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**POLLEN PHYSIOLOGY and FERTILISATION
in *VICIA FAB* L.**

by

Asfaw Telaye

**A thesis submitted in accordance with
the requirement for degree of
Doctor of Philosophy**

**Department of Biological Sciences
Unersivty of Durham, England**

June 1990



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TO NEBIAT TEFAYE

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Abstract

In many legume crop species, early-formed fruits or those located closest to the photosynthate are more likely to mature than other fruits. This is so in the *Vicia faba* crop. Based on physiological, nutritional and genetical aspects of seed development, several hypotheses are documented. However, *Vicia faba* pollen fertility has not been adequately studied. Thus, a series of experiments, consisting of studies of both *in vitro* and *in vivo* pollen viability, pollen tube growth and fertilisation ability were conducted using highly inbred lines (6-13 generations). Some of the *in vivo* experiments were conducted under Ethiopian field conditions.

In some lines, even a low level of stress at 20°C for 4h at 100% relative humidity (RH), caused a dramatic drop in mean percent pollen germination. The overall results indicated that there was considerable variation ($p \geq 0.001$) among the lines studied, in response to high temperature (20 to 35°C) and 100% RH.

Vicia faba pollen showed high variability in fertility level. Overall significant differences exist in fertility ($p \geq 0.01$) among the genotypes studied. A fertility gradient exists along the stem: in most genotypes the fertility declining towards the upper nodes. In all of the genotypes, the first nodes carried more highly fertile pollen than the middle and the last flowering nodes. Also The middle nodes were more highly productive than the last nodes in all the genotypes. Thus, success of fertilisation depends to a degree on the source of pollen used.

In an experiment concerning pollen storage, freshly dehiscent anthers desiccated at 25°C/6h and freeze dried for 45min, stored in either LN2 or at -80°C, gave more than 80% viability after 9 months of storage. Desiccation at RT for 24h and storage in either LN2 or at -80°C and at -20°C, still maintained pollen viability $\geq 80\%$.

Mixed pollination studies conducted indicated that pollen from one or other of each pair of inbred lines, mixed on an equal weight basis, performed better as pollen parents on some maternal lines than on others. The importance of these findings with respect to synthetic variety and hybrid seed production in the text is discussed. The probable existence of genetic self-incompatibility is also discussed.

This thesis is entirely my own work and has not previously been offered in candidature for any other degree or diploma

Asfaw Telaye

June 1990.

No one supposes that all the individuals of the same species are cast in the same actual mould. Therefore, man selects only for his own good: Nature for that of the being which she tends.

Charles Darwin 1898 and 1920.

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List of ABBREVIATIONS

W	Watt
UV	ultraviolet
BV	blueviolet
P	probability
\log^{-1}	antilogarithm
nd	not done
nf	no flowers
CMS	Cytoplasmic male sterility
NMS	Nuclear male sterility
cv	cultivar
min	minutes
h	hours
mm	millimeter
cm	centimeter
mg	milligram
g	gram
ml	millilitre
l	litre
m	metre
km	kilometre
mol	mole
mM	millimolar
M	molar
km	kilo metre
ha	hectare
a.s.l.	above sea level
nr	not recorded
°C	degree Celsius
FCR	fluorochromatic reaction
FDA	fluorescein diacetate
RH	relative humidity
Fig.	figure
Figs.	figures

PTD	Pollent tube distributions
GOT	glutamate oxaloacetate transminase
AAT	aspartate aminotransferase
w/v	weigth/volume
UV	ultra violet
BV	blue violet
PAGE	polyacrylamide gel electrophoresis
TEMED	N,N,N',N,'-Tetramethylethylene-diamine
c.a.	combining ability
ABA	abscisic acid
GA ₃	gibberellic acid
cyt	ctytokinins

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Chapter I

INTRODUCTION

1.1 Biology, classification and origin

The faba bean belongs to the family Leguminosae, sub-family Papilionoidae. It is an annual with generally stiff straw, the stem being hollow except at nodes. Like other legumes, the root system is composed of a strong tap-root and secondary, spreading roots. The growth habit is mainly indeterminate; growth and flower induction continue while the lower part of the stem bears flowers and then pods. Reproductive nodes usually begin between nodes three and five, typically continuing to approximately node twenty to twenty nine. The flowers are borne on racemes which develop acropetally, the pedicel lengths varying considerably. The dominant faba bean flower colour is white with dark spotted wing petals and a dark standard petal but there are also pure white flowers and white flowers with pink streaks on the wing petal. There are cultivars of white flowers with yellow or red spots on the wing petals. The overall height of the stem varies from 45 to over 200 cm; branching at soil level is common but there are also genotypes where branches are produced higher up the stem.

1.1.1 Classification

With certain reservations, the **Muratova** (1931) intraspecific classification mainly based on seed size still provides the basis for *Vicia faba* classification. However, some classifications give *Vicia* and *Faba* distinct taxonomic positions (**Cubero** 1973). According to **Cubero** (1973; 1974; 1981), faba beans are a *Vicia* species but should bear a monospecific, sub-genus status. Therefore the position of *Vicia faba* is under considerable debate. Some authors have classified *Vicia* and *Faba* as two distinct genera. An alternative viewpoint is that *Vicia faba* exhibits pure *Vicia* characters, and there is no reason to place it in a new, distinct

genus (Cubero, 1984). He summarises the alternative taxonomic classifications as follows:

- 1) *Faba bona* medikus (*faba vulgaris* Moench, asynonym) which places faba beans in a separate genus but related to *Vicia*. This has been supported by some flora from Iran.
- 2) Sub-generic rank, placing faba beans as the only species of the sub-genus *Faba* of genus *Vicia*.
- 3) Specific rank in section faba of the genus *Vicia*. Included in this are faba beans as species in the genus *Vicia*, sub-genus (or section) *Faba*, *Vicia narbonesis*, *Vicia galilea*, *Vicia johannis* and *Vicia bithynica*.

Another school of thought (Pickersgill, 1986) contends that the classical intraspecific divisions are becoming almost impossible to maintain. Alternative taxonomic groups based on seed size and seed weight are *Faba major* (broad bean), *F. equina* (horse bean), *F. minor* (tick bean) and *F. paucijuga* (Indian types). Of these, the horse bean and tick bean are often included together as field beans. In this type of classification there are considerable overlaps between the taxa and different authors tend to define them in different ways (Lawes *et al.*, 1983). Sometimes groups are also further sub-divided on the basis of agronomic and/or morphological traits; broad beans for example, may be divided into long pod and Windsor types (Pickersgill, 1986).

1.1.2 Biology

The species *Vicia faba* is characteristically diploid ($2n=12$), although the existence of a tetraploid ($2n=4x=24$) has been described by Poulsen & Martin (1977) after colchicine treatment of mutant lines. These were some of the pollen mutant called "Po-I" lines. In the field, the tetraploid plants are morphogenetically similar to the diploid plants; however, the tetraploid faba bean is agronomically unadapted. It develops late, has a low level of resistance to disease and other adverse environmental conditions, a low rate of tillering and low fertility (Martin & Gonzalez, 1981). Faba bean is taxonomically the most isolated of the *Vicia*

species, with larger and fewer chromosomes than the others. The deoxyribonucleic acid (DNA) content is also greater in *Vicia faba* than any other *Vicia* species (Bond, 1976; Poulsen & Martin, 1977; Cubero, 1982; Lawes *et al.*, 1983).

1.1.3 Origin of the crop

Zohary & Hopf (1973) suggested that *V. faba* might share a common ancestry with *V. narbonensis* and *V. galilaea* but this was rejected by Lawes *et al.* (1983) due to its lack of a close cytogenetic relationship with the two wild species. Based on the assumption that autogamy is the primitive condition, Ladizinsky (1975b) suggested that the *paucijuga* type of India and Afghanistan was more likely to be the ancestor of *V. faba*. However, Cubero's (1973) conclusion that weak allogamy is the more primitive condition and autogamy an adaptation to demanding environments disagrees with this. At present it can only be said that faba bean has evolved from an unidentified or extinct wild ancestor.

The centre of origin of faba bean is also uncertain. Due to inconsistencies in archaeological evidence, Ladizinsky (1975b) rejects the Middle East and instead suggests Central Asia as the centre of domestication of *V. faba*. However, Whitcombe (1981) considers the crop's centre of origin to be S.W. Asia and Hawtin (1981) broadly agrees, naming the Mediterranean-West Asia region. Smart (1984) puts the whole of the Mediterranean basin and the Near East as the areas of domestication. With this in mind, Zohary (1977) claimed that because faba bean was widely distributed by 3000 BC, domestication must have begun around 5000 BC. Hanelt (1972) preferred not to be precise but concluded that the origin of cultivation must have been somewhere in the area between Afghanistan and the Eastern Mediterranean region during the period 7000-4000 BC.

However, in the absence of convincing wild ancestors and strong archaeological evidence, most authorities (Bond, 1976; Hawtin, 1981; Whitcombe, 1981; Hawtin & Hebblethwaite, 1983; Lawes *et al.*, 1983; Pickersgill, 1986) agree with Cubero (1974) that faba bean must have originated in the Near East, from where it later spread;

- i. across Anatolia then through the Danube region to Europe, as far north as Scandinavia
- ii. along the North African coast to the Iberian peninsula
- iii. from Mesopotamia to the Indian sub-continent and Afghanistan
- iv. along the Nile valley as far as the South Ethiopian highland plateau.

After this, secondary centres of diversity must have become established in Afghanistan and Ethiopia (Bond, 1976), both mountainous countries where the faba bean is well adapted. At these high altitudes, cultivation was often in isolated valleys, probably with a limited number of insect pollinators available (Lawes *et al.*, 1983).

Having become established as one of the economically important crops in the grain culture of the world as long ago as 3000 BC (Zohary, 1977), by the Iron Age *Vicia faba* was well established in the vast geographical area from Central Asia to Western Europe and from Ethiopia to Scandinavia (Ladizinsky, 1975; Bond, 1976; Hawtin & Hebblethwaite, 1983; Lawes *et al.*, 1983). There is great uncertainty as to when the faba bean reached parts of the Far East other than India and Afghanistan; mainland China for example. Some authorities (Bond, 1976; Hawtin & Hebblethwaite, 1983; Lawes *et al.*, 1983) however, agree that the arrival of *Vicia faba major* in that country must have been in the 13th century AD with the silk trade. A similar spread must have taken place into the Japanese islands.

Although a new centre of diversity seems to be becoming established now in the mountainous region of Peru (Lawes *et al.*, 1983) faba bean was not present in the New World before Columbus' 1492 exploration. Faba bean is thought to have been introduced to Latin America during the conquest of the continent by the Spanish and Portuguese (Bond, 1976).

1.1.4 Importance of *Vicia faba* as a crop

The nutritional value of Faba bean is considerable; like meat, it is rich in protein, hence the term "poor mans meat" (Ali *et al.*, 1981) but, unlike meat, it is also rich in carbohydrates. The protein is characteristically deficient in the sulphur amino acids cystine and methionine but contains high levels of lysine (Frolich *et al.*, 1974; Griffiths & Lawes, 1977, 1978; Simpson, 1983). A recent report by Griffiths (1984) shows a considerable range (23-39%) in protein content among genotypes and populations of diverse geographical origin, much of the variation reported being due to genetic rather than environmental causes. As a protein source, faba bean is important for both humans and livestock and has been so in the Old World since the Bronze Age (Hyams, 1971).

Vicia faba minor and *equina* are mainly used for livestock in developed countries (Hebblethwaite & Davies, 1971). *Faba major* is used as a green cooked vegetable in the Mediterranean countries (Hawtin, 1981) and also canned or frozen for consumption, for example, in the U.K. Third World countries cannot yet afford to use faba bean seed as livestock feed. In fact, in spite of the large numbers of domestic animals in African countries, their populations are mainly dependent on legumes for their protein. In Ethiopia for example, the large rural population and the poor sector of urban dwellers depend on faba bean for the protein they require. The high price of animal protein compels the urban dwellers to use a cheaper source such as faba beans, while the rural sector always prefer to sell their livestock for much-needed cash. The majority of rural people are long accustomed to the use of faba beans as a staple food (Gabrial, 1981; Ali *et al.*, 1981). Currently, there is encouraging interest in developed countries in the processing of faba beans to produce textured vegetable protein as a meat substitute in the human diet (Simpson, 1983).

Other uses of *Vicia faba* depend on the inherent ability of its root nodule bacteria to fix atmospheric nitrogen as utilizable nitrate; it can for instance contribute 45-552 kg N/ha annually (Roughley *et al.*, 1983). This characteristic of the crop makes it very valuable as a means of replenishing soil nitrate and improving soil quality, particularly in the Third World, for example Ethiopia, where the purchase

of chemical fertilizer is untenable. In developed countries, *V. faba* is used as a break crop where continuous cereal cropping has been practised. This prevents the build up of cereal diseases and pests and reduces the cereal weed population (Hebblethwaite & Davies, 1971; Dyke & Prew, 1983).

Despite its importance as a source of cheap protein and as a soil conditioner, *Vicia faba* has been subject to severe fluctuations in production levels, particularly in the technologically advanced nations. These were due, for example, to the decline in the requirement of feed for draught horses and the lack of ability of the crop to compete with more profitable and reliable crops such as the high-yielding, semi-dwarf wheats available as a result of the Green Revolution in the 1960's (Hebblethwaite & Davies, 1971; Hawtin & Hebblethwaite, 1983). However, in the last 15 years there has been a revival of interest in the crop due to the rapid population increase particularly in the Third World, the rising cost of feed protein and the need for agricultural diversification in Western Europe. This has stimulated the production of a large body of scientific information about faba bean.

In terms of total faba bean production, China is the single largest producer contributing 65% of the world's total, followed by Ethiopia which accounts for only 7% (Hawtin & Hebblethwaite, 1983). In Northern and Eastern Europe the leading producers are Czechoslovakia, the U.K. and France, while in the south Italy is the main producer (Hawtin & Hebblethwaite, 1983). The production of faba bean in the U.K., Italy and France declined in 1970's and early part of the 1980's (Hebblethwaite & Davies, 1971; Hawtin & Hebblethwaite, 1983). Currently it is gaining favour as an alternative crop, a factor responsible for the current increase in production.

Both area planted and tonnes produced in Ethiopia are increasing in relation to chick peas and lentils, even though the output per hectare remains stagnant. Provided it is possible to remove some of the major inherent production problems such as unstable yields, faba bean may have a more promising future.

1.2 Agronomic and economic problems with faba bean production

Of primary importance to faba bean growers is its grain yield; thus the need to achieve stable yields largely determines production practices. Unfortunately, like all other legumes, the grain yield of faba beans is notoriously low and unstable compared, for example, to cereals. The mean harvest index for faba bean is no more than 30% even in developed countries, compared with an average of 45% for cereals (Thompson, 1983). Problems in attaining stable yields, coupled with small profits, result in fluctuations in the area given over to faba bean production. Faba bean production, with details of management, techniques and problems is well documented (Hebblethwaite & Davies, 1971; Dyke & Prew, 1983; Hebblethwaite *et al.*, 1983).

Yields vary from year to year even under the same management and production practices. The abscission of flowers and young pods is the single most important factor contributing to low yields; this will be discussed later (1.2.4). However, pests diseases, weeds and weather conditions also play a major part in determining faba bean yield. For example, adverse conditions such as the winter frosts occurring in the Ethiopian highlands not only reduce plant populations but also weaken those that survive, making them more susceptible to disease and water stress. Thus, quality as well as yield of grain is reduced. Water stress substantially decreases both grain yield and total dry matter production (Hebblethwaite, 1981; Dantuma & Thompson, 1983). In some cases, appropriate irrigation regimes and deep ploughing (Hebblethwaite, 1981) with correct fertilizer application and weed and disease control (Hebblethwaite *et al.*, 1983), have improved grain yield.

1.2.1 Weeds

In general, faba bean competes poorly with weeds but the crop will not be lost totally even if not weeded. The Ethiopian peasant farms are a good illustration; there is no soil preparation and the practices of weeding and hoeing are unknown, yet the crop is still produced. A weed problem prevailed in Western Europe prior to the advent of herbicides (Hebblethwaite & Davies, 1971; Gates, 1978; Smith,

1982; Dyke & Prew, 1983; Hebblethwaite & Hawtin, 1983). Even if annual weeds are controlled there are, as yet, few selective herbicides for the control of broad leaved weeds such as cleavers (*Galium aparine*). This severely restricts production of the crop. However, the use of effectively-translocated, non-selective herbicides such as glyphosate and the recent discovery of other herbicides which selectively control many grass weeds (e.g. common couch, *Agropyron repens*) in the faba bean crop promise to reduce the severity of this problem (Hebblethwaite & Hawtin, 1983).

When total crop failure due to weed infestation occurs it is due to a parasitic weed called broomrape (*Orobanche* spp.), sometimes referred to as "parent parasitic disease" (Cubero, 1983). There are several species of *Orobanche*; the economically important ones are *O. crenata*, *O. ramosa* and *O. aegyptica*. These thrive in cool, humid conditions; thus faba beans grown in the Mediterranean regions, including North Africa, the Near and Middle East, are under threat from devastating infestations of *O. crenata*. In Morocco and Malta, cases are reported of farmers giving up faba bean production completely because of *Orobanche*. *Orobanche* as a weed does not occur in the faba bean growing region of Sudan (personal observation, 1986) but *Orobanche* species are observed in Ethiopia. The possible danger these present has not yet been fully appreciated.

So far, no single effective control for *Orobanche* has been devised although biological control by the fly, *Phytomyza orobancheae*, and incorporation of genetic resistance have been proposed (Cubero 1983; Hebblethwaite *et al.*, 1983). The most effective means found so far involves spraying glyphosate onto the emerging shoot of the parasite (Hebblethwaite *et al.*, 1983). Another chemical means of controlling is by incorporation of Dazomet at 25kg/ha which has been reported to give excellent control of *Orobanche crenata*, if applied before planting (Zahran & Rabeia, 1989).

1.2.2 Pests

Faba bean is prone to damage by several insect pests, the most serious of which

are the *Aphis* species. *Aphis fabae*, the black bean aphid causes the most damage, followed by *Aphis pisum*. Both damage crops by direct feeding on faba bean and by serving as vectors for viral diseases. The extent of damage caused and the population dynamics of these aphids were evaluated by **Cammell & Way** (1983). These insect pests are well established in many parts of the world, from temperate to tropical regions but under Ethiopian conditions *Aphis pisum* is a serious pest only sporadically. The most popular means of controlling aphids is by spraying with systemic organo-phosphorus and carbonate compounds (**Cammell & Way**, 1983; **Hebblethwaite**, 1971). However, faba bean depends predominantly on bee pollinators and systemic insecticide treatment will kill the bees unless care is exercised in spraying. Plant genetic resistance has also been sought (**Bond & Lawes**, 1975).

Other faba bean pests such as the stem nematode *Ditylenchus dipsule* (**Kuhn**, 1857; **Filipjev**, 1936), root lesion nematodes (*Pratylenchus* spp.) and root knot nematodes (*Meloidogyne* spp.) are described, along with methods of control, by **Hooper** (1983).

The third category of economically important faba bean pests has been fully dealt with by **Bardner** (1983) who describes the damage incurred by a diverse assemblage of over 50 pests. Some are the cause of widespread crop losses, while many are only minor pests. Faba bean leaf miners (*Liriomyza congesta*), for example, are very serious pests in Egypt and the Sudan (**Kemal**, 1981; **Siddig**, 1981), while African ball worm (*Hilitus armigera*) can devastate crops in years when early October weather conditions become cloudy and humid. Of the storage pests of faba bean, bruchids (*Bruchidus* spp.) are the most serious problem unless they are chemically controlled, e.g. with 2,5-malathione (**Kemal**, 1981; **Siddig**, 1981). Cow-pea seed beetle (*Callosobruchus maculatus* F) is an important storage pest in Egypt (**Siddig**, 1981). Recently it was observed that an F1 faba bean is more resistant than five parental lines to this pest (**Waly et al.**, 1989).

1.2.3 Disease

All three main classes of disease, i.e. fungal, viral and bacterial, can reduce or totally prevent the production of faba bean. Of these, fungal diseases are the most serious problem. They are widespread in all the major geographical areas where faba bean is grown and may cause substantial crop losses when environmental conditions favour them. Attack by insects predisposes the crop to these fungal attacks.

Fungal diseases may be further sub-divided into diseases of the root and shoot. Root infections of *Vicia faba* frequently involve more than one pathogen; these are often referred to together as the root-rot complex. Stem rot, *Sclerotinia* is a widespread problem and *Fusarium* root and shoot rot even more so. In Sudan high soil temperature has been reported as a predisposing factor for wilt and root-rot disease complex (Saeed *et al.*, 1989). No economic and simple means of fungal control has so far been suggested in the literature (Salt, 1983). Under Ethiopian conditions, the root and stem rot fungi thrive in the black clay soil where drainage is difficult; as a result, production of faba bean in this soil is avoided unless the soil is first formed into a series of ridges 40-50 cm apart to allow drainage.

The major fungal leaf pathogens of *Vicia faba* are *Botrytis* spp., *Uromyces* spp. and *Ascochyta faba*. Severe epidemics of these shoot fungi may cause substantial damage to crops depending on the severity of infection, the time at which infection occurs, the weather conditions and the geographical location (Hebblethwaite & Davies, 1971; Gaunt, 1983).

The two major *Botrytis* species limiting faba bean production are *Botrytis fabae* and *Botrytis cinerea*. *B. fabae* is the more aggressive and can devastate faba bean crops in the Mediterranean region (Mohamed, 1981) and winter beans in Northern Europe (Hebblethwaite & Davies, 1971; Smith, 1982; Gaunt, 1983). It has regularly been a major contributory factor to faba bean grain yield losses. *Botrytis cinerea* is also considered aggressive, occurring as small chocolate spots on the leaves of faba bean grown in most fields. On the Ethiopian highland plateau, in the zone described as the dega zone (coal zone) with an average altitude of 2400

to 3300 m, chocolate spots disease is considered one of the major factors reducing the yield of faba bean crops.

So far, the only means used to control the *Botrytis* diseases has been treatment with fungicides and systemic chemicals such as the benzimidazole group (Gaunt, 1983). Recently, a source of genetic resistance has been identified by Khalil *et al.* (1984). Every year, plants from nursery trials are sent to interested institutions all over the world by ICARDA to test the host pathogenicity reaction under different environmental conditions.

Ascochyta fabae, or leaf spot, has been reported as occurring in most of the faba bean growing regions (Gaunt, 1983). In its virulent condition it attacks all parts of the faba bean plant above the ground, including pods and seeds (Hebblethwaite & Davies, 1971; Gaunt, 1983). The seed then becomes a means of transmission in addition to the pathogen being transmitted by the wind during sporulation. Selection of disease-free seed of a suitable quality probably affords the best prospect for control of this disease. In modern commercial seed production, chemical control is used. Seeds are treated by soaking in thiram or dressing with benomyl and thiobendazole and foliage is sprayed with chlorothalonil (Gaunt, 1983). Recently, sources of genetic resistance have been identified by Khalil *et al.* (1985) from the UK and by Hanounik (1984) from ICARDA, Syria. In Ethiopia, *Ascochyta faba* has been identified but is considered a minor problem in the major faba bean production areas.

Uromyces fabae (rust) is very widely distributed and prevalent in all the geographical areas where faba bean is grown. Infection becomes acute in areas with climatic conditions which are warm but drier than the conditions optimal for *Botrytis faba*. Cases of severe infection may result in total defoliation, leading to the arrest of normal grain-filling processes (Gaunt, 1983). Large crop losses due to *Uromyces* infection have been reported in Upper Egypt by Mohamed (1981) and in the Sudan by Hussien (1981). In Ethiopia, rust is most troublesome in the mid-altitude region of the dega at 1800-2000 m. The fungicide Mancozeb has been mentioned as a means of controlling the fungus by Mohamed (1981) and Gaunt (1983). Genetic resistance has recently been identified by Khalil *et al.* (1984;

1985) and, as for *Botrytis* resistance, ICARDA distributes material for the study of host-pathogen interactions under different climatic conditions.

The less important viral diseases include bean leaf roll virus, bean yellow mosaic virus, broad bean stain virus and broad bean true mosaic virus. These occur in all the major faba bean growing regions including Europe, the U.K., North Africa and the Mediterranean and are mostly transmitted by aphids such as *Acyrtosiphon pisum* (Chapman, 1981; Smith, 1982). Control of the insect and the use of clean seeds are recommended to reduce infection (Cockbain, 1983; Smith, 1982). In Ethiopia, the viral diseases are not considered important in the major faba bean growing regions.

1.3 Flower and young pod abscission

The abscission of flowers and young pods has been ascribed to various causes, one of which is poor pollination. Although some pollen grains are found on the stigmas of undisturbed flowers, there may not be enough to fertilize all the ovules unless the plants are 'tripped' by insect pollinators. Flower morphology, size of the stylar hairs, angle of the style and shape of the keel all determine how soon the mass of pollen can reach the stigma (Kambal *et al.*, 1976). Observations by Soper (1952), Free (1966) and Free & Williams (1976) showed that isolated plants near the edge of a field were frequently visited by pollinating insects to trip the flowers and deposit pollen on the stigmas.

Parthenocarpic pod formation also limits yield of the crop (Kambal, 1969; Kogure *et al.*, 1978; Chapman *et al.*, 1979). Chapman *et al.* found that virtually all the abscised pods they examined contained embryos, supporting the view that after fertilisation the numerous sinks in operation compete with one another to such an extent that the young pods fail to grow. However, Rowland *et al.* (1983) reported that, under field conditions, more than 50% of flowers had no fertilized ovules; these subsequently aborted. They found that overall, the frequency of successful fertilisation was greatest in the ovule position nearest the style, of those flowers towards the base of a particular inflorescence. Depending

on the cultivar, the fertilisation frequency was also generally higher at the upper flowering nodes.

Nevertheless, fertilisation failure is unlikely to be the only major cause of flower abscission (**Kambal**, 1969; 1976; **Free & Williams**, 1976; **Kambal et al.**, 1976; **Chapman et al.**, 1979; **Gates et al.**, 1981; **Rowland et al.**, 1982; **Gates et al.**, 1983). Tripping all the flowers of both autosterile and autofertile lines enhanced the level of pod set but considerable flower abscission was still observed - more than could readily be accounted for by assuming that some of the tripped flowers were not fertilized (**Smith**, 1982c).

Other factors affecting flower abscission include;

- i. genotype
- ii. climatic factors e.g. temperature, light and moisture availability
- iii. agronomic practices such as plant density
- iv. salinity
- v. nutrient supply
- vi. distribution of assimilates
- vii. balance of plant growth regulators

(**Kambal**, 1969; **Chapman & Peat**, 1978; **Chapman et al.**, 1979; **El-Fouly**, 1981; **Hebblethwaite**, 1981; **Peat**, 1983). Some of these will be discussed below.

1.3.1 Plant growth regulators

It has been reported that endogenous plant growth regulators (PGR) can control physiological processes involved in reproduction either directly, or indirectly through various metabolic pathways (**El-Fouly**, 1981; **El-Antably**, 1976a;

1976b). Imbalance in this control can lead to flower drop. The levels of auxin, gibberellic acid (GA_3) and cytokinins (cyt) are high relative to abscisic acid (ABA) early in flowering but not at flower shedding (El-Antably, 1976a; 1976b). El-Beltagy & Hall (1975) found that the endogenous ethylene content increases along with ABA during flower and pod shedding. El-Beltagy *et al.* (1976), Newaz & Lawes (1980), Chapman & Sadjadi (1981) and Smith (1982c) studied the effect of growth regulators on flower and pod retention. No correlation was found between endogenous auxin concentration and abscission; however, the anti-auxin Tri-iodo-benzoic acid (TIBA) enhanced the ability of the inflorescence to compete with the apex for assimilates and prevented flower shedding.

The removal of flower racemes reduced the shedding of flowers on successive nodes (Smith, 1982c); removal of 10 or 20 flowers on the first ten nodes reduced flower shedding by 15% and 35% respectively (Duc & Pickard, 1981). Pod set was also considerably improved by removing the stem apex after flowering at the fifth node, although this did not prevent flower abscission (Chapman *et al.*, 1978; Duc & Pickard, 1981). Removal of the stem apex, a major source of growth regulators, thus, stimulated the development of an effectively-determinate, terminal inflorescence. Mutants where apex senescence is genetically controlled demonstrate a pattern of flower abscission similar to that of non-determinate cultivars; thus, inducing determinacy, either surgically or genetically, does not prevent flower abscission (Gates *et al.*, 1983).

The removal, or complete shading of leaves increases flower drop and has a negative effect on seed yield; this shows the importance of the leaves in the supply of assimilates for pod development (Hodgeson & Blackman, 1957; Tamaki *et al.*, 1972; Kogure *et al.*, 1978; Smith, 1982c).

Although removal of the stem apex increases the number of pods set and the number containing seed (Chapman *et al.*, 1978; Duc & Picard, 1981) it does not increase the seed yield. According to Gehriger & Keller (1980), the total biomass limits grain yield; however, Crompton *et al.* (1981) maintain that the pods set first compete with growing vegetative parts as well as newly formed

pods for assimilates. Others agree, blaming this competition for pod shedding (Jaquierey & Keller, 1978b; Gehriger, 1980; 1982; 1983; White, 1984).

Gates *et al.* (1981) reported that inadequate vascular development in the pedicel and peduncle prevents the pods from competing efficiently with vegetative sinks; thus the pathway for assimilate transport is limiting rather than the amount of assimilate available. Radio-labelled carbon has been fed to plants in an attempt to elucidate the pathway by which assimilates reach the developing pods (Crompton *et al.*, 1981; Smith, 1982c). It was found that although a high proportion of the C¹⁴ accumulated by pods came from the subtending leaf, assimilates could not be transported to nodes below the leaf. There appears to be no direct vascular connection between the subtending leaves and racemes (McEwen, 1972; Gates, *et al.*, 1983).

A new cultivar has been generated with an independent vascular supply (IVS) and improved synchrony of flower anthesis within racemes (Gates *et al.*, 1981). This gives it a competitive advantage during pod fill but does not solve the other major problem of pollen germination and successful fertilisation.

1.4 Breeding systems and objectives

Faba bean has an intermediate breeding system, ranging from total or partial allogamy to complete autogamy according to genotype and conditions. This can be efficiently exploited in breeding programmes. Characteristically, faba bean shows 30-45% outcrossing (Holden & Bond, 1960; Bond & Poulsen, 1983), although a maximum value of 84% (Bond & Poulsen, 1983) and a minimum value of 2.1% (Voluzheva, 1971) have been reported. Most cultivars require the flowers to be tripped by bees or other insects (Free, 1966; Free & Williams, 1976) although some cultivars or land races from North European, Middle Eastern, Indian and African populations will set pods without tripping (Lawes, 1973; Hawtin, 1981; Bond & Poulsen, 1983).

There have been no successful attempts to cross *Vicia faba* with closely related

wild species such as *V. narbonensis*, *V. bithynica*, *V. johannis* and *V. galileae* (Bond, 1976; Ramsay *et al.*, 1984). Such interspecific hybrids would be a useful way of introducing genetic variability into breeding programmes.

The major objectives of breeding programmes are;

- i. increased self-fertility (Lawes, 1981)
- ii. early seed maturity (Aylmer & Walsh, 1979)
- iii. increased yield and yield stability (Sjodin, 1971; Chapman *et al.*, 1978; Chapman, 1981; Naye, 1981; Hawtin, 1981).

Of these, improving the yield and its stability is probably the most important. The factors determining yield are discussed above (1.2) and include; onset and duration of flowering and grain-filling, number of tillers, plant height, harvest index, resistance to pests and diseases, flower abscission and anatomical features such as the independent vascular system for the channelling of assimilates (Hebblethwaite & Davies, 1971; Picard, 1974; Chapman, 1978; De Varies, 1979; Gates *et al.*, 1981, 1983; Depace & Filipettii, 1983; Hebblethwaite, 1983; White *et al.*, 1984).

1.4.1 Temperature effect on pollen germination and fertilisation

Pollen longevity and viability is affected by environmental conditions such as relative humidity, light and above all, temperature regime. Fluctuating temperatures appear to increase flower shedding in a variety of legumes (Coroiness, 1933; Davis, 1945) and high temperatures also adversely affect fruit set, for example in *Phaseolus vulgaris*, where maximum pod set is achieved at 20-25°C (Binkely, 1932; Dale, 1964; Mark & Smith, , 1969; Kuiper, 1972). Dale (1964) found little or no fruit set in *P. vulgaris* at 35°C; any that did occur at this temperature was parthenocarpic. However, Halterlein *et al.* (1980) found that temperatures up to 35°C were unlikely to affect pod set in *P. vulgaris* although Davis (1945) reported that a temperature of 24.5°C reduced pod set in white beans (*Phaseolus vulgaris* L).

This reduction in pollen germination and seed set at elevated temperatures is not characteristic of legumes alone: pollination of *Brassica napus* ssp. *oleifera* is better at 15°C than at 20°C (Coural, 1985); stigma secretion, pollen germination and tube growth are vastly reduced at 35°C in *Acacia* (Marjinson & Sedgely, 1985) and pollen formation and pollination of tomato (*Lycopersicon esculentum*, Mill.) at low temperatures (15°C day/8°C night) gives particularly vigorous progeny (Maisonnevve *et al.*, 1986).

The growth and development of faba bean generally is affected by temperature, as is organ senescence. It is thought that low temperatures (e.g. 14°C) may have more effect on the duration of growth than its absolute rate (Dennter & Elston, 1978). According to von Herzog (1978) the absolute growth rate, which determines morphological features such as the length of the shoot axis and internodes and plant weight, should not be used as a criterion to identify cold tolerance; this is determined rather by CO₂ assimilation and the change in this after cold stress.

Dekhuijzen *et al.* (1981) found that newly formed flowers and pods aborted much more rapidly under a 26°C day/16°C night regime than at a constant temperature of 16°C. Stoddard & Cockwood (1986) believe that pollination failure at low temperatures is due to a selective effect of temperature during the winter and during the flowering season. Pollination failure is not due to loss of pollen viability; Gupta & Munty (1985) showed that *Vicia faba* pollen stored at 4°C for four to five months was still viable while Lim (1979) showed that pollen stored at 20-25°C maintained its viability for only two hours.

1.4.2 Pollen tube growth

Investigators of pollen tube growth biology have historically emphasised either incompatibility response (Heslop-Harrison, 1975) or alteration of expected Mendelian ratios as a result of differential pollen tube growth rates (Pfahler & Linskens, 1972; Currah, 1981). In recent years the importance of differential pollen tube growth rates expressed during gametophytic competition in the ecology and evolution of species has been given considerable attention (Mulcahy *et*

al., 1975). Successful pollen germination, tube growth and fertilisation are vital for seed set in any grain crop. An enormous amount of information is thus available on pollen biology and the physiology and biochemistry of pollen has frequently been reviewed (Johri & Vasil, 1961; Linskens, 1964; Preston, 1965; Heslop-Harrison, 1971; Mascarenhas, 1975; Mulcahy & Ottaviano, 1983). Discussion here will be limited to pollen competition during germination and fertilisation and the effects of temperature on faba bean pollen.

There are many reports in angiosperms of competition between pollen of different genotypes: Pfahler, (1965; 1967a; 1967b), Mulcahy (1971; 1974; 1983), Ottaviano *et al.* (1983) and Yamada & Murakami (1983) discuss this phenomenon in maize; Currah (1983) reports it in onion; Sarr *et al.* (1983) in pearl millet; Barnes & Cleveland (1963) in alfalfa and Mulcahy (1983) in *Geranium*.

As a result of a large number of pollen grains produced by each individual plant and the competition of several pollen tubes in the same style, selection is expected to be more effective on the male gametophyte than on the female counterpart. Male gametophytic fitness arises from paternal plant traits. These are pollen grain yield per plant, shedding time and duration and pollen grain traits such as competitive ability within the anthers, viability, germination time, tube growth rate and selective fertilisation (Pfahler, 1975). A positive response to gametophytic selection has been obtained for specific selective traits such as tolerance of low temperature in *Lycopersicon* (Zamir *et al.*, 1981, 1982; Zamir & Vallejos, 1983), of salinity in *Solanum* (Sacher *et al.*, 1983) and of heavy metals in *Silene dioica* and *Mimulus guttatus* (Searcy & Mulcahy, 1985a, 1985b). Also a positive correlated response for sporophytic traits has been detected in an open-pollinated population of maize (Ottaviano *et al.*, 1982; Ter-Avanesian, 1978); in cotton, wheat and *Vigna sinensis*, Mulcahy, *et al.* (1975); in *Petunia*, Schlichting *et al.* (1987). Also in *Lotus corniculatus* and *Cucurbita pepo*, selection applied to pollen competitive ability resulted in the sporophytic generation for traits that are an expression of plant growth and vigour. These characters are controlled by genes expressed both in the gametophytic and the sporophytic phases (Ottaviano *et al.*, 1988).

Pollen grains contain stored resources. These resources are relatively limited and most pollen tube growth depends on resources obtained from the stylar tissue (**Vasil**, 1974). If many pollen grains are present competition among pollen tubes for limited stylar resources could reduce overall pollen tube growth (**Cruzan**, 1986). On the other hand high pollen tube density could enhance pollen tube growth rates (**Ter-Avanesian**, 1978a; **Cruzan**, 1986; **Bertin**, 1988), provided there is sufficient resource for the pollen tube growth (**Cruzan**, 1986).

The genetic constitution of pollen allows competition during pollen germination and variation in the rate of pollen tube growth according to genotype (**Barnes & Cleveland**, 1963; **Pfahler**, 1965a; 1965b; 1967; **Mulcahy**, 1971; 1974; **Ottaviano et al.**, 1983; **Hill & Vard**, 1986); this is called gametophytic competition. The faster the rate of pollen tube growth, the greater the chance of successful fertilisation of the ovules. Competition between the pollen tubes containing the gametes of different genotypes can occur when pollen of one genotype outnumbers the other (**Mulcahy**, 1975; **Stephenson & Bertin**, 1983; **Willson & Burley**, 1983). The larger number would have more chance of out-competing the smaller number.

When a mixture of pollen arrives on the stigma of a flowering plant, gametophytic competition plays an important part in determining the relative out-crossing success of a genotype (**Bateman**, 1956; **Harding & Tinker**, 1969). In this situation, faster growing pollen naturally achieves higher fertilisation rates which enhances reproductive success. The ability of the gametophyte to compete can give an indication of the fitness of the subsequent sporophyte generation (**Mulcahy et al.**, 1975; **Ottaviano et al.**, 1975; 1980; 1983; **Ter-Avanesian**, 1978; **Mulcahy**, 1974; 1979; **Schennske & Pautler**, 1984). This selection at the gametophytic stage is advantageous, allowing rapid selection of superior gametes which will give rise to superior sporophytes. Pollen competitive ability is also affected by stylar length; the selection pressure exerted is greater with a long stylar column (**Ottaviano et al.**, 1983; **Hill & Lord**, 1983; 1986).

The above concept is supported by evidence of considerable gametophytic gene expression and a large amount of sporophytic genetic overlap. This is to say that a

large proportion of genes are expressed in both phases of the life cycle. **Tanksley et al.** (1981) in tomato and **Sari-Gorla et al.** (1986) in maize have shown that a large proportion (60 and 72% respectively) of a plant's structural genes coding for isozymes in the sporophyte are also expressed in the pollen. Furthermore it has been shown that of 32 different genes controlling endosperm development in maize, 21 (66%) are contributed by the male gametophyte. These are usually expressed in corresponding sporophytes in germination time and tube growth rate (**Ottaviano et al.**, 1988).

In insect-pollinated species such as *Vicia faba*, a large amount of pollen is usually deposited on the stigma. Nevertheless, the number of pollen grains an insect transfers is limited compared with the number that would be deposited on the self stigma as a result of tripping (**Rowlands**, 1958, 1960). This reduces the variation in number of pollen grains present at the start of germination compared with wind-pollinated plants, where the bulk of pollen grains deposited at any one time are nonself-pollen (**Mulcahy & Mulcahy**, 1975).

Both insect pollination and closed carpels in angiosperms are thought to intensify gametophyte competition (**Mulcahy**, 1974); this results in the exclusion of haploid genomes without high metabolic vigour from sporophytes. **Hoekstra** (1983) suggests that the more successful pollen may have transferred some of its synthetic processes, normally carried out on the stigma, to the maturation stage in the anther thus shortening the lag phase of germination.

Pollen competitive ability can be inferred from studies of seed and pod set and seedling vigour as well as pollen germinability and rate of pollen tube growth (**Bookman**, 1984; **Van der Kloef**, 1984). Genetic markers for the quality of pollen have been assessed (**Sari-Gorla et al.**, 1975; **Sari-Gorla & Rovida**, 1980) and electrophoretic screening has also been used (**Marshall & Ellstrand**, 1985).

Pollen germination and its tube growth rate is always affected by pollen genotype (**Pfahler**, 1967; **Pfahler & Linskens**, 1972; **Sari-Gorla et al.**, 1975); maternal genotype (**Pfahler**, 1967; **Sari-Gorla et al.** 1975) and pollen tube density

(**Ter-avanesian**, 1978a; **Gorchov**, 1988). The major external factor, temperature, has a profound effect on pollen germination and pollen tube growth under field conditions (**Chen & Gibson**, 1973; **Mackill et al.**, 1982; **Hoekstra et al.**, 1989 ; **Warrag & Hall**, 1984).

1.5 Summary

Studies of faba bean seed production have been carried out which have led to progress in preventing flower and young pod abortion (**Gates al.**, 1983). Faba bean genetics and genetic variability have been intensively studied (**Afalhein**, 1981; **Chapman**, 1981; 1984). However, little work has been carried out on the effect of varietal differences in pollen germination and competition or on the effect of temperature on pollen tube growth. Such information would be of value in the selection of faba beans for higher, more stable yields.

In *Zea mays* the rate of pollen tube growth is positively correlated with growth of the resultant sporophyte (**Ottaviano**, 1980); this offers a chance to select at the gametophytic level for better adaptive mechanisms. According to **Stoddard** (1986a), abortion is independent of fertilisation in affecting the number of maturing seeds. This study aims to show whether temperature affects pollen competitive ability and fertilisation by looking at its effects on pollen germination, tube growth and effectiveness in carrying out fertilisation. Faba bean inbred lines of diverse geographical origin have been subjected to various temperature stresses *in vivo* and *in vitro* under field conditions in Ethiopia for the whole year of 1988 and *in vitro* during 1987 in Durham, England. Of the total 80 inbred lines field-grown in Ethiopia, eleven were examined for pollen tube growth in their pistils using fluorescence microscopy. A crossing programme both in Durham and Ethiopia has been conducted with nine inbred lines to study pollen competition; equal weights of two different pollen genotypes were used. The resultant F1 hybrids were studied using gel electrophoresis to determine the effect of pollen competition as a result of making crosses with mixed pollen from a pair of genotypes. Also using six Durham and one Ethiopian selections, parameters associated with pollen storage technique have been monitored for nine months in the University of Durham laboratories.

1.6 Objectives of the present study

Vicia faba is notorious for its unstable and low yield. The importance of more gametophytic characters as contributing factors to these yield defects has not been studied in detail. Therefore, the present study was conducted to provide detailed information on six main aspects of *Vicia faba* reproductive biology. These were:

- 1) The effect of temperature stress on flower shedding and pod development under field conditions in Ethiopia.
- 2) The effect of temperature and high relative humidity stress on pollen germination and pollen tube growth under laboratory conditions.
- 3) The effect of storage conditions (including pre-storage desiccation) on pollen longevity under storage.
- 4) Variation in pollen fertility during reproductive growth.
- 5) The extent of potential gametophyte competition between pollen samples from various pairs of *Vicia faba* inbred lines, when these were applied to stigmas.
- 6) The magnitude of extreme temperature stress on pollen germination, pollen tube growth and fertilisation under the Ethiopian growing conditions.

Chapter II

Materials & Methods

2.1 Biological materials

Samples of *Vicia faba* L. varieties, 10 to 13 generations inbred, were supplied by the Botany Department of Durham University. Approximately 4-6 generations inbred lines were supplied by Holetta Research Centre (HRC) (Institute of Agricultural Research, Ethiopia). These are all listed in Table 2.1 below.

2.2 Growth conditions and experimental design

Vicia faba seeds soaked overnight in tap water at room temperature (20°C), were sown in Levington's potting compost inoculated with soil known to contain *Rhizobium leguminosarum* L., one per 14 cm hard plastic pot. To ensure normal drainage facilities, the plastic pots were perforated at the bottom (six holes). Batches of five seeds of each cultivar were planted at 15-20 day intervals to provide a continuous supply of flowers for experiments. Each of the 50 pots was labelled individually and the pots arranged in a randomised block design. Watering, with ordinary tap water, was carried out every three to four days, depending upon the temperature in the glass house which fluctuated between 13 and 26°C.

Seeds sown in the winter when natural day length was less than 12 hours were placed under high pressure 400 watt sodium lamps, type S0NT (Anon, 1973), which were suspended about 1 m above the growing plants. Supplementary lighting was used until the natural day length exceeded 12 hours. Because of relatively low temperatures (13°C) during the early part of the winter season, days to emergence and flowering were somewhat delayed during this period.

Table 2.1: *Vicia faba* lines used for various experimental work, October 1986 to November 1989, in Durham (England) and Ethiopia.

Lines	No. of generation inbred	Geographic origin
STW	10-13	Sudanese
288	10-13	European
NDP	10-13	European
21	10-13	Afganistan
247	10-13	European
18	10-13	European
19	10-13	European
248	10-13	European
11	10-13	European
4/7	10-13	European
20D-2	4-6	Ethiopian
20D-1	4-6	Ethiopian
coll 164/77-2	4-6	Ethiopian
coll 43/78(431)	4-6	Ethiopian
acc.No. 27303-2	4-6	Ethiopian
acc.No. 208103	4-6	Ethiopian
PGRC/E 027052-2	4-6	Ethiopian
77ms88252-1	4-6	ICARDA
75TA26026-2	4-6	ICARDA
78TA26026-1	4-6	ICARDA
TA95-1 (951)	4-6	ICARDA

Cultivars 11 and 4/7 were always very late in both emergence and anthesis, while 19 required scarification or soaking in tap water for 48 hours at room tem-

perature (20°C) to speed up its period to 90% emergence to about 20 days.

2.3 Crossing programmes

The first seeds were sown on 22nd November 1986 in the insect-proof glass house in Durham University Botanic Gardens and flowering started in the first week of February 1987.

To avoid the risk of self-pollination, all flowers to be crossed were emasculated at the stage when the keel petal was properly out of the calyx and the standard clearly visible. This is stage three to four, as described by **Smith** (1982). The emasculation method described by **Gates** (1978) was used; the tip of the corolla was gripped with a pair of forceps and gently pulled. This separates the petals from the calyx and as they slide over the stigma the stamens are ripped off, leaving behind an emasculated flower with an exposed stigma. The method is quick and convenient, and gives similar pod set to the standard emasculation techniques (**Gates**, 1978). Moreover, the damage caused in ripping off the petals induces early formation of stigmatic exudate; this is believed to enhance the success of bud pollination (**Gates**, 1978). However some cultivars, especially 21, 11 and 18 were difficult to emasculate as the whole flower detaches easily at the base of the pedicel, which is very brittle. Flowers of these cultivars had to be gently separated by cutting open the petal and removing the immature anthers. All emasculated flowers were immediately pollinated by depositing a liberal amount of pollen on the stigmatic surface.

2.4 Chemicals

Chemicals used were of analytical reagent grade whenever possible. The following were supplied by Sigma Chemical Co., St. Louis, Mo., USA:

α -naphthyl acetate

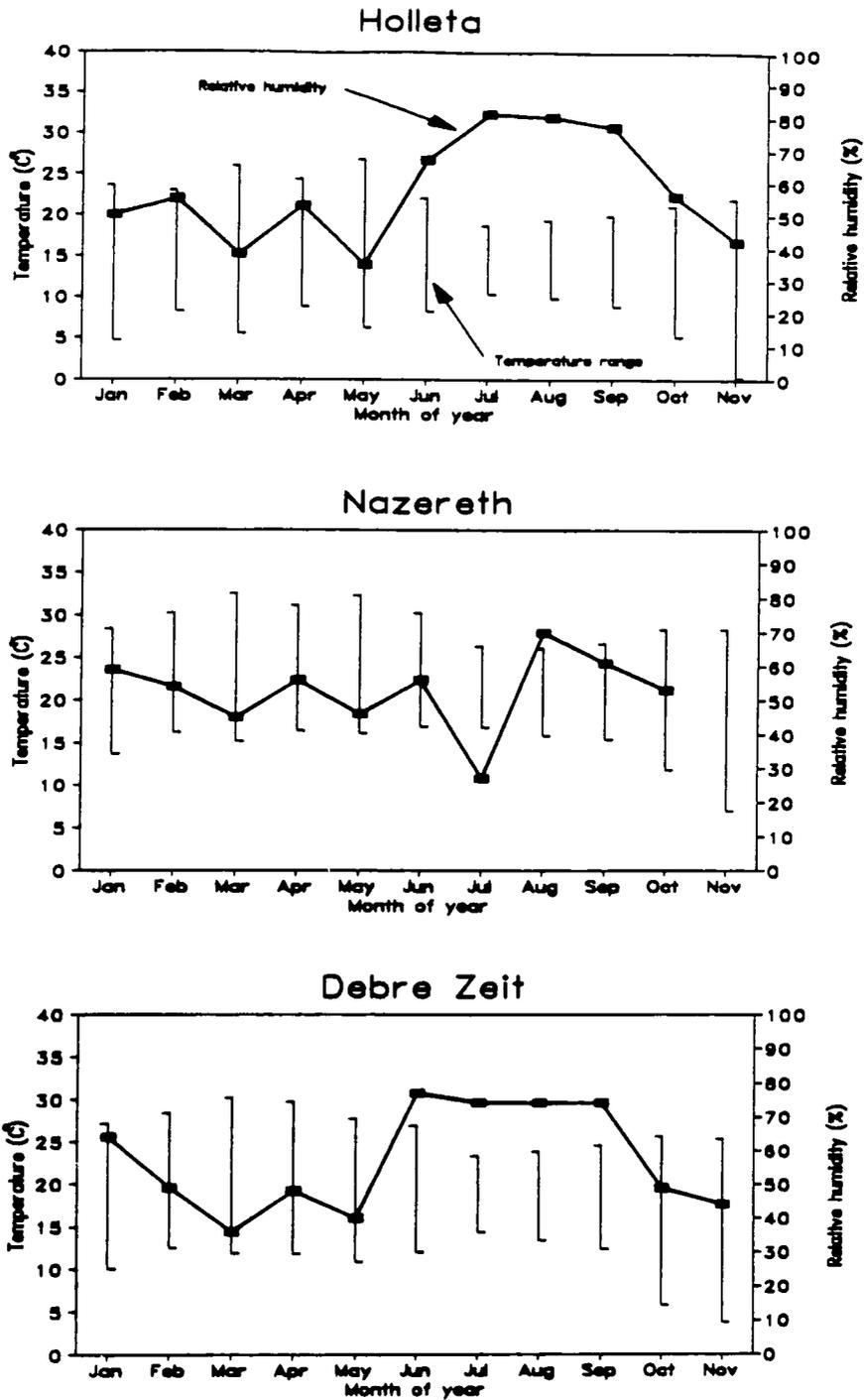
Acrylamide

Fig. 2.1: Ridge patterns used throughout field experiments in Ethiopia, November 1987 to December 1988. Notice the early establishment of *Brassica* seedlings prior to seeding of *Vicia faba* seeds, at Debre Zeit.

Fig. 2.2: *Brassica* seedlings stage after 20 days of *Vicia faba* seed sowing, at Holetta, Ethiopia. Notice the well established *Brassica* seedlings at the time of *Vicia faba* seed sowing.



Fig. 2.3: Monthly mean temperatures and relative humidity (based on four recordings/24 h) at three research centres in Ethiopia (01/88-11/88)



Ammonium persulphate

Aniline blue

Fast blue BB salt

K_3PO_4

Glycine

Methylene-bis-acrylamide (BIS)

$MgSO_4 \cdot 7H_2O$

Starch

N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED)

Tris(hydroxymethyl)-aminomethane

Potassium iodide (KI)

The following were supplied by the British Drug House Ltd., Poole, Dorset:

Acetic acid

Bromophenol blue

$Ca(NO_3)_2 \cdot 4H_2O$

Ethanol

Formaldehyde

H_3BO_3

HCl

KOH

$K_2HPO_4 \cdot 2H_2O$

KNO_3

Methanol

NaCl

Na₂PO₄

NaOH

Sodium acetate

Sucrose

Ethanol

Glacial acetic acid

Fluorescein diacetate

Acetone

2.5 Experimentation

2.6 Harvesting

2.6.1 Seeds

Any pods completely blackened (for STW, dull yellow) and fairly brittle were considered ripe and dry. Prior to shelling and bagging the seeds, pods were allowed to dry in the glass house boiler room to roughly 12% moisture. The seeds from each pod were numbered from the pod apex to the pod base and labelled before storing at room temperature (20°C).

2.7 Pollen viability under different environmental regimes.

Experimental material was grown in the horticultural field of Holetta Research Centre, Ethiopia, between November 1987 and September 1988. The sites were

Holetta Research Centre (HRC), 45km West of Addis Abeba, Debre Zeit Research Centre (DZRC), 45km East of Addis Abeba and Nazereth Research Centre (NRC) 120km South East of Addis Abeba. There were six planting cycles, the first four plantings being planned entirely for furrow irrigation. However, the third and the fourth planting cycles received irrigation at three week intervals from the month of April and at weekly intervals from the month of May 1988. The period from June until mid-September is considered the normal rainy season. Supplementary irrigation for the six planting cycles was given during mid-September to mid-October. For pollen viability studies 12 highly diverse genotypes, inbred for four to nine generations, were studied. Five plants of each of these were grown in replicated 0.4 x 1.25m plots planted on ridges with an initial height of 30 cm (fig. 2.2). Until emergence (20 days), two furrow irrigations every week were provided which were subsequently reduced to only one per week.

Brassica species were planted around the entire block two weeks before faba bean planting (fig. 2.3) and a second batch planted at the same time as faba bean seeds. This was to ensure that *Brassica* plants were in bloom throughout the entire flowering period of the faba bean to keep honey bee activities to a minimum on faba beans and to reduce out-crossing to less than 4% (Roberston 1985, personal communication). The 11 inbred lines used in this study are shown in Table 2.1. Meteorological data were also collected during the period of 1 January 1988 to 30 November 1988 for result data interpretation and discussion (table 2.2 and fig. 2.1). These data were records of 6 am, 12 am, 3 pm and 6 pm every day.

2.7.1 Flower collections

At anthesis, flowers from the 12 genotypes were collected between 08:15 and 15 hrs. These flowers were unopened but mature at collection (James *et al.*, 1986) and were in stage 3-4 as described by Smith (1982). Flowers from each line were collected in premarked plastic bags. The flowers were handled as expeditiously as possible from collection to treatments under appropriate temperature regimes (see below).

Table 2.2: Monthly mean meteorological data during the period of (January 1988- November 1988) *Vicia faba* field research in Ethiopia, based on four recordings/24h.

Month	Holetta			Nazereth			Debre Zeit		
	Temperature °C		R.H.	Temperature °C		R.H.	Temperature °C		R.H.
	Min.	Max.	%	MIN.	Max.	%	Min.	Max.	%
January	4.70	23.60	50	13.70	28.40	59	10.10	27.20	64
February	8.30	23.00	55	16.30	30.30	54	12.50	28.50	49
March	5.60	26.00	38	15.20	32.50	45	11.90	30.30	36
April	8.80	24.40	53	16.40	31.10	56	11.80	29.80	48
May	6.30	26.80	35	16.10	32.30	46	10.90	27.80	40
June	8.30	22.10	67	16.90	30.30	56	12.00	27.00	77
July	10.40	18.70	81	16.80	26.30	27	14.30	23.40	74
August	9.90	19.40	80	15.80	26.10	70	13.40	23.90	74
September	8.90	19.90	77	15.40	26.70	61	12.40	24.60	74
October	5.20	21.20	56	11.80	28.30	53	5.80	25.70	49
November	0.20	22.00	42	7.00	28.30	53	3.80	25.40	44

2.7.2 Temperature and humidity treatments

Fresh flowers were placed in a dry plastic 250 ml beaker which in turn was placed in a 500 ml beaker containing 100 ml of tap water. The larger beaker was covered with aluminium foil to form a chamber with nearly 100% relative humidity. This treatment was provided for 4h at the temperature of 20, 25, 30 and 35°C after which the pollen was transferred to a petri dish containing filter paper and cellophane which were presoaked in the nutrient medium of **Brewbaker et al.** (1963). The components of the nutrient medium are listed below:

H ₃ BO ₃	0.05g
Ca(NO ₃) ₂ .4H ₂ O	0.15g
KNO ₃	0.05g

MgSO₄·7H₂ 0.1g
H₂O (distilled) 100 ml

Then this nutrient medium was mixed with 50% sucrose and distilled water in the ratio of 1:1:3 respectively. The pollen grains were immediately spread gently and allowed to germinate for 2h.

2.7.3 Germination assessment

Pollen germination tests for the treatments were conducted at room temperature (22.5°C). After 2h counts were made under a microscope with fields of view chosen randomly. A total of 20 fields of view were selected to score between 100 and 300 pollen grains per petri dish. Pollen was classified as germinated or non-germinated and tube lengths of the germinated pollen were measured. Pollen was classified as germinated if the intact pollen tubes were at least one grain diameter in length (Cook & Walden, 1965; James *et al.*, 1986). Grains which had the whole of their irregular mass of cytoplasm extruded from the pollen cell wall were defined as burst. Non-germinated pollen grains showed no sign of biological activities (Herrero & Johnson, 1980). Germination results were calculated according to the formula of Herrero & Johnson, 1980).

$$\text{Percent (\%)} \text{ germination} = \frac{\text{Pollen germinated}}{\text{total counts} + \text{burst pollen grains}} \times 100$$

2.8 Pollen storage

2.8.1 Pollen collection

This study was conducted on seven genetically diverse faba beans accessions; STW, 288, NDP, 247, 248, 4/7 and TA95-2. Except for TA95-2, all these lines were inbred for 13 generations. TA95-2 has been inbred for 6 generations, STW had its origin in Sudan, whereas TA95-2 came from ICARDA but was selected in Ethiopia. The remaining five lines were all of European origin.

Pollen collection was from even-aged flowers, at stages 6 to 7 as described by Smith (1982), *ie.* at anther dehiscence, unopened but matured pollen grains (James, Ariyanayagam & Duncan, 1986). On collection, the flowers were immediately enclosed in petri dishes. Flowers were harvested from glasshouse-grown plants at Durham University Botanic Gardens and the pollen plugs immediately dusted on to aluminium foil for the various drying processes.

2.8.2 Drying Process

Four methods of drying before storage were used. Pollen collected on aluminium foil was divided into four samples. One sample was spread on the aluminium foil for 24h. The second sample was put in a desiccator containing silica gel which was pre-dehydrated at 105°C for 24h. This was sealed with wax and placed immediately in the incubator at either 20°C, 25°C or 30°C for 6h. The third sample was dried at room temperature (RT, 20.5°C) for 24h. The fourth sample was stored without dehydration.

2.8.3 Storage

Four temperature regimes were used for storage. These were 4°C, -20°C, -80°C and liquid nitrogen (-196°C). At -20 and -80°C, pollen was stored in gelatin capsules (volume 0.21 ml). In LN2 the gelatin capsules would have burst on return to room temperature and so were not suitable; 2.0ml cryotubes with screw lids proved convenient both for storage in LN2 and for subsequent withdrawal of sub-samples for viability studies (Luza & Polito, 1988; Bajaj, 1987).

The cryotubes were *Nunc Cryogenic* ampoules which were fitted to a metal canister. The metal canister was suspended from the top of a unit hanger and immersed in liquid nitrogen (LN2) or cold nitrogen vapour contained in a Union Carbide CR 30A, liquid nitrogen refrigeration metal cylinder. The liquid nitrogen storage temperature was approximately -196°C. Thus each cryotube containing a pollen sample was placed in an aluminum canister and immediately plunged into LN2 (rapid cooling) (Weatherhead *et al.*, 1978).

2.8.4 Viability studies

Evaluation of pollen viability was carried out by measuring the fluorochromatic reaction (FCR) of pollen treated with a dilution of 2 mgml^{-1} fluorescein diacetate in acetone (Heslop-Harrison & Heslop-Harrison, 1970). The reaction depends on a non-polar, fatty acid ester of fluorescein (fluorescein di-acetate) readily entering living cells through the lipoprotein plasmalemma. This is then cleaved by intracellular esterases to acetate and polar fluorescein. The accumulated fluorescein is observed with the BV filter as bright green fluorescence which develops as the fluorescein di-acetate (FDA) is broken down. This is widely in use for study of cell viability, which in turn depends on cell membrane integrity (Shivanna & Heslop-Harrison, 1981). The reagent was prepared from this stock by adding 20% sucrose until the solution just appeared milky (Shivanna & Heslop-Harrison, 1981). Sucrose prevents pollen bursting due to osmotic effects.

FDA for use was made up freshly each time by adding 2 mgml^{-1} fluorescein diacetate in acetone. All sub-samples of variously dried or fresh pollen to be stored were assessed visually for their viability using the method described above. A pollen grain may be considered viable if it fluoresces bright green under the fluorescence microscope using the blue-violet (BV) excitation filter. In each study 22 samples were taken, with three replicates for each sample. Between 200-1900 pollen grains were counted in each of the three replicates.

Every 30 days, sub-samples were taken from each temperature regime and rehydrated to at least 96% relative humidity in a chamber at 4°C for 45 mins to avoid pollen bursting (Gay, Kerhoas & Dumas, 1987; Hecker *et al.*, 1986). 96% relative humidity was maintained in the petri dish containing pollen sub-samples by including a supersaturated solution of potassium nitrate (Winston & Bores, 1960). This study continued for nine months

2.8.5 *Vicia faba* pollen fertility status between and within nodes

2.8.6 Pollen collection

This study was conducted on three genetically diverse faba beans: STW, 248 and 11. All lines were inbred for 13 generations. STW had its origin in Sudan whereas 248 and 11 were both of European origin.

Pollen collection was from even-aged flowers, at stages 6 to 7 as described by Smith (1982), *ie.* at anther dehiscence, unopened flower but matured pollen grains (James, Ariyanayagam & Duncan, 1986). On collection, the flowers were immediately enclosed in Petri dishes. Flowers were harvested from pre-marked nodes. The first flower node marked as node was No. 1. The middle node as node No. 2, was approximately midway between the first flowering node and the last flowering node (table 3.1). The last flowering node was, node No. 3, the last flowering node in the genotype. Collection of each flower was done sequentially according to maturity stage along the flower pedicels (fig. 2.4) at each flowering node. Pollen plugs were immediately dusted on to glass slides and treated with fluorescein diacetate solution.

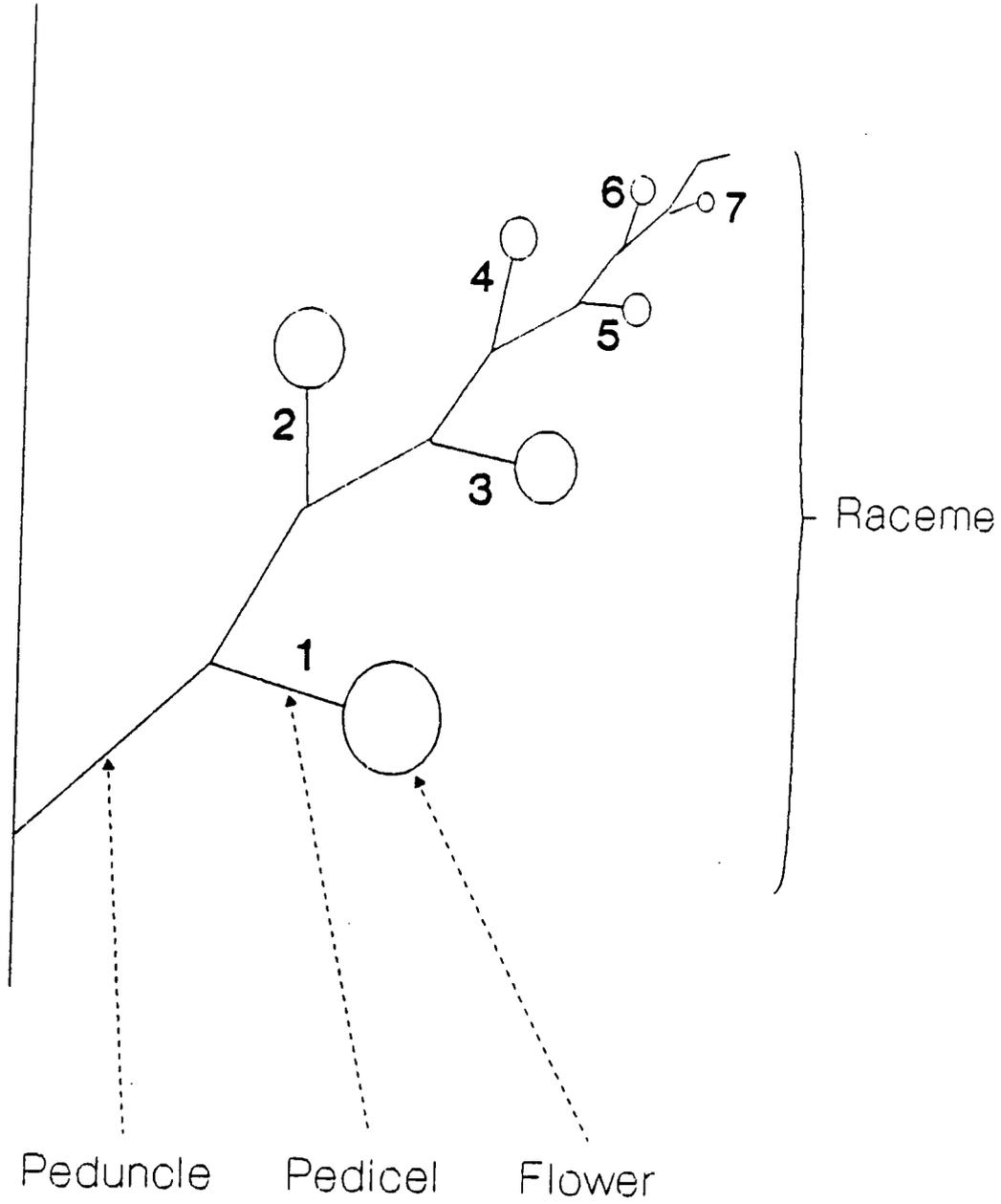
2.9 Pollen competition in *Vicia faba*

2.9.1 Determination of pollen weight

For the study of pollen competition *in vivo*, known weights of pollen were stained with 0.5 % acridine orange after Graman (1982) and, after drying on cellophane at room temperature, were mixed with equal weights of unstained pollen using a mechanical "whirly-mix".

The number of stained and unstained pollen grains in each sample was counted under the fluorescent microscope using a blue excitation filter. Subsequently, the generated data were subjected to a two-way analysis of variance; this showed that the difference in the number of pollen grains in equal weight samples of different genotypes was not significantly different at the level of probability $P < 0.001$.

Fig. 2.4 Schematic diagram of *Vicia faba* raceme showing numbering system used



2.9.2 Isoenzymes for identification of genetic constitution of inbred & their hybrids

2.9.3 Seed analyses

Individual seeds were extracted by soaking them overnight in distilled water then grinding with a small mortar and pestle. The extraction medium and methods used were those described by **Gates** (1978). To remove seed debris, samples were centrifuged at 4200 rpm for 10 minutes.

The formulation of polyacrylamide gels used and the procedures for making up and running them were as described by **Gates** (1978). Polyacrylamide gel electrophoresis (PAGE) has been used to determine isoenzyme patterns of non-specific esterase (EST) for identification of ten faba bean genetic constitutions. Polyacrylamide was chosen because of the high degree of resolution it affords for isoenzyme separations (**Gates**, 1978). It is also stable over a range of physical and chemical conditions and thus gives good reproducibility. The gel and buffers are easily prepared and the availability of relatively inexpensive pure components (**Gates**, 1978) makes the use of polyacrylamide very suitable for this type of study.

As described by **Gates** (1978), quoting **Davis** (1964) and **Smith** (1976), 7.5% discontinuous polyacrylamide gels were prepared for comparison of zymograms of different genotypes. Buffering to pH 8-9 was achieved using Tris-HCl and ammonium persulphate was used as the catalyst, with N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED) as an accelerator.

The apparatus used and details of its operation are described in the laboratory manual for LKB2001 Vertical Electrophoresis Equipment (1982), LKB PRODUKTER AB, Bromma Sweden, pp 6-41.

Gels were cast in glass gel slabs with 5-15 wells per gel. After filling the glass slabs with 7.5% polyacrylamide gels, they were immediately layered with distilled water. On polymerisation which took 1h to 1.30h, the plastic well former was gently removed upwards. Any extra water in the wells was poured off and the

wells were filled with Tris-glycine electrode buffer stock solution (pH 8.3) using a small glass pipette.

The gel run was terminated when the bromophenol blue tracer reached the end of the gels. Of the various enzyme assays described by **Gates (1978)**, glutamate-oxaloacetate transaminase (GOT) (also called aspartate aminotransferase (AAT)) gave the best resolution of bands. After electrophoresis the gel was immersed in the staining solution; keeping it in darkness for 10min gave better resolution of the bands. The staining components of GOT were those listed by **Gates (1978)** which are listed below:

L-aspartic acid	500 mg
α -naphthyl acetate	70 mg
pyridoxal-5-phosphate	10 mg
Fast Violet B salt	200 mg
0.2M Sodium acetate buffer pH 5.0	100 ml

All were supplied by Sigma Chemicals.

For reciprocal cross tests (assays) amylase staining was used. For this assay, the gel was incubated for 2h at room temperature (20.5°C) in 150 ml of 0.2M sodium acetate buffer containing 1.5 g of soluble starch. After two hours the gel was washed twice in 0.1M acetic acid then stained in 100 ml of 0.1M sodium acetate containing 0.1g KI and 20 mg iodine (**Brewbaker et al., 1963**). All gels were allowed to shrink over-night by storing them in 70% ethanol. After a gel was developed to the optimum individual intensity, it was rinsed with distilled water. Gels were placed on a square or rectangular glass. In the gels, individual band migrations, in relation to the bromophenol blue front marker, were measured using a millimeter scale.

2.9.4 Gel drying and scanning:

The shrunken gel was dried under vacuum using Gel Slab Drier GSD-4, (Pharmacia, Sweden). The relative migration distance and band intensity was also

verified by using a Laser Densitometer scanner, (LKB), which has been linked to a line printer, Epson, RX 80, F/T, (Nagano, Japan).

2.10 Factors associated with *Vicia faba* flower drop in Ethiopia

2.10.1 Shed Flower Collections

Flowers were collected as they were shed and put in pre-marked small glass vials (50ml capacity) filled with 98% ethanol and kept at room temperature (18-21°C). Collections were performed twice a day by visiting the fields at 8:30 am and 3:00 pm.

2.11 Factors affecting fertilisation in *Vicia faba* in Ethiopia

2.11.1 Pistil treatments:

For pollen germination and pollen tube growth examination under the epi-florescence microscope, corolla, calyx and staminate sheaths were gently removed, then each pistil was excised by severing at the attachment of the ovary base to the calyx.

For fixation, pistils were transferred to Carnoy's fluid (absolute ethanol : chloroform: glacial acetic acid in the ratio 6:4:1) for 24h at room temperature. After this, they were hydrated in descending concentration of ethanol (Alexander, 1987). The faba bean pistil resists simple squashing so the material was transferred to 0.1M KOH solution and incubated at 32°C for 12h. Each pistil was easily spilt longitudinally using a small pair of tweezers and a laboratory needle, on a slide. This was stained with a solution containing 14.2% (w/v) tripotassium phosphate and 0.5% (w/v) aniline blue (both water soluble SIGMA products). After covering with another glass slide or coverslip, pistils were examined under a Nikon Diaphot-TMD inverted microscope with high-pressure mercury lamp HBO 100W/2 and epi-florescence attachment TMDEF for florescence microscopy. Ani-

line blue stains the callose laid down by pollen tubes, growing down the pistil's conductive tissue (Mulcahy & Mulcahy, 1983) making it fluoresce bright blue, clearly differentiable from phloem tissues using the UV excitation filter.

Ovules were numbered consecutively from the stigmatic end to the base of ovary (fig. 2.5). (Hossaert & Valero (1988); Marshall, (1988) numbered ovule position from the base of the ovary to stylar end).

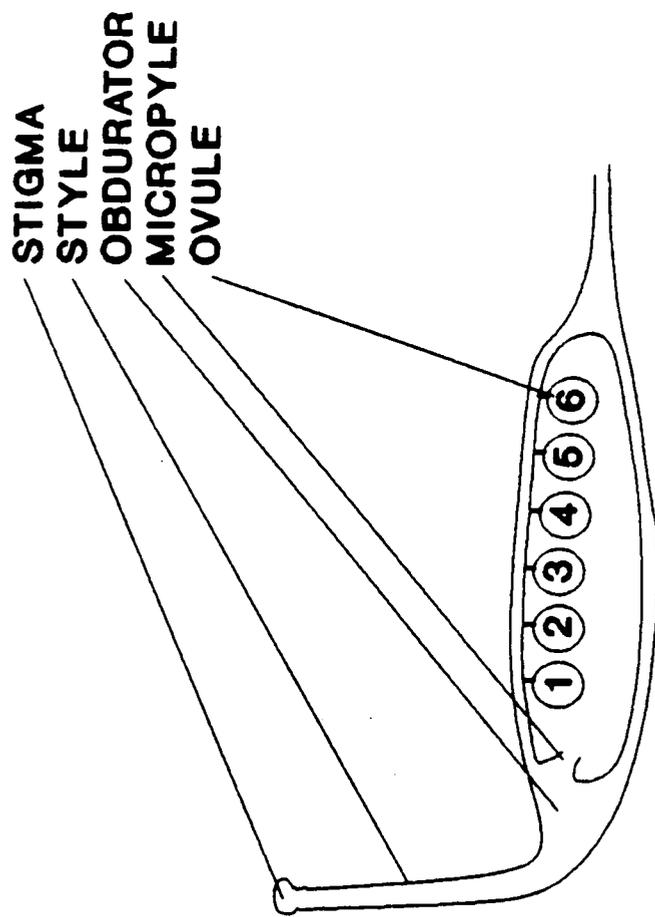
2.11.2 Data Recording

The main interest in this part of the investigation was to study the status of pollen and pollen tube distribution (PTD) whether penetrating the stigma, style, or ovary, and entering the micropyle of the ovule. Therefore data recorded consisted of:

- (1) total number of pollen grains on the stigma. The actual number of pollen grains per stigma, up to a maximum of 300 was recorded. Pollen grain numbers above this figure were estimated and categorised between 500 and 10,000.
- (2) Number of pollen tubes both germinating and penetrating stigma surface
- (3) Pollen tubes in the stylar zone
- (4) Pollen tubes in the ovary
- (5) Number of ovule by-passed, or not reached or penetrated by pollen tubes
- (6) ovules fertilised but dead without further development. This was decided from the colour of an ovule which ranged from bright green to dull black.
- (7) Results were interpreted and discussed in the light of the meteorological data collected during the period (1 January 1988 to 30 November 1988) (table 2.1 and fig. 2.1)

2.12 Microscopy & photography

Fig. 2.5: Diagrammatic median longitudinal section through *Vicia faba* pistil indicating nomenclature used to designate ovule positions.



All photographs, wherever appropriate, were taken using a Nikon FE camera body mounted on the Nikon microscope. For black and white photography, 400 ASA XP1 film was used and for colour photography, 100 ASA Fujichrome film. All films were processed according to the manufacturers' instructions.

2.13 Computing, statistics, graphics and tabulations

The entire text and tabulations were prepared using the \TeX -word processing package (**Knuth**, 1984). Any specific data analyses adopted for a given experimental result is described in an appropriate section(s). Computing facilities used were provided by Northumbrian University Multiple Access Computer (NUMAC), at the University of Durham. The NUMAC machine consists of an Ahmdahl 470/V8 running under the Michigan Terminal System (MTS). The data were analysed on the mainframe, using the Michigan Interactive Data analysis with the SPSSX Software package (**Norusis**, 1985). Statistical analysis was done by relatively straightforward bivariate statistic, eg, ANOVA F-tests and LSD were both available within SPSSX Software. Thus, SPSSX was also used to convert percentage into \log^{-1} in each case for the analyses of variance, regression and correlation analyses (**Kim**, 1987). Various histogram and line graphs were produced using GIMMS Graphics Software (**Carruthers**, 1987). Three-dimensional graphs were produced using Uniras Unigraph Version 4.0 on the Sun Station. After the image was produced it was transferred to MTS for Uniprint on the Laser Printer.

Chapter III

Effect of environmental stress (high temperature and relative humidity) on *Vicia faba* pollen viability

3.1 Introduction

The optimum temperature regimes under which faba bean grows and gives high seed yield normally range from 18°C to 22°C. Temperatures above 25°C can lead to a malfunction of the reproductive organs and, as a result, the crop invariably gives a very low yield. At high elevations in the Tropics or semi-Tropics, where faba is grown under rain fed conditions, high temperatures are usually associated with high relative humidity ($\geq 60\%$). The adverse effect of both high temperature and relative humidity (RH) were investigated under laboratory conditions at Holetta Research Centre (HRC), Ethiopia, to ascertain whether these two environmental conditions have adverse effect in normal pollen germination and pollen tube growth.

3.2 Effect on pollen germination

Fresh flowers were placed in a dry plastic 250 ml beaker which in turn was placed in a 500 ml beaker containing 100 ml of tap water. The larger beaker was covered with aluminium foil to form a chamber with nearly 100% relative humidity. This treatment was maintained for 4h at temperatures of 20, 25, 30 and 35°C after which time the pollen was transferred to a petri dish containing filter paper and cellophane which were pre-soaked in the nutrient medium of **Brewbaker** (1963). The pollen grains were immediately spread gently and allowed to germinate for 2h.

3.2.1 Germination assessment

Pollen germination tests following the treatments were conducted at room temperature (22.5°C). After 2h, counts were made under a microscope with fields of view chosen randomly. A total of 20 fields of view were selected to score between 100 and 300 pollen grains per petri dish. Pollen was classified as germinated or non-germinated and tube lengths of the germinated pollen were measured. Pollen was classified as germinated if the intact pollen tubes was at least one grain diameter in length (Cook & Walden, 1965; James *et al.*, 1986). Grains which had the whole of their irregular mass of cytoplasm extruded from the pollen cell wall were defined as burst. Non-germinated pollen grains showed no sign of biological activities (Herrero & Johnson, 1980). Germination results were calculated according to the formula of Herrero & Johnson, 1980).

$$\text{Percent (\%) germination} = \frac{\text{Pollen germinated}}{\text{total counts} + \text{burst pollen grains}} \times 100$$

3.2.2 Effect on pollen tube growth

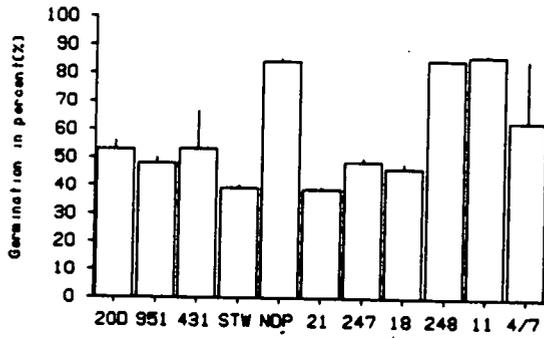
For detail of methodology see under chapter 2.

In this experiment, all imposed treatments were conducted under conditions of 100% relative humidity (RH).

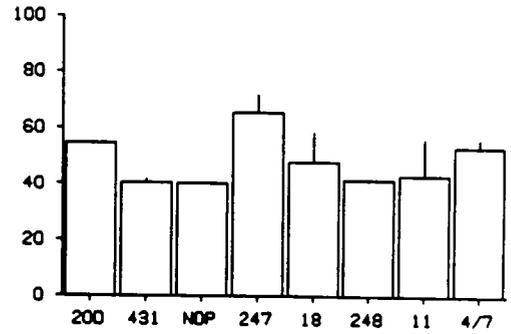
3.3 Varietal pollen germination capacity

Pollen grains not classified as germinated either burst or failed to germinate. In fresh (control) pollen germination, mean percent of the fastest germinating record was from line 11, followed by 248 and NDP. The least value recorded was from STW followed by line 18 (table 3.1 and fig. 3.1-I). Only four lines showed fast germination capacity with values above 60%. The other seven lines all had very low germination and pollen from only two lines gave a score of above 50% germination.

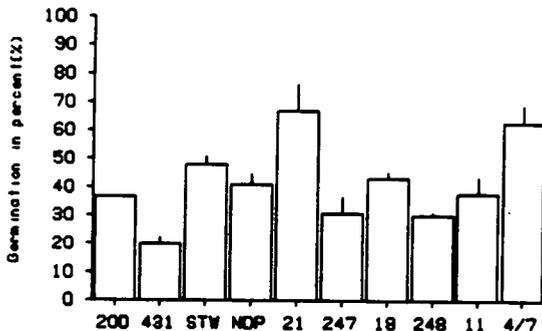
Fig 3.1: Effect of 4h exposure of *Vicia faba* pollen to temperature of 20°C, 25°C, and 30°C on subsequent pollen germination. I= fresh pollen, II=20°C, III=25°C and IV=30°C. All treatments were performed at 100% RH. Incubation period=2h at room temperature (22.5°C).



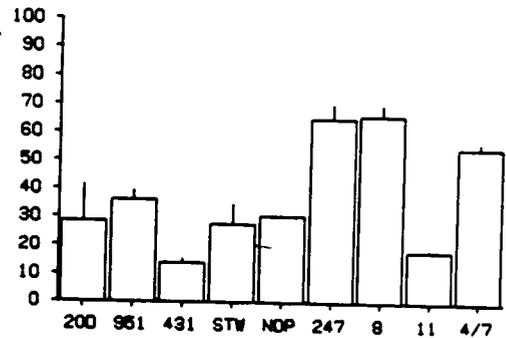
I



II



III



IV

Cultivars

Fig. 3.2: Effect of 4h exposure to 35°C on *Vicia faba* pollen germination (mean percent). Treatment performed at 100% RH. Incubation period=2h at room temperature (22.5°C).

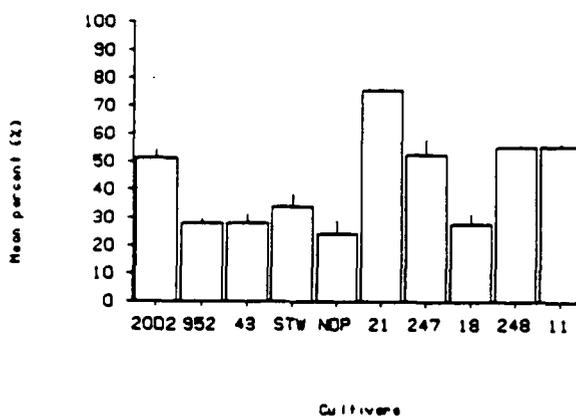


Table 3.1: Mean percent (%) *Vicia faba* pollen germination at room temperature (22.5°C) for 2h after treatment at four temperature regimes and 100% RH.

Lines	Values in %					Means
	***	ns	***	***	***	
	Control	20 C	25 C	30 C	35 C	
20D2	53.00	54.69	36.55	28.48	51.42	42.79
951	47.99	nd	nd	36.13	28.11	32.17
431	53.33	40.24	19.97	14.00	28.09	25.58
STW	39.32	nd	47.78	27.52	nd	37.65
NDP	84.35	40.04	40.50	30.45	33.85	36.21
21	38.78	nd	66.90	nd	24.29	45.60
247	48.77	65.46	30.64	65.01	75.74	59.21
18	46.55	47.82	43.16	66.07	52.64	52.42
248	84.94	41.44	30.14	nd	27.81	33.33
11	86.48	42.99	37.75	18.23	55.23	55.42
4/7	63.20	53.33	62.83	54.91	55.56	56.66
Line means	58.79	48.25	40.16	37.84	43.29	
SE+	3.93	2.80	3.81	4.65	3.81	

ns= not significant at $p \geq 0.05$

*= significant at $P \geq 0.05$

**= significant at $P \geq 0.01$

***= significant at $P \geq 0.001$

nd = means not determined

Thus it was evident that capacity to germinate even at room temperature (22.5°C) showed very large significant differences ($P \geq 0.1\%$) among genotypes.

In contrast, even though there were some differences in germination capacity

at 20°C and 100% relative humidity (RH), these differences were only significant at the 20% level of probability. Nevertheless, germination rates of pollen from line 20D2 showed a slight improvement over the control at 20°C stress, but at 25°C and 30°C, germination rate declined. Mean percent pollen germination from six lines continued to fall in germination capacity as temperature under humidification was raised from 20, 25, and 30°C to 35°C. The effect of 100% RH under the different temperature regimes was that large significant differences ($P \geq 0.01\%$) were observed between treatment parameters.

For genotypes such as 951, 431, NDP, 21 and 248, both 30 and 35°C were damaging. On the other hand pollen from lines 247 and 18 did not seem to have sustained such adverse effects on germination. In effect, a very large significant ($P \geq 0.01\%$) interaction was observed among varieties in response to temperature under high relative humidity (RH).

3.4 Pollen tube growth under the imposed conditions

In the control observation the fastest pollen tube growth was from lines 11, STW and 248, after 2h incubation at room temperature. In this case there were large significant differences detected at the level of $P \geq 0.1\%$. Under these conditions the slowest increase in pollen tube length was in the Ethiopian selection, line 431 (table 3.2 and fig. 3.3).

After the treatments under 100% RH for 4h at 20°C lines 11 and 248 showed more growth, by 44.77% and 51.59% respectively, compared with each respective control. In contrast the slowest pollen tube growth was in line 431, which after similar stress showed an 8.18% increase in growth rate, compared with its respective control. Pollen tube growth from line 247 was also one of the slowest observed in the control experiment, but after similar treatment it showed a faster rate of growth with an increase of 17.78%. Similarly, pollen from line 4/7 also showed some increase in pollen tube growth rate, while pollen grains from 18, NDP and 20D2 lines showed a decrease in the growth rate after stress under identical conditions. However, with respect to pollen germination rate, the analysis of variance indi-

Fig 3.3: Effect of 4h exposure of *Vicia faba* pollen to temperature of 20°C, 25°C, and 30°C on subsequent pollen tube growth. I=frsh pollen, II=20°C, III=25°C and IV=30°C. All treatments performed at 100% RH. Incubation period=2h at room temperature (22.5°C).

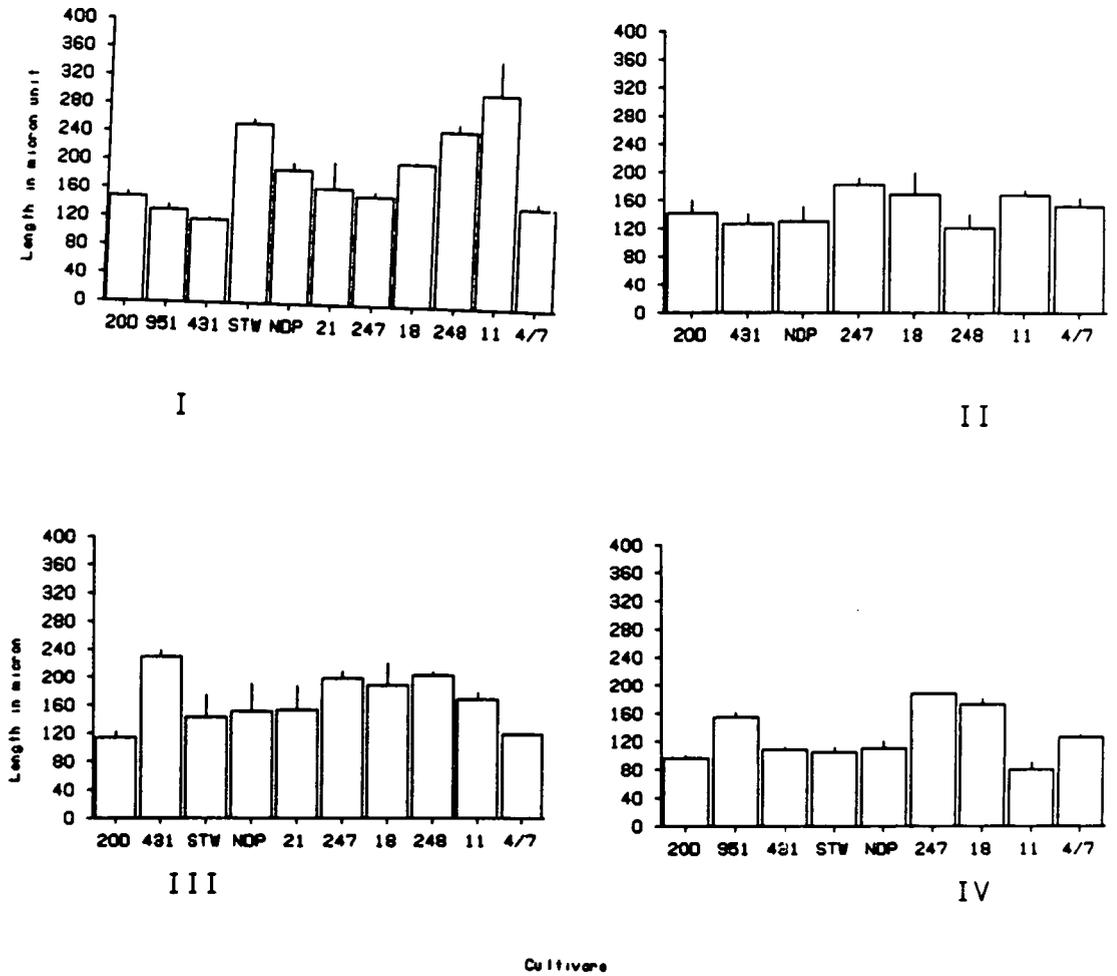
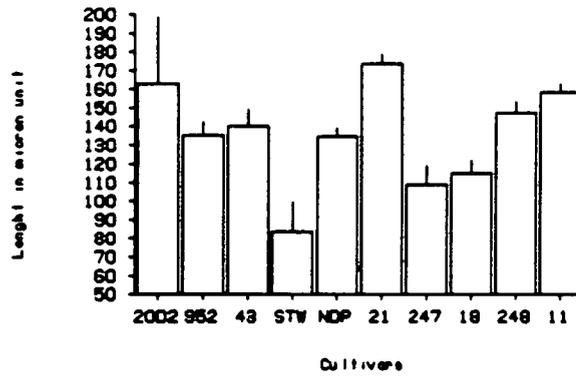


Fig. 3.4: Effect of exposure to 35°C on *Vicia faba* pollen tube growth (mean length in micron). Treatments performed at 100% RH. Incubation period=2h at room temperature (22.5°C).



cated no significant differences at a 5% probability level, but at a 20% probability level, significant differences were observed.

After 25°C exposure at 100% RH, the fastest growing pollen grains from lines 11, STW and 248 were depressed in growth rate by 44.67%, 43.33% and 18.61%, respectively. Similarly, others showing depressed growth rate were those from lines 4/7, 18, 21, and 20D2. In contrast, pollen tube growth rate showed a significant increase (96.93%) in line 431, which showed a very slow growth rate in the control experiment. The analysis of variance in this experiment showed large differences at the 5% level, in response to treatment imposed.

In response to treatments imposed at both 30°C and 35°C with 100% humidity, varietal differences were significant at 0.01% probability. Under 100% humidity for 4h at 30°C, pollen tubes in line 247 showed better growth, compared with the control.

Seven lines showed considerable decreases in pollen tube growth rate, after the exposure to 30°C (table 3.1 and fig. 3.1 I-IV). Also pollen tubes of lines NDP, 21, 248 and 247 continued declining in growth rate at 35°C and 100% RH compared with the growth rate of the control and in the treatments at 20, 25 and 30°C, (table 3.1 and fig. 3.1 to fig. 3.2). However, two Ethiopian lines, namely 20D2 and 431, showed an increase in growth rate over that of their control by 10.05% and 20.29% respectively, after treatment was imposed at 35°C with 100% RH.

Table 3.2: Mean pollen tube length (in microns) of faba bean cvs. germinated at room temperature (22.5°C) for 2h after treatment performed at 20°C, 25°C, 30°C, 35°C and 100% RH for 4h.

Lines	Length in microns unit					Means across Temperatures
	Control	20 C	25 C	30 C	35 C	
20D2	148.11	141.76	114.18	95.53	162.99	128.66
951	129.46	nd	nd	nd	154.06	135.11
431	116.36	125.88	229.15	108.82	139.97	150.98
STW	252.26	nd	142.95	104.66	nd	143.64
NDP	188.28	129.65	150.68	110.41	83.42	118.54
21	163.98	nd	152.97	nd	134.81	143.94
247	154.55	182.03	198.20	188.38	173.80	185.60
18	202.07	168.49	187.98	173.70	108.92	159.81
248	248.99	120.53	202.67	nd	115.07	146.12
11	303.55	167.65	167.95	80.15	147.61	140.86
4/7	143.47	150.07	118.54	126.88	99.00	124.20
Line means	186.40	108.03	166.56	126.98	130.06	
SE+	13.00	13.00	10.22	8.65	6.75	

ns= not significant at $p \geq 0.05$

*= significant at $P \geq 0.05$

**= significant at $P \geq 0.01$

***= significant at $P \geq 0.001$

nd= means not determined

Chapter IV

Pollen fertility variation within and between flower nodes in *Vicia faba* L

4.1 Introduction

Whilst the proportions of crossing incidence vary considerably, on average *Vicia faba* is 33% out-crossing and about 67% self pollinating. (Fyfe & Bailey, 1957; Rowland, 1958; Holden & Bond, 1960; Bond & Poulsen, 1983). However, Porceddu, Monti, Frusvante & Volpe (1980) gave a range of 0% to 50% out-crossing, under Italian environmental conditions. Also Stoddard (1986), reported great variation in the incidence of successful fertilisation among fields in the U.K.

In the inflorescence of *Vicia faba* each flower has the potential to produce seeds. However, the production of flowers usually greatly exceeds the production of viable seeds. Most cultivars are indeterminate in growth habit, in that the stem apex continues to develop vegetatively while a large number of floral nodes also continue to develop and grow. Vegetative growth ceases only with the eventual senescence of the whole plant, coincident with pod ripening.

Most studies on *Vicia faba* reproductive organs have examined seed yield and flower or young pod abscission. No data is available relating to pollen viability between and within flowering nodes. The various scientific publications described above clearly show that successful pod formation is usually at the first, second and third flower nodes, counting upwards from the soil surface (see also chapter 3 of this thesis). Thus by inference, there may be differential viability in the pollen of *Vicia faba* along the stems and flower pedicels.

Fig.4.1: Variation in pollen viability between nodes in *Vicia faba*, mean values of lines STW, 248 & 11

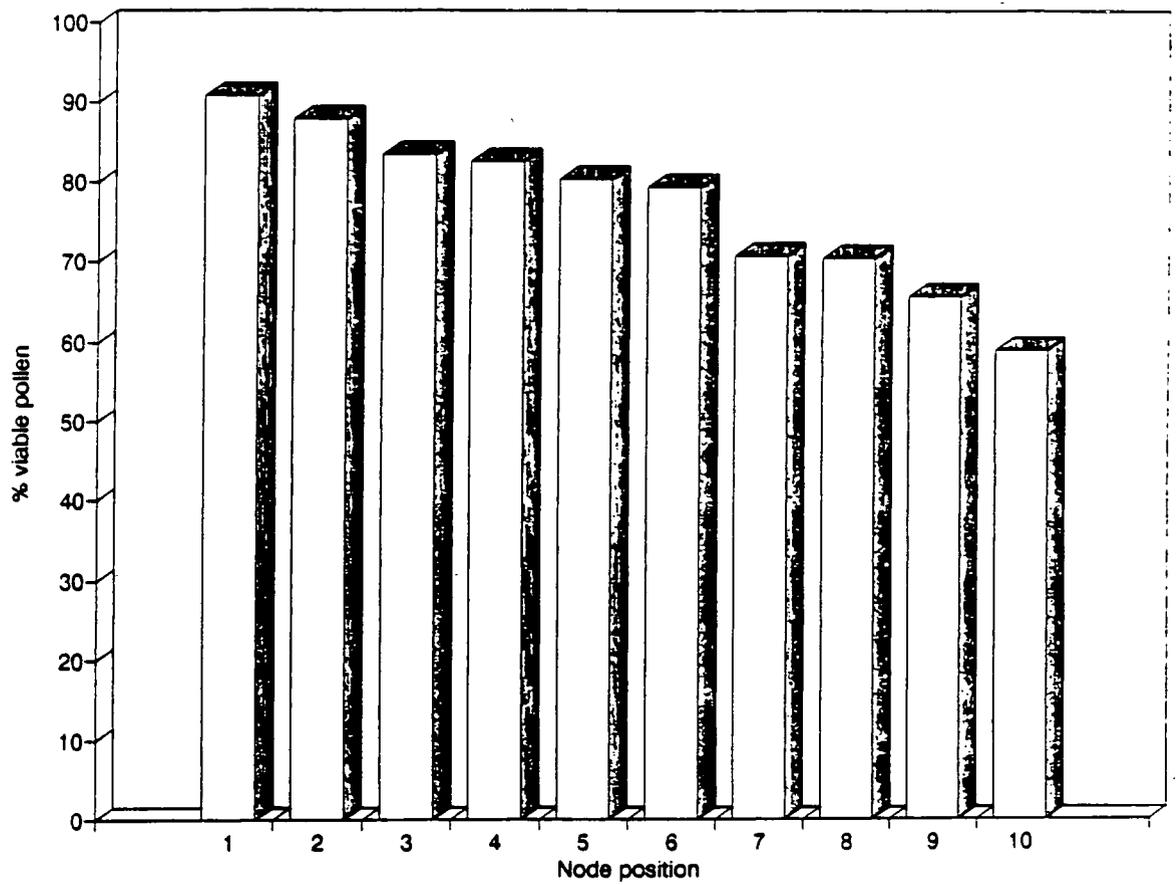


Fig. 4.2: Variation in pollen viability between flowers within flowering nodes. Mean values for all flowering nodes of lines STW, 248 & 11. See fig. 2.4 for schematic diagram of flower numbering system used.



Knowledge of pollen viability status along the reproductive nodes is quite important to breeders choosing the right pollen source during crossing programmes. Such information could also explain why pod bearing is not as good higher up the plant as it is in the lower reproductive nodes of the plant. There is evidence that fertilisation is lower in the upper part of pedicels.

In an attempt to contribute information concerning the fertility status of *Vicia faba* pollen, an experiment on pollen viability was conducted using highly inbred lines.

This study was conducted on three genetically diverse faba bean lines; STW, 248 and 11. All lines were inbred for 13 generations. STW had its origin in Sudan, whereas 248 and 11 were both of European origin. Pollen collection was from homogeneous population of flowers, at stages 6 to 7 as described by **Smith** (1982) i.e. at anther dehiscence, unopened flower but matured pollen grains (**James, Ariyanayagam & Duncan**, 1986). On collection, the flowers were immediately enclosed in petri dishes. Flowers were harvested from premarked nodes. The first flower node was marked as node No. 1; the middle node as node No. 2, some distance between the first flowering node and the last flowering node. Node No. 3, was the last flowering node in the genotype. Collection of each flower was performed according to sequential maturity stage along the flower pedicels at each flowering node. Flowers were collected from glasshouse-grown plants at Durham University Botanic Garden and the pollen plugs immediately dusted on to glass microscope slides and treated with fluorescein diacetate solution.

4.2 Varietal variations

The results from three inbred lines of *Vicia faba* pollen showed that variability in fertility was high. Overall both lines STW and 11 were significantly higher in fertility ($p \geq 0.01$) than line 248. STW and 11 showed differences in their viability, but the difference was not significant at $p \geq 0.05$ confidence level.

Fig. 4.4: Pollen viability comparisons along nodes and pedicels of line 248

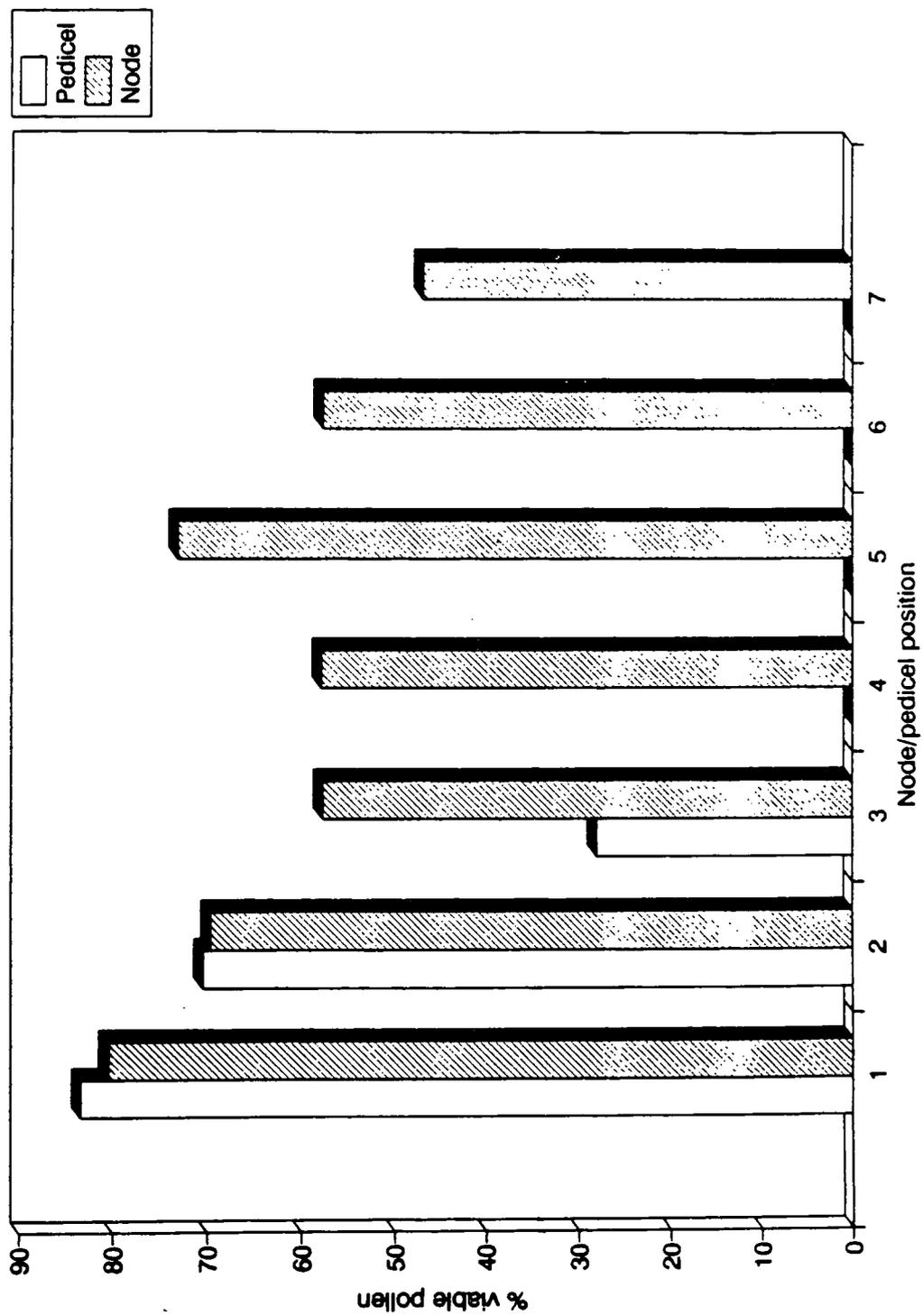


Fig. 4.5: Differences in pollen viability status along nodes and pedicels of line 11

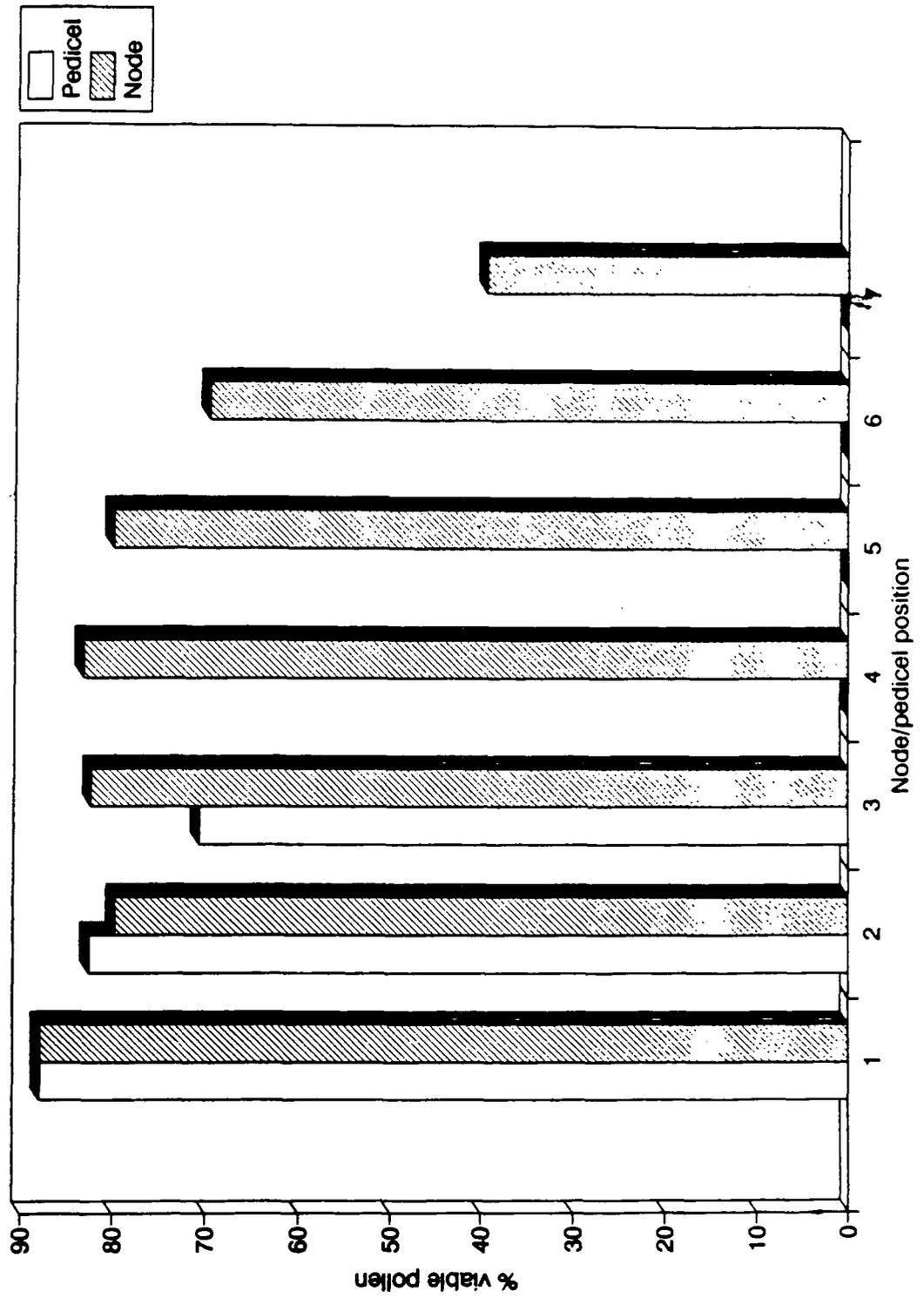


Table 4.1: Varietal pollen fertility variations between nodes of three *Vicia faba* inbred lines.

NODE	L i n e s		
	Mean	percent	(%)
	STW *	248 ****	11 *
4	90.80	nf	nf
6	80.36	nf	nf
10	–	83.40	nf
11	–	–	87.99
12	79.27	–	–
14	nf	70.42	–
21	nf	28.12	70.65
29	nf	nf	70.82

* = values are significantly different at $p \geq 0.05$

**** = values are significantly different at $p \geq 0.0001$

nf= no flowers

– not determined

4.3 Nodal variability

Large variations were observed between both nodes within genotypes and among genotypes. The data are presented in table 4.1. A fertility gradient exists along the stem: in all three genotypes studied, the fertility declining towards the upper nodes (fig. 4.1). In all of the genotypes, the first nodes carried more fertile pollen than the middle and the last flowering nodes. Also The middle nodes were more highly productive than the last nodes in all the genotypes. Further, the last flowering nodes in line 248 produced a mean of 28.12% viability. This was significantly lower ($p \geq 0.001$) than the figures for other flower-producing nodes assessed. Line STW was also lower in pollen fertility at the upper reproductive nodes than line 11 by a mean of more than 15%.

Table 4.2: Variations in pollen fertility within racemes in three inbred *Vicia faba* lines, as tested by FRC (values in mean %)

Flower position	STW **	248 ***	11 ***	Means across flower position
1	90.00	80.48	87.84	86.11
2	82.54	69.63	79.86	77.34
3	63.53	67.82	82.56	71.30
4	nf	57.83	83.39	74.61
5	nf	57.25	80.23	68.74
6	nf	57.68	69.82	63.75
7	nf	46.93	39.57	43.25
Varietal means	78.69	62.52	74.75	

**= Values between pedicels are significantly different at $p \geq 0.01$

*** Values between pedicels are significantly different at $p \geq 0.001$

nf= no flower

4.4 Fertility status within flowering nodes

The recorded data showed conclusively that the basal flowers in racemes have the most fertile anthers (pollen grains) (table 4.2) and fig. 4.2. In the upper flowers within racemes, pollen fertility declined. Again pollen fertility differences were observed within racemes and between racemes of different lines (table 4.2 and figs. 4.1-4.5). In all cases line STW followed by line 11, had the highest mean percentage fertility at the first flowering nodes.

Chapter V

Parameters associated with *Vicia faba* pollen storage

5.1 Introduction

The adverse effects of high temperatures accompanied by high relative humidity and differences in pollen fertility status along the reproductive nodes and peduncles raised a question of pollen conservation for use during unfavourable weather conditions. This study was conducted on seven genetically diverse faba beans: STW, 288, NDP, 247, 248, 4/7 and TA95-2. Except for TA95-2, all these are lines inbred for 13 generations. TA95-2 has been inbred for six generations. TA95-2 came from ICARDA but was selected in Ethiopia. Except for STW, all the five lines were of European origin.

Pollen was collected from even-aged flowers, at stages 6 to 7 as described by Smith (1982), i.e. at anther dehiscence, unopened but matured pollen grains (James *et al*, 1986). On collection, the flowers were immediately enclosed in Petri dishes. Flowers were harvested from glasshouse-grown plants at Durham University Botanic Gardens and the pollen plugs immediately dusted on to aluminium foil for the various drying processes (for more detail see chapter 2).

5.2 Effect of long-term storage on *Vicia faba* pollen viability

5.2.1 Effect of storage duration on pollen viability:

Mean viability values are shown in table 5.1. These were taken after one, six, eight and nine months storage. The analyses of variance for each month data set show large differences between lines in their tolerance to long-term storage. Column 2 of table 5.1 has been included as a control, to compare the degree

Table 5.1: Pollen viability trends under long term storage in seven *Vicia faba* inbred lines (values in mean %), assessed by FCR.

Lines	Control	One month	Six months	Eight months	Nine months
STW	94.32	84.51	76.88	75.17	72.42
loss	0.00	10.40	18.49	20.30	23.22
288	93.05	84.90	78.46	77.37	71.86
loss	0.00	8.67	15.51	16.67	23.59
NDP	93.75	83.80	80.87	76.48	70.37
loss	0.00	10.61	13.74	18.42	24.93
247	96.73	83.04	79.21	79.11	74.73
loss	0.00	14.15	18.11	18.21	22.74
248	89.09	85.78	82.86	82.84	73.50
loss	0.00	3.72	6.99	7.02	17.50
4/7	92.34	84.08	80.73	79.21	67.29
loss	0.00	8.95	12.57	24.78	27.13
TA95-1	87.37	80.34	75.92	74.50	65.64
loss	0.00	8.05	13.11	14.73	24.87

of viability loss as a result of long-term storage at low temperatures, in liquid nitrogen (LN₂), at -80°C, -20°C and at 4°C. In this connection, table 5.1 indicates that the largest viability loss (27.13%) over nine months occurred in line 4/7 and the smallest loss (17.50%) occurred in line 248. The next largest viability losses were seen in lines NDP (24.93%) and TA95-1 (24.87%).

In all lines, except line 248, the loss of viability after 9 months was not less than 22% of the initial value. However, losses of viability between the first and sixth month ranged from approximately 3 to 8%. Thus the highest loss after six months was in line STW (8%) and the lowest loss (3%) was in line 248. In order

Table 5.2: Effect of long-term storage on the percentage of class I pollen as assessed by the FCR

Lines	Fresh (control) ⁴	One month	Six months	Eight months	Nine months
STW	80.64	26.56	7.96	3.07	2.40
loss	0.00	67.06	90.13	96.19	97.47
288	86.61	47.04	14.62	9.86	12.30
loss	0.00	45.69	83.12	85.80	88.62
NDP	73.17	23.81	11.10	8.74	2.15
loss	0.00	77.46	84.83	88.06	97.06
247	75.25	23.35	14.43	6.09	5.41
loss	0.00	67.97	80.82	91.91	92.91
248	83.18	40.39	14.37	7.64	5.09
loss	0.00	51.44	68.81	90.82	93.88
4/7	72.22	27.37	12.64	7.28	4.28
loss	0.00	62.10	82.50	89.92	94.07
TA95-1	59.24	26.17	7.26	6.12	5.93
loss	0.00	55.82	87.74	89.67	89.98

to confirm the viability status *in vivo*, after six months storage at -80°C and in liquid nitrogen, lines STW and NDP were pollinated with stored pollen (figs. 2.1 to 2.2).

5.2.2 Effect of long term storage on fluorescence quality

During evaluation of pollen viability using the fluorochromatic reaction (Heslop-Harrison, 1970) three distinct categories of pollen grains were observed. These were :

Table 5.3: Effect of pre-storage desiccation for 6h on viability of *Vicia faba* pollen (values in mean %).

Lines	Fresh tested**	Stored fresh**	RT 20.5 C ****	20 C **	25 C **	30 C **	FFD **
STW	93.03	78.06	80.55	78.33	77.72	79.81	74.28
288	93.06	77.74	79.26	79.20	86.18	79.71	81.10
NDP	93.75	77.09	79.98	82.22	85.13	78.71	77.35
247	96.73	78.33	81.53	81.03	83.27	77.65	80.12
248	89.10	80.01	84.51	80.55	84.81	80.91	82.79
4/7	92.34	78.97	81.93	79.96	84.16	80.12	80.50
TA95-1	87.384	74.76	74.43	78.68	80.06	76.79	65.40

NOTE: ** shows significant differences among lines at $P \geq 0.01$.

*** shows significant differences among lines at $P \geq 0.001$.

**** shows significant differences among lines at $P \geq 0.0001$.

FFD, fresh pollen frozen for 10min. and freeze-dried for 45 min.

- 1) pollen grains emitting a bright green fluorescence, class I
- 2) pollen grain emitting pale green fluorescence, class II
- 3) no fluorescence from pollen grains, referred to as class III.

Fresh pollen grains in class I from the seven lines, shown in table 5.2, ranged from 59.24% (TA95-1) to 86.61% (288). Within one month of storage at 4°C, -20°C, -80°C and in LN2, the overall class I score had been reduced in all lines. After nine months only two lines, 288 and TA95-1 showed more than 10% of class I pollen grain types (table 5.2). It should be noted that at least in *in vitro* germination, the class II pollen was also as viable as the class I category.

Table 5.4: Influence of storage temperature on viability of *Vicia faba* pollen (values in %).

Lines	Fresh tested	LN2 **	-80 C ****	-20 C ***	4 C ****
STW	93.03	78.83	79.71	78.94	77.08
288	93.06	80.99	81.22	78.79	77.59
NDP	93.75	80.82	80.78	79.42	77.66
247	96.73	80.89	79.30	79.77	79.84
248	89.10	82.09	82.99	81.17	81.56
4/7	92.34	81.56	79.75	81.92	79.37
TA95-1	87.38	75.92	73.97	77.13	76.17

NOTE: ** shows significant differences among lines at $P \geq 0.01$.

*** shows significant differences among lines at $P \geq 0.001$.

**** shows significant differences among lines at $P \geq 0.0001$.

5.2.3 The influence of storage temperature on duration of viability

There were significant differences in viability between the lines in response to storage temperature (table 5.4). However, when data from different storage temperature regimes are compared for each line, there does not seem to be significant differences in response to temperature. This could be partially explained by the unavoidable intermittent monthly thawing and refreezing during the withdrawal of the sub-samples from storage temperatures of -80°C and LN2. This was due to lack of sufficient storage containers for subsamples of each of the seven lines dealt with in the two storage facilities. However, **Hanna et al.** (1983) reported that pollen stored for 78 days at -73°C and thawed for three hours and refrozen for another 55 days gave 100% seed set when used to pollinate cytoplasmic male sterile pearl millet, suggesting that the effect was negligible. Pollen stored at -20 and 4°C was never exposed to temperatures above 4°C at any time. Also the cryotube in the LN2 could not be opened unless it was warmer than its storage temperature. Therefore it seems that this intermittent exposure to room temperature has no significant effect on pollen viability during storage in LN2 or at -80°C .

Table 5.5: Results of Fresh storage* on viability of *Vicia faba* pollen after nine months (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	stored at -20 C	stored at 4 C	Means	% loss F. BV.
STW	93.03	77.50	79.12	79.14	76.62	78.10	1.33
288	93.06	78.10	79.40	77.50	75.87	77.72	2.16
NDP	93.75	81.27	77.22	76.50	71.93	76.73	5.92
247	96.73	79.37	77.19	79.28	77.41	78.31	1.35
248	89.10	81.32	80.18	78.72	79.46	79.92	1.75
4/7	92.34	78.67	75.09	81.90	78.91	78.64	4.15
TA95-1	87.38	79.96	63.25	77.00	72.38	73.15	9.31

Note *=within each treatment there were some values significantly lower at $P \geq 0.05\%$ from all higher or highest values lower at tables 2.5-2.10.

F.BV = from best value.

The mean percent viability of TA95-1 pollen stored at -80°C showed the lowest (73.97%) viability of the varieties stored in/at this temperature regime, after nine months (table 5.4).

5.2.4 Effect of pre-storage desiccation on the viability of pollen

There were six treatments in the experiment, namely control, fresh storage, fresh pollen, left on aluminium foil at room temperature for desiccation (RT, 20.5°C) for 24h and pollen desiccation for 6 h at 20, 25, 30°C . The results in table 5.3 show how different lines responded to the pre-storage treatments. In general, except for STW, the best pre-storage treatment was desiccation of indehiscent anthers at 25°C , and freeze-drying for 45 minutes (Bajaj, 1987). For STW desiccation at RT for 24h was the best treatment. Pollen from freshly dehisced anthers, freeze-dried at 25°C for 6h was the worst treatment for both STW & TA95-1. For the remaining five lines pollen from freshly dehisced anthers was the worst pre-storage treatment. Taking into account all pre-storage treatments,

Table 5.6: Results of 24h RT pre-storage desiccation*** on viability of *Vicia faba* pollen after nine months (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	stored at -20 C	stored at 4 C	Means	% Loss F. BT.
STW	93.03	81.29	85.55	76.17	67.14	77.54	10.33
288	93.06	78.79	81.55	77.25	79.57	79.29	2.50
NDP	93.75	82.82	79.77	80.42	76.45	79.87	3.69
247	96.73	81.67	81.12	83.60	79.25	81.41	2.69
248	89.10	82.78	84.72	86.42	84.00	84.43	2.36
4/7	92.34	85.54	79.04	82.95	79.32	81.71	4.69
TA95-1	87.38	76.70	76.17	74.17	67.14	73.55	4.28

NOTE: *** shows significant differences among lines at $P \geq 0.001$ within treatment.

line 248 pollen had the highest longevity followed by 288 and NDP. Overall, fresh storage of pollen was the worst treatment and desiccation for 6h at 25°C was the best.

Within each pre-storage treatment there was considerable variability among inbred lines in response to treatments imposed. Overall, storage of fresh, undesiccated pollen was damaging for most lines except for 248. For 288, NDP, 247 4/7 and TA95-1, desiccation at RT for 24h was not an optimum treatment for good storage at low temperatures.

5.2.5 Varietal variability in response to pre-storage treatments

5.2.6 Fresh pollen storage

There were large variations in the tolerance of low temperature storage among the freshly-stored genotypes investigated (table 5.5). For pollen grains from NDP, 248 and TA95-1, storage in LN2 was found to be the best low temperature en-

Table 5.7: Influence of pre-storage desiccation at 20°C/6h** on long term viability of *Vicia faba* pollen after nine months (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	stored at -20 C	stored at 4 c	Means Temp.	% Loss F. BV.
STW	93.03	82.32	78.38	79.84	72.80	78.34	5.08
288	93.06	80.41	79.54	79.83	77.16	79.24	1.48
NDP	93.75	83.73	82.24	81.90	81.21	82.27	1.77
247	96.73	79.35	80.17	82.72	81.61	80.96	2.17
248	89.10	84.32	83.15	77.15	78.33	80.74	4.43
4/7	92.34	82.55	79.55	78.02	79.62	79.64	3.65
TA95-1	87.38	79.61	74.96	80.17	79.09	78.44	2.21

NOTE **= shows significant differences among values, within each treatment, at $P \geq 0.01$.

F.BV= from best value.

environment for the long-term preservation of viability. In the least favourable low temperature regime, there was also a high level of variability. For instance, -80°C was recorded as the least favourable environment for pollen grains from TA95-1 and 4/7, but 4°C was the least favourable for NDP pollen.

Viability of pollen grains from STW and 288 was highest at -80°C but lowest at 4°C. There was little difference in viability when fresh pollen from STW was stored at -80°C or -20°C. However, the best storage temperature for fresh pollen grains from 4/7 was -20°C. Fresh pollen grains of 247 showed little variation in levels of viability at different low storage temperatures.

5.2.7 24h desiccation at room temperature (RT, 20.5°C)

Pollen grain desiccation at RT improved storage quality over a long-term period such as nine months, except in lines STW and TA95-1. Line STW showed greater variation in viability under different storage temperatures after this pre-

Table 5.8: Influence of Pre-storage desiccation at 25°C/6h and freeze drying for 45min on long term viability of *Vicia faba* pollen after nine months storage (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	Means across temperatures.	% Loss F. Best
STW	93.03	72.24	81.87	77.06	6.24
288	93.06	88.27	84.84	86.56	1.96
NDP	93.75	84.16	87.84	86.00	2.14
247	96.73	86.31	81.77	84.04	2.70
248	89.10	85.36	86.01	85.69	0.37
4/7	92.34	87.79	80.68	84.24	4.21
TA95-1	87.38	77.17	81.93	79.53	3.02

storage desiccation than line TA95-1 (table 5.6). Thus, pollen viability was better preserved in LN2 or at -80°C than at the remaining two storage temperatures (-20°C and 4°C).

The performance of 248 pollen grains after this treatment, across all storage temperatures, was found to be better than for all the remaining six genotypes. Nevertheless, -20°C and -80°C were the best temperatures for storage of 248 pollen grains, after a pre-storage 24h desiccation at RT. In this respect, 248 and 247 were found to be similar even though their differences with respect to genotypic rate of viability loss could easily be detected (table 5.6).

For 4/7 pollen grains, with this pre-storage treatment, LN2 was the best storage temperature, followed by -20°C. NDP behaved similarly to 4/7, although its viability loss rate was different.

Table 5.9: Influence of Pre-storage desiccation at 30°C* on viability of *Vicia faba* pollen after nine months storage at four low temperature regimes (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	stored at -20 C	stored at 4 C	Means	% Loss F. BT.
STW	93.03	79.08	79.99	80.70	79.57	79.84	1.08
88	93.06	80.16	81.91	80.69	76.35	79.78	2.67
NDP	93.75	77.44	79.62	78.87	79.18	78.78	0.51
247	96.73	78.59	78.34	72.24	81.49	77.67	4.92
4/7	92.34	77.24	80.07	84.16	79.01	80.12	5.04
TA95-1	87.38	71.45	80.97	78.65	77.07	77.04	5.10

NOTE: * shows significant differences among lines at $P \geq 0.05$.

Table 5.10: Influence of fresh freeze-drying for 45min*** on long-term viability of *Vicia faba* pollen after nine month storage (values in mean %).

Lines	Fresh tested	stored in LN2	stored -80 C	Means across temperatures.	% Loss F. Best
STW	93.03	77.98	70.58	74.58	4.04
288	93.06	83.71	80.38	82.05	2.02
NDP	93.75	76.33	78.38	77.36	1.31
247	96.73	83.12	77.12	80.12	3.74
248	89.10	80.61	85.10	82.86	2.78
4/7	92.34	78.42	82.58	80.50	2.58
TA95-1	87.38	69.40	60.61	64.81	7.08

NOTE: *** shows a given value is significantly lower from the highest value(s) within each treatment by $P \geq 0.001\%$.

5.2.8 Indehiscent anthers desiccated at 25°C/6h

The pollen genotypes which stored best after 25°C pre-storage desiccation, were 288 and NDP. However, their optimum storage temperatures were different; LN2 for 288 and -80°C for NDP. Pollen from 4/7, 247 and 288 stored better in LN2 while the remaining four genotypes stored better at -80°C. Of all lines stored in LN2 and at -80°C, line STW showed the lowest level of viability

5.2.9 Effect of pre-storage desiccation at 30°C/6h

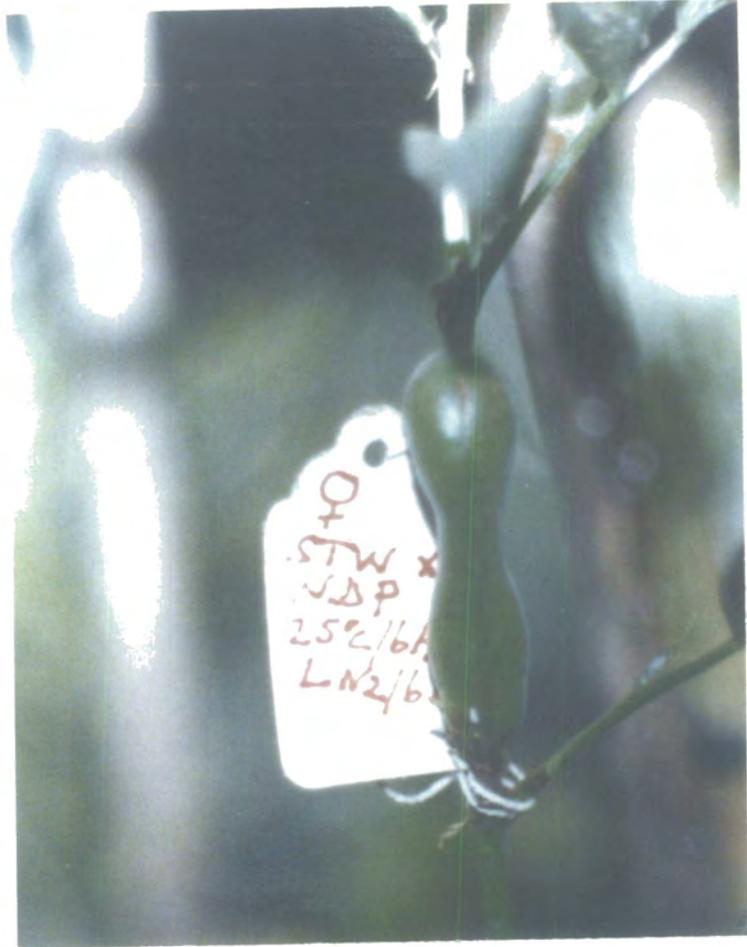
The lowest viability records across the storage temperatures in this treatment were from TA95-1 and 247. In contrast the highest viabilities (81.00% and 80.12%) were recorded in lines 248 and 4/7 respectively. However, the storage temperatures from which their respective high viability values were recorded were 4°C and -80°C. Thus in this treatment 4/7 and STW gave the highest mean percentage viability in -20°C storage. In the same way, for lines 288, NDP and TA95-1 the best storage temperature was -80°C. No genotype gave its highest mean viability value in LN2 storage, while the lowest mean viability value recorded was 71.45%, from pollen of TA95-1 stored in LN2 (table 5.9).

5.2.10 Fresh anthers freeze-dried:

In these treatments pollen viabilities from lines 248 and 288 were the highest after storage in LN2 and at -80°C. The next best genotypes were 4/7 and 247 in this treatment (table 5.10). TA95-1 was the least viable with a value of 64.81%. Three genotypes, 4/7, 248 and NDP gave higher mean percentage viability after storage at -80°C, while the remaining four lines gave better results from storage in LN2.

Fig. 5.1: Successful pod formation after pollinating line STW from NDP stored in LN2 for six months but desiccated at 25°C/6h

Fig. 5.2: Pod formation after pollinating line NDP from line 247 pollen, desiccated at 25°C/6h and stored at -80°C for 6 months.



Chapter VI

Pollen competition in *Vicia faba*

6.1 Introduction

In view of the apparent susceptibility of *Vicia faba* pollen to high temperature, to high RH and because of the variations in pollen fertility along the reproductive nodes, it was postulated that mixed pollination might be advantageous under adverse conditions. This was based on the fact that a heterogenous sporophyte performs better than a homogeneous one. Therefore if mixed pollination performed better than inbred individual lines what would be the practical conditions in which it would be used for faba bean pollination under ordinary field conditions?

In the inflorescence of *Vicia faba*, in which each fertile flower has the potential to produce seeds, the production of flowers normally greatly exceeds that of viable seeds. In the past, this loss in reproductive output has been attributed to the failure of fertilisation or abortion of the zygote. This has prompted various investigators to study the problem of abortion in *Vicia faba* from many different angles.

Vicia faba lines STW, 288, NDP, 247, 248 11, 4/7, 20D-2 and TA95-2 were used in this study. 20D-2 was originated in Ethiopia. Mixed pollination of the first seven lines was carried out between January and June 1987 in glass-houses at the University of Durham Botanic Gardens, England. Mixed pollination of the other two lines was carried out at the Holetta Research Centre, Ethiopia, between March and August 1988. Crossing was either carried out immediately after mixing the pollen or after the vials with their pollen mixture had been stored at 4°C. Pollen stored under these conditions remained as viable as the freshly mixed pollen for up to seven days. Once the pollen mixture was removed from storage at 4°C and had stood for one to two hours in the glass-houses at Durham or in the field at Holetta, pollen viability was drastically and irreversibly reduced. Thus, pollen

mixtures were taken out of storage for use only twice; after this, fresh pollen had to be prepared. The loss of viability was confirmed by a simple test to measure germination *in vitro* (for details see under chapter 2).

Individual seeds were extracted by soaking them overnight in distilled water then grinding with a small mortar and pestle. The extraction medium and methods used were those described by Gates (1978). The formulation of polyacrylamide gels used and the procedures for making up and running them were as described by Gates (1978). Gels were buffered at pH 8.9 using a Tris-HCl system and polymerised using ammonium persulphate as catalyst and N,N,N-tetramethylethylenediamine (TEMED) as an accelerator.

6.2 Terminology

Pollinations were made with a mixture of pollen grains of two inbred parental lines. A pollen mixture was made up of pollen from two inbred lines on an equal weight basis, eg. 288 + 247 inbred parental lines pollen was mixed on an equal weight basis. Thus, in every case, the inbred line quoted in the pollen mixture (eg. 288 in the mixture of 288+247) is referred to as the cross or paternal pollen parent. Likewise, the inbred line quoted second (eg. 247 in the mixture 288+247) is referred to as the self or maternal pollen parent. The pollen mixture was, in each case, deposited on the stigma of the maternal pollen parent.

The maternal parent was an inbred line on whose stigmatic surface mixed pollen loads were deposited in the crossing programme. Thus, one half by weight, of the mixture of pollen grains was derived from the maternal inbred line and the other half from the paternal inbred line.

The term hybrid seed refers to those seeds produced following fertilisation by a pollen tube from the paternal line involved in a particular crossing. A seed was called "selfed" if it had resulted from fertilisation by a pollen tube from the maternal line on whose stigma the mixed pollination was performed.

Fig. 6.1: Ovule position in relation to hybrid seed formation in 10 *Vicia faba* inbred lines in mixed pollination.

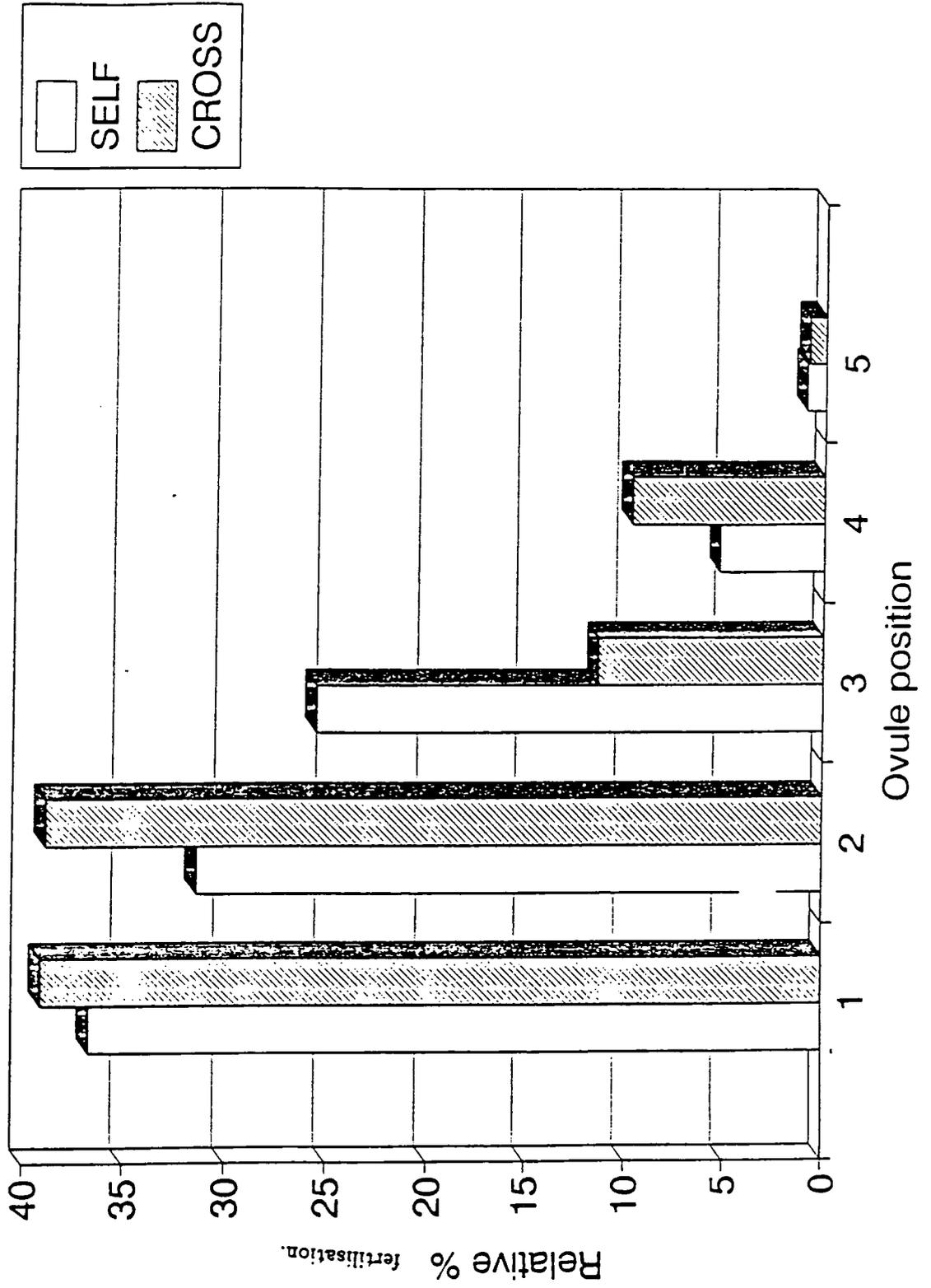


Fig. 6.2: Differences in levels of cross fertilisation after mixed pollination. See table 6.1 for total number of seeds used for each pair of mixed pollination results analyses.

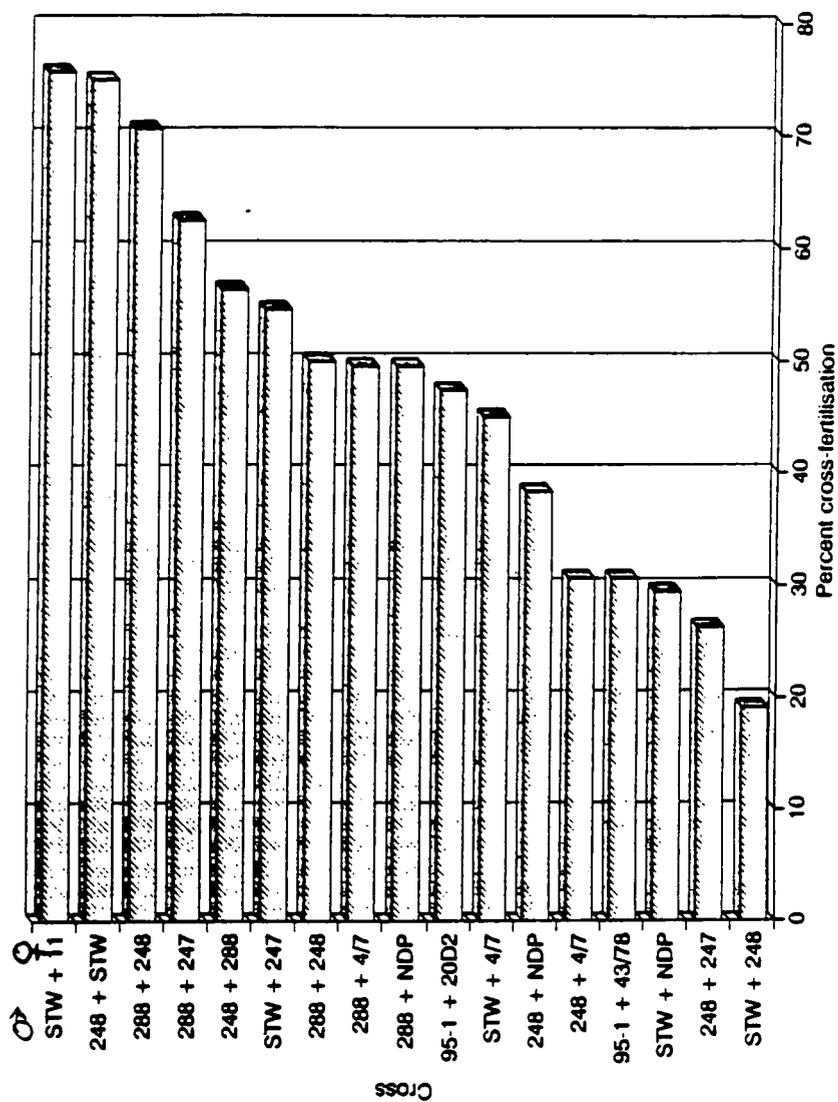


Fig. 6.3: Densitometer-generated graph of aspartate transferase isoenzymes from a single seed, resulting from a pollination of maternal line STW with a pollen mixture containing STW and 248 pollen in equal amounts.

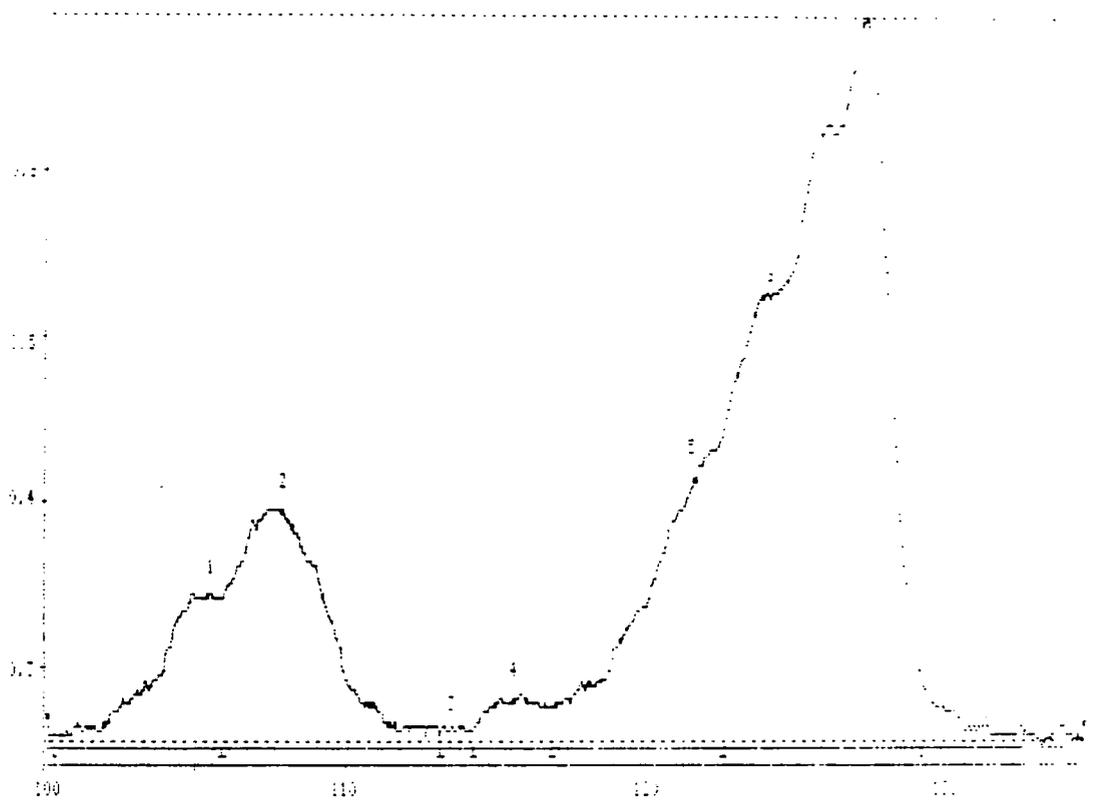


Fig. 6.4: Densitometer-generated graph of F1 single seed from mixed pollination of 248 + STW, where 248 was a adonor parent, gel stained for AAT isoenzymes.

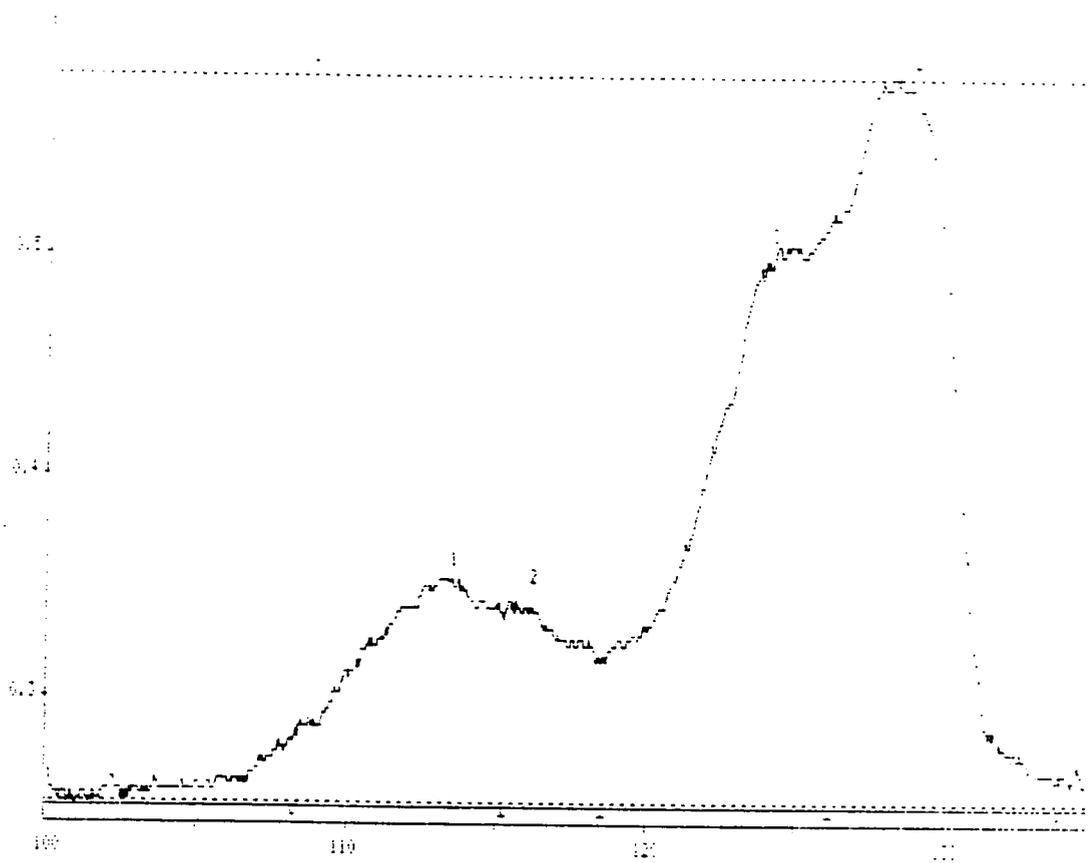


Fig. 6.5: Densitometer-generated graph of inbred STW from a single seed; a gel stained for AAT isoenzyme. STW was used as maternal parent in the mixed pollination.

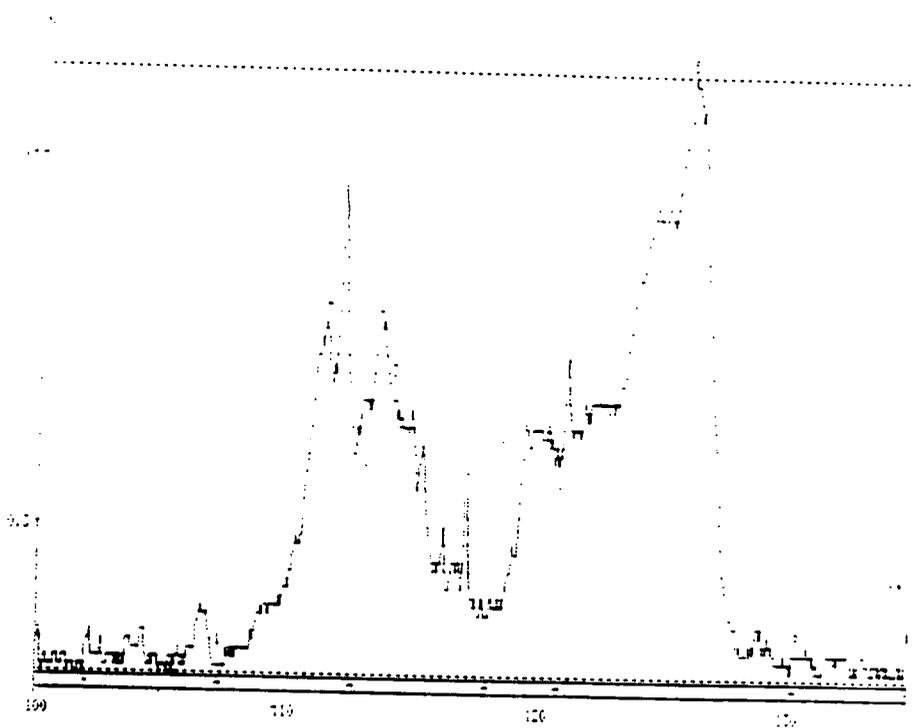


Fig. 6.6: Amylase isoenzyme pattern from single seed. A graph generated by densitometer with beam line smoothed. Inbred line 288 was a pollen donor in the mixed pollination with 248, maternal parent.

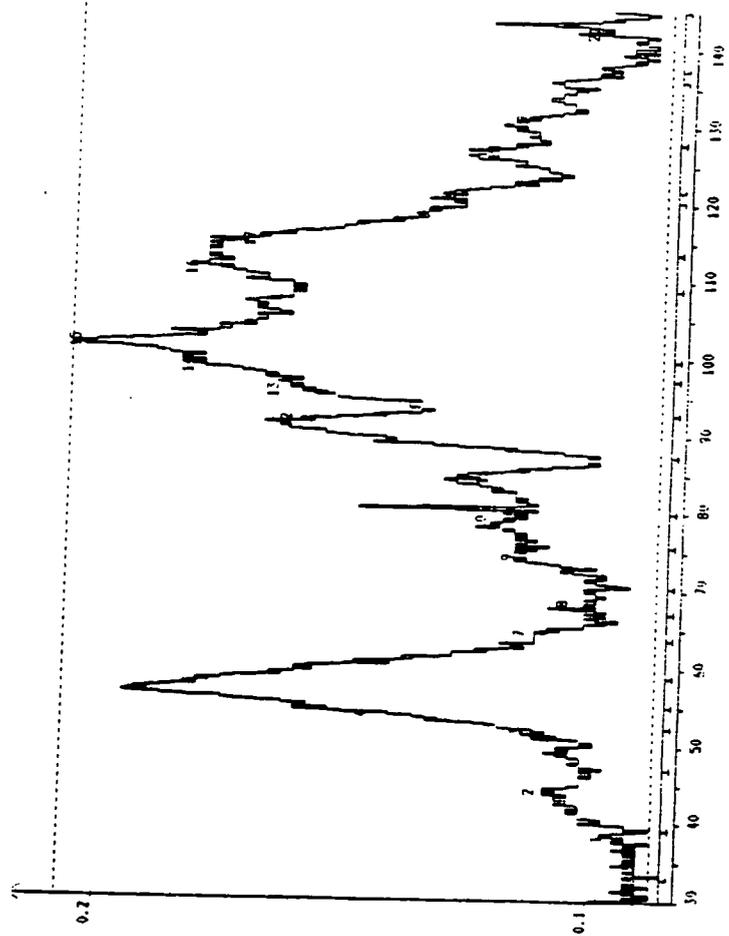


Fig. 6.7: Densitometer-generated graph of F1 hybrid from line 248 in the mixed pollination. Line 248 was used as recipient parent in which pollen from 288 and 248 was mixed in equal amounts. The graph is from a gel of single seed, stained for amylase isoenzyme.

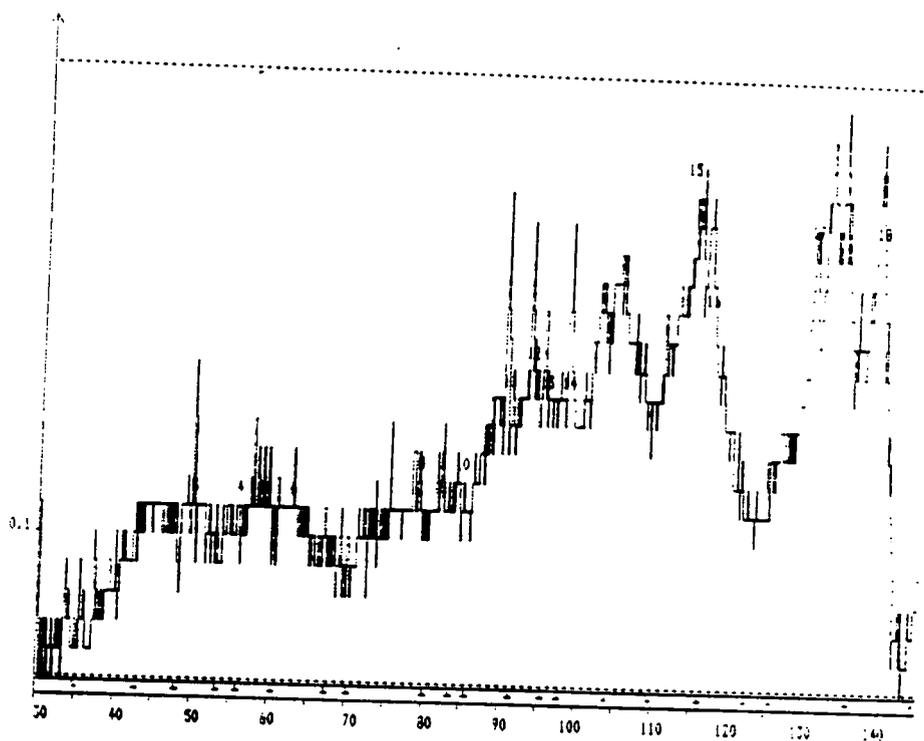


Fig. 6.8: Seed isoenzyme as a gel stained for aspartate aminotransferase (AAT). STW (left hand track) was used as maternal parent; 248 (right hand track) was used as paternal parent. The eight central tracks are from seeds resulting from mixed pollinations with equal mixtures of pollen of STW and 248.

STW

248

6.3 Distribution patterns of the hybrid zygotes in the ovary

Of the several pairs of crosses made in mixed pollinations, 17 are depicted in tables 6.1 and 6.2. From table 6.2 and fig. 6.1 it can easily be seen that there were more hybrid seeds at the first and second ovule positions, while twice as many selfed seeds occurred at the third ovule position than at any other ovule position. At position five, the level of hybridisation and selfing was more or less equal. When individual pairs of crosses were considered, significant variation was apparent.

6.4 Performance of line 248 in the mixed pollinations

6.4.1 Line 248 as a male parent

Pollen of 248 was mixed with pollen of STW, 288, 4/7, NDP and 248. Thus, in each pair of mixed pollination, line 248 was a donor parent. The results shown in table 6.1 reveal that line 248 significantly produced hybrids in all cases. The significance of hybrid seeds formation was larger ($p \geq 0.01$) in the pairs of 248 + 4/7 and 248+247 than in those of 248+NDP and 248 + STW, line 248 being the male parent in all the cases.

6.4.2 Line 248 as female parent

This involved lines 288 and STW. In this experiment line 248 was the recipient of mixed pollination. As table 6.1 shows, a significant ($p \geq 0.01$) number of hybrid seeds was produced, particularly in the case of the STW+248 cross. Line STW was a donor parent and line 248, the recipient parent.

Using line 288 as a donor parent also produced more hybrid than self seeds when its pollen was mixed with that of line 248. This difference was not, however, statistically significant at $p \geq 0.05$ level of confidence.

6.4.3 Performance of STW

6.4.4 Line STW as male parent

Pollen from STW was mixed with pollen from lines 248, NDP, 4/7, 11, and 247 in pair-wise combinations. Mixed with pollen grains from line 11, pollen tubes of STW produced a larger proportion of hybrids than self seeds. (fig. 6.2). The result was not significant at $p \geq 0.05$.

Using 247 as the female and STW pollen mixed as a male parent there were fewer hybrids than self seeds formed. There were, however, no significant differences in the types of seeds produced.

The performance of STW as female parent has already been described above (see also fig. 6.2).

6.5 Performance of TA95-1 (95-1)

6.5.1 Line TA95-1 as a male parent

This involved lines 20D-2 and 43/78. Pollen was mixed as described above. Pollen of 95-1+43/78 on 43/78 stigma gave significantly more self seed formation relative to hybrid production at $p \geq 0.01$ confidence level. Similarly, in crosses involving lines 20D-2 and 95-1, pollen tubes from the former line out-competed the latter in producing more self seeds than hybrids (table 6.2).

Table 6.1: Results of mixed pollination in *Vicia faba* inbred lines STW, 288, NDP 247, 11, 4/7, 248, 20D-2, 43/78 & 95-1.

CROSS	TOTAL SEEDS	RATIO OF		χ^2 values
		CROSSED	SELFED	
248+STW	11	5	1	4.54*
288+248	17	1	1	0.50ns
248+288	19	1	1	1.37ns
248+4/7	12	1	3	7.08**
248+NDP	22	1	3	4.59*
248+247	35	1	7	20.86**
STW+248	23	1	7	20.86**
STW+4/7	21	1	1	1.00ns
STW+NDP	35	1	2	0.66ns
STW+11	19	5	1	2.85ns
STW+247	26	2	1	0.66ns
288+248	34	3	1	0.50ns
288+247	28	3	1	1.25ns
288+NDP	34	1	1	1.00ns
288+4/7	10	5	5	1.00ns
95-1+20D2	11	1	1	1.00ns
95-1+43/78	12	1	3	7.08**

ns= not significant at $p \geq 0.05$

*= significant at $p \geq 0.05$

** significant at $p \geq 0.01$

Table 6.2: Results of mixed pollination of *Vicia faba*
inbred lines STW, 288, NDP 247,11, 4/7, 248, 20D-2, 43/78 & 95-1

Parents	PERCENT BY OVULE POSITIONS									
	SELF					CROSS				
	1	2	3	4	5	1	2	3	4	5
248+STW	11	11	0.0	0.0	0.0	27	45	11	0.0	0.0
288+248	17	13	9	0.0	0.0	30	22	0.0	9	0.0
248+288	19	0.0	13	0.0	0.0	13	38	13	6	0.0
248+4/7	38	10	14	0.0	0.0	10	24	5	0.0	0.0
248+NDP	18	32	18	5	0.0	14	9	0.0	0.0	0.0
248+247	39	24	18	6	0.0	0.0	6	6	0.0	0.0
STW+248	4	4	0.0	0.0	0.0	33	26	15	15	4
STW+4/7	38	10	14	0.0	0.0	10	24	5	0.0	0.0
STW+NDP	23	23	19	6	0.0	6	13	3	6	0.0
STW+11	23	23	19	6	0.0	6	10	3	6	3
STW+247	0.0	11	5	0.0	0.0	58	21	5	0.0	0.0
288+248	17	13	9	0.0	0.0	30	22	0.0	9	0.0
288+247	8	23	0.0	0.0	0.0	27	27	15	0.0	0.0
288+NDP	9	23	14	5	0.0	32	5	9	5	0.0
288+4/7	10	30	30	10	0.0	20	0.0	0.0	0.0	0.0
95-2+20D-2	21	7	7	0.0	0.0	7	21	7	28	0.0
952+43/78	25	17	17	8	8	8	17	0.0	0.0	0.0
Means	18.8	16.1	13.1	2.7	0.5	19.5	19.4	5.7	4.9	0.4

Fig. 6.9: Densitometer-generated graph of a single seed of inbred line 248 used as maternal parent in the mixed pollination. The gel was stained for amylase isoenzyme.

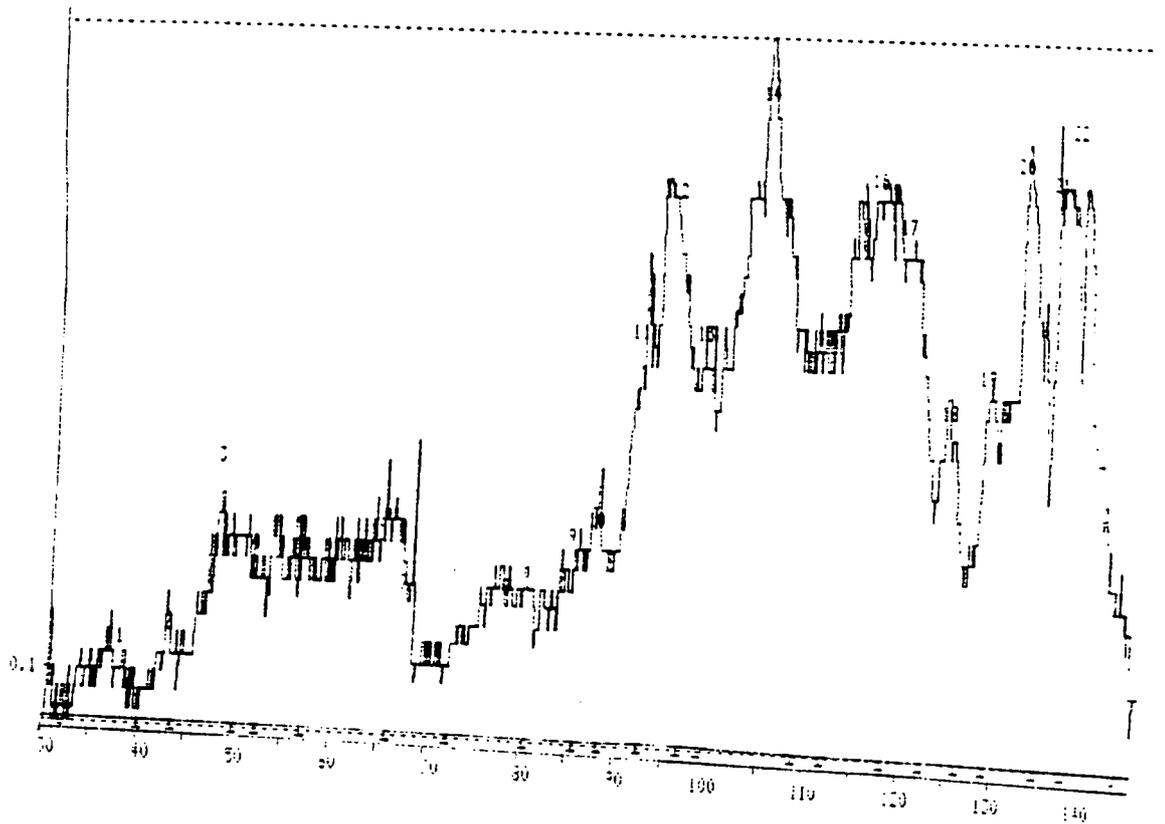
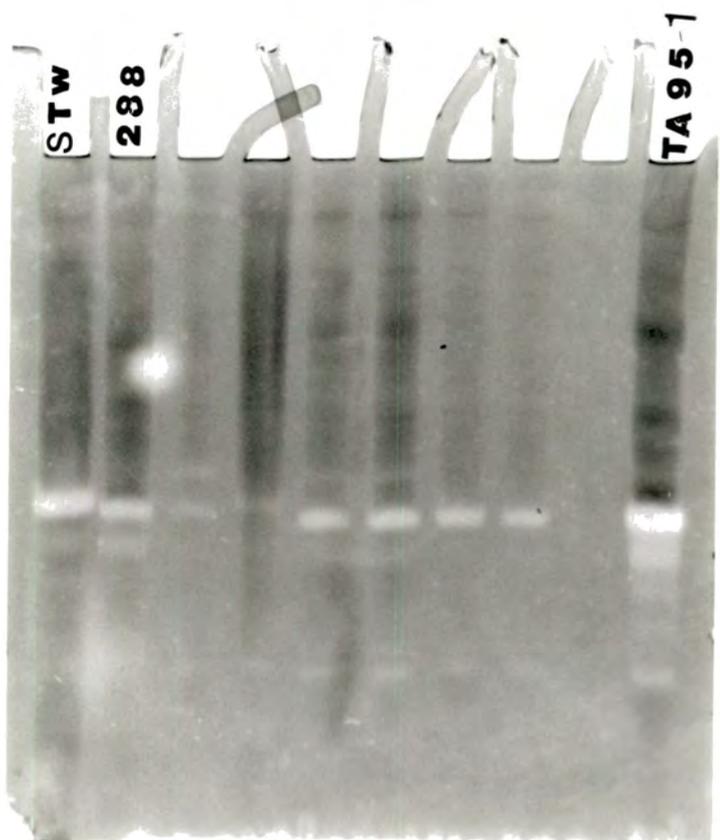
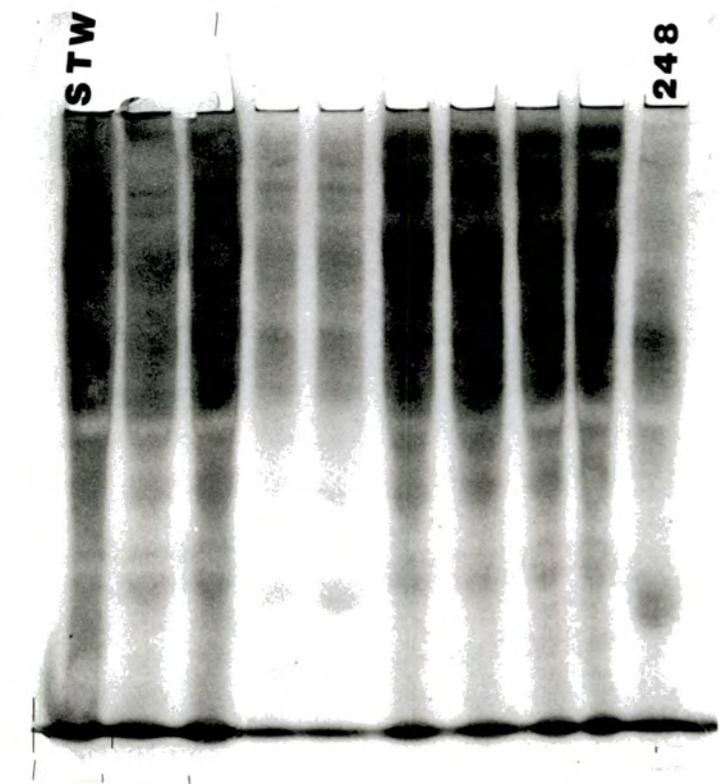


Fig. 6.10: A gel from mixed pollination with respective parents. Notice extreme left is line STW as male parent and extreme right is 248, as a female parent. Stained for amylase enzymes.

Fig. 6.11: A gel stained for amylase enzymes. Notice the longest bands: extreme left band is line STW, extreme right side, line TA95-1. Both these lines were used as male parents in their respective crosses.



Chapter VII

Causes of flower drop in *Vicia faba* under Ethiopian growing conditions

7.1 Introduction

Much data is available on *Vicia faba* flower drop, particularly in Western Europe. One of the key conclusions is that temperature has pronounced effects during the reproductive phase of the crop. Investigations made into pollen germination and pollen tube growth (*in vitro* in Ethiopia and *in vivo* in Durham University laboratories) suggest that low temperature hinders pollen germination and consequently limits fertilisation. Investigations into the problem of flower shedding, using six cycles of field experiments were conducted in Ethiopia between November 1987 and November 1988 (for detailed methodology see under chapter 2).

For the purpose of this study *Vicia faba* lines shown in table 2.1 were used but line 288 was not included. All 21 entries were planted at Holetta Research Centre (HRC) (about 45km west of Addis Abeba), Debre Zeit Research Centre (DZRC) (45km south of Addis Abeba) and Nazereth Research Centre (NRC) (about 120km south of Addis Abeba). The three sites' soil textures are red clay, silt loam, and sand loam respectively. Each entry was replicated three times at each site with plot size 125cm by 50cm. Seeds were placed 25cm apart at an average depth of 3cm. At DZRC and NRC seeds were planted along the sides of the ridges whilst at HRC they were placed on top of the ridges.

Collections of abscised flowers were made in all the three centres, between 9:30 am and 3:00 pm. During these visits plants from which flowers dropped were recorded with respect to flower nodes, counted from the ground level to the top of the plant and the flower pedicel from which the flower was shed. The flower pedicel

Fig. 7.1: Variation in flower and pod counts with pedicel position. Experimental conditions are described in fig. 7.2. Data represent mean counts from all flowering nodes on 21 inbred lines.

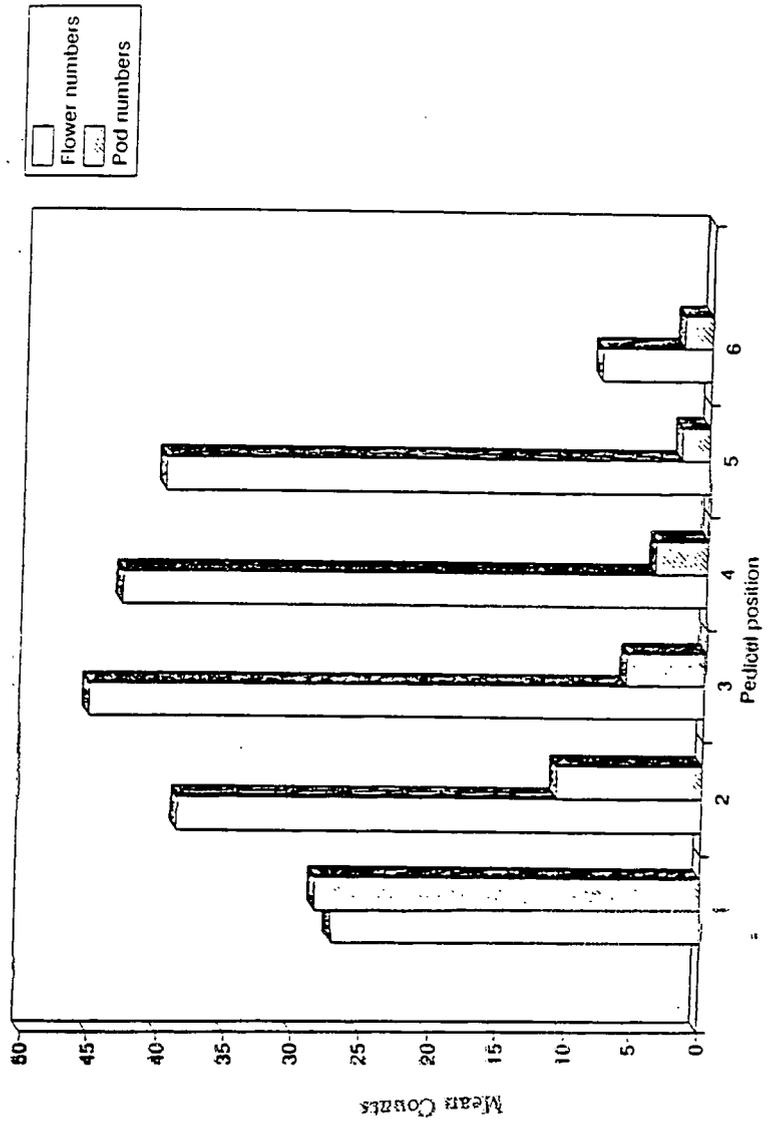


Fig. 7.2: Varietal differences with respect to flower shedding and pod retention to maturity. Experiments conducted Nov. 1987 to Nov. 1988 at Holetta, Debre Zeit and Nazareth research centres, Ethiopia.

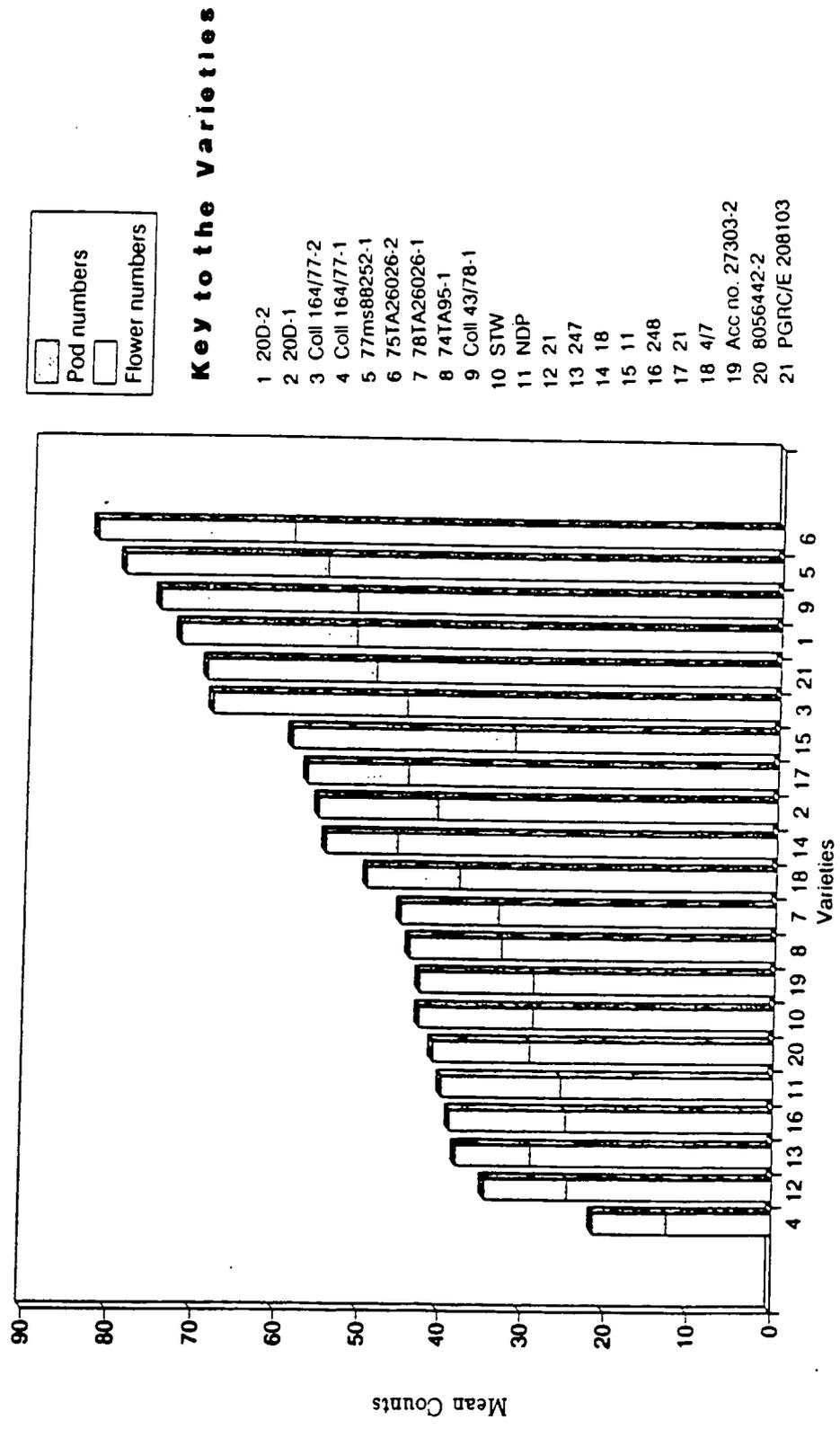
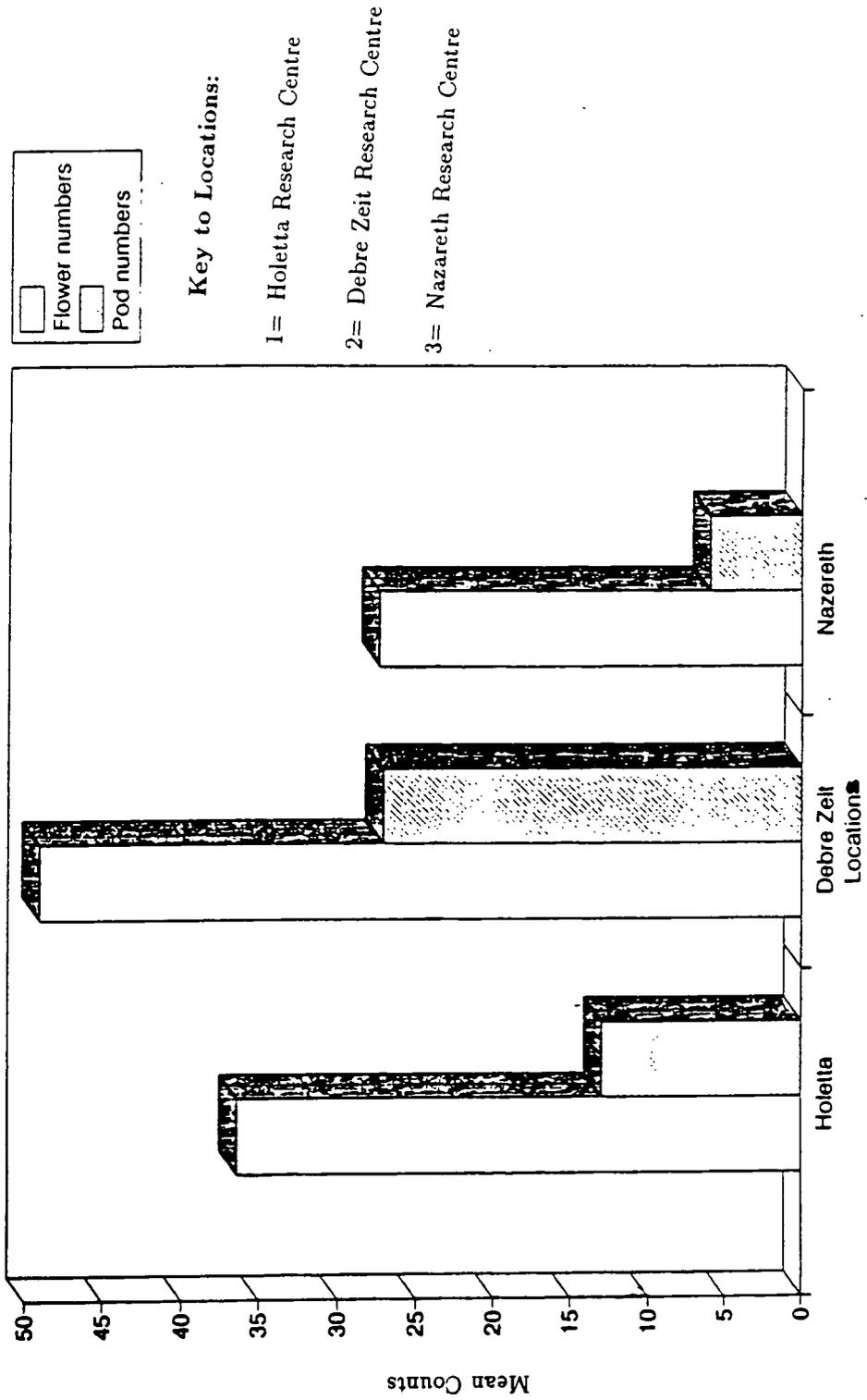


Fig. 7.3: Location environmental (mainly low and high temperatures) effects on the degree of flower shedding and pod retention to maturity. Mean data for 21 inbred lines.



was numbered from the node it subtended to the tip of the peduncles. These data were recorded from late January 1988 to November 1988 at all the three centres.

7.2 Flower shedding and pod development

Trends in flower shedding and pod retention characteristics are shown in figs. 7.5-7.25, for individual lines. Thus, effects of location and season (cycle) on each line could easily be deduced from the figures.

7.2.1 Varietal Trends

The highest flower loss was from a line introduced from ICARDA but selected under the Ethiopian environment. Matured pod counts from this line were just significantly ($p \geq 0.05$) lower than the number of pods produced by line 21. Line 21 was one of the lowest ranked in degree of flower losses. That was very significantly lower ($p \geq 0.00001$) in flower shedding than line 75TA26026-1 (No.7 & 15, fig. 7.2). Overall, highest flower loss was from line 77ms88252-1, an Ethiopian selection but introduced from ICARDA, followed by coll 164/77-1, an Ethiopian line. Lines 20D2, coll 43/78-1 and acc.208103, all Ethiopian lines, ranked third in the magnitude of flower shedding. Lines such as 18 and 11, both from Durham University, were higher in flower losses ($p \geq 0.05$) than line 21.

The following group of lines, even though there were differences among them in flower losses, were not significantly different at $p \geq 0.05$ level. These were STW, 19, 248, NDP, 21, 247, 19, 248, all from Durham University, and acc.27303-2, from Ethiopia.

Highest pod numbers were counted from line STW, across all the three locations. These were significantly different ($p \geq 0.001$) from 16 lines out of the 21 lines under investigation. Coll 164/77-1, 77ms88252-1 and coll 43/78-1 were also significantly ($p \geq 0.05$) higher in number of pods counted than 16 other lines. Thus, among these 16 lines, including line STW, though there were differences among them, the pods counted from each line were not significantly different at $p \geq 0.05$

level.

7.3 Effects of locations

The highest flower losses were recorded from location 2, Debre Zeit, (fig. 7.3). Despite this loss, the highest number of pods harvested was also recorded from this location. The second largest flower losses occurred at Holetta Research Centre, location 1. This site also gave a higher level of harvestable pods than Nazereth. Plants grown at Nazereth, however, were the lowest in flower shedding and also produced the fewest pods (location 3). Thus, statistically, pods produced were significantly fewer ($p \geq 0.0001$) at Nazereth Centre. Pod counts were significantly ($p \geq 0.001$) higher at Debre Zeit than those from Holetta while flower loss at the former site was just significantly higher ($p \geq 0.05$) than at the latter site.

7.4 Influence of raceme position on pod development

Counted from a node upwards, most flowers were shed from the third raceme position in *Vicia faba*. Flower shedding at this position exceeded that at position six ($p \geq 0.0001$) and at position one ($p \geq 0.01$) but was similar to that at position four and five. On the other hand, pod set was greatest at position one exceeding that at positions four, five and six ($p \geq 0.00001$). Pod set at position two was significantly lower than that at position one ($p \geq 0.001$), (fig. 7.1). Therefore, it is clear that all raceme positions can potentially produce pods with matured seeds but positions four, five and six are economically useless as far as marketable seed harvest is concerned.

7.5 Effect of seasons

As can be appreciated from fig. 7.4 *Vicia faba* lines planted in late November and in the early part of December showed moderate flower shedding and consequently the highest number of pods recorded from this planting harvest. Pod harvests from January and mid June plantings were better than those from either

Fig. 7.4: Effect of seasons on flower shedding and pod retention to maturity

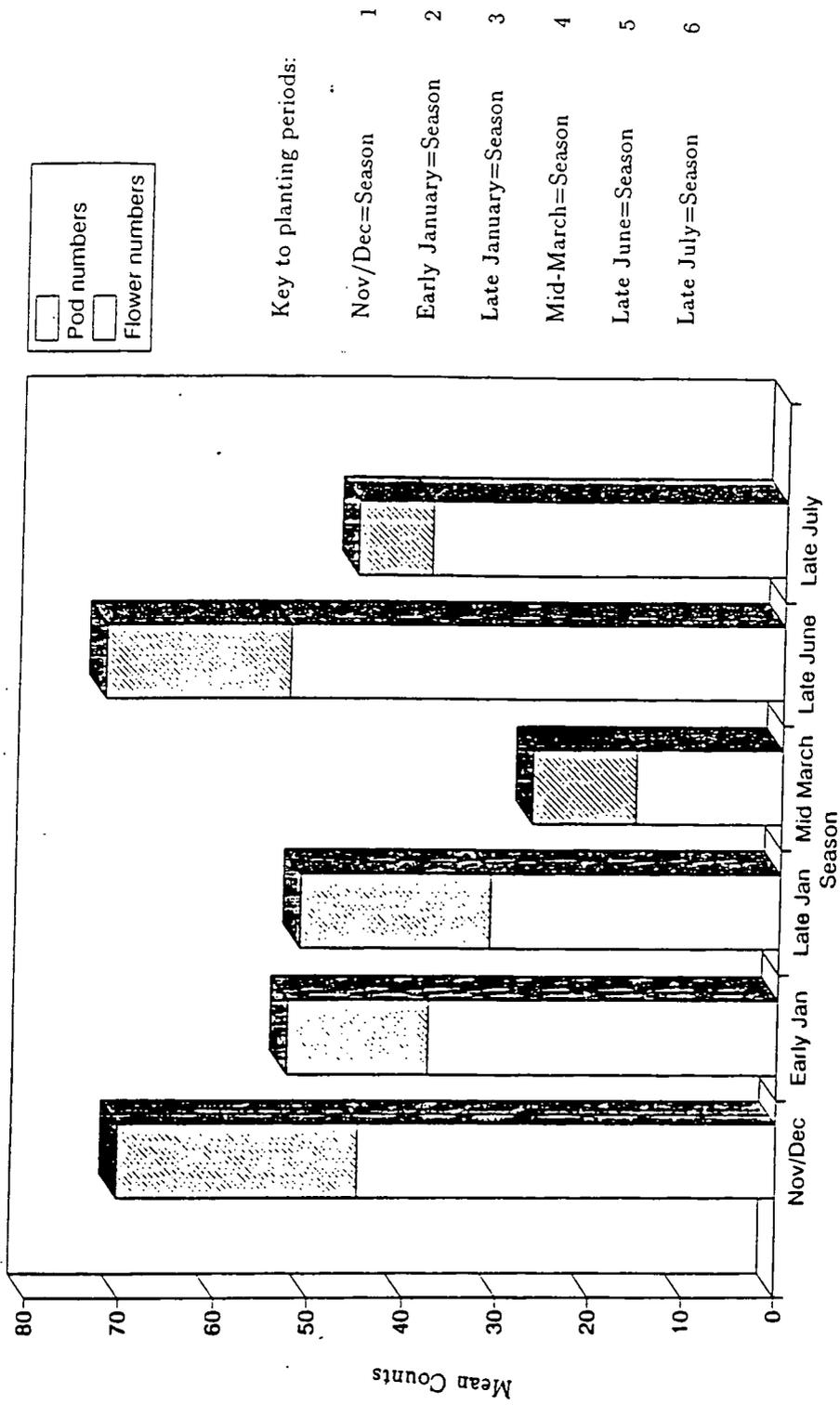


Fig. 7.4: Effect of seasons on flower shedding and pod retention to maturity

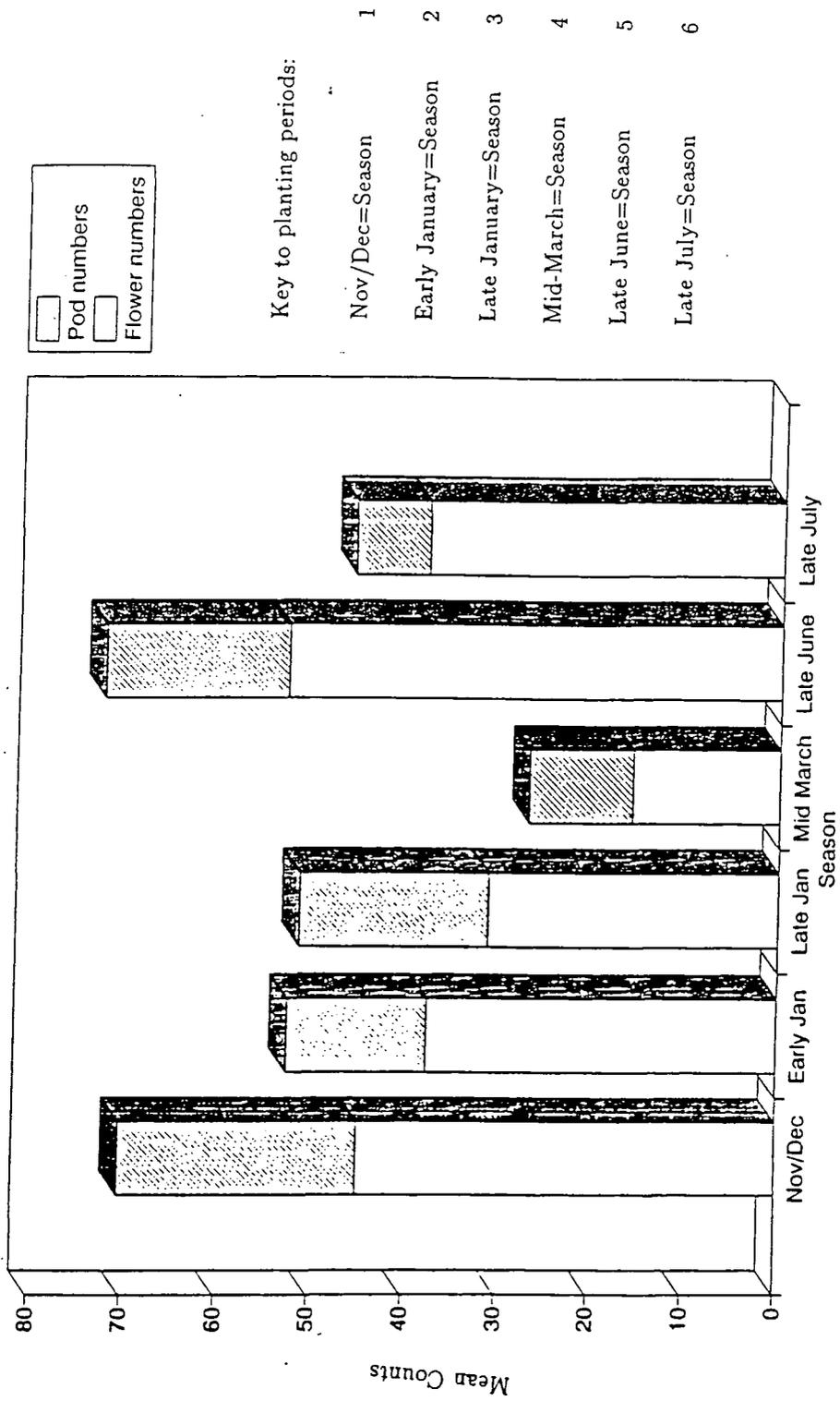


Fig. 7.6: Flower shedding & pod development trends in *Vicia faba*, line NDP, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

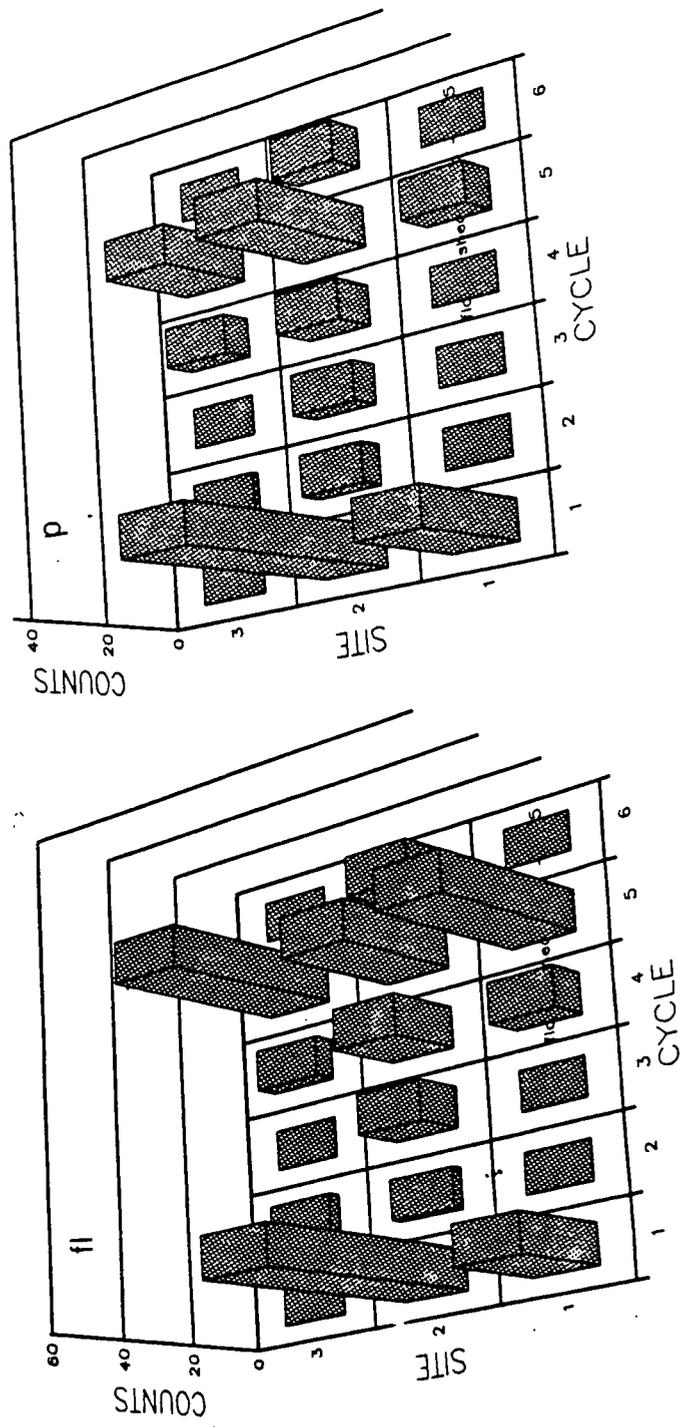


Fig. 7.7: Flower shedding & pod development trends in *Vicia faba*. line 21, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

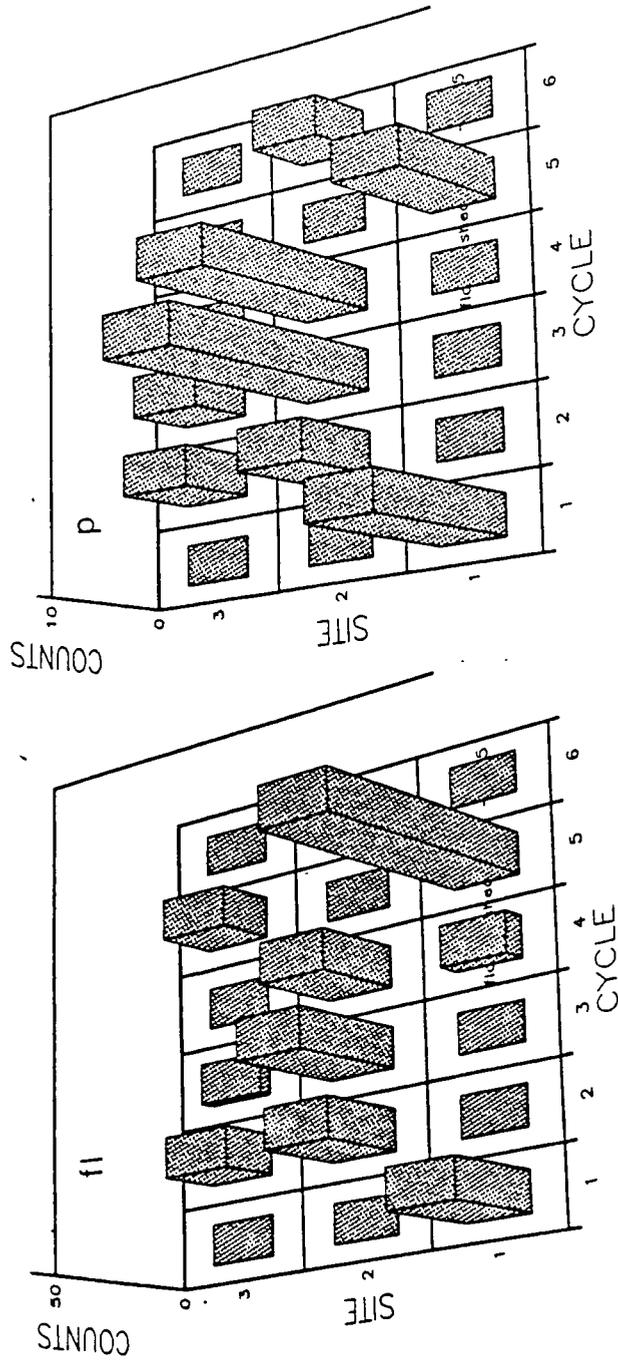


Fig. 7.8: Flower shedding & pod development trends in *Vicia faba*, line 247, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

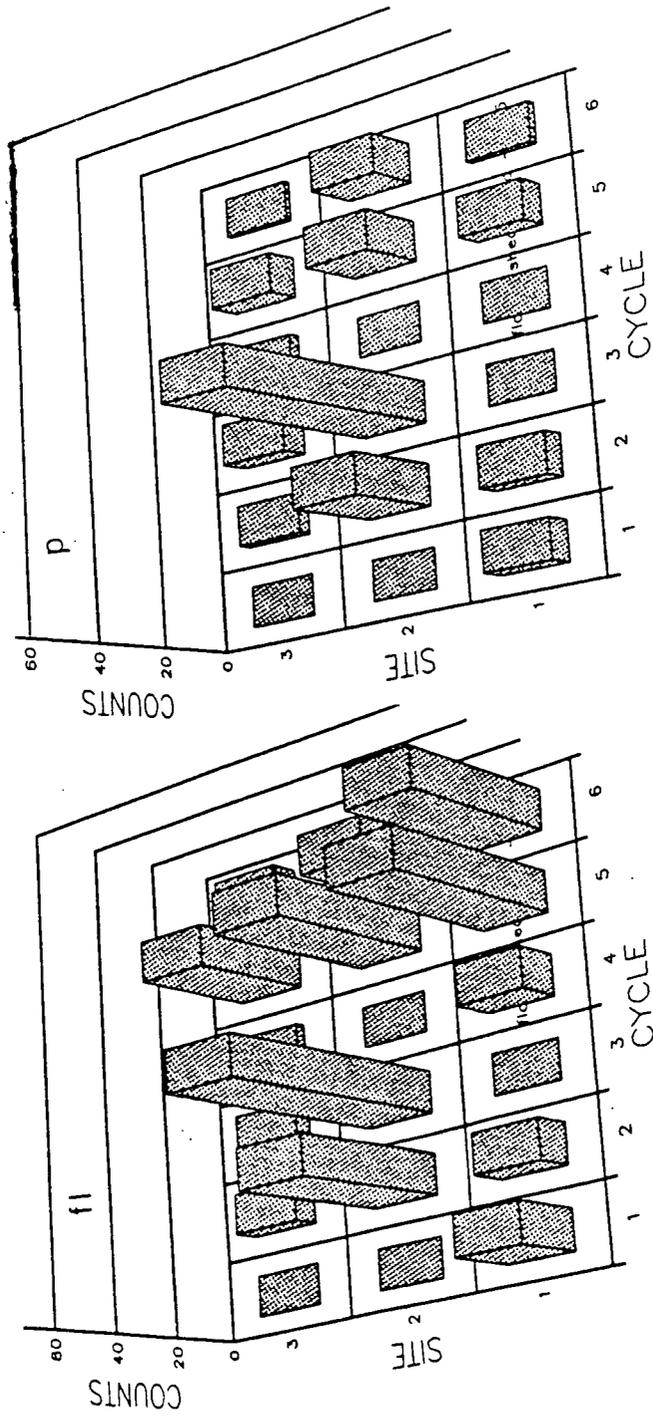
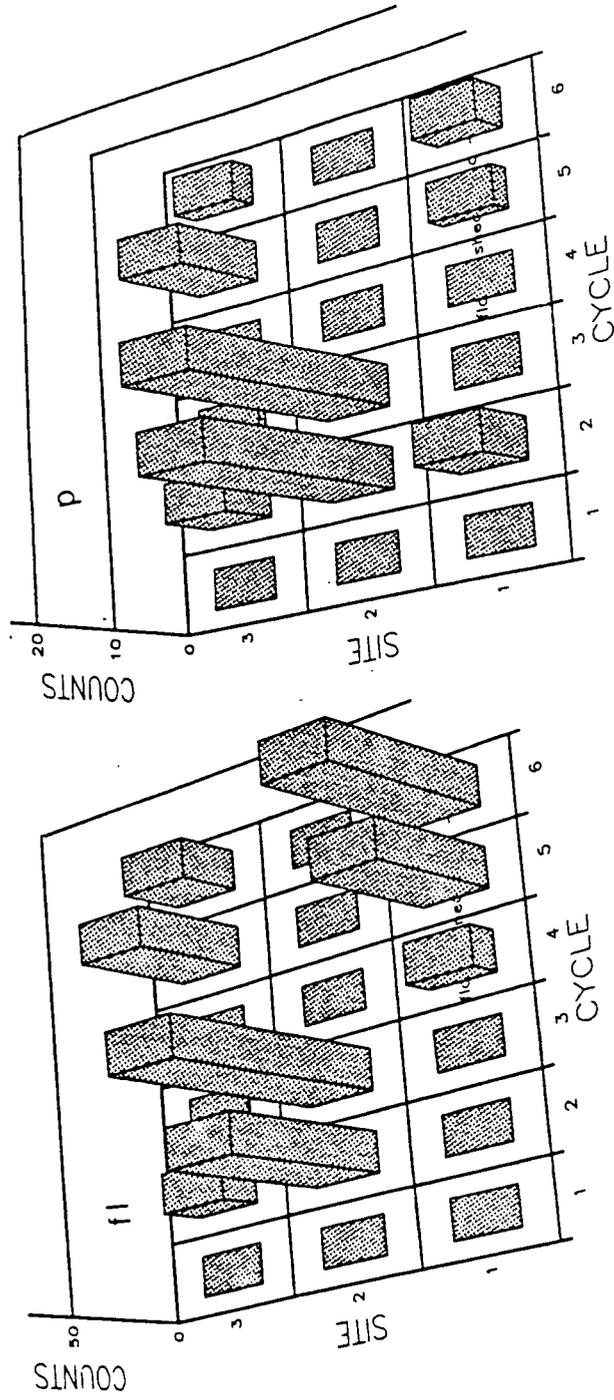


Fig. 7.9: Flower shedding & pod development trends in *Vicia faba*, line 18, at three sites in Ethiopia; β , flower; p , pod. For further details see fig. 7.2.



March or from those mid-July 1988 planting (fig. 7.4). Flower shedding was highest in the planting of mid-July 1988; least flower shedding occurred in plantings between 15th and 19th March 1988 (Season 4). Also, one of the lowest harvestable pod counts was from a planting during this period. Flower shedding levels from planting during the first week of January 1988 (Season 2) and last week of July 1988 (season 6) are not significantly different. However, harvestable pod counts from the season 6 plantings were significantly ($p \geq 0.001$) lower than from planting in the first week of January. Further, flower shedding from the late January planting (Season 3) and from beans planted between 15th and 19th March 1988 (Season 4) were significantly ($p \geq 0.01$) different. Correspondingly their respective harvestable pods were also significantly different ($p \geq 0.01$).

Fig. 7.10: Flower shedding & pod development trends in *Vicia faba*, line 19, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

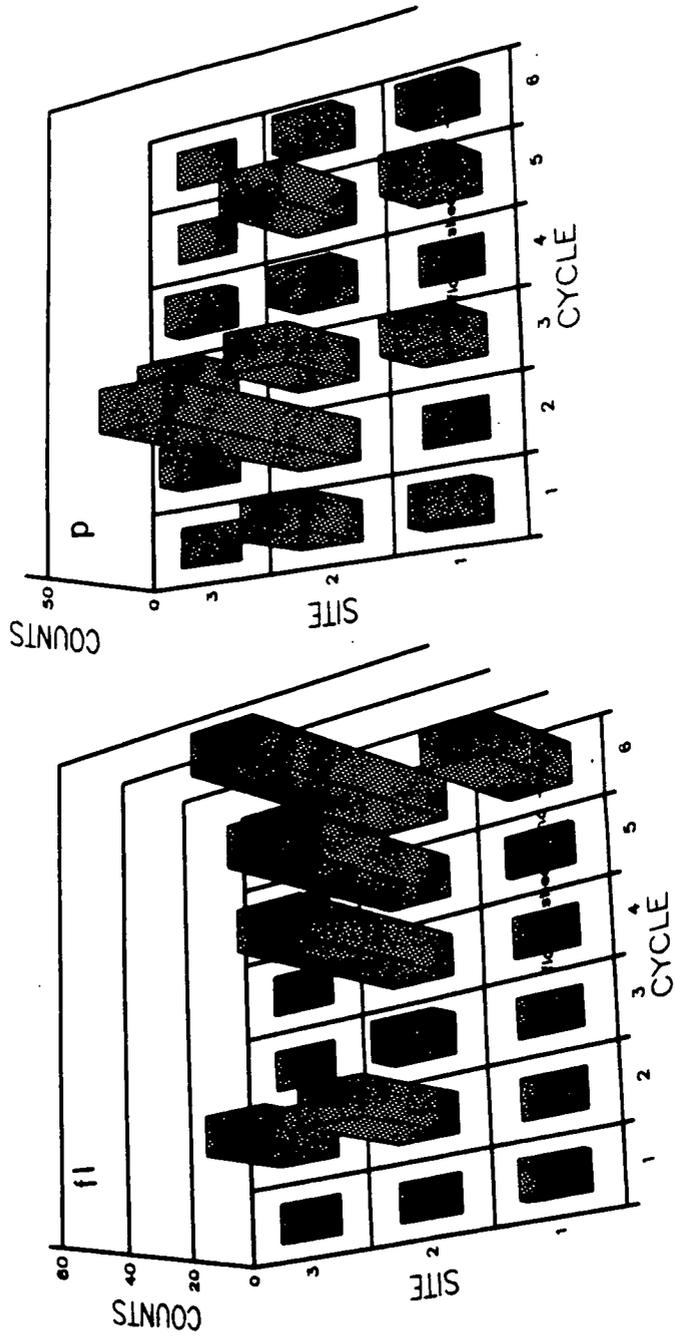


Fig. 7.11: Flower shedding & pod development trends in *Vicia faba*, line 248, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

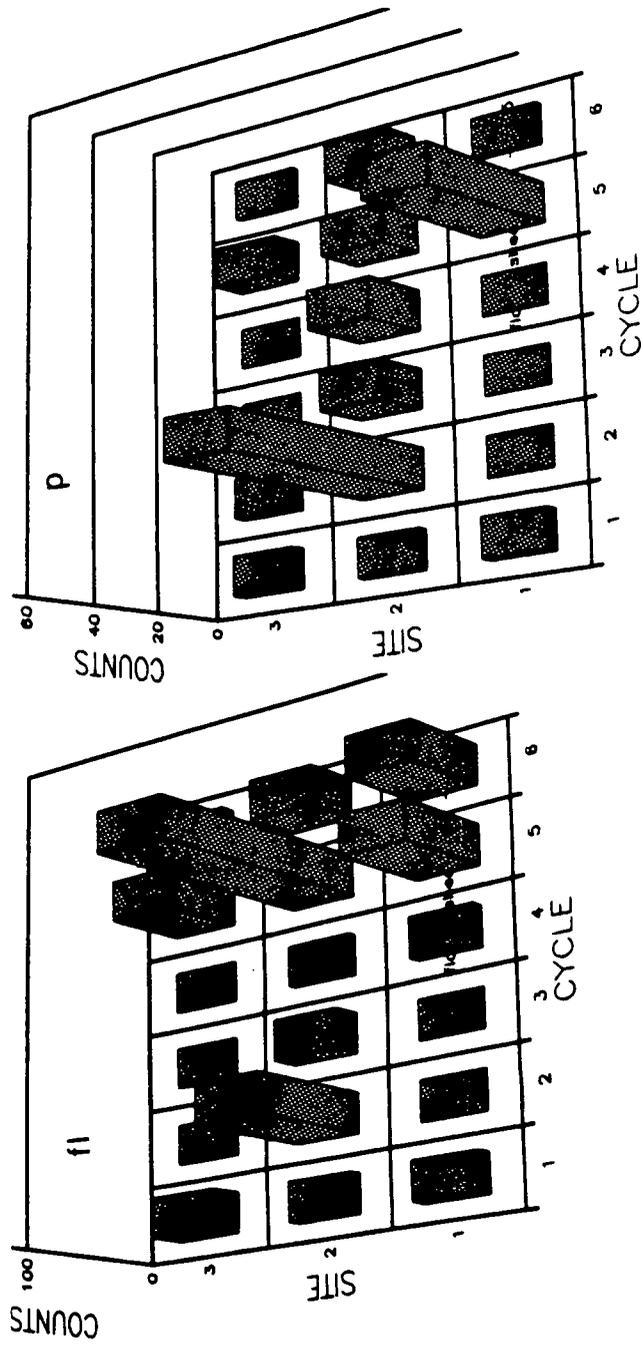


Fig. 7.12: Flower shedding & pod development trends in *Vicia faba*, line 11, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

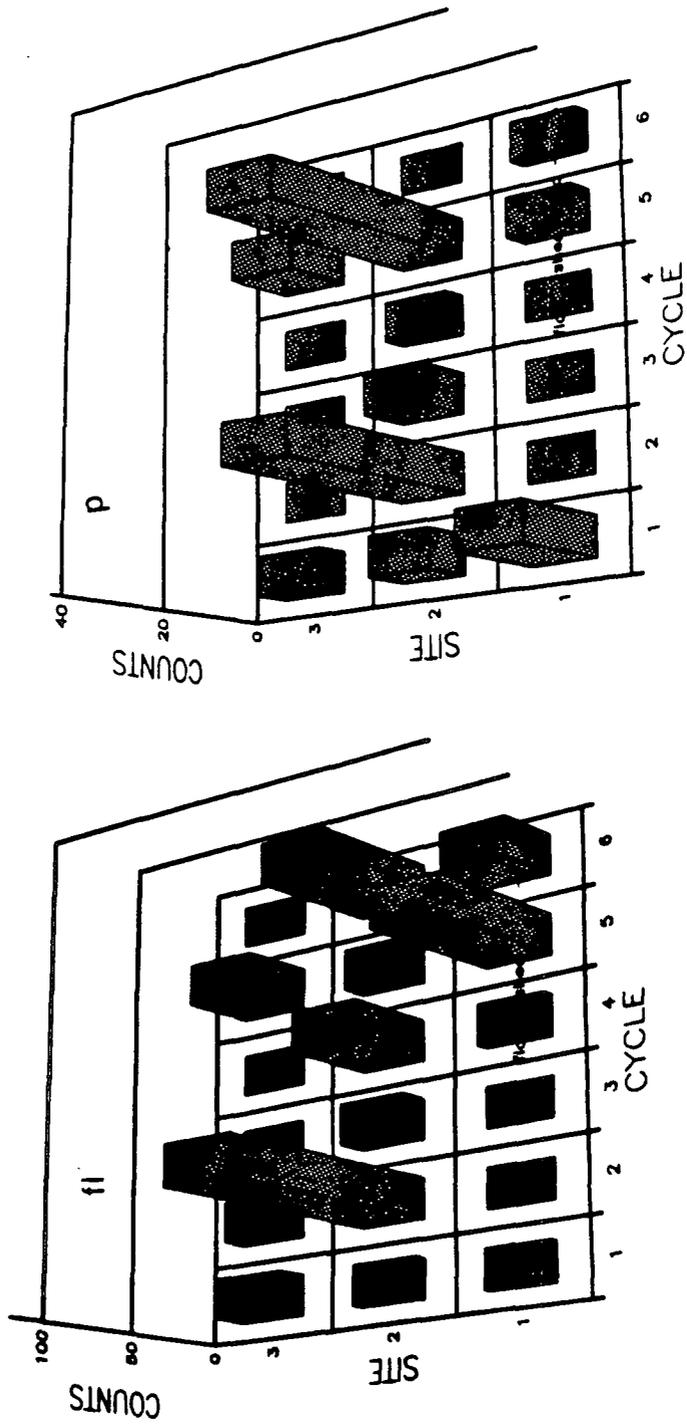


fig. 7.13: Flower shedding & pod development trends in *Vicia faba*, line 4/7, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

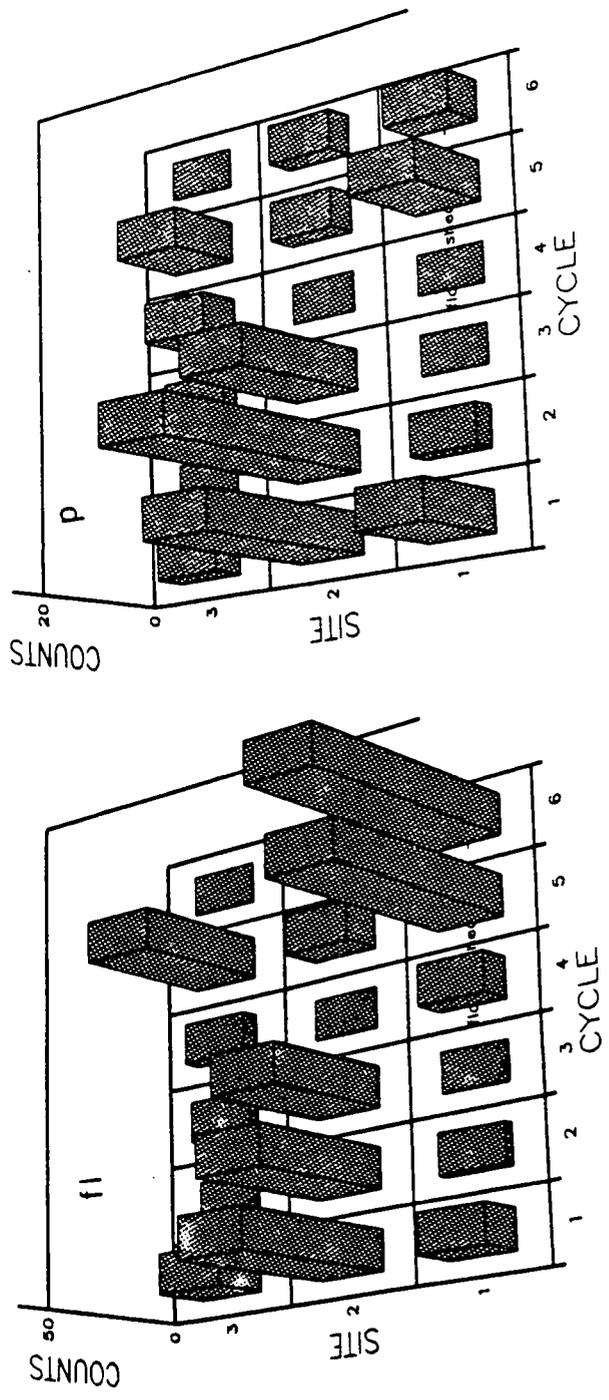


Fig. 7.14: Flower shedding & pod development trends in *Vicia faba*, line 20D-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

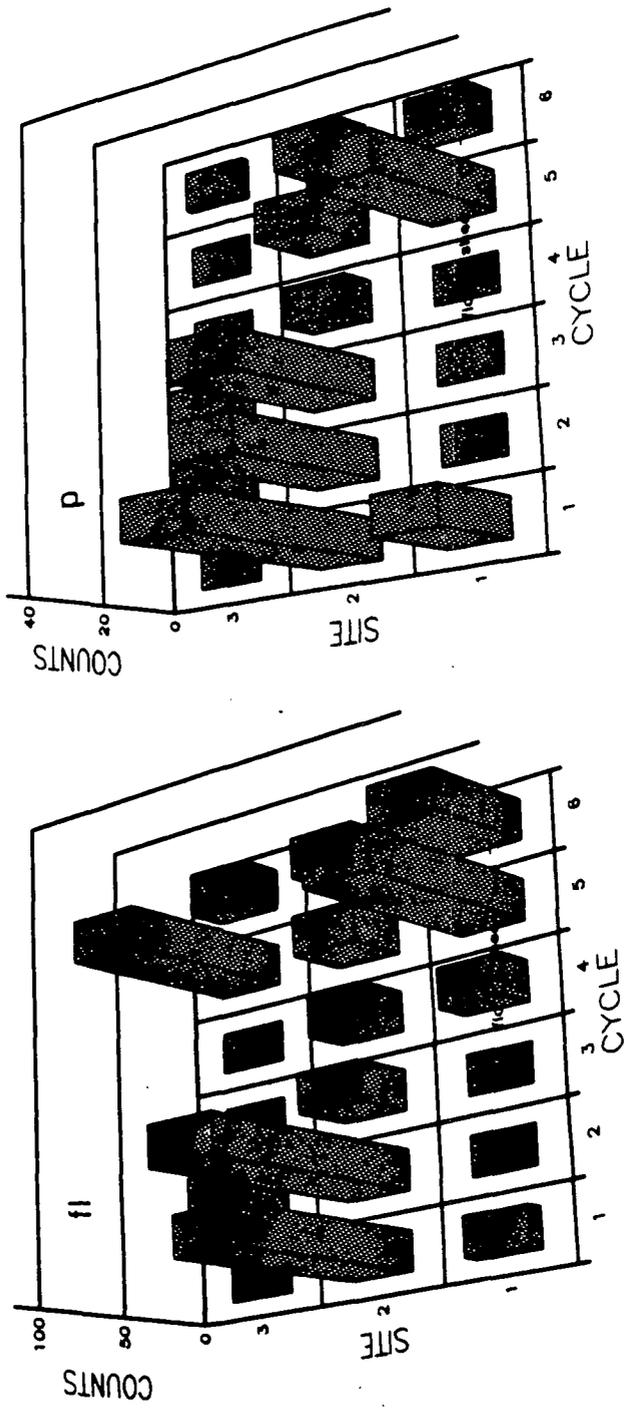


Fig. 7.15: Flower shedding & pod development trends in *Vicia faba*, line 20D-1, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

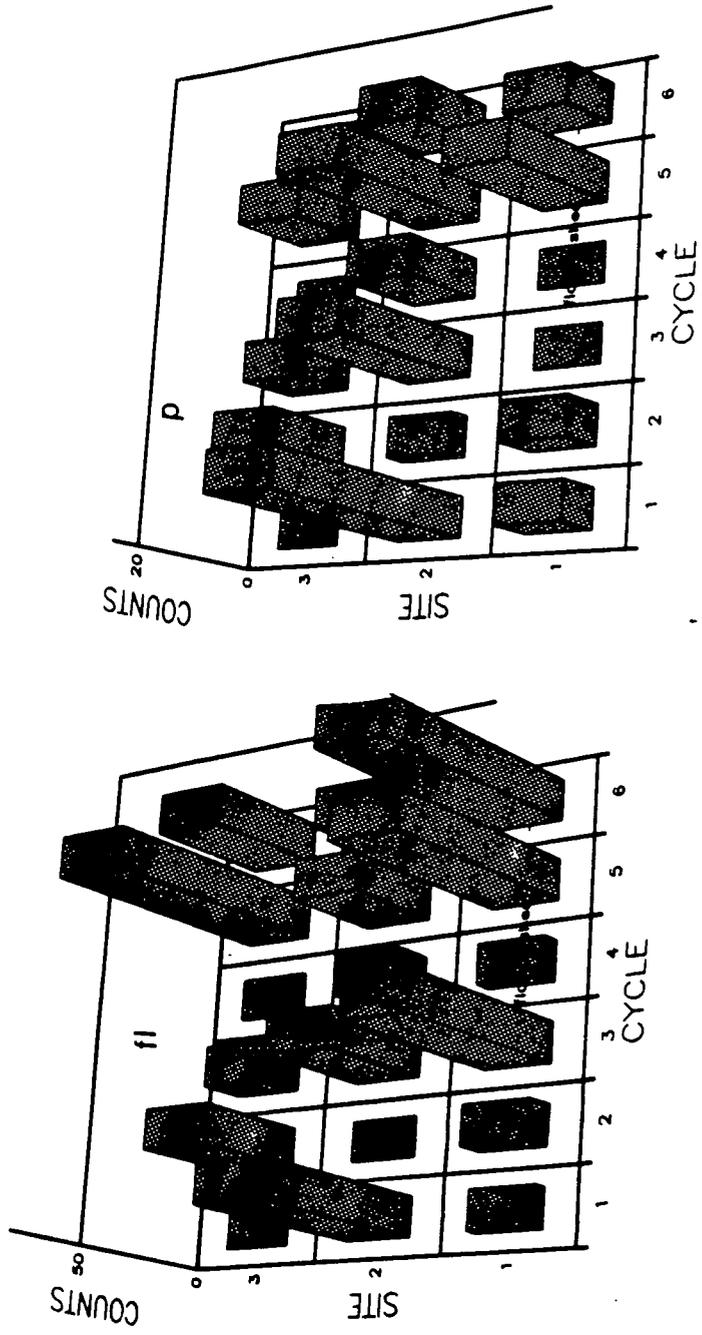


Fig. 7.16: Flower shedding & pod development trends in *Vicia faba*, line coll. 164/77-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

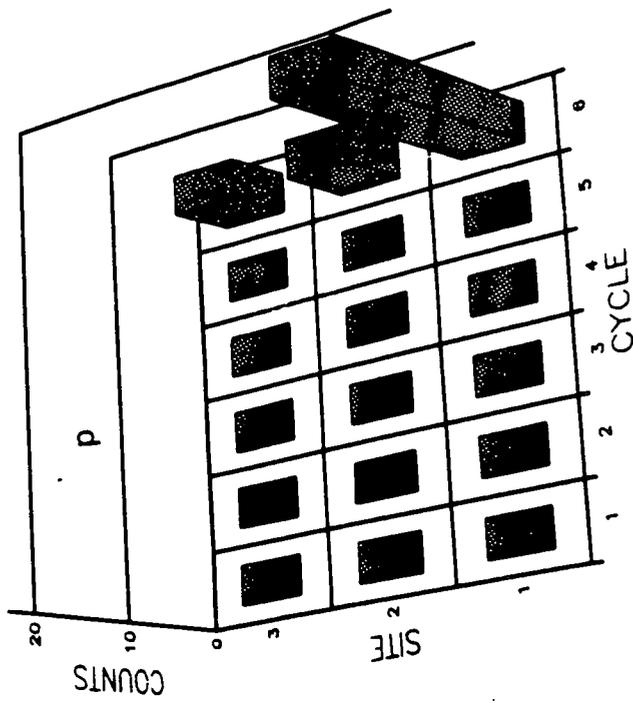
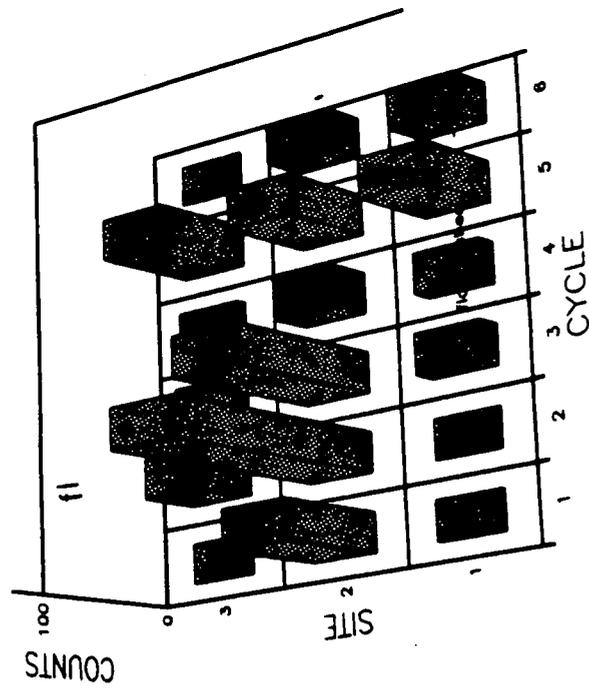


Fig. 7.17: Flower shedding & pod development trends in *Vicia faba*, line coll.43/78(431), at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

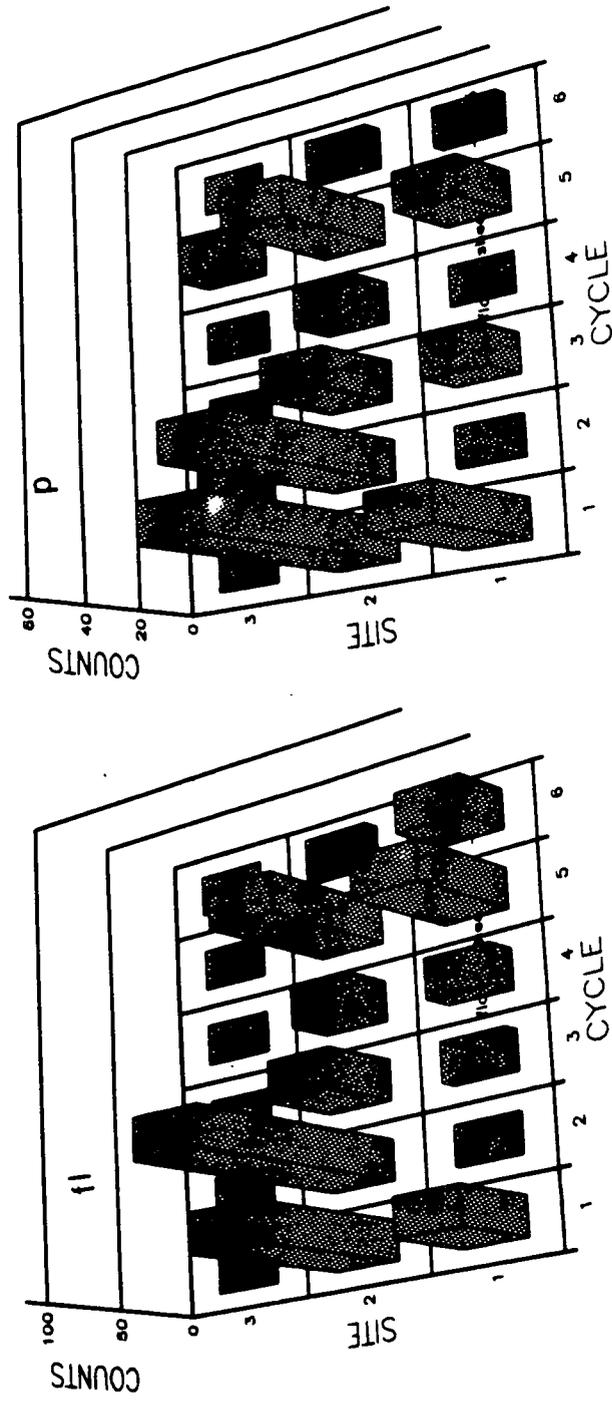


Fig. 7.18: Flower shedding & pod development trends in *Vicia faba*, line, acc. No. 27303-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

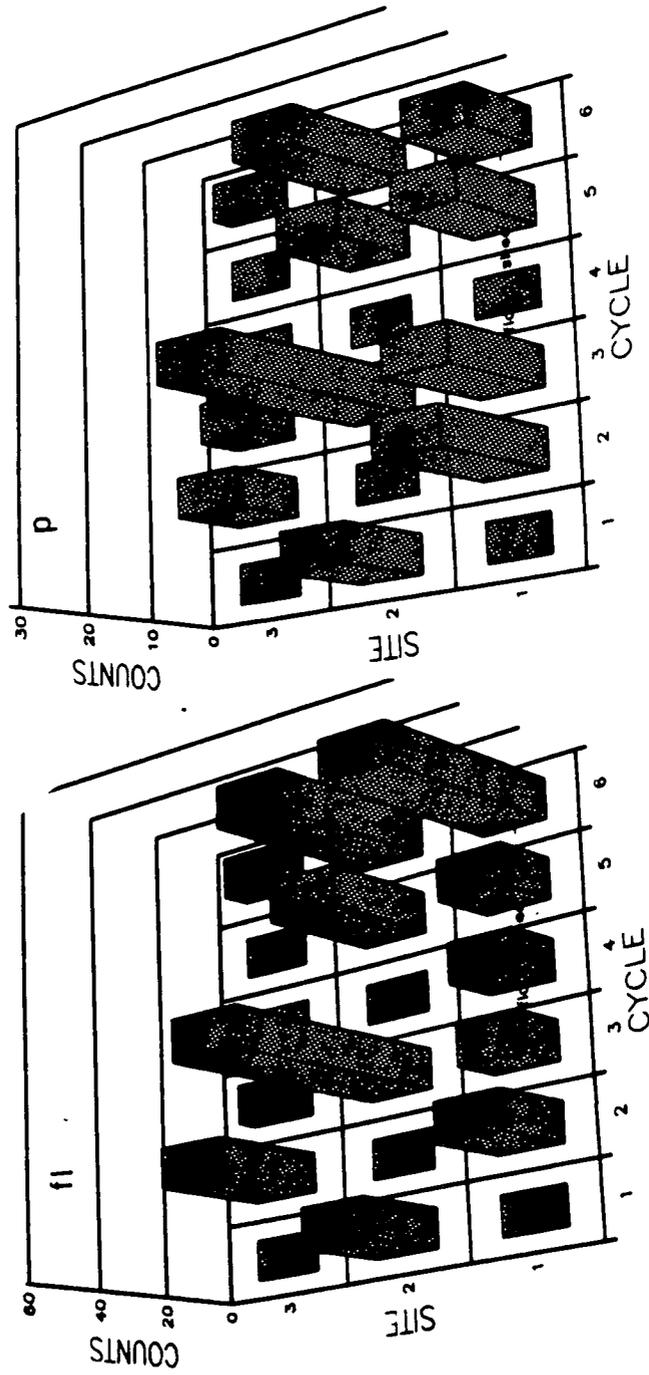


Fig. 7.19: Flower shedding & pod development trends in *Vicia faba*, line acc.No. 0208103, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

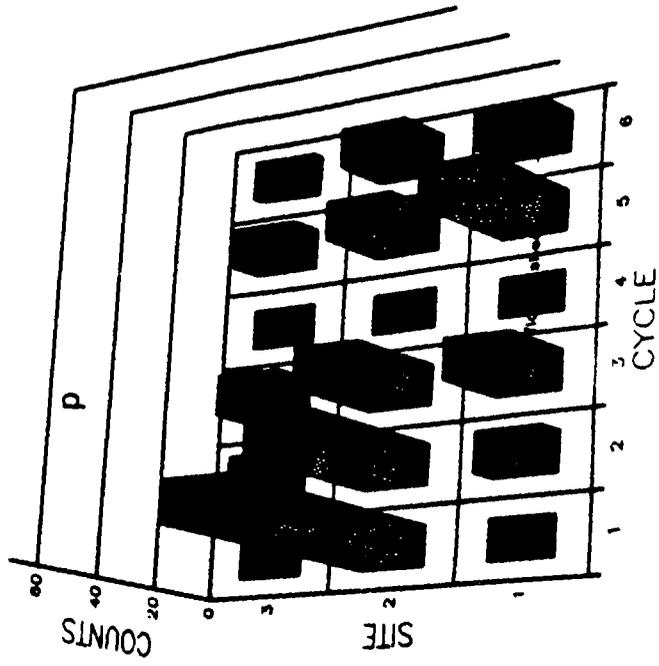
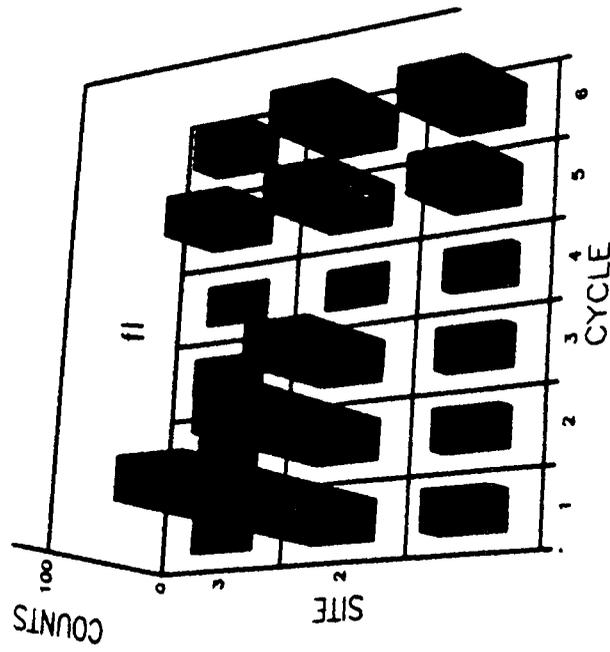


Fig. 7.20: Flower shedding & pod development trends in *Vicia faba*, line PGRC/E 0227052-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

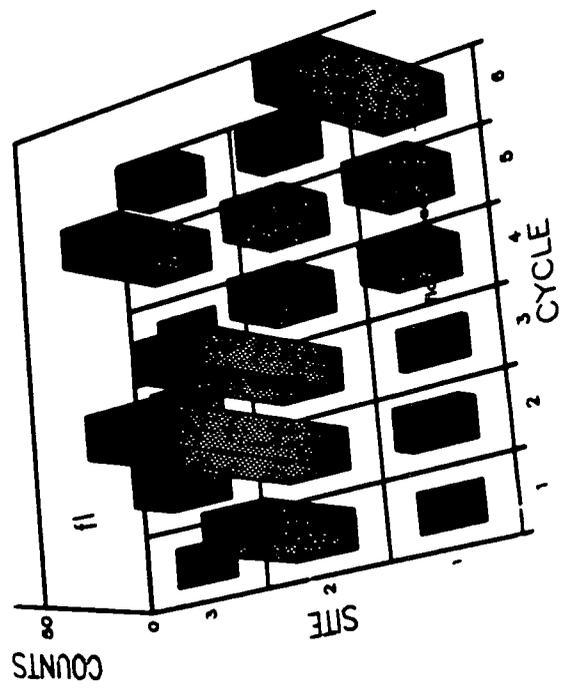
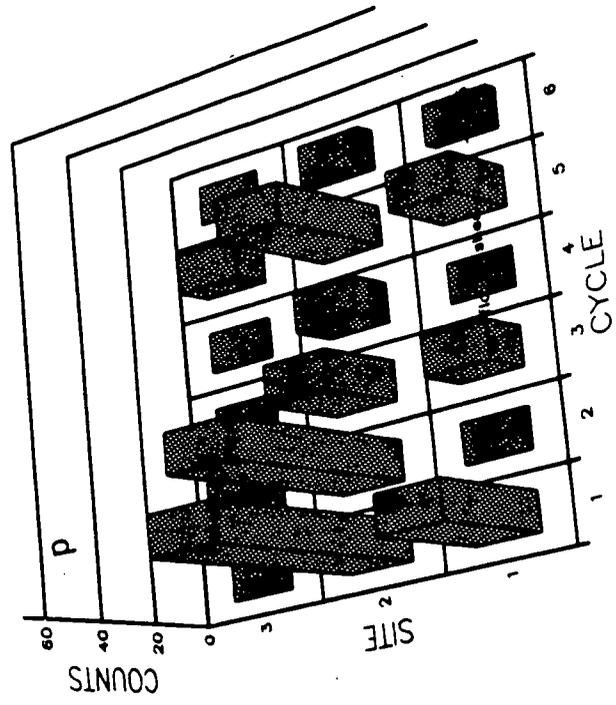


Fig. 7.21: Flower shedding & pod development trends in *Vicia faba*, line 77ms88252-1, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

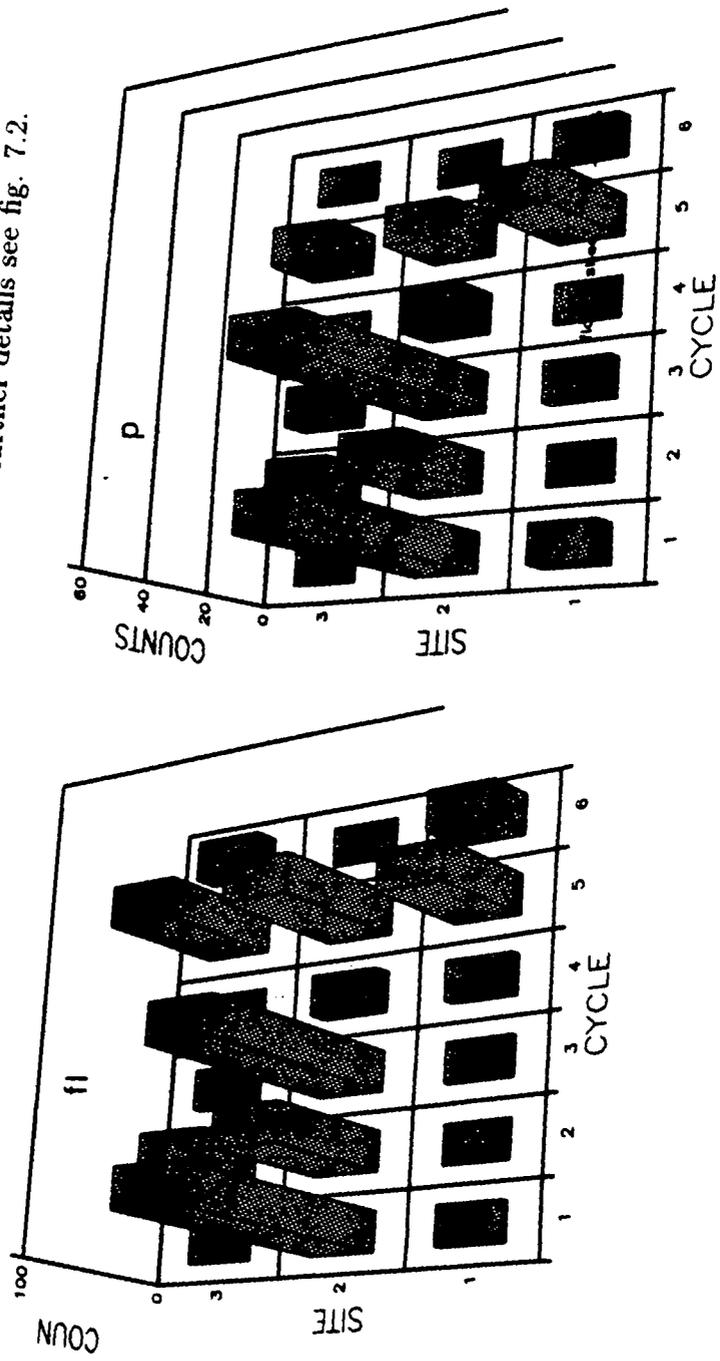


Fig. 7.22: Flower shedding & pod development trends in *Vicia faba*, line 75TA226026-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

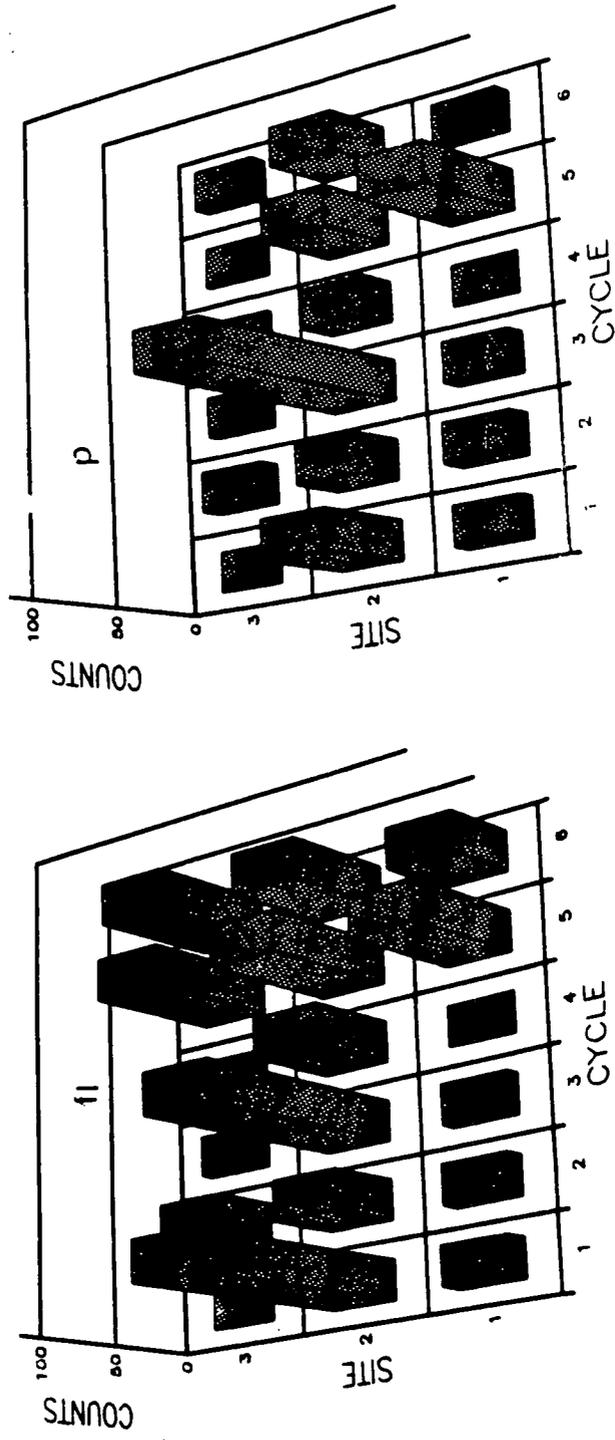


Fig. 7.23: Flower shedding & pod development trends in *Vicia faba*, line 78TA260626-1, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.

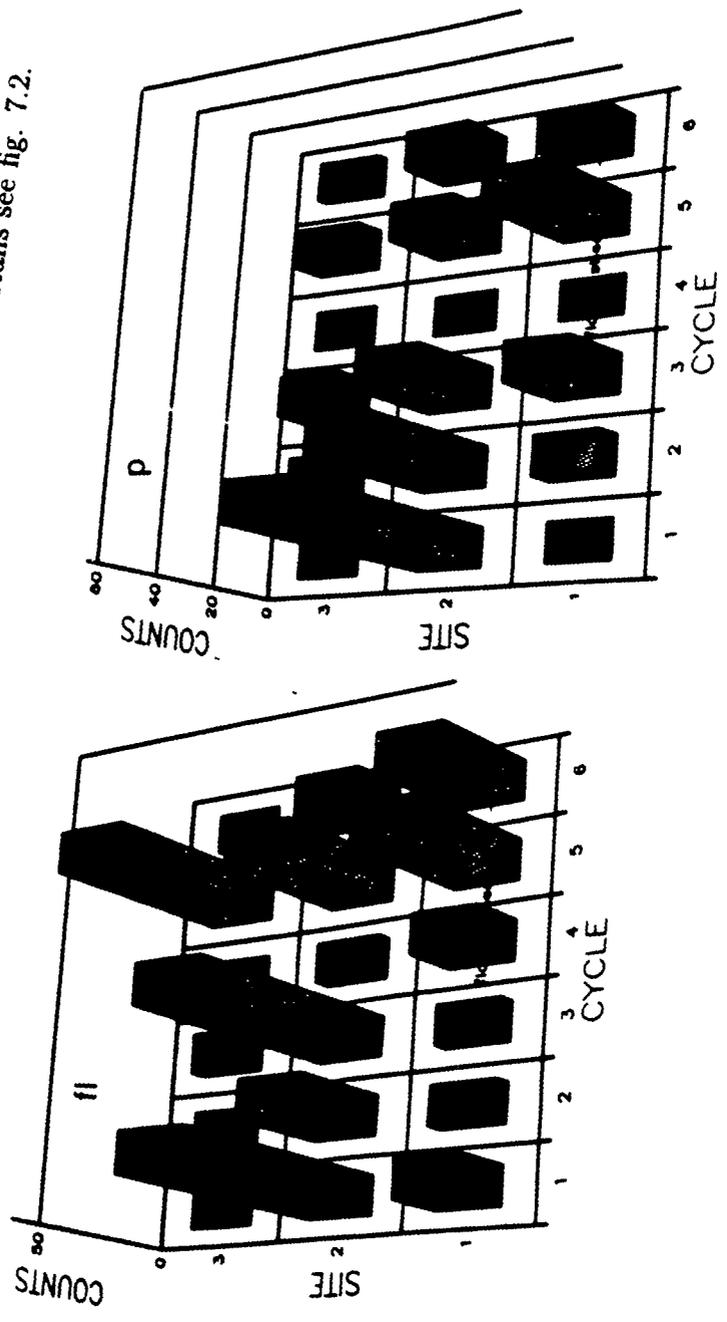


Fig. 7.24: Flower shedding & pod development trends in *Vicia faba*, line 74TA95-1(951), at three sites in Ethiopia; fl, flower; P, pod. For further details see fig. 7.2.

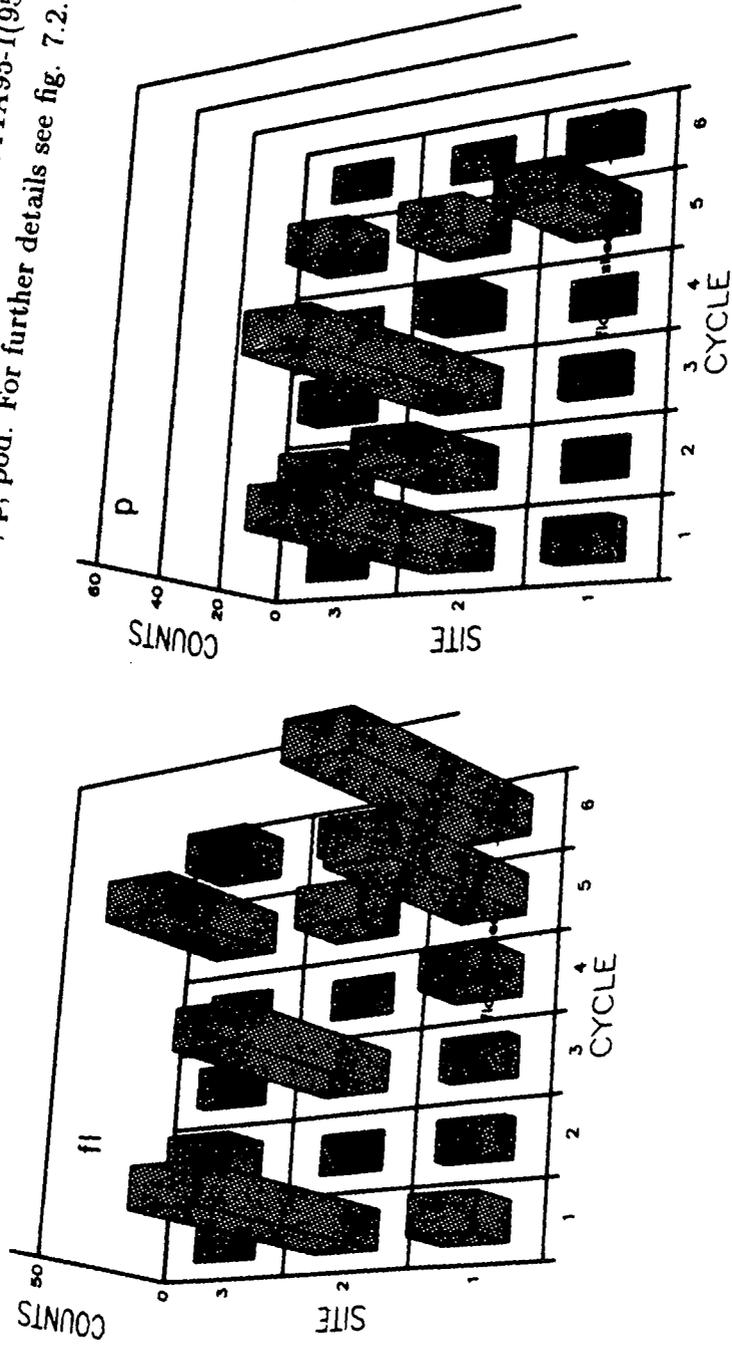
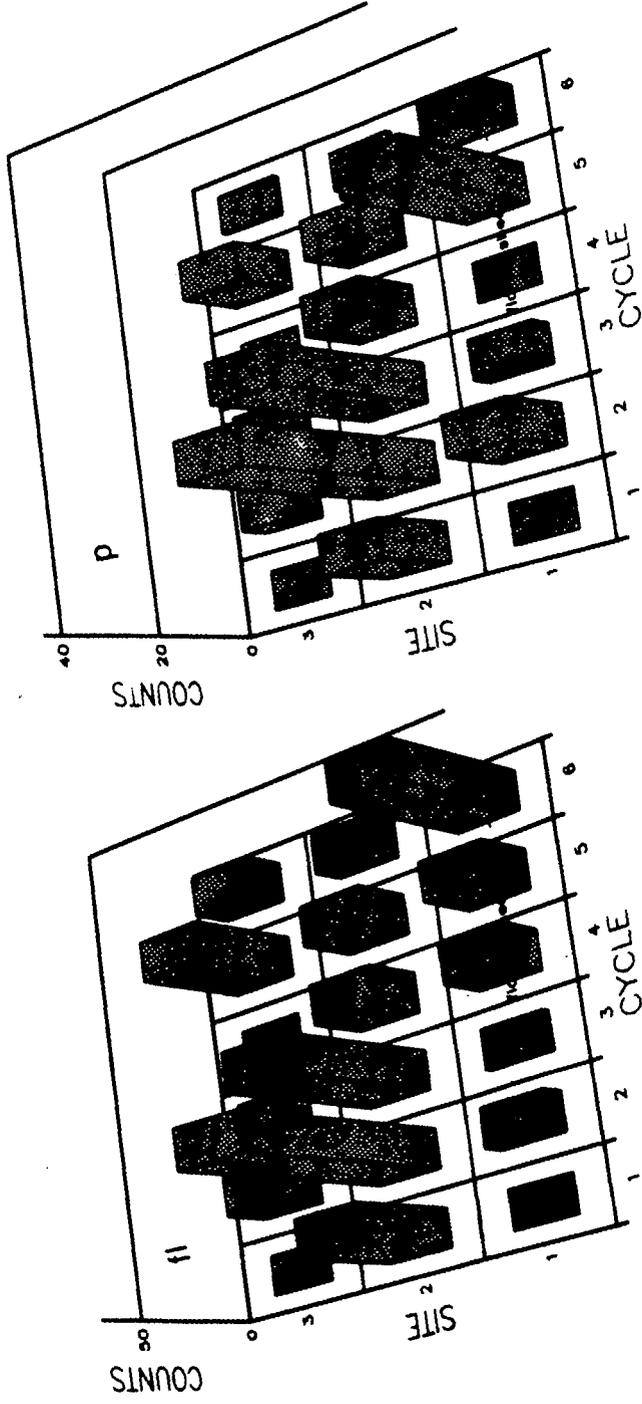


Fig. 7.25: Flower shedding & pod development trends in *Vicia faba*, line 80564422-2, at three sites in Ethiopia; fl, flower; p, pod. For further details see fig. 7.2.



Chapter VIII

Factors affecting pollen fertilisation in *Vicia faba* under Ethiopian growing environment

8.1 Introduction

Flower drop in *Vicia faba* has been given detailed attention particularly with respect to extrinsic factors such as distribution of moisture and the soil nutrients. However results in Chapters 1 and 4 suggest that the intrinsic factors such as male gametophyte failure in germination and fertilisation has a major influence on flower drop. As has been shown stressed pollen is very weak or usually fails to effect fertilisation. Such postulations led to an investigation into the status of pollen on the stigma and fate of pollen tube growth using fluorescence microscopy.

8.1.1 Major parameters considered

What would be the effect of low or high temperature on the pollen germination and pollen tube growth? In an attempt to answer such questions flowers collected during the cold period of late September to mid-October 1988 and those collected during high temperature period, mid-April to early May 1988, were used. As a control, flowers collected during late August and early September, 1988 were included in the study.

Flowers were collected as they were shed and put in premarked small glass vials (50 ml capacity) filled with 98% ethanol and kept at room temperature (18-21°C). Prior to the examination of pollen germination and pollen tube growth under the epi-fluorescence microscope the corolla, calyx and staminate sheaths were gently removed and each pistil was excised by severing at the attachment of the ovary base to the calyx.

fig. 8.3: Late germination of *Vicia faba* pollen as a result of late September to mid October low night temperature regime at Holetta, Ethiopia. Note that magnification for figs. 8.3-8.4, 8.5-8.6, 8.9-8.10, 8.14-8.15, 8.18-8.19 & 8.21-8.22 is 300X.

Fig. 8.4: Pollen tube growth mostly restricted to zone of stylar canal as a result of late September to mid October night low temperature at Holetta, Ethiopia.

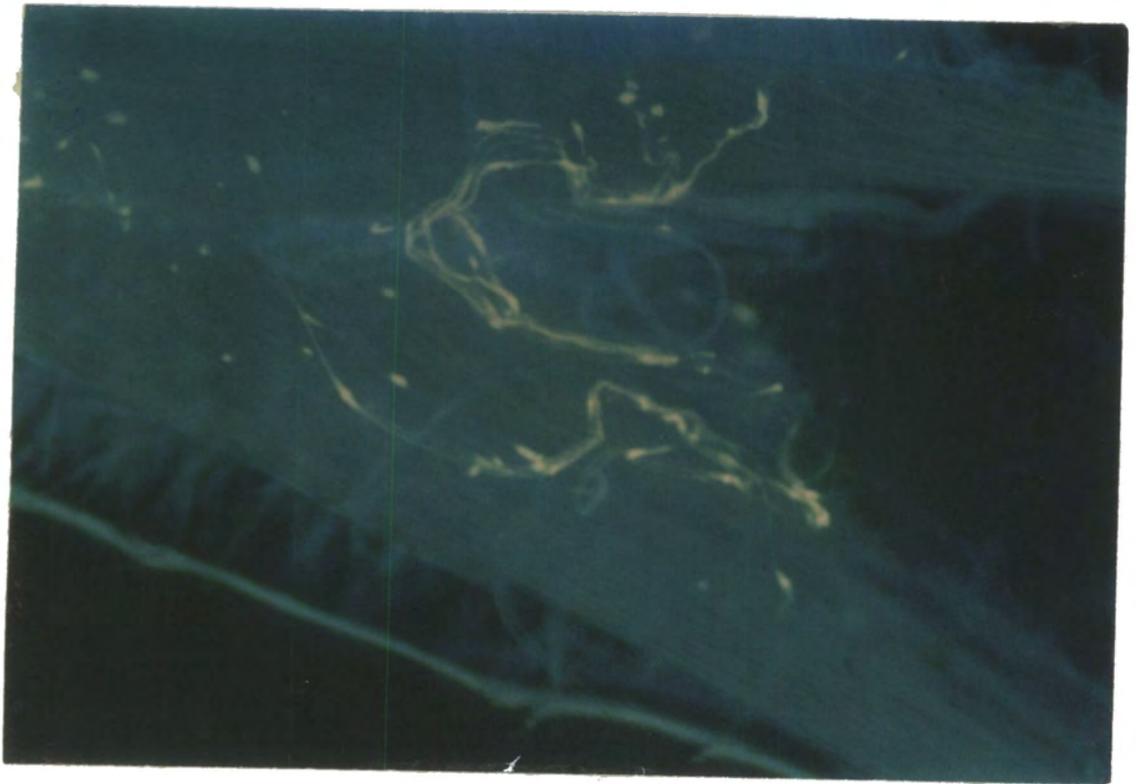
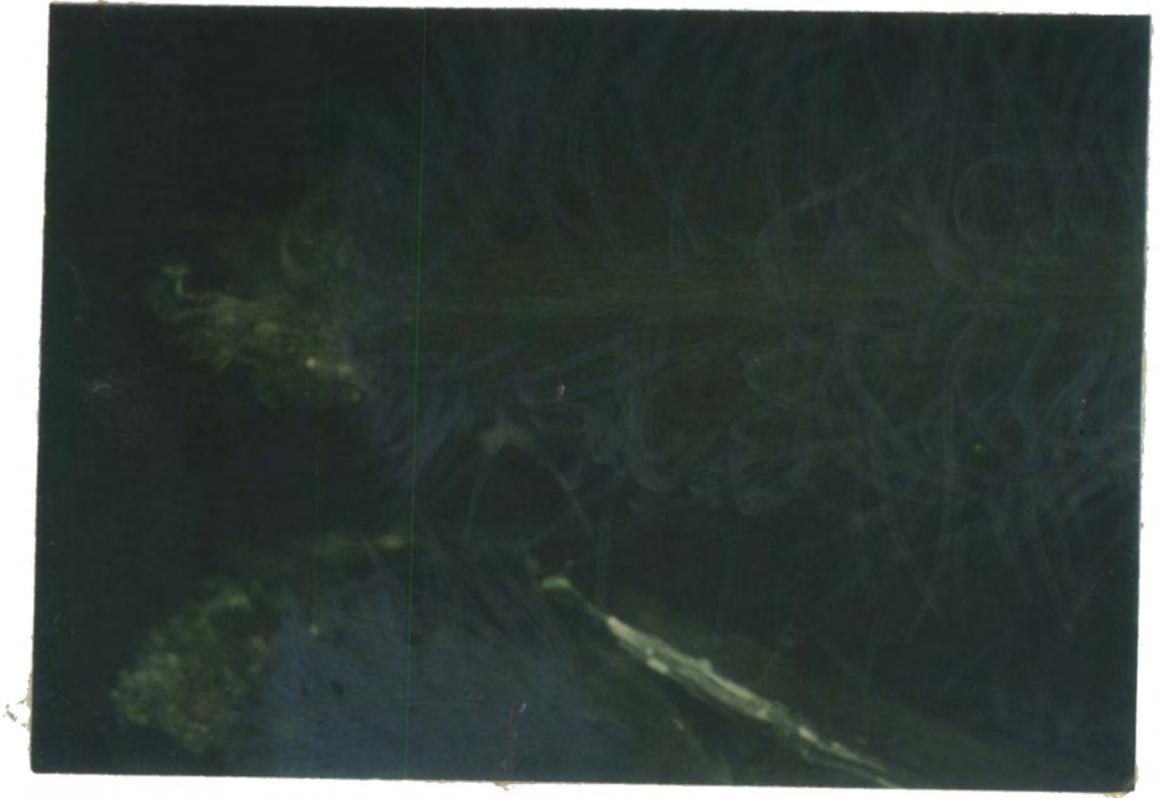


Fig. 8.5: Effect of alternate moderate night and high day temperature regimes (late September to mid October) on mass of pollen tube growth on stigma and in stylar canal, Debre Zeit, Ethiopia.

Fig. 8.6: Abnormal pollen tube growth started on the stigma as a result of day's high temperature between late April and mid May, Holetta, Ethiopia.



Fig. 8.7: Varietal differences in percent (%) fertilisation among abscised flowers. Flowers were collected from location described in fig. 8.1

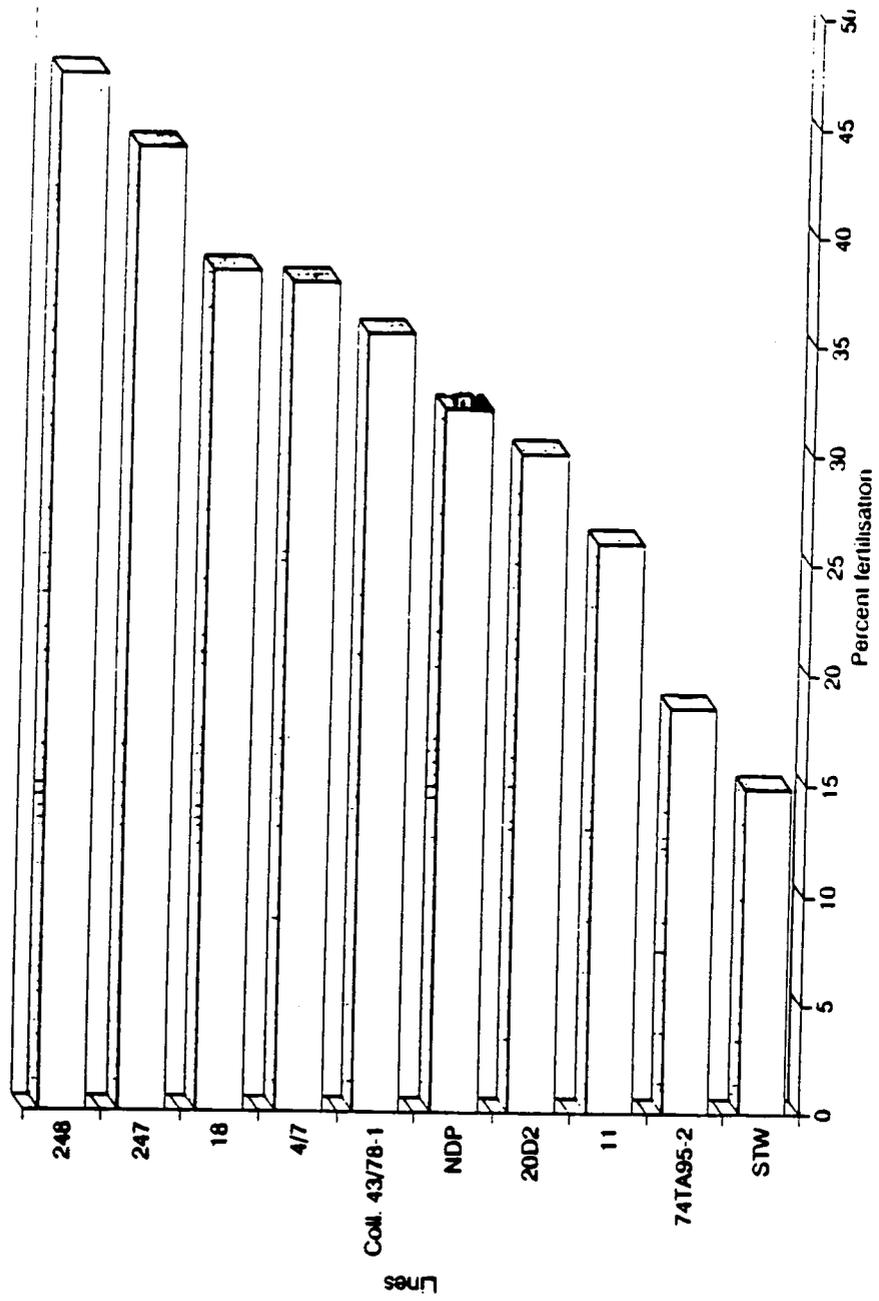


Fig. 8.8: Effect of ovule position on percent fertilisation in line 4/7.

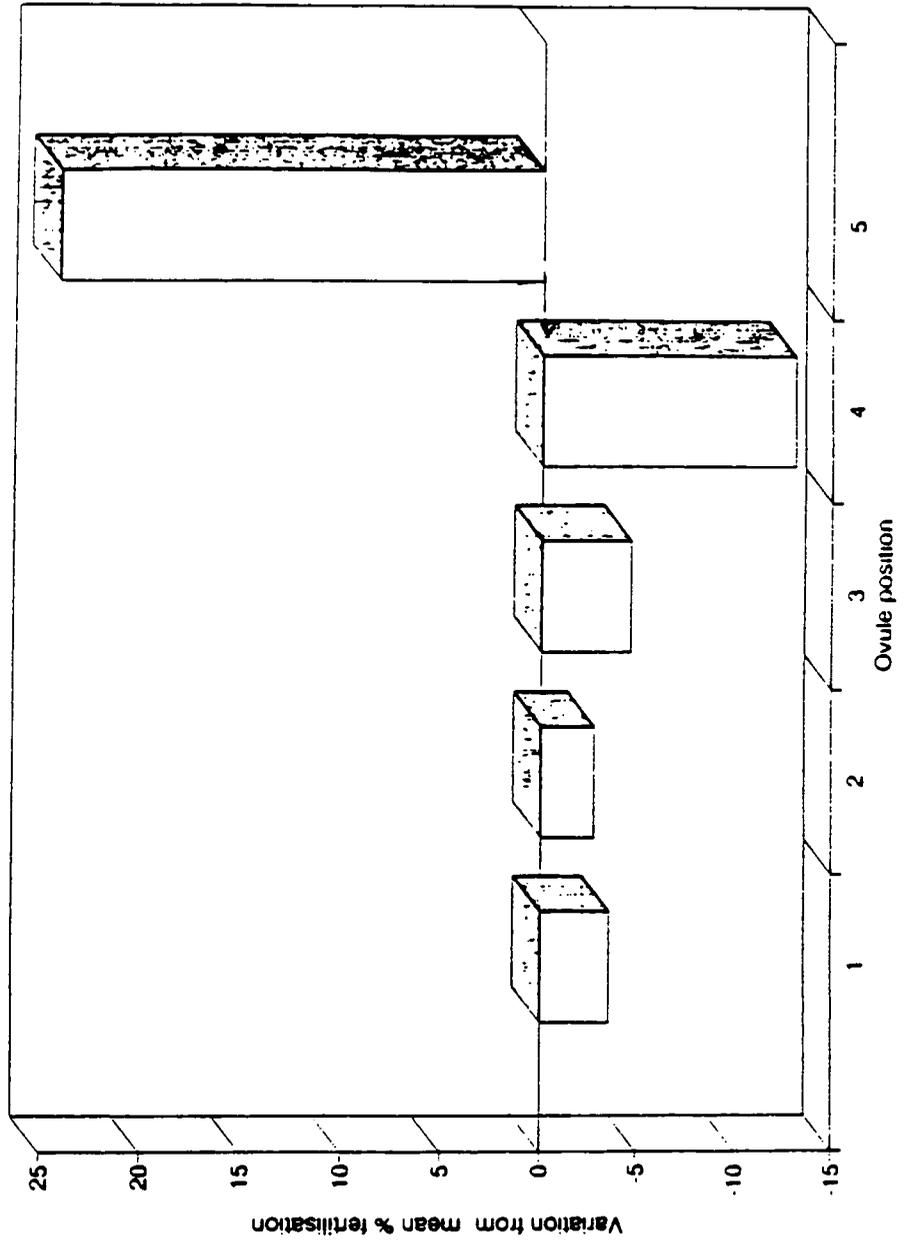
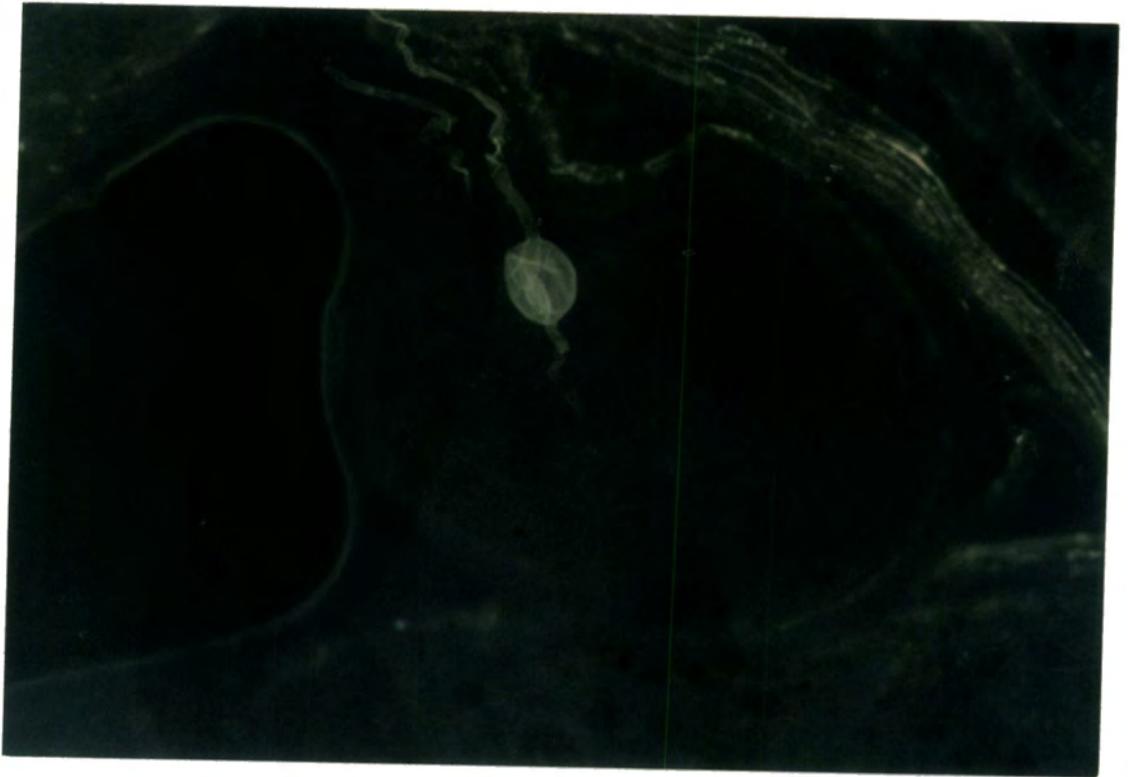
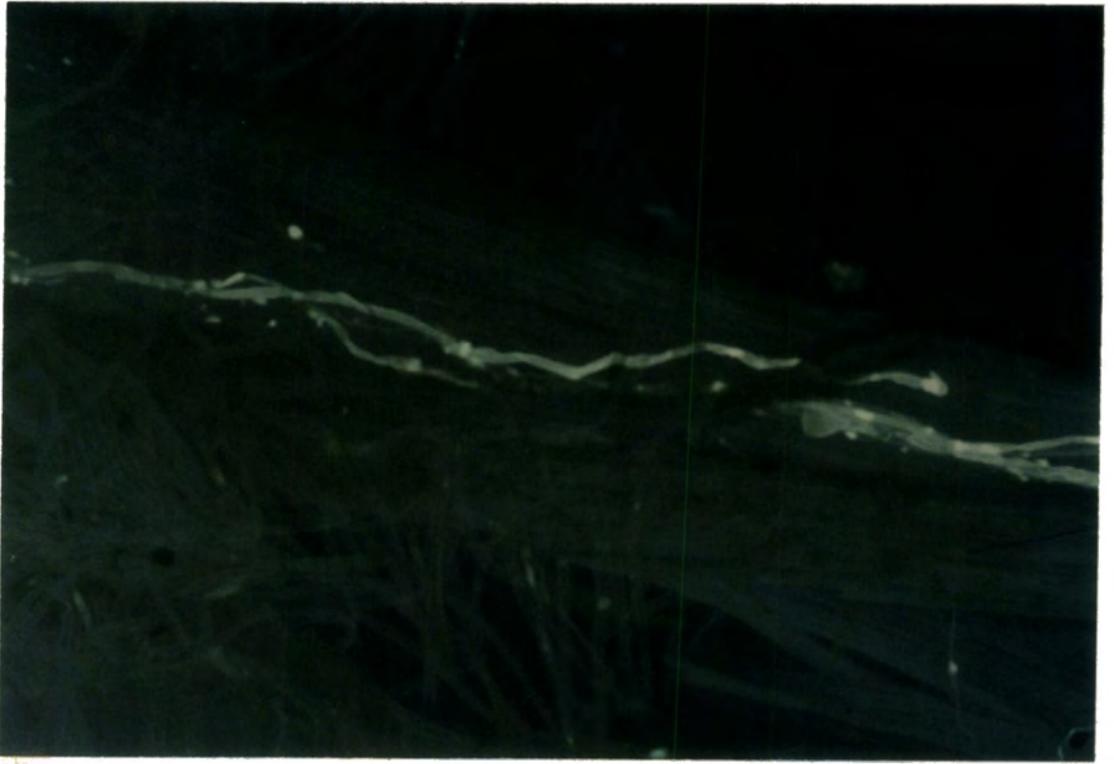


Fig. 8.9: Abnormal pollen tube growth in the style area as a result of high temperatures in late April to mid May, Nazereth, Ethiopia.

Fig. 8.10: Swollen pollen tube growing into an ovule: result of high temperature in late April to mid May at Debre Zeit, Ethiopia.



The material was then stained with an aqueous solution containing 14.2% tripotassium phosphate and 0.5% aniline blue. Aniline blue induces bright blue fluorescence in the callose laid down by pollen tubes grown down the pistil against the back-ground of conductive tissue making them clearly differentiable from phloem tissues using BV excitation filter.

The results of this experiment are those from three planting seasons, where sowings were made in mid-March 1988, mid-June 1988 and late July 1988 (fig.8.1). The mid-June planting served as a control for the other two planting cycles. The mid-March planting came into flower during the hottest period of late April to early May, when the mean monthly temperature would be between 26 and 32°C. The third planting period (late July) was intended to coincide with the coldest period, late August to mid-September. Mid-September may be a period of high humidity.

8.1.2 Effect of season

A hot period, during the plants' reproductive phase was very damaging, (fig.8.1). Thus, the number of unfertilised flowers was significantly higher ($p \geq 0.001$) than in mid-June. There were more unfertilised flowers in the mid-March planting than in the planting in late July ($p \geq 0.05$). It was noted that flowers whose ovules were fertilised were also shed. In this respect, ovule fertilisation in the mid-June planting (normal season which is the main cropping period) was higher ($p \geq 0.01$) than in the mid-March planting. Thus, even though flowers were shed, fertilisation was far better in those flowers planted during the normal cropping period.

8.2 Location effect

When it comes to the question of which ecological growing zone was most unfavourable for fertilisation of the *Vicia faba* ovules, it was at Holetta Research Centre (HRC) where most unfertilized flowers were found. The number of fertilized ovules from Holetta was significantly lower than those from flowers collected from both Debre Zeit, and Nazereth Centres ($p \geq 0.05$). Interestingly, the respective

Fig. 8.11: Effect of ovule position on percent fertilisation in line STW.

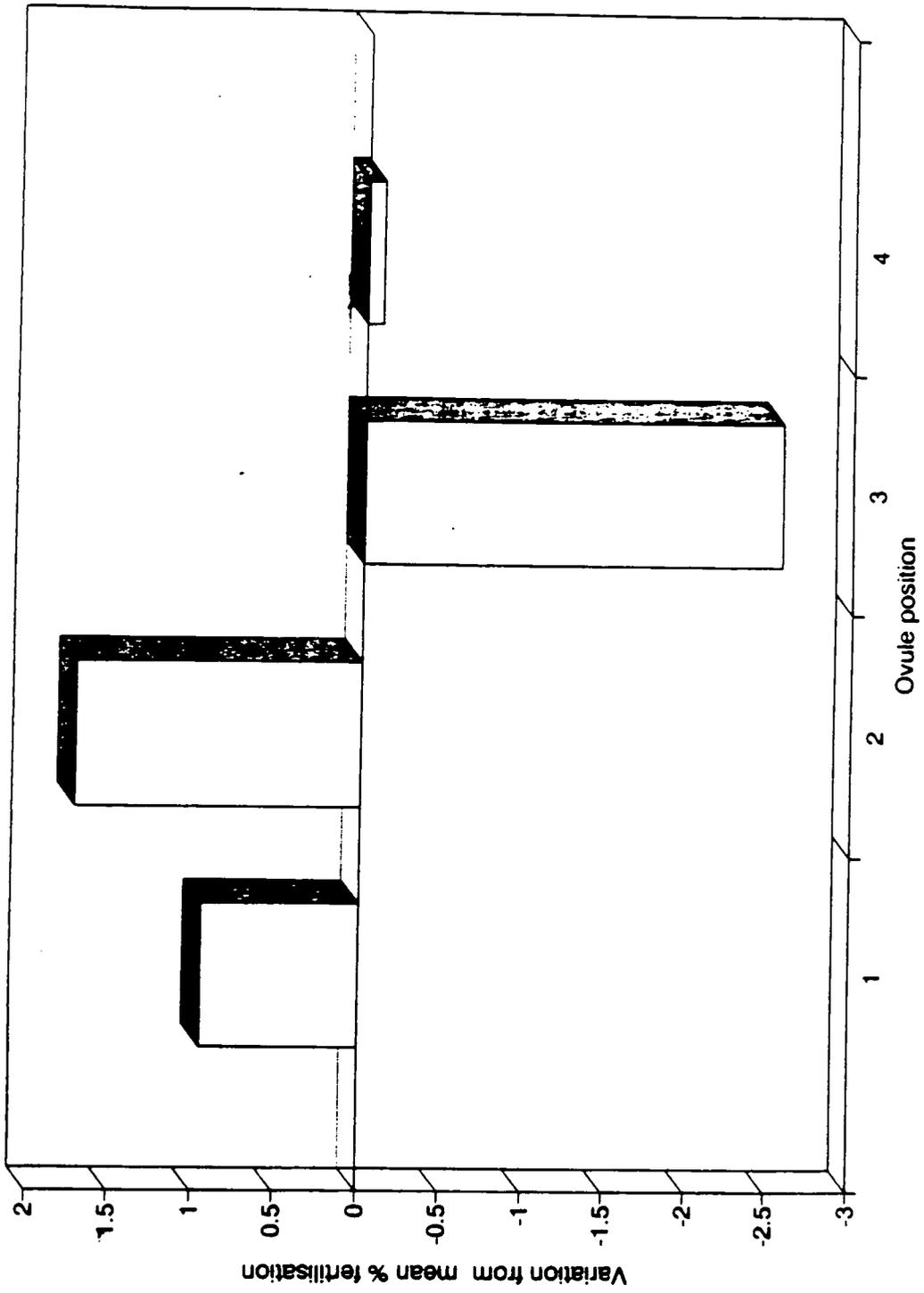


Fig. 8.12: Effect of ovule position on percent fertilisation in line NDP.

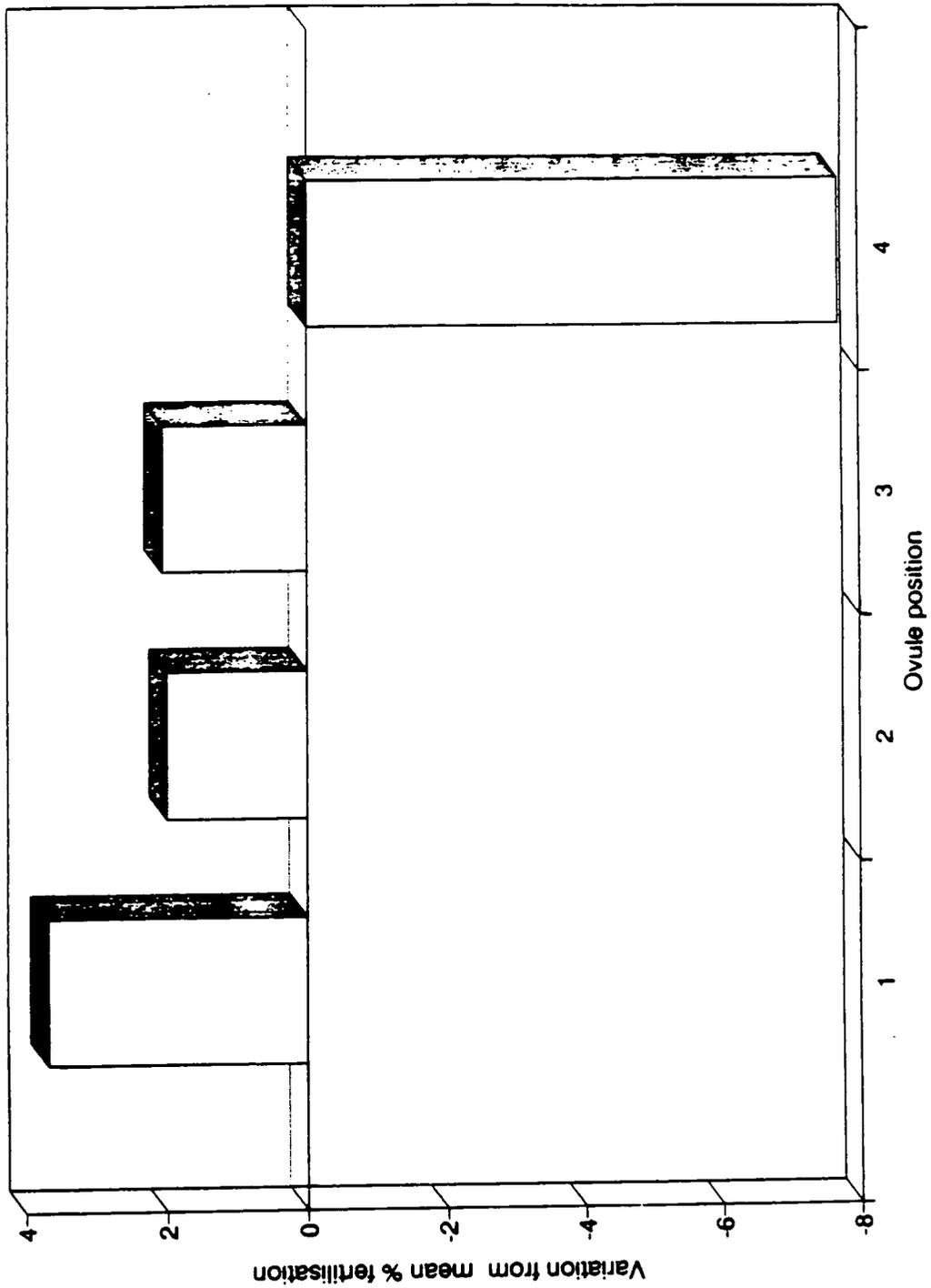


Fig. 8.13: Effect of ovule position on percent fertilisation in line 247.

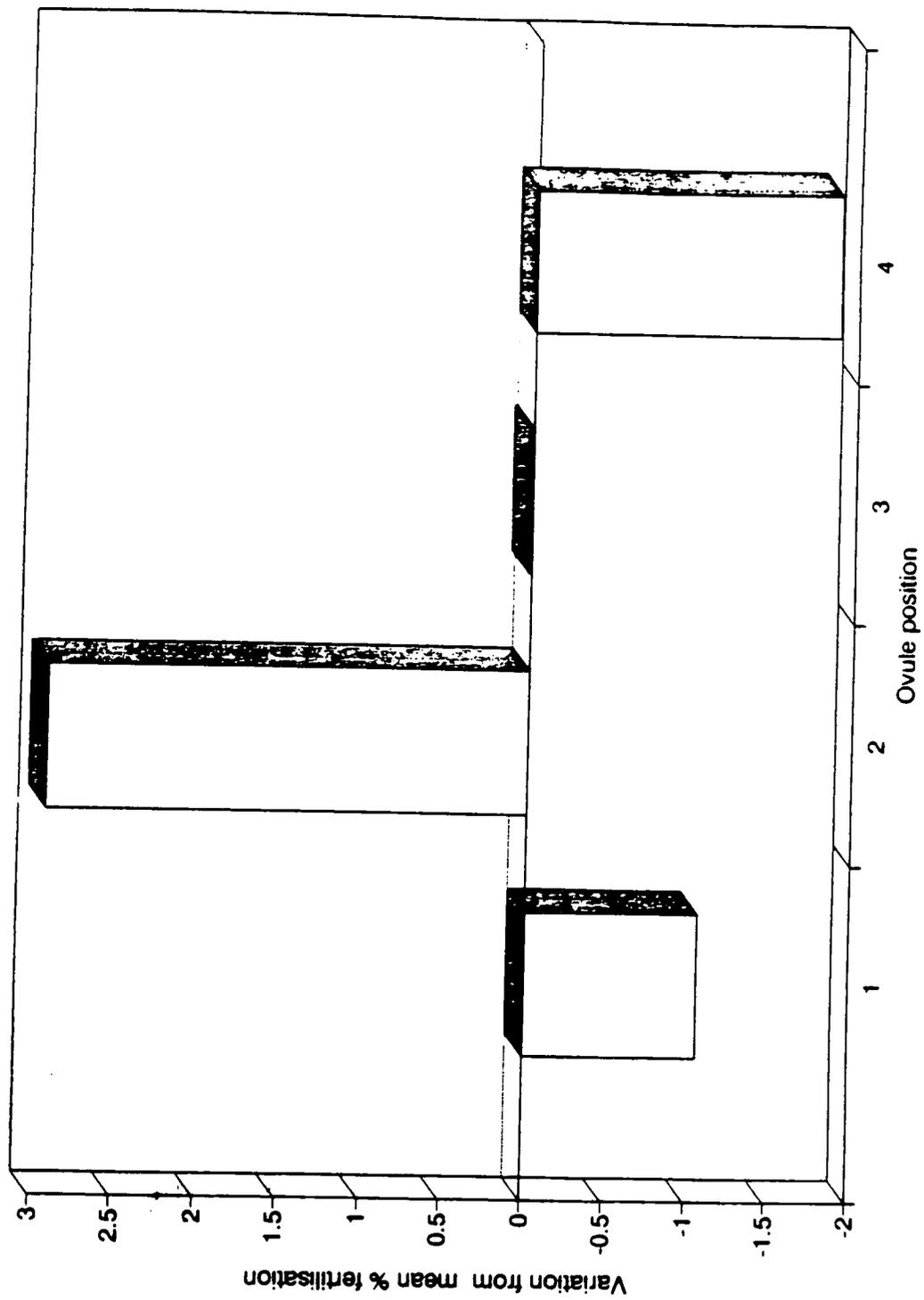


Fig. 8.14: Swollen pollen tube by-passing an ovule while a normal one is approaching the micropyle, location Holetta, Ethiopia.

Fig. 8.15: Pollen tube trying to penetrate an already dead ovule, result of slow rate of pollen tube growth due to low temperature in late September to mid October at Holetta.

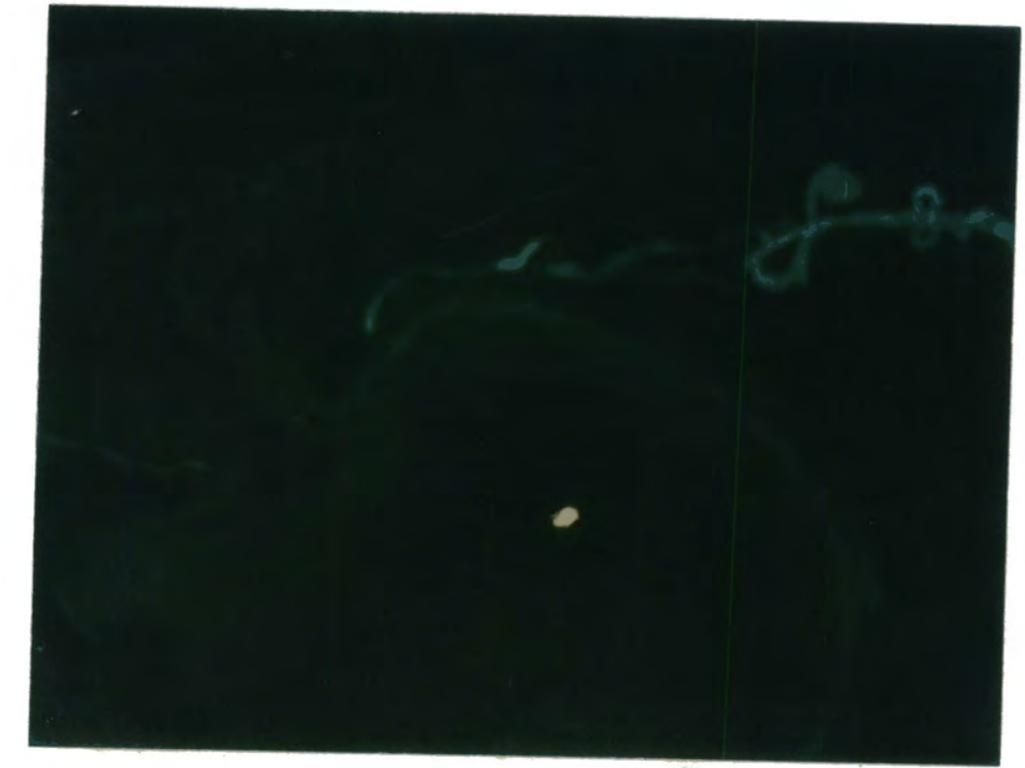
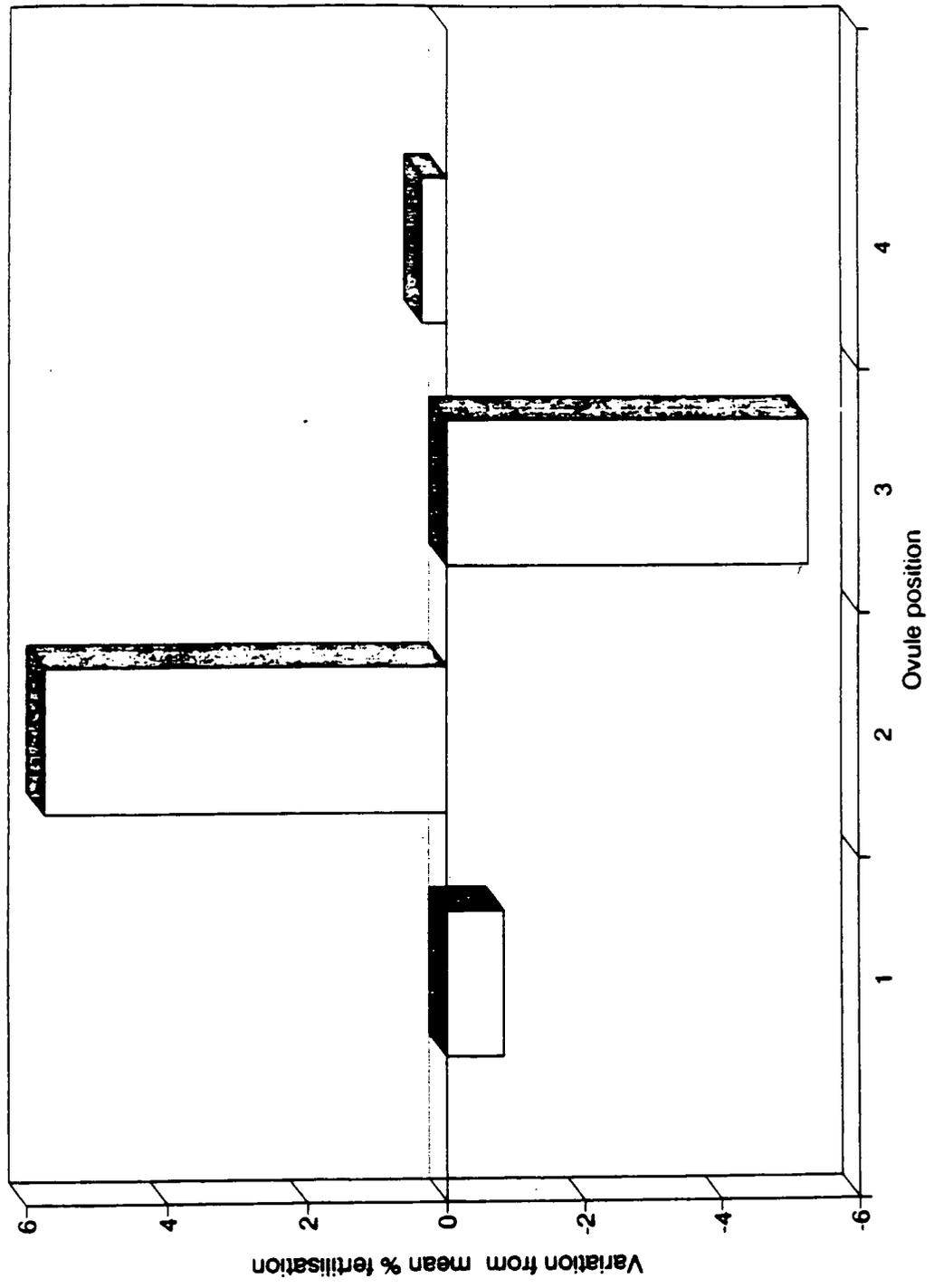


Fig. 8.16: Effect of ovule position on percent fertilisation in line 248.



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Fig. 8.17: Effect of ovule position on percent fertilisation in line 11.

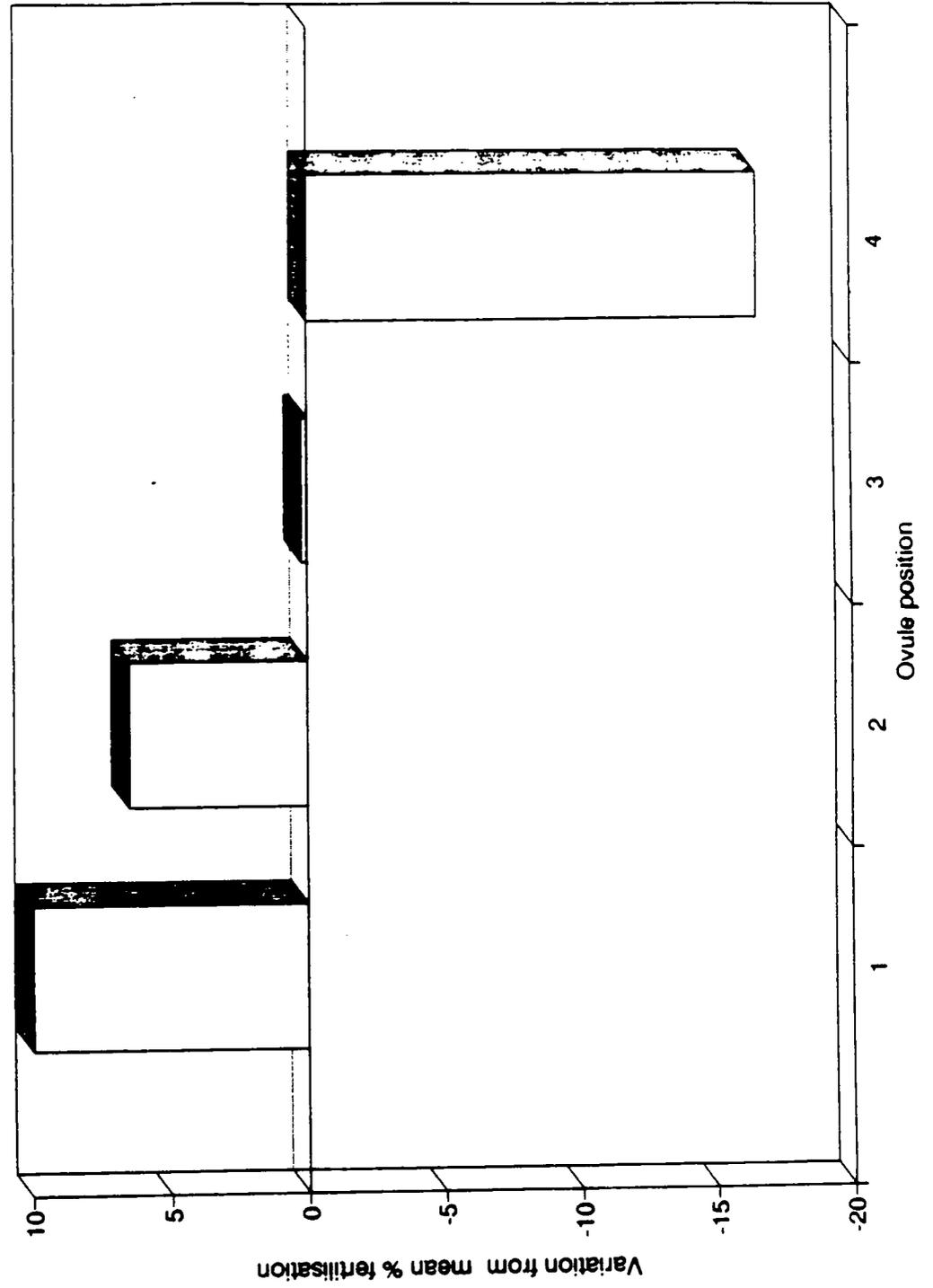
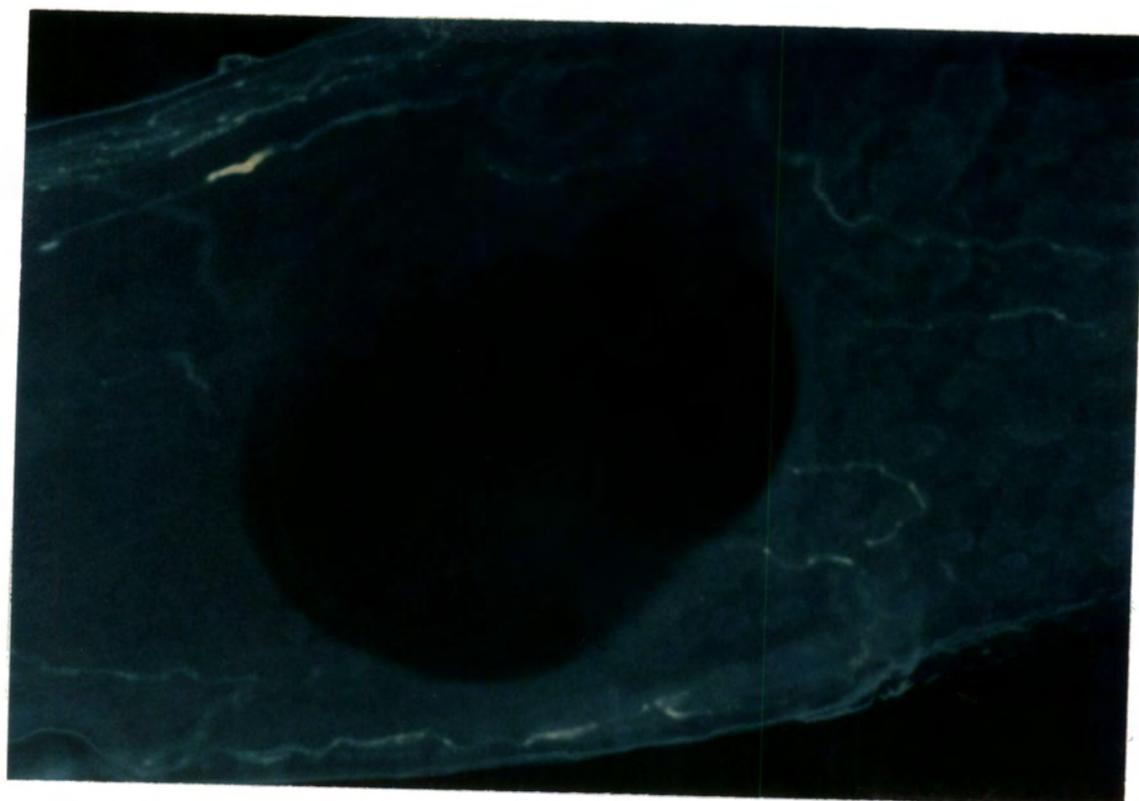
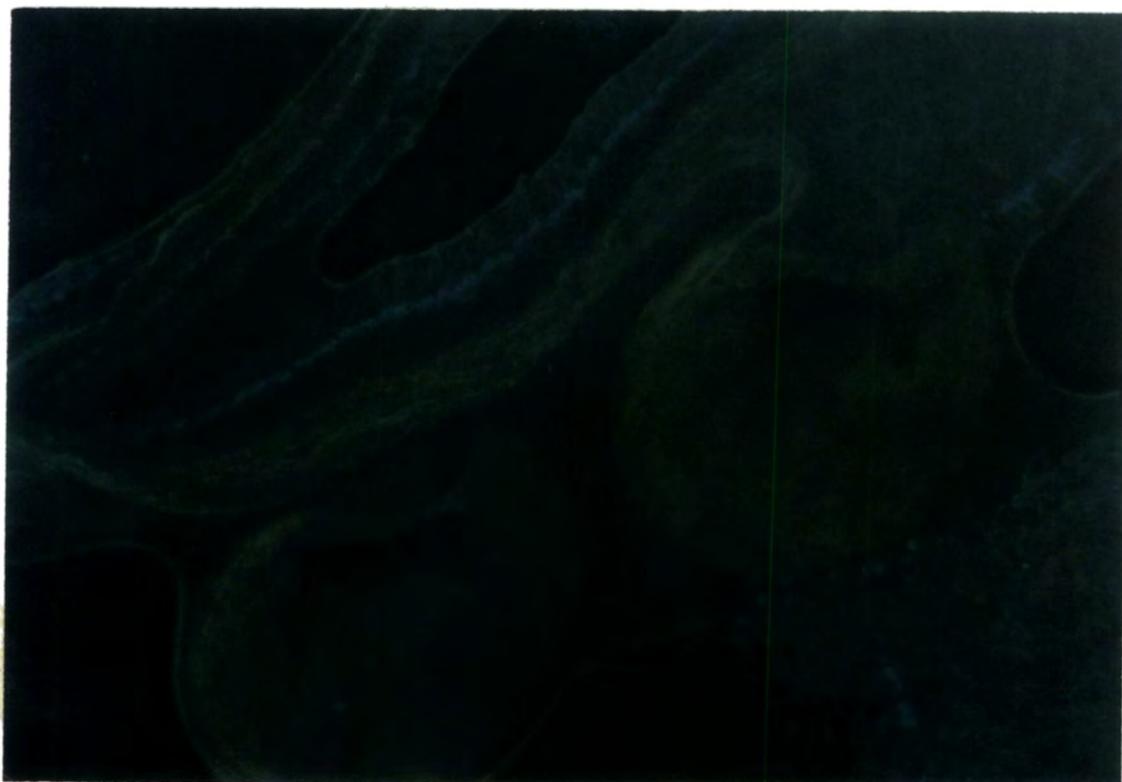


Fig. 8.18: Dead ovules and no pollen tube growth due to high temperature between late April and mid May at Nazereth.

Fig. 8.19: Normal pollen tube by passing a dead ovule due to unsynchronised growth in the low temperature environment in mid October at Holetta.



record of both unfertilized and fertilized flowers from NRC and Debre Zeit Research Centre (DZRC) were not significantly different (fig. 8.2).

8.3 The effect of ovule position

Ovules nearest to the stigmatic surface had the highest level of fertilisation. Thus counting from the style down to the bottom of an ovary, ovules at position one and two showed significantly ($p \geq 0.001$) higher fertilisation than those at positions four and five. Conversely, ovules at position five and four had a higher level of the unfertilized ones than those at position two and one (fig. 8.8).

8.4 Variability among the lines

The mean proportions of fertilized and unfertilized ovules are shown in fig. 8.7 (the values being in percentages). It can be noted that of the examined flowers, the pistils of STW had the highest number of unfertilized ovules among the shed flowers, followed by line TA95-1. Thus among the shed flowers, STW was significantly higher ($p \geq 0.0001$) in unfertilized ovules than the other six lines, exceeding TA95-1, 11, NDP at the $p \geq 0.001$ level of probability. The lines showing the highest numbers of fertilized ovules in shed flowers were lines 247 and 248 both from the University of Durham, England (fig. 8.7). These also showed moderate levels of unfertilised ovules (56 and 52% respectively). These traits are clearly shown in figs. 8.13 and 8.16.

Fig. 8.20: Effect of ovule position on percent fertilisation in line 18.

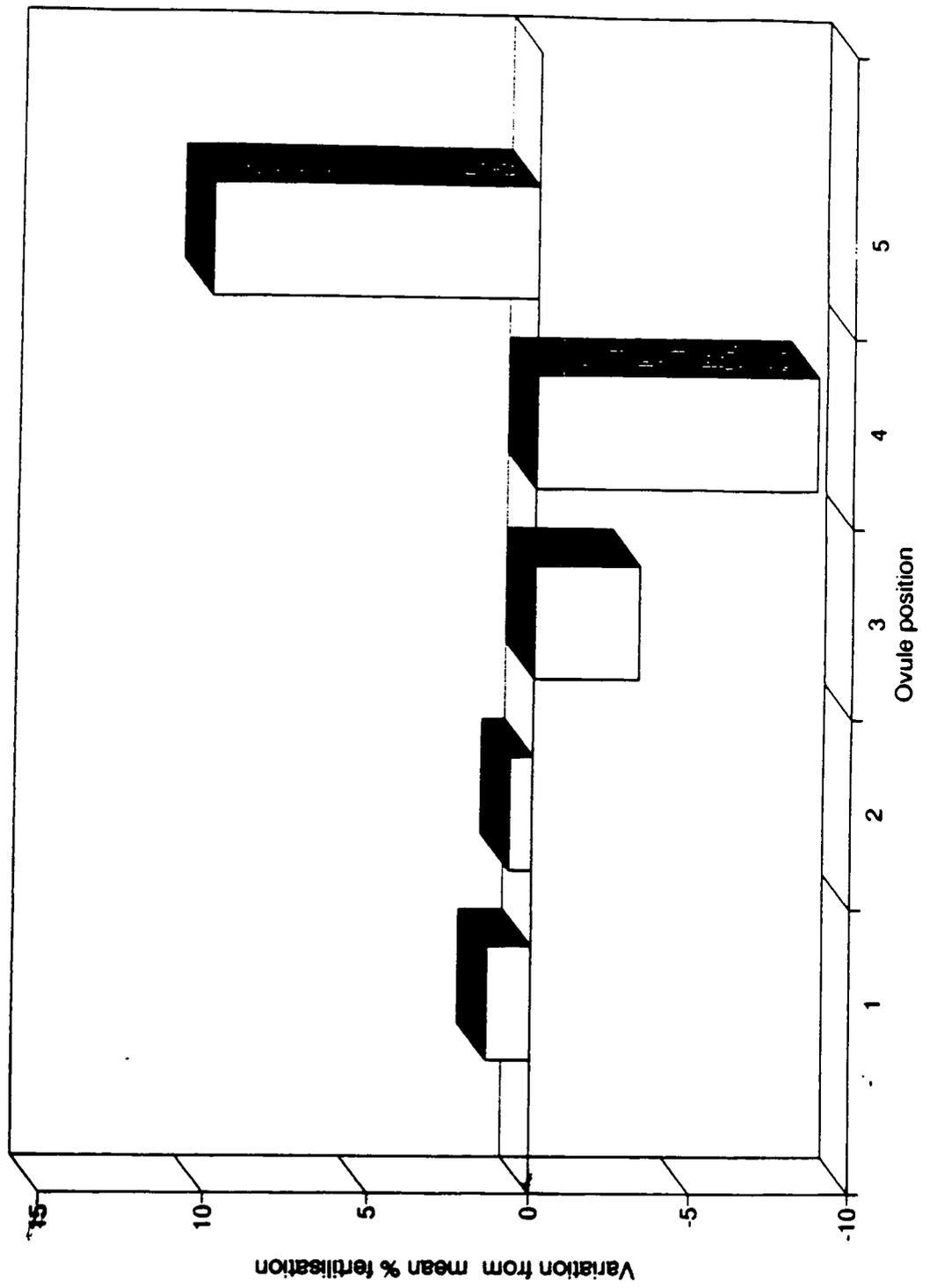


Fig. 8.21: Late attempt of pollen tube to penetrate an ovule. A result of the low temperature effect on pollen tube growth rate at Holetta.

Fig. 8.22: Unoriented pollen tube growth. Result of high day temperature and lower night temperature at Holetta, May 1988.

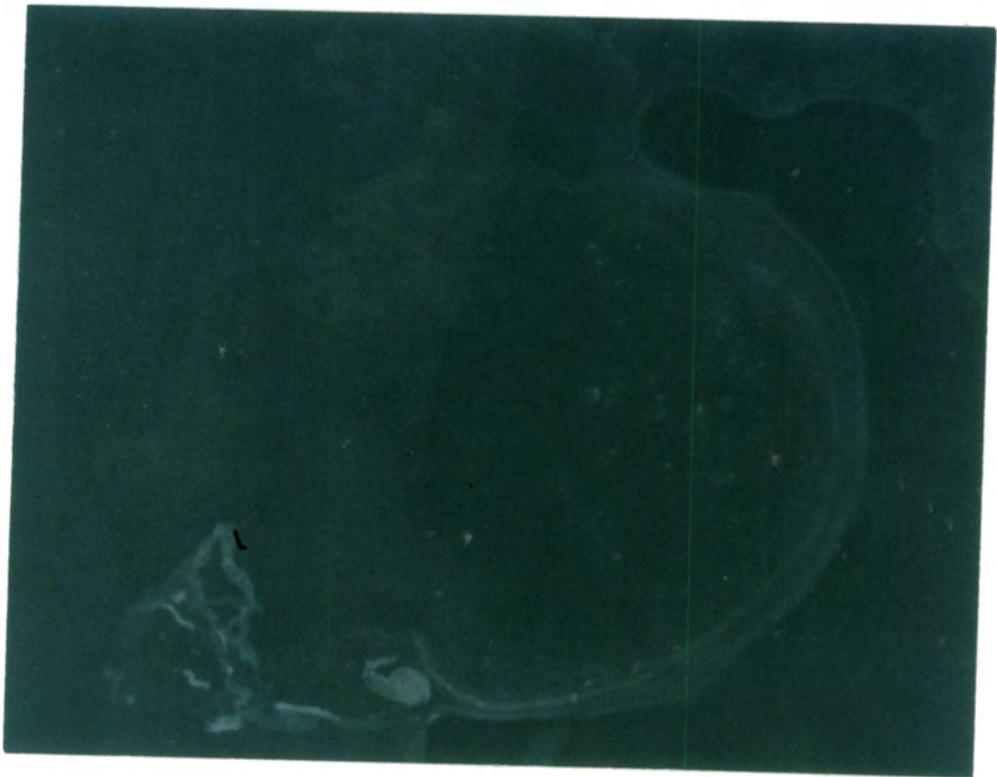
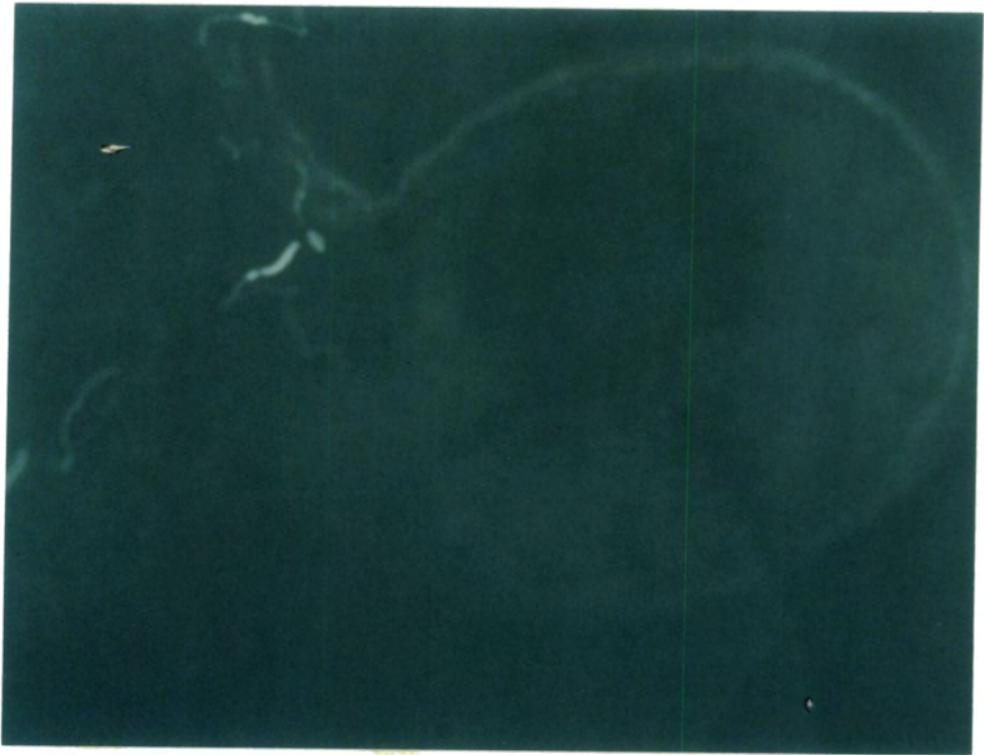


Fig. 8.23: Effect of ovule position on percent fertilisation in line 20D-2.

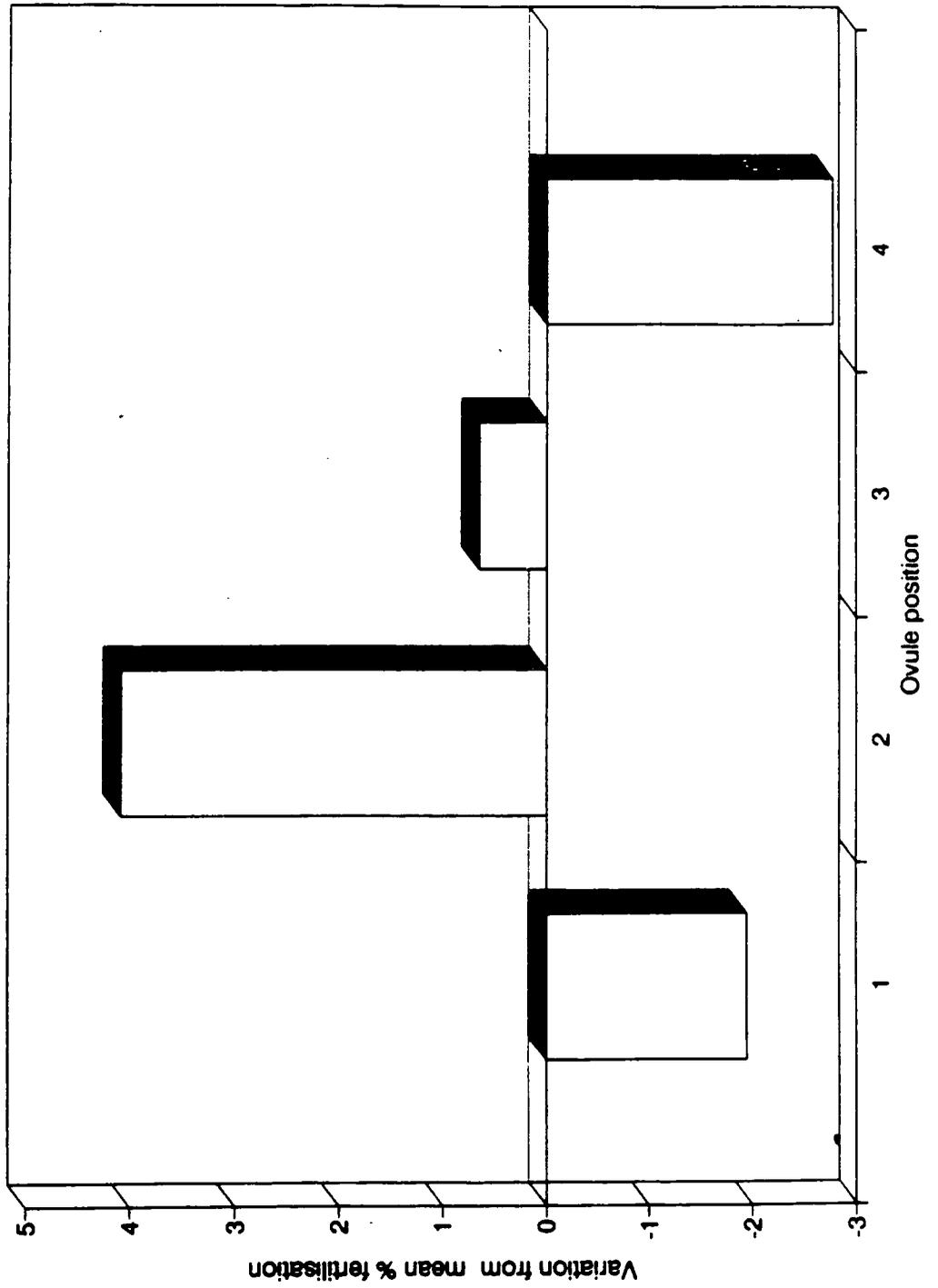


Fig. 8.24: Effect of ovule position on percent fertilisation in line 74TA95-1.

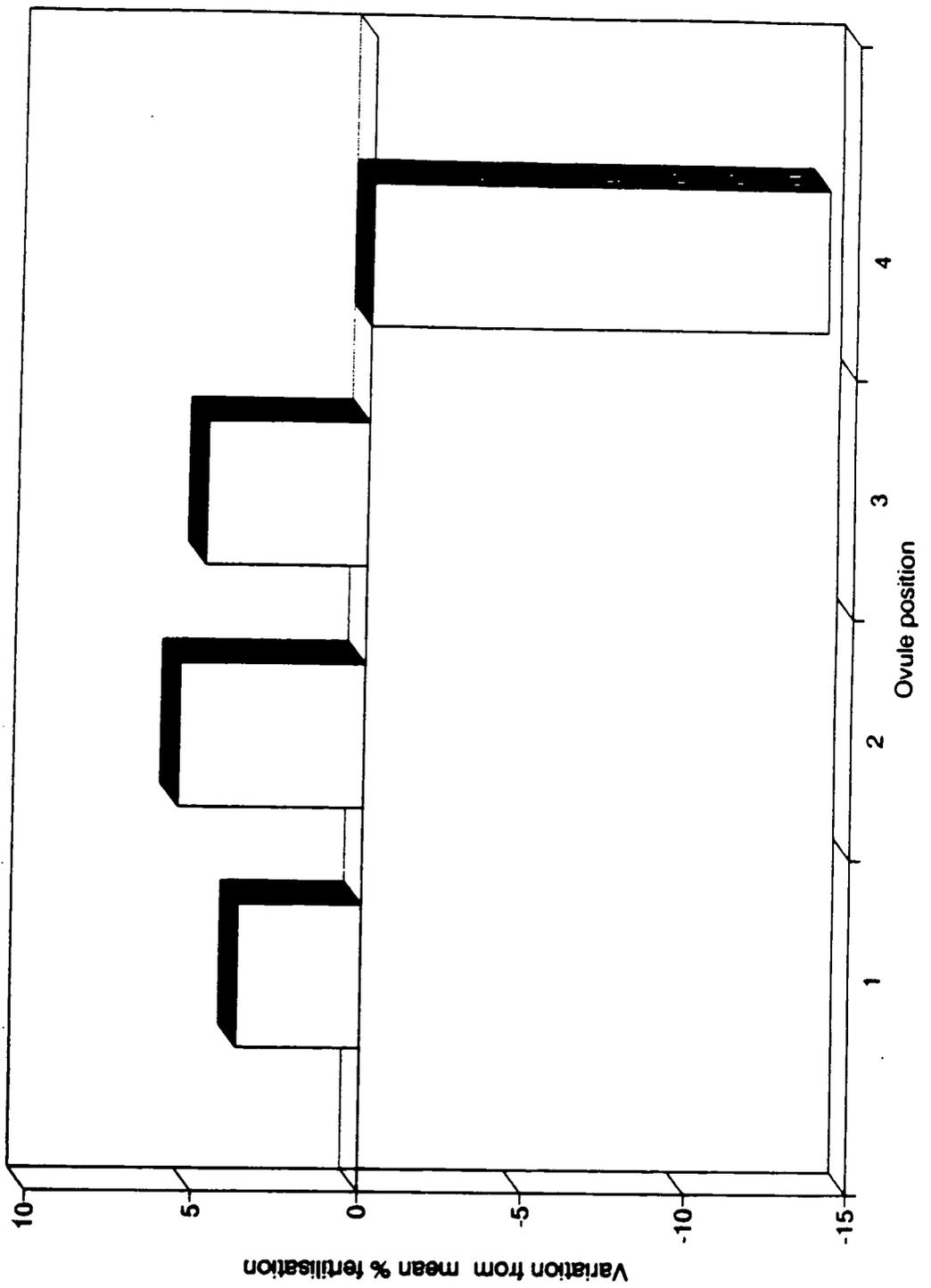
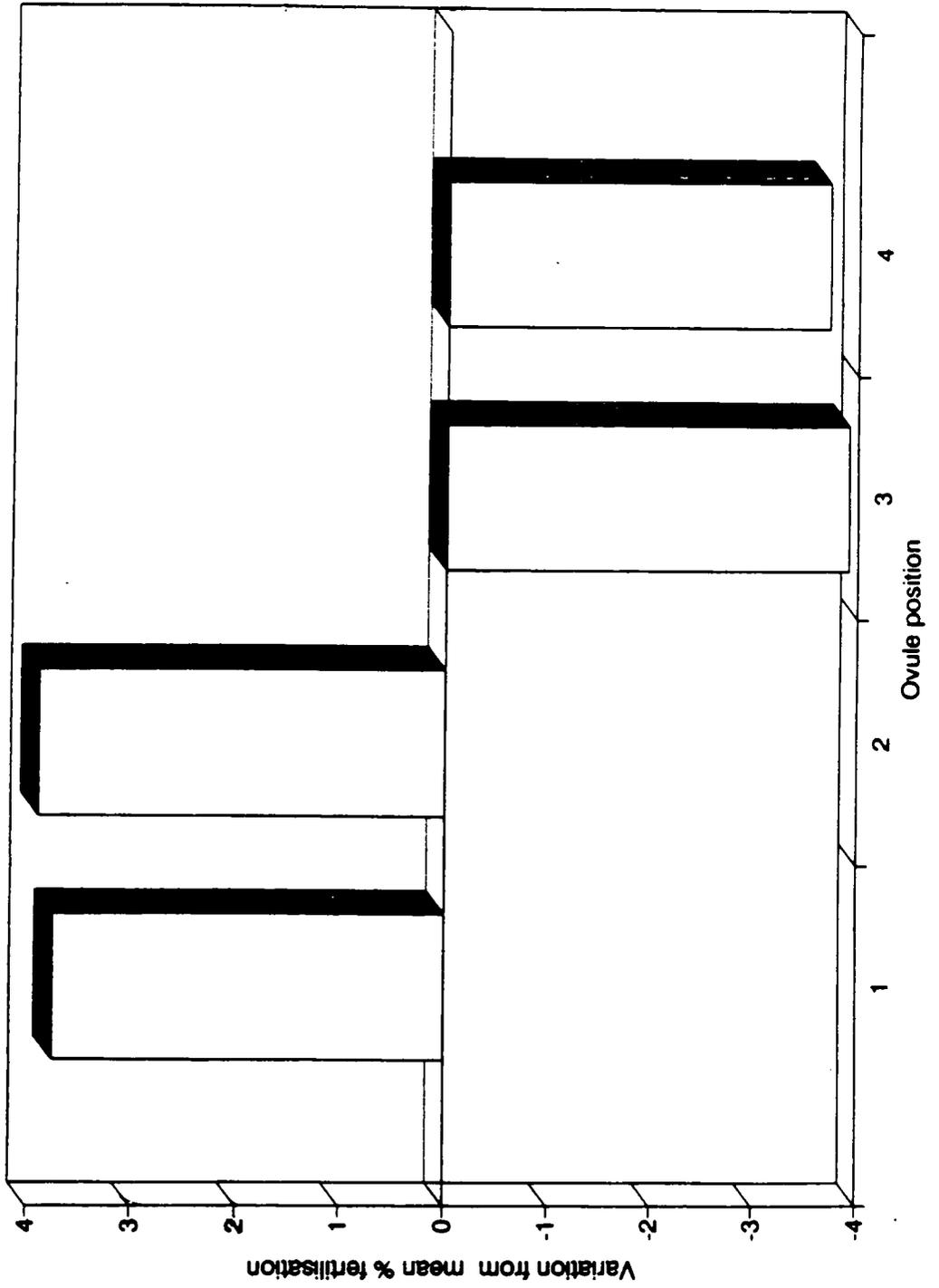


Fig. 8.25: Effect of ovule position on of percent fertilisation in line coll. 43/78-1.



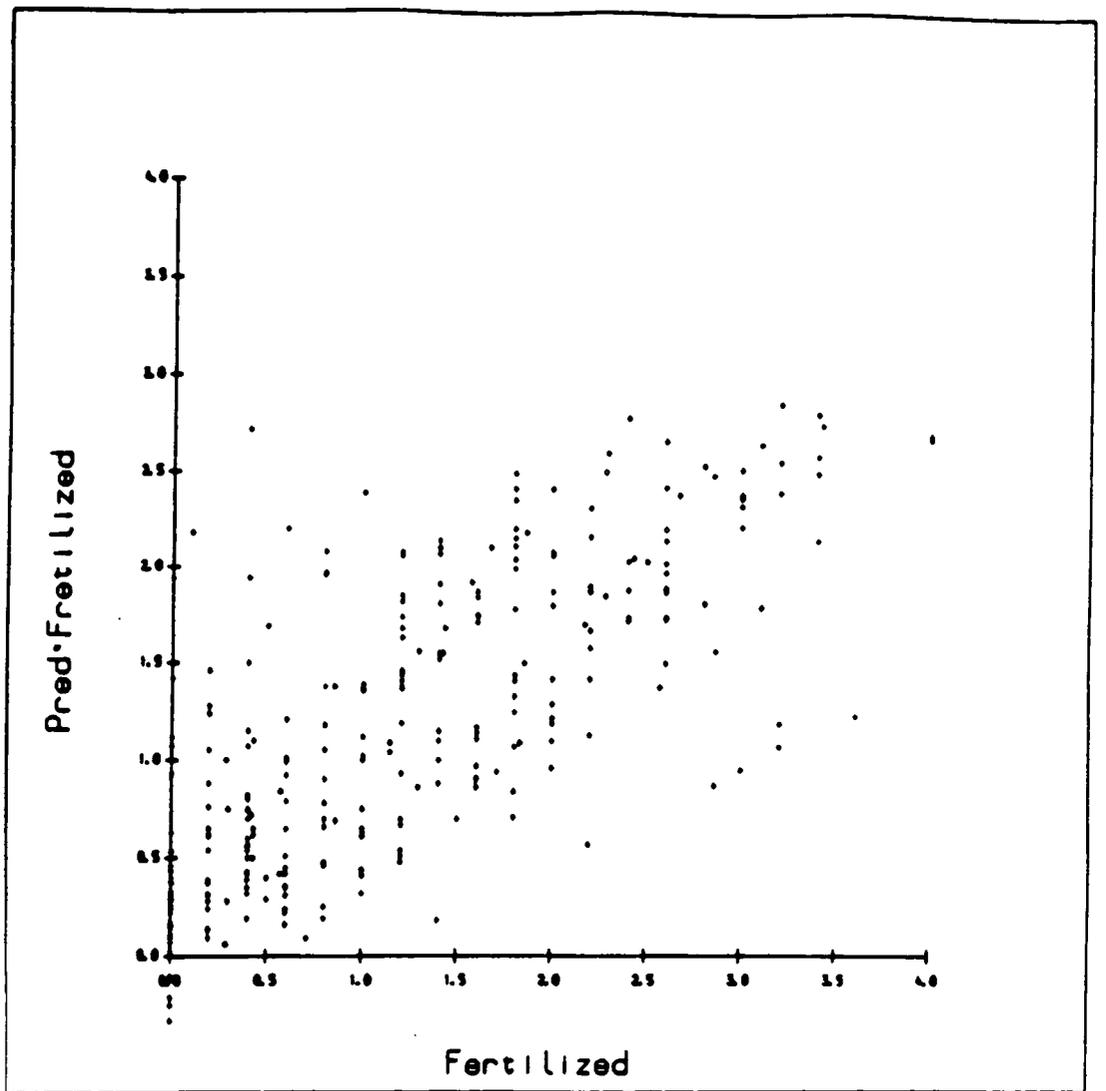


Fig. 8.26: Multivariant regression (adjusted) of predicted on actual incidence of fertilisation in *Vicia faba* under Ethiopian growing conditions. Conditions are described in figs. 8.1-8.2.

Chapter IX

Discussion

9.1 Introduction

This chapter discusses the effect of environmental stress on the germination, pollen tube growth and fertilisation of *Vicia faba* pollen and the investigations into environmental problems related to pollen biology of the crop, encompassed in both *in vitro* and *in vivo* experiments.

I shall discuss the aspects of pollen competition and pollen management associated with the possibility of long-term pollen conservation. Initially investigations into environmental stresses such as temperature and humidity were pursued both *in vitro* and *in vivo* under laboratory conditions. These results led, in turn, to the development of a pollen competition study *in vivo*, and of a study of pollen conservation under various low temperature regimes. Results recorded under field growing conditions also allowed an investigation of *Vicia faba* pollen fertility status along the main reproductive nodes, from the first flowering node to the top reproductive node. From this, I tried to construct a general hypothesis about the main factor responsible for *Vicia faba* flower abscission and consequent low yields.

9.2 Effect of environmental stress on *Vicia faba* pollen *in vitro*

9.2.1 Germination trends

Table 3.1 and figs. 3.1 and 3.2 show that with the fast germinating pollen grains from NDP and 248, the proportion of inviable or non-germinating grain increased as temperature, at 100% RH, was raised. Under field conditions, if pollen grains are subjected to such high humidity at 20°C, 25°C, 30°C or 35°C, during their

peak pollination period, the fastest germinating lines (such as 248, NDP) would lose their viability by approximately 60% and 57% respectively, compared to their extended longevity under optimal conditions of 20°C with low relative humidity.

In contrast pollen grains from genotypes such as 247, 18 and, to a lesser extent, 20D2 seemed to have a tolerance for stress conditions as severe as 35°C with 100% RH, (table 3.1 and fig. 3.2). In this respect the overall mean germination increased over the control by 21.41%, 17.59% and 12.61% respectively for pollen grains from these lines. Therefore pollen grains from these genotypes may not show drastically reduced viability at 35°C with high humidity. **Herrero and Johnson** (1980) tested maize pollen grains, of which about 40% had the highest germination at 32°C.

This tolerance has been attributed to a possible genetic mechanism operating in various genotypes. Under the stress conditions imposed, pollen tube growth declined in all the lines studied relative to the unstressed control. In fact, the decline was a straight line slope except for the 35°C stress condition. This again seemed to be due to pollen tubes from line 247, which scored highest percentage viability (75.74%) under these conditions (table 3.1 and fig. 3.2). At 30°C incubation (no period specified), **Lim** (1979) found 90%, 77.70%, 87.80% of pollen grains burst in 15%, 25% and 35% sucrose concentration, and concluded that germination *in vitro* of *Vicia faba* should be between 10°C and 30°C, below or above which germination would be inhibited. In the present study, of the pollen tubes measured, the maximum percent burst tube was from the line 4/7 (29.55%).

The results of the current study indicate the practicality of pollen selection. This is elimination through selection applied to the male gametophyte prior to pollination of unwanted male gametes (**Hodgkin**, 1987). This would be an attractive prospect to plant breeders (**Zamir & Vallejos**, 1983) and evidence is already being accumulated that many genes are active in both sporophytes and gametophytes (**Tanksley et al.**, 1981; **Willing et al.**, 1984). Furthermore, in crops such as rice it has been observed that heat-tolerant cultivars shed more pollen grains on the stigma under both optimal and maximal temperature regimes, thus compensating for reduced pollen germination at higher temperature. In contrast, the less

tolerant cultivars shed substantially less pollen on the stigma at high temperature (Mackill *et al.*, 1982).

9.2.2 Pollen tube growth trends

Table 3.2 and figs. 3.3 and 3.4 show the effect of temperature on pollen tube growth in lines 248, 18, 4/7, STW, 11, and NDP. These are fast growing lines under room temperature during the incubation period of 2h. Thus, pollen tube growth was inversely proportional to the high temperatures imposed, under 100% RH.

The slower growing lines such as 247 showed only slight declining in pollen tube growth at 35°C. Pollen tube growth from the Ethiopian lines, 20D2 and 951, seemed to be higher at 35°C than at 30°C. Overall the negative effects of high temperature were more pronounced under the condition of high RH on pollen tube growth (table 3.2 and figs 3.3 and 3.4). These results are in accord with Lim (1979) who concluded that the optimum temperature range of pollen tube growth of *Vicia faba* must be between 20 and 25°C.

During the germination process synthesis of protein by the pollen tube during growth is necessary, at least in binucleate pollen (Linsken *et al.*, 1970; Hoekstra & Bruinsma, 1979) and must continue throughout pollen tube growth. In some of the lines, such as 247, it seems that it could be the extent of protein synthesis that gave best tolerance to the stresses imposed, particularly stress under 35°C and 100% RH while the faster growing ones such as NDP, 248, STW, 11, all declined at higher temperature and humidification. Not all pollen from plant species is capable of synthesising heat shock proteins (HSPs) (Scharauwen *et al.*, 1986); Xiao & Mascarenhas, 1985), and therefore the lines which were lacking in this faculty would naturally show lower germination and pollen tube growth rates than those in which this ability was present. Sitch & Snape, (1987c) also reported genotypic different response to ambient temperature by *Hordeum bulbosum*.

This experiment throws some light on why, in certain years when rainfall is

abundant and weather conditions are humid from late August to late September, yield is very much depressed in the highlands of Ethiopia and similarly in the Holetta zone. It is known that standing water or a humid atmosphere causes the bursting of germinating pollen grains (Bair & Loomis, 1941), while high temperature during the reproductive stage could induce sterility (Mackill *et al.*, 1982).

9.3 *Vicia faba* pollen fertility status along reproductive nodes

The results described in the above paragraphs show that low levels of pollen viability between nodes and within racemes of *Vicia faba* are a common feature of the crop, even though the inviability is highly variable between lines. Compared with the remaining two lines, STW pollen grains are less abundant, line 11 is an intermediate and line 248 produces an abundant amount of pollen. In other crop species, such as *Allium cepa* (onion), it has been reported that an excess of pollen is associated with low levels of pollen viability (Ockendon & Gates, 1976). This seemed to be one of the factors correlated with the low level of pollen grain viability of line 248, particularly at the upper nodes and pedicels. In *Allium cepa* great variations in pollen fertility between anthers and flowers in the same inflorescence have been reported (Ockendon & Gates, 1976).

Like most lines of *Vicia faba*, the three lines investigated are indeterminate in their growth habit. Thus the period of vegetative growth is characterised by a gradual increase in the complexity of leaf development while reproductive development is initiated at the axillary meristems (Peat, 1982). Flowers usually develop as short racemes normally 1-4 per node in line STW, while 11 and 248 could have 2-7 flowers per raceme depending upon the nodal position. The stem apex also continues to develop vegetatively so that, particularly in lines 248 and 11, a large number of competitive floral nodes are initiated. Such a situation has been shown to reduce yield due to intra-plant competition (Adcock & Lawes, 1976). As a result, it is believed that intensive competition both intra- and inter-node for metabolites in such lines is bound to occur (Chapman *et al.*, 1978; Chapman & Sadjadi, 1981; Bond & Poulsen, 1983; Peat, 1982). If this is the

case, assimilate starvation must be a common cause of low viability in the upper nodes of line 248 as well as in line 11. A similar conclusion has been reached by **Heslop-Harrison** (1972). Starvation as a result of intensive competition for nutrients between developing flowers and developing bulbs, was also reported as causing pollen sterility in onion (**Ockendon & Gates**, 1976).

In accordance with the hypothesis of vascular independence, lines with only 4-5 flowers per node opening over 1-2 days show very little flower shedding. This is probably a result of fertilisation occurring in the apical flowers, before the hormonal stimulus from recently fertilized basal flowers could trigger the abscission process. In contrast, *Vicia faba* lines with 8 or more flowers per node opening over a period of 5-6 days commonly exhibit 50-60% flower shedding (**Gates et al.**, 1981). Thus, in this hypothesis, a rapid and massive increase in vascular development in the peduncles and pedicels post-fertilisation and prior to pod development/seed growth strengthens the supporting tissue. This vascular development is detectable soon after fertilisation. In this context the vascular and supportive tissues of the racemes are believed to be the primary sink for assimilates at this stage.

Competition for assimilates between competing sinks may be an important factor contributing to lower levels of pollen fertility within and between nodes in the lines 11 and 248. Demand for assimilates created by pod and seed development at lower nodes and by the processes of vascular development and lignification of pedicels and peduncles of fertilised flowers (**White et al.**, 1984) may result in assimilate starvation in developing flowers at upper nodes and in apical flowers within nodes, resulting in increased pollen sterility in these flowers.

It has often been shown that the proportion of flowers fertilised and of pods produced at the lower reproductive nodes of *Vicia faba*, is far greater than it is in the upper nodes. More flower abortion occurs on the upper nodes of the plant than on the lower reproductive nodes. Thus the present study clearly demonstrates that such abortion could be due to lack of fertile pollen grains in the upper nodes of *Vicia faba* lines.

Flower drop due to lack of fertilisation varies depending upon environmental

conditions (Stoddard, 1986b; Stoddard & Lockwood, 1984). The proportion of fertile pollen grains varies both between flowers within a plant and between plants within the progeny. Therefore the proportion of seeds produced is positively related to the number of fertile pollen grains available to a given flower at a particular reproductive node and raceme (Schoen & Stewart, 1986). In *Vicia faba*, due to intra-plant competition, yield usually diminishes as the number of flowering nodes increases (Adcock & Lawes, 1976). To-date, nothing has been reported regarding the status of fertilisation in these positions. It is not difficult to envisage failure of pod set as being due to lack of fertilisation. This remark is in accord with the viability status of pollen grains at the uppermost of flower nodes in line 248, observed in this experiment.

There are also cases where pollen quality can affect pollen germination, and consequently fertilisation. If fertile pollen grains are greatly outnumbered by infertile grains, blockage of the stigma by non-viable grains would pre-empt stigmatic space and substances (Bertin, 1986; Kevan *et al.*, 1987; Herrero & Dickinson, 1980; Herrero & Arbeloa, 1989). By interfering with normal functioning of foreign pollen grains, incompatible self-pollen can increase pollen limited seed set in *Polemonium viscosum*. This led to a reduction in seed yield as the self-pollen grains displaced foreign pollen on the receptive stigmatic surface (Galen *et al.*, 1989).

Also the number of fertile pollen grains in a stigmatic pollen load can influence the success of an individual grain. Thus, a small load is disadvantageous in that it is less likely to stimulate a pollen tube growth race (Brewbaker & Majumder, 1961). Pollen deposited on a *Vicia faba* stigma has been observed to fail to germinate. Cruden (1979) reported that maximum seed set in most plant species requires typically, pollen loads of 2-7 grains per ovule. However, Levin (1975) and Guth & Weller (1986) reported their inability to demonstrate a threshold number of pollen grains necessary for successful pollen tube growth and ovule fertilisation. Thus, failure of fertilisation could occur in any one of the steps leading from pollination to fertilisation. Sensitive stages include pollen germination, penetration of the stigmatic surface by pollen tubes, traversing of the stylar canals and cavities and entry to the ovary/micropyle (Stoddard, 1986b).

Flower position in the raceme is also known to affect the incidence of fertilisation (Rowland *et al.*, 1983). This variation has been reported to be associated largely with the synchrony of anthesis within racemes and with environmental effects (Stoddard, 1986c). Nevertheless, earlier Rowland *et al.* (1983) related the incidence of floral fertilisation with autofertility (ability to trip itself in the absence of insect pollinators or mechanical disturbance, Drayner, 1956 and 1959). Furthermore, variation in incidence of fertilisation among *Vicia faba* lines could also be attributed to individual genetical variations (Stoddard, 1986d) which could in turn be attributed to the overall pollen quality of a genotype. Thus, success of fertilisation depends also on the source of pollen used (Lee, 1988). In other words, the present study also suggests that if, for instance, pollen from the top flower racemes or from the top reproductive node is used for pollination, there could be less success in fertilisation and seed formation than if it is used from the first two reproductive nodes or first two flowers within racemes. This finding is also in accord with the conclusion that a large proportion of *Vicia faba* cultivars produce most of their seeds on the lower reproductive nodes (Adcock & Lawes, 1976).

9.4 Storage of *Vicia faba* pollen for a long period

9.4.1 Viability duration

Pollen viability is tacitly assumed through the season of pollen production and through the life span of individual pollen grains (Yeo, 1975; Waser, 1978a). However, in most plant species pollen grains lose their viability soon after they are shed from anthers (Maheshwari & Rangabwamy, 1965). In this study it was noticed that within one month of storage, pollen viability from three lines dropped by more than 10%, while in another three lines the drop was a little above 8%. In line 248 pollen grain viability only dropped by 4%.

The ability of pollen to germinate and effect fertilisation decreases with age even when stored on the plant for few days (Smith-Huerta & Vasek, 1984). After six months of storage no pollen grains of any line lost viability by as much as

20%. The highest loss in viability in this period was from STW (18.49%) and the least was from line 248 (6.99%). However, after just another three months (nine months) the loss in pollen viability for all six lines was more than 20%.

9.5 Decline in enzymatic activity

Fluorescence quality of pollen grain is an indication of its viability status (Heslop-Harrison & Heslop-Harrison, 1970). The amount of the class I pollen (bright green) loss in the first month was less than 50% from line 288 (table 5.2). Such loss from line 248 was a little above 51%. In the remaining five lines the loss in bright green colour was much higher. By the end of the ninth month, the loss of class I pollen grains was found to be less than 90% only in lines 288 and TA95-1. Thus, as judged by fluorescence intensity, after nine months, pollen grains of most lines were mainly in class II. Such reduction in enzymatic activity (loss in bright green colour) was, partly, attributed to alternate freezing and thawing of pollen grains when subsamples had to be withdrawn for viability test. This was done each month, for the whole of nine months.

9.6 Viability response to storage temperature

The lowest mean percent viability recorded was from pollen grains stored at 4°C. Hecker *et al.* (1986) found that pollen of sugar beet stored at -18°C for one year produced very poor seed set; when stored for one year at 5°C it had essentially lost all its viability. Also fresh pollen of six *Vicia faba* lines stored at 4°C totally lost their viability after 182 days (Gupta & Murty, 1985). Thus, storage temperatures such as 4°C or 5°C are not suitable for pollen storage over a long period. Similar reports in other plant species have also been documented. Hop pollen stored at 3°C for one year was totally non-functional (Haunold & Stanwood, 1985).

Potato pollen stored in LN2 did not show significant decrease in percentage of germination after nine months, whereas samples stored at -20°C for a similar period showed a decrease in germination to about 1% viability (Weatherhead *et*

al., 1978). In the present study, pollen grains from lines 288, NDP, 247, 248 and STW stored in LN2, regardless of pre-storage desiccation treatments, performed best. However, in lines such as TA95-1 and 4/7, the high initial moisture content seemed to be most damaging. Thus, in these lines survival in LN2 storage depended on the moisture content. This observation confirms the report by **Luza & Polito** (1988) that pollen grains of *Juglans regia*, with the moisture content (MC) greater than 7.5%, were killed by freezing in LN2.

9.7 Importance of pre-storage desiccation

For some lines (table 5.2) regardless of which storage temperature was used, pre-storage desiccation proved to be essential for pollen grains to maintain viability above 80%, after nine months storage. In *Juglans regia* fresh stored pollen, out of eight lines only five survived storage in LN2 for one year (**Luza & Polito**, 1988). Thus moisture content of individual pollen samples from a genotype can be important as this is a characteristic of a genotype (**Bajaj**, 1987).

In the 24h RT pre-storage desiccation, only line STW showed better viability after nine months storage in LN2 or at -80°C. However, after storage at -20°C or at 4°C, STW viability declined by 10.33% in this treatment. Thus, its best storage temperature, in this case, should have been either at -80°C or in LN2 (table 5.6).

Reduction in viability as result of the imposition of an inappropriate storage temperature also occurred when pre-storage desiccation at 25°C/6h was carried out. In this case storage should have been only in LN2 and not at -80°C (table 5.8).

STW also reduced in viability when desiccated at 20°C/6h and stored at -80°C, -20°C and 4°C. Its best storage temperature in this case was LN2 (table 5.7). Furthermore, examination of tables 2.5, 2.9 & 2.10 clearly shows that the other pre-storage treatments imposed on STW were detrimental. Thus, since damage had already been incurred as a result of inappropriate pre-storage treatments, in the four low temperature storage regimes no advantage or disadvantage could be concluded statistically.

Fresh storage in LN2 or at 4°C proved to be damaging to pollen from line TA95-1. Pollen from freshly dehiscent anthers and freeze-dried for 45 min from line TA95-1 responded similarly (tables 2.5 and 2.10).

The next most sensitive lines to pre-storage treatments, in relation to storage temperatures, were pollen from lines 4/7 and NDP. Line 4/7 reduced in viability by 5.04% due to pre-storage desiccation at 30°C for 6h and by storing it at either 4°C or in LN2. Its best storage temperature, in this connection, proved to be at -80°C.

Line NDP stored fresh reduced its viability by 5.92% when stored at 4°C (tables 2.5 & 2.9). In the remaining three genotypes, 288, 248 & 247, their respective reduction in viability due to pre-storage treatments was found to be negligible ($\leq 5\%$) and even though there were differences in viability as results of one or all five pre-storage treatments imposed, the differences were not high (tables 2.5-2.10).

Generally, except STW, all the rest of the lines gave highest viability after being desiccated for 6h at 25°C. In sunflower (*Helianthus annuus* L.) 4h drying proved to be adequate for LN2 storage (Roath *et al.*, 1988). On the other hand desiccation at 20°C for 6h proved inadequate, while 30°C for a similar a period of desiccation for all the lines was damaging.

Thus even though a high level of initial moisture content in pollen grain has a decisive influence on its storability, lowering the moisture content excessively can also reduce pollen viability. However, according to Barnabas & Rajki (1976) loss of viability is not directly correlated with water loss from the pollen grains but primarily with the exhaustion of their reserve nutrients. This partly explains the very high variability ($P \geq 0.01$) among the genotypes desiccated for 6h at 30°C (table 5.7).

9.8 Importance of pollen storage

The single most important reason for interest in the preservation of pollen is to use it for breeding purposes as and when desired. Another purpose concerns the

conservation of genetic resources. It has been reported that there would no changes to genetic constitution nor would there be any allelic frequency alteration as result of pollen preservation under low temperature regimes (Roath *et al.*, 1988).

It is thus apparent that successful pollen storage requires two primary procedures to be carefully monitored. These are initial moisture content (MC) and desiccating temperature.

An attempt has already been made, in the above paragraphs, to elucidate the primary objective of reducing the initial MC of pollen grains for preservation. The moisture content of pollen at the time of storage could be the most crucial factor determining the successful storage life of pollen grains of any plant species (Bajaj, 1987; Weatherhead *et al.*, 1978; Hecker *et al.*, 1986; Barnabas & Rajki, 1976; Hanna *et al.*, 1983; Roath *et al.*, 1988; Luza & Polito, 1987, 1988; and Hoekstra *et al.*, 1989). The lower the water content, the longer pollen can survive under low temperature storage conditions as freezing injury is minimised. During the freezing process and storage of fresh pollen, damage could be due to intracellular ice formation directly on the membrane system rather than to indirect effects associated with the loss of liquid water. On the other hand, pre-storage desiccation of cells results in the concentration of solutes which are precipitated and the plasmolysis of cells resulting in reduced ice crystal formation. Hence damage to the cell membrane during the long-term storage of pollen at -80 °C or in liquid nitrogen is minimised (Mazur, 1969).

However, lowering the MC of pollen requires some form of careful desiccation which is in essence heating up of pollen grains. Under natural conditions pollen grains are usually desiccated just prior to dispersal (Heslop-Harrison, 1979) which seems to be an important and necessary step in the maturation of pollen. However, in artificial heating, there is tremendous variation among pollen of various plant species in tolerance of reduced moisture. *Zea mays* is reported to be tolerant to heat shock if its MC is above 7.8% and that of *Pennisetum typhoides* if above 3% (Hoekstra *et al.*, 1989), and *Juglans* spp., if above 4% (Luza & Polito, 1987). There is also a tendency to attribute the tolerance to heat shock to sucrose content

(**Hoekstra et al.**, 1989) and heat shock protein (HSP) synthesis by binucleate pollen (**Munro & Pelham**, 1985; **Cooper et al.**, 1984; **Herpen et al.**, 1989).

Vicia faba seed is a very hardy crop species and seeds maintain viability for as long as five years or more, even under a very crude storage facility in the Ethiopian rural farm community. In the concept of genetic over-lap, selection made for a desirable character in one phase of the life cycle would have positive consequence in the alternate genetic sporophyte or vice versa. Monitoring *Vicia faba* pollen for various storage facilities reveals that even in freshly stored pollen grains viability after nine months is not less than 70%, while those desiccated at 25°C for 6h and stored in LN2 or -80°C maintained their viability above 80%. These figures categorically suggest that the water content of pollen has a decisive influence on its storability at low temperatures such as LN2 or -80°C.

Pollen grains whose fresh moisture content was reduced prior to storage have been reported to remain viable for a year or more and to effect fertilisation as well as fresh pollen. A host of examples include maize pollen (**Barnabas & Rajki**, 1976), pearl millet (**Hanna et al.**, 1983), hop (**Haunold & Stanwood**, 1985), sugar beet (**Hecker et al.**, 1986), English walnut (**Luza & Polito**, 1988) and sunflower (**Roath et al.**, 1988).

Taking into consideration all the above information, *Vicia faba* pollen desiccation and preservation for a longer period than a year should be a practical possibility. Thus, if the concept of genetic overlap (**Mulcahy**, 1974) is to be taken seriously, selection of pollen genotypes must have positive and advantageous consequences for the composition of sporophytic progeny in a *Vicia faba* improvement programme.

In conclusion, it could be pointed out that *Vicia faba* from freshly dehiscent anthers desiccated at 25°C/6h and freeze-dried for 45 min, stored in either LN2 or at -80°C would give more than 80% viability after 9 months of storage.

9.9 Gametophyte interactions

The electrophoretic study of multiple molecular forms of enzymes (isoenzymes), has provided a suitable technique for studying genetic variation in plants. Gates (1978) has listed a wide range of plant species whose cultivars could be identified by the observation of different isoenzyme systems. In this technique two isoenzyme systems, namely aspartate aminotransferase (AAT) and amylase were employed in distinguishing hybrid seeds from selfed progeny in a mixed pollination experiment. The AAT enzymatic activity in line 248 gave the greatest band mobility for all the 10 lines under study (figs. 6.3 and 6.9).

A scanning Densitometer was used to scan the gel lanes to verify the visual identification and to determine the mobility of individual bands. As an illustration, scanned traces for AAT (figs. 6.3 and 6.8) are included.

The amylase isoenzymes resolved into the largest number of bands in lines STW, 288 and TA95-2. Therefore, the application of amylase enzyme analysis to STW or 288 or TA95-1 inbred lines proved to be useful in identifying the hybrids and inbreds from any pair of the crosses, wherever these lines were involved in mixed pollinations. Figs. 6.10 and 6.11 demonstrate the results from some inbred and hybrid seeds with the two inbred parental lines on the extreme left and right tracks of the gels. Figs. 6.6-6.8 illustrate in graphic forms the same phenomenon.

Where STW pollen grains were mixed with maternal lines, 11 or NDP, there were more selfed than hybrids at the first and second ovule positions. Overall, however, there were more hybrids than selfed seeds produced in this cross (table 6.1). On the other hand, where pollen grains of STW were mixed with either 248 or 247, more hybrid seeds were counted at these two positions. Where STW pollen was mixed with that of 4/7, with 4/7 as the maternal parent, more hybrid seeds were counted from ovule position two but there was more selfing at positions one and three in the same mixed pollination.

Interestingly, in lines 248 and 247, where line 288 pollen was mixed as the paternal line with each of the former lines, more hybrid seeds occurred in ovule

positions one and two from each of the maternal parents. With NDP however, hybrids predominated at position one only, while at position four hybrids and selfed seeds were present in equal numbers.

With line 4/7, using 288 as paternal pollen parent in the mixed pollination, there was twice as much crossing at ovule position one, while 100% selfing occurred at positions two to four, and no fertilisation took place at ovule position five.

In two Ethiopian lines 20D-2 and 43/78, most selfed seeds were observed at position one in crosses with pollen from paternal pollen line 95-1. In maternal line 20D-2, however, three times as many hybrid than selfed seeds were counted at position two and the largest proportion of hybrids were observed at position four. No fertilisation occurred at position five. Overall, in maternal line 43/78 there were more selfed seeds than hybrids.

9.10 Pattern of male gametophyte competition

Pollen from 248 was mixed with each of STW, 288, NDP, 247 and 4/7. Line 248 was the paternal pollen parent while the rest were all recipients of the mixed pollination. The result showed that the pollen tubes from 248 were able to produce more hybrids with crosses in mixtures involving 288 and STW. Thus, line 248 was significantly more competitive on the stigmas of STW and 288 only. However, on the stigmas of 4/7, NDP and 247, pollen grains of 248 were significantly out-competed ($p \geq 0.01$) by pollen of each of the maternal genotypes.

Pollen tubes from STW also fathered larger numbers of ovules when mixed with pollen of maternal line 11 (fig. 6.2).

Pollen grains of 95-1 were also significantly ($p \geq 0.05$) less competitive than self pollen on the stigma of line 43/78, but were nearly equal in fathering ability when mixed with the maternal pollen grains of 20D-2.

Pollen grains of 288, as a male donor, did not show any competitive advantage over self pollen on the stigmatic surfaces of 248 and 247. However, 288 was as

competitive as pollen grains from NDP and 4/7, on each stigma of these respective maternal lines.

This pattern was similar with the mixed cross of STW and 4/7, where STW as paternal line fertilized 44.94% of the ovules in the line 4/7. With nearly significant values, pollen from NDP out-competed pollen of STW producing 70% selfed seeds. Thus, the competition pattern in producing hybrid seeds in mixed pollination was highly variable from line to line (table 6.2 and fig. 6.2)

Detailed observation of table 6.2 and fig. 6.2 show that overall mean number of hybrid versus self seeds was nearly equal. However, there were more hybrid than selfed seeds at ovule positions one and two. The results at these two positions accord with the hypothesis that foreign pollen grains grow faster than the maternal grains to effect fertilisation of ovules nearest to the style (Rowlands, 1958, 1960; Huxley, 1940; Ottaviano *et al.*, 1983). Ovules at the fifth position were only fertilized by maternal pollen tube from lines 248, 11 and 43/78, wherever fertilisation was effected. In the case of STW, however, its pollen tubes fathered the most distant ovules in maternal parent 248 and 11, while the fifth ovules in line 43/78 was totally selfed when pollinated with a mixture containing paternal 95-1 pollen grains. Wherever fertilisation of the ovules in positions four and five occurred, there were more self seeds than hybrids (table 6.2 and figs. 6.2 and 6.8).

9.10.1 Position effect

Basically, *Vicia faba* is an entomophilous species. The activities of pollinating bees entail either bringing in pollen from different plants and effecting cross pollination or tripping flowers to ensure positioning of self-pollen grains on the stigmatic surface of a pistil (Rowlands, 1958; Holden & Bond, 1960; Lawes *et al.*, 1983). Therefore, the species is adapted to receive cross or self pollen grains. The amount of foreign pollen transferred is entirely dependent on the activity of the pollinating insects which, in turn, depends on weather conditions. The extent and pattern of fertilisation seems to be very important in seed set and the type of seed distributions within a pod in this mixed pollination experiment.

Post-pollination factors, particularly male gametophyte competition and female choice, may have an influence in the seed types produced (Hossaert & Valero, 1988). Frequency of fertilisation also reflects final seed set (Sitch & Snape, 1986a, 1986b).

The data of table 6.2 indicate that lines such as STW, NDP, 247 and 20D-2 potentially have four ovules that could develop into seeds. However, no ovule developed into a seed in STW and NDP at position four when pollen grains from line 248 were involved in a mixed pollination. Nor was there selfed seed produced from these maternal lines, possibly due to the operation of some form of self-incompatibility system. Lines NDP and 11, each pollinated with a mixture containing STW pollen, gave a one to one (1:1) ratio at the fourth ovule position (table 6.2).

Further, when maternal parents 288 and 4/7 were subjected to mixed pollination involving pollen grains from 248 as paternal pollen parent, no seeds were produced at position five in both maternal parents. However, ovules of lines 248 and 11 were only able to produce hybrid seeds at this position, which were always fathered by paternal pollen parent STW. In this case, pollen tubes produced by STW out-competed other tubes. They were able to reach the fifth ovule position in each of the maternal lines 248 and 11. This observation tends to confirm that of Huxley (1940), that competition among pollen grains results in selection of fast-growing tubes. This phenomenon was also demonstrated by Mulcahy *et al.* (1983) in natural populations of *Geranium* (*Geraniaceae*).

Pollen tubes of 248 were able to out-compete the maternal pollen tubes of STW at position one and three; but were out-competed by those of 247 4/7 and NDP. It was totally out-competed by the pollen tubes of NDP at ovule positions one and two. Therefore if inbreds of STW, 248 and 288 were to be grown together, in a mechanical mixture for random breeding, the resulting hybrids would mostly be those fathered by 248. If on the other hand, inbreds of 4/7, NDP and 248 are mechanically mixed and grown together for random crossing, there would be fewer hybrid seeds fathered by 248. Thus, 248 could only effectively form hybrids if allowed to grow with STW or 288.

Mixing of lines STW, 4/7 and NDP, would likely result in low hybrids fathered by STW. On the other hand in a mixture of 288, 247, NDP and 4/7, line 288 would successfully form hybrids with 247 and NDP, but probably only 1/4 of the case with 4/7. Line STW mixed as paternal pollen parent with line 11 would also produce more hybrid seeds from 11 (fig 6.10).

In similar manner, if TA95-1 were to be mixed with 20D-2 and 43/78, five times as many cross as selfed seeds would be likely to result (table 6.2).

Mixed pollination is an important tool for detecting genotypic pollen vigour in relation its counterpart during an evaluation of random crossing under field conditions or controlled environments where beehive cages could be introduced.

Tripping of *Vicia faba* flowers by insects helps to position self-pollen on the stigmatic surface, and also introduces foreign pollen (Lawes *et al.*, 1983; Holden & Bond, 1960; Rowlands, 1958, 1960 and 1961; Drayner, 1956). Thus, although self-pollen is shed before the flowers open and before the foreign pollen arrives, the former is effectively neutralised by the necessity of tripping before the self-pollen is able to germinate (Rowlands, 1958). This suggests that the crop evolved from a wild, out-breeding species. Due to fast growth of foreign pollen, cross fertilisation would almost always result (Rowlands, 1960, 1961). In the experiment where 248 pollen was mixed with either STW or 288, and pollination carried out on stigmas of the latter lines, evidence for the above conclusions was obtained (table 6.2 and fig. 6.2). However, Guth & Weller (1986) reported that the failure of foreign pollen to effect fertilisation in *Oxalis magnifica* at position one was due to a saturating of the stigma chamber by the self pollen load. Similarly, fewer ovules were penetrated by pollen tubes following self- than cross-pollination in *Eucalyptus woodwardii* (Sedgley & Smith, 1989).

According to the above hypothesis ovules towards the stigma-style should give rise to mainly hybrid seeds. However, from table 6.2, it can be seen that this did not hold true for position one in nine pairs of crosses out of 17, or for position two in six pairs of crosses. This suggests that the highly inbred lines such as 247, NDP, 4/7 have developed differential cross and self fertility (table 6.2). As observed in

the glass-house at the Botanic Garden of the Durham University, from 1986 to 1989, in the absence of any tripping, these latter inbred lines must have developed spontaneous self-fertility (autofertility) (Drayner, 1959), i.e. an ability to self-fertilise readily without a need of tripping such that line 247 would self even if there were pollen grains from 248 in equal proportion on the 247 stigma.

Pollen tubes from 248 as paternal parent out-competed those of STW on the stigma of the latter at ovule positions one and two by factors of three and four respectively. However, this competitiveness was not evident in mixed pollination with pollen of 288, 4/7 or NDP on the respective stigmas of each of these maternal lines. Thus, a donor that shows more success in fathering fruits on one recipient may be less successful on others (Bertin, 1986).

Pollen tubes of 248 were also totally out-competed by the pollen tubes from STW on the stigma of the former, with respect to fertilisation of the ovule at position one. Thus, pollen tubes from STW grew faster on the stigma of 248 such that eight times as many hybrids as selfed seeds were produced. It also totally out-competed pollen tubes of 247 on the latter stigmatic surface for the same ovule position. Furthermore, pollen tubes from 288 out-competed 4/7 and 247 by factors of 2 and 3 on their respective stigmatic surfaces. It also out-competed those of 248 on the stigmatic surface of the latter with the production of 4 and 2 times hybrid seeds in fertilising ovules at position one and two respectively.

An important implication of this is that the resulting seed population can easily be maintained and increased by random mating in an open field, if these inbred lines are used for the generation of a synthetic variety (Lawes *et al.*, 1983; Bond, 1982).

Fathering of the ovules at position one by the paternal pollen tubes came about because these pollen tubes grew faster and effected fertilisation. Similar observations have also been reported by Harding & Tucher (1969); Ottaviano, Sari-Gorla & Arenarai (1983); Lee & Bazzaz (1986); Stoddard (1986b) and Hossaert & Valero (1988). Guth & Weller (1986) reported that the failure of foreign pollen to effect fertilisation in *Oxalis magnifica* at position one was

due to a saturating pollen load on the stigmatic surface. Similarly, fewer ovules were penetrated by pollen tubes following self- than cross-pollination in *Eucalyptus woodwardii* (Sedgley & Smith, 1989).

The results of this experiment demonstrate that the rate of pollen tube growth and fertilisation seem to be more important than distance from maternal resources for seed development in ovules farthest from the peduncular end of the ovary. On the other hand, according to Bawa & Webb (1984), a higher abortion rate among ovules closest to the pedicel may be the result of delayed fertilisation or fertilisation by slower growing, and possibly genetically inferior pollen tubes (table 6.2). Thus, in *Lupinus texensis* even if fertilisation successfully stimulated normal seed development, developed seeds were lighter in weight, despite their advantageous position proximate to nutrient supply, as a result of fertilisation being performed by a slow growing pollen tube (Schaal, 1980). Thus, in Bertin's (1988) view, seeds from unfavoured donors may have more homozygous recessive lethal or deleterious alleles resulting in more zygote (embryo) abortion. In general, this phenomenon seems to be due to genetic inferiority of pollen tubes that fertilised a given ovule.

Sedgely & Smith (1989), on the other hand, argue that in species such as *Eucalyptus woodwardii* where pollen tubes penetrate the stigma and growth proceeds normally, ovarian inhibition must be the major factor for failure of fertilisation. Similar cases were also reported by Seavey & Bawa (1986) and Brewbaker & Gorrez (1967) in *Medicago sativa* and Sayer & Murphy (1966) in alfalfa. Such failure is claimed to be due to the influence of the pistil on pollen tube kinetics. Thus, discontinuous secretion of starch or other pollen tube growth substances at the obturator are a mechanism to control the entrance of pollen tubes into the ovary thence into an ovule (Herrero & Arbeloa, 1989; Herrero & Dickinson, 1980; and Arbeloa & Herrero, 1987).

Most *Papilionaceae*, with some exceptions, are noted for gametophytic self-incompatibility. *Vicia faba* appears to occupy an intermediate position between the two extremes of self fertility and self sterility (Rowlands, 1960). Like other crop species (notably cereals) (Stebbins, 1950), faba bean may have evolved repeatedly from its ancestral cross-fertilising progenitors in response to selection

pressure in favour of immediate fitness (**Rowlands**, 1960). It seems possible that, in accordance with **Rowland's** (1958) observation, where 5% of the population was completely sterile on selfing due to zygotic infertility, the species may have accumulated recessive homozygous deleterious genes. Such zygotic infertility resulting from deleterious recessive characters is usually regarded as the resistance of the system to change. Thus, the zygotic infertility due to deleterious recessive homozygous genes seems a possible factor in lines 247, NDP, 4/7, enabling them to combine only with specific genotypes, where such deleterious genes may not exist in a heterozygous condition in a given line.

The other very important observation from these mixed pollinations is that there was no by-passing of the ovules, as is common in pollination with single genotypes. Thus, table 6.1 reveals that in each pair of mixed pollinations, ovules were always either fathered by maternal pollen tubes or by paternal ones at ovule positions one, two and three, depending on which was the dominant pollen genotype.

Vicia Faba yield is known to vary not only from year to year and location to location, but also at the same farms under similar management practices. Therefore a synthetic line based on high-yielding autofertile lines would most effectively meet the demand for high and reliable seed yield (**Poulsen**, 1975).

The present experiment reveals valuable information for generating synthetic varieties. In this context a synthetic variety has been defined as a variety being synthesised from all possible combinations (intercrosses) among a number of selected genotypes which have been tested for their combining ability. Thus, it is any population which has been constituted from a limited number of distinct and well-evaluated components (**Bond**, 1982).

The advantage of a synthetic variety is that it is expected to be higher yielding than the mean of its components even when the components are themselves synthetics, because the latter possess a buffering effect of one component against another (**Allard**, 1960; **Bond**, 1982; **Lawes et al.**, 1983). Therefore in *Vicia faba*, synthetics may be more autofertile than their inbred components and possibly

more so than their population components, provided crossing between populations brings some extra heterozygosity (**Bond**, 1982; **Lawes et al.**, 1983). As the present study shows, lines 247, 288 and STW would form a good basis for synthetic components, provided that their flowering can be synchronised.

Selfing in faba bean leads to inbreeding depression (**Bond**, 1975; **Picard et al.**, 1982) which can result in 5-13% yield loss (**Poulsen**, 1975). This can only be overcome by increasing heterozygosity, e.g. planting hybrid vigour for higher yields (**Lawes et al.**, 1983).

The other desirable feature of faba bean hybrids is a higher autofertility level than inbreds. Thus, under conditions where pollination is a limiting factor in yield stability, faba bean hybrids would tend to give better results than synthetic or normal populations (**Picards et al.**, 1982; **Lawes et al.**, 1983; **Bond**, 1982).

Currently, faba bean hybrids have a number of drawbacks, especially the lack of sterility stable male sterile lines. Of the two reported forms of male sterility, genetic male sterility and cytoplasmic male sterility (CMS) in *Vicia faba*, the other was reported by **Bond** (1960) as a promising method for hybrid seed production. However, it was found to be highly unstable due to various factors including a reversion to fertility under the influence of elevated temperature (17 to 22°C restores fertility). The problem is confounded by differential characters such as inter- and intra-plant variations, and inter- and intra-line variations in the level of fertility restorations (**Picard et al.**, 1982; **Lawes et al.**, 1983). Factors limiting progress in developing F1 hybrid faba bean include:

- 1) the cost of inbred seed production
- 2) the problem of finding non-restoring fertility male parent which does have good combining ability (c.a.)
- 3) line that gives more stable CMS
- 4) finding interspecific compatibility
- 5) lack of genetic engineering towards achieving CMS parent

In view of these difficulties, the present data on pollen competition suggest the possibility that a female CMS parent with weakly competitive pollen could be selected for commercial hybrid seed production. Thus, from table 6.2 and fig.6.10, a combination of 248 and STW as potential pollen donors should result in hybrid seeds. This however, would produce two types of hybrid mixtures, depending on the source of pollen and stigmatic surface involved. In such a scheme, a mixture of 248 and 288 is another possible choice, or line 11 as female parent with line STW could be a third alternative.

9.11 Factors causing flower drop and pod development

9.11.1 Effect of raceme position

Like many food legume crops, most faba bean cultivars are indeterminate in growth characteristics. As flowers are initiated the stem apex of *Vicia faba* continues to develop vegetatively. Thus a large number of floral nodes can continue to develop until the vegetative growth ceases only with the eventual senescence of the whole plant (Peat, 1982).

Generally, matured pods are usually concentrated at the lower flower raceme (fig. 7.4) while abscission is highest at racemes 3-5. At the sixth flower raceme there seems to be least abscission of flowers as there are fewer flower numbers initiated at this position. The magnitude of abscission usually varies considerably among cultivars (fig. 7.2). In most cases, a greater proportion of early-formed pods or fruits often may proceed to maturity than those late-formed ones (Aker & Udovic, 1981; Lee & Bazzaz, 1982; Doust, 1982; Doust & Eaton, 1982; Webb & Bawa, 1985).

Various investigators have reported different values for percentage flower shed. Gates *et al.* (1983) reported 36-87% shedding from 10 faba bean cultivars. The overall trend regarding the effect of position on flower abscission or on successful pod maturation seems to be that later formed flowers are inhibited by early formed ones as the differential in their stages of development increases.

Four major hypotheses have been advanced to explain this phenomenon:

1) *The source-sink model*: The central point in this model is the effect of sink strength which is defined as the ability of a fruit or seed to locally remove water and solutes from the phloem (Thorne, 1985). This hypothesis proposes the chemical inhibition of aborting fruits and seed by those maturing (Bertin, 1985). Thus, early-formed, proximal fruits may produce a growth inhibitor that induces abortion in late-formed, distal fruits (Cornforth *et al.*, 1966; Huff & Dybing, 1980; Tamas *et al.*, 1979; Van Steveninck, 1957).

2) *Position of flower or fruit on the mother plant*: The temporal advantage of the early fruits or pods may allow them to generate more growth-stimulating phytohormones and thus become more vigorous sinks, pre-empting resources that would otherwise be used by late-formed fruits or pods (Chapman & Sadjadi, 1981; Nitsch, 1950; Peat, 1982). The proximal fruits are in a position to pre-empt resources since photosynthates and nutrients must pass them to reach distal fruits (Stephenson, 1981; Wyatt, 1980).

3) *Morphological constraints*: The distal fruits may also abort due to a poorly developed vascular system in the dorsal portion of the inflorescence (Van Steveninck, 1957). Thus the degree of development of vascular tissue supporting the fruit in relation to other reproductive sink and to the source of photosynthates and nutrients may determine the competitive ability of fruits or flowers (Gates *et al.*, 1981; Gates *et al.*, 1982; White *et al.*, 1984). Such constraints appear to be absent in species such as *Catalpa speciosa* (Stephenson, 1980) and in some varieties of *Vicia faba* (Gates *et al.*, 1981, 1982).

4) *Time of fruit or flower initiation*: This hypothesis is interrelated with that of position of reproductive organ initiation (No. 1) except that it is explained from temporal point of view. Thus early-formed, proximal fruit may produce a growth inhibitor that induces abortion in late-formed, distal flowers or fruits (Gates *et al.*, 1981, 1982; Huff & Dybing, 1980; Sage, 1986; Tamas *et al.*, 1979).

9.12 Seasonal variation

9.12.1 Temperature effect

Environmental factors such as seasonal variation in temperature regimes have profound influences on flower initiation and abscission (fig. 7.1). Figure 7.1 shows that the largest mean percent of flower shedding occurred during the cropping season of mid-June to November (season 5). This period even though it is a normal growing season for *Vicia faba* under Ethiopian conditions, included a period when skies are mostly clouded over, from July to the end of August. Thus, the crop was shaded in the middle of the flowering period, which is a reported cause of major flower shedding (Gates *et al.*, 1982). This period also experiences a very high relative humidity (table 2.2) whose adverse effect on pollen tube growth *in vitro* has been documented (chapter 3.1). In contrast, season 4 (late March to late June) is known for its extreme high temperature (table 2.2) in which whole plant growth was stunted and few reproductive organs were initiated. Thus heat stress led to tremendous losses of flowers and young pods at their early initiating stages. This confirms one of the early reports of Evans (1959) who observed that flower initiation could be inhibited if *Vicia faba* was stressed at $\geq 23^{\circ}\text{C}$ continuously.

The adverse effect of high temperature on cool season food legumes has also been reported by Saxena *et al.* (1988). On the other hand, season 6, where flower initiation occurred in late September, experienced extremely low temperatures (table 2.2) as a result of which the lowest levels of harvestable pods were recorded despite relatively good flower initiation. The highest mean pod percent harvested occurred in the growing season when flowers were initiated in January. During this period, though night temperatures were relatively low, the day temperature regime was within the faba bean optimum range of temperature during the reproductive stage. Relative humidity was also low. Thus, in Ethiopia, faba bean planting in November would give best yield provided water deficit could be overcome.

9.13 Location effect

Figs. 7.5-7.25 illustrate general trends for location (site), period (cycle) and

percent counts of flowers and pods.

Vicia faba may not have any absolute environmental requirement for flower initiation but both low and high temperature regimes would adversely affect the final yield. Under the Ethiopian growing environment, temperature regime is a function of elevation. Nazereth Research Centre is at an altitude of 1650m. above sea level and considered very poorly adapted to faba bean growing (fig. 7.3, location 3). On the other hand, Debre Zeit, location 2, is at an altitude of 1850m above sea level, which is referred to as the Woina Dega (mid-altitude) zone. Both in terms of biomass and grain yield it is the best of the three sites. Thus mean percent pod counts were higher at Debre Zeit than at the remaining two sites.

9.14 Environmental effects on pollen tube performance

An examination of fig. 8.8 reveals a large amount of variation of unfertilized and fertilized ovules in relation to their positions. Thus among lines STW, 247 and 248, there were no significant differences with respect to positions of fertilized ones or unfertilized (figs. 8.9, 8.13 and 8.16). Thus, ovules nearest to the stylar column, and furthest from it had nearly equal frequencies of fertilisation or no fertilisation in these lines. However, among the lines NDP, 11, TA95-1, 20D-2 and coll.43/78, there were large differences between ovules nearest to the style and at the bottom of the ovary in the degree of fertilisation (figs 8.12, 8.17, 8.23 and 8.24). In a third category consisting of lines 4/7 and 18, ovules farthest from the style had a better chance of being fertilized. In these two lines, according to the present study, ovules at the bottom of the ovary were significantly more frequently fertilised than those nearest to the stylar zone (figs. 8.13 and 8.20). However, at ovule position number five, in line 18, the frequency of being or not being fertilized was nearly the same. From positions one to four, however, the gradient of being fertilised declined in the direction of ovule number four (fig. 8.20).

9.14.1 Line STW

This line has been described as possessing independent vascular bundles for

the reproductive organs (Gates *et al.*, 1981). It has minimum levels of flower shedding (see chapter 7).

Under Ethiopian conditions, excessive flower losses were observed during the mid-March to late May 1988 growing period. A fluorescence microscopic study of the fate of pollen grains and pollen tubes showed that in this period no single fertilized ovule was observed at NRC. Between two and ten pollen grains were counted on the stigma, and one to three pollen tubes in the upper style. Pollen tubes in the ovary ranged from 1-2. These observations confirm views of Van Herpen (1981) that high temperature has adverse effect on pollen tube length. Dry and hot conditions are also known to affect pollen germination, due to evaporation of nectar and becoming a highly concentrated sugar solution ($\geq 40\%$) instead of 5-30% concentration (Kevan *et al.*, 1989).

At HRC a similar situation was found, while at DZRC there was moderate fertilisation, about 20% of the 21 pistils studied. Therefore flower shedding was mainly due to lack of fertilisation in this period. Pistil examination of plants grown from late July to late December showed that pollen grains were numerous in stigmas from NRC & HRC, but less so in stigmas from DZRC. Pollen tubes on the stigma from HRC were numerous (figs. 8.6 and 8.10), while at DZRC & NRC these ranged from 1-17 and 1-5 respectively per pistil. In all three centres pollen tubes in the ovary were very sparse. Fertilisation condition during this period was scored as "being fertilised" dominantly, or "unfertilised". Thus, flower drop from this genotype was mainly due to arrested pollen tube growth, caused by alternate high day temperature and low night temperature conditions (table 2.2).

For the mid-June to mid-November cropping season plants were at anthesis, from late August to mid-September. Pollen tubes were found in twelve of the twenty two ovaries examined. This period in Holetta is characterised by heavy rain fall, constant cloud cover and high relative humidity. These conditions are known to affect normal pollen tube growth, as a result of over-shading (Gates *et al.*, 1983). At DZRC and NRC slow pollen tube growth was the dominant factor and therefore ovules were recorded as "being fertilised" in which case abscission might have been mediated already (fig. 8.15).

9.14.2 Lines NDP, 247, 248

The response of pollen tubes of NDP during mid-March to late May flowering period was that ovules were mostly "dead" or "by-passed" at HRC, while "by-passing" or "about to be reached" were the feature at DZRC in this period. The response of NDP during mid-March to late May growth was that ovules were "dead" or "by passed" mostly at HRC, while "by-passing" or "about to be reached" were the common features at DZRC.

Overall the reproductive response of 247 was similar to those of NDP. At NRC also the three lines were recorded either "by-passed" or "unfertilized". In general, at DZRC and NRC during this period germination was very poor and those pollen grains which germinated commonly had swelling tubes on the stigmas, in the style and in the ovary (figs. 8.5 to 8.6 and 8.10 to 8.11). In some cases pollen tubes on the stigma were observed trying to germinate and those which grew down to the ovaries were seen trying to enter ovules which were just a mass of black material with no signs of life (fig. 8.15).

During the relatively low temperature period, from late August to mid-September, the commonest feature of the pollen tubes was very slow growth (figs. 8.3 to 8.4) and as a result ovules were mostly dead by the time the pollen tubes reached them. Therefore "not reached" or "just penetrating" were the common observations recorded (figs. 8.15 and 8.21) or "wandering around" (fig. 22). Therefore, in some cases even if penetration was achieved it seemed that late action and abscission might have been mediated already. Ovule abortion can also occur due to genetic incompatibility between the male and female genome which is a characteristic of some plants (e.g. in the *Rosaceae*) (Weins *et al.*, 1987) or could likely be due to maternal resource limitation (Gorchov, 1988).

Pollen tubes from plantings in mid-June where plants flowered in early August, seemed to have achieved normal growth to the stage of penetration of the ovules. Thus, more records of fertilized ovules were common at all three locations. Therefore, it appeared that the main causes of flowers shedding in that period

could have been male and female incompatibility (Marshall, 1988) or maternal resource limitations (Gorchov, 1988).

9.14.3 Lines 11, 20D-2, 74TA95-1

The pollen tube behaviour during the hot period was such that at NRC and DZRC there was a total lack of the grains on stigma. At HRC there was some degree of fertilisation ($\leq 20\%$ marked as "being fertilized" fig. 8. 21), but much pollen tube bursting prior to entering the ovules was a common feature (fig. 8.11). Therefore, flower drop during this period was mainly due to failure of fertilisation probably as the result of the extremely high temperatures prevailing during the period. On the other hand, because of late or slow germination (figs. 8.3 and 8.4) and sometimes no germination, the majority of the ovules were dead (fig. 8.18) and "by-passing" of the ovules by the pollen tubes was the main feature observed (fig. 8.19) Similar observations were also recorded during the normal flowering period (early August) at HRC. However, both at DZRC and NRC in about 50% of the cases, fertilisation was observed but marked as penetration of ovules and consequently rejection by the ovules could have been the cause of abortion. Pollen tube growth trends in these lines were marked as "not reached" for the fourth and fifth ovules. Figs. 8.17 and 8.23-8.25 demonstrate this phenomenon, in which failure of fertilisation was the highest in the fourth and fifth ovule positions.

9.14.4 Lines 18 and 4/7

Response of pollen tube growth to higher temperature in the Ethiopian fields was characterized as "late", "by-passed" and "ovules dead" a result that most frequently observed ovules at the bottom of the ovary were fertilised late. Generally germination was poor and pollen tube growth was restricted to the upper part of the stylar zones (fig. 8.4).

During the cold period, pollen tubes arrested on the stigma were a common feature at all the sites. Also, due to the late start of the pollen tube germination, "by pass" was common, as though the pollen tubes had failed to receive a necessary

biochemical communication or chemotropic stimulus from the ovules (**Said et al.**, 1986). In the normal growing season, in which the plants flowered from late August to early September, there were fewer unfertilized than fertilized flowers. The main cause for flower drop appears to be due to zygote abortion possibly as a result of late rejection of the male gametophyte.

The analysis of fertilised ovules plotted against the total number of ovules of each of the variants (fig. 8.7) shows that line STW had the least ovules fertilized followed by the Ethiopian selection TA95-1. Looked at from a different perspective in these two lines 85 and 81% of ovules were not fertilised due to environmental stress and consequently flowers were shed. In the multiple correlation-regression analysis of the effect of multiple variables such as the number of pollen tubes on the stigma, in the style and ovary, all were significantly correlated to the degree of fertilisation. Except for the number of pollen tubes in the ovary, all were negatively related to the incidence of fertilisation. Thus in the multiple correlation-regression analysis, the factor of determination showed that in about $\geq 67\%$ of the cases, flowers were shed due to failure of fertilisation. The relationship has been shown in fig. 8.26, and is not distributed normally and so adjustment to non-linear regression after **Gomez & Gomez** (1984) was found necessary.

In the above discussion it has been indicated that the major cause of flower drop ($\geq 67\%$) was mainly attributed to the failure of fertilisation. Thus, about 32% of flower shedding must have been due to other extrinsic and intrinsic factors. **Marshall & Ludlam** (1989) found 50% abortion among the developing seeds in *Lolium perenne*.

Nutrient inavailability (**Stanton & Perston**, 1986; **Sutherland**, 1986; **Van Steveninck**, 1957; **Chapman & Sadjad**, 1981; **Gorchov**, 1986), was probably an important factor.

In view of **Marshall & Ludlam** (1989) however, high rate of abortion can be due to genetic defects associated with outbreeding rather than with a shortage of nutrient factors.

The intrinsic factor such as pollen quality can lead to pollen tube acceptance or rejection. This starts operating at stigma or style ovary and at the zygotic stage (**Bawa & Webb**, 1984; **Wein**, 1984; **Lee**, 1984). In some cases this might mean, particularly for *Vicia faba* which is $\geq 33\%$ out-crossing (**Bond & Poulsen**, 1983), that a high degree of inbreeding (9-13 generations) might have led to less genetic diversity of pollen deposited on the stigma. This in turn might have led to lower competition among potential sires (**Hossaert & Valero**, 1988). In this connection **Charles Darwin** (1900) observed that bean plants which had been protected from insects gave one-third as many pods as were produced by unprotected plants. Therefore *Vicia faba* was a naturally a cross-pollinated species (**Rowlands**, 1958, 1960). In the evolution of *Vicia faba*, although the pollen is shed before the flowers open, this autogamy is effectively neutralised by the necessity of tripping before the pollen is able to germinate, implying that the crop is basically an out-breeding species (**Rowlands**, 1961). Also as foreign pollen grows faster than self pollen, cross fertilisation would almost always result (**Rowlands**, 1958). The genetic system is adjusted to a high hybridity and therefore requires a high rate of out-crossing for its efficient working, although 60-70% of the seeds produced by the crop are the result of self-fertilisation. This in turn is due to the limited number of pollen grains transferred by insects (**Rowlands**, 1960). It therefore follows that both self-and cross-incompatibility must operate to some degree, possibly under polygenetic control, but the participation of major genes in their control cannot be ruled out. Taken in conjunction with differential pollen tube growth (chapter 6), the self and cross-sterility which appear to exist in varying degrees, suggest that some form of incompatibility system exists in *Vicia faba* (**Rowlands**, 1958, 1960). Such a system has been demonstrated by **Whitehead & Davis** (1954) in lucerne (*Medicago sativa*) and by **Galen et al.** (1989) in *Polemonium viscosum*.

Furthermore, flower drop could occur due to zygotic abortion when rejection of the male gametophyte occurs (**Said et al.**, 1986; **Burson**, 1987; **Marshall**, 1988) as the result of a biocommunication link failure between the two gametophytes (**Knox, Gaget & Dumas**, 1987; **Said et al.** 1986). Also post-zygotic abortion is determined genetically and is a characteristic feature of the reproductive biology of out-breeding species (**Weins**, 1984; **Weins et al.**, 1987).

9.15 Conclusions

The seasonal and geographical effects on seed yield in faba bean have been poorly characterised. Despite having a larger above-ground biomass than either wheat and barley it has lower grain yield.

Between the early 1970s and late 1980 a large body of information has been accumulated on the subject of *Vicia faba* low grain yield.

This present investigation has clearly established several important facts about *Vicia faba* male gametophyte biology, particularly with respect to environmental conditions such as extreme temperatures and high relative humidity (RH).

- 1) High temperature accompanied by high RH has detrimental effects on pollen germination and on pollen tube growth *in vitro*.
- 2) There is a great deal of variation in pollen fertility along the reproductive nodes as well as along the flower peduncular racemes. Low fertility levels are characteristics of the upper reproductive nodes and upper parts of flower racemes of peduncles.
- 3) Management of pollen storage techniques under various low temperature regimes showed that if newly dehiscent pollen was desiccated at 25°C /6h and stored either at -80°C or in LN₂, viability would remain at $\geq 80\%$ for more than one year.
- 4) In the mixed pollination, it has been confirmed that pollen from certain lines do out-compete the self pollen on the maternal stigma. However, this competitive ability has been found highly variable from one maternal line to another.
- 5) The studies conducted for 13 months under the Ethiopian field conditions, showed that the major cause of flower drop is lack of fertilisation caused both by high and low temperature conditions. In this study it was found that the planting time for best pod production was between November and mid-March.

High temperatures during the early reproductive phase cause pollen tubes to burst and even if pollen tube growth proceeds it does not pass the stylar canal. Many pollen grains did not germinate, probably due to evaporation of secretion from the stigma at high temperatures and the consequent concentration of sugar ($\geq 40\%$) instead of 5%-30% under normal temperature regime (15-22°C).

About 32% of the flower drop was attributed to various factors including incompatibility as a result of genetic defects in both ovules and pollen tubes. These genetic defects are thought to be from the continuous accumulation of deleterious recessive genes in highly inbred lines (≥ 6 generations).

S U M M A R Y

- 1) *Vicia faba* belongs to the family Leguminosae, sub-family Papillionoidae. It is an annual with generally stiff straw, the stem being hollow except at nodes. The root system is composed of a strong tap-root and secondary, spreading roots. Growth habit of most cultivars is mainly indeterminate; growth and flower induction continue while the lower part of the stem bears flowers and then pods. Flowers are borne on racemes which develop acropetally, the pedicel lengths varying considerably. The dominant faba bean flower colour is white with dark spotted wing petals and a dark standard petal but there are also pure white flowers and white flowers with pink streaks on the wing. Cultivars with white flowers and yellow or red spots on the wing petals have been reported. The height of the stem varies from 45 to over 200 cm; branching at soil level is common.
- 2) Intraspecific classification, mainly based on seed size, is the system used. The groupings of faba widely accepted are:
 - (i) *faba major* (broad bean), these are the horse bean and tick bean often referred to as field beans.
 - (ii) *faba minor* (tick bean)
 - (iii) *faba paucijuga* (Indian types).

There are other alternative viewpoints given by various authorities. Thus the classification of *Vicia faba* is under considerable debate.

- 3) The centre of origin of faba bean is also uncertain. In the absence of convincing wild ancestors and strong archaeological evidence, most authorities seem to

have agreed upon the fact that faba bean must have originated in the Near East, from where it later spread:

- (i) across Anatolia then through the Danube region to Europe, as far north as Scandinavia
 - (ii) along the North African coast to the Iberian peninsula
 - (iii) from Mesopotamia to the Indian sub-continent and Afghanistan
 - (iv) along the Nile valley as far as the South Ethiopian highland plateau.
 - (v) Faba bean is thought to have been introduced to Latin America during the conquest of the continent by the Spanish and Portuguese.
- 4) The nutritional value of Faba bean is considerable; like meat, it is rich in protein, hence the term "poor mans meat", but unlike meat, it is also rich in carbohydrates. The protein is characteristically deficient in the sulphur amino acids cystine and methionine but contains high levels of lysine. Other uses of *Vicia faba* depend on the inherent ability of its root nodule bacteria to fix atmospheric nitrogen as utilizable nitrate. This characteristic of the crop makes it very valuable as a means of replenishing soil nitrate and improving soil quality, particularly in the Third World.
- 5) Despite its importance as a source of cheap protein and as a soil conditioner, *Vicia faba* has been subject to severe fluctuations in production levels.
- (i) The grain yield of faba beans is notoriously low and unstable compared to that of small cereals. However, its total biological yield and its impact on the cereals that follow it is far greater than has usually been acknowledged.
 - (ii) Faba bean competes poorly with weeds but the crop will not be lost totally even if not weeded. This, however, severely restricts production of the crop. The use of effectively-translocated, non-selective herbicides such as glyphosate and the recent discovery of other herbicides which selectively control many grass weeds (e.g. *Agropyron repens*) in the faba bean crop has been reported as reducing the severity of this problem.

- (iii) When total crop failure due to weed infestation occurs it is due to a parasitic weed called broomrape (*Orobanche* spp). These thrive in cool, humid conditions such as the Mediterranean regions, including North Africa, the Near and Middle East.
 - (iv) Faba bean is prone to damage by several insect pests, the most serious of which are the *Aphis* species. *Aphis fabae*, the black bean aphid causes the most damage, followed by *Aphis pisum*. Both damage crops by direct feeding on faba bean and by serving as vectors for viral diseases.
 - (v) All three main classes of disease, i.e. fungal, viral and bacterial, can reduce or totally prevent the production of faba bean. Of these, fungal diseases are the most serious problem. They are widespread in all the major geographical areas where faba bean is grown and may cause substantial crop losses when environmental conditions favour them.
- 6) The abscission of flowers and young pods is the single most important factor contributing to low yields. This has stimulated the production of a large body of scientific information about faba bean. In this field of investigation factors believed to lead to flower abscission include:
- (i) genotype
 - (ii) climatic factors e.g. temperature, light and moisture availability
 - (iii) agronomic practices such as plant density
 - (iv) salinity
 - (v) nutrient supply
 - (vi) distribution of assimilates
 - (vii) balance of plant growth regulators.

Little work has been carried out on the effect of varietal differences in pollen germination and competition, or on the effect of temperature on pollen tube growth. Such information would be of value in the selection of faba beans for higher, more stable yields. The present study was conducted to provide detailed

information on six main aspects of *Vicia faba* reproductive biology. These are described on page 43 of this thesis.

- (7) The present investigation has clearly established several important facts about *Vicia faba* male gametophyte biology.
 - (i) High temperature accompanied by high RH has detrimental effects on pollen germination and pollen tube growth *in vitro*. Fast germinating lines lost their viability by approximately 60% and 57%, respectively after an exposure to 35°C for 4h at 100% RH. In contrast slower germinating pollen genotypes showed tolerance to such treatment. This has been attributed to a possible genetic mechanism operating in these pollen genotypes.
 - (ii) It has often been reported that the proportion of flowers fertilised and of pods produced at the lower reproductive nodes of *Vicia faba*, is far greater than it is at the upper nodes. More flower abortion occurs on the upper nodes of the plant than on the lower reproductive nodes. The present study of fertility status, between flowering nodes in three highly inbred lines clearly demonstrates that such abortion could be due to lack of fertile pollen grains in the upper nodes of *Vicia faba* lines. Therefore the proportion of seeds produced seemed positively related to the number of fertile pollen grains available to a given flower at a particular reproductive node and raceme.
 - (iii) The most important reason for interest in the preservation of pollen is to use it for breeding purposes as and when desired. Another purpose concerns the conservation of genetic resources. The moisture content of pollen at the time of storage could be the most crucial factor determining the successful storage life of pollen grains of *Vicia faba* species. Thus, it has been found that *Vicia faba* from freshly dehiscent anthers desiccated at 25°C/6h and freeze-dried for 45 min, stored in either LN2 or at -80°C would give more than 80% viability after 9 months of storage.

Mixed pollination is an important tool for detecting fast growing pollen grains of two given genotypes competing with each other. This has been envisaged to provide evaluation of random crossign under field conditions or controlled environments where beehive cages could be introduced. In the present mixed pollination,

it has been confirmed that pollen from certain lines do out-compete the self-pollen on the maternal stigma. However, this competitive ability has been found highly variable from one maternal line to another. The importance of this finding with respect to synthetic variety and hybrid seed production has been pointed out.

- (v) Environmental factors such as seasonal variation in temperature regimes have profound influences on flower initiation and abscission. These studies were conducted in Ethiopia, from November 1987 to November 1988. The studies were conducted in three ecological zones representing low, dry altitude at Nazareth, with mean minimum annual range of temperature 15°C and mean maximum 27°C, and Debre Zeit, mid altitude with mean minimum annual range of temperature between 10°C and mean maximum 27°C, and Holetta representing high altitude with mean minimum annual range of temperature of 0°C and mean maximum 24°C. The largest mean percent of flower shedding occurred during the cropping season of mid-June to November (season 5). The results conclusively ($p \geq 0.001$) showed that there were variations in the magnitude of fertilisation from season to season. The number of fertilised ovules from Holetta was significantly lower than from flowers collected from both Debre Zeit, and Nazareth Centres ($p \geq 0.05$). Overall, more than 67% of flower drop was due to lack of fertilisation. Variability among the inbred lines has been identified.

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