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THE USE OF ISOENZYMES  
AS AN AID TO THE  
BREEDING OF FIELD BEANS  
(VICIA FAB L.)

A THESIS  
SUBMITTED IN ACCORDANCE  
WITH THE REQUIREMENTS OF  
THE UNIVERSITY OF DURHAM  
FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

BY

PHILLIP GATES

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OCTOBER 1978

DEPARTMENT OF BOTANY



*To my wife*

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## SUMMARY

A simple, rapid technique has been developed for identifying inbred lines of Vicia faba L. and measuring crossing between them, using vertical polyacrylamide slab gel electrophoresis of isoenzymes isolated from pollen and cotyledons. Six isoenzyme systems were evaluated, and two of these, non-specific esterase (EST) and glutamate oxaloacetate transaminase (GOT), were selected for use in screening genotypes. Neither of these isoenzyme systems, from seed or pollen was found to be affected by environmental variables which included contrasting nitrogen regimes and plant growing conditions. Tissue specificity for EST isoenzymes was found between leaves, pollen, seed cotyledons, and embryos.

It was found that using EST isoenzymes alone a large number of inbred lines could be positively identified by seed or pollen zymograms, and their  $F_1$  hybrids could be detected. Because only small quantities of cotyledon or pollen were required for extraction, the method allowed seeds to be germinated after sampling, and the reproductive capacity of plants was not seriously decreased.

The method was tested for measuring contamination of inbred line and  $F_1$  hybrid seed, and outcrossing was measured in the first synthetic generation of two commercial synthetic varieties. The system was also tested on a diverse range of plant taxa, and found to be widely applicable. The use of isoenzymes in plant breeding, as biochemical markers to measure outcrossing and as a means of identifying breeding material and crop varieties, is discussed.

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## INTRODUCTION

### 1. The Field Bean crop

Vicia faba has been an important seed protein crop in Great Britain at least since Roman times (HEBBLETHWAITE & DAVIES 1971), and in the Eastern Mediterranean, archeological evidence suggest that it has been cultivated since the late Neolithic (SCHULTZ-MOTEL 1972). The centre of origin is generally considered to be the Near East (CUBERO 1974), with secondary centres of origin in Ethiopia and Afghanistan. It has spread in cultivation to cover the area between Central Asia and Western Europe, and between Ethiopia in the South and Scandinavia in the North.

The value of the crop lies in the high protein level of the seeds, ranging from 24% to 35% crude protein, which although characteristically low in the sulphur amino acids cysteine and methionine, is high in lysine (FROLICH, POLLMER & CHRIST 1974); the crop is an important source of protein for animals and humans. Tick and horse beans, V. faba minor and equina respectively, are suitable for feeding cattle (HEBBLETHWAITE & DAVIES 1971; EDEN 1967), pigs (AHERNE & McALEESE 1964), and chickens (DUN & BEER 1974), whilst in the U.K. in particular V. faba major, the broad bean, is canned or frozen for human consumption.

The acreage of Field Beans grown in England has declined steadily throughout the late nineteenth and twentieth centuries, apart from brief increases during the World Wars (HEBBLETHWAITE & DAVIES 1971). In the 1960's interest revived, as the benefits of home-grown protein were recognized and new varieties became available.



The annual fluctuations in yield are the chief reason for the unpopularity of the crop amongst farmers, and research has shown that several factors contribute to this instability.

Before the advent of modern herbicides bean fields were notorious for the presence of weeds, "giving the finest weed display of any crop" (CHURCH 1922), and although the problem of annual weeds has since been reduced, perennial grasses, such as Agropyron repens, have still to be eradicated. Not only do they reduce yields by competition (HEWSON, ROBERTS & BOND 1973), but also flourish in the crop to the detriment of following cereals (HEBBLETHWAITE & DAVIES 1971).

Because of their nitrogen fixing ability, which considerably enhances following cereal crop yields (AMBRUS 1971), and the ability to handle the crop with standard cereal machinery, Field Beans are often grown as a break crop in intensive cereal cultivation (NORTH 1968; SJODIN & ELLERSTROM 1968). Up to 206 Kg N/ha may be fixed, giving yield improvements in following crops equivalent to the direct application of 90 Kg N/ha. Field Beans are classified as either winter or spring varieties depending on sowing date, and the late-maturing spring beans can be difficult to harvest and hinder the sowing of following crops. Early maturity is therefore highly desirable (SJODIN & ELLERSTROM 1968; POULSEN 1977).

The most serious insect pest of Field Beans is Aphis fabae, the Black Bean Aphid. Work seeking resistance to this pest (BOND & LOWE 1975), and also predicting infestation from the levels of overwintering aphid egg populations (CAMMELL & WAY 1977) means that spraying

with expensive insecticides may eventually be rendered of less importance or may be timed to be more economic. A serious difficulty of insecticide treatments is that the crop is dependent on bees for cross pollination, so that care has to be exercised during spraying to prevent their simultaneous destruction.

The major fungal pathogen of the crop is Chocolate Spot, Botrytis fabae, which has led to very serious yield loss in winter beans in recent years and was a major factor in the very poor yields of 1968 (HEBBLETHWAITE & DAVIES 1971). This pathogen mainly attacks winter beans, and spring beans are usually more severely affected by aphids than by fungus.

Although the yield potential of Field Beans is very high, with yields of 7-9 t/ha achieved on experimental plots (ISHAG 1973; SPRENT, BRADFORD & NORTON 1977; THOMPSON & TAYLOR 1977), national average yields are much lower, averaging approximately 3 t/ha. Several physiological factors have a profound effect on yield, including the effect of water stress on nitrogen uptake and dry matter production (SPRENT, BRADFORD & GALLACHER 1978). Recent deep cultivation experiments to improve water and nutrient availability have shown that large increases in dry matter and pod set can result from this treatment (STONE 1978).

A most important factor limiting yield is flower and pod abscission, which may be the result of environmental stress (EL-NADI 1969; EL-BELTAGY & HALL 1974), or a consequence of the pattern of flower development (HARRIS, GATES & BOULTER 1978). Yield is directly related to number of pods per unit soil area (ISHAG 1973; JAQUIERY, GEHRIGER & KELLER 1977), and therefore reliable pod set is an essential prerequisite of good yields.

2. Breeding objectives, breeder's methods, and the breeding system

Until recently V. faba was thought to be exclusively diploid,  $2n = 2x = 12$ , but a tetraploid,  $2n = 4x = 24$ , has now been described (POULSEN & MARTIN 1977). No success has been achieved in attempts to hybridise V. faba with other closely related species, such as V. narbonensis. Such a hybrid would be a useful means of increasing the genetic variability in a crop where this is considered to be somewhat limited (BOND 1976). The wild progenitors of V. faba are unknown, and its evolutionary origins obscure (LADIZINSKY 1975).

The species is subdivided on the criterion of seed size (MURATOVA 1931) and the most recent opinion (CUBERO 1974) recognises four varieties; minor, equina, faba, and ~~pawijuga~~ pawijuga.

Field Beans have an intermediate breeding system, between total autogamy and total allogamy, with outbreeding generally varying between 30-45% (SIRKS 1931; HOLDEN & BOND 1960, inter alia), this figure being subject to variation within wide limits. VOLUZNEVA (1971) recorded a minimum outcrossing rate of 2.1%, whereas HOLDEN and BOND (1960) recorded a maximum outcrossing of 69.8%. The flowers normally require bee visits, to trip the floral mechanism and to effect cross-pollination (FREE 1966; FREE & WILLIAMS 1976). However, some Middle Eastern, Indian and African populations, and heterozygous plants of Northern European populations, exhibit the ability to set pods in the absence of bee visits (LAWES 1973), a phenomenon known as autofertility.

The basis of this behaviour has recently been shown to lie in the timing of the development of stigma receptivity, through the secretion of a stigmatic exudate, relative to the time of anther dehiscence. Stigma exudation in autofertiles occurs before anther dehiscence, whilst in autosteriles, which need bee-tripping of the flower, it does not occur until after anthesis, thus allowing effective cross-pollination before self-pollen can germinate on the stigma (PAUL, GATES, HARRIS & BOULTER 1978).

The breeding system of Field Beans has been manipulated from partial allogamy towards either complete allogamy or complete autogamy, to allow exploitation of standard plant breeding methods. Until quite recently varieties were derived by mass selection, but during the current resurgence of interest in the crop synthetic varieties have become available (BOND & FYFE 1962; BOND 1974), and attempts have been made in Britain and France to breed  $F_1$  hybrids, using male sterility (BOND 1968, 1972; BOND, FYFE & TOYNBEE-CLARKE 1964, 1966).  $F_1$  hybrids have so far proved unsuccessful, due to incomplete male sterility and consequent difficulties of large scale production of seed (PLANT BREEDING INSTITUTE ANNUAL REPORTS 1965-1975; BOND, FYFE & TOYNBEE-CLARKE 1966), although the yield advantages conferred by heterosis and independence from bee pollinators have been shown to be considerable (BOND 1964, 1970).

The most recent breeding strategy, in progress at the Welsh Plant Breeding Station, Aberystwyth, has been to develop varieties which are totally autogamous, using the autofertility characteristic of exotic material, and thus relieving the crop of its dependency on bee pollinators,

a source of yield instability (LAWES 1973, 1974). Initial results have been encouraging. A closed-flower mutant has recently been described which may have a useful application where obligate autogamy is desired (POULSEN 1977 a).

All of these current breeding strategies involve the development of inbred lines for subsequent use in crossing programmes.

### 3. Isoenzymes: Definitions, classifications and occurrence

In the twenty years since HUNTER and MARKERT (1957) first demonstrated esterase isoenzymes separated by zone electrophoresis on starch gels it has been shown that enzymes exhibiting multiple molecular forms are the rule rather than the exception, in almost all organisms studied (SHAW 1965).

The term isoenzyme, synonymous with isozyme, was coined to describe multiple molecular forms of the same enzyme (MARKERT & MØLLER 1959), but it soon became evident that more precise definitions and terminology were necessary, as multiple molecular forms of enzymes could arise in a variety of ways. In addition, non-specific enzymes acting on artificial substrates, such as the esterases, consist of whole groups of enzymes with broadly similar catalytic properties, rather than different molecular forms of the same enzyme. The term isoenzyme has come to be applied, more strictly, to mean various proteins produced by combination of several polypeptides specified by distinct gene loci. PRAKESH, LEWONTIN and HUBBY (1969) introduced the term allozyme to describe variant proteins produced by different alleles at the same locus.

It has also been recognised for many years that the same enzymes isolated from different tissues or different species may exhibit wide variations in their physical and catalytic properties (PAUL & FOTTRELL 1961), and to these the term heteroenzymes has been applied (WIELAND & PFLEIDERER 1962). Problems of definition can easily arise, especially in such enzymes as aspartate aminotransferase (glutamate-oxaloacetate transaminase), which occurs in different forms in the mitochondria and in the supernatant of the same cell (EICHEL & BUKOVSKY 1961; BOYD 1961).

Three more terms must be mentioned. The concept of conformers has been invoked by KAPLAN (1968) to describe conformational variants that are distinct from all other forms of isoenzymes in that their origin does not involve differences in covalent structure of the molecules or detectable alterations in their molecular weights. The only differences among these conformational isoenzymes are the ways in which the polypeptide chains are folded, to produce differences in exposed charges, and consequently alter electrophoretic migration rates. The experimental verification of this theory has depended on the demonstration that large numbers of proteins are capable of undergoing reversible denaturation (EPSTEIN & SCHECHTER 1968). ALLENDORF (1977) and KING (1977) have recently discussed the use of the term electromorph to describe electrophoretic mobility classes. The finding that these mobility classes, until recently merely regarded as the phenotypic expression of alleles, could be increased or decreased in number by alterations in gel concentration or pH, has led to the introduction of this concept. Finally, homoioenzyme, coined to replace isoenzyme, taking into account that isoenzymes differ in their relative affinities for substrate analogues, coenzyme analogues, and inhibitors, has not passed into widespread useage, and is rarely encountered in the literature.

For the correct application of any of the above definitions, careful biochemical investigations of the multiple enzyme forms must be applied. Such investigations were not conceived as a major part of this research project, so that the bands stained on gels for all the enzymes examined are referred to throughout by the term isoenzymes, in the broad sense of the original definition of MARKERT and MØLLER (1959).

When isoenzymes are separated on starch or acrylamide gels differential migration of the proteins depends on net electrostatic charge and to some extent the size or configuration of the molecules involved. Although changes in electrophoretic mobility can occur in a number of ways, perhaps the most important is through amino-acid substitutions to produce changes in net charge (INGRAM 1957; HUNT & INGRAM 1958). Of the twenty standard amino acids, fifteen are neutral in charge, three have a positive net charge and two have a negative net charge. Assuming the amino acids occur in polypeptides in equal amounts, it has been calculated (SHAW 1965) that a single substitution in a nucleotide will only produce a change in net charge in 27.56% of cases, so that almost three-quarters of all such mutants would be electrophoretically undetectable.

Another possible cause of electrophoretic variation affecting net charge of a protein is non-homologous crossing over, resulting in deletion and duplication, but it is not known how many isoenzymes have evolved in this way.

Genetic variation in enzymes is very common. About half of all Drosophila enzymes studied are polymorphic (SHAW 1972). As a gross generalisation, about 30% of structural gene loci are thought to be

polymorphic, and the number of alleles at some loci can be large, the Est-5 locus of Drosophila pseudoobscura having 12 alleles (PRAKESH, LEWONTIN & HUBBY 1969). An average individual in a population is likely to be heterozygous at about 10% of its loci, so every individual has a unique profile of enzyme proteins, and this has been exploited in medico-legal applications (GRUNBAUM 1976), and as genetic signatures in human cell cultures (O'BRIEN, KLEINER, OLSON & SHANNON 1977), but only rarely in the identification of individual plants by their unique enzyme phenotypes (BROWN & ALLARD, 1969 a, b). Isoenzyme techniques have been applied very extensively to microorganisms and animals, but only belatedly to higher plants.

The isoenzyme complements of organisms constitute a natural label, and because electrophoretic techniques require only small amounts of unpurified material, mass screening can be rapid and inexpensive.

#### 4. Electrophoretic techniques applied to plant breeding

The use of electrophoretic techniques in plant breeding has developed in three main directions. As a means of studying the systematics and evolution of crop plants, protein fractions have been used as "fingerprints", and by comparing the protein profiles of related species conclusions have been drawn about their taxonomic positions (BOULTER, THURMAN & TURNER 1966). Examples of taxa to which this technique has been applied include Brassica

(VAUGHAN, WAITE, BOULTER & WAITERS 1966), the Leguminosae (FOX, THURMAN & BOULTER 1964; BOULTER, THURMAN & DERBYSHIRE 1967), Triticum aestivum (JOHNSON, BARNHART & HALL 1967), and Gossypium (JOHNSON & THEIN 1970; CHERRY, KATTERMAN & ENDRIZZI 1970). Similarity of band pattern is often cited as evidence for a close relationship between species, but since the migration of proteins to the same position on gels cannot be proof that they are homologous proteins unless very rigorous biochemical checks are applied (JOHNSON 1977), this evidence is circumstantial, but can be used very effectively to reinforce taxonomic conclusions drawn from older, more conventional methods.

A more extensive application of the technique has been towards the genetic analysis of polymorphisms, a field where isoenzymes have been widely employed. They have been found to be under the control of major genes with simple Mendelian segregation in nearly all instances (LARSEN 1969; GOTTLIEB 1972) and are often controlled by co-dominant alleles (SCHWARTZ 1960; MACDONALD & BREWBAKER 1972; GOTTLIEB 1972), so that the phenotypes of heterozygotes are readily distinguishable from those of homozygotes. Isoenzymes have provided a useful tool for massive surveys of genetic variability (BROWN & ALLARD 1969 a, b), and their use for monitoring the effects of selection on allele frequencies has been discussed by ALLARD and KAHLER (1971). Genotype frequencies have been used by some authors as a varietal characteristic (BASSIRI 1977; HAYWARD, McADAM & THOMAS 1976), but when employed in this way there is a danger that selection for certain alleles may alter allele frequency (WU 1976) and confuse identification.

The major use of electrophoretic methods has been concentrated on cultivar identification. On the basis of total protein profiles cultivars of potato (DESBOROUGH & PELOQUIN 1968; MACKO & STEGEMANN 1969), oats (SINGH, JAIN & QUALSET 1973), wheat (MENKE, SINGH, QUALSET & JAIN 1973; AUTRAN & BOURDET 1975; ELLIS & BEMINSTER 1977; BUSHUK & ZILLMAN 1978), Vicia faba (ADRIAANSE & ROBBERS 1970), barley (FEDAK 1974), Phaseolus vulgaris (ADRIAANSE, KLOP & ROBBERS 1969; ADRIAANSE & ROBBERS 1970), Pisum sativum (ADRIAANSE & ROBBERS 1970), Zea mays (STEGEMANN 1977), and Agrostis palustris and Poa pratensis (WILKINSON & BEARD 1972) have been identified. However, total protein extracts produce very complicated patterns on gels which may be difficult to interpret and require the use of keys to identify varieties (ELLIS & BEMINSTER 1977). Workers using Glycine max (SAVOY 1977) and Euphorbia pulcherrima (WERNER & SINK 1977) have failed to find sufficient differences in protein complement for identification of varieties.

Isoenzymes have found wide favour for cultivar identification. Table 1 lists a wide range of plant species whose cultivars have been identified in this way.

In the field of cytogenetics, isoenzymes have proved to be useful chromosome markers, when their genes can be mapped on specific chromosomes. In this context TANG and HART (1975) were able to use isoenzymes as chromosome markers in wheat-rye addition lines and HART and LONGSTON (1977) located the isoenzyme structural genes in hexaploid wheat. UPADHYA (1968) demonstrated the peroxidase isoenzyme associated with the Aegilops umbellulata chromosome segment transferred to Chinese Spring Wheat, and KOBREHEL (1978)

Table 1. Isoenzymes used in cultivar identification

<u>Species</u>	<u>Support Medium</u>	<u>Tissue</u>	<u>Isoenzymes</u>	<u>Authors</u>
<i>Pisum sativum</i> L.	Starch	Germinated seeds	EST	Frankel & Garber (1965)
<i>Nicotiana</i> spp	Acrylamide	Young leaves	PER EST LAP	Hart & Bhatia (1967)
<i>Solanum tuberosum</i> L.	Acrylamide	Tuber	EST PER	Desborough & Peloquin (1968)
<i>Zea mays</i> L.	Starch	Seedlings	9 systems including PER ACPH	Brown & Allard (1969 a, b)
<i>Dianthus caryophyllus</i> L. <i>D. x plumarius</i> L.	Acrylamide	Leaf, stem	9 systems including PER ACPH EST	McCown, Hall & Beck (1969)
<i>Hordeum vulgare</i> L.	Starch	Plumule	EST	Fedak & Rajhathy (1972)
<i>Pisum</i> spp.	Acrylamide	Roots	PER	Matthews & Williams (1973)

<i>Avena sativa</i> L.	Starch	Seedling	EST PER LAP	Singh, Jain & Qualset (1973)
<i>Triticum aestivum</i> L. <i>T. durum</i> Desf. <i>Triticale hexaploide</i> Lart.	Starch	Germinated seeds Shoots	EST PER	Menke, Singh, Qualset & Jain (1973)
<i>Arachis hypogaea</i> L.	Acrylamide	Dry seeds	EST	
	Starch		INT Oxidase Catalase LAP ACPH	Cherry & Ory (1973)
<i>Hordeum vulgare</i> L.	Agar	Plumule	$\alpha$ Amylase EST ACPH	Fedak (1974)
<i>Hordeum vulgare</i> L.	Starch	Seedlings	14 systems including EST LAP ACPH	Almgard & Landegren (1974)
<i>Hordeum vulgare</i> L.	Starch	Shoot	EST ACPH PER	Bassiri (1976)
<i>Lolium perenne</i> L.	Starch	Leaf	GOT Phosphogluco- isomerase	Hayward (1977)

Carthamus tinctorius L. C. oxycantha Bieb	Starch	10-day seedlings	ACPH PER	Bassiri (1977)
Glycine max (L.) Merr	Acrylamide	6-48h Germinated seed	ACPH Amylase Tetrazolium oxidase Alcohol dehydrogenase	Gorman & Kiang (1977)
Zea mays L.	Acrylamide	Dry seed	EST	Stegemann (1977)
Vicia faba L.	Starch	Germinated seeds	EST PER	Bassiri & Rouhani (1977)
Euphorbia pulcherrima Willd. ex Klotzsch.	Acrylamide	Leaf	PER	Werner & Sink (1977)
Persea americana Mill.	Starch	Mesocarp	GOT Alcohol dehydrogenase Phosphoglucomutase LAP	Torres, Diedenhofen, Bergh & Knight (1978)

has identified the chromosome segments of wheat controlling peroxidase synthesis and used this to identify transfer lines with Agropyron elongatum. Most recently MORIKAWA (1978) has identified monosomic and nullisomic oats using leaf peroxidase isoenzymes.

The isoenzymes of Field Beans have been most extensively studied by BASSIRI and ROUHANI (1977), who used esterases and peroxidases of germinated seeds to identify a number of cultivars. THURMAN, PALIN and LAYCOCK (1965) and FAWOLE (1977), studying L-glutamic dehydrogenase isoenzymes found that these varied between tissues (THURMAN et al 1965) but not between varieties (FAWOLE 1977). MUNTZ (1977) followed changing alpha-amylase patterns during pod development, and PAPACHRISTOS and JOHNSON (1977) found two  $\alpha$  amylase isozymes in germinating V. faba cotyledons. Tissue-specific phosphorylase isoenzymes in cotyledons, young seeds, leaves, and roots were described by GERBRANDY and VERLEUR (1971).

The use of isoenzyme patterns as biochemical markers to supplement or replace conventional morphological markers has been advocated by several authors (McKEE 1973; PEIRCE & BREWBAKER 1973; LARSEN 1969), but has been underexploited despite the advantage of direct phenotypic expression of heterozygotes. The application of the technique to plant breeding problems has been pursued by NIJENHUIS (1968) and WOODS and THURMAN (1976) to measure sib-contamination in  $F_1$  hybrid Brassica oleracea production with success, and by BROWN and ALLARD (1970) to estimate the mating system of open-pollinated maize populations. JAIN (1978) used isoenzymes as an alternative measure of outcrossing in Limnanthes alba, but their use as a method of detecting crossing has yet to become routine. On a minor scale, WEST and GARBER (1967 b) identified hybrids between

Phaseolus vulgaris and P. coccineus on the basis of esterase and leucine aminopeptidase patterns, and outcrossing in Medicago falcata has been measured using total protein profiles (MILLER, SCHONHORST & McDANIEL 1972).

The aim of the work presented here was to develop the method of separation of isoenzymes of Vicia faba on acrylamide gels as a rapid and economical method of identifying inbred lines and measuring crossing in  $F_1$  hybrid and synthetic variety production, and to this end the method was developed requiring the minimum of experimental sophistication compatible with accuracy of identification. Following this, the method was evaluated on samples of Field Bean breeding material.

## MATERIALS AND METHODS

### 1. Biological material

Table 2 lists inbred lines which were tested for their isoenzyme patterns, and between which crosses were made.

Details of the samples of breeding material screened are set out separately in the section dealing with applications of the technique.

### 2. Growth conditions for V. faba plants

#### 2.1 Greenhouse

Seeds were sown in Levington compost, in 5" diameter plastic pots. To ensure nodulation, pots were inoculated with soil in which well-nodulated plants had previously been grown. Under these conditions, large nodules were present on plants at the time of first flower bud formation.

Seeds sown in short days were placed under high pressure 400 W sodium lamps, type SON/T (ANON 1973), which were suspended one metre above the pots, and maintained at that distance from the shoot apex. This supplementary lighting was used to give the plants a 16 h day, until natural daylength exceeded this.

Table 2. Inbred lines of Vicia faba tested for isoenzyme patterns

<u>Accession No.</u>	<u>Source</u>		<u>Identity</u>	
2	P.B.I.	349	Components of synthetic variety . CS "Bulldog" (Equina) winter bean	
3	"	67		
4	"	224		
5	"	51/3		
8	"	157		
9	"	73		
10	"	51/3		
11	"	S45		Fertility restorer line
13	"	73		Male fertile
14	"	73		Male sterile
18	W.P.B.S.	Ch 170	ex. Rahria ser N. Delhi (Paucijuga)	
19	"	Ch 182		
20	"	Ch 468		V. faba major cv. compacta
21	"	Ch 467		Topless mutant
22	P.B.I.	STW		Sudanese triple white
23	"	STW		
41	"	YT 11/2	Selection from Farmer's stock, from Yeldham, Essex (Y) and Stagsden, Beds (S). T = tick bean (minor), H = horse bean (equina). All spring beans	
42	"	YH 73/1		
43	"	ST 47/2		
44	"	ST 26/2		
45	"	YT 70/1		
46	"	YH 105/2		

Table (Cont.)

51	P.B.I.	Af 4/2
52	W.P.B.S.	Ch 4
53	P.B.I.	T 2
54	"	T 51
55	"	AS 19
56	"	AS 1345

P.B.I. = Plant Breeding Institute, Cambridge

W.P.B.S. = Welsh Plant Breeding Station, Aberystwyth

All inbred lines had been self-pollinated for at least seven generations when first tested.

Plants were watered weekly with a commercial nutrient feed containing all necessary elements, including nitrogen, and at all other times with tap water.

Ambient temperature fluctuated between a minimum of 13°C and a maximum of 26°C. For a short period in 1976 the glasshouse temperature rose to 35°C during the period of pod filling.

## 2.2 Field

Seeds were sown directly in open ground, in rows two feet apart, during March. Plants were spaced nine inches apart within rows. No fertiliser treatment was applied.

## 2.3 Controlled environment

Seeds were sown in 5" plastic pots in Levington compost, inoculated with soil known to contain Rhizobium leguminosarum.

Without pre-soaking they took six days to germinate, and no difference in rate of germination was apparent between nine inbred lines and four F<sub>1</sub> hybrids grown.

Pots were placed in a Warren Sherer growth chamber, model CEL 511-38, with the following environmental conditions:

Daylength 16h

Day temperature 22°C

Night temperature 14°C

Relative humidity 75-80%

Light intensity (centre) 2872.4 1/ft<sup>2</sup>

(edge) 1941.8 1/ft<sup>2</sup>

Lighting was supplied by cool white fluorescent tubes, type F48 T12-CW-VH0, and incandescent bulbs, with the following regime to provide dawn and dusk lighting:

- 5 a.m. - 6 a.m. incandescent only
- 6 a.m. - 9 a.m. incandescent + half fluorescent tubes
- 9 a.m. - 5 p.m. incandescent + full fluorescent
- 5 p.m. - 8 p.m. incandescent + half fluorescent
- 8 p.m. - 9 p.m. incandescent only

The bed of the cabinet was lowered as the plants grew, to prevent them touching the lights, and the growing point was finally removed when the plants became excessively tall. Positions of plants were rotated to minimise the effect of lower light intensity at the edges.

All plants were watered daily with tap water, and twice weekly with a nutrient solution containing the elements listed in Table 3.

Table 3. Standard nutrient solution for plants grown in controlled environment conditions

<u>Element</u>	<u>p.p.m.</u>	<u>Element</u>	<u>p.p.m.</u>
N	154	S	16.4
Ca	200	Cu	0.064
K	156	Zn	0.066
Na	40	Mn	0.55
CL	175	Mo	0.019
P	54	B	0.33
Mg	36	Fe	10

Under these conditions the first partial inflorescence often did not appear until at least the twentieth node, and levels of pod set were low. Vegetative growth was rapid, but the change to reproductive growth was delayed in comparison with plants grown in greenhouse or field conditions. Plants which flowered earliest in the greenhouse also did so in the controlled environment.

Table 4. Flowering and pod set under controlled environment conditions

<u>Line</u>	<u>Days to first flower</u>	<u>First flowering node</u>	<u>% Pod set</u>
2	79	27	3.17
3	56	15	3.03
5	70	28	1.86
7	72	25	2.7
8	77	29	-
9	66	23	-
11	64	27	7.15
13	65	21	2.0
14	66	21	-
7x5	65	25	2.17
2x4	90	23	-
4x2	64	24	19.2
3x2	<u>82</u>	<u>25</u>	<u>-</u>
Mean	70.5	24.1	5.16

For greenhouse-grown lines:

mean days to first flower (days after emergence) = 45 days

mean first flowering node = node 9

#### 2.4 Nitrogen regimes

Lines 4 and 5 were both grown under a range of nitrogen treatments in the glasshouse.

Material without root nodules (-Rh + N) was raised by sowing seeds, surface sterilised in 2% hypochlorite solution, in sterilised Levington compost. These non-nodulated plants were supplied with a complete nutrient feed containing 154 p.p.m. nitrogen twice weekly, and at all other times were watered with sterile distilled water. Nodulated plants were grown for comparison from seeds sown in Levington compost inoculated with soil in which nodulated beans had previously been grown. These plants (+Rh - N) were supplied with the same nutrient feed, but minus nitrogen, and were otherwise watered with sterile distilled water.

Plants were also raised with nodules and supplied with a complete feed containing nitrogen (+Rh + N), and in sterile conditions without inorganic nitrogen (-Rh, low N), with no nitrogen supply other than that in the seed cotyledons and in the sterilised nitrogen compost. Leaves of the latter developed clear signs of nitrogen deficiency.

Roots were inspected to check for presence or absence of nodules before leaves, pollen or seeds were harvested. Two plants, which in sterile compost were found to have developed nodules, were discarded.

### 3. Pollination methods

In the first year, 1976, when no insect-proof glasshouse was available, bees were excluded by enclosing inflorescences in muslin. In the two subsequent years an insect-proof glasshouse was used, and seeds were sown in December to flower in March and April, which further reduced the likelihood of bee visits. Bees were excluded from the flowers of field-grown material with muslin.

#### 3.1 Selfing

This was achieved by merely depressing the keel petals, to force pollen onto the stigma. Selfing was carried out as soon as the wing petals separated to reveal the keel petal, the flowering stage 7-8 of PAUL (1977).

#### 3.2 Crossing

Flowers were emasculated at flowering stage 3-4 (PAUL 1977), before the keel petal could be seen, by gently separating the petals and removing the anthers. The flowers were then protected from bees, and cross-pollinated 2-3 days later, when they were fully open.

An alternative method of emasculation used was that recommended by ERITH (1930). At the closed bud stage the whole corolla was gripped with forceps and gently pulled. The petals separated from the calyx base, and as they slid over the stigma and stamens removed the anthers, leaving behind an emasculated flower with an exposed stigma. This was

cross-pollinated immediately. This method proved rapid and efficient, and pod set was similar to that following the standard emasculation technique described above. The damage caused by removal of the petals may induce early exudate formation on the stigma and actually enhance the success of bud pollination (PAUL, GATES, HARRIS & BOULTER 1978).

#### 4. Harvesting

##### 4.1 Flowers

In order to study pollen zymograms pollen was collected from newly opened flowers, at flowering stage 8 (PAUL 1977). The pollen plug was removed from the flower with a mounted needle, separated from the withered anthers, and used in the preparation of extracts.

Samples for storage were dried over  $\text{CaCl}_2$  overnight, and sub-samples of approximately 5 mg were placed in gelatine capsules, which in turn were sealed in glass tubes for storage at  $-20^\circ\text{C}$  (MARTIN 1960). The gelatine capsules allowed removal of sub-samples without the need to thaw the whole sample.

##### 4.2 Seeds

Pods were judged to be ripe when they were completely blackened and brittle. Before storage, seeds were dried at room temperature for two weeks to about 12% moisture, and after being placed in labelled

paper packets were sealed in airtight tins and stored at 4°C. Under these conditions the regeneration time (time taken for viability to drop by 5%) has been quoted as twenty years (HONDELMANN 1976).

Reference samples of all the original accessions, subsequent harvests from inbred lines, and F<sub>1</sub> hybrids were stored under these conditions.

#### 4.3 Leaves

Mature, fully expanded, healthy leaves were harvested, at the transition from vegetative to reproductive growth.

### 5. Biochemical methods

#### 5.1 List of chemicals

All chemicals used were of analytical reagent grade whenever possible.

Enzyme substrates and diazo dyes were supplied by:

Koch-Light Laboratories Ltd., Colnbrook

1-naphthyl acetate A.R.

L-leucyl-β-naphthylamide HCl. puriss. CHR, A.R.

Sigma Chemical Co., St. Louis, Mo., U.S.A.

Fluorescein diacetate

4-methylumbelliferyl acetate (crystalline)

Fast Garnet GBC salt (practical grade)

Pyridoxal-5'-phosphate (crystalline)

Catechol (crystalline)

$\alpha$ -Ketoglutaric acid

L-glutamic acid

Fast Blue  $\beta$  salt (practical grade,  $ZnCl_2$  complex, 20% pure)

George T. Gurr, Div. of Baird and Tatlock, Romford RM1 1HA

Fast Black K salt

Naphthyl A sodium phosphate

Fast Blue  $\beta$  salt (o-dianisidine)

Fast Violet  $\beta$  salt

pH 2-11 carrier ampholytes were obtained from Serva Feinbiochemica

D-6900, Heidelberg 1, P.O. Box 105260, Karl-Benz-Strasse 7.

All other chemicals were obtained from British Drug Houses (BDH) Ltd.,  
Poole, Dorset. Chemicals were used as supplied.

## 5.2 Materials

Visking tubing ( $\frac{18}{32}$ ) was obtained from Medical International,  
49 Queen Victoria Street, London EC4 4SA.

Cellulose acetate membranes were supplied by Beckman Instruments, Inc.,  
Fullerton, CA 92634.

Gel drying membrane was obtained from Pharmacia Ltd., Uppsala,  
Sweden, and uncoated cellophane from W. E. Cannings, Avonmouth Way,  
Avonmouth, Bristol BS11 9DZ

## 6. General methods of gel preparation

Polyacrylamide was chosen as the medium for isoenzyme separation because of the high degree of resolution obtainable, this being particularly important when using small slabs with short migration distances to resolve as many as fifteen bands. The stability of acrylamide over a range of physical and chemical conditions, the range of accurately reproducible concentrations, pore sizes and buffers easily prepared, the availability of relatively inexpensive pure components, the absence of electro-osmotic effects, and the ease of drying the developed gels were all considered as factors favouring the use of polyacrylamide (SARGENT & GEORGE 1975).

The most serious disadvantage of the medium in comparison with starch is that most assay techniques for enzymes have been formulated for use on starch gels, and so the number of enzymes that may be successfully assayed on acrylamide gels is restricted.

### 6.1 Rod gels

Discontinuous 7% acrylamide gels, prepared as described by DAVIS (1964) and SMITH (1976) were used for the comparison of zymograms from different genotypes. These were buffered with Tris-HCl to pH 8.9, and ammonium persulphate was used as a catalyst with N,N,N',N'-Tetramethylethylenediamine (TEMED) as an accelerator for polymerisation. Formulation for the small pore running gel was:

Soln. A	TEMED	0.03 ml	
	1N HCl	6.0 ml	pH 8.9
	TRIS	4.57 g	
	DISTILLED WATER to	12.5 ml	1 volume
Soln. C	ACRYLAMIDE	7.0 g	
	METHYLENE-BIS-ACRYLAMIDE (BIS)	0.183 g	
	DISTILLED WATER to	25.0 ml	2 volumes
Soln. D	AMMONIUM PERSULPHATE	0.07 g	
	DISTILLED WATER to	50.0 ml	4 volumes
	DISTILLED WATER		1 volume

Gels were cast by pouring the prepared monomer solution into perspex cylinders 8 cm long with an internal diameter of 6 mm, and carefully over-laying with distilled water.

When a large pore spacer gel was required, this was polymerised on top of the running gel after the latter had polymerised, using riboflavin as a catalyst.

Spacer gel buffer:

1N HCl	48 ml	
TRIS	5.98 g	HCl titrated
TEMED	0.46 ml	in to pH 6.7
DISTILLED WATER to	100 ml	

3% spacer gel composition:

SPACER GEL BUFFER		1 volume
10 g/100 ml ACRYLAMIDE	monomer stock solution	
2.5 g/100 ml BIS		2 volumes
RIBOFLAVIN (4 mg/100 ml)		1 volume
40% SUCROSE (w/v)		4 volumes

All stock solutions were stored in dark bottles in the cold.

Catalysts were prepared fresh for each gel.

Standard reservoir buffer: TRIS-GLYCINE pH 8.3

TRIS	6.0 g	Diluted 10x for use; used once only
GLYCINE	28.8 g	
DISTILLED WATER to	1000 ml	

0.1% Bromophenol Blue (c. 0.05 ml/l of buffer) was used as a tracking dye for alkaline gels, and methyl green (0.1%) for acid gels.

Acid gel buffer:

	1N KOH	24 ml	
	GLACIAL ACETIC ACID	11.2 ml	
Soln. A	TEMED	2.3 ml	pH.4.2
	DISTILLED WATER to	50.0 ml	

Solns. C and D as for alkaline gels.

Acid gel reservoir buffer:

	GLACIAL ACETIC ACID	2.4 ml	
	GLYCINE	17.25 g	pH 4.0
	DISTILLED WATER to	1000 ml	

Power was supplied by a Shandon Southern SAE 2761 constant current source. Gels were developed at 2 mA/gel for 15 minutes, and thereafter at 4 mA/gel until the front marker reached the bottom of the gel. The gels were then blown from the tubes with a rubber pipette teat, cut at the front marker, pre-incubated in stain buffer at 4°C, stained, destained if necessary, and band positions measured.

Rod gels were used mainly in experiments to investigate assay conditions, extraction methods, and treatments affecting enzyme stability. In these instances the ability to load large volumes of extract and the production of a number of separate gels was a distinct advantage. However, for the comparison of zymograms of different V. faba genotypes, vertical slab gels were employed. For such comparisons, identical running conditions for all samples, and the ease of comparison when they were all assayed in one gel were deciding factors in the use of slabs. Slight differences in shrinkage or swelling of rod gels and small measuring errors could easily produce 3-4% error in the Rf values for bands. Thin slab gels also had the advantage that changing the pH in the pre-incubation solution, from the electrophoresis pH to the assay pH, was rapid, as was enzyme staining. This minimised the diffusion of bands and maximised resolution.

## 6.2 Preparation of slab gels

Vertical slab gels were prepared using the Pharmacia GE-4 system. The gel dimensions were 6.5 cm deep x 7.2 cm wide x 0.25 cm thick, with nine sample wells per slab, each holding a maximum volume of 0.05 ml.

Recommended slab gel preparation involved forcing a sample well former into the polymerising gel. As a modification of this gels were cast inverted, with the former for the sample wells acting as the base. The exposed gel surface was layered with  $\frac{1}{2}$  mm of distilled water, and after polymerisation the gel was turned up the correct way and the sample well former was carefully slid out. All spacers were assembled to the glass plates with paraffin wax, which allowed their easy removal. This method produced perfectly level sample wells in every slab, avoiding inconsistencies arising from layering with water or trapping of air between the spacers and the gel.

Slab gels were developed using a Shandon Southern SAE 2761 constant current source at 25 mA/gel for the first 15 minutes, and then at 50 mA/gel until the tracking dye reached the bottom of the gel.

The buffer was not circulated in the slab gel apparatus, although this facility was available in the Pharmacia system. pH changes measured at the end of electrophoresis showed that from an initial pH of 8.3, the pH of the anode reservoir buffer rose pH 8.8 and that of the cathode reservoir buffer fell to pH 8.2.

### 6.3 S.D.S. gels

- Solutions.
1. 7% acrylamide monomer solution (soln. C)
  2. 0.8% S.D.S.
  3. pH 8.9 TRIS/HCl buffer (soln. A)
  4. Ammonium persulphate (280 mg/100 ml)

5. ACRYLAMIDE	6.0 g	
BIS	0.16 g	
S.D.S	0.2 g	pH 6.8
TEMED	0.15 ml	
TRIS	3.03 g	
DISTILLED WATER to	100 ml	
6. AMMONIUM PERSULPHATE	150 mg	
DISTILLED WATER to	100 ml	

Small pore gel:

4 vols. of soln. 1  
1.5 vols. of soln. 2  
1.5 vols. of soln. 3  
2 vols. of distilled water  
3 vols. of soln. 4

Large pore gel:

Equal volumes of solns. 5 and 6

Electrophoresis buffer:

TRIS	6.06 g	
GLYCINE	28.84 g	pH 8.3
S.D.S.	2.0 g	
DISTILLED WATER to	2000 ml	

6.4 Isoelectric focussing gels

Isoelectric focussing was carried out in glass tubes, 14 cm long with an internal diameter of 6 mm.

Solutions:

1. 2.4 g acrylamide and 6.4 mg N:N'-methylene-bis-acrylamide was made up to 10 ml with distilled water.
2. 0.1 ml N,N,N',N'-Tetramethylethylene diamine (TEMED) made up to 10 ml with distilled water.
3. 15 mg ammonium persulphate in 50 ml distilled water, freshly prepared.
4. Servalyt 40%, pH range 2-11.
5. Servalyt solution for overlaying: 0.1 ml Servalyt pH 2-11. + 200 mg sucrose made up to 4 ml with distilled water.
6. 0.1% 1, 2, Diaminoethane in distilled water.
7. 0.1%  $H_3PO_4$  in distilled water.

The following solution was prepared for the glass tubes:

ACRYLAMIDE (Soln. 1)	7.5 ml
TEMED (Soln. 2)	2.0 ml
SERVALYT (Soln. 4)	0.75 ml
AMMONIUM PERSULPHATE (Soln. 3)	20.5 ml

This mixture was de-gassed in a Buchner flask for one minute and poured into the tubes to a level 2 cm below the top and immediately overlaid with water.

The lower electrophoresis chamber was filled with 0.1%  $H_3PO_3$  (Soln. 7) and the bottom of the tubes lowered into it, taking care to exclude air bubbles.

100  $\mu$ l of cotyledon extract containing 20% sucrose were applied to the rod gels and gently overlaid with overlaying solution 5. This again was overlaid with 0.1% 1,2, Diaminoethane (Soln. 6). Methyl red was applied as front marker and 0.02 M NaOH was used as the cathode electrode solution. A voltage of 300 V was applied initially, and the current was maintained between 1-3 mA throughout the electrophoresis.

#### 6.5 Cellulose acetate membrane electrophoresis

Electrophoresis was carried out using 0.05 M phosphate buffer, in the Beckmann microzone apparatus:

0.05 M  $\text{Na}_2\text{HPO}_4$  488 ml.  
0.05 M  $\text{NaH}_2\text{PO}_4$  312 ml.      pH 7.0

Electrophoresis began at 5 mA, and was halted after 5 or 15 minutes, by which time the current had risen to 7 mA and 12 mA, respectively.

Cellulose acetate membranes were stained with the standard protein and enzyme stains used for acrylamide gels (see below).

### 7. Extraction methods

#### 7.1 Seed meals

These were prepared in a water-cooled coffee mill, using 5 x 15 s bursts with 30 s cooling in between. Meals were prepared with and without the testa.

## 7.2 Fresh extracts from single seeds

Single seed extracts were prepared by homogenising seed material with a little acid-washed silver sand in distilled water or extraction buffer at 4°C in a chilled porcelain mortar and pestle, followed by a brief centrifugation to remove cell debris. Extracts were also prepared at room temperature (c.20°C) to compare enzyme activity at different extraction temperatures. Extracts were rendered dense for loading onto gels by the addition of 20% w/v sucrose.

For comparison of different genotypes half of one cotyledon, weighing about 100 mg, was sufficient material for extraction. With a ratio of 1:5 w/v between tissue and extractant, this yielded 0.5 ml of extract. Smaller volumes of cotyledon extract were more difficult to prepare and dialyse.

A small portion of cotyledon was removed to establish a zymogram, and the remainder germinated and grown on.

## 7.3 Pollen extracts

Pollen extracts were prepared by suspending pollen grains in extraction medium in the ratio 1:3 w/v pollen to extractant. Extracts were rendered dense for loading on gels by the addition of 20% w/v sucrose.

#### 7.4 Leaves

Leaves were extracted by the method of GERBRANDY and VERLEUR (1971). Leaf lamina tissue was cut into small pieces and homogenised in 1:2 w/v extractant at 4°C. The homogenate was pressed through nylon gauze and centrifuged to remove cell debris.

#### 7.5 Storage of extracts

Seed meals were stored at room temperature (c.20°C) and at 4°C. Single seed extracts in distilled water or dialysed salt extracts were stored at 4°C for periods of up to 48 h and dialysed salt extracts with 20% sucrose added were frozen at -20°C for periods of up to 8 days.

### 8. Extraction media

#### 8.1 Seeds

Extraction in 0.4 M NaCl pH 6, followed by dialysis against tap water at 4°C overnight was the standard method employed for preparing extracts to compare zymograms from V. faba genotypes.

For comparison, seed extracts in distilled water and a range of buffers were prepared.

1. TRIS-Glycine electrode reservoir buffer pH 8.3 (stock solution diluted x 20).

2. Phosphate buffer

	pH 5	pH 6	pH 7
0.067 M $\text{KH}_2\text{PO}_4$ (9.08 g/l)	98.8 ml	87.7 ml	39.2 ml
0.067 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (11.88 g/l)	1.2 ml	12.3 ml	60.8 ml

3. 50 mM Borate buffer pH 8

(see section on globulin extraction)

8.2 Pollen

Pollen extracts were routinely prepared by bursting pollen grains in distilled water.

To follow the diffusion of enzymes from the pollen, the material was suspended in pH 5.0 phosphate buffer containing 30% w/v sucrose. The pollen was centrifuged down and samples withdrawn from the extraction medium at regular intervals with a syringe.

8.3 Leaves

Leaves were extracted in 0.1 M sodium citrate buffer pH 6.5, containing 0.4 M sucrose, 0.002 M EDTA, and in order to prevent browning of extracts, 1 ml/100 ml medium of 5 g  $\text{Na}_2\text{SO}_3$  + 3.75 g  $\text{Na}_2\text{S}_2\text{O}_5$  in 100 ml distilled water (GERBRANDY & VERLEUR 1971).

Crude extract was applied to 7% acrylamide gels, with the electrode buffer and gels containing 0.002 M EDTA.

#### 8.4 Seed globulins

Crude globulin fractions were prepared by redissolving the washed precipitate from dialysed salt extracts in 0.4 M NaCl, pH 6.0.

Quantitative extraction of the globulin fraction was achieved by extraction in 50 mM borate buffer pH 8, followed by centrifugation to remove cell debris. The supernatant was dialysed against 33 mM acetate buffer pH 4.5 overnight. The precipitate was separated by centrifugation, resuspended in distilled water, and divided into two parts, one of which was washed by resuspension in distilled water three times, and the other washed similarly six times. The washed precipitates were resuspended in distilled water, titrated against 5 N NaOH in the cold at pH 9.5 to redissolve the globulins and the pH was finally lowered to pH 7.0 before the samples were loaded onto the gels.

#### 8.5 Enzyme subunits

Equal volumes of salt extract of two inbred lines were mixed and treated with 2 M urea in the presence of 20 mM mercaptoethanol to dissociate enzyme dimers and tetramers into monomers. These extracts were subsequently dialysed against tap water to remove the urea and allow random reassociation of the subunits.

The urea extracts were electrophoresed alongside dialysed saline extracts of the inbred lines, with and without urea treatment, equal volumes of the dialysed saline extract mixed, and dialysed salt extracts of seed of reciprocal  $F_1$  hybrids between the two inbred lines. To compensate for the dilution of enzyme concentration when extracts were mixed, the original extracts for these were prepared using half the volume of extractant used for preparing unmixed extracts.

## 8.6 S.D.S. extracts of seed proteins

Seed cotyledons were homogenised in 0.125 M TRIS-HCl buffer pH 6.8 containing 20 mM mercaptoethanol and 4% S.D.S., in the ratio 1:10 w/v. The protein was prepared by allowing the above extracts to stand for 3 h at 37°C, then centrifuging to remove cell debris.

## 9. General gel assay methods

### 9.1 Total protein

Slabs were either immersed for 15 minutes in a 1% solution (w/v) of Amido Black 10 B in 7% acetic acid, or overnight in a 0.025% solution (w/v) of Coomassie Blue R250 in 50% methanol and 7% acetic acid.

Gels stained with Amido Black were destained in successive changes of 7% acetic acid, whilst those stained in Coomassie Blue were destained in 25% methanol containing 7% acetic acid.

Table 5. Enzymes assayed

<u>Enzyme</u>	<u>Abbreviation</u>	<u>International Enzyme Number</u>
Non-specific esterase	EST	E.C. 3.1.1.2.
Acid phosphatase	ACPH	E.C. 3.1.3.2.
Leucine aminopeptidase	LAP	E.C. 3.4.1.1.
Glutamate-oxaloacetate Transaminase (syn. Aspartate aminotransferase)	GOT	E.C. 2.6.1.1.
Peroxidase	PER	E.C. 1.11.1.7.
Pectin methyl esterase	PME	E.C. 3.1.1.11.

## 9.2 Esterase

The standard method for detecting esterase activity employed for comparing genotypes was that of MARKERT and HUNTER (1959), using an  $\alpha$ -naphthyl ester as substrate and coupling the liberated  $\alpha$ -naphthol with tetrazotised o-dianisidine (Fast Blue B salt) or other diazo dyes. A number of variations of assay method were investigated, to assess their relative merits when used in conjunction with acrylamide gels.

### Non-specific esterase assays:

All slabs were pre-incubated before staining in the assay buffer alone for 2 minutes at 4°C, then incubated in the following solutions for 15 minutes at 30°C.

1. 0.2 M sodium acetate buffer pH 5.0  
Fast Blue RR salt (1 mg/ml buffer)  
1% (w/v)  $\alpha$ -naphthyl acetate in 70% (v/v) ethanol  
(0.5 ml/30 ml buffer)
2. As 1, except Fast Blue B substituted for Fast Blue RR.
3. Fluorescein diacetate (FDA) (2 mg/ml in acetone) 1 ml  
0.2 M sodium acetate buffer pH 5.0 20 ml  
Agar 0.5%

After pre-incubation slab gels were laid in contact with the FDA-agar slab at room temperature (c.20°C) and illuminated with an ultra-violet lamp. Fluorescent bands began to appear on the agar surface within 2 minutes. This method was also used to monitor the

esterase distribution in germinating seed cotyledons, by placing their flat inner faces in contact with the FDA-agar slab, and incubating at 25°C. Parallel experiments were conducted using Fast Blue B or 4-methyl umbelliferyl acetate (4 MU) in the agar slab, in place of FDA.

4. Gels were incubated in specific esterase substrate 4-methyl umbelliferyl acetate:

0.2 M sodium acetate buffer pH 5.0

4 MU (2 mg/ml buffer)

### 9.3 Acid phosphatase

Based on the method of RUDOLPH and STAHMANN (1966).

Incubation for 60 minutes at 30°C, after pre-incubation for 2 minutes in assay buffer at 4°C.

1. 0.2 M sodium acetate buffer pH 5.0  
α-naphthyl phosphate 1 mg/ml  
Fast Blue RR Salt 1 mg/ml

2. As 1, but Fast Blue B substituted for Fast Blue RR.

3. Method of WOODS and THURMAN (1976).

0.2 M sodium acetate buffer pH 5.0 150 ml

MgCl<sub>2</sub> 10% (w/v) 1 ml

Fast Garnet GBC 0.1 g

α-naphthyl phosphate 0.1 g

#### 9.4 Leucine aminopeptidase

Pre-incubated in assay buffer for 2 minutes at 4°C, then immersed in assay solution at 30°C until bands visible (30 min - 2 h).

1. Method of SMITH and RUTENBURG (1962).

0.2 M sodium acetate buffer pH 5.0

L-leucyl-β-naphthylamide HCl 0.5 mg/ml

Fast Black K salt 1 mg/ml

2. As 1, but Fast Garnet GBC substituted for Fast Black K.

3. Method of GABRIEL (1971).

L-leucyl-β-naphthylamide HCl (8 mg/ml) 3 ml

0.2 M acetate buffer pH 5.0 30 ml

NaCl (0.85% w/v) 24 ml

0.02 M KCN 3 ml

Fast Blue B 30 mg

Methods 1, 2 and 3 were compared using gels with and without 0.5% starch polymerised into them (BREWBAKER et al 1968).

#### 9.5 Glutamate-oxaloacetate Transaminase

Modified from SHAW and PRASAD (1970).

Incubated for 10 minutes at 20°C.

1. L-aspartic acid 500 mg

α-Ketoglutaric acid 70 mg

Pyridoxal-5'-phosphate 10 mg

Fast Violet B salt 200 mg

0.2 M sodium acetate buffer pH 5.0 100 ml

2. As 1, but Fast Blue RR salt replaced Fast Violet B.

3. As 1, but Fast Blue B salt replaced Fast Violet B.

### 9.6 Peroxidase

1. Modified from MACKO, HONOLD and STAHMANN (1967).

Gels incubated in 0.2 M catechol in 0.2 M sodium acetate buffer pH 5.0 for 10 minutes at 4°C, then transferred to 0.1% H<sub>2</sub>O<sub>2</sub> at 20°C for band development.

2. PALANICHARY and SIDDIQ (1977).

0.2 M sodium acetate buffer pH 5.0 100 ml

o-dianisidine 17 mg

Gels were immersed for 30 minutes at 4°C then transferred to 0.1 M H<sub>2</sub>O<sub>2</sub> at 20°C for band development. Reaction stopped in 7% acetic acid.

### 9.7 Pectin methyl esterase

Method of ROEB and STEGEMANN (1975).

0.025% citrus pectin was included in 7% acrylamide gels prepared as previously described. Electrophoresis was carried out as close to 0°C as possible, and PME activity was detected by incubating gels in the Tris-glycine pH 8.3 electrophoresis buffer containing 0.003% methylene blue.

### 9.8 Controls

As a standard control each assay was carried out without a substrate.

A cotyledon extract from line 5 was included in every gel run, as a standard extract.

### 9.9 pH optima

Esterase, acid phosphatase, and Leucine aminopeptidase assays were each tested over the pH range pH 4 - pH 7 in acetate and phosphate buffers.

The standard pH 5.0 sodium acetate buffer used was prepared as follows:

0.2 M glacial acetic acid (12 g/l)	14.8 ml
0.2 M anhydrous sodium acetate (16.4 g/l)	35.2 ml
Distilled water	50.0 ml

### 9.10 Fixation

After measurement and photography, gels were fixed in 50% methanol containing 7% acetic acid, then shrunk and dried as described below.

## 10. Recording of results and storage of gels

After optimal development of gels in the enzyme assay solution, they were rinsed in the assay solution buffer and photographed on a glass sheet raised above a white reflective background, incorporating a millimetre scale along one edge of the gel. Diffuse daylight was

found to be the most satisfactory form of illumination, and contrast of bands in gels stained for esterase was slightly improved by the use of a 4x orange filter (Kodak Wratten No. 21). Ilford FP4 film rated at 200 ASA and developed in Paterson Acutol developer effectively recorded faintly staining bands.

The migration of bands relative to the Bromophenol Blue front marker was measured using a millimeter scale fixed to a glass plate and mounted above and slightly to one side of the gel. Standardisation of the measuring procedure was rigorously employed, as there were several possible sources of error. Shrinkage and swelling of gels occurred when they were transferred between buffers, and so all gels were allowed to equilibrate in the assay solution buffer for 5 minutes before measurement. Intensely staining bands, which developed rapidly, were often overstained by the time faint bands were clearly visible. Consequently gels were measured at least twice, once when the major bands first appeared and were best resolved, and once when the gels were completely developed. To prevent parallax errors, all measurements were taken from vertically above the slab. The faces of the slab stained first, with stain penetrating to the centre more slowly, and if the gel was viewed from an angle of less than  $90^{\circ}$ , one band appeared as two. The leading and trailing edges of bands were recorded, their relative intensities scored on a three-point scale, and mobilities calculated relative to the front marker.

Rod gels were shrunk and stored in 50% methanol. Slabs were shrunk in several changes of 55% methanol over a period of three days, and then dried in a Pharmacia gel drier, either between pieces of

Pharmacia drying membrane, or with the more economical uncoated cellophane. Attempts to shrink the gels in methanol concentrations higher than 55% caused the gels to become opaque. Shrinkage and drying distorted gels to some extent, but for re-inspection they could be re-hydrated in a methanol series and then showed no distortion compared with their original condition. Dried gels were stored in a card index, attached to an accession card.

It was found that shrunken, dried slabs could be printed onto photographic enlarging paper directly, using them as negatives in a photographic enlarger. This could be used as a precaution in case of fading of the diazo dyes used in the assay procedure. Dried gels stored in dry, dark conditions for two years showed no signs of fading.

## RESULTS AND DISCUSSION

### 1. Enzyme Assay System

#### 1.1 Non-specific esterase

When tested over the range pH 4 to pH 8, all esterase assay systems stained bands most effectively when buffered to pH 5.0. Pre-incubation in the assay buffer, at 4°C to minimise band diffusion, increased the rapidity of staining once the gels were transferred to the assay solution.

Fast Blue RR was found to be less sensitive for gel staining than Fast Blue B, which gave much clearer staining of fainter esterase bands at the expense of some increase in background staining of the gels (Fig. 1). Considerable variation in quality between suppliers and between different batches was found for diazo dyes, and all needed to be added to the assay solution immediately before use, to avoid formation of a precipitate. All assay systems using diazo dyes, except those using Fast Blue B supplied by Sigma Chemicals (Practical Grade, 20% pure, ZnCl<sub>2</sub> complex) needed filtration before use to remove insoluble matter. Varying degrees of precipitation occurred throughout the assay procedure, being most serious when assays were carried out at above pH 5.0, so that several changes of assay solution were needed to prevent coating of the gels with insoluble precipitate.

The fluorescent substrates FDA and 4 MU proved extremely sensitive and rapid assays. Bands appeared within one minute when gels were placed in contact with the agar gel assay block. However, high levels of background fluorescence, particularly when 4 MU was used as a substrate may have masked faint bands, and made gels very difficult to photograph.

No esterase activity was resolved on acid gels, and no bands migrating to the cathode could be detected on alkaline gels when the current was reversed.

### 1.2 Acid phosphatase

Only faint acid phosphatase bands of low mobility could be resolved from seeds or pollen on alkaline gels, although changes appeared during cotyledon development and faint bands of high mobility were present in mature seeds (Fig. 1). Activity of cotyledon extracts persisted unchanged after 16 h storage at 4°C. Clearest banding patterns were obtained at assay pH 6, and no alkaline phosphatase activity could be detected. No bands were resolved moving towards the cathode on alkaline gels. Fast Blue B stained bands more intensely than other diazonium dyes used.

Inbred lines could not be reliably identified on the basis of acid phosphatase banding patterns.

### 1.3 Leucine aminopeptidase

LAP was best resolved on gels containing 0.5% starch (BREWBAKER et al 1968). Assays containing Fast Black K were least satisfactory,

due to the high level of insoluble matter in the stain, which continued to precipitate after filtration and coated the gels. Fast Garnet GBC was more soluble and produced the most intense band staining at pH 5.0. The assay method of GABRIEL (1971) yielded the most intense bands, and the clarity of these was further improved when the gels were shrunk in methanol, bands staining carmine red against an orange-red gel background in all assays.

Only one LAP band was found in pollen, and three were present in young seeds, but two of these decreased in activity during development until in mature seeds all genotypes examined only displayed one clear band at Rf 0.56 (Fig. 1). No cathodal bands appeared on alkaline gels and no activity could be detected on acid gels.

#### 1.4 Glutamate - oxaloacetate transaminase

The method of SHAW and PRASAD (1970), with either Fast Blue B or Fast Blue RR stains, produced very clear bands. Bands began to appear after 30 sec incubation at 30°C. When Fast Violet B salt was used in the assay solution, bands faded during shrinkage of the gels in methanol. This did not occur when the alternative stains were used.

#### 1.5 Peroxidase

Peroxidase bands assayed by both methods described above were of low mobility and poorly resolved. Three bands were detected in cotyledons of inbred line 14 and two from line 11, and F<sub>1</sub> hybrids between the two had patterns which were additive, but which also showed an additional

fast band. As peroxidase bands assayed by both methods faded quickly, gels had to be measured and photographed immediately. When gels were assayed using catechol as a proton donor bands faded completely when gels were shrunk in methanol.

Peroxidase activity was found to persist in extracts which had been stored for up to four days at 4°C.

### 1.6 Pectin methyl esterase

No PME bands were found in extracts of mature seed cotyledon.

## 2. Storage Conditions and Extraction Methods

### 2.1 Seed meals

Although enzyme extracts made from freshly mealed seeds had high initial EST activity, this rapidly declined in meals stored at 4°C, until after 8 days it was scarcely detectable. Stored at room temperature, the meals lost most of their EST activity within 36 h.

### 2.2 Single seed extracts

EST extracts prepared at 4°C and at room temperature (c.20°C) showed no difference in their zymograms, and extraction temperature for this enzyme was not critical.

It was found that up to 75% of the cotyledon of seeds of the large-seeded varieties major and equina could be removed, and the remaining portion germinated, with slight depression of subsequent growth rate (Fig. 2). With smaller-seeded varieties approximately 50% of the cotyledon could be successfully removed. Although flowering was delayed in small seeded varieties sampled in this way, pods were routinely harvested from material grown on after cotyledon excision.

Stored extracts from single seeds, prepared in distilled water or by dialysed salt extraction, showed unchanged EST, ACPH, LAP, PER and GOT isoenzyme patterns after four days at 4°C. Overall activity showed a slight decline, but the zymograms were qualitatively unchanged. Storage at 4°C of extracts containing sucrose was avoided, due to the danger of microbial growth. However, dialysed saline extracts of seed cotyledons with 20% sucrose (w/v) were stored frozen at -20°C for eight days, without change in EST zymogram pattern, but with a general lowering of EST activity, particularly in the two bands Rf 2.1 and Rf 2.5. These frozen extracts were found to contain a precipitate after thawing which could not be redissolved in the original extraction medium.

### 2.3 Pollen storage

Pollen stored at -20°C, as previously described, produced a zymogram indistinguishable from that of fresh pollen when sampled after 6, 12, 18, and 30 months storage. During this time a slow loss of viability occurred (Table 6), but flowers pollinated with pollen stored for twelve months set pods successfully.

Table 6. Loss of pollen viability during storage

<u>Months at</u> <u>-20°C</u>	<u>% germination</u>	<u>% pollen bursting</u>
6	47	5.0
12	49	9.5
18	31	8.5
30	26	18.0

Germination medium: 0.5% agar containing 10% sucrose,  
2000 p.p.m.  $\text{Ca}(\text{NO}_3)_2$ , and 50 p.p.m.  $\text{H}_3\text{BO}_3$ .

#### 2.4 Seed storage

Seeds dried to approximately 12% relative moisture and stored at 4°C throughout the three-year period of this study showed no qualitative change in EST or GOT zymograms. When stored seed of inbred generation 7 ( $I^7$ ) was compared with fresh seed from  $I^{11}$  in line 9, differences in the level of activity of enzyme bands between generations were no greater than those within  $I^{11}$ , and qualitatively the patterns were identical.

#### 2.5 Extraction media

Crude removal of the albumin fraction by homogenising in distilled water yielded active preparations for EST and GOT assay on rod gels, but the contamination with storage proteins also present in these extracts interfered with the slow moving EST bands and caused marked streaking of the gels. This was sufficient to mask some faint bands on slab gels.

Extraction in sodium chloride followed by dialysis against tap water overnight produced samples with clear banding patterns on the thin slab gels, eliminating overloading effects associated with contamination of extracts with quantities of storage protein. This was adopted as the standard method of seed extraction for inter-genotype comparisons.

Extraction in pH 5, pH 6 and pH 7 phosphate and pH 8.3 Tris-glycine yielded zymograms which did not differ significantly from one another, except that undialysed extracts contained increasing proportions of globulin as the pH increased, leading to streaking of the gels. Dialysed pH 8.3 Tris-glycine extracts produced samples which gave the highest level of resolution of EST bands on acrylamide gels.

Zymograms of extracts in pH 8 borate buffer, with and without dialysis are shown in Fig. 3, where the effect of contamination with globulin is clearly illustrated.

## 2.6 Esterase activity in the globulin fraction

Two faint and two clearly visible EST bands were found when the globulin fraction, precipitated after dialysis of salt extracts, was redissolved and developed on acrylamide gels.

To investigate whether genuine enzyme activity was associated with the globulin fraction, a purified globulin extract was prepared and assayed. It was found that slight esterase activity could be

detected, but that this disappeared with repeated washings of the globulin precipitate. The activity was also destroyed by boiling the globulin fraction.(Fig. 3).

### 2.7 Subunit dissociation with urea

Following treatment with urea and subsequent dialysis, slight differences could be detected between the zymograms of the extracts of lines 4 and 9, mixed and treated with urea, and that of the biological hybrid 4 x 9. Two new bands, possibly attributable to randomly associating enzyme monomers, were seen.

Comparison of extracts from line 9, treated and untreated with urea, showed the presence of an additional faint band in the urea-treated extract. The position of this band corresponded with one of the additional bands in the urea-treated mixture of the two inbred lines. No additional bands appeared in the extracts from line 4 prepared by the two different extraction methods (Fig. 4).

## 3. Tissue Specificity of EST Isoenzymes

### 3.1 The testa

No esterase isoenzyme bands were detected in testa extracts. However, high levels of background staining and an additional fast-moving band at Rf 0.97 which stained red, compared with the red-brown staining of esterase bands, appeared after loading crude extracts

including the testa onto acrylamide gels. Testa extracts alone produced an overall red staining gradually decreasing in intensity down the length of the gel when assayed for esterase with assay solutions containing diazo dyes. When esterase isoenzyme changes during seed development were investigated (Fig. 5), a similar red background staining began to appear between Rf 0.09 and Rf 0.15 after day 35 in the 60 day development period, and had increased in intensity by day 45.

Extracts of cotyledons without the testa did not show the Rf 0.97 band.

### 3.2 Embryo and cotyledon

Comparison of the EST zymograms of embryo and cotyledon of line 5 and of cultivar Aquadulce revealed tissue-specific differences (Fig. 6). The intense bands present at Rf 0.58 and Rf 0.64, invariably present in cotyledon extracts, also appeared in the embryo. In the genotypes tested, the embryos, in addition to showing all the isoenzymes detectable in the cotyledons, showed several additional bands which were not observed in cotyledon extracts. These included bands at Rf 0.30 and Rf 0.31 in Aquadulce and at Rf 0.38 in line 5.

Extracts from embryos were generally rather poorly resolved when extracted and electrophoresed under the same conditions as cotyledons, with overloading of protein suggested by "streaking" (Fig. 6).

The distribution of esterase activity within the cotyledon was found to be uniform in dry cotyledons, but was progressively lost

during germination, disappearing from the outer edge first when non-specific EST assay methods were employed. When 4 MU was used as a substrate, the decline in activity was less marked. Esterase activity increased in the embryo during germination (Fig. 7).

### 3.3 Pollen and stigma

Esterase activity was particularly high in the pollen wall and in the region of the germination pore. The cytoplasm of pollen grains which had burst stained less intensely than the pollen wall. When pollen grains were suspended in sucrose, esterase enzymes diffused out from the whole pollen grain surface evenly, and were not released preferentially from the germination pore (Fig. 8).

When pollen was suspended in sucrose, all except two of the isoenzyme bands were detectable within one minute of hydration. The remaining bands began to appear after 90s (Fig. 8).

Three esterase isoenzyme bands, one of very high mobility, were found in stigma extracts (Fig. 9). When esterase isoenzymes of the whole gynoecium were examined, changes occurred during the development of the flower, particularly after pollination (Fig. 9).

Differences were observed when esterase activity was demonstrated histochemically in the stigma, using fluorescent esterase substrates. A peak of esterase activity occurred just before anther dehiscence in autofertile line 19 and in the  $F_1$  hybrid 4 x 2, which did not appear in autosterile line 8 (Fig. 9).

Esterase activity was detectable in the extreme tip of the stigma of line 8, but was concentrated below the tip in the autofertile lines. An exudate was observed on the tip of stigmas of the autofertile material, which stained strongly with Sudan III.

#### 4. EST Isoenzymes and Tissue Ontogeny

##### 4.1 Seed cotyledon development

Three faint isoenzyme bands and one intense band were detected 24 d after pollination (Fig. 5). The intense band lost activity throughout development, but two of the faint bands, at Rf 0.58 and Rf 0.64, present as major bands in all cotyledon extracts examined, increased in activity throughout. The final isoenzyme pattern was established 48 d through the 60 d development period, and thereafter only changes in band intensity occurred.

##### 4.2 Seed size, and position in the pod

Large and small seeds from the same pod, from lines 2 and 8 were compared to investigate the effect of seed size on EST and GOT zymograms. Variation in fresh weight had no effect on the isoenzyme patterns, except that intensity of band staining was higher in smaller seeds.

##### 4.3 Position of the pod on the plant

EST and GOT zymograms from the first and last pods set on plants of line 2 grown in greenhouse conditions were compared, and were found to be identical.

## 5. Environmental Effects

### 5.1 Nitrogen regime

#### 5.1.1 Total seed protein

Under the four contrasting nitrogen regimes, no differences in total proteins separated in acrylamide gels or SDS acrylamide gels were found.

#### 5.1.2 Seed EST and GOT

Zymograms were similar for all nitrogen treatments, except that changes in band intensity were observed for the bands at Rf 0.34 in line 4 and at Rf 0.26 and Rf 0.54 in line 10 (Fig. 10).

#### 5.1.3 Pollen EST

Zymograms were qualitatively identical for all treatments (Fig. 10).

#### 5.1.4 Total leaf protein

Protein banding patterns were similar for all treatments except (low N Rh-), where three additional bands appeared (Fig. 11).

#### 5.1.5 Leaf EST

Isoenzyme banding patterns were subject to variation depending on nitrogen regime. An intensely staining additional band appeared at Rf 0.44, and two fainter bands, occurred uniquely in the (low N Rh-) treatment (Fig. 11). Although qualitatively the patterns for the other three treatments were similar, the staining intensity of bands varied considerably.

## 5.2 Growth environment

Fig. 12 shows seed and pollen EST zymograms from material grown under three different environmental conditions, and it can be seen that no qualitative differences in isoenzyme patterns were manifested. Similar results were obtained for GOT isoenzymes.

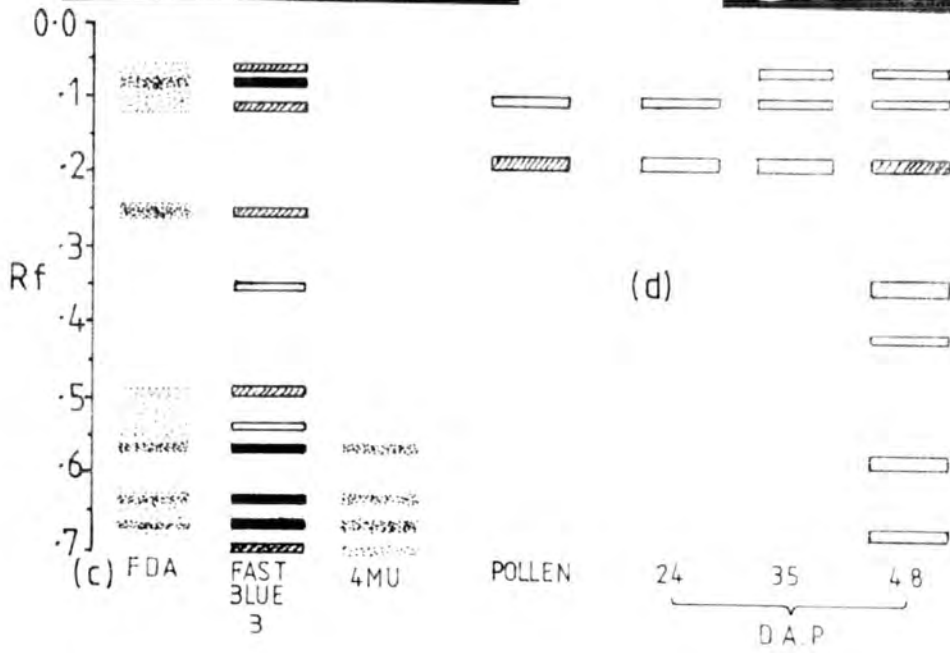
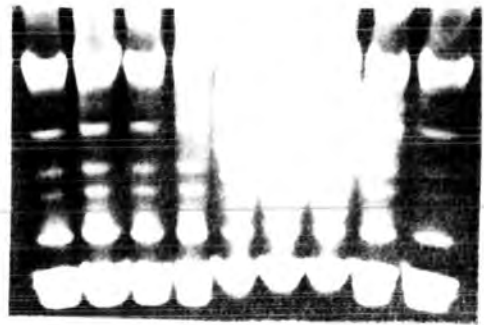
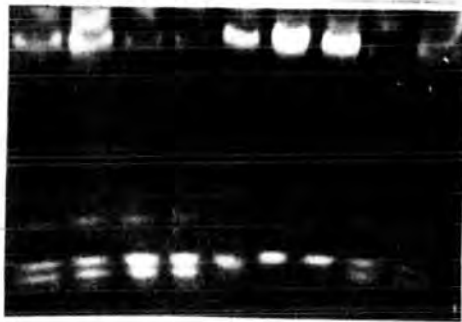
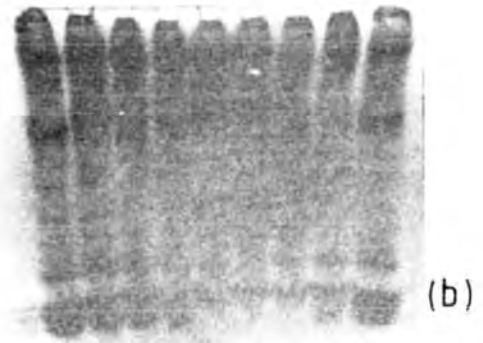
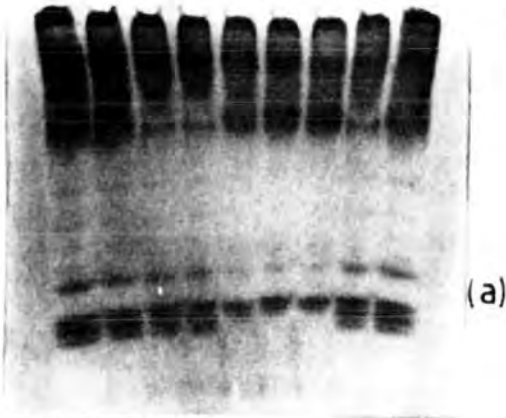
## 5.3 High temperature

Seeds of line 20, subjected in the glasshouse to a maximum daily temperature of 35°C for three days during the middle of the pod filling period had identical EST zymograms to those obtained from material grown in the field (Fig. 12).

Fig.1

- a) Staining of EST bands using the diazo dye Fast Blue RR.
- b) Staining of EST bands using the diazo dye Fast Blue B. Note the intense staining of bands only faintly resolved with Fast Blue RR, and the increased background staining. The upper photographs were made conventionally, using a photographic negative, whilst the lower ones were made by printing the gels directly onto photographic printing paper, and are therefore negative images.
- c) EST isoenzymes stained with Fluorescein diacetate (left), Fast Blue B (centre), and 4-methylumbelliferyl acetate (right).
- d) ACPH of pollen, and of seeds sampled 24, 35 and 48 days after pollination (DAP).
- e) LAP isoenzymes of seeds 24, 35 and 48 DAP, and of pollen.
- f) PER isoenzymes of an  $F_1$  hybrid and its two parental inbreds.

Alternative enzyme assays.



↓ +

↓ +

↓ +

↓ +

Fig.2

Effect of cotyledon excision on plant growth

- a) Two plants of V. faba var. minor, 50% of the cotyledon of the plant on the left was removed before sowing, leading to a reduction in growth rate.
- b) Two plants of V. faba var. major. Although 50% of the cotyledon of the plant on the left was removed, no depression of growth rate was apparent.

(scale in cm.)

Growth of plants after cotyledon excision.



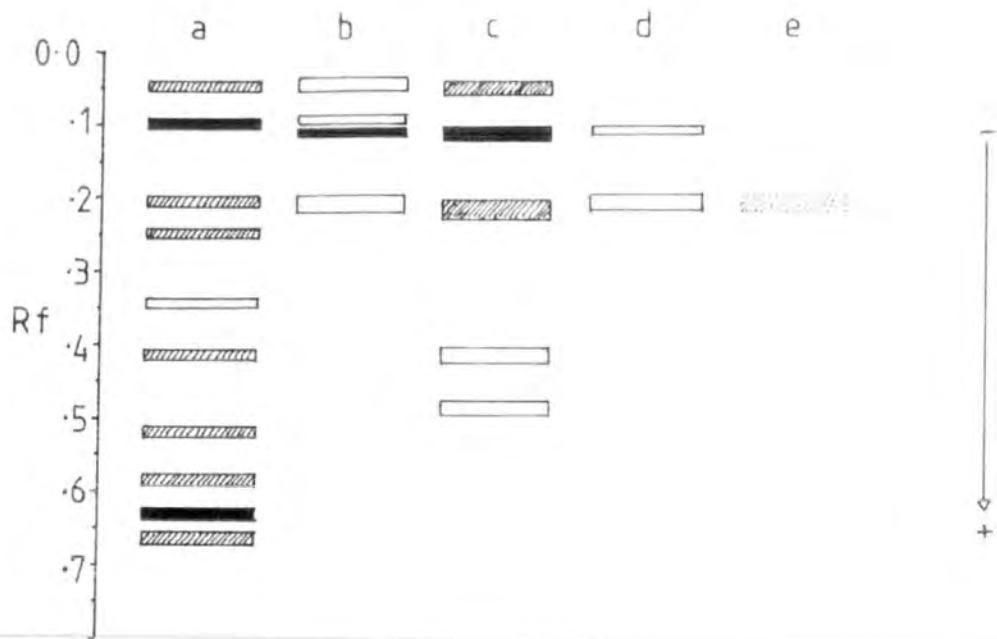
Fig.3

- a) EST isoenzymes in the albumin fraction.
- b) EST activity in the redissolved globulin, precipitated from dialysis against tap water.
- c) Major protein bands of the globulin fraction, stained with Coomassie Blue.
- d) EST activity in a purified globulin extract after three washings.
- e) EST activity in a purified globulin extract after six washings.

NEUCERE (1978) has reported aminopeptidase activity similarly associated with alpha, conarachin in peanuts.

- f) Effect of globulin contamination on resolution of EST isoenzymes Left: cotyledon extract in pH 8 borate buffer Right: the same extract, after 16 h. dialysis against pH 4.5 acetate buffer.

Esterase activity in the globulin fraction.



f

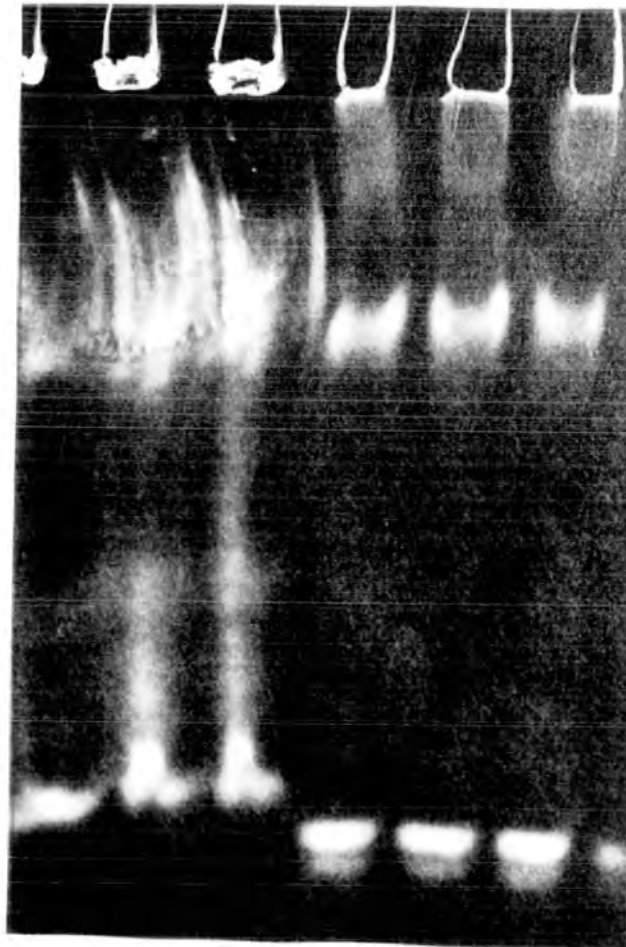


Fig. 4

EST isoenzymes from:

- a) Standard extract of inbred line 224.
- b) Urea-treated extract of line 224.
- c) Mixed standard extracts of lines 224 and 73.
- d) Mixed urea-treated extracts of lines 224 and 73.
- e) Biological hybrid 224 x 73.
- f) Urea-treated extract of line 73.
- g) Standard extract of line 73.

Open circle denotes possible artefact band, caused by urea treatment.

Closed circle denotes a possible hybrid band, derived from reassociation of enzyme subunits.

Esterase subunit structure.

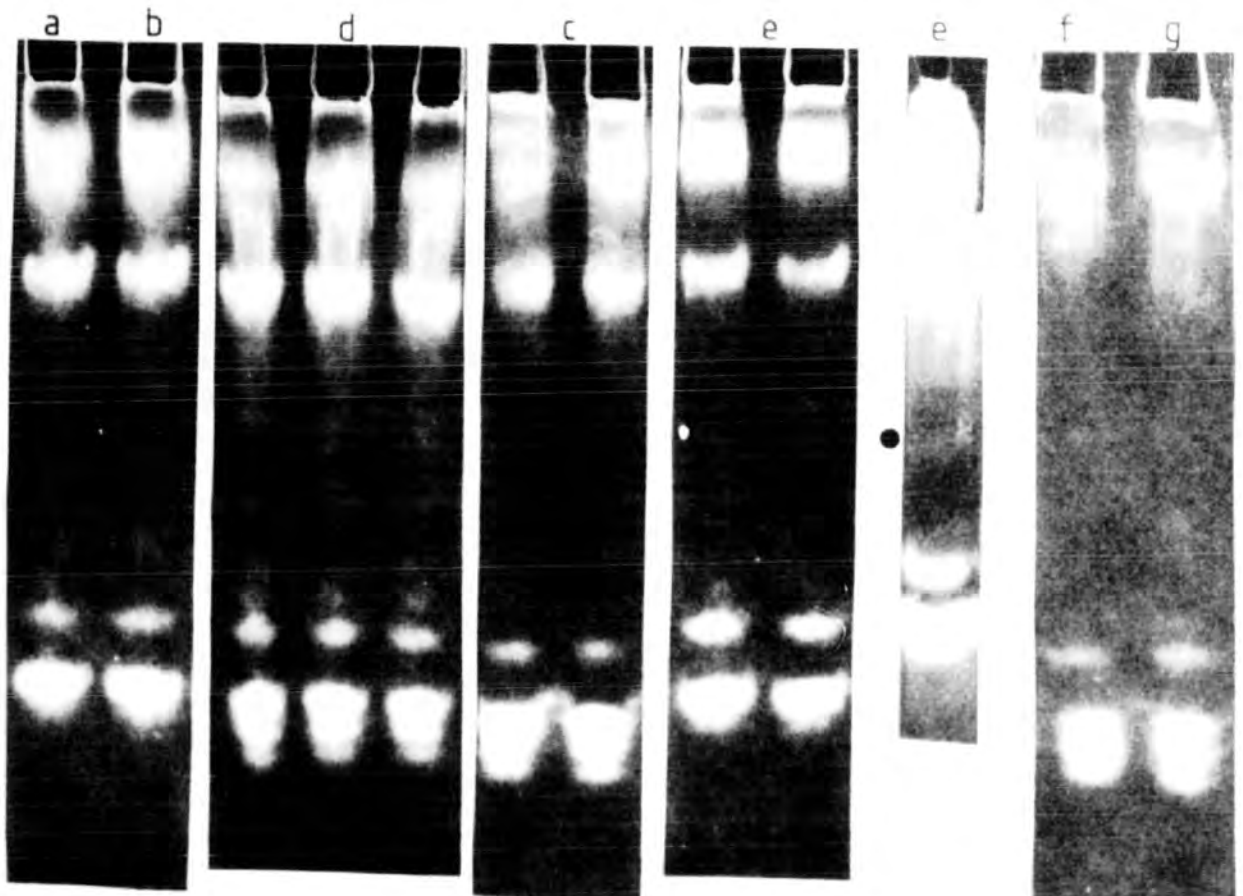
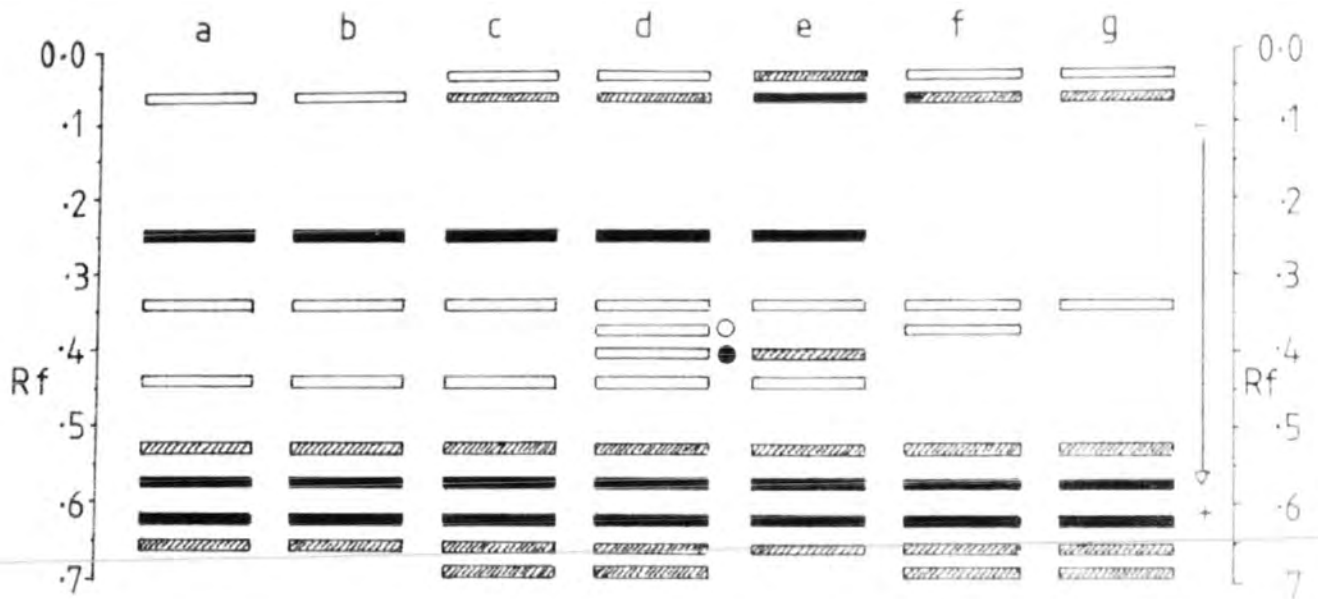


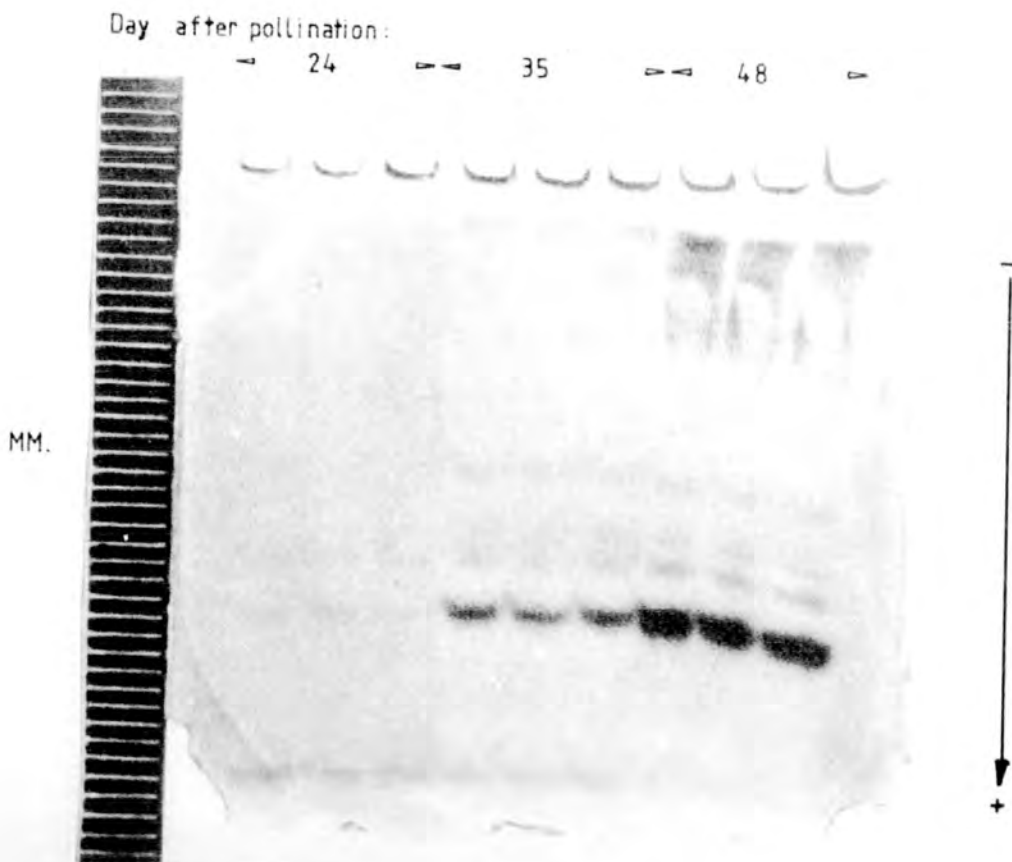
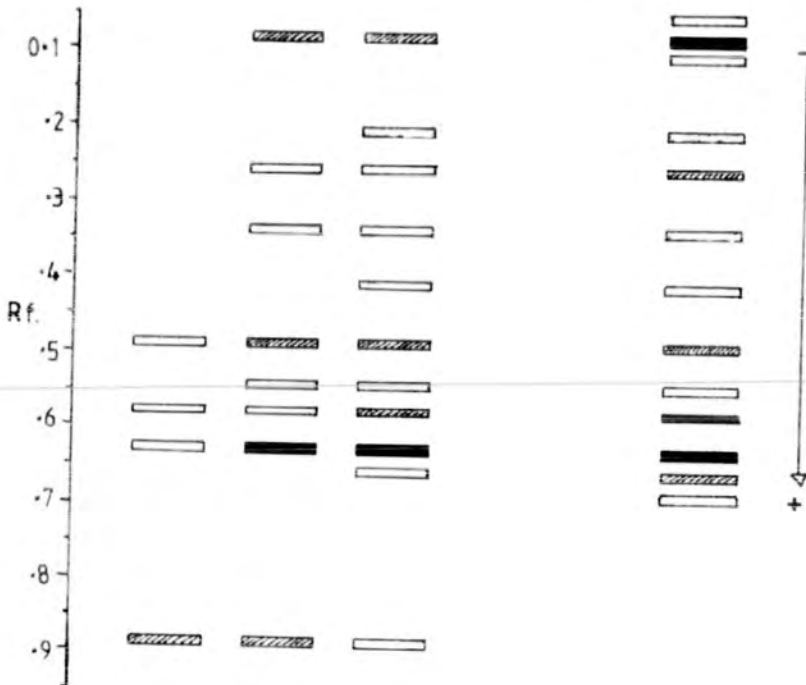
Fig.5

Cotyledon extracts from developing seeds. Standard extraction technique.

Note region of staining near the origin in 48 d. samples, which appeared as a red discolouration of the gel, and represents a reaction between phenolics and the assay solution. The reaction was first detected in 35 d. extracts. Phenolics are known to interact with peroxidases (SRIVASTAVA and VAN HUUSTEE 1977) but there is no evidence that they interfered with EST isoenzymes.

Change in esterase pattern during seed cotyledon development.

	<u>Line 3</u>			
Day after pollination	24	35	48	Mature seed, harvested and dried (12% moisture)
Fresh weight (g)	0.29	1.15	0.84	



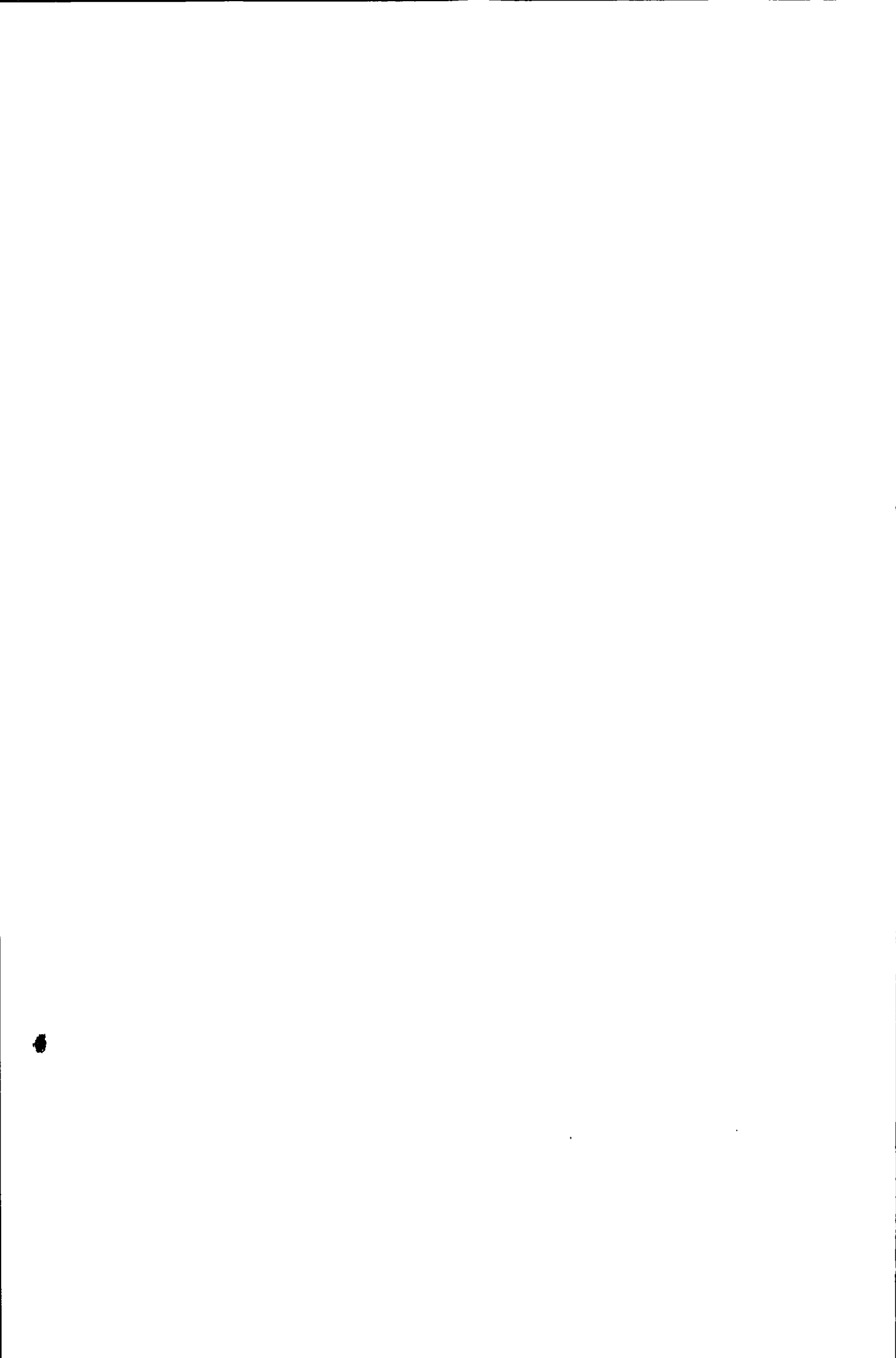


Fig.6

E = Embryo only

C = Cotyledon only

E + C = Extract of embryo and cotyledon tissue

*Non-specific esterases of Vicia faba seed tissues.*

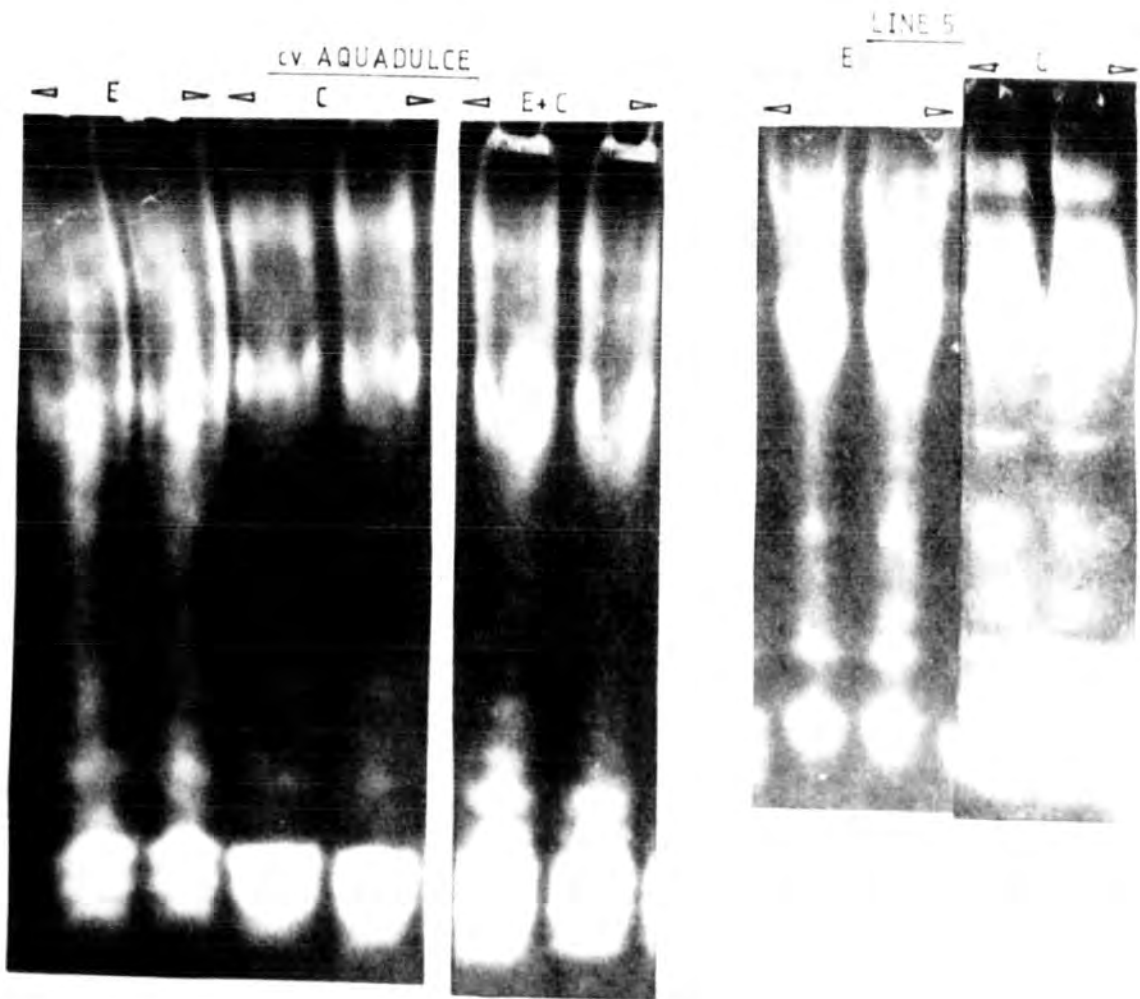
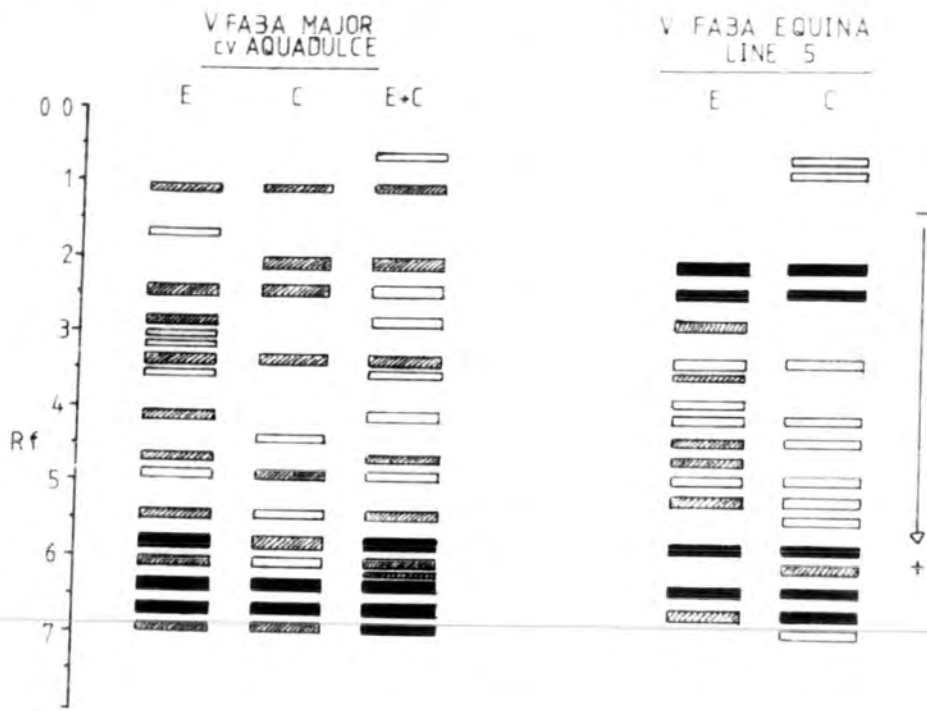


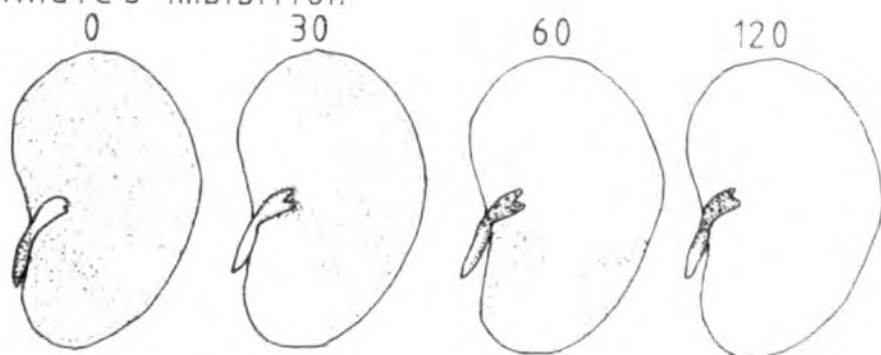
Fig.7

- a) Decline of EST activity (stippled area) during seed imbibition, measured by the reaction with alpha-naphthyl acetate/Fast Blue B.
- b) EST activity during seed imbibition, measured by the reaction with 4-methylumbelliferyl acetate, after 90 min. (upper cotyledons) and 180 min. (lower cotyledons). Embryos had low EST activity, and did not fluoresce. Note uneven EST activity in cotyledons of 180 min. imbibed seeds.

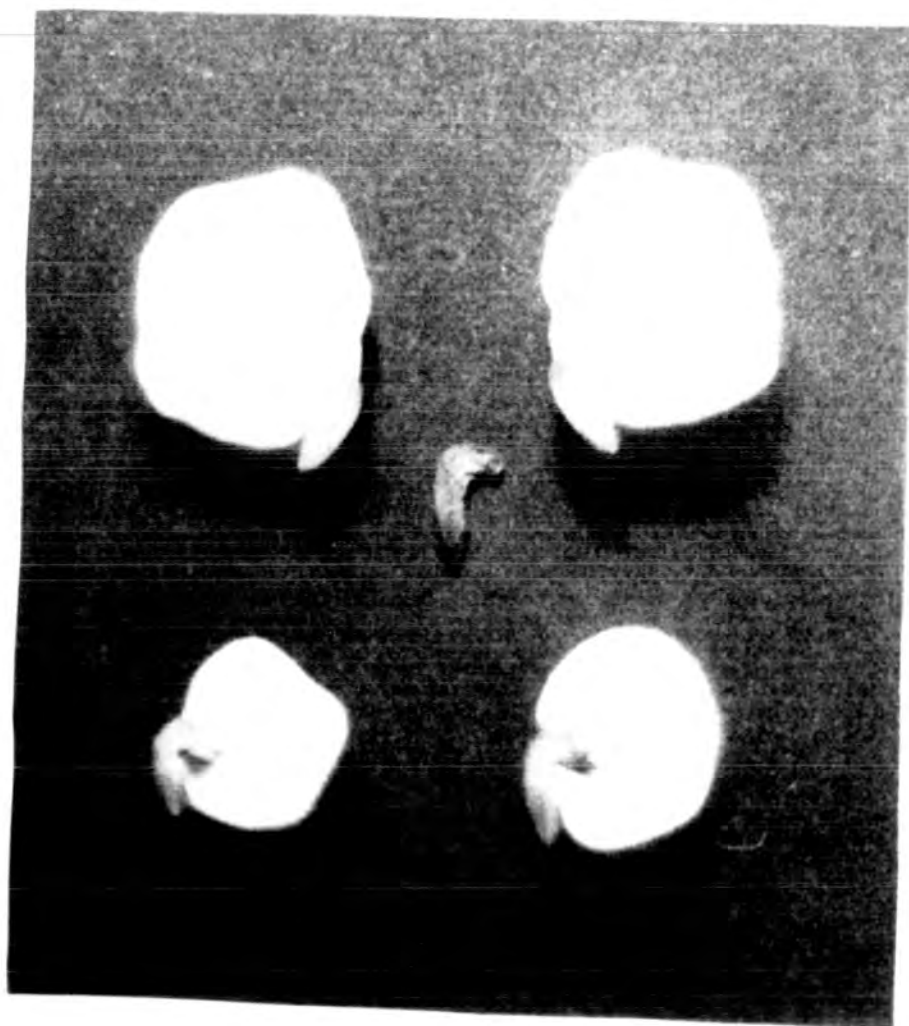
The testa was removed from all seeds before soaking.

Esterase distribution in the cotyledon

a. minutes imbibition



b.



- a) Precipitation of an insoluble diazo dye complex around pollen grains suspended in alpha-naphthyl acetate/Fast Blue B for 10 min. showing even diffusion of EST from pollen grain wall.
- b) Time course of EST isoenzymes diffusing into the sucrose medium from suspended pollen grains.
- c,d,e) Pollen grains stained for EST with alpha-naphthyl acetate/Fast Blue B for 1,3 and 5 min. showing EST activity localised in the pollen grain wall.

Fig. 8.

N.B. Because of difficulties with photographic reproduction, the staining intensities of pollen grains in (c) and (d) appear reversed.

Pollen EST histochemistry.

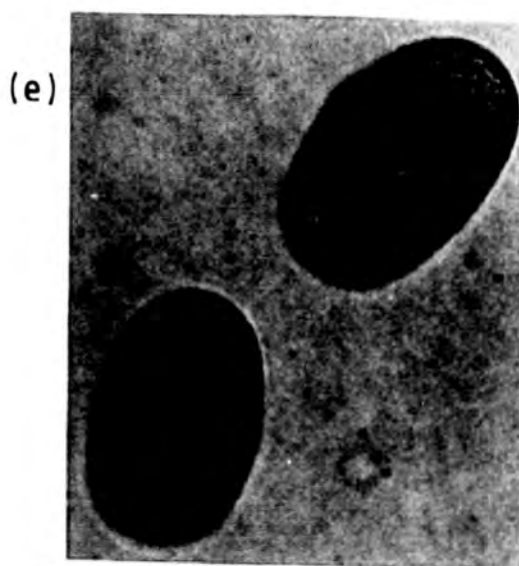
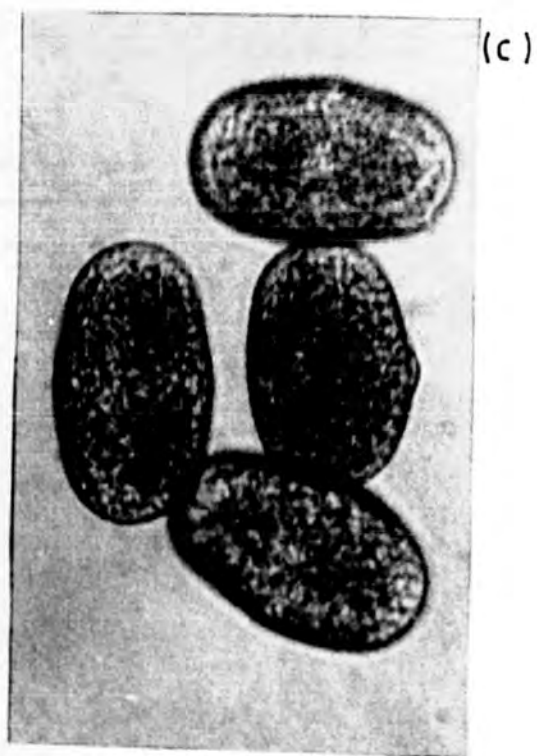
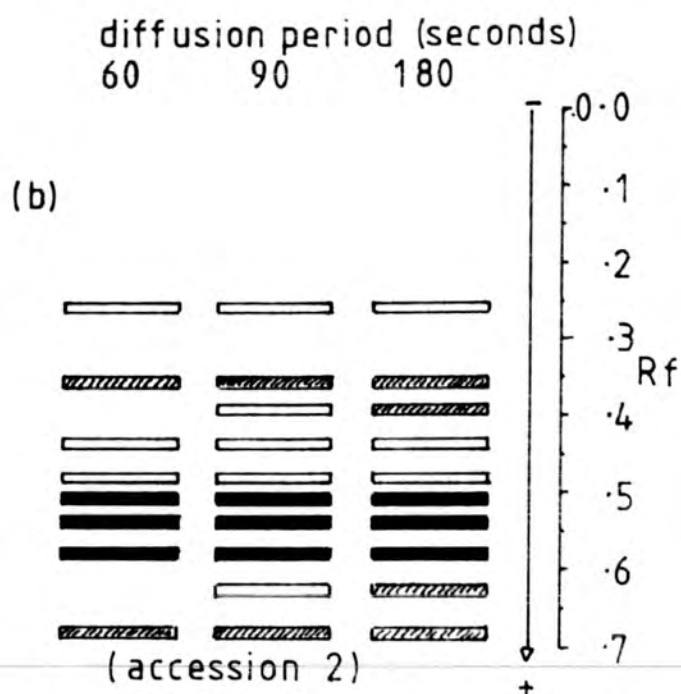
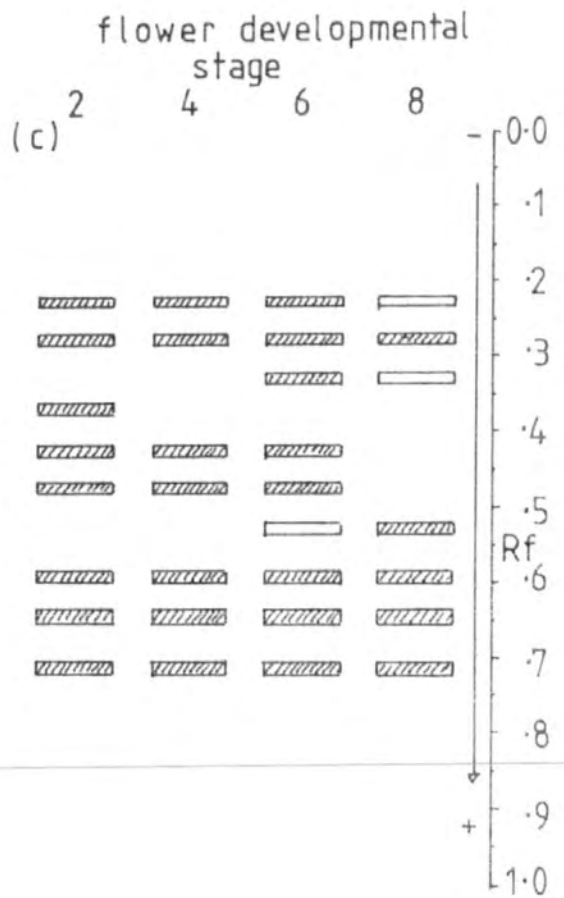


Fig.9

- a) EST activity in a V. faba stigma, detected in vivo using Fluorescein diacetate. Intensely fluorescent stigma tip denotes high EST activity. Little fluorescence was detectable in the stigma papillae.
- b,d) EST isoenzymes of the unpollinated stigma.
- c) EST isoenzymes of the gynaecium at four stages during flower development. Pollination occurred between stages 4 and 6.
- e) EST activity in stigmas of autofertile and autosterile lines during floral development.

Stigma EST histochemistry.

(a)



(d)

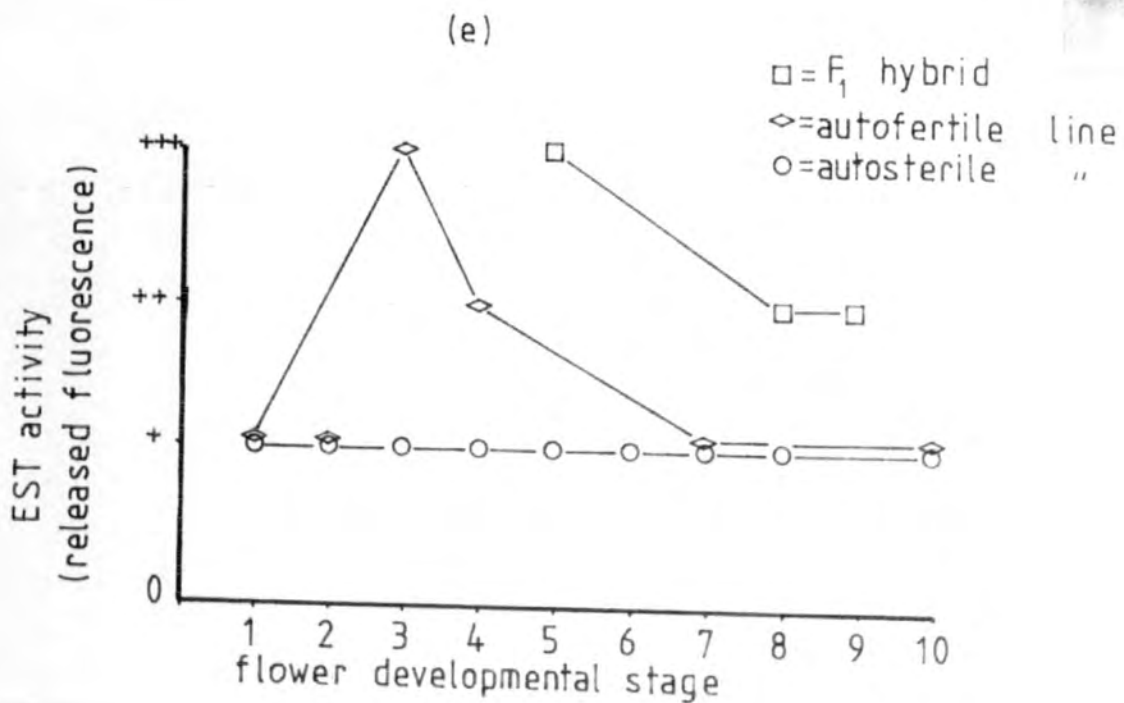


Fig.10

**Treatments:**

- A = nodules present, nitrogen feed supplied (+Rh + N)
- B = no nodules present, nitrogen feed supplied (-Rh + N)
- C = nodules present, no nitrogen feed supplied (+Rh - N)
- D = no nodules present, no nitrogen feed supplied (-Rh, low N)

*Effect of nitrogen regime on esterase patterns.*

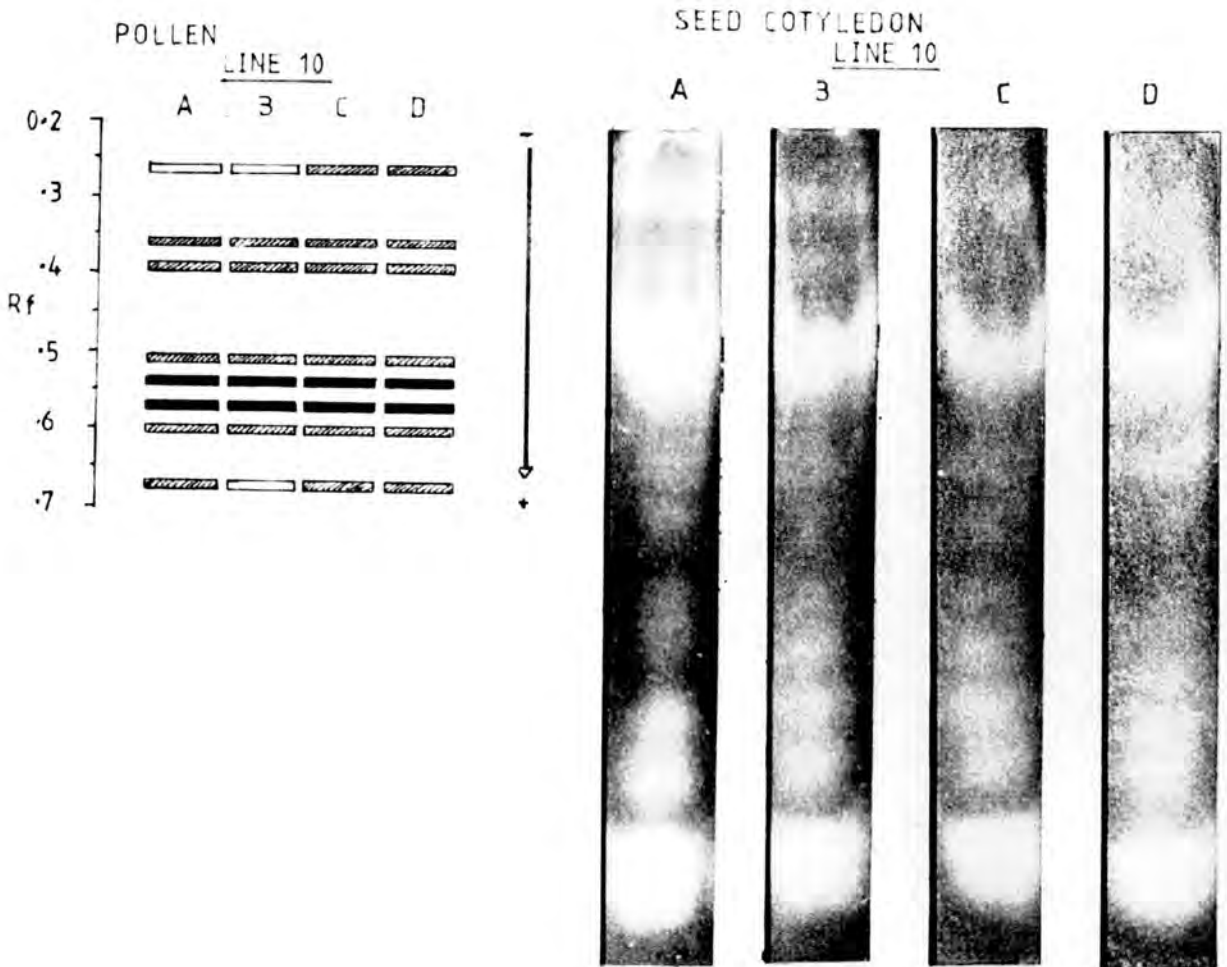
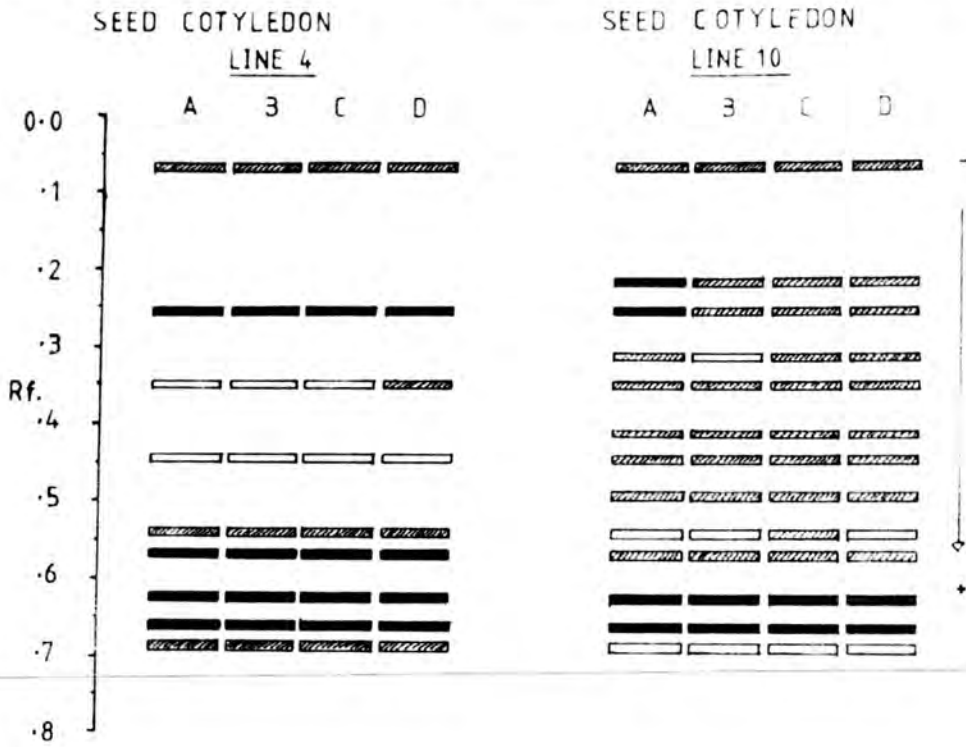


Fig.11

### Treatments

- a) Nodules present, inorganic nitrogen supplied (+Rh + N)
- b) No nodules present, inorganic nitrogen supplied (-Rh + N)
- c) Nodules present, no inorganic nitrogen supplied (+Rh - N)
- d) No nodules present, no inorganic nitrogen supplied (-Rh, low N).

All extracts developed on standard 7% gels.

Total albumin protein gels stained with Coomassie Blue  
Esterase gels assayed as for Fig. 15.

*Leaf proteins and nitrogen regime.*

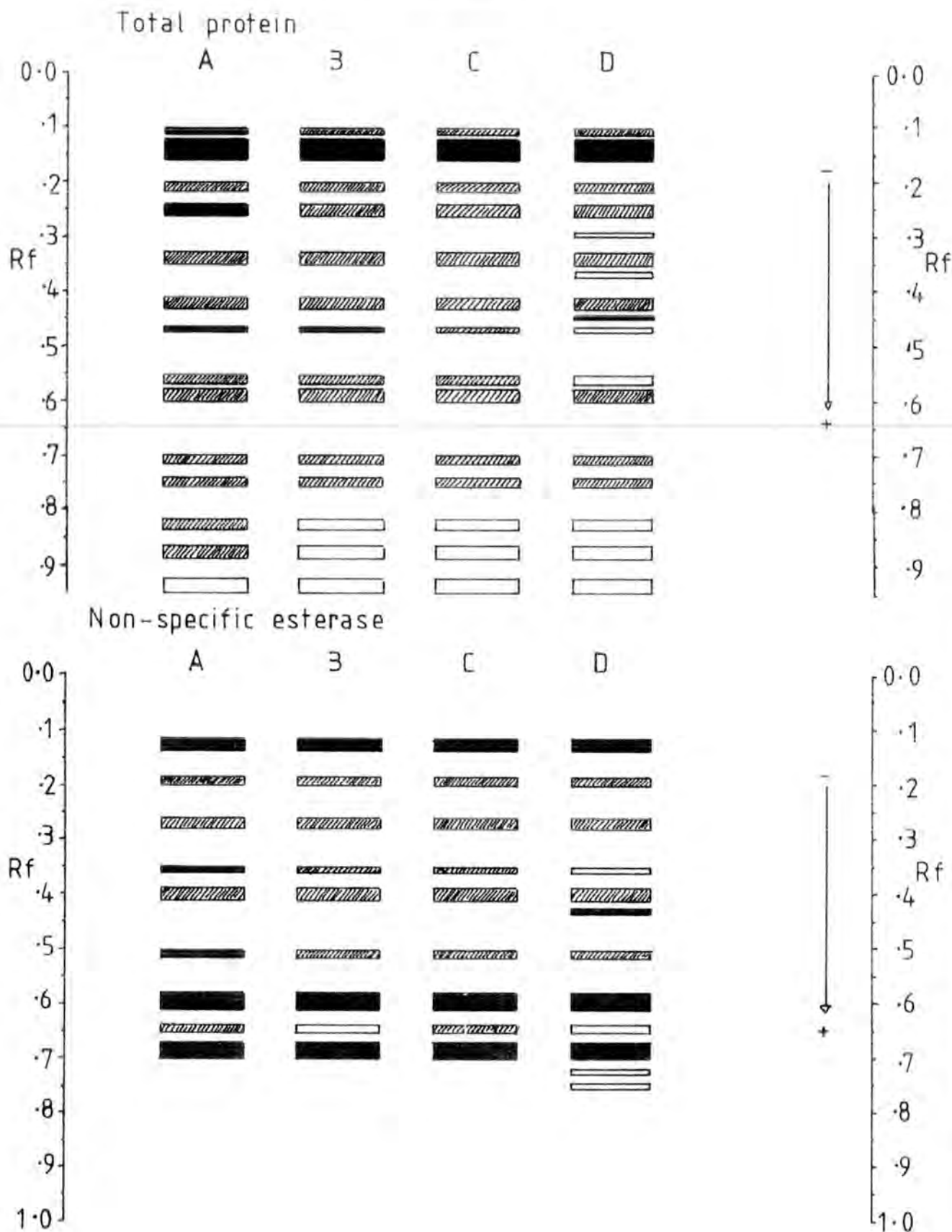


Fig.12

- a) Plants grown in the glasshouse
- b) Plants grown in the controlled environment cabinets
- c) Plants grown in the field
- d) Standard glasshouse conditions
- e) Plant subject to high temperature during pod fill

Effect of growth environment and temperature.

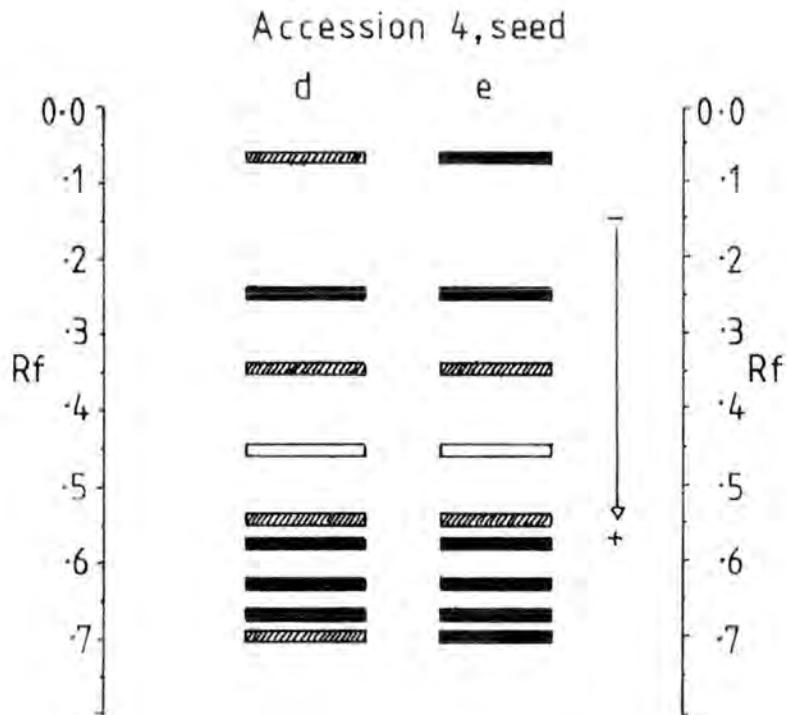
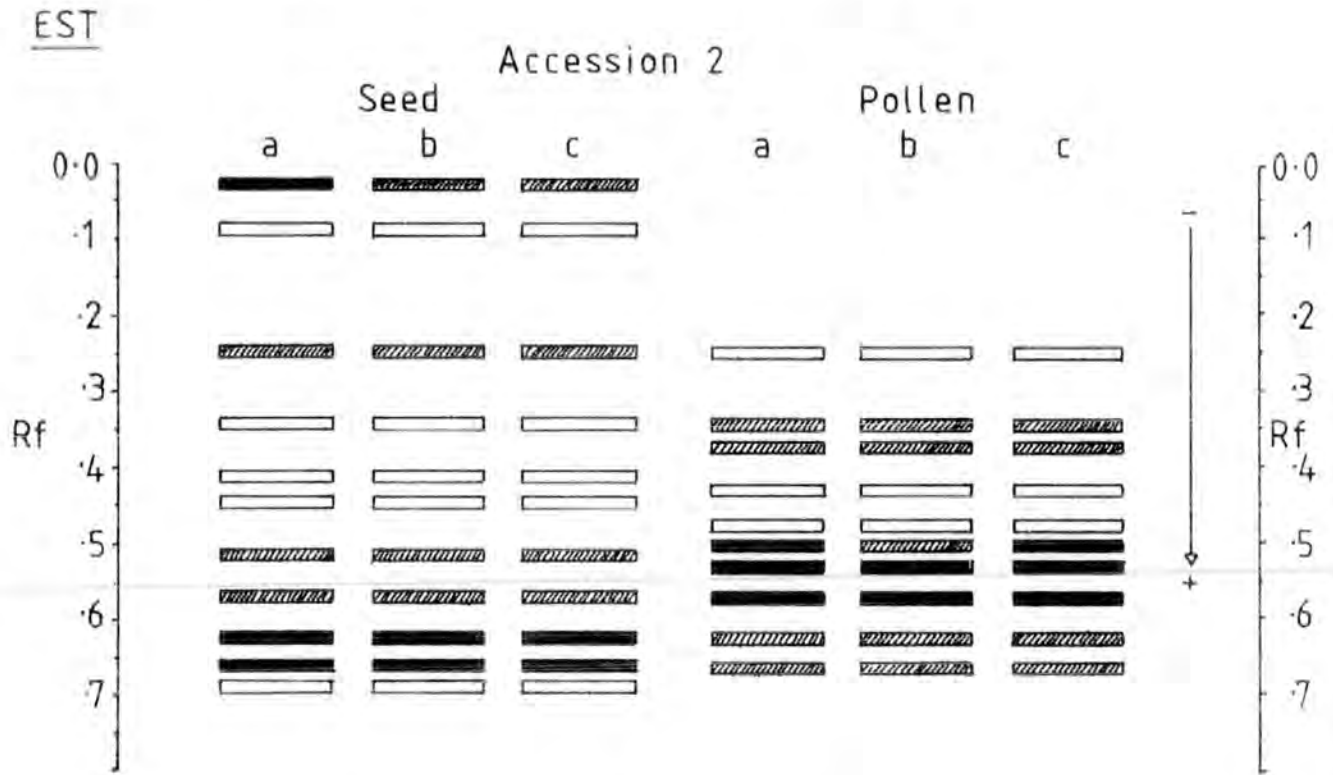


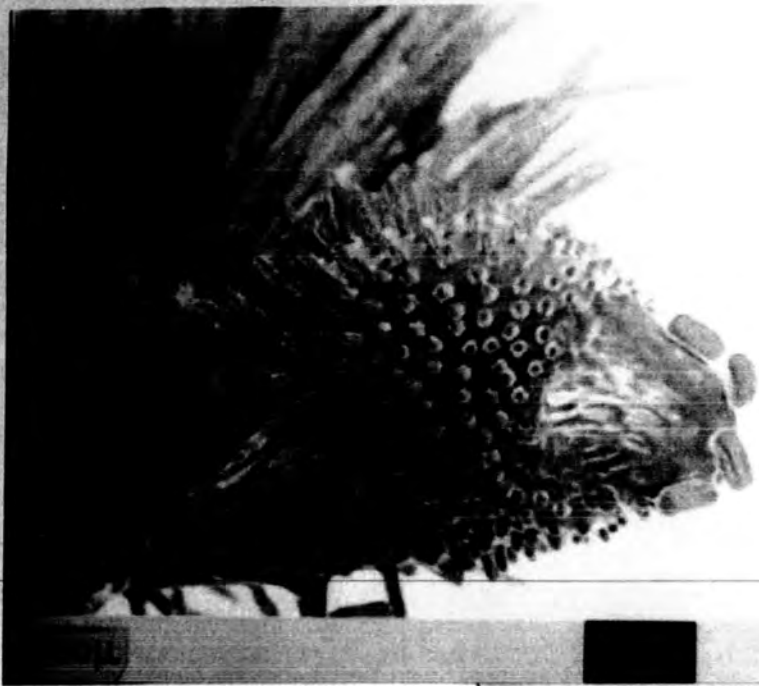
Fig.13

Uncoated specimens 7.5 kV

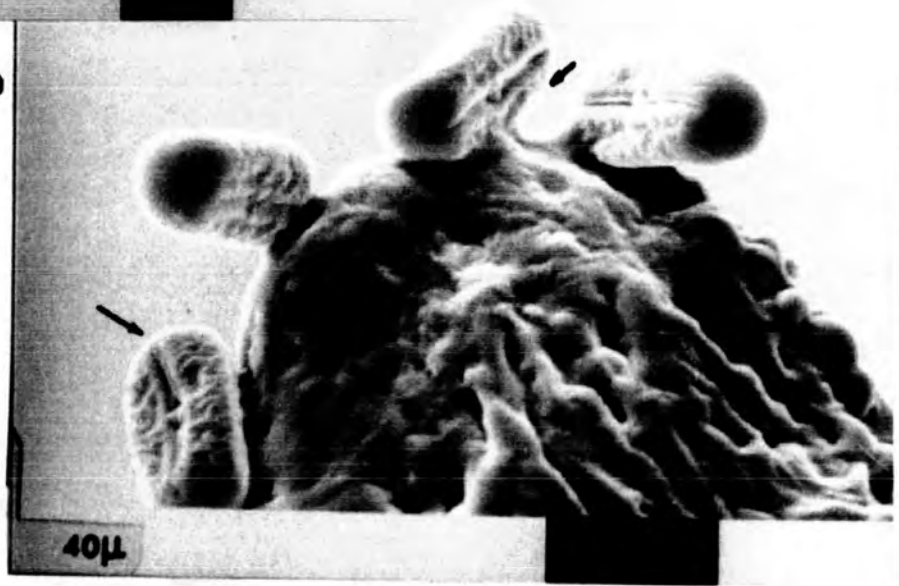
- a) Stigma of an autofertile line, showing pollen grains adhering to the secretion exuding from the stigma tip.
- b,c) Pollen grains embedded in the stigmatic exudate.

The stigmatic exudate.

a



b



c



Fig.14

Standard method employed for screening of genotypes. The procedure for quantitative extraction of albumins and globulins is described in Methods Section 8.4, and that for vegetative tissue in Methods Section 8.3

Standard extraction techniques.

DRY SEED



REMOVE  
TESTA



200mg.  
COTYLEDON



HOMOGENISE  
1 ml. 0.4M NaCl



CENTRIFUGE TO  
REMOVE CELL  
DEBRIS



DIALYSE  
SUPERNATANT  
AGAINST TAP  
WATER OVER-  
NIGHT.



CENTRIFUGE TO  
REMOVE  
GLOBULIN PPT.



ADD 20%  
SUCROSE (w/v) TO  
SUPERNATANT.



LOAD ONTO GEL

POLLEN  
5mg.



BURST IN 0.15ml.  
DISTILLED WATER



ADD 20% SUCROSE (w/v).



LOAD ONTO GEL

#### DISCUSSION: DEVELOPMENT OF THE METHOD

The finding that staining of the enzymes EST, LAP, PER, GOT and ACPH was most satisfactorily achieved on acrylamide gels at between pH 5.0 and pH 6.0 is in agreement with several published accounts of the pH optima of these enzymes. SAHULKA and BENES (1969) found that although variation in pH did not alter the relative staining intensity of V. faba EST Isoenzymes, the best zymograms were obtained at pH 5.0 when  $\alpha$ -naphthyl acetate was used as a substrate, and in the work reported here no EST fractions in the crude extract with distinct pH optima appeared. SAE, KADOUM and CUNNINGHAM (1971) reported that the highest specific activity of Sorghum EST was recorded in pH 5.5 sodium acetate buffer.

Although no measurements of internal pH conditions in gels were recorded during assay, the highly beneficial effects of pre-incubation in pH 5.0 acetate buffer, in terms of rapidity, uniformity and higher resolution of band staining, especially in thick rod gels, were evidence that depression of pH before assay was highly desirable. This was particularly important when assaying multicomponent isoenzyme systems such as EST, with short migration distances on the small gels, where satisfactory band separation could only be achieved if steps were taken to maximise resolution.

The variation of band staining intensity of EST isoenzymes, particularly of fainter bands, when different diazo dyes or sources of the same diazo dye were used, made standardisation of assay technique for

all intergenotype comparisons vital. SAHULKA and BENES (1969) and HUNTER and BURSTONE (1960) also reported differential EST staining, depending on stain used, and attributed this to selective inhibitors in the commercial preparations. Isoenzyme staining techniques were initially applied to starch gel systems, and when the published methods are transferred to starch gels experiments to determine the most reliable staining procedure are generally necessary, as in any genetic analysis of isoenzymes it is of the utmost importance to know whether enzyme activity is absent, or only present at low levels. To determine this, the most sensitive staining method available must be used.

Throughout this study a sample of a standard inbred line (accession number 5) was loaded onto one gel in every experiment, and the banding patterns and intensities of all genotypes tested were related to this. This also provided valuable data concerning reproducibility of technique, and in a total of fifty-one replications of line 5 zymograms, no significant variation in band pattern was recorded. For unambiguous comparisons length of assay period, assay temperature, and source of diazonium dye were standardised.

The use of fluorescent assay methods applied to simple, rapid screening methods of the kind described here was limited by their high sensitivity. Diffusion of enzyme within the gel and streaking of bands contributed to an unacceptable level of background fluorescence, and the subsequent requirement for purified extracts and extremely pure chemical constituents for acrylamide gel preparation was incompatible with the aims of speed and economy. However, the use of FDA as a substrate (GUILBAULT & KRAMER 1964) proved useful in that the acrylamide gel

could be printed onto an agar FDA slab to establish the EST zymogram, and could then be assayed for GOT. This was of value, since 7% acrylamide gel proved very difficult to slice horizontally without extensive damage, and could otherwise only have been assayed for one enzyme system, unlike starch slabs which may be sliced and assayed for several.

4-methylumbelliferyl acetate (4 MU) was used as a substrate to test the practicability of assaying the crude extract for esterases of differing substrate specificities, thus allowing more accurate identification of genotypes using only one enzyme system. 4 MU is one of the brightest fluorescent compounds known, and the use of a series of fatty acid derivatives to detect lipase activity has been described by JACKS and KIRCHER (1967). Only the three most mobile EST bands of line 3 stained with this substrate (Fig. 1), but because of the intense background fluorescence fainter bands may have been masked. 4 MU spontaneously decomposes at above pH 7.0, so that pre-incubation to depress the gel pH was essential, and during this operation enzyme diffusion within the gel may have contributed to its overall fluorescence. In spite of these drawbacks, the method of printing onto an agar/4 MU slab was the most successful for assaying EST on cellulose acetate membranes (GRUNBAUM 1976), as their immersion in a standard assay solution rapidly washed off the soluble enzymes.

Besides the highly variable EST patterns, pilot experiments showed that seed and pollen GOT isoenzymes would be of use in comparing genotypes. Again, choice of diazomium compound was found to influence the results. Fast Violet B salt is specific for oxaloacetate, forming a

chromogenic compound, but this is soluble in most solvents and cannot be used to provide a permanent record (BREWBAKER et al 1968). The substitution with Fast Blue B salt was found to stain the same bands in the gel as did Fast Violet B, but when the gels were shrunk and dried in methanol, additional bands appeared (Fig. 1). The cause of these artefacts was unknown, but their occurrence varied depending on seed genotype, and they proved to be of value in identifying contaminants in  $F_1$  hybrid seed (Table 9). With EST screening, two unidentified contaminants were discovered, but using GOT screening those contaminants could not be identified, due to their having the same GOT pattern as the female line 73. However, when the gels were shrunk in methanol, the artefact bands of two of the ten GOT samples clearly differed from the remaining eight, these two being the unknown contaminants detected by their EST patterns.

EST and GOT were judged to be the most useful isoenzyme systems for screening the genotypes in this study. The ability to distinguish  $F_1$  hybrid from inbred parent with PER patterns was potentially useful, and because of its almost universal occurrence in plant tissues this enzyme has been used by numerous investigators, but the poor resolution, low activity, and ephemeral nature of the staining reaction rendered it inferior to GOT and EST. SIEGEL and GALSTON (1967) found very little PER activity in dry Pisum sativum seeds, and activity in dry V. faba seeds was likewise low. This enzyme has been found to be sensitive to environmental, hormonal and disease effects in several studies, some of which are contradictory and have been criticised by SCANDALIOS (1974) for their lack of genetical analysis. While this is justified criticism,

it must also be pointed out that many genetic and taxonomic studies of isoenzymes have been carried out with scant apparent regard for the non-genetic factors which may control the expression of isoenzymes. The two approaches were inseparable.

The use of ACPH was eliminated as a possible screening method due to the very low activity, which rendered differentiation of genotypes impossible. MURRAY and COLLIER (1977) found ACPH activity to be highest in pea cotyledons before maturity, and this stage would be a more appropriate one to sample, but the attendant danger of confusion of genetic differences with ontogenetic changes would then be present. MURRAY and COLLIER (1977) found that early in seed development the activity of this enzyme was largely confined to the seed coat, which accounts for the low activity found in young seeds (Fig. 1), as the seed coat was not included in the extracts in this case.

BREWBAKER et al (1968) failed to find any alkaline phosphatase activity in a range of plant tissues, and neither was any found in V. faba tissues.

The finding of only one major band of LAP activity, at Rf 0.59, in dry cotyledons precluded the further use of this enzyme. Leucine aminopeptidase is known not to be specific for leucine N-terminals, and the enzyme as assayed in this study should accurately be called L-leucyl- $\beta$ -naphthylamidase. Its activity in this capacity has been measured during Pisum sativum development, and has been found to be at its lowest ebb in dry cotyledons; such tissue was therefore not expected to show

highly diverse isoenzyme activity (COLLIER & MURRAY 1977). The enzyme also proved difficult to assay on acrylamide gels, due to high levels of background staining.

The loss of enzyme activity with storage of seed meals was also noted by K. MUNTZ (personal communication), who found that prepared extracts maintained their activity far longer than meals. McCOWN, HALL and BECK (1969) stored extracts frozen at  $-20^{\circ}\text{C}$  for several weeks, and found no qualitative change in the activity of several enzymes, including PER and ACPH, whilst WALL (1968) also found that crude extracts of Phaseolus cotyledon could be stored at the same temperature for short periods without change in LAP isoenzymes. This stability is by no means universal, and GERBRANDY, SHANKAR, SHIVARAM and STEGEMANN (1975) found conversion between forms of potato phosphorylase when extracts were stored at  $4^{\circ}\text{C}$ , thought to be due to the release of endogenous proteolytic enzymes normally compartmentalised in the cells. Also, freezing and thawing has been used to dissociate tetrameric enzymes into their subunits (MARKERT 1963; SCANDALIOS 1965; HART 1971). The ability to store enzymes unchanged, either at low temperature or deep frozen, is of considerable value in a biochemical screening programme as it allows simultaneous examination of large batches of material and the most efficient organisation of time and materials.

The stability of the EST and GOT complements of seeds and pollen at low temperatures described in this study allows reference samples of these tissues to be retained for at least three years. SCHWARTZ (1960)

stored Zea mays endosperm for over a year at  $-20^{\circ}\text{C}$  with no loss of isoenzyme activity, and seeds stored under these conditions do remain viable for very long periods (HONDELMANN 1976; ROBERTS & ELLIS 1977).

It is well established that binucleate pollen, like that of V. faba (BREWBAKER 1967), can be stored frozen for long periods without serious loss of viability (KING 1965; JOHRI & VASIL 1961). In contrast trinucleate pollen, especially that of the Gramineae (GOSS 1968), rapidly loses viability with storage, and LINSKENS and PFAHLER (1973) recorded changes in amino acid composition of maize pollen after only a few days at  $2^{\circ}\text{C}$ , whilst BREWBAKER (1971) has described slow loss of PER and ACPH activity from stored pollen grains. Pollen storage can allow crossing programmes to be continued without the need to grow all parental material every year.

The appearance of a precipitate in thawed, dialysed salt extracts, and the concomitant loss of EST activity at Rf 0.2 and Rf 0.25 may represent residual globulin protein from incomplete dialysis, as these extracts were only dialysed against tap water. Water extracts also precipitated out protein when held at  $4^{\circ}\text{C}$  overnight. The globulin fraction was associated with EST activity in the Rf 0.2-0.25 region of the gel (Fig. 3), and as it precipitated out it may have carried EST isoenzymes with it, as a result of protein-protein interaction. Redissolved, dialysed precipitates from crude salt extracts showed the association of these with EST when they were separated on acrylamide gels, and even with highly purified extracts of globulin some EST activity could still be detected unless the globulin was thoroughly washed. This *in vitro* association of V. faba storage proteins and EST activity may be a sensitive measure of the purity of globulin extracts.

The sensitivity of protein and enzyme patterns on acrylamide gels to extraction method has been referred to by several authors (BARBER, WOOD & STEWARD 1967; MAKINEN & MACDONALD 1968), and in this study this largely depended on the extent to which the extractant dissolved other cellular constituents in addition to the albumins. A progressively more serious interference of the EST isoenzyme patterns with increase in globulin contamination was noted, and although EST differences between inbred lines were still apparent when large volumes of such contaminated extracts were loaded onto rod gels, dialysis of salt extracts to remove most of the globulin was a superior method for inter-genotype comparison. CHERRY and ORY (1973) also found it necessary to remove crude arachin from peanut extracts with ammonium sulphate precipitation and dialysis before EST could be satisfactorily resolved on acrylamide gels.

Subunit dissociation was carried out in an attempt to gain insight into whether any of the EST isoenzymes were dimeric or tetrameric, and to predict whether hybrid bands might be expected in heterozygotes. Dissociation by freezing and thawing in 1 M NaCl has been used to confirm genetic evidence that beef Lactate Dehydrogenase is a functional tetramer (MARKERT 1963), maize Catalase a tetramer (SCANDALIOS 1965) and Alcohol Dehydrogenase a dimer (HART 1971). Studying the EST-5 locus of Drosophila pseudoobscura BERGER (1974) found that hybridisation between monomers occurred spontaneously at 25°C, and still occurred slowly at 4°C. Freezing treatment of dialysed extracts failed to reveal any changes in isoenzymes banding patterns in V. faba, and so dissociation was carried

out using urea, and by freezing in sodium chloride. MARKERT and APPELLA (1963), working with LDH, lost enzyme activity after urea treatment, but in this study no loss of EST isoenzyme activity could be detected. One set of extracts was subjected to three cycles of freezing and thawing in 0.4 M sodium chloride. Zymograms from these treatments showed the presence of two new bands, in addition to those found in the biological hybrid (Fig. 4). An extra band found in the biological hybrid also appeared in the in vitro hybrid, suggesting that dimeric esterases were present in the extracts. Both dissociation methods gave similar results. The reasons for the appearance of additional in vitro bands can only be speculated on; it is likely that these may have resulted from induced conformational change or protease activity, and were artefacts caused by the treatments applied. The appearance of an additional band in the zymogram of inbred line 9 would tend to confirm this supposition. Other authors who have investigated the subunit structure of EST (SCHWARTZ 1960; NARISE & HUBBY 1966; BERGER 1974) have described dimeric esterase isoenzymes.

Tissue specificity of isoenzymes has been reported for a wide range of isoenzyme systems in a broad spectrum of plant tissues (BREWBAKER et al 1968; HALL, McCOWN, DESBOROUGH, McLEESTER & BECK 1969; GERBRANDY & VERLEUR 1971). JACOBSEN, SCANDALIOS and SCULLEY (1966) separated the developing barley embryo from the rest of the seed and showed that the total complement of PER isoenzymes was a composite of contributions from the embryo and the endosperm, and that the two tissues had very few PER isoenzymes in common. The finding in V. faba of differential EST isoenzyme complements between embryo and cotyledon confirmed the importance of sampling comparable tissues, especially if any conclusions as to the genetic control of the isoenzymes were to be recorded.

The Rf 0.57 and Rf 0.63 EST bands of seed cotyledon and pollen ran to the same position on the gel when extracts from the two tissues were co-electrophoresed, suggesting that they may be homologous isoenzymes, but without further biochemical analysis of the isoenzymes this cannot be considered proven (JOHNSON, 1977).

The basis for tissue specificity of isoenzymes is uncertain, but some studies have shown that tissues with different isoenzyme patterns grown in tissue culture and exposed to identical conditions develop a uniform pattern of isoenzymes (DAVIDSON 1964), suggesting that the cytoplasmic environment may cause differential gene activity or repression of isoenzyme activity. Isoelectric focussing PER patterns from tobacco tissue cultures indicated that a basic similarity in PER complement occurred in different tissues and organs, but that large quantitative differences occurred (RUCKER & RADOLA 1971). The focussing principle, which concentrates very small amounts of components at their pI allowed the detection of bands which by other methods would have passed unobserved, due to their low concentration in the sample.

The absence of EST activity in the Field Bean testa was also noted by FROSSARD and VOSS (1978). ACPH (MURRAY & COLLIER 1977) and L-leucyl- $\beta$ -naphthylamidase activity (COLLIER & MURRAY 1977) has been found in the seed coat of peas in the early stages of development, and high and low peroxidase activity in the testa of Glycine max has been used by BUTTERY and BUZZELL (1968) as a means of dividing cultivars into groups for identification purposes. Although no EST activity

was found in the testa of V. faba, testa extracts did induce a general red staining of the gel, caused by tannins reacting with the diazonium dye to produce a red complex. This reaction has been described by JENSEN and HAUG (1952) and HAUG and LARSEN (1958), who have used it in the taxonomy of the Phaeophyceae. The appearance of this reaction product after day thirty-five in the developing cotyledon (Fig. 5) suggests that some phenolics accumulate in this tissue from this stage of development onwards. This resulted in a region of red staining on the gels, but was never severe enough to warrant removal of phenolics from extracts of cotyledons before electrophoresis.

As EST enzyme activity was evenly distributed throughout the dry cotyledon (Fig. 7), sampling of particular regions of dry cotyledon was not necessary. In studies of EST isoenzymes of Pisum sativum FRANKEL and GARBER (1965) found that the two cotyledons possessed identical isoenzyme arrays, and this was later confirmed in Phaseolus vulgaris by WEST and GARBER (1967a). Since the same result was found when V. faba cotyledons were compared, about 25% of the upper portion of both cotyledons was taken as a standard sample.

The uneven disappearance of EST activity from the cotyledons during germination (Fig. 7) indicates that whole cotyledons should be sampled in germinating seeds. Using the substrate 4 MU the uneven distribution of EST during germination was less marked, which may have indicated qualitative changes in the isoenzyme spectrum, but more probably was a reflection of the higher sensitivity of this assay to low enzyme activity. In either case, EST activity measured in this way again decreased unevenly across the cotyledon faces during germination, and was found to be relatively low in dormant embryos (Fig. 7).

The necessity for only a small portion of cotyledon to be used for extraction allowed the remainder of the seed to be germinated and grown on for breeding purposes, an advantage also exploited by FRANKEL and GARBER (1965), WEST and GARBER (1967a), and WALL (1968). A large percentage of the cotyledon tissue could be removed without excessive depression of the reproductive potential of the plant. CHIN, NEALES and WILSON (1977) found that cotyledon excision in soya beans brought about a growth reduction in the first ten weeks after planting, and that the longevity of plants was extended through delayed leaf senescence. Similar observations were made <sup>on</sup> V. faba plants subjected to this treatment.

The developmental changes found in EST isoenzyme patterns found in V. faba cotyledons, parallel to those found by CHERRY and ORY (1973) in peanuts, underlined the importance of the choice of dormant tissue for the comparison of isoenzymes from different genotypes. The presence of the final isoenzyme complement after 48 d of the 60 d development period would allow the screening of material before maturity, and the increase in intensity of staining of enzyme bands during this later period of maturation probably merely reflected that the seed was dehydrating, and therefore, since extraction was carried out at a constant fresh weight to extractant volume ratio, enzyme concentration in the extract was increasing. A necessary additional precaution when comparing unripe seed tissues would be only to compare genotypes with similar overall ripening periods, as between early and late maturing genotypes chronological and physiological development would be unrelated. NIJENHUIS (1968) found that ACPH zymograms of Brassica seeds could be compared

before maturity, and the finding of MURRAY and COLLIER (1977) that the activity of this enzyme is highest just prior to seed maturity makes sampling at this stage desirable for this enzyme. Accounts in the literature show that other enzymes, including PER (SIEGEL & GALSTON 1967) and LAP (SCANDALIOS 1965; COLLIER & MURRAY 1977) have low isoenzyme variability and activity in dry seeds, and the latter enzyme has been reported by MIKOLA and KOELEHMAINEN (1972) to be characteristic only of actively growing tissue in barley. This represents the major disadvantage in choosing dry seed tissue for screening, and is one which must be balanced against the absence of ontogenetic change.

Allied to developmental stability, and of paramount importance in the identification of material on the basis of isoenzyme polymorphism, is the question of environmental influence. V. faba seeds and pollen from plants grown under contrasting nitrogen regimes, and in a range of environments, showed only slight quantitative changes in isoenzymes under these conditions; insignificant differences compared with genotypic variation (Figs. 10, 12). This stability of some seed proteins to environmental perturbations has also been described by JOHNSTON, BREWSTER and DAVIES (1977) for the influence of nitrogen treatments on pea proteins, and by ADRIAANSE, KLOP and ROBBERS (1969) for the effect of nitrogen fertilisation, climatic conditions and soil properties on Phaseolus vulgaris proteins. Seed EST patterns of wheat (MENKE, SINGH, QUALSET & JAIN 1973) and peanut (CHERRY & ORY 1973) from different environments and harvest years have also been shown to be constant.

Arguments against seed composition being affected by plant nutrition and environment have been advanced by DUNNILL and FOWDEN (1965). No similar published data appears to exist for pollen, but it seems unlikely that the gametophyte, which matures within a nurse tissue, would be highly sensitive to environmentally-induced compositional changes which might prejudice its viability.

In contrast numerous studies have revealed that in vegetative tissue, and in leaves in particular, isoenzymes are not only subject to pronounced developmental change (SIEGEL & GALSTON 1967; HART & BHATIA 1967; HAMILL & BREWBAKER 1969; McCOWN et al 1969; PAWAR & GUPTA 1975; THOMAS & BINGHAM 1977), but are also sensitive to environmental fluctuation. WILKINSON and BEARD (1972) found that for cultivar identification in Agrostis palustris and Poa pratensis it was essential that plant material be grown under uniform temperature, light, moisture and nutritional conditions prior to leaf sampling. Such requirements are often difficult to fulfil and render large-scale screening uneconomic.

MATSUMOTO, OKAMURA and TAKAHASHI (1976) found that only a 4 d period of nitrogen stress was sufficient to induce changes in the activity and isoenzymic composition of cucumber leaves. Reference to Fig. 11 shows the extent of leaf protein and EST variation induced by nitrogen regimes. The pattern of change observed in leaves may represent the onset of senescence, induced by nitrogen deficiency. The lack of nitrogen may have resulted in early mobilisation of metabolites from the leaves sampled at the end of the vegetative period of growth, to meet the requirements for reproductive growth,

so that in the absence of adequate root-derived nitrogen, nitrogenous compounds were recycled within the plant. ABU-SHAKRA, PHILLIPS and HUFFAKER (1978) have reported observations which lend support to this contention. They found that soybean lines which exhibited prolonged nitrogen fixation also showed a delay in leaf senescence, thus linking this with nitrogen stress. There is also considerable evidence that nodulated plants show a restricted rate of growth compared with those supplied with mineral nitrogen (RYLE, POWELL & GORDON 1978), partly due to the respiratory burden imposed by nodulated roots consuming 10-15% of assimilates. Nodulated plants exhibited earlier leaf senescence than plants fed with nitrate, according to these authors. LAWES, MYTTON, EL-SHERBEENY and SORWI (1977) have also reported superior performance of plants fed with inorganic nitrogen, compared with those depending on nitrogen fixation, and have also described very specific V. faba plant variety x Rhizobium strain interaction. Since all the available evidence indicated dependency of leaf physiology on nitrogen status in legumes, leaves were not considered suitable material for further use in this study.

Leaf isoenzymes are also known to be affected quantitatively (SIEGEL & GALSTON 1967; JAIN, TALWAR, SOPORY & GUHA-MUKHERJEE 1978), and qualitatively (KARATAGLIS 1977) by light conditions during development, and WARNER and UPADHYA (1968) reported changes in EST and LAP in Citrus shoots dependent on daylength. The activation and repression of leaf isoenzymes by hormones has been discussed by GALSTON (1967), and ethylene was shown by HERRERO and HALL (1960) and MORGAN and FOWLER (1972) to modify peroxidase isoenzyme patterns in cotton leaves.

Besides developmental changes in leaves already alluded to, EST isoenzymes from legumes root nodules also change during ontogeny (FOTTRELL 1968), and root and leaf ACPH isoenzymes vary quantitatively and qualitatively depending on levels of lead in the soil (MAIER 1978).

Evidence concerning the effects of disease has also been accumulating (SHANNON 1968; PEIRCE & BREWBAKER 1973), and peroxidase, the enzyme most commonly investigated in this connection, shows compositional changes after infection very similar to those found in wounded tissue. In some instances, isoenzymes extracted from infected tissue have been found to originate from the pathogen as well as the host tissues (STAPLES & STAHMANN 1963), so that any material selected for sampling must be free from fungal and bacterial infection.

Seasonal variation in isoenzyme patterns has been investigated in several perennials, including Juniperus scopulorum, where KELLY and ADAMS (1977) found that EST patterns showed qualitative changes correlated with seasonal growth. Similar results were obtained by McCOWN et al (1969) with leaves of Dianthus species, where seasonal changes in the form of lowering of temperature led to the loss of EST bands. This kind of variation means that care must be exercised when comparing populations to assure phenological similarity. No similar changes have been reported in the leaves of V. faba, and this factor was not investigated in this study, but it would assume some importance if the leaf enzymes of winter beans were to be sampled, as these might be affected by the intervening cold period between emergence in autumn and resumption of growth in spring.

Effects of temperature are much less apparent in seeds, and ELLIS and BEMINSTER (1977) detected no change in wheat gliadin banding patterns after subjecting seeds to temperatures in the range 30-100°C. Stability in seed isoenzyme patterns was described by PAPACHRISTOS and JOHNSON (1977), who found no change in V. faba cotyledon  $\alpha$ -amylases subject to high temperature. In this study, no effect of elevated temperature during the middle of the pod-fill period could be discerned on V. faba seed EST and GOT (Fig. 12).

In 1967 HART and BHATIA concluded that leaf tissue was not an ideal material for interspecific isoenzyme comparisons, finding that in Nicotiana species soluble protein patterns were profoundly influenced by environmental conditions, and that leaves collected from plants of the same genotype, raised under different environmental conditions, showed variability in peroxidase patterns which even transcended interspecies variability. Reference to Table 1 shows that leaf proteins and enzymes have continued to be used extensively for cultivar identification. Unless environmental conditions and stage of sampling are carefully standardised there is clearly a risk of error, and because of this dormant seeds are a superior starting material. If these fail to exhibit sufficient isoenzyme variability, for reasons already outlined above, or if the seeds are too small to allow sampling without their complete destruction, pollen represents an excellent alternative. The pollen from about fifteen V. faba flowers (c. 5 mg) was found to be ample for the preparation of extracts, but with careful handling pollen EST zymograms could be prepared using the pollen from only two flowers.

BREWBAKER (1971) has published an extensive list of enzymes reported to be active in pollen grains, and in V. faba extraction of pollen was found to be particularly straightforward, as simple osmotic rupturing of pollen in suspension yielded enzyme extracts of high activity.

Attempts to follow the diffusion of EST isoenzymes out of the pollen grain met with very limited success, as this occurred with great rapidity. MAKINEN and BREWBAKER (1967), using Oenothera organensis pollen detected changes in EST isoenzymes diffusing into the medium over a period of 19 h., but in view of the findings of HESLOP-HARRISON, KNOX and MATTSON (1974) that pollen proteins, particularly those held in the exine, are released from the pollen grain within minutes of hydration, it seems probable that they also observed the products of protein synthesis. It was hoped that from observations of diffusing EST isoenzymes, those of sporophytic origin held in the exine (HESLOP-HARRISON 1975) could be distinguished from those synthesised in the haploid gametophyte and residing in the intine and cytoplasm (KNOX & HESLOP-HARRISON 1969, 1970), but release of EST isoenzymes was so rapid that the late-appearing components can only be tentatively proposed as being of gametophytic origin. Microscopical observations showed that although highest EST activity appeared to be associated with the colpus and germination pore, diffusion of enzyme activity occurred evenly from the whole surface of the pollen grain (Fig. 8). That esterase activity was present in the pollen cytoplasm was confirmed by staining with FDA, by the method of HESLOP-HARRISON and HESLOP-HARRISON (1970). Fluorescence enzymatically-released within the cytoplasm was retained in the pollen grain by the cell membrane while EST activity in the pollen wall was lost to the medium.

EST activity has been described as an almost invariable feature of stigma surfaces (HESLOP-HARRISON & SHIVANNA 1977). The differential EST activity observed in stigmas of autofertile and autosterile lines (Fig. 9) may be linked to the time of secretion of the stigmatic exudate, which has been shown to control the expression of the autofertility character (PAUL, GATES, HARRIS & BOULTER 1978). Some EST activity was found to be associated with this exudate when stained with Fast Blue B and  $\alpha$ -naphthyl acetate, but the intense staining with Sudan III revealed that it was largely lipidic. HESLOP-HARRISON, HESLOP-HARRISON and BARBER (1975) found very high EST activity dispersed in the surface stigma exudates. The evidence from V. faba was that most EST activity resided within the stigma, but since large vesicles of the exudate form in this tissue (HARRIS, PAUL, GATES & BOULTER, unpublished), the EST activity could have been confined in this secretion. At least three EST isoenzymes occurred in the stigma (Fig. 9).

The role of pollen and stigma esterases is still a matter for speculation. LEWIS (1965) proposed that the recognition event between the pollen and stigma involved the formation of a tetrameric molecule from dimeric precursors in pollen and stigma, and esterases have been considered for this role, but the experiments of NISHIO and HINATA (1977) have indicated that this is unlikely. The spectrum of EST isoenzymes present in pollen and stigma may have individual roles associated with pollen germination and pollen tube growth through the stigma surface. AHOKAS (1976) described a barley pollen EST specific for hydrolysing the sporopollenin at the germination pore, and other pollen esterases might act in a complementary fashion with stigma EST

to bring about lysis of the stigma cuticle by the pollen tube, thus constituting an active cutinase of the type recently demonstrated in Tropaeolum majus pollen by SHAYK and KOLATTUKUDY (1977). CHRIST (1959) first suggested that pollen cutinases were necessary for stigma cuticle penetration, and this hypothesis has recently been modified in the light of more recent evidence by HESLOP-HARRISON (1975), who considered that an unspecified stigmatic factor might enhance the activity of a pollen-borne cutinase.

The role of the stigma exudate in V. faba is uncertain, but one possibility is that it represents a hydrophobic blanket over the stigma, preventing desiccation, and under which enzymic reactions between pollen and stigma might occur. KONAR and LINSKENS (1966) demonstrated a thin film of water under the stigmatic exudate of Petunia hybrida and favoured this explanation. Evidence for this comes from the observation that V. faba pollen does not germinate on stigmas in the absence of exudate, even when close contact occurs between the germination pore and stigma surface. However, attempts to induce pollen germination on immature stigmas under a biochemically inert layer of silicone oil, to simulate the exudate, failed, so that some active constituent of the secretion may be involved in pollen germination, and CHEN and UEDA (1977) have recorded an in vitro stimulation of pollen germination by Narcissus stigma extracts which might support this contention. Petunia pollen enzymes are apparently unable to degrade stigma exudates (KONAR & LINSKENS 1966).

The changes in EST isoenzymes with flower development recorded in Fig. 9 may be of use in defining stages of flower development for physiological studies, complementing the system devised by PAUL (1977). BREDEMEIJER (1973) recorded changes in PER isoenzymes, analogous to those shown for EST in Fig. 9, during growth and senescence of unpollinated styles of tobacco plants. The changes in EST pattern noted after pollination are similar to those for the activity of a range of enzymes described by ROGGEN (1967), who found a "wave of enzyme activity down the style" preceding the growing pollen tube, and activation of a stylar enzyme (GDH) by a pollen component.

After considering the results discussed here, a standard method of pollen and seed enzyme extraction was evolved (Fig. 14), and this was used in conjunction with carefully defined assay conditions for the screening of genotypes described in the following section.

## RESULTS

### 6. Zymograms of V. faba inbreds and F<sub>1</sub> hybrids

#### 6.1 Seed EST

Of twenty-nine inbred lines screened, all could be identified on the basis of their seed EST patterns (Fig. 15, Table 7), with the exception of accessions 5 and 10, 22 and 23, and 13 and 14, which were very similar pairs. Accessions 5 and 10 were subsamples of the same inbred line, this being a common component of the two synthetic varieties. 22 and 23 originated from two plants of the same inbred progeny, from material which had been selfed over a number of generations, and were expected to be similar. Accessions 13 and 14 were male fertile and male sterile versions respectively of the same inbred line, and did not differ significantly in their EST patterns.

A total of eighteen esterase bands were found in all lines examined, the number present in any one line ranging from 5 to 13.

Accessions 4 and 7 were subsamples of the same inbred line, but because of the presence of a contaminant, the (presumed) genuine line 224 and the contaminant were maintained separately, and labelled as accessions 4 and 7 respectively.

A diffuse area of staining in zymograms of accession 43 occurred between Rf 0.1 and 0.2, and was treated as an artefact.

## 6.2 Pollen EST

The lines which could be identified and those which were similar pairs were essentially the same as those for seed EST. The two monomorphic pollen EST bands occupied the same position in the gel as those in seed zymograms (Fig. 16, Table 8).

Pollen from a yellow-flowered mutant of accession 18 had an identical zymogram to a normal-flowered plant of the same inbred line.

A total of 17 clearly-staining bands was observed in all inbred lines examined, the number present in any one line ranging from 4 to 12.

## 6.3 Seed F<sub>1</sub> hybrid EST

Seed zymograms of F<sub>1</sub> hybrids essentially displayed the sum of the bands of the inbred parents, but some differences were observed in reciprocal crosses (e.g. 2 x 4, 4 x 2, 7 x 9, 9 x 7). The reciprocal differences were most often differences of band intensity in minor bands, but one band (Rf 0.71) was totally absent in some hybrids having a parent with this isoenzyme (e.g. 3 x 8, 7 x 9).

Bands also appeared in hybrids which were not detected in either inbred, these probably representing hybrid bands between enzyme subunits, and one such band appeared in the same position (Rf 0.42) as a hybrid band produced by urea-dissociated enzyme subunits (see results section 2.7).

#### 6.4 Pollen F<sub>1</sub> hybrid EST

In pollen from F<sub>1</sub> hybrids the patterns observed were additive, representing the sum of the maternal and paternal complements (Fig. 18).

#### 6.5 Seed GOT: inbred lines and F<sub>1</sub> hybrids

Four different GOT patterns were observed in fifteen inbred lines examined for this enzyme system (Fig. 21), with F<sub>1</sub> hybrids having additive patterns. The three-banded pattern typified by line 5 was the zymogram most frequently observed. Artefact bands, caused by shrinkage in methanol, were genotype-specific.

#### 6.6 Pollen GOT: inbred lines and F<sub>1</sub> hybrids

In a limited number of lines examined only two different zymogram patterns were seen. F<sub>1</sub> hybrid patterns were additive.

### 7. Contamination of F<sub>1</sub> hybrids

#### 7.1 Esterases

The F<sub>1</sub> hybrid 73 x 545 was readily distinguished from the inbred parents, having a zymogram which included all parental bands. The double band at Rf 0.35-0.36 in the F<sub>1</sub> hybrid and male parent was highly characteristic.

Two contaminants of unknown origin, presumably originating from chance cross-pollination, were detected (Fig. 23).

## 7.2 GOT

The GOT pattern of the  $F_1$  hybrid contained all parental bands, but the contaminants could only be detected on the basis of artefact bands.

## 8. Crossing in synthetic varieties

Pollen and seed zymograms of syn-1 seed were identified with the aid of a reference set of zymograms of inbreds and all possible  $F_1$  hybrids, and considerable inequality was found between the proportions of individual inbred lines in the syn-1 generation.

Because of the highly characteristic GOT zymograms of  $F_1$  hybrids involving accession 8, all hybrids involving this line could be rapidly identified. Results are presented in Table 9.

## 9. Enforced inbreeding in autofertiles and autosteriles

Using EST isoenzyme patterns, the higher level of heterozygosity in the initial generation of the autosterile material was clearly shown, despite the very small sample size, and after eight generations of inbreeding two thirds of the plants sampled from either genotype were totally homozygous.

10. Esterases of other taxa (Fig. 24)

10.1 Triticum aestivum (Gramineae)

Each variety tested had a distinct zymogram, although the patterns of Ranger and Mardler were more similar to each other than to the remaining varieties. Freeman had a highly characteristic double band at Rf 0.72-0.73, not seen in the other varieties. The Hungarian variety differed from the other three in the slower migrating group of esterases.

Background staining in gels loaded with wheat extracts was notably lower than with extracts of other taxa.

10.2 Citrus sinensis cv. Ortanique,

C. limon and C. paradisi (Rutaceae)

Zymograms of each species were clearly distinguishable, with EST bands finely resolved.

In gels loaded with C. limon extracts, and stained with Fast Blue RR, bands stained either grey-brown or red-brown, which might have been an indication that esterases of differing specificities were present, or might have been attributable to an interaction with a component of the crude extract (e.g. a phenolic).

10.3 Pisum sativum, Tetragonolobus purpureus (Leguminosae)

Both genera exhibited a number of EST bands, and the large number of EST bands in T. purpureus may have been a reflection of the genetic heterogeneity of individual seeds in the composite seed sample extracted, unlike the sample of the pea variety, which would have been relatively homozygous.

10.4 Castanea sativa (Fagaceae)

The EST bands of this genus, stained with Fast Blue RR, showed the same colour variation observed in Citrus limon. Twenty bands were clearly resolved.

10.5 Malus sylvestris cv Russet (Rosaceae)

Ten bands were identified, which showed colour variation already noted for Castanea sativa and Citrus limon.

10.6 Raphanus sativus (Cruciferae)

EST bands of this taxon were clearly resolved, but all were of low mobility in the gel system, and a high level of background staining appeared near the gel origin.

10.7 Vicia narbonensis, V. cracca (Leguminosae)

V. narbonensis possessed a band not seen in any V. faba genotypes examined, whilst V. cracca differed strongly from all other Vicia material.

Fig.15

**Standardised assay method:**

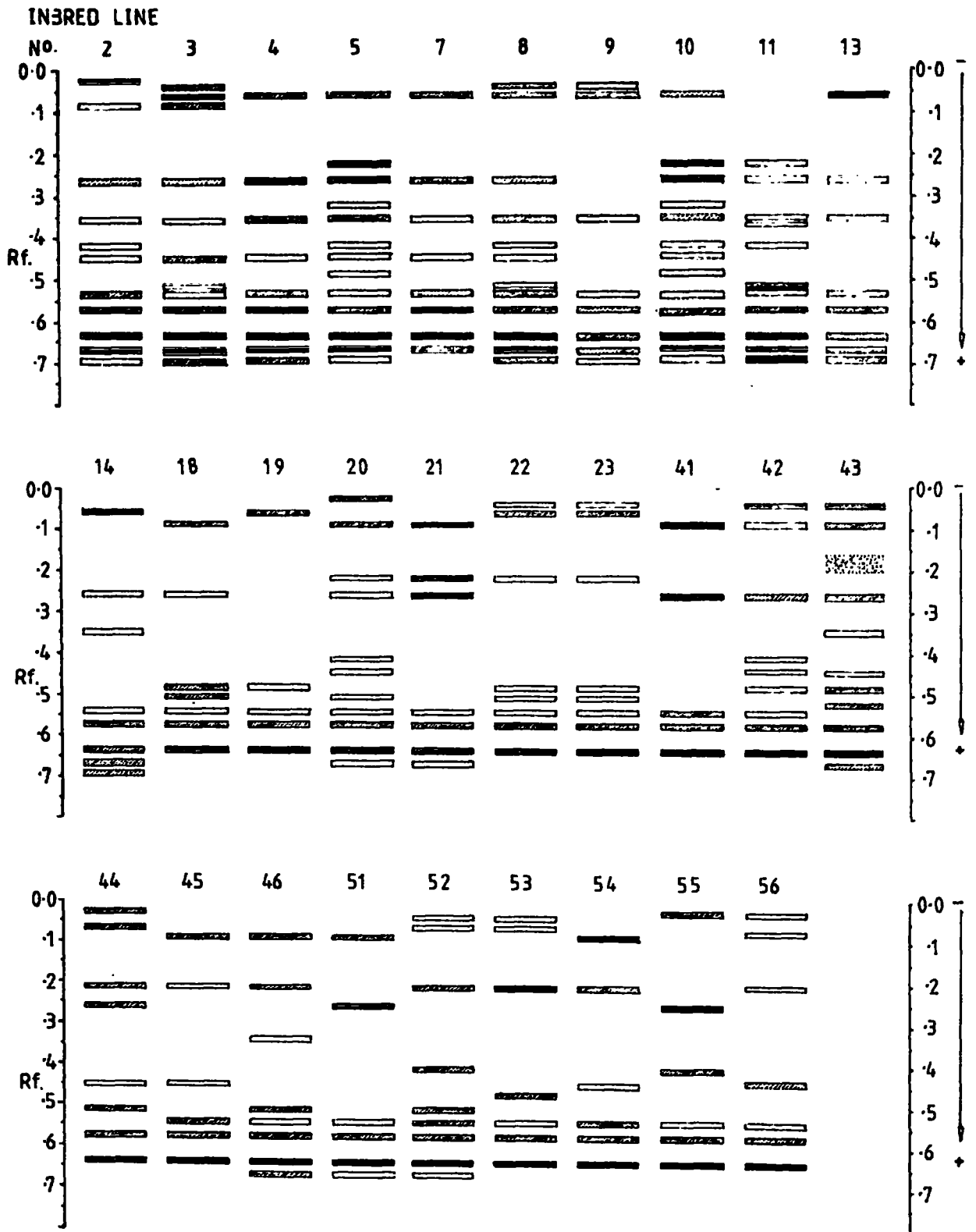
**Substrate:**  $\alpha$ -naphthyl acetate (5 mg/30 ml)

**Diazo dye:** Fast Blue B (1 mg/ml)

**Assay period:** 15 min. with one change of assay solution  
after 5 min.

**Artefact band in accession 43 shown stippled.**

**NON-SPECIFIC ESTERASES OF VICIA FA3A : SEEDS.**



7% acrylamide, spacer gel omitted.

Fig.16

Assay conditions as for Fig. 15



SEED ESTERASES.

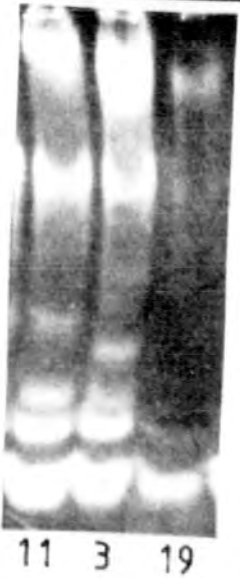
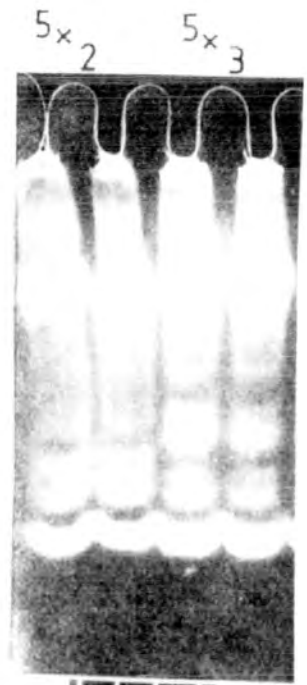
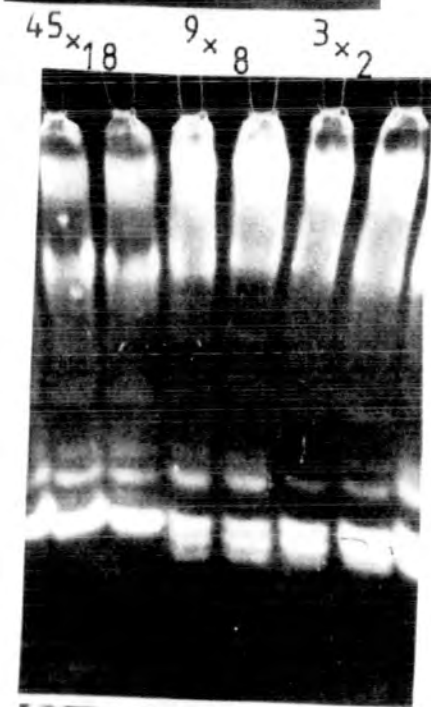
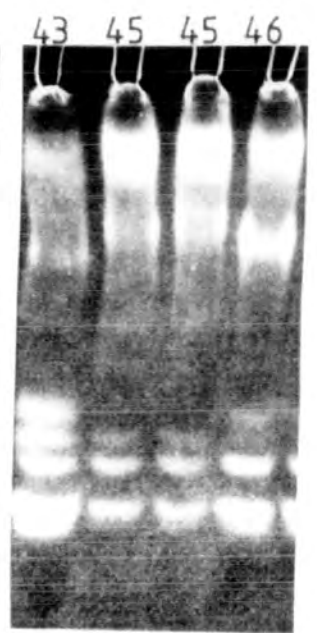
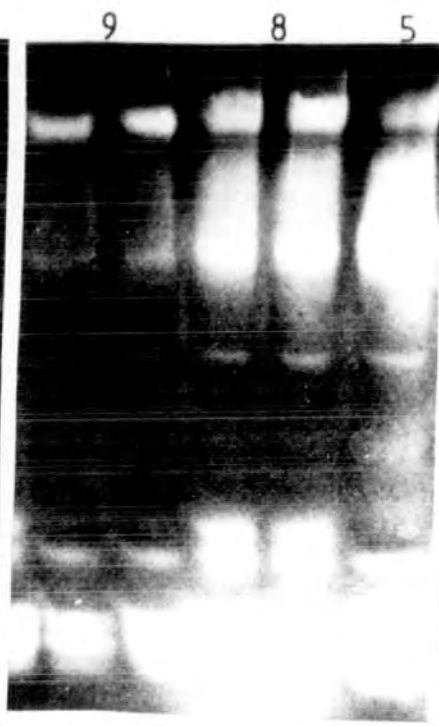
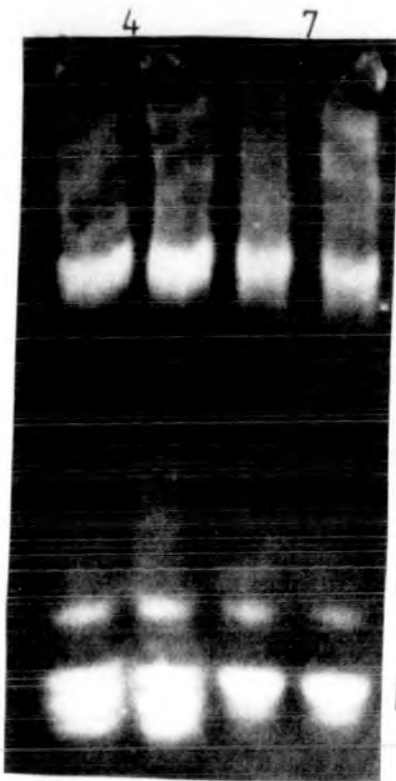
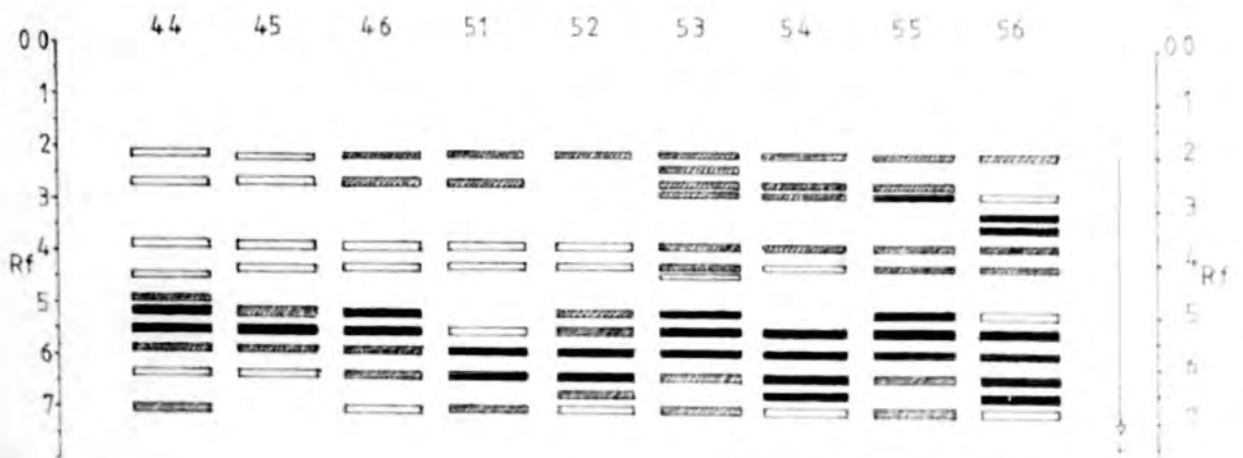
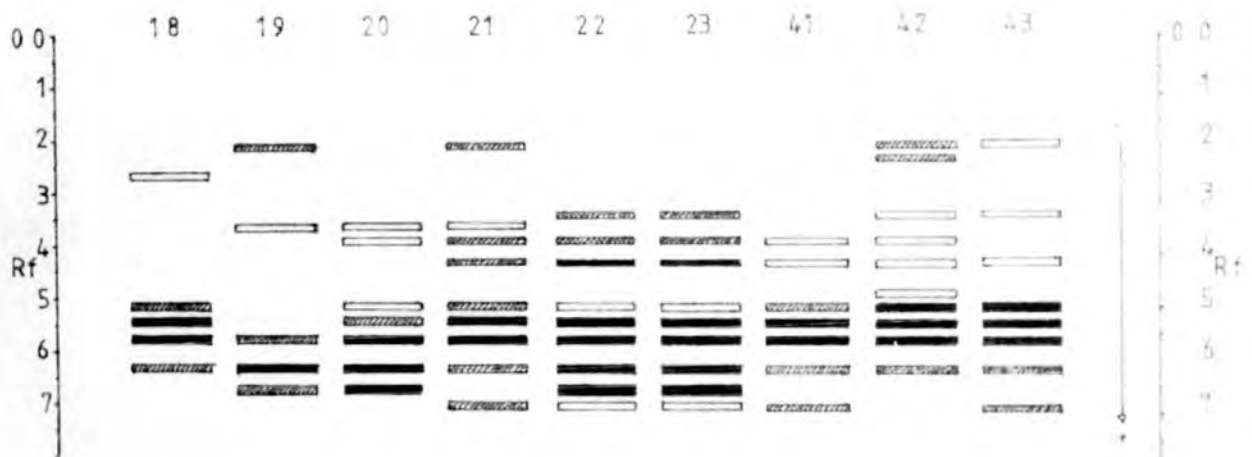
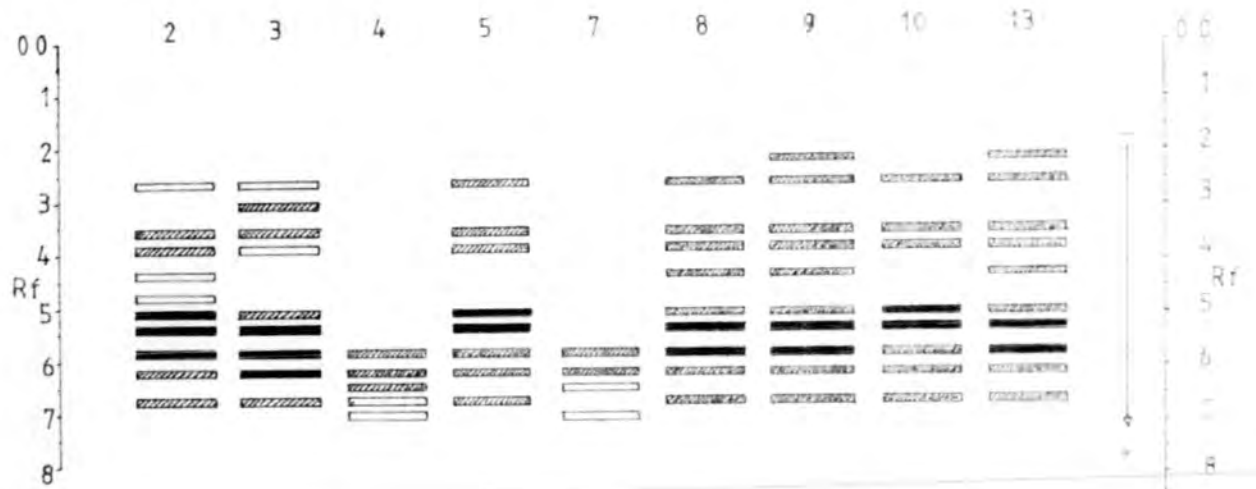


Fig.17

Assay conditions as for Fig. 15

NON-SPECIFIC ESTERASES OF VICIA FABA POLLEN.

Inbred lines.



7% acrylamide, spacer gel omitted

POLLEN ESTERASES.

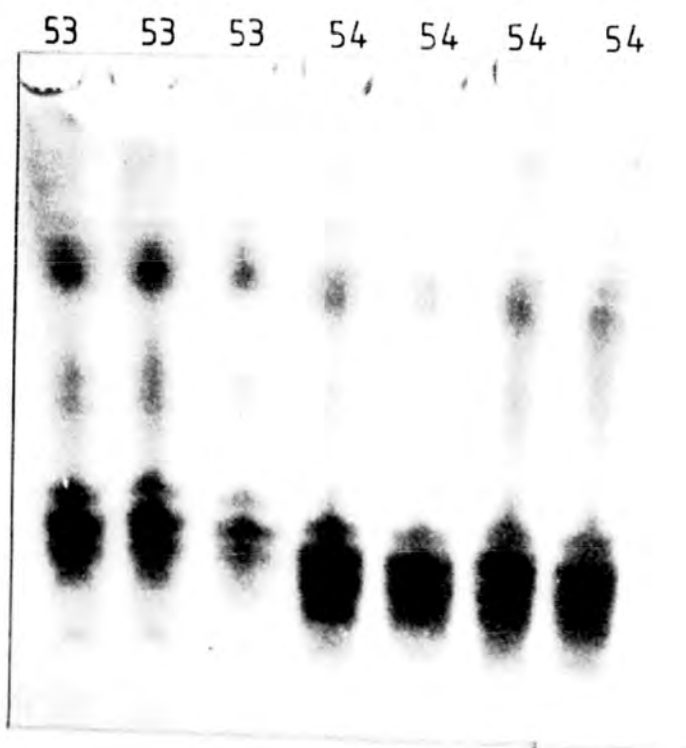
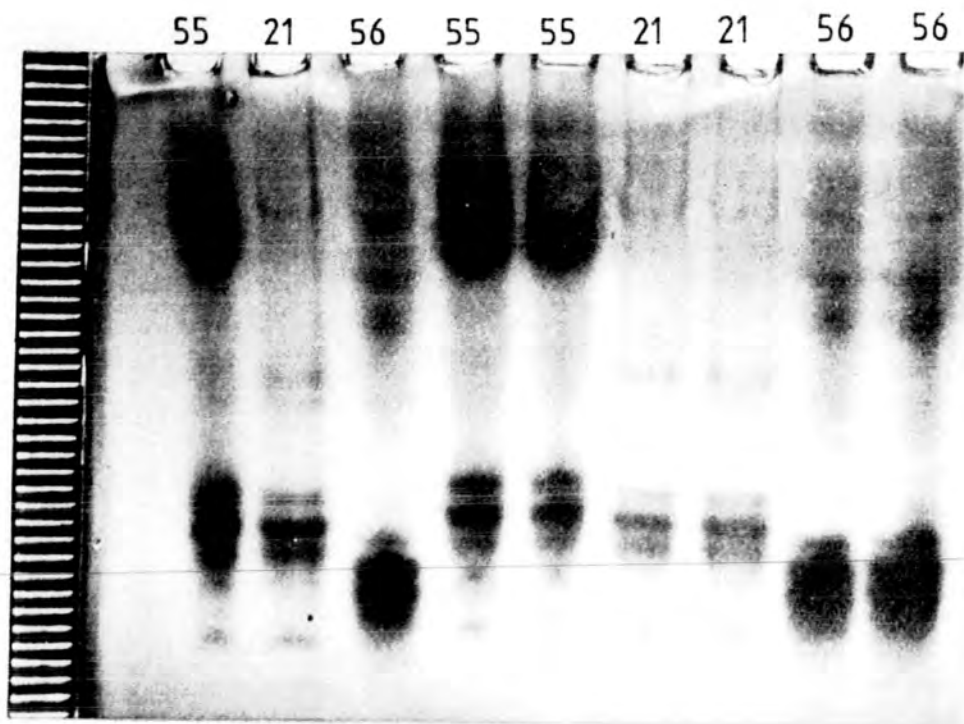
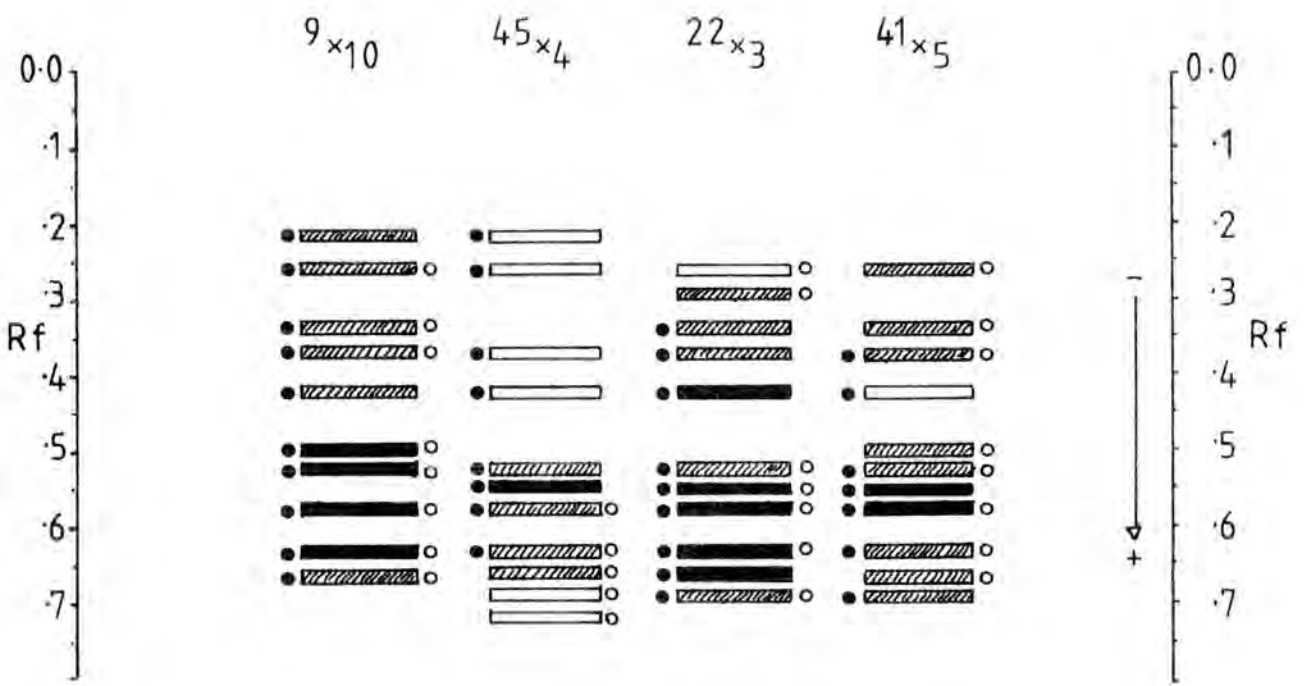
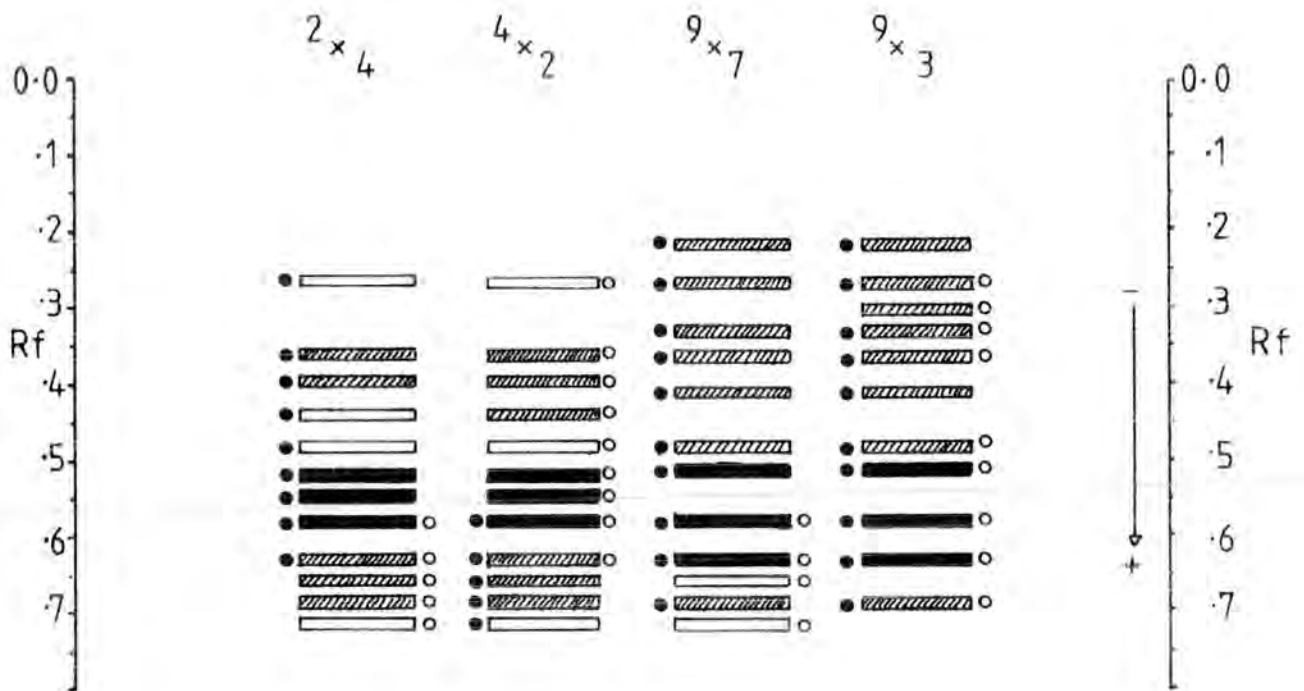


Fig.18

Assay conditions as for Fig. 15.

Non-specific esterases of *Vicia faba* F<sub>1</sub> hybrids: Pollen.



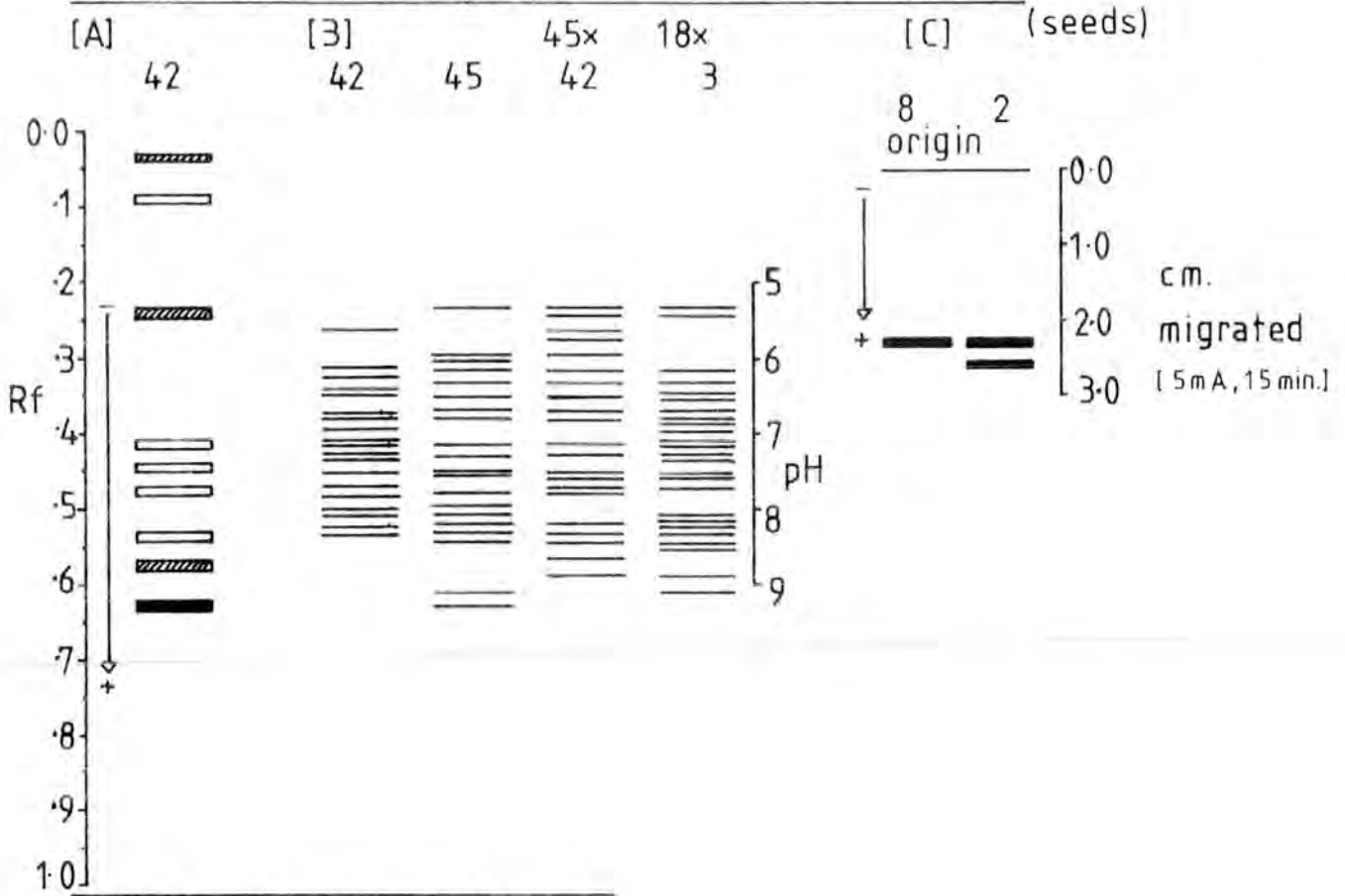
• = band derived from female parent  
 ○ = " " " male "

7% acrylamide, spacer gel omitted.

Fig.19

- A) Inbred line EST developed on standard 7% gels.
- B) Isoelectric focussing gels of esterases.
- C) CAM separation of total protein, stained with Coomassie Blue.
- D) Pollen soluble proteins from inbred lines.
- E) Seed albumins stained with Coomassie Blue (left) and Amido Black (right), after separation on standard 7% gels.
- F) Seed albumins of inbred lines, stained with Coomassie Blue after development on standard 7% gels.

Isoelectric focussing and CAM electrophoresis.



Seed and pollen proteins.

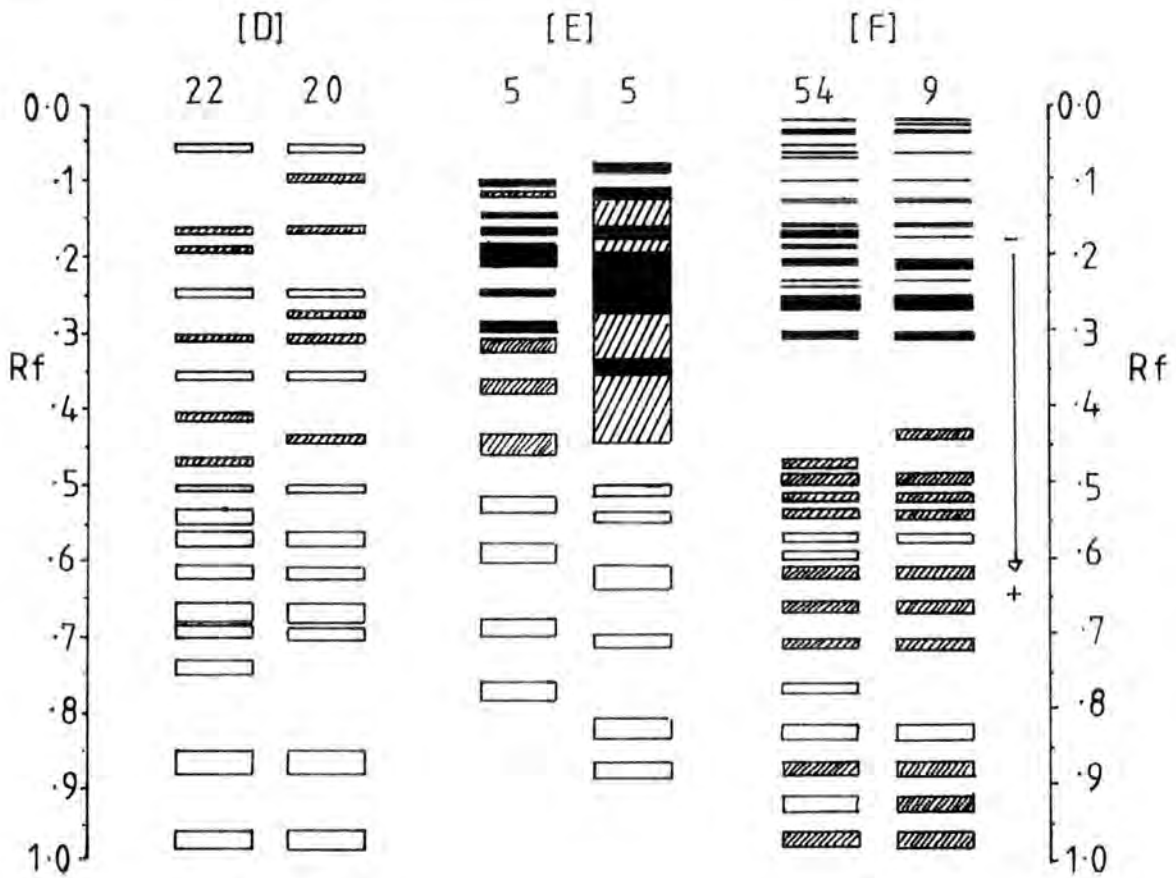
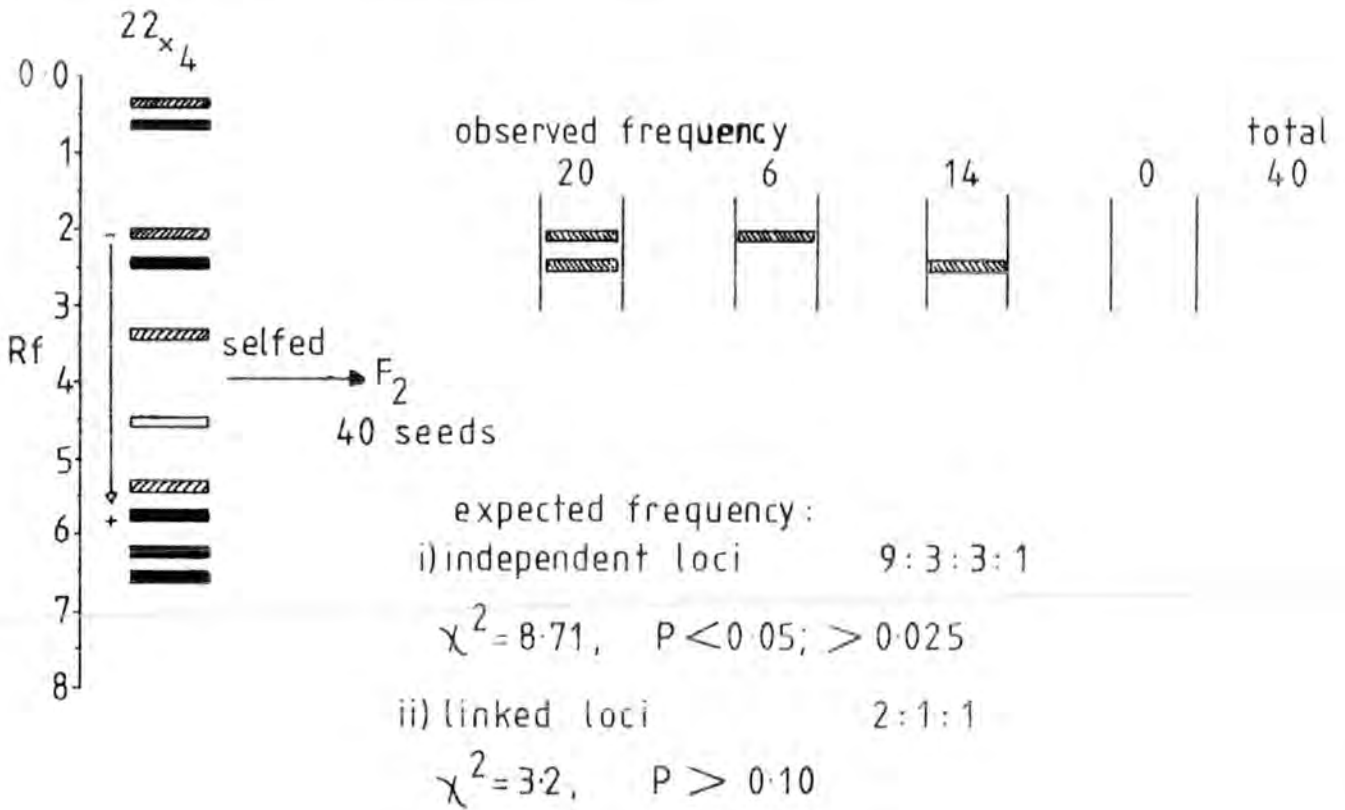


Fig.20

- A) Occurrence of EST bands in 40 seeds of an F<sub>2</sub> generation.
- B) Esterase zymograms of 4 seeds of Maris Bead, chosen at random.

F<sub>2</sub> esterase isoenzyme patterns.



Maris Bead esterase isoenzyme patterns.

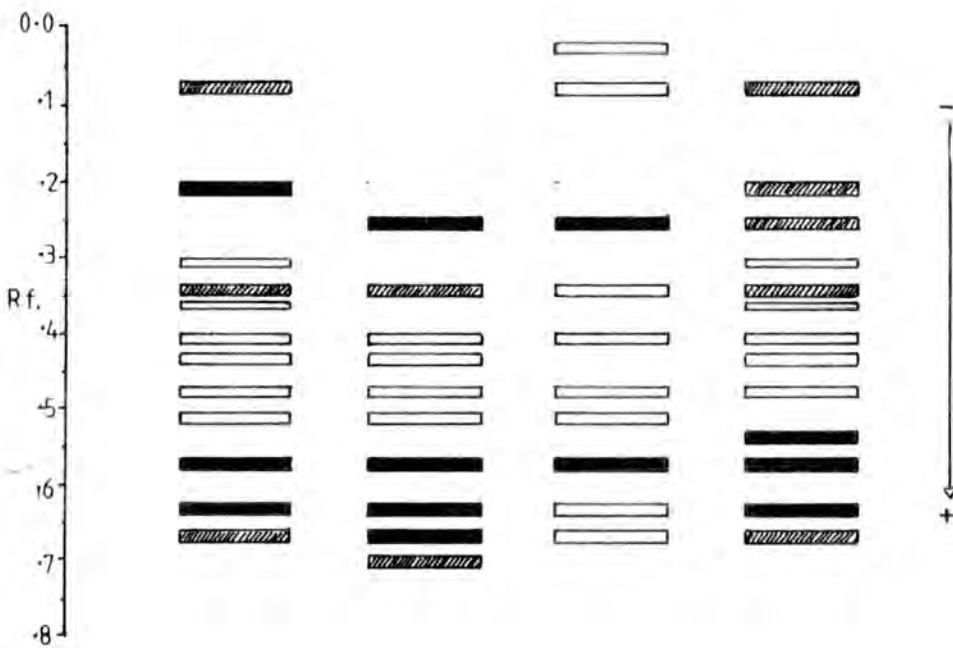


Fig.21

Assayed using the standard GOT assay (Methods section 9.5), with Fast Blue B as the diazo stain. Stained for 10 min. at 20°C.

- a) Gel photographed immediately after incubation.
- b) The same gel, after shrinking in 55% methanol, showing the presence of genotype-specific artefact bands.

GOT isoenzymes of *Vicia faba*: seeds.

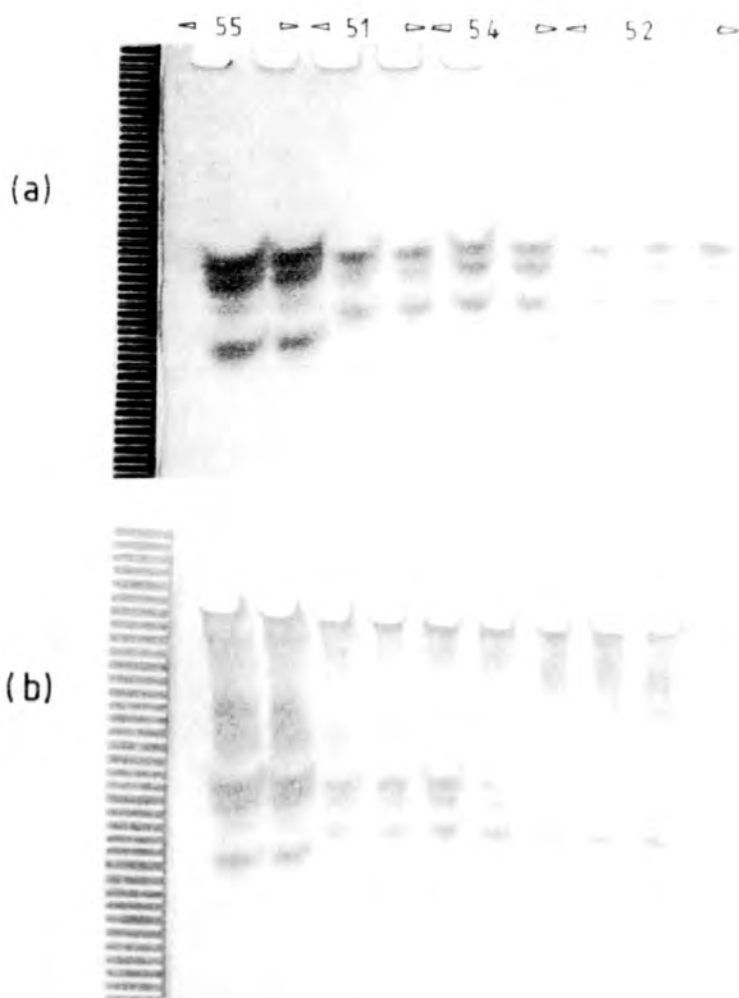
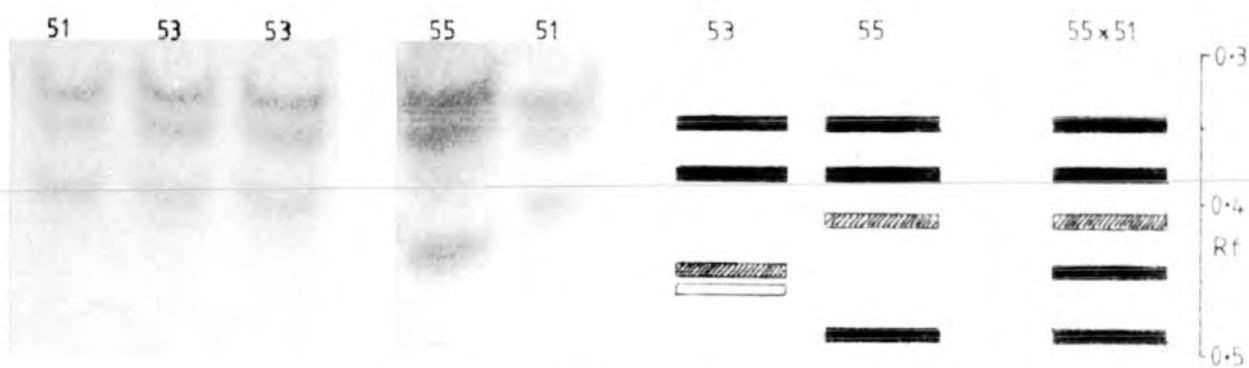
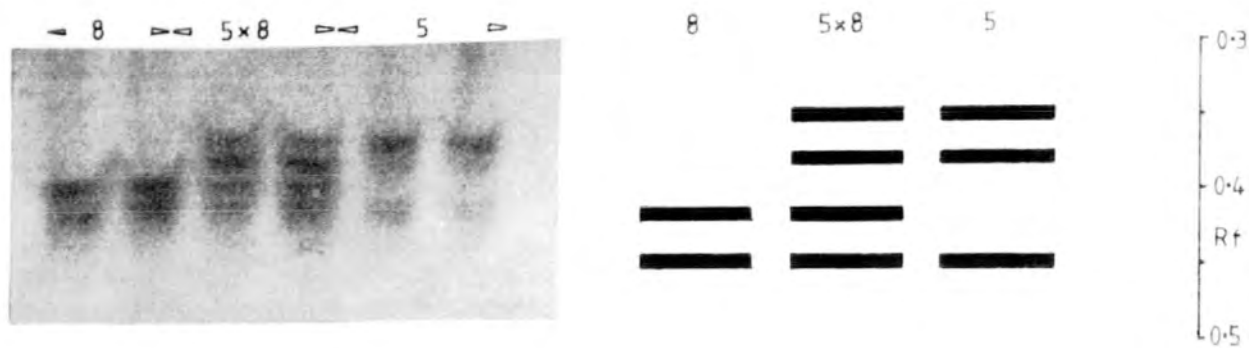
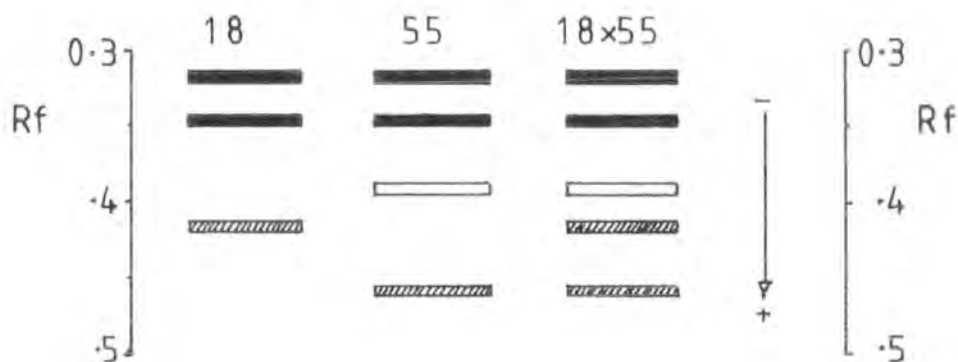


Fig. 22

Assay details as for Fig. 21.

GOT isoenzymes of *Vicia faba*: Pollen.



DISTRIBUTION OF GOT BANDING PATTERNS AMONGST ACCESSIONS

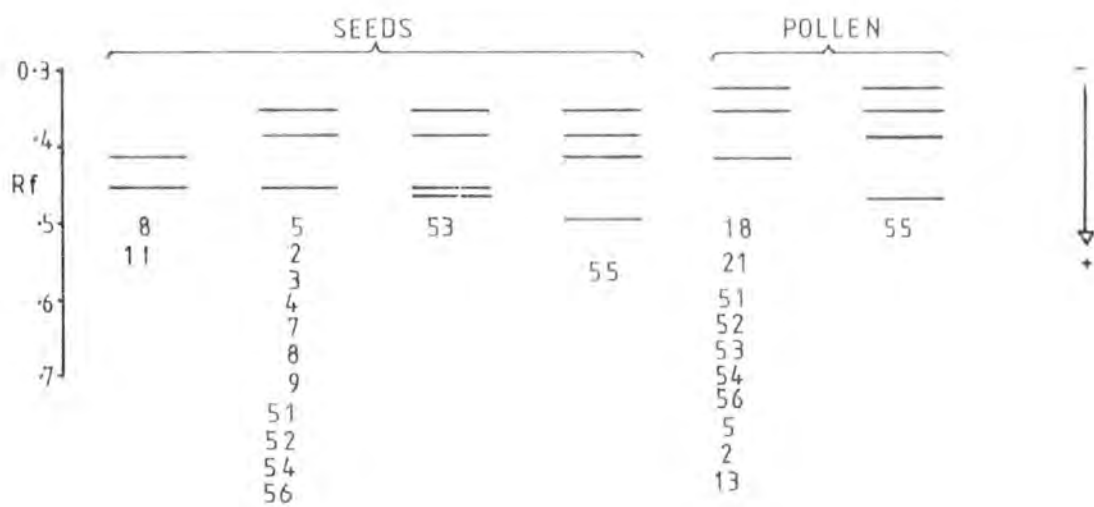
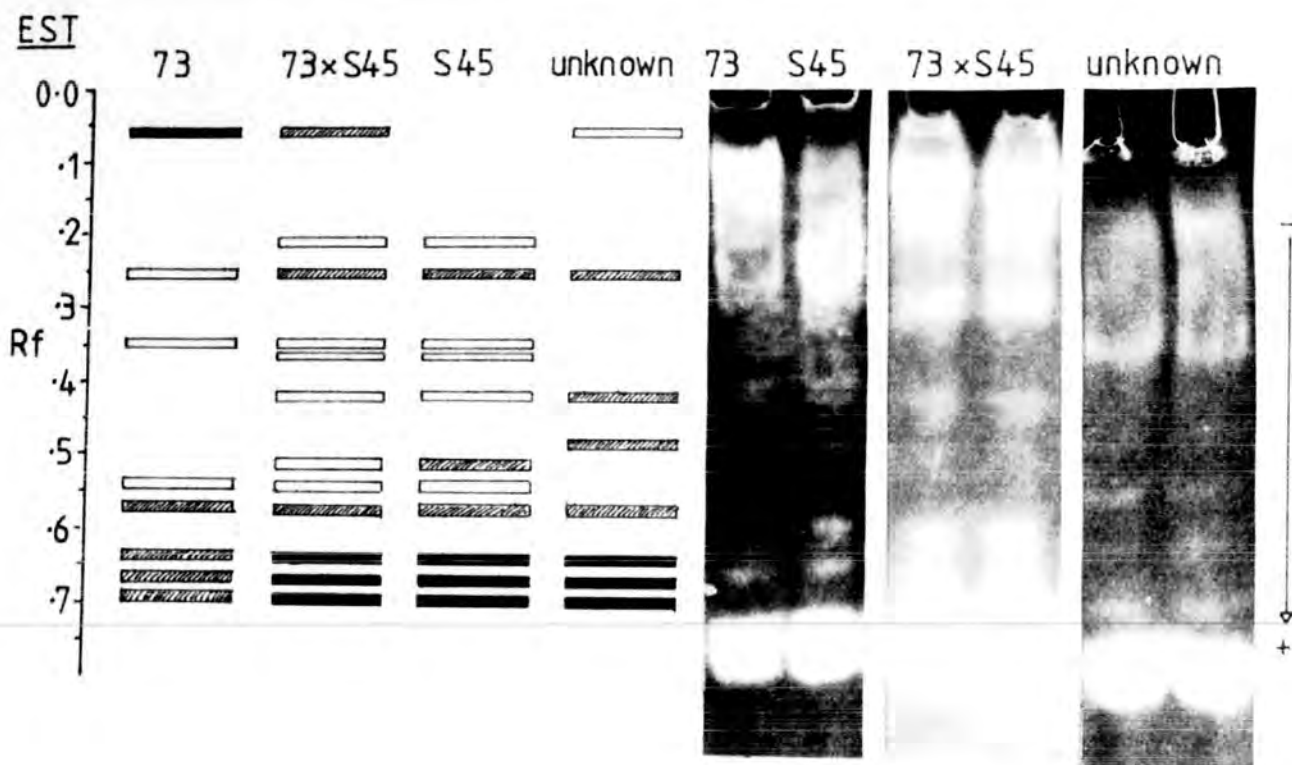


Fig .23

The hybrid was easily identified, as it showed bands derived from both parents, including the characteristic double bands at Rf 3.5 and 3.6.

Contamination of  $F_1$  hybrid seed.



<i>genotype</i>				
<i>enzyme</i>	$F_1$	Female parent.	Unknown contaminant.	% hybrid
EST	36	8	2	78.3
GOT	36	10	-	78.3

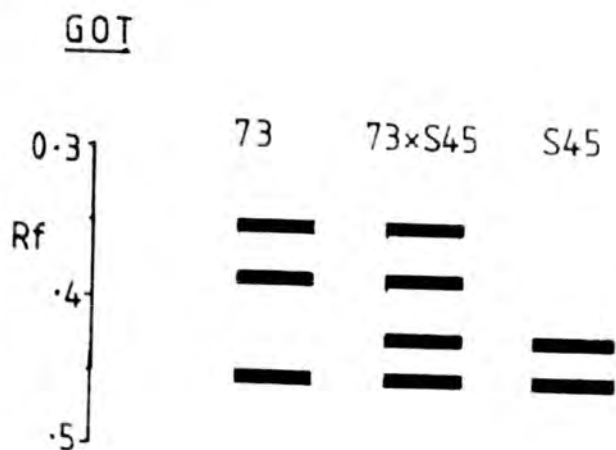
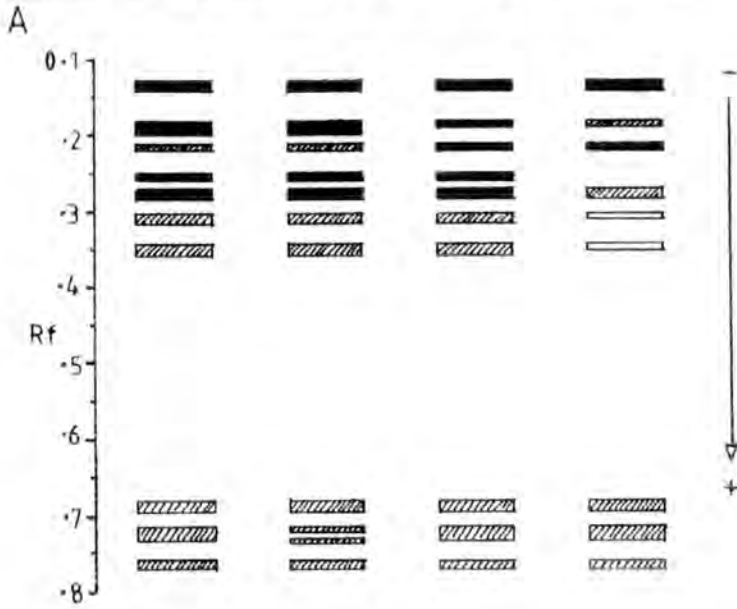


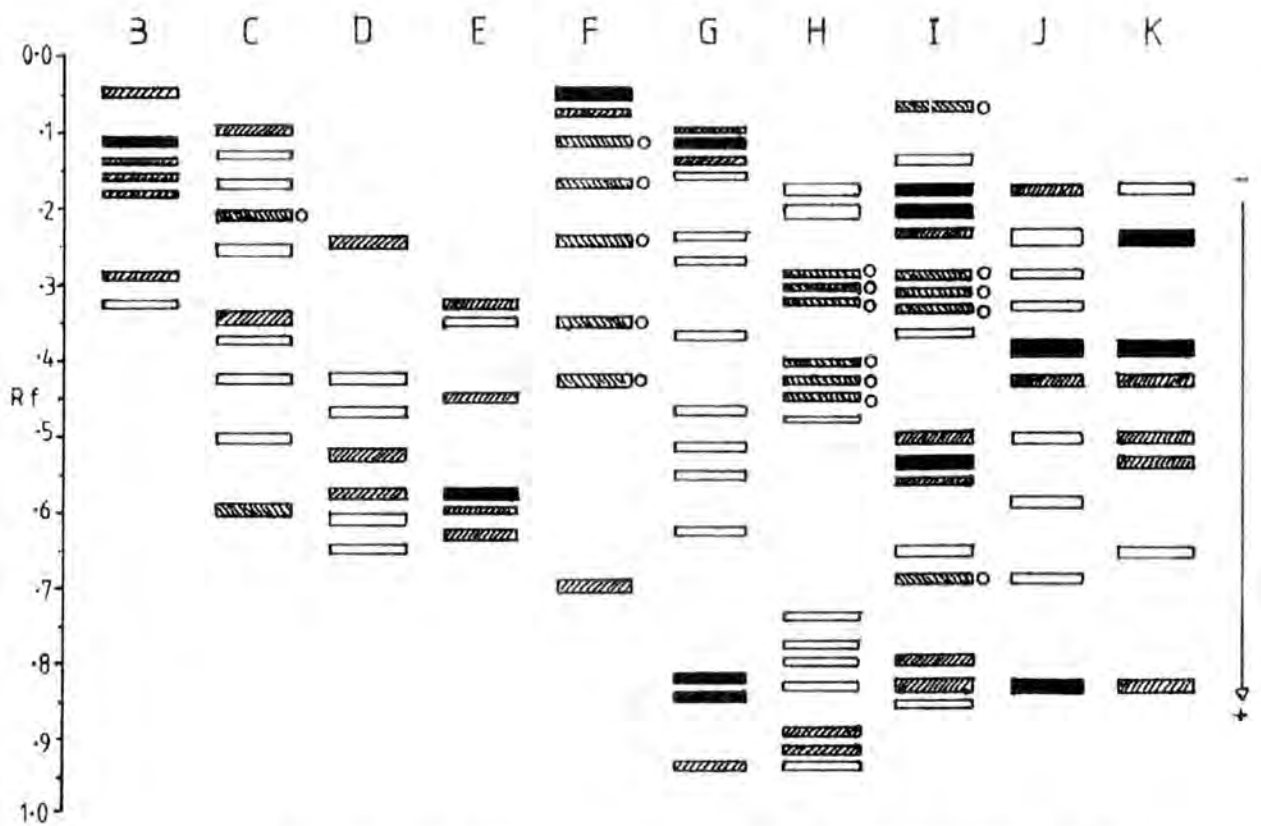
Fig .24

- A) Triticum aestivum varieties (left-to-right)  
Ranger, Freeman, Mardler, Hungarian.
- B) Raphanus sativus
- C) Malus sylvestris cv. Russet
- D) Vicia cracca
- E) V. narbonensis
- F) Pisum sativum
- G) Tetragonolobus purpureus
- H) Castanea sativa
- I) Citrus limon
- J) C. paradisi
- K) C. sinensis cv. Ortanique

Non-specific esterase zymograms of four Wheat varieties.



Non-specific esterase zymograms of diverse taxa.



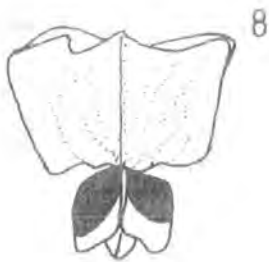
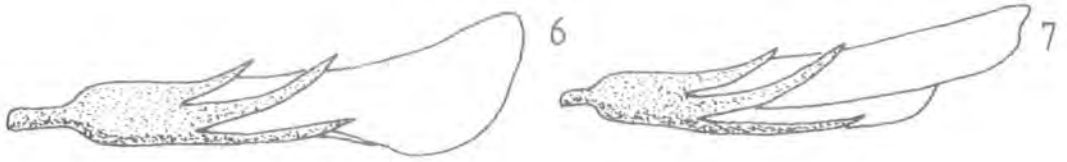
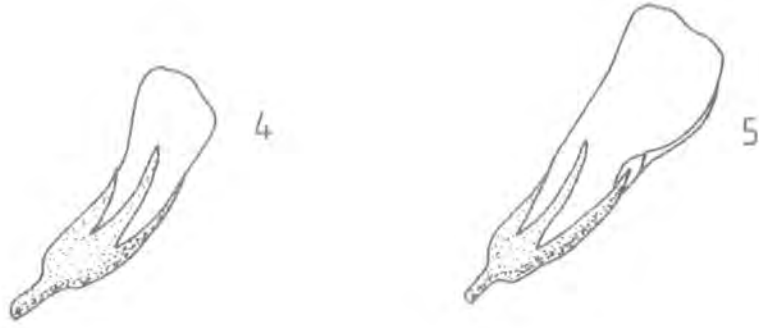
o = bands staining red-brown with Fast Blue RR

Fig.25

Key to visual corolla development during flower maturation  
in *Vicia faba* L. (PAUL 1977)

1. Corolla not yet extruded from calyx.
2. Standard petal visible but not longer than calyx tips.
3. Rolled-in standard petal longer than calyx tips. Keel petal not visible even when lifting basal calyx tip.
4. Keel petal visible only when lifting basal calyx tip.
5. Basal calyx tip does not cover bulging-out keel petal any longer, but still reaches its most distal visible point.
6. Standard petal still rolled in, but most lateral visible point not reached by basal calyx tip any more.
7. Standard petal fully unrolled, but not yet erected. Wing petals not yet visible.
8. Standard petal erected, but its sides not proximally reflexed.
9. Standard petal erected and sides proximally reflexed.
10. Standard petal collapses.

Stages of flower development in *Vicia faba*.



1 cm.

Table 7

Data derived from Fig. 15.

Staining intensities:

1 = low

2 = intermediate

3 = high

Most lines identifiable on the basis of strongly-staining  
bands (underlined) alone



Table 8

Data derived from Fig. 16.

Staining intensities as for Table 7.



Table 9

Buccaneer

Deviation from equality of proportions of each inbred line significant at the 2.5% level

$$(\chi^2 = 10.229, p = < 2.5\%, > 1\%)$$

Bulldog

Deviation from equality of proportions of each inbred line not significant

$$(\chi^2 = 0.7037, p > 10\%)$$

Buccaneer

Deviation from expected frequency (50%) of crosses involving lines 7 and 8 not significant

$$(\chi^2 = 2, p > 10\%)$$

Composition of syn-1 generations.

<u>BUCCANEER</u>	7	8	9	10	F <sub>1</sub>	TOTAL
POLLEN	15	4	7	11	13	50
SEED	12	6	5	8	19	50
TOTAL	27	10	12	19	32	100

<u>BULLDOG</u>	2	3	4	5	F <sub>1</sub>	
POLLEN	8	7	5	7	23	50
TOTAL	8	7	5	7	23	50

<u>BUCCANEER</u> F <sub>1</sub> HYBRIDS.	EST	GOT
7 × 8	5	} 20
8 × 9	8	
8 × 10	7	
7 × 9	5	} 12
7 × 10	3	
9 × 10	4	
TOTAL	32	32

Table 10

Twenty seeds from each plant were sampled, except from plant 10-2-20, where only 15 seeds were available.

Enforced inbreeding in autofertiles and autosteriles.

<u>GENERATION 0</u> <u>AUToFERTILES</u>	<u>Plant No.</u>	<u>No. of different</u> <u>band patterns.</u>
	10-2-14	4
	19-1-27	5
<u>AUToSTERILES</u>		
	19-1-8	7
	2-2-8	8
	10-2-20	2
<u>GENERATION 8</u> <u>AUToFERTILES</u>		
	17-1-26	1
	17-1-1	2
	17-1-5	1
<u>AUToSTERILES</u>		
	17-1-18	1
	17-1-10	1
	17-1-3	2

Table 11

Calculated using the formula from GONELLA and PETERSON (1978)

$$\text{Relatedness index} = \frac{\text{similarities}}{\text{similarities} + \text{differences}} \times 100\%$$

where similarities were taken as presence (+/+) or absence (-/-) of each band in both lines.

W = Winter inbreds

S = Spring inbreds



Table 12

Cost quoted is for a shrunken, dried slab, assuming each sample replicated twice (= 18 samples per experiment using Pharmacia GE-4 apparatus). Costs were those prevailing in November 1977.

Cost analysis

EXTRACT	COST / 100 SAMPLES.
_____	£ _____
Seed/undialysed	0.63
Seed/dialysed	1.80
Pollen	0.58

DISCUSSION: SCREENING OF GENOTYPES AND APPLICATION  
OF THE METHOD

The stable EST and GOT patterns obtained for the inbred lines screened in this study allowed easy identification of each accession. Most could be identified from the major seed EST patterns alone (Table 7), and by preparing a reference set of zymograms for all accessions, each sample could be compared with these for accurate and swift identification, without the need for measurements or spectrophotometric scanning of gels. This approach was only strictly valid for checking lines from a limited collection of known material, because to prepare a reference collection of all possible genotypes would be highly time consuming and impractical, unless only very few polymorphic loci were involved. However, by using more than one polymorphic enzyme system, it has been shown that the number of genotypes which may be identified can be enormous. BROWN and ALLARD (1969 a, b), using nine isoenzyme loci calculated that a total of 19,683 different genotypes of barley could be positively identified. In practice, for handling a limited number of breeding lines, two or three isoenzyme systems each governed by two or three loci with codominant alleles, allowing between 81 and 19,683 genotypic classes to be assigned, would provide for the unequivocal identification of all the accessions in most breeding programmes with a high degree of confidence.

The potential value of the system was demonstrated by the detection of a contaminant in one of the inbred accessions. Two samples of P.B.I. line 224 were obtained (accession numbers 4 and 7), and five seeds were sown from each. All subsequent material used in this study were derived from progeny of this sowing. Seeds harvested from the two accessions showed that those from 4 displayed an extra fast band at Rf 0.7 when compared with those from 7 (Fig. 15), whilst slight differences in pollen isoenzymes were also noted (Fig. 16). Initially it was supposed that the two samples might have been derived from different harvests, plots or populations, and that contamination had occurred during inbreeding in one sample. However, it was found that they were subsamples from the same harvest (D. A. BOND, personal communication). The most likely explanation for the difference observed was that the contaminant was amongst the small sample of seeds sown from accession 4 at the beginning of the experiments, and was maintained in subsequent inbred generations in the glasshouse at Durham within this accession, but not within accession 7. As no segregation was seen amongst the isoenzyme bands in 4, the contaminant was probably due to mechanical mixture rather than to cross-pollination in the original multiplication plot. Re-examination of the two original seed samples revealed that types with and without the extra band were present in accession 4, but only the type without the extra band in accession 7. Once recognised, these two variants were treated as different inbred lines in subsequent crosses, and labelled as in Figs. 15 and 16.

The original seed supplied was not guaranteed one hundred percent pure (D. A. BOND, personal communication), not having been derived from hand pollination, and any contaminants suspected from morphological variations were routinely discarded, but in the case described above the two genotypes were morphologically indistinguishable. One obvious use of the finding described above is that such screening provides a means of ensuring that synthetics or  $F_1$  hybrids are always reconstituted from the same parental inbreds, guarding against genetic drift. The problems associated with multiplication of inbred lines in isolation plots have been discussed by POPE and BOND (1975), and the technique described here is a rapid method which might be applied to measuring contamination of isolation plots, and to the screening out of contaminants from the seeds harvested, as the sampling method does not impair the germination of the seeds.

A major consideration of a screening method is that of economy, and the system as described was developed to be simple, rapid and cheap. Cost is of paramount importance, but is often not satisfactorily stressed (McKEE 1973). Table 12 shows the total cost of all material used, and taking into account that the screening was carried out on a small scale, and that scaling up of the operation would inevitably reduce the unit cost, the method was a relatively inexpensive means of measuring outcrossing. The speed of the operation meant that accurate information could be produced in a matter of a few hours. Although conventional morphological markers can often be scored rapidly, they are not always available or easily incorporated into

breeding material, and may require the growing and harvesting of plants with the attendant expense, before results can be obtained. The ideal of the isoenzyme system applied to plant breeding is that the plant breeder need not make a special effort to attach a unique marker to lines or varieties (LARSEN 1969), and by using several isoenzyme systems this might be achieved in practice.

The initial cost of commercially-produced electrophoresis equipment can be high, but the Pharmacia equipment used here was over-elaborate for the purpose to which it was applied. Several authors (see SMITH 1976) have described the design and construction of polyacrylamide gel electrophoresis equipment, and a more efficient, purpose-built slab gel apparatus for screening work could be built inexpensively. It could usefully incorporate wider gels, with more sample wells, and several such units could be run in series, allowing simultaneous electrophoresis of large numbers of samples under identical conditions.

With only one operator the equipment used allowed a rate of screening of the order of 72 seed samples and 144 pollen samples per day, but with two operators producing large batches of extracts, equipped with ideal apparatus, this figure could be multiplied several-fold. Seed extraction constituted the longest single operation, and the dialysis step accounted for a large proportion of the total cost, but by using either water extraction or extraction in an acid buffer to minimise globulin contamination, this process could be eliminated

for the simple screening out of contaminants or for identifying inbred lines. It was most necessary when identifying crosses between several inbred lines simultaneously, when material sometimes showed only small differences in banding pattern.

The use of small, thin slabs minimized the materials required, both for gel monomer components and assay solutions. The latter consideration assumes considerable importance when assaying for enzymes requiring expensive substrates.

On the grounds of cost, both cellulose acetate membrane electrophoresis (CAM) and isoelectric focussing (IEF) compared unfavourably with plain polyacrylamide gels. CAM revealed general protein differences between inbred lines (Fig. 19), but was not a successful method when applied to isoenzymes, due to difficulties of assay. The soluble proteins rapidly diffused in the assay solution, and the most satisfactory method was to place the membrane in contact with an agar slab containing a fluorescent substrate. In addition to inferior resolution, the elaboration of the technique decreased the advantage of rapidity inherent in CAM.

IEF, with its high resolution of bands, was an excellent method of distinguishing inbred lines from one another, and for identifying their hybrids, but was slow and expensive compared with the standard technique. IEF gels resolved a large number of bands, many of which were not detected on standard slab gels, and this discrepancy may have been due to at least two causes.

During enzyme extraction, the action of proteases normally compartmentalised within the cell may have removed part of the polypeptide chain from the enzyme molecules. Large sections of polypeptides can be removed from some enzymes with no impairment of catalytic activity, especially when a non-specific enzyme system is assayed using a synthetic substrate, so that such partial hydrolysis of EST isoenzymes would result in mobility changes without loss of enzyme activity. Such molecules identified on IEF gels would fall outside of any definition of isoenzymes, but would still be of value if they were reproducible in vitro artefacts. GERBRANDY et al (1975) explained interconversion of potato phosphorylase isoenzymes on this basis.

An alternative explanation is that the IEF technique concentrated the isoenzyme bands to an extent where those which were diffuse and invisible on the acrylamide slabs, either through low concentration or high background staining of the gel, became visible. A similar argument has been advanced to account for the finding that the tissue specificity of peroxidase band patterns shown on starch or acrylamide gels was not found when the same extracts were run on IEF gels (RUCKER & RADOLA 1971). Isoenzyme activity may be differentially expressed in different cellular environments, so that levels of activity of some isoenzymes might have been low enough to escape detection by methods other than IEF. The finding that prolonged staining in certain diazo dyes revealed bands unresolved by less sensitive dyes might also support this interpretation. Quite possibly the increase in bands resolved on IEF gels was in part a result of in vitro protein changes and partly a result of concentration of small amounts of enzyme activity.

The yellow wing petal spot, observed in one plant of accession 18, has been described by SJODIN (1971) and has been attributed to the homozygous recessive gene  $dp-b^1$ . Since only one plant was found exhibiting this character in accession 18, in over fifty progeny from two  $I^7$  seeds and subsequent inbred generations, it seems likely that this variant may have arisen by mutation. No difference could be found between the pollen zymograms from the yellow-petalled plant and the standard accession 18 material, all of which had black wing petal spots (Fig. 15). This highlights a possible application of the isoenzyme technique in the identification of material for certification purposes under Plant Breeders' Rights Acts, in that material from two different sources showing the same mutation could be proven to have been developed independently if the isoenzyme patterns for each type were markedly different. Substantial isoenzyme differences could not themselves be explained on the basis of mutation, as the loci controlling these have been shown not to be especially mutable, having mutation rates of the order of less than  $\frac{1}{105}$  (ALLARD & KAHLER 1971).

An important philosophical problem in the application of biochemical, as well as other techniques, for testing of varieties or lines for legal purposes is that whereas it is often relatively easy to prove that a given seed lot or group of plants does not conform to the established characteristics of a reputed line or variety, it is difficult to prove that a given lot of seeds are of the reputed variety. This problem arises not only because no genotype can be tested against all possible genotypes of a crop species, but also because it can be argued that protein bands migrating to the same position on a support medium are not inevitably homologous. To prove them to be so would involve the

use of complex and expensive physical and chemical determination (JOHNSON 1977). However by the combined use of several polymorphic enzyme systems, an individual or group of homozygous individuals can be assigned to one of a vast number of genotypic classes (BROWN & ALLARD 1969 b) and thus identified with a considerable degree of certainty. Combinations of blood group tests and enzymatic analyses of blood are used in this way in medico-legal applications (GRUNBAUM 1976), and the system used in forensic science could be applied to plants with greater ease than to humans, as it is often possible to determine the pedigrees of plant material submitted for varietal identification.

The failure to detect segregation within lines when seed and pollen material from generations  $I^7$ ,  $I^9$  and  $I^{11}$  was compared showed that all the EST loci were homozygous at this stage, and as breeding lines for  $F_1$  hybrids and synthetic varieties would be carried through at least seven generations of inbreeding before use, the breeder could be confident that the isoenzyme patterns by this generation were fixed. By  $I^7$  the inbred lines should theoretically be homozygous for a minimum of over ninety-nine percent of their loci. MITRA, JAGANNATH and BHATIA (1970) have shown a lack of variation in barley pure lines similar to that in V. faba, as have HAMILL and BREWBAKER (1969) with peroxidases from maize inbreds. WILLIAMSON, KLEESE and SNYDER (1968) found electrophoretic variation in EST isoenzymes within commercial varieties of oats, but SINGH, JAIN and QUALSET (1973) found that seven out of ten commercial oat varieties were electrophoretically homogenous. The origin of the variability observed by WILLIAMSON et al (1968) is

unclear, but it might have arisen from contaminated initial samples, storage effects, ontogenetic changes in the four-day-old seedlings sampled, or environmental effects. By sampling dry dormant seeds, two of these pitfalls can be avoided.

As this study revealed that the isoenzyme patterns were genotype specific and unaffected by environment, the assumption that V. faba esterases were genetically controlled was a safe one. Their value as markers would have been diminished, but not destroyed, had they been total biochemical artefacts, because of their highly stable and repeatable nature. It would be valid to use the electrophoretic variation as a phenotype without genetic analysis, but by considering the genetic control of the patterns, the bands might be equated with alleles and information about the structure of the isoenzymes themselves obtained, especially when considered in conjunction with the results of the subunit dissociation. Other advantages of genetic analysis included the possible prediction of expected band patterns in  $F_1$  hybrids, which would allow the assessment of the suitability of applying the technique to measuring crossing between different inbred lines, and also it would allow some distinction to be made between bands which were genetically determined and those which were preparative or electrophoretic artefacts.

On the small gels used for screening, the fastest migrating band only moved about four centimetres, and overlapping of some bands were inevitable. For identification of various lines and hybrids, only those bands which were clearly visible were used,

and faintly staining or doubtful bands were ignored. For example, it was always possible to score two major seed EST bands at Rf 0.2 and 0.25, but other faint bands occurred in this region, possibly associated with storage proteins (see above), which were indistinct and therefore not taken into account. The system was not developed as one for genetic analysis, as the study was not originally conceived in these terms, and during the course of experiment it was found that the small slabs were unsuited to genetic studies, but data from  $F_1$  hybrids and  $F_2$  segregation of seed EST isoenzymes allowed some conclusions to be drawn (Fig. 17, 20).

The major bands at Rf 0.2 and Rf 0.25 occurred in all inbreds except 9 and 19. Some inbred lines (5, 21, 20, 44, 11) exhibited both bands, indicating that they could not be controlled by codominant alleles at one locus, but must have originated from two loci. The  $F_1$  seeds from the cross 22 x 4 had both bands, one from each parent, and the  $F_2$  segregated in the ratio twenty seeds with both bands, fourteen with only the fast, six with only the slow, and none without any bands. A second  $F_2$  of the cross 46 x 42 showed a similar deficiency in seed without either band. Applying  $\chi^2$  significance test showed that it was doubtful whether the observed data could be accounted for on the basis of codominant alleles, with a null allele, at two independent loci, but that there was no reason to reject the hypothesis that the loci were linked.

Examination of the remaining bands in the inbred lines revealed no pairs of bands which could be considered as allelic, suggesting that the presence or absence of the bands could be explained on the

basis of codominant alleles, with a null allele, at a rather large number of loci. On this basis the total number of loci involved may have been as many as eighteen, which would allow  $2^{16}$  possible combinations of bands scored on a presence or absence basis, as two bands were monomorphic (Rf 0.57 and 0.63), occurring in every genotype examined. In accounting for this large number of loci, it must be borne in mind that the esterase reaction demonstrates a wide variety of functionally dissimilar enzymes, probably including lipases, phosphatases, proteases and ribonucleases and assaying these individually with their specific substrates would simplify genetic analysis enormously.

The cotyledons of legumes are diploid and arise from one paternal and one maternal genome. Examination of the  $F_1$  hybrids showed that seed cotyledon EST isoenzyme patterns were the sum of the parental inbred patterns, but that in reciprocal crosses the intensity of staining of bands could vary dramatically. This may indicate that the expression of alleles depends on the cytoplasmic environment in which they are expressed, and that some paternal alleles are suppressed in the maternal tissue. As an example of this, the Rf 0.07 band of 4 x 2 and the Rf 0.46 band of 7 x 9 were both expressed more intensely in these rather than in the reciprocal crosses. Another expression of this was in the non-appearance of the fastest migrating band in some crosses with lines not exhibiting this band, e.g. 3 x 18, 9 x 18. The possibility that repressor loci might be involved was not explored experimentally, but should be considered

as these have been invoked in other isoenzyme systems (MACDONALD & BREWBAKER 1972), and DAVIES (1973) favoured this explanation to account for maternal inheritance of globulin patterns in peas. Similarly, reciprocal differences in EST isoenzymes of Zea mays have been discussed by SCHWARTZ (1963) in terms of differential activation or repression.

Several bands occurred in  $F_1$  hybrids which did not appear in either parent or in any inbred lines (e.g. Rf 0.47 in 4 x 9; Rf 0.29 in 9 x 8; Rf 0.37 in 3 x 2; Rf 6.2 in 45 x 18). The Rf 0.47 band also appeared in the subunit dissociation experiment, and the evidence suggests that this arose from the recombination of subunits of a dimeric enzyme, although it is not clear which parental bands were involved. The other bands listed might have arisen either from subunit recombination or as biochemical artefacts, but it must be stressed that the gel system under which these determinations were made rendered conclusions about genetic control difficult, and these should be reserved for a separate study, where overlapping bands and bands with faint activity can be separated clearly.

To summarise, the results indicate that the non-specific esterases in V. faba seeds are controlled by at least sixteen polymorphic loci, two of which are linked, and two monomorphic loci, and that isoenzymes are controlled by codominant alleles, with a null allele. Null alleles have been widely reported in isoenzymes (MACDONALD & BREWBAKER 1972), and in view of the fact that gene duplication has been put forward as a likely mode of isoenzyme evolution (WATTS & WATTS 1968; SHAW 1969), the presence of linked loci might be expected.

The EST patterns observed in the pollen of  $F_1$  hybrids always represented the sum of those of the parental lines, and no reciprocal differences occurred. As noted in seed EST, the two bands at Rf 0.57 and 0.63 were monomorphic, whilst the remainder were present or absent with a range of frequencies (Table 8). Again, a large number of gene loci were involved, but no genetic analysis of the pollen isoenzymes was attempted due to the limitations of the gel system.

At its simplest level the method described here allows the verification of hybridity in experimental hybrids without seriously impairing their germination, if sampling is carried out on the seed, or reproductive potential, if sampling is carried out on the pollen. The concept of using isoenzymes to measure outcrossing is not new, and has been used by MARSHALL and DOWNES (1977) to check for apomixis in Sorghum, by BROWN and ALLARD (1970) as part of a method to estimate the mating system of open-pollinated maize populations, and as a means of measuring outcrossing in Limnanthes (JAIN 1978), Acacia (PEIRCE & BREWBAKER 1973) and Citrus (TORRES & SOOST 1978). Most work has fallen into the categories of either small-scale experimental studies or evolutionarily-orientated studies in population genetics, and the potential large scale use of the system as a means of tackling general plant breeding problems has not been developed (PEIRCE & BREWBAKER 1973).

The potential use of isoenzymes to measure contamination of  $F_1$  hybrid seed has been demonstrated by NIJENHUIS (1968) and WOODS and THURMAN (1976). Both of these studies used ACPH isoenzymes, assayed on starch gels, which only allowed the Brassica parental inbred lines to be classified into three or two groups respectively. This made ACPH a rather limited marker, as the parental inbred lines had to be selected for different bands to allow identification of the  $F_1$  hybrids.

Using the highly polymorphic EST system described here, the very large number of different band combinations when bands were scored on a presence or absence basis ( $2^n$  different combinations, where  $n$  = number of polymorphic bands), indicates that the likelihood of two parents having the same band patterns would be small. The widely recognised observation that heterosis is most strongly expressed when the parents differ extensively (WU 1939; HAYES & JOHNSON 1939; JOHNSON & HAYES 1940) also means that the parental lines would be unrelated, so that isoenzyme patterns could be expected to be dissimilar. These factors contributed to the usefulness and flexibility of the EST isoenzymes applied to the screening of  $F_1$  seed, and by using more than one enzyme system, for example EST and GOT, the system could be applied to a very large range of inbred parents.

Fig. 23 shows that estimates of contamination of  $F_1$  hybrid seed of V. faba were identical when either EST or GOT isoenzyme systems were used, but that the GOT system failed to detect the unknown contaminant revealed by EST screening. However, these could be detected using the GOT artefact bands, as previously described and illustrated in Fig. 21. The original  $F_1$  sample supplied, derived from the cross nearly male sterile line 73 x S45 fertility restorer line (accessions 14 x 11), was expected to be approximately eighty percent hybrid and twenty percent female parent, and the actual findings from a sample of forty-six seeds corresponded quite closely with this expectation (Fig. 23).



BOND (1968) has stated that contamination of  $F_1$  hybrid seed with female parent inbred seed can be tolerated at a level of as much as 25% with only limited economic loss in yield. The ability to rapidly and accurately measure such contamination by the isoenzyme technique might be applied to the production of  $F_1$  seed. On the premise that increasing the plant density would tend to cause the weaker inbreds to be eliminated by interplant competition, the seed rate could be adjusted when Field Beans were sown depending on the degree of contamination. This would be possible because of the ease with which contamination can be measured by the isoenzyme technique, and such a system might make the marketing of impure  $F_1$  seed practical, especially if a price adjustment could be applied depending on the degree of contamination.

Syn-1 generations, from the synthetic varieties "Buccaneer" (accessions 7 x 8 x 9 x 10) and "Bulldog" (accessions 2 x 3 x 4 x 5) were screened using EST and GOT isoenzymes, and it was found that the proportions of each inbred component of this generation, after one cycle of crossing, could rapidly be determined by comparing pollen or seed zymograms with those of the inbred parents (Table 9). The proportions of each of the possible  $F_1$  hybrids was also determined in the syn-1 of "Buccaneer", but this operation was complicated by the differential intensity of stain and suppression of some bands in reciprocal crosses. For a synthetic variety composed of four inbred lines, the preparation of reference zymograms from all twelve reciprocal hybrids was necessary, and scoring was more difficult. Since no reciprocal differences in GOT patterns were noted, and the GOT patterns of all hybrids involving accession 8 was highly characteristic (Fig. 21) this system alone could be used

successfully to measure the crosses involving this accession (Table 9). The other hybrids in the syn-1 of "Buccaneer" all had identical GOT patterns, but the artefact bands for all the crosses differed.

On the basis that all inbreds would be expected to be present in the syn-1 in equal proportions if random crossing had occurred and equal fertility in selfed syn-0 inbreds applied, a  $\chi^2$  test showed that the proportions of inbreds differed significantly from equality. The proportion of  $F_1$  seed from crosses involving accessions 7 and 8 (highest and lowest inbred classes) did not differ significantly from the expected fifty percent on the small sample tested, so it might be concluded that the imbalance of inbreds in the syn-1 was the result of differential self fertility rather than non-random crossing. To verify this a larger sample would be required.

The question of sample size is dependent on the number of classes of progeny expected and the level of accuracy required, and can be determined by applying the relevant significance tests to the data. To obtain an estimate of the level of contamination of  $F_1$  hybrid seed, or to measure the level of outcrossing in a syn-1, where only two classes of progeny need to be scored, samples of as few as 24 seeds have been proposed (NIJENHUIS 1968) whereas to measure the proportions of all sixteen progeny classes in a syn-1 with reciprocal differences in  $F_1$  hybrids would require a correspondingly large sample. Clearly, the level of accuracy required and value of the information must be balanced against the overall cost of the operation.

It would be impractical to follow outcrossing in generations after the syn-1, as the number of possible progeny classes would rapidly multiply and in the syn-2 would include  $F_1 \times F_1$ ,  $F_2$ ,  $BC_1$ ,  $F_1$  and I types. The method is therefore practically limited to the syn-1 generation, but could be applied to a range of useful investigations, including checking for random intercrossing in different years and in different environments, which might give data relevant to the selection of inbred components, or for measuring crossing in different areas of the growing crop, where it has been shown to vary considerably (BOND & POPE 1974). The ability to identify genotypes from pollen zymograms may also have application in detecting, early in flowering, differential winter kill amongst winter synthetic variety components.

EST isoenzyme patterns can be successfully used for cultivar identification (Table 1), and could be put to this use in V. faba if genetically uniform varieties, either in the form of  $F_1$  hybrids or autofertile varieties, are developed in the future. BASSIRI and ROUHANI (1977) used EST and PER to identify a number of traditional cultivars, presumably composed of unknown proportions of autofertile and autosterile plants, using leaf tissue pooled from about ten plants to establish characteristic zymograms. The validity of taking pooled samples from a crop with an intermediate and variable level of outbreeding could be challenged from several viewpoints.

Pooled samples applied to such varieties may cover up a considerable amount of variation within the sample, and this disadvantage must be balanced against the more laborious sampling

of individual plants or seeds and basing cultivar identification on band (allele) frequencies. Fig. 20 shows the variation encountered when four seeds of cultivar "Maris Bead", selected at random, were sampled, and identification on the basis of allele frequency rather than on band patterns of bulk samples in material with such high levels of isoenzyme polymorphism would increase the number of cultivars which could be identified. However, the danger of using either technique on crops which are not wholly autogamous or allogamous is that the allele frequencies might not be stable when samples were grown under different environmental conditions, and the high fluctuation in levels of outcrossing reported in V. faba (HOLDEN & BOND 1960) might lead to changes in allele frequencies in populations over a number of years. ALLARD, KAHLER and WEIR (1972) demonstrated substantial changes in gene and genotype frequencies at four EST loci in a composite cross of barley over twenty-five generations, due to directional selections favouring particular alleles, and similar changes could be expected to occur in any heterozygous population.

The isoenzyme technique was also applied to autofertile and autosterile material that had been subject to enforced inbreeding over a number of generations by growing in isolation from bee pollinators in an experiment conducted by M. H. POULSEN at the Plant Breeding Institute, Cambridge (BOND, POPE & TOYNBEE-CLARK 1977). The isoenzymatic variation was compared between the initial populations and the eighth inbred generation in both autofertile and

autosterile genotypes, all originally derived from the cultivar Francks Ackerperle. The results in Table 10 clearly illustrate the higher level of isoenzyme heterozygosity in the autosterile material at the beginning of the experiment, and the homozygosity of both types after eight generations of selfing. Differences in heterozygosity at generation eight between autofertile and autosterile genotypes were not apparent, but this might have been due to the small samples tested. This application of the method, to compare and monitor declining levels of heterozygosity, is one which might profitably be extended, in combination with more precise genetic analysis of the EST isoenzymes.

Two separate hypotheses have been advanced to account for isoenzyme polymorphism (GOTTLIEB 1972). The selectionist hypothesis (PRAKASH et al 1969) supports the contention that isoenzyme polymorphisms have definite selective value, and are maintained and modified by natural selection, whilst the iso-allelic hypothesis (KIMURA 1968; KIMURA & CROW 1969; KING & JUKES 1969) states that isoenzymes arise and are maintained by mutation and genetic drift, and that they have no selective value. Consequently a number of authors have sought correlations between isoenzyme polymorphisms and ecological, physiological, and in some cases agronomic characteristics, with generally limited success. Positive correlations have been found between mosquito (Aedes triseriatus) EST alleles and ecological variables (SAUL, SINSKO, GRIMSTAD & CRAIG 1978), and EST alleles of a freshwater fish (Catostomus clarkii) and water temperature variation over the ecological range of the species

(KOEHN 1969). In the botanical field ALLARD and KAHLER (1971) detected allelic differentiation for a variety of enzymes over geographical ranges, with sharp EST differentiation between populations of inbreeders, whilst CLEGG and ALLARD (1972) demonstrated that Avena barbata populations were fixed for different isoenzyme complexes depending on environmental conditions, and HAMRICK and ALLARD (1975) discussed a correlation between quantitative characters and enzyme genotypes in the same species. MITRA, JAGANNATH and BHATIA (1970) failed to find any relationship between EST patterns of Hordeum vulgare and geographic origin, a result confirmed by BASSIRI (1976) for this species, and by BASSIRI & ROUHANI (1977) for V. faba.

It has been reported that characteristic EST isoenzyme changes are associated with photoinduction of Citrus (WARNER & UPADHYA 1968) and exposure to low temperature in Dianthus species (McCOWN, HALL & BECK 1969), and isoenzymes may provide useful markers for physiological changes.

There is only limited published work available showing association between isoenzyme patterns and agronomic characters, that would allow the use of isoenzyme patterns as a selection criterion, although VALIZADEH and VALDEYRON (1978) reported the existence of a relationship between allozymes of ACPH and precocity of fructification in Ficus carica. GONELLA and PETERSON (1978) sought a correlation between the relatedness of maize inbred lines, based on their isoenzyme similarities, and the performance of their hybrids, without success. Using the same index of relatedness employed by these authors, thirteen accessions of V. faba were compared (Table 11). It can be seen that the relatedness

indices are generally high for the P.B.I. winter synthetic variety components (Accessions 2-9), compared with the six selected spring bean lines. Between accessions 41-46, there is no clear relationship between the different origin of the lines and their relatedness indices.

The general methods applied in this study could potentially be extended to any self-pollinated crop variety, or any breeding programme where inbred lines were an integral part of the breeding cycle. For the method to be valid, either as a genetic marker or as a means of taxonomic distinction between crop varieties, the material under test should be genetically uniform, and exhibit the necessary isoenzyme polymorphism, unaffected by environmental conditions. Even if the last condition cannot be fulfilled, the method can still be used for plants grown in uniform environmental conditions.

To briefly test the feasibility of applying the gel system developed for V. faba on a range of other crop plants, seed extracts of Pisum sativum cv. "Little Marvel", Tetragonolobus purpureus, Malus sylvestris cv. Russet, Citrus limon, C. paradisi, and C. sinensis var. Ortanique, Castanea sativa, Raphanus sativus cv. "Suttons Scarlet Globe", and four varieties of Triticum aestivum were prepared and EST zymograms developed under the standard screening conditions used for V. faba. In addition, EST of V. cracca and V. narbonensis were compared with those of Field Bean. Fig. 24 shows that in these diverse taxa a large number of EST isoenzymes were resolved using the standard system.

The four varieties of T. aestivum could readily be identified on the basis of their EST isoenzyme patterns, and this enzymic character may be valuable in the identification of varieties which are morphologically very similar. MENKE et al (1973), using starch gel electrophoresis of vegetative tissue, have shown that wheat varieties have characteristic EST patterns which are not affected by environmental conditions. The system might be used to monitor the changing frequencies of components of mixed or multiline varieties over successive generations or at different locations, without the need to resort to incorporating morphological markers. Several authors have used gliadin patterns to identify wheat varieties (AUTRAN & BOURDET 1973; ELLIS & BEMINSTER 1977; BUSHUK & ZILLMAN 1978), where complicated protein patterns were resolved, and EST isoenzymes could profitably be investigated as a viable alternative.

Application of isoenzyme techniques to Citrus varieties has already been advocated by TORRES and SOOST (1978), who pointed out that by means of isoenzyme patterns, recombinant seedlings of potential interest to breeders, could be distinguished from valueless nucellar seedlings which commonly occur in this genus. Isoenzymes of vegetative tissues of Citrus and their relationship to environmental variation have been described by WARNER and UPADHYA (1968). Citrus, Malus sylvestris and Castanea sativa varieties are propagated vegetatively, and varietal clones and rootstocks could easily be identified by their isoenzyme patterns.

The work of FRANKEL and GARBER (1965) has shown that the EST isoenzymes of Pisum sativum are polymorphic, so that the bands resolved on the acrylamide gels could be used in this species for varietal identification and crossing studies.

Comparison of the three Vicia species showed that V. narbonensis exhibited an EST band not seen in any V. faba genotypes, and V. cracca had a banding pattern distinct from both of these species. The use of albumin protein patterns by LADIZINSKY (1975) showed that the seed protein profile was characteristic for Vicia species, and on the basis of the results reported here this may apply to EST patterns also.

A factor which must be constantly considered throughout isoenzyme studies such as this is that the conditions under which the enzymes are examined represent a highly artificial environment, and one that is totally unlike that in which the isoenzymes naturally function. In isoenzyme assays, the interactions between plant and environment which render some morphological markers unreliable is often eliminated, but there is still an environmental interaction during extraction, electrophoresis and assay, so that during these operations techniques must be carefully standardised. That the environment can be controlled in the laboratory represents a major advantage of the technique.

Secondary metabolic products in plants have been widely used in taxonomy, and have been employed to identify V. faba populations (POLIGNANO & SPLENDIDO 1978), but since proteins are the primary

products of genes and reflect the sequence of bases in the DNA, they are a more direct and accurate tool for detecting genetic variability in plants. In current synthetic varieties of Field Bean, and in future breeding methods, where the crop is likely to be pushed towards genetic uniformity either through the production of  $F_1$  hybrids or autofertile varieties, isoenzyme polymorphisms have a potentially useful role, allowing the breeder to identify material and monitor the progress of crossing programmes, and this study has shown that the technique can be reduced to a level of simplicity where the factors of cost and speed are reduced to acceptable levels. The method has wide application in the breeding of other crops.

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