to dedicate this dissertation to my parents and my wife Monika for unconditional support and patience during my Ph.D. program.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

Part 1, faculty committee recognition

This work was supervised by a dissertation committee consisting of Dr. Dirk B. Hays and Dr. Bir B. Singh, Dr. and William Rooney of the Department of Soil and Crop Sciences and Professor Dr. Kevin Crosby of the Department of Horticulture

Part 2, student/collaborator contributions

Isolation of DNA from the mapping population and RAD-Seq libraries were constructed by Chantel F. Scheuring. The cowpea linkage map construction, described in Chapter 3, was accomplished with help of Professors Dr. Hong-Bin Zhang, Dr. Meiping Zhang, and Dr. Yadong Zhang. I am also grateful to graduate students Laura Masor, Trevis Huggins, Suheb Mohammed, Richard Burton, Alfredo Delgado, Henry Awika, Fatima Castillo, and Xiangkun Gu, visiting scientist Ravindra Prasad, and student worker Marco Roque for helping in planting and harvesting.

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NOMENCLATURE

SWT	Seed weight per plant
PODWT	Pod weight per plant
PODN	Pod number per plant
SEEDN	Seed number per pod
FL	Days to flowering
HT	Plant height
QTL	Quantitative trait loci
RIL	Recombinant inbred line

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cowpea (*Vigna unguiculata* (L.) Walp.), also known as black-eyed pea, southern pea, crowder pea, zipper pea (USA), kunde (East Africa), wake and niebe (Africa), and lobia (India), is widely grown in drier regions of the tropics and sub-tropics of 65 countries. In the United States, Southern Europe, and the Middle East "black-eyed pea" cowpea is dominant, and it is characterized by its large grain and white seed coat with a prominent black pigmented eye around the hilum. Cowpea was one of the earliest domesticated crops and remains an important annual grain and forage legume. *Vigna sp.* are members of the Leguminosae (Fabaceae) family which consist of 757 genera and over 20,000 species (Lewis et al., 2005). The first identifiable legumes appeared in the fossil records about 56 million years ago, and all legumes were believed to share a common ancestor, which existed about 59 million years ago (Lavin et al., 2005). Most of the legume crops belong to either Papilionoideae (temperate climate, such as *Medicago*) or Phaseoloid/millettioids (warm season legumes as *Glycine* and *Phaseolus*) clade. The origin of cowpea and its subsequent domestication is associated with the cultivation of pearl millet and sorghum in Africa. Harlan (1992) reported that cultivated cowpea plants were domesticated from their wild progenitors about 10,000 years ago. Several morphological and physiological changes occurred during the domestication of cowpea. Selection pressures by humans resulted in a loss of pod shattering characteristics, fast

and rapid germination, determinate plants, larger seeds, and inflorescences by intentionally selecting for higher yield components (Gepts, 2010).

Cowpea is a warm-season and drought-tolerant crop, well-adapted to drier regions of the tropics where other legumes do not perform well. It belongs to Phaseoloid clade along with the common bean, pigeon pea, and soybean (Doyle and Luckow, 2003). Cowpea is widely grown in drier regions of the tropics covering over 14 million ha, and the world production of cowpea in 2013 was 7.2 million tones. Cowpea originated in southern Africa and countries in Africa still plant the largest acreage of cowpea. However, the grain yields of cowpea in Africa are lower compared to countries in other continents such as China, Peru, and the United State of America (Table 1). One of the most important reasons behind lower yield in Africa is significantly lower use of fertilizers. It is an important food and forage legume in Sub-Saharan Africa, Southern Europe, the Southern United States, Central and South America, and some parts of Asia (Singh, 2005; Timko et al., 2007; Timko and Singh, 2008). It has been dispersed worldwide and is common in Brazil, West Indies, India, Burma, Sri Lanka, Yugoslavia, and Australia. In the United States, cowpea is primarily grown in the southern states, with major production area in Texas and California. Nigeria, Niger, Brazil, Burkina Faso, and United Republic of Tanzania are the top five producers of cowpea (Table 1). Since 1961, cowpea production has increased seven-fold, from one million to over seven million tonnes (Fig. 1; Singh, 2014).

Table 1. Top 20 cowpea producing countries ranked by area harvested

Country	Area harvested (million ha)	Production (million tonnes)	Yield (kg/ha)
Niger	5.32	1.59	298.2
Nigeria	3.70	2.14	577.6
Burkina Faso	1.18	0.57	486.2
Mozambique	0.38	0.10	274.8
Kenya	0.28	0.14	492.0
Sudan	0.26	0.08	307.7
Cameroon	0.26	0.17	676.0
Mali	0.24	0.15	609.1
URT	0.20	0.19	940.4
Senegal	0.15	0.06	418.5
DRC	0.15	0.08	518.5
Myanmar	0.13	0.12	872.7
Malawi	0.08	0.04	464.7
Caribbean	0.04	0.03	722.5
Haiti	0.04	0.03	711.8
Mauritania	0.04	0.01	364.3
Uganda	0.03	0.01	404.0
China	0.01	0.01	1038.5
Peru	0.01	0.02	1376.6
USA	0.01	0.02	1685.2

URT = United Republic of Tanzania; DRC = Democratic Republic of the Congo; USA = United States of America

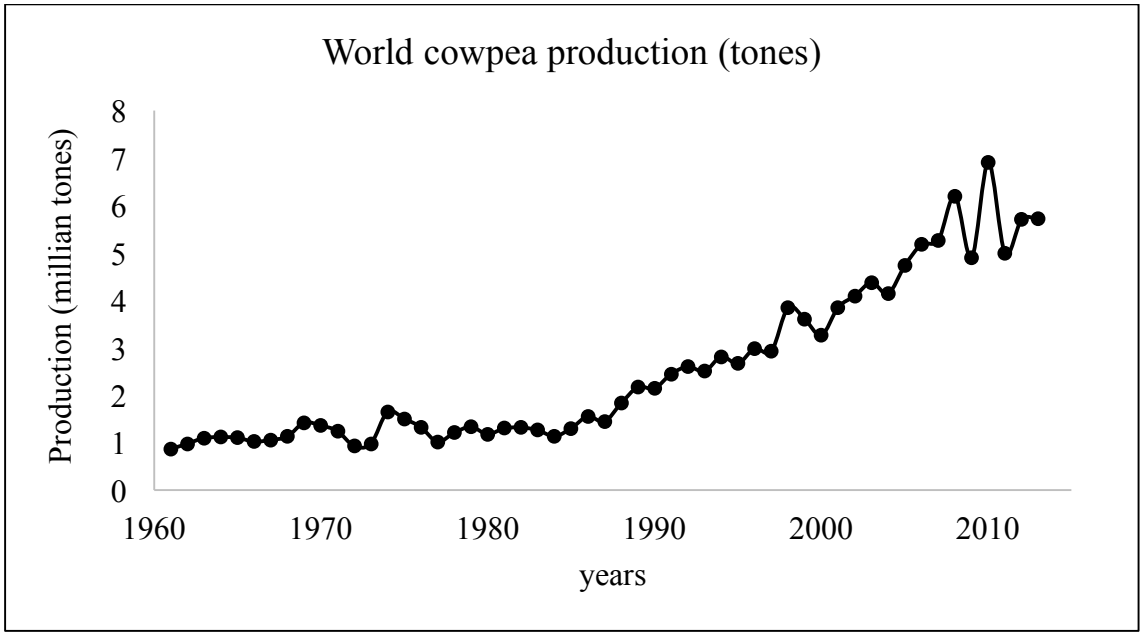

Fig. 1. World cowpea production (tones) trend over years (FAO, 2013)

Cowpea is a multifunctional crop; it can be used as seeds, vegetables, cover crops, and fodder (Quaye et al., 2009). In any capacity, it is very nutritious, serving as an inexpensive source of protein, amino acids (lysine and tryptophan), vitamins, and minerals in the daily diets of millions of people (Bressani, 1985). It also enhances the quality of cereal grains due to its high lysine content, balanced with the high content of methionine and cysteine in cereals (Hall et al., 2002). In addition to nutritional supplementation in the diet, cowpea improves cropping systems and soil fertility by reducing soil erosion, suppressing weeds, and working as green manure and a nitrogen-fixing crop, which contributes to the yield of nitrogen demanding crops growing amid or subsequent to it (Tarawali et al., 2002). It can grow in poor soils which have more than 85% sand and less than 2% organic matter (Andargie et al., 2013). It is also shade

tolerant and compatible as an intercrop with maize, millet, sorghum, sugarcane, and cotton. These features make it an important crop of the complex and subsistence cropping system of the dry savannas in Sub-Saharan Africa (Ogbuinya, 1997; Tarawali et al., 2002; Sanginga et al., 2003). Some recently improved varieties of cowpea have a short life cycle between 55 to 65 days from seed to seed. Such a short life cycle increases its potential to be an important crop in existing cropping systems (Singh, 2014).

In light of rising global temperatures, there is a general concern that crop yields may be adversely affected in the coming decades. The Intergovernmental Panel on Climate Change (IPCC) and Jones et al. (2006), predicted that global mean temperatures will rise by 0.3 ˚C per decade, reaching to approximately 3 ˚C above the present value by the year 2100. For some crops, increases in growing season temperatures are linked to a decrease in agriculture yield (Lobell and Asner, 2003). Heat causes several physiological, phenological, and molecular changes in plants, which negatively affects the reproductive growth of plants. Therefore, concerted efforts are underway to develop heat-tolerant crop varieties and adopt appropriate management practices.

Cowpea is inherently adaptable to dry land production with varying levels of drought and heat because it originated in the semi-arid region of Africa. However, above normal temperatures can significantly reduce cowpea productivity. In cowpea, reproductive organs, flowers in particular are most affected by heat. Flower abscission can be induced by both high nighttime temperatures, and high daytime temperature (Warrag and Hall, 1984a). High nighttime temperatures reduce pollen viability and grain

yield in long-day environments (Elhers and Hall, 1998) and increase indehiscence of anthers (Warrag and Hall, 1984a). Therefore, systematic efforts are being made to identify sources of heat-tolerance in cowpea for introgression into improved cowpea varieties. Considerable genetic variability has been observed for heat tolerance in cowpea and several lines with high levels of heat tolerance have been identified (Hall et al., 1993; Singh B.B., 2014; and Abdelbagi et al., 1999). However, studies to elucidate the inheritance and identify the QTL (quantitative trait loci) regulating heat-tolerance are limited, thus making it difficult to identify appropriate breeding strategies to develop improved varieties. The aim of this project is to identify sources of heat tolerance in cowpea germplasm, elucidate inheritance and map the quantitative trail loci (QTL) regulating heat tolerance for marker-assisted selection protocol, and ultimately develop improved heat-tolerant cowpea varieties. The central hypothesis of this study is that genetic variability exists among cowpea germplasm, which can be utilized to elucidate genes/QTL conditioning heat tolerance and incorporate this trait in improved cowpea varieties. The *following objectives were used to test our hypothesis*:

I. Define the genetic variability for heat tolerance in cowpea germplasm
II. Identify lines with high levels of heat tolerance
III. Elucidate the inheritance of heat tolerance
IV. Map QTL responsible for the regulation of heat tolerance and other agronomic traits

CHAPTER II

GENETIC VARIABILITY FOR HEAT TOLERANCE IN COWPEA

Introduction

The effect of high temperature on cowpea

Hot weather conditions in the tropics and subtropics can have detrimental effects on reproductive organs and yields of several crops (Hall, 1992). Since cowpea is primarily grown in the semi-arid regions of Sub-Saharan Africa, South-East Africa, and Central and South America, it is often exposed to high temperatures. Several systematic and controlled experiments testing a combination of 21-36 ˚C daytime temperatures and 16-31 ˚C nighttime temperatures have shown that maximum dry matter production occurs with an optimum combination of 27 ˚C daytime and 22 ˚C nighttime temperatures. Other experiments and field observations have shown that nighttime temperatures above 25 ˚C and daytime temperature above 40 ˚C cause significant flower abortion, as well as a reduction in the number of pods and seed yield. (Craufurd et al., 1996, 1998; Turk et al., 1980; Warrag and Hall, 1983; Nielsen and Hall, 1985a, b; Ismail and Hall, 1999). The reproductive stages in cowpea are especially sensitive to high temperatures, particularly high nighttime temperatures (Hall, 1993). For each degree centigrade increase in minimum nighttime temperature above 16 ˚C, a decrease in grain yield of 4-14% has been reported (Nielsen and Hall, 1985b; Ismail and Hall, 1999). During the reproductive growth stage, heat-tolerant cowpea genotypes were selected under hot temperatures and long days (Ehlers et al., 2000; Hall, 1992; and Hall, 1993)

and they observed that greater grain yields obtained in hot environments were associated with greater pod set, higher harvest index, reduced flower bud abortion, reduced vegetative biomass, and shorter plants (Ismail and Hall, 1999). The number of pods set is severely reduced by high-nighttime temperatures (30 ˚C) primarily because of anther indehiscence, low pollen viability, and flower drop. High temperatures in later stages of floral bud development don't influence flower production, but reduce pollen viability and result in reduced pod set (Warrag and Hall, 1984b). High daytime temperatures (36 ˚C) also reduces the number of pods set, but not as severe as the high nighttime temperatures (Hall, 1993). The critical developmental stage is 9 to 7 days before anthesis (Warrag and Hall, 1984b; Ahmed et al., 1992). High temperatures during this period may cause a drastic reduction in yield.

Singh et al. (2010) conducted a study having Six contrasting genotypes of cowpea representing differential sensitivity/tolerance to heat, 'California blackeye (CB)-5' and 'CB-46' (both heat sensitive), 'CB-27' (heat tolerant), 'Mississippi Pinkeye' (heat effect is not known), 'Prima' (heat tolerant), and 'UCR-193' (heat tolerant), and their results revealed that the combined effect of UVB radiation and temperature cause severe reduction in grain yields (Hare, 1991; Fang et al, 2007; Warrang and Hall, 1983).

Genetic variability for heat tolerance in cowpea germplasm

Cowpea germplasm is very diverse, especially considering several biotic and abiotic stresses. Warrag and Hall (1983) evaluated several cowpea genotypes under hot conditions and found that TVu 4552, PI 204647, and Prima produced a substantial number of pods, while the other 55 genotypes produced few or no pods. Patel and Hall

(1990) conducted another study in Imperial Valley, California, under hot temperatures (mean daily max/min 41/24 °C) and in Riverside, California, under optimal temperatures (35/17 °C). They observed that the development of cowpea reproductive organs was normal at the Riverside site compared to that of Imperial Valley. Based on the study, they grouped the cowpea genotypes according to heat tolerance. Genotypes in the tolerant group exhibited normal peduncle elongation, early flowering, and produced many flowers and pods, whereas genotypes in the susceptible group did not produce visible flower buds. They reported TVu 4552 as the most heat-tolerant, followed by Prima, CB5, and 7964, respectively. Another study conducted by Ehler and Hall (1998) showed similar results. They tested the heat tolerance of 20 cowpea lines under hot short days as well as under moderate temperatures and reported wide variability among the germplasm. The percentage grain yield of the germplasm ranged from 88% for H8-9-3 to as low as 22% for Vita 1. All of these studies showed that genetic variability for heat-tolerance exists in the cowpea germplasm.

Materials and methods

Plant material

A total of 41 cowpea varieties were planted on the Horticulture farm at College Station on June 12, 2011. These varieties were collected from different parts of the world including Africa, USA, and Brazil.

Planting methods and data collection in the field

All 41 cowpea varieties were planted in plots at the Horticulture farm in College Station, Texas, USA in 2011. Ridges and furrows were made before planting and then the seeds were planted on the ridges. Each variety was planted in a 4-row plot using a four-row planter. The row length was 7 m and row-to-row distance was 1m with 20 cm plant-to-plant spacing within each row. A furrow irrigation method was performed to water the plots. The test was irrigated after planting, and later, irrigations were applied as necessary. Pre-plant and pre-emergence herbicides were applied to control weeds in the field.

The vegetative growth of all 41 varieties was normal but major differences were noticed among the varieties at the flowering and early pod development stages due to continuous high temperatures in the months of July and August, 2011. Severe flower and pod abscission were noticed in the heat-susceptible varieties but not in the resistant ones. To study the effect of heat on pollen development, young buds and flowers were collected from each variety and the extent of pollen development and pollen viability was analyzed using Sudan IV dye (Ying et al., 2004).

For assessing the effect of heat on yield and yield contributing characters, five plants were randomly selected and harvested manually from each plot at maturity, and each plant was treated as a replication. Data on pod weight per plant (PODWT), seed weight per plant (SWT), number of pods per plant (PODN), number of seeds per pod (SEEDN), and 100 seed weight (HSWT) was measured. Lucas et al. (2013) used an average number of pods per peduncle as an estimate of heat tolerance, and Samba et al.

(2004) reported that pod production is positively correlated with grain yield in cowpea. Therefore, SWT and PODN were used as an estimate of heat tolerance in the genetic variability study.

Based on the results from 2011, a total of 23 varieties representing "highly heat-tolerant" and "highly heat-susceptible" reactions were selected for planting in a 2012 field trial. The field trial was planted on May 10, 2012, at Texas A&M University AgriLife Research Station, Snook, Texas, USA, to reconfirm the reaction to heat effect. The same plant-to-plant and row-to-row distances were followed as in the previous year, and the same methodologies were used to harvest and measure traits. Data were also recorded for the same traits studied in 2011.

Screening for heat tolerance in the greenhouse

The same 23 selected cowpea varieties were planted in a controlled greenhouse in a completely randomized design with three replications. Two seeds of each variety were planted in two-gallon pots filled with Sun Gro Metro-Mix 900 Grower Mix with RESiLIENCE as potting mixture. Two plants were planted in each two-gallon pot and replicated three times. The potting mixture contained 50-60% bark, Canadian sphagnum peat moss, perlite and vermiculite, starter nutrient charge (with gypsum) and slow release nitrogen, dolomitirc limestone, and a long-lasting wetting agent. The varieties were subjected to a temperature range from 35-43 ˚C during the day and from 25-30 ˚C during the night to mimic the field conditions of the summer 2011 study. The traits measured in the greenhouse were the same as in the field, and SWT was used as an estimate of heat tolerance.

Statistical analysis

As appropriate, the PROC CORR procedure of SAS 9.4 was used to measure the correlation between the traits.

Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Bartlett's test, respectively, in JMP statistical software, and normality and homogeneity of variances couldn't be achieved even after using different transformation methods. Thus, it was ignored for the analysis.

Data were analyzed for each environment separately, as well as combined, over 2011 field, 2012 field, and greenhouse. Statistical analysis was carried out using PROC GLM for 2011 and combined analysis of both years (SAS v9.4, SAS Institute Inc., Cary, North Carolina, USA). The data from the 2011 field trial was analyzed using all fixed model of $Y_{ij} = \mu + t_i + \varepsilon_{ij,}$ where Y_{ij} = observation response, μ = overall mean, t = genotype (i = 1… 41), ε = error, and the combined data was analyzed defining genotypes and environments as fixed. The reason for using fixed model is that varieties were not selected randomly and results would not be applicable to other population or cowpea varieties. The model of $Y_{ijk} = \mu + t_i + \gamma_j + (t\gamma)_{ij} + \varepsilon_{ijk}$ was used, where Y_{ij} is the observed response, μ is an overall mean, t_i is one treatment effect, and γ_j is the environmental effect. The term $(t\gamma)_{ij}$ represents the interaction between treatment and environment, and a deviation from the additive response. And the last term ε_{ijk} is the error. Expected mean squares and F-test methods are explained in Table 2. Variance components were estimated from the analysis to calculate broad sense repeatability (*R*)

on entry mean basis using the formula $R = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gxe}^2}{e} + \frac{\sigma_e^2}{re}}$. Confidence intervals were calculated for these repeatability estimates using the procedure described by Knapp et al. (1985).

Expected mean squares and F test was conducted to test the significant difference in factors, which created the variation in the population (Table 13). Tukey method of mean separation was carried out using SAS 9.4 software. It is a good technique for carrying out all pairwise comparisons. It enables us to rank mean separation, and put them into significance groups while controlling maximum experiment-wise error rate. It uses the distribution of studentized range statistics.

Table 2. Expected mean squares estimates for combined (2011 field, 2012 field, and 2012 greenhouse) analysis of 23 selected varieties

Source	Mean Square	Expected Mean Square	F test
Environment	MSl	$\sigma^2_e + gr\sigma^2_l$	MSl/ MSe
Genotype	MSg	$\sigma^2_e + rly\sigma^2_g$	MSg /MSe
Genotype X Environment	MSgl	$\sigma^2_e + r\sigma^2_{gl}$	MSgl/MSe
Error	MSe	σ^2_e	

Results and discussion

Crop season 2011

Climate

Based on climatic data, the 2011 summer was one of the hottest years at College Station. The daytime temperatures ranged between 33 to 43 ˚C and the nighttime temperatures ranged between 23 to 27 ˚C during the months of July and August which coincided with flowering time and early pod development stages (Fig. 2). According to OSC (Office of the State Climatologist) reports, average temperatures for June to August 2011 were over 2 ˚F above the previous Texas record were reported as the hottest statewide summer temperatures ever recorded in the United States (Nielson-Gammon, 2011). This temperature range was similar to what Lucas et al. (2013) had reported earlier for their heat tolerance study of cowpea varieties. They observed a great deal of variation in the grain yield per plant among cowpea lines when the daytime temperatures ranged between 35 to 43 ˚C and the nighttime temperatures were between 25 to 30 ˚C during the flowering stage. Thus, the extremely hot 2011 crop season at College Station provided an excellent opportunity to screen cowpea germplasm for heat tolerance.

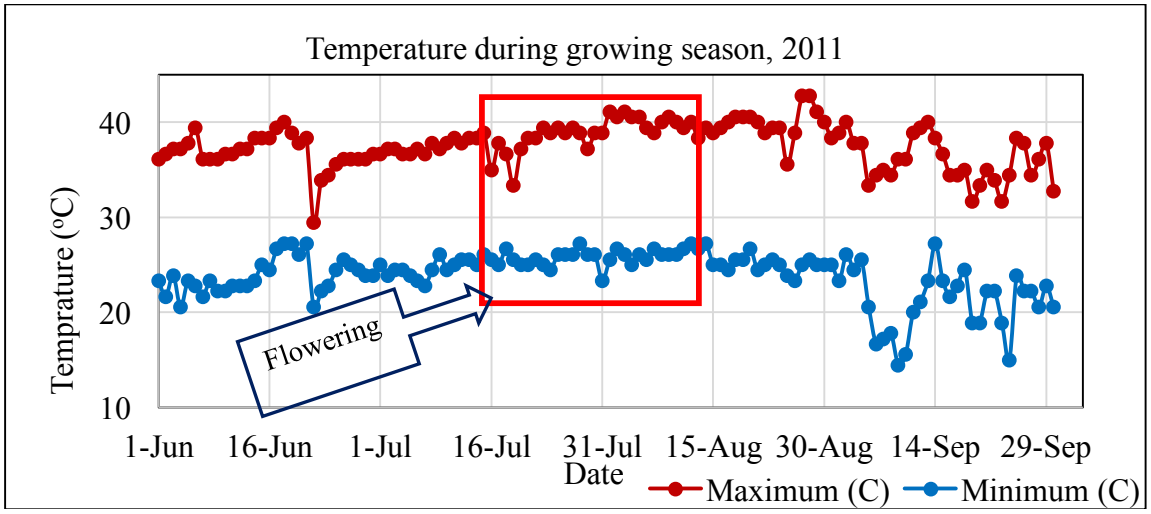

Fig. 2. College Station temperature during growing cowpea season, 2011

Effect of heat on flowering and pod set

Each cowpea variety had normal vegetative growth without any noticeable variability among the varieties. However, the effect of heat began to appear at the onset of flowering and pod setting. Some varieties showed severe flower abscission and little or no pods while others showed normal flowering and pod growth (Fig. 3). Most of the peduncles on the heat-susceptible entries had a few or no pods with fewer SEEDN, giving the appearance of severe sterility. In contrast, the peduncles on the heat-tolerant plants had normal pod density and pods had a higher number of seeds (Fig. 3).

Fig. 3. Normal podding and flower pod drop under field condition in 2011

Pollen characteristics

The flowers and young buds were collected from the field (2011) during the hottest time of the day and the pollen viability was measured using a light microscope and Sudan IV dye (Ying et al., 2004). The pollen grains from the heat-susceptible plants showed a range of size differences and sterility (indicated by a clear unstained cytoplasm) whereas the pollen grains from the heat-tolerant lines were completely normal (Fig. 4). The adverse effect of heat on pollen development has been observed in many crops and therefore, these observations were expected.

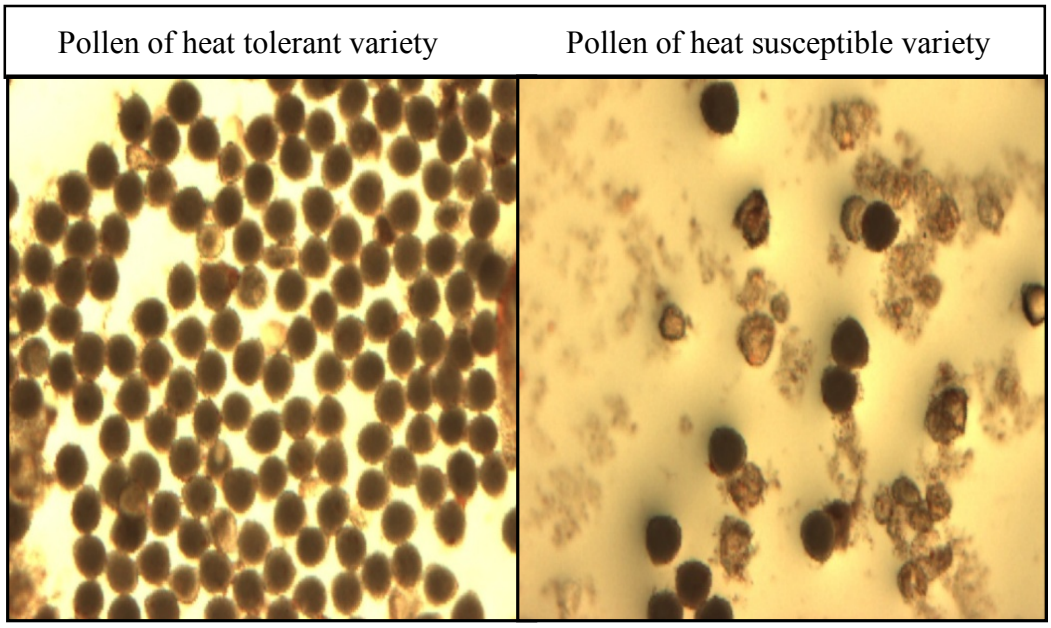

Fig. 4. Pollen of heat tolerant and susceptible varieties, dark stained pollens are viable and hollow stained pollen are inviable

Effect of heat on yield and yield contributing traits

As expected, a great deal of variability for SWT was observed ranging from over 52 g/plant in some varieties to no seed yield from others (Fig. 5). The reaction to heat was independent of days-to-maturity since highly tolerant and highly susceptible varieties were observed in both early and medium maturing varieties. The seed SWT and related traits in heat-tolerant and heat-susceptible varieties corresponded well with floral abscission and pod production. PODN ranged from 0 to 42 pods/plant and HSWT ranged from 0 to 25.43 g. A summary of data on yield and yield components from early and medium maturity groups are presented in Table 3 and 6.

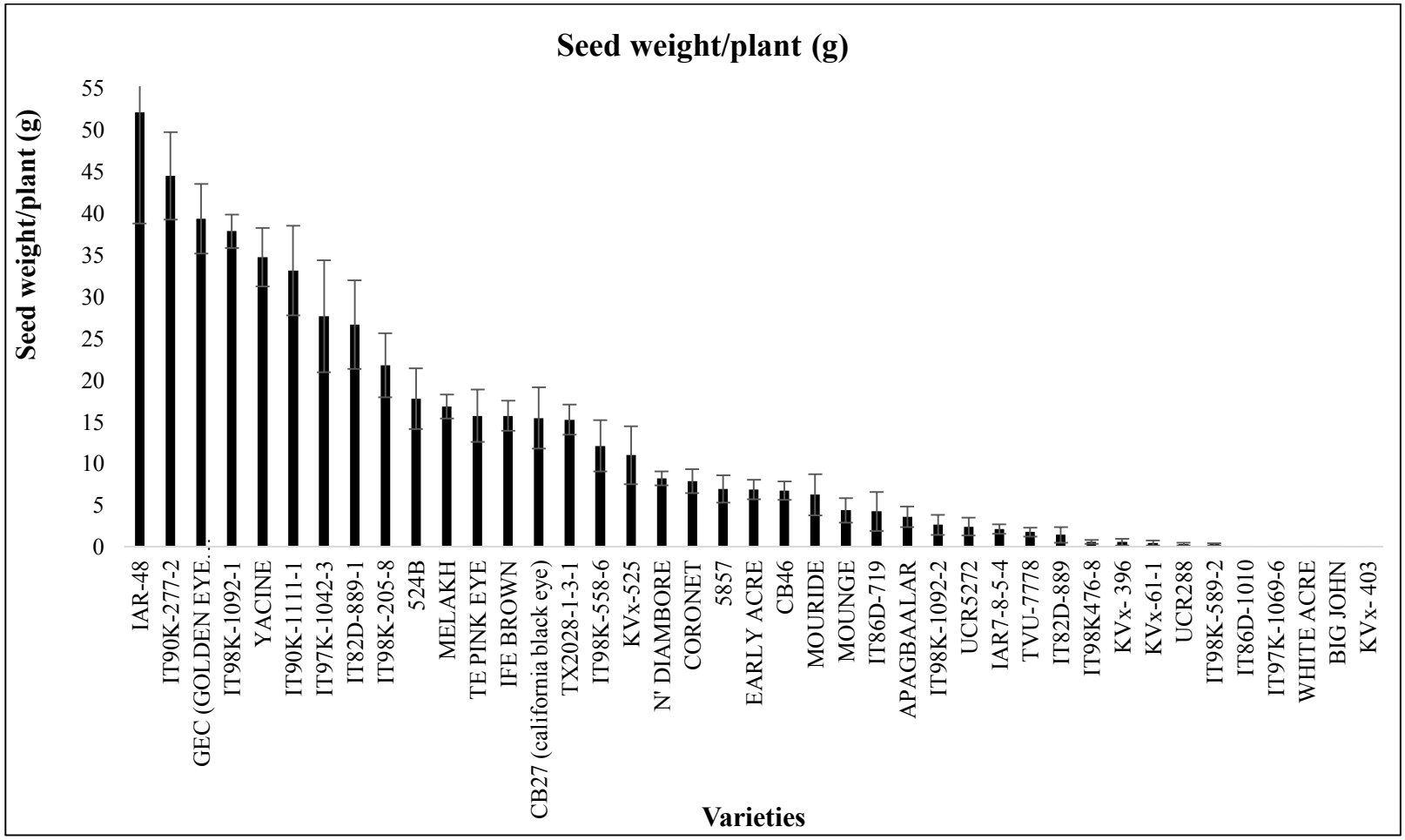

Fig. 5. Seed weight per plant of total 41 cowpea varieties screened for heat tolerance, 2011

Mean, range, and repeatability estimates

In the 2011 study, some lines were sterile, while some produced optimum grain yield (Fig. 3). Averages of SEEDN, PODN, PODWT, SWT, and HSWT were 5.4, 13.7, 18.1 g, 12.3 g, 12.9 g, respectively. SWT, PODN, SEEDN, PODWT, and HSWT ranged from 0 to 52 g, 0 to 42, 0 to 11, 0 to 73 g, and 0 to 25 g, respectively. Repeatability (*R*) values were very high for all traits ranging from 73% to 93% (Table 3). Such high levels of repeatability for quantitative traits are expected only if the differences among the varieties are due to major gene effects and when all the yield-contributing traits are affected by the same genes. As mentioned earlier, the high temperatures caused different levels of floral sterility, abscission and pod abortion which determined the overall pod set and seed yield in different varieties. Thus, these seed traits were highly correlated (Table 4).

Table 3. Mean, range, and heritability of 41 cowpea varieties, 2011

Traits	Mean	Range	*R*
SEEDN	5.41	0-11.16	0.79
PODN	13.71	0-42	0.73
PODWT	18.12 g	0-72.64 g	0.77
SWT	12.27 g	0-51.99 g	0.77
HSWT	12.85 g	0-25.43 g	0.93

SEEDN = number of seeds/pod; PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plant; HSWT = 100 seed weight; *R* = repeatability

Pictures taken from the field (2011) showed a clear difference between heat-tolerant and heat-susceptible varieties (Fig. 6). The picture on the left in Fig. 6 is a heat-tolerant variety, which produced optimum yield under high temperature conditions, and the picture on the right shows a drop of almost all the flowers, and this was confirmed with the range of SWT in 2011 crop season (0 to 51.99 g). Fig. 7 shows a gradient from heat tolerance to heat susceptibility. The left peduncle produced three pods, compared to the rightmost peduncle, which produced nothing. Similarly, heat-tolerant varieties produced longer and filled pods compared to the susceptible varieties (Fig. 7).

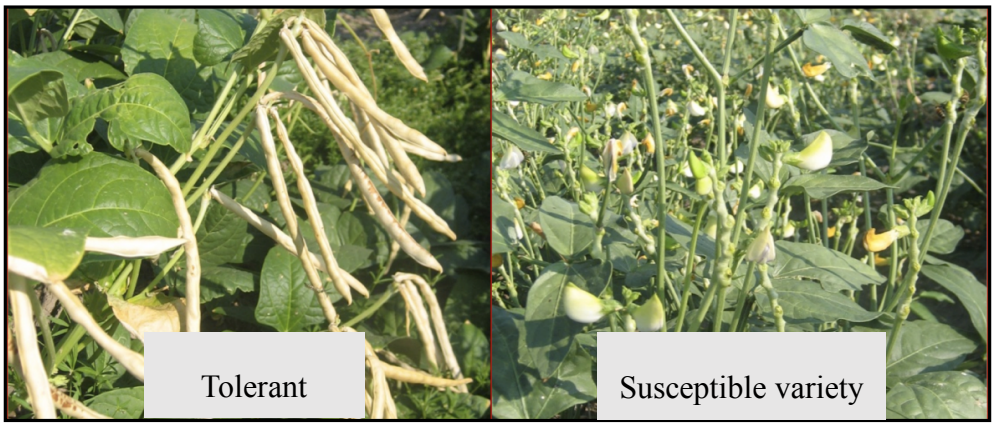

Fig. 6 A heat tolerant and a susceptible variety from field study 2011

Fig. 7. Heat tolerant to susceptible varieties performance in the field study 2011

Correlation analysis

All the traits showed high significant positive correlations at 0.001 significance level in the 2011 field study. The SWT showed a lower correlation to SEEDN (0.6132) and HSWT (0.5161) compared to other traits, SWT and PODN were highly correlated (0.9169), which agrees with the results reported by Samba et al. (2004) (Table 4).

Table 4. Correlation between seed traits of heat screening trial, 2011

Traits	PODN	SEEDN	PODWT	SWT	HSWT
PODN	1	0.5789***	0.9418***	0.9061***	0.4521***
SEEDN		1	0.6187***	0.6132***	0.6454***
PODWT			1	0.9914***	0.5017***
SWT				1	0.5161***
HSWT					1

SEEDN = number of seeds/pod; PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plant; HSWT = 100 seed weight
**A significant correlation exists between traits at significance level 0.001

Table 5. Analysis of variance of genetic variability of cowpea, 2011

Source	df	Sum of	Mean Square	F test	P value
PODN					
Total	204	42862			
Varieties	40	33532	838	14.74	<.0001***
Experimental	164	9329	56		
SEEDN					
Total	204	2277			
Varieties	40	1981	49	27.45	<.0001***
Experimental	164	295	2		
PODWT					
Total	204	94713			
Varieties	40	76644	1916	17.39	<.0001***
Experimental	164	18069	110		
SWT					
Total	204	48486			
Varieties	40	39454	986	17.91	<.0001***
Experimental	164	9032	55.07		
HSWT					
Total	204	8182			
Varieties	40	7748	193	66.12	<.0001***
Experimental	164	433	3		

SEEDN = number of seeds/pod; PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plant; HSWT = 100 seed weight
***Significantly different at 0.001 alpha level

Analysis of variation

A highly significant difference existed among the 41 cowpea varieties for all the traits in 2011 at the 0.001 significance level, and that means a significant genetic variability for all the seed traits. Mean square value was least for SEEDN (49.54) and highest for PODWT (1916.10). Mean square values of experimental error were lower

than the mean square values of varieties for all the traits, and that indicates higher efficiency of the experiment conducted in 2011 study (Table 5).

There were two groups, early maturing and medium to late maturing, among 41 varieties planted in the 2011 study. Table 6 shows both groups consisting of contrasting varieties for SEEDN, PODN, PODWT, SWT, and HSWT. The most heat-tolerant varieties in the early group were IT82D-889-1, IT98K-205-8, and IT98K-1111-1, and most heat susceptible were IT98K-589-2, IT82D-889 based on SWT. Similarly, the most heat-tolerant varieties in the medium maturing group were IT98K-1092-1, Ife Brown, Yacine and the most heat-susceptible were Big John, UCR-288, and IT98K-1091-2 based on SWT. PODN, SWT, and HSWT ranged from 0 to 42, 0 to 52 g, and 0 to 23.44, respectively (Table 6). The seeds per pod (SEEDN) were significantly lower in the heat-susceptible varieties compared to heat-tolerant varieties except for KVx396, which produced 9 SEEDN. This indicates that heat-susceptible varieties experienced either pollen sterility or seed abortion.

Table 6. Over all means of seed number per pod per plant, pod number per plant, pod weight per plant, seed weight per plant, and 100 seed weight in cowpea from College Station 2011 variety trial

Early maturing cowpea varieties					
Highly heat tolerant					
Variety	**SEEDN**	**PODN**	**PODWT**	**SWT**	**HSWT**
IT82D-889-1	10.92 ^{A}	29.80 ^{ABCD}	39.32 ^{BCDE}	26.58 ^{BCD}	9.92 ^{DEFG}
IT98K-205-8	7.96 ^{AB}	18.00 ^{CDEF}	30.64 ^{CDEF}	21.70 ^{CDE}	16.48 ^{BCDE}
IT98K-1111-1	7.64 ^{ABC}	36.60 ^{ABC}	45.88 ^{ABCDE}	33.04 ^{ABCD}	16.33 ^{BCDE}
TX PINK EYE	5.76 ^{BCDE}	24.80 ^{ABCDE}	26.05 ^{DEFGH}	15.65 ^{DEF}	13.90 ^{CDEF}
TX 2028-1-3-1	6.64 ^{BCD}	18.60 ^{BCDEF}	23.59 ^{DEFGH}	15.18 ^{DEF}	16.74 ^{BCDE}
Highly heat susceptible					
Variety	**SEEDN**	**PODN**	**PODWT**	**SWT**	**HSWT**
IT98K-589-2	2.00 ^{FG}	0.80 ^{F}	0.60 ^{H}	0.25 ^{F}	14.28 ^{EFGH}
IT82D-889	4.76 ^{BCDEF}	2.40 ^{F}	2.83 ^{FGH}	1.39 ^{EF}	10.33 ^{DEFG}
IT86-D-1010	0.00 ^{G}	0.00 ^{F}	0.00 ^{H}	0.00 ^{F}	0.00 ^{H}
Medium maturing cowpea varieties					
Highly heat tolerant					
Variety	**SEEDN**	**PODN**	**PODWT**	**SWT**	**HSWT**
IT98K-1092-1	11.16 ^{A}	37.80 ^{ABC}	56.71 ^{ABC}	37.75 ^{ABC}	9.86 ^{DEFG}
IEF BROWN	5.60 ^{BCDE}	34.40 ^{ABCD}	29.36 ^{CDEFG}	15.64 ^{DEF}	10.42 ^{CDEFG}
YACINE	6.40 ^{BCD}	24.60 ^{ABCDE}	49.27 ^{ABCD}	34.64 ^{ABCD}	23.44 ^{AB}
IAR-48	8.12 ^{AB}	42.00 ^{A}	72.64 ^{A}	51.99 ^{A}	18.25 ^{ABCD}
CB-27	5.72 ^{BCDE}	16.00 ^{DEF}	19.83 ^{EFGH}	15.39 ^{DEF}	18.96 ^{ABC}
IT98K-277-2	8.48 ^{AB}	38.40 ^{AB}	59.73 ^{AB}	44.37 ^{AB}	17.95 ^{ABCD}
GEC	8.56 ^{AB}	24.20 ^{ABCDE}	46.99 ^{ABCDE}	39.26 ^{ABC}	25.43 ^{A}
Highly heat susceptible					
Variety	**SEEDN**	**PODN**	**PODWT**	**SWT**	**HSWT**
BIG JOHN	0.00 ^{G}	0.00 ^{F}	0.00 ^{H}	0.00 ^{F}	0.00 ^{H}
UCR288	5.00 ^{DEFG}	0.60 ^{F}	0.49 ^{H}	0.30 ^{F}	9.53 ^{FGH}

Table 6 Continued

Variety	SEEDN	PODN	PODWT	SWT	HSWT
IT98K-1092-2	3.92 ^{DEFG}	6.80 ^{EF}	5.47 ^{FGH}	2.61 ^{EF}	10.84 ^{EFG}
IT98K-1069-6	0.00 ^{G}	0.00 ^{F}	0.00 ^{H}	0.00 ^{F}	0.00 ^{H}
KVx396	9.00 ^{EFG}	0.40 ^{F}	1.00 ^{GH}	0.53 ^{EF}	8.00 ^{FGH}
TVu7778	4.85 ^{BCDEFG}	5.00 ^{EF}	2.56 ^{FGH}	1.74 ^{EF}	6.89 ^{FGH}
UCR5272	4.77 ^{CDEFG}	3.80 ^{F}	3.65 ^{FGH}	2.38 ^{EF}	12.55 ^{DEFG}
IT98K-476-8	1.70 ^{EFG}	1.00 ^{F}	0.98 ^{GH}	0.53 ^{F}	11.40 ^{CDEF}

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant
Numbers following different alphabets are significantly different at significance level 0.05

Crop season 2012

The daytime temperatures ranged from 30 to 41 ˚C and the nighttime temperatures ranged from 20 to 27 ˚C (Fig. 9) in 2012 (source: National Oceanic and Atmospheric Administration). The temperature ranges were not as high as the previous year, but they were high enough to recreate the trend between heat-tolerant and heat-susceptible varieties (Fig. 8).

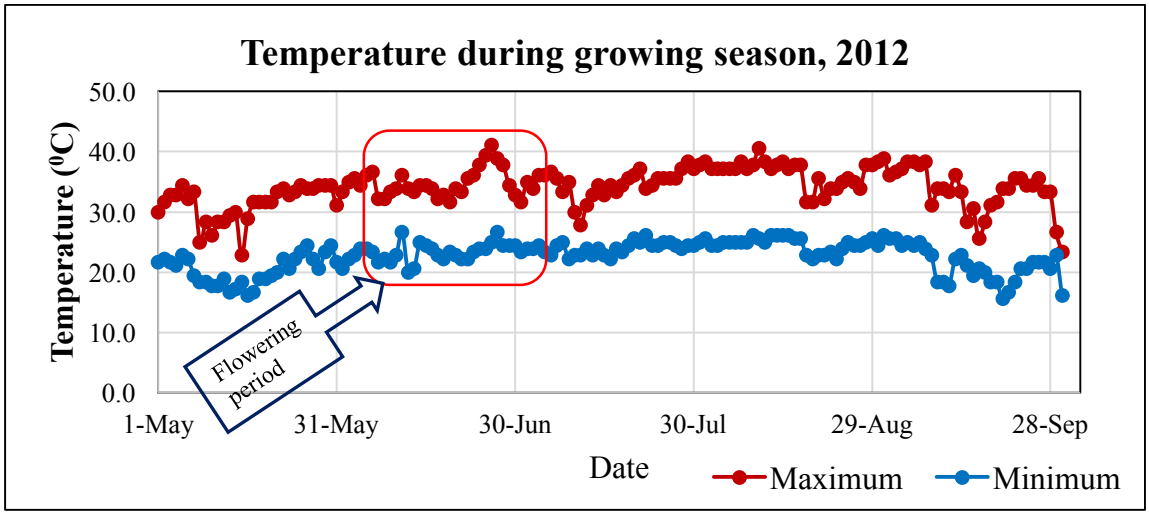

Fig. 8. Temperature during growing season, 2012

The mean PODN, PODWT, SWT, and HSWT were 10, 17.5 g, 13.5 g, and 15.6 g., respectively. Similar to other studies, the repeatability estimates were high for all traits (Table 7) and extreme SWT producing varieties were significantly different for all the traits at significance level 0.05, and a significant genetic variability was observed for all the measured traits. However, all the varieties managed to produce SWT unlike the 2011

crop season and 2012 greenhouse study, this was likely due to the lower temperatures in the 2012 field study. Variety IT98K-476-8, selected for further studies, was able to produce SWT in 2012 because of lower temperature compared to crop season 2011.

Table 7. Mean, range, and heritability of 41 cowpea varieties, 2012 field

Traits	Mean	Range	*R*
PODN	10	4 – 15 g	0.66
PODWT	17.5 g	3.9 – 29.4 g	0.72
SWT	13.5 g	2.6 – 23.5 g	0.76
HSWT	15.6	3.3 – 27.4 g	0.96

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant; *R* = repeatability

Greenhouse study 2012

In the greenhouse, the daytime temperature was maintained between 35 to 43 ˚C, and the nighttime temperature was maintained between 24 to 28 ˚C at the flowering stage, to mimic the field conditions of 2011.

The mean of PODN, PODWT, SWT, and HSWT of the 23 varieties planted in the greenhouse were 4.72, 5.53 g, 4.24 g, and 17.04 g., respectively, and PODN and SWT ranged from 0 to 8 and 0 to 6.63 g respectively. Similar to the 2011 field study, the results showed very high heritability estimates (Table 8). Similar to the previous studies, a significant genetic variability was observed for all the measured traits. SWT was lower in the greenhouse compared to the field study, and that was expected.

Table 8. Mean, range, and heritability of 23 cowpea varieties, greenhouse

Traits	Mean	Range	*R*
PODN	4.73	0-8	0.93
PODWT	5.50 g	0-7.88 g	0.90
SWT	4.19 g	0-6.63 g	0.92
HSWT	10.00 g	0-27.27 g	0.98

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant; *R* = repeatability

Combined analysis 2011 field, 2012 field, and 2012 greenhouse

A combined analysis was carried out using common varieties (23 varieties) in 2011 field, 2012 field, and 2012 greenhouse.

Performance of varieties across the studies based on SWT

The result of the analysis showed varieties GEC, IT90K-277-2, Yacine, IT90K-1111-1, and IT98K-1092-1 were consistently heat-tolerant, and varieties Big John, IT98K-589-2, IT98K-476-8, CB-46, and White Acre were consistently heat-susceptible in both the 2011 and 2012 field and the 2012 greenhouse trials (Fig. 9). This indicated the consistency of these varieties across environments as sources of heat tolerance and susceptibility for further heat tolerance research and breeding efforts.

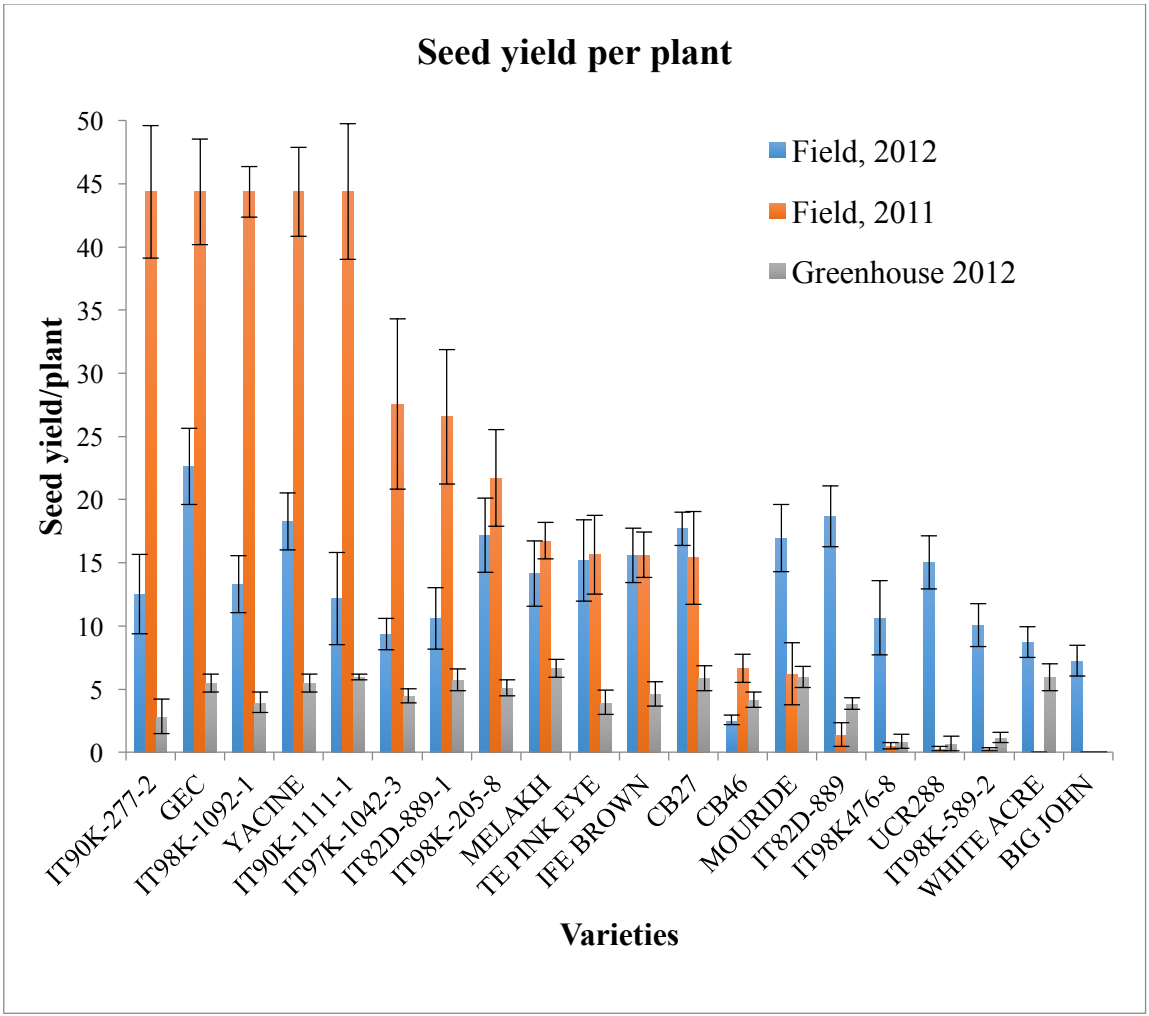

Fig. 9. Seed yield per plant for 2011 field, 2012 field, and 2012 greenhouse

Correlation analysis

As expected, the yield components PODN, PODWT, HSWT, and SWT showed highly significant positive correlations in the combined analysis (Table 9).

Table 9. Correlation of pod number per plant, pod weight per plant, 100 seed weight and seed weight per plant in cowpea from a variety trials. These values were obtained via a combined analysis across all the three environments used in the study (CS 2011, CS 2012, and greenhouse)

	PODN	PODWT	HSWT	SWT
PODN	1.0000	0.9241***	0.1199*	0.8795***
PODWT		1.0000	0.1965***	0.9849***
HSWT			1.0000	0.2365***
SWT				1.0000

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant
***A significant correlation exists between traits at significance level 0.001
*A significant correlation exists between traits at significance level 0.05

Analysis of variance

Combined analysis of all three environments showed a significant interaction between genotypes and environments for all traits. As such, we separated the individual effects. We have noticed that the genotype effect was significant in all individual analysis and this remained the same in the combined analysis. Similar to the individual analysis, there was a lower error mean square in the combined analysis compared to other effects, which indicates consistency among the replications and studies. A high R^2 also existed for all traits (Table 10) in the combined analysis.

Table 10. Mean squares from the analysis of variance for pod number, pod weight, and seed weight per plant in cowpea from a variety trial. These values were obtained via a combined analysis across all three environments used in the study (CS 2011, CS 2012, and Greenhouse 2012)

Source	d.f.	PODN		PODWT		SWT		HSWT	
G	22	581	***	1482	***	842	***	376	***
E	2	5242	***	10891	***	5093	***	263	***
G x E	42	379	***	849	***	425	***	34	***
Error		31		84		47		9	
R^2		0.75		0.73		0.71		0.82	

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant; G = genotype; E = environment
***The effect is significantly different at significance level 0.001

Repeatability estimates

PODN, PODWT, SWT, and HSWT exhibited 0.30, 0.40, 0.49, and 0.93 repeatability values, respectively, in the combined analysis of all three environments (Table 11). The repeatability values for all the traits were lower in the combined analysis compared to the individual analysis except for the HSWT (0.93).

Table 11. Repeatability for PODN, pod PODWT, SWT, and HSWT in cowpea from combined analysis of CS 2011, CS 2012, and Greenhouse 2012. Confidence intervals are provided in parenthesis (0.05 – 0.95)

Trait	Repeatability
PODN	0.30 (0.26 – 0.64)
PODWT	0.40 (0.11 – 0.68)
SWT	0.49 (0.30 – 0.72)
HSWT	0.93 (0.82 – 0.95)

PODN = number of pods/plant; PODWT = pod weight/plant; SWT = seed weight/plan; HSWT = 100 seed weight per plant

Top performing varieties based on SWT

IAR-48, GEC, and IT98K-272-2 produced higher SWT, and IT98K-476-8, IT98K-589-2, and Big John produced consistently lower SWT across the three environments. Results showed a high consistency, for SWT, among top and low performing varieties across different environments (Table 12).

Table 12. Top ten performing entries (RILs) for SWT within each environment from a population derived from a cross between GEC x IT98K-476-8. Three environments are represented, CS 2011, CS 2012, and Greenhouse 2012, as well as the combined mean for all of these environments

	Combined		2011		2012		Greenhouse	
Entry	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**
IAR 48	1	37.75 ^{A}	1	51.99 ^{A}	1	23.52 ^{AB}	.	.
GEC	2	22.44 ^{B}	3	39.26 ^{ABC}	2	22.59 ^{AB}	9	5.47 ^{A}
IT98K-277-2	3	20.85 ^{BC}	2	44.37 ^{AB}	14	12.50 ^{ABCD}	7	5.67 ^{A}
YACINE	4	18.79 ^{BCD}	5	34.64 ^{ABCD}	7	16.24 ^{ABC}	8	5.48 ^{A}
IT98K-1092-1	5	18.34 ^{BCD}	4	37.75 ^{ABC}	13	13.29 ^{ABCD}	16	3.97 ^{ABCD}
IT98K-1111-1	6	17.05 ^{BCD}	6	33.04 ^{ABCD}	15	12.15 ^{ABCD}	2	5.97 ^{A}
IT98K-205-8	7	14.66 ^{BCD}	9	21.70 ^{CDE}	5	17.17 ^{ABC}	10	5.10 ^{A}
IT82D-889-1	8	14.40 ^{BCD}	8	26.58 ^{BCD}	17	10.86 ^{ABCD}	6	5.75 ^{A}
IT97K-1042-3	9	13.78 ^{BCD}	7	27.57 ^{BCD}	20	9.32 ^{BCD}	13	4.47 ^{ABC}
CB-27	10	12.98 ^{BCD}	13	15.39 ^{DEF}	4	17.69 ^{ABC}	5	5.87 ^{A}
MELAKH	11	12.51 ^{BCD}	10	16.74 ^{DEF}	12	14.14 ^{ABCD}	1	6.63 ^{A}
IFE BROWN	12	11.94 ^{BCD}	12	15.64 ^{DEF}	8	15.56 ^{ABC}	11	4.63 ^{BC}
TPE	13	11.60 ^{BCD}	11	15.65 ^{DEF}	9	15.18 ^{ABC}	15	3.98 ^{ABCD}
MOURIDE	14	9.67 ^{BCD}	15	6.20 ^{EF}	6	16.83 ^{ABC}	3	5.97 ^{A}
IT82D-889	15	7.96 ^{BCD}	17	1.39 ^{F}	3	18.65 ^{ABC}	17	3.85 ^{ABCD}
2028	16	7.79 ^{BCD}	.	.	16	11.06 ^{ABCD}	12	4.52 ^{ABC}
MOUNGE	17	7.34 ^{BCD}	16	4.32 ^{EF}	11	14.54 ^{ABCD}	18	3.15 ^{ABCDE}
UCR-288	18	5.33 ^{CD}	19	0.30 ^{F}	10	15.00 ^{ABC}	21	0.68 ^{DE}
WHITE ACRE	19	4.88 ^{CD}	22	0.00 ^{F}	21	8.71 ^{CD}	4	5.94 ^{A}

Table 12 Continued

	Combined		2011		2012		Greenhouse	
Entry	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**
CB-46	20	4.46 ^{CD}	14	6.66 ^{EF}	23	2.55 ^{D}	14	4.17 ^{ABCD}
IT98K-476-8	21	4.01 ^{CD}	18	0.53 ^{F}	18	10.63 ^{BCD}	20	0.87 ^{CDE}
IT98K-589-2	22	3.82 ^{D}	20	0.25 ^{F}	19	10.04 ^{BCD}	19	1.17 ^{BCDE}
BIG JOHN	23	2.41 ^{D}	21	0.00 ^{F}	22	7.24 ^{CD}	22	0.00 ^{E}

CHAPTER III

GENOTYPE X ENVIRONMENT INTERACTION, GENETIC MAP CONSTRUCTION, AND QTL MAPPING FOR HEAT TOLERANCE IN COWPEA

Introduction and literature review

According to Intergovernmental Panel on Climate Change (IPCC 2007), the global climate temperature will rise by 2-4 ˚C at the end of 21st century. The predictions, based on the study of Battisti Naylor (2009), suggest that the worst sufferer will be the tropical and subtropical parts of the world. The maximum temperature is rising by 0.3˚C and minimum temperature is rising by 0.2 ˚C per decade based on a linear trend reported by Lobell and Gourdji (2012). As a result of the temperature rise, phenologies of plant species are altered (Ibanez et al., 2009; Li et al., 2014), and yields of some crops are reduced. Thus, rising temperature is an alarming situation which may impact the global food security. However, plants develop different mechanisms such as regulating networks/pathways by various gene expressions and physiological and biological alterations, but the mechanism is a complex phenomenon. Heat response effects are of a complex genetic nature and that poses a challenge to the scientist (Blum, 1986). Furthermore, the magnitude of genotype by environmental interaction and epistasis of genes increase the complexity (Cossani and Reynolds, 2012).

High nighttime temperatures during the growing season have detrimental effects on reproductive organs and grain yields in several crops (Hall, 1992). Most of cowpea cultivars are heat susceptible during the reproductive stage (Patel and Hall, 1990) and

can exhibit 13.5% reduction in the grain yields per degree centigrade increase in daily minimum nighttime temperatures above 16.5 ˚C (Neilson and Hall, 1985; Ismail and Hall 1999). The reduction in number of pods is one of the main reasons for lower grain yield under hot field conditions (Neilson and Hall 1985a).

Heat effects in cowpea can be defined in two stages. In stage I heat effects, floral bud initiation is inhibited by heat (Dow El-Medina and Hall 1986). In Stage I, the effects are influenced by photoperiods which are regulated by phytochromes (Mutters et al., 1989a and Hall 1992). Stage II effects occur during later stage of floral bud development, and cause male sterility, which results in fruit abortion (Warrang and Hall, 1984a). Ahmed et al. (1992) studied anther tissue development during high nighttime temperatures, and reported a distortion in microspore cells, the tapetal layer, and the endothecium. These abnormalities in reproductive tissue may reduce translocation of proline from anther to pollen (Ahmed et al., 1992), and that had been associated with male sterility in cowpea (Mutters et al., 1989b).

Heat tolerance in cowpea has been associated with high grain yield and pods per plant, as well as reduced flower abortion, vegetative biomass, and plant height (Ismail and Hall, 1999). Selection for heat tolerance can be performed in the reproductive stage based on low flower abortion and high pod set in extreme hot daytime field conditions (Hall, 1992), and in the greenhouse with high nighttime temperatures and long days (Hall, 1993). Hall (1993) reported a single gene responsible for flower production, and Marfo and Hall (1992) reported a single gene for pod set. All of these studies indicate that heat tolerance, which is measured based on high grain yield, pod set and reduced

flower abortion is governed by a few genes. SWT, PODN, and visual heat tolerance scores, based on pod set and flower fertility, can be used as an estimate of heat tolerance (Lucas et al., 2013; Samba et al., 2004).

Cowpea belongs to the genus *Vigna* Savi. in the Phaseoleae (Marechal et al., 1978), and consists of a total of 22 diploid chromosomes (Barone and Saccardo, 1990; Pignone et al., 1990; Saccardo et al., 1992). Several efforts have been made in the past using molecular markers to develop an informative and dense genetic linkage map (Menendez et al., 1997; Ouedraego et al., 2002; Muchero et al., 2009a). Prior to 1993, only limited cowpea genetic map information was available in scientific literature. Fatokun et al. (1993) and Menancio-Hautea et al. (1993) developed a genetic map from a cross between an improved cultivar and a putative wild progenitor (*Vigna unguiculata* ssp. dekindtiana). The map consisted of 87 random genomic, five cDNA RFLPs (restriction fragment length polymorphism), five RAPDs (random amplification of polymorphic DNA), and two morphological loci that culminated in eight linkage groups covering 684 cM of the cowpea genome. However, genetic map construction from wide crosses has a disadvantage of identifying loci that may be polymorphic only between more divergent genotypes but not between closely related genotypes and have a limitation in cowpea breeding programs. To make up for the shortcoming, Menendez et al. (1997) constructed the first genetic map using a cultivated gene pool of cowpea. They used an RIL population (94 lines) derived from a cross between IT84S-2049 and 524B. The developed genetic map consisted of 133 RAPDs, 19 RFLPs, 25 AFLPs, three morphological markers, and a biochemical marker. These markers clustered and

produced 12 linkage groups spanning 972 cM with an average distance of 6.4 cM between markers. Ubi et al. (2000) used 79 RAPD and three morphological markers to construct another genetic map. They used a RIL population derived from a cross between IT84S-2246-4, an improved cowpea line, and TVNu 110-3A. Their map spanned 669.8 cM, comprising 80 mapped loci clustered into 12 linkage groups with an average distance of 9.9 cM between markers (ranged from 0.7 to 26.7 cM). These linkage maps could not offer much utility to cowpea breeding programs because of the low number of markers utilized and the large gaps between markers. Ouedraogo et al. (2002) identified 242 new AFLP markers in the mapping population (94 individuals) utilized by Menendez et al. (1997). They utilized these newly developed markers in addition to 181 markers developed by Menendez et al. (1997) to construct a higher resolution genetic linkage map. This improved the genetic map of cowpea which consisted of 11 linkage groups spanning a total of 2670 cM, with an average distance of 6.43 cM between markers. Muchero et al. (2009b) published another genetic map utilizing 306 AFLP markers placed on 11 linkage groups spanning a total genetic distance of 643cM. In the same year, for the first time, a consensus genetic map was reported by Muchero et al. (2009b) based on EST-derived SNPs. They used six by-parental populations (741 inbred lines) ranging from 79 to 114 individuals in each population. Their individual map size ranged from 601 to 665 cM and the number of SNPs ranged from 288 to 436 markers per population. They performed SNP mining from 183,118 ESTs sequenced from 17 cDNA libraries yielding about 10,000 high confidence SNPs. Then an Illumina 1,536-SNP GoldenGate genotyping array was

developed and applied to 741 RILs. After analysis, 928 markers, placed in 645 bins, were incorporated into a consensus genetic map spanning 680 cM with 11 linkage groups with an average marker to marker distance of 0.73 cM. This map was more dense compared to previously reported maps and projected its utility in cowpea breeding programs across the globe. Lucas et al. (2011) published an improved consensus map using 1293 individual lines representing 13 mapping populations. They used an Illumina 1536 GoldenGate assay to construct a consensus map which contained 1107 EST-derived SNP markers in 856 bins and 11 linkage groups spanning over 680 cM of the cowpea genome. This improved map presented 33% more bins and 19% more markers compared to the previously published consensus map. The cowpea genetic map was compared to the reference soybean legume, and extensive macrosynteny encompassing 85% of the cowpea map was revealed (Muchero et al. 2009b). These results support the recent evolutionary closeness between cowpea and soybean.

A limited number of studies have been conducted on the inheritance and identification of QTL responsible for heat tolerance in cowpea. Marfo and Hall (1992) conducted a study on TVu 4552 and Prima cowpea varieties to identify the number of genes responsible for heat tolerance in cowpea. They grew F1, F2, and backcross progenies under field conditions in Imperial Valley, California, during the summers of 1987 and 1988. They selected heat-tolerant and heat-susceptible F2 plants in 1987 and evaluated F3 families in 1988. They concluded that heat tolerance is governed by a single dominant gene in both Prima and TVu 4552 based on pods per peduncle and number of tolerant plants. In another study, Lucas et al. (2013) conducted a study to map

the QTL responsible for heat tolerance using RILs population developed using parents CB27 (heat-tolerant) and IT82E-18 (heat-sensitive), and they reported five major QTL for heat tolerance. They used pods per peduncle as an estimate of heat tolerance and used a threshold LOD (logarithm of the odds) of 3 to identify the significant QTL. These five QTL explained 11.5 to 18% phenotypic variation, spanning a total of 61.42 cM, which covers 9% of the cowpea genome. Identified QTL were mapped to linkage groups 2, 3, 6, 7, and 10. Favorable alleles for heat tolerance of four QTL out of five were donated by the heat-tolerant parent CB 27. QTL studies on other abiotic stresses of cowpea such as drought tolerance have been conducted in the past. One of the studies conducted by Muchero et al. (2009b) on drought tolerance QTLs reported 10 QTL associated with seedling drought tolerance in cowpea RILs. Andargie et al. (2013) conducted a study on the molecular mapping of QTL for domestication-related and agronomic traits. They reported seven QTL detected on LG1, LG2, LG3, LG7, and LG10 for seed weight, and three QTL were mapped for days to flowering on LG1, LG2, and LG7. In conclusion, these studies indicate that heat tolerance in cowpea is governed by a few major genes/QTL.

Materials and methods

Plant material and data documentation

Two parents, GEC and IT98K-476-8, were crossed to produce a RIL (recombinant inbred lines) population. These varieties differ in genetic background and are highly polymorphic for several traits. GEC (Golden Eye Cream) was developed in the USA (Creighton et al., 2006). GEC is a high yielding medium maturity (66 to 72

days) variety (Creighton et al., 2006) whereas IT98K-476-8 is a high yielding medium to late maturing (70 to 80 days) variety (Saidou1et al., 2007). GEC was developed from a cross between TX17032 (advance breeding line from Texas program) and US432 (released as germplasm from US Dept. of Agriculture) in the greenhouse in College Station, Texas, in the fall of 1984 (Creighton et al., 2006). IT98K-476-8 was developed by IITA (International Institute of Tropical Agriculture) in Africa in 1998, corresponding to a genetic gain of 1.96%. It was developed as a dual purpose variety with increased grain and fodder yield (Kamara et al., 2014). GEC is susceptible and IT98K-476-8 is tolerant to low phosphorus. IT98K-476-8 is a cultivated variety in West Africa and used for grains and fodder. GEC produces light brown small eye seeds and IT98K-476-8 produces dark brown Watson eye seeds. They also differ in seed size, plant type, flower color, leaf type, and joint pigmentation at the nodes.

F1 hybrid was developed crossing GEC/IT98K-476-8 in a greenhouse and advanced to the F8 generation to develop a RIL (175 lines) population using single seed descent.

The RIL population (F8 generation) was planted on July 10, 2015, in the greenhouse in completely randomized design in two replications with two plants in each replication for heat tolerance screening. Plants were grown in two-gallon pots (two plants in each pot) using the potting mixture, and optimum growing conditions were maintained in the greenhouse. Sun Grow Metro-Mix 900 Grower Mix with RESiLIENCE was used as a potting mixture. The potting mixture contained 50-60% bark, Canadian sphagnum peat moss, perlite and vermiculture, starter nutrient charge

(with gypsum) and slow release nitrogen, dolomitic limestone, and a long-lasting wetting agent. SWT, PODN, SEEDN, PODWT, HSWT, and days to flowering traits were measured.

The developed RIL population was also planted in a complete randomized block design with two replications at Agronomy farm on July 16, 2014, in College Station, Texas. Each plot consisted of 10 plants and both parents were repeated after every 20 lines as checks. Seeds were planted manually by hand on ridges. Two seeds were planted adjacent to each other and were thinned to one plant after 10 days of planting to achieve 10 uniform plants in each plot. A distance of 75 cm row-to-row and 20 cm plant-to-plant were maintained leaving 75 cm between two ranges. Five plants were uprooted randomly from each plot in each replication to measure SWT, PODN, SEEDN, PODWT, and HSWT, and average of these five plants was used in the analysis. In addition, plant height and days to flowering were documented in the field. Plant height of three plants was measured from each plot and average was used for the analysis. SWT, PODN, and visual scoring (1-5; 1 being highly heat-susceptible, and 5 being highly heat-tolerant) based on pod and flower fertility were used as an estimate of heat tolerance (Lucas et al., 2013; Samba et al., 2004).

The RIL population trial was repeated in 2015 at two locations – Corpus Christi and Weslaco, Texas. The trial in Corpus Christi was planted on June 3, 2015, and in Weslaco on June 24, 2015. The same plot sizes were maintained as in 2014 trial, and the randomized complete block experimental design was followed with two replications. Three plants were selected randomly from each plot and pods were harvested manually

to measure SWT, PODN, SEEDN, PODWT, and HSWT from both locations. Ismail and Hall (1999) reported that heat tolerance in cowpea was associated with higher grain yield and pods per plant, and reduced flower abortion, and plant height (Ismail and Hall, 1999). Based on the studies conducted on cowpea heat tolerance, SWT, PODN, and visual ratings based on flower and pod fertility were used as an estimate of heat tolerance (Lucas et al., 2013; Samba et al., 2004). Visual ratings for heat tolerance were assigned between 1-5 (1 = highly heat-susceptible and 5 = highly heat-tolerant) based on the number of flowers and pod fertility, which is positively correlated to grain yield in cowpea (Samba et al., 2004). In addition, plant height and days to flowering were documented.

Statistical analysis

PROC CORR procedure of SAS was used to measure the correlation between the traits. Data were tested for normality and homogeneity of variances using Shapiro-Wilk test in JMP statistical software, and normality and homogeneity of variances couldn't be achieved even after using different transformation methods. Thus, it was ignored for the analysis.

Statistical analysis was carried out using PROC MIXED (SAS v9.4, SAS Institute Inc., Cary, North Carolina, USA). Randomized complete block design was followed and the data was combined across all three locations because there was no obvious reason to remove a dataset. Dataset was tested for normality and homogeneity of variances using Shapiro-Wilk and Bartlett's test, respectively. Different transformation methods failed to achieve normality, hence, transformation was ignored

for the analysis. Variance components were estimated from the analysis to calculate broad sense heritability (H^2) on an entry mean basis using the formula $H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gxe}^2}{e} + \frac{\sigma_e^2}{re}}$. Confidence intervals were calculated for these heritability estimates using the procedure described by Knapp et al. (1985).

An all random (genotype, location, and replications) model was chosen to analyze the data. The following statistical model was used to analyze the data: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + y(\beta)_{jk} + \varepsilon$, where Y_{ijk} = response of i^{th} genotype in j^{th} environment and in k^{th} replication, μ = overall mean, α = genotypes effect (i = 1,2,…172), β = locations effect (j = 1,2,3), y = replications effect (k = 1,2), and ε = error

Expected mean squares and F test was conducted to test the significant difference in factors, which created the variation in the population (Table 13). Tukey method of mean separation was carried out using SAS 9.2 software. It is a good technique for caring out all pairwise comparisons. It enables us to rank mean separation, and put them into significance group while controlling maximum experiment-wise error rate under. It uses the distribution of studentized range statistics.

Table 13. Sources of variation and expected mean squares

Source	Mean Square	Expected Mean Square	F test
L	M1	$\sigma^2_e + r\sigma^2_{gl} + g\sigma^2_{r(l)} + rg\sigma^2_l$	(M1+M5)/(M2+M4)
Rep (L)	M2	$\sigma^2_e + g\sigma^2_{r(l)}$	
G	M3	$\sigma^2_e + r\sigma^2_{gl} + rl\sigma^2_g$	M3/M4
G x L	M4	$\sigma^2_e + r\sigma^2_{gl}$	M4/M5
Error	M5	σ^2_e	

Rep = replication; G = genotype; L = location

Procedure of DNA Isolation and ddRAD-seq libraries construction

Two RAD-seq methods have been developed are in use. One digests the sample DNA with an 8- or 6-bp restriction enzyme and then physically shears the restricted fragments for smaller fragment size selection and sequencing library construction (Davey et al., 2011). The other named ddRAD-seq (Peterson et al. 2012) or GR-RSC (genome-reduction restriction site conservation, Maughan et al. 2009) double-digests the sample DNA with an 8- or 6-bp enzyme and a 4-bp enzyme for size selection and sequencing library construction. In this study, the ddRAD-seq method was used to genotype the parents and the mapping population. Although both methods are well suited for SNP discovery and genotyping in the species with or without reference sequences, the ddRAD-seq method (Peterson et al. 2012), by comparison, has several advantages over the restriction/physical shearing method (Davey et al., 2011). The ddRAD-seq method is easier, inexpensive, rapid and well-suited for high-throughput

library construction and allows sequencing a large number of samples per sequencer lane with low cost.

Leaf tissues were collected in liquid nitrogen from each of the 175 RILs and both the parents from the greenhouse planting at seeding stage and nuclear DNA (deoxyribonucleic acid) was isolated and ddRAD-seq libraries were constructed with a combination of restriction enzymes, *Bam*HI and *Mlu*CI. The quality and quantity of the libraries were checked using the Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Santa Clara). The libraries were multiplexed and sequenced on HiSeq 2000 (Illumina, Inc., San Diego) with 100SE (100 nucleotide single end) at BGI America (Cambridge, MA). Fig. 10 shows the steps followed to construct the ddRAD-seq libraries.

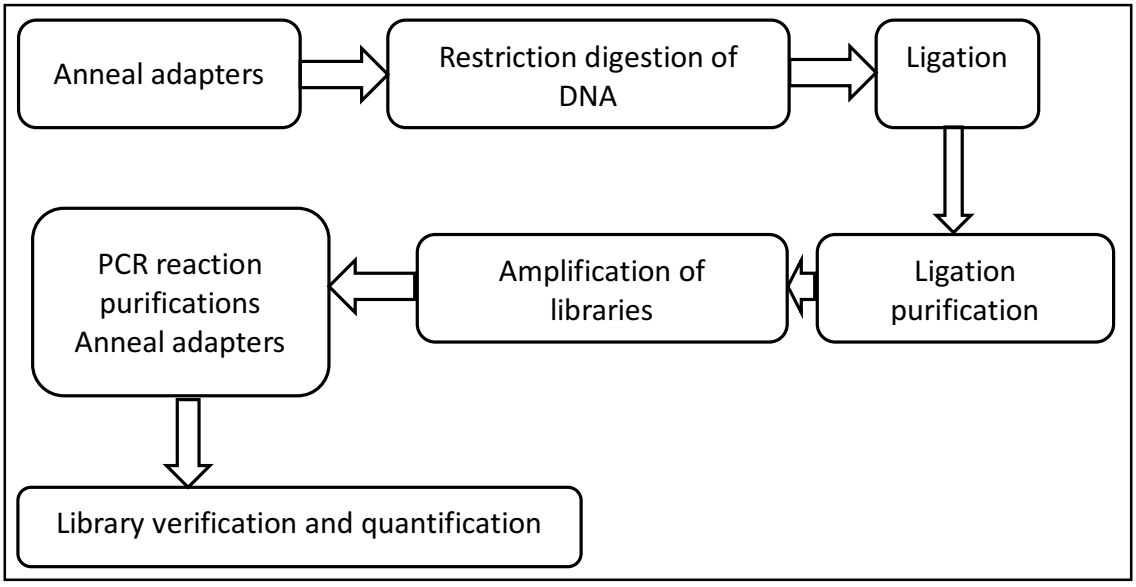

Fig. 10. Steps followed to construct ddRAD-seq libraries of the RIL population for development of SNPs

All the 175 RILs and 2 parents of the population were sequenced in only 3 - 4 lanes of HiSeq 2000. The sequencing clean reads of each sample were extracted using the Illumina pipeline and sorted according to its nucleotide sequence index.

Genotype (SNPs) calling

SNPs were identified and the mapping population was genotyped using the STACKS software (Catchen et al. 2011). STACKS is especially developed to use short-read sequence data, such as RAD-seq data, to identify and genotype loci in a set of individuals either de novo or by comparison to a reference genome using a maximum likelihood statistical model. Then, the SNP genotyping results were subjected to quality filtration by missing data and Chi-square test. Only the SNPs that passed the filtration were used for SNP genetic map construction. Since cowpea has a relatively smaller genome of 620 Mb/haploid and is diploid, the SNP discovery and genotyping was straightforward.

Linkage map construction

IciMapping software was used to construct genetic linkage map and map QTL of measured traits in cowpea heat trial. This software is capable of constructing a linkage map from millions of DNA markers. It first clustered the DNA markers that are co-segregating into "bin" and then used one of the markers from each bin to construct a genetic map with the function of "MAP". The SNPs were then examined one by one and filtered for those significantly distorted in genetic segregation.

A file with extension *.bip* was created, which had general information about the population, including population type, mapping function, marker space type, number of markers, and population size, genotypic data at all markers for all individuals in the population, and anchor group information for all markers. The Kosambi mapping function was used to convert recombination frequencies to cM. The file was uploaded to the IciMapping software, and the Binning function was used to remove redundant markers. Grouping, Ordering, and Rippling steps were used to construct the genetic map. The Grouping function was used to group markers. After grouping of all the markers correctly, the Ordering function was performed to make the genetic linkage map. If there are many markers, there is no guarantee that the final identified orders from the algorithms represent the global optimum solution. Therefore, the Rippling function was used after ordering; each marker sequence needs to be rippled for fine tuning.

QTL mapping

Genotype calls and phenotypic data collected from GEC X IT98K-476-8 population were combined for QTL analysis with the developed genetic map. LOD scores for threshold level were generated using 1000 permutations in IciMapping software. Only QTL exceeding the threefold LOD score are reported.

For each trait, BLUPs (best linear unbiased predictions) of random effects were extracted from mixed linear model using META-R (multi environmental trial analysis R) software based on R scripts in Macintosh operating system. The META-R is developed by CIMMYT (International Maize and Wheat Improvement Center), and is a set of R programs that performs statistical analyses to calculate BLUPs, heritability entry mean

basis, and genetic correlations. BLUPs are used in linear mixed models for estimation of random effects. BLUPs were first derived by Henderson (1975) for animal breeding and were not in use until 1962, but have been proved useful in animal as well as plant breeding. Based on the knowledge from previous studies, BLUPs values were used to detect QTL for the measured traits in our study. QTL for individual locations were also mapped along with BLUPs values.

For individual location, BLUEs (best linear unbiased estimates) were generated, using META-R software. However, the QTL results did not change with BLUEs values. Hence, actual data were used to map QTL in individual location.

A number of statistical methods are available for QTL detection and effect estimation. All the methods for QTL mapping are based on three broad cases – regression, maximum likelihood, and Bayesian model.

Simple interval mapping is based on maximum likelihood parameter estimation and provides a likelihood ratio test for QTL location. Regression interval mapping approximates maximum likelihood interval mapping to save computation time at one/multiple genomic locations. Simple interval mapping is known to bias the estimation of locations and effect of QTL, and composite interval mapping (CIM) combines simple interval mapping with multiple marker regression analysis, which control the effect of QTLs on other linkage groups or chromosomes on the tested QTL, thus, increases the precision. However, CIM is not able to control the effect of epistasis, which is a common phenomenon in QTL mapping of complex traits. In the current study, ICIM (inclusive composite interval mapping) procedure was used to detect the QTL using

IciMaping software (Meng et al., 2005). The principle of ICIM mapping lies under the assumption of additivity of QTL effects on phenotype of the trait, the additive effect of a QTL is absorbed by two flanking marker variables, and epistasis effect of two QTLs is absorbed by four marker-pair multiplication variables between two flanking marker pairs. Marker variables are considered in a linear model for additive mapping, and both marker variables and pairs multiplication was simultaneously considered for epistasis mapping. ICIM has a fast convergence speed and less computing intensive. ICIM is advantages of CIM over interval mapping, and avoid the possible increase of sampling variance and complicated background marker selection process in CIM. ICIM increases detection power, reduce false detection rate, and less biased estimates of QTL effects compared to CIM in additive mapping, and remains an efficient method of epistasis mapping (Wang et al., 2011). ICIM-EPI (inclusive composite interval mapping of digenic epistasis) functionality of IciMapping software was used to test the interaction (epistasis) between QTL (Meng et al., 2005).

Files with *.bip* (analyzes individual environment and additive, dominant, and digenic epistasis) and *.met* (analyzes QTL by environmental interactions) extension were generated to advance QTL mapping process. The *.bip* and *.met* file consisted of general information about the population, including population type, mapping function, marker space type, marker space unit, number of chromosomes, population size, number of phenotypic traits, marker number information, including name of the linkage group or chromosome, number of markers on the group or chromosome, linkage map information including marker name, group or chromosome ID, position or interval of the marker,

genotypic data at all markers for all individuals in the population, and phenotypic data (collected from the field trials) for all traits.

Finally, *.met* and *.bip* files were developed and after uploading the file and setting all the parameters including 1000 permutations for LOD score calculation, when the "Start" item in the menu bar was clicked, the MAP functionality was activated, and the QTL mapping results were shown in the project window (Meng et al., 2015).
All marker trait association were discovered in the bi-parental, F8-RIL population GEC x IT98K-476-8 consisting of 175 individuals. We mapped QTL for HEATR, seed traits, FL and plant height using the SNPs genetic map and phenotypic data collected from the field and greenhouse studies. For heat tolerance and other traits mapping, the phenotypic data were collected from both greenhouse (2015) and field conditions (2004 and 2015) in College Station, Corpus Christi, and Weslaco, Texas.

Results

Field studies 2014 and 2015

Climate

In Corpus Christi, Texas, during the reproductive growth stage of cowpea, the day temperature ranged from 32 to 37 ˚C, and night temperatures ranged from 20 to 26 ˚C (Fig. 11). The day temperature in Weslaco ranged from 28 to 37 ˚C, and night temperatures ranged from 21 to 26 ˚C (Fig. 12). These temperature ranges during the reproductive growth stage were high enough to elicit the phenotypic differences in heat tolerance.

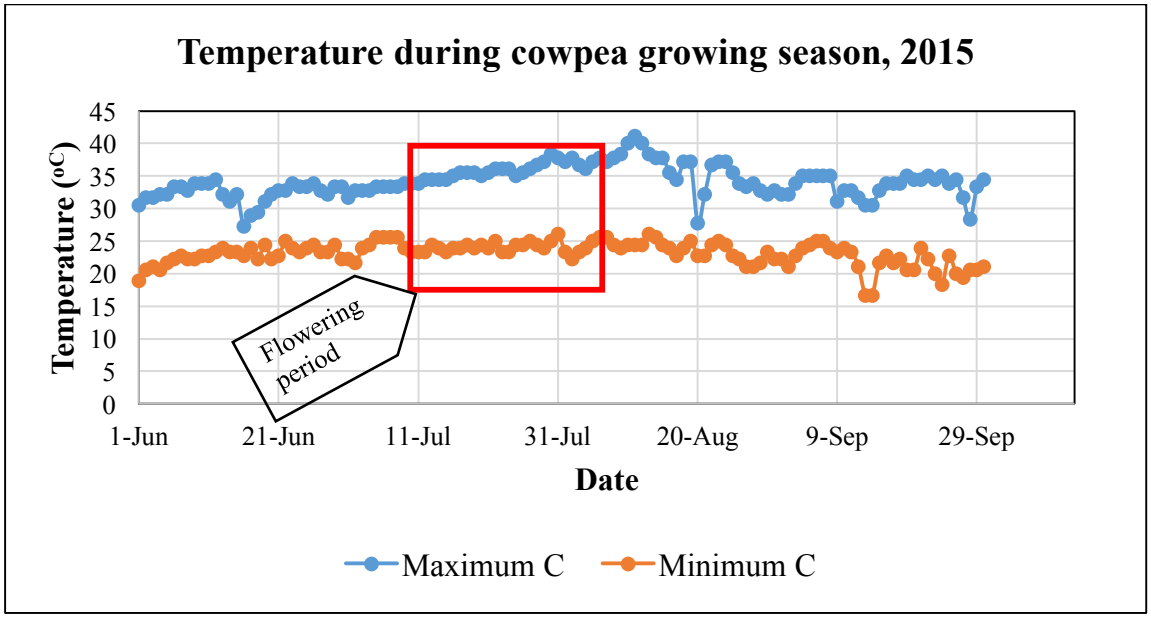

Fig. 11. Temperature during cowpea growing season, Corpus Christi, 2015

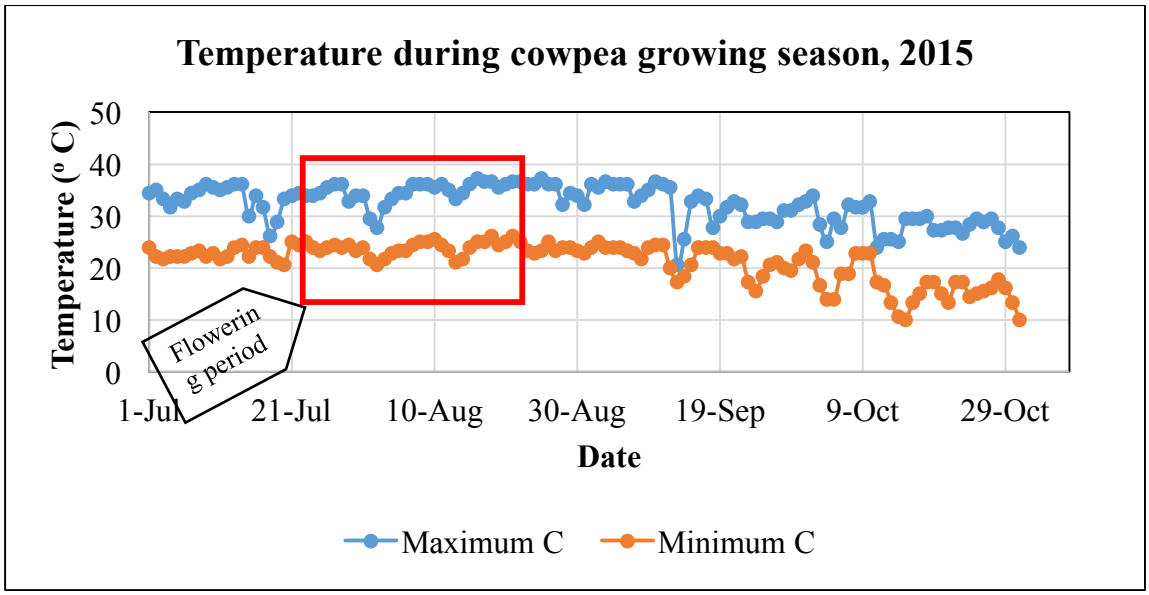

Fig. 12. Temperature during cowpea growing season, Weslaco 2015

Variability for heat tolerance and population means and ranges

We noticed a clear difference between heat-tolerant and heat-susceptible RILs in the College Station, Corpus Christi, and Weslaco field trials. Fig. 13 and 14 show some of the heat-tolerant and heat-susceptible RILs from the field. The heat-tolerant RILs produced more pods and dropped fewer flowers compared to the heat-susceptible RILs.

Fig. 13. Heat tolerant and heat susceptible lines from Weslaco field trial, 2015

Fig. 14. Heat tolerant and heat susceptible RILs from Corpus Christi trial, 2015

The parents were significantly different for all traits in all locations except for SEEDN trait in College Station and Corpus Christi, and HSWT at the Corpus Christi location. That indicated that the parents were polymorphic for most of the traits (Table 14). GEC was very consistent across environments. It produced significantly higher PODWT, SWT, PODN, and HSWT across all three environments. GEC was significantly shorter and flowered earlier compared to IT98K-476-8. This indicated the ability of GEC to produce well under high heat conditions (Ismail and Hall, 1999), and the visual rating supports this statement. The average visual rating of GEC was 3.9 compared to 2 for IT98K-476-8 in the combined analysis. In all the field studies, the ranges of RILs for PODWT, SWT, SEEDN, and PODN started with zero, which

indicated that some of the RILs could not produce any grain because of high temperatures during the growing period. Corpus Christi had the highest temperature among all the studies and that is evident within the results, showing the lowest mean for PODWT, SWT, SEEDN, and PODN among the three field studies. The combined RILs mean of PODWT, SWT, SEEDN, PODN, and HSWT were 15.2 g, 11.2 g, 6.5, 9.1, and 17.5 g, respectively. The combined days to flowering of RILs ranged from 33 to 69 days, which indicated the genetic variability among the RILs for flowering date which confounds the heat tolerance data. Similarly, a significant variability for height (17 to 152 cm) was noticed in the combined analysis (Table 14).

Table 14. . Summary of population means obtained from a population of RILs derived from a cross between GEC X IT98K-476-8. Data from College Station (CS), Corpus Christi (CC), and Weslaco (WE) is included. The mean for the two parents and their progeny are shown, as well as the range for the progeny from lowest to highest value

Trait	Genotype	Combined	CS	W	CC
PODWT	GEC	21.8 A	19.8 A	28.4 A	17.1 A
	IT98K-476-8	36.6 B	10.9 B	14.3 B	11.4 B
	RILs	15.2	15.1	20.5	9.9
	Range of RILs	0.3-37.7	0-52	0-80	0-37
SWT (g)	GEC	17.9 A	16.8 A	23.3 A	13.7 A
	IT98K-476-8	8.10 B	7.20 B	9.60 B	7.50 B
	RILs	11.2	11.4	15	7.2
	Range of RILs	0.2-27.4	0-37	0-57	0-29
SEEDN	GEC	7.7 A	7.1 A	8.40 A	7.7 A
	IT98K-476-8	6.9 A	7.1 A	6.96 B	6.7 A
	RILs	6.5	7.1	6.8	5.5

Table 14 Continued

Trait	Genotype	Combined	CS	W	CC
	Range of RILs	0.4-10.1	0-12	0-12	0-12
PODN	GEC	12.1 A	12.8 A	14.9 A	8.7 A
	IT98K-476-8	7.90 B	10.3 B	7.30 B	6.0 B
	RILs	9.1	11	11	5.4
	Range of RILs	0.3-20.1	0-35	0-40	0-17
HSWT (g)	GEC	24.1 A	23.7 A	24.5 A	24.1 A
	IT98K-476-8	16.6 B	16.7 B	16.8 B	16.4 A
	RILs	17.5	16.9	19.8	15.8
	Range of RILs	2.7-26.6	0-25	0-31	0-30
FL (days)	GEC	38.7 A	35 A	42 A	39 A
	IT98K-476-8	48.3 B	43 B	50 B	52 B
	RILs	46.9	40	50.4	49.8
	Range of RILs	32.7-68.7	23-64	37-72	29-82
HT (cm)	GEC	29 A	31 A	31 A	25 A
	IT98K-476-8	88 B	37 B	175 B	52 B
	RILs	57.6	39.5	83.8	50.2
	Range of RILs	17.4-151.8	15-124	15-245	13-170
HEATR	GEC	3.9 A	NA	4.2	3.6
	IT98K-476-8	2 B	NA	2	2.1
	RILs	2.67	NA	2.79	2.66
	Range of RILs	1-4.5	NA	1-5	1-5

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HT = plant height; HEATR = visual heat rating; CS = College Station, TX; W = Weslaco, TX; CC = Corpus Christi, TX; NA = that particular trait was not measured
Means followed by the same letter within a column are not significantly different by Tukey's test ($p < 0.05$)

Heritability estimates

Entry mean basis broad sense heritability values ranged from 0.33 to 0.86. The values were high for SEEDN (0.73), HSWT (0.73), flowering date (0.80), and visual heat rating (0.86), indicating selection for these traits should be very effective. The heritability for height (0.33) was low because heavy rainfall in Weslaco, Texas, (Table 15) in the later season of growth increased plant height significantly, and that is reflected in high genotype mean square variation of 4502.63 for plant height (Table 18).

Table 15. **Entry-mean heritability (H^2) estimates for all the measured traits. Confidence intervals are provided in parenthesis (0.05 – 0.95). These values were calculated using phenotypic traits taken from a population of RILs derived from a cross between GEC x IT98K-476-8. Heritability estimates were calculated across three environments (CS, CC, and W in Texas)**

Traits	H^2
PODWT	0.58 (0.46 – 0.65)
SWT	0.59 (0.47 – 0.66)
SEEDN	0.72 (0.64 – 0.80)
PODN	0.58 (0.45 – 0.65)
HSWT	0.73 (0.65 – 0.80)
Flowering	0.80 (0.72 – 0.81)
Height	0.33 (0.14 – 0.45)
HEATR	0.86 (0.78 – 0.88)

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HT = plant height; HEATR = visual heat rating; H^2 = broad sense heritability entry mean basis

Table 16. Correlation of the traits in combined analysis of College Station, Corpus Christi, and Weslaco, TX

Traits	PODWT	SWT	SEEDN	PODN	HSWT	FL	HT	HEATR
PODWT	1.0000	0.9939***	0.6714***	0.9325***	0.4584***	-0.2133***	-0.0409	0.3222***
SWT		1.0000	0.6728***	0.9235***	0.4592***	-0.2258***	-0.0440	0.3224***
SEEDN			1.0000	0.6332***	0.7518***	-0.4126***	-0.2316***	0.4526***
PODN				1.0000	0.4314***	-0.3076***	-0.1025**	0.3543***
HSWT					1.0000	-0.3853***	-0.1866***	0.3675***
FL						1.0000	0.3388***	-0.3704***
HT							1.0000	-0.2206***
HEATR								1.0000

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HT = plant height; HEATR = visual heat rating
Color from blue to red indicates positive to negative correlation
** and *** are significant at $p < 0.01$, and < 0.001, respectively.

Correlation analysis

A correlation analysis was performed by combining all field locations. All the seed traits (PODWT, SWT, SEEDN, PODN, and HSWT) showed significant positive correlations with each other at a 0.05 significance level in the combined analysis. FL showed significant negative correlation with all the seed traits. HT was negatively correlated (significant at 0.05 alpha) with SEEDN, PODN, and HSWT. However, HT was not significantly correlated with PODWT and SWT in the combined analysis; however, in the individual analysis, HT showed significant negative correlation with all seed traits at the College Station and Corpus Christi locations (Table 16). A significant

positive correlation between HT and FL indicated taller RILs flowered late and shorter RILs flowered earlier. The analysis also indicated that late flowering and taller genotypes produced lower PODWT, SWT, SEEDN, PODN, and HSWT compared to early flowering and shorter genotypes, and showed susceptibility to heat.

Top performing entries based on HEATR

Table 17 shows the top performing RILs across Corpus Christi and Weslaco based on HEATR. The top three heat-tolerant RILs were 97, 100, and 65, based on HEATR (Table 17).

Table 17. Top ten performing entries (RILs) for visual heat rating trait within each environment from a population derived from a cross between GEC x IT98K-476-8. Two environments are represented, CC = Corpus Christi and W = Weslaco in Texas, as well as the combined mean for both the environments

	Combined		W		CC	
Entry	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**
97	1	4.50 ^{A}	3	4 ^{A}	1	5 ^{A}
100	2	4.50 ^{A}	4	4 ^{A}	2	5 ^{A}
65	3	4.25 ^{A}	5	4 ^{A}	3	4.5 ^{A}
145	4	4.25 ^{A}	1	4.5 ^{A}	13	4 ^{A}
76	5	4.25 ^{A}	2	4.5 ^{A}	14	4 ^{A}
134	6	4.25 ^{A}	6	4 ^{A}	4	4.5 ^{A}
167	7	4.25 ^{A}	7	4 ^{A}	5	4.5 ^{A}
121	8	4.00 ^{A}	8	4 ^{A}	15	4 ^{A}
79	9	4.00 ^{A}	9	4 ^{A}	16	4 ^{A}
123	10	4.00 ^{A}	10	4 ^{A}	17	4 ^{A}

HEATR were given 1 to 5, where 1 indicate highly heat susceptible and 5 indicate highly heat tolerance genotype
CS = College Station, TX; W = Weslaco, TX; CC = Corpus Christi, TX; NA = that particular trait was not measured

Analysis of variance

Effects due to genotype, environment, and genotype x environment were analyzed for all the measured traits by combining phenotypic data across all three field locations. A significant interaction between genotype and environment were observed for all traits. However, we analyzed individual genotype and environmental effects ignoring the significant interaction. Visual heat ratings of the RILs were very consistent and we noticed the lowest genotypic variation (2.39) for the traits. The highest mean square variation of genotype (4502.63) and environment (176013) were noticed for plant height trait, and the reason for the large variation is heavy rain during the late growth stage of RILs in Weslaco, Texas. If we remove the plant height data of Weslaco, Texas, from the analysis, RILs were consistent for plant height for the remaining two locations (College Station and Corpus Christi), and produced lower mean square variation. The environment had a low effect on HEATR (0.01) and SEEDN (53.78) traits. RILs were more consistent for these traits compared to others. Error variance was low for SEEDN (3.72), FL (7.12), and HEATR (0.01). R^2 values were high for all traits (Table 18).

Table 18. Mean squares from the analysis of variance traits taken from a population of RILs derived from a cross between GEC x IT98K-476-8. These values were calculated after combining the phenotypic data across three environments (CS, W, and CC)

Source	d.f.	PODWT		d.f.	SWT		d.f.	SEEDN	
Genotype	170	404.19	***	170	232.32	****	170	8.5	***
Environment	2	9023.79		2	4858.71		2	53.78	
Genotype*Environment	328	181.7	***	328	101.06	***	286	3.73	**
Rep (Environment)	3	2321.99	***	3	1248.68	***	3	42.11	***
MS Error	441	112.69		441	62.9			3.72	
R^2		0.76			0.76			0.78	
Source	**d.f.**	**PODN**		**d.f.**	**HSWT**		**d.f.**	**FL**	
Genotype	170	110.36	***	170	162.97	***	170	296.38	***
Environment	2	3428.22		2	1104.97		2	9453.32	***
Genotype*Environment	333	50.5	***	330	46.29	***	326	66.62	***
Rep (Environment)	3	579.65	***	3	218.26	***	3	30.72	**
MS Error	441	29.43		432	26.35		418	7.12	
R^2		0.77			0.78			0.97	
Source	**d.f.**	**HT**		**d.f.**	**HEATR**				
Genotype	171	4502.63	**	152	2.39	***			
Environment	2	176013	***	1	0.01				
Genotype*Environment	336	3069.57	***	136	0.4	***			
Rep (Environment)	3	1951.51	***	2	0.14				
MS Error	487	85.11		227	0.24				
R2		0.98			0.89				

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HT = plant height; HEATR = visual heat rating; MS = Mean Square
*, **, and *** are significant at $p < 0.05$, < 0.01, and < 0.001, respectively.

Greenhouse studies 2015

Climate

Greenhouse temperatures were raised during the reproductive growth stage of cowpea to mimic the hot field conditions. Daytime temperatures were maintained between 38 to 42 ˚C, and nighttime temperatures were maintained between 24 to 28 ˚C. The temperature ranges were high enough to differentiate between heat-tolerant and heat-susceptible lines and the parents.

Population means and ranges

The susceptible parent (IT98K-476-8) dropped all of the produced flowers and produced no grains; whereas, the tolerant parent (GEC) produced optimum grain yield (6.56 g) in the greenhouse. The IT98K-476-8 parent flowered 43.4 days (average) after planting and the GEC parent, which is an early to medium maturing variety, flowered 38 days (average) after planting. Visual heat ratings, the same as field, were assigned to all RILs and parents, and they aligned well with all seed traits. RILs show transgressive segregation for all traits (Table 19). The results of the study indicate that both the parents were significantly different at 0.05 significance level for all the measured traits in the greenhouse.

Table 19. Trait mean of both parents, trait mean of RILs, and trait ranges of RILs in Greenhouse 2015

Traits→ Genotype ↓	PODWT (g)	SWT (g)	SEEDN	PODN	HSWT (g)	FL (days)	HEATR
GEC	8.34 A	6.56 A	6.7 A	6.6 A	25 A	38.0 A	3.1 A
IT98K-476-8	0 B	0 B	0 B	0 B	0 B	43.4 B	1 B
RILS	3.7	2.89	4.09	2.89	13.54	40.13	2.56
Range of RILs	0-17.85	0-14.20	0-11.4	0-12.5	0-28.18	26-66	1-5

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; HEATR = visual heat rating
Different alphabet followed by numbers indicate a significant difference at 0.05 alpha level

Fig. 15 shows a clear phenotyptically different response between parents for heat stress. The left side parent is GEC, which produced significantly greater pods and dropped a fewer number of flowers compared to the right side, heat-susceptible parent (IT98K-476-8), which produced no pods. The left-most picture is one of the heat-tolerant RILs, and the right-most picture is one of the very high heat-susceptible lines. A clear difference in pod production and flower drop between them could be noticed visually (Fig. 15).

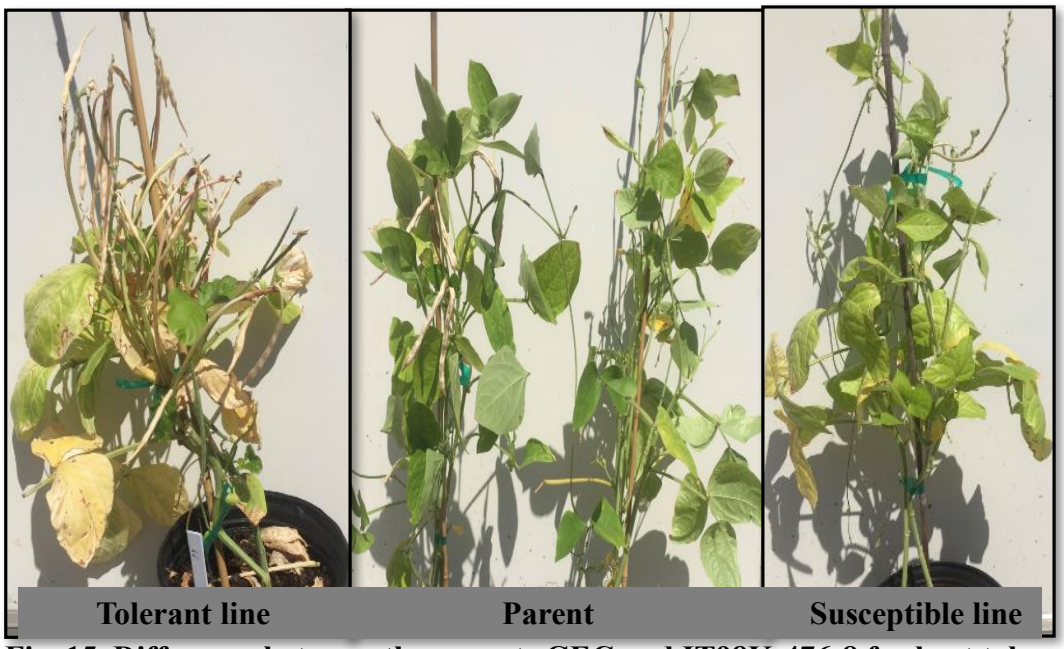

Fig. 15. Difference between the parents GEC and IT98K-476-8 for heat tolerance, and high heat tolerant and susceptible RIL

Heritability estimates

Broad sense heritability estimates of FL (0.99) and HEATR (0.91) were very high compared to other measured traits. Heritability estimates of all the traits ranged from 0.43 to 0.99 in the 2015 greenhouse study (Table 20).

Table 20. Entry-mean heritability (H2) estimates for all the measured traits. These values were calculated using phenotypic traits taken from a population of RILs derived from a cross between GEC x IT98K-476-8. Heritability estimates are from RILs trial in greenhouse 2015

Traits	H^2
PODW	0.46
SWT	0.46
SEEDN	0.54
PODN	0.47
HSWT	0.43
FL	0.99
HEATR	0.91

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flowering; HEATR = visual heat rating; H^2 = heritability entry mean basis

Correlation analysis

A highly significant correlation was observed between all traits at significant level 0.001. The FL trait negatively correlated with HEATR (-0.526) and all other seed traits, which explains that the late maturing RILs produced lower PODWT, SWT, SEEDN, and HSWT, and showed higher heat-susceptible compared to the early maturing RILs in the greenhouse. All the seed traits showed high significant positive correlation between them (Table 21). The results are similar to the field combined analysis.

Table 21. Correlation analysis of all the traits, greenhouse 2015

	PODWT	SWT	SEEDN	PODN	HSWT	FL	HEATR
PODW	1	0.9965***	0.821***	0.9362***	0.6421***	-0.4822***	0.7081***
SWT		1	0.8122***	0.922***	0.6337***	-0.4765***	0.7042***
SEED			1	0.7523***	0.7655***	-0.4188***	0.5915***
PODN				1	0.607***	-0.5404***	0.7416***
HSWT					1	-0.2948***	0.4368***
FL						1	-0.526***
HEAT							1

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; HEATR = visual heat rating
*** is significant at < 0.001

Top performing RILs

We have considered SWT, PODN, and HEATR as an estimate of heat tolerance. The top 20 lines were ranked based on SWT, and the ranks were compared to PODN and HEATR. Top performing lines based on SWT also produced higher PODN and were assigned higher HEATR. Tope five performing RILs were 165, 71, 36, 105, and 74 (Table 22).

Table 22. Top ten performing entries (RILs) for SWT, PODN, and HEATR trait in greenhouse, 2015, from a population derived from a cross between GEC x IT98K-476-8

	SWT		PODN		HEATR	
Entry	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**
165	1	12.00 ^{A}	10	7 ^{A}	5	3.5 ^{A}
71	2	11.80 ^{A}	13	6 ^{A}	1	5 ^{A}
36	3	11.75 ^{A}	12	7 ^{A}	1	5 ^{A}
105	4	11.80 ^{A}	2	9 ^{A}	2	4.5 ^{A}
74	5	11.35 ^{A}	17	6 ^{A}	3	4 ^{A}
50	6	10.43 ^{A}	41	5 ^{A}	3	4 ^{A}
124	7	11.25 ^{A}	3	8 ^{A}	3	4 ^{A}
167	8	9.38 ^{A}	7	7 ^{A}	2	4.5 ^{A}
4	9	9.37 ^{A}	15	6 ^{A}	5	3.5 ^{A}
29	10	9.85 ^{A}	18	6 ^{A}	4	3.75 ^{A}
35	11	9.00 ^{A}	14	6 ^{A}	4	3.75 ^{A}
164	12	9.20 ^{A}	4	8 ^{A}	4	3.75 ^{A}
40	13	8.30 ^{A}	32	5 ^{A}	7	3 ^{A}
100	14	9.04 ^{A}	16	6 ^{A}	2	4.5 ^{A}
103	15	9.03 ^{A}	22	6 ^{A}	1	5 ^{A}
70	16	8.80 ^{A}	11	7 ^{A}	5	3.5 ^{A}
156	17	8.73 ^{A}	5	8 ^{A}	3	4 ^{A}
84	18	8.05 ^{A}	19	6 ^{A}	3	4 ^{A}
18	19	8.60 ^{A}	24	5 ^{A}	9	2.5 ^{A}
27	20	8.00 ^{A}	33	5 ^{A}	6	3.25 ^{A}

SWT = seed weight/plant; PODN = number of pods/plant; HEATR = visual heat rating
Means followed by the same letter within a column are not significantly different by Tukey's test ($p < 0.05$)

Analysis of variance

RILs were significantly different for all traits, and very high R^2 was observed for FL and HEATR. Error means square values ranged from 2.02 to 50.57 for all the traits, that indicated very low error variance in the experiment compared to genotype effect variance (Table 23). Highest mean square value was noticed for FL (198.60) and lowest was noticed for HEATR (2.89). That indicated that there was high variation for FL, but that didn't affect response of RILs to high temperatures.

Table 23. Mean squares from the analysis of variance traits taken from a population of RILs derived from a cross between GEC x IT98K-476-8, greenhuouse 2015

Source	**d.f.**	**PODWT**		**d.f.**	**SWT**		**d.f.**	**SEEDN**	
Genotype	160	17.72	**	160	11.14	**	160	10.46	***
Error		10.81			6.73			5.62	
R^2		0.67			0.68			0.70	
Source	**d.f.**	**PODN**		**d.f.**	**HSWT**		**d.f.**	**FL**	
Genotype	159	9.09	**	158	84.81	**	155	198.60	***
Error		5.49			50.57			2.02	
R^2		0.67			0.68			0.99	
Source	**d.f.**	**HEATR**							
Genotype	164	2.89	***						
Error		0.29							
R^2		0.92							

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HEATR = visual heat rating

** and *** are significant at $p < 0.01$ and < 0.001, respectively

Linkage mapping and QTL analysis

Linkage map construction

SNPs were developed for all the 175 RILs and both the parents. A range of 380,399 - 2,267,979 100-nucleotide clean reads were obtained for each line, with an average of 1,449,966 100-nucleotide clean reads per line. Given that cowpea has a genome size of 620 Mb/1C and an estimation of one *Bam*HI site in approximately 10 kb, there are approximately 62,000 *Bam*HI sites in the cowpea genome. Because we sequenced both flanking sides of all *Bam*HI sites, the coverage of the sequences for each *Bam*HI site ranged from 6.1x to 36.6x genome coverage for each line, with an average coverage of 11.7x for each site in the genome of each line. Therefore, the RAD sequences of the population should be sufficient for the construction of a SNP genetic map for cowpea and for mapping of the genes controlling drought and heat tolerances in the species. In 2015 we analyzed the RAD sequences using our RAD-seq sequence analysis pipeline and identified 6,001 SNPs for the RIL population. Then we examined the SNPs one-by-one and filtered those significantly distorted in genetic segregation. Consequently, we obtained a total of 4,154 high-quality SNPs. We constructed a SNP genetic map for cowpea from the 4,154 SNPs using QTL IciMapping software (Meng et al., 2015). The software first grouped the SNPs that are genetically co-segregating into bins and then constructed them into the genetic map. This resulted in a total of 531 bins, with each bin consisting of an average of 7.8 SNPs (Table 24). We constructed the SNP genetic map using four different sets of parameters. The results showed that the SNP genetic maps constructed using all four sets of parameters were almost identical.

Therefore, we selected the genetic map constructed with the fourth set of parameters, named v4.0, for trait QTL mapping.

The map v4.0 consists of 11 linkage groups (LGs; Fig. 16) and 531 bins containing 4,154 SNPs. The map collectively spans 1,084.65 cM having a density of one SNP marker in approximately 0.26 cM or 149 kb. The largest linkage group was 2 (164.9 cM) and the shortest was 1 (63 cM). Highest marker density was detected on linkage group 8 (0.37) and lowest was on linkage group 1 (0.19). The largest interval of 12.97 cM was detected on linkage group 4. The total number of markers on linkage groups were ranged from 299 on linkage group 6 and 608 on linkage group 4 (Table 24 and Fig. 16).

Because the SNPs in every bin are genetically co-segregating, any of them could be used for marker-assisted selection of the trait QTL for enhanced breeding. The multiple SNPs flanking each QTL significantly broaden the utility of the markers in different breeding programs, because as long as one of them is polymorphic in a breeding population, it will be applicable to the breeding population for marker-assisted selection. Therefore, the SNP bins are much more applicable for different breeding programs than a single marker mapped for a QTL. Because the SNPs in every bin are genetically co-segregating, any of them could be used for marker-assisted selection of the trait for enhanced breeding. The multiple SNPs flanking each QTL significantly broaden the utility of the markers in different breeding programs because as long as one of them is polymorphic in a breeding population, it will be applicable to the breeding population for marker-assisted selection.

Table 24. Summary statistics cowpea genetic linkage map constructed using the RIL population derived from a cross between GEC and IT98K-476-8

Linkage group	**1**	**2**	**3**	**4**	**5**	**6**
Length (cM)	63.01	164.96	113.21	142.06	84.09	97.4
Number of Markers	328	523	335	608	322	299
Marker density (cM)	0.19	0.32	0.34	0.23	0.26	0.33
Largest interval	10.13	10.99	19.71	12.97	10.14	12.07
Linkage group	**7**	**8**	**9**	**10**	**11**	**Total**
Length (cM)	63.21	111.92	82.02	94.82	67.95	1084.65
Number of Markers	322	304	395	390	328	4154
Marker density (cM)	0.20	0.37	0.21	0.24	0.21	0.26
Largest interval	6.43	10.36	6.29	8.55	7.2	12.97

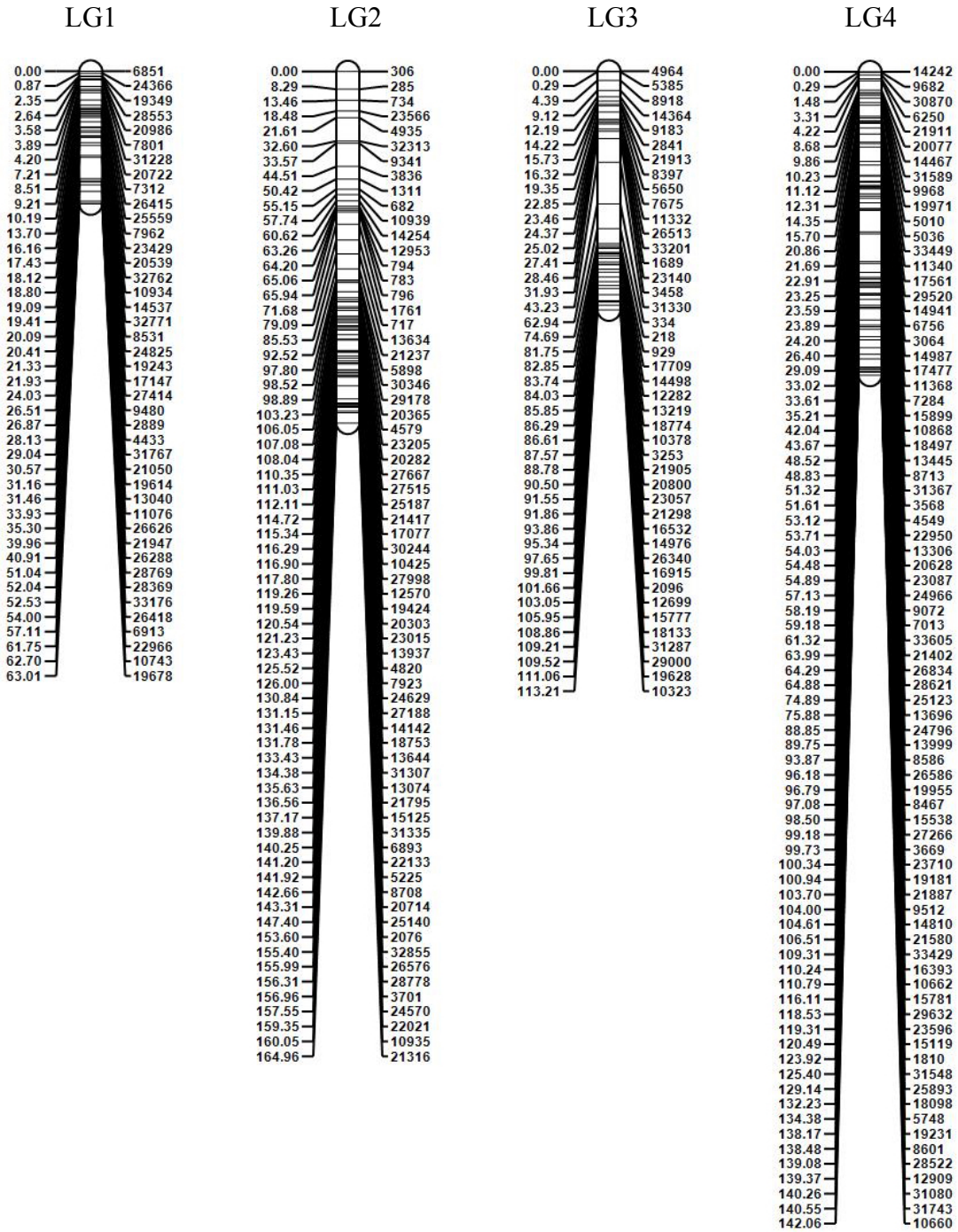

Fig. 16. A total of 11 linkage groups constructed using a mapping population derived from GEC and IT98K-476-8

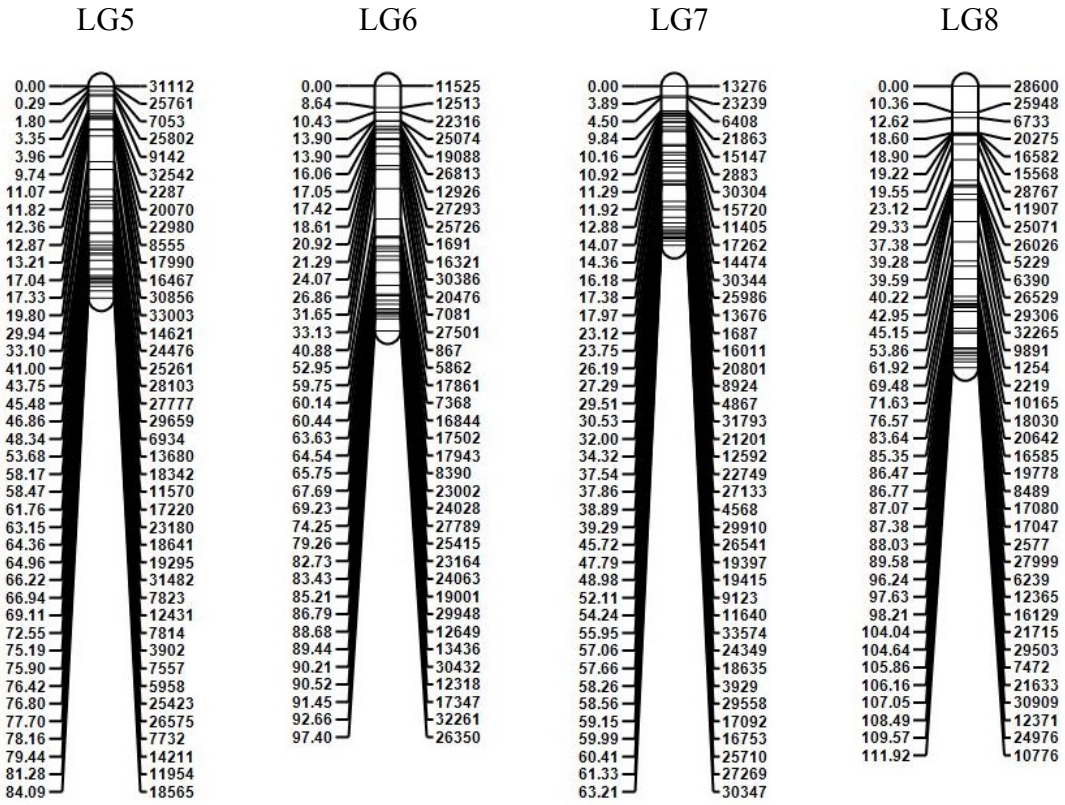

Fig. 16 Continued

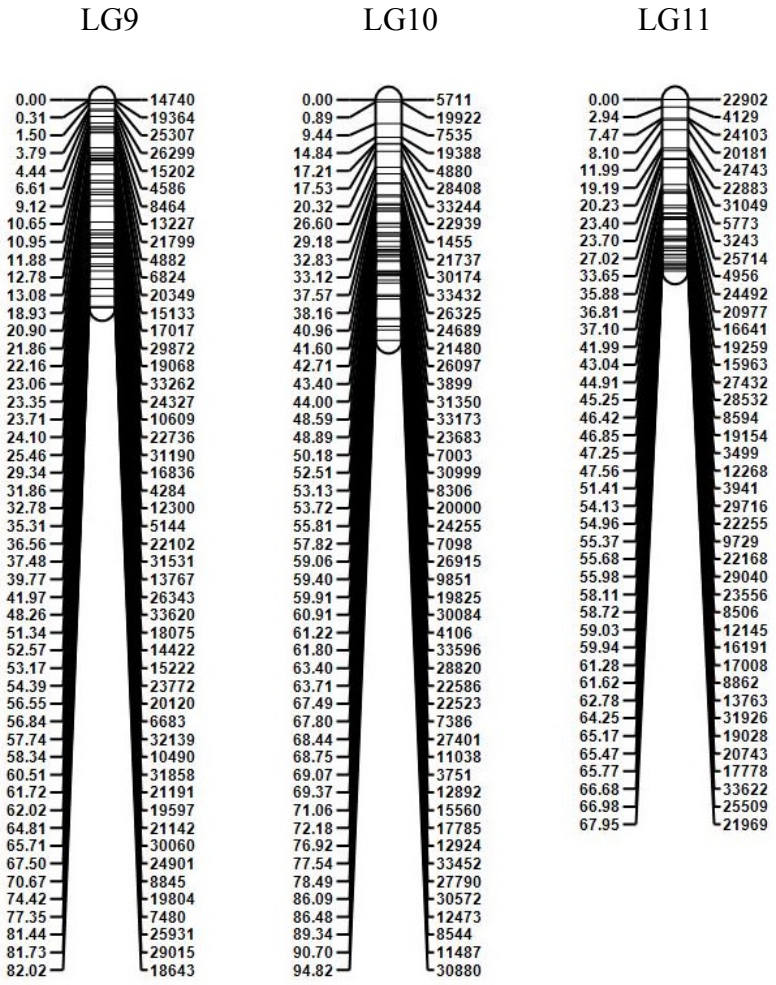

Fig. 16 Continued

QTL analysis

For all the seven traits, a total of 47 QTL were detected in the field and the greenhouse location. Results showed maximum QTL for all traits on linkage groups 3 and 10 (Table 25).

Heat rating QTL analysis

QTL were mapped for visual heat tolerance score (HEATR). A single QTL at 28 cM position on linkage group 3 was detected using Corpus Christi data, and we were not able to detect any QTL at Weslaco. We have detected another QTL for heat tolerance using greenhouse data at 72 cM position on linkage group 10. We could not detect a consistent QTL for heat-tolerant visual score; however, when we combined visual score data from Corpus Christi, Weslaco, and the greenhouse, we detected the same QTL at linkage group 3 at position 28 cM and also a QTL on linkage group 10 at position 70 cM (Table 25).

Pod number, seed weight, pod weight per plant, number of seeds per pod, and 100 seed weight QTL analysis

In the combined analysis of all field locations, three QTL for PODN at positions 83, 54, and 94 cM on linkage groups 3, 10, and 10 were located, respectively. In Corpus Christi we found two QTL for PODN on linkage groups 3 and 10 and both were detected at position 87 cM. In Weslaco and College Station, we again found QTL for PODN at positions 16 and 23 cM on linkage group 3. Results indicate that QTL on linkage group 3 seems more consistent compared to other QTL (Table 25).

In the combined analysis (BLUPs), two QTL for SWT were detected at positions 84 and 93 cM on linkage groups 3 and 10, respectively. In individual analysis, Corpus Christi showed QTL on linkage groups 3 (position 86 cM) and 10 (position 87 cM), Weslaco again showed QTL on linkage groups 3 (position 83 cM) and 10 (position 94

cM), and College Station showed QTL on linkage groups 3 (position 27 cM), 9 (position 22 cM), and 10 (position 71 cM). Results showed that QTL on linkage group 3 seems consistent for SWT across Corpus Christi and Weslaco. QTL for PODWT were mapped very close to the QTL for SWT (Table 25), and that was expected because of a very high correlation between SWT and PODWT. In the combined analysis (BLUPs) of SEEDN data, a total of five QTL were detected on linkage groups 3, 6, and 10. After careful analysis of individual locations for SEEDN, QTL on linkage group 10 was consistently detected at about 81 cM position (Table 25).

For HSWT, two QTL were detected on linkage groups 3 and 10 at 28 and 71 cM position, respectively, in combined analysis using BLUPs. A QTL was found on linkage group 3 (position 28) at Corpus Christi and we again located a QTL on linkage group 3 but at 85 cM position in Weslaco. We were not able to find similar QTL in College Station, but results indicate that QTL on linkage group 3 was consistent (Table 25).

Days to flowering QTL analysis

We have mapped only one QTL at position 72 cM in combined analysis (BLUPs) on linkage group 10, and that QTL was consistently detected in Corpus Christi, College Station, and greenhouse studies at the same position on the same linkage group. We have also detected a QTL for flowering at the Weslaco location on the same linkage group but it was at 70 cM position. In addition to this consistent QTL, we also found QTL for flowering on linkage groups 1 and 5 in the greenhouse, linkage group 1 in College

Station, and linkage group 3 in Weslaco (Table 25). QTL analysis of FL showed a consistent QTL on linkage group 10 at about 72 cM.

Plant height QTL analysis

We have detected two consistent QTL for plant height on linkage groups 3 and 10. In the combined analysis (BLUPs) of field data, two QTL were detected at 27 and 70 cM on linkage groups 3 and 10, respectively. In Corpus Christi we have detected plant height QTL at 28 and 70 cM positions on linkage groups 3 and 10, respectively. We again located two QTL on linkage groups 3 and 10 at positions 23 and 70 cM, respectively, in College Station. Our results indicated that these two QTL on linkage groups 3 and 10 were consistent (Table 25) across both the Corpus Christi and College Station locations.

Table 25. QTL of seed traits, heat tolerance, height, and flowering, position, percent heritable phenotypic variation explained, logarithm of odds, additive effect, and confidence intervals in College Station (2014), Corpus Christi (2015), Weslaco (2015), greenhouse and average across all the fields

Environment	Trait	Linkage group	Position	Left Marker	Right Marker	LOD	PVE(%)	Add	Left CI	Right CI
Combined	PODW	3	83	17709	14498	9.70	19.67	-2.26	81.5	83.5
Combined	PODW	10	93	11487	30880	5.95	12.26	1.78	90.5	94.0
Combined	SWT	3	84	14498	12282	7.86	17.05	-1.59	83.5	85.5
Combined	SWT	10	93	11487	30880	5.07	11.37	1.29	90.5	94.0
Combined	SEEDN	3	28	1689	23140	7.30	7.33	-0.53	25.5	28.5
Combined	SEEDN	3	87	10378	3253	17.43	20.17	-0.88	86.5	87.5
Combined	SEEDN	3	94	16532	14976	7.80	7.79	0.55	92.5	95.5
Combined	SEEDN	6	59	5862	17861	4.63	4.59	0.42	54.5	60.5
Combined	SEEDN	10	84	27790	30572	8.92	10.09	0.62	81.5	86.5
Combined	PODN	3	83	17709	14498	11.43	22.93	-1.28	81.5	83.5
Combined	PODN	10	53	30999	8306	3.34	5.93	0.65	52.5	53.5
Combined	PODN	10	94	11487	30880	4.04	7.62	0.73	90.5	94.0
Combined	HSWT	3	28	1689	23140	12.81	25.27	-2.10	26.5	28.5
Combined	HSWT	10	71	12892	15560	3.63	6.14	1.03	69.5	71.5
Combined	FL	10	72	15560	17785	12.60	29.75	-3.31	71.5	74.5
Combined	Height	3	27	33201	1689	3.21	7.02	2.35	25.5	28.5
Combined	Height	10	70	12892	15560	8.13	19.25	-3.87	69.5	71.5
College Station	HSWT	5	13	8555	17990	3.79	9.97	-1.45	12.5	13.5
College Station	HSWT	8	19	16582	15568	2.99	7.78	-1.19	18.5	19.5
College Station	FL	1	36	26626	21947	2.62	6.49	2.11	33.5	39.5
College Station	FL	10	72	15560	17785	7.79	19.33	-3.64	71.5	75.5
College Station	Height	3	23	7675	11332	4.13	7.40	5.48	19.5	23.5

Table 25. Continued

Environment	Trait	Linkage group	Position	Left Marker	Right Marker	LOD	PVE(%)	Add	Left CI	Right CI
College Station	Height	10	70	12892	15560	12.80	27.08	-10.54	69.5	71.5
College Station	PODN	1	20	32771	8531	2.96	5.72	1.42	19.5	20.5
College Station	PODN	3	23	7675	11332	6.36	13.04	-2.14	20.5	23.5
College Station	PODN	6	7	11525	12513	4.16	9.64	-1.84	3.5	10.5
College Station	PODN	9	11	21799	4882	3.06	5.90	1.44	10.5	12.5
College Station	PODW	3	27	33201	1689	5.40	10.47	-3.47	25.5	28.5
College Station	PODW	9	22	29872	19068	3.33	6.35	2.68	20.5	22.5
College Station	SEEDN	10	81	27790	30572	3.98	11.71	0.71	77.5	86.5
College Station	SWT	3	27	33201	1689	4.11	9.91	-2.37	25.5	28.5
College Station	SWT	9	22	29872	19068	3.04	7.39	2.03	20.5	22.5
College Station	SWT	10	71	12892	15560	2.51	5.87	1.80	69.5	72.5
Corpus Christi	HSWT	3	28	1689	23140	13.00	28.44	-4.71	27.5	29.5
Corpus Christi	FL	10	72	15560	17785	8.69	22.35	-5.05	71.5	74.5
Corpus Christi	HEATR	3	28	1689	23140	2.78	7.66	-0.30	27.5	30.5
Corpus Christi	Height	3	28	1689	23140	4.34	8.39	11.17	25.5	28.5
Corpus Christi	Height	10	70	12892	15560	13.36	29.46	-20.85	69.5	71.5
Corpus Christi	PODN	3	87	10378	3253	8.70	18.57	-1.87	86.5	87.5
Corpus Christi	PODN	10	87	12473	8544	3.91	7.99	1.22	86.5	89.5
Corpus Christi	PODW	3	86	13219	18774	9.70	20.63	-3.92	85.5	86.5
Corpus Christi	SEEDN	3	28	1689	23140	11.70	24.79	-1.71	27.5	29.5
Corpus Christi	SEEDN	10	87	12473	8544	3.65	7.01	0.90	86.5	88.5
Corpus Christi	SWT	3	86	13219	18774	8.93	19.30	-2.78	85.5	86.5
Corpus Christi	SWT	10	87	12473	8544	2.72	5.52	1.48	86.5	89.5
Weslaco	HSWT	3	85	12282	13219	4.82	12.63	-2.33	83.5	85.5

Table 25. Continued

Environment	Trait	Linkage group	Position	Left Marker	Right Marker	LOD	PVE(%)	Add	Left CI	Right CI
Weslaco	HSWT	5	75	7814	3902	3.44	8.62	1.92	72.5	75.5
Weslaco	FL	3	28	1689	23140	3.10	6.69	2.22	27.5	30.5
Weslaco	FL	10	70	12892	15560	9.42	22.33	-4.04	69.5	71.5
Weslaco	PODN	3	16	21913	8397	4.14	9.99	-2.59	15.5	19.5
Weslaco	PODN	10	54	20000	24255	3.50	8.50	2.39	53.5	55.5
Weslaco	PODW	3	83	17709	14498	6.09	12.67	-6.25	81.5	83.5
Weslaco	PODW	10	53	30999	8306	3.96	8.00	4.94	52.5	53.5
Weslaco	SEEDN	3	83	17709	14498	11.24	19.88	-1.26	81.5	83.5
Weslaco	SEEDN	5	20	33003	14621	4.29	6.93	-0.77	17.5	25.5
Weslaco	SEEDN	5	81	14211	11954	2.92	4.62	0.62	79.5	84.0
Weslaco	SEEDN	9	21	17017	29872	3.06	4.78	0.62	18.5	21.5
Weslaco	SEEDN	10	84	27790	30572	4.72	8.52	0.82	80.5	86.5
Weslaco	SWT	3	83	17709	14498	5.00	11.54	-4.21	81.5	83.5
Weslaco	SWT	10	94	11487	30880	3.64	8.56	3.62	90.5	94.0
Greenhouse	PODN	10	73	17785	12924	3.64	10.50	0.75	71.5	76.5
Greenhouse	FL	1	22	17147	27414	3.97	7.19	2.85	21.5	24.5
Greenhouse	FL	5	20	33003	14621	3.11	5.63	-2.60	17.5	27.5
Greenhouse	FL	10	72	15560	17785	11.24	22.90	-5.07	71.5	75.5
Greenhouse	HEATR	10	72	15560	17785	3.86	10.64	0.41	69.5	72.5

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HEATR = visual heat rating
BLUPs = Best linear unbiased prediction calculated using all the field locations

Discussion

The climate is changing and the weather is becoming unpredictable. In this scenario, there is a need to develop more crop varieties which can tolerate harsh weather conditions. According to Lane and Jarvis (2007), the amount of productive land is going to decrease by 2.6% in Sub-Saharan Africa, which is the major cowpea growing region of the world. Heat-tolerant varieties of cowpea need to be developed to sustain rising temperatures around the globe.

The availability of advanced molecular techniques could be vital in identifying DNA regions responsible for heat tolerance as well as developing improved heat-tolerant cowpea varieties. DNA regions host genes which are known to play an important role in stress resistance, and researching these regions will provide a framework for cloning and characterizing the action of underlying genes.

Marfo and Hall (1992) conducted a study on the inheritance of heat tolerance and reported two dominant genes controlling heritable heat tolerance in cowpea. They developed F1, F2, and backcross population from two parents (Prima and TVu 4552), and studied the population for heat tolerance. The study developed F1 from sensitive x sensitive, tolerant x sensitive, and tolerant x tolerant parents, and reported that F1 from sensitive x sensitive were more tolerant than either of the sensitive parents, F1 from tolerant x sensitive were less tolerant than the tolerant parent, and F1 from two tolerant parents were more tolerant than either of the tolerant parents. Results from some F2 populations did not match the ratios of one or two tolerant genes. In another study on cowpea heat tolerance, Lucas et al. (2013) studied RIL population developed from a

heat-tolerant and a heat-sensitive parent. They reported five QTL on linkage groups 2, 7, 6, 10, and 3. In the current study, a QTL (LOD 3.8) for heat tolerance on linkage group 3 was detected based on the visual score in Corpus Christi, Texas. We also detected a QTL in the greenhouse study; however, it was detected on linkage group 10 (LOD 3.8). We have observed in previous studies that PODN and SWT were also considered an estimate of heat tolerance. In the combined analysis (BLUPs) of the field studies, we have detected three QTL for PODN on linkage groups 3 and 10. QTL on linkage group 3 was more consistent compared to others across the locations. Similarly, a consistent QTL for SWT on linkage group 3 was detected at Corpus Christi and Weslaco locations. These results suggest the presence of major QTL on linkage group 3.

For the days to flowering trait, we have detected a consistent QTL across all locations including the greenhouse on linkage group 10 at about 70 cM position with LOD score ranging from 7.79 to 11.24. For plant height, we detected two major QTL on linkage groups 3 and 10 and they were consistent at the College Station and Corpus Christi locations. However, the QTL on linkage group 10 was detected with a higher LOD score (LOD score at College Station – 12.80, Corpus Christi – 13.36) compared to the QTL on linkage group 3 (LOD score at College Station – 4.13, Corpus Christi – 4.34). The QTL for plant height and days to flowering on linkage group 10 was overlapped. That indicated a similar genetic region controlling both traits, and that is also confirmed by phenotypic data, taller plants flowered late and a significant negative correlation existed between plant height and days to flowering.

QTL interact with each other positively or negatively to exhibit phenotype. Results showed that the genetic regions on linkage group 3 and linkage group 10 interacted the most with each other. A total of 25 QTL interacted with each other with an LOD score ranging from 5 to 25, and percent variation explained 4 to 57%. QTL for days to flowering trait showed highest LOD score, and interacted QTL were detected on linkage groups 3 and 10 at positions 80 and 70 cM, respectively (Table 26).

Table 26. QTL interaction of seed traits, heat tolerance, height, and flowering, position, percent heritable phenotypic variation explained, logarithm of odds, and confidence intervals in College Station (2014), Corpus Christi (2015), Weslaco (2015), greenhouse, and average across all the field locations

Env	Trait Name	Linkage group 1	Pos 1	Left Marker1	Right Marker1	Linkage group 2	Pos 2	Left Marker2	Right Marker2	LOD	PVE (%)
Combined	PODW	3	20	5650	7675	10	70	12892	15560	6.60	39.67
Combined	SWT	3	20	5650	7675	10	70	12892	15560	7.08	39.18
Combined	SEEDN	3	25	26513	33201	10	70	12892	15560	13.63	49.94
Combined	PODN	3	25	26513	33201	10	70	12892	15560	5.80	35.35
Combined	HSWT	3	25	26513	33201	10	70	12892	15560	18.04	50.20
Combined	FL	3	80	218	929	10	75	17785	12924	22.54	50.01
Combined	Height	3	40	3458	31330	10	80	27790	30572	6.54	27.56
Corpus Christi	PODW	3	25	26513	33201	10	70	12892	15560	6.99	30.92
Corpus Christi	SWT	3	25	26513	33201	10	70	12892	15560	7.72	30.40
Corpus Christi	SEEDN	3	25	26513	33201	10	70	12892	15560	11.97	45.11
Corpus Christi	PODN	3	25	26513	33201	10	70	12892	15560	6.53	29.65
Corpus Christi	HSWT	3	30	23140	3458	10	65	22586	22523	14.85	56.96
Corpus Christi	FL	3	25	26513	33201	10	70	12892	15560	9.15	38.66
Corpus Christi	Height	3	80	218	929	10	70	12892	15560	23.14	57.30
Weslaco	SWT	2	30	4935	32313	2	35	9341	3836	5.01	4.03
Weslaco	HSWT	3	30	23140	3458	3	40	3458	31330	5.04	16.44
Weslaco	HSWT	8	5	28600	25948	8	15	6733	20275	5.24	15.19
Weslaco	HSWT	3	80	218	929	10	70	12892	15560	9.04	11.48
Weslaco	FL	3	80	218	929	10	70	12892	15560	25.11	55.30
Weslaco	Height	2	30	4935	32313	2	40	9341	3836	5.61	5.15
College Station	PODW	3	30	23140	3458	10	75	17785	12924	5.42	30.33
College Station	SWT	3	20	5650	7675	10	70	12892	15560	7.01	28.50

Table26. continued

Env	Trait Name	Linkage group 1	Pos 1	Left Marker1	Right Marker1	Linkage group 2	Pos 2	Left Marker2	Right Marker2	LOD	PVE (%)
College Station	SEEDN	3	80	218	929	10	75	17785	12924	7.24	30.35
College Station	HSWT	2	70	796	1761	2	75	1761	717	6.11	18.21
College Station	HSWT	3	80	218	929	10	75	17785	12924	11.85	14.14
College Station	FL	6	60	17861	7368	6	70	24028	27789	5.33	20.77
College Station	FL	3	25	26513	33201	10	65	22586	22523	5.14	15.01
College Station	Height	1	45	26288	28769	1	50	26288	28769	7.01	7.02
College Station	Height	2	120	19424	20303	2	125	13937	4820	7.50	7.68
College Station	Height	4	85	13696	24796	4	90	13999	8586	5.98	8.48
College Station	Height	5	55	13680	18342	5	60	11570	17220	5.70	7.02
College Station	Height	3	80	218	929	10	70	12892	15560	17.80	23.22

PODWT = pod weight/plant; SWT = seed weight/plant; SEEDN = number of seeds/pod; PODN = number of pods/plant; HSWT = 100 seed weight; FL = days to flower; HEATR = visual heat rating; Env = Environment; Pos = position
BLUPs = Best linear unbiased prediction calculated using all the field locations

CHAPTER IV

CONCLUSION

In combined analysis (2011, 2012, and greenhouse study), a significant genetic variability was noticed. IAR-48, GEC, IT98K-277-2, Yacine, and IT98K-1092-1 were heat-tolerant varieties, and Big John, IT98K-589-2, It98K-476-8, CB-46, and White Acre were consistently showed heat-susceptibility across all the locations. Heritability ranged from 0.30 (PODN) to 0.93 (HSWT) for all seed traits. High heritability values were observed from all traits. We have also noticed a significant correlation among all traits.

Heat tolerance can be conferred by many factors but we observed pollen viability as one of the major causes for heat tolerance under high-temperature conditions.

Based on the results of the genetic diversity studies for heat tolerance and other traits, we conclude that a significant genetic variability exists in the cowpea germplasm for heat tolerance (based on SWT and PODN), PODWT, and HSWT, and that it could be utilized to develop genetic populations to study the inheritance of heat tolerance, genetic and environmental variation, genotype x environment interaction, and QTL mapping.

Both parents (GEC and IT98K-476-8) were significantly different for all the measured traits except SEEDN in RILs field trials. RILs were also significantly different for all measured traits in the combined analysis of three field environments, and we have observed similar results in the greenhouse study. The heritability estimates analysis

resulted in high heritability for all traits. SEEDN (0.72), HSWT (0.73), days to flowering (0.80), and HEATR (0.86) traits showed higher heritability values compared to PODN (0.58), SWT (0.59), PODWT (0.58), and plant height (0.33). A significant positive correlation was observed between the seed traits and HEATR. However, FL and plant height traits showed significant negative correlation with the seed traits, but a significant positive correlation between them, that indicated that taller plants flowered late and produced fewer SWT compared to shorter plants, and were heat-susceptible. Visual heat ratings (HEATR) were consistent across all locations. Based on HEATR, entry numbers 97,100, 65, 145, and 76 were the top performing (heat-tolerant) RILs.

Genetic map construction resulted in 11 linkage groups and 531 bins (each bin approximately contained 7.8 SNPs) containing 4,154 SNPs, and spans over 1084.65 cM, having a density of one SNP marker in approximately 0.26 cM. The genetic map provides a foundation for future genetic studies.

Linkage groups 3 and 10 housed a maximum number of consistent QTL. A QTL on linkage group 3 (position 28cM) was detected at the Corpus Christi location, and another QTL on linkage group 10 (position 72 cM) was detected in the greenhouse study for HEATR. SWT is another estimate of heat tolerance, and we noticed two consistent QTL: one on linkage group 3 and another on group 10 at about 84 cM and 90 cM, respectively.

Consistent QTL were detected for days to flowering on linkage group 10 at about 70 cM position and that explained phenotypic variations from 19 to 23% across all studies. For plant height, we have noticed two consistent QTL on linkage groups 3 and

10 at positions 28 and 70 cM, respectively, at College Station and Corpus Christi locations. QTL on linkage group 3 explained about 7.3% phenotypic variation in College Station, and 8.4% in Corpus Christi. QTL on linkage group 10 explained 27.1% phenotypic variation in College Station and 29.5% in Corpus Christi.

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