MAKERERE UNIVERSITY

COLLEGE OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES

SCHOOL OF AGRICULTURAL SCIENCES

Genetics of Cowpea Resistance to Bruchid (*Callosobruchus maculatus* Fab.)

By

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Reg No. 2014/HD02/2699X

A THESIS SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN PLANT BREEDING AND BIOTECHNOLOGY OF MAKERERE UNIVERSITY

JANUARY 2019

DECLARATION

The work presented in this thesis is my own research and has not been presented for the award of degree or diploma in any other University.

Sign:..... Date:.....

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This thesis has been submitted for examination with our approval as the university supervisors.

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DEDICATION

This work is dedicated to my mother Hadas Lema, my sisters Fana Yemane and Bethelihem Tilahun, and my brothers Kurabachew Belay and Getnet Gemechu for their encouragement during the entire study.

ACKNOWLEDGEMENTS

My sincere gratitude goes to my supervisors, Prof. Patrick R. Rubaihayo and Prof. Samuel Kyamanywa for their supervision, mentoring, guidance and encouragement that pushed me to complete my research and write-up on time. Apart from my two supervisors, I acknowledge the other members of my doctoral committee for their time and guidance in shaping this work. Special thanks to Dr. Solange Uwituze and Prof. Ekwamu Adipala for their encouragement to complete my PhD on time. My special thanks go also to Dr. Geoffrey Maxwell Malinga from Gulu University for his continuous technical support and encouragement. I am also grateful for the full doctoral funding received from DAAD through the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM). I would like to thank a capacity building competitive grant Training "the next generation of scientists" of Carnegie Cooperation of New York for providing partial fund to the project. Special thanks to the PI for RUFORUM sponsored students in Makerere University, Dr. Sylvia Mkandawire and Mrs Molly Akello, for their prompt actions in putting in requisitions for stipends, research and other funds for a smooth stay in Uganda. Special thanks to the Makerere University Regional Center for Crop Improvement (MaRCCI) for its immense support in providing cowpea germplasms and allowing me to use their screen house for crossing and advanced generations. Furthermore, I would like to thank again MaRCCI for providing genotyped mini-core cowpea accession, without which it would have been hard to complete the research on time. I am also grateful to the Biochemistry section of the National Crops Resources Research Institute for hosting the laboratory work.

I am thankful to all my colleagues in the Plant Breeding and Biotechnology programme for sharing and critiquing to improve the research and write-up. Finally, my sincere gratitude goes to my mother, Hadas Lema and all siblings for their encouragement and support.

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ABSTRACT

Cowpea *Vigna unguiculata* (L.) is an important indigenous legume crop providing dietary protein, minerals, carbohydrates, fats, vitamins and income to many people in Africa, Asia, and Central and South America. However, its production is limited by insect pests in general and bruchids attack in particular. Bruchids particularly Cowpea weevil (*Callosobruchus maculatus)* is the most destructive pest causing complete storage loses in quality and quantity. This makes the grain unfit for consumption, marketing and planting. To minimize losses due to bruchids infestations, the majority of farmers in Sub-Saharan Africa are using chemical pesticides. The use of pesticides however is expensive, pose health hazards to farmers and consumers, and their continuous use can lead to development of insecticide resistant bruchids. The use of resistant genotypes is, therefore, a promising alternative control method to the hazardous pesticides for the management of *C. maculatus*. However there is a paucity of information on genetics and sources of cowpea resistance to bruchids. Thus the objectives of this study were to: (i) Identify new sources of cowpea germplasm that are resistant to bruchids; (ii) determine the biochemical traits involved in the resistance to bruchids; (iii) determine the mode of inherence of resistance to cowpea bruchids and combining ability and (iv) identify candidate genes that control bruchids resistance traits in the cowpea association mapping panel using individual SNP markers. The study was conducted at Makerere University Agricultural Research Institute - Kabanyolo (MUARIK) between May 2015 and March 2018. One hundred and forty five (145) cowpea genotypes were evaluated, in a completely randomized design (CRD), for their reaction to *C. maculatus.* As a result 18 genotypes (ACC23 × 3B, NE39 × SEC4, ALEGI×5T, ACC2 × ACC12, 3B × 2W, SEC1 × SEC4, IT84s-2246, TVu-2027, IT97K-499-35, IT95K-207-15 and IT90K-76) were identified as being resistant, suggesting that they could serve as donor source/parent to breed for cowpea resistance to bruchids. To study the mechanism of cowpea resistance to bruchids, different biochemicals were extracted from seed coat and cotyledons of seven cowpea genotypes, four susceptible and three resistant to bruchids. The results indicated that none of the studied seed coat biochemicals but α-amylase inhibitor and carbohydrate content extracted from the cotyledon was responsible for cowpea resistance to *C. maculatus*. Nine selected parents, comprising of four susceptible and five resistant ones, were crossed using a full diallel mating design. F_2 plants and the parents were evaluated at MUARIK to study the heritability and gene action controlling resistance to *C. maculatus*. Additive gene effects were more important for all of the resistance traits (Baker's ratio > 0.5). Parents 2419, TVu-2027

and IT84s-2246 showed significant negative GCA effects for number of eggs, and insect emergence and holes, and positive effect for median development period suggesting that the parents could be selected for breeding of cowpea resistance to bruchids. Likewise, crosses IT84s-2246 × 2419, 2419 × MU9, TVu-2027 × SECOW2W, 2419 × IT90K-76, 2419 × WC69, 2419 × SECOW5T and 2419 × SECOW2W showed negative SCA effects for number of eggs, insect emergence and holes and positive values for median development period indicating that the crosses were the best and could be used for further breeding. To dissect the genetic basis of resistance to bruchids at molecular level, 217 mini-core accessions were genotyped at the university of California using 51,128 SNPs and also phenotyped for their reaction to bruchids at Kabanyolo (MUARIK). Genome wide association studies identified 11 genomic regions associated to bruchids resistance. Further analysis of gene prediction using Phytozome, identified six candidate genes associated with the resistance traits which included (i) gene *Vigun08g132300* control number of eggs, holes and insect emergence; (ii) *Vigun08g158000* and (iii) *Vigun06g053700* for number of eggs; (iv) *Vigun02g131000*, (v) *Vigun01g234900* and (vi) *Vigun01g201900* for median development period. The identified candidate genes are influencing resistance through their involvement in carbohydrate and protein biosynthesis, and their being regulatory element. The negative allelic effects of their corresponding SNP for number of eggs, holes and insect emergence, and positive effect on median development period was also another evidence for their involvement in resistance. Therefore, the information generated from genome wide association study could be used as a tool for analyzing the inheritance of the resistant genes, for monitoring the transmission of the resistance genes or genomic regions from parents to progeny, and for map-based cloning of those genes

LIST OF PUBLICATIONS

The following papers were generated from the thesis.

Published papers.

Chapter 3: Miesho B., Hailay M., Ulemu M., Khalid, A., Geoffrey M., Sadik. K., Odong T. L., Rubaihayo P. and Kyamanywa S. 2018. New sources of cowpea genotype resistance to cowpea bruchids *Callosobruchus maculatus* (F.) in Uganda. *International Journal of Agronomy and Agricultural Research* 12(4), 39-52.

Chapter 4: Miesho B., Msiska U., Hailay M, Malinga G., Odong T., Edema R., Gibson P., Rubaihayo P. and Kyamanywa S. 2017. Biochemical Basis of Cowpea Resistance to Bruchids, *Callosobruchus Maculatus* (F.). *International Journal of Advanced Research* 5(10), 219-227.

Chapter 5: Miesho B., Msiska U., Mehari H., Malinga M., Ongom P., Rubaihayo P. and Kyamanywa S.; Inheritance and combining ability of cowpea resistance to bruchids (*Callosobruchus maculatus* F.). Journal of Plant Breeding and Crop Science 10(9), 228-238.

Paper Submitted.

Chapter 6: Miesho B., Mehari H., Msiska U., Awio B., Ongom P., Malinga M., Edema R., Gibson P., Rubaihayo P. and Kyamanywa S. ; Identification of candidate genes associated with Resistance to Bruchids (*Callosobruchus maculatus* Fab) in Cowpea. *Journal of Plant Breeding*.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

1.1.1 Importance of cowpea in Africa

Cowpea, *Vigna unguiculata* (L.) Walp. (Fabaceae), is a commonly grown and consumed grain legume in Sub- Saharan Africa, and it is particularly well adapted to the dry Savanna region where many other crops could fail or perform very poorly due to water stress caused by irregular and short seasonal rainfall as well as poor soil fertility (Boukar *et al.*, 2016). According to a survey from four African countries, Malawi, Uganda, Rwanda and Tanzania, cowpea is among the highly appreciated leafy vegetable species due to its nutritional benefits (Keller, 2004). Its grain provides high amounts of proteins (Frota *et al.*, 2017), carbohydrates, vitamins and minerals (Khalid *et al.*, 2012), and is an income source for the rural and suburban inhabitants (Boukar *et al.*, 2016). Drought tolerance, short growing period and its multi-purpose use make cowpea a very attractive alternative for farmers who cultivate in marginal, drought-prone areas with low rainfall and less developed irrigation systems, where infrastructure, food security and diminishing malnutrition are major challenges (Hallensleben *et al.*, 2009). However, despite its regional importance, cowpea has been widely neglected in research and improvement programmes of many African countries (Schippers, 2000).

1.1.2 Cowpea Production

Cowpea is grown in all countries in sub-Saharan Africa and in Asia, South America, Central America, the Caribbean, the United States of America (USA) and around the Mediterranean Sea (Hall, 2004). However, the vast majority of the world's cowpea production is from Africa, with about 12.61 million hectares under cultivation in 2014 (FAOSTAT, 2016). In Africa, cowpea is the second most important grain legume (NRC, 2006) after beans; and in Uganda it ranks fourth after beans, groundnuts and soybean (Ronner and Giller, 2013). Cowpea is grown by approximately 2.2 million smallholder farmers, mainly in eastern and northern regions using traditional methods (Ddungu *et al.*, 2015) with an annual total area coverage and production of 114,210 ha and 71,181.82 tons, respectively (FAOSTAT, 2012).

1.1.3 Status of cowpea research in Uganda

Cowpea improvement programme in Uganda was initiated in the late 1960s at Makerere University with the collection of local and exotic accessions that were screened for yield

potential (Rubaihayo *et al.,* 1973). Likewise, the Rockefeller Foundation initiated a preliminary research on cowpea during 1990s (Anonymous, 2013). Considering the nutritional and economic value of cowpeas for many Ugandans, scientists at the Uganda National Agricultural Research Organization (NARO) and Makerere University began research on cowpea in 1993. Over the last 14 years, up to seven new cowpea varieties have been developed and released in Uganda for production (NARO, 2015). However, all the cowpea research in the country has often been targeting towards improving the yield potential and entomological studies have concentrated more on field pests than storage pests, the bruchids.

1.1.4 Constraints of cowpea production

Cowpea yield potential of 3000 kg ha-^{1} is achievable in Uganda but farmers' yields are constantly very low, averaging 300-500 kg ha-^{1} due to several production constraints (Anonymous, 2013). Insect pests, plant diseases, parasitic flowering plants and drought are among the yield reducing factors (Terao *et al.,* 1997). Moreover, lack of improved cowpea varieties resistant to pest is a bottleneck for cowpea production. The majority of farmers in developing counties in general, and Uganda in particular, have limited access to improved cowpea varieties (Mundua, 2010). Consequently, only about 5% of African area under cowpea is planted to improved cowpea varieties (Anonymous, 2015).

The nature and magnitude of these constraints differ among production areas. However, the major constraints for cowpea grain production in most cowpea producing nations are insect pests (Ronner and Grill, 2013), including cowpea aphids (*Aphis craccivora* L.), leafhoppers (*Empoasca* spp), thrips (*Megalurothrips sjostedti*-Synonym: *Taeniothrips sjostedti* L.), flower eating beetles (*Mylabris* spp. and *Coryna* spp.), blister beetles (*Hycleu slugens*) and green stink bugs (*Nezara viridula* L.). The bruchids (*Callosobruchus maculatus* F.) is the most devastating postharvest insect pest (Adams *et al.*, 2018), which attacks cowpea in the field and final products in the store leading to economic losses.

1.1.5 Impact of bruchid on cowpea grains and associated problems to human health

In the dry zones of sub-Saharan Africa, cereals and legumes once harvested must be stored throughout the long dry season to ensure food security as well as to enable households sell their grains at higher prices later in the season. Small scale farmers often, however, find that the grains are severely damaged during storage by the bruchids *(*Anonymous, 2013).

Bruchids is regarded as the most important and most common storage pest of cowpea both in Africa (Fakayode *et al.*, 2014) and Asia (Deshpande *et al.*, 2011) causing up to 80.2%% grain damage one month after infestation (Adams *et al.*, 2018) and if there is control measure all the stored cowpea can be consumed by bruchids in the first 10 to 12 months of storage (Gómez, 2004). In Uganda, bruchids is amongst the major constraints to cowpea production (Ronner and Giller, 2013) and is more pronounced for small-scale farmers (Jackai and Daoust, 1986). According to NARO (2012), up to 71,000 metric tons of dry cowpea grains are produced annually in Uganda and about 27.5 % of this production is damaged in storage by bruchids (*C. maculatus* F.). The huge post-harvest losses in quantity and quality deterioration caused by this insect are major obstacles to achieving food security in developing countries such as Uganda.

The impact of this storage pest induces reduction in seeds, loss or conversion of nutrients which lead to reduced germination rate and nutrient level of seeds (Swella and Mushobozy, 2007). It also causes loss in quality as a result of contamination with filthy materials composed of insect fragments, exuviae, excreta and moulds (Musa and Adeboye, 2017). All these damages reduce and diminish the degree of usefulness, making the seeds unfit either for planting or human consumption (Ali *et al.*, 2004).

1.1.6 Management and control of bruchids in cowpea

The use of insecticides or fumigants to protect cowpea seeds from bruchids is effective, but these chemicals pose health hazards to farmers and consumers, cause environmental pollution, and are expensive. Furthermore, insects develop resistance to insecticides, necessitating the application of larger amounts (Boyer *et al.*, 2012). For these reasons, non-chemical approaches for control of bruchids have been adopted. These methods include triple bagging using plastic bags (Tarver *et al.*, 2007), mixing seeds with ash in the storage containers (Songa and Rono, 1998), solar treatment (Kitch *et al.*, 1992), use of various botanical insecticides, e.g., neem (Bottenberg and Singh, 1996), use of different storage structures such as Earthen wave Granaries, Steel Drums/Tins, Polythene bags, Silos, and Pit method (Yakubu *et al.*, 2012), and storage in sealed containers (Singh *et al*., 1977). In northern Uganda, for example, over 63% households use synthetic pesticides to manage storage bruchids whereas in the southwest and eastern regions over 80% and 58%, respectively, use local options. However, these management practices are not effective, safe or sustainable especially when large quantities of seeds are involved (MBAZARDI, 2014).

For example, Yakubu *et al.* (2012) reported mud rumbus are effective only when small quantities of cowpea grains, between 1.0-5.0 metric tonnes, are stored. Likewise, when seeds are stored in pits, the seeds could be eaten up by termites and rodents (Adejumo and Raji, 2007). This suggests the need for another bruchids management practice which is cheap, easy to apply, and safe to the environment and consumers.

Breeding of cowpea for resistance to bruchids is considered an environmentally friendly, and economically feasible and effective method for controlling the pest in stored cowpea grains. Efforts have been made elsewhere to breed cowpea resistant to bruchids. For example, Singh *et al.*, (1985) identified lines TVu-2027 and TVu-11952 and they were used by IITA, Nigeria to develop resistant varieties IT90K-76, IT90K-59, and IT90K 277-2 (Singh, 1999) for production. However, the impact is challenged due to changes in pest populations and lack of durability of resistance to bruchids (Johnson, 1984). Amusa *et al.* (2013) also reported the breakdown of genetic resistance for some improved varieties to this insect pest, highlighting the need to search for new sources of resistance.

1.2 Problem Statement

There is currently no improved cowpea cultivar which is resistant to the bruchids in Uganda and yet, *C. maculatus* which attacks seeds in storage has the capacity to cause complete seed destruction. Seven improved cowpea varieties (SECOW I T, SECOW 2W, CPKUNDE, CP WHITE, SECOW 3B, SECOW 4 W and SECOW 5T) were released from 2003 - 2011 by the National Semi-Arid Resources Research Institute (NaSARRI, Serere) for cultivation in Uganda. However, none of them are resistant to *C. maculatus*.

In Uganda, cowpea breeding programmes so far have not focused on identifying and breeding of bruchids resistant genotypes and cowpea entomological studies have concentrated mostly on field pests rather than storage pests. This has resulted in the development of cowpea varieties with considerable levels of resistance to, or tolerance for, other biotic and abiotic constraints, but that are more vulnerable to bruchids invasion in storage.

In Uganda breeding of cowpea resistant to cowpea bruchids is difficult because identification of genotypes resistant to bruchids has not yet been undertaken. Moreover, the globally identified bruchids resistant genotypes have been found to lack durability due to their monogenic nature of resistance and existence of different bruchid biotypes (Shade *et. al.*,

1999). Understanding of the mechanisms of resistance to the bruchids is therefore one of the key pre-requisites for designing effective and efficient strategies to breed resistant genotypes. Different outcomes on the mechanism of how cowpea seed overcomes bruchids attack have been suggested. For example; Lattanzio *et al.* (2005) reported elevated levels of α-amylase inhibitor as the main mechanism for resistance to bruchids. Gatehouse *et al.* (1979) reported that elevated level of trypsin inhibitor in TVu-2027 seeds was responsible for resistance to *C. maculatus*. However, Baker *et al.* (1989) found no significant correlations between the levels of trypsin inhibitor and *C. maculatus* development time or mortality. There are also conflicting reports on the effect of seed coat tannin content on the oviposition and survival of *C. maculatus* (Swain, 1977; Lattanzio *et al*. 2005).

The genetic control and heritability of cowpea resistance to *C. maculatus* have not been studied in Uganda and yet it is important for optimizing breeding pipeline for bruchids resistance.

Furthermore, there is no any genome wide association study of cowpea resistance to bruchids (Tan *et al.*, 2012). However, few studies on QTL related to bruchids resistance are reported from elsewhere. For example, Fatokun (2000) reported SSR marker Vm50 that is closely linked with the delay in emergence of *C. maculatus* explaining 20% of the variation. Fatokun (2002) also identified four QTLs associated with bruchids resistance, major QTL accounting 80% of the variation. However GWAS of cowpea resistance to bruchids at global level in general and under Uganda conditions in particular are lacking and it is hindering marker assisted selection.

1.3 Justification

In Uganda, there is huge collection of cowpea genotypes which is not yet tapped for breeding (Afutu *et al*., 2017). Therefore, breeding program against bruchids has to be initiated by identifying bruchids resistant genotypes which could be used either for production and/or breeding. For effective breeding and selection of genotypes, there is also need to investigate the biochemical basis of cowpea resistance to bruchids, both from the seed coat and cotyledon.

To develop successful breeding strategy, the accurate phenotyping for bruchids resistance should be followed by the study of gene action and trait inheritance of bruchids resistance genes in local and improved sources. Such knowledge would significantly accelerate the

introgression of bruchids resistance genes to farmers' preferred, high-yielding and adapted cultivars. The study of genome wide association would help to identify the location of underlying candidate genes and to understand the nature of gene action. The molecular markers linked to key bruchids resistance traits would also help in marker assisted selection of resistant parents. The study reported in this thesis aimed to achieve some of these. Information generated from this study should be used up in monitoring the transmission of resistance genes or genomic regions from parents to progeny, and for map-based cloning of those genes.

1.4 Objectives of the Study

The overall objective of this study was to contribute to the reduction in cowpea storage losses through elucidation of cowpeas genetics of resistance to bruchids. The specific objectives were to:-

1. Identify new sources of cowpea germplasm that are resistant to cowpea bruchids in Uganda
2. Determine the biochemical traits of cowpea seeds involved in the resistance to bruchids
3. Determine the mode of inherence and combining ability of cowpea resistance to bruchids.
4. Identify candidate genes that control bruchids resistance traits in cowpea

1.4.1 Hypotheses

In undertaking this research, the following hypothesis were set out and tested:

1. There is genetic variability in cowpea for resistance to the bruchids that could be exploited to improve local materials.
2. There are biochemicals in the seed of resistant cowpea genotypes associated with resistance to bruchids.
3. The mode of inheritance for resistance to bruchids is quantitative and primarily additive, with parents expressing different combining abilities.
4. There exist genes in the genome of cowpea associated with resistance to bruchids which could be exploited for marker assisted breeding.

1.5 Thesis Structure

This thesis has been written in a composite form and thus there are overlaps either in context or literature citations. The objectives are discussed in chapters 3-6. Chapter One provides

general thesis introduction, Chapter Two covers literature review related to previous efforts in breeding of cowpea to bruchids resistance, Chapter Three deals about screening of cowpea genotypes resistance to *C. maculatus* (F.), Chapter Four presents the biochemical basis of cowpea resistance to bruchids (*Callosobruchus maculatus* (F.), Chapter Five presents the inheritance and combining ability studies of resistance to cowpea bruchids (*Callosobruchus maculatus* F.), Chapter Six describes identification of candidate genes in Cowpea associated with resistance to bruchids (*Callosobruchus maculatus* Fab) and chapter seven provides a brief overview of the main findings, implications for breeding, and recommendations for future research.

CHAPTER TWO

LITERATURE REVIEW

2.1 Sources of Cowpea Resistance to Bruchids

The use of resistant genotypes to manage bruchids was reported by Amusa *et al.* (2014) in cowpea. The approach is cheap, eco-friendly and specific against a particular insect pest. The approach enables seeds to restrict, retard or overcome bruchids infestation, thereby improving the quality of the harvested cowpea seeds (Kumar, 1984). In view of this, considerable progress has been made elsewhere in developing cowpea varieties resistant to storage insects (Singh *et al.*, 1985; Amusa *et al.*, 2013 & 2014). To achieve this, several cowpea genotypes were screened for resistance to bruchids by different researchers. For example, Singh *et al.* (1990) and Amusa *et al.* (2014) identified TVu-2027, TVu-11952 and TVu-11953 as resistant to bruchids. Using a combination of field and laboratory screening, Van Boxtel *et al.* (2000) and Singh *et al.* (2002) identified IT82D- 889, IT83S-818, IT86D-880, IT86D-1010, IT84S-2246-4, IT89KD-889, IT90K-59, IT90K-76, and IT90K- 277-2, IT90K-284-2, IT97K-207-15, IT97K-499-35 and IT98K-205-8 as potential breeding lines against bruchids attack. Obiadalla-Ali *et al*. (2007) screened 21 cowpea genotypes for resistance to bruchids and classified them into susceptible, moderately resistant and resistant genotypes. The identified resistant lines showed a high percentage pest tolerance, reduced seed weight loss, low seed damage and delayed bruchids development period and a moderate oviposition preference, and were recommended as potential sources of resistance for cowpea breeding against bruchids.

Badii *et al.* (2013) screened 22 cowpea genotypes, comprising eighteen elite, one local and three improved cultivars for their susceptibility to infestation and highest number of eggs, adult emergence, percentage weight loss and insect growth index were recorded from the improved than the elite lines and local varieties. Musa and Adeboye (2017) evaluated seven improved cowpea varieties in Nigeria for susceptibility to bruchids and all were found to be susceptible to the pest. Similar results were also reported by Augustine *et al.* (2016). The above findings implied the necessity of incorporating resistance genes to improved cowpea varieties.

Several improved bruchids resistant cowpea varieties popular in different countries have been bred and released for production elsewhere in the world. Amongst the several popular

varieties; IT90K-76, IT90K-59 and IT90K 277-2 (Singh *et al.,* 1996) and; IT97K-207-15, IT95K-398-14 and IT 98K-506-1(Singh, 1999) were with a high level of resistance to bruchids. Similarly, IT90K-277-2, IT93K-452-1, IT94K-437-1, IT97K-569-9, IT95K-222-3, IT97K-837, and IT97K-499-38 were also promising bruchids resistant varieties (Fatokun *et al.*, 2002), and were introduced to East, Southern, West and Central Africa countries for production (Timko and Singh, 2008). The sources of resistance were from TVu-2027, but time and evolutionary pressure lead bruchids to overcome its resistance (Srinives *et al.*, 2007). Shade *et al.* (1999), for example, reported a virulent bruchid biotypes capable of breaking resistance to the already established resistant line TVu-2027. Amusa *et al.* (2013) also reported the breakdown of genetic resistance of some improved varieties to this insect pest. These highlight the need to look for new sources of resistance from landraces, and improved and introduced cowpea genotypes with acceptable levels of resistance.

2.2 Mechanisms of Bruchid Resistance

An understanding of the mechanisms of cowpea resistance to the bruchids is one of the key pre-requisites for designing effective and efficient strategies to breed resistant genotypes. The mechanisms of resistance could have either direct impact on the insect pest through antibiosis, or indirect effect in which the seeds are not preferred by the colonizing bruchids hence displaying non-preference by the insects (War *et al.*, 2012). Antibiosis is associated with the presence of adverse metabolites in the seed of the resistant genotypes. The metabolites could be phenolic compounds, such as phenolic acids, and tannins and flavonoids found in the seed coat (Egounlety and Aworh, 2003; Duenas *et al.*, 2005) and/or α-amylase inhibitor and seed carbohydrate content (Ajeigbe *et al.*, 2008) in the cotyledon of the seed. These biochemicals can act either additively or synergistically against the bruchids (War *et al.*, 2017) and may cause resistance through their inhibitor activity to *C. maculatus* (Ojwang *et al.*, 2012). These biochemicals also play a role in resistance by reducing oviposition and insect emergence, and elongating insect development period (Lattanzio *et al.*, 2005).

There are conflicting reports on the effects of seed coat biochemicals on oviposition and seed resistance to *C. maculatus*. Baker *et al*. (1989) obtained high tannin contents from susceptible genotype VITA 7 and low content from resistant genotype TVu-2027, indicating correlation between seed tannin content and seed resistance to bruchids attack. Nonetheless, Swain (1977) found negative associations between seed tannins and seed resistance parameters. On the contrary, Lattanzio *et al*. (2005) reported lack of association between seed tannin content

and resistance to bruchids. Edde and Amatobi (2003), in their experiments using 22 cowpea genotypes found no significant association between number of eggs deposited, adult mortality and mean developmental periods on cowpea with and without seed coat suggesting that seed coat had no value in protecting cowpea seeds against attack by *C. maculatus*, but rather the resistance factors were carried in the cotyledon and embryo of the seed.

Plant α-amylase inhibitors are abundant in cereals and Leguminosae, suggesting their role in protecting plants against insect pests (Pedra *et al.,* 2003). Low concentrations of α-amylase inhibitors in beans were proven to be lethal to the larvae of cowpea weevil and adzuki bean weevil (Pedra *et al.,* 2003). Lattanzio *et al*. (2005) reported positive association between the level of cowpea seed α-amylases inhibitor and seed bruchids resistance parameters, and suggested that elevated levels of α-amylases inhibitor as the main mechanism of cowpea resistance to bruchids. Singh *et al*. (1990), however, suggested elevated levels of trypsin inhibitor as the main mechanism of resistance in cowpea. Thus, it seems that cowpea seeds do not rely on one type of chemical defense only, implying that resistance might be due to the accumulation of several biochemicals.

A review by Lattanzio *et al.* (2005) showed that cowpea seeds do not rely on one type of chemical defense only, but rather that the accumulation of several chemicals could be responsible for increasing their defense levels. Therefore, the high resistance of some cultivated or wild *Vigna* species to *C. maculatus* may be due to the presence of multiple chemical factors with additive or synergistic action to protect seeds from predation (Lattanzio *et al.*, 2005). For effective breeding and selection of genotypes, there is thus a need to investigate impact of cowpea seed nutritional and antinutritional factors in increasing seed resistance to bruchids.

2.3 Gene Action and Inheritance of Bruchid Resistance Genes in Cowpea

The existence of genetic variability among cowpeas for resistance to bruchids is a good asset for breeding (Amusa *et al.,* 2013). Although several authors have identified cowpea genotypes that are resistant to bruchids, comparatively few studies have been done so far with respect to the genetics of cowpea resistance to bruchids at global level in general and Uganda in particular. The lack of such information has meant the development of cowpea varieties with considerable levels of resistance or tolerance to other biotic and abiotic constraints, but which are more vulnerable to bruchids attack during storage.

The genetic control of resistance to storage insect pests has been studied on other legume crops elsewhere. Chen *et al.* (2007) and Somta *et al.* (2007), for example, studied the inheritance of resistance of mungbean to *C. chinensis* and *C. maculatus* and found resistance to be governed by either monogenic, oligogenic or polygenic genes. Somta *et al.* (2006, 2007, 2008) reported that the inheritance of bruchids resistance genes is governed by additive and dominant genes with a few cases of cytoplasmic gene governing the inheritance of resistance to storage insect pest in many leguminous crops. Nevertheless, few studies have been done on the inheritance of cowpea resistance to bruchids.

Redden *et al.* (1983) studied the inheritance of cowpea seed resistance to bruchids using F_1, F_2, F_3 and F_4 families and the F_2 generations did not provide evidence of reciprocal differences. Similarly, evidence of reciprocal differences and possible cytoplasmic inheritance was not observed in their F3 generation (Redden *et al.,* 1983). Redden *et al.* (1983), in the same experiment from the F_2 crosses, found evidence of one to two loci with major gene effects and loci with minor gene effects on bruchids resistance. In the same experiment but using F_3 crosses, Redden *et al.* (1983) reported evidence of digenic main effect with unequal gene expressions, plus modifier genes for the genetics of resistance inheritance. In some other crosses, they also observed monogenic basis plus modifier genes and hypothesised the possibility of the presence of maternal and minor gene expressions in the embryo of the resistance parent (Redden *et al.,* 1983).

Adjadi *et al.* (1985) bio assayed parental, F_1, F_2, and backcross populations using no-choice experiment for bruchids reaction to elucidate the genetics of cowpea resistance to bruchids and the F_1 and the maternal parent responded similarly, indicating the existence of maternal effect on the resistance of cowpea to bruchids infestation. Dobie (1981) in his experiment obtained resistance cross when bruchids resistant female parent was used and vice versa. The same author also obtained resistant seed in a backcross involving the resistant cowpea as female parent, indicating the presence of maternal genes. Adjadi *et al.* (1985), from their F_2 plants, showed segregation ratio of 15 susceptible: 1 resistant, implying digenic inheritance of seed resistance to bruchids which was supported by data from backcross, generated from F_1 plants of the resistant parent, which provided a segregation ratio of 3 susceptible: l resistant. Similar results were reported by Dobie (1981) and Singh *et al*. (1985). However, Dobie (1981) hypothesized that either dominant or interactive effects were more important than additive types of gene effects. To exploit the information for breeding against

bruchids, there is need to obtain clear information on the genetics of cowpea resistance to bruchid under Uganda conditions.

2.4 Molecular Markers Associated with Bruchids Resistance Traits

Conventional breeding method depends on selection of genotypes based on their response to bioassay tests using controlled pest infestation of cowpea seeds (Redden *et al*., 1983). This approach, because of its sole dependence on phenotype, is slow, inefficient and less accurate. This highlights the need for techniques, such as molecular approaches, that enable identification of variation at the nucleotide level which may reduce the heavy dependence on phenotyping and improve breeding efficiency and precision.

Molecular approach should be utilized in cowpea breeding for resistance to bruchids. The approach could help in enhancing fast, precise and accurate selection of genotypes resistant to bruchids and localize the resistance gene. This would reduce the heavy dependence on phenotyping for screening and could improve the efficiency and precision of conventional plant breeding via marker-assisted selection (MAS) (Collard and Mackil, 2008). Due to quantitative inheritance of bruchids resistance traits, few efforts have been made to understand the genetic basis of such complex traits (Redden, 1983; Babura and Mustapha, 2012). The molecular markers are a promising tool in dissecting the complex genetic traits in cowpea.

Restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs or microsatellites) are widely employed to construct linkage maps and mapping of important agronomic traits in many crop plants. In *Vigna* species, for example, SSR markers have been developed in cowpeas (Li *et al*., 2001) and azuki bean (Wang *et al*., 2004). The markers have been applied to map quantitative trait loci (QTL) associated to seed weight in cowpea, and bruchids and powdery mildew resistance traits in mungbean (Somta *et al*., 2008). The existence of genomic regions conferring resistance to bruchids was reported in cowpeas (Fatokun, 2000, 2002), rice bean (Venkataramana *et al.*, 2015) and mungbean (Mei *et al*., 2009; Wanga *et al.*, 2016). However, the application of molecular markers to study cowpea resistance to bruchids is in its exploration stage (Tan, *et al.*, 2012) and yet few QTL associated to bruchids resistance traits have been identified. Fatokun (2002) reported four QTL associated with resistance to bruchids with major QTL accounting for 76% of the variation in the trait. Similarly, the same author identified minor QTL from the susceptible

parent contributing to resistance. Fatokun (2000) reported SSR marker Vm50, explaining 20% of the variation which were closely associated with the delay in the emergence of *C. maculatus*.

High-resolution genetic maps provide breeders with powerful tools for analyzing the inheritance of genes of interest, for monitoring the transmission of specific genes or genomic regions from parents to progeny, and for map-based cloning of those genes (Kumar, 1999). At the University of California 51,128 single nucleotide polymorphisms (SNP) have been developed for the cowpea genome using the "Cowpea iSelect Consortium Array" (Munoz-Amatriain *et al.,* 2016). However, no one has used them for genome wide association study of cowpea resistance to bruchids. A genome-wide association has been attempted in this research

The identified gaps on sources, mechanisms and inheritance of resistance to bruchids have hampered cowpea breeding. Moreover, the progress of breeding is affected by lack of information on the application of molecular markers in cowpea improvement resistance to bruchids. Therefore, to breed cowpea resistance to bruchids, there is a need to search additional sources of resistance which could be used as donor parents; study mechanisms of cowpea resistance to bruchids which could be used as biochemical markers; study gene action and inheritance of resistance genes to bruchids in local and introduced sources to design effective breeding strategy. Moreover, to initiate marker assisted breeding, there is a need to identify SNPs and candidate genes associated with resistance to bruchids. The knowledge generated from the study will hence significantly help to introgress resistant genes to the high yielding but susceptible cowpea varieties. Therefore, the focus of this study was to reduce cowpea storage losses through elucidation of cowpea genetics of resistance to bruchids.

CHAPTER THREE

NEW SOURCES OF COWPEA GENOTYPES RESISTANCE TO BRUCHIDS (*CALLOSOBRUCHUS MACULATUS* F.)

3.1 Introduction

Cowpea (*Vigna unguiculata (L.)* is the second most important and most widely consumed grain legume in semi-arid and subtropical regions of Africa (NRC, 2006). It is cultivated primarily as a pulse, vegetable and as a cover and fodder crop (Faye, 2005). Cowpea seeds provide a rich source of protein, carbohydrate, minerals and vitamins, particularly for the poorer sectors in many developing countries (Gonçalves *et al.,* 2016). Globally, more than 12.32 million hectares of cowpea are harvested, 98.1% being from Africa (FAOSTAT, 2016). In Uganda, it is the fourth most important legume crop after beans, groundnut and soybean (Ddungu *et al.,* 2015) and it is grown by approximately 2.2 million smallholder farmers on total area of 77,000 ha harvested annually (FAOSTAT, 2012). However, cowpea production in these producing countries is limited by insect attacks (Beck and Blumer, 2007).

In storage, cowpea weevil *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) is the most destructive pest (Deshpande *et al.,* 2011). The females deposit their eggs on seed coat, and embryogenesis is completed after 3 to 5 days (Beck and Blumer, 2007). After eclosion, the larvae penetrate the cotyledons where they develop by consuming the energy reserves of cotyledons, reducing both the quantity and quality of seeds, and making them unfit for planting, marketing and human consumption (Ali *et al.,* 2004). Adult emergence occurs after 25-30 days (Oliveria *et al.,* 2009). The loss in quality is due to contamination with insect exudate, eggs, dead insects and holes and conversion of seed contents (Ali *et al.,* 2004). The loss in quantity is attributed to seed weight loss (Maina *et al.,* 2012).

In Sub-Saharan Africa, chemical control using insecticides is a common practice used by the majority of farmers to minimize losses due to bruchids infestations (Olakojo *et al.,* 2007). However, the method is expensive, pose health hazards to farmers and consumers, and their continuous use can lead to development of insecticide resistant bruchids (Boyer *et al.,* 2012). The use of resistant genotypes offers a promising alternative control method to the hazardous pesticides for the management of *C. maculatus,* especially where large quantities of grains are involved (Cruz *et al.,* 2016). Several studies have assessed the performance of *C. maculatus* infesting different genotypes (Singh *et al.,* 1985; Shade *et al.,* 1999). In Nigeria, for example, out of the 8000 germplasm lines screened, only three *C. maculatus* resistant

lines (TVu-2027, TVu-11952 and TVu-11953) were identified by the International Institute for Tropical Agriculture (IITA), and *C. maculatus* showed decreased survival and increased developmental times during infestation of those seeds. However, the use of resistant genotypes is affected by the durability of resistance (Appleby and Credland, 2004), which is rapidly overcome by changes in pest populations (Keneni *et al.*, 2011) and by lack of high-resistance sources (Leach *et al.*, 2001). A study in Nigeria, for example, showed the identified bruchids resistant genotype, TVu-2027, has already succumbed to the pest population (Shade *et al.*, 1999). Such breakdown of resistance in improved cowpea genotypes to bruchids highlights the need to search for new sources of resistance from different cultivated varieties and wild species.

In Uganda, information on sources of local and improved cowpea bruchids resistant genotypes is scarce. Therefore, in this study, the susceptibility and resistance of 145 *V. unguiculata* genotypes to infestation and damage by *C. maculatus* was investigated. The aim was to identify new sources of cowpea genotypes resistant to bruchids for the improvement of cowpeas in the breeding programme.

3.2 Materials and Methods

Seeds of 145 cowpea genotypes (130 from Uganda, one Kenya and 14 from IITA Nigeria) were used for the study (Table 3.1). To generate sufficient seeds for laboratory testing, each of the genotypes were planted in screen house in 5 five litter plastic buckets at the Makerere University Agricultural Research Institute Kabanyolo (MUARIK) (0°28'N and 32°37'E, approximately 1200 m a. s. l.), between May and December 2015. MUARIK has deep ferrallitic soils with pH range of 5.2 to 6.0. Average temperature during experiment ranged from 25˚C - 31˚C while relative humidity was from 71% - 90%.

3.2.1 Bruchids laboratory culture

Adult *C. maculatus* (F.) were obtained from the National Agricultural Research Laboratory, Kawanda. A permanent laboratory culture of the insect was established at MUARIK by allowing the insects to lay eggs on a susceptible inbred line IT71. Insects were reared on 12 kg seeds kept in four transparent plastic buckets of five litre capacity whose tops were covered with muslin cloth to provide aeration but prevent the insects from escaping. The insects were allowed to oviposit and their progeny maintained by regularly replacing the infested seeds with fresh seeds.

Table 3.1: Cowpea genotypes evaluated for bruchids resistance.

Genotype	Cultivar type	source	Genotype	Cultivar type	source	Genotype	Cultivar type	source
182	Landrace	Uganda	MU9	Landrace	Uganda	5T - 3B	Inbred line	Uganda
2282	Landrace	Uganda	NE13	Landrace	Uganda	5T × Acc12	Inbred line	Uganda
2309	Landrace	Uganda	NE15	Landrace	Uganda	5T×4W	Inbred line	Uganda
2392	Landrace	Uganda	NE19	Landrace	Uganda	ACC12 × 3B	Inbred line	Uganda
2419	Landrace	Uganda	NE23	Landrace	Uganda	ACC12 × 2W	Inbred line	Uganda
2434	Landrace	Uganda	NE30	Landrace	Uganda	ACC2× ACC12	Inbred line	Uganda
3306	Landrace	Uganda	NE37	Landrace	Uganda	ACC2 × IT	Inbred line	Uganda
IT109	Improved	IITA	NE39	Landrace	Uganda	ACC23 × 4W	Inbred line	Uganda
IT97	Landrace	IITA	NE39 × SEC2	Inbred line	Uganda	ACC25	Landrace	Uganda
KVU-27-1	Improved	Kenya	NE39 × SEC4	Inbred line	Uganda	ACC26 x ACC2	Inbred line	Uganda
NE20	Landrace	Uganda	NE4	Landrace	Uganda	ALEGI x 4W	Inbred line	Uganda
NE51	Landrace	Uganda	NE40	Landrace	Uganda	ALEGI	Local	Uganda
3B x 2W	Inbred line	Uganda	NE44	Landrace	Uganda	ALEGI×3B	Inbred line	Uganda
ACC12 x 5T	Inbred line	Uganda	NE48	Landrace	Uganda	ALEGI×5T	Inbred line	Uganda
ACC23 x 3B	Inbred line	Uganda	NE5	Landrace	Uganda	ALEGI × ACC2	Inbred line	Uganda
ACC26 * IT	Inbred line	Uganda	NE51 × SEC3	Inbred line	Uganda	CIG	Inbred line	Uganda
EX-1Seke	Landrace	Uganda	NE51 × SEC4	Inbred line	Uganda	EBELAT×NE39	Inbred line	Uganda
IT × ACC23	Inbred line	Uganda	NE55	Landrace	Uganda	EBELAT×NE51	Inbred line	Uganda
IT ×ALEGI	Inbred line	Uganda	NE67	Landrace	Uganda	WC32 × SEC5	Inbred line	Uganda
IT2841 x BROWN	Inbred line	Uganda	NE70	Landrace	Uganda	IT71	Inbred line	IITA
MU17	Landrace	Uganda	NYBOLA	Landrace	Uganda	IT84	Improved	IITA
MU20B	Landrace	Uganda	OBONQ1	Landrace	Uganda	IT889	Improved	IITA
MU24C	Landrace	Uganda	SEC1 × SEC4	Inbred line	Uganda	MU15	Landrace	Uganda
NE21	Landrace	Uganda	SEC5 × SEC2	Inbred line	Uganda	WC5	Landrace	Uganda
NE31	Landrace	Uganda	SEC5 × NE39	Inbred line	Uganda	WC55	Landrace	Uganda
NE32	Landrace	Uganda	SECOW2W	Improved	Uganda	WC60	Landrace	Uganda
NE36	Landrace	Uganda	SECOW5T	Improved	Uganda	WC44	Landrace	Uganda
NE41	Landrace	Uganda	UW × 5T	Inbred line	Uganda	WC46	Landrace	Uganda
NE45	Landrace	Uganda	2W×Acc2	Inbred line	Uganda	WC62	Landrace	Uganda
NE46	Landrace	Uganda	4W × 5T	Inbred line	Uganda	WC63	Landrace	Uganda
NE49	Landrace	Uganda	W10	Landrace	Uganda	WC64	Landrace	Uganda
NE50	Landrace	Uganda	W32	Landrace	Uganda	WC67	Landrace	Uganda
NE53	Landrace	Uganda	WC10	Landrace	Uganda	WC674	Landrace	Uganda
NE6	Landrace	Uganda	WC13	Landrace	Uganda	WC67B	Landrace	Uganda
NE71	Landrace	Uganda	WC15	Landrace	Uganda	WC68	Landrace	Uganda
SEC1×SEC3	Inbred line	Uganda	WC16	Landrace	Uganda	WC684	Landrace	Uganda
SEC5× SEC1	Inbred line	Uganda	WC17	Landrace	Uganda	IT82D - 716	Improved	IITA
WC2	Landrace	Uganda	WC18	Landrace	Uganda	IT84s-2246	Improved	IITA
WC29	Landrace	Uganda	WC19	Landrace	Uganda	IT97K-499-35	Improved	IITA
WC35C	Landrace	Uganda	WC21	Landrace	Uganda	TVu-2027	Improved	IITA
WC42	Landrace	Uganda	WC26	Landrace	Uganda	IT90K-277-2	Improved	IITA
WC52	Landrace	Uganda	WC27	Landrace	Uganda	IT90K-76	Improved	IITA
WC58	Landrace	Uganda	WC30	Landrace	Uganda	IT95K-207-15	Improved	IITA
WC69	Landrace	Uganda	WC32A	Landrace	Uganda	IT98K-205-8	Improved	IITA
WC7	Landrace	Uganda	WC35A	Landrace	Uganda	IT99K-1399	Improved	IITA
WC8	Landrace	Uganda	WC35D	Landrace	Uganda			
WC41	Landrace	Uganda	WC36	Landrace	Uganda			
2W x IT	Inbred line	Uganda	WC37	Landrace	Uganda			
SEC5 x SEC2	Inbred line	Uganda	WC48	Landrace	Uganda			
SEC5 x NE39	Inbred line	Uganda	WC48A	Landrace	Uganda			

3.2.2 Infestation and data collection

Seeds of each of the 145 cowpea genotypes were dried in an oven at 40^{o}C for 24 hours to eliminate any bruchids infestation coming from the field and to keep moisture level of the seeds uniform (Amusa *et al.*, 2014). Ten randomly selected seeds for each genotype were initially weighed and put into a petri-dish of size 90mm diameter and 10mm depth. The petri-

dishes containing the seeds were arranged at MUARIK animal lab. After arranging them in lab table, each petri-dish was infested at the same day with two pairs of newly emerged male and female adult bruchids and covered to prevent the insects from escaping. The insects were left undisturbed in the petri-dishes for three days to allow for mating and oviposition, after which they were removed (Amusa *et al.*, 2013). The experiment was laid in a completely randomized design with three replications per genotype. Data on number of eggs (eggs counted on seeds three days after bruchids infestation), number of exit holes, number of damaged and undamaged seeds, initial seed weight (g), residual seed weight (g), were recorded and percentage weight loss and percentage pest tolerance (ratio of total number of undamaged seeds to total number of initial seeds) were computed using the method of Amusa *et al.* (2014). The number of emerged adult bruchids was recorded daily until no more adults emerged for five days.

3.2.3 Insect growth index and Bruchids resistance rating

Insect growth index (GI) (Badii *et al.*, 2013) was calculated by combining the data on the number of eggs, percentage adult bruchids emergence and the median development period (Sharma and Thakur, 2014) for each genotype using the formula;

$$Adult\ emergence\ (\%) = \frac{Number\ of\ adults\ emerged}{Total\ number\ of\ eggs\ laid} x100$$

$$GI = \frac{Adult\ emergence\ (\%)}{Median\ deelopment\ period}$$

At the end of the experiment, Dobie Susceptibility Index (DSI) was calculated for each genotype using the data on total number of adult bruchids that emerged on each genotype and their median development period (i.e., the time from the middle of oviposition to the emergence of 50% of adult bruchids) using the formula of Dobie (1974);

$$DSI = \frac{\text{Loge F1} \times 100}{MDP}$$

F1– total number of emerging adults and

MDP –median developmental period (days).

The susceptibility index ranging from 0 to 11 was used to categorize the cowpea genotypes; where; 0-3 = resistant, 4-7 = moderately resistant, 8-10 = susceptible and ≥10 = highly susceptible (Dobie, 1974).

3.2.4 Data analysis

One-way analysis of variance (ANOVA) was used to examine differences in the performance of different cowpea genotypes for resistance to bruchids and Fisher's LSD test was used to separate the means. Pearson correlation was used to examine the association among resistance parameters including the DSI for the genotypes. Multiple linear regression analysis was used to identify which traits (number of eggs, number of holes, seed weight loss and pest tolerance) were better predictors of resistance (DSI). All analyses were conducted using GenStat Discovery, 16.1th Edition statistical package.

3.3 Results

3.3.1 Cowpea genotype resistance to bruchids

The results of performance of cowpea genotype resistance to bruchids are presented in Table 3.2.

Table 3.2: Mean squares for the performance of cowpea genotypes to *Callusbrocus maculatus* infestation.

Source of variation	Traits							
	df	NE	GI	MDP	ANH	PWL	PPT	DSI
Genotype	144	2302.92**	2.089**	27.66**	13.18**	196.88**	1718.00**	8.23**
Residual	290	51.65	0.12	0.87	0.15	2.96	64.83	0.05

NE= Number of eggs; GI=Growth index; MDP= Median development period; ANH= Average number of holes; PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

Significant differences (P< 0.001) were found in the number of eggs laid (NE) by *C. maculatus*, median time to adult bruchids emergence (MDP), insect growth index (GI), average number of holes (ANH), percentage weight loss (PWL), percentage pest tolerance (PPT) and Dobie susceptibility index (DSI) amongst the 145 cowpea genotypes.

3.3.2 Effects of *V.unguiculata* genotypes on growth performance of adult *C. maculatus*

The cowpea genotypes showed significant (P< 0.001) impacts on all bruchids growth parameters (Table 3.3). The mean number of eggs laid by bruchids ranged from 0-147.7. The top four genotypes in terms of mean number of eggs laid were NE32 (147.7), WC19 (141), WC69 (141) and EBERAT×NE51 (137.7). There was a significant (P<0.001) reduction in oviposition on genotypes IT84s-2246, IT95K-207-15 and TVu-2027.

The median development period to adult emergence of all the genotypes ranged from 20.8 to 44 days. The shortest period was recorded for genotypes IT889 (20.8 days) and SECOW5T (21.1 days) while the longest was for genotype IT84s-2246 (44 days).

The highest bruchids growth index was recorded for genotypes SECOW2W (3.92), MU9 (3.82), WC67B (3.69), IT889 (3.67), IT71 (3.56) and SECOW5T (3.5) whereas genotypes 2419 (0.03), WC42 (0.23), IT97K-499-35 (0.23), TVu-2027 (0.37), IT84s-2246 (0.38), ACC23×3B (0.46) and WC16 (0.51) showed least growth index values.

Table 3.3: Means of genotypic performance under bruchids infestation.

Genotype	NE/10 seeds	MDP (days)	GI	ANH/seed	PWL	PPT (%)	DSI
2419	39.7	42	0.03	0	0	96.7	0
IT84s-2246	0.7	44	0.38	0	0.2	96.7	0
TVu-2027	7	42	0.37	0.1	0	93.3	0.2
IT97K-499-35	19.7	29.2	0.23	0.1	3.7	90	0.3
WC42	17.3	32	0.23	0.1	0.5	90	0.3
IT95K-207-15	6	28.3	1.41	0.2	1.7	86.7	1.3
ACC23 × 3B	31.7	29.8	0.46	0.4	10.7	66.7	2.1
182	23.3	29.2	3.43	0.4	1	80	2.2
IT90K-76	12.7	29.2	1.34	0.4	0.7	80	2.3
WC16	36.3	32.3	0.51	0.6	2.2	60	2.4
NE39×SEC4	13.7	25	1.16	0.4	2.7	70	2.4
NE4	17	29.3	1.13	0.5	2.5	50	2.5
ACC2×ACC12	54.7	32	0.41	0.7	1.6	70	2.6
ALEGI×5T	77.3	29.3	0.25	0.6	1	70	2.6
WC67	18.3	28.8	1.19	0.6	3.4	60	2.7
SEC1×SEC4	14	25	1.62	0.6	1.3	76.7	3
3B×2W	28	30	0.95	0.8	3.4	66.7	3
WC48	12	25.2	1.88	0.6	6	53.3	3
2W×IT	38	30	0.76	0.9	4.4	46.7	3.1
ALEGI×ACC2	39.7	30.5	0.74	0.9	2.2	56.7	3.1
CIG	49.3	29.2	0.61	0.9	13.9	40	3.2
4W × 5T	21.3	29	1.63	1	6.4	33.3	3.4
WC13	23.7	29	1.41	1	3.5	70	3.4
IT98K-205-8	39.7	28.3	0.9	1	3.9	53.3	3.5
2434	33	30	1.15	1.1	10.3	46.7	3.5
2392	48	30.2	0.85	1.2	12.3	40	3.6
ALEGI×3B	54.7	33.2	0.87	1.5	12.2	20	3.6
WC32×SEC5	36	29	1.08	1	1.3	66.7	3.6
NE53	47	28.3	0.78	1	5.7	46.7	3.6
WC58	39.3	32.2	1.28	1.6	1.7	43.3	3.7
WC35D	54.7	30	0.8	1.3	4.8	30	3.7
WC8	43.7	29.3	0.96	1.2	15	40	3.7
IT×ACC23	29.7	29.5	1.57	1.4	10.9	46.7	3.8
ACC23x4W	20.3	29.8	2.33	1.3	8.9	36.7	3.8
NE39	22.7	29.2	2.01	1.3	9.8	20	3.8
MU20B	27	31.3	1.84	1.5	22.7	30	3.8
ACC12×3B	56	29.8	0.91	1.4	10.5	53.3	3.9
WC5	43	29.5	1.1	1.4	11.5	26.7	3.9
5T×3B	33	29.7	1.62	1.6	12.2	30	4
WC48A	40.3	30.5	1.38	1.7	12.7	13.3	4
NYBOLA	29.7	30.5	1.85	1.6	8.6	20	4
NE51×SEC3	45.7	31.8	1.35	1.8	17.7	30	4
IT99K-1399	56	32	1.1	1.9	9.1	33.3	4
WC27	18.7	27.2	2.39	1.2	10	50	4
NE41	37.3	29.2	1.32	1.4	10.3	20	4

Genotype	NE/10 seeds	MDP (days)	GI	ANH/seed	PWL	PPT (%)	DSI
WC7	50.7	29.2	1.09	1.5	11.2	23.3	4.1
WC68	48	29	1.11	1.5	8.9	30	4.1
NE44	52	30.5	1.1	1.7	10.4	30	4.1
KVU-271	46	30	1.4	1.9	8.5	53.3	4.2
WC674	38	33.8	2.05	2.5	9.7	20	4.2
NE18	50.3	29.5	1.15	1.7	12.5	10	4.2
2309	41.3	30	1.56	1.9	22	16.7	4.3
ACC12 × 2W	46.7	31.5	1.52	2.2	21.2	36.5	4.3
WC60	52.7	29.7	1.19	1.7	18.1	26.7	4.3
NE19	75	29.7	0.96	2.1	25.2	26.7	4.4
IT2841×BROWN	57.3	33.2	1.56	2.9	13.7	20	4.4
NE49	38	29.8	1.8	2	22.6	3.3	4.4
WC55	45.3	31	1.59	2.2	16.1	36.7	4.4
ACC26×ACC2	39	26.2	1.54	1.5	12.6	50	4.5
NE70	44	29	1.6	2	10	40	4.5
NE40	35	27.8	1.81	1.7	16.9	20	4.5
WC35A	52	29	1.32	1.9	16.5	0	4.5
ACC12×5T	83	34.5	1.4	4	37.6	6.7	4.6
MU17	35.3	29.5	2.25	2.3	10.2	10	4.6
NE37	38.7	29.7	2.08	2.4	7.5	50	4.6
SEC1×SEC3	48.7	28.8	1.59	2.1	15.7	10	4.6
IT90K-277-2	26.3	28.8	2.98	2.1	5.6	13.3	4.6
SEC5×SEC2	57.3	28.5	1.27	2	15.9	10	4.6
WC35C	54.7	28.5	1.33	2	14.3	20	4.6
WC67B	44.7	29.8	3.69	2.3	19.5	26.7	4.6
IT82D-716	33	30.8	2.8	2.6	14.4	0	4.7
NE51	49.3	28.8	1.6	2.2	10.9	36.7	4.7
NE50	48.7	29.3	1.85	2.5	9.6	10	4.8
W10	63	30.7	1.59	3	7.8	46.7	4.8
WC15	44.3	31	2.32	3	13.7	50	4.8
WC29	54	29	1.54	2.3	13.8	10	4.8
MU24C	51.3	29	1.8	2.7	5.1	60	4.9
NE39 × SEC2	30.7	29.3	3	2.6	11.7	23.3	4.9
NE45	39.7	28.8	2.53	2.7	7.7	30	5
WC44	63	29.8	1.73	3.1	9.2	50	5
NE23	50	29.5	2.09	3	10.8	30	5
5T×4W	37	30.2	2.96	3.2	7.7	10	5
NE31	50.3	28.2	1.81	2.6	12.7	16.7	5
W32	55.7	30.8	1.98	3.3	10.9	6.7	5
ACC26×IT	38.7	29.5	2.6	2.8	23.5	10	5
NE71	53	29.7	2.11	3.3	11.9	30	5.1
NE51×SEC4	58.3	31.2	2.13	3.8	6.9	26.7	5.1
UW×5T	51.3	28.3	1.89	2.6	6.9	36.7	5.1
NE32	147.7	33.8	1.83	9.2	25.6	0	5.2
NE36	55.7	29.3	2.28	3.4	14.7	20	5.2
NE46	68	28.5	1.63	3.1	14.2	40	5.2
MU15	47.7	29.7	2.55	3.5	18.9	30	5.2
NE6	72.7	29.5	1.64	3.5	9.7	30	5.2
ALEGI×4W	47.7	25.8	1.82	2.3	5.7	56.7	5.2
ITxALEGI	53.7	30.2	2.3	3.5	18.1	16.7	5.2
5T×Acc12	45.7	29.7	2.57	3.4	6.6	36.7	5.2

Genotype	NE/10 seeds	MDP (days)	GI	ANH/seed	PWL	PPT (%)	DSI
WC30	50	29	2.22	3.2	6.1	36.7	5.2
WC17	75.3	30.2	1.62	3.6	41.8	10	5.2
NE67	39.3	27.5	2.5	2.6	14.8	10	5.2
WC2	78.3	29.5	1.44	3.2	19.7	20	5.2
NE30	73.3	31.8	2.09	4.7	19.9	10	5.3
WC18	64	31.2	2.28	4.4	12.3	10	5.3
WC63	49.3	29.7	2.53	3.7	10.2	23.3	5.3
SEC5×SEC1	76	29.8	1.65	3.6	10.9	13.3	5.3
WC684	64.7	29.8	1.93	3.6	20.7	30	5.3
ACC2 × IT	67	30	2.12	4.1	8.6	13.3	5.4
EBERAT×NE51	137.7	31.3	1.16	2.1	26.6	10	5.4
NE21	72	32.8	2.46	5.7	18.9	3.3	5.4
WC46	53	27.7	2.09	3	7.3	16.7	5.4
ACC25	57	28.7	2.29	3.7	7	20	5.5
NE13	82	29.5	1.83	4.3	12.9	10	5.6
WC37	56.3	27	2.1	3.2	20.2	13.3	5.6
SEC5×NE51	98	29.7	1.69	4.9	14.8	10	5.7
NE55	66.3	28	2.09	3.8	13.2	20	5.7
WC32A	96.3	29.5	1.87	5.3	7.6	26.7	5.8
3306	72.7	27	1.87	3.7	17	3.3	5.8
WC21	45.7	26.2	3.13	3.7	8.7	13.3	6
NE5	119.3	29.5	1.88	6.6	22.3	3.3	6.1
WC62	91.3	27.5	1.92	4.7	13.1	23.3	6.1
NE20	95.7	28.5	2.27	6.1	28.9	3.3	6.3
NE48	61.3	27.3	3.11	5.2	13.4	3.3	6.3
2W×ACC2	53.3	25.3	3.13	4.2	10.9	33.3	6.4
WC64	74.7	26.5	2.53	4.9	21.6	3.3	6.4
WC26	55.7	24.2	2.63	3.5	6.5	46.7	6.4
EX-1Seke	95.3	28.2	2.37	6.2	12.1	20	6.4
EBERAT×NE39	94	28	2.3	5.9	14	6.7	6.4
NE15	71	24.2	2.16	3.6	14.8	33.3	6.5
WC36	67.7	27.5	3.21	5.9	10.9	26.7	6.5
ALEGI	103.7	24.8	1.69	4.3	10	36.7	6.6
WC10	83	26.2	3.02	6.5	10	23.3	6.9
OBONQ1	122.7	25.5	2.22	6.9	7	26.7	7.2
IT97	67.3	24.5	0.49	5.7	9.9	0	7.2
2282	88.3	25.2	3.19	7	16.7	3.3	7.3
IT84	86	24.5	3.3	6.9	13.8	10	7.5
SEC5×NE39	80.7	24	3.4	6.5	19.6	0	7.6
IT889	61	20.8	3.67	4.5	15.5	3.3	8
IT71	87	22.8	3.56	6.9	44.7	0	8.1
MU9	75.7	22.5	3.82	6.5	16.7	0	8.1
SECOW5T	68.3	21.2	3.5	5.1	27.8	0	8.1
WC19	141	23	2.42	7.8	22.3	0	8.2
WC69	141	23	2.42	7.7	35.9	0	8.2
SECOW2W	87.3	22.8	3.92	7.7	24.2	0	8.3
IT109	124	21.5	2.92	7.8	27.6	0	8.8
LSD	11.4	1.5	0.55	0.5	2.7	12.9	0.4
% CV	13.13	3.22	18.6	13.77	13.93	26.72	4.81

ACC = Accession; NE = Northern and Eastern; WC = Western and Central; Inbred lines at F7 generation; MU=Makerere University and IT = International Institute of Agricultural Research; NE= Number of eggs; MDP= Median development period; GI=Growth index; ANH= Average number of holes; PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

3.3.3 Genotypic variation of cowpea seeds to bruchids damage

Bruchids attack caused significant (P< 0.001) effects on seeds of cowpea genotypes (Table 3.3). The lowest mean number of holes and the highest percentage pest tolerance were observed on four cowpea genotypes including genotype 2419 (0 and 96.7%), IT84s-2246 (0 and 96.7%), TVu-2027 (0.1 and 93.3 %) and WC42 (0.1 and 90%). Meanwhile, the highest number of holes and lowest percentage pest tolerance was recorded on genotype NE32. The weight loss in different genotypes ranged from zero to 44.7 percent. The highest weight loss was recorded on genotype IT71 (44.7%) after 22.8 days followed by WC69 (35.9%) while the lowest was recorded from genotype 2419 and TVu-2027 (0.0%), IT84s-2246 (0.2%) and WC42 (0.5%) (Table 3.3). Based on the Dobie susceptibility index, genotypes IT84s-2246, 2419, TVu-2027, WC42, IT97K-499-35, IT95K-207-15, ACC23 × 3B, 182, IT90K-76, NE39 × SEC4, WC16, NE4, ALEGI × 5T, ACC2×ACC12, WC67, WC48, 3B × 2W and SEC1× SEC4 were considered resistant, whereas IT109, SECOW2W, WC19, WC69, IT71, MU9, SECOW5T and IT889 were susceptible to the pest (Table 3.3).

The results of frequency distribution of the 145 genotypes based on the DSI, showed that 12% were resistant, 79.3% moderately resistant and 8.7% susceptible (Table 3.4).

Table 3.4: Classification of genotypes based on Dobie susceptibility index.

Class	Resistance Class	No. Of Genotypes	NE/10 seeds	GI	MDP (days)	ANH/ seed	PWL (%)	PPT (%)	DSI
1	Resistance	18	0.7-77.7	0.03-3.43	25-44	0.0-0.8	0.0-3.7	50-96.7	0.0-3.0
2	Moderately resistance	114	22.7-147	0.74-3.69	24.2-34.5	0.9-6.6	1.3-28.9	0.0-66.7	3.1-6.9
3	Susceptible	13	61-141	0.49-3.82	20.8-25.5	4.5-7.8	7-44.7	0.0-26.7	7.2-8.8

NE= Number of eggs; GI=Growth index; MDP= Median development period; ANH= Average number of holes; PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

3.3.4 Correlation and regression analysis

The correlation coefficients (r) of cowpea resistance parameters screened are presented in Table 3.5. The percentage grain weight loss was significantly (P<0.001) positively correlated with the number of eggs (r = 0.55) and number of holes (0.54). Pest tolerance showed significant (P<0.001) negative correlations with number of eggs (-0.56), insect growth index (-0.50), number of holes (-0.66) and seed weight loss (-0.66). Dobie Susceptibility index showed significant (P<0.001) and negative correlations with insect development period (-0.63) and pest tolerance (-0.75); and positively correlated with number of eggs (0.72), growth index (0.7), number of holes (0.88) and weight loss (0. 57).

Table 3.5: Correlation coefficients (r) for cowpea genotype under *Callosobruchus maculatus* artificial infestation.

Trait	NE	GI	MDP	ANH	PWL	PPT	DSI
NE	1						
GI	0.21**	1					
MDP	-0.30**	-0.48**	1				
ANH	0.81**	0.61**	-0.42**	1			
PWL	0.55**	0.34**	-0.20**	0.54**	1		
PPT	-0.56**	-0.50**	0.29**	-0.66**	-0.66**	1	
DSI	0.72**	0.70**	-0.63**	0.88**	0.57**	-0.75**	1

NE= Number of eggs; GI=Growth index; MDP= Median development period; ANH= Average number of holes; PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

Dobie Susceptibility index was predicted by a multiple linear regression analysis which was performed with number of eggs, number of holes, seed weight loss and pest tolerance as predictor variables. The results of multiple regression analysis indicated that these variables accounted for 82.32 % of the total variability among the genotypes for their resistance to bruchids (Table 3.6), but the significant (P<.001) and better predictors of DSI were number of holes and pest tolerance (Table 3.6).

Table 3.6: The results of multiple regression analysis for cowpea genotypes under *Callosobruchus maculatus* artificial infestation.

Parameter	Regression coefficient (b)	Adjusted R-square	P-value
Regresie (Dobie susceptibility index)	3.778***		.001
NE	0.000ns		.661
ANH	0.543***	82.32	.001
PWL	0.002ns		.672
PPT	-0.021***		.001

***= significant at P< 0.001 level, ns=non-significant; NE= Number of eggs; GI=Growth index; MDP= Median development period; ANH= Average number of holes; PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

3.4 Discussion

The study demonstrated the existence of new sources of cowpea resistance to bruchids which could be used to introgress resistance into farmers' preferred but susceptible cowpea cultivars. Substantial variations were observed among the tested cowpea genotypes on their bruchids resistance parameters (Table 3.2) such as DSI (Dobie, 1974). According to Dobie (1974), the susceptibility index is linearly correlated with the intrinsic rate of increase and the logarithm of the number of insects that emerge over a given time period hence it provides a reliable estimate of resistance levels. Several studies have used Dobie susceptibility index as

a measure of resistance to cowpea bruchids (Singh *et al.*, 1985; Singh, 2002; Singh, 2005). Genotypes that were identified as resistant based on DSI included 2419, 182, WC42, WC16, NE4, WC67, WC48, ACC23 × 3B, NE39 × SEC4, ALEGI×5T, ACC2 × ACC12, 3B × 2W, SEC1 × SEC4, IT84s-2246, TVu-2027, IT97K-499-35, IT95K-207-15 and IT90K-76. Similar results were obtained by Singh *et al*. (1985); Singh (2002) and (Singh, 2005). However, IT98K-205-8 and IT82D-716, introduced from IITA, Nigeria as resistance sources were found moderately resistant to the bruchids attack, suggesting existence of bruchid biotypes which may have overcome the resistance of earlier reported resistant genotypes (Shade *et al.*, 1999).

Evidence of the resistance of cowpea genotypes to *C. maculatus* was clearly confirmed by reduced rate of oviposition in the resistant cowpea genotypes. Earlier work (Tripathi, 2012) showed a negative relationship between the number of eggs laid by bruchids and the level of resistance to bruchids, suggesting the existence of physical and/or biochemical factors which could either limit the insect from accessing the grain or make the seeds difficult for eggs to adhere to it. Sharma and Thakur (2014) also reported similar findings on the role of physical and biochemical factors of seed of resistant varieties in reducing oviposition rate. Amusa *et al.* (2014) also reported significant reduction in oviposition of bruchids on resistant cowpea genotypes.

Differences between the genotypes were apparent with the days to adult emergence. The resistant genotypes were characterized by extended adult emergence period while adult emergence in susceptible lines was rapid. In case of resistant genotypes, the time to adult emergence was long for example 44 days in case of IT84s-2246 compared to 20.8 days, for the susceptible line IT889. This was accompanied by lower growth index values observed on resistant genotypes compared to susceptible ones (Table 3.3) with the insect progeny development taking a longer time in a resistant than in susceptible genotypes (Jackai and Asante, 2003; Amusa *et al.*, 2014). This significant delay in development of *C. maculatus* on the resistant genotypes could suggest the difficulty the insect was facing to infest the seeds and to cause damage. Badii *et al.* (2013) recorded extended adult emergence and low growth index value from the resistant cowpea genotypes and reported that growth index was the most reliable indicator of resistance of cowpea to bruchids. Another reason for the delay in bruchids development on resistant varieties could be due to biochemical factor in the variety inhibiting normal development (Lattanzio *et al.*, 2005).

Number of holes is one of the reliable indicators of cowpea resistance to bruchids attack. High number of holes per seed was recorded from susceptible genotypes (4.5-7.8/seed) compared to the resistant genotypes (0-0.8/seed) (Table 3.4). This could suggest the existence of physical barrier in the seeds of resistant genotypes which could affect larval penetration (Laphale *et al.*, 2012) resulting in lowered number of holes. Similar results were reported by Appleby and Credland (2003) who observed reduced number of holes in resistant cowpea genotypes. This could also be related to the seed's biochemical compounds reducing rate of multiplication of bruchids and hence reducing the number of holes and hence suggesting antixenosis as the key component of resistance in the resistant varieties (Sales *et al.*, 2005). Oviposition cues utilized by female bruchids may be more related to the presence or absence of certain chemical factors in the seed coats of these resistant cultivars (Epino and Rejesus, 1983). As reported by Sharma *et al.* (1997) and Afzal *et al.* (2009), seed biochemicals are involved in feeding and oviposition stimulation and deterrency which renders the seed undesirable to be bad host for rather an easy invasion to the insect (Dhaliwal and Arora, 2003). It is possible that the genotypes identified as resistant in this study may have an elevated level of certain chemical deterrents or a reduced level of certain oviposition stimulants in their seed coats than the susceptible genotypes.

The result also showed wide variability among the cowpea genotypes with respect to seed weight loss (0.0% for the resistant to 44.70% for the susceptible) (Table 3.3). Low reduction in seed weight by the bruchids could be attributed to low insect growth index and seed damage. It was observed that, genotypes that had low weight loss generally had fewer eggs deposited, low growth index, reduced number of holes and increased percentage pest tolerance. It has been reported that variables such as weight loss, number of holes and growth index are the most reliable indicators for resistance of cowpea to damage by *C. maculatus* (Jackai and Asante, 2003). This study indicated that the genotypes which were least preferred by the *C. maculatus* for oviposition recorded less per cent weight loss (0-3.7%) compared to the highly preferred genotypes (16.7-44.7%) (Table 3.4). Similar reports were given by Jackai and Asante (2003) and Badii *et al.* (2013).

The extent to which the studied traits contributed to increase resistance to bruchids was given by information obtained through correlation studies supplemented by multiple regression analysis. Correlation analysis indicated weight loss was positive and significantly ($P<0.001$) associated with the number of eggs laid, average number of holes and DSI, and negatively correlated with percentage pest tolerance (Table 3.5). This suggests seeds that permit higher

number of holes leads to higher weight loss and Dobie's susceptibility value thereby resulting greater susceptibility. Similar correlation results were reported by Shade *et al.* (1999).

The results also indicated that number of eggs and number of holes and weight loss could be used as reliable indicators for identifying cowpea genotypes resistant to bruchids damage. Dobie susceptibility index showed significant ($P<0.001$) positive correlation with average number of holes but negatively correlated with percentage pest tolerance and median development period. This indicates that the longer the insect development period, the lesser the seed weight loss during storage due to low rate of insect multiplication as confirmed by a lower number of holes compared to susceptible genotypes. Similar results were reported by Shade *et al.* (1999), Lephale *et al.* (2012); Tripathi (2012) and Amusa *et al.* (2014) on cowpea and Mwila (2013) on common beans.

The results of multiple regression analysis also indicated that number of holes and pest tolerance were major contributors for genotypic variation (Table 3.6). The positive correlation between number of holes and number of eggs indicated that these two traits could be controlled by similar, overlapping, linked genetic loci (Acquaah, 2012). This information could guide breeders on how to improve resistance in cowpea genotypes by focusing on reducing number of holes and eggs. The regression and correlation results also indicated that the number of holes and pest tolerance could be considered essential while selecting bruchids resistant genotypes, because they had strong correlations and higher contributions to variation of genotypes for their resistance to bruchids attack.

CHAPTER FOUR

BIOCHEMICAL BASIS OF COWPEA RESISTANCE TO THE BRUCHIDS (*CALLOSOBRUCHUS MACULATUS* (F.)

4.1 Introduction

Understanding the mechanisms of resistance of cowpea to bruchids (*Callosobruchus maculatus* F.) is one of the key pre-requisites for designing effective and efficient strategies to breed resistant genotypes. Many studies have indicated that chemical factors, such as phenolic acids, tannins, and flavonoids and α-amylase inhibitor may confer cowpea resistance to *C. maculatus* (Ojwang *et al.*, 2012). For example, Lattanzio *et al.* (2005) reported elevated levels of α-amylase inhibitor as the main mechanism for resistance to bruchids. Gatehouse *et al.* (1979) reported elevated levels of trypsin inhibitor in seeds of TVu-2027 were responsible for resistance to *C. maculatus*. In contrast, Baker *et al.* (1989) found no significant correlation between the levels of trypsin inhibitor and *C. maculatus* development time or mortality.

There are also conflicting reports on the effect of seed coat tannin content on the oviposition and survival of *C. maculatus*. For instance, Lale and Makoshi (2000) reported a positive association whereas Lattanzio *et al.* (2005) found no significant association between seed coat tannin content and seed resistance to bruchids. Edde and Amatobi (2003), in their experiments using 22 cowpea varieties also, found no significant association between number of eggs deposited, adult mortality and mean developmental periods on cowpea with and without seed coat suggesting that seed coat had no value in protecting cowpea seeds against attack by *C. maculatus,* and the resistance factors were carried in the cotyledon and embryo of the seed.

It seems therefore that cowpea seeds do not rely on one type of chemical defense only, implying that resistance might be due to the accumulation of several biochemicals. Direct evidence of the protective roles of these compounds against bruchids is, however, limited. In view of this, the aim of the present study was to examine the influence of biochemical attributes including protein, carbohydrate, phenolic acids and their anti- oxidant activity, flavonoid, tannins and α-amylase inhibitory activities in three resistant and four susceptible cowpea genotypes in resistance to *C. maculatus*.

4.2 Materials and Methods

Seven cowpea genotypes differing in response to bruchids infestations were used in this study (Table 4.1). These genotypes were identified out of 145 cowpea genotypes that were screened for response to bruchids under no-choice laboratory bioassay (Miesho *et al.* 2018a). All the genotypes except TVu-2027 were procured from the Makerere University Regional Centre for Crop Improvement (MaRCCI).

Table 4.1: Materials used in the study

Genotypes	NE	MDP (days)	GI	ANH	PWL	PPT (%)	DSI	Resistance status	Cultivar type	Origin
IT109	124	21.5	2.92	7.8	27.6	0	8.8	Susceptible	Improved	IITA
SECOW2W	87.3	22.8	3.92	7.7	24.2	0	8.3	Susceptible	Improved	Uganda
WC69	141	23	2.42	7.7	35.9	0	8.2	Susceptible	Landrace	Uganda
IT71	87	22.8	3.56	6.9	44.7	0	8.1	Susceptible	Inbred line	IITA
WC42	17.3	32	0.23	0.1	0.5	90	0.3	Resistant	Landrace	Uganda
TVU 2027	7	42	0.37	0.1	0	93.3	0.2	Resistant	Improved	Nigeria
2419	39.7	42	0.03	0	0	96.7	0	Resistant	Landrace	Uganda

NE: Number of eggs, MDP: Median development period, GI: Growth index ANH: Average number of holes, PWL: percentage weight loss; PPT: percentage pest tolerance and DSI: Dobie susceptibility

4.2.1 Experimental design and data collection

Three replicates of 40g seeds from each genotype were used for estimation of seed coat and cotyledon biochemicals. To separate seed coat from cotyledons, the seeds were soaked in phosphate buffered saline for one day and dehulled manually. The experiment was laid in a completely randomized design (CRD) replicated thrice.

Extraction and quantification of biochemical parameters

A. Seed coat biochemical analysis

i. Anti-oxidant assay of extracted phenolics

The seed coat biochemical attributes were assessed by refluxing defatted seed coat flour in 80% hexane using a soxlet apparatus (Sreerama *et al.*, 2012). Total phenolics, flavonoids, free and condensed tannins were extracted, quantified and recorded. Total phenolic content was extracted with 80% aqueous methanol containing 1% HCl (1:50, w/v) by refluxing in a boiling water bath for 30 minutes. The refluxed material was concentrated under vacuum in a rotary flash evaporator (RU 10 C SO99, IKA, Germany) and used for determining the total phenolic content (TPC) (Sreerama *et al.*, 2012) and total flavonoid content (TFC) (Sreerama *et al.*, 2012). The TPC of each extract was determined using the method described by

Chandrasekara and Shahidi (2010) and the contents expressed in mg of gallic acid equivalents (GAE) per gram of defatted flour (Sreerama *et al.*, 2012). The TFC of flour was measured by the aluminium chloride colorimetric assay method described by Kim *et al.* (2003) by reading the absorbance at 510 nm (Biowave ii+, Cambridge, England). Total flavonoid content of flour was expressed as mg of catechin equivalent (CE) per gram of defatted flour.

ii. Free and condensed tannins

To determine free and condensed tannins, about 0.1g of cowpea defatted seed coat flour was placed in a 1.5ml Eppendorf tube and 0.5ml acetone (70%): ascorbic acid (1%) solution was added. The solution was shaken for 20 minutes using an orbital shaker (Unimax 1010 DT, Germany). Thereafter, petroleum ether (0.5ml) was added and the solvent left to evaporate. Distilled water (0.3ml) was then added and the sample centrifuged at 10 rpm for 10 min. An aliquot of 0.1ml was taken and 0.4ml of HCl-butanol solution (5%v/v) added. The tube was placed in a water bath at 80^0C for 70 minutes. Absorbance was then read at 550 nm to quantify the amount of free tannins. For condensed tannins, the remaining solution was drained from the tubes and distilled water (0.2ml) and 0.8ml of acid-butanol solution were added and the tube placed in a water bath (80^oC) for 70 minutes. Absorbance, which is directly proportional to the tannin content, was read at 550 nm (Biowave ii+, Cambridge, England).

iii. Ferric ion-reducing capacity assay (FRC)

The extracted phenolics were assayed for their antioxidant activities following the method of Pownall *et al.* (2010), with slight modifications. Briefly, various sample dilutions (500 µL) in 50 mM phosphate buffer (pH 7.0) were mixed with 250 µL of 1% (w/v) potassium ferricyanide solution and incubated for 20 min at 50ºC. Thereafter, 500 µL of 10% (v/v) trichloroacetic acid was added and centrifuged at 3000 r/minute for 10 minutes. The supernatant was mixed (500 µL) with 100µL of 0.1% (w/v) ferric chloride (freshly prepared) and 500 µL of distilled water followed by additional incubation for 10 min, absorbance was immediately measured at 700 nm (Biowave ii+, Cambridge, England) against a blank consisting of phosphate buffer and the appropriate volume of solvent, treated in the same manner. The results were expressed as absorbance units at 700 nm, which was considered as a measure of reducing power (Stanisavljević *et al.*, 2014). Higher absorbance of the reaction mixture indicated greater reducing power (Stanisavljević *et al.*, 2014).

B. Cotyledon biochemical analysis

i. Total protein assay

Total protein was quantified using the Bradford method (Bradford, 1976). A sample of 0.1g of cotyledon flour was added into a falcon tube containing 5ml of distilled water. The solution was agitated for 30 minutes at 50^{o}C on a thermomixer (Eppendorf AG, Hamburg, Germany). From this protein solution, 0.1 ml was pipetted off and stained with 3 ml of Bradford reagent (Commassie brilliant blue + 95% Ethanol + 85% Phosphoric acid + Deionized water). The stained proteins were quantified by measuring absorbance in a spectrophotometer (Biowave ii^{+}, Cambridge, England) at a wave length of 595nm against standard albumin.

ii. Total carbohydrate

Total carbohydrate was determined as total starch and sugar content by hydrolysis of 0.1g sample with 5ml of 10% (v/v) Sulphuric acid at 80^{o}C in a waterbath (Grant TXF 200, England) for 30 minutes. The sample was left to cool to room temperature and the resultant sugar as well as the original sugar was quantified using the method of Dubois *et al.* (1956). 0.5 ml of solution was diluted with 1ml of deionized water and dehydrated using 1ml of concentrated sulphuric acid. The resulting furfural compound was estimated by adding 0.5ml of 5% phenol and the resulting coloured compounds quantified by measuring its absorbance at a wavelength of 490 nm in a spectrophotometer (Biowave ii^{+}, Cambridge-England) against a starch soluble standard.

iii. The α-amylase inhibitors assay

To determine the α-amylase inhibitory activity, ten grams of finely ground de-hulled seeds were incubated in an eppendorf thermomixer (Eppendorf AG, Hamburg, Germany) at 200 rpm with 0.15M NaCl (1:5, w/v), followed by centrifugation at 12100 × *g* for 60min (Lattanzio *et al.*, 2005). The supernatant was buffered by adding 0.2M Na-succinate, 0.1M CaCl2 (pH 3.8) (110 µlml−1) and was heated in a water bath at 70^{o}C for 15min. The protein precipitate was removed by centrifugation (12100 × *g* for 60min) and the clear supernatant was brought to pH 5.6 with NaOH. Ethanol (19% final concentration) was added to this solution and the mixture was stirred for 3.5h at 4^{o}C and then centrifuged (Moreno *et al.*, 1990). The protein inhibitory activity of *α*-amylase inhibitor extracts was determined using the Bradford method using bovine serum albumin as a standard (Bradford, 1976). A sample of 10µl of extracted α-amylase inhibitors was added to 50µl of standard *α*-amylase enzyme

(extracted from *Aspergillus Oryzea* and supplied from SIGMA) in a total volume of 1.2 ml of barbital buffer solution, pH 6.5. The mixture was incubated at 37^{o}C for 10 minutes followed by the addition of 0.2ml of substrate solution (0.1% potato starch solution in water). After incubation at 20^{o}C for 10 min, the reaction was stopped with 0.2ml 3M HCl. The undigested starch was determined by adding 0.4ml of potassium iodide (I2–KI) solution (1.2 and 1.8mM, respectively) and by measuring the change in absorbance at 620 nm. Controls without inhibitors were included to determine the amylase activity of each preparation (expressed as amylase units, i.e., the amount of enzyme that gave 50% hydrolysis of the added starch) (Silano *et al*., 1975). The α-amylase inhibitory activity (percentage of control) was expressed as a percentage of the α-amylase activity values in the absence of pre-incubation with the seed extract (Lattanzio *et al.*, 2005).

4.2.2 Data analysis

A one way analysis of variance (ANOVA) was used to test differences in biochemical characteristics among cowpea genotypes. Least significant difference (LSD) test was used to separate means. Pearson correlation was used to examine the association between each biochemical characteristic and seed resistance to bruchids parameters (number of eggs, median development period, growth index, average number of holes, percentage weight loss, percentage pest tolerance and Dobie susceptibility) for the studied genotypes. All analyses were conducted using GenStat Discovery, 16.1th Edition statistical package.

4.3 Results

4.3.1 Cowpea seed metabolites associated with resistance to bruchids

The results of seed coat and cotyledon biochemicals are presented in Table 4.2.

There were significant differences ($P< 0.001$) in cotyledon (total protein, total carbohydrate and α- amylase inhibitor activity) and seed coat biochemical factors (free tannins, condensed tannins, phenolic compounds and their ferric ion-reducing capacity assay (FRC), and flavonoids) among the cowpea genotypes.

Table 4.2: Mean squares for differences in seed biochemical traits among the studied cowpea.

Source of variation	df	Mean squares of traits							
		CT	FT	FLVN	TPC	FRC	Protein	Carb	α-AIs
Genotype	6	11.4***	0.44***	1.07***	278.03***	1498.08***	86.68***	43.84***	360.93***
Residual	14	0.06	0.00	0.00	1.70	0.06	0.44	0.31	7.76

CT= condensed tannins, FT= free tannins, FLVN= flavonoids, TPC= Total phenolic compounds, FRC= Ferric ion-reducing capacity assay of the extracted phenolic compounds, Carb= Carbohydrate, and α-AIs= α-amylase inhibitory activity. *** P<0.001

4.3.2 Cowpea seed anti-nutritional contents and their effect on *C. maculatus*

The cowpea genotypes showed significant differences in their seed anti-nutritional (seed coat biochemicals and α-amylase inhibitory enzyme) contents (Table 4.3).

Table 4.3: Estimates of seed biochemical constituents in resistant and susceptible cowpea genotypes to bruchids.

	Seed coat biochemicals					**Cotyledon biochemicals**		
Genotype	CT mg TA/g	FT mg TA/g	FLVN %GAE	TPC mg GAE/g	FRC	Protein %	Carb %	α-AIs (µl)
IT109	4.71	1.18	0.67	15.04	22.74	29.0	61.9	24.59
SECOW2W	4.37	2.19	0.83	13.21	27.56	28.4	63.5	32.53
WC69	7.62	1.10	1.80	13.32	76.76	19.4	64.3	44.97
IT71	2.91	1.38	0.84	23.67	25.61	14.8	59.0	33.28
WC42	7.10	1.16	1.83	38.48	67.42	22.2	69.5	17.04
TVu-2027	3.03	1.14	0.70	11.09	27.02	27.5	65.5	20.79
2419	3.16	1.27	0.26	15.57	33.12	26.9	65.0	13.35
LSD	0.45	0.10	0.09	2.28	0.44	1.2	1.0	4.88
% CV	5.40	4.30	5.20	7.00	0.60	2.8	0.83	2.72

CT= condensed tannins, FT= free tannins, FLVN= flavonoids, TPC= Total phenolic compounds, FRC= Ferric ion-reducing capacity assay of the extracted phenolic compounds, Carb= Carbohydrate, and α-AIs= α-amylase inhibitory.

Susceptible genotype WC69 (7.62mgGA/g) had the highest amount of condensed tannins whereas the lowest (2.91mgGA/g) was recorded also from susceptible genotype IT71. The amount of free tannins ranged from 1.10 mgGA/g (susceptible, WC69) to 2.19mgGA/g in susceptible genotype SECOW2W. Flavonoids ranged from 0.26%GAE/g in resistant genotype 2419 to 1.83%GAE/g in resistant genotype WC42. The highest phenolic compound was recorded from resistant genotype WC42 (38.48mgGAE/g) and the lowest from resistant genotype TVu-2027 (11.09mgGAE/g). The highest Ferric ion-reducing capacity assay (FRC) was recorded from susceptible genotype WC69 (76.76) and the lowest again from susceptible IT109 (22.74). Highest α-amylase inhibitory activity was recorded on resistant genotype 2419 (13.356µl) and the lowest was recorded from susceptible genotype WC69 (44.97µl) (Fig 4.1A).

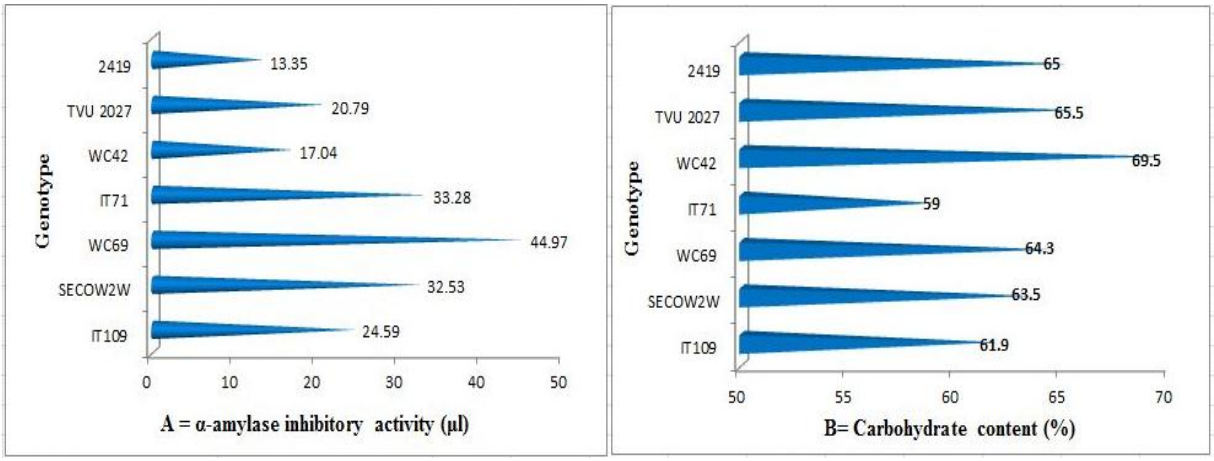

Figure 4. 1: Mean values of α-amylase inhibitor activity and carbohydrate content (%). A=µl of α-amylase inhibitor that gives 50% inhibition of insect α-amylase enzyme; and B= carbohydrate content in the studied cowpea genotypes.

4.3.3 Cowpea seed nutritional contents and resistance to *C. maculatus*

The studied cowpea genotypes varied significantly ($P < 0.001$) in their seed nutritional contents, protein and carbohydrate contents, (Table 4.3). The highest protein content was recorded from susceptible genotype IT109 (29.0%) whereas the lowest was obtained from another susceptible genotype IT71 (14.8%). The highest total carbohydrate content was recorded from resistant genotype WC42 (69.5%) and the lowest from susceptible genotype IT71 (59.0 %) (Fig. 4.1 B).

4.3.4 Relationship among cowpea biochemical attributes and resistance parameters to bruchids

The extent to which the studied traits contributed to increase resistance to bruchids was given by information obtained through correlation studies. Total carbohydrate content was positively and significantly ($P<0.001$) correlated to median development period ($r = 0.82$) and pest tolerance ($r = 0.79$); and negatively correlated to PWL ($r = -0.85$), number of eggs ($r = -0.72$), average number of holes ($r = -0.78$), insect growth index ($r = -0.79$) and Dobie susceptibility index ($r = -0.79$) (Table 4.4).

Table 4.4: Correlation between cowpea seed biochemical traits with phenotypic bruchids resistance parameters.

Traits	CT	FT	FLNDS	TPC	FRC	Protein	carb	α-AIs
PWL_	0.09^{ns}	0.19^{ns}	0.14^{ns}	-0.17^{ns}	-0.04^{ns}	-0.55^{**}	-0.85^{***}	0.81^{***}
NE	0.32^{ns}	0.11^{ns}	0.16^{ns}	-0.35^{ns}	0.11^{ns}	-0.20^{ns}	-0.72^{***}	0.75^{***}
MDP	-0.22^{ns}	-0.35^{ns}	-0.07^{ns}	0.06^{ns}	-0.01^{ns}	0.24^{ns}	0.82^{***}	-0.55^{**}
ANH	0.16^{ns}	0.38^{ns}	0.11^{ns}	-0.32^{ns}	-0.08^{ns}	-0.23^{ns}	-0.78^{***}	0.80^{***}
PPT	-0.15^{ns}	-0.37^{ns}	-0.12^{ns}	0.27^{ns}	0.09^{ns}	0.27^{ns}	0.79^{***}	-0.80^{***}
GI_value	-0.03^{ns}	0.57^{**}	-0.00^{ns}	-0.25^{ns}	-0.27^{ns}	-0.21^{ns}	-0.79^{**}	0.71^{***}
DSI	0.14^{ns}	0.36^{ns}	0.09^{ns}	-0.29^{ns}	-0.11^{ns}	-0.23^{ns}	-0.79^{***}	0.78^{***}

Ns = non-significant; values with *, ** and *** implies significant at $P = .05$, $P < .01$ and $P < .001$ respectively

The α-amylase inhibitory activity was positively and significantly (P<0.001) correlated to the percentage weight loss (0.81), NE (0.75), average number of holes (r = 0.80), insect growth index (r = 0.71) and Dobie susceptibility index (r = 0.78); and negatively correlated to median development period (r = -0.55) and percentage pest tolerance (r = 0.80).

4.4 Discussion

Results of the study confirm the importance of certain biochemical attributes for cowpea resistance against cowpea bruchids. Among the biochemical attributes, only total carbohydrate content and α-amylase inhibitory activity of the seeds were associated with resistance to bruchids (Table 4.4). Although all the analyzed genotypes contained varied quantities of proteins, condensed and free tannins, flavonoids and phenolic compounds and ferric ion-reducing capacity assay, none of them was associated with seed resistance to bruchids (Table 4.4). For example, cowpea line TVu-2027, an accession classified as bruchids resistant line, showed low concentrations of condensed and free tannins, whereas WC69 and SECOW2W, the susceptible lines contained very high concentrations of condensed and free tannins. Additionally, the highest and lowest flavonoid contents were recorded only from resistant genotypes WC42 and 2419, respectively. Likewise, the highest phenolic compounds and ferric ion-reducing capacity were recorded for resistant genotypes WC42 and TVu-2027, respectively (Table 4.3). The lack of correlation between all of the individual seed coat biochemical attributes and the seed resistance parameters found in this study showed that seed coat biochemical attributes were not effective barriers against *C. maculatus*. Similar results were reported by Baker *et al.* (1989) and Lattanzio *et al.* (2005). Edde and Amatobi (2003) also, found no significant correlation between numbers of eggs deposited, adult mortality and mean development periods on cowpea seeds with and without

seed coat. These findings, as well as the results of this study suggest, that the seed coat may not be a useful aspect to consider for breeding of cowpea resistant to bruchids but rather that the resistance factors are carried in the cotyledon of the seed.

Additionally, the results of the study provides strong evidence that elevated levels of α-amylase inhibitory activity in the cotyledons of cowpea genotypes is responsible for conferring resistance of cowpea genotypes to *C. maculatus* (Fig. 4.1 A). Previous work by Lattanzio *et al.* (2005) also found a positive relationship between Dobie susceptibility index and the level of α-amylase inhibitory activity, suggesting the negative effect of α-amylase inhibitory activity on bruchids growth and development. Also, α-amylase inhibitory activity has been shown to prolong insect developmental period, cause reduction in the levels of adult emergence and retardation in insect growth by inhibiting enzymes responsible for starch digestion resulting in carbohydrate starvation (Macedo *et al.*, 2004).

Amongst the studied cowpeas, the concentration of α-amylase enzyme in 2419, a genotype showing resistance to the pest, was found to be about three times higher than in WC69, a susceptible genotype (Fig. 4.1A). When the amount of α-amylase inhibitor was considered, resistant genotypes 2419 and WC42 required 13.35 and 17.04 µl levels of α-amylase inhibitors to inhibit 50% of the insect α-amylase enzyme, respectively. On the other hand, susceptible genotypes IT71 and WC69 needed 33.28 and 44.97µl of α-amylase inhibitor. The results also showed that, amongst the different genotypes of the same resistance level, a great variability in the inhibitory activity could be detected, indicating the existence of different types of α-amylase inhibitor in the tested genotypes (Franco *et al.*, 2002). Lattanzio *et al.* (2005) working on cowpea and Wisessing *et al.* (2010) on mungbeans had reported α -amylase inhibitory activity as the main factor of seed defense against bruchids infestation.

The higher total carbohydrate content in seeds of resistant genotypes compared to those of susceptible ones (Fig. 4.1 B) is an indication that carbohydrate content could also offer seed defense against bruchids infestation. Carbohydrate content increases resistance by increasing seed hardness (Ajeigbe *et al.*, 2008), thereby making seed penetration by the insect difficult. Greater roles of carbohydrate in imparting seed resistance to bruchids damage was fully reflected by its strong correlation with seed resistance parameters and Dobie susceptibility index, a measure of resistance to bruchids damage (Dobie, 1974) (Table 4.4).

Correlation analysis results also confirmed the role of α-amylase inhibitory activity and seed carbohydrate content in conferring resistance to bruchids. The negative correlation relationship between α-amylase inhibitory activity and median development period, one component of DSI, indicated that prolonging insect development period might be due to α-amylase inhibition. Similarly, the negative relationship between carbohydrate content and median development period also could indicate that carbohydrate is responsible for prolonging insect development period in addition to offering physical barrier. On the other hand, the negative association between seed carbohydrate content and α-amylase inhibitory activity suggest that the two traits are controlled by different, overlapping; linked genetic loci (Acquaah, 2012).The results of this study will help to guide breeders on how to improve resistance in cowpea genotypes by focusing on elevating the levels of seed α-amylase inhibitory activity and crude carbohydrate contents.

CHAPTER FIVE

INHERITANCE AND COMBINING ABILITY OF COWPEA RESISTANCE TO BRUCHIDS (*CALLOSOBRUCHUS MACULATUS* F.)

5.1 Introduction

To develop an appropriate breeding strategy, the search for sources of resistance to bruchids in cowpea must be followed with the study of the inheritance of resistant genes. In an earlier study by Miesho *et al.* (2018a), 18 bruchids resistant genotypes were identified from local and introduced cowpea genotypes; for example, 2419, IT84s-2046 and TVu-2027. However, knowledge regarding the genetic control and heritability of the resistance to *C. maculatus* was not studied and yet it is needed to be able to optimize breeding pipeline for bruchids resistance (Barelli *et al.*, 1999; Viana *et al.*, 1999).

Previous genetic studies using TVu-2027 as donor suggested that maternal genes were involved in the inheritance of resistance to bruchids (Dobie, 1981). The same study highlighted involvement of a major recessive gene and modifiers, and also noted that either dominant or interactive effects were more important than additive types of gene effects (Dobie, 1981). Redden *et al.* (1983) also reported paternal and embryonic genotypic effect in certain backcross combinations of F_3 generation and digenic control of resistance in one of their cross and monogenic control in another cross, in conjunction with one or more modifier or minor gene loci. In contrast, Adjadi *et al.* (1985) reported that resistance to bruchids resulted from two recessive genes.

In Uganda, studies on inheritance of resistance to bruchids are scarce. It is important to understand the heritability of resistance to bruchids character and the gene action controlling it to help breeders select suitable parents for a breeding program. Therefore, the aims of the present study were to estimate the level of inheritance and identify mode and estimate the gene effects as well as identify parents and crosses with good combining abilities for cowpea resistance to bruchids under the Uganda growing condition.

5.2 Materials and Methods

5.2.1 Experimental procedures and diallel mating scheme

Nine cowpea genotypes comprising of five resistant (IT90K-76, IT97K-499-35, TVU-2027, 2419 and IT84s-2246) and four susceptible (SECOW2W, WC69, MU9 and SECOW5T) lines

selected from the study reported earlier (Chapter Three) were used as parents (Table 5.1). These genotypes were selected based on their adaptation to wider agro-ecology, preference by farmers and resistance to other biotic and abiotic stresses.

Table 5.1: Characteristics of the genotypes used in 9 × 9 diallel mating.

Genotype	Cultivar type	Origin	Yield potential (Tonnes/ha) #	Resistance Rating*
SECOW2W	Improved	Uganda	2.222	Susceptible
WC69	landrace	Uganda	1.481	Susceptible
MU9	landrace	Uganda	0.922	Susceptible
SECOW5T	Improved	Uganda	2.741	Susceptible
IT90K-76	Improved	IITA	-	Resistant
IT97K-499-35	Improved	IITA	-	Resistant
TVU-2027	Improved	IITA	-	Resistant
2419	landrace	Uganda	0.505	Resistant
IT84s-2246	Improved	IITA	-	Resistant

Yield data according to Afutu *et al.*, 2016; *Resistance rating according to Miesho *et al.*, 2018a; WC= Western and Central, MU= Makerere University and IT= International Institute of Agricultural Research

The nine cowpea parental lines were each planted separately in a five-litter bucket (two seeds per bucket) in December 2015 in a screen house at Makerere University Agricultural Research Institute Kabanyolo (MUARIK), Uganda, (0°28'N and 32°37'E, approximately 1200 m a. s. l.). Each line was hand emasculated before pollen shedding and crossed at flowering in all possible combinations following Griffing's (1956) Method 1 approach to produce 36 F_1 plants and 36 reciprocal crosses. The F_1 seeds and the reciprocal crosses were selfed to produce F_2 generation in a screen house. The F_1 seeds were planted along with their parents to identify true crosses. The F_2 seeds were harvested and bulked for each of the 36 crosses and 36 reciprocal crosses.

5.2.2 Bruchids laboratory culture

Bruchids used in this study were cultured and multiplied in accordance with the procedure outline in Chapter 3 (Section 3.2.1) of this thesis.

5.2.3 Screening of cowpea seeds for resistance to *C. maculatus*

To evaluate for resistance to bruchids, 10 F_2 generation seeds of each of the 36 F_1 and 36 reciprocal crosses and the nine parents were weighed and separately put in a petri-dish of size 90mm diameter and 10mm depth. Thirty seeds were randomly selected from each of the

bulked F_2 and parental seeds and oven dried at 40^{o}C for 24 hours to destroy any insects or eggs that could have been present and to standardize moisture levels of the seeds (Amusa *et al.*, 2014). The experiment was laid in randomized complete block design with three replications. Time was used as blocking factor and infestation was done once to each replicate at an interval of eight days in order to ease data collection.

To each petri dish containing the ten seeds, two pairs of three-day old male and female adult bruchids from laboratory culture were introduced and the top covered to prevent the insects from escaping. The insects were left undisturbed in the petri-dishes for three days to allow for mating and oviposition and then removed (Amusa *et al.*, 2013). Data on the different bruchids resistance traits were collected and Dobie susceptibility index calculated following the methods described in section 3.2.3. Accordingly, data were collected on number of eggs, number of emerged insects, average number of holes, median development period, and percentage weight loss, percentage pest tolerance and Dobie susceptibility index computed.

5.2.4 Data analysis

General analysis of variance, using GenStat Discovery, 16.1th Edition statistical package was performed for all quantitative data.

Diallel analysis was performed for number of eggs, insect emergence and median development period of the populations developed by Griffing's (1956) Method 1 using Genetic Designs in R (AGD-R) Version 3.0 (Rodríguez *et al.*, 2015). In this model, genotypes were considered as a fixed effect whereas replication effects were regarded as random.

Estimation of heritability, general and specific combining ability, reciprocal and maternal effects, and Bakers ratio

The general combining ability (GCA) effects were analysed for each parent. Specific combining ability (SCA) and reciprocal effects were analysed for the F_2 crosses and their reciprocals, respectively. Similarly, maternal effects were analysed for each parent. Coefficient of genetic determination in the narrow (CGD-NS) and broad sense (CGD-BS), analogues of the narrow sense (h^2) and broad sense heritability (H^2), respectively were also estimated. All the analysis was done using Genetic Designs in R (AGD-R) Version 3.0 (Rodríguez *et al.*, 2015). Confirmation of the adequacy of the additive and non-additive variances was estimated (Baker, 1978).

5.3 Results

There were significant differences in the responses of the parents and the F_2 segregating cowpea populations to bruchids infestation for all the traits measured (Table 5.2).

Table 5.2: Analysis of variance for resistance of cowpea genotypes to *C. maculatus*.

Source of variation	df	NE	NEI	ANH	MDP	PWL	PPT	DSI
Genotype	80	1831.45^{***}	888.50^{**}	9.14^{***}	155.00^{***}	543.23^{**}	2766.76^{**}	16.45^{**}
Replication	2	16.807^{ns}	5.94^{ns}	0.75^{*}	4.49^{*}	0.45^{ns}	153.10^{ns}	0.95^{***}
Residual	160	7.32	3.55	0.64	1.45	1.96	61.42	0.11

NE= Number of eggs, NEI= Number of emerged insects, ANH= Average number of holes, MDP= Median development period, PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index. ***, **, * and ns; significant at $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.05$ and non-significant, respectively.

The observed significant differences among progenies were for number of eggs laid (NE), number of holes per seed (ANH), median development period (MDP); and adult bruchids emergence (NEI), percentage seed weight loss (PWL), percentage pest tolerance (PPT) and Dobie susceptibility index (DSI).

Total number of genotypes identified as susceptible, moderately resistant and resistant based on the DSI value were 15, 38 and 28, respectively (Table 5.3), indicating the continuous distribution of resistance in different classes.

Table 5.3: Evaluation of F_2 generation and parental seeds for resistant to bruchids.

Genotypes	Type of cross	NE	NEI	ANH	MDP	PWL	PPT	DSI	Resistance status
2419 × IT84S-2246	R×R	0	0	0	44	0	100	0	
IT84S-2246	Parent	0	0	0	44	0	100	0	
IT84S-2246 × 2419	R×R	0	0	0	44	0	100	0	
TVu-2027	Parent	4	0	0	44	0	96.67	0	
TVu-2027 × 2419	R×R	14	0	0	44	0	100	0	
TVu-2027 × IT97K-499-35	R×R	0	0	0	44	0	100	0	
2419	Parent	43	0.67	0.07	40.67	0.09	96.67	0.24	
IT84S-2246 × TVu-2027	R×R	6	2	0.2	33	0.1	86.67	0.91	
IT97K-499-35	Parent	21	2	0.2	26	2.68	83.33	1.16	
TVu-2027 × IT84S-2246	R×R	12	3	0.3	38	4	70	1.26	
WC69 × 2419	S×R	5	2	0.2	21	5	70	1.43	R
2419 × MU9	R×S	13	4	0.4	37	3.47	73.33	1.63	
IT97K-499-35 × TVu-2027	R×R	12	4	0.4	36	1.66	76.67	1.67	
TVu-2027 × SECOW2W	R×S	16	5	0.5	34	5	60	2.06	
IT84S-2246 × IT90K-76	R×R	12	4	0.4	27	1.3	73.33	2.23	
IT90K-76	Parent	13	5	0.5	31	1.19	53.33	2.25	
TVu-2027 × IT90K-76	R×R	12	5	0.5	28	1	76.67	2.5	
2419 × IT90K-76	R×R	43	12	1.23	42.33	7.7	40	2.55	
2419 × SECOW2W	R×S	12	8	0.8	34.67	3.94	50	2.6	
2419 × SECOW5T	R×S	12	9	0.8	38	2.37	56.67	2.63	

Genotypes	Type of cross	NE	NEI	ANH	MDP	PWL	PPT	DSI	Resistance status
2419 × WC69	R×S	25	10.67	1.07	37.67	6.36	43.33	2.72	
IT90K-76 × 2419	R×R	30	10.67	1.07	37.5	14.89	16.67	2.74	
2419 × IT97K-499-35	R×R	43	13.67	1.33	41.17	9.3	30	2.76	
2419 × TVu-2027	R×R	8	5	0.5	25	2.13	66.67	2.8	
IT90K-76 × IT84S-2246	R×R	10	7	0.7	30	2.99	73.33	2.82	
IT97K-499-35 × IT84S-2246	R×R	29	12	1.2	27.67	3.95	60	2.98	
IT90K-76 × TVu-2027	R×R	26	10	1.03	33.5	17.7	30	2.98	
IT84S-2246 × IT97K-499-35	R×R	14	9	0.9	32	2.1	60	2.98	
TVu-2027 × WC69	R×S	14	12	1.2	34	4	53.33	3.17	
IT84S-2246 × MU9	R×S	45	24	2.3	41	6.8	20	3.37	
WC69 × IT90K-76	S×R	9	7	0.7	24	5	70	3.52	
IT97K-499-35 × SECOW5T	R×S	14	10	1	28	12.32	10	3.57	
TVu-2027 × SECOW5T	R×S	26	18	1.8	35	23	20	3.59	
IT84S-2246 × SECOW2W	R×S	22	12	1.2	30	5	36.67	3.6	
IT90K-76 × SECOW5T	R×S	15	7	0.7	23	3.74	76.67	3.67	
IT90K-76 × SECOW2W	R×S	20	13	1.3	28	10.11	40	3.98	
IT84S-2246 × WC69	R×S	32	13	1.3	27	18.49	20	4.13	
IT97K-499-35 × MU9	R×S	13	11	1.1	25	7.93	33.33	4.17	
IT97K-499-35 × SECOW2W	R×S	20	10	1	24	4.32	26.67	4.17	
SECOW2W × IT84S-2246	S×R	33	23	2.3	31	14.51	40	4.39	
IT97K-499-35 × IT90K-76	R×R	20	15	1.5	26	14.51	20	4.52	
SECOW5T × IT90K-76	S×R	38	10	0.8	22	8	50	4.55	
SECOW5T × 2419	S×R	28	14	1.4	25	14.18	50	4.58	
TVu-2027 × MU9	R×S	42	24	2.2	30	11	16.67	4.6	
IT90K-76 × IT97K-499-35	R×R	38	20	2	27	6.53	23.33	4.82	MR
SECOW5T × IT97K-499-35	S×R	29	14	1.2	23	7	46.67	4.98	
IT90K-76 × MU9	R×S	24	18	1.8	25	17.5	6.67	5.02	
SECOW2W × TVu-2027	S×R	32	15	1.6	23	5.42	43.33	5.11	
SECOW5T × IT84S-2246	S×R	34	19	2	25	23	36.67	5.12	
IT90K-76 × WC69	R×S	53	25	2.5	27	7.23	33.33	5.18	
IT97K-499-35 × WC69	R×S	44	26	2.5	27	37.29	0	5.24	
WC69 × IT97K-499-35	S×R	42	21	2.1	25	12	0	5.29	
IT84S-2246 × SECOW5T	R×S	48	23	2.4	25	25	20	5.45	
SECOW2W × IT97K-207-15	S×R	60	27	2.4	25	25.79	26.67	5.73	
IT84S-2246 × SECOW5T	R×S	48	23	2.4	25	25	20	5.45	
SECOW2W × IT97K-207-15	S×R	60	27	2.4	25	25.79	26.67	5.73	
MU9 × TVu-2027	S×R	42	32	3.1	26	16	20	5.79	
WC69 × TVu-2027	S×R	45	35	3.4	26.5	22	16.67	5.83	
SECOW2W × 2419	S×R	40	27	2.9	24.5	15.63	30	5.84	
SECOW5T × TVu-2027	S×R	43	22	2	23	20	23.33	5.84	
IT97K-499-35 × 2419	R×R	45	29	2.9	24	24.81	3.33	5.94	
WC69 × IT84S-2246	S×R	42	38	3.8	26.5	20	16.67	5.96	
SECOW2W × IT90K-76	S×R	42	29	2.9	24	22.44	20	6.09	
SECOW2W × MU9	S×S	50	31	3	24	37.65	20	6.21	
MU9 × IT97K-499-35	S×R	35	27	2.6	23	11	40	6.22	
MU9 × IT90K-76	S×R	61	35	3.5	24	22	16.67	6.43	

Genotypes	Type of cross	NE	NEI	ANH	MDP	PWL	PPT	DSI	Resistance status
MU9 × SECOW2W	S×S	63	49	5	25.5	50	3.33	6.63	
MU9 × 2419	S×R	74	39	3.9	23	12	20	6.92	
SECOW5T × WC69	S×S	45	36	3.5	22	38	3.33	7.07	
MU9 × IT84S-2246	S×R	37	33	3.3	21	13	40	7.23	
MU9 × SECOW5T	S×S	57	55	5.4	24	22	3.33	7.25	
SECOW2W × SECOW5T	S×S	60	51	5.13	23.5	26	10	7.27	
SECOW5T × MU9	S×S	49	36	3.4	21	22.36	0	7.41	
WC69 × SECOW2W	S×S	59	43	4.3	22	50	0	7.42	
SECOW2W × WC69	S×S	60	53.33	5.27	22	54.49	3.33	7.57	
MU9 × WC69	S×S	98	47	4.8	22	14	10	7.6	S
WC69 × MU9	S×S	98	40	4.2	21	45	6.67	7.63	
WC69 × SECOW5T	S×S	51	40	3.7	21	45.27	3.33	7.63	
SECOW5T × SECOW2W	S×S	64	52.67	5.13	22	26	16.67	7.83	
SECOW5T	Parent	61	55	5.2	22	26	6.67	7.91	
SECOW2W	Parent	95	69	6.9	23	30	3.33	7.99	
MU9	Parent	70	65	6.5	22	19	10	8.24	
WC69	Parent	130	61.33	6.2	21.17	35	0	8.46	
LSD		4.36	3.04	1.29	1.94	2.26	12.64	0.54	
% CV		1.3	1.3	4.4	0.8	0.5	3.6	2.4	

R= Resistant, MR=moderately resistant S= susceptible, NE= Number of eggs, NEI= Number of emerged insects, ANH= Average number of holes, MDP= Median development period, PWL= percentage weight loss; PPT= percentage pest tolerance and DSI= Dobie susceptibility index.

The Highest Dobie susceptibility index and adult bruchids emergence were recorded from the parental genotypes WC69, MU9, SECOW2W and SECOW5T, and were higher than for their crosses and reciprocals. The Dobie susceptibility index ranged from zero for the resistant (TVu-2027 × IT97K-499-35) to 8.46 for the susceptible genotype (WC69). The number of emerged insects ranged from zero (2419 × IT84s-2246, IT84S-2246, IT84S-2246 × 2419, TVu-2027, TVu-2027 × 2419 and TVu-2027 × IT97K-499-35) to 69 (SECOW2W). Similarly, number of holes per seed was low for the resistant and high for the susceptible genotypes.

5.3.1 Combining ability and maternal effects

The results of diallel analysis for the parents and F_2 segregating populations and the different genetic variance components for number of eggs, adult bruchids emergence and median development period are presented in Table 5.4.

Table 5.4: Combined ANOVA for GCA and SCA, heritability and degree of dominance of F_2 population and parents' diallel analysis for *C. maculatus* resistance traits.

Source	DF	NE	NEI	MDP
GCA	8	10056.36***	6031.5***	917.94***
SCA	36	1242.39***	379.94***	46.29***
Reciprocal	36	592.76***	234.49***	94.37***
Maternal	8	867.85***	577.82***	231.05***
Residual	160	7.32	4.6	1.52
$\delta^2 GCA$		186.09	111.61	16.97
$\delta^2 SCA$		205.84	62.56	7.46
BR		0.64	0.78	0.82
CGDNS (%)		64.12	77.69	80.99
CGDBS (%)		99.74	99.92	98.53

*** Data significant at P ≤ 0.001; GCA the general combining ability; SCA the specific combining ability; Reciprocal the reciprocal crosses; BR the Baker's ratio; $\delta^2 GCA$ = variance of general combining ability; $\delta^2 SCA$ = variance of specific combining ability of parents; CGDNS the coefficient of genetic determination – Narrow-sense heritability estimates; CGDBS the coefficient of genetic determination – broad sense heritability estimates, NE= Number of eggs, NEI= Number of emerged insects, MDP= Median development period

The GCA and the SCA effects were both significant (P ≤ 0.001). Highly significant (P ≤ 0.001) difference was also observed among the reciprocal crosses for the traits measured, indicating significant diversity among the genotypes. Additionally, maternal effect was significant (P ≤ 0.001) for NE, NEI and MDP. The number of eggs laid by the bruchids, adult bruchids emergence and median development period accounted for 52.42%, 64.34% and 51.51% of the sum of squares for the parents and 29.15%, 18.24% and 11.69% of the sum of squares for the crosses, respectively (Table 5.4). The result also provided evidence for the existence of wide variation among both the parents and the resultant crosses, suggesting a high potential for selection for improvement in the resistance to bruchids. Values of Baker's ratio estimated for all the traits were greater than 50%, suggesting the predominance of additive over non-additive gene action in the expression of these traits.

High coefficient of genetic determination – broad sense (H^2) was observed. The coefficient of genetic determination – narrow sense (h^2) estimates for number of eggs (64.12%), emerged insects (77.69%) and median development period (80.99%) were also high (Table 5.4).

5.3.2 General Combining Ability (GCA) effects

Estimates of the general combining ability effects for the nine selected parents for resistance to bruchids traits are shown in Table 5.5.

Table 5.5: Estimates of general combining ability effects for median development period, adult bruchids emergence and number of eggs laid by the bruchids in the F2 population diallel analysis.

Parent	NE	NEI	MDP
SECOW2W	11.66***	10.97***	-3.35***
WC69	19.99***	11.58***	-3.68***
MU9	16.95***	13.34***	-3.17***
SECOW5T	5.47***	8.10***	-4.02***
IT90K-76	-7.80***	-7.64***	-1.29***
IT97k-499-35	-7.71***	-7.20***	-0.15ns
TVu-2027	-15.06***	-10.03***	4.98***
2419	-9.16***	-10.55***	6.19***
IT84s-2246	-14.34***	-8.57***	4.49***

***, **,* and ns; significant at P ≤ 0.001, P ≤ 0.01, P ≤ 0.05 and non-significant, respectively, NE= Number of eggs, NEI= Number of emerged insects, MDP= Median development period

All the parents, except IT97k-499-35 for median development period, showed significant (P ≤ 0.001) GCA effects for number of eggs laid by the bruchids, adult bruchid emergence and median development period, suggesting a greater contribution of additive gene effects in determining resistance to *C. maculatus* among the studied cowpea genotypes. Lines 2419, TVu-2027 and IT84s-2246 contributed significant (P < 0.001) GCA effects of -10.55, -10.03 and -8.57 for number of emerged insects and 6.19, 4.98 and 4.49 for median development period, respectively, suggesting that the genotypes performed far better in the crosses for these specific traits. Conversely, genotypes SECOW2W, WC69, SECOW5T and MU9 contributed significant (P ≤ 0.001) and positive GCA effects of 10.97, 11.58, 13.34 and 8.10 for number of emerged insects and negative GCA effects -3.35, -3.68, -3.17 and -4.02 for median development period, respectively, indicating their negative contribution to resistance.

5.3.3 Specific Combining Ability (SCA) and maternal effects

The majority of the F_2 generation seeds showed significant (P < 0.001) SCA effects for median development period, adult bruchids emergence and number of eggs laid by the bruchids (Table 5.6).

Table 5.6: Estimates of specific combining ability effects for median development period, adult bruchids emergence and number of eggs laid by the bruchids in the F_2 population diallel analysis.

Female	Male	MDP	NEI	NE
SECOW2W	SECOW2W	2.47*	18.45***	36.27***
WC69	SECOW2W	0.42ns	5.55***	-4.07***
WC69	WC69	-0.01 ns	17.11***	52.27***
MU9	SECOW2W	1.83***	-4.71***	-7.86***
MU9	WC69	-0.27 ns	-1.82*	25.97***
MU9	MU9	-0.19 ns	13.25***	-0.99ns
SECOW5T	SECOW2W	1.81***	12.40***	11.62***
SECOW5T	WC69	-0.58***	-2.58***	-13.05***
SECOW5T	MU9	1.91***	2.16***	-4.68***
SECOW5T	SECOW5T	2.19***	15.39***	12.30***
IT90K-76	Secow2W	0.28***	-5.23***	-7.10***
IT90K-76	WC69	1.6***	-8.51***	-15.60***
IT90K-76	MU9	-0.41***	0.40***	-0.23ns
IT90K-76	SECOW5T	-0.80 ns	-12.53***	-5.58***
IT90K-76	IT90K-76	-0.95ns	0.22ns	-6.14***
IT97k-499-35	SECOW2W	-0.86ns	-7.01***	-0.36ns
IT97k-499-35	WC69	1.21**	0.22ns	-2.53**
IT97k-499-35	MU9	-1.54***	-10.38***	-20.99***
IT97k-499-35	SECOW5T	-0.61ns	-10.47***	-13.01***
IT97k-499-35	IT90K-76	-1.76ns	11.77***	11.27***
IT97k-499-35	IT97k-499-35	-1.56*	-4.34***	2.01ns
TVu-2027	SECOW2W	-2.66***	-9.84***	-8.68***
TVu-2027	WC69	1.17***	1.38ns	-9.84***
TVu-2027	MU9	-2.34***	2.96***	5.36***
TVu-2027	SECOW5T	-0.15ns	0.36ns	7.84***
TVu-2027	IT90K-76	-0.72ns	4.27***	7.45***
TVu-2027	IT97k-499-35	6.64***	-1.34ns	-4.64***
TVu-2027	TVu-2027	5.51***	-0.67ns	0.05ns
2419	SECOW2W	-2.79***	-2.99***	-14.08***
2419	WC69	-1.21*	-14.93***	-29.92***
2419	MU9	-0.72***	-3.52***	-0.71ns
2419	SECOW5T	0.72ns	-5.79***	-10.73***
2419	IT90K-76	6.48***	8.46***	17.38***
2419	IT97k-499-35	-1.99***	16.85***	22.12***
2419	TVu-2027	-5.04***	2.51***	0.30ns
2419	2419	-0.25ns	1.36ns	26.90***
IT84s-2246	SECOW2W	-0.50ns	-6.64***	-5.73***
IT84s-2246	WC69	-2.34***	3.59***	-3.23***
IT84s-2246	MU9	1.73***	1.66*	4.14***
IT84s-2246	SECOW5T	-4.49***	1.06ns	15.29***
IT84s-2246	IT90K-76	-3.73***	1.142ns	-1.44ns
IT84s-2246	IT97k-499-35	0.46ns	4.70***	6.14***
IT84s-2246	TVu-2027	-2.42***	0.36ns	2.16*
IT84s-2246	2419	4.79***	-1.95*	-11.25***
IT84s-2246	IT84s-2246	6.49***	-3.93***	-6.07***

***, **,* and ns; significant at $P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.05$ and non-significant, respectively, MDP= Median development period, NEI= Number of emerged insects, NE= Number of eggs

Significant SCA effects for median development period (MDP) were also observed in 25 crosses, indicating the presence of non-additive gene effects. The lowest SCA values for MDP were observed from crosses 2419 × TVu-2027 (-5.04), IT84s-2246 × SECOW5T (-4.49), IT84s-2246 × IT90K-76 (-3.73) and 2419 × SECOW2W (-2.79), and the highest were recorded from TVu-2027 x IT97K-499-35 (6.64). Likewise, Significant SCA effects for number of emerged insects were observed in 29 crosses ranging from -14.93 (2419 x WC69) to18.45 (SECOW2W) (Table 5.6). These results suggested that resistance of these cowpea genotypes was higher or lower than would be expected from the average resistance of their respective parents. Therefore, these crosses could be selected for the improvement of resistance to bruchids.

All parents, except IT84s-2246, showed significant ($P \leq 0.01$) maternal effects on median development period (Table 5.7).

Table 5.7: Estimates of maternal effect of parents on median development period, adult bruchids emergence and number of eggs laid by the bruchids in the F_2 population diallel analysis.

Parent	MDP	NEI	NE
SECOW2W	-1.14^{***}	3.61^{***}	5.26^{***}
WC69	-1.54^{***}	0.13^{ns}	-1.30^{***}
MU9	-1.94^{***}	6.70^{***}	6.96^{***}
SECOW5T	-2.01^{***}	-0.68^{*}	2.19^{***}
IT90K-76	0.39^{*}	-0.17^{ns}	-0.5^{ns}
IT97k-499-35	-0.68^{*}	-0.65^{*}	-3.07^{***}
TVu-2027	3.45^{***}	-2.89^{***}	-3.83^{***}
2419	3.19^{***}	-3.19^{***}	-4.48^{***}
IT84s-2246	0.28^{ns}	-2.87^{***}	-1.22^{***}

***, **, * and ns; significant at $P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.05$ and non-significant, respectively, MDP= Median development period, NEI= Number of emerged insects, NE= Number of eggs

Meanwhile, SECOW2W, MU9, TVu-2027, 2419 and IT84s-2246; and IT97k-499-35 and SECOW5T showed reciprocal effects on number of emerged insects at $P \leq 0.001$ and $P \leq 0.01$, respectively. Similarly, all genotypes, except IT90K-76, showed significant ($P \leq 0.001$) maternal effect on number of eggs laid by bruchids (Table 5.7).

Most crosses showed significant ($P \leq 0.001$) reciprocal differences for number of eggs, number of emerged insects and median development period (Table 5.8).

Table 5.8: Reciprocal effects for median development period, adult bruchids emergence and number of eggs laid by the bruchids in the F_2 population diallel analysis.

Female	Male	MDP	NEI	NE
WC69	SECOW2W	0.92***	4.17***	1.00ns
MU9	SECOW2W	0.33ns	-8.67***	-5.17ns
MU9	WC69	-0.58***	-2.83***	0.00***
SECOW5T	SECOW2W	0.54***	-1.54***	-0.50ns
SECOW5T	WC69	0.083ns	1.83***	-1.17***
SECOW5T	MU9	1.92***	8.33***	6.83***
IT90K-76	SECOW2W	-2.17***	8.17***	10.50***
IT90K-76	WC69	-0.83***	-9.50***	-20.67***
IT90K-76	MU9	0.33ns	8.17***	17.67***
IT90K-76	SECOW5T	-0.92***	1.67***	11.50***
IT97k-499-35	SECOW2W	0.17***	8.83***	18.33***
IT97k-499-35	WC69	-1.08***	-3.67***	-2.83***
IT97k-499-35	MU9	-1.33***	7.50***	10.33***
IT97k-499-35	SECOW5T	-2.25***	-0.17***	6.17***
IT97k-499-35	IT90K-76	0.67***	4.33***	10.50***
TVu-2027	SECOW2W	-5.17***	6.17***	6.00***
TVu-2027	WC69	-3.33***	10.33***	14.50***
TVu-2027	MU9	-3.00***	2.33***	-3.67***
TVu-2027	SECOW5T	-5.33***	0.50***	7.00***
TVu-2027	IT90K-76	2.00***	2.33***	9.00***
TVu-2027	IT97k-499-35	-4.00***	2.50***	7.33***
2419	SECOW2W	-6.08***	10.50***	12.83***
2419	WC69	-7.83***	-3.50***	-9.00***
2419	MU9	-6.50***	16.00***	28.83***
2419	SECOW5T	-6.58***	2.83***	9.67***
2419	IT90K-76	-2.42***	-0.67*	-5.83***
2419	IT97k-499-35	-8.58***	6.50***	0.67ns
2419	TVu-2027	9.33***	-3.00***	3.17***
IT84s-2246	SECOW2W	1.17***	4.83***	4.33***
IT84s-2246	WC69	0.67***	12.67***	8.50***
IT84s-2246	MU9	-9.08***	6.50***	-2.50***
IT84s-2246	SECOW5T	-0.50*	-2.33***	-9.50***
IT84s-2246	IT90K-76	-0.33ns	1.00***	0.83*
IT84s-2246	IT97k-499-35	2.67***	2.00***	6.83***
IT84s-2246	TVu-2027	2.92***	1.17**	2.50***
IT84s-2246	2419	0.00ns	0.00ns	0.00ns

***, **, * and ns; significant at $P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.05$ and non-significant, respectively, MDP= Median development period, NEI= Number of emerged insects, NE= Number of eggs

Overall, 31 reciprocal crosses showed differences in median development period and number of eggs, and 35 reciprocal crosses showed differences for number of emerged insects. Low reciprocal combining ability (reciprocal effect) for median development period was also recorded for crosses IT84S-2246 × MU9 (-9.08), 2419 × IT97K-499-35 (-8.58) and for crosses 2419 × SECOW2W (-7.83) (Table 5.8). Crosses 2419 × IT97K-499-35 (9.33), IT84S-2246 × IT97K-499-35 (2.92) showed the highest reciprocal combining ability for median development period. Likewise, the lowest reciprocal combining ability for number of emerged insects was recorded from IT90K-76 × WC69 (-9.5), MU9 × SECOW2W (-8.67) and IT97K-499-35 × WC69 (-3.67) (Table 5.8).

5.4 Discussion

The study demonstrated the existence of phenotypic differences among cowpea parents and segregating F_2 generations for resistance to bruchids which could be useful to select the best parent or cross for production or further breeding. For instance, there was wide variation among genotypes for susceptibility index, a measure of resistance to bruchids damage (Dobie, 1974), and other traits (Table 5.2). Zero DSI and 44 days of MDP were recorded from genotypes IT84S-2246, IT84S-2246 × 2419, 2419 × IT84S-2246, TVu-2027, TVu-2027 × 2419 and TVu-2027 × IT97K-499-35 (Table 5.3), suggesting that these genotypes were resistant. These results are in line with previous findings which suggested that resistant cowpea genotypes often show reduced insect emergence and delayed insect development (Amusa *et al.*, 2018; Miesho *et al.*, 2018a). In the contrast, the highest number of insects was recorded from WC69 (8.46), suggesting susceptibility. Similar results were obtained by Amusa *et al.* (2018) and Miesho *et al.* (2018a).

The genetics of insect development period (MDP), insect emergence (NIE) and number of eggs (NE) laid by bruchids were evaluated for the parents and the segregating F_2 population. Number of emerged insect and bruchids development period, which were strongly correlated to Dobie susceptibility index (DSI) (Table 3.5), were considered as the most important parameters to measure bruchids resistance in the tested cowpea genotypes (Redden and McGuire, 1983; Jackai and Asante, 2003; Miesho *et al.*, 2018a).

The study further demonstrated the existence of genetic variability among the tested genotypes in their resistance to bruchids**.** GCA and SCA analysis revealed significant differences ($P < 0.001$) among genotypes for number of eggs, insect emergence and median development period (Table 5.4), suggesting the importance of additive and non-additive gene effects in determining the inheritance of resistance to cowpea bruchids. Dobie (1981) and Redden *et al*. (1983) also reported significant GCA and SCA effects for insect emergence and median development period. A 6x 6 diallel analyses in common bean revealed significant GCA and SCA effects in the study of heritability of resistance genes to *Acanthoscelides obtectus* (Kananji, 2007.). Mwila (2013), using North Carolina Design II involving crosses among two resistant and six susceptible bean lines to *C. maculatus*, also reported similar GCA and SCA effects.

The results also showed that the inheritance of traits for number of eggs, number of insect

emergence and median development period were predominantly controlled by additive gene action (Table 5.4). GCA effects accounted for 52.42% (number of eggs), 64.34% (insect emergence) and 51.51% (median development period) of the sum of squares for the crosses, and large (>50%) GCA/SCA ratios indicated the predominance of the additive gene action to the inheritance of resistance to bruchids (Baker, 1978).

Negative combining ability values for NE and NEI, and positive values for MDP are indicators of seed resistance to bruchids. Genotypes that presented negative GCA values for number of eggs and insect emergence were TVu-2027, IT84s-2246, 2419, IT90K-76 and IT97k-499-35 (Table 5.6). Whereas, genotypes that presented the highest positive general combining ability for MDP were 2419, TVu-2027 and IT84s-2246. The negative GCA values of number of eggs and emerged insects and positive GCA values of median development period indicated that the cowpea parents contributed to reduced number of eggs, number of emerged insects and contributed to delayed insect emergence; thereby providing positive contribution to resistance, and indicating that these genotypes could be selected as good parents for breeding resistance to bruchids. Kananji (2007) and Mwila (2013) reported similar results in respect to the resistance of beans to *A. obtectus* and *C. maculatus* respectively.

Parents 2419, TVu-2027 and IT84s-2246 were identified as promising general combiners for resistance to bruchids. Similarly, the specific combining ability effects were used to identify specific crosses with desirable traits (Acquaah, 2012). Accordingly, crosses IT84s-2246 × 2419, 2419 × MU9, TVu-2027 × SECOW2W, and 2419 × IT90K-76, 2419 × WC69, 2419 × SECOW5T and 2419 × SECOW2W which revealed lowest number of eggs and insect emergence and elongated insect development period were the best specific crosses for bruchids resistance (Table 5.6). The selection of parents based on data obtained from combining ability and understanding the genetic parameters controlling trait inheritance ensures the efficiency of breeding program (Sleper and Poehlman, 2006; Sharma *et al.*, 2015).

The majority of the cowpea crosses were affected by maternal genes in their resistance to bruchids (Table 5.7). Maternal effects are common in sexually reproducing crops, and these can be detected by investigating the existence of differences between individuals of the forward and reverse crosses (Eizadshenass, 2013). The maternal effects were significant among the reciprocals for number of eggs, insect emergence and median development period.

Fewer numbers of eggs and insect emergence and extended insect development period were observed on the forward crosses involving the resistant parents as females than their counter reciprocals. The direction of crossing revealed influence of maternal effects on the number of eggs, insect emergence and insect development period. The existence of maternal effect was also confirmed by the significant effects of reciprocal crosses and their varied SCA effects (Tables 5.8). Similar results were reported by Redden (1983) and Adjadi *et al.* (1985) in cowpea; and in mungbean (Somta *et al.*, 2007).

Bruchids resistance traits had low magnitude of dominance variances, indicating higher broad and narrow-sense heritability. Narrow sense heritability of 64.12%, 77.69% and 80.99% were recorded for number of eggs, emerged insects and median development period, respectively (Table 5.4). This implied that the heritability of the traits from the parents was highly predictable, thus explaining the very high values obtained for the narrow sense heritability. The results provide evidence for the presence of additive and non-additive gene effect on the inheritance of cowpea resistance to bruchids. High heritability estimates indicated higher frequency of genes controlling the traits (Ma-Teresa *et al.*, 1994) and expression of the reliability with which phenotypic value guides the breeding value. In the improvement of self-pollinated plants such as cowpea, additive variation is of great importance and makes it possible to successfully select better individuals in segregating populations (Warner, 1952). For this reason, backcross, pedigree or single-seed descent selection methods are recommended for advancing the segregating populations as proposed by Bernado (2003).

CHAPTER SIX

IDENTIFICATION OF CANDIDATE GENES IN COWPEA ASSOCIATED WITH RESISTANCE TO BRUCHIDS (*CALLOSOBRUCHUS MACULATUS* FAB)

6.1 Introduction

Cowpea is susceptible to field insect pests and *Callosobruchus maculatus* during storage (Boukar *et al.*, 2012). The inheritance of bruchids resistance traits (number of eggs, insect emergence and insect development period) is quantitative and complex and little efforts have been made to understand the genetic basis of such complex traits (Tripathy, 2016). It is challenging to improve complex traits through conventional breeding approaches, but the use of molecular markers enhances the selection process although it requires a good understanding of how variations at the DNA level relate to phenotypic variations. Genome-wide association studies (GWAS) has emerged as an effective tool to identify multiple related candidate genes regulating important traits in both self- and cross-pollinated crops (Lucas *et al.*, 2011).

GWAS use collections of diverse lines that have been genotyped and phenotyped traits of interest. The technique is used for identifying genomic loci that are linked with quantitative traits (Varshney *et al*., 2014). In comparison to quantitative trait loci (QTL) studies that are achieved using pedigrees (e.g., bi-parental crosses), GWAS have the advantage of detecting smaller chromosomal regions affecting a trait and provide precise estimates of the size and direction of the effects of alleles in known loci (Abdel-Shafy *et al*., 2014).

The development of genomic resources for cowpea has been more recent than for the majority of other crops and recent efforts have focused on molecular diversity and genetic linkage mapping (Boukar *et al*., 2016). Currently, molecular work on cowpea have focused on the study of quantitative trait loci (QTLs) governing many agricultural and adaptive traits such as root architecture, seed size, and resistance to *C. maculatus* (Burridge *et al*., 2016; Egbadzor *et al*., 2013). Genetic mapping has been done using a range of methods, such as restriction fragment length polymorphism (RFLP) (Fatokun *et al.*, 1992), simple sequence repeat (SSR) (Fatokun, 2000) and single-nucleotide polymorphism (SNP) (Egbadzor *et al*., 2013; Burridge *et al*., 2016). Molecular markers together with phenotypic data can generate more reliable data than use of phenotype alone in crop improvement.

Studies of quantitative trait loci (QTL) related to key bruchids resistance in cowpea are scarce (Tan *et al*., 2012). Fatokun (2000) reported a closely linked SSR marker Vm50 closely associated with the delay in the emergence of *C. maculatus* explaining 20% of the variation. Fatokun (2002) also identified four QTLs associated with resistant to bruchids, with major QTL accounted for 76% of the variation in the trait.

The application of GWAS in cowpea improvement both at the global level in general and under the Uganda condition in particular has been limited to the study of agronomic and nutritional traits and its use for the study of resistance to *C. maculatus* are scant. The aim of this study was therefore to identify genomic regions, candidate genes and their functions that control the resistance to bruchids parameters such as number of eggs (ANE), number of emerged insects (NEI), number of holes (ANH), median development period (MDP) and Dobie susceptibility index (DSI) from cowpea mini-core collection SNP markers.

6.2 Materials and Methods

A total of 217 mini-core cowpea accessions, from ~60 countries across six continents representing a worldwide diversity of cultivated cowpea (Muñoz-Amatriaín et al., 2016), collected and genotyped by the University of California were procured through the Makerere University Regional Center for Crop Improvement (MaRCCI). To generate sufficient seeds for laboratory testing, each of the accessions was grown at the Makerere University Agricultural Research Institute Kabanyolo (MUARIK) (0°28'N and 32°37'E, approximately 1200 m. a. s. l.), between September and December 2017. The mean annual temperature at MUARIK ranges between 15-35.5$^{o}_{C}$ with annual average rainfall of 1150 mm and humidity ranging between 13-96%.

6.2.1 Bruchids laboratory culture

Bruchids used in this study were cultured and multiplied in accordance with the procedure outline in Chapter 3 (section 3.2.1) of this thesis.

6.2.2 Screening of cowpea seeds for resistance to *C. maculatus*

To evaluate for resistance to bruchids, experiment was conducted at MUARIK animal lab from September 2017 to March 2018, seeds from each of the 217 minicore cowpea accessions were weighed and separately put in a petri-dish of 90mm diameter and 10mm depth. Twenty seeds were randomly selected from each of the minicore cowpea accessions

and oven dried at 40^0C for 24 hours to destroy any insects or eggs that could have been present and to standardize moisture levels of the seeds (Amusa *et al*., 2014). The experiment was laid in complete randomized design replicated twice.

To each petri dish containing the ten seeds, two pairs of three-day old male and female adult bruchids from laboratory culture were introduced and the top covered to prevent the insects from escaping. The insects were left undisturbed in the petri-dishes for three days to allow for mating and oviposition and then removed (Amusa *et al.*, 2013). Data on the different resistance traits to bruchids were collected and Dobie susceptibility index calculated following the methods described in section 3.2.3. Accordingly, data were collected on number of eggs, daily insect emergence, number exit holes, median development period and Dobie susceptibility index.

6.2.3 Genotyping

The 217 mini-core collections were genotyped at the University of California with the "Cowpea iSelect Consortium Array" available from Illumina (Illumina Inc., San Diego, CA, USA; http://www.illumina.com/areas-of-interest/agrigenomics/consortia.html) containing 51,128 SNPs (Muñoz-Amatriaín et al., 2016). A total of 41, 948 polymorphic and non-redundant SNP markers, with > 5% minor allele frequency (MAF) and missing data lower than 20% filtered using TASSEL 5.2.1.5 (Bradbury *et al*., 2007) were used for subsequent analysis. Heterozygous markers were treated as missing data according to Boukar *et al*. (2012)

6.2.4 Data analysis

Phenotypic data

One-way analysis of variance (ANOVA) was used to examine differences in the performance of different minicore cowpea accessions for resistance to bruchids and Fisher's LSD test was used to separate the means. All analyses were conducted using GenStat Discovery, 16.1^{th} Edition statistical package.

Association mapping

Genome-wide association analysis was performed on the number of eggs per seed (ANE/S), number of emerged insects per seed (NEI/S), number of holes per seed (ANH/S), median development period (MDP) and Dobie susceptibility index (DSI) using the mixed linear

model (MLM) in the program TASSEL 5.2.1.5, incorporating Q matric and kinship data (K) (Zhang *et al*., 2010) as follows:

$$Y = X\beta + Wm + Qv + Zu + e$$

Where y = vector of phenotypic observations

β = vector of unknown fixed effects except for the SNP marker under testing

m = is a vector of fixed marker effects (i.e., SNP)

v = is a vector of sub-population effects

u = is a vector of unknown random effects, and

e = is a vector of residual effects

Q= is an incidence matrix of principal component scores of marker-allele frequencies

X, W and Z = incidence matrices of ones and zeros relating y to β, m and u, respectively.

The covariance of u is equal to KVA, where K is the kinship matrix that was estimated with a random set of SNPs using the Tassel program and VA is the additive variance estimated with the restricted maximum likelihood (REML). The kinship matrix estimation and principal component analyses were performed using the TASSEL package. The optimum number of principal components/covariates included in the model for each trait was three. SNPs with a LOD score greater than 3.5 were treated as a significant threshold for marker-trait association analysis (Contreras-Soto *et al*., 2017; Burridge *et al*., 2016). The single trait-single environment association mapping procedure (Egbadzor et al., 2013; VSN International, 2012) was followed to identify SNP markers that are linked with the traits resistance to bruchids. Quantile-quantile (QQ) plots were used to assess the presence of spurious associations.

Gene prediction

To identify possible genes underlying the association signals detected by GWAS, the cowpea reference genome annotation accessible through Phytozome was exploited (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Vunguiculata_er).

The physical positions of significant SNPs were searched on the genome browser to discover the relevant genes in the SNP vicinity. Annotated functions of the surrounding genes were investigated for their involvement in resistance to bruchids.

6.3 Results

6.3.1. The response of the mini-core accessions to bruchids attack

Uniform levels of bruchids damage were recorded in all the replicates, resulting in distinct susceptibility and resistance responses to bruchids attack among cowpea genotypes. Frequency distributions of the phenotypic data were continuous, suggesting additive gene effect. Furthermore, the distribution of the accessions to the different resistance traits and resistant classes were fitting to normality (Fig. 6.1), indicating the existence of additive gene effect.

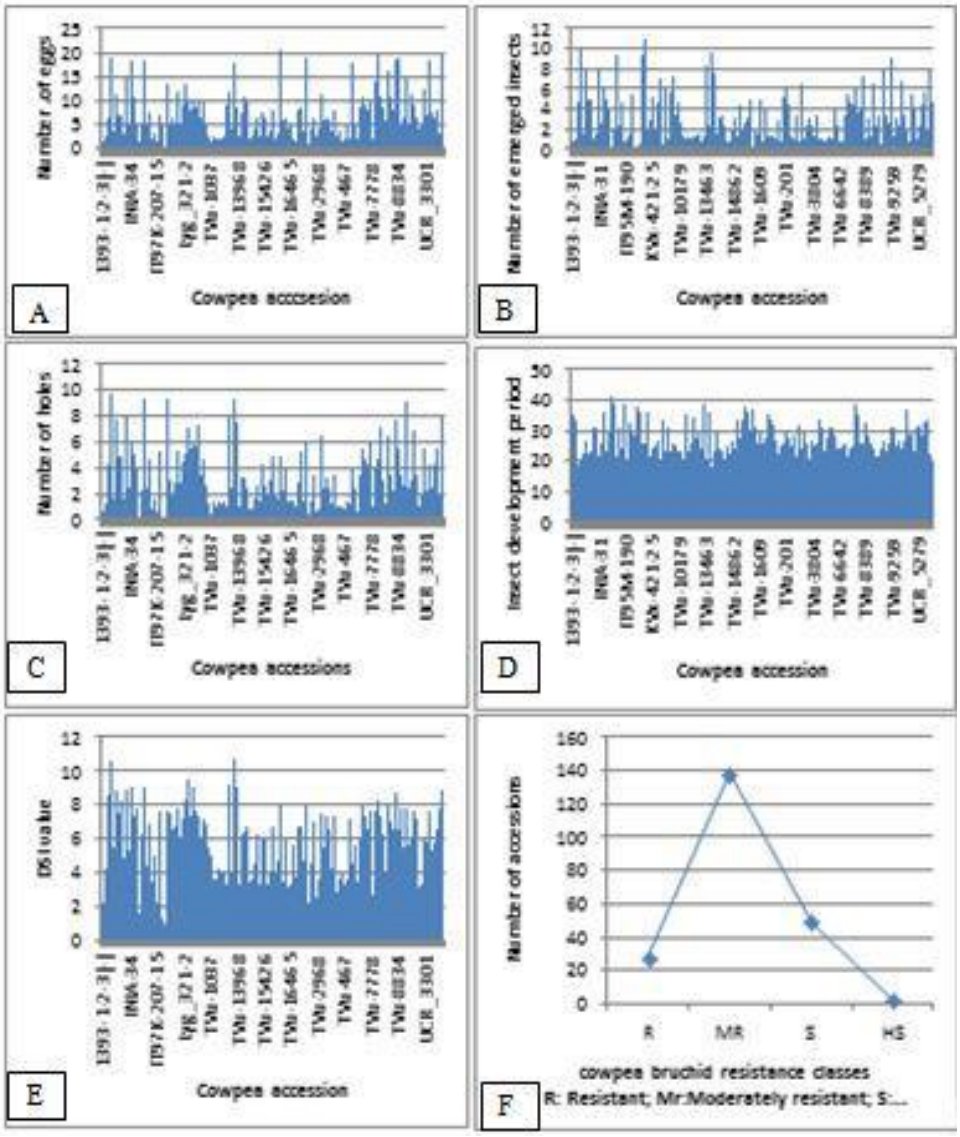

Figure 6. 1**:** Distribution of cowpea accessions in the different bruchids resistance traits and classes. A=number of eggs; B=number of emerged insect, C=Number of holes; D=Median development period; E=Dobie susceptibility index; F=resistance classes.

The resistant accessions exhibited little bruchids damage whereas the susceptible accessions showed more damage. These differences were confirmed by ANOVA, in which *F* values for phenotypic differences were highly significant (P< 0.001) and coefficient of variation (CV) ranged from 3.9 to 8.3% (Table 6.1).

Table 6.1: Analysis of variance for resistance of cowpea accessions to *C. maculatus* infestation.

Source of variation	df	Variables				
		ANE	ANI	ANH/S	MDP	DSI
Genotype	216	44.91***	10.66***	9.97***	51.10***	9.45***
Residual	217	0.11	0.05	0.05	1.07	0.05
Mean		5.75	2.63	2.58	26.25	5.06
Minimum		0.25	0.10	0.10	18.50	0
Maximum		20.65	11.00	9.80	41.50	10.71
CV%		5.80	8.20	8.30	3.90	4.5
SEM		0.23	0.15	0.15	0.73	0.16

ANE= Number of eggs per seed, ANI= Average number of emerged insects per seed, ANH= Average number of holes per seed, MDP= Median development period, and DSI= Dobie susceptibility index.

6.3.2 SNP-based association analyses

For model fit evaluation of mixed linear models with Q (structure) matrices showed a better fit for the model that consider Q for all the traits (ANE, ANI, ANH, MDP and DSI) (Fig. 6.2), indicating SNPs traits association were not due to spurious associations, and the detected significant SNP hits are not false.

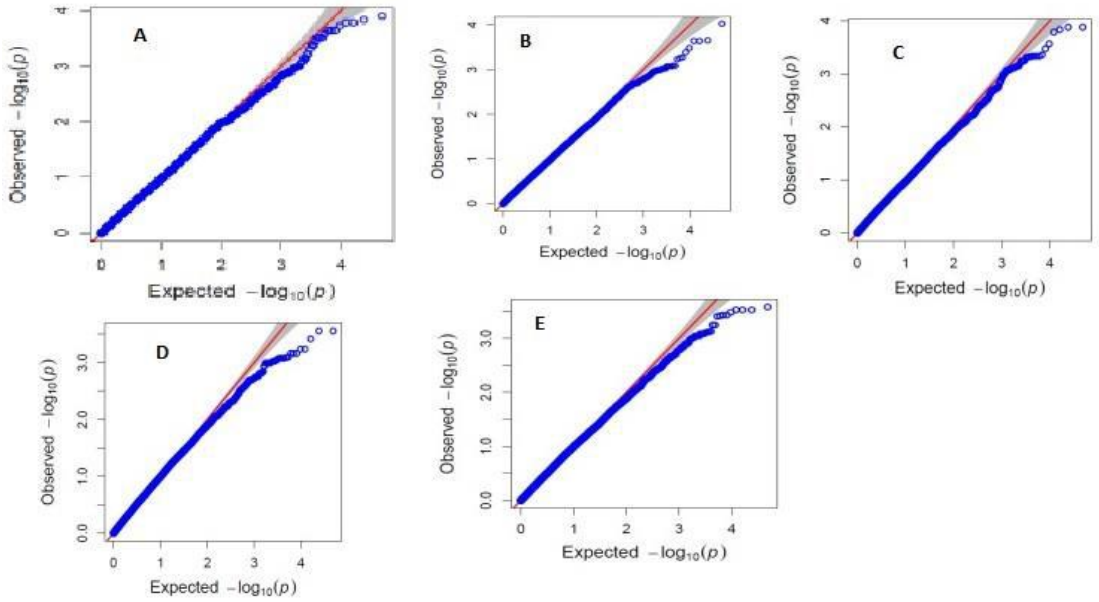

Figure 6. 2: QQ plots of GWAS for ANE (A), ANI (B), ANH (C), MDP (D) and DSI (E) evaluated in a cowpea association mapping panel

Eleven significant regions (LOD > 3.5) associated with the resistance cowpea traits to bruchids were identified on six chromosomes (Table 6.2). The SNPs *2_36050* on chromosome 8, *2_37236* on chromosome 6, *2_38306* on chromosome 7 and *2_38338* on chromosome 9 had allelic effects of -6.5, -6.4, -6.2 and -3.1 on the number of eggs, respectively. SNPs *2_36627* on chromosome 7 had -2.1 allelic effects on number of emerged insects. SNPs *2_32564* and *2_37091* on chromosome 1; and *2_15764* on chromosome 2 had 2.1 and 5.1; and 1.8 allelic effects on insect development period, respectively. Likewise, SNPs 2_*54737* and *2_35230* on chromosome 7 had -1.4 and -1.5 allelic effects on DSI, respectively. SNP 2_20268 on chromosome 8 had allelic effects on the number of eggs (-1.5), emerged insects (-0.7) and number of holes (-0.7).

Table 6.2: QTLs (LOD >3.5) associated to resistance traits to bruchids and their effects to the resistance traits.

Trait	SNP marker	Chromosome	Position (cM)	Allelic[a] effects	R^2 (%)	LOD	Favorable allele	Alternative allele
ANE	*2_20268*	8	30339110	-1.5	9.1	3.8	T	C
ANH				-0.7	10.7	3.9		
ANI				-0.7	8.5	3.6		
ANE	*2_36050*	8	33041542	-6.5	9.2	3.8	T	C
ANE	*2_37236*	6	17949201	-6.4	8.8	3.6	T	C
MDP	*2_15764*	2	28200370	1.8	7.1	3.5	A	G
MDP	*2_32564*	1	40651151	2.1	7.4	3.6	C	G
MDP	*2_37091*	1	37818038	5.1	7.4	3.6	G	T
NEI	*2_36627*	7	19304251	-2.1	9.3	4.0	G	A
ANE	*2_38306*	7	12810789	-6.2	9.4	3.9	G	A
ANE	*2_38338*	9	40479817	-3.1	9.0	3.7	T	A
DSI	*2_54737*	7	19387024	-1.4	10.6	3.6	C	A
DSI	*2_35230*	7	19399548	-1.5	10.5	3.5	G	A

a Marker additive effect, all significance at P > 0.001, *downstream gene; ANE= Average number of eggs per seed; ANH= Average number of holes per seed; ANI=Average number of emerged insects per seed; MDP= Median development period; DSI= Dobie susceptibility index.

Several regions with minor allelic effects on the phenotypic expression of the resistance traits were detected (Table 6.2). 45.5% of the proportion of phenotypic variance for the number of eggs was explained by five genomic regions (*2_20268*, *2_36050*, *2_37236*, *2_38306* and *2_38338*). 21.1% of the variation among accessions in their DSI values was accounted for by SNPs *2_54737* and *2_35230*. Similarly, SNP *2_20268* explained 10.7%, 9.1% and 8.5% of the phenotypic variations on the number of holes, number of eggs and number of emerged insects, respectively, indicating strong linkage among the traits. Likewise, 21.9% of the

phenotypic variation among accessions on their median development period was accounted for by SNPs *2_15764*, *2_32564* and *2_37091*.

From this study, five candidate genes and one upstream gene involved in the inheritance of seed resistance to bruchids (Table 6.3) were identified. *Vigun08g132300* on chromosome 8 linked to SNP *2_20268* was involved in reducing the number of holes, insect emergence and number of eggs. *Vigun08g158000* on chromosome 8 linked to *2_36050* was involved in reducing the number of eggs. *Vigun06g053700* on chromosome 6 linked to 2_37236 was also involved in reducing oviposition. Candidate gene *Vigun02g131000* and a downstream gene *Vigun01g201900*, 1482bp away from SNP *2_37091*, and *Vigun01g234900* on chromosome 1 were involved in elongating the insect developmental period.

Table 6.3: Identified candidate genes, and their molecular and biological function.

Trait	SNP marker	Chromosome	Candidate genes	Molecular function[#]	Biological Function[#]
ANE ANH ANI	*2_20268*	8	*Vigun08g132300*	Chloroplast envelope transporter (Tic110)	biosynthesis of carbohydrates and proteins
ANE	*2_36050*	8	*Vigun08g158000*	Interacting selectively and non-covalently with any protein or protein complex	cellular transport process
ANE	*2_37236*	6	*Vigun06g053700*	WRKY DNA -binding domain (WRKY)	Regulation of transcription
MDP	*2_15764*	2	*Vigun02g131000*	Alpha/Beta Hydrolase fold-containing protein (ABH)	Glycerol biosynthesis
MDP	*2_32564*	1	*Vigun01g234900*	Glutamate-prephenate aminotransferase	The chemical reactions and pathways resulting in the formation of substances; typically the energy-requiring part of metabolism in which simpler substances are transformed into more complex ones.
MDP	*2_37091*	1	*Vigun01g201900*[*]	Any molecular function by which a gene product interacts selectively and non-covalently with DNA (deoxyribonucleic acid). / PTHR	Transcription factor activity

[*]Downstream gene; ANE= Average number of eggs per seed; ANH= Average number of holes per seed; ANI=Average number of emerged insects per seed; MDP= Median development period. [#]biological and molecular function is from cowpea reference genome accessible through Phytozome

6.4 Discussion

The study demonstrated the existence of candidate genes conferring resistance to bruchids which could be incorporated into farmers' preferred but susceptible cowpea cultivars. The existence of genomic regions conferring resistance to bruchids was previously reported on cowpeas (Fatokun, 2000; 2002), rice bean (Venkataramana *et al*., 2015) and in mungbean (Mei *et al*., 2014; Wanga *et al.*, 2016). The identification of eleven genomic regions associated with resistance to bruchids may also indicate the predominance of additive gene action in conferring resistance to bruchids (Miesho *et al*., 2018b).

Five significant genomic regions associated with the number of eggs were identified on chromosome 6, 7, 8 and 9 (Table 6.2). The highest allelic effect was recorded from SNP *2_36050* (-6.5) and the lowest from *2_20268* (-0.7). The negative allelic effect indicated the involvement of the alternative alleles (cytosine and adenine) in reducing the number of eggs (Burridge *et al*., 2016) thereby enhancing resistance. Similarly, SNPs *2_20268* and *2_36050* which are co-localized on chromosome 8 accounted for 18.3% of the variation in the number of eggs highlighting the need to target this chromosome to discourage oviposition. The other SNPs distributed on chromosomes 6, 7 and 9 contributed 27.2% of the total phenotypic variation. The results also suggested that SNP *2_20268* is in linkage disequilibrium with *2_36050*, and indeed the two SNPs cover a narrow interval (around 30338080–33041839 bp) flanked by SNPs on chromosome 8 (Table 6.3), and within this region, there was one chloroplast envelope transporter,Tic110, gene (*Vigun08g132300*) and one PTHR gene (*Vigun08g158000*) (Table 4) involved in reducing oviposition through their ability to control carbohydrate (Block *et al*., 2007) and functional protein biosynthesis (Lindemose *et al*., 2013). In addition, WRKY DNA-binding domain (WRKY) was closely associated to SNP *2_37236* and encoded by candidate gene *Vigun06g053700* involved in reducing eggs through its ability to modulate transcription (Pedra *et al*., 2003). Negative allelic effect of the SNPs associated with the number of eggs was also another evidence for their involvement in resistance (Burridge *et al*., 2016). Similarly, gene Ortholog to *Vigun06g053700* in soybean (*Glyma.08G320200*) was involved in a wide range of developmental and physiological processes, particularly in the plant response to biotic and abiotic stresses (Yu *et al*., 2016). Genomic regions associated with the number of emerged insects identified on chromosome 7 and 8 (Table 6.2) are of particular interest because of their importance in reducing insect emergence. SNP *2_20268* on linkage group (LG) 8 which is linked to chloroplast envelope transporter (*Vigun08g132300*) gene is co-located with SNP for the number of holes and eggs

in which the SNP haplotype confer a reduced number of insects. The strong correlation between the traits (Table 3.5) is an evidence for the existence of a common gene among them (Miesho *et al*., 2018a).

Chloroplast envelope transporter (Tic110) was involved in enhancing resistance to bruchids through its ability in reducing insect emergence, holes, oviposition and elongating insect development period. The chloroplast envelope membranes are the permanent membrane structure of the different types of plastids (proplastids, chloroplasts, chromoplasts, etioplast) and an important structure for the integration of plastid metabolism within the cell (Block *et al*., 2007). Chloroplasts are crucial for photosynthesis and are the sites of carbon dioxide reduction and its assimilation into carbohydrates, amino acids, fatty acids, and terpenoid compounds (Block *et al*., 2007). The higher the carbohydrate assimilated by the plant, the higher will be seed carbohydrate leading to increased seed hardness (Ajeigbe *et al*., 2008), thereby making seed penetration by the insect difficult, resulting into reduced insect population and number of holes, and elongated insect development period (Miesho *et al*., 2017). Similarly, the higher the amino acid biosynthesis, the higher will be the seed inhibitory enzymes and the better the seed resistance to bruchids (Westermann and Craik, 2010). The involvement of inhibitory enzymes in enhancing seed resistance to bruchids was previously reported by Lattanzio *et al.* (2005) and by Miesho *et al*. (2017) on cowpea and Wisessing *et al*. (2010) on mung beans.

In beans, for example, α - amylase inhibitor is found only in the seeds (Moreno *et al*., 1990). This is because more efficient glycosylation in the seeds than the other parts of the plant (Obiro *et al*., 2008). The better the carbohydrate biosynthesis the more efficient will be the glycolysis leading to a more efficient synthesis of α-amylase inhibitor. High concentration of α-amylase inhibitors reduces number of eggs, insect emergence and number of holes and, the longer the insect development period (Miesho *et al*., 2017). This is because α-amylase inhibitors target α-amylase enzyme in the insect guts and the insect will suffer from reduced availability of carbohydrates that serve as energy resource (Westermann and Craik, 2010).

Two candidate genes (*Vigun01g234900* and *Vigun02g131000*) and one downstream gene (*Vigun01g201900*), 1482bp far from SNP 2_37091, associated with median development period were identified (Table 6.3). Aspartate transaminase encoded by candidate gene *Vigun01g234900* involved in extending insect development period through its ability to synthesize carbohydrate and different essential amino acids (Torre *et al*., 2014). Alpha/beta

hydrolase family (ABHs), encoded by candidate gene *Vigun02g131000*, is associated with housekeeping roles and participate in the breakdown and recycling of cellular metabolites, processing of external nutrients and detoxification of xenobiotics (Long and Cravatt, 2011). It was also involved in increased insect development period (Chang and Hartman, 2017) through its regulatory roles in metabolism and modulating protein lifetime, function, and turnover (Van der Hoorn, 2008). The positive allelic effect of the SNPs on the median development period is also another evidence for their involvement in resistance (Burridge *et al*., 2016). Jui Lin *et al*. (2016) reported *At2g22250* gene, Ortholog to *Vigun02g131000*, in Arabidopsis thaliana involved in contributing for resistance to biotic stress. Similarly, the downstream candidate gene, *Vigun01g201900*, encoding transcriptional factors were also involved in extending insect development period through its ability to modulate protein synthesis. Chang and Hartman (2017) reported the involvement of genes, encoding transcription factors, for the resistance of soybean to Potato leafhopper and soybean looper. Similarly, in Arabidopsis genes with the same function were also reported to enhance insect resistance (Misra *et al*., 2010). Therefore, the identified gene (*Vigun01g201900*) could also be involved in enhancing cowpea seed resistance to bruchids.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSSIONS AND RECOMMENDATIONS

7.1 General Discussion

The results of the first study (Chapter 3), that is, identification of resistance sources to bruchids were obtained following laboratory evaluation of one hundred forty five (145) cowpea genotypes from among the local collections, improved cultivars and introduced ones. Based on DSI values, 18 resistance sources, comprised of 13 new sources from Uganda genotpes and 5 earlier reported resistance sources, were identified (Chapter 3). Out of the 13 Uganda resistance sources, genotype 2419 and WC42 performed equally with the globally known sources of resistance to bruchids, IT84s_2246 and TVu-2027. Therefore, the identified sources of resistance could be used by cowpea breeders to develop cultivars with relatively high resistance to cowpea bruchids.

DSI which correlated positively with number of eggs and number of holes, and negatively with pest tolerance demonstrated its role in determining resistance. The multiple regression analysis indicated that 82.32% of the variation among genotypes on their DSI were explained by number of eggs, holes and weight loss. Number of holes and eggs were positively correlated, which indicated that the traits could be controlled by similar, overlapping, linked genetic loci (Chapter 3 & 6).

From the 145 cowpea genotypes evaluated at Kabanyolo (MUARIK), seven cowpea genotypes, comprising of four susceptible (IT109, SECOW2W, WC69 and IT71) and three resistant (WC42, TVu- 2027 and 2419), were selected to study the contribution of seed coat and cotyledon biochemicals in enhancing cowpea seed resistance to bruchids (Chapter 4). Out of the seven genotypes, similar study was previously reported only on genotype TVu-2027. Results of the study indicated that none of the seed coat biochemicals and seed protein contents were involved in enhancing seed resistance to bruchids. Both highest and the lowest quantities of the seed coat biochemicals and protein content were recorded on genotypes irrespective of the resistance classes, and none of the biochemicals was correlated to any of the seed resistance traits. However, seed carbohydrate content and α-amylase inhibitory enzyme were involved in enhancing seed resistance to bruchids. Seed carbohydrate was involved in resistance to bruchids by increasing seed hardness thereby making seed penetration by the insect difficult (Ajeigbe *et al*., 2008). The strongly correlations of DSI and

all the resistance traits with carbohydrate content were another evidence for its contribution in imparting seed resistance to bruchids. Likewise, α-amylase inhibitory enzyme was involved in enhancing seed resistance to bruchids by inhibiting bruchids α-amylase enzyme resulting in carbohydrate starvation (Macedo *et al*., 2004). The negative correlation of the enzyme with pest tolerance and insect development period is another supporting evidence for its involvement in enhancing seed resistance to bruchids. Both biochemicals enhanced resistance by prolonged their development period and retarding insect population growth.

When the IITA resistance source was compared with the Uganda resistance sources, the seed carbohydrate content of TVu-2027 was significantly lower than the Uganda line WC42 but was at par with 2419. Although, the concentration of seed α-amylase inhibitory enzyme of TVu-2027 was lower than the Uganda genotype WC42, it was at par with 2419. The biochemical data supported the phenotypic data (Chapter 3), indicating that the Uganda resistance source was better in its resistance than the IITA source, it was therefore recommended as good source of resistance to bruchids damage. On the basis of this study, seed carbohydrate and α-amylase inhibitory enzyme could be used as biochemical markers for quick and accurate selection of cowpea genotypes resistant to bruchids.

From the 145 cowpea genotypes evaluated in study 3, nine potential parents comprised of four susceptible (SECOW2W, WC69, MU9 and SECOW5T), Uganda origin, and five resistant (IT90K-76, IT97K-499-35, TVu-2027, 2419 and IT84s-2246) IITA supplied and Uganda were selected and crossed in full diallel mating design to study combining abilities, gene actions and the heritability for bruchids resistance traits namely; number of eggs, insect emergence and median development period (Chapter 5). Except TVu-2027, the remaining resistant and susceptible genotypes have never been used for similar study. Results showed significant general combining ability effects for all the traits, indicating that parents express different combining abilities. Furthermore, significant GCA effects indicated the presence of additive gene effect on the expression of the traits. High values of Baker's ratio for all the bruchids resistance traits indicated the preponderance of additive over non-additive gene action in the expression of all the bruchids resistance traits (Baker, 1978). High coefficients of genetic determination in narrow sense for all the traits were also another supporting evidence for the predominance of additive over non additive action.

Significant negative GCA effects for number of eggs and insect emergence, and positive effects for median development period were observed on parents 2419, TVu-2027 and IT84s-2246, suggesting that these parents' transmitted genes for resistance to bruchids and therefore were considered as good general combiners and are recommended for breeding of cowpea resistance to bruchids. Likewise, Significant SCA effects observed for number of eggs, insect emergence and median development period, suggested the existence of non-additive gene effects on the inheritance of the traits. Significant negative SCA effects for number of eggs, insect emergence, and positive effects for median development period were observed on crosses IT84s-2246 × 2419, 2419 × MU9, TVu-2027 × SECOW2W, 2419 × IT90K-76, 2419 × WC69, 2419 × SECOW5T and 2419 × SECOW2W. The DSI value of the crosses was within the resistance class, therefore, were recommended as best crosses for breeding. Significant reciprocal effects were observed in majority of the reciprocal crosses suggesting significant maternal effects and therefore, it was recommended to use the resistant parent as female parent.

The study was designed to identify candidate genes and their function in controlling the inheritance of cowpea bruchids resistance traits namely; number of eggs, number of insect emergence, number of holes, median development period and dobie susceptibility index (Chapter 6). Total of 217 "mini-core" cowpea accessions, genotyped using 51,128 single nucleotide polymorphisms (SNPs), was phenotyped for their reaction to bruchids infestation. The study identified eleven significant genomic regions associated with the resistance traits, suggesting the presence of additive gene effect on the expression of the traits (Chapter 5). Further analysis of gene prediction using Phytozome identified six candidate genes (*Vigun08g158000, Vigun06g053700, Vigun02g131000, Vigun01g234900* and *Vigun01g201900*) associated to the resistance traits. The identification of multiple candidate genes involved in resistance to bruchids is also another supporting evidence for the predominance of additive over non additive gene action (Chapter 5).

Some of the identified candidate genes are involved in the synthesis of carbohydrate and proteins, indicating that seed carbohydrate and protein (could be inhibitory enzymes) are part of the mechanism of cowpea resistance to bruchids (Chapter 4). Candidate gene *Vigun08g132300* controlled the inheritance of number of eggs, insect emergence and number of holes. Strong positive correlation between number of eggs and holes (Chapter 3) was evidence for the existence of a common gene.

7.2 Conclusions

This study indicated genotypes IT84s-2246, 2419, TVu-2027, WC42, IT97K-499-35, IT95K-207-15, ACC23 × 3B, 182, IT90K-76, NE39 × SEC4, WC16, NE4, ALEGI × 5T, ACC2×ACC12, WC67, WC48, 3B × 2W and SEC1× SEC4 as bruchids resistant sources. The study on the mechanism of resistance demonstrated that α-amylase inhibitor enzyme and carbohydrate contents, which could be used as biochemical markers for screening resistance sources, were the main actors responsible for cowpea resistance to bruchids. Furthermore, the study provided evidence that the inheritance of seed resistance to bruchids is governed predominantly by additive gene effects. Accordingly, parents 2419, TVu-2027 and IT84s-2246 were indicated as good general combiners for all the resistance parameters to bruchids; and seven best crosses (IT84s-2246 × 2419, 2419 × MU9, TVu-2027 × SECOW2W and 2419 × IT90K-76, 2419 × WC69, 2419 × SECOW5T and 2419 × SECOW2W) which showed better SCA effects for all the resistance parameters were selected as valuable genetic materials for breeding. The existence of eleven genomic regions and six candidate genes associated to resistance is supporting the predominance of additive over non additive gene action.

7.3 Recommendations

Genotypes 2419, WC42, TVu-2027and IT84s_2246 which showed better performance for resistance to bruchids during phenotyping, biochemical and inheritance study were recommended as donor parents to develop cowpea variety resistance to *C. maculatus*. The information from genome wide association study could be used as a tool for analyzing the inheritance of the resistance genes, monitoring the transmission of the resistance genes or genomic regions from parents to progeny, and for map-based cloning of those genes. The molecular cloning and functional analysis for the candidate genes should be carried out to confirm the roles of the candidate genes in conferring resistance to bruchids.

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