INHERITANCE PATTERNS OF MORPHOLOGICAL CHARACTERS AND THE KARYOTYPE OF *Crotalaria* SPECIES IN KENYA

MERCY ACHIENG WASONGA

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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award.

Signature: ..... Date: .....
Mercy Achieng' Wasonga
Department of Biological Sciences
B520/1224/2018

This thesis has been submitted for examination with our approval as University Supervisors

Signature: ..... Date: …….
Prof. Nancy Budambula
Department of Biological Sciences
University of Embu

Signature: ..... Date: …….
Dr. Edith Esther Arunga
Department of Agricultural Resource Management
University of Embu

DEDICATION

I dedicate this work to the Almighty God for giving me the chance and strength to complete this study. I also dedicate it to my father Edward Wasonga, my mother Josephine Wasonga and my siblings. Your prayers, endless encouragement and firm support made it possible to complete this study.

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LIST OF ABBREVIATIONS

ANOVA Analysis of variance

BC	Backcross
$Ca(NO3)_2$	Calcium nitrate
CRD	Completely randomized design
$CuSO_4$	Copper(II) sulfate
F_1	First filial generation
F_2	Second filial generation
GCA	General combining ability
H_2MoO_4	Molybdic acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium nitrate
$MgSO_4$	Magnesium sulfate
$MnCl_2$	Manganese(II) chloride
NH_4H2PO_4	Ammonium dihydrogen phosphate
RCBD	Randomized complete block design
SAS	Statistical analysis software
SCA	Specific combining ability
$ZnSO_4$	Zinc sulfate

ABSTRACT

Slender leaf (*Crotalaria* spp.) is an African indigenous leafy vegetable with high nutritional benefits. However, despite these benefits, this vegetable has been neglected in terms of research, and information on breeding techniques is scanty. This study aimed to determine the inheritance patterns of morphological characters and the karyotype of *Crotalaria*. *Crotalaria* accessions from Kakamega, Bungoma, Vihiga, Busia, Siaya, Homa Bay, Kisumu, Migori and Kisii were subjected to inheritance studies under similar field conditions. The experiments were carried out at the University of Embu Farm. Two landraces FKK 0039 and FHB 0211 were used to develop an artificial pollination protocol. The experiment was set up in a completely randomized design (CRD) in a greenhouse with *Crotalaria ochroleuca* as the female parent and *Crotalaria brevidens* as the male parent. Six plants were planted in pots replicated three times for both male and female parents. Six-day-old flower buds of the female parent were emasculated and pollen from a freshly opened flower was rubbed over the stigma of the emasculated flower. The pollinated stigma was inserted back into the keel petal and covered by the wing and standard petal. Data on crossing success rate, pod and seed production was subjected to analysis of variance. Eleven parents of *Crotalaria* were used in a diallel cross to determine genetic control of morphological traits. The experiment was laid out in a triple lattice design with 110 hybrids and 11 parents. The data were evaluated for the inheritance of six traits using Hayman's method and Griffing's model 1 for estimation of gene action, general and specific combining abilities. A mean separation was done by Tukey's HSD test at 5% probability level. Twenty *Crotalaria* seeds were grown in petri dishes for karyotype studies. Chromosome morphology was observed and the chromosome numbers were noted. Chromosome positioning at different mitotic stages were observed under a compound microscope. The developed artificial pollination method showed 75% success rate in the interspecies cross of *C. ochroleuca* and *C. brevidens*. Time of crossing did not significantly influence the success rate, pod and seed production. Data showed significant additive and dominance gene effects for two and six traits, respectively. The general combining ability and specific combining ability were significant for plant height and leaf length. This study recorded a diploid chromosome number of 2n = 2x = 16 for five *Crotalaria* species; *C. trichotoma*, *C. brevidens*, *C. ochroleuca*, *C. spectabilis* and *C. intermedia*. The diploid chromosome number of 2n = 2x = 16 was reported for the first time in *C. trichotoma*. The study demonstrated artificial interspecific pollination of *Crotalaria* by rubbing method involving keel petal incision. This protocol lays the foundation for genetic studies and improvement of *Crotalaria* spp. The study showed the importance of general combining ability for parental selection and specific combining ability in hybrid production.

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Crotalaria species are annual herbs found in old world tropical areas where they colonize open fields, altered places and roadsides (Jacobi, 2005). Over 600 species in genus *Crotalaria* are distributed worldwide and are commonly known as rattle pod or rattle box (Soyewo and Omiyale, 2015). The name is derived from the sound produced when the dry pod is shaken. *Crotalaria* are leguminous crops that plays a role in soil improvement because the roots support nitrogen fixing bacteria. Slender leaf (*Crotalaria* spp.) is an indigenous vegetable commonly cultivated in Kenya, Uganda, Tanzania and Sudan. It is also known as 'marejea' in Swahili and 'mitoo' in some local languages (Nduhiu, 2017). Slender leaf includes *C. ochroleuca* and *C. brevidens*. The vegetables provide 100% of the dietary requirement for vitamin C, vitamin A, calcium, iron and proteins when 100 g of the fresh weight are consumed (Buleti, 2017).

The genus *Crotalaria* includes crops cultivated for direct human consumption and as forage suitable for cattle consumption (Bhandari *et al.*, 2016). Economically, the genus has a wide range of uses. *C. tetragona* is used as an edible vegetable in the North Eastern India while *C. retusa* is used as an animal poison due to the presence of alkaloids. The seeds of *Crotalaria* yield gum which is used in the textile and dye industry. Flowers of *Crotalaria* have oil which has potential application in cosmetic industries. The genus also contains important secondary metabolites, macrocyclic pyrrolizidine alkaloids which are used as bio-pesticides (Subramaniam & Pandey, 2014). *Crotalaria micans* is used as edible vegetables in North Eastern India (Subramaniam and Pandey, 2013). *C. juncea* is a native of India but also grown in other countries like Brazil, Bangladesh, Pakistan, China and Korea (Bhandari *et al.*, 2016). The *Crotalaria* species produce fibre and is identified as a major source of fibre in India (Sarkar *et al.*, 2015). The fibre is long-lasting, light coloured and equally resistant to micro-organisms, moisture and mildews (Chaudhury *et al.*, 2000). In addition, *C. juncea*, is used as green manure and cover crop thus reducing nematode, weed and invasions (Marshall, 2002).

Slender leaf is of medicinal importance and has been used in the treatment of stomach ailments and malaria. It performs well in nitrogen stressed soil. Its early leaves and

shoots are cooked as vegetables. The young shoots and leaves can be boiled or used as herbs in soups. The leaves can be cooked alone or maybe mixed with other vegetables such as jute mallow. Addition of milk is recommended to reduce the bitter taste of *C. brevidens*. Further, slender leaf has potential use in management of the striga weed (*Striga hermonthica*) and *Meloidogyne* nematodes. This is attributed to its suicidal induction of germination of striga seeds and suppression of nematode populations (Fischler *et al.*, 1999; Krueger *et al.*, 2008; Abukutsa-Onyango, 2016; Nduhiu, 2017).

C. ochroleuca is characterized by its pale yellow flower colour compared to the bright yellow flower colour of *C. brevidens*. Its leaves are bright green and the crop can grow to a height of 250 cm. The pods are bigger or wider in diameter than those of *C. brevidens* (Abukutsa-Onyango, 2016). *C. brevidens* has bluish green leaves, growing to a height of 210 cm and bright yellow flowers. The pods are small and narrow or slender. The seeds of *C. ochroleuca* are pale yellow to brown while seeds of *C. brevidens* are pale yellow turning orange to dark red (Al-snafi, 2017). The key distinguishing features between *C. ochroleuca* and *C. brevidens* are the pod size and the taste. *C. ochroleuca* has a mild taste with wide big pods while *C. brevidens* is bitter with narrow slender pods (Nduhiu, 2017).

Plant breeding is a continuous process which results in continual creation of new varieties and plant hybrids. One of the most effective methods for enhancing breeding is selection followed by hybridization (Konarev *et al.*, 2000). Improving productivity of the crop is the main goal of the breeding process. Quite often productivity is subdivided into separate structural elements, which, in turn, provide an opportunity to study the inheritance and variability of each element separately and in general (Adebo *et al.*, 2015). Information on magnitude and nature of gene action governing quantitative characters is essential for effecting genetic improvement. Genetic enhancement is one of the key tools used to improve productivity. Increased variation in a population gives a prospect for selection of a variety with anticipated characters. Greater variability in breeding enables better opportunity of creating desired forms of a crop (Lachyan *et al.*, 2016).

Chromosome numbers of the genus *Crotalaria* are recognized for about one third mostly from Africa and Asia. Information on cytogenetics of some *Crotalaria* species are well documented (Tapia-Pastrana *et al.*, 2005; Almada *et al.*, 2006; Flores *et al.*,

2006). Most *Crotalaria* species have a diploid chromosome number of 2n = 2x = 16, 2n = 2x = 14 or 2n = 2x = 32. *Crotalaria ferruginea*, has been reported to have 2n =4x = 48 (Mangotra and Koul, 1991). The basic number of chromosome for *Crotalaria* has been reported to be x = 8 (Gupta, 1978; Polhill 1981; Mangotra and Koul 1991), while *x* = 7 is considered to have been derived later on in a few species such as *C. incana* (Mondin *et al.*, 2007). The genus *Crotalaria*, is said to have uniformity in size, symmetry and chromosome morphology (Raina *et al.*, 1979). However, other studies assert that even though there might be a similarity, karyotypes are not mostly symmetrical. This support the fact that interspecific karyotypic modifications can be used in the characterization of species (Almada *et al.*, 2006; Tapia-Pastrana *et al.*, 2005). Conventional squash method for karyotype studies of *Crotalaria* species, shows mostly sub-metacentric and metacentric chromosomes (Almada *et al.*, 2006), with only one or two sub-telocentric. Understanding chromosome structure and karyotypic patterns is important to determine mechanisms involved in the evolution of *Crotalaria* species. Cytogenetic studies in *Crotalaria* have provided more information in relation to chromosome progression within the genus. Information on chromosome banding and number have been used for making inferences in *Crotalaria* chromosome evolution (Flores *et al.*, 2006; Mondin *et al.*, 2007).

1.2 Statement of the Problem

The genus *Crotalaria* contains an important group of African indigenous vegetables with both nutritional and potential medicinal value. However, *Crotalaria* has received little research or breeding attention. Two species; *C. brevidens* and *C. ochroleuca* are consumed in Kenya as indigenous vegetables and their production is largely limited to the coastal and western regions of Kenya. A potentially limiting factor to the popularity of these species as a vegetable is their observed low biomass due to small leaflet size. Additionally, there is limited information on *Crotalaria* breeding techniques, therefore, their potential has not been fully exploited. This emphasizes the need for development of an artificial pollination protocol. Further, few studies are available regarding genetics of different traits in *Crotalaria* species in Kenya and there is no documented research on cultivar improvement. Some features of *Crotalaria* such as complex breeding behavior including lack of genetic variation and presence of self-incompatibility could be some of the reasons that have hindered development of superior varieties. The genus *Crotalaria* has been reported to have diploid number of

chromosomes, that is, 2n = 2x = 14, 2n = 2x = 16, while some species are polyploid with a chromosome number of 2n = 4x = 48 and 2n = 4x = 54. Although karyotyping of *Crotalaria* species has been previously reported, little is acknowledged about the chromosome architecture of *Crotalaria* species in Kenya.

1.3 Justification

The unavailability of quality seed of *Crotalaria* prompts the need for improvement of the plant by developing an artificial pollination protocol. An artificial pollination protocol for *Crotalaria* species is important for genetic improvement and suitable production of the crop. Improved hybridization techniques will help in development of improved varieties in *Crotalaria* with novel traits like high yield. Successful hybridization is important for the utilization of genetic diversity of *Crotalaria* gene pools to transfer suitable and desirable genes within and between species. Crop improvement by artificial pollination method can therefore be achieved in a shorter time as compared with unplanned natural pollination which takes a longer time to achieve the desired results. Knowledge on inheritance of morphological characters and patterns in *Crotalaria* is important in breeding programmes, genetic resource conservation as well as maintenance and preservation of intraspecific and interspecific variations. The knowledge of gene action of quantitative traits will help in understanding inheritance patterns of *Crotalaria* characters and in the selection of suitable parents for crop improvement (Fasahat, 2016). There is therefore need for in depth studies on inheritance of qualitative and quantitative traits of *Crotalaria* species. Knowledge on the chromosome morphology including; size, position of the centromere, banding and staining regions provide knowledge on the relationship between various species. Most of research done on cytogenetic of *Crotalaria* species has not reported on the optimum time for preparing the slides to carry out the procedure. Therefore, this study also sought to determine the optimum time for carrying out cytogenetic procedures. In addition, karyotyping of *Crotalaria* species will help identify which species have diploid and polyploid number of chromosomes which is key for successful crosses hence, important in breeding programmes. This study therefore, aimed to describe inheritance patterns of morphological characters and karyotype of *Crotalaria* species in Kenya.

1.4 Research Questions

1. What is the suitable protocol for artificial pollination of *Crotalaria* species?
2. What is the pattern of inheritance of morphological characters of *Crotalaria*?
3. What is the karyotype architecture of *Crotalaria* accessions in Kenya?

1.5 Objectives

1.5.1 General Objective

To evaluate inheritance of morphological characters and the karyotypic architecture of *Crotalaria* species in Kenya.

1.5.2 Specific Objectives

1. To develop an artificial pollination protocol for *Crotalaria* species in Kenya.
2. To determine the inheritance patterns of morphological characters in the *Crotalaria* species.
3. To karyotype selected *Crotalaria* accessions in Kenya.

CHAPTER TWO
LITERATURE REVIEW

2.1 Taxonomy, Origin and Distribution of *Crotalaria* Species

The genus *Crotalaria* belongs to the family Fabaceae which comprises three sub-families namely; caesalpinioideae, mimosoideae and papilionoideae (Polhill, 1981; Bandyopadhyay, 2018). The division into three sub-families is based on the floral parts for instance symmetry of the flower, size of the flower, arrangement of the petals in flower bud, number of stamens and presence of root nodules (Mosjidis and Wang, 2011). After intensive molecular phylogenetic studies of the family, it has been updated to be 727 genera, 36 tribes and 19, 327 species (Ninkaew *et al.,* 2017). The tribe of Crotalarieae (Benth) is a sub-family and is known to be the largest tribe in the papilionoid legumes comprising of about 51% of the genistoid legumes (Yaradua, 2018). The sub-family contains flowers having a zygomorphic symmetry and are commonly adapted to bee pollination. The flower comprises standard petal, wing petal and keel petal. The genus *Crotalaria* occurs in the open and wooded grassland and is infrequently found in seasonal swamps and on termite mounds.

2.2 Salient Features of Selected *Crotalaria* Species

Slender leaf (*Crotalaria* spp.) is an erect herbaceous annual plant with branching on upper portion of the stem. The stems are cylindrical having ridges which end on an inflorescence (Nduhiu, 2017). The plant produces deep yellow flowers with a fragrance and nectar glands which encourages cross pollination (Subramaniam & Pandey, 2013). The main distinguishing features between *C. brevidens* and *C. ochroleuca* are taste and pod size (Abukutsa-Onyango, 2007). *C. ochroleuca* has a mild taste with bright green leaves, grows to a height of 250 cm with pale yellow flowers, seeds are light yellow and has big pods that are wide in diameter (Sahou *et al.*, 2014). *C. brevidens* has a bitter taste, bluish green leaves, bright yellow flowers and grows to a height of 210 cm. The seeds are light brown in colour and pods are narrow and small. *C. brevidens* is a short-lived perennial legume with a height of 0.4 – 2 m. The stems are rising with short hairs. The leaves are trifoliate with lanceolate to linear leaflets. The pods are cylindrical, narrow and pubscent pods. The seeds are smooth, small and inconstant in colour from medium yellow to orange yellow, red to dark brown or dark grey- blue (Abukutsa-Onyango, 2004; Mosjidis and Wang, 2011).

Sunn hemp is annual erect shrubby herbaceous legume growing to the height of 1.4 m. *C. juncea* has spiral leaves arranged along the stems, hairy, elliptical or oblong-lanceolate in shape (FAO, 2011). The pod is hairy, cylindrical, many seeded and bears showy, deep-yellow papillonaceous flowers (FAO, 2017). *Crotalaria retusa,* an herbaceous plant can grow up to 1.2 – 1.5 m tall. The plant has simple leaves, obovate with rounded leaf tip and wedge- shaped leaf base. The flowers are bright yellow consisting of 1 large triangular petal and are arranged in terminal spike inflorescences located at the branch tip (FAO, 2017). *C. retusa* has a cylindrical, hairless pod with a colour change from greenish to dark brown to black when mature (FAO, 2017).

2.3 Economic Significance of *Crotalaria* Germplasm

Various species in the genus *Crotalaria* can adapt to different climatic conditions. The *Crotalaria* species suitable for varied purposes are commonly found in tropical regions (Rockinger *et al.*, 2017). Members of the genus are characterized by early maturity, good nodulation, pest resistance and high seed setting which makes them suitable for different purposes. The plants are also used as fodder, however, use as fodder is limited due to toxicity by pyrrozidinial alkaloids produced at flowering and seed formation (Williams *et al.*, 2019). *Crotalaria* is prominently used in coffee plantations as a green manure and cover crop. *Crotalaria* has good produce in sloppy areas and hence it can be used to control soil erosion (Araujo *et al.,* 2013). *C. brevidens,* promotes suicidal germination of *Striga* seeds. In presence of *Crotalaria,* seeds of *Striga* germinate and die later due to absence of appropriate host plant (Sahou *et al.*, 2014). The roots of *Crotalaria* have nematocidal property hence they are important in temperate regions where they are used as plants antagonistic to parasitic nematodes while at the same time promoting free living nematodes in the soil (Soyewo and Omiyale, 2015). Currently *Crotalaria* is produced in Kenya as a vegetable for domestic and commercial purposes as the surplus is sold for additional income (Abukutsa-Onyango, 2007) .

2.4 Nutritional Importance of *C. brevidens* and *C. ochroleuca*

The value of indigenous leafy vegetables has been acknowledged in many developing countries due to their nutritional and medicinal benefits. Slender leaf is a food legume whose young shoots are cooked as vegetable, either boiled or fried in stews or in soups (Abukutsa-Onyango, 2007). The vegetable is rich in zinc, protein, Beta carotene, calcium, iron and ascorbic acid (Sahou *et al.*, 2014). In addition, slender leaf is also rich

in vitamins A and C (Buleti, 2017). The stage of maturity has no significant effect on protein and calcium content of slender leaf. Conversely, there is significant difference in the values of moisture, ash, iron and zinc in the slender leaf leaves at different stages of maturity (Sahou *et al.*, 2014).

2.5 Cultivation and Harvesting of *Crotalaria* Species.

Crotalaria brevidens and *Crotalaria ochroleuca* are vegetables of small-scale production. *Crotalaria* spp. grow naturally in damp grassland, mainly in depressions, floodplains, swamps, roadsides, bushes and open areas. The genus flourishes in the open with adequate sunshine and an altitude of 300 – 2000 m (Cotias, 2000). *Crotalaria* can thrive in different ecological zones with varied soil types. It is also hardy and drought resistant crop growing in poor soils which should be rather well drained (le Roux & van Wyk, 2013). It prefers warm conditions and after development of long taproots and strong lateral roots, it can tolerate relatively dry conditions (Nduhiu *et al*., 2015). The plant is cultivated in moderate and well distributed rainfall at least 50 -75 mm during vegetative flowering. *Crotalaria* seeds are sown by drilling or broadcasting. Drills are made in 30 cm spacing apart within rows, their germination takes 5 to 6 days (Nduhiu, 2017). Thinning is done after two weeks to maintain a spacing of 15-20 cm between the plants (Sikuku and Musyimi, 2007). The young shoots are ready for harvesting in eight weeks (Soyewo and Omiyale, 2015). Harvesting may continue for up to four months when the plant starts flowering and podding (Nduhiu, 2017). Slender leaf matures in about eight weeks and has the ability to produce seed under tropical conditions (Abukutsa-Onyango, 2010).

For market purposes, slender leaf is harvested by uprooting of the whole plant just before flowering when it is 8 weeks old and the stems are about 40 cm (Oluoch *et al*., 2009). The shoots and leaves are utilized for home consumption. Thinning, is carried out as the first harvest after 6 weeks (Oluoch *et al*., 2009). The first shoot maybe plucked at 8 weeks-stage which leads to development of lateral shoots which are subsequently harvested. The main shoot is cut at 15 cm above the ground leaving three leaves. The new shoots are harvested after two weeks. When the rainfall is adequate, harvesting may be done about fifteen times (Nduhiu, 2017).

2.6 Reproductive Biology and the Flower Structure of *Crotalaria* spp.

The reproductive biology and flower structure have been fairly studied in the genus *Crotalaria*. The genus has zygomorphic flowers. The *Crotalaria* flower consists of the standard, wing and keel petals. The androecial filaments are fused into a tube that is open on the upper side (Jacobi, 2005). Some species have special floral features thus increasing protection of the ovary and optimize pollen dispersal for a longer period of time (Le Roux and Van Wyk, 2012). *C. bolista* which has helically coiled keel is exceptional. The 700 *Crotalaria* species that are found in Africa and Madagascar are grouped into two. An unspecialized group having rostrate keel with untwisted tip and presence of standard is the first group. The specialized group has rostrate keel with callosities on standard petal blade and has trichome distributed along two sides on the style (Rockinger *et al.*, 2017). *Crotalaria* inflorescence has a terminal raceme of up to 50 cm long. The flowers are bisexual, hairy to glabrous with short and long anthers. The wing petals are long with 2.5 cm beak and ten stamens connected to an open sheath at the base with a curved style and small stigma (Tripathi *et al.*, 2013). *C. brevidens* is closely related to *C. ochroleuca* however, *C. brevidens* can be distinguished by the colour of its flower (bright yellow) while *C. ochroleuca* is pale yellow or creamish yellow (Nduhiu, 2017). Both *C. brevidens* and *C. ochroleuca* have two types of stamens, that is, long and short stamens, each comprising five anthers, summing to a total of 10 stamens (Wasonga *et al.*, 2020).

Facultative xenogamy is the type of pollination that occur in the genus *Crotalaria.* This is delayed self-pollination which occurs in absence of visitors (Etcheverry *et al.*, 2003). In *Crotalaria*, pollen is supplied by a pump mechanism which involves keel as the cylinder. The anthers act as the piston where the pollen is pumped out in a brush action when the flower is faltered hence enabling pollination process (Le Roux and Van Wyk 2012). Four types of pollen presentation have been identified in *Crotalaria* namely; valvular, explosive, brush and pump types. Brush type has been described based on the structure of the trichome. An erect trichome that is closely arranged on the distal end of the style at anthesis brushes the pollen from the keel beak after anthesis hence promoting delayed self-pollination and xenogamy (Jacobi, 2005). *C. brevidens* and *C. ochroleuca* are self-compatible however, some species of *Crotalaria* such as *C. juncea* can be cross pollinated by insects such as the *xylocopa* species (Etcheverry *et al*., 2003). In *C. juncea*, pollen is masked and is presented only when authentic pollinators such as

large bees, for example, the carpenter bee which can easily weaken the keel, uncover the stigma and collect excess pollen (Yaradua, 2018).

2.7 Pollination of *Crotalaria* Species

Crossing is an important part of plant breeding work (Buishand, 1956). A correct method of pollination results in a high percentage of successful crossings. Many species in the genus *Crotalaria* are widely spread and benefit from exotic or native pollination services even though the rates of spreading in the family varies significantly (Krueger *et al.*, 2008). Pollination of the self-pollinated *Crotalaria* species is ensured by dehiscence of the anthers before anthesis while cross-pollinated species of *Crotalaria* especially in *C. juncea* is ensured by having yellow, showy flower structure which promotes the possibility of entomophily that is, pollination aided by insects (Jacobi, 2005). The bright colour and corolla structures aid in cross-pollination by insects such as *Anthophila* and *Danaus plexippus* in *C. juncea* (Etcheverry *et al*., 2008). Fertilization takes place after the stigmatic surface has been injured by mechanical means or bees. Although, *Crotalaria retusa* has been identified to be self-pollinated, its annual life cycle exposes the species to the possibility of pollinators (Shi *et al.*, 2008). The increased self-pollination in *C. retusa* is due to lack of a membrane on the stigma. However, in some species of the genus *Crotalaria*, there are low rates of self-pollination because the stigma has a membrane, which has to be rubbed before self-pollination occurs (Jacobi, 2005).

2.8 Qualitative and Quantitative Inheritance

A structured study of the hybrids in several generations of selfing can provide genetic evidence on the type of gene action controlling the inheritance patterns of quantitative traits. Such information is necessary to ascertain the inheritance of superior characters for subsequent hybridization schemes. One way to attain this goal is by using a diallel mating design. This method allows performance of lines to be separated into mechanisms relating to general combining ability (GCA) and specific combining ability (SCA) (Fasahat, 2016). Gene action and combining ability of quantitative characters are of importance for studying the inheritance of characters for selection of proper parents for hybridization program and finding required segregants (Lachyan *et al.*, 2016). Scientific plant classification is based on morphological traits, some of which may serve as genetic markers suitable for plant germplasm management. Quantitative traits are controlled by many genes each with a small but additive effect while

qualitative traits are controlled by major genes each with major effect. Quantitative traits are highly influenced by environment while qualitative inheritance is not much affected by environment. Studies on inheritance of quantitative and qualitative traits can be accomplished due to the genotypic and environmental effects (Stuessy, 1990; Arshard *et al*., 2005; Janis *et al*., 2007; Kumar *et al.,* 2012; Gibbs, 2014).

Diallel is a mating system involving lines in all possible combinations to recognize parents as best or poor general combiners by GCA and the specific cross combinations by SCA (Kumar *et al.,* 2012). Complete diallel cross designs entail the occurrences of equal numbers of each of the different crosses among *p* inbred lines. Partial diallel mating design is a type of diallel design where a portion of required cross is made (Kand *et al.,* 1995). The most frequently used methods in the diallel analysis are Griffing's diallel procedures. Diallel methods used in plants include; Method 1 which involves parents, F_1 and reciprocals. Method 2 include parents and F_1's. Method 3 involves F_1's and reciprocals only while Method 4 involve F_1's only. The North Carolina designs is a factorial mating designs or schemes where certain groups of parents are designated male and others female for use in crosses. North Carolina designs are useful for studying combining ability in fixed model experiments and gene action when random models are applied. North Carolina design is sub-divided into three; North Carolina design I, one male is crossed with a different subset of female parents, thus females are nested within males. In the North Carolina design II, each member of a group of male is mated to each member of a group of females. North Carolina design III is to cross the i^{th} individual of an F_2 population to both parental lines (Franco *et al.*, 2001; Fasahat, 2016; Rukundo *et al.*, 2017).

Diallel mating design is used by plant breeders and geneticists to obtain genetic information such as estimation of general and specific combining ability variance for a population. Identification of GCA and SCA effects of traits is important for estimation of additive and non- additive gene effects. Estimation of general combining ability and specific combining ability provide information on how parental lines perform in hybrid combinations (Kumar *et al.*, 2012; Senbetay, 2015). The diallel crossing also provides the information on inheritance of gene action in generations to breeders for development of hybrids (Hayman, 1954; Jinks, 1954). The system provides genetic information about the traits of interest through random and fixed selection sets of

parental lines within a short time (Hayman, 1954; Griffing, 1956). The diallel mating system therefore is useful in the identification of desired lines to increase the frequency of targeted alleles in hybrids. The importance of additive and non-additive type of gene actions is also determined from analyzed diallel. Griffing's diallel approach (1956) is often used for estimation of combining ability of inbred lines making selection easy.

2.9 Role of Qualitative and Quantitative Characters in Genetic Improvement of Crops

The type of gene action and the importance of the environmental expression of the genes controlling a plant character determines whether it is qualitative or quantitative character. Qualitative trait inheritance shows discrete variation controlled by major genes that are not affected by the environment while quantitative trait inheritance exhibits continuous variation controlled by many genes that are influenced by the environment (Hughes *et al.*, 2020). In the study of how morphological characters are inherited, pure parental lines showing contrasting characteristics of traits of interest are developed then crosses are made to generate the first filial generation (F_1) and second filial generation (F_2). Backcrosses to each of the parents are also made (Lachyan *et al.*, 2016). The choice of appropriate qualitative traits such as leaf shape, pod colour and quantitative traits such as pod size, leaf length plus their utilization for hybridization is key to improving the productivity of any crop (Senbetay, 2015). Structured study of the hybrids in more than one generation of selfing can provide genetic information on the type of gene action controlling the inheritance patterns of characters (Thirupathi *et al.*, 2013). Studies of combining ability on qualitative traits such as leaf colour and quantitative traits such as leaf length and plant height can provide information for selection in crop improvement program (Kand *et al.*, 1995). The diallel mating system helps to identify the superior characters for hybridization and development of improved cultivars. This method allows the progeny performance to be separated into mechanisms relating to GCA and SCA. The GCA represents the average performance of a line in a hybrid combination. The SCA refers to the cases in which certain combinations do comparatively better or worse than would be expected on the basis of the average performance of the lines involved (Griffing, 1956). Combining ability of inbred lines provides information about the genetic nature of quantitative traits and can be used for selection of most appropriate parents in heterosis breeding (Fasahat, 2016). The effect is said to be additive when each additional gene enhances the expression of

the other by equal increments. Dominance gene action describes the relationship of alleles at the same locus. Epistatic gene actions involve interactions of alleles at different loci (Upadhyaya *et al.*, 2006). Variance due to general combining ability (GCA) indicates the extent of additive gene action while variance due to specific combing ability (SCA) provides the extent of non-additive gene action (Rukundo *et al.*, 2017).

2.10 Inheritance of Qualitative and Quantitative Characters of *Crotalaria* Species

Few genetic studies on *Crotalaria* are available due to the absence of morphologically distinct characters. The only distinct character observed for most *Crotalaria* species is the seed colour that is either black or yellow (Thimmaiah *et al.*, 2018). The inheritance patterns of the anthocyanin pigmentation in flowers and hypocotyl in *Crotalaria* species such as *C.juncea* is controlled by single gene with anthocyanin pigmentation being dominant and absence of pigmentation being recessive form (Bhandari *et al.*, 2016).

Additive gene action and non-additive gene actions are essential for genetic expression of related traits (Mashilo, *et al.*, 2016; Kumar *et al*., 2012). Selection of an appropriate breeding program for maximum genetic improvement is based on relative values of general and specific combining ability (Hayman, 1954; Griffing, 1956). Dominance and additive gene actions are important in the improvement of hybrids ((Kumar *et al.*, 2012; Fasahat, 2016; Rukundo *et al.*, 2017). Tripathi *et al.* (2005) reported high and positive correlation in the basal diameter in *Crotalaria* and prevalence of SCA was reported for quantitative traits in *C. juncea*. The general combining ability was significant only for plant height.

Crotalaria flower shape and colour is classified as qualitative trait. The *C. brevidens* and *C. ochroleuca* flowers are bisexual and have papilionaceous corolla, with four petals and are yellow in colour (Wasonga *et al.*, 2020). *Crotalaria capensis* have large flowers with bright yellow petals which are often edged with red. In *C. juncea* the seed size and weight vary with the genotype and the environmental conditions. Colour polymorphism of some *Crotalaria* species has been related to seed morphology (Carreras *et al*., 2001). *C. juncea* has two different seed types; highly pigmented seed coat and seeds lacking anthocyanin pigments on the coats (Kumar *et al*., 2012). Mwakha *et al.* (2020) described quantitative and qualitative traits of *C. brevidens* and *C. ochroleuca* including leaf type, seed colour and flower colour. The flower colour

varied from pale yellow to creamish yellow with the former being more dominant in *C. brevidens* while the latter was more dominant in *C. ochroleuca*. Quantitative traits include; leaf length, plant height, pod length and leaf width.

Leaf shape feature overlaps in the genus from lanceolate to oblanceolate and ovate (Soyewo and Omiyale, 2015). *Crotalaria colorata*, *C. giessii*, *C. meyeriana*, and *C. pearsonii* have obovate-elliptic leaflets (le Roux and van Wyk, 2013). The leaf shape comprises of lanceolate, ovate and linear types in slender leaf. *C. ochroleuca* have more leaf type variations compared to *C. brevidens* in which the lanceolate type dominates. *Crotalaria* species have trifoliate leaves. In *C. excise* and *C. humilis,* the length of the petiole was reported to be longer than terminal leaflet though shorter than terminal leaflet in all the other species (le Roux and van Wyk, 2013).

The seed colour is a variable qualitative trait within the species ranging from pale yellow to reddish to black and a mixture of either pale yellow and reddish colour or pale yellow, reddish and black colours (Mwakha *et al.*, 2020). A study of 38 *Crotalaria* species by Subramaniam and Pandey (2013) reported seed colour that ranged from black, brown, steel grey, pale yellow and orange red. These studies indicate intraspecific variability in seed colour among and within the species in genus *Crotalaria*. Significant variation has been reported in leaf area index (LAI) and pod width. Quantitative traits such as petiole length, pod length and width are phylogenetically significant in the morphological characterization of *Crotalaria* (Le Roux and Van Wyk, 2012).

2.11 Morphological Characterization of *Crotalaria* spp.

Morphological characters are beneficial in characterization of germplasm of any plant (Reddy *et al.*, 2016). Variation in morphological characters is important in the identification and classification of plant materials into different groups. The most useful traits for morphological characterization of indigenous vegetables are; leaf size and shape, number of seeds/ pods, plant height, the leaf number per plant, length of the leaf, number of primary and secondary branches per plant (Muasya *et al.*,, 2012; Adebo *et al.*, 2015). The revision of the classification of American species of *Crotalaria*, was based on the leaf length, width and shape (Yaradua, 2018). One group of the plants endemic to India *C. paraguayensis* had broad elliptic leaves and short inflorescence whereas a second set from Brazil *C. martiana* had narrow, elongate leaves with short

inflorescence (Daimon, 2006; Leverett *et al.*, 2012; Smýkal *et al.*, 2015; Loumeren, 2016).

High morphological diversity of jute mallow accession was determined by multivariate analysis of quantitative and qualitative traits (Ngomuo *et al.,* 2017). A study by Kumar *et al*. (2019) on brinjal (*Solanum melongen*a) landraces revealed a wide range of variability in quantitative and qualitative characters. Considerable variability is reported to exist in qualitative characters of Bambara groundnut including the growth habits which vary from bunch, semi bunch and spreading (Ntundu *et al.,* 2006). Nwangburuka *et al.* (2011) reported that plant height, days to flowering, branches per plant, pod width, pod length, seeds per pod and 100 seed weight had the highest contribution to the morphological diversity of cultivated okra. From a study of 49 different *Crotalaria* species, the principal component analysis showed that the more diverse factors were petiole length, width of leaflet and pod length (Yaradua *et al.*, 2019). Study of the flower structure of *Crotalaria* identified six flower types including; pump, gullet, hugging, saddle, tunnel and brush (Le Roux & Van Wyk, 2012).

2. 12 The Karyotype of *Crotalaria* Species

Chromosomes are carriers of genetic material and consist of DNA and proteins. Genetic information is stored in the DNA of the chromosome whose selective expression regulates the genetic diversity (Wang and Yu, 2016). Karyotyping is the study of an organism's chromosome to determine the chromosome structure or morphology (Flores *et al*., 2006). The structure, number and behavior of chromosomes correlates with hereditary and traits of variation (Shi *et al*., 2008). Chromosome preparation is a crucial cytogenetic procedure for analyses, like genomic *in situ* hybridization (GISH), FISH (Younis *et al.*, 2015) and karyotyping. Understanding the relationships of the genus *Crotalaria* can be applied in introduction of new variation into the species. Mitotic chromosomes are prepared from fast growing tissues because they contain actively dividing cells. The chromosome structure and number has been previously described in the genus *Crotalaria*. Flores *et al.* (2006) reported chromosome numbers for 15 Brazilian species of *Crotalaria. C. incana* had 2n = 2x = 14 while *C. calcynae* had 2n = 4x = 54 which is the highest number of chromosome reported in the genus. Most *Crotalaria* species have a diploid chromosome number of 2n = 2x = 16 for example; *C. juncea, C. pumilla, C. sagittalis* (Tapia-pastrana, 2005). The basic chromosome number

of the genus *Crotalaria* is n = x = 8 and n = x = 7, the former being the predominant one. From an evolutionary perspective, n = x = 8 is the primary base number from which n = x = 7 has evolved (Subramaniam & Pandey, 2014). Many cytogenetic analyses in the genus *Crotalaria* records the number of chromosome and morphology based on convectional analyses of chromosome (Flore *et al.*, 2006).

The karyotype of most *Crotalaria* species consists of metacentric and sub- metacentric chromosomes with a secondary constriction in the chromosome (Almada *et al.*, 2006). Characterization of four American *Crotalaria* species by Tapia-pastrana (2005) recorded metacentric chromosomes in the species indicating that *Crotalaria* species are highly symmetrical. This was in agreement with Cotias-de-Oliveira *et al.* (1999). However, the karyotype of Indian *Crotalaria* species recorded sub-metacentric and sub-telocentric chromosomes (Gupta, 1978; Raina *et al.*, 1979) showing these species to be asymmetrical. Tapia-pastrana (2005) reported that, secondary constrictions and primary constrictions can be easily identified by a little chromosome section corresponding to nucleolus of chromosomes. Knowledge on the number of chromosome and chromosomal banding are important for determining relationship in *Crotalaria* species (Shi *et al*., 2008).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Site

Field experiments were carried out at University of Embu Farm to study the inheritance patterns of *Crotalaria* species. The University of Embu lies at 0° 30' 76" S and 37° E 27' 30") at 1494 m above the sea level. The mean annual temperature in Embu is 19.5 °C with a maximum temperature of 25 °C and a minimum of 14.1°C. Embu receives an average rainfall of up to 1230 mm annually with long rains in March through June and short rains in October to December (Jaetzold *et al.*, 2007). The major soils are *Humic Nitisols* which are derived from basic volcanic rocks. *Nitisols* are deep weathered with friable clay texture and moderate to high inherent fertility (FAO, 2011).

3.2 Germplasm

This study used *Crotalaria* accessions that were previously collected from seven Kenyan Counties namely; Vihiga, Busia, Siaya, Homa Bay, Kisumu, Nandi and Nairobi. The accessions of *Crotalaria* species with different features of the leaf type, shape and pod size were used for studies of inheritance patterns of morphological characters and karyotypic architecture of *Crotalaria* species (Table 3.1). The experiments to study the inheritance of traits on *Crotalaria* species and development of artificial pollination protocol were set up at the University of Embu Farm and greenhouse. An experiment to determine the karyotype was also set up at the University Research Laboratory.

3.3 Development of an Artificial Pollination Protocol

The two distinct genotypes; *C. brevidens* and *C. ochroleuca* landraces from Homa-Bay and Kakamega counties were used. *C. ochroleuca* was used as the female parent while *C. brevidens* was used as the male parent. The two species were used due to their distinctive feature on leaf shape, pod shape and plant height. *C.brevidens* (FKK 0039) is early flowering plant with an intermediate height of 1.3 m to 1.5 m, pod diameter of 0.82 cm to 1.0 cm which is described as a slender or small pod and leaf length of 5.0 cm to 6.0 cm identified as slender leaf shape (Plate 3.1). *C. ochroleuca* (FHB 0211) is late flowering with an intermediate height of 1.37 m to 1.5 m, pod diameter of 1.0 cm to 1.8 cm referred to big pod and leaf length of 4.5 cm to 6.5 cm recognized as oblanceolate leaf shape.

Table 3.1: *Crotalaria* accessions used for inheritance and karyotype studies

S/N	Description Accession number	County	Height	Pod size	Leaflet
1.	FBS 0061	Busia	Dwarf	Big	Linear
2.	FKK 0099	Kakamega	Dwarf	Big	Linear
3.	FKS 0165	Kisumu	Dwarf	Big	Linear
4.	FVH 0119	Vihiga	Tall	Big	Elliptic
5.	FVH 0160	Vihiga	Tall	Big	Elliptic
6.	FKK 0129(a)	Kakamega	Tall	Big	Elliptic
7.	FBS 0064	Busia	Intermediate	Big	Oblanceolate
8.	FSY 0151	Siaya	Intermediate	Big	Oblanceolate
9.	FHB 0211	Homa-Bay	Intermediate	Big	Oblanceolate
10.	FKK 0039	Kakamega	Intermediate	Slender	Linear
11.	FKK 0097	Kakamega	Intermediate	Slender	Linear
12.	FND 0181	Nandi	Intermediate	Slender	Linear
13	FKK 0067	Kakamega	Intermediate	Big	Elliptical
14	FBS 0047	Busia	Intermediate	Big	Elliptical
15	FNB 0284	Nairobi	Tall	Big	Elliptical
16	FKS 0201	Kisumu	Dwarf	Slender	Linear
17	FVH 0121	Vihiga	Tall	Big	Elliptical
18	GBK 5639	GBK	Intermediate	Big	Oblanceolate
19	GBK 5279	GBK	Intermediate	Slender	Linear
20	GBK 5440	GBK	Dwarf	Slender	Linear

Key

Plant height:	Below 0.69m (short), 0.7m -1.39m (intermediate), 1.4m and above (tall)
Pod circumference:	2.5cm and below (slender), 2.6cm and above (big)
Pod diameter:	0.795cm and below (slender), 0.82cm and above (big)

3.3.1 Experimental Layout and Management

The experiment to develop an artificial pollination protocol, was laid in July 2019 in a completely randomized design (CRD) with six treatments replicated thrice. The experimental units comprised of 20 cm diameter plastic containers containing 3 kg medium made up of farm soil, farm yard manure and sand in the ratio of 3:2:1. Three *Crotalaria* seeds were planted in the medium and later thinned to one plant.

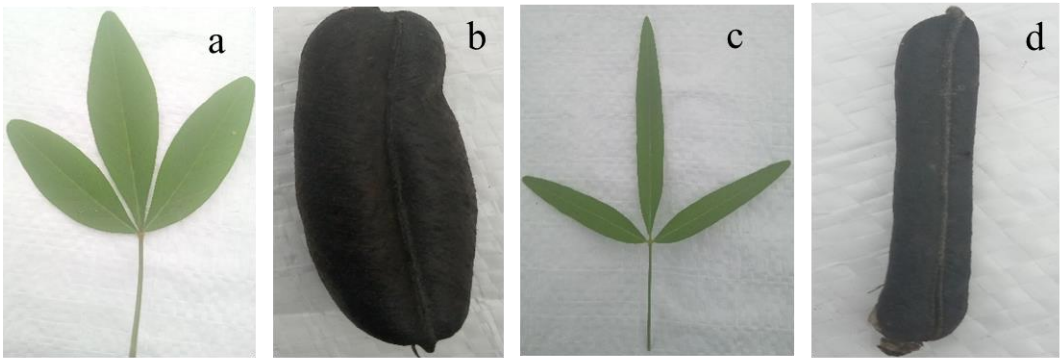

Plate 3.1: Leaf shape and pod type of *Crotalaria* genotypes used for pollination.
a and **b** oblanceolate leaf shape and big pod of *C. ochroleuca* (female parent). **c** and **d** slender leaf shape, slender pod of *C. brevidens* (male parent)

3.3.2 Emasculation

Six day old flower buds that were about to open were selected (Plate 3.2) and mechanically emasculated using a pair of forceps. The bud was gently grasped to prevent any kind of stress or injury. A small fine pointed pair of forceps was used to make a cut at the bottom of the bud to allow access to the anthers and the stigma. The flower was held by the thumb finger supported by the index and the middle finger. Using a sharp tip of a scalpel, an incision was made along the center of the keel. This exposed the stamens and the pistil of the flower granting easy access to the male and female parts of the flower. Each of the 10 anthers; 5 long and 5 short were carefully removed one by one without damaging the stigma. The pair of forceps was sterilized by dipping it in 70% ethanol between crosses to avoid cross contamination (Plate 3.3).

3.3.3 Pollination

Artificial pollination was carried out immediately after emasculation. Freshly opened anthers were obtained from desirable males and used immediately. To expose the anther sac, the inner petals of an open flower were slipped downwards using a pair of forceps to expose the mass of pollen grains. Thick pollen emerged after the wings were pressed downwards (Plate 3.4). The pollen grains were finely rubbed on the stigma of the female plant using forceps to ensure that the pollen sticks on the stigma. The stigma was returned to the keel and closed with the wing and the standard petals immediately after the pollination process to avoid desiccation. To study the effect of pollination time on pollen viability and hybridization success, pollination was done in the morning

(08:00 hours) and in the evening (17:00 hours) and the data for the two events were recorded separately.

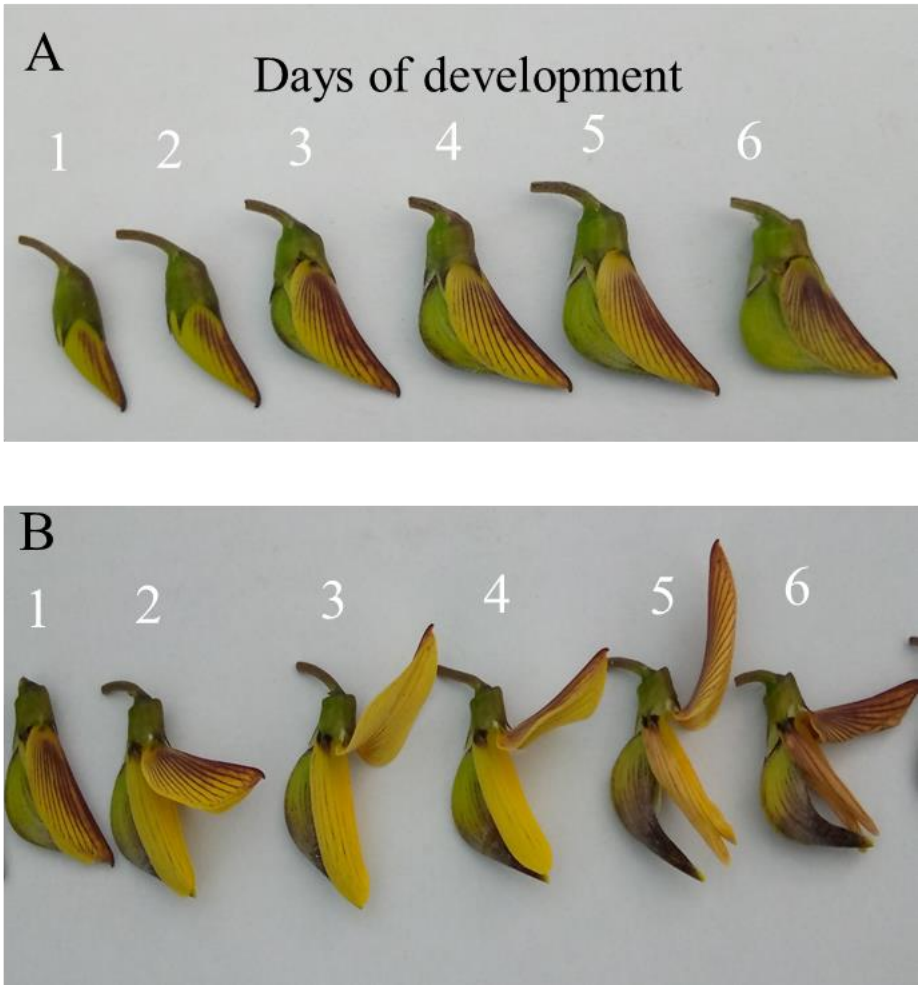

Plate 3.2: Developmental stages of *Crotalaria* flower.

A- Bud stages for emasculation. **B-** Flower bud stages for pollen collection. **A 1-2**, closed bud stage where the flower stigma is immature and the anthers are at the base. **3-4**, the stigma is immature with partially closed petals. **5**, stigma is receptive. **6**, best bud for emasculation. **B 1-2**, pollen shedding but immature. **3**, fully open flower for pollination. **4-6**, self-pollination has taken place.

Plate 3.3: Emasculation process of *Crotalaria* by keel petal incision method.
a, flower bud chosen for emasculation. **b**, removal of the standard and the wing petal. **c**, incision of keel at the bottom to expose the inner part of the flower.

Plate 3.4: Pollination of emasculated *Crotalaria* bud by rubbing method.
a, chosen *Crotalaria* flower for pollen source. **b,** pollen collected from the male parent. **c**, rubbing of pollen on the female stigma. **d**, pollinated stigma returned inside the keel to form a natural case

3.3.4 Tagging and Protection of Flower Buds

The emasculated and artificially pollinated flowers were tagged and labelled for identification. The tags were attached to the flower pedicel. The name and identification code of the female parent and male parent, date of emasculation and pollination time and initials of the pollinator were recorded on the tag using a pencil. The pollinated flowers were then covered using glassine bags and pinned using staples (Plate 3.5). This was to discourage other pollinators from visiting the flower bud. The greenhouse was sprayed weekly using insecticides to discourage insects such as ants which are mostly

attracted to nectaries and flying insects from visiting the plant and accessing the flower bud.

After the artificial hybridization process, non-emasculated buds (self-pollinated) were covered with a glassine bag to serve as positive controls while the negative controls comprised emasculated buds without artificial pollination. Both positive and negative controls were carried out at the same time as the artificial pollination process. After emasculation and pollination procedures were carried out, the flower buds were keenly monitored and observed for changes that occurred from the time of pollination to pod formation and maturity. Pod development was closely monitored for 30 days. Photographs were taken on day 0, 3, 6, 9, 12, 15, 21, 27 and 30. The hybrid seeds were then planted for confirmation of hybridity and the plants were once again monitored till maturity. This was important in order to determine the pod type produced from a cross between big podded *Crotalaria* as the female (*C. ochroleuca*) and the slender podded as the male parent (*C. brevidens*).

Plate 3.5: Tagging and protection of pollinated flower bud.
a, placing a tag on the pollinated bud indicating the female and male parent, time, date and pollinator initials. **b**, protecting the pollinated bud from other pollinator by covering with a glassine bag. **c**, protected bud.

Data on the number of mature pods and number of seeds per pod were documented. Artificial hybridization success rate was determined as per the following formula.

$$\text{Success rate} = \frac{\text{total number of successful mature pod}}{\text{total number of cross done}} \times 100$$

3.3.5 Data Analysis

Data on number of pods and number of seeds were subjected to ANOVA using a Statistical Analysis Software (SAS) version 9.4 (SAS Institute, 2013). The significant means were compared using Tukey's test procedure at 5 % probability level.

3.4 Inheritance Studies of *Crotalaria* Species

3.4.1 Plant Material and Experimental Design

Eleven genetically diverse parental genotypes of *Crotalaria* species were used for studies on inheritance of quantitative and qualitative traits. The eleven genotypes namely; FBS 0061, FKK 0099, FKS 0165, FVH 0119, FND 0181, FKK 0129a, FBS 0064, FSY 0151, FHB 0211, FKK 0039, FKK 0097 were crossed in diallel mating design. The botanical description of the parents is provided in Table 3.1. From the diallel crossing, 110 F_1 populations were obtained from a full diallel mating design for quantitative traits studies. Hybrid seeds were planted along with 11 parents to raise F_1 population. Observation on inheritance of qualitative and quantitative traits were made on the different generations. The experiment was laid out in a triple lattice design with the 110 F_1 combinations, together with the 11 parents, allocated in an experimental unit of 3 m row of 10 plants and replicated three times. Sowing of the *Crotalaria* seeds was done at a spacing of 30 cm apart within rows and 50 cm between rows then covered thinly. Other practices such as weeding and pest control were carried out to maintain a weed free field.

3.4.2 Data Collection

Both qualitative and quantitative data were collected and recorded using descriptors adopted from (Schippers, 2002) (Table 3.2). Qualitative traits such as flower colour, stem colour and hairiness of the stem were recorded at 50% flowering stage. Dry pod colour was recorded at maturity. Quantitative traits collected included; plant height, number of branches, days to flowering, leaf length, pod circumference and days to pod maturity.

Table 3.2: Descriptors of *Crotalaria* species (Schippers, 2002)

S/No.	Descriptor Name	Descriptor State
1.	Plant height	Plant height measured at ground surface at 50% flowering (cm)
2.	Leaf length	Leaf blade length excluding petiole length (cm)
3.	Leaf type	1. Lanceolate; 2. Ovate; 3. Other
4.	Petiole length	Length of leaf stalk (cm)
5.	Days to 50% flowering (50 FLR)	Number of days from sowing to 50% flowering
6.	Number of primary branches (PB)	Number of branches from main stem
7.	Number of Secondary Branches (SB)	Number of branches from the secondary stem
8.	Plant Canopy	Plant width taken at widest point (cm)
9.	Flower colour	1. Yellow; 2. Other
10.	Pod Length	Length of mature fruit excluding the pedicel
11.	Days to First Mature Pods (DMP)	Number of days from sowing to mature of first pods
12.	Number of Leaves	Counted from individual plant during flowering
13.	Biomass Yield (BY)	Plant total weight on the ground surface (gm)
14.	Number of Pods/plant (NPP)	Counted from individual plant at maturity stage
15.	Weight of 1000 Seeds (W1000S)	Measured in weighing balance after counting
16.	Stem colour	1. Green; 2. Other
17.	Hairiness of stem	1. Absent; 2. Present
18.	Leaf colour	1. Dark green; 2. Light green
19.	Dry pod colour	1. Black; 2. Brown; 3. Other
20.	Seed colour	1. Yellow; 2. Brown; 3. Red
21.	Number of seeds per pod	Counted from individual pods

3.4.3 Data Analysis

Quantitative data was then subjected to ANOVA to check for the genotypic differences. For estimation of GCA and SCA effects, Griffing's (1956) approach, Model 1 (fixed effects), was used. In addition, Hayman's (1954) approach was used to partition the components of variation into additive (a), dominance (b), maternal (c) and non-maternal reciprocal effects. Griffing's and Hayman's analyses were conducted using

DiallelAnalysisR Package in R version 3.6.1 (R, 2018). The statistical model for Griffing's (1956) Method 1 Model 1, is as follows:

Combining abilities analyses:

$\boldsymbol{Y_{ij}=m+g_i+g_j+s_{ij}+r_{ij}+1/bc\Sigma\Sigma eijkl}$ (Griffing, 1956).

where:

$\boldsymbol{Y_{ij}}$ = mean of the ijth genotype over k and l.

$\boldsymbol{m}$ = grand mean.

$\boldsymbol{g_i}$ = general combining ability effect on the ith parent.

$\boldsymbol{r_{ij}}$ = reciprocal effect.

$\boldsymbol{s_{ij}}$ = interaction, i.e., specific combining ability effect.

$1/bc\Sigma\Sigma eijkl$ = mean error effect.

The model used for Hayman's (1954) approach is as follows:-

$\boldsymbol{Y_{rs}= m+J_r+l+l_r+l_s+K_r-K_s+K_{rs}}$ **(**Singh and Chaudhary, 1985**)**

where:

$\boldsymbol{Y_{rs}}$ = entry in *rth* row and *sth* row.

$\boldsymbol{m}$= grand Mean.

$\boldsymbol{J_r}$ = the mean deviation of *rth* parents from the grand mean.

$\boldsymbol{J_{rs}}$= remaining discrepancy due to *rth* reciprocal sum.

$\boldsymbol{l}$ = mean dominance deviation.

$\boldsymbol{l_s}$ = dominance deviation (additional) due to *rth* parent.

$\boldsymbol{l_{rs}}$ = remaining discrepancy in *rsth* reciprocal sum.

$\boldsymbol{2k_r}$ = differences when *rth* line is used as male and female.

$\boldsymbol{2K_{rs}}$ = discrepancy in *rsth* reciprocal differences.

3.6 Karyotyping of *Crotalaria* Species

A total of 20 seeds of 20 different *Crotalaria* accessions obtained from different counties in Kenya were grown in Petri dishes with cotton wool soaked in water. The *Crotalaria* accessions were; FBS 0061, FKK 0099, FKS 0165, FVH 0119, FVH 0160, FKK 0129a, FBS 0064, FSY 0151, FHB 0211, FKK 0039, FKK 0097, FND 0181, FKK 0067, FBS 0047, FNB 0284, FKS 0201, GBK 5639, GBK 5279 and GBK 5440. A different set of the 20 *Crotalaria* accessions were grown in test tubes soaked with a plant nutrient solution known as Hoagland's solution. Mitotic cells were prepared as

previously described by Kihlman (1975) with some modifications such as squashing of stained root tips using a thumb. The best preparations with distinct mitotic stages were photographed (Plate 3.6).

3.6.1 Preparation of Hoagland's Solution

Hoagland's solution was prepared as follows; 0.75g of 2 M NH_4H2PO_4 was dissolved in 10.1g of 2M KNO_3 nutrient solution. Nutrient solutions with 11.8g of 2M $Ca(NO3)_2$ and 24.65g of 2M $MgSO_4$ were added. To the mixture, 0.143g of H_3BO_3, 0.0905g of $MnCl_2$.$4H_2O$, 0.011g of $ZnSO_4$.$7H_2O$, 0.004g of $CuSO_4$. $5H_2O$, 0.0045g of H_2MoO_4. H_2O and 6.8g of 1M KH_2PO_4 micronutrients were added and the solution was mixed well. The solution was used to water the plants after three days.

3.6.2 Chromosome Slide Preparation

The squash technique is a common method for chromosome preparation mainly for fast chromosome count done directly on softened root tips. This method involved placement of a cover slip over a dissected root tip and spread of the chromosome. Squash method of chromosome preparation was recommended because it is cheap and easily done however, requires technical skills. Preparation of Aceto orcein stain was done as follows; a mixture of 45ml of glacial acetic acid and 1g of Orcein powder were boiled. The mixture was then cooled at room temperature. A 100ml measuring cylinder was filled with 55ml of distilled water. The acid was then added to water to avoid splashing and mixed well. The solution was stored in a refrigerator for one week before use (Dyer *et al.*, 1963).

Seeds collected from different counties in Kenya were grown in Petri dishes with a wet cotton wool lined in it and test tubes under natural light and room temperature. Root tips were obtained from germinated *Crotalaria* seeds after four days. Fresh, plump, about 2 mm long root tip was cut and placed in a fixative solution (70% ethanol) at room temperature (20^oC - 25^oC). One root tip was then placed on a watch glass with 4 – 5 drops of 1M HCl and heated at 60^o C for 2 minutes to soften the cell wall and weaken cellular connection for easy squashing. Three (3) to four (4) drops of Aceto-orcein stain were added and heated for 5 - 6 minutes to allow penetration of the stain into the tissues. The stained, dark-purple tips (2 mm to 4 mm) was then cut off on a slide and a drop of Aceto Orcein stain was added then covered with a cover slip.

Plate 3.6: Chromosome preparation from root tips of *Crotalaria*.
a, growth of the seeds in a test tube; **b**, growth of the seeds in a petri dish; **c**, four days old seeds after planting; **d**, heating root tip with 1M HCl; **e**, addition of Aceto-Orcein stain; **f**, prepared slide for observation under a microscope.

The root tip was then squashed using a thumb. A paper towel was used to remove excess stain after squashing (Plate 3.6). The slide was then observed under a microscope. Chromosome preparation were carried out at different times of the day (8 am, 9 am, 10 am and 11 am) in order to identify the optimum time for cell division especially metaphase stage which enables easy counting of the number of chromosomes of the species involved.

3.6.3 Data Collection and Presentation

Slides of intact cells with well spread chromosomes that were not overlapping and with same contraction were photographed from each collection using a Moti-connect camera (Camera Moticam X 2.0 resolution) which was connected to a compound microscope by moticam Wi-Fi resolution of 1280 X 1024 1.3MP (Speed Fair Co., Ltd, China). To determine the chromosome number, different mitotic stages of cell division were observed. Data on the number of chromosomes at different mitotic stages were recorded for different *Crotalaria* species.

CHAPTER FOUR

RESULTS

4.1 Floral Architecture of *C. brevidens* and *C. ochroleuca*

Dissection of the flower revealed that *C. brevidens* and *C. ochroleuca* flowers are bisexual and have papilionaceous corolla, which contains four petals. The petals include one standard petal, two lateral wing petals and one keel petal that covers both female and male floral organs. The stigma is globose and surrounded by anthers. *C. brevidens* and *C. ochroleuca* have two stamens that is, long and short stamens each comprising five anthers summing to a total of ten stamens (Plate 4.1).

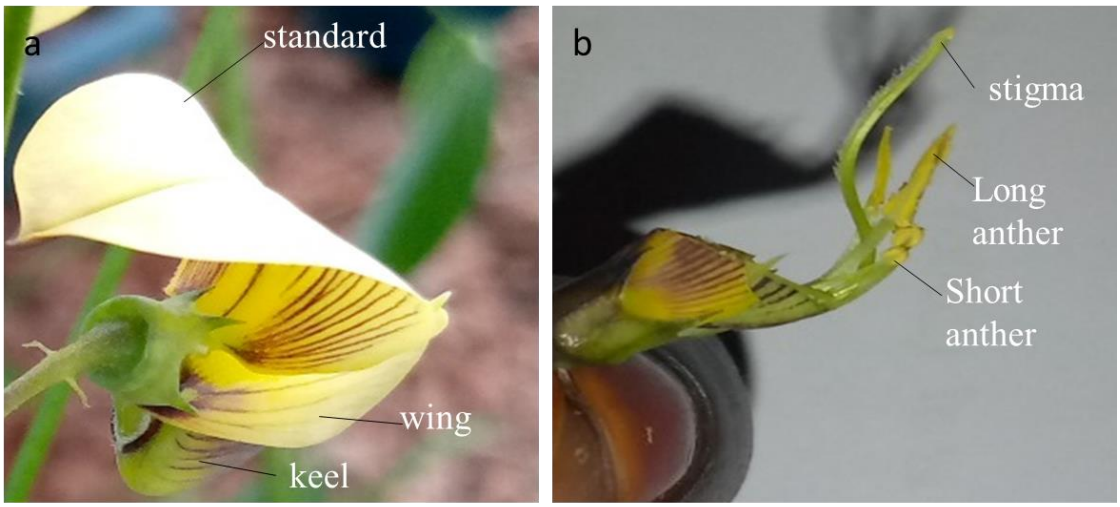

Plate 4.1: Flower structure of *Crotalaria*.

A, outer part of *Crotalaria* flower. **B**, inner part of *Crotalaria* flower bud.

4.2 Developmental Stages of *C. brevidens* and *C. ochroleuca* Flowers and Optimum Flower Bud Stage for Crossing

The *C. brevidens* and *C. ochroleuca* flowers undergoes different developmental stages from day 1 to day 6 after bud formation. On the first two days of bud formation, the stigma was immature and the anthers were still at the base of the bud with closed flower petals. On day three and four, the stigma was immature with partially closed petals. The stigma was receptive on day five and six with moderately closed flower petals. On the 6^{th} day the bud measured 6.0 mm and was appropriate for hand pollination before pollen shedding. A fully fresh open flower bud was best for pollen production due to high viability and was observed on the day three. It was observed that pollen was shed the

first and second day although it was not mature enough for pollination. Self -pollination took place between day four and day five while formation of the first pod began on day six. After successful fertilization, ovaries enlarged within two to three days and the pods were formed (Plate 4.2). If feritilization did not occur, the flower abscised after three days. Abortion of pods was also observed in late stages of pod development (Plate 4.3). Mostly, pod abortion were observed after nine to fifteen days. An intermediate pod was obtained from a cross between the two *Crotalaria* species thus confirming hybridity (Plate 4.4).

Plate 4.2: Pod development from pollinated flower to pod maturity.

a, day zero. **b**, day 3. **c**, day 6. **d**, day 9. **e**, day 12. **f**, day 15. **g**, day 21. **h**, day 27. **i**, day 30.

Plate 4.3: Flower and pod abortion in *Crotalaria*.

a, aborted flower bud after three days. **b**, aborted pod after 9 days. **c**, pod abortion after 30 days. **d**, dry aborted pod with no seed.

Plate 4.4: Pods of parents and intermediate pod of hybrid.

a female parent (FHB 0211), **b** hybrid, **c** male parent (FKK 0039**)**

4.3 Cross-Pollination Success

The hybridization success between the two species was 58.33%, which was relatively higher than the negative control (0.00) but significantly lower ($P < 0.05$) than the positive control, which had a success rate of 81.82%. The successfully hybridized plants produced a mean of 6.56 pods, which was significantly lower than the positive control, whose mean number of pod was 9.00 but significantly higher than the negative control, which did not produce any mature pods. Based on the number of seeds produced for

the positive control, 220.33 seeds were significantly higher than the hybrids which produced a mean of 64.86 seeds lower than the negative control (Table 4.1). The combined effect of time and species hybridization showed a mean success rate of 61% for the morning crosses. A success rate of 55% was recorded for the evening crosses. The positive control had a success rate of 82% in both morning and evening crosses. Higher numbers of mature pods and seeds were also reported on the positive control. The mean number of pods produced by the hybrids at 8 am and 5 pm was 5.352 and 5.028, respectively, whereas the positive controls produced a mean of 9 pods (Table 4.2).

Table 4.1: Means of crossing success rate, seed and pod production of interspecies cross between *C. ochroleuca* and *C. brevidens*

Combinations	**Success rate (%)**	**Number of pods produced**	**Number of seeds produced**
FHB 0211 × FKK 0039	58.33[b]	6.56[b]	64.86[b]
Emasculation without pollination	0.00[c]	0.00[c]	0.00[c]
No artificial pollination	81.82[a]	9.00[a]	220.33[a]
Mean	46.72	5.19	95.06

Means sharing the same letter in one column are not significantly different at $P < 0.05$ according to Tukey's test.

Table 4.2: Means ± standard error representing the effects of time of pollination on hybridization success between *C. ochroleuca* and *C. brevidens*

Time	**Success (%)**	**No. of pods**	**No. of seeds**
8am	0.48 ± 0.13	0.13 ± 1.42	104.19 ± 37.19
5pm	0.46 ± 0.12	5.03 ± 1.34	85.94 ± 30.48

Means ± *SE*

The mean number of seeds from the hybrids in the morning was 69.25 and 60.47 in the evening, compared to 243.33 and 197.33 at 8 am and 5 pm, respectively, in the self-pollinated controls (Table 4.3).

Table 4.3: Effects of interaction between combinations and time of interspecies cross between *C. ochroleuca* and *C. brevidens*

Combinations	Time	Success rate (%)	No. of pods	No. of seeds
Fhb 0211 × Fkk 0039	8am	0.61 ± 0.07	7.06 ± 0.75	69.25 ± 0.55
Fhb 0211 × Fkk 0039	5pm	0.55 ± 0.04	6.08 ± 0.43	60.47 ± 3.39
Emasculation without pollination	8am	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Emasculation without pollination	5pm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
No artificial pollination	8am	0.82 ± 0.10	9.00 ± 1.15	243.33 ± 29.63
No artificial pollination	5pm	0.82 ± 0.05	9.00 ± 0.58	197.33 30.31

Means ± *SE.*

4.4 Inheritance of Qualitative and Quantitative traits

4.4.1 Qualitative and Quantitative Trait Variations

Flower colour is an important identifier of *Crotalaria* species. Yellow flower colour was observed in all the crosses. Therefore, this trait did not have any variation among the parents or the hybrids. The *Crotalaria* parents studied lacked hair on the stem and this was also the case in the hybrid plants. Stem colour was observed for both the hybrids and the parents. Green stem colour was observed for all the parents and F_1 plants. Pod colour was assessed for all the plants and F_1s at maturity that is after 120 days. Black dry pod colour was recorded for all the species studied hence, there was no variation for pod colour among the species. Significant variations ($P < 0.05$) between the genotypes were observed for plant height, pod circumference and leaf length. The genotypes were not significantly different ($P < 0.005$) for number of branches, number of days to pod maturity and days to flowering. The estimates of genotypic means between the selected quantitative traits are given in appendix 2.

4.4.2 Pearson Correlations between Quantitative Variables

Weak and moderately significant correlations were observed among the traits. The number of branches was not correlated to days to flowering ($r = 0.05$, $P = 0.05$). Leaf length was weakly negatively correlated to days to flowering ($r = -0.19$, $P = 0.001$). Days to mature pod was not correlated to number of branches ($r = -0.05$, $P = 0.38$) and weakly negatively correlated to leaf length ($r = -0.241$, $P = 0.001$). Pod circumference was negatively correlated to pod maturity ($r = -0.19$, $P = 0.03$). Plant height was

moderately correlated to leaf length (r = 0.28, P = 0.001) and days to mature pod (P = 0.10, r = 0.04) as shown in Table 4.4.

Table 4.4: Pearson's correlation of selected quantitative traits of *Crotalaria*

Variables	Days to flowering	No. of branches	Leaf length	Days to mature pod	Pod circum.
Days to flowering					
No. of branches	**0.10***				
Leaf length	**-0.19***	-0.02			
Days to mature pod	0.004	-0.05	**-0.241***		
Pod circumference	0.013	-0.09	-0.072	**-0.19***	
Plant height	0.08	0.02	**0.28***	**0.10***	0.09

*Significant at (P < 0.05)

4.4.3 Genetic Control of Quantitative Traits in *Crotalaria*

Additive gene effects were significant for leaf length (P < 0.05) and plant height traits (P = 0.03) while dominance effects were highly significant for all the traits observed (plant height, leaf length, pod circumference, number of branches and pod maturity). Maternal effects were significant for number of branches and leaf length while non-maternal reciprocal effects were significant for plant height, number of branches and leaf length (Table 4.5).

Table 4.5: Mean squares of Hayman's analysis for quantitative traits of *Crotalaria* from 11× 11 diallel cross

Genetic parameter	d.f	Number of branches	Days to flowering	Pod maturity	Pod circ.	Leaf length	Plant height
additive	10	6.32	31.40	40.00	1.21	1.42***	527.10*
Non additive	55	146.76***	11502.60***	16490.90***	65.21***	87.28***	13424.40***
b1	1	5.89	11.90	1.80	0.04	7.87***	100.60
b2	10	4.84	28.70	17.30	1.14	1.22**	366.10
b3	44	182.21***	14371.50***	20609.60***	81.26***	108.64***	16695.00***
maternal	10	11.42**	27.50	102.90	0.77	3.49***	354.60
reciprocal	45	7.09*	44.70	77.70	1.26	2.80***	657.90***

***,*, ** significant at (P < 0.001), (P < 0.01) and (P < 0.05) respectively

b1, b2, b3; additive×additive, additive×dominance, dominance×dominance

Table 4.6: GCA, SCA and reciprocal means squares of quantitative traits of *Crotalaria* from 11 × 11 diallel cross

Source of variation	d.f	Number of branches	Days to flowering	Pod maturity	Pod circ.	Plant height	Leaf length
GCA	10	2.11	10.46	13.32	0.43	175.90*	0.47***
SCA	55	1.47	11.65	13.30	0.33	135.82*	0.79***
reciprocals	55	1.97	10.39	20.56	0.29	150.68*	0.73***

*, *** Significant at P < 0.05, 0.001 respectively

4.4.4 Combining Ability of Quantitative Traits

Analysis of variance for combining ability showed significant ($P < 0.05$) GCA, SCA and reciprocal effects for plant height ($P < 0.05$) and leaf length. Different sets of parents combined differently for the traits under study. For plant height and leaf length, two genotypes were confirmed to be best combiners with significant GCA effect. These were; FBS 0061(*C. ochroleuca*) and FND 0181(*C. brevidens*). The estimates for GCA, SCA and reciprocal effects are shown in Table 4.7, 4.8 and 4.9, respectively.

Table 4.7: Estimates of GCA effects for quantitative characters of *Crotalaria* from 11×11 diallel cross

Combination	D. pod maturity	leaf length	plant height	N. of branches	D. to flowering	pod circ.
FBS 0061	0.37	-0.28*	-4.08	0.14	0.65	0.03
FKK 0099	-0.37	-0.23	1.36	0.59	0.34	0.16
FKS 0165	4.81	0.04	-0.76	0.14	-0.81	-0.38
FVH 0119	-2.06	0.12	-2.08	2.77	0.47	-0.24
FND 0181	-1.66	0.20	4.45*	-1.77	0.52	0.12
FKK0129a	0.24	0.00	0.35	-0.47	-1.55	-0.01
FBS 0064	1.21	-0.04	-2.39	-0.31	0.22	0.18
FSY 0151	1.79	-0.05	2.80	0.08	-0.29	0.27
FHB 0211	-1.44	0.02	2.34	-0.35	0.69	0.08
FKK 0039	0.20	0.14	1.79	0.07	0.01	0.01
FKK 0097	-3.09	0.08	-3.78	0.00	-0.25	-0.21

*significant $p < 0.05$

Table 4.8: Estimates of SCA and reciprocal effects for plant height, pod maturity and number of branches for *Crotalaria* spp.

		Days to mature pod		L. length		Plant height	
S/N	Combination	Direct	Rec.	Direct	Rec.	Direct	Rec.
1.	FBS 0061 × FKK 0099	-4.41	6.33	-0.49	-0.32*	-4.26	3.56
2.	FBS 0061×FKS 0165	5.24	-12.83	-0.67*	0.51*	5.26	-4.51
3.	FBS 0061 × FVH 0119	2.77	-10.00	0.29	-0.67*	-6.69	-3.32
4.	FBS 0061 × FND 0181	-1.96	-1.83	-0.67*	-0.32*	-3.55	-11.07*
5.	FBS 0061 × FKK 0129a	-1.03	-2.5	0.16	0.35*	-0.59	7.60*
6.	FBS 0061 × FBS 0064	-2.34	3.33	-0.19	-1.53*	-3.48	-6.07
7.	FBS 0061 × FSY 0151	-0.41	5.83	-0.52	0.17	3.51	-1.08
8.	FBS 0061 × FHB 0211	0.15	7.50	-0.02	0.05	10.29	13.03*
9.	FBS 0061 × FKK 0039	-2.33	13.83	-0.64*	-0.74*	-5.71	-6.43
10.	FBS 0061 × FKK 0097	2.81	11.00	2.03*	0.65*	5.24	0.92
11.	FKK 0099 × FKS 0165	-0.19	-2.22	0.07	-0.97*	-6.21	-16.97*
12.	FKK 0099 × FVH 0119	0.85	-5.00	-0.52	-0.19	-6.77	-15.36*
13.	FKK 0099 × FND 0181	-1.22	0.50	0.04	-0.12	19.74*	-6.92
14.	FKK 0099 × FKK 0129a	-2.29	-4.62	0.16	-0.82*	6.34	-11.95*
15.	FKK 0099 × FBS 0064	5.91	-0.88	0.38	0.18	7.74	10.06*
16.	FKK 0099 × FSY 0151	-6.17	-10.00	-0.29	0.93*	-12.94*	-3.87
17.	FKK 0099 × FHB 0211	-3.94	-6.17	0.31	0.83*	4.33	-9.88*
18.	FKK 0099 × FKK 0039	2.58	2.50	0.88*	0.52*	3.47	-6.72
19.	FKK 0099 × FKK 0097	3.88	3.33	-1.06*	-0.63*	-3.14	-1.23
20.	FKS 0165 × FVH 0119	-3.50	-5.67	0.57*	0.26	2.15	-8.93*
21.	FKS 0165 × FND 0181	3.26	-1.67	0.91*	1.42*	16.32*	3.82
22.	FKS 0165 × FKK 0129a	-6.97	-0.17	-0.01	-0.04	-6.40	2.68
23.	FKS 0165 × FBS 0064	-4.28	-1.00	-0.22	0.56*	-6.21	-6.68
24.	FKS 0165 × FSY 0151	-2.52	-0.17	-0.23	0.06	1.14	5.28
25.	FKS 0165 × FHB 0211	0.54	-5.5	-0.59	-0.18	-14.43*	-11.79*
26.	FKS 0165 × FKK 0039	6.24	-3.33	-0.44	0.04	0.30	9.93*
27.	FKS 0165 × FKK 0097	-2.46	-11.5	0.87*	1.02*	4.65	-8.65*
28.	FVH 0119 × FND 0181	5.63	-14.17	-1.38*	-0.48*	-9.55	-15.64*
29.	FVH 0119 × FKK 0129a	-4.32	-1.50	-0.48	0.06	9.34	-7.23*
30.	FVH 0119 × FBS 0064	0.09	5.00	-0.39	0.46*	-3.76	20.95*
31.	FVH 0119 × FSY 0151	1.02	2.00	0.68*	0.07	6.89	0.07
32.	FVH 0119 × FHB 0211	-1.42	-4.17	-0.04	0.28*	5.79	-15.58*
33.	FVH 0119 × FKK 0039	0.27	-0.17	1.20*	-0.68*	-3.46	10.08*
34.	FVH 0119 × FKK 0097	-3.09	3.67	0.27	0.49*	-6.09	16.23*
35.	FND 0181 × FKK 0129a	0.54	1.19	-0.43	-0.47*	-10.04	-11.89*
36.	FND 0181 × FBS 0064	-1.31	-0.83	0.32	-0.23	7.45	-11.65*
37.	FND 0181 × FSY 0151	-4.22	-1.00	0.78*	0.20	-2.78	3.86
38.	FND 0181 × FHB 0211	0.51	1.50	0.11	0.02	-4.30	-6.07
39.	FND 0181 × FKK 0039	-1.14	17.00	-0.09	-0.85*	-7.06	8.08*
40.	FND 0181 × FKK 0097	0.67	4.17	-0.25	-0.58*	-0.23	8.22*
41.	FKK 0129a × FBS 0064	-0.54	-6.18	0.03	0.22	-3.48	-5.84
42.	FKK 0129a × FSY 0151	8.71	4.22	-0.17	-0.49*	-9.39	7.13*
43.	FKK 0129a × FHB 0211	2.45	0.83	0.07	-0.22	-1.22	-1.26
44.	FKK 0129a × FKK 0039	-0.31	1.67	0.09	-0.45*	1.40	6.33
45.	FKK 0129a × FKK 0097	1.65	4.82	-0.71*	-0.17	-1.22	1.68
46.	FBS 0064 × FSY 0151	7.07	7.5	0.17	-1.69*	13.42*	-4.53
47.	FBS 0064 × FHB 0211	-1.72	-1.83	-0.27	-0.09	8.02	4.97
48.	FBS 0064 × FKK 0039	-3.01	2.67	0.25	0.74*	-7.13	9.31*
49.	FBS 0064 × FKK 0097	-2.37	2.50	0.19	-0.49*	-3.77	-0.39
50.	FSY 0151 × FHB 0211	-0.28	-5.00	-0.02	-0.28*	11.25	1.14
51.	FSY 0151 × FKK 0039	2.25	1.21	-0.76*	0.68*	0.07	-1.97
52.	FSY 0151 × FKK 0097	-1.11	3.5	-0.48	-0.46*	-5.24	3.47
53.	FHB 0211 × FKK 0039	0.29	6.67	-0.58*	0.77*	-15.79*	-0.21
54.	FHB 0211 × FKK 0097	2.28	4.83	-0.19	0.47*	-1.99	-0.07
55.	FKK 0039 × FKK 0097	1.31	4.17	-0.61*	0.09	13.63*	11.11*

*significant $P < 0.05$, L. length – leaf length

Table 4.9: Estimates of SCA and reciprocal effects for pod circumference, days to flowering and leaf length of *Crotalaria*

S/N	Combination	No. of branches		Days to flowering		Pod circumference	
		Direct	Rec.	Direct	Rec.	Direct	Rec.
1.	FBS 0061 × FKK 0099	1.64	0.23	-2.49	0.67	0.47	-0.04
2.	FBS 0061 × FKS 0165	-0.41	-0.27	-0.84	-3.50	-1.14	1.38
3.	FBS 0061 × FVH 0119	-4.41	-0.35	-2.61	-1.17	0.68	1.62
4.	FBS 0061 × FND 0181	3.03	-3.17	1.34	-0.67	0.18	0.11
5.	FBS 0061 × FKK 0129a	-0.63	0.23	0.07	0.17	0.19	0.22
6.	FBS 0061 × FBS 0064	-1.12	-0.63	2.64	-1.67	0.12	-0.08
7.	FBS 0061 × FSY 0151	0.83	-1.52	2.15	-2.00	-0.10	-0.07
8.	FBS 0061 × FHB 0211	-1.01	0.47	1.83	-0.33	0.29	-0.01
9.	FBS 0061 × FKK 0039	-0.53	1.37	-1.89	2.50	0.43	-0.12
10.	FBS 0061 × FKK 0097	-0.22	-2.50	0.27	-1.67	-1.11	-0.98
11.	FKK 0099 × FKS 0165	-1.22	-1.70	1.81	4.67	-1.02	-0.24
12.	FKK 0099 × FVH 0119	5.66	1.23	1.53	-0.83	0.50	-0.09
13.	FKK 0099 × FND 0181	-1.48	0.00	6.32	0.83	0.11	0.15
14.	FKK 0099 × FKK 0129a	0.24	-1.62	-1.79	3.82	0.16	1.35
15.	FKK 0099 × FBS 0064	-0.45	1.25	-2.05	-1.11	-0.15	0.30
16.	FKK 0099 × FSY 0151	1.21	1.57	-4.04	-2.00	-0.24	-0.26
17.	FKK 0099 × FHB 0211	-0.77	-0.17	2.47	-0.67	-0.40	-0.22
18.	FKK 0099 × FKK 0039	-0.29	1.67	-0.84	-2.33	0.05	-0.20
19.	FKK 0099 × FKK 0097	1.08	1.27	0.58	3.17	0.3	0.03
20.	FKS 0165 × FVH 0119	6.88	8.88	-0.48	2.33	0.85	-0.59
21.	FKS 0165 × FND 0181	-4.57	0.23	0.63	0.17	0.85	-0.12
22.	FKS 0165 × FKK 0129a	0.39	-0.50	-3.97	3.00	0.51	-0.12
23.	FKS 0165 × FBS 0064	0.04	-1.26	-2.57	3.17	0.64	-0.28
24.	FKS 0165 × FSY 0151	1.26	1.00	0.45	-1.50	0.62	0.02
25.	FKS 0165 × FHB 0211	0.68	-8.88	-1.71	-4.00	0.19	1.10
26.	FKS 0165 × FKK 0039	-1.01	-3.33	1.15	2.50	0.31	0.06
27.	FKS 0165 × FKK 0097	0.50	1.33	-0.26	-5.33	0.25	-0.03
28.	FVH 0119 × FND 0181	4.93	0.57	4.86	0.67	1.22	-0.39
29.	FVH 0119 × FKK 0129a	-0.50	-0.30	0.27	0.67	-1.24	0.29
30.	FVH 0119 × FBS 0064	1.44	0.60	-3.01	-1.33	0.32	0.25
31.	FVH 0119 × FSY 0151	0.32	1.20	1.34	-2.00	0.11	0.19
32.	FVH 0119 × FHB 0211	-1.02	1.43	1.18	4.50	-1.33	-1.33
33.	FVH 0119 × FKK 0039	-0.47	8.33	-1.29	-0.67	-0.12	-0.32
34.	FVH 0119 × FKK 0097	-0.13	1.37	-1.70	1.50	-0.81	0.27
35.	FND 0181 × FKK 0129a	-0.46	-0.53	-0.01	-0.77	0.16	0.28
36.	FND 0181 × FBS 0064	0.76	-0.26	-0.56	-1.28	-0.43	-0.01
37.	FND 0181 × FSY 0151	-1.16	-0.06	-4.88	-1.75	-0.13	-0.30
38.	FND 0181 × FHB 0211	-0.03	1.23	-4.37	-1.67	0.54	-0.16
39.	FND 0181 × FKK 0039	1.07	0.27	-2.38	0.83	-1.03	0.47
40.	FND 0181 × FKK 0097	-1.08	1.67	-1.09	1.67	-0.61	-0.48
41.	FKK 0129a × FBS 0064	0.29	4.22	-0.99	1.96	0.14	-0.94
42.	FKK 0129a × FSY 0151	-1.33	0.25	3.35	1.11	-0.27	-0.89
43.	FKK 0129a × FHB 0211	1.13	-0.77	-1.46	5.33	0.20	0.33
44.	FKK 0129a × FKK 0039	-0.14	-0.5	-1.18	1.50	-0.75	0.25
45.	FKK 0129a × FKK 0097	0.00	-1.17	2.09	-0.22	0.54	-0.18
46.	FBS 0064 × FSY 0151	0.81	-0.55	3.26	2.83	-0.31	-1.33
47.	FBS 0064 × FHB 0211	-0.74	0.07	0.37	-1.50	0.23	0.08
48.	FBS 0064 × FKK 0039	-0.19	1.97	1.29	0.83	0.22	0.52
49.	FBS 0064 × FKK 0097	-0.63	-7.33	1.54	-0.67	0.37	-1.68
50.	FSY 0151 × FHB 0211	0.14	1.33	1.73	3.33	-0.08	-0.97
51.	FSY 0151 × FKK 0039	-0.57	-0.68	-0.69	-0.21	0.02	-0.3
52.	FSY 0151 × FKK 0097	-1.16	-0.52	-2.44	0.67	0.42	0.14
53.	FHB 0211 × FKK 0039	-0.42	-0.1	1.26	0.83	0.07	0.12
54.	FHB 0211 × FKK 0097	0.13	1.37	2.24	1.50	0.12	-0.06
55.	FKK 0039 × FKK 0097	0.84	-0.03	0.42	4.00	0.00	0.12

*Significant at $P < 0.05$.

4.5 Karyotype of *Crotalaria* Species

4.5.1 Chromosome Number of *Crotalaria* Species

This study was able to determine chromosome numbers of *C. ochroleuca*, *C. brevidens*, *C. trichotoma*, *C. spectabilis* and *C. intermedia*. The squash method of chromosomal preparation using Aceto orcein stain worked well. The method was used in the laboratory due to its simplicity and speed especially for fresh material. Obtaining roots from seeds grown in tubes filled with Hoagland's solution was problematic due to algae formation after three days and mitotic phases were not clearly observed under a microscope. Root tips collected from seeds grown in Petri dishes were found appropriate as chromosomes were visible and could be counted. Different mitotic phases were observed under a compound microscope such as; interphase, prophase, metaphase, anaphase and telophase (Plate 4.5). The chromosomes were easily observed at different mitotic stages and the metaphase stage was easily seen. Although chromosome counting was possible, satellites due to constriction could not be detected in the observed *Crotalaria* species. Details like positioning of the centromere and exact length of the chromatids were not documented due to the limited resolution (Camera Moticam X 2.0 MP) of the microscope. A diploid chromosome number of $2n = 2x = 16$ was observed in five *Crotalaria* species (Plate 4.6).

4.5.2 Optimum Time for Cytogenetic Analysis of *Crotalaria*

Chromosome preparation done at different times showed that different stages were observed at different times from 8.00 am to 11.00 am. Interphase was mostly recorded at 8.00 am and 11.00 am. Interphase was also observed at 10.00 am. Chromatin began to condense into chromosomes as the nuclear envelope broke down (prophase) at 8.00 am. The chromosome alignment at the equatorial plate or at the center (metaphase) was mostly observed for slides fixed at 9.00 am. The sister chromatids separation from each other was observed at 9.30 am to 9:45 am (anaphase). Chromosomes with nuclear envelope were observed in slides fixed at 10 am in sets at separate poles (telophase).

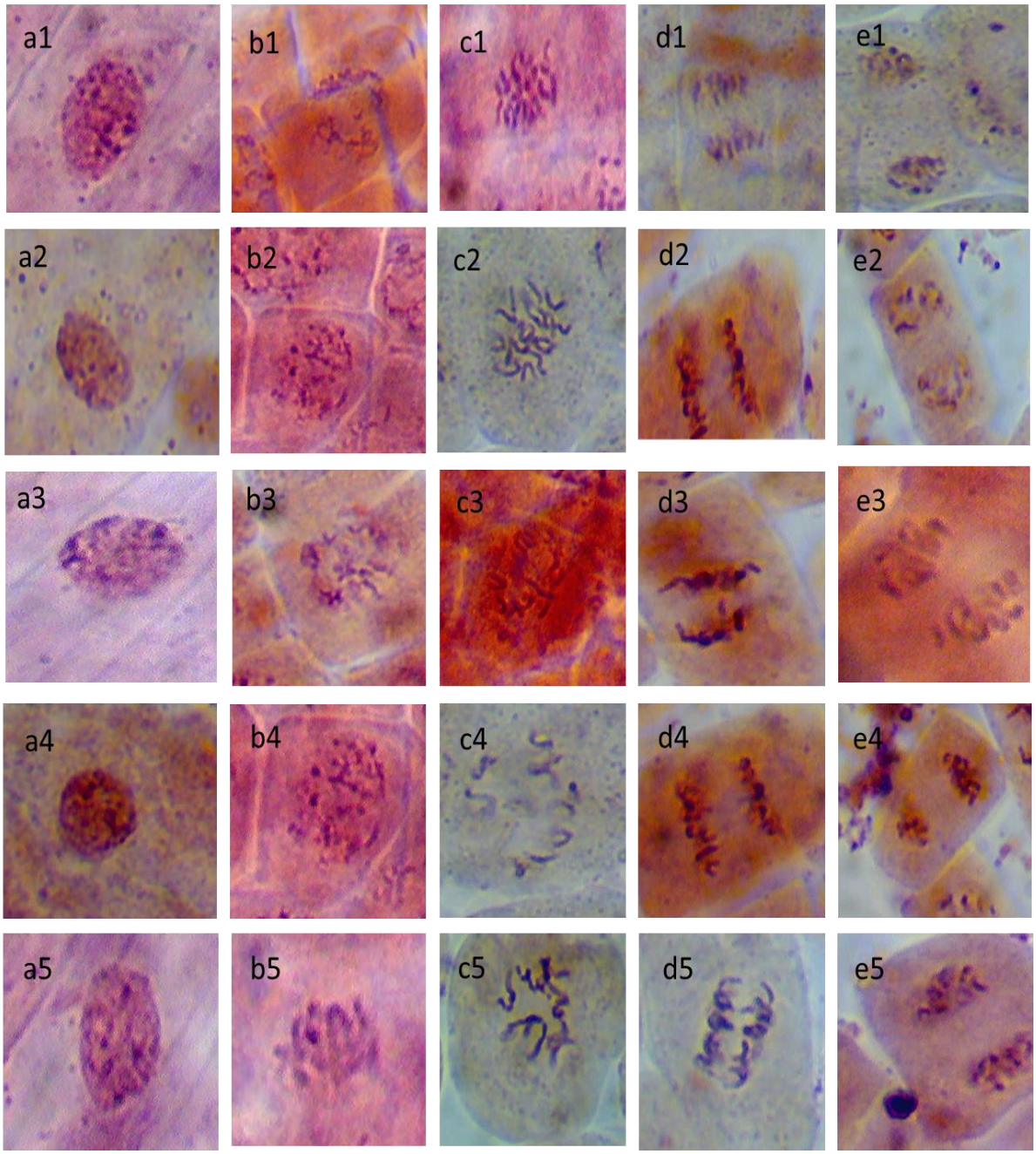

Plate 4.5: Chromosomes of *Crotalaria* species at different mitotic stages.

1- *C. intermedia*, **2-** *C. spectabilis*, **3-** *C. trichotoma* **4-** *C. ochroleuca*, **5-** *C. brevidens*

a interphase, **b** prophase, **c** metaphase, **d** anaphase, **e** telophase

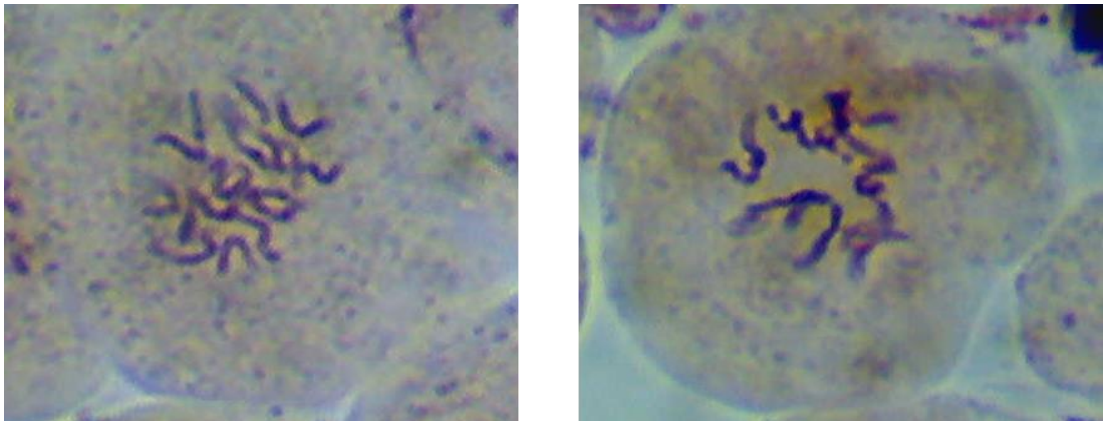

Plate 4.6: Chromosome numbers of *Crotalaria* species

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Artificial Pollination Protocol of *Crotalaria* Species

This study, successfully crossed two *Crotalaria* species; *C. ochroleuca* and *C. brevidens* with distinct differences in leaf shape and pod size. Pollination success has not been previously reported on crossing *C. ochroleuca* and *C. brevidens.* However, artificial pollination has been previously reported in other leguminous crops such as bean, chickpea and Bambara. Emasculation method by petal cutting and pollination was successfully carried out from 3 pm to 10 pm in *Vigna subterranea* (Suwanprasert *et al.*, 2006). Hybridization was attained when pollination was conducted within one hour of initiation of pollen shedding (Suwanprasert *et al.*, 2006). Drayner (1959) reported that flower manipulation and cross pollination in *Vicia faba* was effective in stimulating seed setting. Crossing by keel petal incision has been previously reported in *Cicer arietinum* with a high crossing success rate (Kalve and Tadege, 2017).

The *Crotalaria* corolla is pale to bright yellow with purple stripes. Flower colour may change from yellow to other shades of orange or brown after pollination (Arroyo, 1981). The anthers of *Crotalaria* species are often dimorphic with filaments that are fused into a tube (Le Roux and Van Wyk, 2012). The present study reports that *C. brevidens* and *C. ochroleuca* flowers are bisexual with papilionaceous corolla, which has four petals including; one standard petal, two lateral wing petals and one keel petal, covering both female and male floral organs. The stigma is globose and surrounded by anthers. Both *C. brevidens* and *C. ochroleuca* have two types of stamens. Cross pollination of *C. ochroleuca* was favoured by its flower structure.

This study explored two methods of artificial pollination; rubbing method of crossing and hooking method of crossing as no artificial pollination method for *Crotalaria* had been previously reported. The rubbing method was found to be appropriate for artificial pollination of *Crotalaria* species. In the current study, the hook method was not appropriate because the *Crotalaria* stigma is straight and the bud is curved which allows storage of pollen thus causing self-pollination. The stigma has a membrane that allows pollen to stick easily (Le Roux and Van Wyk, 2012). The rubbing method was therefore found to be the most appropriate method for artificial pollination of *Crotalaria* species. In other legumes like common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer*

arietinum) (Anbessa *et al*., 2005) two methods; hooking and rubbing methods are commonly used for artificial hybridization (Genchev, 2007).

The six-day old flower bud was observed to be the best flower developmental stage for emasculation and pollination. Pollination was not carried out until the 6th day, a day before pollen shedding. Choosing the correct bud for pollination determined whether pollination took place or not. Pollination was attempted on day four, five and six. Most of the flower buds pollinated on day four and five aborted. Therefore, the developmental stage of the flower bud influenced the success rate of artificial pollination in *Crotalaria* species. It is also important to choose appropriate flower for pollen production. The ideal flower for pollen supply was a fully fresh open flower petal obtained after three days. A fresh open flower was found to have sufficient pollen viable enough for pollination. The pollen was yellow in colour and evenly mature for pollination. The importance of the developmental stage of the flower bud has been previously discussed by Veerappan *et al.*, (2014) who described the developmental stages of chickpea flower and identified the bud stages for artificial pollination.

In this study, the appropriate position for handling the flower bud depended on the correct handling of forceps. Correct positioning prevented the flower from falling and minimized injury of the stigma during emasculation. Additionally, holding forceps in the correct way reduced the risk of injury to the pistil. Observations from the present study indicate that for artificial pollination of *Crotalaria* species, the forceps should be held obliquely pointing upwards to ensure that the pollinator gets the correct position of holding the flower bud. This observation is in agreement with previous studies which documented the importance and procedures of holding forceps correctly during artificial pollination (Free, 1966; Raju *et al.*, 2017).

Tagging and bagging were important precautions in the artificial pollination of *C. ochroleuca* and *C. brevidens*. This was done to avoid any confusion of the artificially pollinated flowers from the non-pollinated flowers. This technique has been previously used and documented (Massawe *et al.*, 2003). The artificially pollinated flower in this study was covered with a glassine bag to protect it from insects such as ants to reduce chances of contamination by other pollination agents. Other workers such as Etcheverry, (2001) recommended the protection of the pollinated flower bud within the

greenhouse and the field from insects which are common pollinators of *Crotalaria* species.

Emasculation and pollination process was carried out in the morning at 8:00am and in the evening at 5.00pm. This was done in order to determine an appropriate time for cross pollination of *Crotalaria* species because it has not been previously reported. However, time of cross pollination has been reported in other leguminous crops such as chickpea to be either in the morning 8.00 am or evening at 5.00 pm (Lachyan *et al.*, 2016). In the present study, the time of artificial pollination did not influence the success rate of pollination in *Crotalaria* species. Therefore, this study affirms that cross pollination of *C. ochroleuca* and *C. brevidens* can be carried out either in the morning 8.00 am to 10.00 am or in the evening 5.00 pm to 7.00 pm without affecting success rate of pollination.

The lower seed production by artificially pollinated flowers of *C. ochroleuca* was probably due to inadequate pollen. Deposition of inadequate pollen on the stigma can cause low seed production. Low production of seed by amount of pollen supplied takes place when too little pollen grains are deposited on the stigma for fertilization. Limitation by pollen quality occur when low quality pollen is deposited on stigma and subsequent reduction in production of pollen (Vaughton and Ramsey *et al.*, 2010).

The total number of pods ranged from six to nine in artificial pollination compared to seven to eleven pods per cross in the free pollination treatment. The high percentage (80%) of the seeded pods attained from the free pollination experiment showed that *Crotalaria* is self-compatible. Pod abortion in *C. brevidens* and *C. ochroleuca* mostly occurred after 9 to 15 days. Pod abortion has been previously reported in *C. juncea*. Thus, this observation is similar to that of Krueger *et al*. (2008) who reported pod abortion in a study of natural and artificial pollination of sunn hemp by insects. Pollination failure can occur at different stages especially pre-dispersal, dispersal and post-dispersal (Wilcock *et al.*, 2002).

5.2 Inheritance of Quantitative and Qualitative traits

This study examined the inheritance of quantitative and qualitative characters of *Crotalaria brevidens* and *C. ochroleuca*. Four of the studied qualitative traits, were similar in the parents and hybrids. These are; dry pod colour, colour of the stem,

presence of hair on the stem and flower colour. Similarity in the qualitative traits could have occurred because the species are closely related to each other. These qualitative traits are controlled by major genes hence the discontinuous variation of the traits observed in the hybrid generations. This is in agreement with previous studies which reported similarity of qualitative characters among *C. brevidens and C. ochroleuca* (Schippers, 2002; Raj and Britto, 2011).

According to le Roux *et al.* (2013) growth habit, leaf type and quantitative characters such as petiole length, pod width, and length are phylogenetically essential in the morphological characterization of *Crotalaria* species. Significant variations between the accessions was observed for plant height, pod circumference and leaf length. Significant variations among genotypes for plant height had been previously reported in *Phaseolus vulgaris* (Arunga *et al.*, 2010). Mwakha *et al.* (2020) reported significant variations for plant height, days to pod maturity and leaf length in *Crotalaria* species. Other quantitative traits studied such as number of branches, days to pod maturity and days to flowering were not significantly different between the species.

Positive correlation was observed among quantitative variables. This indicated that some variables can be indirectly targeted for the improvement of other related variables specifically when the target provides better grounds for selection. Negative correlation was also observed among the traits indicating that these traits can be independently improved. From this study, a weak correlation in plant height and leaf length, days to mature pod was reported suggesting presence of pleotropic relationship for genes in control of height and pod, leaf parameters (Baranda *et al.*, 2017). Leaf length is an important trait to the consumers or farmers because the leaves are the ones that are edible. Therefore, this trait could be embrased for improvement of the crop. Significant correlation has been reported between plant height and number of branches in other leafy vegetables such as *Amaranthus* L. Species (Gerrano *et al.*, 2015). Baranda *et al*, (2017) also observed positive correlation of plant height and leaf length in *Vigna unguiculata*. Negative correlation in days to flowering and leaf length, days to mature pod and leaf length indicated that these traits cannot be utilised in simultaneous selection for the improvement of *Crotalaria* species.

The present study recorded significant non-additive gene effects for plant height, pod circumference, days to mature pod, days to flowering, leaf length and number of

branches. Additive gene action was significant for plant height and leaf length only. Information on gene action and trait expression are essential in plant breeding (Rukundo *et al.,* 2017). Kumar *et al.* (2012) reported a significant additive and non-additive differences among quantitative traits such as plant height in *C. juncea*. From this study, additive genes can be considered for the development of breeding materials using recurrent selection for improving the crop. Further, interaction (b_3) was observed in all the traits studied and b_1, b_2 for plant height and leaf length. This could be due to epistasis among the genes of *Crotalaria* species. Epistatic effect has been observed for plant height in *C. juncea* (Kumar *et al.,* 2012) and other leguminous crops such as *Cicer arietinum* (Anbessa, 2006; Upadhyaya *et al.*, 2006).

The present study examined combining abilities and reciprocal effects of quantitative traits. The GCA, reflects the average performance of a genotype in hybrids. Additive variance was significant for plant height and leaf length. Since the leafy parts of slender leaf are eaten as vegetables, plant height and the leaf length are of interest for *C. brevidens* and *C. ochroleuca* breeders. Data from the present study indicates that GCA can be used in the recurrent selection of parents for improving the plant height and leaf length of the crop. The selection of parents on the basis of combining ability, and considering the genetic control of major traits determine the efficiency of a breeding programme (Sleper and Poehlman, 2006; Rukundo *et al.,* 2017).

The specific combining ability provides important information about the performance of hybrids relative to the parents. Non-additive gene effects are important for selection of materials to be used in hybrid production in crop improvement programs (Fasahat *et al.,* 2016). According to Franco *et al.* (2001) estimated values of SCA provide vital information about the hybrid performance compared to its parents and the importance of non-additive interactions due to a larger or minor gene effect. Rawlings and Thompson (1962) stated that SCA effect is due to genes with dominance and epistatic effect. Specific combining ability, is identified when specific hybrid combinations perform better or worse than what would be expected based on the average performance of the parental lines. Although GCA and SCA gene effects were significant for plant height and leaf length, the SCA was predominant. Kumar *et al.* (2012) documented the effects of non-additive gene action on plant height in *C. juncea.*

The consumption of *Crotalaria* species is valuable due to its nutritive and medicinal value. As a result, alleles that increase the leaf length and days to flowering would be needed for the genetic improvement of the crop. Crosses with negative SCA effects for flowering are desirable as they contain the gene that enhances late flowering which is advantageous to the farmer. The significance of SCA and GCA therefore suggests that, for improvement of *Crotalaria* hybrids with respect to plant height and leaf length and lines with highest SCA value can be selected. However, for identification of average performance of lines based on plant height and leaf length, parental lines with highest GCA values would be ideal.

The maternal effects were also significant for number of branches and leaf length. This was confirmed by the significant effects of reciprocal crosses and their SCA effects. Maternal effects commonly occur in sexually reproducing crops, and are distinguished by computing the differences of individuals from direct and reciprocal crosses (Grami and Stefansson, 1977). According to Weiner, *et al.* (1997*)*, reciprocal pairs have same nuclear genetic contributions however, differences in reciprocal pairs performance can be observed in presence of maternal effect. Maternal effects may differ among closely related species. The existence of maternal effects is important for *Crotalaria* since it directs breeders on particular crosses to be improved for a particular trait. Large genetic maternal effects for leaf area has been highly reported in family pioaceae (Roach and Wulff, 1987). Maternal effect has been previously reported in *Phaseolus vulgaris* on plant height (Arunga *et al.*, 2010)

5.3. Karyotype of *Crotalaria* Species

Various chromosome preparation methods have been reported (Wang and Yu, 2016). The squash and splash methods are the commonly used. In this study, a best procedure/method for chromosome preparation from the root tips of *Crotalaria* was determined as the squash method of chromosome spread. The method involves application of various chemicals and physical agents for pre-treatment and fixation of the dividing cells (Wang and Yu, 2016). Squashing technique permitted a good spread of the chromosomes during the squashing process. Other karyotype studies on *Crotalaria* have used the splash method of chromosome preparation for karyotyping (Mondin *et al.*, 2007; Tapia-pastrana, 2005). Chromosome preparation is important in cytogenetic technique for subsequent analyses, for example karyotyping. Good

chromosome preparation produces sufficient, well-spread, and flattened chromosomes with no chromosome damage (Wang and Yu, 2016).

Root tips were obtained from seeds grown in petri dishes. The root tips were preferred because they were easy to handle, the mitotic cells were large and the chromosomes were clearly observed unlike root tips grown in test tube filled with nutrient solution. In this study, 2 mm to 3 mm of root tip was cut and used in the cytogenetic studies. The 2 mm to 3 mm root tip was preferred because cell division occurs at the lower part of the root tip. In addition, the length was used because older tissue has a smaller proportion of mitotic cells. Fast growing plant tissues such as root tips, flower buds, and callus are good sources of materials for metaphase chromosome preparations (Cotias, 2000). The root tip is the apical growing part of a root having the root cap, region of cell division, region of elongation and region of maturation (Wang and Yu, 2016). The growing point is inside the distal 1 mm to 2 mm region, and is the region of mitosis (She *et al.*, 2017). Tapia-pastrana (2019) obtained least meristems from 2 mm to 6 mm root tip for chromosome study of four American *Crotalaria* species.

The major reason for somatic chromosome preparation is to determine the chromosome numbers and study chromosome morphology. Aceto orcein stain was preferred because of its bright visibility on the chromosome enabling easy and clear observation of the chromosomes. Aceto orcein schedule is simple and the most widely used method for somatic chromosome preparation (Almada, 2006). The use of Aceto orcein stain has been reported in the family Cruciferae (Soliman, 2002). This study reports the use of aceto orcein stain for cytogenetic studies in *Crotalaria* species for the first time. Other studies have used the Feulgen stain for chromosome staining of chromosome in *Crotalaria* instead of aceto orcein stain (Hook *et al.*, 1991; Cotias, 2000; Mondin *et al.*, 2007; She *et al.*, 2017; Tapia-pastrana, 2005).

Chromosome preparation was done at different times and the cells were observed under the microscope at different times from 8.00 am to 11.00 am. The time the root tips were fixed was essential because it influenced the proportion of mitotic cells. Therefore, this study identified morning hours (8.00 am, 9.00 am, 10.00 am and 11 am) to be best for preparing *C. ochroleuca* and *C. brevidens* chromosomes and aceto orcein stain to be best for staining them. The optimum time for carrying out chromosome preparation in order to obtain the metaphase stage for easy count of chromosome has not been

previously reported for studies of *Crotalaria* species. This study therefore, reports the best time for preparing *Crotalaria* root tip for cytogenetic studies.

More studies have been carried out on the genus *Crotalaria*, but numbers of chromosome are known for only a few species of the genus. This study observed 2n = 2x = 16 chromosome number among five different *Crotalaria* species; *C. ochroleuca*, *C. brevidens*, *C. intermedia*, *C.trichotoma* and *C. spectabilis*. The genus has a dibasic number of $x = 7$ and $x = 8$. $x = 8$ is recurrent in the genus (Chennaveeraiah and Patil, 1973; Gupta, 1978; Cotias, 2000; Mondin *et al.*, 2007; Tapia-pastrana, 2005). The genus has few tetraploids with more diploids distributed mainly in American species (Mondin *et al.*, 2007). This study did not record tetraploids from the studied *Crotalaria* species. The somatic number 2n = 2x = 16 with one pair of sub-metacentric chromosomes has been previously reported in some *Crotalaria* species such as *Crotalaria mollicula* (Mondin *et al.*, 2007).

In the current study, the diploid chromosome number *C. spectabilis* was 2n = 2x = 16 which is in agreement with previous studies (Palomino *et al.*, 1991; Almada *et al.*, 2006). *C. intermedia* from different counties in Kenya was also observed to have a chromosome number of 2n = 2x = 16. The chromosome number of 2n = 2x = 16 in *C. intermedia* has been previously reported (Atchison, 1950; Raina *et al.*, 1979; Chautá-mellizo *et al.*, 2012). The chromosome number of *C. ochroleuca* in the present study was observed to be 2n = 2x = 16 which is in agreement with Morales and Mondin, (2012) who documented 12 m+ 4 am in *C. ochroleuca*. A diploid chromosome number of 2n = 16 chromosome number was observed for *C. brevidens*. A previous study on genus *Crotalaria* reported the 2n = 2x = 16 diploid number in *C. brevidens* (Gupta, 1978). Further, the diploid chromosome number for *C. trichotoma* was determined to be 2n = 2x = 16 thus a basic chromosome number of n = 8. The chromosome number in *C. trichotoma* has not been previously reported. Therefore, this study affirms the 2n = 2x = 16 chromosome number of *C. trichotoma*. The basic chromosome number of n = 8 is in agreement with many studies carried out in the past on karyotype of different *Crotalaria* species (Chennaveeraiah and Patil, 1973; Raina *et al.*, 1979; Palomino *et al.*, 1991; Cotias, 2000; Almada *et al.*, 2006; Mondin *et al.*, 2007; She *et al.*, 2017; Tapia-pastrana, 2005). The karyological characterization in the current study provides

information relevant to the phylogenic relationships and evolutionary tendencies of the *Crotalaria* species in Kenya.

5.4 Conclusion

This study successfully developed an easy and efficient artificial interspecies-pollination protocol for *C. ochroleuca* and *C. brevidens*. The artificial pollination protocol was successful in an inter-species cross but can also be used in intraspecies crosses. The study determined that the rubbing method of pollination was ideal for use in *Crotalaria* species because of the position and structure of the stigma. A six-day-old flower bud was best for emasculation and pollination whereas a fresh fully open flower was best for pollen supply. The study demonstrated that artificial pollination can be carried in the morning or evening without affecting pollination success. The development of this artificial pollination protocol is an important step in hybridization and genetic improvement of the *Crotalaria* species. The study determined the importance of GCA in the parental selection for crop improvement based on plant height and leaf length. In addition, SCA was also ideal with significance in all the traits showing their importance in hybrid production. Significant maternal effect in the number of branches and leaf length could help *Crotalaria* species breeders identify crosses for improvement of the trait. Inheritance studies of *Crotalaria* species in Kenya is important in identification of selection patterns and breeding procedures for the crop improvement. This study identified a diploid chromosome number of 2n = 2x = 16 for five *Crotalaria* species in Kenya. The squash method of chromosome preparation was determined to be the best method for cytogenetic studies of *Crotalaria* species. The study identified optimum time for carrying out the cytogenetic studies of *Crotalaria* species to be from 8.00 am to 10.00 am. This study also determined the chromosome number for the first time for *C. trichotoma* to be 2n = 2x = 16.

5.5 Recommendations

The artificial pollination protocol described in this study can be used as a practical guide for artificial pollination of *Crotalaria* both in the field and in the greenhouse. Plant height and leaf length can be used in the selection procedures for *Crotalaria* species in Kenya. This study determined *C. trichotoma* to have a chromosome number of 2n = 2x = 16. Molecular cytogenetic characterization and comparison can be done to determine chromosome length, symmetry and positioning of the centromere in Kenya *Crotalaria* species.

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APPENDICES

Appendix 1: Mean squares showing genotypic differences for plant height, leaf length, days to flowering, pod circumference, days to pod maturity and number of branches from 11 × 11 diallel cross.

		Mean Squares					
Sources	**d.f**	**Plant height**	**Leaf length**	**Days to pod maturity**	**Pod circ.**	**Days to flowering**	**No. of branches**
Replication	2	5334.8**	8.38***	600.8***	3.35***	37.7	108.29***
treatment	120	437.9***	2.21***	158.4	2.14***	32.9	5.26

*** Significant at P< 0.001; ** Significant at P <0.05

Appendix 2: Means of quantitative traits in *Crotalaria* species obtained from 11× 11 diallel experiment

Combination	**Leaf length**	**Plant height**	**Days to flowering**	**No. of branches**	**Pod circ.**	**Pod maturity**
FBS 0061×FBS 0061	6.35±0.53	68.25±11.01	73.00±3.00	9.87±0.64	5.32±0.09	83.33±1.67
FBS 0061×FKK 0039	5.59±0.30	72.28±6.51	68.33±4.41	7.63±0.41	5.30±0.21	93.33±1.67
FBS 0061×FKK 0097	6.30±0.12	69.27±5.15	76.33±1.33	6.70±1.49	4.23±0.81	91.67±1.67
FBS 0061×FKK 0099	4.86±0.37	73.00±6.80	71.33±5.78	10.60±1.83	5.76±0.26	90.00±2.89
FBS 0061×FKS 0165	5.78±0.34	72.33±14.83	67.67±4.33	7.60±0.31	5.21±0.16	84.67±2.60
FBS 0061×FVH 0119	6.00±0.00	52.50±0.00	70.00±0.00	4.80±0.00	5.90±0.00	86.67±1.67
FBS 0061×FND 0181	5.60±0.33	72.16±16.85	72.67±6.33	6.73±0.93	5.58±0.13	91.67±1.67
FBS 0061×FKK 0129a	5.09±0.49	55.13±4.73	76.00±3.00	5.33±0.24	5.28±0.11	94.00±2.08
FBS 0061×FBS 0064	6.60±0.13	62.60±4.27	73.33±1.67	8.60±1.75	5.66±0.27	94.33±2.33
FBS 0061×FSY 0151	5.29±0.29	81.33±1.21	77.67±1.33	8.53±1.56	5.39±0.19	95.33±0.33
FBS 0061×FHB 0211	5.95±0.22	77.75±4.96	76.00±3.00	6.47±1.04	6.01±0.33	91.67±1.67
FKK 0039×FBS 0061	5.19±0.24	64.56±3.89	72.67±6.33	7.73±2.24	6.08±0.04	81.67±1.67
FKK 0039×FKK 0039	7.14±0.13	100.28±14.15	76.67±1.20	9.87±0.75	6.15±0.18	85.00±5.00
FKK 0039×FKK 0097	5.87±0.28	99.17±22.49	76.33±1.33	8.87±1.85	5.27±0.15	81.67±1.67

FKK 0039×FKK 0099	6.95±1.49	89.11±16.70	73.33±1.67	7.13±1.21	5.72±0.39	80.00±0.00
FKK OO39×FKS 0165	6.77±0.14	69.67±1.45	71.67±1.67	6.93±0.84	4.81±1.16	80.00±0.00
FKK 0039×FVH 0119	8.22±0.77	64.44±1.55	69.67±4.67	6.20±0.42	5.47±0.13	81.39±1.39
FKK 0039×FND 0181	6.21±0.27	81.45±8.40	68.33±4.41	8.53±1.44	5.38±0.01	81.67±1.67
FKK 0039×FKK 0129a	6.90±0.42	72.83±6.56	68.33±4.41	7.20±1.91	5.48±0.20	80.00±0.00
FKK O0039×FBS 0064	6.74±0.09	69.94±3.47	68.33±1.67	8.33±0.47	5.43±0.18	80.00±0.00
FKK 0039×FSY 0151	5.96±0.33	74.75±12.86	69.67±5.49	8.07±0.53	5.40±0.20	80.00±0.00
FKK 0039×FNHB 0211	5.93±0.28	63.08±6.83	74.33±4.67	8.47±1.52	5.68±0.24	85.00±5.00
FKK 0097×FBS 0061	9.70±0.62	78.33±22.19	70.00±2.89	8.47±1.16	5.36±0.22	80.00±0.00
FKK 0097×FKK 0039	5.69*0.55	76.94±4.82	68.33±4.41	8.93±0.55	5.02±0.52	80.00±0.00
FKK 0097×FKK 0097	6.28±0.52	67.00±3.51	70.00±2.89	8.67±0.77	5.44±0.23	81.67±1.67
FKK 0097×FKK 0099	5.06±0.37	65.89±8.04	74.33±4.67	9.60±0.60	5.51±0.29	80.00±0.00
FKK 0097×FKS 0165	6.44±0.34	67.22±8.61	70.00±2.89	6.67±1.07	4.48±1.00	80.00±0.00
FKK 0097×FVH 0119	7.15±0.27	64.87±2.96	71.33±4.09	8.87±1.77	5.76±0.18	85.00±0.00
FKK 0097×FND 0181	6.49±0.37	75.72±6.51	68.00±5.69	6.60±0.70	4.63±1.08	83.33±3.33
FKK 0097×FKK 0129a	4.86±0.37	73.75±4.73	72.67±6.33	8.20±2.36	5.97±0.12	81.67±1.67
FKK 0097×FBS 0064	6.88±0.39	63.00±1.15	74.67±2.60	7.80±1.11	5.36±0.27	80.00±0.00
FKK 0097×FSY 0151	4.96±0.52	70.42±7.95	68.33±4.41	7.00±1.10	5.71±0.35	82.50±1.44
FKK 0097×FHB 0211	5.61±0.17	73.06±2.27	73.33±1.67	6.40±1.83	4.49±1.00	88.33±8.33
FKK 0099×FBS 0061	5.51±0.71	65.89±5.21	70.00±5.00	10.13±1.31	6.02±0.23	91.67±1.67
FKK 0099×FKK 0039	6.99±0.29	76.97±10.44	70.00±2.89	9.60±1.53	4.58±1.04	98.33±3.33
FKK 0099×FKK 0097	4.88±0.29	75.83±9.39	71.33±5.78	9.73±1.49	5.68±0.22	95.00±0.00
FKK 0099×FKK 0099	6.24±0.36	70.83±6.09	71.33±5.78	8.67±1.80	5.89±0.44	93.33±1.67
FKK 0099×FKS 0165	5.39±0.06	67.50±4.33	71.67±1.67	7.43±0.35	4.50±1.04	96.00±1.00
FKK 0099×FVH 0119	5.89±0.46	76.53±4.28	74.67±2.60	9.67±1.78	4.78±1.15	95.00±0.00
FKK 0099×FND 0181	6.25±0.00	115.00±0.00	79.00±0.00	7.40±0.00	5.73±0.00	98.33±3.33
FKK 0099×FKK 0129a	5.93±0.34	69.11±3.87	68.33±3.33	9.60±1.31	5.57±0.32	98.33±3.33
FKK 0099×FBS 0064	7.13±0.27	73.25±8.39	70.00±2.89	7.67±1.33	5.31±0.18	95.00±0.00
FKK 0099×FSY 0151	6.18±0.53	60.97±7.15	71.33±4.09	8.62±2.57	5.25±0.13	95.00±0.00
FKK 0099×FHB 0211	6.74±0.38	105.42±19.79	74.33±4.67	8.07±1.27	5.43±0.19	95.00±0.00

FKS 0165×FBS 0061	4.76±0.12	81.35±16.68	74.67±2.60	8.13±1.79	4.52±1.03	72.67±32.84
FKS 0165×FKK 0039	5.36±0.31	91.38±7.63	73.75±1.25	9.50±2.09	5.89±0.26	87.50±4.33
FKS 0165×FKK 0097	7.91±0.26	85.83±6.13	71.67±1.67	10.60±1.71	5.52±0.18	91.67±3.33
FKS 0165×FKK 0099	6.73±0.31	74.13±5.48	74.67±2.60	7.87±0.85	4.32±0.92	101.67±3.33
FKS 0165×FKS 0165	6.01±0.29	78.33±12.28	76.33±1.33	7.80±1.44	4.59±1.05	95.00±0.00
FKS 0165×FVH 0119	5.39±0.45	69.67±14.95	69.67±4.67	8.47±1.16	5.49±0.33	103.33±8.33
FKS 0165×FND 0181	6.60±0.23	90.00±13.23	75.00±0.00	8.87±1.68	4.75±1.14	104.33±7.88
FKS 0165×FKK 0129a	6.09±0.28	62.69±3.25	66.67±1.67	8.07±0.97	5.61±0.17	98.33±3.33
FKS 0165×FBS 0064	6.49±0.26	60.33±2.22	66.67±1.67	9.53±0.88	5.58±0.34	101.67±6.67
FKS 0165×FSY 0151	5.93±0.00	76.67±0.00	65.00±0.00	9.20±0.00	5.38±0.00	115.00±0.00
FKS 0165×FHB 0211	5.75±0.09	65.23±5.85	68.75±1.25	8.90±1.12	5.54±0.21	94.50±1.66
FVH 0119×FBS 0061	6.64±0.42	74.64±7.25	71.33±5.78	11.13±1.79	5.67±0.19	85.00±5.00
FVH 0119×FKK 0039	7.06±0.16	80.89±7.62	73.00±3.00	9.53±1.54	4.51±1.03	81.67±1.67
FVH 0119×FKK 0097	6.15±0.18	64.08±4.43	70.00±2.89	7.40±1.25	3.85±0.77	85.00±5.00
FVH 0119×FKK 0099	5.21±0.53	61.33±6.96	74.33±4.67	9.20±1.96	5.55±0.17	84.67±2.60
FVH 0119×FKS 0165	8.44±0.45	81.80±14.36	73.00±4.16	9.73±1.71	5.65±0.06	96.33±4.67
FVH 0119×FVH 0119	6.20±0.22	84.43±18.61	73.00±3.00	7.60±0.72	5.67±0.39	86.67±4.41
FVH 0119×FND 0181	5.78±0.41	70.17±8.85	76.33±1.33	8.33±1.73	4.47±1.00	88.33±1.67
FVH 0119×FKK 0129a	5.00±0.14	72.08±5.53	74.67±2.60	7.63±0.94	5.54±0.35	89.67±0.33
FVH 0119×FBS 0064	5.24±0.29	66.98±5.48	73.00±4.16	10.67±1.64	5.63±0.32	80.00±0.00
FVH 0119×FSY 0151	6.76±0.36	72.24±8.43	69.67±5.49	8.67±0.85	5.59±0.21	80.00±0.00
FVH 0119×FHB 0211	6.56±0.41	66.90±10.51	79.00±0.00	8.33±1.33	3.56±1.03	85.00±0.00
FND 0181×FBS 0061	5.26±0.61	74.33±11.05	76.67±6.67	9.78±0.36	5.71±0.11	85.00±0.00
FND 0181×FKK 0039	6.66±0.12	69.76±10.02	71.33±4.09	10.45±1.83	2.51±0.01	93.33±7.26
FND 0181×FKK 0097	5.94±0.48	78.00±6.51	74.67±2.60	6.87±1.31	3.67±1.08	85.00±0.00
FND 0181×FKK 0099	6.14±0.28	88.95±8.03	79.67±5.78	6.47±0.79	5.74±0.11	83.33±1.67
FND 0181×FKS 0165	8.07±0.46	102.87±16.85	70.00±2.89	6.13±0.41	6.04±0.19	81.67±1.67
FND 0181×FVH 0119	4.47±0.30	68.33±22.84	79.67±5.78	8.83±2.68	6.42±0.26	80.00±0.00
FND 0181×FND 0181	7.24±0.69	79.33±6.85	73.33±1.67	8.67±1.23	5.73±0.32	85.00±2.89
FND 0181×FKK 0129a	6.14±0.36	81.25±1.91	70.00±2.89	8.13±0.85	5.92±0.22	85.00±5.00

FND 0181×FBS 0064	6.93±0.29	77.00±2.00	74.67±2.60	8.27±0.27	4.62±1.07	80.00±0.00
FND 0181×FSY 0151	7.16±0.22	90.83±7.92	70.00±2.89	7.40±1.22	5.66±0.11	90.00±5.00
FND 0181×FHB 0211	5.84±0.51	89.00±9.54	68.33±1.67	8.27±1.07	5.77±0.11	88.33±3.33
FKK 0129a×FBS 0061	7.02±0.44	89.07±11.39	66.67±3.33	8.73±1.49	5.76±0.32	86.67±1.67
FKK 0129a×FKK 0039	5.92±0.13	87.09±19.53	69.67±5.47	7.97±0.79	5.04±0.79	80.00±0.00
FKK 0129a×FKK 0097	6.22±0.35	69.81±3.63	71.33±4.09	6.27±0.27	5.33±0.18	80.00±0.00
FKK 0129a×FKK 0099	6.30±0.66	99.83±21.09	70.00±2.89	7.13±1.41	5.74±0.32	84.67±2.60
FKK 0129a×FKS 0165	6.34±0.43	76.53±9.21	65.00±5.00	8.07±1.41	5.31±0.19	93.33±7.26
FKK 0129a×FVH 0119	6.64±0.45	95.99±13.62	66.67±3.33	7.93±1.38	4.99±1.25	85.00±5.00
FKK 0129a×FND 0181	5.77±0.24	61.14±9.09	71.33±4.09	4.73±0.27	5.28±0.15	80.00±0.00
FKK 0129a×FKK 0129a	7.44±0.47	9240±14.17	72.67±6.33	8.07±2.40	5.68±0.11	81.67±1.67
FKK 0129a×FBS 0064	7.58±0.78	74.72±4.90	70.00±5.00	7.73±1.96	5.54±0.25	85.00±5.00
FKK 0129a×FSY 0151	6.98±0.37	61.53±5.65	68.33±4.41	7.60±2.00	5.31±0.11	91.67±11.67
FKK 0129a×FHB 0211	6.68±0.88	94.13±20.87	71.33±5.78	9.67±2.94	5.88±0.39	80.00±0.00
FBS 0064×FBS 0061	4.73±0.16	70.33±10.65	77.67±1.33	5.13±0.57	5.90±0.40	85.00±5.00
FBS 0064×FKK 0039	6.31±0.28	67.42±2.09	79.00±0.00	6.80±0.31	6.08±0.03	88.33±4.41
FBS 0064×FKK 0097	5.95±0.41	69.94±4.12	74.33±4.67	6.53±1.29	5.82±0.23	85.00±5.00
FBS 0064×FKK 0099	5.47±0.76	93.00±22.28	71.33±5.78	8.00±1.15	5.75±0.12	85.00±0.00
FBS 0064×FKS 0165	5.44±0.71	73.78±7.37	71.33±4.09	6.20±0.53	5.98±0.19	80.00±0.00
FBS 0064×FVH 0119	6.50±0.21	69.42±7.80	66.67±1.67	8.13±0.44	5.56±0.24	91.67±3.33
FBS 0064×FND 0181	6.40±0.26	94.86±4.41	70.00±2.89	8.27±0.74	5.80±0.21	81.67±1.67
FBS 0064×FKK 0129a	4.75±0.35	67.08±1.96	69.67±4.67	7.27±1.17	5.77±0.29	85.00±5.00
FBS 0064×FBS 0064	5.83±0.42	62.83±6.43	72.67±6.33	7.13±1.21	4.54±1.06	81.67±1.67
FBS 0064×FSY 0151	5.78±0.19	74.61±2.45	76.00±3.00	9.13±0.59	5.08±0.81	85.00±5.00
FBS 0064×FHB 0211	5.42±0.99	72.50±6.05	72.67±6.33	6.07±0.97	6.11±0.27	90.00±2.89
FSY 0151×FBS 0061	5.37±0.14	75.97±4.89	71.67±3.33	9.53±0.35	5.63±0.08	83.33±1.67
FSY 0151×FKK 0039	5.06±0.34	87.42±13.12	72.67±6.33	7.07±1.57	5.89±0.10	83.22±1.61
FSY 0151×FKK 0097	6.50±0.00	70.00±0.00	70.00±0.00	6.80±0.00	5.94±0.00	83.10±1.55
FSY 0151×FKK 0099	5.06±0.01	74.33±11.46	65.00±2.89	11.13±2.15	5.81±0.35	85.00±5.00
FSY 0151×FKS 0165	5.88±0.23	74.33±9.97	73.00±4.16	8.47±1.51	5.83±0.37	80.00±0.00

FSY 0151×FVH 0119	7.11±0.39	95.83±15.1	77.67±1.33	8.67±1.66	5.38±0.18	88.33±4.41
FSY 0151×FND 0181	7.08±0.64	70.97±4.88	65.00±5.00	8.07±1.79	5.53±0.24	80.00±0.00
FSY 0151×FKK 0129a	4.93±0.38	78.83±6.17	79.00±0.00	4.93±0.67	4.68±1.09	81.67±1.67
FSY 0151×FBS 0064	6.75±0.35	105.89±4.97	74.67±2.60	8.00±1.97	5.87±0.26	81.61±1.67
FSY 0151×FSY 0151	6.91±0.25	76.11±17.75	71.33±5.78	6.80±0.46	5.85±0.45	80.00±0.00
FSY 0151×FHB 0211	5.90±0.51	81.17±17.55	73.00±4.16	7.60±1.33	5.60±0.31	85.00±5.00
FHB 0211×FBS 0061	5.84±0.47	92.20±19.09	74.67±2.60	7.07±0.52	5.42±0.14	83.33±3.33
FHB 0211×FKK 0039	5.58±0.31	66.43±7.93	74.67±2.60	5.67±0.55	5.17±0.09	80.00±0.00
FHB 0211×FKK 0097	6.55±0.29	72.92±8.61	76.33±1.33	9.13±1.58	5.28±0.15	80.00±0.00
FHB 0211×FKK 0099	5.83±0.55	63.50±2.02	76.33±1.33	7.23±1.12	4.68±1.12	80.00±0.00
FHB 0211×FKS 0165	5.57±0.49	63.50±8.09	72.33±2.33	7.27±1.27	4.68±1.13	88.09±1.90
FHB 0211×FVH 0119	5.99±0.54	98.06±26.51	70.00±2.89	5.47±0.29	5.18±0.06	88.33±8.33
FHB 0211×FND 0181	7.19±0.05	68.83±8.58	69.67±5.49	6.60±0.42	6.41±0.19	81.48±1.48
FHB 0211×FKK 0129a	5.85±0.21	61.67±0.88	68.33±4.41	6.93±0.74	5.34±0.09	80.00±0.00
FHB 0211×FBS 0064	6.36±0.18	96.27±10.64	75.00±0.00	7.00±0.81	5.47±0.15	84.26±0.01
FHB 0211×FSY 0151	6.36±0.15	104.47±15.69	76.00±1.53	9.00±1.97	5.66±0.04	80.00±0.00
FHB 0211×FHB 0211	7.46±0.43	79.17±3.89	70.00±2.89	9.20±1.06	4.74±1.12	80.00±0.00
G mean	6.181	76.423	72.132	8.005	5.373	86.43
SE	0.054	1.004	0.332	0.122	0.05	0.477
CV	11.679	23.695	9.068	29.047	16.880	8.984

± Standard deviation, $P < 0.05$