HISTOCHEMICAL AND IMMUNOLOGICAL CHARACTERISTICS IN THE LARVAE OF SOUTHERN COWPEA WEEVIL, *CALLOSOBRUCHUS MACULATUS* REARED ON SUSCEPTIBLE AND RESISTANT VARIETIES OF BLACKEYED PEAS

A THESIS

Presented to the Faculty of Science, Ain Shams University for the Award of the Ph. D. Degree

(IN ENTOMOLOGY)

By

MOHAMED OMAR MOHAMEDABOKERSH

(B. Sc., M. Sc.)

Department of Entomology
Faculty of Science
University of Ain Shams
Cairo
2012

THESIS EXAMINATION COMMITTEE

NAME	TITLE	SIGNATURE
………………….	………………….	………………….
………………….	………………….	………………….
………………….	………………….	………………….
………………….	………………….	………………….

SUPERVISORS:

Dr. Mohamad Saad Hamed
Professor of Entomology, Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt

Dr. Emad Mahmoud Said Barakat
Professor of Insect Physiology, Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt

Dr. Karam Teleb Huessein
Professor of Entomology, Department of Zoology, Faculty of Science, Zagazig University, Egypt

Dr. Samir Abdel Azeem Nassar
Assistant Professor of Histology & Histochemistry,
Department of Zoology, Faculty of Science, Zagazig University, Sharkia, Egypt

Dr. Somyia Ahmed Abbassi
Lecturer of Entomology, National Center for Radiation Research & Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt

Biography

Name:	Mohamed Omar Mohamed Abo Kersh
Date and place of birth:	17- November 1974, Libya
Degree awarded:	B. Sc. Entomology, 1997, Faculty of Science, Nasar University, Lybia
	M. SC. Entomology, 2005, Faculty of Science, 7th October University, Libya
Occupation:	Demonstrator in Department of Zoology, Faculty of Science, El- Margeb University, El- Margeb, Libya
Date of registration for the Ph. D. degree:	May 2009.

ACKNOLEDGMENTS

Thanks to **ALLAH** who enabled me to complete this work, despite of all problems that faced me throughout my study.

I wish to express my profound, sincere appreciation and supreme gratitude to Dr. Mohamad Saad Hamed, Professor of Entomology, Faculty of Science, Ain Shams University for suggesting the point of study, his continuous encouragement, fruitful supervision and criticism of the manuscript, to Dr. Emad Mahmoud Said Barakat, Professor of Insect Physiology, Department of Entomology, Faculty of Science, Ain Shams University, for designing the plan of work, his continuous support, faithful guidance during practicing and writing the thesis, to Dr. Karam Teleb Hussein, Professor of Entomology, Faculty of Science, Zagazig University, for fruitful supervision, helpful advice and for reading and correcting the draft. to Dr. Samir Abdel Azim Nassar, Assistant Professor of Zoology, Faculty of Science, Zagazig University, for helpful advice and faithful technical guidance during practicing and writing the thesis, and to Dr. Somyia Ahmed Abbassi Lecturer of Entomology, Radiation Technology Research Institute, Atomic Energy Authority, Cairo, Egypt, for helpful advice and for reading and correcting the draft.

I want to express my sincere thanks to the Head, staff members and colleagues of Department of Entomology, Faculty of Science, Ain Shams University, for providing laboratory facilities, the kind supply of the materials of study.

I feel deeply grateful to the Head, staff members and colleagues of Zoology Department, Faculty of Science, Zagazig University, for providing laboratory facilities and for helping me in histochemical study.

Appreciation is also expressed to Dr. Hammam B. Hammam, Professor and Senior Researcher at the Food Legume Research Section, Plant Protection Research Institute, Agricultural Research Center, Egypt for his kind supply of the different varieties of cowpea seeds, *Vigna unguiculata* (L.)

The Study Mission, Higher Education Ministry, Libya is acknowledged due to financing the project by the scholarship and deal with Ain Shams University, Egypt.

DEDICATION

This thesis is dedicated to the sprit of my father,

and to the women in my life,

My mother,

My sister, and

My wife

TABLE OF CONTENTS

	List of tables	I
	List of figures	iii
	Abstract	Viii
I.	INTRODUCTION	1
	Aim of the work	5
II.	LITREATURE REVIEW	6
	1- The cowpea seed, *Vigna unguiculata*	6
	1.1- Taxonomy of cowpea	6
	1.2- Origin, domestication and diversity of cowpea	6
	1.3- Morphological description of cowpea	7
	1.4- Social-economic importance of cowpea	8
	1.5- Production constraints of cowpea	8
	2- The cowpea weevil, *Callosobruchus maculatus*	9
	2.1- Biology of cowpea weevil	9
	2.2- Host planet preference and oviposition decision by female cowpea weevil	11
	2.3- Association of cowpea weevil with cowpea seeds	13
	2.4- Control of cowpea weevil	15
	3- Screening methods (traits) used for the identification of cowpea resistant varieties	17
	4- Limitations of breeding legume crops for storage pest resistance	19
	5- Mechanisms of seed resistance to storage insect pests	20
	6- Physical and chemical characteristics of the cowpea seed varieties and resistance to cowpea weevils	23
	6.1- Physical characteristics of the cowpea seeds	23
	6.2- Chemical characteristics of the cowpea	25
	6.2.1- Cowpea phenolic compounds	26
	6.2.2- Cowpea lectins	29
	6.2.3- Cowpea trypsin inhibitor	32
	6.2.4- Cowpea α-amylase inhibitors	33
	6.2.5- Cowpea oligosaccharides	33
	6.2.6- Other non-nutritional storage proteins	34
	7- Chemical basis of resistance in pulses to *C. maculatus*	35
	7.1- Proteins make up the reserves of seeds	36
	7.2- Vicilins, legumins and arcelins	37
	7.3- Arcs belong to the lectin-like defense protein	39
	7.4- Protease inhibitors as a defense molecule	41
	8- Seed proteins impair development of cowpea weevil	45
	8.1- Binding of vicilins to midgut chitinous structures	45
	8.2- Uptake of Arc into the haemolymph of bruchid larvae	46
	9- Effect of cowpea storage proteins on the internal structure of bruchid larvae	84
III.	MATERIALS AND METHODS	50
	1- Insect culture	50
	2- Seed varieties	50

	3- Seed partitioning and pellet making	50
	4- Determination of some physical characteristics of seed varieties	51
	4.1- Seed color, texture and its helix color	52
	4.2- Major and minor diameter	52
	4.3- Seed coat thickness	52
	4.4- Seed weight	52
	5- Determination of some chemical contents of seed varieties	52
	5.1- Nutritional constituents of cowpea seed varieties	53
	5.1.1- Moisture content (%)	53
	5.1.2- Total crude protein (%)	53
	5.1.3- Total fat (%)	54
	5.1.4- Crude fiber (%)	54
	5.1.5- Ash (%)	55
	5.1.6- Crude carbohydrate (%)	55
	5.2- Non-nutritional constituents of cowpea seed varieties	55
	5.2.1- Tannins	55
	5.2.2- Total phenols	56
	5.2.3- Hemagglutinin activity	57
	5.2.4- Trypsin inhibitor activity	58
	6- Estimation of infestation potential of *C. maculatus* on cowpea seeds	60
	6.1- Determination of developmental time	61
	6.2- Determination of adult emergence (%)	61
	6.3- Determination of susceptibility index (SI)	61
	6.4- Determination of seed weight loss (%)	62
	7- Determination of *C. maculatus* larval damage on cowpea seeds	62
	7.1- Natural seed system assay	62
	7.1.1- The distance traveled by the larva inside the seed	63
	7.1.2- The seed weight consumed by the larva infesting the seed	63
	7.1.3- The seed volume consumed by the larva infesting the seed	64
	8- Biological studies of cowpea seed resistance	64
	8.1- Artificial seed system assay	64
	8.1.1- Developmental time in different seed zones	65
	9- Biochemical studies of cowpea seed resistance	65
	9.1- Extraction and fractionation of proteins	65
	9.2- Determination of protein concentration	65
	9.3- Protein electrophoresis	67
	10- Immunological studies	71
	10.1- Preparation of vicilins from cowpea seeds	71
	10.2- Vicilin quantification	71
	10.3- Demonstration of vicilin in internal organs of larval *C. maculatus*	72
	11- Histological and histochemical studies	74
	11.1 - Histological technique	74

	11.2 - Histochemical techniques	74
	11.2.1 - Histochemical demonstration of carbohydrates	74
	11.2.2 - Histochemical demonstration of proteins	75
	11.2.3 - Histochemical demonstration of DNA content	76
	11.2.4 – Quantitative histochemical technique	76
	12- Statistical analysis	77
IV.	RESULTS AND DISCUSSION	78
	1- Physical characteristics of the seeds varieties	78
	2- Chemical characteristics of the cowpea seed varieties	83
	2.1- The nutritional chemical constituents of cowpea seeds	83
	2.2- The non-nutritional chemical constituents of cowpea seeds	86
	3- Infestation potential of *C. maculatus* and seed varietal resistance	94
	4- Larval growth and development on susceptible and resistant seeds	99
	4.1- Shape of larval burrows	99
	4.2- The distance traveled by the larva inside the seed	101
	4.3- The seed weight consumed by the larva infesting the seed	102
	4.4- The seed volume consumed by the larva infesting the seed	103
	4.5- Size and growth patterns of larva infest the seed	104
	5- Biological studies of cowpea seed resistance	109
	5.1- Developmental time of *C. maculatus* feeding in different seed zones	109
	5.2- Cowpea seed protein analysis	110
	5.2.1- Quantification of total proteins in seed tissues	110
	5.2.2- Protein electrophoresis	111
	6- Immunological studies	117
	6.1- Vicilin concentration	117
	6.2- Detection of vicilins in internal organs of larval *C. maculatus*	118
	7- Histological observations	124
	7.1- Normal larvae (bruchid- susceptible)	124
	7.2- Variant larva (bruchid- resistant)	127
	8- Histochemical observations	149
	8.1- Carbohydrates	149
	8.2- Total protein:	157
	8.3- Deoxyribonucleic acid (DNA)	166
	9. Discussion of histological and histochemical observations	147
V	SUMMARY AND CONCLUSION	180
VI.	REFERENCE CITED	192
	ARABIC SUMMARY	

I. INTRODUCTION

In tropical and subtropical regions, the production of cereal grains and legumes is especially important because a high percentage of the population depends on these crops as its primary source of food. Legumes are the most important source of protein for economically-disadvantaged people of less-developed countries, in large part because high agronomic yields make them relatively inexpensive sources of proteins.

The cowpea (*Vigna unguiculata* L., Walp., Leguminosae) is the most popular legume cultivated in these countries because of its high nutritional value. Nutritional properties make them an excellent food supplement of the cereal grains (Singh, 2005). The mature grain contain 23-25% protein, 50-67% starch, B vitamins such as folic acid which is important in preventing birth defects, and essential micronutrients such as iron, calcium, and zinc (Kebe and Sembene, 2011). An added advantage of cowpea is that the plants can be harvested as fodder for livestock.

A significant portion of the total harvest of cowpea is stored for subsistence purposes using traditional methods. Cowpea traditionally is stored in sacks. These sacks usually are stacked in the same rooms used for living purposes. In the walls of these rooms there are cracks and crevices where insects can hide. Metal barrels also are used to some extent as storage units either inside or outside the house (De Breve *et al.*, 1984). Storage of cowpea and other pulses under adverse conditions is known to cause quality losses and therefore, shortened shelf-life; one of the most relevant causes of losses has been reported to be insect infestation (Hohlberg *et al.*, 1991).

Almost all arthropod species attacking stored products (i.e., insects and mites) are widely distributed throughout major climatic zones wherever these commodities are stored (Haines, 1984). Several insect pests attack food grains, including cowpea in storage (Southgate, 1958). Insects not only consume these materials but also contaminate them with body fragments and feces. Collectively, damage and contamination constitute major sanitation and quality control problems (Cotton and Wilbur, 1982).

The family Bruchidae includes major pests of legume seeds. Bruchidae includes some 850 species, several of which are considered of primary economic importance affecting stored cowpea (Southgate, 1958).

The cowpea seed beetle or cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) is one of the most destructive pests of stored leguminous seeds in tropical and subtropical countries, particularly the black-eyed seeds; the most favorite host to the cowpea weevil. Yet other varieties of cowpea, peas and bean are also liable to its attack and furnish suitable breeding sources for it (El- Sawaf, 1956). It infests seeds of wild and cultivated legumes and it multiplies rapidly in storage, giving rise to a new generation every month in grain at 25°C (Fox, 1993). Their feeding causes sever weight losses and reduce nutritional value of the seeds. Farmers storing cowpea confront significant economic losses from either this species or a combination of other beetles. Consumers have a strong aversion to grain that has been damaged by weevils, but it still can be effective as seed, although, germination percentage may have been reduced.

In the aim at reducing the losses caused by the weevils, many control strategies were considered. Insecticides for the control of bruchid weevils are rarely available to, or affordable by the subsistence farmer. When an insecticide is available, there is appreciable danger of abuse because there is a general lake of knowledge about proper use. Many people of the world need ready access to safe and reliable control against seed beetles (Quentin *et al.*, 1991).

Among the strategies, there is the use of cowpea varieties which are resistant to weevils' attack. Where chemical control is expensive or unavailable, the use of resistant varieties has been considered an alternative for reducing bruchids damage and losses. However, a few researches are made to show the relation between the levels of infestation on cowpea in proportion to the places where the cowpeas are stored and the cultivated cowpea varieties.

The objective of this study is to evaluate the level of cowpea infestation by weevils in proportion to the kind of cowpea variety. The larval penetration and the rate of emergence of insects are also studied for the different varieties. The major purpose is to improve knowledge on the physical characteristics and biochemical basis of defensive strategies of cowpea against bruchids, thus allowing plant breeders to increase the insect resistance of cultivated varieties.

Plants possess an array of physical or chemical defensive molecules against herbivores. As examples of physical defenses, plant cells have rigid cell walls, usually rich in lignin and suberin, trichomes or needles, or they produce gums and waxes with repulsive potential against predators (Broglie and Broglie, 1993). Chemical defenses frequently involve the participation of secondary metabolites, such as phytoalexins, non-protein amino acids, phenols, etc. They may also include proteins such as proteinase inhibitors, lectins, α-amylase inhibitors, hydrolytic enzymes such as chitinases and glucanases, ribosome-inactivating proteins, thionins, defensins (Shewry *et al.*, 1993). However, despite this arsenal, plant killing by insects and microorganisms is an inexorable reality and a threat to crops used as food and feed.

Biochemical studies suggested that variant storage proteins, vicilins (Macedo *et al.*, 1993; Fernandes and Xavier-Filho, 1998; Yunes *et al.*, 1998 Sales *et al.*, 2000;) and arcelins (Osborn *et al.*, 1988; Cardona *et al.*,

1990) from cowpea seeds their toxic properties may be related to their recognition and interaction with glycoproteins and other membrane constituents along the digestive tract of the insect. Immunolocalization studies have shown that certain *V. unguiculata* seed proteins bind to the larval midgut epithelium. This ability to bind, together with the low digestibility of these proteins and their absorption through the midgut, may be associated with the deleterious effects of these proteins (Firmino *et al.*, 1996; Fernandes and Xavier-Filho, 1998; Sales *et al.*, 2000).

Plant protease inhibitors function as defense molecules against insect predation have shown the great potential in delaying insect growth and development (Mosolov *et al.*, 2001a). The defensive function of these inhibitors is attributed to their ability to suppress insect digestive enzymes, consequently causing insect starvation. Alternative activities other than simple protease inhibition, such as lipid transfer protein-like activity, may also play a role in their anti-insect function (Mosolov *et al.*, 2001b). Many coleopteran insects utilize their major digestive enzymes such as serine, cysteine, aspartate and metallo-proteases for food protein degradation (Zhu-Salzman *et al.*, 2003). Pharmacological and molecular studies indicated that proteases that are present in the guts of numerous insects can also function to degrade plant defense proteins. The potential use of protease inhibitors for plant protection against insect pests is, however, complicated by the ability of insects to circumvent plant defenses. Available biochemical and molecular evidence indicates that some insects adapt to protease inhibitors by overproduction of existing digestive proteases (De Leo *et al.*, 1998), while others selectively induce inhibitor-insensitive proteases,

In view of the variation observed in damage due to feeding by *C. maculatus* larvae on various cowpea seed varieties, the present study was planned to identify chemical factors that are responsible for imparting resistance and/or the possible strategy used by larvae to overcome the host defense mechanisms.

Aim of the work:

The overall aim of the present study is to determine some histochemical and immunological characteristics associated with feeding of cowpea weevil larvae, *C. maculatus* on susceptible and resistant seed varieties of cowpea, *V. unguiculata*; hopping that the varietal differences which could be obtained may provide important information for designing a control strategy for this important pest of storage products.

To accomplish this aim, six major objectives were developed and covered the following points:-

1- Determination of some physical and chemical characteristics of susceptible and resistant varieties of cowpea, *V. unguiculata*

2- Determination of the infestation potential of *C. maculatus* on both seed varieties.

3- Analysis of seed variability to study the chemical basis of resistance to bruchid infestation.

4- Investigation of the toxicity of cowpea storage proteins isolated from both seed varieties on the development of the weevil.

5- Evaluation of the capability of these proteins to bind to the midgut epithelium of larvae fed on susceptible and resistant cowpea seeds.

6- Assessment of the impact of toxicity of cowpea storage proteins in both seed varieties on the histological and histochemical structure of the bruchid larvae. This will be investigated in an attempt to explain the mode of action of these toxic proteins at the cellular level and to know how plants can defend themselves against pests and pathogens with a variety of physical and chemical strategies.

II. LITERATURE REVIEW

1- The cowpea seed, *Vigna unguiculata*:

1.1- Taxonomy of cowpea:

Cowpea [*Vigna unguiculata* (L) Walp.] is a dicotyledonous crop in the order: *Fabaceae*, subfamily: *Faboideae* (Syn. *Papillionoideae*), tribe: *Phaseoleae*, subtribe: *Phaseolinae*, genus: *Vigna* and section: *Catiang*. It is a diploid plant containing 22 chromosomes (Timko and Singh, 2008) and its nuclear genome size is estimated to cover 620 million base pairs (Mbp) (Timko *et al*. 2008). The genus was divided into subgenera based upon morphological characteristics, the extent of genetic hybridization and geographical distribution of the species. The major groups consist of the African sub-genera: *Vigna* and *Haydonia*, the Asian sub-genus: *Ceratotropis,* and the American subgenera: *Sigmoidotropis* and *Lasiopron* (Timko and Singh, 2008). *V. unguiculata* sub-species *unguiculata* includes four cultivated groups: *unguiculata biflora* (or *cylindrical*), *sesquipedalis*, and *textilis* (Ng and Maréchal, 1985). *V. unguiculata* subspecies *dekindiana*, *stenophylla*, and *tenuis* are intermediate wild progenitors of cultivated cowpea and form the major portion of the primary gene pool of cowpea. Fatokun and Singh (1987) pointed out that, wild subspecies like *pubescence* that do not readily hybridize and show some degree of pollen sterility form a secondary gene pool.

1.2- Origin, domestication and diversity of cowpea:

The precise origin of cultivated cowpea is not known. However, Asia and Africa were discussed as domestication sites of this crop. Recently, Asia has being questioned as a center of domestication due to the lack of wild ancestors. By reason of the highest genetic diversity of the crop and the presence of the most primitive form of wild cowpea, (Padulosi, 1993), Southern Africa is the most probable center of domestication. The determination of the origin and domestication of cowpea had been based on morphological and cytological evidence, information on its geographical distribution and cultural practices (Ng 1995; Ng and Maréchal 1985). Padulosi and Ng (1997) suggested Southern Africa to be the center of origin, while domestication occurred in West Africa. The cultivated cowpea (*V. unguiculata*) evolved through domestication and selection from the annual wild cowpea (ssp. *dekindtiana*), and during this process seed dormancy and pod dehiscence was lost (Ng, 1995). The distribution of diverse wild cowpea from Ethiopia to South Africa lead to the proposition that East and Southern Africa are primary centers of diversity, while West and Central Africa are secondary centers of diversity (Baudouin and Maréchal, 1985).

1.3- Morphological description of cowpea:

Based on the investigation conducted by Padulosi and Ng (1997) and supported by (Baudouin and Maréchal, 1985; Padulosi, 1993), about the range of variation and number of varieties found in wild cowpeas as well as their primitive characteristics, such as perenniality, hairiness, small size of pods and seeds, pod shattering with pronounced exine on the surface of the pollen, out-breeding and bearded stigma, the highest genetic diversity and most primitive forms of wild *V. unguiculata* occur in southern Africa.

Variability in morphology of different cowpea accession is very high. There are three types according to their uses: for grain, forage or dual purpose. *V. unguiculata* is an herbaceous, prostrate, climbing or sub erect annual plant, growing 15-80 cm high. Leaves are alternating trifoliate with petiole 5-25 cm long. The lateral leaflet is opposite and asymmetrical, while the central leaflet is symmetrical and ovate. The inflorescence are racemose, flowers are white, cream, yellow or purple.