INHERITANCE OF *Bt* TRANSGENE CONTROLLING THE LEGUME POD BORER (*Maruca vitrata* [Geyer]) IN COWPEA(*Vigna unguiculata* L. Walp)

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DECLARATION

I declare that the work in the thesis entitled INHERITANCE OF *Bt* TRANSGENE CONTROLLING THE LEGUME POD BORER *(Maruca vitrata* [Geyer]) IN COWPEA (*Vigna unguiculata* L. Walp) has been performed by me in the Department of Plant Science under the supervison of Professor M.F. Ishiyaku, Drs U.S. Abdullah and M.D. Katung. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma in any university.

MOHAMMED, Baba Saba ---------- ----------

Signature Date

CERTIFICATION

This thesis entitled ‘‘INHERITANCE OF *Bt* TRANSGENE CONTROLLING THE LEGUME POD BORER *(Maruca vitrata* [Geyer]) IN COWPEA (*Vigna unguiculata* L. Walp) ” by MOHAMMED, Baba Saba meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to the late Professor Balarabe Tanimu; Former Director, Institute for Agricultural Research, Ahmadu Bello University Zaria. May the mercy and compassion of Allah be upon his soul (Ameen).

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ABSTRACT

The inheritance of *Bt* transgene controlling the legume pod borer [*Maruca vitrata* (Geyer)] in cowpea (*Vigna unguiculata* L. Walp.) was studied using two transgenic lines namely; 709 and 711. These were crossed to three non-transgenic lines viz; IT97K-499-35, IT93K-693-2 and IT86D-1010 to produce six hybrids. Field trial was conducted at confined field trial site (CFT), Samaru, involving F_1, F_2, backcross populations and the five parental materials. The study was undertaken to understand the mode of inheritance, segregation and stability of *Cry1Ab* gene in different cowpea genetic backgrounds. The results showed 3:1 and 1:1 segregation for F_2 and first backcross generations respectively. These demonstrated the existence of Mendelian inheritance of *Cry1Ab* gene in cowpea genome. *Cry1Ab* gene was inherited as a single dominant gene and segregates in the Mendelian fashion. Therefore, classical cross and backcross can introgress the *Cry1Ab Maruca* resistant gene from the transgenics into conventional cowpea varieties. Quantitative analysis for *Cry1Ab* gene resistance with the use of artificial infestation showed that the inheritance of *Cry1Ab* gene was governed by a dominant gene action. The performance of the transgenic lines and their hybrids in comparison with the non-transgenic parents regarding some key paramters were conducted. The transgenics and the hybrids did not sustain pod damage due to *Maruca* feeding while the non-transgenic genotypes sustained various degrees of pod damage as a result of feeding activities by the *Maruca*. In addition, the impact of genetic transformation of cowpea on the expression of some morphological traits of the transgenic lines were investigated. In the present study, comparison made between the cowpea transgenic line 709 and original parental type (IT86D-1010) showed non-significant differences in all the parameters compared while, comparison made between cowpea transgenic line 711 showed highly significant differences for days to first flowering, days to first pod maturity and plant height. However, the phenotypic data showed no pattern of changes that would support the hypothesis that the introduction of *Cry1Ab* gene had any unintended impact on the productive performance of cowpea plants, besides conferring resistance to *Maruca* pest.

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List of Abbreviations

AATF	African Agricultural Technology Foundation
ANZFA	Australia New Zealand Food Standards Agency, Canberra, Australia
ARCN	Agricultural Research Council of Nigeria
BC_1P_1	First Backcross to Parent one
BC_1P_2	First Backcross to Parent two
Bt	*Bacillus thuriengiensis*
CBD	The Convention on Biological Diversity
CERA	Center for Environmental Risk Assessment
CFT	Confined Field Trial
CGIAR	Consultative Group on International Agricultural Research
CSIRO	Commonwealth Sciencitific and Industrial Research Organization
GE	Genetic Engineering
GLM	Generalized Linear Model
F_1	Fisrt Filial generation
F_2	Second Filial generation
GCDT	Global Crop Diverstiy Trust
IAR	Institute for Agricultural Research
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
IITA	International Institute of Tropical Agriculture
MAS	Marker Assisted Selection
NGICA	NGICA Network for Genetic Improvement of Cowpea in Africa
PD	Number of pods Damaged Per Plant
NUPs	Number of Undamaged Pods per Plant
OECD	Organisation for Economic Co-operation and Development
P_1	Parent one
P_2	Parent two
SAS	Statistical Analysis System
TNPs	Total Number of Pods Per Plant
ZAMSEED	Zambia Seed Company

CHAPTER ONE

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an annual legume, commonly referred to as southern pea, blackeye pea, crowder pea, lubia, niebe, coupe or frijole which originated in Africa and widely grown in Africa, Latin America, Southeast Asia and in the southern United States (Davis *et al*., 1991). It is chiefly used as a grain crop, for animal fodder and as a vegetable. The history of cowpea dates to ancient West African cereal farming, five to six thousand years ago, where it was closely associated with the cultivation of sorghum and pearl millet (Davis *et al*., 1991). Outside Africa, the main producing areas are in central and south America and in Asia, with several smaller areas spread over southern Europe, southern USA, and Oceania (Quin, 1997). Among countries, the main producers are Nigeria, Niger and Brazil (Quin, 1997).

Cowpea *(V. unguiculata L. Walp)* is considered the most important food grain legume in the dry savannas of tropical Africa (NGICA, 2002). It is the most important indigenous African legume for both home use and as a cash crop (Kushwaha *et al.,* 2004) and it is especially important for the Sahel because of its drought tolerance (Kushwaha *et al.,* 2004). It is rich in quality protein and has energy content almost equivalent to that of cereal grains, it is a good source of quality fodder for livestock and also provides cash income (Bressani, 1985; Walker, 1982). The protein content ranges from 23-38 % in the grain and 29-43 % in the leaves (Bressani, 1985; Walker, 1982). Averagely, the cowpea grain contains about 24.8% protein, 63.6% carbohydrate, 1.9% fat, 6.3% fibre, 0.00074% thiamine, 0.00042%

riboflavin, and 0.00281% niacin (Davis *et al.,* 1991). For this reason, cowpea has been referred to as "the poor man's meat" (Aykroyd and Doughthy, 1982).

All parts of the plant that are used for food are nutritious, providing protein, vitamins (notably vitamin B) and minerals (Singh *et al*., 2002). The protein in cowpea seed is rich in the amino acids, lysine and tryptophan as compared to cereal grains; however, it is deficient in methionine and cystine when compared to animal proteins, therefore, cowpea seed is valued as a nutritional supplement to cereals and as an extender of animal proteins (Singh *et al.,* 2002).

Nearly 200 million people in Africa consume the crop (AATF, 2010; NGICA, 2002). Cowpea is consumed in many forms; the young leaves, green pods, and green seeds are used as vegetables. Dry seeds are used in various food preparations, the haulms are fed to livestock as nutritious supplement to cereal fodder and being a fast growing crop, cowpea curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contributes to soil fertility (Singh *et al*., 2002).

An estimated 14.5 million ha of land is planted to cowpea each year worldwide; with total annual production being approximately 6.2 million metric tonnes (MT) (Abate *et al*., 2012). Nigeria is the largest producer and consumer of cowpea with about 5million hectares (ha) and over 2 million tonnes produced annually (Singh *et al.,* 2002). The current average yield is estimated at about 454 kg per ha (Abate *et al*., 2012) while the world cowpea area,

yield and production grew at an annual rate of 4.5%, 1.4% and 5.9%, respectively (Abate *et al*., 2012).

Cowpea yield is very low, with grain yield in Nigeria ranging between 100 – 300 kg/ha, under subsistence practices where it is grown on poor soils and poor management practices (Darshna *et al.*, 2007). However, improved varieties grown under proper management practices have yielded up to 1500 kg/ha (Darshna *et al.*, 2007).

1.1 Research Problem

The overall productivity of its existing traditional genotypes is low due to their prominent susceptibility to insect pests and pathogens apart from obvious genetic inadequacies such as viny growth habit, compulsive photoperiodism, low flowering and seed setting abilities (Darshna *et al.*, 2007). Insect pests are the major constraints in cowpea cultivation and among the most damaging insects are aphids (*Aphis cracivora*), flower thrips (*Megaluthrips djsotedti*), cowpea pod borer (*Maruca vitrata [Geyer]*), pod sucking bugs (*Clavigralla* spp), and the cowpea weevils (*Callosbrochus maculatus*) (Darshna *et al.*, 2007). Parasitic weeds especially Striga, Alectra constitutes major biotic factors militating against cowpea cultivation.

The cowpea pod borer *(M. vitrata)* is a serious lepidopteran pest that inflicts severe damage to Cowpea on farmers' fields. In severe infestations yield losses of between 70–80% have been reported (AATF, 2010). Control through spraying with insecticide has not been fully adopted by farmers due to the prohibitive costs, causing resource-poor farmers to opt for cheaper but more toxic alternatives that impact their health (AATF, 2010). Also, farmers

are not well equipped to protect themselves when using such toxic chemicals, in some farming communities, adulterated chemicals that do not control the pests are sold to farmers (Fatokun, 2009). The pod borer (*M. vitrata*) has persistently affected the yield of cowpea (*V. unguiculata*) in most West African countries and there is need to acquire a resistant variety. Although great progress has been made in cowpea improvement, there is a need to protect the cowpea crop with 2–3 sprays of insecticide from *Maruca* pod borer for which no resistance has been found (AATF, 2010). Much progress has not been made in host plant resistance in cowpea, especially *M. vitrata*. Using conventional breeding, several hundreds of accessions of cultivated cowpea and its wild relatives have also been screened for resistance to this pest. Accessions belonging to *Vigna vexillata* were found to be resistant to *M. vitrata*. These accessions were found to be closest to cowpea in a phylogenetic study of diversity in the *Vigna* species. The study was based on data obtained after DNA genotyping. Efforts were made to cross cowpea with *V. vexillata* but without success. This strong cross-incompatibility makes gene exchange between the two species impossible. This is where biotechnology comes to the rescue of the crop (Fatokun, 2009).

1.2 Justification

Laboratory studies have shown that the *Bt* gene confers resistance on cowpea to the pod borer (Fatokun, 2009) and the transfer of the *Bt* gene into improved cowpea varieties, will reduce the need for insecticide sprays in cowpea and smallholder farmers can substantially increase their yields and greatly enhance their nutritional and economic status (AATF, 2010). This led to genetic transformation of an advanced breeding line (IT86D-1010) with *Cry1Ab* gene at the Commonwealth Scientific and Industrial Research Organization

Laboratory, Canberra, Australia (Fatokun, 2009; Ishiyaku, 2010). There have been a number of concerns raised from different people regarding the use of *Bt* crops. For example, it is alleged that the resistance to insect pests in biotechnology-derived crops may pose new or different human safety concerns in comparison to conventionally bred crops with similar traits. The U.S. Environmental Protection Agency has determined that the numerous toxicology studies conducted with *Bt* microbial products show no adverse effects and has concluded that these products are not toxic or pathogenic to humans (McClintock *et al.,* 1995; EPA, 1998). EPA, in its1998 re-registration eligibility decision, concluded that microbial *Bt* products pose no unreasonable adverse effects to humans or the environment and that all uses of those products are eligible for re-registration (EPA, 1998).

Cowpea is the first grain legume to be genetically transformed with *Bt* gene, and the inheritance and segregation of foreign gene (s) have not been reported. This step is indispensable for the future utilization of cowpea plants in breeding programs. The inheritance and stable expression of *Cry1Ab* transgene has been studied in different transgenic plants such as cotton, rice, maize (Wang *et al.,* 2012; Wu *et al.,* 2002). However, it is very important to understand the inheritance, segregation and stability of foreign gene (s) for a given transgenic crop. Stable inheritance and expression of transgenes in transgenic crop plants has important role on successful employment of genetic engineering in traditional breeding programs (Wu *et al.,* 2002). Transformation of cowpea with genes from a soil Bacterium *Bacillus thuringiensis* (*Bt*) is a common approach used in other crops like maize, cotton, rice etc to confer resistance to insect infestations providing an opportunity for plant breeders to utilize genes from other species. Once a gene is stably

introduced into a plant, it could be utilized for development of new varieties through classical breeding programs.

Breeding for insect resistance with the aid of phenotypic selection is time consuming, laborious and relatively expensive. In addition, most crops have a high level of heterozygosity that makes visual selection difficult but selection based on allele composition will avoid this problem. Ability to select breeding progeny early at the seedling stage is another advantage of using allele-based selection tools. The number of plants that are needed to be maintained in a crop breeding programme can be reduced by eliminating progenies that do not carry the desirable allele at the seedling stage, saving space, time, labor and other resources. Genetic engineering is a regulated technology and there is need to understand the stability of the foreign gene across generations in different cowpea genetic backgrounds because of linkage with undesirable genes. With the aforementioned importance of cowpea, constraints posed by *M. vitrata*, inability to source resistant genes to pod borer in the collected germplasm and time factor involved in phenotypic based selection, justified this research.

1.3 Objectives

The present study was designed and conducted in order to understand the mode of inheritance, segregation and stability of *Cry1Ab* gene in transgenic cowpea genetic backgrounds as the main objective while the specific objectives are;

i) to study the inheritance pattern of *Cry1Ab* gene in generations of cowpea lines,

ii) to evaluate the two transgenic lines and their hybrids regarding some key parameters,

iii) To carry out a comparative assessment between transgenics and untransformed parent, and

iv) To conduct Quantitative genetic analysis for *Cry1Ab Maruca* resistant gene.

1.4 Research Hypotheses

The study was carried out to test the following hypotheses that;

(i) The inheritance of *Cry1Ab* gene in First filial generation is governed by a dominant gene action,

(ii) The inheritance of *Cry1Ab* gene in segregating populations follows the Mendelian expected ratio of 3:1 and 1:1 in the F_2 and backcross generations respectively,

iii) The performance of transgenic lines and their hybrids differed from the non-transgenic lines,

(iv) The genetic modification of cowpea with *Cry1Ab* gene has no effect on its morphological features, and

(v) The precision of quantitative genetic analysis is the same with the analysis based on *Cry1Ab Bt* strip screening.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Distribution of Cowpea

Cowpea (*V. unguiculata* L. Walp.) is a member of the Phaseoleae tribe of the fabaceae family. Members of the Phaseoleae include many of the economically important warm season grain and oilseed legumes, such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), and mungbean (*Vigna radiata*) (Timko *et al.,* 2007). Cowpea is synonymous as *Vigna sinensis* (L.) Savi; *Vigna sinensis* Endl (Anonymous, 2012c, Anonymous, 2012d and Bruce, 2008) and *Vigna catjang* (Burm.) (Anonymous, 2012a and Anonymous, 2012d). There is a difference of opinion regarding the valid name for this plant, however, *V. unguiculata* has been preferred with the several cowpeas throughout the world as cultivars (Anonymous, 2012a).

The name cowpea probably originated from the fact that the plant was an important source of hay for cows in the southeastern United States and in other parts of the world. Some important local names for cowpea around the world include “Niebe,” “Wake,” and “Ewa” in much of West Africa and “Caupi” in Brazil. In the United States, other names used to describe cowpeas include “southern peas,” “black-eyed peas,” “field peas,” “pinkeyes,” and “crowders.” These names reflect traditional seed and market classes that developed over time in the southern United States (Timko *et al.,* 2007). Classification of cultivated cowpea is now based on five so-called cultivar-groups (cv-gr., also “cultigroups”): *Unguiculata*,

Biflora, *Catjang*, *Sesquipedalis* (the former subspecies *Unguiculata*, *Catjang* and *Sesquipedalis*), *Textilis* and *Melanophtalmus* (Anonymous, 2012d).

Cowpea most certainly evolved in Africa, as wild cowpeas only exist in Africa and Madagascar (Steele, 1976). Although the centre of domestication for cowpea is uncertain, all evidence points to its originating in Africa. Ethiopia, Central, Southern and West Africa have all been considered probable centers of domestication (Padulosi and Ng, 1997). Many centres of origin have been speculated for cowpea and the precise location of the centre of origin of a species is rather difficult to determine. Previous speculation on the origin and domestication of cowpea had been based on botanical and cytological evidence, information on its geographical distribution and cultural practices, and historical records (Ng and Marechal, 1985; Ng, 1995). Based on the range of variation and number of varieties found in wild cowpea, as well as their primitive characteristics, such as perenniality, hairiness, small size of the pods and seeds, pod shattering, with pronounced exine on the surface of pollen, out-breeding, and bearded stigma, the highest genetic diversity and most primitive of the wild *V. unguiculata* occur in southern Africa in the region encompassing Namibia from the west, across Bostwana, Zambia, Zimbabwe, and Mozambique to the east, the republic of south Africa and Swaziland to the south (Padulosi *et al.* 1997). However, the center of maximum diversity of cultivated cowpea is found in West Africa, in an area encompassing the savanna region of Nigeria, southern Niger, part of Burkina Faso, northern Benin, Togo, and the northwestern part of Cameroon (Ng and Marechal, 1985; Ng, 1995).

Recent studies of more than 10,000 accessions of the world cowpea collections at International Institute of Tropical Agriculture (IITA) revealed that germplasm accessions from Nigeria, Niger, Burkina Faso and Ghana showed greater diversity than those from East Africa, with the centre of diversity in Nigeria. These studies provide further evidence that West Africa was the primary centre of domestication. Cowpea was introduced from Africa to the tropical Americans in the 17th century by the Spanish in the course of the slave trade; it has been grown in Southern USA since the early 18th Century (Padulosi and Ng, 1997).

In another report, it is said that cowpea as a crop has its origins in ancient West African cereal farming, 5,000-6,000 years ago. It was closely associated with the domestication and cultivation of sorghum and pearl millet. It is now grown on a commercial scale in 33 countries in Asia, Africa, and Central and South America, as well as parts of southern Europe and the United States (GCDT, 2012). West Africa where the highest genetic diversity and the most primitive forms of wild *V. unguiculata* occur, was probably the primary centre of domestication. Some evidence that domestication occurred in northeastern Africa, based on studies of amplified fragment length polymorphism (AFLP) analysis, has also been presented (Coulibaly *et al*., 2002). The wild cowpea *Vigna unguiculata* ssp. *Unguiculata* var. *spontanea* is the likely progenitor of cultivated cowpea (Pasquet, 1999). It is likely that the crop was first introduced to India during the Neolithic period, and therefore India appears to be a secondary center of genetic diversity (Pant *et al.,* 1982). "Yard-long beans," a unique cultivar group (Sesquipedialis) of cowpea that

produces very long pods widely consumed in Asia as a fresh green or "snap" bean, apparently evolved in Asia and is rare in African landrace germplasm (Timko *et al*., 2007).

Today, cowpea is cultivated throughout the tropics and subtropics between 35ºN and 30ºS, across Asia and Oceania, the Middle East, southern Europe, Africa, southern USA, and Central and South America (Anonymous, 2012d).

2.2 Botany

Cowpea is an herbaceous, warm-season annual crop that is similar in appearance to common bean except that leaves are generally darker green, shinier, and less pubescent. Cowpeas also are generally more robust in appearance than common beans with better developed root systems and thicker stems and branches (Timko *et al*., 2007). Its growth habit can be erect, semi-erect, prostrate (trailing), or climbing depending mostly on genotype, although photoperiod and growing conditions can also affect plant stature. Most cowpea accessions have indeterminate stem and branch apicies. Early flowering cowpea genotypes can produce a crop of dry grain in 60 days, while longer season genotypes may require more than 150 days to mature depending on photoperiod. Flowers are borne on racemes on 15 to 40mm peduncles that arise from the leaf axils. Two or three pods per peduncle are common, and often four or more pods are carried on a single peduncle if growing conditions are very favorable the presence of these long peduncles is a distinguishing feature of cowpea, and this characteristic also facilitates hand harvesting (Timko *et al*., 2007).

Variability in plant morphology of the different accessions is high. There are three types according to their uses: for grain, forage or dual-purpose. *V. unguiculata* grows between 15-80cm high depending on the genotype and growing conditions. Leaves are alternate, trifoliolate with petioles 5-25 cm long. The lateral leaflets are opposite and asymmetrical, while the central leaflet is symmetrical and ovate. Inflorescence racemose, flowers white, cream, yellow, mauve or purple. There are accessions with determinate and with indeterminate growth habit. Pods are 10-23 cm long with 10-15 seeds/pod. Seeds are variable in size and shape, square to oblong and variously coloured, including white, brown, maroon, cream and green, commonly in the range of 5,000-12,000 seeds/kg (Anonymous, 2012d).

The plant architecture and growth habits of cowpeas are diverse because genotypes and environments interact to influence both the numbers and the lengths of their branches. At one extreme are bushy, erect types with short branches, at the other are prostrate, spreading or sometimes twining and climbing forms with five or more orders of branching. Each axillary inflorescence is a compound raceme of several simple racemes carried on a grooved peduncle 5-60cm (Zamseed, 2012). As the floral buds develop, elongation of the peduncle occurs rapidly (Ehler and Hall, 1997). Each simple raceme has between six and twelve flower buds, but only the lower, first formed pair develops while the rest degenerate to form extra-floral nectarines between the paired flowers (Zamseed, 2012).

The floral buds of cowpea are borne on peduncles arising in axils of the main stem or branches, floral buds complete their development in one to two weeks, typically each floral

raceme produces 2 to 4 flowers sequentially over several days. After two to four pods are set, further development of other floral buds on the apex of the peduncle (raceme) is arrested until the first set pods become mature, the length of peduncles typically doubles after anthesis (Ehlers and Hall, 1997). In the axil of each leaf there are three buds. Only the central bud normally expands to produce either a potentially indeterminate, monopodial branch or a racemose inflorescence. Consequently, the number of branches is the complement of the number of inflorescence. Some determinate cowpea genotypes have terminal inflorescence on their main stem and branches, such types may have more than one inflorescence and a reduced branch at a single node.

The cleistogamous flowers are typically papilionaceous and are large, with a standard petal 2-3cm wide which is either white or with anthocyanin pigmentation in shades or pale mauve or pink to dark purple, with or without a "V" shaped distribution at the top centre of the petal (Zamseed, 2012).

Mature fruits vary widely in size, shape, colour and texture. Fruit lengths range between 12-20 cm and are straight, curved or coiled. They may lack pigmentation (tan or straw coloured) or have varying intensities of anthocyanin pigmentation from pink purple or almost black. They contain between six and twenty-one kidney shaped, oval or, rarely, almost spherical seed whose dimensions is within the range 5-12 mm and with individual weights between 50 mg and 340 mg. The testa may be smooth or wrinkled and rough. Testas may be white, buff brown, red and black (Zamseed, 2012). Pods are cylindrical and may be curved or straight, with between 8 and 15 seeds per pod. Seed may also be speckled or patterned. Seeds of well-known cowpea types, such as "blackeye pea" and "pinkeye," are

white with a round irregular-shaped black or red pigmented area encircling the hilum, giving the seed the appearance of an eye (Timko *et al*., 2007).

Emergence is epigeal (similar to common Bean and Lupin), where the cotyledons emerge from the ground during germination. This type of emergence makes cowpea more susceptible to seedling injury, since the plant does not regenerate buds below the cotyledonary node. The open display of flowers in and above the canopy and the presence of extrafloral nectaries contribute to the attraction of insects. Cowpea is highly self-pollinated in most environments, the result of a cleistogamous flower structure and simultaneous pollen shed and stigma receptivity (Ehlers and Hall, 1997).

2.3 Economic Importance

Cowpea is of major importance to the livelihoods of millions of people providing nourishment and an opportunity to generate income, it is an important food legume and an essential component of cropping systems in the drier regions of the tropics covering parts of Asia and Oceania, the Middle East, Southern Europe, Africa, Southern USA and Central and South America (Padulosi and Ng, 1997). It is consumed by approximately two hundred million people in sub-Saharan Africa and is a source of income for millions of low resource farmers (NGICA, 2002, AATF, 2010).

Being a fast growing crop, cowpea curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contribute to soil fertility (Singh *et al.,* 2002). Cowpea is consumed in many forms: the young leaves, green pods, and green seeds are used as

vegetables; dry seeds are used in various food preparations; and the haulms are fed to livestock as nutritious supplement to cereal fodder (Singh *et al.,* 2002). Trading fresh produce and processed food and snacks provide rural and urban women with the opportunity for earning cash income and, as a major source of protein, minerals, and vitamins in daily diets; it positively impacts on the health of women and children. The bulk of the diet of rural and urban poor Africa consists of starchy food made from cassava, yam, plantain and banana, millet, sorghum, and maize (Singh *et al.,* 2002). The addition of even a small amount of cowpea ensures the nutritional balance of the diet and enhances the protein quality by the synergistic effect of high protein and high lysine from cowpea and high methionine and high energy from the cereals, this nutritious and balanced food ensures good health and enables the body to resist infectious diseases and slow down their development (Singh *et al.,* 2002).

Early maturing cowpea varieties can provide the first food from the current harvest sooner than any other crop (in as few as 55 days after planting), thereby shortening the "hungry period" that often occurs just prior to harvest of the current season's crop in farming communities in the developing world (Timko *et al*., 2007). Because of its superior nutritional attributes, versatility, adaptability, productivity, cowpea was chosen by the United States National Aeronautical and Space Administration as one of few crops worthy of study for cultivation in space stations (Ehlers and Hall, 1997).

Cowpea is a drought-tolerant crop and thrives in warm weather. It is well-adapted to the drier regions of the tropics, where other food legumes do not perform well. It grows well in poor soils with more than 85% sand and with less than 0.2% organic matter and low levels

of phosphorus. In addition, it is shade tolerant, and therefore, compatible as an intercrop with Maize, Millet, Sorghum, Sugarcane, and Cotton. This makes Cowpea an important component of traditional intercropping systems, especially in the complex, subsistence farming systems of the dry savannas in sub-Saharan Africa (AATF, 2010).

Cowpea is now a broadly adapted and highly variable crop, cultivated around the world primarily for seed, but also as a vegetable (for leafy greens, green pods, fresh shelled green peas, and shelled dried peas), a cover crop, and fodder. The Cowpea haulm is also a great source of livestock feed, and therefore of great value to farmers. It also is used as a green manure crop (Singh *et al.,* 2002).

2.4 Major Constraints of Cowpea Production

Many biotic and abiotic factors greatly reduce cowpea productivity in the traditional African farming systems, among these constraints is the Pod borer, *Maruca vitrata,* which perennially damages Cowpea pods on farmers' fields (AATF, 2010). According to the International Institute of Tropical Agriculture (IITA) in Nigeria, the major pests attacking cowpea plants are flower thrips (*Megalurothrips sjostedti*), pod borer (*Maruca vitrata*), and pod sucking bugs (GCDT, 2012).

Drought (or low moisture stress) and low soil fertility affect the well-being of all tropical legumes across the countries in the Asia and sub Saharan Africa. Drought is not necessarily a lack of moisture but rather it is the result of erratic distribution of rainfall in many

situations. Different crops have different critical periods in their demand for adequate rainfall. Some crops like pigeonpea are particularly sensitive to low moisture stress, especially near maturity stage. In many circumstances (e.g. common bean), the impact of drought is exacerbated by low soil fertility and soil pathogens (Abate *et al*., 2012). Drought may be expressed in the form of mid-season gaps of rains, or rains ending too early or low amounts of rainfall, or rains coming late. Extreme heat is another abiotic factor that threatens the development of tropical legumes. At present, chickpea and cowpea are the most affected crops. The importance of heat is expected to worsen with the impact of climate change (Abate *et al*., 2012).

The witch weed *Striga gesnerioides* and *Alectra vogelii* are two most important parasitic weeds that pose great challenges to the production of cowpea in western Africa. An IPM approach based on developing host plant resistance, combined with crop rotation, is the recommended method of management (Abate *et al.,* 2012).

2.5.0 Biology, Effects and Economic Importance of *Maruca vitrata*

The legume pod borer, *M. vitrata* [Geyer] (Lepidoptera: Crambidae) is a serious pest of grain legumes in the tropics and subtropics because of its extensive host range, destructiveness and distribution (Mohammad, 2007). The pod borer has a very wide range of distribution throughout Africa and Asia, South America and southern states of Australia (Mohammad, 2007; Anonymous, 2012b). *M. vitrata* is an important pest of grain legumes such as cowpea, pigeonpea, mungbean, common bean, soybean, adzuki bean, groundnut, hyacinth bean, field pea, country bean, broad bean kidney bean, and lima bean etc (Sharma *et al.,* 1999). In the absence of host plants in the off-season the populations of *M. vitrata*

survive on alternative plants like wild leguminous shrubs and trees, the larvae feed on about 39 host species, the majority of which belong to leguminaceae (Sharma *et al.,* 1999).

2.5.1 Biology of *Maruca vitrata*

Eggs are normally deposited on flower buds and flowers, although oviposition on leaves, leaf axils, terminal shoots, and pods has also been recorded (Taylor, 1967). A female lays between 6 and 189 eggs, although 200-300 ova have been observed per female. It has also been reported that a female may lay up to 400 eggs in batches of 2-16 (Okeyo-Owuor and Ochieng, 1981; Jackai *et al.,* 1990). The effect of temperature on oviposition and adult longevity of *M. vitrata* was examined by Chi *et al*., (2005) at four constant temperatures with 14 hours Light: 10 hours Darkness photoperiod. The mean numbers of eggs laid per female were 838, 856, 777 and 644 at 27ºC, 25ºC, 22ºC and 20ºC, respectively. Female adult longevity and pre-ovipositional period were shortened with increasing temperature. The egg laying period lasts an average of 3 days at 24-27ºC (Ramasubramanian and SundaraBabu, 1989a). Eggs are light yellow, translucent, and have faint reticulate sculpturing on the delicate chorion, and measure 0.65 to 0.45mm. Eggs are usually deposited singly or in batches of 2 to 16 (Sharma *et al.,* 1999). Females live for 4-8 days. The incubation period ranges from 2 to 4 days with an average of 3.31 days (Sureja *et al.,* 2010; Vishakantaiah and JagadeeshBabu, 1980). Eggs hatch in about five days and there are five larval instars: I lasts for 3.7±0.2 days, II for 3.2±0.14, III for 2.5±0.16, IV for 2.4±0.15, and V for 4.5±0.16 days (Sureja *et al.,* 2010; Odebiyi, 1981; Das and Islam, 1985). The total larval period is completed in 13.37 ± 1.35(Sureja *et al.,* 2010).

The larvae are translucent and shining, and have six rows of black spots running from thorax to abdomen (Sharma *et al.,* 1999). Because of the prominent black spots on the larva, it is also called a spotted caterpillar. The head of the larva is dark brown, the larvae are very active and tend to fall off the webbed flowers and pods with the slightest disturbance, by spinning a silken thread. The prepupal and pupal period lasts for about three and seven days respectively (Sureja *et al.,* 2010).

Pupation occurs in a silken cocoon amongst webbed leaves/pods or in soil (Sharma *et al.,* 1999). The life cycle is completed in 18 to 35 days (Sureja *et al.,* 2010; Taylor, 1967 and Akinfenwa, 1975). Adults are brown to black with a white patch on the wings. In their normal resting posture, the moths hold the wings in a horizontal position, unlike other moths which rest with folded wings. There is no diapause in this insect, and the populations during the off-season are maintained on wild hosts such as *Vigna triloba, Crotalaria* spp, or *Phaseolus* spp (Taylor 1967). Under laboratory conditions, eggs hatch in 3-4 days. Adults begin to lay eggs after a preoviposition period of 5 days. Jackai *et al.,* (1990) observed that four or five nights pairing resulted in the highest mating percentage and oviposition. Some males mated more than once, while the majority of the females mated only once. A one-to-one ratio (10 males: 10 females) gave best results for mating and oviposition. Mating took place between 2100 hour 0500 hour, when temperatures ranged between 20° to 25°C and relative humidity (RH) over 80%. Peak moth activity has been observed between 0200 and 0300 h (Sharma *et al.,* 1999).

Mature larvae are 17-20 mm long. The head capsule is light to dark brown, and the pro-thoracic plate is dark brown and divided dorsally. The body is whitish to pale green or pale brown, with irregular brownish black spots; the spots become indistinct immediately before pupation. Early instars are dull white, but the later instars are black-headed, with irregularly shaped brown or black spots on the dorsal, lateral and ventral surfaces of each body segment. Pupation takes place within a cocoon inside the soil and lasts 5-10 days. Pupae are 11.5 x 2.5 mm, within a silken cocoon. The adult moth has light brown forewings with white patches, and white hind wings with an irregular brown border. It often rests with the wings outspread measuring up to 25 mm. The wings are brownish-black with clear, white markings. Adults live, on average, 6-10 days. They are inactive during the day and can be found at rest with outspread wings under the lower leaves of the host plants. In expressing mating readiness, the male approaches the females sideways, and then hovers around for several minutes. This behaviour was reported to be repeated several times with different females until a 'receptive' female was found (Jackai *et al.,* 1990). The seasonal flowering pattern of the different host plants on a South-North gradient has been found to influence the migration of *M. vitrata* from the coast to the dry Savannas of West Africa (Bottenberg *et al.,* 1997). During this migration, the population of *M. vitrata* finds favourable conditions for multiplying on a succession of different host plants, thereby increasing the size of each generation. In fact, over 1500 moths per night were caught during the cowpea growing season in Kano, northern Nigeria, using light traps (Bottenberg *et al.,* 1997). The life cycle is completed in 18-35 days depending upon temperature (Sureja *et al.,* 2010; Sharma *et al.,* 1999).

2.5.2 Nature of Damage by *Maruca Vitrata* [Geyer] on Cowpea

The importance of *M. vitrata* as a pest of grain legumes results from its early establishment on the crop (Sharma *et al.,* 1999). After hatching, the young larvae of *M. vitrata* (1st, 2nd and 3rd instars) especially injure the terminal shoots and the flower buds whereas the older larvae (4th and 5th larval instars) particularly damage the open flowers and the pods (Atachi, 1998). The larvae web the leaves and inflorescence, and feed inside on flowers, flower buds, and pods. This typical feeding habit protects the larvae from natural enemies and other adverse factors including insecticides (Sharma *et al.,* 1999). Older larvae are highly mobile, feeding continuously on flowers and newly formed pods and causing severe damage throughout the reproductive cycle of the crop (Singh and Jackai, 1988). Larvae move from one flower to another, and each may consume 4-6 flowers before larval development is completed (Taylor, 1967). Usua and Singh (1979) studied the larval behaviour and found that larvae are most active on the plant surface from 1800 to 0600 hr, with a peak period of activity from 2200 to 0600 hr. The infested pods exhibit irregular bored holes and webbed insect frass. Sharma (1998) reported that the extent of infestation was in the order: flowers > flower buds > terminal shoots > pods. Karel (1985) also observed more larvae (52.3%) on flowers than on pods (37.8%) and leaves (9.9%). The host plant undergoes a high energetic loss following the damage inflicted to the vegetative parts by *M. vitrata* infestation. This fact has been revealed by a study of the biomass dynamics of the *Pyralidae* (Atachi, 1998).

The flower bud stage is preferred most for oviposition, and it is at this stage that the young larvae cause substantial damage, and reduce the crop potential for flowering and fruit

setting (Sharma *et al.,* 1999). The young larvae bore into the flower buds, and cause flower shedding by destroying the young flower parts enclosed in the sepals, the successful establishment of this pest at the flower bud stage is significant in relation to subsequent damage, reduction in grain yield, and efficiency of control (Sharma *et al.,* 1999). Young larvae feed on the style, stigma, anther filaments, and ovary, besides a limited feeding on the internal components of the corolla little or no feeding has been observed on the anthers (Sharma *et al.,* 1999). At this stage the damage is largely internal and there is little or no Sign of damage externally, usually more than one larva is present in each flower (Sharma *et al.,* 1999). These subsequently disperse to other flowers and flower buds on the same or other adjoining peduncles. The larval movement is facilitated by the silken threads, which are used as bridges between flowers. After initial dispersal, larval development is completed on several flowers/pods. The larvae move from one flower to another as they are consumed, and a larva may consume 4-6 flowers before larval development is completed. Third to fifth-instar larvae were capable of boring into the pods, and consuming the developing grains (Taylor, 1967).

The moths and larvae of *M. Vitrata* are nocturnal (Usua and Singh, 1979). The larvae, which are photo-negative, emerge early in the evening and feed on the plant throughout the night. In dual-choice assays, the third-instar larvae preferred pods rather than flowers or young leaves, and flowers rather than leaves (Sharma *et al.,* 1999). First-instar larvae showed a strong preference for flowers over pods and leaves (Sharma *et al*., 1999).

2.6.1 Mechanisms of Resistance of Host Plants to *Maruca Vitrata*

Information on the biology of M. *vitrata* has been generated for cowpea, comprehensive information is needed on the population dynamics of this insect, and the factors that lead to rapid population build up. This information will be useful to screen for resistance under natural infestation, development of resistance screening techniques, and appropriate management strategies for controlling this pest. Such information can be generated through light traps, and sequentially planted crops. A few susceptible and resistant cultivars may be included in such studies to quantify the role of plant resistance in minimizing the damage by this pest. Some information are available on insect density-yield loss relationships (Sharma *et al.,* 1999). This will be useful for estimating economic thresholds, the level of insect infestation needed to screen for resistance, and the desirable levels of resistance needed in the commercial cultivars to minimize losses due to this insect; screening for resistance has been carried out using natural infestation, and multi- and no-choice tests under greenhouse and laboratory conditions. Laboratory/greenhouse tests are useful for confirming the resistance observed under field conditions. Ten eggs per plant are adequate to screen for resistance to this insect in cowpea (Sharma *et al.,* 1999). The larvae can be reared on natural hosts/artificial diet in the laboratory. Procedures for infestation and evaluation of resistance under field and greenhouse conditions should be standardized and adopted across locations in crop improvement programs (Sharma *et al.,* 1999).

Considerable information has been generated on genotypic resistance/susceptibility to *M. vitrata* in cowpea. Levels of resistance seem to be repeatable across seasons. Important sources of resistance to other yield-reducing traits should be evaluated for resistance to the

pod borer to identify lines with multiple resistance to insects and other yield-reducing factors (Sharma *et al.,* 1999). Several plant characteristics such as stem and pod wall thickness, and podding habit (clusters versus spread out pods, pod angle, etc) contribute to decreased susceptibility to *Maruca.* Some of these characteristics such as growth habit, pods exposed above the foliage, days to complete flowering, and time required for pod maturity can be used to select genotypes as possible candidates for resistance to *Maruca.* The relative contribution of these traits should be assessed in a diverse array of genotypes with resistance to *Maruca.* This material can also be used to quantify the contribution of non-preference, antibiosis, and tolerance mechanisms of resistance. This will also help to identify lines with different mechanisms of resistance, which can be used in the resistance breeding program to increase the levels and diversify the bases of resistance to *M. vitrata* (Sharma *et al.,* 1999).

2.6.2 Non preference

M. vitrata females showed oviposition preference for Hyacinth bean, followed by cowpea and pigeonpea (Ramasubramamian and Sundara Babu, 1989b). The maximum number of eggs was laid three days after mating on the preferred host, while on cowpea and pigeonpea the highest number of eggs were laid on the fourth day after mating, greenhouse experiments in a choice situation have clearly shown non-preference for oviposition as a component of resistance in cowpea (Macfoy *et al.,* 1983). However, Valdez (1989) did not observe any oviposition non-preference in cowpea cultivars. Non-preference for larval

feeding has been observed by Echendu and Akingbohungbe (1990). Attraction and arrest-stay of first-instar larvae contributed to the resistance of TVu 946 and VITA 5 to the legume pod borer (Okech and Saxena, 1990). Significant differences in oviposition preference have been observed under multi-choice conditions on different pigeonpea cultivars (Sharma *et al.,* 1999).

2.6.3 Antibiosis

Larval survival in cowpea is low on assession TVu 946, and this has been attributed to nutritional and antibiotic factors (Macfoy *et al.,* 1983). Valdez (1989) observed only a slight effect of the host on larval Survival. Okech and Saxena (1990) indicated that antibiosis was a component of resistance in cowpea assession TVu 946 and VITA 5 stems and pods. Highest larval weight gains has been recorded on assession TVu 3 and least in CES 15-27. Consumption index (CI) was higher on TVu 1248 and TVu 1 compared With CES 15-27, TVu 1611-2, TVu 461, TVu 946, TVu 1016-1, and TVu 1499-1 (Sharma *et al.,* 1999).

There are significant differences in consumption and utilization of flowers by the third instar larvae and pods by the fifth-instar larvae. Third-instar larvae consumed 27.0 – 47.2 mg food on the flowers, and had growth rates of 1. 47% on pigeonpea assession ICPL 88020 to 207.3% on ICPL 85010 (Sharma *et al.,* 1999). The consumption index was greater on ICPL 90011 compared with that on ICPL 88020, ICPL 85010 and ICPL 88007. Approximate digestibility was lower on ICPL 85010 than on ICPL 90011. Efficiency of conversion of digested food into body matter was lower on ICPL 90011 and ICPL88020 as

compared with ICPL 85010 and ICPL 88007. The fifth-instar larvae consumed between 52.3 and 80.6 mg of food on pods, and showed growth rates of 30.1 to 41.8%. Food consumption was lowest on ICPL 85010, and maximum on ICPL 90011. Efficiency of conversion of ingested food into body matter was lowest on ICPL 90011, followed by that on ICPL 88020, ICPL 88007, and ICPL 85010 (Sharma *et al.,* 1999).

2.6.4 Tolerance

The ability of plants to recover from insect damage is an important component of resistance to insects in crop plants (Sharma *et al.,* 1999). In pigeonpea, there was no relationship between *Maruca* damage and recovery resistance. However, pigeonpea variety ICPL 88034 and MPG 679, which recorded low *Maruca* damage (10 to 25%), showed excellent recovery. Although larval counts and pod damage were similar on resistant and susceptible cultivars, the grain yield was significantly greater in the *Maruca* resistant cultivars than the susceptible ones (Saxena *et al*., 1998). This suggests that some genotypes recover quickly following *Maruca d*amage (Sharma *et al.,* 1999).

2.6.5 Plant architecture as a factor associated with resistance

Infestation and damage by *M. vitrata in* cowpea is influenced by plant architecture. Canopy structure and pod position together or independently exert a profound effect on cowpea resistance to the pod borer. Cultivars with pods held within the canopy suffer significantly greater damage than the cultivars where the pods are held in the normal position (Sharma *et al.,* 1999). Selection and breeding cowpea cultivars with less dense foliage and long

peduncles holding the reproductive structures above the canopy may increase resistance to M. *vitrata* (Oghiakhe *et al.,* 1991a, Usua and Singh, 1979). A negative relationship has been observed between pod angle and percentage pod damage, as well as the seed damage index in two cowpea cultivars (Oghiakhe *et al.,* 1992a). Pods with wide angles (>89°) were damaged on one, but rarely on both sides. The eventual pod size and rate of pod growth appeared to be important factors in cowpea susceptibility to the pod borer (Tayo, 1988).

Tall and intermediate type cultivars (non-determinate type) of pigeonpea have fewer flowers per cluster than shorter cultivars (determinate type), and a disproportionately lower number of pod borer larvae per 100 flowers (Sharma *et al.,* 1999). Genotypes with branching and loose flower arrangements were less susceptible to legume pod borer damage (Fellows *et al*., 1977). Saxena *et al*., (1996) also observed that non-determinate type pigeonpea lines were less susceptible to *Maruca* damage than the determinate types. The average score of the determinate lines was 7.1, while the corresponding value for the non-determinate group was 5.3. This suggests that, in general, the determinate lines having a clustered inflorescence are more prone to *Maruca* damage than the nondeterminate genotypes which have long fruiting branches and a loose inflorescence (Sharma *et al.,* 1999). Lateef and Reed (1981) also suggested that determinate types suffered greater pod borer damage than the non-determinate types. In the case of cowpea, lines having clustered pods have been found to be more susceptible (Usua and Singh, 1979). However, there was a large variation for *Maruca* damage within each growth type in the determinate group, only four lines (MPG 359, 531, 532, and 566) suffered a damage rating of <3, while in the non-determinate group, 12 lines showed a damage rating of <3. None of the non-determinate types showed 100% *Maruca* damage. On the contrary, 18 determinate types

suffered complete damage. In the non-determinate group, 56% of the lines tested showed <50% damage, while in the determinate group, 85% of the lines had >50% damage, therefore, factors other than the flowering habit may also be important in resistance to *Maruca* (Sharma *et al.,* 1999).

2.6.6 Anatomical characteristics as a factor associated with resistance

Anatomical features of the stem and pod wall were associated with resistance to *M. Vitrata* in cowpea (Oghiakhe *et al*., 1991b). The anatomical micro-environment of the area immediately beneath the stem epidermis seemed to impose severe limitations on larval movement and feeding within the tissue. Although stem anatomy was considered to be an important factor, this did not appear to be the case in pod wall resistance to *M. vitrata,* the toughness of non-intact and intact pod walls increased with age, but the rate varied at different growth stages of the pod as well as between cultivars (Oghiakhe *et al*., 1992b). It has been observed that in two wild cowpea *(Vigna vaxillata)* accessions (TVNu 72 and TVNu 73), feeding and development were deterred on the pods with or without trichomes compared with the susceptible variety IT84E-124, a Similar effect has also been observed on seeds and flowers (Sharma *et al.,* 1999).

Maruca larvae fed and developed better when the trichomes were removed. The growth Index was 13x less when the trichomes were left intact both on TVNu 72 and TVNu 73. It appears that the resistance of these lines is based on trichomes and phyto-chemicals. Thick and compact collenchyma cells in the stems and fibrous tissues on the petal surface

contributed to resistance. Trichomes varied in length and density, but not in type on different plant parts (Oghiakhe *et al* 1992c). Trichome density decreased with plant age. Significant correlations have been observed between trichome density and pod borer damage, but trichome length may be less important than density (Sharma *et al.,* 1999). In pigeonpea, trichomes have been shown to be associated with resistance to *H. armigera* (Shanower *et al.,* 1996). However, there is no information on the role of trichomes in pigeonpea in imparting resistance to *M. vitrata* (Sharma *et al.,* 1999).

2.6.7 Biochemical factors as associated with resistance

There are no specific studies on biochemical mechanisms of resistance to legume pod borer in pigeonpea. However, the secondary plant substances present in pigeonpea, which affect the plant suitability to other insects, are likely to affect the growth and development of M. *vitrata.* Sugar and protein content of different genotypes may also influence the nutritional value of different genotypes for the growth and development of larvae. Sugar content in the pod walls of cowpea cultivar TVNu 72 was greater than in IT82D-716, and phenol content was lower in the pod wall of TVNu 72, but the reverse was true for fresh and dry seeds. Neither sugars nor phenols seemed to be involved in the resistance of TVNu 72 to M. *vitrata* (Oghiakhe *et al.,* 1993). Phenol concentration varies significantly between different plant parts, and generally decreases with an increase in plant age. Otieno *et al.,* (1985) indicated that an ethyl-acetate soluble fraction of methanol extracts of stems of TVu 946 showed greater feeding inhibition than the extract from lCG 1 (Sharma *et al.,* 1999).

2.7.0 Control Methods for *Maruca Vitrata*

Several natural enemies have been reported on *M. vitrata.* Pathogens have been reported to be most important as population regulating factors in the field. In this regard, the usefulness and effectiveness of *Bacillus thuringiensis, Nosema,* and *Aspergillus* may be explored for integrated management of this pest. Cultural practices such as intercropping, weeding, time of sowing, density of sowing, and pruning have been shown to reduce the damage by the legume pod borer (Sharma *et al.,* 1999). However, the results are not consistent over seasons or locations. Such studies should be repeated involving large plots, and possibly including genotypes that are less susceptible to this insect. Several insecticides have been evaluated for the control of this insect. Various control options for minimizing the losses due to *M. vitrata* should be tested in farmers' fields in collaboration with the National Agricultural Research Services and other organizations. A Network of International Agricultural Research Centers working on *Maruca* may be established to share the information and technology for integrated management of *M. vitrata* (Sharma *et al.,* 1999).

2.7.1 Use of Natural enemies

Several parasites and predators have been recorded on *M. vitrata* by Barrion *et al.,* (1987), Usua and Singh (1977), Okeyo-Owuor *et al.,* (1991), lCRISAT (1978), Subasinghe and Fellows (1978), Odindo *et al.,* (1989), and summarized by Sharma (1998). Parasites recorded on larvae/pupae include Tachinids *[Aplomya metallica* (Wiedemann), *Exorista xanthaspis* (Wiedemann), *Palexorista solenins* (Walker), *Peirbaea orbata* (Wiedemann), *Zygobothria atropivora* (Robineau- Desvoidy), *Zygobothria ciliata* (Wulp), *Thelairosoma* sp, *Pseudoperichaeta laevis* (Villeneuve), *Pseudaperichaeta* sp, and *Thecocarcelia*

incedens (Rondani)], Braconids *[Apanteles teragamae* Vierek, *Apanteles* sp, *Bracon greeni* Ashmead, *Bracon* sp, *Braunsia* sp, *Cardiochiles philippinensis* Ashmead, *Chelonus* sp, *Snellenius manilae* Ashmead, *Phanerotoma handecasisella* Cameron, and *Phanerotoma* sp], chalcidids *[Antrocephalus* sp nr *subelongatus* Kohl, *Antrocephalus* sp, and *Brachymeria* sp], Eulophids *[Nesolynx thymus* (Girault), *Tetrastichus sesamiae* Risbec, and *Tetrastichus* sp], Ichneumonids *[Caenopimpla arealis* (Cushman), *Charops nigrita* Gupta and Maheswary, *Meloboris sinicus* (Holmgren), and *Metopius rufus browni* Ashmead], Pteromalids *[Trichomalopsis* sp**],** Scelionids *[Telenomus* sp], Mites *[Dinothrombius* sp], Nematodes, Protozoa *[Mettessia* sp, *Nosema marucae* sp n and *Nosema* sp], and Bacteria *[Baccillus* sp and *Clostridium* sp] (Sharma *et al.,* 1999).

Predators include Dermapterans *[Diaperastichus erythrocephala* Olivier], Mantids *[Polyspilota* sp and *Spodromantis* sp**],** Carabids *[Chlaenius* sp and *Cicindela lacrymosa* (Fabricius)], Coccinellids *[Coccinella repanda* (Thunberg), *Menochilus sexmaculatus* (Fabricius), and *Synharmonia octomaculata* (Fabricius)], Anthocorids *[Orius tantillus* Motschulsky], Formicids *[Camponotus sericeus* Fabricius and *Camponotus rufoglaucus* (Jerdon)], vespids *[Ropilidia flavopicta flavobrunnea* van der Vecht], Selenopids *[Selenops* sp], Araneids *[Nephila maculata* (Fabricius)], Oxyopids *[oxyopes javanus* Thorell], Salticids *[Evarcha* sp, *Marpissa bengalensis* Tikader, and *Marpissa calcutaensis* Tikader], and Sparassids *[Heteropoda venatoria* (Linnaeus)] (Sharma *et al.,* 1999).

Okeyo-Owuor *et al.,* (1991) carried out key-factor analysis of *M. vitrata* populations in Kenya. The total mortality from egg to adult stage was nearly 98 to 99%, and highest

mortality occurred between the egg stage and the third-intar larvae, whilst the fourth-instar larvae suffered lowest mortality. The causes of mortality were disappearance, followed by disease. Parasitism contributed minimally to *M. vitrata* mortality. There was no correlation between population density and mortality at the same stage (Okeyo-Owuor *et al.,* 1991). A pupal endoparasitoid, *Antrocephalus* sp, was the predominant natural enemy, while *Nosema* sp and *Bacillus* sp caused the highest natural mortality. Parasitoids and pathogens contributed 40.7% to the total generation mortality (K) at Site 1 and 35.6% at Site 2. Parasitism only contributed 3.3% of the total generation mortality at Site 1 and 3.8% at Site 2. Mortality due to disappearance, which also included predation, accounted for 59.4 and 64.8% of *K* at the respective Sites. Life table data and survival curves for the pest revealed high generation mortality (about 98%), most of which occurred m the early life stages of the pest. The results suggested a high potential for utilizing biocontrol agents for the management of this pest (Sharma *et al.,* 1999).

Information on the role of various natural enemies in regulating the legume pod borer populations is scanty or unavailable. Published information indicates that parasitoid contribution to the total natural mortality is very low. Pathogens seem to play a major role in the control of pod borer populations under field conditions. This area of research needs to be pursued in future to exploit natural enemies for the management of this pest (Sharma *et al.,* 1999).

2.7.2 Use of Cultural practices

Pod borer populations tended to build up over the season, and the pod borer infestation increased on the late sown crops (Alghali, 1993a). Grain yield also decreased in late-sown crops. Simultaneous sowings of maize and cowpea increased pod borer infestation in cowpea (Ezueh and Taylor, 1984), whereas sowing cowpea 12 weeks after maize reduced the legume pod borer damage (Sharma *et al.,* 1999).

Pod borer damage in monocrops was greater than the maize - cowpea - sorghum inter/mixed crops (Omolo *et al.,* 1993). Pod borer incidence was significantly lower in intercropped, and at higher plant populations than in pure stands, and in lower plant populations of common bean, *Phaseolus vulgaris* (Karel, 1993). Flower and pod damage was significantly lower in an intercrop combination of one third bean - two thirds maize. However, Alghali (1993b), Natarajan *et al.,* (1991), and Patnaik *et al.,* (1989) reported no effect of intercropping on *M. vitrata* damage Cowpea weeded 2, 3, or 4 times had less flower infestation by *M. vitrata* than the non weeded plots (Ofuya, 1989). However, effects of weeding frequency on pod damage by *M. Vitrata* are not consistent (Sharma *et al.,* 1999).

2.7.3 Use of Chemicals

Effective control of the pod borer on cowpea has been achieved with endosulfan (applied at 35 DAS twice at weekly intervals) (Jackai, 1983), one spray of Cypermethrin, Biphenthrin, Cyhalothrin, and in combination with dimethoate (Amatobi, 1994), a mixture of Cypermethrin + Dimethoate using an Electrodyn sprayer (Jackai *et al.,* 1987), or two

applications of cypermethrin + dimethoate at 10-day intervals (beginning at bud formation) (Amatobi, 1995). Atachi and Sourokou (1989) reported that a sequence of Deltamethrin - Dimethoate Deltamethrin sprays resulted in the highest grain yield (1.37 t/ha^{-1}). Future studies should focus on insecticide application based on economic thresholds, and timing of insecticide application. Emphasis should also be placed on using the biorational pesticides for integrated management of this insect. Spray regimes which terminated early offered better protection against the pod borer, but were inadequate for controlling sucking insects. Calendar-based sprays resulted in less borer infestation than when sprays were based on economic thresholds (Afun *et al.,* 1991). However, differences in grain yield between the calendar-based sprays and those based on economic thresholds were not significant. Crop monitoring reduced the number of sprays by half compared with those based on calendar schedules (Sharma *et al.,* 1999).

Decamethrin, Cypermethrin, and fluvalinate caused the highest mortality of the legume pod borer larvae three days after spraying under laboratory conditions (Bhalani and Prasana, 1987). Plots sprayed with synthetic pyrethroids, except Fenvalerate at 0.01%, showed least damage to the pods at harvest. Significantly greater grain yield was recorded in plots treated with Fluvalinate, followed by those treated with Cypermethrin, Decamethrin, and Fenvalerate at higher dosages. Samolo and Patnaik (1986) reported that of the six insecticides tested, Monocrotophos and Endosulfan (0.5kg a.i ha^{-1}) were most effective, and three applications of Endosulfan starting at flower initiation (at 20 days interval) were most effective. Foliar application of Cypermethrin (0.008%) or Dimethoate (0.07%) at flowering or when egg numbers reached 2 per meter row, and then repeated at 10-15 days interval

provided effective protection against *M. vitrata* (Rahman, 1991). Cypermethrin (75g a.i ha^{-1}) sprayed three times, has been found to be effective against pod borers, followed by Decamethrin (12.5g a.i ha-1), Fenvalerate (150g a.i ha-^{1}), and Endosulfan (400g a.i ha-^{1}) (Sontakke and Mishra, 1991). The latter showed the highest cost-benefit ratio. Sprays of 0.07% Traizophos or Endosulfan, and 0.04% Monocrotophos resulted in maximum reduction in pod borer damage (Sundara Babu and Rajasekaran, 1984). Dust formulations of Phoxim, Endosulfan, and Phosalone (4%) also gave effective control of the legume pod borer. Venkaria and Vyas (1985) reported that the least number of pods were damaged in plots treated with Fenvalerate (0.01 %), Endosulfan (0.07%) + Miraculan (a plant growth stimulant), followed by those treated with Fenvalerate (0.01 %), Endosulfan (0.07%) + Miraculan, and Monocrotophos (0.04%). Thiodicarb (613 ppm) and Ethofenprox (125 ppm) were as effective as Methamidophos (200 ppm) for the control of legume pod borer on pigeonpea in Sri Lanka (Dharmasena, 1993). Insecticide application increased the grain yield by 28%. Thiodicarb sprays resulted in maximum increase (43%) in grain yield over two seasons (Sharma *et al.,* 1999).

Four sprays of Cypermethrin 0.008% (1st spray at initiation of flowering, 2nd spray at 50% flowering, 3rd spray at 100% flowering, and 4th spray at 100% pod setting) were effective for protecting the pigeonpea crop against *Maruca* (Rahman and Rahman, 1988). This schedule also offered the highest benefit-cost ratio (6.23). Dimethoate was not as effective as Cypermethrin. The number of flowers, pods, and seeds per plant was significantly greater in plots treated with insecticides based on the economic threshold level of 10 larvae per 100 flowers (3 insecticide applications) than in the untreated plots. The differences in

the number of flowers, pods, and seeds per plant were not significant between plots sprayed 3 and 4 times. It has been concluded that 10 larvae per 100 flowers can be considered as a tentative threshold for M. *vitrata* on pigeonpea (Dharmasena *et al.,* 1992).

2.7.4 Use of Natural/Biopesticldes

Bacillus thuringiensis (*Bt*) (Karel and Schoonhoven, 1986), neem seed powder and neem kernel extract (Singh *et al.,* 1985, Jackai *et al.,* 1992) are effective against legume pod borer. Flower infestation was not influenced by 5 and 10% neem leaf extracts in cowpea (Bottenberg and Singh, 1996). Neem leaf extract applied four times on Cv 715 resulted in less pod borer damage than on Cv 941. Neem application reduced pod damage by 12% in Cv715, and by 16% in Cv 941 (Sharma *et al.,* 1999). In pigeonpea, trials conducted to assess the utility of *Maruca* resistant cultivars for managing this pest revealed that pod borer-resistant lines can reduce the number of insecticide sprays at least by one under certain conditions (Saxena *et al.,* 1998).

2.7.5 Mechanism of Resistance of *Cry1Ab* gene

There are multiple theories about how these active toxins cause cell death, however there is general agreement that the first step is binding of specific receptors on the plasma membrane of midgut epithelium cells in susceptible insects (Aronson and Shai, 2001, OECD, 2007). The most popular theory holds that, once bound to receptors, the toxin is able to insert into the plasma membrane through the formation of oligomeric transmembrane pores (Aronson and Shai, 2001; Bravo *et al.,* 2007; OECD, 2007). There is evidence that some *Cry* proteins have multiple receptors, or may bind to multiple sites on a

single receptor and it has been demonstrated that receptor binding is necessary but not sufficient for toxicity (Aronson and Shai, 2001; Jenkins *et al.,* 1999; OECD, 2007). There is also some evidence based partly on experiments using sub-lethal concentrations, that there may be other relevant interactions between *Cry* proteins and their insect targets (Aronson and Shai, 2001).

The objective of inserting the *Cry1Ab* gene into a crop is to provide protection from feeding damage by lepidopteran pests. Other organisms that are not pests in the agricultural system may also be exposed to the *Cry1Ab* protein, and are considered "non-target organisms" (NTOs). Such exposure could be direct, from deliberate or incidental feeding on crop tissues such as leaves, ears, silks and pollen or decaying leaf material, or be indirect, from feeding on other herbivores that feed on the crop. Although, the potential for harm to Non target organisms (NTOs) has been considered as a part of regulatory risk assessments for genetically engineered (GE) plants that express *Cry1Ab*, with special consideration to beneficial NTOs that perform valuable functions as well as threatened endangered and charismatic species (Khan, 2008). There is a long history of use for microbial preparations of *B. thuringiensis*, along with early characterization of the *Cry1A* proteins as being harmless to vertebrate species and having specific activity against only a subset of lepidopterans (CERA, 2011).

2.8 *Bacillus thuringiensis* and the Cry δ endotoxins

Bacillus thuringiensis is a rod-shaped, gram positive *Bacterium* capable of forming long-lived endospores. It is often referred to as a soil *Bacterium*; although it is ubiquitous in the

environment (Hofte and Whiteley, 1989; Schnepf *et al.,* 1998; OECD, 2007). There is tremendous variation within the species with regard to the type or class of pesticidal proteins that differ in mode of action, target specificity and mechanism of expression (Hofte and Whiteley, 1989; Schnepf *et a*l., 1998; OECD, 2007). Pesticidal proteins expressed by *B. thuringiensis* strains include antifungal compounds, β exotoxins2, vegetative insecticidal protein (VIP), and the δ endotoxins which include the *Cry* (crystalline) proteins and the structurally unrelated Cyt (cytolytic) proteins (Hofte and Whiteley 1989, Schnepf *et al.,* 1998; OECD, 2007). Most of these have been shown to contribute to insect toxicity and some (notably β exotoxins and Cyt proteins) have a wide spectrum of activity (Hofte and Whiteley, 1989; Schnepf *et al*., 1998; OECD, 2007).

The *Cry* protein δ endotoxins are so named because they are the primary component of the protein parasporal crystals that are characteristic of spore formation in *B. thuringiensis* (Hofte and Whiteley, 1989; Schnepf *et al.,* 1998; OECD, 2007). A systematic nomenclature for identifying and differentiating Cry proteins was proposed in 1989 and widely adopted (Hofte and Whiteley, 1989; OECD, 2007). Under this nomenclature, the *Cry* proteins were grouped into four initial classes I, II, III, and IV based on their toxicity to particular orders of insect. *CryI* proteins were those toxic to only Lepidoptera, *CryII* proteins were those toxic to Lepidoptera and Diptera, *CryIII* proteins were toxic to Coleoptera and *CryIV* proteins were those toxic to Diptera. This system has been subsequently updated to account for additional *Cry* proteins and expanding knowledge of their molecular structure and function and relatedness, leading to some minor discrepancies in naming relative to earlier literature (Crickmore *et al.,* 1998, 2005; OECD, 2007).

B. thuringiensis was first isolated from diseased larvae of Mediterranean meal moth (*Ephetia kuhniella*) (Sharma *et al.,* 2004). Genes from *B. thuringiensis*, *B. spharicus,* Protease inhibitor, Plant lectins, Ribosome inactivating proteins, secondary plant metabolites and small RNA viruses have been used alone or in combination with conventional host plant resistance to develop crop cultivars that suffer less damage from insect pests (Hilder and Boulter, 1999). Genes conferring resistance to insects have been inserted into crops plants such as maize, rice wheat, sorghum, sugarcane, cotton, potato, tobacco, broccoli, cabbage, chickpea, pigeon pea, cowpea, groundnut, tomato, and soybean (Sharma *et al.*, 2000). Genetically transformed crops with *Bt* genes have been deployed for cultivation in USA, Argentina, Canada, China, South Africa, Australia, Romania, Mexico, Bulgaria, Spain, Germany, France, Uruguay, Indonesia, Ukraine, Portugal and India (Sharma *et al.,* 2004).

While, several transgenic crops with insecticidal genes have been introduced in the temperate regions, very little has been done to use this technology for improving productivity in the harsh environments of the tropics, where the need for increasing food production is most urgent (Sharma *et al.,* 2004). The most urgent need to use this technology is in the tropical region, where soils fertility, water availability, insect pests, and diseases severely constrain crop production (Sharma *et al.,* 2004). However, some African

countries in collaboration with foreign donor organizations are adopting genetic transformation technology to combat the challenges posed by pest problems.

For example, a *Bt* cowpea is being developed at the Institute for Agricultural Research, Ahmadu Bello University, Zaria; in collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO) laboratory, Australia (Ishiyaku, 2010). *Bt* toxins have been widely used as natural insecticides for many years with no reports of spontaneous development of resistance in the insect populations, unfortunately, *Bt* sprays are often washed away by rain, degrade under solar ultra violet radiation and are not optimally targeted against certain insect pests that live within plant tissues. The limitations associated with the use of conventional methods in effectively dealing with cowpea's pest problem makes the application of biotechnological procedures for overcoming the constraints to cowpea production particularly attractive (Sharma *et al.,* 2004).

However, with the dramatic increases in the presence of *Bt* toxins in the ecosystem via transgenic crops, this may significantly increase the pressure on insect populations to evolve resistant biotypes, create secondary pest problems, gene escape into the environment etc one of the approaches to overcome these problems is to develop a new generation of

transgenic with better genes, and use combinations of gene to delay the development of resistance in insect populations (Sharma *et al.,* 2004).

2.9 Genetic Transformation of Cowpea

Until recently cowpea remained one of the last major grain legume species for which an efficient genetic transformation/regeneration system had not been developed, despite substantial efforts for more than ten years by several groups of researchers (Machuka *et al.,* 2002). With advances in molecular and cellular biology, it is now possible to engineer into plant genomes the genes that encode expression of crystal proteins, thus providing to the plant built-in protection against Lepidopterans such as *Maruca* Pod Borer (Stella *et al*., 2008). Ikea *et al*., (2003) reported the successful genetic transformation of cowpea using the particle-gun bombardment of shoot meristems. They were able to isolate several plants in the T3 generation that showed strong expression of the transgene "bar" that confers resistance to the herbicide Basta. An efficient and stable cowpea transformation/regeneration system has been developed recently (Popelka *et al*., 2006). Cowpea transformation based on *Agrobacterium* mediated system aided by an antibiotic selectable marker (*nptII*) has been developed at the Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia (Popelka *et al.,* 2006). The choice of the system was based on freedom to operate (FTO) and the fact that *nptII* allows useful differentiation between transformed and untransformed tissue. The use of *nptII* with geneticin selection has increased efficiency of transformation (Stella *et al*., 2008). Transgenic resistance to insects has been demonstrated in plants expressing insecticidal genes such as δ-Endotoxin from *B. thuringiensis* while transgenic plants with introduced *Bt*

genes have been deployed in several crops on a global scale (Sharma *et al.,* 2004), the alternative genes have received considerably less attention. The protease inhibitor and lectin genes largely affect insect growth and development and in most instances, do not result in insect mortality (Sharma *et al.,* 2004).

Transgenic approaches should be undertaken to develop varieties of cowpeas with strong resistance to insect pests. Insect-resistant cowpeas would dramatically increase cowpea productivity in many developing countries and reduce costs, safety hazards, and environmental risks in virtually all cowpea producing countries. Traditional plant breeding has made only limited progress in breeding for resistance to the major insect pests of cowpea and "new genes" are apparently needed to protect cowpea. The development and successful deployment of transgenic cultivars with genes conferring resistance to insects will be a major achievement (Timko *et al.,* 2007). The best current options for developing insect resistant cowpeas is to employ *Bt* technology against the *Maruca* pod borer (*M. Vitrata*) and the alpha-amylase inhibitor gene from common bean that provides effective control of cowpea weevil (*Callosobruchus maculatus).* The soybean cysteine protease inhibitor soya cystatin N (scN) and alpha- amylase inhibitor (alphaAI) from wheat have synergistic effects against the cowpea weevil in artificial seed systems and are also potential genes that could be used to develop cowpea cultivars with resistance to this pest. Several different *Cry1Ab, Cry1C,* and *CryIIA* proteins that are produced by different forms of *Bt* genes are toxic to *Maruca* pod borer and using these *Bt* genes in cowpea is considered a high priority for transformation. Genes producing plant lectins and plant proteinaceous inhibitors (PIs) of insect proteinases (serine, cysteine, aspartic, and metallo-proteinases) are

also considered potential candidates for gene transfer for resistance to *Maruca* pod borer (Timko *et al.,* 2007).

In brief, the protocol of *Agrobacterium*-mediated transformation of cowpea expressing the *Cry1Ab* gene consists of the following steps: (1) Imbibition of sterilized mature seeds overnight in water, (2) explants are derived either by longitudinally bisecting embryonic axes with shoot and root apices removed but still attached to the cotyledon, or by isolating intact embryonic axes without cotyledons (3) infection with *Agrobacterium* strain *AGL*1 aided by local wounding in the meristematic regions, (4) co-culture on medium containing thiol compounds over 4 days at 25°C in light, (5) shoot initiation and elongation on medium containing selective agents, (6) shoots are usually rooted *in vitro* or occasionally grafted onto non-transgenic seedlings and transferred to greenhouse conditions for flowering and seed setting, (7) PCR or enzyme analysis of putative transgenic plants, and (8) screening of next generation progeny by PCR or enzyme activity (Higgins *et al.,* 2009). The progeny of transgenic T_0 plants are normal in phenotype. The transgenes are transmitted to the progeny and homozygous T_2 plants, can be identified by screening their T_3 progeny for enzyme activity or by PCR. Using this transformation system, about 1-3 transgenic plants per 1000 explants can be produced, similar to other legume systems (Popelka *et al.,* 2004). Depending on the cultivar or line to be transformed, this protocol requires 5-8 months from explant preparation to harvested T_1 seeds. The transformation system is now being used to introduce insect protection and other genes into cowpea germplasm (Higgins *et al.,* 2009).

Adenubi *et al.,* (2008) has reported successful *Cry1Ab* transgenic cowpea transformation by nodal Electroporation. Electroporation-mediated genetic transformation was used to introduce *Cry1Ab* insecticidal gene into cowpea. Nodal buds were electroporated *in planta* with a plasmid carrying the *Cry1Ab* and antibiotic resistance *nptII* genes driven by a 35S CaMV promoter. T_1 seeds derived from electroporated branches were selected *in vitro* on a medium containing geneticin. PCR and Southern blot analyses confirmed the stable integration of the *Cry1Ab* gene into genome of transgenic T_1 cowpea plants. Copy numbers of the gene in cowpea were estimated to be between one to three per genome. However transgene integration occurred at high molecular weights and corresponded to the hybridization bands obtained for plasmid control. When tested for resistance to *Maruca vitrata,* T_1 plant progenies reduced larval survival to as low as 11% three days after infestation (DAI). T_2 progenies of *Cry1Ab* transgenic lines also positively hybridized with *npt II* probes when subjected to southern analyses. T_3 progenies significantly reduced larval survival and larval weight and inhibited *M. vitrata* feeding on cowpea leaves (Adenubi *et al.,* 2008). The fact that T_3 plants with *Maruca* pod borer (MPB) resistant phenotypes were obtained suggests that the transgene is stably inherited in cowpea. Although the use of *in planta* electroporation for cowpea transformation has not become widespread, it has been shown to be a simple means of generating transgenic cowpea (Adenubi *et al*., 2008).

2.10 Artificial Hybridization of Cowpea

Cowpea is highly self-pollinated in most environments (Ehlers and Hall, 1997). However, outcrossing of rates as high as 5% have been recorded (Timko *et al.,* 2007). Artificial hybridization is relatively easy due to the large size of the keel, anthers, and style, and the

fact that the keel is straight, unlike many other grain legumes which have small flowers and curved or coiled keels and stigmas. The keel of cowpea is easily opened with tweezers revealing nine readily accessible anthers. Greater than 90% hybrid pod set using the following method described below in a greenhouse has been recorded; in the late afternoon of the day prior to pollination, floral buds that are destined to open the following morning are identified and emasculated. Emasculation is performed by opening the sepals enclosing the keel, opening the keel and removing the anthers. All operations are performed with tweezers (forceps), care must be taken that anthers are not broken, which could result in self-pollination since the stigma is receptive by this time. Emasculated flowers are left uncovered and tagged for easy identification the following morning. In early to mid-morning, pollination is achieved by taking open flowers of the desired male parent and brushing pollen from the burst anthers and style of the male parent onto the emasculated flower. Male flowers should be chosen that are shedding copious pollen. Pollinations performed after 10am are less successful than pollinations performed between 7-10am (Ehlers and Hall, 1997). An alternative method is to collect open flowers of the male parent in the early morning, refrigerate them at 4^{o}c to 10^{o}c until late afternoon. At this time, mature but unopened floral buds are chosen, emasculated, and the pollinated with the floral flowers (Ehlers and Hall, 1997). Night temperatures greater than 20^{o}c impair Microsporogenisis leading to formation of indehiscent anthers and pollen with low viability. Thus, hybridization efforts are most effective in environments with night temperatures cooler than 20^{o}c. Greenhouses with night temperature between 13 -18^{o}c have provided effective environments for artificial hybridization of cowpea (Ehlers and Hall, 1997).

Cowpea flowers are large, about 2cm in length and breadth when open. The style and stigma are surrounded by anthers tightly enclosed in a straight keel. Anther dehiscence and pollination of a particular flower normally occurs in the early morning on the day that the flower opens i.e. when the corolla colour is visible (Ehlers and Hall, 1997). Flower open only once, and remain open for several hours. Stigma become receptive about 12 hours before anther dehiscence, which is useful in making artificial hybrids. The low level of outcrossing can occur in breeding nurseries and seed production fields, which may be due to visitation by large bees (Ehlers and Hall, 1997). Bumble bees can also cause outcrossing of cowpea (Asiwe, 2009). Nevertheless, out-crossing in cowpea is often assumed by breeders to be very low and insignificant because cowpea is self-pollinated (Asiwe, 2009). Asiwe (2009) reported outcrossing in cowpea of 0.5 to 0.85% when cowpea was planted in alternate rows than in concentric rows (0.01 to 0.13%) and outcrossing was found to have occurred in a random fashion beyond 13m. For this reason, provision of containment structures for Genetically Transformed cowpea under experimentation and observation should not be based on isolated minimum distance, open containment structures at a given minimum distance from cowpea plots, or fencing. Rather, genetically transformed cowpeas being tested for a release should be given adequate protection from pollen carriers. Such genetically transformed cowpeas should only be planted in well-netted screen-houses to prevent the entrance or exit of bees or insects that are capable of moving pollens (Asiwe, 2009).

2.11 The Prospective Impact of *Bt* Cowpea on the Economy of Sub Saharan Countries

Cowpea is indigenous to the region and the region accounts for about 80% of the world trade as well as 69% of the production of the crop (Langyintuo and Lowenberg-DeBoer, 2006). As a crop it provides an important source of protein in the local diets and can command a relatively high price in the markets of the region.

Producing a variety of cowpea that resisted all of the pests that reduce its yield could increase yields over four-fold (Foltz, 2007). The *Bt* technology addresses the *Maruca* insect that attacks the maturing cowpeas on the plant. There exist within the germplasm of the cowpea some natural resistances to the other insects in the growth of the crop, but none for the *Maruca*. Thus the *Bt* technology is a necessary addition to the technology mix in order to make cowpea resistant to insects. But in addition to *Bt* technology, there will need to be a lot of work with other conventional breeding or marker assisted selection techniques to produce the type of four-fold increase in the crop that is possible for cowpea (Foltz, 2007). In judging the *Bt* technology by itself it still plays an important role in increasing the potential yield of cowpea in West and Central Africa. Two estimates have been provided for *Bt* technology because of uncertainty as to how much of the yield boost can be related to the biotechnology improvements and how much would come from other improvements. Foltz (2007) reported that from discussions with experts it has been estimated, that *Bt* cowpea technology can eliminate the *Maruca* problem entirely meaning an increase in yields of 50%. Also a 20% yield increase estimates was provided, which would be appropriate, if it were only 40% effective against *Maruca* (Foltz, 2007). These represents conservative estimates compared to that proposed in Langyintuo and Lowenberg-DeBoer

(2006), where they propose a baseline scenario of a doubling of production 100%. This estimate would seem appropriate for the kind of cowpea seed that will likely see the market, which will include multiple resistances. But a conservative estimate of the returns is more appropriate, although both estimates (100% and 40% estimate of the returns for *Bt* cowpea) are provided, in comparisons, a 50% yield increase estimates was used by Foltz (2007).

There is little direct evidence on adoption rates for new cowpea varieties, but given the visible problems that insects produce in cowpea production and the important trade economy in cowpea that makes it somewhat like a cash crop, a reasonably fast adoption path for this technology is expected. It is expected that cowpea adoption will reach 100% within 8 years of its introduction (Foltz, 2007). Overall the benefits to producers from producing *Bt* cowpeas are high in the West and Central Africa region, with the Sahel taking the lead with the highest benefits followed by the coastal region. Benefits are highest in Nigeria and Niger, where much of the cowpea production is intended for trade with Nigeria. Some other countries that have significant benefits include Burkina Faso, Cameroon, and the DR Congo (Foltz, 2007). There are fairly low estimates for Mali, much lower than what one would expect from the published literature, which may be because of some measurement problems in the data. Overall the data used by Foltz (2007) is likely to understate the potential cowpea acreage since the pest problems are so great as to mean that many fields are never harvested. Thus there may well be greater amounts of production that are not fully captured in the report by Foltz (2007).

Availability of *Bt* cowpea lines with resistance to *Maruca* will contribute significantly to: (1) increased production and incomes, (2) improved nutrition, (3) enhanced soil fertility, (4) increased storability, and (5) decreased pesticide use. A study was initiated by African Agricultural Technology Foundation and executed by the International Institute of Tropical Agriculture (IITA) and Purdue University (Foltz, 2007) aims to: 1) Elicit consumer preferences, acceptability, willingness to pay and adaptability of *Bt* cowpea to local conditions in west Africa. 2) Assess the competitiveness and potential market niches for *Bt* cowpea. 3) Identify strategies for capacity building of West African seed organizations for *Bt* cowpea. 4) Assess the *ex-ante* economic impact of *Bt* cowpea at farm, country and region levels, and the preliminary results showed that: 1) Information exchange and awareness are important for the adoption and large diffusion of *Bt* cowpea. 2) There is a high willingness to pay for *Bt* cowpea seed by farmers 3) Given the potential of reducing health hazards by lowering the use of toxic synthetic pesticides, both farmers and consumers are willing to pay a premium price for *Bt* cowpea as an alternative to harmful cotton pesticides. The opportunity costs of using cotton insecticides include the economic losses encountered by the farm household when a family member is sick due to the misuse of chemical insecticides. 4) Urban consumers in regional markets believe that *Bt* cowpea may be safer than conventional cowpea treated with chemicals. 5) *Bt* cowpea will raise incomes substantially at farm, household, community and regional levels (Ousmane *et al*., 2008).

In Nigeria, 90% of the sampled producers were not aware of Genetically Modified food. Only 10% reported some information on GM food. But 84% of producers would buy *Bt*

cowpea seed at current market prices. About 70% of producers were willing to pay a premium of 30% (US$ 0.20/kg) for *Bt* cowpea seed over conventional cowpea seed price. A small group of farmers were willing to pay more than 60% of premium price for *Bt* cowpea seed (Ousmane *et al*., 2008). Results showed that only 16% of rural consumers are aware of GM food compared to 33% in urban areas in northern Nigeria. Rural consumers (82%) prefer *Bt* cowpea. Three main reasons were given by rural consumers to justify their preference for *Bt* cowpea. Their opinions are that *Bt* cowpea should be: Safe for human consumption (95% of consumers who accept *Bt* cowpea), Easy to cook for rural households (94%) and be able to increase the income of the rural farm household and therefore increase its welfare. The rural cowpea consumer tends also to be a cowpea producer. The perceptions of consumers are similar in urban zones. About 33% of consumers think that *Bt* cowpea is quite safe for human consumption while 43% reported that it will be easy to cook. The willingness to pay for consumers changed according to expected *Bt* cowpea price. Only a small proportion of consumers (16%) were willing to pay a premium of up to 30% over current price of conventional cowpea for *Bt* cowpea (US$ 0.6/kg equivalent to N90/kg) (Ousmane *et al*., 2008).

Given the above assumptions, the average urban consumers in Nigeria would like to pay for *Bt* cowpea. In two out of the three cities where interviews were held, consumers preferred *Bt* to conventional cowpea. For example consumers in Maiduguri would discount *Bt* cowpea while consumers in both Sokoto and Kano liked *Bt* cowpea and were ready to pay a premium for it (Ousmane *et al*., 2008). The producers and consumers in Nigeria would experience a welfare gain of about US$ 5.1 billion once *Bt* cowpea is available in Nigerian

rural markets (Foltz, 2007). Results from Foltz, (2007) suggest a gross welfare gain of about US$ 3.3 billion for all urban consumers in Nigeria once *Bt* cowpea is made available in Nigerian urban markets. This assumption is based on the survey results which were conducted in Kano, Sokoto and Maiduguri.

The results of the study by Ousmane *et al.,* (2008) showed that the majority of producers and rural consumers are not aware of GM food or GMO products. In urban areas, the level of information and awareness of consumers is much higher. Information exchange, sensitization and awareness are important elements for the adoption and large diffusion of *Bt* cowpea when developed. The average farmer is willing to pay a higher price for *Bt* cowpea seed as it would reduce chemical pesticide use and/or solve its non-availability. Expectations are that *Bt* cowpea would reduce potential health hazards to both farmers and consumers by reducing the use of harmful cotton pesticides. Health benefits will be linked to the reduction in health costs and/or a decrease in the use of harmful cotton insecticides. *Bt* cowpea will significantly increase the profitability for farmers (Ousmane *et al*., 2008).

Langyintuo and Lowenberg-DeBoer (2006) analyzed the potential effect of *Bt* cowpea on regional trade. In particular, they considered the scenario of *Bt* cowpea being adopted in Nigeria well before other countries in the region. Their results suggested that if *Bt* cowpea roughly doubled yields, Nigeria would become a net exporter of cowpea only when *Bt* cowpea was planted on more than 80% of the cowpea area. Nigeria is currently the largest cowpea importer in the world. If Nigeria became a net exporter, this could seriously disrupt the economies of some of its neighbors, particularly Niger.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Site

The Research was conducted under the confined field conditions between July, 2011 to August, 2012 at the Institute for Agricultural Research (IAR), Samaru. Samaru is located on latitude $11^{0}11^{1}$N and longitude $07^{0}38^{1}$E in the Northern Guinea savannah ecological zone, at an Altitude of 686m above sea level. The average annual rainfall of the area is about 1058mm which is distributed within 160 days (May – September) and the dry season period starts at about the middle of October to the end of April, the temperature distribution is fairly uniform with a mean temperature of 27^{0}c. The soil type is mostly sandy loam (Kowal and Knabe, 1972).

3.2 Experimental Materials

Two genetically engineered cowpea line; cowpea transgenic line (CTL) 709 and 711 and three non-transformed cowpea genotypes; IT97K-499-35 (Sampea 10), IT93K-693-2 and IT86D-1010 were used in this study.

Table 3.1: The Pedigree and Description of the Parental materials used in the Experiment

Genotypes	Pedigree	Description
709	Transformation Event derived from IT86D-1010.	Sourced from CSIRO, transgenic line resistant to *M. vitrata.*
711	Transformation Event derived from IT86D-1010.	Sourced from CSIRO, transgenic line resistant to *M. vitrata.*
IT97K-499-35	Derived from a cross of IT93K-596-9-12 X IT93K-2046-1	Its a medium maturing variety (about 75 d) with semi-erect growth habit. Its heat tolerant and photo-insensitive variety with erect growth habit. It has large white seeds (about 18g 100 seeds-^{1}). In addition to being resistant to Striga and Alectra, it has combined resistance to major diseases and insect pests (Singh *et al.,* 2006).
IT86D-1010	Derived from a cross between TVx4659-03E X IT82E-60	It's an advanced breeding line, medium maturity (71days), photo-insensitive variety with semi- erect plant growth habit. It has combined resistance to cowpea yellow mosaic, blackeye cowpea mosaic and many strains of cowpea aphid borne mosaic, Cercospora, smut, rust, Septoria, scab, Ascochyta blight, bacterial blight, anthracnose, nematodes, Striga, Alectra, ahpid, thrips and bruchid (Van Boxtel *et al*., 2000; Singh *et al*., 2002; Lale and Kolo, 2007).
IT93K -693-2	IT88D-867-11 X IT89KD-374-57	IT93K-693-2 is resistant to Alectra as well as all five strains of Striga reported in West Africa (Singh, 2002). IT93K-693-2 is an extra-early maturing (about 60 d) photo-insensitive and heat tolerant variety with semi-erect growth habit. It has green plants without purple pigmentation. It has medium size seeds (about 14g 100 seeds-^{1}) with brown color and rough seed coat texture. It has combined resistance to major diseases and insects (Singh *et al.,* 2006). This has been used as a parent in crosses for identifying DNA markers for *S. gesnerioides* resistance (Boukar *et al.,* 2004).

3.3 Data Collection

The following qualitative and quantitative data were taken for further analysis;

Days to first flowering; this was taken as the number of days from planting to the day a flower fisrt opened (i.e. first corolla color visible),

Days to first pod maturity; this was the number of days from planting to the day a pod was seen matured on the plant (i.e. the day the first straw color pod was seen on the plant),

Plant height at maturity; the height in centimetres from the base of the plant to the node of terminal bud of the main growing stem of the plant,

Stem, Peduncle, and Pod pigmentations; these refer to their colours during the vegetative stage,

Number of primary branches at vegetative stage; the number of branches steming directly from the main stem,

Total number of pods per plant; this was taken as the total number of pods on the plant at maturity,

Number of damaged pods per plant; the number of pods that were damaged by *Maruca* larvae and

Screening for *Cry1Ab* gene with the aid of *Cry1Ab Bt* strip kits,

3.4 *Cry1Ab* Inheritance Studies

The inheritance of *Cry1Ab* gene was studied in filial generations. And, to study the Mendelian inheritance of the gene, filial generations were developed through crossing between transgenic and non-transgenic lines.

3.4.1 Development of the Genetic Population

The cowpea transgenic lines 709 and 711 along with three non-transgenic genotypes; IT97K-499-35, IT93K-693-2 and IT86D-1010 (the original parent of the transformed lines having the same genetic architecture except the *Bt* gene) were crossed using Biparental mating as described by Sharma (2006) to generate F_1 population. The F_1 seeds were advanced to second filial generation (F_2) population by self pollination and first Backcross populations were generated by crossing the F_1 progenies to their respective parents (parent 1 and parent 2) which formed first Backcross to parent one (BC_1P_1) and first Backcross to parent two (BC_1P_2) respectively. Population development was carried out at the confined field trial site (CFT) of *Maruca* Resistant *Bt* cowpea project at the IAR farm between July, 2011 to November, 2011. Backcross populations were generated between November, 2011 to June, 2012. All the agronomic practices were carried out during the growing period of the plants.

3.4.2 Emasculation

In all the flowers of *Vigna* species, anthesis takes place just before, or simultaneously with, the opening of papilionaceous corolla. Hence, flower buds destined to open the following morning are ready for emasculation. These buds have reached their maximum unopened size and have started to pale slightly from the deep rich green of earlier development (Myers, 1996). Emasculation was performed by opening the petals enclosing the keel, opening the keel and removing the anthers. All operations were performed with forceps, and care was taken that anthers were not broken, which could result in self-pollination since

the stigma is receptive by this time. Stigma becomes receptive about 12 hours before anthessis (Timko *et al*., 2007).

3.4.3 Pollination

During the crossing period, pollens were taken from male parent (transgenics) opened flowers and dabbed on the stigma of emasculated flower buds (mother plant i.e. non transgenics), after which the date and time of pollination were clearly written on the crossing tag and the cross documented as parental genotypes (mother x father).

3.4.4 Combinations of crosses

The following six combinations of crosses were made:-

Cross	Female parent	Male parent
1. IT97K-499-35 x 709	IT97K-499-35	709
2. IT97K-499-35 x 711	IT97K-499-35	711
3. IT93K-693-2 x 709	IT93K-693-2	709
4. IT93K-693-2 x 711	IT93K-693-2	711
5. IT86D-1010 x 709	IT86D-1010	709
6. IT86D-1010 x 711	IT86D-1010	711

3.4.5 Inheritance Studies in F_1 Generation

The F_1 plants were grown at CFT between June to August, 2012. The plants were screened with the aid of *Cry1Ab Bt* strips and the transfer of *Cry1Ab* gene from a *Bt* plant to a non-*Bt* plant was checked. The number of positive plants were taken to infer the behaviour of the transgene whether dominant or recessive.

3.4.6 Inheritance Studies in Segregating Generations

The segregating generations were grown at the CFT between June to August, 2012. Adequate sample size was taken from each F_2, BC_1P_1 and BC_1P_2 families and analyzed with the aid of *Cry1Ab Bt* strips. Since the gene is expected to segregate in F_2, BC_1P_1 and BC_1P_2 generations, the plants were clearly classified as *Bt*–positive or *Bt*-negative regarding the *Cry1Ab* expression. The *Cry1Ab Bt* strip test was found to be the most suitable in order to rapidly analyze large number of plants in lesser time and to differentiate between the two groups. The data were subjected to chi-square goodness of fit test against the Mendelian ratio 3:1 and 1:1 for the F_2 and BC_1P_1/BC_1P_2 generations respectively (Kiani *et al.,* 2009).

3.5. Field Evlauation

The parents, F_1, F_2, and the backcrossed progenies were evaluated under field conditions during the 2012 raining season at CFT Samaru. The trial was planted using Randomized Complete Block Design (RCBD) with three replications. The plant to plant and row to row spacing was kept at 30cm by 75cm respectively. The plot size was 3m x 5m for all entries except F_2 plants which were 6m x 5m. No insecticidal spray against lepidopteran insects was applied. The crop was however protected from the activities of the aphids by hand killing of the insects.

3.6 Assessment of some morphological traits of transgenics and the untransformed parent

Each transformation event results in a different change in the genetic architecture of the cells. The site of gene insertion and the copy number are not always the same. There may be up-regulation or vice-versa of some native genes due to the insertion of a foreign gene. Similarly, the expression of one or more gene(s) may be masked or enhanced otherwise. Some native genes may become silenced or the silenced genes may become active due to the pleiotropic effects of foreign gene (Khan, 2008). In brief, each transgenic plant has its unique genetic architecture and level of expression. It was therefore imperative that transformed plants be studied for any unintended change in the plants i.e. assessment of possible impact of genetic transformation on the Morphological expression of transgenic cowpeas.

3.6.1 Comparison of some Quantitative characters of *Bt* lines & the untransformed parent

The plants of cowpea line IT86D-1010 and the transgenic lines 709 and 711 were grown under field conditions between June to August, 2012. The data were recorded on individual plant basis for the following characteristics:-i) days to first flowering, ii) Days to first pod maturity iii) Plant height (cm), iv) Number of primary branches at vegetative stage, v) Total Number of pods per Plant.

3.6.2 Comparison of some Qualitative characters of *Bt* lines and the untransformed parent

The observations on the following qualitative characters of *Bt* and non- *Bt* cowpeas were also taken during the present studies:-

I) Leaf Shape, II) Pod Shape, III) Node pigmentation, VI) Flower colour, v) Stem pigmentation, VI) Peduncle pigmentation, VII) Pod pigmentation, VIII) Reaction to *Maruca*.

These characters were compared between the *Bt* lines and the original genotype (IT86D-1010), this was done to see if any unintended changes occurred in the *Bt* cowpeas lines after transformation.

3.7.0 *Cry1Ab Bt* strip screening versus Field Bioassay with *Maruca* larvae

3.7.1. *Cry1Ab Bt* strip screening

The screening was carried out with the aid of *Cry1Ab Bt* strips (Envirologix, 2008) to check for the presence of *Cry1Ab* gene in the genetic populations (P_1, P_2, F_1, F_2, BCP_1 and BCP_2). Detection of *Bt* Proteins (*Cry1Ab*) on cowpea involved assaying plant leaves for expression of the *Bt* gene. A quick *Bt* strip test was used to confirm the expression of the *Cry1Ab* protein in cowpea transgenic lines. Briefly, this was achieved by placing leaf discs in test tubes containing Buffer and the *Bt* strips was slowly lowered into the Buffer. Then, formation of a single line in the test tube proved that the test was working while, appearance of a second lower line showed that *Cry1Ab* protein was present (Envirologix, 2008).

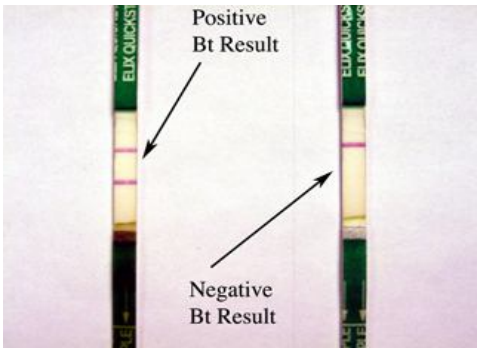


Plate 1: *Bt* strips showing positive and Negative results



Plate2: *Bt* strips In Test tubes

3.7.2 Fied Bioassay with *Maruca* Larvae (Artificial Infestation)

Artificial infestation with 1st instar larvae of *M. vitrata* was used to substantiate the *Bt* strips test for the classification of the genotypes as either resistant or susceptible.

The generations (parents, F_1 and F_2 and Backcross plants) of a cross of IT97K-499-35 x 709 were subjected to artificial infestation of *Maruca* larvae. For the purpose of field infestation, a large number of *Maruca* larvae were reared in the *Maruca* rearing laboratory of IAR/ABU *Maruca* Resistant *Bt* Cowpea project situated in the Department of Crop Protection, Ahmadu Bello University Zaria. Six infestation events were carried out at the interval of four days each. Ten 1st instar larvae, pre-fasted, were placed on a flower of each of the F_1 and on all the F_2 plants and Backcross plants and were allowed to feed on the plants. Different levels of pressure were applied to the plants with 1st instar *Maruca* larvae viz; 10 larvae/plant for the 1st infestation, 20 larvae/plant 2nd infestation and 30 larvae/plant for 3rd to the 6th infestation events. Larvae were placed on flowers of the plants with the help of soft hair brush; larvae were expected

to feed and develop to pupae in the field, grow into adult, lay eggs and damage the plants at larval stage.

The infestation started on the 45th day after planting and continued till the sixth infestation event on the 69th day after planting. The data on insect damaged were taken on the 77th day after planting. The plants were categorized as:-Resistant plants (0% pod damaged) or susceptible plants (1% - 100%) pod damaged. Two scoring were done in relation to *Cry1Ab Bt* strips test classification of positive and negative plants as resistant and susceptible plants respectively. A pod which was completely or partially damaged (at least 1%) by any *Maruca* larvae was counted damaged while pods which were totally undamaged were counted as healthy pods (undamaged pods).

3.7.3 Estimates of Genetic effects from Quantitative data of Artificial Infestation

Measurement of phenotypic values of six related generations (Parents, F_1, F_2, and Backcrosses) allows for the simultaneous analysis of both Mendelian and quantitative traits (Wehner, 2007)**.** Analysis of phenotypic data for estimating genetic effects and heritability for quantitative traits is an important statistical tool for plant breeders (Wehner, 2007). Quantitative methods partition the total variance into genetic and environmental variances and the genetic variance into additive and dominance components, whenever the population structure and composition allows (Nyquist, 1991; Holland *et al*., 2003). For this present study, quantitative methods were used for the genetic analysis for the data set (Number of pods damaged per plant due to artificial infestation with *Maruca* larvae and Number of

undamaged pods per plant at maturiy) collected from artificial infestation screening with *M. vitrata* for all the six related generations of IT97K-499-35 x 709 cross.

3.8.0 Statistical Analysis

3.8.1 Chi-square goodness of Fit Test

Data thus recorded for the genetic segregation of *Cry*1Ab transgene were analyzed with the help of chi-square (X^2) goodness of fit test, the most commonly used statistics to test hypothesis concerning the frequency distribution of one or more populations, to determine whether the observed data conforms to the expected Mendelian 3:1, 1:1, 1:1 ratios for F_2, BC_1P_1 and BC_1P_2 segregating populations of each cross. A SAS program that uses *Proc Freq* for a chi-square test of goodness of fit by Mcdonald (2009) was used.

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where; O = Observed value, E = Expected value and $\sum$ = Summation (Gomez&Gomez, 1984).

3.8.2 Analysis of Variance for different characters of parental genotypes and F_1 hybrids evaluated at the Confined Field Trial site 2012.

The data pertaining to number of pods damaged due natural infestation of *Maruca* larvae, number of pods per plant, days to first flowering, days to first pod maturity and number primary branches at vegetative stage per plant were subjected to analysis of variance using the general linear model (GLM) procedure of the Statistical analysis System (SAS) programme. Duncan Multiple Range Test was used to separate the means where there was significant difference.

The standard linear model for an RCBD with both the block and treatment effects fixed as follows was used: $Y_{ij} = \mu + R_i + B_j + E_{ij}$ (Shieh and Jan, 2004). Where Y_{ij} = denotes the response for the experimental unit with the i^{th} treatment in the j^{th} block, μ = is the overall mean, R_i = is the treatment effect, B_j = is the block effect, and E_{ij} = is the random error with i =1,…,a and j = 1,…,b. a = the number of treatments; b = the number of blocks.

3.8.3 Assessment of some morphological traits of transgenics and the untransformed parent

Paired-wise comparison were made for some quantitative morphological traits viz; i) Days to first flowering, ii) Days to first pod maturing, iii) Plant Height in (cm), iv) Number of primary branches at vegetative stage and v) Total number of pods per Plant at maturity.

3.8.4 Genetic Analysis of Quantitative Traits from Artificial Infestation Screening

Genetic analysis for a cross viz; IT97K-499-35 x 709 was carried out for six generations constituting six treatments (P_1, P_2, F_1, F_2, BC_1P_1, and BC_1P_2). As required by the genetic models used, combined summary statistics were computed in order to estimate genetic factors and their effects.

3.8.4.1 Computational Procedure for the Genetic Analysis

Variance of the F_2 provides an estimate of phenotypic variance, whereas the mean variance of the non-segregating generations (P_1, P_2 and F_1) provides an estimate of environmental variance (Chahal and Gosal, 2002; Wright, 1968). Estimates of phenotypic (P), Environmental (E), genotypic (G), additive (D) and dominance (H) effects from generation

variances as well as Sum cross products of additive and dominance effects of the segregating genes (F) were computed as;

$V_{F2} = {}^{1}/_{2}D + {}^{1}/_{4}H + E$, $V_{B1} + V_{B2} = {}^{1}/_{2}D + {}^{1}/_{2}H + 2E$

$D = 2V_{F2} - (V_{B1} + V_{B2})$, $H = V_{F2} - {}^{1}/_{2}D - E * 4$

$F = V_{B2} - V_{B1}$ (Chahal and Gosal, 2002).

Where; V_{F2} = Variance of F_2 population= Phenotypic Variance,
D = Additive component of variance, H = Dominance component of variance,
E = Variance of non-segregating populations, V_{B1} = Variance of BC_1P_1,
V_{B2} = Variance of BC_1P_2,
F = Sum of the cross products of Additive and Dominance effects of genes that are segregating.

The broad and narrow sense heritability were calculated from the available estimates of genetic, additive, and phenotypic variances, using the following formulae (Allard, 1960; Chahal and Gosal, 2002).
$H_b = V_D + V_H/V_{F2}$, $H_n = V_D/V_{F2}$. Hb = Broad Sense heritability, Hn = Narrow Sense heritability.

CHAPTER FOUR

RESULTS

4.1.1 Inheritance of *Cry1Ab* gene in F_1 Generations

The results of the six set of F_1 plants analyzed with the aid of *Cry1Ab Bt* strips to study the transmission and expression of the transgene have been given in (Table 4.1). It was found that all the F_1 plants were positive to *Cry1Ab Bt* strip test. It thus means that; the gene was successfully transferred from *Bt* lines to non-*Bt* lines and the gene was dominant.

4.1.2 Inheritance Studies in segregating populations

The results have been given in (Table 4.2 to 4.4). The Tables revealed that Mendelian segregation ratios existed in all the six cross combinations for the F_2 (3:1) and the first backcross generations (1:1).

The F_2 populations of these crosses segregated into plants with positive and negative *Cry1Ab* gene indicating the presence and absence of the *Maruca* resistant gene respectively, with a good fit to the Mendelian ratio of 3:1 with non significant Chi-square values (X^2) for F_2 plants of the following crosses; IT97K-499-35 x 709 ($X^2 = 0.18$; $P = 0.67$) in (Table 4.2), IT97K-499-35 x 711 ($X^2 = 0.15$, $P = 0.70$) in (Table 4.2), IT93K-693-2 x 709 ($X^2 = 0.22$; $P = 0.64$) in (Table 4.3), IT93K-693-2 x 711 ($X^2 = 0.31$; $P = 0.58$) in (Table 4.3), IT86D-1010 x 709 ($X^2 = 0.26$; $P = 0.61$) in (Table 4.4), IT86D-1010 x 711 ($X^2 = 0.0041$; $P = 0.95$) in (Table 4.4).

Four of the BC_1p_1 populations of the crosses segregated into plants with positive and negative *Cry1Ab* gene indicating presence and absence of the *Maruca* resistant gene respectively, with a good fit to the Mendelian ratio of 1:1 with non significant Chi-square values of the following crosses as follows; 709 x (IT97K-499-35 x 709) (X^2 = 3.20; P = 0.07) in (Table 4.2), 711 x (IT97K-499-35 x 711) (X^2 = 2.78, P = 0.09) in (Table 4.2), 711 x (IT93K-693-2 x 711) (X^2 = 1.00; P = 0.32) in (Table 4.3), 711 x (IT86D-1010 x 711) (X^2 = 0.20 ; P = 0.65) in (Table 4.4) while a backcross of 709 x (IT93K-693-2 x 709) (X^2 = 10.89; P = 0.001) in (Table 4.3) produced highly significant chi-square value indicating deviation from the Mendelian expected ratio and a backcross of 709 x (IT86D-1010 x 709) (X^2 = 0; P =0) in (Table 4.4) produced non-segregating progenies, where all the backcrossed progenies tested positive indicating skewness towards the resistant parent.

Similarly, all the six BC_1P_2 populations of the following crosses had non-significant Chi-square values; IT97K-499-35 x (IT97K-499-35 x 709) (X^2 = 1.13; P = 0.29) in (Table 4.2), IT97K-499-35 x (IT97K-499-35 x 711) (X^2 = 0.22, P = 0.64) in (Table 4.2), IT93K-693-2 x (IT93K-693-2 x 709) (X^2 = 1.29; P = 0.26) in (Table 4.3), IT93K-693-2 x (IT93K-693-2 x 711) (X^2 = 3.90; P = 0.05) in (Table 4.3), IT86D-1010 x (IT86D-1010 x 709) (X^2 = 0.05; P = 0.82) in (Table 4.4) and IT86D-1010 x (IT86D-1010 x 711) (X^2 = 1.29; P = 0.26) in (Table 4.4).

Table 4.1: Inheritance of *Cry1Ab* gene in F_1 populations of transgenic cowpea crosses

Cross (Female x Male)	No. of Plants Tested	Positive	Negative	Expected ratio
IT86D-1010 x 709	23	23	0	1:0
IT86D-1010 x 711	23	23	0	1:0
IT97K-499-35 x 709	30	30	0	1:0
IT97K-499-35 x 711	28	28	0	1:0
IT93 693-2 X 709	25	25	0	1:0
IT93 693-2 X 711	23	23	0	1:0

Positive: *Cry1Ab* gene is present i.e. resistant to *M. vitrata,* Negative: *Cry1Ab* is absent i.e. susceptible to *M. vitrata*

Table 4.2: *Cry1Ab* gene Goodness of Fit Test for populations derived from IT97K-499-35

Generation	N	Positive	Negative	Expected Ratio	(X^2)	D.F.	P(0.05)
IT97K-499-35 x 709							
709	50	50	0	1:0			
IT97K-499-35	28	0	28	0:1			
IT97K-499-35 x 709	30	30	0	1:0			
(IT97K-499-35 x 709)F_2	89	65	24	3:1	0.18	1	0.67^{NS}
709 x (F_1)	20	14	6	1:1	3.20	1	0.07^{NS}
IT97K-499-35 x (F_1)	32	13	19	1:1	1.13	1	0.29^{NS}
IT97K-499-35 x 711							
711	25	25	0	1:0			
IT97K-499-35	28	0	28	0:1			
IT97K-499-35 x 711	28	28	0	1:1			
(IT97K-499-35 x 711)F_2	111	85	26	3:1	0.15	1	0.70^{NS}
711 x (F_1)	9	7	2	1:1	2.78	1	0.09^{NS}
IT97K-499-35 x (F_1)	18	8	10	1:1	0.22	1	0.64^{NS}

N = Total number of plants tested, NS = Not Significant, Positive: *Cry1Ab* gene is present i.e. resistant to *M. vitrata,* Negative: *Cry1Ab* is absent i.e. susceptible to *M. vitrata,* P(0.05) = Probability at 5%.

Table 4.3:*Cry1Ab* gene Goodness of Fit Test for populations derived from IT93K-693-2

Generation	N	Positive	Negative	Expected Ratio	(X^2)	D.F.	P(0.05)
IT93K-693-2 x 709							
709	50	50	0	1:0			
IT93K-693-2	23	0	23	0:1			
IT93K-693-2 x 709	25	25	0	1:0			
(IT93K-693-2 x 709)F_2	75	58	17	3:1	0.22	1	0.64^{NS}
709 x (F_1)	18	16	2	1:1	10.89	1	0.001**
IT93K-693-2 x (F_1)	28	17	11	1:1	1.29	1	0.26^{NS}
IT93K-693-2 x 711							
711	25	25	0	1:0			
IT93K-693-2	23	0	23	0:1			
IT93K-693-2 x 711	23	23	0	1:0			
(IT93K-693-2 x 711)F_2	131	101	30	3:1	0.31	1	0.58^{NS}
711 x (F_1)	4	1	3	1:1	1.00	1	0.32^{NS}
IT93K-693-2 x (F_1)	31	21	10	1:1	3.90	1	0.05^{NS}

N= Total number of plants tested, Positive: *Cry1Ab* gene is present i.e. resistant to *M. vitrata,*
Negative: *Cry1Ab* is absent i.e. susceptible to *M. vitrata,* NS = Not Significant, ** Highly Significant, P(0.05) = Probability at 5%.

Table 4.4: *Cry1Ab* gene Goodness of Fit Test for populations derived from IT86D-1010

Generation	N	Positive	Negative	Expected Ratio	(X^2)	D.F.	P(0.05)
IT86D-1010 x 709							
709	50	50	0	1:0			
IT86D-1010	19	0	19	0:1			
IT86D-1010 x 709	23	23	0	1:0			
IT86D-1010 x 709(F_2)	105	81	24	3:1	0.26	1	0.61^{NS}
709 x (F_1)	5	5	0	1:1	--	1	---
IT86D-1010 x (F_1)	19	10	9	1:1	0.05	1	0.82^{NS}
IT86D-1010 x 711							
711	25	25	0	1:0			
IT86D-1010	19	0	19	0:1			
IT86D-1010 x 711	23	23	0	1:0			
IT86D-1010 x 711(F_2)	81	61	20	3:1	0.004	1	0.95^{NS}
711 x (F_1)	5	3	2	1:1	0.20	1	0.65^{NS}
IT86D-1010 x (F_1)	7	5	2	1:1	1.29	1	0.26^{NS}

N= Total number of plants tested, NS = Not Significant, Positive: *Cry1Ab* gene is present i.e. resistant to *M. vitrata,* Negative: *Cry1Ab* is absent i.e. susceptible to *M. vitrata,* P(0.05) = Probability at 5%.

4.2 Evaluation of the transgenic lines and their Hybrids at Confined Field, Samaru, 2012

The two transgenic lines and their hybrids derived through crosses with the susceptible genotypes were evaluated regarding some agronomic parameters under field conditions. The data were subjected to analysis of variance, which revealed that the genotypes had highly significant differences among themselves (Table 4.5). The results of mean performance of the parental materials and thier hybrids used in this study are presented in (Table 4.6). The mean number of days to first flowering (DFF) showed significant differences among the parental lines as well as the hybrids, the DFF ranged from 45 to 49 days for IT86D-1010 and IT93K-693-2 respectively (Table 4.6). The DFF was early in IT86D-1010, which was not statistically different from 709, IT97K-499-35 x 709, IT97K-499-35 x 711, IT93K-693-2 x 711. The F_1s of IT86D-1010 x 709 and IT86D-1010 x 711 had moderate number of days to first flowering. The days to first flowering was later in IT93K-693-2 and was not significantly different from IT97K-499-35, 711, and IT93K-693-2 x 709 (Table 4.6).

The days to first pod maturity (DFPM) showed significant differences among the genotypes (Table 4.6). It ranged from 63.43 to 71.10 days after planting for IT93K-693-2 x 711 and IT97K-499-35 respectively. The DFPM was lowest in IT93K-693-2 x 711, which was statistically not different from DFPM of IT86D-1010, 709, IT86D-1010 x 709. Genotypes 711, IT93K-693-2, IT97K-499-35 x 711, IT93K-693-2 x 709, IT97K-499-35 x 709, were statistically thesame in regards to DFPM while IT97K-499-35 had the highest number of DFPM among the genotypes comaperd (Table 4.6). Similarly, the plant height showed significant differences among the genotypes comapared. Plant height ranged from 24.79cm

to 160.28cm for IT93K-693-2 and IT86D-1010 respectively. The plant height of IT86D-1010 was not statistically different from 709, IT86D-1010 x 709, IT86D-1010 x 711, IT97K-499-35 x 709, and IT97K-499-35 x 711 while the plant height of IT93K-693-2 was not significantly different from that of IT93K-693-2 x 711 (Table 4.6).

Statistical significant differences existed between the total number of pods per plant, it ranged from 26.00 to 69.03 for 711 and IT97K-499-35 x 709 respectively. IT86D-1010 was not signifcantly different from 709, 711, IT97K-499-35, IT93K-693-2, IT86D-1010 x 709, IT86D-1010 x 711 and IT97K-499-35 x 711 in regards to mean total number of pods per plant while IT97K-499-35 differed statistically from IT93K-693-2 x 709 (Table 4.6). The number of primary branches per plant taken at vegetattive stage was statistically the same among all the genotypes compared (Table 4.6) while the number of pods damaged per plant due to natural infestation of *Maruca* was statistically the same for the two transgenic lines and all the F_1 hybrids while the IT97K-499-35, and IT93K-693-2 were the same in regards to pod damaged but differed in the degree of pod damaged from IT86D-1010 (Table 4.6). The susceptible genotypes sustained various degree of pod damaged due to natural infestation of *Maruca* larvae (Table 4.6).

Table 4.5: Analysis of variance: Mean Squares for different characters of the field evaluation at CFT, Samaru, 2012.

Source of Variation	Days to First Flowering	Days to First Pod Maturity	Plant Height in (cm)	Total Number of Pods	Number of Primary Branches	Number of Pods Damaged
Replication	1.27^{NS}	42.95**	7805.65*	1326.52*	0.66^{NS}	14.12^{NS}
Genotypes	28.27**	78.89**	31445.15**	6193.75**	2.09*	322.61**
Error	3.98	5.38	1624.36	391.50	0.91	7.79

*Indicates significant differences at P<0.05 probability level, ** Indicates significant differences at P<0.01 probability level, NS indicates non significant difference

Table 4.6: Mean performance of the parental materials and F_1 hybrids evaluated under natural infestation of *Maruca* at CFT, Samaru, 2012

Genotypes	Days to First Flowering*	Days to First pod maturity*	Plant Height in (cm)*	Total number of Pods per plant*	Number of Primary Branches per plant*	Number of Pods Damaged per plant*
IT86D-1010	44.73^{e}	64.28^{e}	160.28^{a}	34.29^{def}	5.13^{ab}	11.07^{a}
709	45.96^{de}	64.48^{e}	137.29^{ab}	43.79^{cd}	5.04^{ab}	0.00^{c}
711	47.65^{abc}	69.25^{b}	82.99^{cd}	26.00^{efg}	5.40^{a}	1.92^{c}
IT97K-499-35	48.40^{ab}	71.10^{a}	56.94^{d}	23.00^{fg}	5.20^{ab}	5.36^{b}
IT93K-693-2	48.93^{a}	68.70^{b}	24.79^{e}	19.91^{g}	4.13^{b}	5.00^{b}
IT86D-1010 x 709	46.62^{dc}	65.27^{de}	125.81^{b}	35.00^{def}	5.07^{ab}	0.08^{c}
IT86D-1010 x 711	46.93^{bcd}	66.47^{cd}	106.51^{bc}	39.08^{de}	4.60^{bc}	0.00^{c}
IT97K-499-35 x 709	45.87^{de}	67.80^{bc}	120.75^{b}	69.03^{a}	5.33^{ab}	0.04^{c}
IT97K-499-35 x 711	46.28^{cde}	69.00^{b}	133.75^{ab}	45.95^{bcd}	5.28^{ab}	0.00^{c}
IT93K-693-2 x 709	47.60^{abc}	68.45^{b}	56.40^{d}	58.64^{ab}	5.00^{ab}	0.00^{c}
IT93K-693-2 x 711	44.87^{e}	63.43^{e}	52.75^{de}	56.10^{bc}	4.93^{ab}	0.08^{c}

* Means with the same Letter in a column are not statistically different according to Duncan multiple range text (DMRT).

4.3.1 Assessment of some Morphological traits of Transgenics and Untransformed parent

Days First Flowering

The days to first flowering data have been given graphically in Figures (1 and 2). Statistically, the 709 and IT86D-1010 had non-significant differences among themselves in the number of days it takes to first flowering (Table 4.6), while 711 and IT86D-1010 had highly significant differences in relation to days to first flowering (Table 4.6). It means that the *Bt* gene had exerted no significant positive or negative effect on days to first flowering of the 709, as was as expected. However, the *Bt* gene exerted negative effect on the line 711, delaying flowering later than the original genotype (Table 4.6).

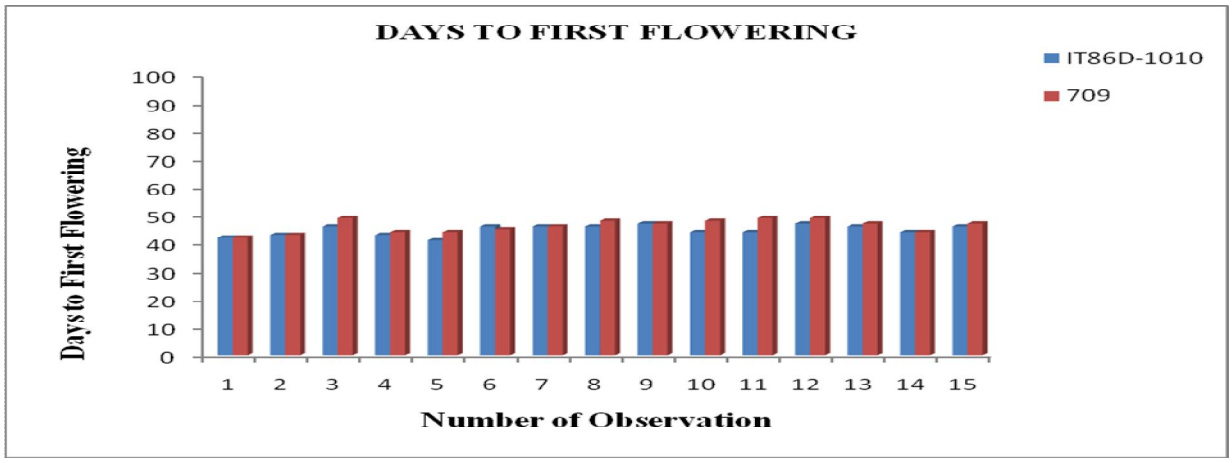


Figure 1: Days to First Flowering for IT86D-1010 and Transgenic line 709

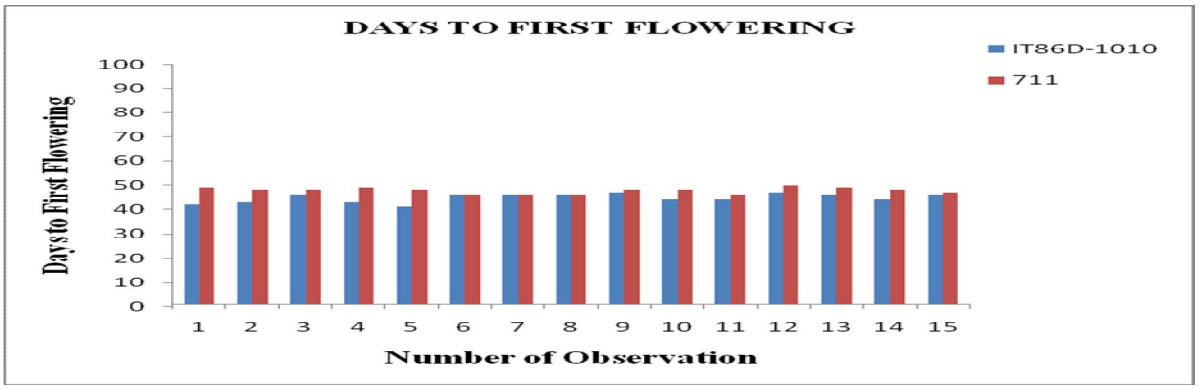


Figure 2: Days to First Flowering for IT86D-1010 and Transgenic line 711

Days to First Pod Maturity

The Days to first pod maturity data have been given graphically in Figures (3 and 4). Statistically, the 709 and IT86D-1010 had non-significant differences among themselves in the number of days it takes to first pod maturity (Table 4.6), while 711 and IT86D-1010 had highly significant differences in relation to days to first pod maturity (Table 4.6). It means that the *Bt* gene had exerted no significant positive or negative effect on the number of days to first pod maturity of the 709. However, the *Bt* gene exerted negative effect on the line 711, making it to mature later than IT86D-1010 (Table 4.6).

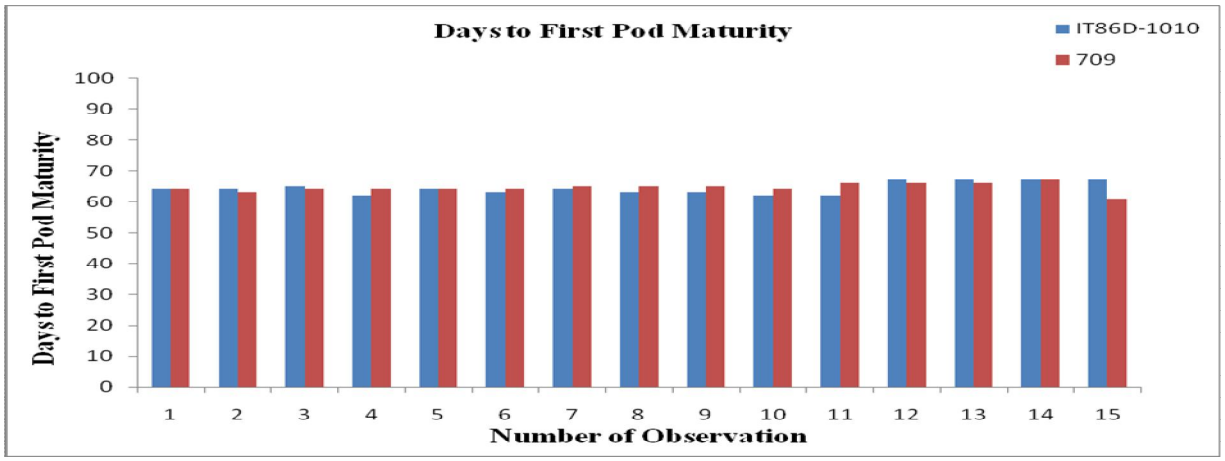


Figure 3: Days to First Pod Maturiy for IT86D-1010 and Transgenic line 709

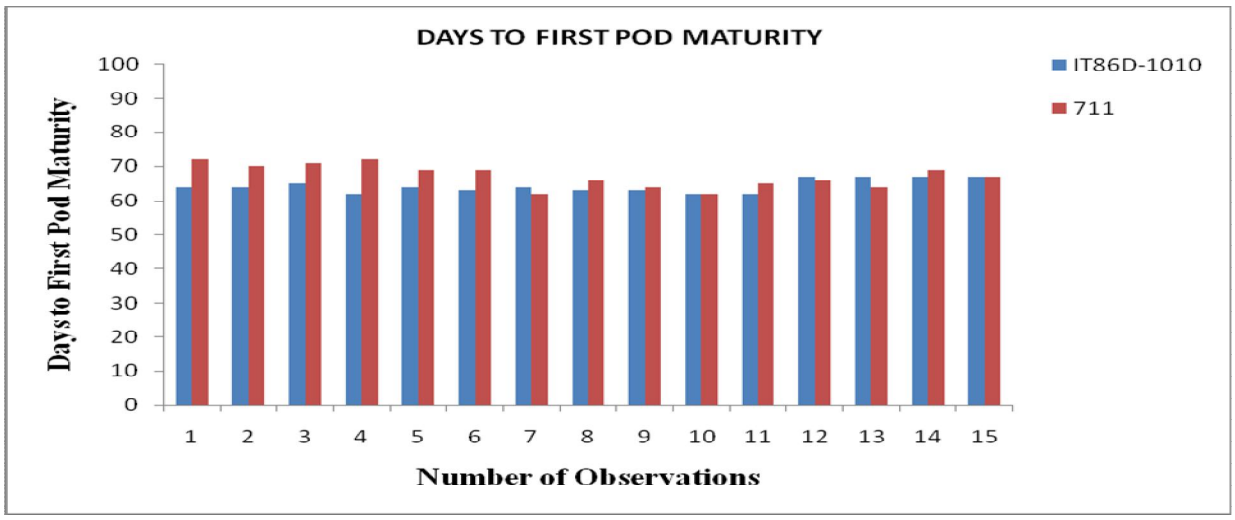


Figure 4: Days to First Pod Maturity for IT86D-1010 and Transgenic line 711

Plant Height

The plant height of the genotypes (709, 711 and IT86D-1010) under comparison has been represented graphically in Figures (5 and 6). The data were subjected to DMRT, which revealed that differences among the genotypes were not significant statistically for 709 and IT86D-1010 (Table 4.6). It means that the *Bt* gene had exerted no effect on the height of the plants. Line 711 and IT86D-1010 had highly significant differences in relation to their heights (Table 4.6). The insertion of *Bt* gene has affected the height of the line 711, making it generally shorter than the IT86D-1010.

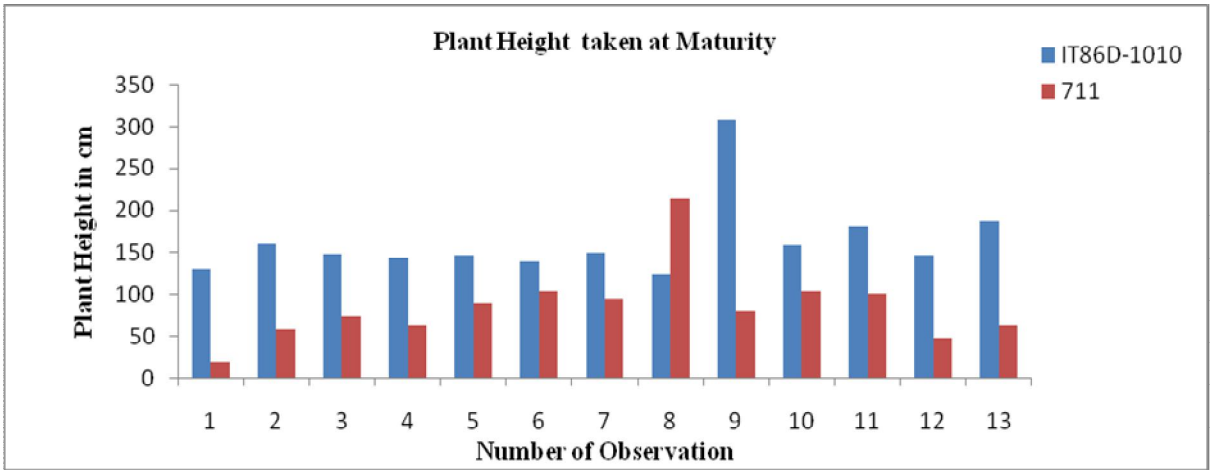


Figure 5: Plant Height data of IT86D-1010 and Transgenic line 711

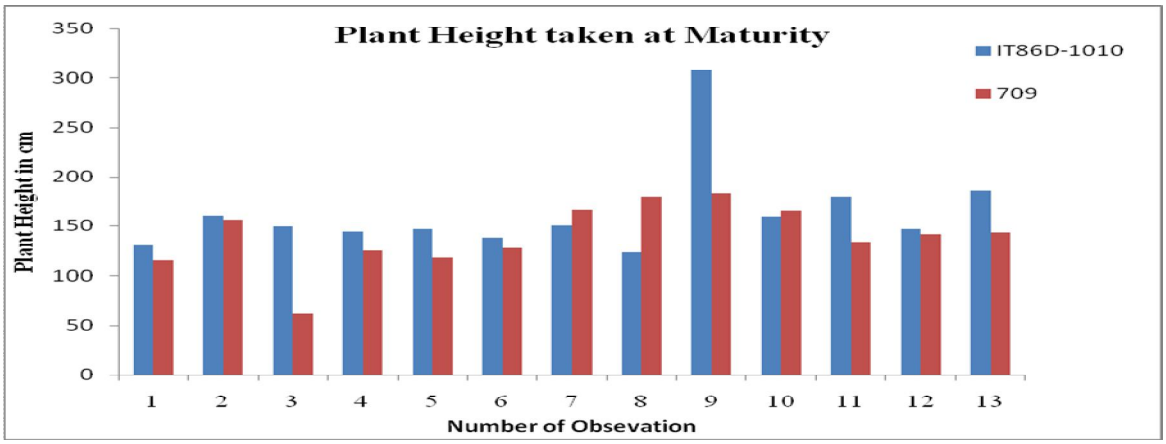


Figure 6: Plant Height data of IT86D-1010 and Transgenic line 709

Number of primary Branches per Plant at vegetative stage

A perusal of the (Table 4.6) indicates that the lines (709 and IT86D-1010) did not differ in respect of number of primary branches per plant. Also, in (Table 4.6), line 711 and IT86D-1010 did not also differ in respect to number of primary branches. The results clearly indicated that the transformation had not affected this character of the plants. The number of primary branches data has been given graphically in Figures (7 and 8).

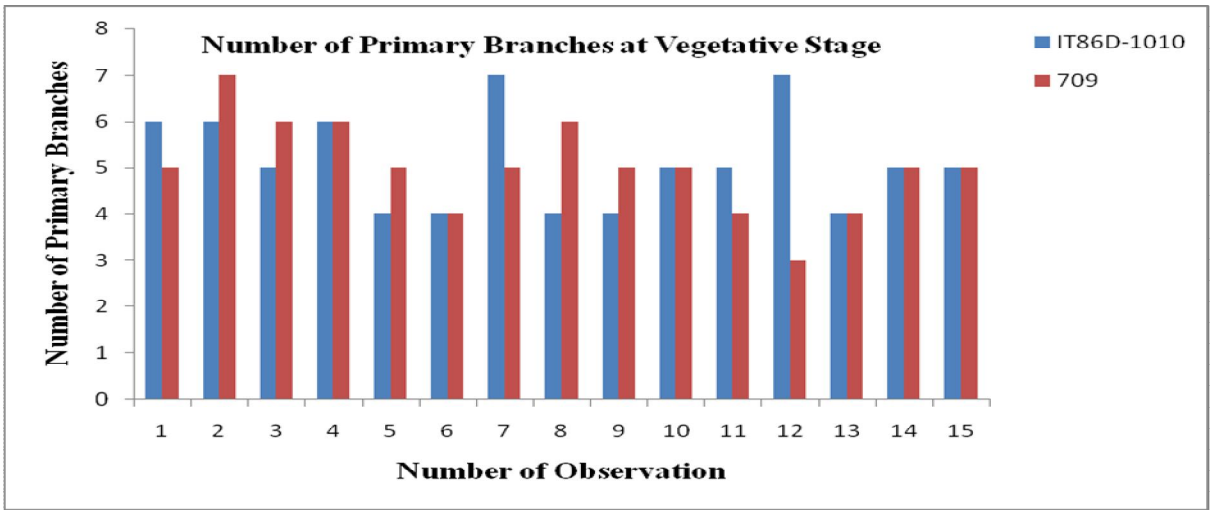


Figure 7: Number of Primary Branches at Vegetative stage for IT86D-1010 & 709

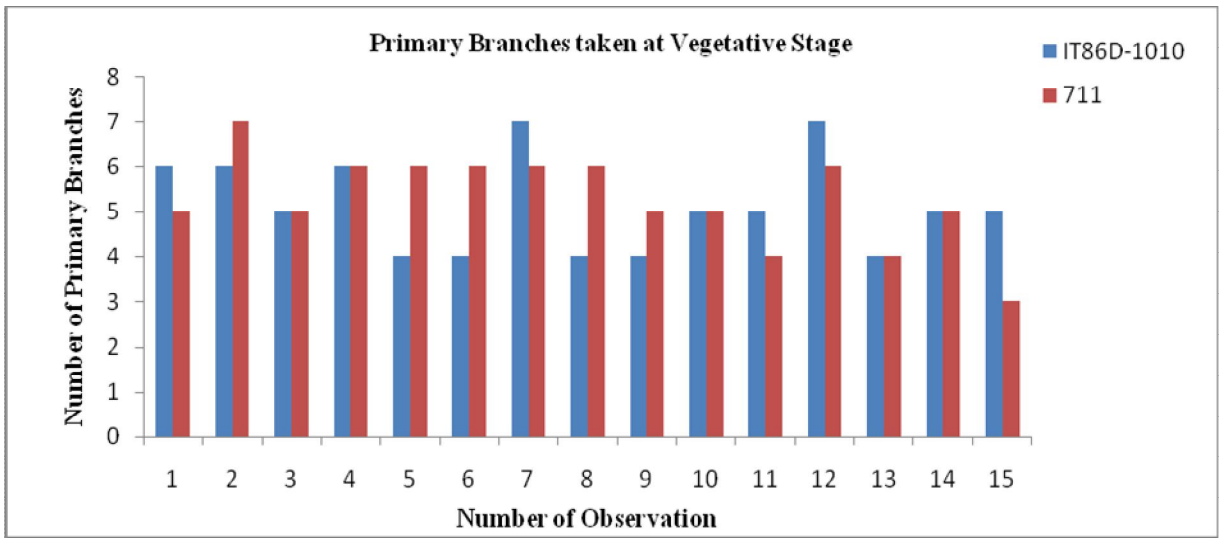


Figure 8: Number of Primary Branches at vegetative stage for IT86D-1010 & 711

Total Number of Pods at maturity

The total numbers of pods data have been given graphically in Figures (9 and 10). Statistically, the genotypes had non-significant differences among themselves in relation to the total number of pods per plant (Table 4.6). It means that the *Bt* gene had exerted no significant positive or negative effect on the number of pod produced per plant. It was as per expectations because the *Bt* gene is not a yield contributing gene. However, theoretically the *Bt* genotypes might have higher yield than the non- *Bt* check due to the enhanced insect resistance capability of the *Bt* plants-as an indirect effect.

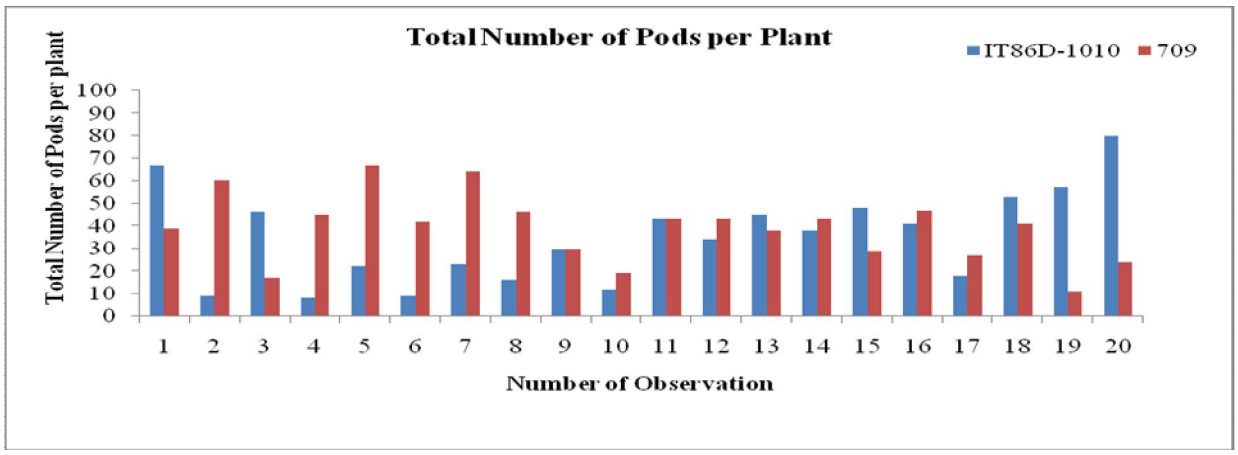


Figure 9: Total Number of Pods for IT86D-1010 and Transgenic line709

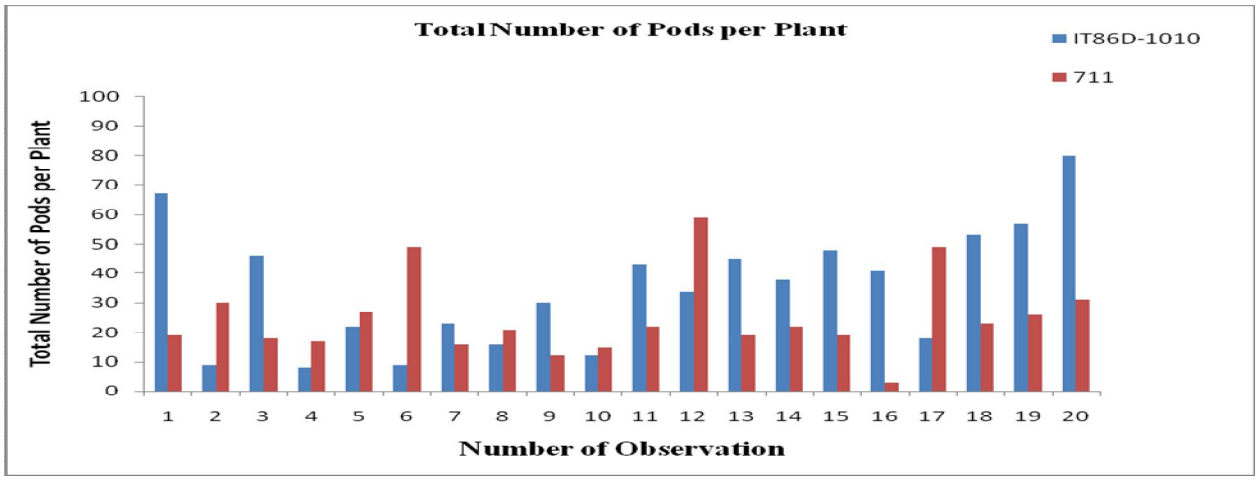


Figure 10: Total Number of Pods for IT86D-1010 and Transgenic line711

4.3.2 Comparison of some Qualitative traits of Trangenics and the untransformed parent

The comparisons of *Bt* and non- *Bt* Parent have been given in (Table 4.7). The Hastate (Narrow) leaf shape of the genotypes remained unchanged after transformation. Similarly, pod shape remained erect even after transformation. The stem, pod, peduncle, pigmentations were the same in the transgenic lines as in the original genotype. The plant reaction to *Maruca* was found to be susceptible in case of non- *Bt* genotype. It was however, resistant in case of *Bt* lines (Table 4.7).

Table 4.7: Comparison of some Qualitative traits of Transgenics and the untransformed parent

Feature	709	711	IT86D-1010 (Non-*Bt* line)
Leaf shape	Hastate	Hastate	Hastate
Pod shape	Erect	Erect	Erect
Stem pigmentation	Green	Green	Green
Peduncle pigmentation	Green	Green	Green
Flower color	White to light purple	White to light purple	White to light purple
Pod pigmentation	Green	Green	Green
Node pigmentation	Purple	Purple	Purple
Reaction to *Maruca*	Resistant	Resistant	Susceptible

4.4 Field Bioassay with *Maruca* Larvae

Table 4.8: Summary Statistics for IT97K-499-35 x 709 under artificial infestation of *Maruca*

Generations	Total Number of Pods	Number of Pods Damaged	Number of Undamaged Pods	% Pods Damaged	% Pods Undamaged
(709) P_1	1226	0	1226	0	100
(IT97K-499-35) P_2	666	153	513	23	77
(IT97K-499-35 x 709) F_1	1816	0	1816	0	100
(IT97K-499-35 x 709) F_2	2257	136	2121	6	94
709 x (IT97K-499-35 x 709)	1151	47	1104	4	96
IT97K-499-35 x (IT97K-499-35 x 709)	1093	102	991	9	91

The results of the screening of resistance to *M. vitrata* of different populations derived from the IT97K-499-35 X 709 cross involving parents with varying reaction to *Maruca* pod borer are presented in (Tables 4.8 and 4.9).

The Mean Total number of pods per plant (TNP) showed differences among the parental materials, the Mean TNP produced per plant were drastically reduced by *Maruca* larvae in P_2 generation (IT97K-499-35) as compared with the Mean TNP of P_1 generation (709) (Table 4.8) while considerable number of pods were obtained in the F_2, BC_1P_1 and BC_1P_2 generations (Table 4.8). The F_1 Mean for TNP was higher than the parental indicating better performance over both of the parents (Table 4.8).

The variation between the percentage of damaged pods (PD) scored by the two parental genotypes confirms the earlier *Bt* strips screening of transgenic line 709 being resistant (Table 4.2), while IT97K-499-35 being susceptible genotype as observed in the percentage PD, with 709 having (0% pod damaged and 100% undamaged pods per plant) and IT97K-499-35 (23% pods damaged and 77% of pods undamaged) (Table 4.8). The F_1 hybrid scored percentage PD of (0% pod damaged and 100% pods undamaged); this also confirms the *Bt* strips screening of F_1s being resistant (Table 4.1). The percentage PD for the backcross involving 709 and IT97K-499-35, viz; BC_1P_1 (4% pods damaged and 96% pods undamaged) and BC_1P_2 (9% pods damaged and 94% pods undamaged) (Table 4.8). In

addition, about 9% pods damaged were recorded among the F_2 population while 91% of the F_2 plants had undamaged pods due to *Maruca* feeding (Table 4.8).

4.4.1 Estimates of Genetic Effects and Heritabilities for Number of Damaged and Undamaged Pods per plant

Estimates of additive and dominance gene effects (D and H) for number of pods damaged and number of undamaged pods per plant were obtained by combining the Arcsine transformed variances (Appendix 3) of genetic populations with different number of individuals rated per generation.

4.4.2 Estimates of Gene Effects

The nature of gene action involved in the resistance of cowpea to *Maruca* pod borer confirmed that dominant gene action was the most important gene action governing the inheritance of *Cry1Ab* resistance to *M. vitrata*. The genetic analyses for the quantitative data (Damaged and Undamaged Pods per plant) taken from the artificial infestation of a cross of cowpea involving transgenic and susceptible lines showed that the dominant alleles contributed most to both the number of damaged and undamaged pods. This was indicated by a positive value of the sum of cross products of additive and dominant effects for segregating genes 'Parameter F' (462.26 and 477.58 for the number of damaged and undamaged pods respectively) (Table 4.9), the positive estimate of parameter 'F' indicated that the higher scoring parent (P_1) has more dominant alleles controlling the traits studied (Chahal and Gosal, 2002).

4.4.3 Heritability Estimates

A perusal of the (Table 4.9) revealed that the Broad Sense Heritability (BSH) were high (59%) for both number of damaged and undamaged pods per plant while the Narrow Sense Heritability (NSH) was high (-59%) for number of pods damaged and moderate (-44%) for undamaged pods per plant. However, the NSH estimates were in negative directions for the traits studied, these were due to negative additive variance for the said traits (Table 4.9).

Table 4.9: Estimates of Gene Effects for a Cross of IT97K-499-35 x 709 Evaluated under Artificial Infestation of *Maruca* larvae at CFT Samaru, 2012

Trait(s)	Additive component	Dominance component	F Estimate	%Broad sense heritability (Hb)	%Narrow sense Heritability (Hn)
Damaged pods	-129.38	258.36	462.26	59.01	-59.18
Undamaged pods	-105	247.83	477.58	59.9	-44.03

F= Sum of the cross products of additive and dominance effects of the genes that are segregating (Chahal and Gosal, 2002)

CHAPTER FIVE

DISCUSSION

5.1 Inheritance of *Cry1Ab*

Gene segregation analysis is one of the fundamental methods in plant breeding and genetic research (Liu *et al.,* 1997). The genetic segregation and pattern of inheritance of *Cry1Ab* gene in the genetically modified cowpea were studied in six crosses of cowpea involving transgenic and non-transgenic lines. In the present study, the segregation of *Cry1Ab* gene was found to be in Mendelian fashion in all the six cowpea crosses, the results indicated that the resistant trait was controlled by a single dominant gene in the crosses that were examined. The transgenic lines carried the dominant gene while the recessive allele resides in the susceptible genotypes. In the F_1 generation studies, the *Bt* gene was found to be successfully transferred from *Bt* to non- *Bt* lines and it was dominant. These results are in agreement with earlier research works on genetically modified *Bt* crops with *Cry1Ab* transgene; that *Cry1Ab* transgene is inherited as single dominant gene, for example; in *Bt* maize where the *Cry1Ab* conferred resistance to stem borer (*Ostrinia nubilalis*) (Murenga *et al.,* 2012), *Bt* Rice containing resistant gene to striped stem borer (*Chilo suppressalis*) (Kiani *et al*., 2009, Wang *et al.,* 2012), in crosses of transgenic Rojolele Rice (Sulistyowati *et al.,* 2008) and in *Bt* Cotton; where Khan (2008); Zhang *et al*. (2000) separately studied the inheritance and segregation of foreign *Bt* (*Bacillus thuringiensis* toxin) and tfdA genes in cotton. Their results confirmed that inheritance and segregation of both resistance characters were governed by a single dominant nuclear gene, and was not affected by cytoplasm.

On the other hand, the present findings are partly in disagreement with the research reports of Wu *et al*., (2002), who reported both Mendelian and distorted segregation ratios in some selfed and crossed F_2 populations of cotton genetically modified with *Cry1Ab* gene. Canming *et al*., (2000) also reported segregation of resistant and susceptible plants in Mendelian 3:1 ratio in six F_2 populations whereas in one F_2 population the segregation was non-Mendelian. Typically, a single foreign-gene insert in the host genome often leads to the expected 3:1 segregation ratio in the selfed population (Shimamoto *et al.,* 1989; Datta *et al.,* 1990; Christou *et al.,* 1991; *Peng et al.,* 1992; Rathore *et al.,* 1993) and 1:1 in the backcross population (Peng *et al.,* 1992; Hiei *et al.,* 1994; Casas *et al.,* 1995; Dillen *et al.,* 1997). However, the situation has been otherwise in the studies of Altman *et al*., (1996) who analyzed F_2 progenies to ascertain the inheritance pattern of *Bt* genes *Cry1Ab* and *Cry1Ac*. Their data indicated that the mode of inheritance was not always Mendelian in different genetic backgrounds.

The genetic background could have a great effect on the segregation pattern. Under particular circumstance, the segregation of foreign genes with a single insert in transgenic plants may deviate from the classic one-locus Mendelian segregation ratio (Peng *et al.,* 1995; Sachs *et al.,* 1998).

Some mechanisms responsible for this phenomenon have been reported, these include the lower viability of transgenic pollen and lower fertilization ability (Zhang *et al.,* 1996) or transgene inactivation (Spencer *et al.,* 1992; Walters *et al.,* 1992), the recessive lethal (Scott *et al.,* 1998).

5.2 Field evaluation of the transgenic lines and hybrids for some key parameters

The field evaluation was conducted to study the performance of the *Bt* lines, their hybrids in comparison with the non-*Bt* parental lines. The results revealed that the lines containing the *Bt* gene produced enough toxin to kill and inhibit the lepidopteran insect (*Maruca*). The data collected on natural infestation of *Maruca* larvae as number of damaged pods per plant gave a clear proof of the achievement of the goal of the genetic transformation of cowpea lines with *Cry1Ab* gene. The Duncan multiple range test clearly indicated the *Bt* lines and hybrids to be better in insect resistance than the non-*Bt* genotypes. In the present studies, the non-*Bt* genotypes had higher number of pods damaged by *Maruca* larvae, these were similar to the results already reported by various workers, a few of which are described below; Perlak *et al.,* (1990) and Barton (1989) reported total protection from insect (lepidopteran) damage of leaf tissue of cotton plants expressing *Cry1Ab* and *Cry1Ac* genes. Ishiyaku *et al.,* 2010 reported complete protection of some transgenic cowpea lines expressing *Cry1Ab* gene from *Maruca* damage under field conditions. Sachs *et al.,* (1998) reported that cotton plants of different genetic backgrounds that posssessed the *Cry1Ab* insecticidal protein were more resistant to tobacco budworm larvae than plants with other traits. Also, Estruch *et al.,* (1997), Halcomb *et al.,* (1996), and Van Rie (2000) evaluated plant stands of *Bt* cotton and non-*Bt* cotton, they found that the attack of bollworms and tobacco budworm was less on *Bt* cotton plants as compared to non-*Bt* cotton plants.

The *Bt* lines and the hybrids were also studied for a number of other key characters besides the primary insect resistance traits. The lines were found to be statistically the same with

the oringinal IT86D-1010 for most of the parameters compared except for *Bt* line 711 that had significant differences in regards to days to first flowering, days to first pod maturing and plant heght. The *Bt* lines were not different from their non-*Bt* counterpart in the number of primary branches at vegetative stage. It is thus evident that these characters of the plants were not affected by the transformation event and remained at their parental level. This is much desirable from the breeder's point of view. A plant breeder always wishes to get a variety transformed for insect resistance but keeping the other characters of the plant intact (Khan, 2008).

5.3 Comparative Assessment of Transgenics and the Untransformed Parent

Information about the phenotype of genetically engineered plants expressing *Cry1Ab* is important for plant breeders and regulatory agencies to: (1) identify any intentional changes to the phenotype that might impact the environmental safety of the plant and (2) to identify any unintended changes to the biology of the plant. This information includes phenotypic characteristics of the plant that might contribute to its survival or persistence (*i.e.* potential weediness), or that negatively affect agricultural performance (*e.g.,* disease susceptibility and yield data) (ANZFA, 2000a, 2000b, 2000c) and are usually sourced from laboratory, greenhouse and field trial studies (ANZFA, 2000a, 2000b, 2000c).

In the present study, comparison made between transgenic cowpea line 709 and original parental type (IT86D-1010) showed non-significant differences in all the parameters compared namely; days to first flowering, days to first pod maturity, number of primary branches at vegetative stage, plant height at maturity and the total number of pods per

plant. These results are in conformity with the earlier research report of Khan (2008) that *Cry1Ab* did not affect the number of monopodial branches in cotton. The non-alteration of the characters other than for which transformation was done is highly desirable (Khan, 2008). It is thus evident that these characteristics of the plants were not affected by the transformation event and remained almost at their parental level (Khan, 2008). Since the seeds used for transformation were of an approved breeding line, it was therefore a highly positive aspect of this transformation that most of the varietal characters remained un-altered. Similarly, comparison made between cowpea transgenic line 711 had significant to highly significant differences for days to first flowering, days to first pod maturity and plant height while non-significant differences were observed for number of primary branches at vegetative stage, total number of pods per plant at maturity and number of peduncles per plant at maturity. Khan, (2008) reported significant changes in cotton transformed with *Cry1Ab* gene; that the *Bt* plants had got other positive effects as well in addition to insect resistance, for example; the percentage Ginning out-turn and Number of Sympodial Branches per Plant had increased significantly when compared with the non- *Bt* parent (Khan, 2008). The most important character in a crop-improvement programme is yield, the result indicated that the genotypes (711, 709 and IT86D-1010) had non-significant differences among themselves regarding total number of pods per plant.

Also, statistically significant differences have been seen between genetically engineered (GE) plants expressing *Cry1Ab* and controls in many cases, but these differences were small and fell within the reported range for maize (ANZFA, 2000a, 2000b, 2000c). Collectively, the phenotypic data showed no pattern of changes that would support the

hypothesis that the introduction of *Cry1Ab* protein had any unintended impact on the gross morphology or phenotypic characteristics of plants, besides conferring insect resistance to *Maruca* pest (CERA, 2011).

The *Cry1Ab* protein is intended to provide resistance to target insect pests; this is taken into account when phenotypic observations are made, some of the collected data are quantitative (*e.g.* plant height or % seed germination) while other data are qualitative and observational (*e.g.*, no differences in disease susceptibility) (ANZFA, 2000a, 2000b, 2000c). The weight of evidence from analyses of phenotypic and compositional data demonstrates that *Cry1Ab* expression in approved Genetically engineered (GE) maize events did not alter the gross physiology of the plant, and that these plants are not more likely to become weedy or invasive than their conventional counterparts (CERA, 2011).

The *Cry1Ab* protein expressed in insect resistant GE plants is derived from the common soil bacterium *Bacillus thuringiensis* and is specifically toxic to Lepidoptera (CERA, 2011). Environmental risk assessments related to the introduction of (GE) plants are conducted on a case-by-case basis taking into account the biology of the plant, the nature of the transgene and the protein it produces, the phenotype conferred by the transgene as well as the intended use of the plant and the environment where it will be introduced (*i.e.* the receiving environment). These assessments are comparative by necessity, and typically involve comparisons to an untransformed parent line or closely related isoline (CBD, 2000a, 2000b). The point of these comparisons is to identify potential risks the GE plant might present beyond what is already accepted for like plants in the environment (CERA, 2011).

5.4 Quantitative Genetic Analysis from artificial infestation screening for *Cry1Ab*

Plant breeders are interested in the analysis of phenotypic data to measure genetic effects and heritability of quantitative traits (Wehner, 2007). Our results showed that dominance effect was more pronounced for the two traits studied.

The variation in any character in a segregating or mixed population is due to both genetic and environmental factors (Khan, 2008). The genetic factor is the most important in plant breeding since it can be used to improve the population. The greater the proportion of total variability that is due to environmental factors, the more difficult it will be to select for inherited differences (Khan, 2008). The estimates of additive and dominance variances represent the summed contribution of any number of genes acting independently (Chahal and Gosal, 2002). The parameter 'F' which is the sum of the cross products of additive and dominance effects of genes that are segregating, determines which of the parent carry more number of dominant alleles (Chahal and Gosal, 2002).

The Broad Sense Heritability of *Cry1Ab* transgene was found to be high in number of damaged and undamaged pods. The findings are in agreement with Zhang *et al*., (2001) who reported high Broad Sense Heritability for *Cry1Ab* in all crosses except one. Ahmad *et al.,* (2003) reported very high estimates of Heritability for bolls per plant and boll weight. Their data suggested selection for improvement of these traits due to presence of sufficient genotypic variability. Since, the efficiency of selection would depend upon the magnitude

of variability that is heritable and caused by genetic factors, therefore, the high values of heritability for the characters studied should be quite valuable.

In the present studies, high Heritability was observed in number of damaged and undamaged pods per plant. The findings of the present study suggest that the trait exhibiting high heritability estimates have sufficient genetic variability worth of exploitation and effective selections can be done in early generations for *Cry1Ab* resistant gene. However, the narrow sense heritability estimates were moderate (-44.03%) to high (-59.18%) for the two traits but were in negative directions, these were due to negative additive effects, which were as a result of low variance observed for the F_2 population in comparison with sum variances of the backcross populations. The low heritability estimates does not mean that there would be no progress, but improvement, in this case, would be slow and gradual for this trait (Khan, 2008).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

The study of the inheritance of *Bt* transgene controlling the legume pod borer in cowpea involved the use of two transgenic lines namely; 709 and 711 that were crossed to three non-transgenic lines viz. IT97K-499-35 (Sampea 10), IT93K-693-2 and IT86D-1010 to produce six hybrids namely: IT97K-499-35 x 709, IT97K-499-35 x 711, IT93K-693-2 x 709, IT93K-693-2 x 711, IT86D-1010 x 709 and IT86D-1010 x 711. The filial generations were then screened for the presence and absence of the *Cry1Ab* gene, grouping the plants as either *Cry1Ab* positve or negative plants. The present results have demonstrated the existence of Mendelian inheritance of *Cry1Ab* gene in cowpea. *Cry1Ab* gene can be steadily inherited and expressed in commercial transgenic cowpea cultivars. The insect-resistance is governed by a single dominant nuclear *Bt* gene. Therefore,classical cross and backcross can introgress the *Cry1Ab Maruca* resistant gene from the transgenics into conventional cowpea varieties, and one can breed new cowpea varieties carrying desired characters such as insect resistance, disease resistance and so on.

In this study, the use of *Cry1Ab* strips and artificial infestation screening of six related generations of a cross involving the transgenic cowpea lines confirmed the stable inheritance and expression of the C*ry1Ab* gene under field conditions. Combining the segregation results in the F_2 population, it could be concluded that the *Cry1Ab* gene was integrated into the cowpea genome as one insert. The distorted segregation ratio in the

BC_1P_1 populations derived from the cross between IT93K-693-2 x 709 and IT86D-1010 x 709 may be due to small sample size of the generations.

No results on the inheritance and expression of the *Cry1Ab* gene in transgenic cowpea under natural field conditions have been reported so far in the scientific world, whereas this step is indispensable for the future utilization of transgenic cowpea plants in breeding programs. In this experiment, all the transgenic cowpea lines conferred a very high degree of resistance to *M. vitrata* under field conditions. As the *Cry1Ab* gene expression remained stable in progenies through successful sexual generations and conferred a high degree of resistance to cowpea pod borer, the transgenic cowpea should be used as a precious insect-resistant line to be employed in traditional breeding programs. Meanwhile the results presented here provide primary data for further research on *Bt* transgenic cowpea, including the potential risk assessment, sustainable utilization and implementation of integrated pest management in *Bt* transgenic cowpea.

The use of *Cry1Ab Bt* strips as a screening tool of transgenic lines containing *Cry1Ab* gene is strongly recommended, this has an importance in the hybridization programme where genotypes having *Bt* gene can be distinguished at seedling stage at lesser time.

The performance of the transgenic lines and their hybrids in comparison with the non-transgenic parents regarding some key paramters were conducted. The data were recoreded on various characters and the results from the analysis of variance showed significcant differences among the genotypes. The Duncan multiple range test at (5% level) was applied to establish the level of significance among the various genotypes for all the

parameters compared. The transgenics and the hybrids derived from them through hand crosses with the non-transgenic parents were statistically the same regarding the number of pod damaged due to natural infestation of *Maruca* larvae. The transgenics and along with the hybrids did not sustained pod damaged due to *Maruca* feeding. It therefore, means that the two transgenic lines used and F_1s derived from them were resistant to *Maruca* while the non-transgenic genotypes sustained various degrees of damaged as a result of feeding activities by the *Maruca*.

The possible impact of genetic transformation of cowpea on morphological expression of the transgenic lines used in this study were investigated to identify any unintended changes to the biology of the plant that might negatively affect agricultural performance of these lines such as yield and disease susceptibility. In the present study, comparison made between GE cowpea line 709 and original parental type (IT86D-1010) showed non-significant differences in all the parameters compared, the un-alteration of the characters other than for which transformation was done is highly desirable. Similarly, comparison made between cowpea transgenic line 711 had significant to highly significant differences for days to first flowering, days to first pod maturity and plant height. Statistically significant differences have been seen between GE plants expressing *Cry1Ab* and controls in many cases, but these differences were small and insignificant. In summary, the phenotypic data showed no pattern of changes that would support the hypothesis that the introduction of *Cry1Ab* protein had any unintended impact on the gross morphology or phenotypic characteristics of cowpea plants, besides conferring insect resistance to *Maruca* pest.

The precision level of the *Cry1Ab Bt* strips screening were compared with the conventional screening for insect resistance using the artificial infestation approach. The *Cry1Ab Bt* strips were able to categorize the plants into resistant and recessive groups with good level of precision. The strip screening clearly grouped the F_1 plants as resistant plants just like the transgenic parents and the segregating progenies of F_2 were seen clearly behaving as hypothesized into 3:1 Mendelian test ratio and the backcross plants clearly segregated into 1:1 ratio except for two backcrossed generations that deviated due to small sample size. Using the *Cry1Ab Bt* strips, the screening were done at seedling stage with good precision, this saves time and resources. However, with the conventional screening, the plants were screened successfully but not with the same level of precision as *Cry1Ab Bt* strips. All the transgenic parental plants and F_1 plants were resistant just as they did with the *Cry1Ab Bt* strips aided selection. The segregating progenies were seen falling into resistant and susceptible cowpea plants from scoring of plants as damaged and undamaged pods. However, this is not too reliable since some of the plants would have escaped attack by the *Maruca* larvae and therefore be counted as resistant while it's a susceptible in real sense. The conventional screening was time consuming, since the plants were grown and maintained till their maturity before screenings were done.

Therefore, the use of molecular based tools is strongly advocated for the segregation studies involving transgenic cowpeas, this is faster, easier, and produce output with high level of precision. Elite and promising plants can be faithfully screened and selected at seedling stage particularly during the development of backcross population, aimed towards development of transgenic cowpea varieties.

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Appendix 1: Producer Benefits to *Bt* Cowpea

Region	Country	Producer Benefits (in $) 20% yield increase	Producer Benefit (in $) 50% yield increase
Coastal			
	Guinea	0.0	0.0
	SierraLeone	36,464.7	96,214.2
	CotedIvoire	2,181.2	5,710.9
	Ghana	3,953.3	10,350.0
	Togo	0.0	0.0
	Benin	0.0	0.0
	Nigeria	709,652.3	1,860,432
Central			
	Cameroon	176,001.2	465850.5
	CAR	0.0	0.0
	Gabon	0.0	0.0
	Congo Rep	7,355.9	19462.8
	D.R. Congo	91,461.1	242, 028.4
Sahel			
	Burkina Faso	143,846.7	368844.2
	Chad	54,590.0	140103.9
	Gambia	0.0	0.0
	Guinea Bissau	0.0	0.0
	Mali	17,497.0	44873.1
	Mauretania	52,404.2	134490.6
	Niger	680,609.6	1746714
	Senegal	33,719.7	86544.4
Coastal	Total	752,251.5	1,972,707.4
Central	Total	274,818.2	727,341.7
Sahel	Total	982,667.2	2,521,570.0
Whole region		**2,009,736.9**	**5,221,619.1**

(Data from Foltz, 2007).

Appendix 2: Gross benefits from *Bt* cowpea in Nigeria

	Benefits in Naira/Year	Benefits in US Dollar/Year ($)
Rural producers and consumers	658,038,817,706	5,131,907,332
Urban consumers	422,491,532,978	3,294,923,244
Total benefits from *Bt* cowpea in Nigeria	1,080,530,350,684	8,426,830,576

(Ousmane *et al*., 2008).

Appendix 3: Generation Means and Variances of IT97K-499-35 x 709 for percentage Damaged and Undamaged Pods per plant

	Original scale		Arcsine transformed		Original scale		Arcsine transformed	
Generations	**Mean of damaged pods**	**Variance of damaged pods**	**Mean of damaged pods**	**Variance of damaged pods**	**Mean of undamaged pods**	**Variance of undamaged pods**	**Mean of undamaged pods**	**Variance of undamaged pods**
709	0.00	0.00	18.99	16.38	44.00	283.00	71.07	16.62
IT97K-499-35	5.00	8.00	33.31	228.31	18.00	139.00	56.55	245.96
IT97K-499-35 x 709	0.00	0.00	23.86	24.19	67.00	606.00	66.18	24.33
(IT97K-499-35 x 709)F_2	2.00	29.25	21.88	218.61	35.00	299.21	68.13	238.47
709 x (F_1)	2.00	24.00	23.32	52.17	58.00	780.00	66.70	52.18
IT97K-499-35 x (F_1)	3.00	8.00	29.91	514.43	32.00	627.00	59.98	529.76