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THE BREEDING SYSTEM OF *SESBANIA SESBAN* (L.) MERR. (Leguminosae)

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ABSTRACT

The floral biology of *Sesbania sesban* (L.) Merr. (Leguminosae: Papilionoideae) was studied in general, and in particular the level of out-crossing was determined under conditions of open-pollination. Floral biology studies were conducted on one cultivated population (ILRI Accession No. 15022) of *S. sesban* at the compound of the International Livestock Research Institute (ILRI) in Addis Ababa. For the purpose of estimating out-crossing rate, seeds were collected from *S. sesban* populations at Addis Ababa (Ethiopa), Debrezeit (Ethiopia) and Zambia.

Stigma receptivity and anther dehiscence time vary considerably for different flower bud sizes. Stigma become receptive before shedding of pollen grains and /or maturity of pollen grains. Therefore, the flowers of this species were determined to be protogynous. There were significant differences between mean bud size (mm) with receptive stigma ($\bar{x} = 14.1 \pm 1.46$) and those with non-receptive ones ($\bar{x} = 12.1 \pm 1.84$). Also between mean size of buds with dehiscing anthers ($\bar{x} = 15.39 \pm 2.28$) and those with non-dehiscing anthers ($\bar{x} = 10.68 \pm 2.36$) at $P < 0.001$ level of significance. Further more, pollen tubes were found to be abundant in more mature flower buds ($\bar{x} = 16.53$ mm) from deliberate tripping. Germination of self-pollen under natural condition takes place at a very late bud stage i.e. when a flower bud size of 22 mm and above is attained. On the other hand, tripping of flowers indicated that pollen germination could take place at an early bud stage (stage 2, $\bar{x} = 14$ mm). Pollen tube growth was more abundant in those styles, which received hand tripping treatment than with forceps or toothpick tripping. Seed production (no./ pod) was also higher due to hand tripping ($\bar{x} = 29.9 \pm 1.27$) than due to bagging alone ($\bar{x} = 23.67 \pm 1.84$) or toothpick tripping ($\bar{x} = 24.11 \pm 2.25$), although not statistically significant. However, seed production from open-pollination ($\bar{x} = 34.11 \pm 2.60$) was significantly higher than from the tripping and bagging treatments.

Because of difficulties in preparing an optimum medium for maximum pollen grain germination in vitro and due to short hydration time (6 hours of incubation), 49.43% germination was the average score obtained in 20% sucrose. On the other hand, with fluorochromatic reaction test an average of 90.51% viability was scored from flower buds that fall in the range of 11 to 22 mm. Flower bud size was significantly negatively correlated ($r = -0.61$, $p = 0.000$) with pollen grain viability and pollen grain viability was also found to correlate ($r = -0.39$, $P = 0.000$) inversely with germination. There was a significant positive correlation ($r = 0.87$, $P = 0.000$) between flower bud size and germination at $P < 0.05$.

Although the floral structure of *S. sesban* could favour self-pollination, the delay in self-pollen germination under natural condition rather seems to favour out-crossing in the presence of appropriate pollinators. Based on the electrophoretic assay of a large number of progenies on one polymorphic locus of Malate dehydrogenase (Mdh), on average, an estimated 64% of the seeds were found to be derived from out-crossing, ranging from 0.395 to 1.105 among populations. The genotype frequencies of the populations of Accession 15022 and Accession 15019 were in line with those of the Hardy-Weinberg expected genotype proportions, whereas genotype frequencies of the Zam-R024 population deviated from the expectations indicating the occurrence of non-random mating in this population.

It is possible to conclude that *S. sesban* can under go complete self-fertilization in the absence pollinators and also complete out-crossing in the presence of suitable pollinators.

1. INTRODUCTION

The genus *Sesbania* is a member of the family Leguminosae, sub-family Papilionoideae in the tribe Robinieae (Lavin, 1987). The genus is composed of both annual and perennial species which are distributed in tropical and sub-tropical regions (Evans and Rotars, 1987; Steinmuller, 1995). There are 12 species known in Ethiopia (Thulin, 1989) and the 13th species is recently described by Bidgood and Friis (1998) as *Sesbania melanocaulis* in Kefa and Wellega regions. It is similar to *S. sesban* in many respects but differs mostly by it's dark purplish brown to almost black bark of the stems and uniformly yellow flowers (dark dotted in *S. sesban*). Six of these species are woody and the rest are semi-woody herbs. According to Thulin (1989), there are 50 species in the tropics and sub-tropics, the majority (30 species) of which are found in Africa and 33 species of the total are already described (Steinmuller, 1995).

S. sesban (L.) Merr. (2n = 12) (*Am =Girangre*) is one of the perennial shrub or tree species and is widely distributed in Sub-Saharan African countries (Azene *et al.*, 1993). It has a wide adaptation ranging from low land (altitude 300 m) to highland (altitude 2000 m). Semi-arid and Sub-humid zones with annual rainfall between 500 - 2000 mm are suitable for its growth (Heering, 1994). *S. sesban* tolerates both alkalinity and salinity conditions including water logging and flooding but, susceptible to frost (Shelton, 1994). It grows by the side of streams or on the borders of fresh water lakes. According to Lavin (1987), the species typically inhabits areas that are periodically inundated with water. In Ethiopia, it is found at the lake shores of Zwai, Abaya, Langano, Awassa and in some other locations (Kidest, 1991). A wide variety of soils, ranging from loose sandy to heavy clay soils, are suitable for its growth. It is among the five priority species (together with *Calliandra calothyrsus, Leucaena diversifolia, Grevillea robusta, Gliricidia sepium, Casuaiana junghuniana andMarkhamia lutea*) identified for genetic improvement programme in agroforestry systems in the Eastern and Central African highlands (Owino, 1992).

The seeds of *Sesbania* can germinate and grow with minor drawback up to pH 9 but decline beyond pH 10 (Ghai *et al.,* 1985). Some accessions of *S. sesban* can give moderate shoot yield at lower pH (e.g. pH of 4.1) (Mugwira and Haque, 1993). Odul and Akundo (1988) reported the possibility of vegetative propagation from stem cuttings. *In vitro* techniques for rapid propagation of seedlings was also attempted (Hanson and Ruredzo, 1992).

Besides its wide adaptations, *S. sesban* serves the following purposes (Evan and Rotar, 1987; Thulin, 1989; Kusekawa *et al.,* 1993; Onim and Oteino, 1993; Oteino, 1993; Evans and Macklin, 1990; Woodward, 1988; Azene *et al.*, 1993; Weigand *et al.*, 1995):

1. It has been used in alley farming as a source of green manure supplying nitrogen and as wind breaks.

2. It provides rapidly degrading nitrogen for animals, e.g. ruminants on poor quality diets can be supplemented with leaves of *S. sesban.*

3. It serves as a source of pulp fibers, fuelwood and wood for construction.

4. In some countries, its various parts (leaves, roots, barks and seeds) have been used for medicinal purposes for both human and livestock.

S. sesban has been grown to shade coffee, cacao and tumeric in Uganda and as wind break in Africa and India and for fruit orchards in Israel (Karani, 1983). Increases in soil productivity and thus to yield of crops has been obtained when it is planted in alleys or when the foliage is incorporated into soil. With applications of 4.4 t DM/ha of foliage, the yield of rice increased from 1.9 to 3.9 t/ha. When the mulch of 13 t DM/ha was applied into maize and bean fields, the yield has increased consistently for 3 consecutive years (Weerakoon, 1990; Onim *et al.*, 1990).

S. sesban is one of the most promising agroforestry species in the tropics (NAS, 1983; Yamoah and Bruleign, 1990). When it is supplemented to cereal crops residues, it improves live weight gains in ruminant animals such as sheep. Its potential for high nutritive value as leaf fodder for animals makes this species more advantageous than other forage crops (ILCA, 1986). The leaves contain high protein ranging between 20-25 % of the dry matter depending on the age and soil condition. It is now commonly used as a supplement to low quality roughage's to ruminants (Heering, 1996). Where it occurs as an element of riverine vegetation in Africa, it is browsed by cattle (Evans and Rotar, 1987).

Difference in phenolics (soluble tannins and insoluble tannins) concentration in *S. sesban* accessions was reported by Heering *et al.* (1996). Tannins could affect the nutritive value of this species. Rams fed on leaves of some accessions had lower digestibility due to higher concentrations of condensed tannins (Weigand *et al.,* 1995). Saponins from this species were tested for contraceptive activities, and a concentration of 100 µg/ml was found to inhibit the mobility of human spermatozoid (Hostettman and Marston, 1995).

In the highland African agriculture, there is an increasing interest in this species due to three main reasons (Steinmuller, 1995):

- fast growth and coppicing ability
- use to reclaim areas with alkaline and saline soils and
- potential to grow in areas prone to seasonal water logging and flooding.

Regarding the reproductive strategy of *S. sesban*, there were different views and suggestions by different researchers. The 1961 FAO report suggested that, all *Sesbania* species are self-compatible. Brubidge (1965) reported that the flower structure of *S. sesban* as well as the availability of pollen and nectar attract insects which initiate cross-pollination. Similarly, Brewbaker (1990) assumed that *Sesbania* species are largely out-crossing and therefore, the occurrence of self-incompatibility would be common. Heering (1994) reported the non-existence of any stigmatic or stylar self-incompatibility. On the other hand he suggested that, there might be ovular inhibition which needs further investigation Therefore, this study was intended to be clear on these opposing views.

Baker and Huden (1968) had listed some important components that are required to be focused on while studying floral behavior and/ or the breeding system of a species.

These are:

- conditions for pollen grain germination
- how long stigma stays receptive?
- how long pollen grains remain viable under natural condition?
- are the flowers protandrous or protogynous?
- the existence of self-incompatibility

In addition, it is also important to determine the level of out-crossing. The rate of out-crossing was found to vary for a species depending on environmental conditions. Different rates of out-crossing were obtained in mesic and xeric areas for populations of *Hordeum spontaneum* (Brown et al., 1978). Out-crossing rate could also be dependent up on pollen competition. The gametophytic competition theory suggests that foreign pollen tubes may fertilize the majority of the female gametes by growing faster than self-tubes thus increasing the level of out-crossing (Hessing, 1986).

S. sesban has been found to undergo interspecific hybridization artificially with *S. kinensis and S. geotzei*. Its potential as a forage crop is significantly immense but its forage quantity and quality reduces from year to year, due to its susceptibility to an insect pest (*Mesoplatys ochroptera*) (Heering, 1994). However, it might be possible to transfer insect resistance characteristics of *S. geotzei* (which is less palatable) to *S. sesban.* Before embarking on any hybridization programme, the floral biology and pollination aspects mentioned above should be investigated. Knowledge on the biology of flowering as well as the processes of pollination and fertilization represents the foundation for establishing appropriate breeding methods for whatever plant species (Wojciechowska, 1972).

1.1 Objective of the study

Information on the extent of self- or cross-pollination which is needed: 1. for its management in the germplasm banks and 2. to formulate an effective breeding strategy for improvement of this forage crop is not available. The present investigation was, therefore, intended with an objetive of studying the floral biology in general and to estimate the level of out-crossing in *S. sesban* using allozymes as a genetic marker.

2. LITERATURE REVIEW

2.1 Breeding systems

Breeding system refers to the mechanism (sexually) by which an organism (plant or animal) is producing the future generation or progenies. In a very broad sense, it includes all aspects of the sex expression in plants that affect the relative genetic contribution to the next generation of individuals within a species (Wyatt, 1983). Knowledge of the breeding system provides an indispensable framework for understanding the complexities of patterns found in natural reproduction (Briggs and Walters, 1984). Successful plant breeding programme pre-requisites an understanding of the breeding system of a species (Sedgley *et al.*, 1992).

The breeding behavior of many leguminous plants have been studied. For example, *Desmodium sandwicence* E. Mey (Rotar *et al.*, 1967), *Acacia auriculiformis* and *A. mangium* (Sedgley *et al.*, 1992), *Leucaena* species (Brewbaker, 1982), Egyptian clover (Beri *et al.*, 1985). The study of floral (pollination) biology involves observation and collection of data on flowers and identification of pollinating agents. The carpenter bees *(Xylocopa flavorufa* and *X. somalica*), honey bees (*Apis melifera*), mason bees (*Megachile bituberculata*) and leaf cutters (*Chalicodoma sp.*) were identified as visitors of the flowers of *S. sesban*, at Zwai, in the rift valley of Ethiopia (Heering, 1994). These insects are variously assumed to be the factors for out-crossing in *S. sesban*. High degree of out-crossing in *Cardus nutans* (Asteraceae) is due to visitation from *Bombus*, *Apis* and various lepidoptera species (Smyth and Hamrick, 1984). Thus structural and functional aspects of flowers must be studied and comprehended for proper appraisal of the pollination mechanisms.

2.1.1 Flower development

Sexual reproduction in angiosperms pre-requisites the development of a flower and its essential parts. After a plant is fully grown, there is a stage or positional information which initiates the growth of floral parts. In most species, flower development takes place at the shoot apex with a transition from vegetative to reproductive stage, i.e., when the vegetatively growing apex of a branch is transformed to a flowering apex or when the flowering signal reaches it. This phenomenon takes place at a certain distance from the base of the branch at certain time depending on the growth rate of the branch and the propagation rate of the flowering signal (Lindenmayer, 1984).

2.1.2 Flowering period

Flowering period is the length of time a population of flowers maintain exposed anthers and stigma. Photoperiod determines the flowering condition of many species. Some other species flower independent of the light period. Sedi and Humphreys (1992) reported that *S. sesban* flowers independent of day length over the range of 11-13 hours but, flowering is arrested by extreme temperatures. It can flower many times in a year as far as the environmental conditions are conducive. Climate variables such as rain, wind, air humidity, temperature and light intensity are factors that regulate the timing of flowering in plants (Sedi and Humphreys, 1992). In *Chamaeystisus palmensis* (tagasaste), flowers are much more longer lived in cool overcast weather than in the hot sunny weather (Webb and Shand, 1985).

Flowers usually open during warm hours of the day and anthers dehisce under desiccated condition (Shivanna and Johri, 1985). The exact hour of flower opening is subject to weather condition and often also to the age of the flower. According to Frankel and Galun (1977), flowering persistence is shorter in plants producing abundant pollen grains than in plants producing moderate or small amount of pollen grains.

2.1.3 Flower structure

In Leguminosae (Papilionoidae), e.g., alfalfa, the flower is determinate and produces calyx, corolla, 10 stamens and a pistil (Barnes *et al.*, 1972). The flower structure of *S. sesban* is that of the typical papilionoid Leguminosae. In Papilionoidae, the corolla is highly evolved and consists of five petals, a large standard, two lateral wing petals and two fused petals that form the keel.

Flower structure is one of the main factors that determines the breeding behavior of a species. If the position of anthers and stigma does not correspond in a hermaphrodite species, self-pollination becomes an impossible task. Thus, the major mechanism of reproduction in such species would be out-crossing. Heterostyly is the most widely recognized anti-selfing structure, but the functional relations between heterostyly and self-incompatibility is far from clear (Faegri and Van der Pijl, 1979). The architectural set up of flowers in many species acts as a secondary attractant to insect pollinators. By texture of the colour and/ or smell, the flowers create deceptive devices that affect the sense of the visitors. Some species of Orchidaceae display their flowers (imitating the female) attracting a male hymenopter (e.g. *Ophrys speculum, Ophrys blossom*) (Faegeri Van der Pijl, 1979)

2.1.4 Cross-pollination and self-pollination

Self-pollination is a condition of pollen transfer from an anther of one flower to the stigma of the same flower (autogamy) or pollen transfer from anther of one flower to the stigma of another flower on the same plant. Cross-pollination refers to pollen transfer from the flower of one plant to the flower of another plant (allogamy). This process normally requires agents of pollination such as insects, wind or animals. It is in very rare cases that a plant may be absolutely self-pollinating or cross-pollinating. For example, the most self-fertilizing species, *Hordeum vulgare,* is 99% self-pollinating. However, it allows some level of cross-pollination as far as other varieties of *Hordeum* are present at a reasonable distance. Plants are classified as self-fertile if they set 90% or more seeds upon self-pollination. Several ecological factors affect the rates of variation in out-crossing but above all the mode of pollination has the major impact (Brown *et al.*, 1989).

In entomophilous species gene flow is affected by (Handle *et al.*, 1984; Faegri and Van der Pijl, 1979):

- pollinator guild
- pollinator behaviour
- dynamics of pollen carryover among plants
- spatial arrangement of plants within a population and
- morphological properties of flowers and inflorescence.

Insects visit the flowers of Papilonoideae either for nectar and/or for pollen collection. In *Chamaecytisus palmensis,* the chance of cross-pollination is minimized as self-pollination will take place before the flowers open. On the other hand, there is a possibility that cross pollen grains could have a chance to pollinate the stigma if the latter stays receptive and self-pollen germination delays until late bud stage (Webb and Shand, 1985).

A high level of flower and fruit abortion is common in shrubs and trees with hermaphrodite flowers and need not be attributed to lack of compatibility or limitation of resources (Bawa and Webb, 1984). The flower structure in Papilionoideae allows the possibility of self-pollination, and unless there is some mechanism preventing cross-pollination, the chances of self-fertilization is very high. In *Chamaeystisus palmensis* all anthers were found already shedding their pollen at late bud stage before the flowers have even begun to open. Therefore, in this species, self-pollination is the dominant mode of reproduction with little chance of cross-pollination and all plants are self-compatible (Webb and Shand, 1985).

In some plant species self-pollination may not lead to self-fertilization due to the existence of self-incompatibility.

2.2 Evolution of self-incompatibility (SI)

2.2.1 Self-incompatibility

The term self-incompatibility is defined as a failure following pollination or mating of a male and a female gamete to achieve fertilization. But, on the other hand, each of them is capable of uniting with other gametes of the breeding group after similar pollination or mating (Barnes *et al.*, 1972). Different authors have more or less related opinion with regard to the definition of self-incompatibility.

Mechanisms which prevent self-fertilization were evolved in either space (herckogamy and monoecism) or time (dichogamy) (Lande and Schemske, 1985). Furthermore, genetic polymorphism such as dioecism, heterostyly and self-incompatibility were evolved to restrict self-fertilization (Baret and Eckert, 1990). Once a gene arises which permits self-fertilization in a normally out-crossing species, it will have an immediate evolutionary advantage. And unless it is disfavoured due to selection the species will finally evolve towards self-fertilization (Phillips and Brown, 1977)

The evolution of two SI systems might be a result of conflict created by dissimilar reproductive requirements of the gametopyte and sporophyte generations (Beach and Kress, 1980). Stigmatic recognition and rejection of self-pollen is proposed as an evolutionary advancement by the sporophytes generation (Shivanna and Johri, 1985).

According to Lande and Schemske (1985), inbreeding depression is the major selective force maintaining out-crossing to avoid recessive deleterious mutations in homozygotes occurring due to evolution of self-fertilization. Selections that control inbreeding are most likely to affect those characteristics most directly associated with gene flow in flowering plants (pollination and seed dispersal) (Olmstead, 1986). The role that SI plays in the avoidance of selfing contributes to the ability of flowering plant populations to maintain high levels of inbreeding while minimizing the ill effects of the deleterious recessive alleles (Partridge, 1983).

The rise of a very efficient out-breeding mechanism (i.e. self-incompatibility) was indeed likely to occur with the evolution of specialized pollinating insects and therefore at a very early date in the history of angiosperms (de Nettancourt, 1977). The results of Darwin experiments in 1877 were a land mark in the study of breeding behaviours. Based on his results, it was possible to see how the range of floral types and physiological differences (maturity of anthers and stigma) on the same flower could be viewed as an adaptation to ensure cross-fertilization (Baret and Eckert, 1990).

Self-incompatibility has been viewed traditionally as a mechanism to promote out-crossing and minimize inbreeding in flowering plants (Lawrence, 1971). It has been estimated that SI is present in more than 3,000 species of plants (Olmstead, 1986). SI is common among cultivated plants and can be found in many species of forage crops such as legumes, cereals, root crops, fruit trees, vegetables and ornamentals (de Nettancourt, 1977). It is common among herbaceous genera, e.g., *Trifolium*, *Lotus*, *Melilotus*, *Medicago* and *Phaseolus* (Brewbaker, 1982). For example, retarded growth rates of self pollen tubes have been demonstrated for *Lotus corniculatus* and inhibition of pollen tube growth on stigma was shown for both *L. corniculatus* and *L. tenuis* (Lundqvist, 1993).

SI in higher plants can be manifested by failure of self-fertilization due to (Heslop-Harisson, 1975; Frankel and Galun, 1977):

1. Pollen - style reaction, a characteristics of many gametophytic SI control systems

2. Pollen - stigma reaction which is depicted in many sporophytic SI control systems

3. Pollen tube - ovule interaction within the ovarian cavity e.g. *Medicago.*

4. Failure of syngamy within the embryo sac e.g. *Theobroma cacao*

Self-incompatibility system together with other devices promoting cross-pollination regulate the breeding system of many plant species. This will ensure a greater or less amount of out-crossing in a population. Out-crossing facilitates the formation of heterozygous individuals. A certain degree of heterozygosity is essential to guarantee the capacity for response to selection and to evolutionary potential of a species (Heslop-Harrison, 1975).

Incompatibility could be either intraspecific (within species) or interspecific (between species). The latter phenomenon occurs due to pre-zygotic isolating mechanism in both plants and animals. Here, the review focuses only on intraspecific incompatibility which is common in higher plants. SI has been studied in various species of plants such as *Lotus tenuis,* (Leguminosae) (Lundqvist, 1993), *Sinapis arvensis,* (Cruciferae) (Ford and Kay, 1985), *Medicago sativa,* (Leguminosae) (Barnes *et al.,* 1972), *Nicotiana alata,* (Solanaceae) (Harris *et al.,* 1985), *Cicer arietinum,* (Leguminosae) (Lather and Dahiya, 1992), *Guizotia abyssinica* (Asteraceae) (Sileshi Nemomissa *et al.*, 1999).

The study of self-incompatibility pre-requisites the distinction between a gametophytically and sporophytically determined characters (Lewis, 1978). Beach and Kress (1980) suggested that gametophytic self-incompatibility is the primitive and fundamental type of self-incompatibility and sporophytic SI as a derived system.

2.2.1.1 Sporphytic self-incompatibility (SSI)

The pollen grains that land on the surface of a stigma from self-pollination will either germinate or abort depending on the pollen-stigma interaction. If they fail to germinate, the inhibition is said to be sporophytic because the incompatibility phenotype of the pollen is determined by the genotype of the pollen producing plant (sporophyte). According to Dafni (1992), the pollen-stigma relationship depends on pollen viability, stigma receptivity and genetic interaction of both partners as dictated by the incompatibility system (if any).

Stigmatic incompatibility is restricted to sporophytic system and is a characteristics of species with trinucleate pollen grains (e.g. homomorphic gametophytic grasses and many sporophytic species) (Barnes *et al.*, 1972; de Nettancourt, 1977). Single locus sporophytic system is more likely universal in the Brassicaceae. In the SSI system certain events occur after the rejection of an illegitimate pollen grains. The reactions are cell to cell and often between pollen wall and papillae of stigma (Ford and Kay, 1985). According to Jackson and Linskens (1990), in sporophytic type of incompatibility the reaction between the molecules involved takes place on the outer stigma surface between the substances carried by pollen grains in their pollen wall and the substances deposited by the maternal genotype in the pellicle outside the stigmatic cuticle. A relatively dry stigmas are found in the two main SSI families, Cruciferae and Compositae that discourages pollen landing on stigma (Heslop-Harisson, 1975). SSI was reported in *Sinapis arvensis* (Cruciferae) (Ford and kay, 1985), in *G. abyssinica* (Sileshi Nemomissa *et al.*, 1999).

Sporophytic SI systems associate pollen rejection with a number of differences in floral morphology which reinforce the out-breeding mechanism. That is, the condition of heterostyly which directly contributes to the prevention of self-pollen from landing on the stigma. In gametophytic SI systems, SI is expressed with the capacity of pollen to germinate through the stigma and or the style (de Nettancourt, 1977).

In general, in both SI systems, viable pollen grains (de Nettancourt, 1977; Shivanna and Johri, 1985):

- may fail to germinate.
- germinate but with very short pollen tubes and sometimes with callose at the end.

- protrude longer abnormal tubes that can not penetrate the stigmatic surface, instead coil or twist.
- produce pollen tube that initially penetrates normally but ceases soon forming callose.

Callose (β-1,3 glucan) deposition on the stigmatic papillae was reported to be an exclusive feature of SSI systems (Heslop-Harrison, 1975). It is usually detected as a material which fluoresces when treated with aniline blue. This synthetic fluorochrome is primarily specific for (1-3)-β-D-glucan although some (1-3) and (1-4)-β-D-glucan bind the fluorochrome as well (Harris *et al.*, 1985). Harris *et al.* (1985) observed this phenomenon in the pollen tubes of *Nicotiana alata*. The absence of fluorescence at the pollen tube tip means the absence of (1-3)-β-D-glucan at the tip.

2.2.1.2 Gametophytic self-incompatibility (GSI)

Pollen-style interaction is a characteristics of gametophytically controlled SI systems (Barnes *et al.*, 1972). An important feature of the GSI system is that pollen tube is inhibited from growing after it has penetrated the stigma and style (Lewis, 1978). In this type of SI system, the genotype of the individual micro-spore determines the phenotype of the pollen grains (de Nettancourt, 1977). That is, the molecule that produces the inhibition protein are contained in the intine of micro-spores and will not be exposed until the pollen tube elongates into the style. Those species with GSI system have smooth stigma or wet papillae and bears plentiful fluid secretions. This facilitates the landing of pollen grain and pollen tube penetration into the style (Heslop-Harrison, 1975).

Generally, de Nettancourt (1977) has classified the incompatibility system in to two groups, depending on the time of gene action, the site of expression, the association with morphological characteristics, the cytology of the pollen and presence or absence of pollyallelic series.

Table 1. The difference between sporophytic and gametophytic self-incompatibility systems

Sporophytic	**Gametophytic**
Diallelic	Polyallelic
Heteromorphic	Homomorphic
Trinucleate	Binucleate
Stigmatic	Stylar
One or two loci	One or two loci

The sporophytic and gametophytic incompatibility mechanisms are distinguished by several cytological and physiological features. In both systems the recognition and rejections reactions on the sporophyte side involves the stylar or stigmatic tissue of the pistil. On the pollen side, however, GSI involves the activity of the recognition factor coded by the haploid genome. In contrast in SSI, the pollen grains receive the gene products of the sporophytes S locus (loci) which are deposited extracellularly in the pollen grain wall (Beach and Kress, 1980).

A study made by Sharma and Shivanna (1986) and Sharma *et al.* (1985) on *Nicotiana* and *Petunia* species respectively suggested that gametophytic incompatibility can be analyzed *in vitro* with pistil extracts. In the extract, it was shown that self-pollen were selectively recognized and inhibited. According to Williams *et al.* (1982), components isolated from styles of *Prunus avium* were found to influence the growth of pollen tubes in bioassay. The effect of both stylar diffusate and extracts on pollen germination were tested on 3 cultivars of *Mallus domestica*. The result showed that the style extract and diffusate had an inhibitory effect on self-pollen with reduced percent germination (Speranza and Calzoni, 1988). In *Ranunculus sp.* the stylar extract was found to inhibit the germination of self-pollen while having less effect on cross-pollen (Jackson and Linskens, 1990). Therefore, *in vitro* bioassay technique have become an important tool in studying self-incompatibility mechanisms chemically. However, none of the *in vitro* bioassay described up to 1990 were considered convincing although they can be valuable in specific cases (Jackson and Linskens, 1990).

Gametophytic type of SI system is to be expected in the species with binucleate pollen, some of which are members of the family Leguminosae (Brewbaker, 1982; Lundqvist, 1993). When the 3 sub-families of Leguminosae (Caesalpinoidae, Mimosoidae, Papilionoidae) are compared SI is proportionately less frequent among Papilionoidae. However, the tendency here is that, woody groups are relatively with high frequency of SI than their herbaceous counter parts (Arroyo, 1981). For example, Shivanna and Owens (1989) reported that self-incompatibility in *Giliricidia sepium* is a common occurrence.

Autogamous Papilionoidae legumes are noteworthy for many interesting adaptations associated with the breeding system. When compared to related out-crossing species the flowers of the former are generally reduced in size. In some of these species selfing is facilitated by automatic rupturing of the stigmatic membrane (Arroyo, 1981). In autogamous plants, a number of species have a reproductive system which exhibit devices that ensure self-pollination, e.g. cleistogamy. In *Lettuce* species, juxtaposition of anther and stigma favor self-pollination, where the anthers dehisce as the stigma protrudes along (Lawrence, 1971).

2.2.1.3 Late acting self-incompatibility

In most cases, the incompatibility reaction doesn't proceed until the pollen tubes have reached the ovary. This phenomenon is often termed as ovarian inhibition. SI systems that operate in the ovary have been assumed to be extremely rare. However, in some notable cases the incompatibility reaction delays until the pollen tube enters the ovule (Frankel and Galun, 1977).

The late acing self-incompatibility is divided into 4 components (Seavey and Bawa, 1986).

1. Ovarian inhibition of incompatible pollen tubes before the ovule is reached.
2. Pre-fertilization inhibition in the ovule.
3. Pre-zygotic rejection of the embryo

4. Ovular inhibition for which the cytological details have not been established.

The genetic picture for late acting self-incompatibility is virtually unknown. It can be proposed that there are many obstacles to the analysis of events within the ovule. However, there is a compelling evidence for recognizing ovular self-incompatibility as a phenomenon distinct from classical SSI and GSI. Evidence from many plant species is presented to show that self-incompatibility barriers in the ovary are not as uncommon as once thought (Seavey and Bawa, 1986). Tangmicharoen and Owens (1997) reported that, pollen tube inhibition occurred in the lower portion of the ovary in *Tectona grandis*; a form of late acting self-incompatibility. In *Theobroma cacao,* the release of sperm nuclei have partially failed to fertilize the ovule (Seavey and Bawa, 1986).

2.2.1.4 Genetics of self-incompatibility systems

Self-incompatibility systems are based on the fact that the inherited character of the flower is capable of rejecting its own pollen. This is possible because the rejection phenomenon involves the recognition of identical gene products in the pollen and style (Jackson and Linskens, 1990; de Nettancourt, 1977; Lawrence, 1971). That is, the incompatibility reaction occurs between like alleles in the pollen and style. The same opinion was held as an hypothesis of oppositional factor by Heslop-Harrison (1975). The hypothesis states that, any pollen grain carrying an S allele (incompatibility allele) identical with one present in the pistil would be rejected. In most of the SSI systems, a single locus with an allelic series control the behavior of a plant in order to maintain out-breeding. It means, in SSI, pollen grains receive the gene products of the sporophyte's S-locus (loci), whereas GSI involves the activity of a recognition factor to be coded by the haploid genome (Beach and Kress, 1980).

In general, crossing of plants with different genetic constitution or selfing or inbreeding may be; 1. completely incompatible 2. half compatible and 3. fully compatible (Borojevic, 1990). In some instances, the incompatible system may spontaneously shift to compatibility. This may happen if the S gene mutates either to another active allele or to an allele which does not function. In this case the pollen tube carrying the mutated allele will grow down its own style and fertilize the ovule (Lewis, 1978).

The system of self-incompatibility is a common occurrence in diploid species as opposed to polyploids. Polyploidy generally leads to the breakdown of self rejection. For example, diploid potatoes are wholly or partially self-incompatible while tetraploids are not. Diploid species of most *Leucaena* species are self-sterile, where as the polypolid *Leucaena leucocephala* is self-fertile (Brewbaker, 1982). According to Brewbaker (1982), polyploids which produce pollen grain having two S alleles will under go "competition interaction", which will breakdown the self-incompatibility barrier. In a similar way, induced tetraploids of *Petunia*, *Trifolium* and other genera often are self-fertile immediately after doubling their chromosome, even though their parent diploids are self-incompatible (Brewbaker, 1982).

Compatibility depends in part on parental genotypes at the self-incompatibility locus and elsewhere in the genome. It may also be affected by temperature, age of pollen and pistil. Exposure of pollen to water and water status of the pistillate plant contributes to compatibility condition (Pitman and Levin, 1986).

In general, self-incompatibility is not invariably absolute; in fact it varies from 100% to a very slight preference for foreign pollen (Faegri and Van der Pijl, 1979).

2.2.1.5 Methods of overcoming self-incompatibility

In cultivated crops incompatibility effects could be overcome by artifices (Faegri and Van der Pijl, 1979). There are a number of reports that indicate the methods employed to avoid self-incompatibility barriers. These include some physical treatments which can change the physiological conditions of the stigma. Physical stresses such as electric-shock, high temperature, and applications of organic acids (like cycloheximide) on stigma breakdown SI. As soon as pollen is attached onto the surface of the stigma quick CO_2 treatment has been found to effect self-fertility in some self-incompatible species (Hinata and Okazaki, 1985).

Other physiological and genetical changes that could modify the behavior of self-incompatibility have been shown to occur spontaneously or after experimental manipulation (de Nettancourt, 1977). Sharma *et al.* (1985) have used lectins and sugar to overcome SI in *Petunia* and *Eruca* species. Other treatments include the use of irradiation, application of hormones and inhibitors and pistil grafting (Hinata and Okazaki, 1985).

Intra-ovarian pollination or fertilization of ovary cultures is also one method of overcoming SI (Faegri and Van der Pijl, 1979). When the site of the incompatibility reaction is confined to the region of stigmatic surface, removal of this part may permit adequate rate of pollen tube growth (Lawrence, 1971).

In most cases, the incompatibility phenotype is determined when the flower bud is at anthesis. It means the stage at which the plant determines its incompatibility phenotype coincides with the maturity of the flower. The incompatibility factor is expected to weaken in old and young flowers (Frankel and Galun, 1977). Therefore, based on the age of flowers, researchers have succeeded in breaking down SI by initiating pollination either on young flower buds or on old flowers.

2.2.1.5.1 Bud pollination

Bud pollination may allow slow growing pollen tubes enough time to reach the ovule (Lawrence, 1971). Many workers have successfully managed to prevent SI on both gametophytic and sporophytic systems through bud pollination. As a result, some useful inbred lines for hybrid seed production has been possible in *Brassica* species (de Nettancourt, 1977).

2.2.1.5.2 Pollination of old flowers

It has been found out that there is a gradual loss of the capacity to produce active incompatibility substances in older flowers. Therefore, aged stigma and/or pistils which are self-pollinated will allow the incompatible pollen to germinate and reach the ovary to accomplish fertilization (Shivanna and Johri, 1985). This has been evident in species of *Lilium* and *Brassica* (de Nettancourt, 1977).

2.3 Pollen grain viability and pollination

The production of pollen grains is a characteristics of all angiosperms and is by definition an integral part of the pollination processes. A pollen grain is a highly reduced male gametophyte and is generally shed in a desiccated condition (Shivanna and Johri, 1985).

Immediate and small scale weather effects may act as constraints to pollination. That is microclimate influences the timing of flower opening, anther extrusion and dehiscence. Anther dehiscence is largely mediated by environmental humidity levels such that it often takes place during the driest time of the day. Survival and germination of pollen grains and receptivity of stigma can also be affected by the microclimate around a flower bud (Corbet, 1990).

Regardless of the pollen size, pollen intensity and mode of dispersal, pollen viability is a key factor in the process of pollination and viability can be estimated using various techniques such as pollen germination method and fluorochromatic reaction test (Dafni, 1992).

2.3.1 Pollen grain viability

Maintenance and testing of pollen grain viability is an important aspect of plant breeding (Sorensson and Nagahara, 1989). Pollen grains contained in an anther are supposed to be of high quality (viable) and will be able to germinate upon landing on the stigma. However, the condition of the parent plant (physiological status) and weather conditions in general affect pollen quality. The life span of pollen of different species under natural condition varies from few hours to more than a year (Frankel and Galun, 1977).

Methods that reflect the competence of the pollen grain to perform its normal function in the pistil has been devised (Shivanna *et al.*, 1991). Pollen quality assessment for a species is some times necessary before one embarks to conduct a breeding programme and when pollen is under artificial storage condition. The two commonly used methods of testing pollen grain quality are *in vitro* germination and the fluorochromatic reaction (FCR) test (Martin, 1959). The former method is rather preferred for determining pollen vigor and the latter is more reliable for investigating pollen viability. FCR also shows correlation's with potential germinability (if mature pollen is used) (Dafni, 1992). However, this direct relationship between FCR viability and *in vitro* germination results can be affected with adverse weather conditions. For example, pollen grains of *Agave* species subjected to high relative humidity at 48 °C showed over 80% FCR, but failed to germinate *in vitro* (Shivanna *et al.*, 1991). With FCR test two properties of pollen grains are assessed (Shivanna and Johri, 1985):

- the intact condition of the plasmalemma and,
- the activity of esterase capable of cleaving the fluorescein ester, fluorescein diacetate.

FCR test can be used for both binucleate and trinucleate pollen grains. Three celled pollen grains are difficult to germinate *in vitro,* therefore, difficult to determine their viability in this way (Shivanna *et al.*, 1991). Fluorescein diacetate (FDA) method does not seem to be applicable for testing the viability of pollen grains of *Leucaena* species as both dead and live pollen fluoresce equally (Sorensson and Nagahara, 1989).

Pollen grains can germinate in water or aqueous solution of sucrose with no additives (exception, tri-cellular pollen grains need special substrate). Sucrose solutions with concentrations ranging between 0 to 50% have been used for germination tests. Optimal concentrations for pollen grains of a particular species depends on osmotic relations (Dafni, 1992), which will be identified empirically. For example a medium of 10% sucrose solution was found to be optimal for *in vitro* germination of *S. sesban* pollen grains. An optimal sucrose solution for pollen grain germination can be used to evaluate the maximal pollen grain germination rate as indicator of pollen viability (Owuor and Owino, 1993).

Pollen germination rate *in vitro* could be low, but enough for satisfactory seed set. Thus, *in vitro* results of pollen germination do not necessarily count for pollen capacity to fertilize ovules to produce seeds (Dafni, 1992).

Sources of intraspecific variation in pollen germination and viability tests might be due to pollen age, weather effect, location of flower on the canopy, time of the season, genetic variation between individuals. According to investigations on pollen physiology, longevity depends on variables such as atmospheric humidity, temperature, air composition and pressure as well as on pollen vigour. The latter may be modified by plant nutrition, viruses and other pathogens (Frankel and Galun, 1977).

It was reported that conditions such as relative humidity (RH) and extreme temperatures affect pollen vigour before affecting pollen viability (Shivanna *et al.*. 1991). RH is obviously a very important factor controlling pollen germination and the growth of pollen tubes (Pritchard and Mannetje, 1967). The same authors found out that a high humidity (93-98% R.H.) was essential for good pollen grain germination and growth in *Trifolium* species. According to Shivanna *et al.* (1991), pollen grain viability was not affected when pollen grains were treated with high relative humidity/ high temperature (98/ 45 °C) stress, but pollen vigour was significantly reduced. Comparison of pollen tube growth *in vitro* and *in situ* indicated that a pollen parent that produced long pollen tubes *in vitro* fertilize more ovules than a pollen parent that produced short pollen tubes (Shivanna and Johri, 1985).

Dafni (1992), suggested that pollen viability tests are generally used for:

1. evaluation of the fertility of a given parent plant,
2. monitoring the pollen state as a function of storage condition,
3. evaluation of the chance of pollen germination in studies of pollen stigma interaction and
4. studies of incompatibility and fertility.

Pollen grains of many crops can be stored viable for quite a long time at 4-5 °C if properly dried under vacuum and maintained in sealed containers (Frankel and Galun, 1977). Preserved pollen grains are used when fresh pollen grains are not available especially in crop breeding programs (Shivanna and Johri, 1985).

2.3.2 Receptivity of stigma

A receptive stigma is capable of permitting hydration of pollen and penetration by pollen tubes. It is likely that the stigma could be vulnerable to water loss and infection by pathogens. It is often protected or exposed briefly. In Papilionoideae, the stigma is enclosed in the keel petals. Receptivity of stigma is highly dependent on the physiological conditions of the parent plant. Moreover, the intra-floral micro-climate influences the maturation and emergence of stigma (Corbert, 1990). Stigma receptivity is a crucial stage in the maturation of the flower which may greatly influence the rate of self-pollination. The duration of stigma receptivity varies from a few hours up to 10 days. The age of the flower, the time of the day and the presence or absence of stigmatic exudate may all influence receptivity (Dafni, 1992).

A receptive stigma should receive mature pollen grains for germination and fertilization to occur. The amount of pollen grains that lands on the surface of the stigma will determine the amount of ovules to be fertilized, hence the amount of seeds to be developed (Tangmitcharoen and Owens, 1997). Pollination intensity is usually positively correlated with the number of seeds per fruit (Lee, 1988). In a particular species, the stigma of some of the flowers receive excess pollen grains and some may receive less. In others, even if there is adequate amount of pollen grains, the pollen load reaching the stigma may be low due to extended stigma. Such species may require some form of tripping that ruptures the stigma and facilitates pollen landing and receptivity. This is the case in alfalfa (*Medicago sativa*) which require mechanical pressure applied by insects for tripping the flowers (Barnes *et al.,* 1972). Environmental factors such as wind, rain, heat and cold will also influence tripping. According to Barnes *et al.* (1972), stigma of alfalfa was not covered with pollen prior to tripping. This may be due to shortage of pollen or due to an extension of stigma above the pollen mass.

2.4 Mating systems

Mating system is a description of the way in which gametes are brought together to produce offspring's of a population (O' Malley and Bawa, 1987). Therefore, the mode of transmission of genes from one generation to the next is determined by mating systems. Mating system plays a crucial role in shaping the genetic composition of populations (Hamrick, 1989). Such systems are expressed in plant species in a great variety of ways. Their genetic impact is measurable in different approaches one of which is the segregation of marker genes (e.g. isoenzymes) in progeny arrays (Brown, 1990). The mating system by which a given population reproduces plays an important role in determining its genetic structure (Brown and Allard, 1970). Detailed knowledge of the mating system is required to understand the genetic composition and evolutionary potential of plant populations (Smyth and Hamrick, 1984).

Plant species fall in one of the following five mating classes (Brown, *et al.*, 1989).

1. Predominantly self-fertilizing.

2. Predominantly out-crossing.
3. Mixed selfing and out-crossing (mixed mating).
4. Partially apomictic.
5. Partially selfing of gametophytes.

Predominant selfing and predominant out-crossing are found to be an alternative stable states of the mating system in plant populations (Lande and Schemske, 1985). Isozyme markers have been used to measure mating system parameters in natural and planted populations (Brown *et al.*, 1985).

2.4.1 Isozyme electrophoresis

The term isozyme (synonymous with isoenzyme) is defined as a multiple molecular forms of an enzyme, derived from a tissue of a single organism. Such enzymes share a common substrate but differ in electrophoretic mobility (Wendel and Weeden, 1989). The terms isozymes and allozymes have been used as synonymous although they are slightly different in their definitions. Isozymes are different forms of the same enzyme that are encoded by different gene loci, where as allozymes are different forms of the same enzyme encoded by different alleles at the same gene locus.

The basic principle behind electrophoresis is that any charged ion could migrate when placed in an electric field. Since proteins carry a net charge at any pH other than their isoelectric point, they will migrate and separate (Sargent and George, 1975). Before the technique of isozyme electrophoresis was developed, morphological and agronomic evaluation were used for cultivar identification and/or characterization. Although phenotypic evaluations from morphological and agronomic data are always important, they are not easily understood at the gene level. Isozyme electrophoresis was found to be useful in obtaining direct genetic information, such as markers which may be used to identify particular genotypes (Simpson and Withers, 1986). For example, Maass *et al.* (1993) used isozyme markers to characterize the germplasm of *Arachis pintoi*. Isozyme polymorphism was used to study the genetic diversity in the germplasm of *Arachis glabrata* (Maass and Ocampo, 1995). Isozyme data was used to asses genetic diversity within and among plant populations and examine variation between *Leucaena shannonii* varieties (Chamberlain *et al.*, 1994)

Isozymes are reliable genetic markers than those previously available. They generally exhibit Mendelian inheritance, and co-dominant expression (Brown *et al.*, 1989). Because isozymes are proteins, they can directly reflect alteration in the DNA sequences through changes in the amino acid composition (Weeden and Wendel, 1989). A major advantage of isozyme electrophoresis over morphological evaluation is that, there is no need to grow plants for so long as samples for electrophoresis can be obtained from very young seedlings. In addition to characterization or identification of cultivars or accessions, techniques of isozyme analysis have been serving as a tool in determining mating systems, gene flow and paternity within and between populations (Baret and Eckert, 1990). Furthermore, isozymes have been used as a means of distinguishing hybrid genotype from parental types (Scandalios, 1974). It has also been used in the area of systematics. Studies of zymogram (banding) pattern have verified the taxonomic value of isozyme analysis (Crawford, 1989; Feret and Bergmann, 1976). Difference in enzyme composition between several species of *Nicotiana* was demonstrated (Feret and Bergman, 1976). However, isozymes are usually expressed at a specific stage in the life of an individual plant and in materials grown under controlled conditions (Brown and Allard, 1970).

The level of genetic diversity within and among accessions can be estimated and changes in the diversity can be monitored on gene bank collections. For example, Abebe Demissie and Bjornstand (1997) used isozyme markers for genetic diversity assessment in Ethiopian land race barleys. Isozymes can also provide data on the genetic relationship within and between natural populations and indicate most efficient approach to future sampling (Simpson and Withers, 1986). The identification of clones using isozyme bands could be useful for identifying the identity of suspected or mislabeled clones. Seed certification has also used the techniques of isozyme electrophoresis which is directly dependent on the genetic (allelic) composition of a population (Feret and

Bergmann, 1976). The clear genetic basis of isozyme variability has made them a more favourable markers than low molecular weight markers such as flavonoids or phenolics (Abebe Demissie, 1996). However, there are some inherent problems to detect all changes that occurred at DNA level, which makes isozymes handicapped. Generally, it seems probable that for years to come isozyme electrophoresis might continue to be the cheapest and easiest to use for studying variation and solve many important problems in population biology (Pasteur *et al.*, 1988).

Generally, among electrophoretic techniques, isozyme analysis has been widely used for its relative efficiency and cost effectiveness, particularly in studies of intraspecific variability (Kephart, 1990).

2.4.2 Isozymes in plant mating systems

The development of isozyme markers has opened up a new era in genetic studies of plant mating systems. Isozyme electrophoretic technique utilizes the polymorphic condition of isozymes that are common and readily detectable. Using such markers, the level of inbreeding and out-crossing have been determined for a large number of plant species (Table 2). Isozyme markers have gained popularity in determining out-crossing rates in plant populations due to their co-dominant expression and high degree of polymorphism (Brown *et al.*, 1989).

The proportion of progenies resulting from self-fertilization (s) and from out-crossing ($t = 1\text{-}s$) each generation are the parameters most commonly used to describe the mating systems (Shaw and Allard, 1982). The probability that an out-crossed individual will be detected on maternal genotype depends on whether the pollen grains carries a non-maternal allele at any one of the loci assayed (Hamrick and Godt, 1990).

Table 2. Some plant species for which mating system was studied using isozyme markers. *NP = Natural population, PP = Planted population, t % = % out-crossing*

Species	**Family**	**No. pop.**	**No. Loci**	**t %**	**Source**
Eucalyptus pauciflora	Myrtaceae	7NP	4	63	Philips & Brown 1977
Hordeum spontaneum	Poaceae	26NP	22	1.6	Brown *et al.*, 1978
H. vulgare	Poaceae	1PP	4	1	Wagner & Allard, 1991
Carduus nutans	Asteraceae	2	2	1	Smyth & Hamrick, 1984
Medicago sativa	Leguminosae	3PP	2	76	Knapp & Teuber, 1993
Pithecellobium pedicellare	Leguminosae	1NP	4	95	O' Malley & Bawa, 1987
Pseudotsuga menziessii	Pinaceae	8NP	11	90	Shaw & Allard, 1982
Sorghum bicolor	Poaceae	2PP	1	46	Ellstrand & Foster, 1983
Pinus radiata	Pinaceae	1PP	4	98	Moran *et al.*, 1980
Zea mays	Poaceae	2PP	9	97	Brown & Allard , 1970
Cordia alliodora	Boraginaceae	2NP	4	97	Boshier *et al.*, 1995
Helianthus annus	Asteraceae	5NP	1	73	Ellstrand *et al.*, 1978

Results from studies of mating systems have been helpful for understanding the evolutionary potential and their impact on population structure of species (Hamrick, 1989). A small amount of out-crossing has a profound effect on a predominantly self-fertilizing species. For example, species such as *Hordeum vulgare* L., *Phaseolus lantus* L. and *Avena fatua* L. are assumed to be predominantly selfing, but it has been variously shown that the rate of out-crossing in these species ranges between 1 to 10%. This level of out-crossing could play a significance role in the genetic structure of plant populations (Jain, 1979).

Ritland and Jain (1981) reported that, the genetic structure and population dynamics are fundamentally affected by the breeding system. Therefore, an accurate estimate of out-crossing rate helps in evaluating the various hypotheses regarding the effects of breeding systems as well as for planning breeding programmes.

Out-crossing rate has been estimated based on morphological markers for a large number of plant species. Indeterminate growth habit and normal floral morphology were used as dominant markers. The rate of out-crossing (0.14 - 19 %) was estimated as percentage of the observed hybrid plants for cleistogamous *Cajanus cajan* (pigeonpea) lines (Saxena *et al.*, 1994). Similarly, flower colour was used as a marker to estimate out-crossing rate in many tropical forage legume species such as *Chamaecrista rotundifolia* (13%), *Centrosoma virginianum* (18%), *Codariocalyx gyroides* (23%), *Desmodium heterocarpon* (4%), and *Galactia striata* (13%) (Maass and Torres, 1998). Beri *et al.* (1985) also used flower colour to study the extent of natural out-crossing (2.63%) in Egyptian clover. Morphological markers may sometimes lead to biased out-crossing rate estimates. Pedersen *et al.* (1972) estimated **t** for alfalfa as high as 96% which is quite high when compared to that estimate (t = 70%) obtained by Knapp and Teuber (1993) based on isozyme markers. Jain (1979) has noted that the use of several methods of out-crossing estimation even on the same population may not lead to concordant results. For example, out-crossing estimates in barley using morphological and isozyme loci seem to be significantly different (Jain, 1979).

Difference in out-crossing rate was noted on seeds collected from the lower and upper portion of the crown of douglas-fir (*Pseudotsuga menziesii*) (Shaw and Allard, 1982). They also found out that some trees selfed at a much higher rate than other trees. In this particular species out-crossing rate (t = 0.90) for natural stands and cultivated plants (seed orchards) was found to be the same. Similarly, out-crossing rates within natural stands of wild barley were found to be equal to rates observed within cultivated populations of barley (Wagner and Allard, 1991).

Plant mating systems should be just as variable as any other characteristics and therefore should vary among populations and years and several studies have indicated that estimates of out-crossing vary greatly among populations (Hamrick, 1982).

According to Saxena *et al.* (1994), out-crossing rate varied from environment to environment on the population of *Cajanus cajan*. Similarly, Brown *et al.* (1978) have found out that t is high in populations growing in more mesic regions (2.1%) than in the xeric (0.4%) environment using isozyme markers for *Hordeum spontanium* populations. In contrast, Ellstrand *et al.* (1978), reported that t is higher in more dry sites than wet ones, although the difference is not significant. Out-crossing rate can also be variable depending on population structure. Out-crossing rate of *Sorghum bicolor* was estimated to be 0.22 and 0.46 for populations which were stratified and over-dispersed respectively (Ellstrand and Foster, 1983). So the physical arrangement of individuals in the population will definitely have an impact on the rate of out-crossing. In barley which is highly self-fertilizing species, out-crossing occurs between adjacent plants and rare out-crosses are expected at distances of 1 m or more. The physical contact between plants increases the average out-crossing rate (Wagner and Allard, 1991). There is also variation in out-crossing rate estimates among natural populations of the same species at different locations or over years within the same population (Hamrick, 1982). Generally, out crossing rates of plants are not unchangeable. Rates can vary between different flowers on the same plant, different plants in the same population and between different population of the same species (Brown *et al.*, 1989).

Different researchers have used varying number of isozyme marker loci and populations for estimating out-crossing rate for different species of plants (see Table 2). Various enzyme systems have been assayed and those which were polymorphic were selected and used as markers. For example, Moran *et al.* (1980) assayed 22 allozyme loci and obtained 10 polymorphic ones to determine out-crossing rate of *Pinus radiata*. Ellstrand and Foster (1983) used one polymorphic locus of Alcohol dehydrogenase (Adh) to estimate out-crossing in 2 cultivated populations of

Sorghum bicolor. The choice of number of loci for estimating **t** depends on whether a species is predominantly selfing or out-crossing. Shaw and Brown (1982) have indicated that the maximum efficiency for estimation of out-crossing in highly inbreeding species comes from assaying large number of samples on few polymorphic loci. Based on this assumption, one to many polymorphic loci have been used as markers to estimate out-crossing for different species. According to Shaw and Brown (1982), once the most polymorphic locus is identified it will be more efficient to score more plants on this locus than fewer plants on more loci; except in a highly out-crossing population. These authors have further strengthened their assumptions by saying that " The experimental effort required to assay one plant at two loci is the same as that required to assay two plants at one locus". When there is no limit in attaining more polymorphic loci, Ritland and Jain (1981) advice that three or four loci will give more accurate estimates and more often this will provide the minimum possible variance.

Currently, knowledge in molecular genetics such as random amplified polymorphic DNA (RAPD) nulliplex analysis are being used to estimate out-crossing rates. Using this technique, the rate of out-crossing for auto-tetraploid alfalfa was determined (Gjuric and Smith, 1995). Such results might be relatively more accurate, as they are reflections of changes that are occurring directly at the DNA level. DNA markers (RAPD and rDNA, intergeneic spacer length polymorphism) were used to study genetic variability, mating systems and population genetic structure of three species of *Centrosoma* (Penteado, 1996).

3. MATERIALS AND METHODS

Floral biology studies were conducted on mature plants (4 years old) of one accession of *S. sesban* var. bicolor (origin Rwanda, ILRI Accession No. 15022). The plants were established in 1993 on 600 m^2 area at the compound of the International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia.

3.1 Maturity of flower buds: Anther dehiscence and stigma receptivity

Ten very young flower buds (approximately < 10 mm) were tagged on each of 5 different randomly selected trees and frequent observation was conducted to study stages in flower maturity. Differences in size of the buds and maturity of the flowers were recorded on a daily basis. The variation in development was detected by measuring the size of each bud and observing changes in colour. At the different stages of maturity, some of the flowers were opened using fine forceps and observed the internal floral (stigma and anthers) arrangement and the length of the pistils was noted.

To determine the relationship between size of bud, anther dehiscence and stigma receptivity 10 flower buds were picked from 10 different trees. The size of each bud was measured and simultaneously opened to check for pollen shedding and stigma receptivity using a hand lens (BAUCH & LOMB Magnifier) or a binocular microscope (CARL-ZEISS 4750 03-9901). Anther dehiscence intensity was considered as equal in the various flower buds. Pollen shedding buds were recorded as positive (+) and those with intact anthers as negative (-). Flower buds with receptive stigma and non-receptive stigma were also scored as positive and negative respectively. The experiment was done at peak flowering period.

Test of receptivity of stigma was also performed through controlled *in vivo* pollination. Flower buds with intact anthers but not too young were tagged on 10 randomly selected trees. 10 flower buds were considered from each tree. Those buds which had already shed their pollen were disregarded.

The size of each bud was measured and self-pollination was initiated using forceps on each flower bud. Other group of flowers of the same size were emasculated and pollinated with mature pollen grains from different flowers on the same trees. This was done to avoid the confusion that pollen grain germination failure could be either due to immaturity of the pollen grains or non-receptivity of the stigma. Self-pollinated flowers were then tagged and bagged for 24 hours. These buds were collected and pistils were excised using forceps and scalpel blades. Pistils were fixed in ethyl-alcohol : acetic acid (3:1) solution for 24 hours. This solution was poured off and pistils were softened in 4 M sodium hydroxide overnight. After pouring off the NaOH solution the pistils were soaked for 4 hours in 0.1% solution of aniline blue dissolved in 0.1 M tri-potassium orthophosphate. Each pistil was placed on a microscopic glass slide and squashed gently. Finally, pollen tube growth into the style was observed under fluorescent microscope (OLYMPUS-BX60) (Martin, 1959). Pollen tube penetration was scored for each style by 0 for no pollen tube growth, 1 for few pollen tubes, 2 for many pollen tubes and 3 for abundant pollen tubes (Hinata and Okazaki, 1985). Pictures showing pollen tube growth within styles were taken using an Olympus Camera (C-35AD-4) fitted onto the fluorescent microscope and an Olympus Automatic Photomicrographic System (model PM-10A DS).

3.2 Effect of tripping flowers

To study the effect of tripping flowers of *S. sesban,* 30 flower buds were tagged on 5 randomly selected trees. Their size was measured and two methods of tripping were employed to initiate self-pollination (Barnes *et al.,* 1972). Ten flowers were used for each treatment and the control (bagging) on a tree and 50 flower buds were used in total for each treatment.

a) The petals were opened and toothpicks were inserted at the position of anthers and rotated inside to initiate pollen shedding and stigma receptivity.

b) Tripping by hand was performed by rubbing the tip of the flowers (at the position of anthers) between the thumb and index fingers. The flowers were tagged and bagged after the tripping treatment.

c) Non-tripped flowers were bagged for the same period of time. After 24 hours, the style of each flower was checked for pollen tube penetration (Martin, 1959). Rate of pollen tube abundance was recorded for each style. Pollen tube abundance was used as an indicator of the best form of tripping.

3.2.1 Tripping and seed production

Sixty flower buds of the same size were tagged on each of 2 different trees and 2 mechanisms of tripping were employed to study their effect on the amount of seed production. For each treatment 30 flower buds were used on each tree, and 60 flowers for each treatment in total.

1. Toothpick tripping. A sharp pointed toothpick was inserted at the position of anthers and rotated gently inside to break the anthers.
2. Finger tripping. The thumb and index fingers were used to rub the flower buds at the tip. After each treatment the flowers were bagged and tagged. The bags were removed after 120 hours, the time lapse which was found to be enough for fertilization to have occurred. Those flowers which formed pods were left with their tags until the pods were ripe enough for harvest. Seeds from each pod were counted and their mean number from each treatment was compared with seeds produced from open-pollination and bagging on the same trees.

3.2.2 Self-pollen germination and pod development

Germination of self-pollen grains and pod development was studied under natural condition (without tripping). Flower buds that fall in the range of 16 and 25 mm were picked from a tree. 20 flower buds were examined for each measurement. Bud size in mm, internal floral conditions (anther dehiscence, pollination of stigma) were noted. Pistils were then excised and soaked to detect pollen grain germination (Martin, 1959). Number of flowers that allowed pollen tube penetration were recorded.

Abortion of flowers was assessed by tagging (but not bagged) flowers on different trees and followed their development until mature pods were developed. Three inflorescence carrying varied number of flower buds were tagged on 10 randomly selected trees (n = 261 flower buds). Observation of pod development was performed every three days for the first 2 weeks, then once in a week until the formed pods were fully mature. Number of flowers that dropped before pod formation were considered as aborted. This experiment was conducted between September and December 1998 to avoid the effect of heavy rain on flower dropping.

3. 3 Test for self-incompatibility (SI)

3.3.1 Reciprocal cross (*in vivo* test)

Reciprocal cross was performed on 10 randomly selected trees of one accession (ILRI No.15022) of *S. sesban* plants. Flower buds which were at the right stage (14 – 15 mm) for emasculation were selected. Emasculation was done between 9 and 12 AM by first carefully opening the standard, wing and then the keel petals with fine forceps. All the 10 anthers were carefully removed without causing damage on the pistil as much as possible. Any suspicion of anther dehiscence before and after emasculation was checked by a hand lens. Artificial cross-pollination was completed the next morning. The inflorescence bearing pollinated flowers were tagged and covered with pollination bags. The bags were made with minute holes to allow the movement of air but small enough to prevent the entrance of insects. Flowers were collected after 24 hours and checked for pollen tube growth in the pistil (Martin, 1959). Data was recorded by assigning positive (+) for pollen penetration into the style and negative (-) for no pollen tube in the style.

3.3.2 *In vitro* test for self-incompatibility

Ten stigmas, 10 styles and 10 ovaries were excised and collected separately in ice-cold pestle and mortar. These parts were crushed with pollen grain germination solution (medium) prepared from 15% sucrose + 0.01% Boric acid dissolved in Calcium phosphate buffer (pH 5.9). The stigma, styles and ovaries were crushed in 1ml, 2.5 ml, and 10 ml, of the same solution respectively (Sharma and Shaven, 1986) in proportion to the size of the tissues. Few pollen grains were picked from mature flowers and mixed with 2 drops of the extract on microscopic glass slide. The glass slides were covered in a tray and incubated in a seed germination incubator (CONSOLE-STYLE GERMINATOR D-7100) for 3 hours at 30 °C and 90% relative humidity (RH). Germinated pollen grains were counted using an Olympus Microscope (Model-Olympus BH2) with a magnification of X100, under 15 field of view. Ovules were also excised from 10 ovaries and the extract was made with 1.5 ml of the germination medium and the effect was tested on cross- and self-pollen germination.

Extract of leaf samples (equal weight to the ovary sample) was also tested for pollen grain germination and finally freeze dried samples of the ovaries and leaves (0.1 g of each) were analyzed for tannin content. This experiment was performed to investigate that chemical compounds present in either the ovary or leaf are capable of inhibiting pollen grain germination and the result will prove that the ovary extract is free of incompatibility substances.

3.4 Assessment of the quality of pollen grains

Pollen grain quality can be assessed either through pollen grain germination technique or the fluorescence method (Dafni, 1992).

3.4.1. Germination of pollen grains in sucrose

Sucrose concentrations of 5%, 10%, 15%, 20%, 25%, and 30% were tested. Each concentration was made by dissolving the sugar in Calcium phosphate buffer (0.015M and pH 5.9) containing Boric acid (0.01%). From 3 randomly selected trees, 3 flower buds of different size (17-24 mm) were picked and the size of each bud measured. Then each bud was opened using a fine forceps and few pollen grains were picked and placed on 2 drops of each sucrose concentration on a microscopic glass slide. The pollen grains were uniformly dispersed in the drops and incubated in an incubator at 30 °C and 90% RH for 3 hours. The number of germinating pollen grains were counted using an Olympus microscope under X100 magnification and 20 field of view for each concentration. A pollen grain was scored as germinated if an entire tube produced is greater than the grain diameter (Roberts *et al.*, 1982).

3.4.2 Fluorochromatic reaction test (FCR)

Twelve flower buds ranging in size between 11 and 22 mm were collected from a tree. Pollen grain viability test was performed using fluorescein diacetate (FDA) solution. To prepare FDA solution, 0.01g of FDA was first dissolved in 10 ml of acetone. Then 0.5 M of sucrose was prepared by dissolving 17.1 g in 100 ml of water. 0.1ml of FDA in acetone was mixed with the sucrose solution. This final solution (10^{-6} M FDA) was used as a working solution of FDA (Kasten, 1984). Few pollen grains were picked from each bud with forceps and placed in 2 drops of the solution on microscopic glass slides. Pollen was uniformly dispersed in 2 drops of this solution and the count of fluorescing and the non-fluorescing grains was taken within 1 hour from 20 field of view. Those pollen grains which fluoresce bright golden yellow were counted as viable. Empty, undeveloped or dead pollen grains did not fluoresce at all. Those which are loosing their viability due to age or pathogen infection will have a dim fluorescence (Dafni, 1992) and were counted as not viable. The same range of flower buds were simultaneously assessed for pollen grain germination in 20% sucrose solution. Pollen grains were allowed to germinate for 6 hours under 30 °C and 95% relative humidity. The incubation time was extended to 6 hours to obtain the maximum germination possible. More pollen grains germinate in a medium with increased hydration time (Shivanna and Johri, 1985). Pollen grain viability with FCR was compared with pollen grain germination in 20% sucrose.

Pollen grain viability was also determined under natural condition using FCR test. For this experiment, flower buds which were in the verge of opening were selected and tagged. 10 buds on each of three different trees were used at different times. 10 microscopic glass slides, 10 fine forceps and fluorescein-diacetate working solution were taken to the field near a tree. Two drops of the solution were put on each slide. Few pollen grains were carefully picked at a time from individual buds using forceps and mixed with the solution on each slide. These were then taken to the laboratory and pollen grains were checked for viability using fluorescence microscope. Viability of pollen grain from each flower was tested 2 times (at 10 AM and 4 PM) during a day. This was continued until the percentage viability was highly reduced in each flower bud. The result from this experiment was used to determine approximately for how long pollen grains remain viable after the flower was opened.

3.5 Isozyme electrophoresis

3.5.1 Equipment

Vertical gel electrophoresis apparatus from SIGMA-ALDRICH and electrophoresis power supply (EPS 500/400) from PHARMACIA were used for electrophoresis. The power supply unit could provide constant voltage, making possible the regulation of the amount of heat that could be produced. A cold incubator (Minicoldlab-2023) which was adjusted to 4 °C was used to house the electrophoretic apparatus providing a cool environment suitable for isozyme electrophoresis. A pair of glass plates, spacers, clips and a silicon rubber gasket (all from SIGMA) were used to form slab gels.

3.5.2 Seed collection

Seeds were collected from Ethiopia and Zambia. Seeds from Zambia (collection number ZAM-R024) were collected from natural stands of *S. sesban* population growing along the shore of Lake Bangweula. The lake is situated at an altitude of 1120 m (latitude 11.20' S and longitude 29.35' E). The collection was made by the Gene bank of ILRI in 1993 for the purpose of isozyme electrophoresis. 20 families (individual trees) were randomly selected and 20 pods per tree were collected and seeds bulked from each tree.

In Ethiopia seeds were collected from 2 sites, Debrezeit and Addis Ababa. At Debrezeit (altitude 1850 m, latitude 8° 44' N and longitude 38° 58' E) ILRI research station, one accession of *S. sesban* var. nubica, (origin Zaire, ILRI Accession No. 15019) representing one population was cultivated for seed increase in 1994. Since the number of trees were limited here, only 10 trees were considered. 20 pods per tree were picked and their seeds bulked separately. In Addis (altitude 2380 m, latitude 9° 00' N longitude 38° 45' E), at ILRI compound one accession (ILRI Accession No. 15022) was planted in 1993. In 600 m^2 area there were about 80 plants, out of which 20 trees were randomly selected and 20 pods were picked from each and seeds were mixed from a tree. When sampling was done during pod collection all possible directions on the canopy of each tree was taken in to consideration to finally homogenize the seeds.

A number of seeds were assayed from each family (Table 3) to determine the maternal tree genotypes and to assess the banding pattern of the progeny arrays, and finally the progeny genotypic data was used for estimation of out-crossing rate (Ritland, 1994).

3.5.3 Seed germination and sample extraction

Seeds were gently scarified using sandpaper and set to germinate under aseptic condition in a controlled environment in an incubator (GALLENKAMP cooled incubators IR 211 GA) adjusted to 25-29 °C with 12 hours light and 12 hours darkness. Four days old seedlings were used to extract the enzymes. Individual seedlings were crushed in ice-cold pestle and mortar with extraction buffer. The extraction buffer was prepared from sucrose 15%, Polyvinyl-pyrrolidone (PVP-40,000) 5%, Triton (X-100) 0.5% and Mercaptoethanol 100 ul. These were dissolved in Tris-HCl 0.05 M and the pH was adjusted to 8.3. Mercaptoethanol was added after the pH was adjusted under fume-hood to avoid the bad smell. The ratio of sample (seedling weight) to volume of extraction buffer was 1g to 0.6 ml (Hussain *et al.*, 1988). Each crashed sample was collected in micro-centrifuge tubes (1.5 ml, Fisher brand) and centrifuged (EPPENDORF MICRO-CENTRIFUGE 5415 C) at 12000 rpm for 10 minutes. The supernatant from each tube was collected separately in small eppendorf tubes (0.5 ml) and then mixed (VORTEX-Gennie 2) with a drop of bromophenol blue (tracking dye). This final extract can be used immediately or can be kept at -20 °C for few days.

A minimum of 30 seeds were assayed for Mdh from each family of Addis (Acc. No. 15022) and Zambia (Zam-R024) populations and 60 seeds were assayed from each family of Debrezeit (Acc. No. 15019) population (Table 3).

Table 3. *S. sesban* populations and the number of progeny arrays used in the electrophoretic assay. (*Acc. No. = Accession Number*).

Population	No. of Trees Sampled	No. of pods Collected	No. of progenies assayed
Acc. No. 15019	10	20	10 x 60
Acc. No. 15022	20	20	20 x 30
Zam-R024	20	20	20 x 30

3.5.4 Gel preparation and the buffer systems

Polyacrylamide gels were prepared from the following chemicals. All chemicals were bought from BDH chemical company.

1. Acrylamide solution was prepared by dissolving 30 g of acrylamide and 0.8 g of bis-acrylamide in 70 ml of distilled deionized water (DDW) and the final volume was brought to 100 ml.
2. Resolving gel buffer was prepared from 36.3 g Tris (hydroxymethyl) methylamine + 35 ml of 1 M HCl and adjusted to pH 8.8 and the final volume was filled to 100 ml with DDW.
3. Stacking gel buffer was prepared from 6.0 g Tris + 40 ml water (DDW) + 35 ml of 1 M HCl and adjusted to pH 6.8 and the final volume was brought to 100 ml.
4. Sodium deodecyl sulphate (10 % SDS) solution. It was used as supplied by the company.
5. Ammonium per sulphate was bought in powder form and 0.15 g was dissolved in 10 ml of water and kept as stock solution at 4 °C for few days or it could be prepared fresh just before use because of its instability.
6. Water (DDW)
7. TEMED (NNN'N'-Tetramethylethylene diamine) solution. It was used as supplied by the company. Acrylamide, resolving gel buffer, and stacking gel buffer stock solutions were filtered with Whatman No. 1 filter paper and stored in a refrigerator (4 °C). The volume to be taken from each ingredient is decided based upon the required concentration of a gel. In this study the final concentration of acrylamide

was preferred to be 10% and the gel was 1 mm thick. The reservoir or electrode buffer was prepared from Tris and glycine. Three gram of tris and 14.4 g of glycine were dissolved in 800 ml of distilled water and pH was adjusted to 8.3 and the final volume was brought to 1000 ml (Hames, 1990).

Once the ingredients were mixed, the resolving gel solution was first poured in the space available between a pair of glass plates held by clips leaving some space for stacking gel buffer solution. Distilled and deionized water (DDW) was poured over the resolving gel solution to avoid oxygen interference in gel polymeraization. Polymeraization required about 20 minutes. After the resolving gel was polymeraized the water was poured off and the stacking solution was poured in the remaining space. Then, a comb was inserted immediately to form the wells. Gels prepared were allowed to cool at 4 °C before they were set for sample loading. The discontinuous buffer system was used for all the electrophoretic runs.

The chemicals were weighed using a sensitive balance (METTLER AE 200) and pH was measured by a digital pH-meter (HANNA Instruments 8581). The recipe for gel and buffer preparations were followed from that of Hames (1990) and Hussain *et al.*, (1988).

3.5.5 Sample loading and gel staining

The gel with pre-formed wells was mounted onto an electrophoretic apparatus with upper and lower buffer compartments. The compartments were filled with electrode buffer. A sample volume of 20 µl was applied in each well using eppendorf gel loader tips fitted onto an eppendorf micropipette (5 – 20 µl). A constant voltage of 225 V was used for 2 - 3 hours. Under this electrophoretic condition the tracking dye was expected to move 8 cm, a distance within which most of the enzyme loci could be resolved.

10 seeds from some of the families in each population were initially assayed and gels were screened (stained) for five enzyme systems. (1) Peroxidase (Prx) - 1.11.1.7 (2) Esterase (Est) - 3.1.1.1 (3)

Malate dehydrogenase (Mdh) – 1.1.1.40 (4) Acid phosphatase (Acp) – 3.1.3.2 and (5) Alcohol dehydrogenase (Adh) – 1.1.1.1] (Manchenko, 1994) to assess polymorphism. These were the enzyme systems for which specific substrates, cofactors and dyes were available. Finally, the populations that showed heterozygous bands were further assayed (Brown *et al.*, 1975) for Malate dehydrogenase (Mdh) for increased number of seeds.

Staining for Mdh requires (Pasture *et al.*, 1988):

Tris-HCl (pH 7-8)	20ml
Mg Cl_2 (0.5 M)	1.5ml
Malic acid 0.2 M (pH 7)	1.0ml
NADP (1% in DDW)	0.1ml
MTT (1% in DDW)	0.2ml
PMS (1% in DDW)	0.1ml

After a gel was immersed in the staining solution in a staining tray (plastic tray with a lid) it was incubated at 37 °C in dark for 1 hour and blue bands were detected. Mdh also Known as Malic enzyme is dimeric (Manchenko, 1994).

3.5.6 Data collection

The first locus at 3.5 cm was the only polymorhic locus of Mdh, which was scored and used in the estimation of the mating system parametres in *S. sesban*. The bands at this locus were assigned their respective genotypes. Bands are expressions of several genes at several loci or expressions of several alleles of a given gene at a given locus (Pasteur *et al.*, 1988). Homozygous individuals revealed one band which was either fast (FF) or slow (SS) and the heterozygotes showed 3 bands and were assigned FS (fast and slow). The middle band in the heterozygotes is considered to be a hybrid resulting from the joint action of the two alleles F and S (Pasteur *et al.*, 1988).

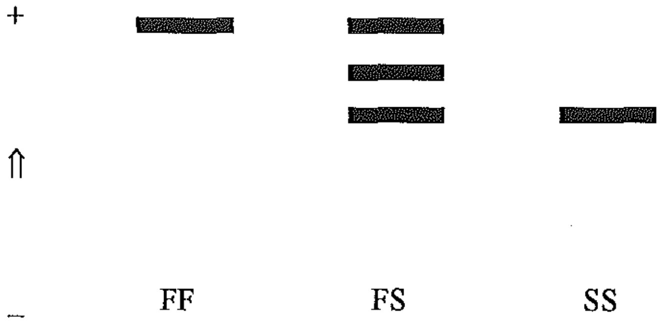

Figure 1. Symbolic representation of banding pattern of a dimeric enzyme and genotype assignment.

3.5.7 Data analysis

Means ± the standard error of means were determined where necessary using analysis of variance (ANOVA). Duncan's multiple range test in SAS/ stat. (1997) was used to compare the means if there was significance difference among the variables. Multi-locus mating system (MLTR) programme of Ritland (1994) was employed to analyze the genotype data of the progeny arrays to estimate mating system parametres. Variances for population out-crossing estimates were based on 100 bootstraps using the Newton Raphson (NR-) method (Ritland, 1994). For convenience, in the analysis of out-crossing rate, numeric assignment of the genotypes is a prerequisite for the MLTR computer programme. Therefore, fast bands (FF) were assigned 11, slow bands (SS) as 22 and the heterozygotes (FS) were designated 12. These numeric assignments were entered in to the format of the programme and paternal pollen and ovule allele frequencies and most likely maternal genotypes were determined. This genotype data of the progeny arrays from the polymorphic Mdh-1 locus was used at the same time to estimate the apparent out-crossing rate for the 3 populations of *S. sesban*.

To determine whether the observed genotype frequencies correspond to the panmictic value of the Hardy-Weinberg genotype frequencies, the expected genotypic frequencies and the corresponding expected number of individuals in the 3 populations were calculated. Finally, Chi- square values were calculated for goodness of fit test.

4. Results

4.1 Stages in flower maturity

Four stages of a flower can be recognized from the floral observation of *S. sesban.*

Stage one: At early bud stage (< 11 mm), three fourth of the length of the flower bud was covered with sepals and the colour generally looks green. The tip of the style was curled and the whole pistil from the base of the ovary to the stigma measured between 9 and 10 mm. Some of the anthers lie slightly above the stigma and somehow in contact with it.

Stage two: This stage was characterized by the gradual turning of the flower colour from green to pale yellow and the average size was 14 mm. Half of the length of the bud was covered with sepals. At this stage the anthers were intact and were in line with the stigma with an alternate arrangement. The pistil measured between 12 and 13 mm.

Stage three: As the flower bud gets elongated the sepals cover one third of the bud length only and the standard petals were in the verge of opening for some flowers. The petals become fully mottled and the flowers at this stage ranged in size between 16 and 18 mm. The pistil measured about 15 to 17 mm. The anthers had started shedding and in some flowers the surface of the stigma was seen slightly above the anthers and in some cases the stigma was partly and / or wholly self-pollinated.

Stage four: The standard petal had begun opening and the buds measured between 19-22 mm for different trees. The anthers had fully burst and the stigma was fully covered with pollen mass except in some 'extended stigmas'. In more mature flowers that measured up to 25 mm, the standard petals were completely opened and the anthers were completely withered. In old flowers where fertilization had occurred, the petals collapsed and come together due to lose of turgidity.

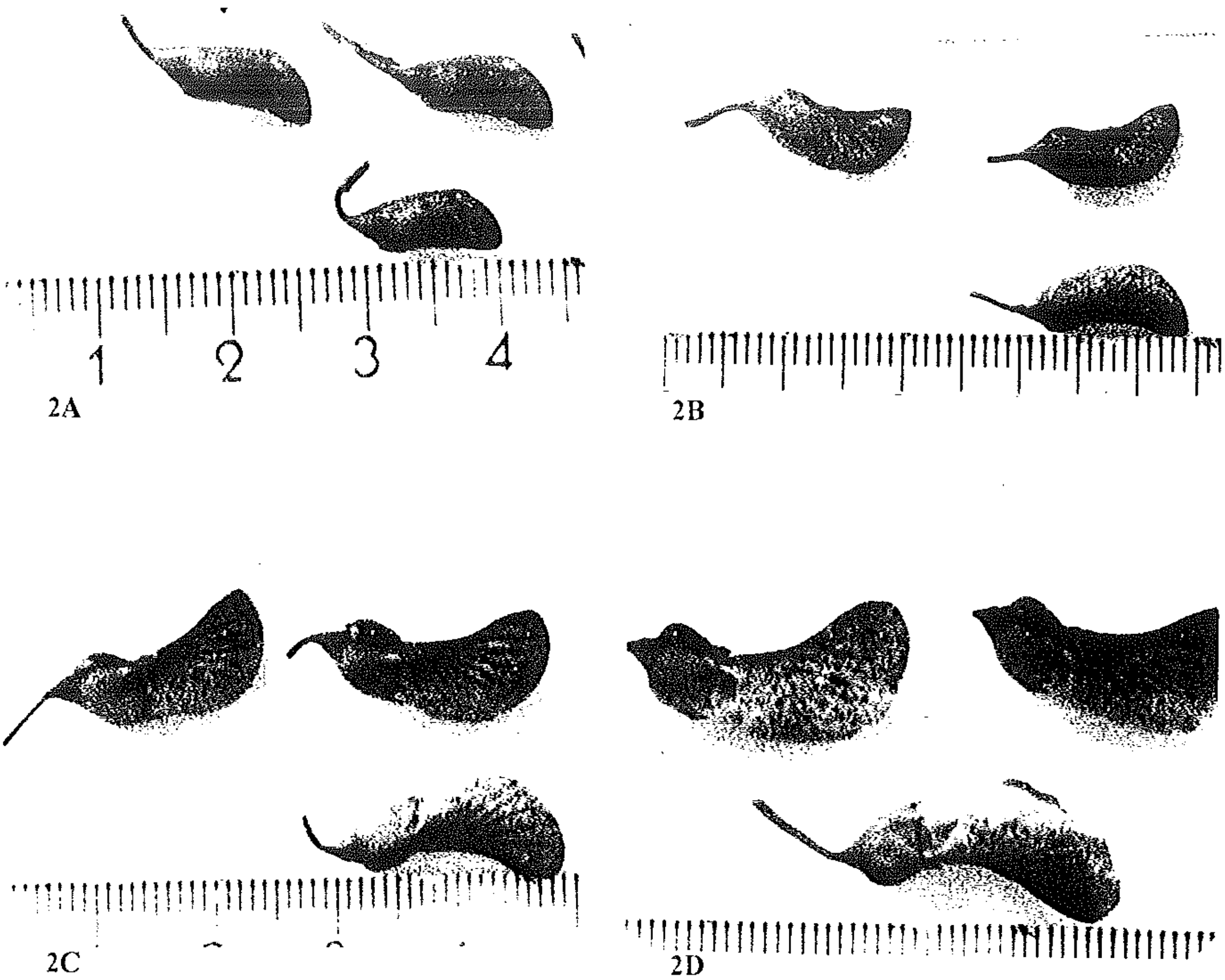

Figure 2 (A-D). Appearance of flower buds at different stages of maturity (2A. stage 1, 2B. stage 2, 2C. stage 3 and 2D. stage 4).

In Figure 3 the internal floral structures of some flowers of *S. sesban* are displayed. The variation in their developmental stage is indicated by the differnce in size of the respective parts (standard, wing, keel petals and the pistils).

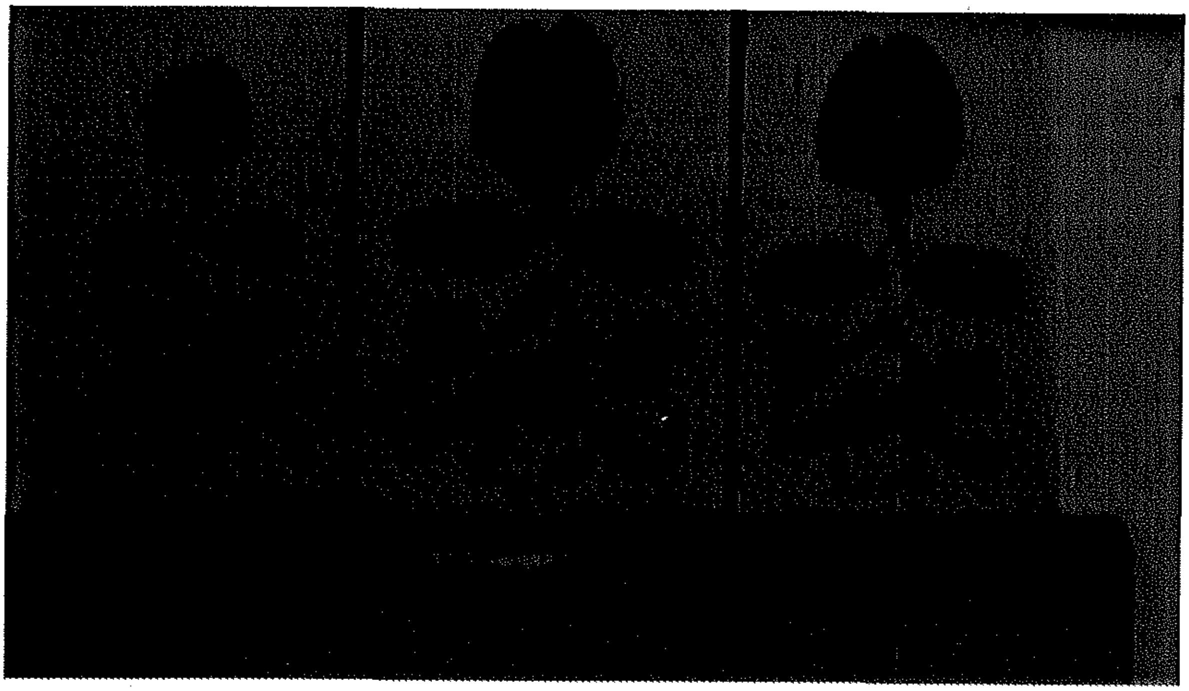

Figure 3. Internal floral structures of *S. sesban* at stage 2, 4 and 3 showing the standard, wing and keel petals and pistils from top to bottom.

A certain size of bud will be at a stage where the stigma is receptive, and in the same flower bud the anthers may or may not dehisce, e.g. some of the 14 mm buds usually have receptive stigma but the anthers were not dehiscing (Figure 4). This characteristic indicated the protogynous condition of the flowers of *S. sesban*.

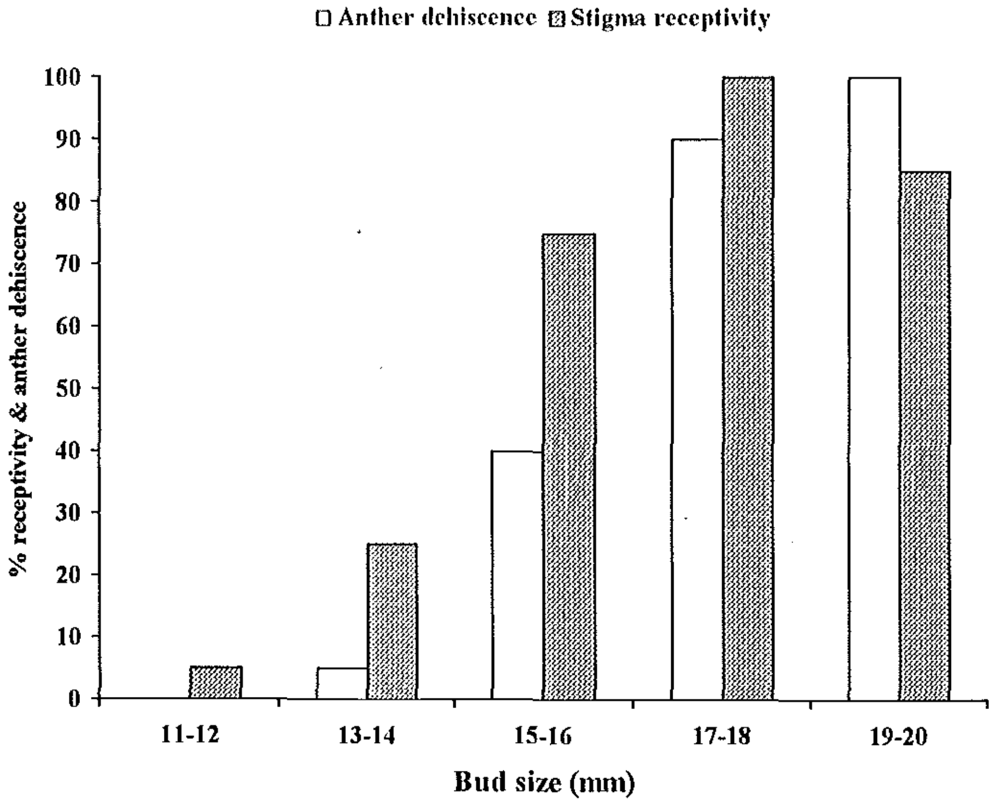

Figure 4. Range of flower bud sizes compared for anther dehiscence and stigma receptivity.

In Figure 4 it is shown that anther dehiscence occurred in few number of flower buds before anthesis, i.e., stigma becomes receptive before pollen shedding. Similarly, after the same size of flower buds (13 – 15 mm) were pollinated with mature pollen and some with immature pollen, it was possible to detect receptivity in the former ones. This indicated that stigma receptivity is also a head of pollen grain maturity. A receptive stigma displays a shiny surface which is absent in younger flower buds (e.g. < 13 mm) and fades away as the flower gets older. It was found that stigma stays receptive for at least 5 days and pollen grains could remain viable for at least 72 hours after the beginning of flower opening.

Controlled self-pollination indicated that there was statistically significant mean size difference between the flower buds with receptive stigma ($\bar{x} = 14.0 \pm 1.46$) and those with non-receptive ($\bar{x} =$

12.1 ± 1.84) stigma at $P < 0.001$. Abundant pollen tube growth was also observed in mature flower buds ($\bar{x}$ = 16.53 mm). Those flower buds which allowed few number of pollen tube penetration were the younger ones and their mean size ($\bar{x}$ = 13.50 mm) was statistically different from those which accommodated more pollen tubes from deliberate self-pollination (Table 4).

Statistically there was also a significant difference between means size of pollen shedding flower buds ($\bar{x}$ = 15.39 ± 2.28) and non-shedding ones ($\bar{x}$ = 10.68 ± 2.36) at $P < 0.001$ level of significance.

Table 4. Mean size of flower buds and pollen tube abundance in the style from deliberate self-pollination using forceps.

Pollen tube abundance	**Mean size of buds (mm)**
3 (abundant pollen tubes)	16.53^{a}
2 (many pollen tubes)	$15.93^{a,b}$
1 (few pollen tubes)	$15.22^{b,c}$
0 (no pollen tubes)	13.50^{c}

Means with the same letter superscript are not significantly different at $P < 0.001$.

The degree of receptivity of stigma varies from flower to flower depending on the developmental stage of each flower bud. Hence, the number of penetrating pollen tubes is different for different flower buds as shown in Figure 5 (A-C). Few number of pollen grains germinate on the stigma of less receptive flower buds and no pollen grain germinates on non-receptive stigma (Table 4).

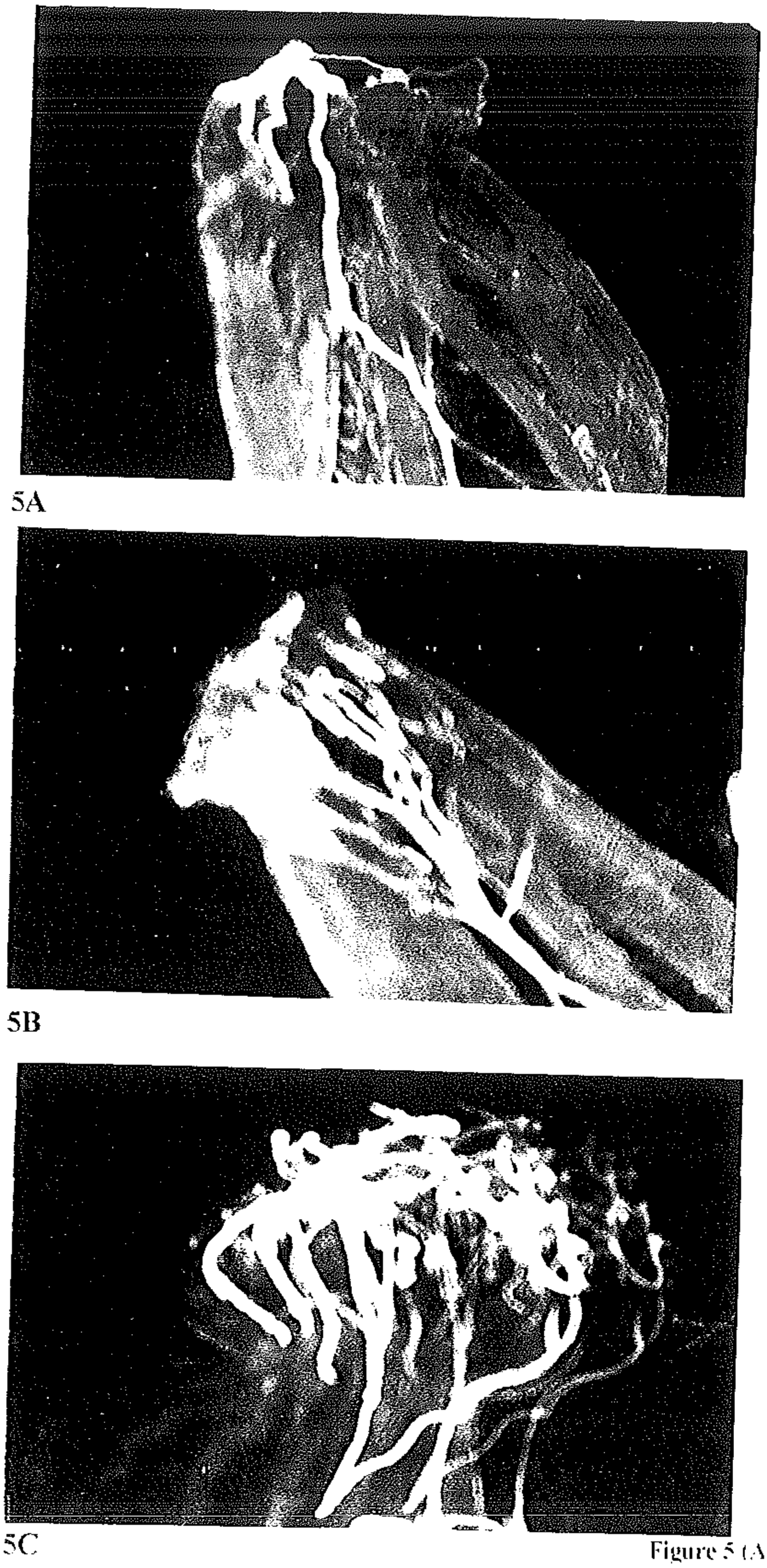

Figure 5 (A-C). Abundance of pollen tubes in the styles of different sizes of flower buds: A. few pollen tubes (1), B. many pollen tubes (2) and C. abundant pollen tubes (3).

4.2 Self-pollen germination and pod development

Under natural condition self-pollen germination was found to delay until late bud stage. Pollen tube was observed in those flowers which ranged in size between 20 and 25 mm. However, high percentage of flower buds that showed self-pollen germination were the ones which were very old (25 mm) as shown in Table 5. But during deliberate self-pollination (tripping), flowers as small as 14 mm allowed pollen grain germination.

Pistils excised from the same size of flower buds showed different number of pollen tubes as shown from pollen tube abundance rating in row 7 of Table 5. In some of the flower buds, stigma surface which was slightly above the anthers was observed.

Table 5. Flower bud size (stage) when self-pollen germinates without artificial pollination.

Abundance rating of pollen tubes in the style: 3 = abundant pollen tubes, 2 = many pollen tubes, 1 = few number of pollen tubes, 0 = no pollen tube (no pollen grain germinated), fp = fully pollinated.

Bud size (mm)	No. of buds observed	Internal floral condition	No. of buds with pollen tubes	Pollen tube abundance rate	% of buds with pollen tube
25	20	Anthers withered/ fp.	18	3	90
24	20	Anthers withered/ fp.	12	3	60
23	20	Fully pollinated (fp.)	8	3	40
22	20	Pollinated/ extended stigma	4	3	20
21	20	Pollinated/ extended stigma	4	3	20
20	20	Pollinated/ extended stigma	3	3/2/1	15
19	20	Pollinated/ extended stigma	4	1/3	10
18	20	Pollinated/ extended stigma	-	-	-
17	20	Partly pollinated	-	-	-
16	20	Partly pollinated	-	-	-

The majority of the flowers open mainly in the afternoon and remain fresh for 4 to 5 days. Then the petals lost turgidity and the anthers have withered. During this time, some of the flowers have aborted and dropped off and others developed into pods. A flower bud tagged at the beginning of anthesis required 7 to 10 days to form a pod and about 90 days to develop into a mature pod with mature seeds but not dry enough for harvest. In the first week of observation, about 23.46% of the flowers aborted before forming pods and after 90 days, 54.41% of the flowers were able to develop into mature pods.

Tripping by either hand (fingers) or toothpick was found to enhance self-pollen germination before late bud stage. Abundant pollen tubes were observed more commonly in styles of finger tripped flower buds. The control, in this case, bagging flowers (for only 24 hours) resulted in the germination of very low number of pollen grains in few flower buds although the size of flower buds that were bagged were almost similar to those subjected to tripping. It is shown that the mean size ($\bar{x}$ =16.62 mm) of flower buds which allowed many (rate 2) or abundant pollen tubes (rate 3) are not different. Flowers buds with a mean size of 16.83 mm but which were bagged for only 24 hours did not allow pollen tube penetration.

But those flower buds whose styles accommodated the growth of few pollen tubes had lower mean bud size ($\bar{x}$ = 15.90 mm) which is significantly different from those that allowed the penetration of more pollen tubes.

After tripping flower buds of similar size they were left until mature pods developed. Pods which had as few as 8 seeds and those which contained a maximum of 48 seeds were encountered. The former was obtained from toothpick treatment and the latter from open-pollination (control) as shown in Table 6.

When toothpick tripping and finger tripping were compared for their efficiency in seed production, the latter initiated the production of larger amount of seeds ($\bar{x}$ = 29.39 ±1.27) than the former ($\bar{x}$ =

24.11 ±2.25) although not significantly different at $P < 0.05$. When the amount of seeds produced by either toothpick tripping or bagging were compared with that produced through open-pollination the latter was significantly more productive ($\bar{x}$ = 34.11 ± 2.60). The mean number of seeds produced through hand tripping ($\bar{x}$ = 29.39 ± 1.27) is not significantly different from that produced through open-pollination ($\bar{x}$ = 34.11 ± 2.60) (Table 6).

Table 6. Mean (±SE) number of seeds produced through tripping, bagging and open-pollination.

Treatment	Mean no. of seeds	Minimum	Maximum	SD	SE
Tooth-pick tripp.	24.11^{a}	8	42	9.55	2.25
Finger tripp.	$29.39^{a,b}$	21	42	5.40	1.27
Bagging	23.67^{a}	9	39	7.81	1.84
Open-poll.	34.11^{b}	14	48	11.01	2.60

Means with the same letter superscript are not significantly different at $p < 0.05$.

4.3 *In vivo* test for self-incompatibility

The incompatibility diagonal in Table 7 indicated that there is no self-incompatibility in *S. sesban*. However, in few crossings and selfings abnormal pollen tube growth (no growth) was observed in the style and on the stigma and were noted as negative (-).

Table 7. Reciprocal cross to detect self-incompatibility

Ξ X	01	02	04	06	15	16	17	19	27	36
01	+	+	_	+	+	+	+	+	+	+
02	+	+	+	_	_	+	+	+	+	+
04	+	+	_*	+	+	+	_	_	+	+
06	+	+	_	+	+	+	_	_	+	_
15	+	+	+	+	+	+	+	+	_	+
16	+	+	+	+	+	+	_	+	+	+
17	+	+	+	+	+	+	+	+	+	+
19	+	+	+	_	+	+	+	+	+	+
27	+	_	+	_	+	_	_	+	+	_
36	+	_	+	+	+	+	+	+	+	+

4.4 *In vitro* test for self-incompatibility

Stigma, style and ovule crude extracts did not inhibit both self- and cross- pollen germination. On the other hand, there was complete cessation of pollen grain germination in the ovary extract of both cross- and self-pollen grains as shown in Table 8.

Table 8. Effect of stigma, style, ovule and ovary extract on self- and cross-pollen germination (+) = *pollen germinated,*(-) = *no pollen germinated (+ for stigma, style and ovule and - for ovary extract).*

	01	07	35	06	04	15	36
01	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
07	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
35	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
06	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
04	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
15	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -
36	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -	+ + + -

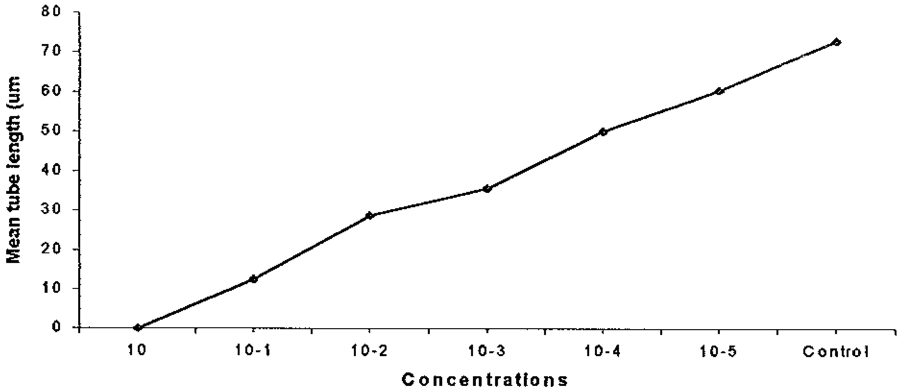

Figure 6. Effect of serial dilution of the ovary extract on pollen tube length

When 10 ovaries were crushed in 5 or 10 ml of the pollen germinating medium, the extract was strong enough to inhibit the germination of both self- and cross-pollen. But as soon as it was diluted by a factor of 10, pollen grains resume germination and as the extract becomes more diluted pollen tube length increased as shown in Figure 6. Pollen grains germinate normally in the germination medium (Figure 7) as well as in the extracts of stigma, style and ovules.

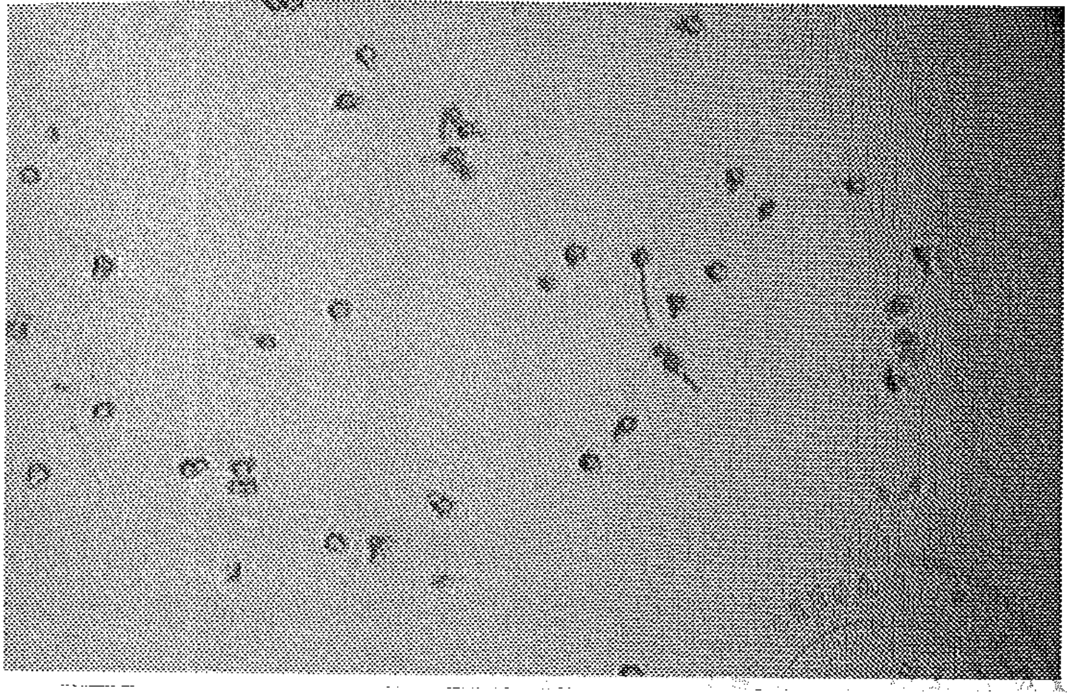

Figure 7. Germination of pollen grains in the germination medium.

Freeze dried ovary samples of 0.1 g were found to contain about 28% soluble tannins. Such chemicals might have an inhibitory effect on pollen grain germination.. The extract from the same amount of leaf sample also inhibited pollen grain germination. Freeze dried leaf samples of 0.1 g contained an equivalent amount of soluble tannins like the ovary.

4.5 Viability of pollen grains

From the 6 concentrations of sucrose solutions tested for pollen grain germination, 20% was found to favour higher number of germination ($\bar{x}$ = 37.56%) within a period of 3 hours incubation although it was not significantly different from 5, 10 and 15% at $P < 0.05$ significant level. Low number of pollen grains germinated ($\bar{x}$ = 10.18%) in 30% sucrose (Figure 8).

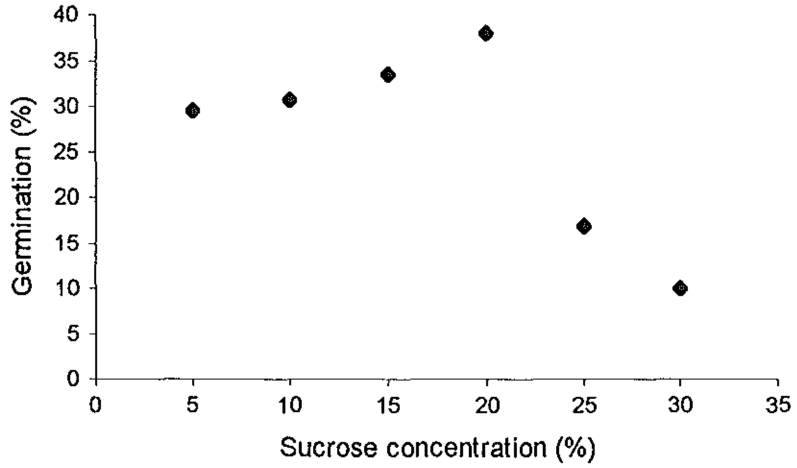

Figure 8. Pollen grain germination in different concentrations of sucrose

Flower buds that range in size between 11 and 22 mm were used to test pollen grain viability with FCR technique and pollen grain germination in 20% sucrose. Pollen grain viability percentage ranged from 63.20 to 97.28 with an average of 90.51% where as percentage pollen grain germination scores varied between 0.00 and 84.26 with a mean of 49.43%.

Flower buds that fall between 11 and 19 mm showed high viability scores (90 - 97%). Flower buds which were greater than 19 mm (19 - 22 mm) do also contain high number of viable pollen grains (63 - 89%), but percentage viability was variable from bud to bud with increase in size and viability (intensity of fluorescence) generally decreased in older flower buds. On the other hand, pollen grain germination in 20% sucrose solution was found to be low especially in young flower buds. However, the germination percentage some how increased with increase in size of buds. This trend is clearly shown in Figure 9. As a result, bud size was found to correlate (r = - 0.61) inversely with viability (P = 0.000) and there was also a negative (-0.39) correlation between viability and germination (P = 0.000), whereas a significant positive correlation (r = 0.87) was found between bud size and germination at P < 0.05.

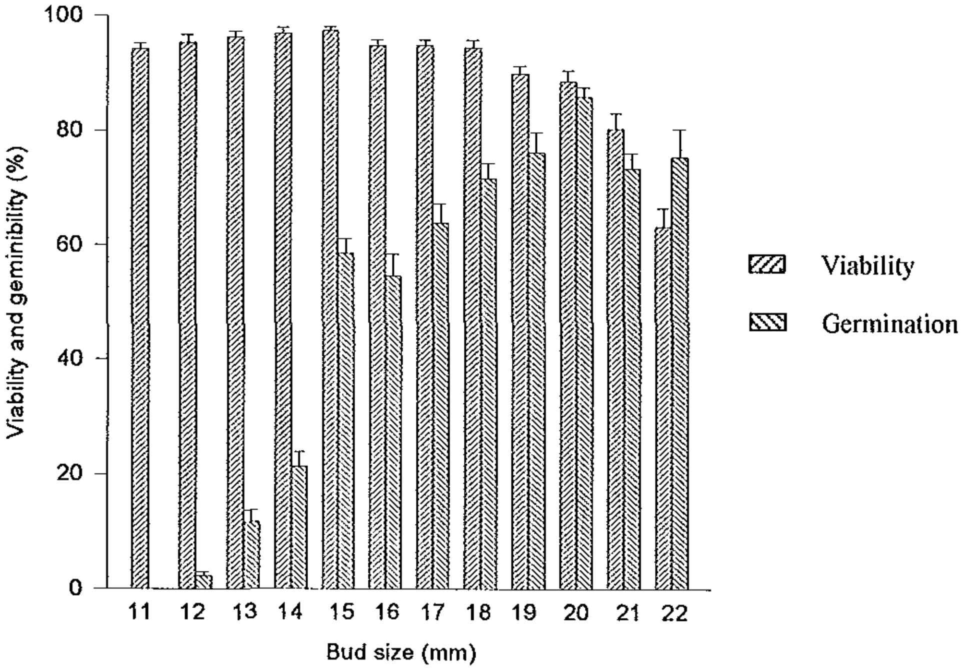

Figure 9. Comparison of pollen grain viability with FCR test and pollen grain germination in 20% sucrose

Under natural condition although viability decreases with age of flower buds, some amount of pollen could remain viable until late bud stage (23-25 mm). For example, pollen grain viability test in the field condition indicated that, about 20 to 30% of the pollen grain remained viable for 72 hours after the flowers were opened.

4.6 Estimation of out-crossing rate

Apart from estimation of out-crossing rate, other mating system parameters such as progeny heterogeneity, most likely maternal genotypes, pollen and ovule gene frequencies were determined for the 3 populations of *S. sesban* (Tables 9 and 10).

Peroxidase and Esterase showed too many bands making scoring difficult. Some of these bands were considered to be artifacts (ghost bands) and were generally excluded from the data. Acid phosphatase and Alcohol dehydrogenase were monomorphic for individuals tested from each family in the 3 populations. Therefore, out-crossing estimation was based on the genotype data of the progeny arrays scored on one polymorphic locus of Malate dehydrogenase. As a result, on average, an estimated 64% of the seeds were found to be derived from out-crossing and 36% of them from self-fertilization.

Table 9. Proportion of heterozygous progeny and most likely maternal genotypes of each family in the 3 populations of *S. sesban*.

	Populations					
	Acc. No. 15022		Zam-R024		Acc. No. 15019	
Family	Mat. genotyp.	Prop. of hets.	Mat. genotyp.	Prop. of hets.	Mat. genotyp.	Prop. of hets.
1	22	.266	22	.166	22	.133
2	22	.033	22	.066	22	.033
3	22	.033	21	.000	22	.150
4	21	.566	22	.133	22	.050
5	21	.500	22	.000	22	.100
6	22	.066	21	.000	22	.018
7	22	.200	21	.033	22	.117
8	22	.300	22	.266	22	.083
9	22	.166	22	.366	22	.050
10	22	.000	22	.400	22	.018
11	22	.100	22	.366	-	-
12	22	.066	22	.133	-	-
13	22	.033	22	.366	-	-
14	22	.300	22	.333	-	-
15	21	.466	22	.033	-	-
16	22	.000	22	.000	-	-
17	22	.000	22	.033	-	-
18	22	.066	22	.033	-	-
19	22	.033	21	.333	-	-
20	22	.000	21	.133	-	-

In *S. sesban* the proportion of heterozygous genotypes present in the families are variable from 0.033 to 0.566 as shown in Table 9. Population average heterozygosity were 0.160, 0.075 and 0.160 for Acc. 15022, Acc. 15019 and Zam-R024 respectively which were obtained by dividing the total heterozygous genotypes in each population by the number of families assayed in each population, and mean heterozygosity of the three populations was 0.132.

Table 10. Out-crossing rate (t), pollen and ovule gene frequencies of *S. sesban* populations based on the single polymorphic locus of Mdh

	Populations					
	Acc. 15019 (t = 0.395 ± .061)		Zam-024 (t = 0.428 ± .105)		Acc. 15022 (t =1.105 ± .255)	
Allele/locus	Pollen allele frequency	Ovule allele frequency	Pollen allele frequency	Ovule allele frequency	Pollen allele frequency	Ovule allele frequency
Mdh 1 (F)	.171 ± 0.024	.048 ± .001	.213 ± .070	.125 ± .050	.089± .028	.075 ± .046
Mdh 2 (S)	.829 ± 0.024	.952 ± .001	.787 ± .070	.875 ± .050	.911± .028	.925 ± .046

The allele designated as '2' or 'S' is occurring with very high frequency in the pollen pool and in the maternal ovule of the three populations as shown in Table 10. In Figure 10 a typical banding pattern of malate dehydrogenase is presented.

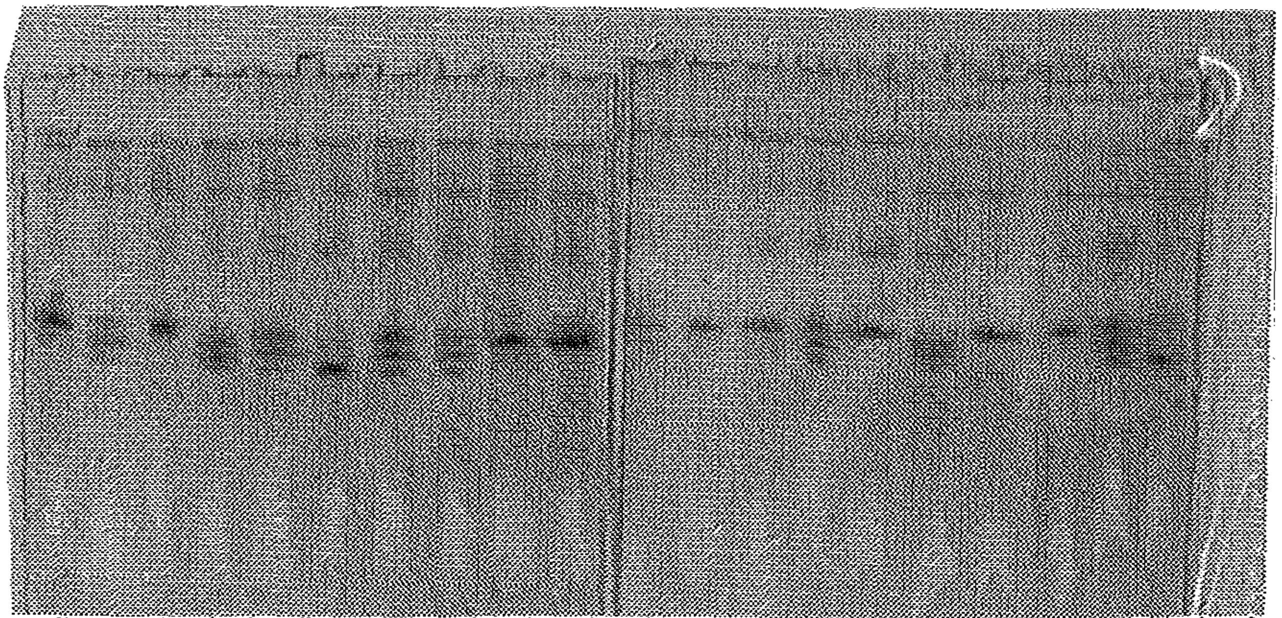

Figure 10. The banding pattern of Malate dehydrogenase enzyme system for some individuals of Accession No. 15022 population.

The populations from the three sites are composed of individuals with different genotype frequencies. To find out whether these genotype frequencies were in accordance with the Hardy-Weinberg frequencies, the allele frequencies of F (p) and S (q) were calculated. These were found to be 0.09 and 0.91 respectively for Acc. 15022, 0.04 and 0.96 for Acc. 15019, 0.14 and 0.86 for Zam-R024 population. Based on these allele frequencies the expected Hardy-Weinberg (H-W) genotype frequencies and the corresponding expected number of individuals in each genotype in the 3 populations are shown in Table 11.

Table 11. Observed and expected genotype frequencies and the number of progeny arrays.

Accession 15022				Accession 15019				Zam-R024			
Ob. Allele Freq.	Obs. No. of Ind.	Exp.ect. Geno Freq.	Expect. No. of Ind.	Obs. Allele Freq.	Obs. No. of Indv.	Expect. Geno. Freq.	Expect. No. of Indv.	Obs. Allele Freq.	Obs. No. of Indv.	Expect. Geno. Freq.	Exp. No. of Indv.
0.088 (p)	FF 5	0.01	6	0. 04	0	0. 00	0.00	0.14	36	0.02	12
	FS 96	0.16	96		43	0. 08	48		96	0. 24	144
0.912 (q)	SS 499	0.83	498	0. 96	557	0. 92	552	0.86	468	0. 74	444

Based on the number of observed and expected number of individuals, Chi-square (x^2) values were calculated to be 0.17, 0.57 and 65.30 for Acc. 15022, Acc. 15019 and Zam-R024 respectively. For 1 degree of freedom the corresponding probability value at 5% confidence interval was 3.84.

5. Discussion

5.1 Stigma receptivity and pollen grain maturity

In many hermaphrodite flowers self-fertilization will only be possible if pollen maturity and stigma receptivity coincide and moreover the flower should be homomorphic. In developing flowers of *S. sesban,* there exist a condition conducive for self-pollination to occur. When flower buds were at sizes ranging between 16 and 18 mm anther dehiscence was common before flower opening (at stage 3 of the flower buds, Figure 2). Stigma were found to be receptive before anther dehiscence especially at stage 2 of the flowers. In young tripped flowers (e.g. 14 mm) pollen germination was automatic and pollen tubes were observed even after 4 hours of tripping. Application of mature and immature pollen grains on stigma of the same size but different flower buds indicated that stigma becomes receptive before the maturity of pollen grains. As a result, the flower of *S. sesban* was suggested to be protogynous, a condition that was also reported by Owuer and Owino (1993).

Under natural condition pollen tube germination was observed from those flowers which have opened and then closed their standard and wing petals and mostly whose anthers were withered. In the pistils of flowers picked at very late bud stage, pollen tube penetration was fully apparent (Table 5). This means, even if self-pollination occurs, the germination process is delayed under natural condition. This could be a mechanism by which *S. sesban* favours the occurrence of cross-pollination. Pollen grain germination at late bud stage was reported for *Chamaecytisus palmensis* (Papilionoideae) (Webb and Shand, 1985). In very few cases, before late bud stage, there was observed germination of pollen grains and penetration of pollen tubes in to the stylar canal (Table 5). However, some of the tubes were not growing in to the style despite their germination on the surface of the stigma. A similar condition was reported for *Ornithopus sp.,* which is an autogamous or even cleistogamous form (Wojciechowska, 1972).

The range of flower bud size used for detecting receptivity and anther dehiscence was similar but anther dehiscence was delayed until after the stigma becomes receptive as shown in Figure 3. Those buds with receptive stigma existed with higher percentage at early and medium stage (range 12 –16 mm bud size) of the flower buds, but, later on more or less equal proportion of buds with receptive stigma and anther dehiscence were detected. There was a decline in proportion of buds with receptive stigma in old flowers (e.g. 19 – 20 mm). The stigma could have lost its receptivity at this stage. While examining the flowers for anther dehiscence careful observation of the internal parts was very essential because, the larvae of some insects were found harboring inside and fastened anther dehiscence.

The degree of stigma receptivity was found to vary from flower to flower depending on their stage of maturity or size. Those flower buds with average size of 16. 5 mm had allowed more number of pollen tubes upon tripping indicating probably, that they were more receptive (Table 4). But under natural condition more abundant pollen tubes were detected in more mature flower buds, e.g. > 22 mm (Table 5). Apart from stages of maturity, difference in pollen tube abundance could also be attributed to the non-uniform dispersal of pollen grains on the surface of stigma, i.e., some stigma could receive abundant pollen grains while others receive only a few. For *Ornithopus sp.* single pollen tube and also bundles of tubes were observed in the style at time of profuse germination of pollen grains on the stigma (Wojciechowska, 1972).

Stigma stays receptive until late bud stage (for at least 5 days until after the flower is opened). About 20 to 30% of the pollen grains were also found to remain viable after the flower had started opening for about 72 hours with a potential to germinate and fertilize the ovule. This time could be shorter when thrips enter the flower buds. These insects enter in to a flower bud in large numbers when the flower just starts to open and the quantity and quality of the pollen grains were reduced. They were observed moving from one flower bud to the other on the same plant upon depletion of pollen grains. The study of their movement from one plant to the other was difficult. They do carry some pollen on their body but still difficult to consider them as cross pollinators. Forbes *et al.* (1971) reported that, thrips *(Franklincella spp.)* were not considered as pollination vectors for blue lupins (*Lupinus angustifolius* L.) although they may render some flowers male sterile by eating all the pollen.

At the time of anthesis when the standard petals are opened for the flowers of *S. sesban* there is a chance of cross pollen to land on the stigma due to insect visitation. For *Chamaecytisus palmensis* it was reported that, selfing is the dominant mode of reproduction but, when cross pollen is available the production of more out-crossed progeny is favoured (Webb and Shand, 1985). Webb and Shand (1985) reported that this species is largely pollinated with *Bombus terestris*. A similar group of insects were observed frequently visiting the *S. sesban* flowers of the Addis population (Acc. 15022).

In general, morphological observation and *in vivo* controlled pollination tests showed that in developing flowers of *S. sesban* there exist conditions favorable for self-pollination and /or self-fertilization. Therefore, it can be suggested that the processes of autogamy are in preponderance. Nevertheless, the estimation of out-crossing indicated that the chance of cross-pollen in fertilizing a given ovule could be very high in the presence of adequate number of pollinators.

In the tripping experiment, mean size ($\bar{x}$ = 16.83 mm) of the control buds that were randomly selected and bagged was not significantly different from those mean bud sizes ($\bar{x}$ = 16.62 mm) of the treatments However, pollen tube penetration in these non-treated buds (control) was very poor or nil at least for the treatment period, i.e., 24 hours. Flower bud mean size ($\bar{x}$ = 15.90 mm) that allowed few pollen tube penetration (rated as 1) was significantly smaller from those of the other treatments and the control. The expectation here was that 0 rating would be an indicator of smallest bud sizes but the same rating could be obtained in bigger flowers which were not tripped but simply bagged for a period not more than 24 hours. Those buds which fall in the range of 16 - 17 mm may be considered to be at high receptivity stage as they allowed more pollen tube penetration upon tripping.

Although tripping was found to initiate early pollen grain germination in young buds of *S. sesban*, it does not seem necessary for more seed production because, seed production was higher under natural condition although not significantly different from finger tripping as indicated in Table 6. On the other hand, Barnes *et al.* (1972) reported that hand tripping (rolling or squeezing flower buds between fingers) was a more efficient technique and gave more seed production in *Medicago sativa* (alfalfa), but no comparison was made with seeds from open-pollination. However, Barnes *et al.* (1972), had confirmed that some form of mechanism (tripping) by insects is necessary for alfalfa (a partially self-incompatible species) efficient seed production. He reported also that, cross pollination of alfalfa resulted in a higher % of flowers forming pods and a larger number of seeds per pod than does self-pollination because, the species is partially self-incompatible. The mean number of seeds produced through bagging was significantly lower from that of open-pollination. This reduction in the number of seeds could not be a result of SI in *S. sesban*, rather it could be due to the effect of bagging or tripping which might have changed the microclimate around the inflorescence.

In some flowers of *S. sesban* and at stage 3 and 4, the surface of the stigma was found slightly above the anthers. Such condition may not allow the stigma surface to be completely covered with pollen grains, indicating the delay in time until the stigma becomes fully pollinated. In *Medicago sativa* (alfalfa), the stigma is not some times covered with pollen grains. This was attributed to the fact that in some flower buds the amount of pollen is highly reduced. Moreover, most of the pollen grains might be defective and some times stigma extends beyond the pollen mass (Barnes *et al.* 1972).

Generally, self-fertilization was found to delay under natural condition unless some form of tripping is applied on the flowers of *S. sesban*. Pollen tube penetration was apparent within 12 hours of artificial tripping of young flowers (15 - 16mm) (Table 4), whereas untripped flowers of 18-19 mm size did not show pollen tube growth. (Table 5).

5.2 Pollen grain viability

Pollen grains of *S. sesban* are binucleate and have three germination pores (tricolpate) (Figure 7). The viability of both binucleate and trinucleate pollen can be assessed through FCR test. High pollen grain viability scores were obtained from those flower buds ranging in size between 11 and 19 mm. Most of the pollen grains from this range of bud sizes depicted very intense bright golden yellow fluorescence which is a sign of viability. Viability of pollen grains seemed to have reduced at late bud stage, when only those with strong fluorescence intensity were considered as viable as shown in Figure 9. Pollen grain germination scores were found to be higher than the viability scores in some of the mature flower buds, because, those mature pollen grains with dim fluorescence which were counted as non-viable must have germinated.

Pollen production is abundant in *S. sesban*. Therefore, even if there is a reduction in viability of pollen, the few numbers that remained viable until late bud stage could be sufficient for adequate amount of ovule fertilization. Pollen germination *in vitro* could be low, but may be sufficient for satisfactory seed set *in situ* (Dafni, 1992). The amount of pollen grain that lands on the surface of stigma determine the amount of ovules to be fertilized and hence the amount of seeds to be produced (Tangmitchroen and Owens, 1997). Similarly, Lee (1988) reported that, pollination intensity is usually positively correlated with the number of seeds per pod.

Many studies have shown that the FCR test reflects the ability of the pollen to germinate. Under this experiment viability scores were found to be higher for those pollen grains derived at young and medium bud stages (11-15 mm). On the other hand, pollen germination scores were found to be better at late bud stage than at young bud stage. According to Dafni (1992), FCR test could show correlation with potential germinability if mature pollen is used and recommended that FCR is more reliable in investigating pollen grain viability. In mature flower buds there appears to be a direct correlation between pollen grain viability with FCR and pollen germination in sucrose solution (e.g. bud sizes 19 and 21 mm). When pollen grains are subjected to a kind of stress for a limited period of time there will be a reduction in FCR and a significant delay in germination (Shivanna *et al.*, 1991). According to Shivanna *et al.* (1991), pollen grains exposed to stress lost their vigor before they lost viability. Therefore, *in vitro* germination tests have been used to asses the vigor of pollen grains (Shivanna *et al.*, 1991).

Shivanna and Johri (1985) reported that, fluorescence is caused when FDA solution enters the cell cytoplasm and enzymatic cleavage releases fluorescein. This will not happen in non-viable pollen as the plasmalemma can not hold the solution inside and the activity of an enzyme (esterase) is reduced in such cells. The integrity of the plasmalemma is closely associated with viability, thus proving FCR to be an effective method for assessing pollen viability (Heslpo-Harrison and Heslop-Harrison, 1970). Some young undeveloped pollen grains in bud sizes below 11 mm and those after the stage of anthesis (> 20 mm) may lack intact plasmalemma and as a result some of the pollen grains did not fluoresce or showed dim fluorescence. Similarly, pollen grains from young (11 - 14 mm) flower buds, even though they show high viability scores they either did not germinate at all or did not readily germinate in the medium within a period of 6 hours. The germination percentage was very low in 20% sucrose for pollen grains derived from some flower buds as shown in Figure 9. This may be; 1. The pollen hydration time (germination time) was short (extended hours of germination will also be a problem in scoring, as the dense mat of tubes wiil hinder accurate counting) 2. In most cases viability in FCR test does not coincide with germination in sucrose unless mature pollen grains are used (Dafni, 1992) and 3. The difficulty in obtaining the best artificial medium optimal for pollen germination (Shivanna *et al.*, 1991). Heslop-Harrison (1982) reported that water relationships of the pollen grains and the germination medium can affect pollen membrane hydration and thus significantly affecting their germination. An optimal sucrose solution for pollen grain germination could be used to evaluate the maximal pollen grain germination rate as an indicator of pollen viability (Owour and Owino, 1993). They reported that 10% sucrose was the best for *S. sesban* pollen germination. However, under this experiment 20% was found to be the best in the range of 5

- 30% sucrose (Figure 8).

Heslop-Harrison and Heslop-Harrison (1970) had stated that, "fluorescence is likely to be correlated with the viability of the vegetative cell of the pollen grain : and while it does not follow that a living male gametophyte will necessarily be able to form a pollen tube and effect fertilization, certainly one that is dead will never do so." It means, all pollen grains that are considered viable (fluoresce) may not germinate forming tubes. This could be one of the reasons, why viability scores with FCR and germination scores in sucrose did not show a direct correlation.

Pollen grains of *S. sesban* can remain viable for about 72 hours after the flower was just opened and stigma could stay receptive for at least 5 days. In wet season stigma stays receptive for 3 days after anthesis for *Cordia alliodora* (Boshier and Lamb, 1997b). In *Cucurbita pepo*, *Festuca arundinacea* and *Mercurilis annua* all pollen grains have become inviable within 72 hours after anthesis (Pacini, 1997). Tangmitcharoen and Owens (1997) reported that, under *in vitro* experiment pollen viability was high just after anthesis, but decreased gradually and after 3 days (84 hours) of flower opening, pollen grains were no longer viable in *Tectona grandis*. Pollen viability is lost within minutes for graminae while in other taxa viability could last for months (Shivanna and Johri, 1985).

Depending on the microclimate of a particular flower bud and due to the physiological condition of the plant, as well as infections with pathogens and insects, pollen viability could be reduced. An average percentage viability of 94.25% was obtained for those flower buds that measured between 11 and 19 mm. This score of pollen grain viability could have been reduced if the plant was under stress conditions. According to Pitman and Levin (1986), pollen viability may be affected by temperature, age of pollen, exposure of pollen to water and water status of the parent plant. So it is necessary to test pollen germination before starting any breeding work. To ensure the success of hand pollination on *Tectona grandis*, Tangmitcharoen and Owens (1997) had determined pollen viability before applying pollen grains on stigma.

5.3 Self-incompatibility in *S. sesban*

Controlled *in vivo* pollination on the trees was found to be the best method in assessment of self-incompatibility in *S. sesban*. The size of buds that were chosen for emasculation were mature enough (but with intact anthers) and had receptive stigma. According to Owuor and Owino (1993), flowers younger than those at the stage of anthesis were selected for emasculation. Sedgley (1992) reported also that, self-pollination can be prevented if flowers are emasculated prior to anther dehiscence. Cross-pollination performed on the day of emasculation or the next day were not different for *S. sesban* when flower buds with receptive stigma were used. However, despite the careful handling of the flowers during emasculation and pollination abnormal pollen tube development was detected on either the stigma or in the style of both self- and cross-pollinated flowers. Such abnormal pollen tube growth may be due to defective pollen or defective stigma or the effect of the weather condition prevailed during the time of flower manipulation.

Abnormalities of pollen tube growth such as meandering tubes, irregular tubes, forked and swollen tips were observed along the style of the flowers of *Tectona grandis* (Tangmitcharoen and Owens, 1997). Hessing (1986) reported that from crossings and selfings he made, six cross pistils and three self pistils either had no pollen grain attached or no pollen tube growth for *Geranium caespitosum* James. Similarly, pollen tube inhibition either due to bursting or bulb formation occurred in the stigma and style respectively after cross-pollination for *Cichorium intybus* L (Compositae) (D' Eeckenbrugge, 1986). D' Eeckenbrugge (1986) noted that such phenomenon was the result of interaction of competing tubes or when the tube is arrested by the transmitting tissue in the style. Schlichting (1986) reported that environmental variation influences not only the quantitative production of the plants male and female gametes, but also the quality of the male function as well. In *Phlox drummondii* Hook. cross compatibility was affected by the macro-environmental variation experienced by the parental plant (Pittman and Levin, 1986). They reported also that plants cross more successfully with some plants than with others, and that the "best mates" are different for different individuals. Therefore, it should be one or two or more of the above factors that resulted in abnormal pollen tube development from the reciprocal crosses made for *S. sesban* as shown in Table 7.

Seed development in bagged flowers of *S. sesban* was quite abundant confirming the absence of self-incompatibility in this species. The experiment had confirmed that there was significant amount of seed production from self-fertilization in *S. sesban* when compared to the amount of seeds produced through open-pollination (Table 6). Isolated plants in the greenhouse have also produced large amount of seeds, an evidence which could also support the absence of self-incompatibility.

Assay of stigma, style, and ovule extracts indicated that there is no inhibition of self- and cross-pollen germination. However, non-specific inhibition of pollen grain germination was observed in the ovary extract. Similarly, a highly concentrated (20 styles in 1.5 ml of germination medium) extract of styles did also inhibit pollen germination. On the other hand, pollen grains germinated profusely in a germinating medium in which the extract was not included. Bioassay of self-incompatibility has been performed for a number of species. Crude extracts of pistils of *Petunia* and *Nicotiana* selectively inhibited self-pollen germination (Jackson and Linskens, 1990). According to Jackson and Linskens (1990), there is as yet no proof that these glycoproteins that inhibit pollen germination are S-gene products. And also difficulties in obtaining clear cut effects from bioassay test of self-incompatibility was reported by Speranza and Calzoni (1988). Therefore, in this study, the inhibition in either the ovary or the style extract can not be considered as the reaction of self-incompatibility, since the inhibition did not discriminate between self- and cross-pollen. The inhibition could be due to certain chemicals (soluble tannins) that might affect pollen tube growth. A 0.1 g freeze dried sample of the ovary was found to contain about 28% of soluble tannins. According to Speranza and Calzoni (1988), in the absence of self-incompatibility factors, the inhibition in the extracts may be due to non-specific inhibitors and/ or low molecular weight substances. Treatments that affect the integrity of the cell membrane drastically changes the response of the pollen grains. For example, saponins at a concentration of 0.05% were found to stimulate loss of fluorescein from cells (Heslop-Harrison and Heslop-Harrison, 1970). It means saponins could damage the plasmalemma of the pollen grains. These chemical substances are also present in *S. sesban* plants. Many saponins have detergent properties, show haemolytic activity, have a bitter taste and are toxic to fish (Hostettmann and Martson, 1995). Therefore, these might be the substances that inhibited pollen grain germination in the ovary extract. Serial dilution of the ovary extract allowed the germination and elongation of pollen tubes as shown in Figure 6 indicating that the inhibition is concentration dependent. According to Seavey and Bawa (1986), the genetic picture of late-acting systems is virtually unknown.

The breeding strategy of *S. sesban* was identified by artificial crossing and selfing of flowers on different trees within an accession and except for the immaturity of pollen grains or defective sigma or the effect of weather, pollen tube penetration within the styles of flowers was apparent in most cases. Generally, from the floral biology studies and *in vitro* and *in vivo* assessment of

incompatibility, it can be concluded that this species can undergo self-fertilization and also has the potential for out-crossing in the presence of suitable insect pollinators.

5.4 Estimation of the mating system

The 2^nd^ locus of Mdh (Mdh-2) which was closer (1.5 cm) to the origin was monomorphic for all the progenies assayed. The first locus (Mdh-1) at 3.2 cm from the origin was polymorphic for individuals assayed from Acc. 15019, Acc. 15022 and Zam-R024 *S. sesban* populations (Figure

10). Brown *et al.* (1975) reported that Mdh-2 was polymorphic only for those populations at more elevated sites, but monomorphic for other populations of *Eucaluptus obliqua.* Acid phosphatase and Alcohol dehydrogenase were monomorphic for individuals in the three populations of *S. sesban*. Peroxidase and Esterase showed some level of polymorpism for these same populations but the banding patterns were too variable to be used in the data interpretation. For example, Boshir *et al.* (1995) obtained 11 polymorphic loci for *Cordia alliodora* progeny arrays but only 4 were used in the data analysis because, the rest of the loci were showing inconsistent banding pattern. Some enzyme systems even though they show polymorphism, they lack consistency or show non-interpretable variation.

Therefore, the single polymorphic locus of Mdh was used in this experiment to assay a large number of samples from the three populations. According to Shaw and Brown (1982), best out-crossing estimates will be obtained when only the most variable single locus is used and as many plants are scored for this locus. They also suggested that small number of samples could be assayed on many loci for plants which are largely out-crossing for improved statistical efficiency. Therefore, an experimenter limited by the time and resource should be able to choose between assaying a large number of samples at a few loci or assay a restricted number of individuals but, at many loci (Shaw and Brown, 1982).

Based on the single locus of Mdh out-crossing rate (abbreviated as t) was estimated to be 0.395, 1.105 and 0.433 for Acc. 15019, Acc. 15022 and Zam-R024 populations respectively (Table 10). The out-crossing rate estimates for Zam-R024 and Acc. 15019 populations are close to each other, whereas that of Acc. 15022 is significantly higher from both. The high out-crossing rate for Acc. 15022 (Addis) population indicates that this species can under go complete out-crossing under certain circumstances. The out-crossing rate estimated as 1.106 for *Giliricidia sepium* differ significantly from one indicating the species to be strongly out-crossing (Dawsen and Chamberlain, 1996). It is important to note here that, the *S. sesban* plants of Acc. 15022 were found being surrounded by other populations or accessions probably with different genotypes but with a possibility of mating with this population. This might be one of the reasons that resulted in increased heterozgosity hence an increased out-crossing rate for Acc. 15022 .

It is not uncommon to find the value of **t** close to 1.0 or exactly 1.0 or exceeding unity. According to Ellstrand *et al.* (1978), random out-crossing rate can be greater than 1.0 when different genotypes mate at higher frequency than expected, i.e. if there is disassortative mating (preferential mating between different genotypes) (Boshier and Lamb, 1997a). Out-crossing estimates of Acc. 15019 and Zam-R024 populations were found to be less than one. Values of **t** less than 1.0 occurs when like genotypes mate among themselves more frequently than expected, as a result of selfing (positive assortative mating). It is likely that related individuals could be spatially clumped in natural plant populations and that consanguineous mating of related neighbors should be common (Ellstrand and Foster, 1983).

Several studies have shown that plant mating systems are variable like any other characteristics, therefore, should vary among populations and years (Hamrick, 1982). Levin *et al.* (1979) reported that out-crossing estimation for the 10 populations of *Oenothera organensis* varied from 0.74 to 1.27. For a predominantly self-incompatible tree *Eucalyptus obliqua,* t was found to range from 0.42 to 1.05 with an overall mean of 0.76 for the 4 populations assayed (Brown *et al.* 1975). In three populations of *E. pauciflora* t ranged from 0.30 to 0.85 with a mean of 0.63 overall (Philips and Brown, 1977). Out-crossing rate of *Helianthus annuus* was found to vary between 0.54 and 0.91 from five different populations based on one variable locus of Adh-1 (Alcohol dehydrogenase) (Ellstrand *et al.*, 1978)

The presence of high proportion of heterozygous individuals in a population could increase the estimation of apparent out-crossing. But, heterozygosity at a marker locus in maternal plants markedly reduces the contribution of statistical information made by that locus to the estimate of out-crossing. Such loci are much less likely to be diagnostic of out-crossing events (Shaw and

Brown, 1982). This phenomenon might be one of the factors that reduced the estimate of out-crossing rate for Zam-R024 population which contains five heterozygous maternal genotypes (most likely maternal genotypes, Table 9). Reduced out-crossing estimate for Acc. 15019 and Zam-R024 populations could also be attributed to the non-random mating of individuals. Ellstrand, *et al.* (1978) reported that, plant populations are unlikely to be panmictic regardless of their mating systems due to restricted pollen and seed flow. For example, most gene flow seem to involve related plants that will reduce the effective rate of out-crossing in *Helianthus annuus,* i.e., plants of similar genotype cross with increasing frequency so that the level of heterozygosity deviates from that of the Hardy-Weinberg expectation for random mating, thus resulting in reduced out-crossing.

In table 10 and 11, it is shown that allele '2' or 'S' (slow allele) is occurring at a higher frequency. Its occurrence at this frequency may be attributed to the fact that 1. Directional selection has favored it because the genotypes that contain it have a better chance of survival (Pasteur *et al.*, 1988) 2. When trees of related genotypes (with similar alleles) are clumped together they tend to pollinate with one another more frequently (positive assortative mating) than expected in the absence of self-incompatibility, thus favoring this allele which is already occurring in the pollen pool and maternal ovule and 3. Mutation could have introduced multiple copies of this allele into the population (Brown *et al.*, 1975).

Despite the high frequency of allele 2, the proportion of the observed progeny arrays are in accordance with Hardy-Weinberg proportion for the two populations (Acc. 15019 and Acc. 15022) at 5% significant level as shown in Table 11. Proportion of observed individuals of the population of Zam-R024 deviates significantly from Hardy-Weinberg proportion. Departure from Hardy-Weinberg expected proportions could be due to population sub-structuring resulting either from the introduction of germplasm from other populations or from mating between related individuals (Dawsen and Chamberlain, 1996).

The effect of selfing could also increase the rate of out-crossing as a result of increased chance of cross zygote survival. Due to unequal viability of cross- and self-zygotes, the out-crossing rate estimation could be unexpectedly higher for some species. This phenomena was reported by Stevens and Bougourd (1988), when they estimated t as 0.91 for *Allim schoenoprasum* L. which is a clonal, self-compatible hermaphrodite plant with apparently ample opportunities for geitonogamous selfing. According to Barnes *et al.* (1972), the frequency of ovule abortion is greater in zygotes and embryos resulting from self-pollination than from cross-pollination. A report from Phillips and Brown (1977) has indicated that a portion of seeds derived from self-fertilization do not germinate as readily as the seeds derived from out-crossing. As a result, the presence of heterosis is probably the force which prevents the evolution towards higher level of self-fertilization in populations of *Eucalyptus pauciflora.* In this study, it was found out that about 45.6% of the flowers abort before forming pods and some of the seeds that were set to germinate either do not germinate readily or germinate very poorly. Complete or selective abortion of ovules was also reported by Heering (1994) for species of *Sesbania* (*S. sesban, S. kinensis* and *S. geotzei*). Similarly, Moran *et al.*, (1980) indicated that the level of selfing (10%) for *Pinus radiata* does not necessarily reflect how much selfing occurred during fertilization. According to Moran *et al.*, (1980), it is generally believed that there can be considerable embryo abortion between fertilization and seed germination and many of the zygotes that die are progenies of self-fertilization. The effect of environment on the extent of out-crossing is also not negligible which could affect both pollen and ovule production and pollinators behaviour.

The apparent variation in out-crossing rate for the populations of *S. sesban* could be the effect of pollinators behaviour. According to Brown *et al.* (1989), out-crossing rate in self-compatible species can depend on pollinator activity and behavior especially in species where selfing will eventually occur in the absence of pollinators. For example, entomophilous species display more variation in out-crossing rate both within and between species and it can also vary greatly among and within populations. For *Lupinus nanus, L. sculentus* and *Collinsia sparsiflora* the large variation in out-crossing rate is apparently due to fluctuations in pollinators activity (Brown *et al.* 1989). Although there was no chance to observe pollinators activity at Debrezeit and Zambia *S. sesban* populations, in Addis, large crowd of bumble bees (*Bombus spp.*) were seen actively moving from flower to flower and/or from plant to plant in the peak flowering period. This might be one of the main reasons for the unexpected high estimate of **t** for Addis population (Acc, No. 15022). *Bombus canariensis,* which is closely related to *B. terrestris* is the probable pollinator of *Chamaesytisus palmensis* (Papilionoideae) These are the type of insects which can easily manipulate the Papilonoideae flowers for either nectar or pollen (Webb and Shand, 1985).

It seems that *S. sesban* has the potential to undergo complete self-fertilization in the absence of insect pollinators (e.g. abundant pod development was observed from plants protected from pollinators in the green house). If all the zygotes that would be formed from self-fertilization fully survive and develop into mature seeds the apparent out-crossing rate would have been negligible. But under open-pollination, the effect of inbreeding, the presence of pollination vectors and natural selection disfavor the survival of self-fertilized zygotes, hence increasing the number of out-crossed progenies. In entomophilous species, the presence of adequate number of pollinators enhance out-crossing. Smyth and Hamrick (1984) have concluded that *Carduus nutans* was predominantly out-crossed when pollen and pollinators were available.

In general, the average out-crossing rate (0.64) of the three *S. sesban* populations based on allozyme marker locus may not be an exact specification of the mating system. According to Brown *et al.*, (1975), this could be simply the net effect of deviation from random mating due to gametic selection and/ or early self-zygotic lethality. According to Smyth and Hamrick (1984), out-crossing leads to high levels of heterozygosity in individuals as well as in populations which might be leading to potentially adaptable populations. In *S. sesban* the frequency of heterozygous genotypes present in individual family is so variable (range from 0.033 to 0.560 Table 9). Such heterozygous genotypes have the ability to adapt to various environmental situation. This may be the reason why *S. sesban* populations are found growing in a range of altitudes (300 to 2000 m) and soil conditions.

6. CONCLUSION AND RECOMMENDATIONS

The flower of *S. sesban* has many characteristics that make selfing possible. These are; anther dehiscence before flower opens, the alignment of anthers with the stigma (homomorphic) and the almost coincidence of stigma receptivity with pollen maturity. In contrast, colour of the flower, availability of nectar and pollen attracts insects that facilitate cross-pollination. In addition, although self-pollen grains land on the stigma, pollen germination was found to delay until late bud stage by the time when most flowers could be visited by insects which effect cross-pollination. Controlled *in vivo* pollination and visual observation supported the protogynous condition of the flowers of *S. sesban*, i.e., the stigma becomes receptive before anthers dehisce. Controlled *in vivo* pollination was found to be the best method of detecting receptivity of stigma.

S. sesban may not require tripping, because, self-fertilization and seed development was apparent from untripped and bagged flowers. However, artificial tripping in *S. sesban* pollination may be useful if one intends to initiate pollen germination before the flowers open. This will make sure that self-pollen germinates and pure seeds can be produced from a particular elite genotype by repeated controlled self-pollination. Abundance of pollen tubes and the amount of seeds produced had supported that finger tripping was more efficient than toothpick tripping. However, seed production through open pollination was found to be higher although not significantly different from finger tripping.

Inhibition of self-pollen germination was not detected from *in vitro* bioassay test and a considerable amount of seeds was produced from bagged flowers. Therefore, both *in vivo* and *in vitro* tests confirmed the absence of either gametophytic and/or late-acting self-incompatibility in this species. However, few abnormal pollen tube development was observed in some crosses and selfs which could be the result of defective stigma or defective pollen grains or the effect of weather condition prevailed in the experiment period.

Flower bud sizes which are less than 15 mm are suitable for emasculation. It is advisable to use flowers buds for any breeding purpose in peak flowering season when the buds attain a size large enough for easy manipulation. FCR test indicated that high percentage of viable pollen grains could be obtained from those flower buds that range in size between 11 and 19 mm.

The single locus average out-crossing estimate of *S. sesban* (0.64) may not be different from multi-locus estimates. However, further work may be required to confirm the situation. Boshier *et al.* (1995) reported that, for *Corida alliodora* the single locus out-crossing estimates were not different from the multi-locus estimates. Similarly single locus and multi-locus out-crossing estimates for open-pollinated populations of douglas fir was reported to be 0.925 ± 0.024 and 0.932 ± 0.013 respectively, which does not seem to be significantly different at 95% confidence interval (Ritland and El-Kassaby, 1985). The mating system estimation procedure used in this study can handle both multi-locus and single locus isozyme data to estimate mating system parameters. Further more, the value of the standard deviation obtained in estimation of out-crossing rate of *S. sesban* is low enough (Table 10) confirming the validity of the statistical analysis used.

Information on the breeding system of plants is crucial for maintenance of accessions in germplasm banks. Moreover, if the breeding mechanism of a species is more or less determined, future work regarding its evolutionary genetic structure and a need to improve its potential as a multipurpose tree through either selection or breeding would be easier.

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