ASPECTS RELATING TO THE OCCURRENCE OF AN INHIBITOR

OF TISSUE PLASMINOGEN ACTIVATOR IN *ERYTHRINA*

CAFFRA THUNB. PLANTS AND

IN VITRO CULTURES

by

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Erythrina caffra Thunberg

PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from January 1986 to December 1989 under the supervision of professor J. van Staden.

These studies, except the acknowledged work of others are the result of my own investigations.

H. J. MEYER

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ACKNOWLEDGEMENTS

material of various members of the genus *Erythrina*.

ABSTRACT

A double sandwich enzyme-linked immunosorbent assay (ELISA) was developed to quantify the proteinaceous inhibitor of tissue plasminogen activator (t-PA) which occur in the tissue of *Erythrina caffra* Thunb. Using the ELISA the t-PA inhibitor could be detected in nanogramme quantities on the microtiter plate.

The concentration of the t-PA inhibitor was determined in different tissues of *Erythrina caffra*. t-PA inhibitor concentrations in the order of 1 000 microgrammes per gramme protein were found in the seeds. Relatively small quantities of t-PA inhibitor, in the order of 10 to 50 microgrammes per gramme protein, occurred in root, shoot, leaf and living bark material.

The t-PA inhibitor was found to accumulate in a similar way to the storage proteins in developing seeds. The accumulation of the inhibitor is at a relatively low level during the early period of seed development but increases exponentially just before the seeds reach their maximum size.

The t-PA inhibitor content of the cotyledons decreased drastically during the process of germination and subsequent seedling development. The disappearance of the inhibitor can be the result of total degradation of the molecule or

partial proteolysis with the modified molecule still being present in the tissue.

An attempt was made to increase the t-PA inhibitor content of excised leaves of *Erythrina caffra* with protein inducing substances such as polyamines, precursors of ethylene and phytic acid. The protein inducing compounds included cell wall hydrolysates of *Erythrina caffra*, the marine alga *Ecklonia maxima* Osbeck (Papenfuss) as well as *Lycopersicon esculentum* Mill. which induced the synthesis of proteinase inhibitors suggested to be involved in the defense mechanism of plants. None of the substances used, increased the t-PA inhibitor content of excised leaves or *in vitro* cultures of *Erythrina caffra*. It is suggested that the t-PA inhibitor is probably not involved in a defense mechanism of *Erythrina caffra*.

A callus and suspension culture derived from shoot tissue was developed to determine the occurrence of the t-PA inhibitor *in vitro*. The optimal nutrient medium for the growth of callus was the salts and vitamins of MURASHIGE and SKOOG (1962). The medium was supplemented with 3 % sucrose, 0.1 gramme per litre meso-inositol, 10 micromoles per litre benzyladenine and 5 micromoles per litre 2,4-dichlorophenoxyacetic acid. Different auxins and cytokinins had a similar growth stimulatory effect on the growth of callus derived from a number of organs of *Erythrina caffra*. The callus from different organs did however, grow at different rates on the same nutrient medium. Callus derived

from leaf, shoot, and cotyledonary tissue grew at similar rates on the nutrient media of MURASHIGE and SKOOG (1962), SCHENK and HILDEBRANDT (1972) and B5 (GAMBORG, MILLER and OJIMA, 1968) despite large differences in the concentration of the nutrients in the three nutrient media. The source of nitrogen and ratio of nitrate to ammonium was critical to the growth of callus cultures. The optimal concentration of nitrate and ammonium was 30 millimoles per litre. The growth of callus from different organs was significantly affected by the concentration of sucrose in the nutrient medium. A concentration of 3% was optimal for callus growth. Temperature had a significant effect on the growth of callus. The optimal temperature for callus growth was 25 $^{\circ}$C.

A shoot cell suspension culture was established and maintained at the same temperature and on the same medium as the callus cultures but with a ten times lower concentration of growth regulators. A low shake speed was essential for the growth of the suspension culture. Maximum growth was obtained at a shake speed of 60 rpm. Relatively low quantities of t-PA inhibitor, in the order of 1 to 5 microgrammes per gramme protein, was detected in the suspension cultures. An attempt was made to increase the t-PA inhibitor content of the suspension cultures with the protein inducing compounds used on excised leaves, but without success. However, the t-PA inhibitor content of the suspension culture was significantly increased with a ten times increase in the sulphate content of the nutrient medium.

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CHAPTER 1

INTRODUCTION

1.1 DESCRIPTION OF *ERYTHRINA CAFFRA*

Erythrina caffra Thunb. is a member of the family Leguminosae (Fabaceae) and falls in the subfamily Papilionoideae. It resides in the tribe Phaseoleae and the subtribe Erythrinae (COLLETT, 1941). COLLETT (1941) describe *Erythrina caffra* also known as the coastal coral tree as one of nine members of the genus *Erythrina* in Southern Africa. *Erythrina caffra* is a tree that can attain a height of seven to seventeen meters. The branches are woody, leafy and armed with thorns. The tree which is deciduous has scattered or clusterd leaves at the end of the branches. The leaves which are armed with thorns in the seedling stage are glabrous, trifoliate broadly ovate and frequently broader than long. The racemes are borne terminal and is densely many flowered. The flowers are brilliant scarlet-orange, showy and appear before the leaves. The pods are black when mature and subligneous. They are about twelve centimeters long, stalked, unarmed and deeply constricted between the seeds. The pods are densely tomentose when young, becoming glabrous when old. The seeds are elliptic and bright red with a linear scar of attachment.

1.1.2 DISTRIBUTION

Erythrina caffra is distributed mainly along the eastern coastal region of Southern Africa with a southernly limit near Humansdorp in the Eastern Cape province. It occurs north up to Kosi Bay along the coast. The coastal coral tree occurs inland in its most southern distribution in the Eastern Cape as far as the ditricts of Bathurst and Albany. The tree is also found inland in the Transvaal lowveld as well as on the highveld in the Pretoria district. It also occurs to the north in the Soutpansberg and Pietersburg districts. *Erythrina caffra* is mainly associated with coastal forest and wooded stream banks where it apparently occurs as a transitional species on the forest margin.

1.1.3 ECONOMIC AND MEDICINAL IMPORTANCE

The genus *Erythrina* is not a genus with economic importance. The bright red seeds of *Erythrina caffra* and other members of the genus are used for necklaces by the local indigenous tribes. The most important economic use of *Erythrina caffra* is as ornamental street- and garden trees (COLLETT, 1941).

The genus has no medicinal importance in the western world. A leaf paste of *Erythrina caffra* is one of the

ingredients in a poultice used by the Zulus for bladder infection (WATT and BREYER-BRANDWIJK, 1962).

1.2 TRYPSIN AND OTHER PROTEINASE INHIBITORS

1.2.1 DESCRIPTION

Plants contain a number of low molecular mass proteins which have the the property of forming reversible stochiometric complexes with various proteolytic enzymes. This brings about competitive inhibition of their catalytic functions. One group of proteinase inhibitors is the inhibitors of trypsin. The trypsin inhibitors do not exclusively inhibit trypsin but they can also inhibit the catalytic activity of chymotrypsin (JOUBERT, CARLSSON and HAYLETT, 1981 ; JOUBERT, 1982a ; JOUBERT, 1982b) and other serine proteinases such as elastase (WILSON and LASKOWSKI, 1975), thrombin (WARSY, NORTON and STEIN, 1974), plasmin and kallikrein (SAKATO, TANAKA and MISAWA, 1975) and tissue plasminogen activator (HEUSSEN, JOUBERT and DOWDLE, 1984 ; JOUBERT, MERRIFIELD and DOWDLE, 1987). Some potent inhibitors of trypsin are inactive or only weakly active against chymotrypsin (WILSON and LASKOWSKI, 1973; JOUBERT, 1982a ; JOUBERT, 1982b) and vice versa (JOUBERT, 1982a ; JOUBERT, 1982b). In some cases the reactive site of the inhibitor is the same for trypsin, chymotrypsin (JOUBERT, 1982a ; 1982b) and tissue plasminogen activator (JOUBERT, MERRIFIELD and DOWDLE, 1987). This type of inhibitor is referred to as a monovalent

or single headed KUNITZ type inhibitor, named after KUNITZ (1945) who first isolated and crystallised it from *Glycine max* Merr. It was demonstrated in earlier studies on trypsin inhibitors in soybeans that more than one trypsin inhibitor are present in soybeans which could be readily distinguished from the KUNITZ inhibitor on the basis of their solubility in ammonium sulphate, trichloroacetic acid, alcohol and acetone. The acetone soluble inhibitor was subsequently purified by BIRK and coworkers (BIRK, 1961; BIRK, GERTLER and KHALEF, 1963). These BOWMAN-BIRK type inhibitors have two active sites for the independent inhibition of trypsin and chymotrypsin. The typical KUNITZ inhibitor has a polypeptide chain of 181 residues and only two disulphide bridges (Figure 1.1). The folding of the polypeptide backbone of the *Glycine max* KUNITZ inhibitor and a model of the three dimensional conformation of the molecule is illustrated in Figures 1.2 and 1.3 respectively. The molecular mass of these inhibitors range from 20 000 to 60 000. The BOWMAN-BIRK inhibitor has seven disulphide bridges which contributes to the higher stability of the molecule (Figure 1.4). It has only 70 amino acid residues and the molecular mass of this class of inhibitor ranges between 6000 and 12000 (LIENER and KAKADE, 1969). These molecules have two regions of homology on the same peptide chain which coincides with the two reactive sites. These regions of homology are linked by disuphide bonds. This is not the case with the KUNITZ inhibitors. Differences were also found in the kinetics of the two types of inhibitors. With the KUNITZ inhibitor it was found that a second order

- 5 -

Figure 1.1 The primary structure of the KUNITZ
trypsin inhibitor isolated from *Glycine
max* Mill. The shaded areas represent the
S-S bonds between cysteine residues
(KOIDE and IKENAKA, 1973).
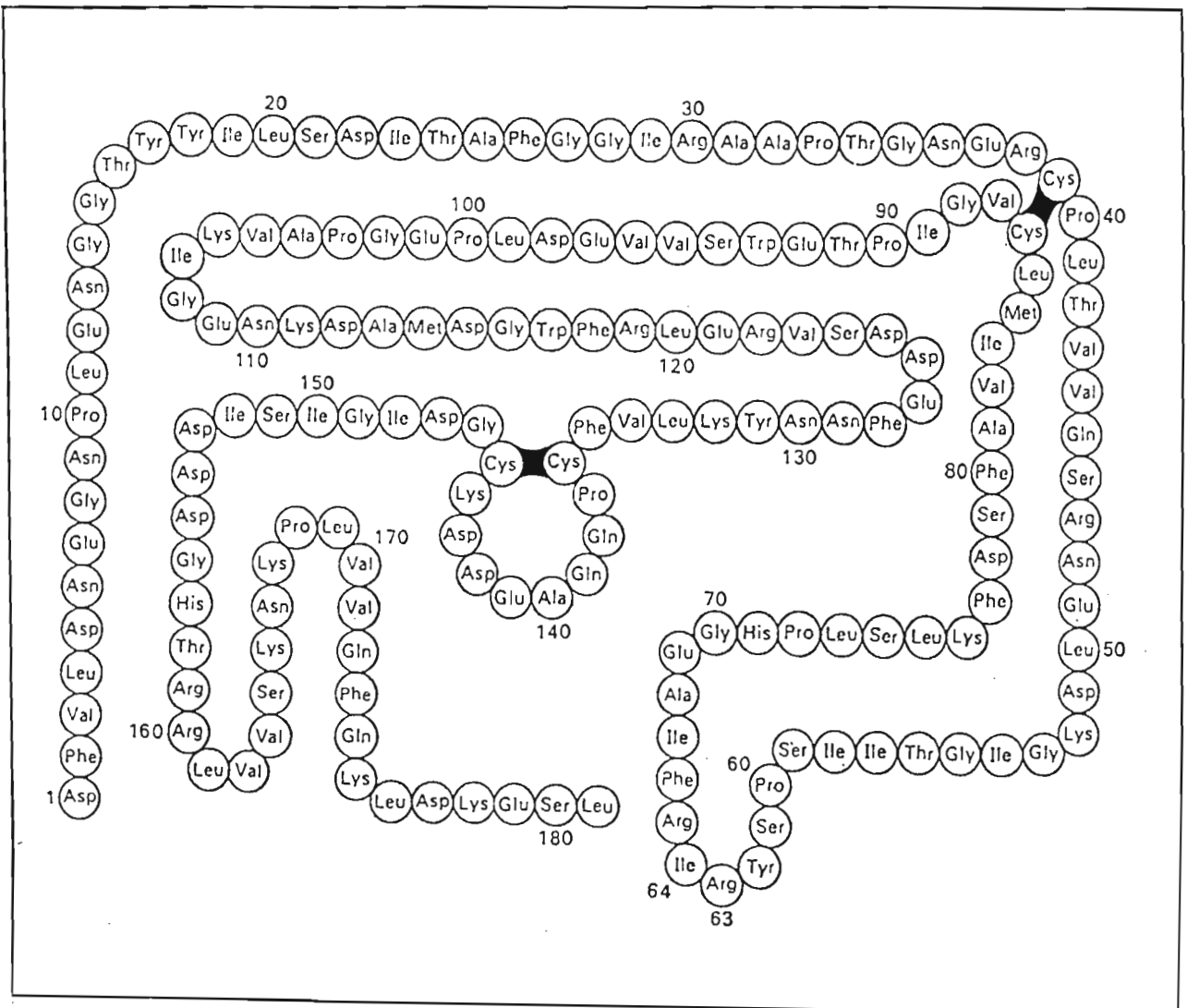
Figure 1.2   The secondary structure   of   the   KUNITZ
             trypsin inhibitor isolated from   *Glycine*
             max Mill.   The   shaded   areas   represent
             amino acids in intimate   contact with the
             trypsin molecule during the bonding
             process   (SWEET,   WRIGHT,   JANIN,   CLOTHIS
             and BLOW, 1974).
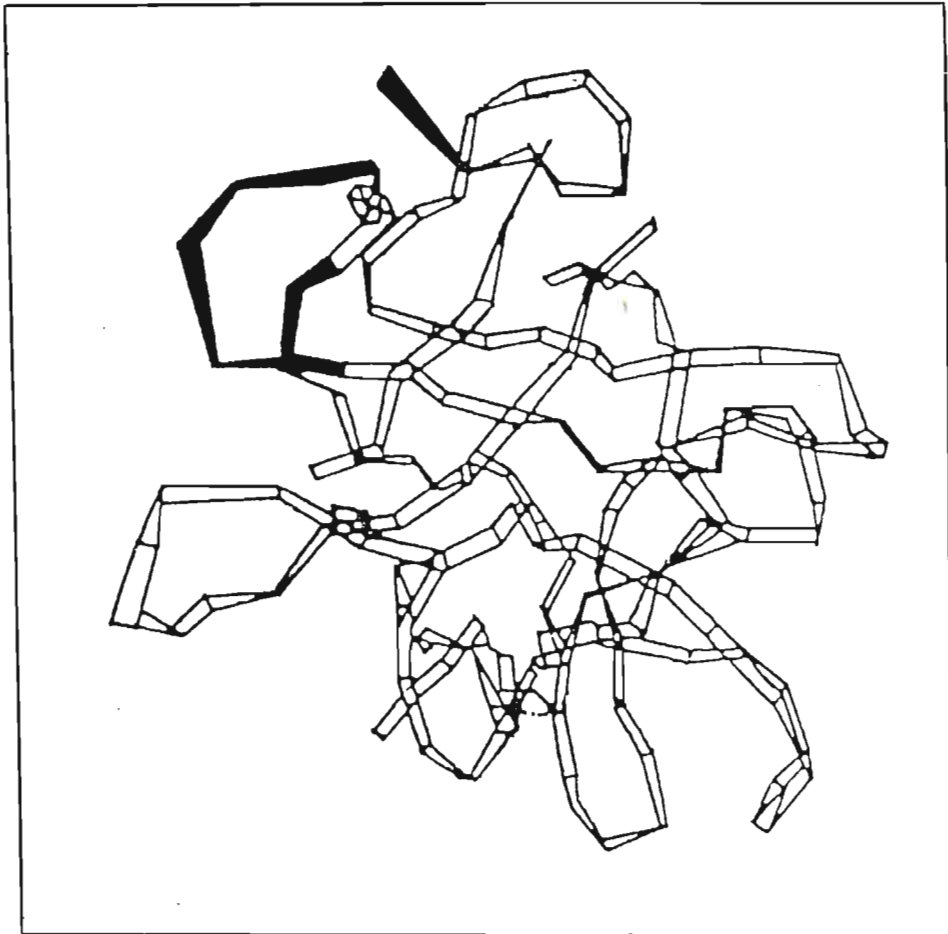
Figure 1.3   Model of the bonding between the KUNITZ
             trypsin inhibitor isolated from *Glycine*
             max Mill. and trypsin.   Shaded area
             represents the trypsin molecule (SWEET,
             WRIGHT, JANIN, CLOTHIS and BLOW, 1974).
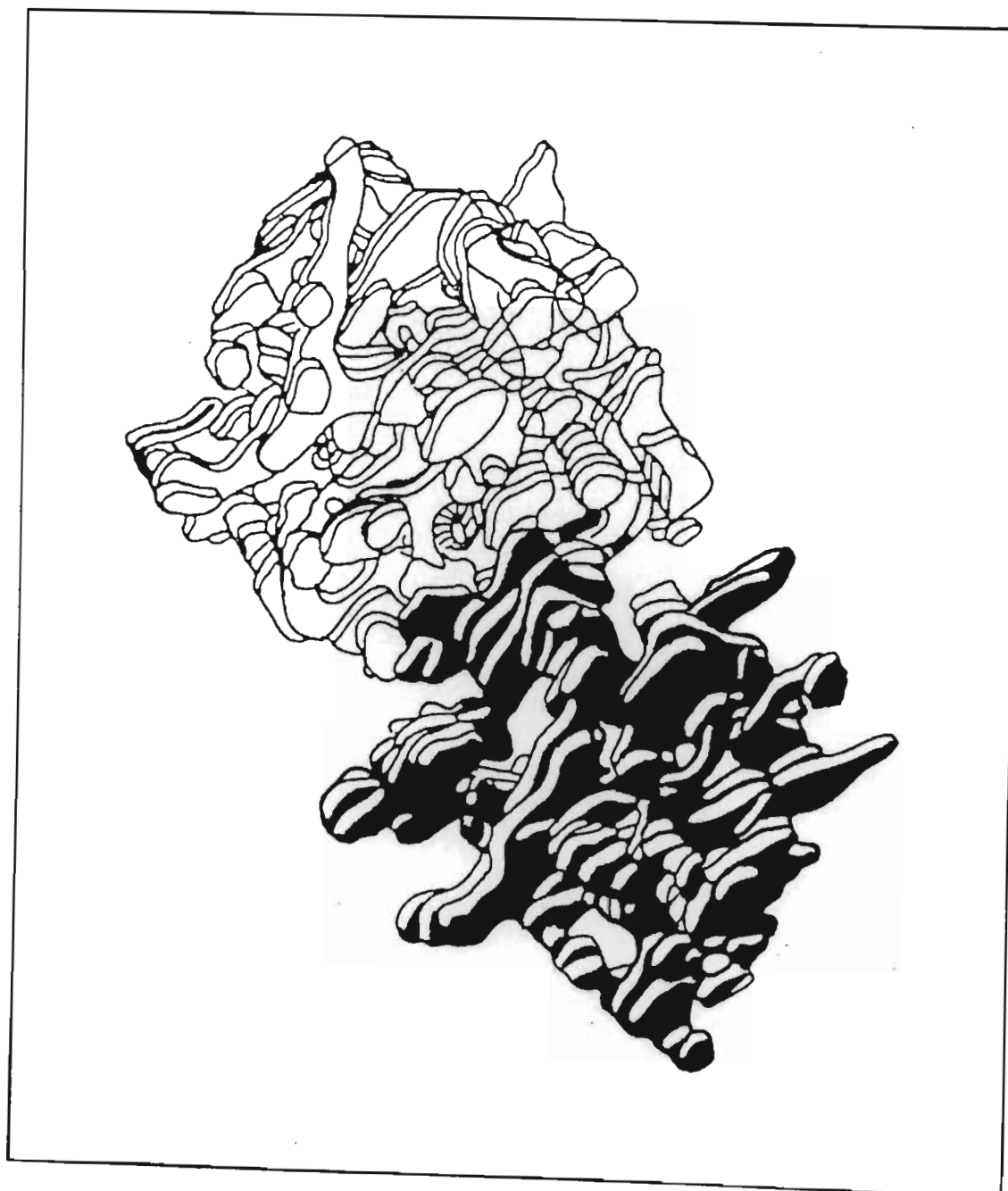
Figure 1.4   The primary   structure of   a BOWMAN-BIRK
              trypsin   inhibitor.   The   shaded   links
              between the   amino   acids   represent   the
              S-S bonds between cysteine residues.   The
              shaded   amino   acids   represent   the
              reactive   sites   for   trypsin   and
              chymotrypsin (KOIDE and IKENAKA, 1973).
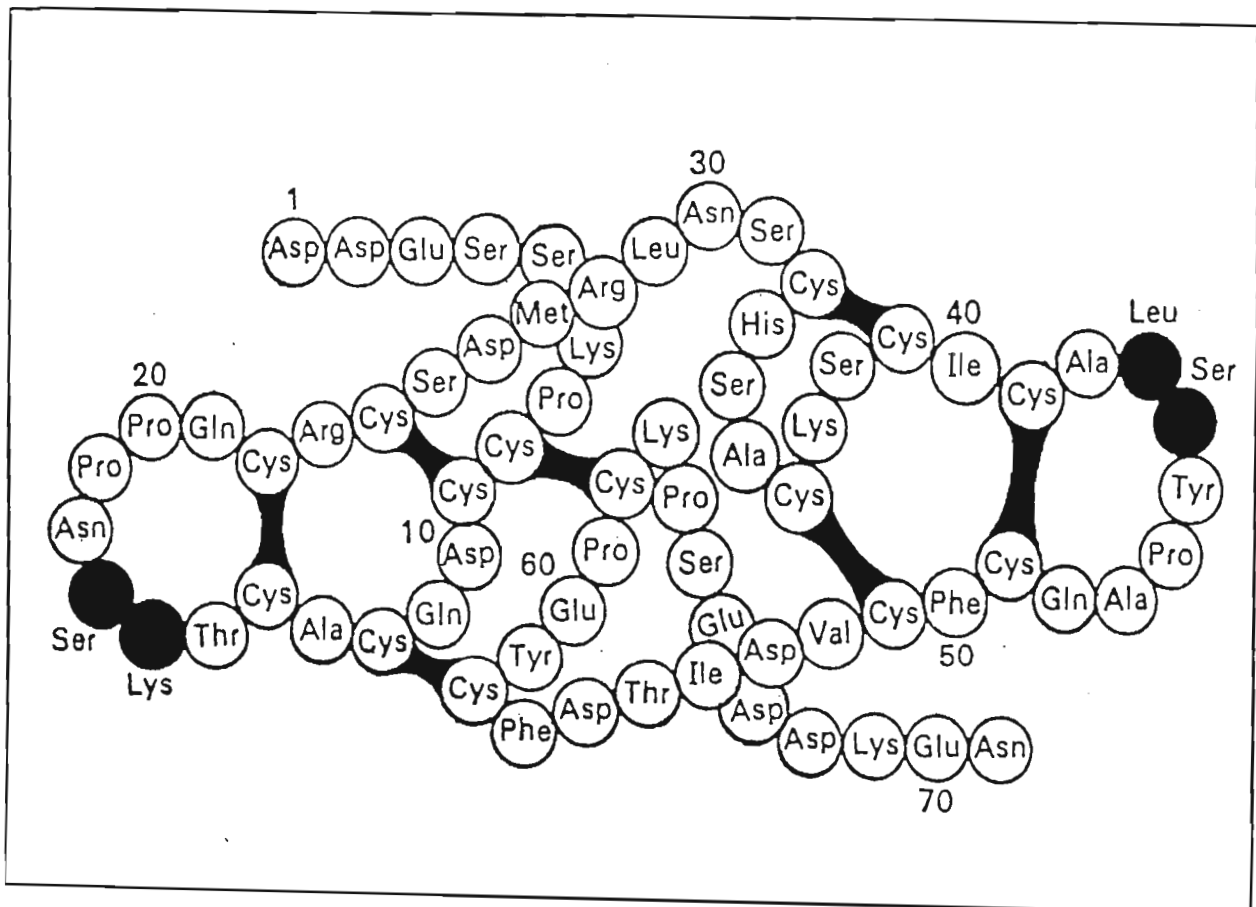
process followed by a first order process was sequentially involved in a stable complex formation with trypsin. The postulated mechanism of interaction is as follows:

$$T + I == T-I == C == T-I* == T + I*$$
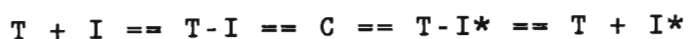
where T = trypsin

     I = inhibitor

     T-I and T-I* = loose non-covalent complexes

     I* = modified inhibitor

The rate of association of the BOWMAN-BIRK inhibitor with trypsin was also biphasic. It changed from a second order reaction at low reactant concentrations to a first order reaction when the reactant concentrations were raised. This was explained by postulating that the initial loose complex underwent a subsequent intermolecular rearrangement to form a stable compound (BIRK, 1976). The suggested model for the complete process according to BIRK (1976) is :

$$T + I == C* == C$$

Where T = trypsin

     I = inhibitor

     C* = initial complex

     C = final complex

The molecular relationships between trypsin inhibitors from

different plants were elucidated with the comparison of the amino acid sequence of the different inhibitor molecules. An example of such comparisons is illustrated in Table 1.1. Unexpected large regions of homology were detected between trypsin inhibitors of plants within the same family. An interesting observation was the similarity in the amino acid sequence of the active site of different KUNITZ inhibitors. Inhibitors of proteinases from members of the same genus seemed to have very similar amino acid sequences. In members of the genus *Erythrina* a high degree of inter-specific homology exists in the amino acid sequence around the active site of inhibitors of chymotrypsin, (Table 1.2) trypsin (Table 1.3) and inhibitors of both trypsin, chymotrypsin and tissue plasminogen activator (Table 1.4). Although the inhibitors inhibited the catalytic activity of different proteinases it is very noticeable that in the inhibitors of trypsin, chymotrypsin and tissue plasminogen activator the active site possesses exactly the same amino acid sequence of arginine and serine. The only exceptions were the replacement of serine with threonine at the active site of the trypsin inhibitor of *Erythrina lysistemon* Hutch. and the chymotrypsin inhibitor of *Erythrina abyssinica* Lam. DC. This indicates that not only the active site of the proteinase inhibitor was determinant in site recognition but that the three dimensional conformation of the inhibitor molecule also plays a role in site recognition and steric hinderances between substrate and inhibitor. It is evident from Tables 1.2, 1.3 and 1.4 that a large degree of inter specific homology exist in the amino acid sequence

Table 1.1   Comparison of the amino   acid sequence of
members of the KUNITZ trypsin inhibitor
family. Abbreviations for the trypsin
inhibitors are: WBI = inhibitor from
Psophocarpus tetragonolobus DC. ; ELI = DE3
inhibitor from *Erythrina caffra* Thunb. STI =
KUNITZ trypsin inhibitor from *Glycine max*
Mill.; PAI = trypsin inhibitor from *Peltophorum*
africanum Sond; AJI1 and   AJI2 = partial
sequences of the   two-chained inhibitors
AII and BII from *Albizzia julibrissin* L.; API =
two-chained inhibitor DE5 from *Adenanthera*
pavonina L.; AEI and ASI = partial sequences of
the two-chained inhibitors   from *Acacia elata*
Cunn. and *Acacia sieberana* DC. respectively;
BASI and WASI   = inhibitors of subtilisin and
endogenous alpha-amylases from *Hordeum vulgare*
L. and wheat respectively; RPI = inhibitor from
Oryza sativa L. Vertical arrows (⬍) indicate
the reactive sites of the inhibitors.
Unidentified amino acid residues are indicated
by an asterisk (*). Gaps introduced for the
alignment of amino acid residues are indicated
by a horisontal line (-). The N-terminal
positions of the second chain of the two-
chained inhibitors are indicated by a vertical
line (|). Abbreviations for amino acids for
Tables 1.1   to   1.5 are : A=alanine,
C=cysteine, D=aspartic acid, E=glutamine,
F=phenylalanine, G=glycine, H=histidine,
I=isoleucine, K=lysine, N=asparagine,
P=proline, Q=glutamate, R=arginine, S=serine,
T=threonine, V=valine and Y=tyrosine (GARCIA-
OLMEDO, SALCEDO, SANCHEZ-MONGE, GOMEZ, ROYO and
CARBONERO, 1987).

```
                  1                                                                              50
WBI    E P L L D S E G E L V R N G G T Y Y L L P D R W A L G G G I E A A A T G I E T C P L T V V R S
ELI      V L L D G N G E V V Q N G G T Y Y L L P Q V W A Q G G G V Q L A K T G E E T C P L T V V Q S
SII    D F V L D N E G N P L E N G G T Y Y I L S D I T A F - G G I R A A P T G N E R C P L T V V Q S
PAI    D F V L D A E G K F L - N G E I Y Y I L P
IIIb   K E L L D A D G D I L L N G G • Y Y I V
SIIb   S N L L L D T D G N L L E D G G • Y Y I L P A
API    R E L L D V D G N F L R N G G S Y Y I V P A F R G Y G G G L E L A R T G S E T C P P T V V Q A
BEI    K G L L D A D G D I L
ISI    • • L L D A D G D L L • S G Y L Y Y I L
BASI   A D P P P V N D T D G H E L R A D A N Y Y V L S A N R A H G G G L T M A P G H G R H C P L F V S Q D
WASI   D P P P V H D T D G N E L R A D A N Y Y V L P A N R A H G G G L T M A P G H G R R C P L F V S G E •
RPI    A P P P V Y B T Z S H G L S A B G S Y Y V L P A


                  51                    P3 P2 P1 P'1 P'2 P'3                                      100
WBI    P N E V S V G E P L R I S S Q L R S G F I P D Y - - - S - L V R I G F A N P P K C A P S P - W W T V
ELI    P N E L S D G K P I R I E S R L R S T F I P D D - - - D - E V R I G F A Y A P K C A P S P - W W T V
SII    R N E L D K G I G T I I S S P Y R I R F I A E G H P L S - L K F D S F A V I M L C V G I P T E W S V
API    P A E Q S R G L P A R L S T P P R I R Y I G P E F Y L T - I E F E E - Q K P P S C L R D S N L Q W K
BASI   P N G Q H D G F P V R I T P Y G V A P S D K I I R - L S T D V R I S F R A Y T T C L Q - S T E W H I
WASI   A D G Q R D G L P V R I A P H G G A P S D K I I R - L S T D V R I S F R A Y T T C V Q - S T E W H I


                  101                                                                            150
WBI    V E D Q P Q Q P S V K L - S E L K S T K F D Y - - - L F Y F E K V T S - K F S S Y K L K Y C - A K R
ELI    V E D E Q E G L S V K L - S E D E S T Q F D Y - - - P F K F E Q V S D - K L H S Y K L L Y C - E G K
SII    V E D L P E G P A V K I - G E N K D A M - D G - - - W F R L E R V S D D E F N N Y K L V F C - P Q Q
API    V E E E S Q I - - V K I - A S K E E E Q L F G - - - S F Q I K P Y R D D - - - - Y K L V Y C E P Q Q
BASI   D S E L A A G R R H V I T G P V K D P S P S G R E N A F R I E K Y H G A E V S E Y K L M S C - - - -
WASI   D S E L V S G R R H V I T G P V R D P S P S G R E N A F R I E K Y S G A E V H E Y K L M A C - - - -


                  151                                                                      193
WBI    - - - D T C K D I G I Y R D Q - G Y - A R L V V T D E N P L - V V I F - K - K V E S S
ELI    - - H E K C A S I G I N R D Q K G Y - R R L V V T E D N P L T V V L - - K - K D E S S
SII    A E D D K C G D I G I S I D H D D G T R R L V V S K N K P L - V V Q F Q K L D K E S L
IIIb   K D D H C K D L G - S I D D D E N
SIIb   N R D C K D L G I S T D D D N
API    G G R P E C K D L G I S I D D D N N - R R L A V K E G D P L - V V Q F V N A D R E G N
BEI    D D E S C K D L G I S I D D - E N
ISI    S • • E • C Q D L G I • V D D - E
BASI   - - G D W C Q D L G V F R D L K G G A W F L G A T E P Y H V - V V - F K K A P P A
WASI   - - G D S C Q D L G V F R D L K G G A W F L G A T E P Y H V - V V - F K K A P P A
```

Table 1.2 Partial amino acid sequence of chymotrypsin inhibitors isolated from members of the genus *Erythrina* (JOUBERT, MERRIFIELD and DOWDLE, 1987).

Species	Amino acid sequence
E. caffra	R**S--T--F--I--P--N--G--S--P
E. latissima	R**S--T--F--I--P--N--G--N--L
E. abyssinica	R**T--T--F--E--P--N--N--E--K

** Reactive site.

Underlined text indicates amino acids invariant from those of *Erythrina caffra*.

Table 1.3 Partial amino acid sequence of trypsin inhibitors isolated from members of the genus *Erythrina* (JOUBERT, MERRIFIELD and DOWDLE, 1987).

Species	Amino acid sequence
E. caffra	R**S--Y--F--I--P--K--G
E. abyssinica	R**S--Y--F--I--P--K--G
E. decora	R**S--Y--F--I--P--K--G
E. latissima	R**S--Y--F--I--P--K--G
E. lysistemon	R**T--Y--F--I--P--V--G

** Reactive site.

Underlined text indicates amino acids invariant from those of *Erythrina caffra*.

Table 1.4 Partial amino acid sequence of inhibitors of
tissue plasminogen activator isolated from
members of the genus *Erythrina* (JOUBERT,
MERRIFIELD and DOWDLE, 1987).

Species	Amino acid sequence
E. caffra	R**S--A--F--I--P--N--G--S--K
E. lysistemon	R**S--A--F--I--P--N--G--S--K
E. zeyheri	R**S--A--F--I--P--N--G--S--K
E. acanthocarpa	R**S--T--F--I--P--N--G--S--K
E. decora	R**S--T--F--I--P--N--G--S--E
E. latissima	R**S--T--F--I--P--N--G--S--E

** Reactive site.

Underlined text indicates amino acids invariant from those
of *Erythrina caffra*.

Table 1.5   Partial amino acid sequence of inhibitors of
trypsin, chymotrypsin and tissue plasminogen
activator isolated from *Erythrina caffra*.
(JOUBERT, MERRIFIELD and DOWDLE, 1987).

Inhibitor	Amino acid sequence
Trypsin	R**S--Y--F--I--P--K--G
Chymotrypsin	R**S--T--F--I--P--N--G
t-PA	R**S--A--F--I--P--N--G

** Reactive site.

Underlined text indicates amino acids invariant from those
of *Erythrina caffra*.

between inhibitors of a similar proteinase. On the other hand it is clear from Table 1.5 that more pronounced inter specific differences occur at the active sites between inhibitors of different proteinases. The existing homology between trypsin inhibitors from different genera and families raises the question about the evolutionary development of these proteinase inhibitors. Such homology could point to a very early existence of these inhibitors and a common ancestor for these plants. Further research on proteinase inhibitors was mainly stimulated by the realization of the possible deleterious nutritional effects they may have when present in the diet of husbandry animals.

1.2.2 THE OCCURRENCE OF PROTEINASE INHIBITORS IN LIVING ORGANISMS

Inhibitors of proteinases are ubiquitous. They are present in tissues of mammals (VOGEL, TRAUTSCHOLD and WERLE, 1968), snails, cuttle fish and mussels (TSCHESCHE and DIETL, 1976), ascarids (KASSEL, 1970b), sea anemones (WUNDERER, BERESS, MACHLEIDT and FRITZ, 1976), higher plants, algae, fungi and bacteria (VOGEL, TRAUTSCHOLD and WERLE, 1967). After the discovery of trypsin inhibitors in soybeans, trypsin, chymotrypsin and other protease inhibitors were isolated mainly from plant families of economic significance, such as the Leguminosae, Solanaceae, Poaceae and to a lesser extent in the Chenopodiaceae, Brassicaceae and Rosaceae (VOGEL, TRAUTSCHOLD and WERLE, 1968 ; LIENER and KAKADE, 1969 ;

CHEN and MITCHELL, 1973). Of these families most attention has been devoted to the Leguminosae. This is ascribed to the recognition of legume seeds as an excellent source of protein. Trypsin and chymotrypsin inhibitors have been found in 66 species from 43 genera of the subfamily Papilionoideae, 26 species from 16 genera of the subfamily Caesalpinoideae and 27 species from 9 genera of the subfamily Mimosoideae. This accounts for 60 %, 50 % and 80% of the total species content of the Papilionoideae, Caesalpinoideae and Mimosoideae respectively (WEDER, 1981). Apart from trypsin- and chymotrypsin inhibitors members of the genus *Erythrina* contain the unique inhibitor of tissue plasminogen activator (t-PA). Inhibitors of trypsin, and chymotrypsin were isolated from *E. latissima* E. Mey. (JOUBERT, CARLSSON and HAYLETT, 1981), *E. acanthocarpa* E. Mey. (JOUBERT, 1982a), *E. caffra* Thunb. (JOUBERT, 1982b), *E. humeana* Spreng. (JOUBERT, 1982c), *E. lysistemon* Hutch. (JOUBERT, 1982d), *E. Zeyheri* Harv. (JOUBERT, 1984), *E. cristagalli* L. and *E. corallodendron* L. (JOUBERT and SHARON, 1985), *E. decora* Harms. and *E. abyssinica* Lam. DC. (JOUBERT, 1986). An inhibitor of t-PA was isolated from local *Erythrina* species such as *E. caffra*, *E. latissima* and *E. lysistemon* and other members of this genus (Tables 1.6 and 1.7). Commercial crop plants from which trypsin and chymotrypsin inhibitors have been isolated include a large number of legumes, for example *Arachis hypogeae* L., *Phaseolus aureus* Roxb. (VOGEL, TRAUTSCHOLD and WERLE, 1968), *Cajanus indicus* Spreng. and *Dolichos lablab* L. (JAFFE, 1950), *Faba vulgaris* Moench. (SOHONIE and BHANDARKAR, 1954),

Phaseolus vulgaris L. (BOWMAN, 1944) and *Pisum sativum* L. (CHATTAPADHYAY and BANNERJEE, 1953). Other species from which trypsin and chymotrypsin inhibitors have been isolated include *Solanum tuberosum* L. (SOHONIE and AMBE, 1955), *Ipomoea batatas* Poir. (SOHONIE and HONOVAR, 1956) *Zea mays* L. (HOCHSTRASSER, MUSS and WERLE, 1967), *Lycopersicon esculentum* Mill. (PEARCE, LILJEGREN and RYAN, 1987), *Spinacea oleracea* L., *Cucumis sativus* L., *Raphanus sativus* L., *Lactuca sativa* L., *Daucus carota* L., *Asparagus officinalis* L., *Prunus persica* Sieb. and Zucc., *Prunus domestica* L., *Persea americana* Mill.and various varieties of *Brassica oleracea* L. (CHEN and MITCHELL, 1973).

1.2.3 THE DISTRIBUTION OF PROTEINASE INHIBITORS IN PLANTS

Most proteinase inhibitors have been found in seeds of various plants such as *Glycine max* (KUNITZ, 1945), *Albizzia julibrissin* Durazz. (ODANI, ODANI, ONO and IKENAKA, 1979), various *Erythrina* species from Africa (Tables 1.6 and 1.7) and *Acacia* species (WEDER and MURRAY, 1981) to mention but a few. However, these inhibitors are not restricted to this organ of the plant. Trypsin inhibitors were isolated from the leaves of *Ipomoea batatas* (HONOVAR and SOHONIE,1955), *Solanum tuberosum* (RYAN and HUISMAN, 1967) and *Lycopersicon esculentum* (WALKER-SIMMONDS and RYAN, 1977a). The stems of *Phaseolus aureus* Roxb. (HONOVAR and SOHONIE, 1955) and *Dolichos lablab* (AMBE and SOHONIE, 1956) also contain

Table 1.6 Inhibitors of chymotrypsin and t-PA isolated from members of the genus *Erythrina* (JOUBERT, MERRIFIELD and DOWDLE, 1987).

Species	Fraction	INHIBITOR ACTIVITIES		
		Trypsin ($U.mg^{-1}$)	Ch-trypsin ($U.mg^{-1}$)	t-PA ($IU.mg^{-1}$)
E. abyssinica	DE-1	3160	7280	ND
E. acanthocarpa	DE-2	190	9870	1070
E. caffra	DE-2	910	9230	ND
E. corallodendron	DE-2	0	6530	ND
E. cristagalli	DE-2	0	9330	ND
E. decora	DE-1	440	9910	1590
E. latissima	DE-1	160	8160	110
E. lysistemon	DE-1	0	9960	300
E. zeyheri	DE-2	0	7870	ND

ND = not determined. Ch-trypsin = Chymotrypsin.

Table 1.7 Inhibitors of trypsin and t-PA isolated from members of the genus *Erythrina* (JOUBERT, MERRIFIELD and DOWDLE, 1987).

Species	Fraction	Inhibitor activities		
		Trypsin $(U.mg^{-1})$	Ch-trypsin $(U.mg^{-1})$	t-PA $(IU.mg^{-1})$
E. abyssinica	DE-6	8100	2500	600
E. caffra	DE-4	10410	930	740
E. corallodendron	DE-6	8440	2140	ND
E. cristagalli	DE-1	8550	3230	30
E. decora	DE-4	10900	1590	1590
E. latissima	DE-2	6040	2720	1170
E. lysistemon	DE-2	7360	6400	1450
E. zeyheri	DE-5	8650	6400	700

ND = not determined. Ch-trypsin = chymotrypsin.

trypsin inhibitors. Inhibitors of trypsin and chymotrypsin were also isolated from the roots, endosperm and embryos of *Hordeum vulgare* L. (KIRSI, 1983) and the roots of *Sagittaria sagittifolia* L. (CHI, LIN, TAN and WANG, 1985).

The inhibitory activity of proteinase inhibitors is not the same in the different organs of the same plant or at different stages of the growth cycle of the plant. High inhibitor activity was found in the cotyledons and leaves of *Phaseolus aureus*. However, the inhibitory activity in the stem and leaves of the same plant was considerably lower (HONOVAR and SOHONIE, 1955). During the early phase of the growth cycle plant organs such as leaves seem to contain higher levels of inhibitor than the older organs. This is the case with *Ipomoea batatas* (HONOVAR and SOHONIE, 1955) and *Solanum tuberosum* (RYAN and HUISMAN, 1967).

Differences in inhibitor concentration were sometimes found in different parts of the same organ. It was established that the concentration of inhibitor was higher in the outer parts than in the inner parts of the cotyledons of *Phaseolus vulgaris* and *Cicer arietinum* L. (VOGEL, TRAUTSCHOLD and WERLE, 1968).

1.2.4 THE PHYSIOLOGICAL SIGNIFICANCE OF PROTEINASE INHIBITORS

The physiological significance of protease inhibitors is well established in animals and conversely to plants the inhibitor in an animal has a direct function as an enzyme regulator *in vivo*. For example proteinase inhibitors in blood plasma reduce blood clotting and affect the biosynthesis and metabolism of hormones. Secretory trypsin inhibitors in the pancreas prevents premature activation of trypsinogen and trypsin inhibitor-al protect the lung tissue against excessive proteolysis (LASKOWSKI and KATO, 1980).

The physiological significance of proteinase inhibitors in plants is still obscure since little direct evidence of it's function has been reported. Sometimes these inhibitors are considered as artifacts of the evolutionary process. More recent evidence indicate that they may play a role as protective agents against insects and pathogenic fungi.

The occurrence of proteinase inhibitors in high concentrations in seeds focused the attention of many researches on the seed in an attempt to explain the function of these proteins in terms of a regulator of proteinase activity or simply as a storage protein. The fate of trypsin inhibitors in the seed and especially in the cotyledons is not clearly understood at this time. In some seeds the first event in proteinase inhibitor metabolism during germination seems to be an increase in the proteinase

content of the cotyledons as was found in *Phaseolus vulgaris* cultivars (PUSZTAI, 1972 ; KIRSI, 1974), *Glycine max* (COLLINS and SAUNDERS, 1976) and *Vigna sinensis* (L.) Savi ex Hassk. (XAVIER-FILHO and NEGEIROS, 1979). However, there is overwhelming evidence that a decrease in proteinase inhibitor content occur during and after germination of the seeds of plants such as *Vigna radiata* (BAUMGARTNER and CHRISPEELS, 1976 ; CHRISPEELS and BAUMGARTNER, 1978), *Hordeum vulgare* (MIKOLA and ENARI,1970), *Lactuca sativa* (SHAIN and MAIER, 1968), *Oryza sativa* L. (HORIGUCHI and KITAGISHI, 1971) and *Phaseolus vulgaris* (PALMER, McINTOSH and PUZTAI, 1973).

Changes in the trypsin inhibitor content during germination does not only occur in the cotyledons but also in the embryo and the resulting seedling. A decrease was observed in the trypsin inhibitor content of the embryonic axis of *Vigna sinensis* cultivar Sendo during the first twenty four hours of germination. In contrast with this observation the trypsin inhibitor concentration of the cotyledons increased during the same period (XAVIER-FILHO and NEGEIROS, 1979). In *Hordeum vulgare* the trypsin inhibitor disappeared from the endosperm during germination but the total amount of trypsin inhibitor in the embryo was not affected and was the same as in the emerging seedling. However, five days after germination no trypsin inhibitor could be detected in the seedling (KIRSI and MIKOLA, 1971). The total disappearance of trypsin inhibitor from seedlings a few days after germination was also reported with *Phaseolus vulgaris*

(NIELSON and LIENER, 1988). These differences in behaviour of trypsin inhibitors in the seed during developmental processes may be an indication of functional differences of the inhibitors between plant parts and organs.

A closer examination of the effect of germination on the class of trypsin inhibitor revealed that the KUNITZ and BOWMAN-BIRK trypsin inhibitors reacted differently and independently of each other. It was observed that the KUNITZ trypsin inhibitor in *Glycine max* cultivar Fiskeby 5 decreased slower than the BOWMAN-BIRK trypsin inhibitor during germination. New KUNITZ trypsin iso-inhibitors also appeared during germination. BOWMAN-BIRK trypsin iso-inhibitors both increased and decreased during the earlier period of germination but in contrast to KUNITZ trypsin iso-inhibitors no BOWMAN-BIRK trypsin iso-inhibitors were found in cotyledons 13 days after inbibition (TAN-WILSON, RIGHTMERE and WILSON, 1982). Apart from the class of trypsin inhibitor the types of proteinase inhibitor also behave differently from each other during germination. In *Vigna sinensis* cultivar Sendo trypsin and chymotrypsin inhibitor content of the cotyledons decreased during the 24 hours of imbibition. After this period however, the trypsin inhibitor content increased up to the seventh day of germination but the chymotrypsin inhibitor level kept on declining until the end of the experiment (XAVIER-FILHO and NEGEIROS, 1979).

Trypsin inhibitors do not only seem to behave quite

differently in different species but also in cultivars of the same species. It was observed that the total trypsin inhibitor content was unaffected by germination in seeds of *Phaseolus vulgaris* cultivar C20 but in the cultivar Pinkdak an increase in trypsin inhibitor was detected at the third day of germination followed by a small decrease up to the sixth day (CHANG and HARROLD, 1988). Taking into account that various authors used different cultivars of the different species in their research the conflicting results found in the literature especially with Phaseolus *vulgaris* could partly be attributed to cultivar differences. The basis on which the trypsin inhibitor data was expressed also affected the interpretation of the final results. Expressing the fate of trypsin inhibitors during germination of *Phaseolus vulgaris* seed on a total protein or dry mass basis resulted in no change being found in the trypsin inhibitor content of the cotyledons. However, when the trypsin inhibitor content of the cotyledons was determined per cotyledon a decrease in the trypsin inhibitor content was detected during germination (NIELSON and LIENER, 1988).

The decrease in the activity of trypsin inhibitors during germination raises the question of proteolytic degradation of the inhibitor. It was reported that trypsin inhibitors are hydrolysed by endogenous proteinases but the degradation could not be correlated with the events which took place during the germination process. It was suggested that trypsin inhibitor degradation could be catalysed in legumes by the abundant enzyme peptidohydrolase (CHRISPEELS and

BAUMGARTNER, 1978). A possible function of proteinase inhibitors in the seed is the regulation of the proteolytic activity during germination. OFELT, SMITH and MILLS (1955) established that trypsin inhibitors do not inhibit endogenous proteolytic enzymes in *Glycine max*. This observation was confirmed by BIRK and WALDMAN (1965). However, it was observed that the proteinase activity of a proteotylic enzyme in *Lactuca sativa* was inhibited by an endogenous proteinase inhibitor. This inhibitor later disappeared during germination as an increase in proteolytic activity of the enzyme was observed (SHAIN and MAIER, 1965).

The occurrence of proteinase inhibitors in large quantities in the cotyledons of many plants raises the question whether the inhibitors do not merely function as storage proteins. Proteinase inhibitors could represent as much as six percent of the total protein in *Glycine max* (RACKIS and ANDERSON, 1964) and up to ten percent of the total protein in grains of *Hordeum vulgare* (MIKOLA and KIRSI, 1972). This may however, be a simplistic approach to the problem. It is quite possible that proteinase inhibitors can be multifunctional proteins which also serve as storage proteins. They may regulate the activity of some hydrolytic enzymes and may be involved in the defense mechanism of plants as will be dicussed later. The function of proteinase inhibitors *in vivo* is still obscure. At this stage it appears as if the change in inhibitor activity can not be correlated with any specific event during germination.

However, the progress being made with the detection of the appearance and disappearance of iso-inhibitors during the germination process is a positive indication that proteinase inhibitors do fulfill some role during germination. In the assays for trypsin inhibitor activity the total amount of trypsin inhibitor has been determined and no discrimination has been made between different trypsin inhibitors in the same tissue. However, when different trypsin inhibitors in the same tissue were considered it was evident that the different iso-inhibitors that exist in the same dormant seed could change in levels independently from each other during germination. Some of the trypsin inhibitors might even disappear totally from the tissue (PUZTAI, 1972). With a similar observation new trypsin iso-inhibitors were reported to appear during the germination of seeds of *Glycine max* cv. Fiskeby 5 (TAN-WILSON, RIGHTMERE and WILSON, 1982).

Since to date no clear indication exists as to the function of trypsin inhibitors in the metabolism of the plant, the question arises whether trypsin inhibitors are essential for the existence of plants which produce them. Seeds of *Glycine max* without trypsin inhibitors have been found in nature. These seeds germinated similar to seeds containing trypsin inhibitors. The plants which developed from the seedlings grew and flowered like normal plants (HYMOWITZ, 1980). Although these results cannot necessarily be extrapolated to other species it seems from this observation as if the trypsin inhibitors did not develop

through evolutionary processes to fulfill an essential role in the metabolism of the plant.

Recent evidence indicated that proteinase inhibitors are probably involved in the defense mechanism of the plant. APPLEBAUM (1964) first proposed that proteinase inhibitors in the legumes evolved as a defense mechanism against insects. He suggested that protein digestion in insects should be considered an important factor in plant host selection. An accumulation of data around this subject do in fact strongly suggest that proteinase inhibitors are insect deterrents (APPLEBAUM, 1964 ; GREEN and RYAN, 1972). Plants release a polysaccharide elicitor from the cell walls when a wound is afflicted mechanically, or after being attacked by chewing insects. This elicitor induces the synthesis of trypsin inhibitors in plants like *Lycopersicon esculentum* and *Solanum tuberosum* (GREEN and RYAN, 1972 ; NELSON, WALKER-SIMMONDS, MAKUS, ZUROSKE, GRAHAM and RYAN, 1983). Two to three days after the insect attack the trypsin inhibitors accounted for over one percent of the total protein in the leaves.

It has been established that trypsin inhibitors inhibit the growth of larvae from insects which feed on legume seeds such as *Trilobium confusum* Duval. (LIPKE, FRAENKEL and LIENER, 1954) and *Trilobium castaneum Herbst.* (BIRK and APPLEBAUM, 1960). In other cases it seems as if the insect had already counteracted this plant defense mechanism through evolutionary progression. The growth of weevils

feeding on cowpea and cornborer larvae parasitising corn were not affected by the trypsin inhibitors of the host plant. However, their growth was severely inhibited by trypsin inhibitors from non-host plants (RYAN, 1981). Trypsin inhibitors have an adverse effect on physiological processes other than growth inhibition of larvae. It was established that trypsin inhibitors not only inhibited the midgut proteolytic enzymes of the mosquito *Aedes aegypti* L. but it also inhibited egg development in the female mosquitoes (BOROVSKY, 1988). More evidence of insect deterrent ability of trypsin inhibitors was the observation that the resistance of a variety of *Vigna unguiculata* to the bruchid *Callosobruchus maculatus* F. is biochemical. This resistance correlated very well with the levels of the trypsin inhibitors in the resistant variety (GATEHOUSE and BOULTER, 1983). Trypsin inhibitors from various sources showed a marked species specificity towards insects in their activity towards trypsin-like enzymes. Trypsin inhibitor from *Vigna unguiculata* for example effectively reduced the larval growth and significantly increased the mortality of *Callosobruchus maculatus*. However, feeding the larvae on trypsin inhibitors from *Glycine max* or *Vigna radiata* had no effect on the growth or mortality of the larvae (GATEHOUSE and BOULTER, 1983). The growth reduction of larvae caused by trypsin inhibitors were first ascribed to the inhibition of the trypsin-like enzymes in the midgut of the larvae with the consequent decrease in protein digestion (BIRK and APPLEBAUM, 1960). Recent evidence however, indicated that the growth reduction was due to stress on

the proteolytic enzyme producing organ in the larvae and the depletion of sulphur containing amino acids from the body tissue of the larvae (BROADWAY and DUFFEY, 1984).

It has been suggested that proteinase inhibitors do repel microorganisms by inhibiting the action of the digestive enzymes secreted by pathogens during invasion of the plant (MIKOLA and SOULINA, 1971 ; RYAN, 1973 ; MOSOLOV, LOGINOVA, FEDURKINA and BENKEN, 1976 ; PENG and BLACK, 1976). It is well established that plant pathogenic fungi release various hydrolytic enzymes including proteinases (KUC and WILLIAMS, 1962 ; BURGUM, PRESCOT and HARVEY, 1964 ; PORTER, 1966 ; PORTER, 1969). The proteinases are reported to be secreted extracellularly and are also found in the infected tissue of the host plant (KUC and WILLIAMS, 1962 ; REDDY, STUTEVILLE and SORENSEN, 1971). It was suggested that these proteinases catalysed the breakdown of hydroxyproline-rich structural protein in plant cell walls (GINSBURG, 1961 ; LAMPORT, 1970 ; RIES and ALBERSHEIM 1973). Evidence does exist that proteinase inhibitors inhibit proteinases from plant pathogens. Proteinase inhibitors from *Hordeum vulgare* inhibited proteolytic enzymes secreted by *Aspergillus oryzae* Ahlb., *Bacillus subtillus* (Cohn) Praz., *Streptomyces griseus* Krain and *Alternaria tenuissima* (Fr.) Wiltsh. (MIKOLA and SOULINA, 1971). These results were confirmed with the inhibition of proteolytic enzymes from *Fusarium solani* Appel and Woll. strain 68 by a proteinase inhibitor from *Hordeum vulgare* (MOSOLOV, LOGINOVA, FEDURKINA and BENKEN, 1976). As with insects it was reported

that plants react to fungal infection by producing elevated levels of proteinase inhibitors. For example the levels of proteinase inhibitor increased in *Lycopersicon esculentum* when it was infected with a non-compatible race of *Phytophthora infestans* De Bary (PENG and BLACK, 1976).

1.2.5 THE NUTRITIONAL SIGNIFICANCE OF PROTEINASE INHIBITORS

The wide distribution of trypsin inhibitors in those very plants that constitute the most important source of dietary protein throughout the world has stimulated considerable research regarding their possible nutritional significance. With the recognition of the presence of a trypsin inhibitor in *Glycine max* it was tempting to conclude that the growth inhibition which it evoked in animals and insects is due to the inhibition of the digestion of dietary protein by trypsin and chymotrypsin in the intestinal tract. However, this hypothesis came under scrutiny when it was found that preparations of trypsin inhibitor were capable of inhibiting growth even when incorporated into diets containing predigested protein or free amino acids (LIENER, DEUEL and FEVOLD, 1949). It was also established that with the intake of trypsin containing diets supplemented with the amino acid methionine no substantial malnutrition occurred in the test animals (LIENER, DEUEL and FEVOLD, 1949 ; PALMER, MCINTOSH and PUZTAI, 1973). These observations ruled out the inhibition of proteolysis as the sole factor contributing

to growth inhibition or malnutrition. Similar observations were made with the addition of methionine or cysteine to trypsin containing diets of larvae of *Callosobruchus maculatus* (GATEHOUSE and BOULTER, 1983).

A significant observation which ultimately led to a better understanding of the mode of action of trypsin proteinase inhibitors was the finding that raw soybeans and the trypsin inhibitor itself caused hypertrophy of the pancreas. The hypertrophy of the pancreas was the result of stress on the pancreas to continually produce abnormally high levels of proteases (CHERNICK, LEPKOVSKY and CHAIKOV, 1948). The production of proteases in the intestine was found to be regulated by a negative feedback mechanism in animals (GREEN and LYMAN, 1972 ; LYMAN, OLDS and GREEN, 1974) and in man (LIENER, GOODALE, RESHMUKH, SATTERBERG, WARD, DIPIETRO, BANKEY and BOMER, 1988) mediated by cholecystokinin (LOESER, FOELSCH, MUSTROPH, CANTOR, WUNDERLICH and CREUTZFELDT, 1988). The complex formation between trypsin or chymotrypsin and the inhibitor molecule ultimately resulted in the activation of the negative feedback mechanism and the over production of proteinases by the pancreas. Intraluminal trypsin inhibits pancreatic trypsin secretion by inhibiting the release of the hormone cholecystokinin from the intestinal mucusa, whereas the complex formation of trypsin with trypsin inhibitors results in the unfettered release of cholecystokinin. This continuous release of cholecystokinin at excessive levels is responsible for the hypertrophy and hyperplasia of the pancreas of rats and

chickens (LIENER, 1986). These findings led to the suggestion that the decrease in growth caused by trypsin inhibitors might be the consequence of the endogenous loss of essential amino acids being secreted as proteases by the pancreas. After complex formation between trypsin and the trypsin inhibitor the complexed enzyme was not reabsorbed to be recycled as amino acids (LYMAN and LEPKOVSKY, 1957 ; BOOTH, ROBBINS RIBELIN and DeEDS, 1960). Pancreatic secreted enzymes are particularly rich in sulphur containing amino acids. Thus the overproduction of proteolytic enzymes and loss from the normal circulation by complex formation caused a depletion of the body tissue of methionine and cysteine (LIENER, 1972). This resulted in the malnutrition effect observed with the intake of trypsin inhibitors by mammals. The growth reduction observed in insect larvae after feeding them on a diet which contained trypsin inhibitor was also ascribed to a depletion of amino acids and hypertrophy of the proteinase producing organ (BROADWAY and DUFFEY, 1984).

1.2.6 THE SIGNIFICANCE OF PROTEASE INHIBITORS IN HUMAN NUTRITION

Essentially all research dealing with the physiological effects of trypsin inhibitors on animals has been done with soybean trypsin inhibitors and virtually all the *in vitro* work has been done with bovine trypsin (LIENER, 1972). Although the pancreas of mice, rats, hamsters, young guinea

pigs and chickens is sensitive to soybean trypsin inhibitor other animals such as dogs, calves and pigs do not display this sensitivity (LIENER, 1986). Because of this difference in response between species any prediction regarding the effect of trypsin inhibitors on humans is uncertain. It has been demonstrated that human trypsin is not inhibited by the soybean trypsin inhibitor (FEENY, MEANS and BIGLER, 1969 ; TRAVIS and ROBERTS, 1969). Despite the body of evidence which implicate trypsin inhibitors as a factor contributing to the poor nutritive value of raw legume diets, they may

1.3 TISSUE PLASMINOGEN ACTIVATOR

1.3.1 DESCRIPTION

The trypsin inhibitor from *Erythrina caffra* dealt with in this study also inhibits tissue plasminogen activator (JOUBERT and DOWDLE, 1987). Two types of plasminogen activator are presently known, e.g. tissue plasminogen activator and urokinase plasminogen activator. Tissue plasminogen activator is a single chain glycoprotein serine protease with a molecular weight of approximately 68 000 daltons. Human tissue plasminogen activator consists of an A-chain, containing either 278 amino acid residues with glycine at the N-terminal or 275 amino acids with serine at the N-terminal. It also has a B-chain which contains the active site (PENNICA, HOLMES, KOHR, HARKINS, VEHAR, WARD, BENNET, YELVERTON, SEEBURG, HEYNEKER, GOEDDEL and COLLEN,

1983) depicted in Figure 1.5. When purified in the absence of proteinase inhibitors a mixture of single chain and double chain tissue plasminogen activator is obtained (RIJKEN, HOYLAERTS and COLLEN, 1982). Single chain tissue plasminogen activator can be converted into the double chain form through limited proteolysis (WALLEN, POHL, BERGSDORF, RANBY, NY and JORNVAL, 1983). Plasminogen, the proenzyme of the major fibrinolyte plasmin, is present in the blood as a single chain glycoprotein. The activation of the single chain plasminogen molecule into the two chain disulphide bonded serine protease plasmin is done by tissue type plasminogen activator (EMEIS, BROMMER, KLUFT and BRAKMAN, 1985).

1.3.2 THE SITE OF SYNTHESIS OF TISSUE PLASMINOGEN ACTIVATOR

Tissue type plasminogen activator is synthesised *in vivo* by endothelial cells of the blood vessels and then released into the blood (LOSKUTOF and EDGINGTON, 1977 ; LAUG, 1981). This activator of plasminogen has also be extracted from many other animal and human tissues such as mucosae (CORASANTI, CELIK, CAMILIO, MITTELMAN, EVERS, BARBASCH, HOBIKA and MARKUS, 1980), rat and human uterine tissue (RIJKEN, WIJNGAARDS, ZAAL, DE JONG and WELBERGEN, 1979 ; PELTZ, KATZENELLENBOGEN, KNEIFFEL and MANGEL, 1983), rat liver (SHARONI, TOPAL, TUTTLE and BERGER jr, 1982), hog kidney (SOEDA and NEGAMATSU, 1981) and rodent brain (SOREQ

Figure 1.5 Primary structure of tissue plasminogen
            activator (t-PA).   S-S links within and
            between the heavy and light chain are
            represented by a line (-) (BACHMAN and
            KRUITHOF, 1984).
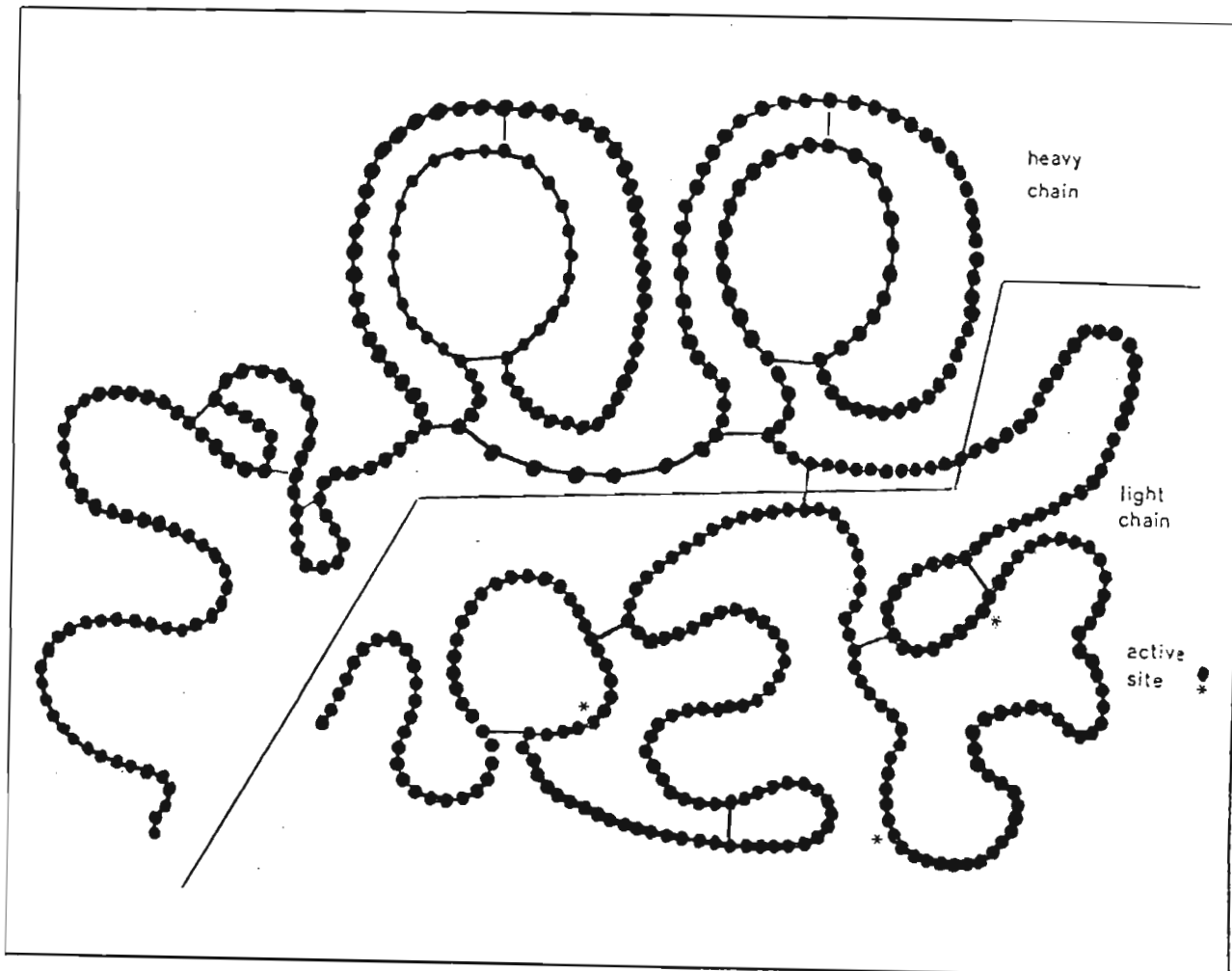
and MISKIN, 1981). It has also been isolated *in vitro* from endothelial cells (BOOYSE, SCHEINBUKS, RADEK, OSIKOWICZ, FEDER and QUARFOOT, 1981; RIJKEN, WIJNGAARDS and COLLIN, 1985), neurons (KRYSTOSEK and SEEDS, 1981), embryonic cells (MAROTTI, BELIN and STRICKLAND, 1982), trophoblast cells (MARTIN and ARIAS, 1982) and malignant cells (RIJKEN and COLLEN, 1981 ; COLLEN, RIJKEN, VAN DAMME AND BILIAU, 1982 ; GRONOW and BLIEM, 1983 ; WILSON, BECKER, HOAL and DOWDLE, 1983).

1.3.3 THE PHYSIOLOGICAL SIGNIFICANCE OF TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator has such a high affinity for fibrin that the activator will almost quantitatively bind to fibrin during blood coagulation (THORSEN, GLAS-GREENWALT and ASTROP, 1972). The action of tissue plasminogen activator was specifically directed at the lysis of fibrin and no degradation of fibrinogen was observed (GUREWICH, HYDE and LIPINSKI, 1975 ; KORNINGER and COLLEN, 1981). This is probably due to the low plasminogen activating potential of the activator in the absence of fibrin. In the presence of fibrin the catalytic efficiency of tissue plasminogen activator to convert plasminogen into plasmin was increased more than 500 fold (RANBY, 1982). The low plasminogen activating potential of the activator in the absence of fibrin, the high specificity of tissue plasminogen activator for fibrin and the marked increase in

plasminogen activation by fibrin fixed activator explain the negligible fibrigenolysis but excellent fibrinolytic effect of tissue plasminogen activator (BACHMANN and KRUITHOF, 1984). Although tissue plasminogen activator is primarily known as a fibrinolytic enzyme it also plays a major role in inflammation, ovulation, trophoblast implantation and neoplasia (REICH, 1978 ; OSSOWSKI, BIEGEL and REICH, 1979 ; MARTIN and ARIAS, 1982). Several factors operating during clot formation triggered the release of tissue plasminogen activator from the vascular wall. Some of these factors were thrombin, (KITAGUCHI, HIJIKATA, and HIRATA, 1979), bradykinin (KLOCKING, 1979) and activated protein C (COMP and ESMON, 1981). Blood clots prepared *in vitro* from tissue plasminogen activator rich blood did not dissolve spontaneously, even though the activator adsorbed quantitatively to the clot during coagulation. However, clots prepared from plasma containing little or no free tissue plasminogen activator incubated in tissue plasminogen activator rich plasma lysed gradually. This phenomenon was postulated to be the result of continuous adsorption of tissue plasminogen activator by the blood clot. This mechanism ensured normal homeostasis and the formation of a solid thrombus even in the presence of a high concentrations of circulating tissue plasminogen activator. It facilitated slow thrombolytic degradation of a thrombus in the presence of elevated local concentrations of tissue plasminogen activator even if circulating levels of the activator were very low (BACHMANN and KRUITHOF, 1984). From research results to date it is clear that the ratio of thrombolytic

to fibrinolytic potential of tissue plasminogen activator is far superior to that of urokinase and streptokinase (CAMIOLO, THORSREN and ASTRUP, 1971 ; GUREWICH, HYDE and LIPINSKI, 1975 ; COLLEN, 1980 ; KORNINGER and COLLEN, 1981). *In vitro* and *in vivo* studies have demonstrated that the thrombolytic activity of tissue plasminogen activator was between five and ten times higher than that of urokinase while causing no or little fibrinogenolysis (MATSUO, RIJKEN and COLLEN, 1981 ; KORNINGER, MATSUO, SUY, STASSEN and COLLEN, 1982). Tumor tissue in general produced more tissue plasminogen activator than the corresponding normal tissues (REICH, 1978 ; OSSOWSKI, BIEGEL and REICH, 1979 ; WILSON, BECKER, HOAL and DOWDLE, 1980 ; GRONOW and BLIEM, 1983). Further research revealed that tissue plasminogen activator plays an important role in the metastasis of tumor cells. Metastatic tumor cells were able to penetrate blood vessels, a property ascribed to the copious production of tissue plasminogen activator (FELDMAN and EISENBACH, 1988).

1.3.4 ASSAY FOR TISSUE PLASMINOGEN ACTIVATOR

The traditional fibrin plate and clot lysis as well as the more sensitive plasminogen rich radio active iodine marked fibrin coated microtiter well assay do not distinguish between tissue plasminogen activator and urokinase (WIJNGAARDS, 1979 ; KRUITHOF, RASIJN and BACHMAN, 1982). Several investigators have developed assays that were based on the potentiating effect of fibrin on the tissue

plasminogen activator mediated plasminogen activation (RANBY, NORRMAN and WALLEN, 1982 ; WIMAN, MELBRING and RANBY, 1983). A few authors have developed radioimmunometric assays to determine the concentration of tissue plasminogen activator antigen in blood and body fluids (HOLMBERG, KIRSTOFFERSSON, LECANDER, WALLEN and ASTEDT, 1982 ; RIJKEN, JUHAN- VAGUE and COLLEN, 1983). As with fibrin, the inhibitor of tissue plasminogen activator isolated from *Erythrina* species forms a complex with tissue plasminogen activator but not with urokinase. With this property of *Erythrina* trypsin inhibitor in mind an assay can be developed to determine the concentration of tissue plasminogen activator in relatively unpurified extracts without interference by urokinase.

1.4 PURPOSE OF THE CURRENT RESEARCH

The occurrence of an inhibitor which apart from trypsin and chymotrypsin also inhibits tissue plasminogen activator, appears to be unique to members of the genus *Erythrina*. No research has been done on any aspect of the occurrence or function of this inhibitor in *Erythrina caffra* or any other member of the genus *Erythrina*. It is possible that the t-PA inhibitor will behave in the same way as trypsin and chymotrypsin inhibitors from other plants since it also inhibits trypsin and chymotrypsin.

The first object of the study was to determine the

occurrence and distribution of the t-PA inhibitor in the different organs of *Erythrina caffra*. The results would be an indication whether or not the t-PA inhibitor could be economically extracted from any other part of the plant than the seed.

The second objective was to investigate the possibility of producing the t-PA inhibitor *in vitro* in a callus or cell suspension culture.

A third objective was to determine whether it was possible to increase in the production of t-PA inhibitor *in vitro* with the inclusion of various eliciting compounds into the nutrient medium. The fourth object was to determine whether the inhibitor of t-PA occurred in other members of the genus *Erythrina* than the Southern African species.

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

Mature trees of *Erythrina caffra* Thunb. was identified and selected on the campus of the University of Durban-Westville. The trees were identified by a numbering system and appropriate trees were selected on their seed bearing capacity and condition of health. Plant material was used mainly from one tree which was a prolific producer of seeds. The seeds of this tree also seemed to be superior in quality since they were much larger than seeds collected from any other tree. Seeds were also obtained from trees in their natural habitat in the Tharfield area and Alexandria district in the Eastern Cape.

Ripe seeds were collected after the mature pods opened. At this stage the seeds were in their natural state for dissemination and had a hard dry testa. Green seeds were collected while the pods were still green with the seeds fully swollen. These seeds were creamy in colour. Seeds not fully swollen were green in colour. To determine the stage of development of the seeds seed length was determined during the course of the maturation process.

Leaf, root and shoot material was collected from seedlings grown from seeds collected from a tree at the University of Durban-Westville. The seeds were surface sterilised under aseptic conditions for 20 minutes in 0.2 % mercuric chloride. The seeds were subsequently rinsed with 500 millilitre autoclaved water. This sterilisation step was necessary to prevent fungal growth on the seeds during germination. The seeds were scarified by immersing them in concentrated sulphuric acid for one hour at room temperature. The seeds were sufficiently scarified when small black spots appear on the surface of the testa. After scarification the sulphuric acid was decanted and the seeds were rinsed in one litre autoclaved tap water. The seeds were imbibed overnight in distilled water at room temperature. The imbibed seeds were germinated on peat moss at 27 ^{o}C in an incubator. The seedlings emerging from the seeds were grown in a greenhouse at a day and night temperature of 27 ^{o}C and 15 ^{o}C respectively. The seedlings were kept free from insects by spraying them regularly with Metasystox (BAYER S.A. (Pty) Ltd.), Red Spidersprey (WONDER Horticultural Products) and Parashoot (FBC Industrial Chemicals (Pty) Ltd.). The plants were watered once a week with Multifeed (Haifa Chemicals Ltd.) which is a commercial nutrient formulation. Plant material for experimentation was collected from three to six-months-old seedlings.

2.2 PURIFICATION OF CELL WALLS FOR ELICITOR RECOVERY

It has been reported that hydrolysates of purified cell walls act as an elicitor which induced the synthesis of a trypsin inhibitor in a cell suspension of *Lycopersicum esculentum* (WALKER-SIMMONDS and RYAN, 1986). To obtain cell wall material from *Nicotiana tabacum* and *Lycopersicum esculentum* plants these two species were grown under the same conditions described for the growth of *Erythrina caffra*. Leaf material from fully expanded, undiseased leaves of *Erythrina caffra*, *Nicotiana tabacum* and *Lycopersicum esculentum* were collected from these plants. A thallus extract of *Ecklonia maxima* Osbeck (Papenfuss) marketed as KELPAK 66 was also used. All plant material was purified with modifications according to the method of HAHN, DARVILL and ALBERSHEIM (1981) to obtain purified cell wall extracts (Table 2.1). With the exception of the *Ecklonia maxima* extract the fresh mass of all other plant material collected was determined. The material was dried at 80 $^{\circ}$C until a constant mass was obtained. The dry mass of the leaf material was then determined. The rest of the fresh leaf material was kept frozen at -20 $^{\circ}$C.

Table 2.1 Protocol for the purification of cell walls.

1. Pulverise frozen tissue in mortar with a pestle.
2. Suspend 35 g powder in 250 ml phosphate buffer.
3. Homogenise the suspension with a homogeniser.
4. Centrifuge suspension at 16 000 X g for 15 minutes.
5. Suspend the pellet in 1.0 l phosphate buffer.
6. Centrifuge suspension at 16 000 X g for 15 minutes.
7. Repeat steps 5 and 6.
8. Suspend the pellet in 1.0 l distilled water.
9. Centrifuge suspension at 16 000 X g for 15 minutes.
10. Boil the pellet for 1 hour in 1.0 l ethanol.
11. Vacuum filtrate through Whatman fibre glass filter.
12. Resuspend the residue in 1.0 l 96 % ethanol.
13. Vacuum filtrate through Whatman fibre glass filter.
14. Repeat steps 12 and 13 with 1.0 l acetone.
15. Air dry the residue overnight at room temperature.
16. Store the purified cell walls at room temperature.

Reagents

1. Phosphate buffer
     Di-sodium hydrogen orthophosphate   70.98 g
     Distilled water   1.0 l
     Dissolve in 500 ml distilled water. Adjust pH to
     7.0 with 1 N HCl and 1 N NaOH. Adjust the volume
     to 1 l with distilled water.

2. Ethanol (96 %)   2.0 l
3. Acetone   1.0 l
4. Distilled water   1.0 l

2.3 HYDROLYSATION OF THE CELL WALLS

Ten grammes of purified cell wall material were suspended in 100 millilitres of distilled water. The suspension was then autoclaved for one hour at 121 $^{\circ}$C to partially hydrolyse the cell walls. The hydrolysate was cooled and centrifuged at 16 000 X g for 15 minutes. The supernatant was decanted and stored at -20 $^{\circ}$C. The supernatant was used as the cell wall hydrolysate.

2.4 DEFATTING OF SEED MATERIAL

Seeds were defatted according to the method described in Table 2.2. Seeds were cleaned with liquid soap, rinsed with tap water and dried with paper toweling. The seeds were cracked with pliers and the seed coats were separated from the cotyledons and embryos. The seed coat was removed from the cotyledons since the pigments in the red seed coat binds to protein and might interfere with the protein- and enzyme-linked immunosorbent assays used during this study. The seed material could be satisfactorily defatted with acetone. Material containing chlorophyll was depigmented using a similar protocol as was described for the defatting of seeds in Table 2.2. The chlorophyllous tissue was homogenised in acetone and then stirred in acetone for one hour. The acetone was filtered off and replaced with fresh acetone. The procedure was repeated until all the pigment had been extracted. The acetone was then filtered off and

Table 2.2 Protocol for the defatting of seeds of *Erythrina caffra*.

1. Crack seeds with pliers and separate the seed coat from the rest of the seed.
2. Homogenise 10 g of cotyledons on ice in 100 ml cold acetone. Homogenise 5 times for 1 minute with a 1 minute cooling period in between.
3. Vacuum filter the homogenate through Whatman no 1 filter paper.
4. Wash the residue 5 times with 200 ml acetone.
5. Dry the residue 3 times with 100 ml ethyl ether.
6. Air dry overnight at room temperature.
7. Store the fat free powder at room temperature.

Reagents

1. Acetone   300 ml
2. Ethyl ether   100 ml

the residue dried as outlined in Table 2.2.

2.5 EXTRACTION OF PROTEIN

Apart for a few modifications, protein was extracted according to the method of JOUBERT (1982b). According to the original method protein was extracted with 0.5 moles per litre sodium chloride only. The first modification from the original extraction procedure was to buffer the sodium chloride solution at pH 8.0 with 0.1 mole per litre Tris-HCl buffer.

No browning of protein from extracted seed tissue was observed. However, protein extracted from leaf tissue according to the method of JOUBERT (1982b) was brownish in colour presumably due to quinones which formed by the oxidation of polyphenolic substances in the leaf tissue during the extraction process. The extraction procedure was adapted to minimise the oxidation of endogenous polyphenols by adding mercapthoethanol at a concentration of 40 millimoles per litre to the extraction buffer. The modified extraction procedure was used for the extraction of protein from all plant material. The reagents used and the protocol for the protein extraction is described in Tables 2.3 and 2.4 respectively.

Table 2.3   Reagents for the extraction of protein from
            Erythrina caffra plant material.

1. Protein extraction buffer

   Tris-HCl   12.11 g
   Sodium chloride   29.22 g
   Mercaptoethanol   2.8 ml
   Distilled water   1.0 l

   Dissolve chemicals in 500 ml distilled water and
   adjust pH to 8.0 with 1 N HCL and 1 N NaOH. Make up
   to 1.0 litre with distilled water.

2. Ammonium sulphate   50 g

3. Sodium chloride solution
   Sodium chloride   2.922 g
   Distilled water   1.0 l
   Dissolve in 500 ml distilled water and   adjust   pH
   to 8.0 with 1 N HCL and 1 N NaOH. Make up to 1.0 l
   with distilled water.

Table 2.4 Protocol for the extraction of protein from
Erythrina caffra plant material.

1. Add 100 ml extraction buffer to 10 g defatted, pulverised plant material.
2. Extract overnight at 10 °C.
3. Filter slurry through four layers of muslin cloth.
4. Centrifuge filtrate at 5 000 X g for 30 minutes.
5. Decant the supernate and determine its volume.
6. Stir the supernate and add slowly 0.39 g ammonium sulphate to each 1.0 ml of supernate.
7. Leave for 3 hours at 10 °C to denature protein.
8. Centrifuge suspension at 5 000 X g for 30 minutes.
9. Decant the supernate and dissolve the precipitate in 10 ml of 50 mM sodium chloride.
10. Dialyse overnight at 10 °C in 20 1 distilled water.
11. Lyophilise the dialysate.
12. Store the protein air tight at 4 °C.

2.6 DETERMINATION OF PROTEIN

The concentration of protein in plant samples was determined with Serva Blue according to the method of READ and NORTHCOTE (1981). This is a modification of the protein determination method described earlier by BRADFORD (1961). The reagents used and the protocol for the determination of protein is described in Table 2.5. With this method protein concentrations as low as two milligrammes per litre can be determined reliably. Protein concentrations between 0.2 and 2 microgrammes per litre can be determined with the micro assay of READ and NORTHCOTE (1981). The only modification in the method described in Table 2.5 for the micro assay was the relative quantities of protein solution and reagent used. A volume of 0.7 millilitres protein solution was mixed with 0.3 millilitres of reagent. The protocol in Table 2.5 was then followed.

2.7 GERMINATION OF SEEDS AND GROWTH OF SEEDLINGS

Seeds were obtained from *Erythrina caffra* trees growing on the campus of the University of Durban-Westville. Seeds from trees growing in the Eastern Cape were obtained through Technifin. The seeds were surface sterilised for 20 minutes with 0.2 % mercuric chloride containing 0.1 % Tween 20 as a surfactant. Fifty seeds were rinsed five times under aseptic conditions with a total volume of 500 millilitres autoclaved distilled water. Before imbibition the seeds were

Table 2.5 Protocol for the determination of protein.

---

1. Add 0.05 ml of standard or sample solution to 0.95 ml colour reagent.
2. Mix by vortexing for 5 seconds.
3. Leave 5 minutes for colour to develop.
4. Blank with mixture of 0.95 ml colour reagent and 0.05 ml sample buffer at a wavelength of 595 nm.
5. Read the absorbance at 595 nm.

Preparation of colour reagent

Reagents

1. Serva Blue (Sigma)	100 mg
2. Ethanol (96 %)	50 ml
3. Phosphoric acid (16 M)	100 ml
4. Distilled water	850 ml

Method

Dissolve 100 mg Serva Blue in 50 ml ethanol.
Add 100 ml phosphoric acid to the solution and mix.
Make the reagent up to 1.0 l with distilled water.
Filter the reagent through Whatman no 1 filter paper.
Store at room temperature.

scarified for 60 minutes at 25 ^{o}C with concentrated sulphuric acid as outlined in section 2.1. The appearance of black spots on the red seed coat indicated the end of the scarification procedure. Care has to be taken not to scarify the seeds for an extended period since the acid can damage the cotyledons. After scarification the seeds were rinsed five times in a total of one litre autoclaved distilled water. The scarified seeds were imbibed for 18 hours under aseptic conditions in autoclaved distilled water at 25 ^{o}C. Imbibed seeds were placed in a petri dish on five layers of moist Whatman no 1 filter paper. They were germinated under aseptic conditions in the light at 27 ^{o}C. Most seeds germinated within 24 hours after imbibition. Germination was considered to be completed with the appearance of the tip of the radicle from the seed coat. The seedlings were planted in a 1:1 mixture of milled pine bark and peat moss and grown in a greenhouse under natural daylight conditions. The day temperature was 25 to 32 ^{o}C and the night temperature was not lower than 15 ^{o}C. The plants were watered once a week with 2 grammes per litre Multifeed.

The material and methods used for the development of the enzyme-linked immunosorbent assay and the establishment of the callus and suspension cultures *in vitro* are described in Chapters 3, 4 and 5 respectively.

CHAPTER 3

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION

AND QUANTIFICATION OF t-PA INHIBITOR

3.1 INTRODUCTION

The first enzyme-linked immunosorbent assays (ELISA) were developed in the field of medical research as a substitute for the more expensive and hazardous radio immunoassays (ENGVALL and PERLMANN, 1971 ; VAN WEEMEN and SCHUURS, 1971). The potential of immunodiagnostic procedures in botanical research has only been realised recently with the development of radioimmunoassays (KAHN, HUMAYUN and JACOB, 1977 ; PENGELLY and MEINS, 1977 ; WALTON, DASHEK and GALSON, 1979) and enzyme-linked immunosorbent assays (ELISA) for the detection of plant hormones (DAIE and WYSE, 1982 ; ATZHORN and WEILER, 1983) and trypsin inhibitor (BRANDON, BATES and FRIEDMAN, 1988). Other phytochemicals which can be detected with immunological methods such as phytochrome, phytoalexin, nitrate reductase, proteins and latex is dicussed by LINSKENS and JACKSON (1986).

3.2 ACQUISITION OF THE t-PA INHIBITOR

Purified samples of t-PA inhibitor was a generous gift from dr. O. SAFRIEL of Technifin.

3.3 QUANTIFICATION OF THE t-PA INHIBITOR

The t-PA inhibitor could not be quantified through a trypsin inhibition assay since three known inhibitors of trypsin occur in the seed of *Erythrina caffra* (JOUBERT, 1982b). It was therefore decided to quantify the t-PA inhibitor through an enzyme-linked immunosorbent assay (ELISA) since it would be specific for the inhibitor of t-PA and a relative unpurified extraction of t-PA inhibitor could be used in the assays. Although t-PA inhibitor could also be quantified through a t-PA inhibition assay the cost involved in the purchasing of t-PA ruled out this alternative.

3.4 RAISING OF ANTIBODIES AGAINST t-PA INHIBITOR

3.4.1 PREPARATION OF ANTIGEN EMULSION

The t-PA inhibitor antigen was dissolved in physiological saline which was prepared by dissolving 9 grammes of sodium chloride (MERCK Analar grade) in one litre distilled water. The emulsion for the initial immunization was

prepared by mixing one volume of 3 milligrammes per millilitre antigen in physiological saline with two volumes of FREUND'S complete adjuvant. The two phases were thoroughly mixed in a LEUR-lock syringe to obtain a good emulsion of saline and adjuvant. A final mass of one gramme antigen was injected per rabbit. A mass of 0.5 milligramme antigen in FREUND'S incomplete adjuvant was injected per rabbit for the subsequent booster injections.

3.4.2   IMMUNIZATION OF THE RABBITS

Rabbits were obtained from the Natal Blood Transfusion Services. The dorsal area of the rabbits was shaved and disinfected with 70 % ethanol. The rabbits were immunized intradermally by injecting approximately 0.1 millilitre antigen emulsion between the layers of the skin until a small pocket of emulsion was visible. Approximately one millilitre of emulsion was injected at about ten sites along the upper sides of the rabbits. After four weeks the first boost injection was given subcutaneously similar to the first injection.

3.4.3   BLEEDING OF THE RABBITS

Fourteen days after the boost injection the rabbits were bled from the ear to obtain serum. Thirty to 50 millilitres of blood was collected in a test tube from each rabbit.

3.4.4 PREPARATION OF THE SERUM

As soon as the blood clotted in the tube the edges of the clot was rimmed with a glass rod. This step was critical to obtain the maximum amount of serum from the blood. The blood was allowed to clot for one hour at 37 ^{o}C and then left overnight at 4 ^{o}C. The blood clot was brought to room temperature and the serum decanted. The serum was centrifuged at 1000 X g for 10 minutes to remove the red blood cells.

3.4.5 PARTIAL PURIFICATION OF THE IMMUNOGLOBULINS

The immunoglobulins were precipitated with ammonium sulphate at 60 % saturation. An amount of 0.3 gramme of ammonium sulphate was slowly added per millilitre of serum while the serum was vortexed. Precipitation was allowed to occur for one hour while stirring the serum. The serum was centrifuged at 5000 X g for 10 minutes. The precipitated immunoglobulins were dissolved in a small as possible volume of distilled water and dialysed overnight at 10 ^{o}C against 5 litres of distilled water. The dialysate was centrifuged at 5000 X g for 10 minutes. The supernatant was lyophilised and the gamma globulins stored at -20 ^{o}C. The lyophilised immunoglobulins were used in the ELISA techniques.

3.5 THE CONJUGATION OF PEROXIDASE WITH THE IMMUNOGLOBULINS

Peroxidase was used in the conjugation procedure to label the antibody. The peroxidase conjugation procedure of WILSON and NAKANE (1978) as modified by CONRADIE (pers. comm.) was followed. The reagents used and protocol followed is outlined in Tables 3.1 and 3.2. All reagents used were of analytical grade. Peroxidase of the highest grade of purity (RZ E403/E275 = 3.2 - 3.3) was used. A critical step in the procedure is the collection of the activated peroxidase. Care should be taken not to collect too much of the activated peroxidase since the separation between the peroxidase and the unreacted periodate in the Sephadex G-25 column is critical. Any unreacted periodate in the activated peroxidase will lead to inactivation of the conjugated product. The conjugate was stored at 4 $^{\circ}$C. The conjugate was active for three to six months.

The efficiency of the conjugation was determined by performing the assay with a serial dilution of the conjugated peroxidase-antibody. The dilutions ranged from 1:400 in Tris buffer with Tween 20 (TST) (Table 3.3) up to as low as 1:3200. A second assay was done with a concentration range around the optimal of the first assay. Eight replications were used for each treatment. The treatments were randomly assigned to the rows and wells of the microtiter plate. The absorbance was read at 492 nm with a spectrophotometer and the data statistically analysed

Table 3.1 Reagents for the conjugation of peroxidase with immunoglobulins raised against the t-PA inhibitor from *Erythrina caffra*.

1. Peroxidase (EC 1.11.1.7) RZ = 3.2 - 3.3

   Dissolve 0.02 g peroxidase in 1 ml distilled water immediately before use.

2. Sodium periodate activator.

Sodium periodate	220 mM	0.047 g
Distilled water		1.0 ml

   Dissolve the sodium periodate in the water immediately before use.

3. Equilibration buffer.

Sodium acetate	1 mM	0.0164 g
Distilled water		200 ml

   Dissolve the sodium acetate in the water immediately before use.

   Adjust the pH to 4 with HCl and NaOH.

4. Coupling buffer.

Sodium carbonate	200 mM	0.021 g
Distilled water		1.0 ml

   Dissolve the sodium carbonate in the water before use.

   Adjust the pH to 9.6 with HCl and NaOH.

Table 3.1 continued.

5. Conjugate stabilizer.

    Tris-HCl   50 mM   0.606 g

    Sodium chloride   100 mM   0.584 g

    BSA   1.0 %   2.0   g

    Thimerosal   0.02 %   0.4   g

    Glycerol   100   ml

    Distilled water   100   ml

    Dissolve the Tris, sodium chloride and thimerosal

    in the water. Adjust the pH to 8.0 with HCl and NaOH.

    Add the BSA and let it dissolve completely.

    Add the glycerol and mix well.

    Store at 4 °C.

6. Antibody.

    Use antibody described in text.

7. Chromatography column.

    Columns (PD-10) 8 cm long and 1.7 cm in diameter

    packed with Sephadex G-25 (Pharmacia).

Table 3.2   Protocol for the   conjugation of peroxidase with
            immunoglobulins   raised   against   the   t-PA
            inhibitor   from   *Erythrina*   *caffra*.

---

1. Dissolve 0.016 g peroxidase in 0.8 ml distilled water.
    Ratio of peroxidase:water 1:50 (m/v).

2. Add 0.08 ml sodium periodate activator and vortex.
    Let stand at room temperature for twenty minutes.
    Ratio of activator:peroxidase (1:10 v/v).

3. Equilibrate Sephadex G-25 column with four times the
    dead volume equilibration buffer.

4. Apply the activated peroxidase to the Sephadex column
    to remove excess activator. Collect the peroxidase
    when the first brown drop emerges from the column.
    Stop collecting the activated peroxidase when the
    effluent becomes lighter in colour.

5. Dissolve 0.0256 g antibody in 2.56 ml coupling buffer.
    Ratio of peroxidase:antibody 1:1.6   (m/m).

6. While vortexing, add the activated peroxidase slowly
    and   leave   the   mixture for two   hours at room
    temperature.

7. Add the conjugate to the conjugate stabiliser at a
    ratio of 1:30 conjugate:stabiliser (v/v).
    Store as 1 ml aliquots at 4 °C.

as a complete random design. The concentration of peroxidase-antibody conjugate resulting in the maximum absorbance obtained from such an assay was not necessarily the optimal dilution. It was found that relatively high concentrations of conjugate resulted in undesirable high non-specific binding values (Figure 3.2). The conjugate concentration used in the ELISA for the t-PA inhibitor was the concentration at which the difference between the lowest and highest detectable concentration of antigen has a value of more than 1.0 absorbance units and with a non-specific binding value of less than 0.1 absorbance units.

3.6 PREPARATION OF STANDARDS AND SAMPLES

The t-PA inhibitor standards and the samples were made up in the reaction buffer with the same composition as the TST washing buffer used for the ELISA (Table 3.3). A stock solution of 10 milligrammes per litre t-PA inhibitor was prepared by dissolving 1.0 milligramme inhibitor in about 50 millilitres of reaction buffer. The solution was made to 100 millilitres in a volumetric flask and dispensed as one millilitre aliquots. The one millilitre aliquots were stored at -20 $^{\circ}$C. The standards were made up by serial dilution of the stock solution with reaction buffer. The standards covered a concentration range of 0.005 to 0.2 milligramme per litre. Fresh standards were prepared weekly, filter sterilized through a 0.22 micron filter and stored at 4 $^{\circ}$C.

3.7 THE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR t-PA INHIBITOR

The enzyme-linked immunosorbent assay (ELISA) for the t-PA inhibitor was developed in consultation with Dr. P. HOFMAN of the Department of Horticultural Sciences, University of Natal and Dr. J. D. CONRADIE of the Natal Blood Transfusion Services, Pinetown. A protocol has been established for a competitive ELISA for the detection of t-PA inhibitor by Professor E. DOWDLE (pers. comm.) but it was decided to establish a double sandwich ELISA. The advantages of the double sandwich ELISA over the competitive ELISA is the exclusion of possible non-specific reactions between substances in the sample which could bind to the antibodies in the competition phase of the assay. The substrate concentration, incubation time and temperature used in the ELISA were adopted from ELS, GOVENDER, MARIMUHTU, BUBB and CONRADIE (1984). The reagents and protocol used for the ELISA is set out in Tables 3.3 and 3.4 respectively. The ELISA used was an indirect double sandwich technique illustrated in Figure 3.1. The solid phase used was NUNC polystyrene microtiter wells (Weil Organisation). The ELISA was based on the principle that the immunogobulin containing t-PA inhibitor antibody was adsorbed to a solid phase. The microtiter well was then washed with 50 millimoles per liter saline Tris buffer with Tween 20 (TST) to remove antigen not attached to the solid phase. The antigen-containing solution was then incubated with the

Figure 3.1 An illustration of the indirect double
            sandwich enzyme-linked immunosorbent assay
            used for the quantification of the t-PA
            inhibitor of *Erythrina caffra*.

1 ANTIBODY ADSORBED TO PLATE

   WASH

2 TEST SOLUTION CONTAINING
   ANTIGEN ADDED

   WASH

3 ADD ENZYME LABELLED
   SPECIFIC ANTIBODY

   WASH

4 ADD ENZYME SUBSTRATE
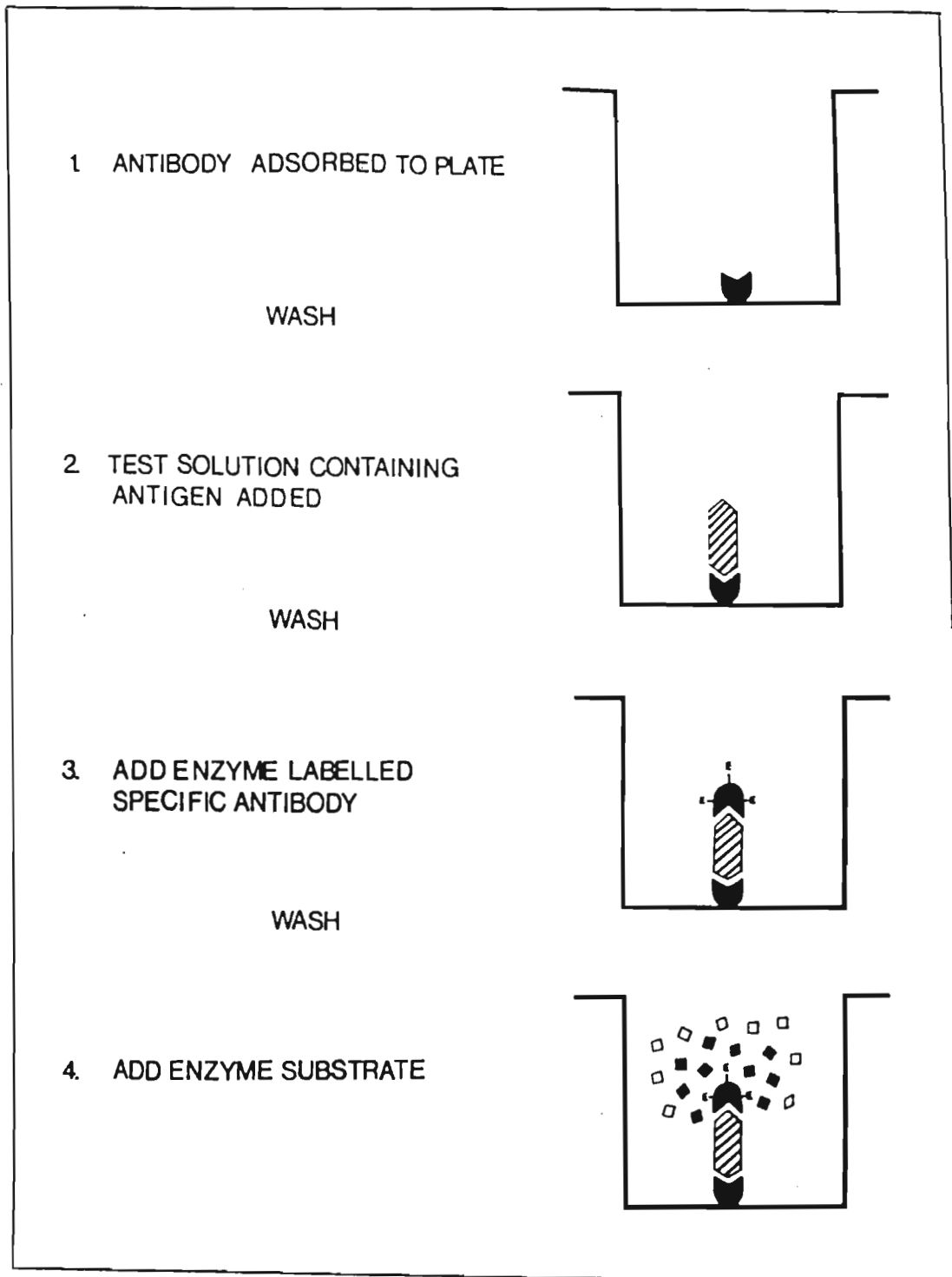
Table 3.3   Reagents for the enzyme-linked immunosorbent

assay for the t-PA inhibitor from *Erythrina*

caffra.

1.   **Washing buffer (TST) pH 7.4   (Ten times stock).**

Tris-HCl	50 mM	60.6 g
Magnesium chloride	100 mM	20.3 g
Sodium chloride	100 mM	58.4 g
Tween 20	0.5 % (v/v)	5.0 ml
Distilled water		995.0 ml

Dissolve the constituents in about 750 ml distilled

water, adjust the pH to 7.4 with 10N HCl and 10N

NaOH and make up to a volume of 1000 ml with

distilled water.

Filter sterilise and store at 4 $^{\circ}$C.

To   make a working solution add one volume of stock

solution to nine volumes of distilled water and

adjust the pH to 7.4 with 1N NaOH and 1N HCl.

2.   **Coating buffer   pH 9.6**

Sodium carbonate	50 mM	0.53 g
Distilled water		100.0 ml

Dissolve the sodium carbonate in 70 ml distilled

water. Adjust the pH to 9.6 with 1N HCl and 1N NaOH

and adjust the volume to 100 ml. Filter sterilise

the solution and store it at 4 $^{\circ}$C for not more than

two weeks.

Table 3.3   continued

3.   Chromogenic substrate buffer   pH 5.0

Anhydrous borax   0.093 g

Succinic acid   0.093 g

Urea hydrogen peroxide   0.01 g

Double ·distilled water   25.0   ml

Dissolve the borax and urea hydrogen peroxide in 20

ml double distilled water.

Adjust the pH to 5.0 by adding succinic acid.

Adjust the volume to 25 ml with double distilled

water.

Make up fresh solution for every assay.

4.   Preparation of chromogenic substrate

Orthophenylenediamine-   0.018 g

dihydrochloride (OPD)

Chromogenic substrate buffer   15.0   ml

Add OPD to buffer just before use.

Table 3.4   Protocol for the enzyme-linked immunosorbent assay for the t-PA inhibitor from *Erythrina caffra*.

1. Coating of plate with antibody

    Dissolve T-PA inhibitor antibody in coating buffer to obtain a concentration of 6.0 mg per litre.

    Dispense 0.1 ml into each well of the assay plate.

    Cover plate and incubate overnight at 4 $^{\circ}$C or incubate for two hours at 37 $^{\circ}$C.

    Flick out the antibody solution.

    Wash plate 5 times with washing buffer.

    Dry plate by tapping it 10 times on paper toweling.

2. Addition of standards and samples to wells.

    Dispense 0.1 ml t-PA inhibitor standards, blanks, controls and samples in quadruplicate to the wells.

    Cover the plate and incubate for one hour at 37 $^{\circ}$C.

    Flick out the contents of the wells.

    Wash plate 5 times with washing buffer.

    Dry plate by tapping it 10 times on paper toweling.

Table 3.4   continued

3.   Addition of peroxidase-labelled antibody.

   Dispense into each well 0.1 ml peroxidase-labelled

   antibody.

   Cover the plate and incubate for one hour at 37 $^{\circ}$C.

   Flick out the contents of the wells.

   Wash plate 5 times with washing buffer.

   Dry plate by tapping it 10 times on paper toweling.

4.   Addition of chromogenic substrate.

   Dispense into each well 0.1 ml OPD.

   Cover the plate and incubate for exactly 30 minutes

   in the dark at room temperature.

   Dispense 0.1 ml 0.75 N sulphuric acid into each well

   to stop the reaction.

   Read the optical density at 492 nm.

sensitised solid phase which resulted in a very specific bond formation between the antibody attached to the solid phase and the antigen in solution. All unreacted antigen was subsequently separated from the bound fraction by another wash in TST. A peroxidase-labelled polyclonal antibody specific to the antigen was then incubated with the solid phase. The microtiter wells were washed again in TST buffer to remove the unreacted labelled antibody. The peroxidase substrate orthophenylenediamine dihydrochloride (OPD) (SIGMA) was then pipetted into the microtiter wells. The colour change was a measure of the amount of conjugate fixed. This is proportional to the amount of antigen in the test sample. The absorbance was subsequently read in a Titertek Multiskan spectrophotometer at a wavelength 492 nanometres. The mass of antigen per well was determined by analysing the data with the IRMA programme in the Securia computer programme designed for immunoassays. The data was statistically analysed as a complete random design (SNEDECOR and COCHRAN, 1972) with the Genstat Statistical Package.

3.8 DETERMINATION OF THE STANDARD WORKING RANGE OF THE ELISA FOR THE t-PA INHIBITOR

The range of concentrations over which the standards could be used to determine the concentration of unknown samples was determined by performing the ELISA as outlined in Table 3.4 with concentrations of t-PA inhibitor standards from

0.005 to 0.2 milligramme per litre. The ideal standard curve should look like the curve in Figure 3.2. At the lower end of the concentration range (L) the lowest concentration should be determined at which no antigen can be detected. The highest antigen concentration (H) should indicate a saturation point at which no difference in absorbance is obtained with further increases in the antigen concentration. The working range (W) is indicated by the linear region of the standard curve. Linearity of the standard curve is obtained with a logit transformation of the data for the standard curve.

3.9 NON-SPECIFIC REACTION IN THE ELISA

A source of experimental error is the non-specific reaction (non-specific interference) encountered in immunodiagnostic assays. Two types of non-specific reactions can occur. Substances in the sample extract can interfere with the assay by binding reversibly or irreversibly to the gamma globulins, inactivating the antibody or altering its affinity for the antigen. This will result in a decrease in the sensitivity of competitive assays (JONES, 1987). Non-specific interference has been found to be more pronounced in plant tissue with a high concentration of phenolic compounds (CLARK and BAR-JOSEPH, 1984 ; ROSHER, JONES and HEDDEN, 1985). Another type of non-specific interaction known as non-specific binding occurs with the non-specific binding of protein   and other substances to the solid

Figure 3.2 An ideal dose-response curve of an indirect double sandwich enzyme-linked immunosorbent assay. H and L indicate the concentration ranges outside the range (W) suitable to estimate the antigen concentration.
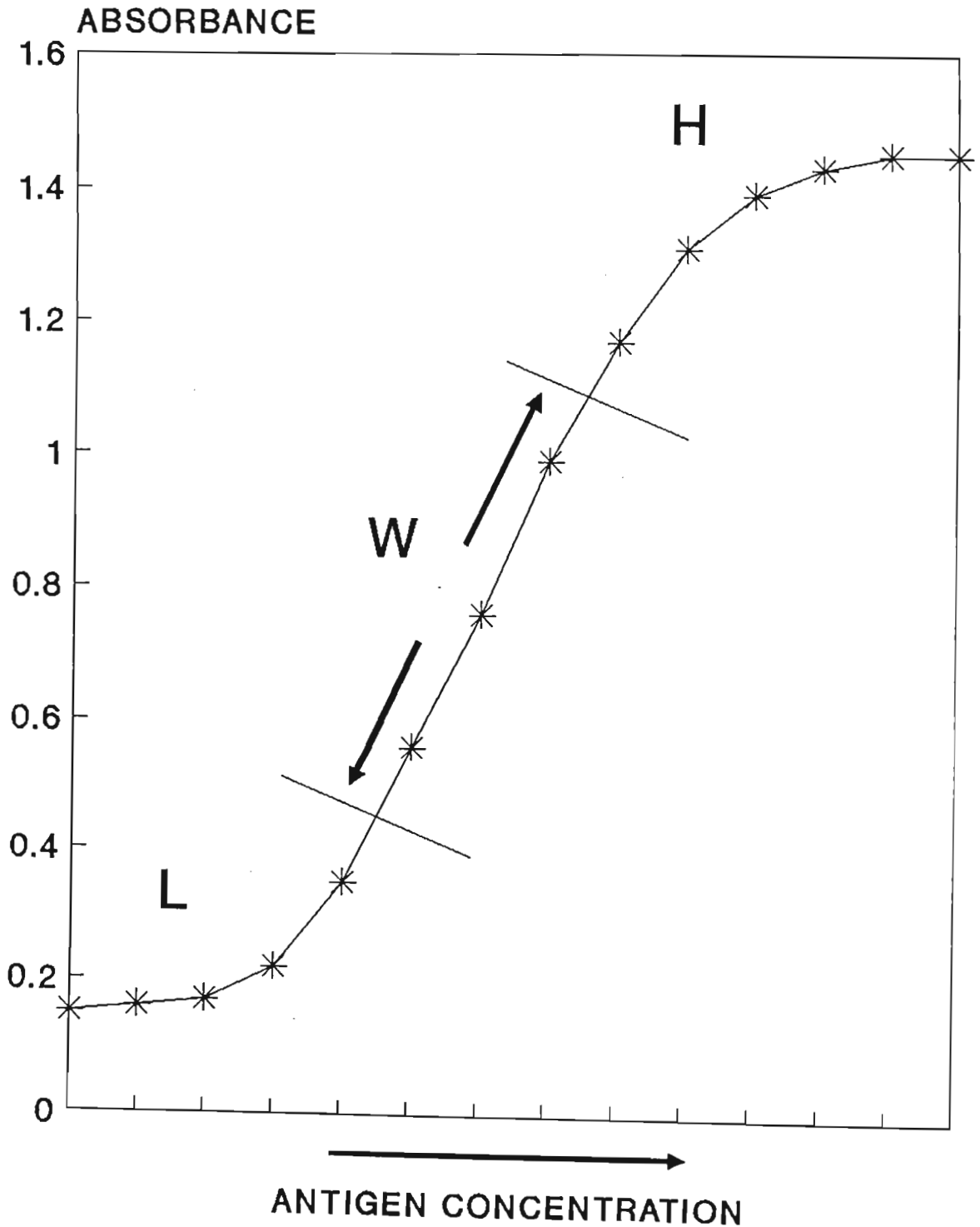
phase. These proteins in the plant extract can be structurally similar to the antigen and can therefore increase the background noise of the assay (JONES, 1987). The extent of non-specific binding in a sandwich ELISA is determined by substituting the antigen in the assay with reaction buffer. The non-specific reaction can be reduced by adding a non-ionic detergent such as Triton X-100 or Tween 20 to the reaction- and washing buffers. The detergents prevent post-coating adsorption of protein to the solid phase by preventing the formation of hydrophobic bonds between the protein and the solid phase (CLARK and ENGVALL, 1980). Non-specific binding can also be reduced by including additional protein, normally an albumin, in the test sample to block the still available reactive sites on the solid phase (VOLLER, BIDWELL and BARTLETT, 1979). Gelatin (0.5 % to 1 %) and 1 % bovine serum albumin (BSA) are widely used today to reduce the non-specific binding (KEMENY and CHALLACOMBE, 1988).

3.10 BLOCKING OF THE SOLID PHASE

A common problem in enzyme-linked immunosorbent assays is the undesirable binding of different serum or proteins to the assay plate. To determine whether blocking would reduce the non-specific binding in the ELISA for t-PA inhibitor two tests were conducted. In the first test the efficiency of albumin derived from different sources was determined as blocking agents. The reactive sites on the solid phase was

blocked in four treatments with 1 % BSA , goat serum albumin, human serum albumin and chicken egg albumin respectively. The albumin was dissolved in reaction buffer. In the control treatment the albumin was replaced with reaction buffer. The blocking treatment was incorporated after the coating of the wells.

In the second test the efficiency of BSA and Tween 20 were determined as blocking agents. This experiment was a 4 X 4 factorial with both Tween 20 and BSA at levels of 0, 1, 2, and 4 %. The Tween 20 was dissolved in assay buffer. Apart from the blocking step the assay plate was washed with washing buffer containing 1 % Tween 20.

In both experiments the blocking with albumin was included in the assay just after the coating of the wells. Blocking was done for 30 minutes at 37 $^{\circ}$C. After the blocking step the assay was performed as described in Table 3.4. The blocking treatments were randomly assigned to the wells. Each treatment was replicated eight times on the assay plate. The experiment was a completely randomised design and analysed accordingly. The results were expressed as a decimal fraction of the control value and was therefore transformed with an arcsin transformation to obtain normality.

RESULTS AND DISCUSSION

From the results of the first experiment it is clear that blocking of the wells in the assay with various sources of albumin did not decrease the non-specific binding substantially (Tables 3.5 and 3.6). It was emphasized previously that effective blocking of the assay plate could not be achieved in all the assays performed. (CLARK and ADAMS, 1977 ; KEMENY and CHANTLER, 1988). From the second experiment it is however, evident that the inclusion of Tween 20 in the washing and reaction buffers of the ELISA reduced the non-specific binding to the wells significantly, irrespective of the BSA concentration (Table 3.8). From the results in Table 3.9 it is evident that BSA seemed to reduce the non-specific binding to the assay wells considerably. However, examining the results in Table 3.10 it is clear that the effect of BSA was associated with the Tween 20 treatments. This is also clear from the significance of the interaction between BSA and Tween 20 (Table 3.7). The reduction of the non-specific binding by Tween 20 in the absence of BSA was 75 %. In contrast BSA could not reduce the non-specific binding significantly in the absence of Tween 20. Considering the high efficiency of Tween 20 alone as a blocking agent and the relative high price of BSA only Tween 20 was used to reduce the non-specific binding of the ELISA.

Blocking coated plates with protein can reduce the

Table 3.5 Analysis of variance of the effect of blocking the microtiter plate with albumin from different sources.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatments	0.0110	5	0.0022	0.61
Residual	0.0224	7	0.0032	
Total	0.0334	47		

Table 3.6 Multiple range analysis of the blocking of the plate with albumin from different sources.

Treatment Albumin source	95% Tukey intervals Blocking efficiency	Homogeneous groups
Rabbit	1.11	*
Human	1.10	*
Chicken	1.07	*
Goat	1.05	*
Bovine	1.03	*
No albumin	1.00	*

* In different columns indicate significant differences at a 95% level of confidence.

Table 3.7 Analysis of variance of the effect of Tween 20 and bovine serum albumin (BSA) on the non-specific binding in the ELISA for the t-PA inhibitor.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Tween 20	3.7424	3	1.2475	1000.0*
BSA	0.0356	3	0.0119	19.6*
Interaction				
Tween 20 X BSA	0.0194	9	0.0021	3.6*
Residual	0.0290	48	0.0006	
Total	3.8265	63		

* Indicate significant differences at a 95% level of confidence.

Table 3.8 Multiple range analysis of the main effects of Tween 20.

Treatment	95% Tukey intervals	Homogeneous groups		
Tween 20 (%)	Absorbance			
4	0.121	*		
2	0.204		*	
1	0.263			*
0	0.442			*

* In different columns indicate significant differences at a 95% level of confidence.

determinants on the coated proteins. However, the benefits
in reducing non-specific or background binding compensates
for this effect (KEMENY and CHALLACOMBE, 1988). Bovine serum
albumin or other common dietary proteins used to block
plates may bind some antibodies in the test sample since
extracts from animal origin especially may contain small
quantities of antibodies to the coating protein (KEMENY and

Table 3.9   Multiple range analysis for the main effects of
bovine serum albumin.

Treatment BSA (%)	95% Tukey intervals Absorbance	Homogenous groups	
2	0.313	*	
4	0.314	*	
1	0.329	*	
0	0.371		*

*   In different columns indicate significant differences at
a 95 % level of confidence.

CHANTLER, 1988). High background interference can be caused
by specific proteins in the sample solution. It has been
found that cationised proteins resulted in a very high
background   due   to   non-specific   binding   of   the
immunoglobulins to the charged protein. This non-specific
binding was minimized by the addition of polyanions such as
heparin to the sample dilution buffer (PESCE and MICHAEL,
1988).

Table 3.10   Multiple   range   analysis   of   the   effect   of
Tween   20   and   bovine   serum   albumin   on   the
non-specific   binding   in   the   ELISA   for   t-PA
inhibitor.

Treatment		95% Tukey Interval	Homogeneous groups					
Tween 20 (%)	BSA (%)	Absorbance						
4	4	0.102	*					
4	2	0.117	*	*				
4	0	0.132	*	*	*			
4	1	0.132	*	*	*			
2	4	0.177		*	*	*		
2	2	0.183			*	*		
2	1	0.202				*	*	
2	0	0.236				*	*	*
1	0	0.246					*	*
1	4	0.253					*	*
1	1	0.282						*
1	2	0.286						*
0	1	0.421							*
0	2	0.442							*
0	4	0.448							*
0	0	0.523							*

* In different columns indicate significant differences at
a 95% level of confidence.

3.11 COATING OF THE SOLID PHASE

The optimal antibody concentration for the coating of the solid phase was determined by coating the wells with a serial dilution of the antibodies to t-PA ranging from 0.25 to 16 milligrammes per litre. The assay plates were incubated overnight at 10 ^{o}C. A concentration of 0.01 milligrammes per litre antigen was used in the second step of the assay. The assay was conducted as described in Table 3.4. Four replications were used per treatment. The experiment was analysed as a complete randomised design.

RESULTS AND DISCUSSION

The results in Figure 3.3 show that the wells were optimally coated at concentrations of 4 to 16 milligramme per litre antibody. It was previously recommended to coat the solid phase with a protein concentration slightly higher than the optimal concentration to minimise the effect of differences in binding capacity between microtiter plates (KEMENY and CHALLACOMBE, 1988). For this reason the microtiter plates were coated with a concentration of 6 milligrammes per litre antibody. The coating of the assay plate takes place as the binding of a monolayer of molecules to the surface of the plate. The efficiency of the coating depends mainly on the concentration of the coating protein (CONRADIE, GOVENDER and VISSER, 1983). The highest

Figure 3.3 The effect of different concentrations of antibodies on the coating efficiency of the microtiter plate.
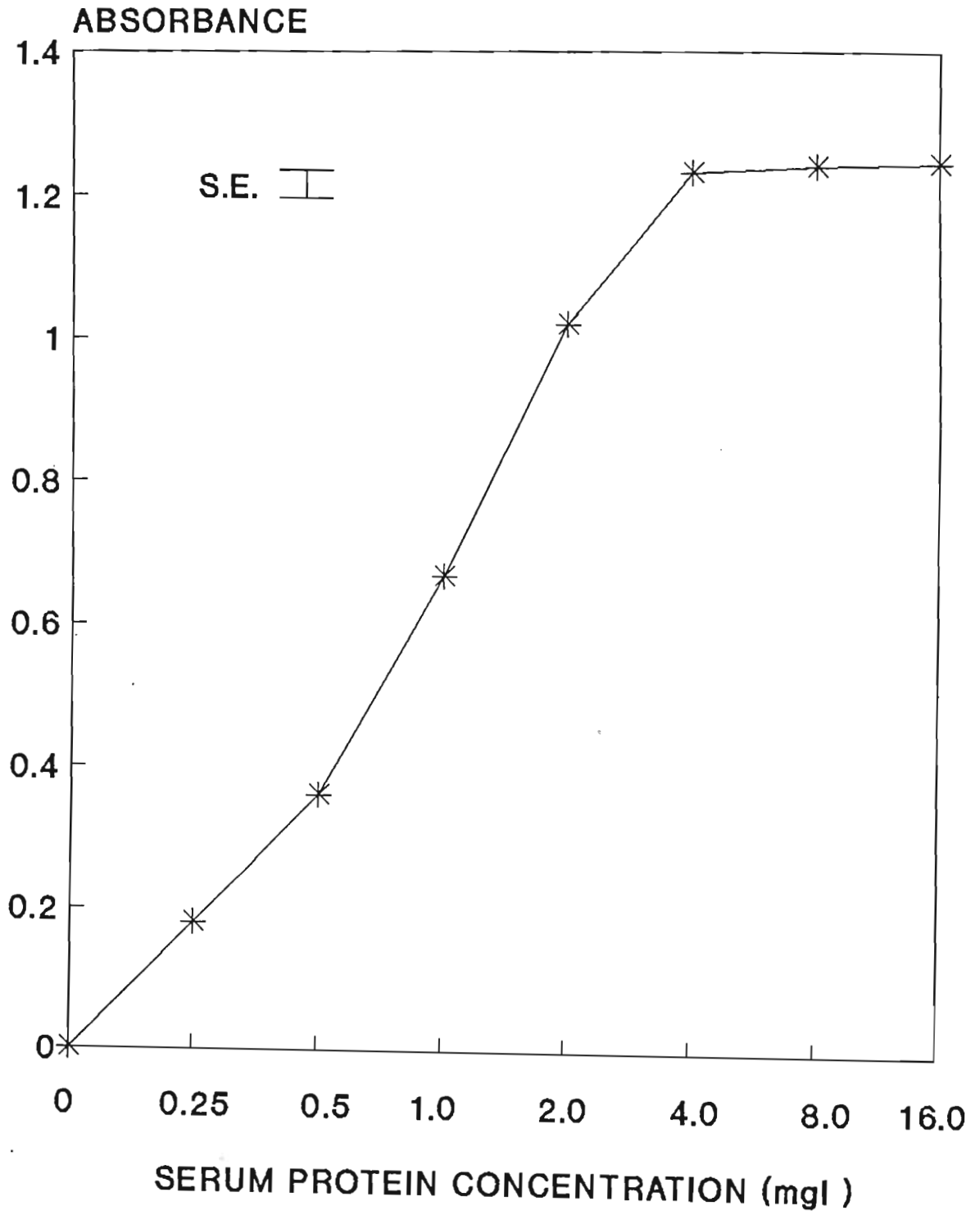
concentration of most proteins required to form a monolayer on the microtiter wells is about 1 milligramme per litre (KEMENY and CHALLACOMBE, 1988). The use of excessively high concentrations of gamma globulins to coat the solid phase result in the prozone effect in which the non-specific background intensifies while antigen-specific reactions diminish. The reason for this effect is not well understood but it may be related to a decrease in the stability of attachment of the antibodies to the solid phase or to steric effects (CLARK, 1981).

The binding of proteins to microtiter plates is independent of the pH and ionic strength of the coating buffer (KEMENY and CHALLACOMBE, 1988). A pH of 9.6 is recommended for the maximum binding of most proteins to the surface of microtiter plates (VOLLER, BIDWELL and BARTLETT, 1979). This is the optimum pH for the binding of gamma globulins (KEMENY and CHALLACOMBE, 1988). Binding of proteins to the solid phase is dependent mainly on temperature and time. Coating is completed normally within two hours at 20 °C to 25 °C but for convenience plates can be coated overnight at 4 °C (VOLLER, BIDWELL and BARTLETT, 1979). Excessive long periods of coating can result in the desorption of protein from the solid phase resulting in a decrease in the sensitivity of the assay (LETHONEN and VILJANEN, 1980). It was first thought that the above mentioned coating conditions are sufficient for most proteins but recent evidence indicated that the conditions for optimal coating should be determined for each assay (SALONEN and VAHERI, 1979 ; SCHUURS and VAN

WEEMEN, 1977). Various factors have been shown to affect the binding efficiency of proteins to the solid phase. To illustrate the effect of pH on coating it was reported that coating microtiter plates at a low pH of 1.5 to 4 was three times more effective than coating the wells at a pH of 9.6 (CONRADIE, GOVENDER and VISSER, 1983). Coating of the microtiter plate can be improved in some cases by altering the properties of the coating protein or by covalent binding the protein to the surface of the wells. Partial denaturation of gamma globulins with a heat treatment or with urea improved the coating efficiency of the assay plate (CONRADIE, GOVENDER and VISSER (1983). The efficiency of binding protein to the solid phase can also be improved by cross-linking the coating protein with glutaraldehyde (ROTMAN and SCHEVEN, 1984) or treating the microtiter plate with glutaraldehyde and then binding the coating protein covalently to the plate (SUTER, 1982).

3.12 THE EFFECT OF THE RINSING INTENSITY ON THE ELISA

The ELISA plate has to be rinsed after each incubation period before the following reactant is dispensed into the wells. Since the extent to which proteins bind to the wells is determined by the protein, it is clear that the optimal number of rinses for an ELISA has to be determined for every assay which is developed. Too few rinses will result in the transfer of reactant to the next step. If the wells are rinsed too many times it will result in the desorption

of the coating protein from the surface of the microtiter well. In both cases this will result in an underestimation of antigen in samples and an increase in the variablilty of the assay (CLARK and ADAMS, 1977 ; KEMENY and CHALLCOMBE, 1988). The effect of the number of rinses on the efficiency of the antigen assay was determined by conducting six assays as described in Table 3.4. One standard antigen solution was used in the assays with a concentration of 0.01 milligramme per litre. The number of rinses for the assays varied from three for the first assay to eight rinses for the last ELISA with an increase of one rinse per assay. Each ELISA was randomly assigned to a microtiter plate. The experiment was analysed as a complete randomised design.

RESULTS AND DISCUSSION

No significant differences were found in the absorbance values for the different number of rinses per assay (Tables 3.11 and 3.12). It is clear that the microtiter wells were sufficiently washed with three rinses and no cross-contamination occurred between the different incubation steps. However, as many as eight rinses after each incubation step did not result in the desorption of the coating protein from the wells. Three rinses between incubation steps was sufficient for the ELISA and was routinely used in the ELISA for t-PA inhibitor.

Table 3.11 Analysis of variance of the effect of the number
of rinses of the solid phase in the ELISA for
t-PA inhibitor.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatments	0.0068	5	0.0014	0.9
Residual	0.1454	90	0.0016	
Total	0.1522	95		

Table 3.12 Multiple range analysis of the effect of the
number of rinses of the solid phase in the ELISA
for t-PA inhibitor.

Treatment	95% Tukey intervals	Homogeneous groups
Number of rinses	Absorbance	
3	0.795	*
4	0.801	*
5	0.802	*
6	0.807	*
7	0.808	*
8	0.808	*

* In different columns indicate significant differences at
a 95% level of confidence.

3.13 THE EFFECT OF THE INCUBATION PERIOD ON THE ELISA

The effect of the incubation period during the performance of the ELISA was determined by conducting four assays as outlined in Table 3.4. One standard antigen concentration of 0.04 milligramme per litre was used. The incubation periods for the four assays were 30, 60, 90 and 120 minutes respectively. Each incubation period was randomly assigned to an assay plate. Eight replications randomly assigned to the wells on the plate were used per treatment. The experiment was a complete randomised design with eight replications per treatment and was analysed as described before.

RESULTS AND DISCUSSION

According to the results a substantially lower estimate of the antigen concentration was found with the 30 minutes incubation of the microtiter plates (Tables 3.13 and 3.14). No differences were found between the absorbance values of the 60 and 120 minutes incubation periods. It is clear that the variability of the assays was the same for all the incubation periods (Table 3.15).

The length of the incubation periods of the ELISA is critical. A too long incubation period can lead to the

Table 3.13 Analysis of variance of the effect of different incubation periods of the ELISA for t-PA inhibitor.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatments	0.3419	3	0.1140	59.1 *
Residual	0.1155	90	0.0019	
Total	0.4574	95		

* Indicate significant differences at a 95% level of confidence.

Table 3.14 Multiple range analysis of the effect of different incubation periods of the ELISA for t-PA inhibitor.

Treatment Incubation period (h)	95% Tukey intervals Absorbance	Homogeneous groups	
0.5	0.795	*	
1.0	0.801		*
1.5	0.802		*
2.0	0.807		*

* In different columns indicate significant differences at a 95% level of confidence.

desorption of the coating protein from the wells and an

Table 3.15 Analysis of the homogeneity of variance
between different incubation periods of the
ELISA for t-PA inhibitor.

Treatment	95% Tukey intervals	Homogeneous groups
Incubation period (h)	Variance	
0.5	0.00168	*
1.0	0.00102	*
1.5	0.00168	*
2.0	0.00152	*

*   In different columns indicate significant differences at
    a 95% level of confidence.

increase in the non-specific binding of proteins in the
different incubation steps of the ELISA (CLARK and ADAMS,
1977). Incubating the microtiter plate for a too short
period of time can result in uneven heat distribution on the
assay plate. Since the wells at the perifery of the assay
plate are at a lower temperature than the other wells the
reaction rate is slower in the periferal wells than in the
other wells. This results in the edge effect with lower
absorbance values being obtained for the periferal wells.
This effect results in an increase of the variability of the
assay and the erroneous estimations of antigen (CLARK and
ADAMS, 1977) .

3.14 THE EFFECT OF THE MICROTITER PLATE ON THE ELISA

Microtiter plates from different manufacturers do not bind the coating protein to the same extent (CLARK, 1981). The effect of different microtiter plates on the ELISA was determined by conducting the assay in plates obtained from different manufacturers. The assay was carried out as is described in the ELISA protocol (Table 3.4). A concentration of 0.06 milligramme per litre t-PA inhibitor was used in the assay. Only one plate of each manufacturer was used. Eight replications of one well each for the antigen concentration and the non-specific binding control were randomly assigned to each plate. The antigen concentration was determined as described before and the data was analysed as a complete random design.

RESULTS AND DISCUSSION

A substantial difference in the capacity of different brands of plates to adsorb the antibody during the coating phase was observed (Figure 3.4). The NUNC plate gave the best results and was used in all subsequent experiments. These findings confirm the observations of KEMENY and CHALLACOMBE (1988) who observed that the binding capacity of different plates for bee venom phospholipase A2 was substantially different from each other with the highest binding capacity obtained with NUNC plates. The

Figure 3.4 The effect of microtiter plates from
different manufacturers on the
sensitivity of the ELISA for the t-PA
inhibitor from *Erythrina caffra*.
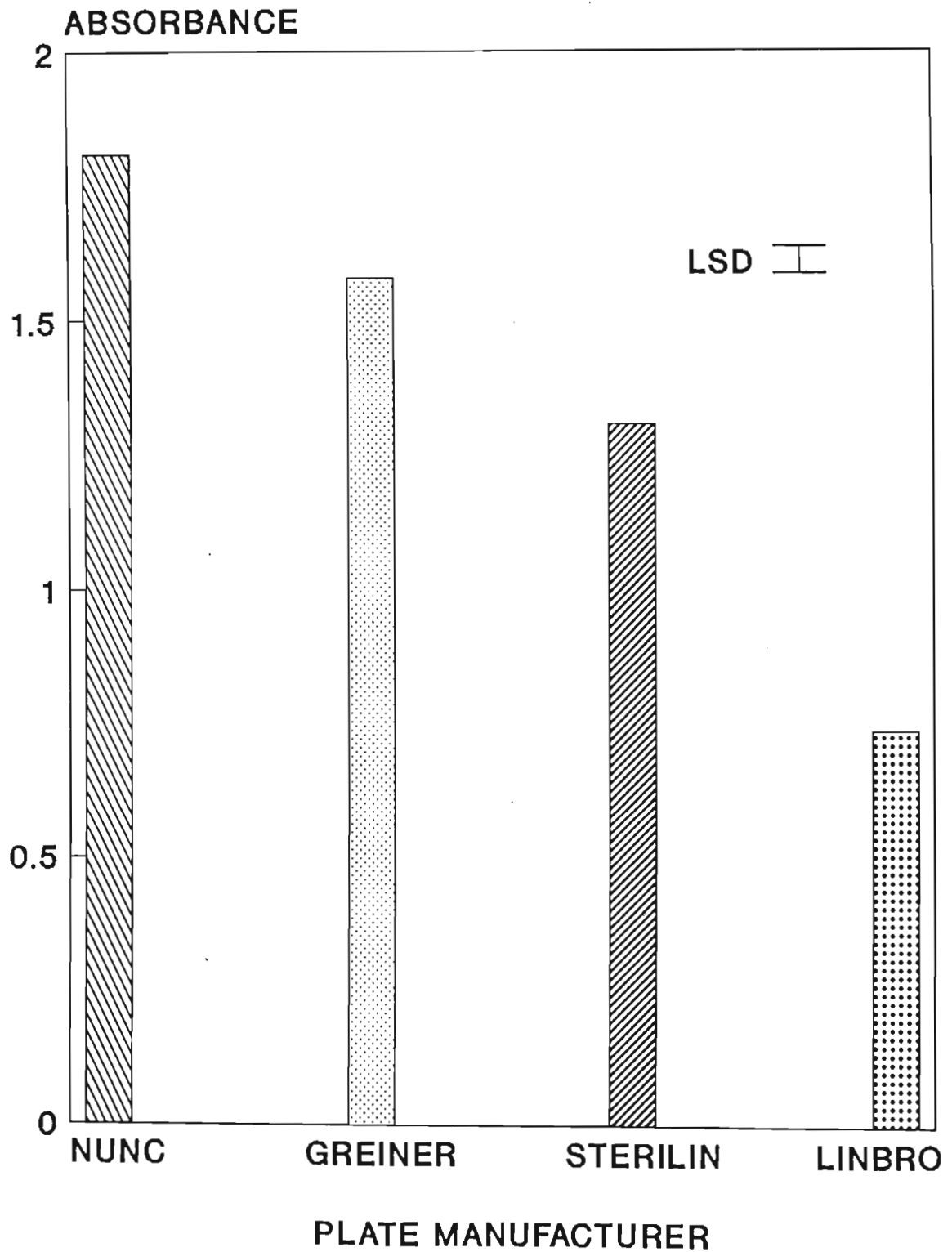
variability in results on a microtiter plate is critical since high variability will decrease the accuracy of the assay. With an absorbance value of about 1.0 an acceptable standard deviation is about 5 % (VOLLER, 1980).

3.15 REPRODUCIBILITY OF THE ELISA

The reproducibility of the ELISA was determined by conducting the assay as described in Table 3.4 on different days with different batches of buffers and antigen but with the same peroxidase-antibody conjugate. A standard curve was produced with the data of ten different assays. From the results in Figure 3.5 it is clear that the variability of the assay as indicated by the standard deviations of the bound fraction was low. The antigen concentration range over the linear part of the curve covers concentrations from approximately 0.005 to 0.015 milligramme per litre. It was found that different batches of antibody from different rabbits and different batches of peroxidase-antibody conjugate affected the slope and intercept of the standard curve. It has to be stressed however, that the standard curve changed within limits with each new batch of antibodies and peroxidase conjugated gamma globulins being used. The effect of any deviations in the standard curve was taken into account by including antigen standards in each assay.

Figure 3.5 A standard curve for the ELISA for the t-PA inhibitor from *Erythrina caffra*. The curve was constructed from data of ten consecutive assays.
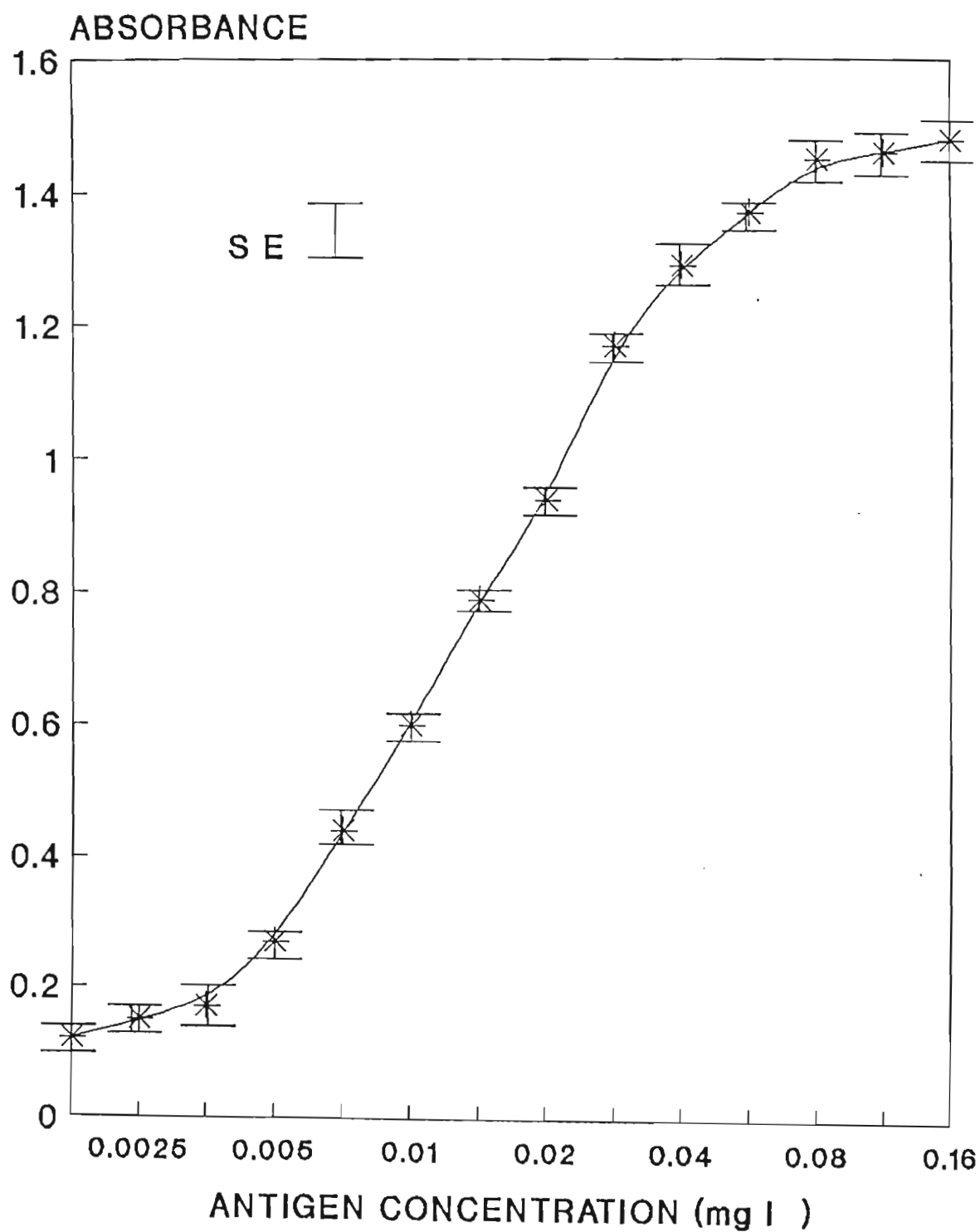
3.16 CROSS-REACTION IN THE ELISA

The seeds of *Erythrina caffra* contains apart from the t-PA inhibitor two other trypsin inhibitors with amino acid sequences very similar to that of the t-PA inhibitor (JOUBERT, 1982b ; JOUBERT and DOWDLE, 1987 ; JOUBERT, MERRIFIELD and DOWDLE, 1987). These two trypsin inhibitors could cross-react with the antibodies raised to the t-PA inhibitor. The possibility of cross-reaction between the different trypsin inhibitors in *Erythrina caffa* and pure t-PA inhibitor was determined by conducting a parallel ELISA test. Parallelism in the ELISA is the property of the dose-response curves generated by standard and test analytes which do not cross react so as to be parallel to each other. When cross reaction does occur the dose-response curves of the cross reacting analytes will not be parallel and will meet each other. The ELISA was conducted as described before with the standards and a series of dilutions of protein extractions from cotyledonary, leaf and callus material of *Erythrina caffra*. The dose-response curves were then plotted to determine whether cross reaction occurred.

RESULTS AND DISCUSSION

From the results in Figure 3.6 it is clear that the dose-response curves of the protein extracts from cotyledonary and leaf tissue were parallel to the standard curve up to

Figure 3.6   Dose-response curves for protein extracts
of cotyledonary, leaf and callus tissue
in a parallelism test to determine the
presence of cross-reactivity in the ELISA
for t-PA inhibitor.
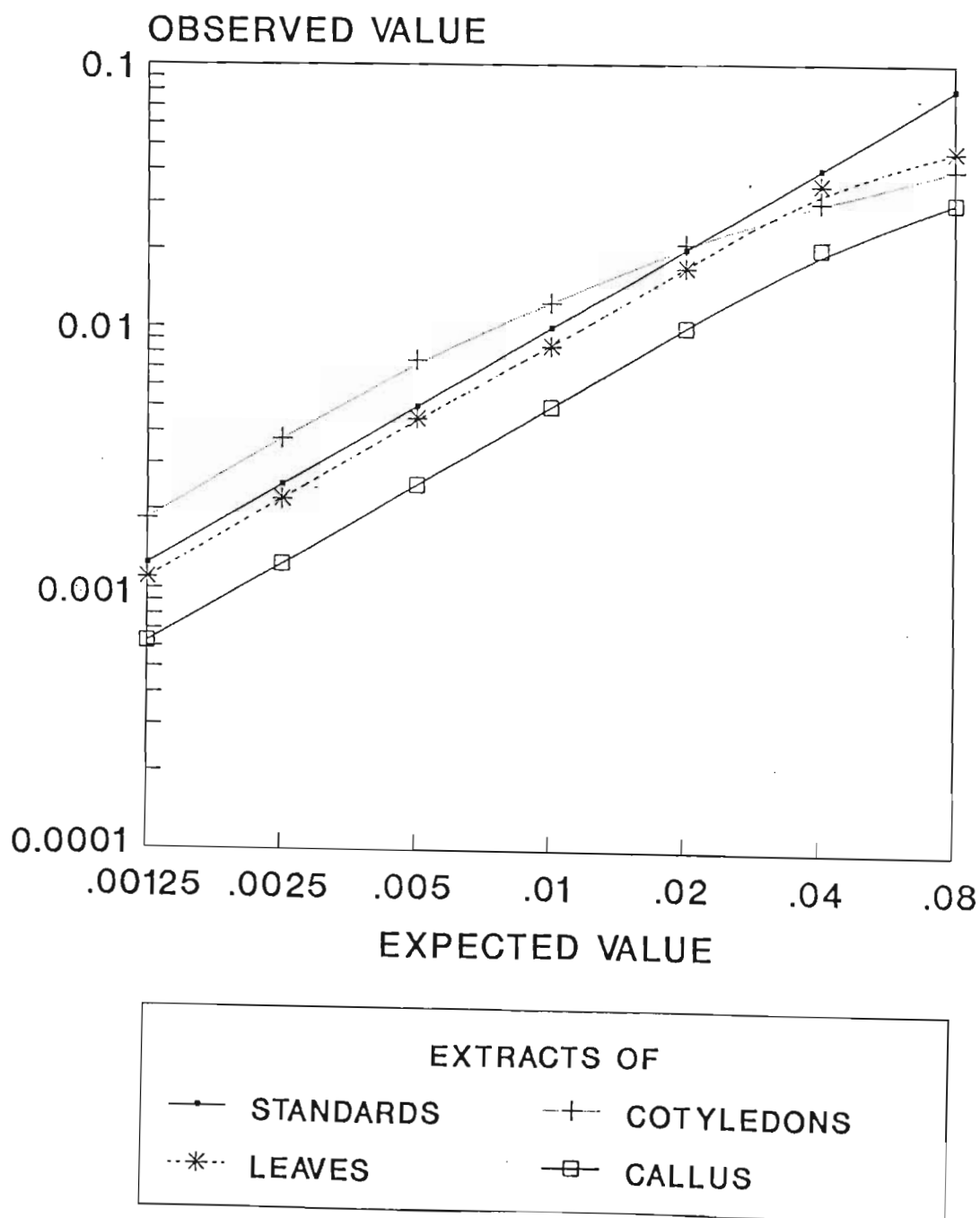
the standard concentrations of 0.005 and 0.02 milligrammes per litre respectively. Cross-reaction occurred with more concentrated samples. The dose-response curve for the protein extract from the callus tissue was parallel to the standard curve up to a concentration of the standard of 0.04 milligrammes per litre. The results indicate that cross reaction was more pronounced with the samples from the intact plant tissue than with the protein extract from the callus tissue. This suggests that quantitative and qualitative differences are present in the chemical composition of the different tissues. These affect the t-PA inhibitor differently in the different tissues. Non-parallelism of the callus and leaf protein extracts fell outside the usefull range of the ELISA and did not affect the preparation of samples. However, the cross-reaction of the cotyledonary sample occurred mainly with high sample concentrations which restricted the working range of the ELISA to the lower standard concentration range. Although this ensured the exclusion of spurious results it did not exclude the cross-reaction of other trypsin inhibitors present in the tissue on the ELISA.

The basic assumption underlying the use of standards to determine the quantity of an antigen in a non-competitive immunoassay is that the antigen in the standards and test samples should exhibit equivalent ability to bind to the receptor on the solid phase. Ideally the antigen in the standards and test samples should be identical in their molecular configuration. The diluent for the standards

should closely resemble that of the sample matrix and the diluted sample matrices should be similar in chemical composition. This is difficult to achieve since the matrix of different organs of the plant is not the same. These variations in the matrix give rise to non-specific effects and can result in artifacts that can alter the antibody-antigen reaction (HAMILTON and ADKINSON, 1988). The cause of the cross-reactions in the ELISA for the t-PA inhibitor is not clear. The other trypsin inhibitors in the tissue of *Erythrina caffra* may be involved in the cross- reaction. However, other factors causing artifacts such as antigen-polyphenolic interactions and partial hydrolysis of the t-PA inhibitor or glucose moieties on the inhibitor molecule may also be relevant. The results imply that the absolute values obtained for the t-PA inhibitor in plant material should be viewed with caution and inferences about the occurrence of the t-PA inhibitor in plant tissue can only be made on a relative basis. The practical importance of parallelism is that any test sample dilution analysed will generate the same final estimate.

Factors that can affect the occurrence of cross-reaction are the homology in the amino acid sequence that exists between the proteinase inhibitors in *Erythrina caffra*. With the use of polyclonal antiserum cross-reactivity can occur. Since every antibody recognises a specific epitope on the antigen it is possible that one of the epitopes on the t-PA inhibitor may be similar to an epitope on the other trypsin inhibitors of *Erythrina caffra*. This will result in

a cross-reaction of the gamma globulin with related antigen species. The selection of monoclonal antibodies specific to non-homologous regions on the antigen molecules of the different trypsin inhibitors would eliminate cross-reaction with these molecules. The inability to obtain absolute pure protein with column chromatography also contribute to the raising of antibodies against the impurities. Since the immunogenicity of different substances such as proteins can be very different it is possible to raise more antibodies with a higher avidity and affinity to unwanted proteins occurring at a much lower concentration than the target protein. In such a case different degrees of cross-reactivity can occur resulting in inaccurate antigen determination.

3.17 CONCLUSIONS

The double sandwich ELISA developed for the estimation of t-PA inhibitor had a high degree of sensitivity to detect differences in the inhibitor concentration of 0.025 milligramme per litre. This implies that t-PA inhibitor in plant tissue can be determined in nanogramme quantities. Non- specific binding to the microtiter plate could not be reduced with albumin from various sources. The detergent Tween 20 was the most effective agent in reducing non-specicfic binding and was subsequently used. The microtiter plates were coated effectively with antibodies against t-PA inhibitor at a concentration of 2 milligrammes per litre.

The microtiter plates should be coated with a slightly higher concentration than necessary so that any minor dilutional errors will not upset the assay. Cross-reaction occurred in the ELISA but reliable results can be obtained by selection of the proper dilution range for the samples to fall within the range of parallelism of the dose-response curve. It is not clear what caused the cross-reaction but it is possible that proteinase inhibitors in the samples closely related to the inhibitor of t-PA contributed to the cross-reaction. The ELISA as outlined in Table 3.4 was subsequently used to quantify the t-PA inhibitor.

Throughout the assay it was found that the purity of chemicals and distilled water used was critical, especially in the last step of the assay with the reaction of peroxidase with the chromogenic substrate. Peroxidase was found to be sensitive to fumes of various volatiles used in the laboratory, especially ethyl acetate. These assays should be performed in a well ventilated room set aside for this kind of assay.

CHAPTER 4

IN VITRO CULTURE OF *ERYTHRINA CAFFRA.*

4.1 INTRODUCTION

During the last fifteen to twenty years substantial progress has been made in the *in vitro* culture of legumes. In contrast to the successes attained with forage crops limited regeneration have been achieved with the large seed grain legumes, commonly known as pulses (Table 4.1).

Of the forage crops *Medicago sativa* L. is probably the best researched *in vitro*. Other fodder crops which have been intensively studied *in vitro* include members of the genera *Trifolium*, *Trigonella*, *Stylosanthes* and *Melilotus* (HAMMATT, GHOSE and DAVEY, 1986). Examples of the pulses that were cultured *in vitro* are *Pisum sativum* L. (HILDEBRANDT, WILMAR, JOHNS and RIJKER, 1963), *Cajanus cajan* (L.) Millsp. (PHILLIPS and COLLINS, 1979), *Cicer arietinum* L. (MUKHOPADHYAY and BHOJWANI, 1978) and the peanut *Arachis hypogeae* L. (VERMA and VAN HUYSTEE, 1970). Beans that have been cultured *in vitro* include *Glycine max* Mill. (GAMBORG, MILLER and OJIMA, 1968), *Vicia faba* L. (BINDING and NEHLS, 1978), *Phaseolus vulgaris* L. (LIAU and BOLL, 1970). Other legumes cultured *in vitro* are mentioned in Table 4.1.

Table 4.1 Members of the Leguminosae cultured *in vitro*

(GEORGE and SHERRINGTON, 1984).

Species	Explant	Morphogenesis
Seed Legumes		
Arachis		
hypogaea L.	Pericarp	Roots
	Cotyledons	Multiple shoots
	Shoot tips	Shoot elongation
	Leaves	Adventitious shoots
	Root discs	Roots
Arachis		
pusilla Benth.	Root disk	Proembryos; Shoots
Cajanus		
cajan (L.) Millsp.	Hypocotyl	Plantlets
Canavalia		
ensiformis (L.) DC.	Callus	Callus
Ceratonia		
siliqua L.	Roots	Callus
	Cotyledons	Callus
	Hypocotyls	Callus
Cicer		
arietinum L.	Embryo	Multiple buds
	Leaflets	Roots
	Shoot tips	Plantlets
	Shoot apices	Plantlets

Table 4.1 Continued.

Species	Explant	Morphogenesis
Stylosanthes		
humilis H.B.K.	Hypocotyl	Adventitious shoots
	Leaves	Adventitous shoots
Trifolium		
alexandrinum L.	Hypocotyl	Adventitious shoots
Trifolium		
alpestre L.	Shoot apices	Axillary shoots
Trifolium		
incarnatum L.	Hypocotyls	Adventitious shoots
	Shoot apices	Axillary shoots
Trifolium		
medium L.	Shoot apices	Axillary shoots
Trifolium		
pratense L.	Hypocotyl	Adventitious shoots
	Flower heads	Adventitious shoots
	Shoot apices	Axillary shoots
Trifolium		
repens L.	Shoot tips	Axillary shoots
	Shoot nodes	Roots
Trifolium		
rubens L.	Shoot apices	Axillary shoots
Trifolium		
subterraneum L.	Shoot apices	Axillary shoots

Table 4.1 Continued.

Species	Explant	Morphogenesis
Vigna sinensis (L.) Savi. ex Hassk.	Leaves	Shoots, roots
	Hypocotyl	Shoots, roots
Vigna unguiculata (L.) Walp.	Shoot callus	Shoots
Forage legumes		
Coronilla varia L.	Leaflets	Embryogenesis
	Hypocotyls	Embryogenesis
Lathyrus sativus L.	Shoot apices	Plantlets
	Leaflets	Plantlets
Lotus corniculata L.	Anthers	Embryogenesis
	Shoot apices	Axillary shoots
	Leaflets	Embryogenesis
Lupinus albus L.	Cotyledons	Multiple shoots

Table 4.1 Continued.

Species	Explant	Morphogenesis
Medicago		
sativa L.	Hypocotyl	Shoots
	Ovaries	Shoots, roots
	Stems	Shoots, roots
	Petioles	Shoots, roots
	Shoot tips	Axillary shoots
	Leaves	Embryoids, buds
	Cotyledons	Adventitious buds
	Shoot buds	Adventitious shoots

Very few leguminous trees have been cultured *in vitro* since relatively few legume trees are of economical importance. The trees and woody shrubs cultured *in vitro* were mostly utilised for timber, ornamental purposes or as fodder crops such as *Acacia koa* Gray (SKOLMEN and MAPES, 1976), *Ceratonia siliqua* L. (MARTINS-LAUCOU and RODRIGUEZ-BARRUECO, 1981), *Dalbergia sissoo* Roxb. (MUKHOPADHYAY and MOHAN RAM, 1981), *Prosopis cineraria* Gray (GOYAL and ARYA, 1981), *Mimosa pudica* L. (GHARYAL and MAHESWARI, 1982), *Albizzia lebbeck* L. (GHARYAL and MAHESWARI, 1983), *Dalbergia latifolia* Roxb. (DATTA, DATTA and PRAMANIK, 1983) *Dalbergia lanceolaria* Moon (ANAND and BIR, 1983), *Sesbania grandiflora* Poir (KHATTAR and MOHAN RAM, 1983), *Sesbania sesban* (L.) Merill (KHATTAR and MOHAN RAM, 1984), *Sesbania cannabina* Poir. (XU, YANG, WEI and GAO, 1984), *Leucaena leucophylla* (Lam) de Wit (NATARAJA and SUDHADEVI, 1984) and *Acacia melanoxylon* R. Br. (MEYER and VAN STADEN, 1987).

The aim with the *in vitro* culture of *Erythrina caffra* was to obtain a callus and suspension culture to establish whether the inhibitor of t-PA could be produced *in vitro*.

4.2 NUTRIENT MEDIUM AND GROWTH CONDITIONS

The basal medium used for the regeneration of callus (Table 4.2) consisted of the macro- and micro nutrients, vitamins and meso-inositol of the medium developed by MURASHIGE and SKOOG (1962). To this medium was added 3 % sucrose.

Table 4.2 Composition of the EC nutrient medium modified
         from the nutrient medium of MURASHIGE and SKOOG
         (1962) for the *in vitro* culture of callus derived
         from tissues of *Erythrina caffra*.

Compound	Mass	Mole
Macro nutrients	mg	mmole
NH_4NO_3	1650	20.6
KNO_3	1900	18.8
$CaCl_2.2H_2O$	440	2.99
$MgSO_4.7H_2O$	370	1.5
KH_2PO_4	170	1.25
Micro nutrients	mg	umole
KI	0.83	5.0
H_3BO_3	6.2	100.0
$MnSO_4.4H_2O$	22.3	100.0
$ZnSO_4.4H_2O$	8.6	29.9
$NaMoO_4.2H_2O$	0.25	1.0
$CuSO_4.5H_2O$	0.025	0.1
$CoCl_2.6H_2O$	0.025	0.1
FeEDTA	36.75	100.0
Organic substances	mg	umole
Meso-inositol	100.0	555.00
Nicotinic acid	0.5	4.06
Pyridoxine.HCl	0.5	2.43
Thiamine.HCl	0.1	0.30
Carbon source		
Sucrose	30 g	
Gelling agent		
Gelrite	2 g	

Amount per litre

Different amounts of growth regulators were added to the medium depending on the experiment being conducted. The basal medium was solidified with two grammes per litre Gelrite. The pH of the basal medium was adjusted to 5.7 before autoclaving. The nutrient medium was autoclaved at 121 ^{o}C for 20 minutes. Two types of containers were used for the incubation of explants. One type of container was a glass vial with a diameter of 25 millimetres and a length of 100 millimetres. These vials into which was added 10 millilitres of nutrient medium were capped with metal caps and sealed with parafilm. The second type of container used was a glass jar 55 millimetres in diameter and 110 millimetres high. These jars contained 50 millilitres of nutrient medium. The jars were tightly sealed with a polycarbonate screw cap. The cultures were incubated at the standard conditions of 25 ^{o}C and a quantum flux (400 to 700 nm) of 5 to 10 μM m^{-2}sec^{-1}.

In the first experiments 20 explants were used per treatment and not less than ten observations were used in the statistical analysis of the data. After enough data had been collected to determine the minimum number of replications needed to obtain consistent results a minimum of ten replications were used for the statistical analysis. *In vitro* culture experiments were completely randomised or factorial designs and the data analysed with the analysis of covariance using the initial inoculation mass of callus as the covariate. Experiments in which it was not possible to determine the initial fresh mass were analysed according

to the statistical design described. The data obtained from the experiments done were not normally distributed. A log transformation was used to normalise the data. The analysis of variance presented in the text is not the transformed values. Differences between treatment means were determined with the least significant difference between means (LSD) as designed by TUKEY (STEEL and TORRIE, 1960). The data was analysed with the Genstat (Long Ashton Agricultural Research Station, U.K.) and Statgraphics (Statistical Graphics Corporation, U.S.A.) statistical computer packages.

4.3 THE SOURCE OF EXPLANTS AND CALLUS

Seeds were obtained from *Erythrina caffra* trees growing on the campus of the University of Durban-Westville. The seeds were germinated and raised as described in Chapter2, section 2.7.

Explants were taken from the leaves and shoots of three-month- old plants. Healthy, young, fully expanded leaves which were free of diseases were used as explant material. Green, non-lignified shoots from the apical internodes of the seedlings were used as explants. Cotyledons of imbibed seeds were used as explants. Seeds were aseptically imbibed as described in Chapter 2, section 2.7. Cotyledons were removed aseptically from the imbibed seeds and sterilised for 10 minutes in 0.2 % mercuric chloride

containing 0.1 % Tween 20. The cotyledons were then washed five times in 100 millilitre aliquots of autoclaved distilled water. The leaf and shoot material were surface sterilised in the same way as the cotyledons. Air bubbles tended to form on the surface of the shoots and leaves when they were submerged in the sterilant or water. The material had to be shaken vigorously to get rid of the air bubbles. The sterile material was kept in a stoppered Schott reagent bottle for further use. A shoot explant consisted of an internode section approximately one centimetre long. The leaf explants were approximately one square centimetre in size and were cut from the centre of the leaf.

Root explants were obtained from aseptically grown seedlings. Aseptic seedlings were obtained by sterilising, scarifying and imbibing seeds as described in Chapter 2 section 2.7. The seeds were germinated aseptically and grown on the salts of MURASHIGE and SKOOG (1962) described in Table 4.2. The seeds germinated within two days and the seedlings grew well on the nutrient medium. Roots obtained from the aseptically grown seedlings were used as explants. All explants were transferred aseptically onto nutrient medium for *in vitro* culture.

Root, shoot, leaf and cotyledonary explants were incubated on a callus inducing medium to determine from which part of the plant the best callus could be obtained. An acceptable callus was defined as a non-brown, fast growing, friable callus. The friability of a callus was determined

qualitatively by mechanically disturbing the callus with a pair of forceps. Callus which disintegrated into small aggregates upon touching was considered as friable. Explants were placed on the nutrient medium of MURASHIGE and SKOOG (1962) prepared and sterilised as described in section 4.2. Benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were added to the medium at a concentration of 10 micromoles per litre each. The fresh mass and colour of the callus from 10 replicates of each organ were determined. The data was analysed as described before.

The best callus was selected on the basis of growth rate combined with colour and friability. White, friable fast growing callus was obtained from shoot explants (Table 4.3 ; Plate 4.1). The callus developed from the xylem, phloem and cortex of the shoot. No callus was formed by the pith cells (Plate 4.2). Callus on leaf explants was white but turned browned after the first subculture (Plate 4.3). Callus derived from leaf tissue did not grow as vigorously as shoot callus (Table 4.3 ; Plate 4.4). The leaf callus was not as friable as the shoot callus. The callus obtained from cotyledonary explants was white but was hard and had a slower growth rate than the shoot derived callus (Table 4.3 ; Plate 4.5). Callus from roots were brown and had a spongy appearance. Root callus grew less vigorously than shoot callus (Table 4.3 ; Plate 4.6). The callus from shoot explants was used to determine the optimal growth regulator concentration for callus growth.
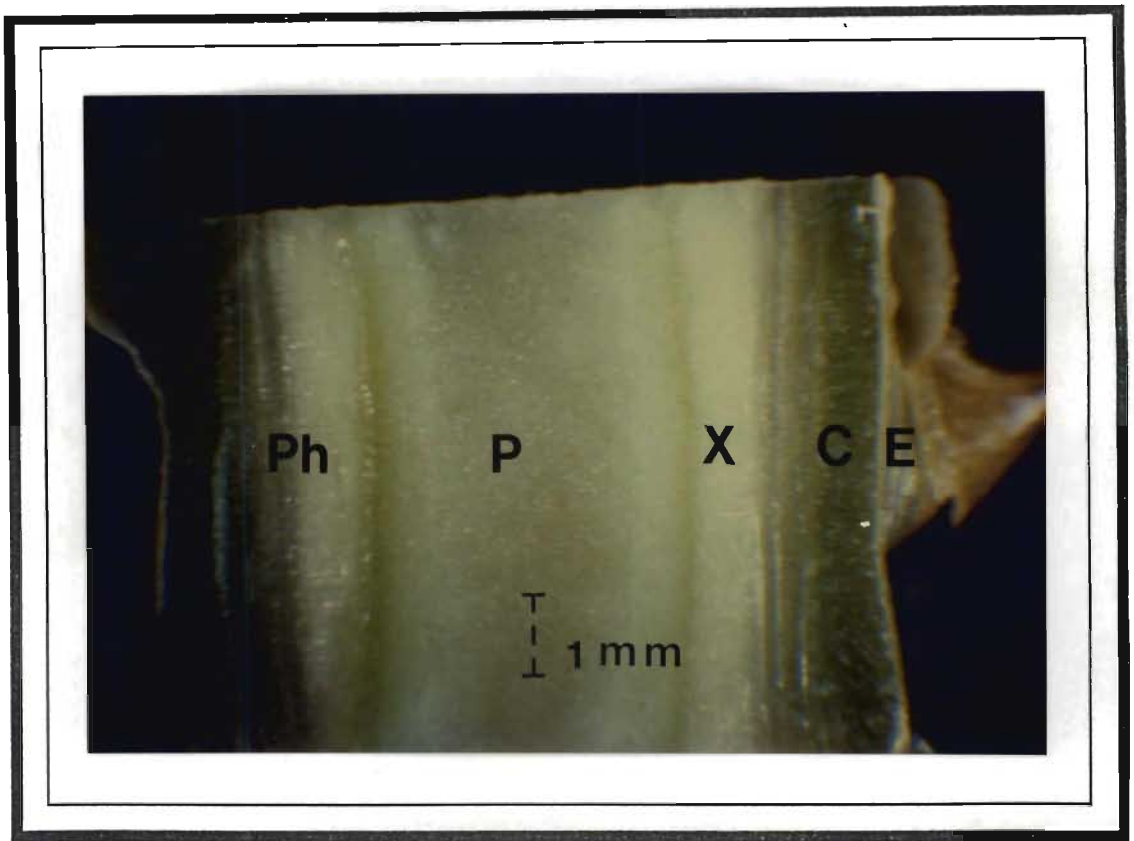
Plate 4.1 Radial section of a shoot explant of
Erythrina caffra with epidermis (E),
phloem (Ph), xylem (X), cortex (C) and
pith (P).
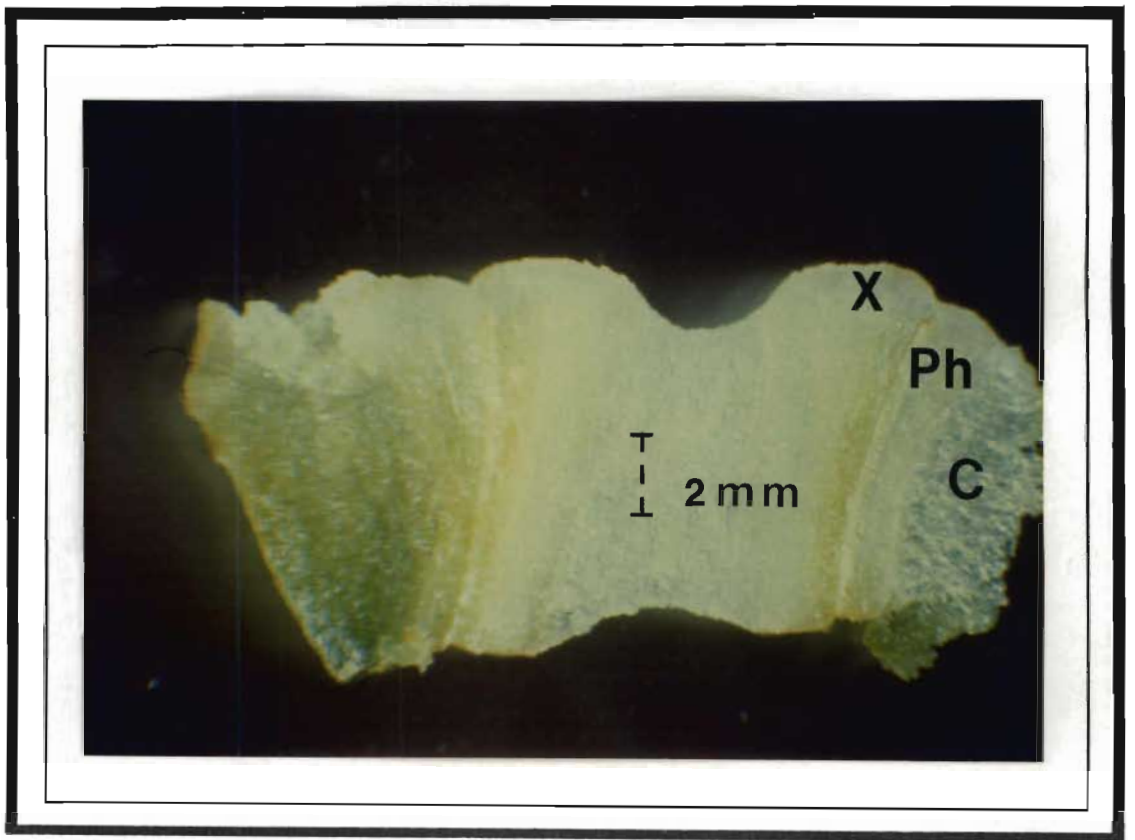
Plate 4.2 Radial section of a shoot explant of
          Erythrina caffra. White friable callus
          developed from the xylem (X), phloem (Ph)
          and cortex (C). No callus was formed by
          the pith tissue.
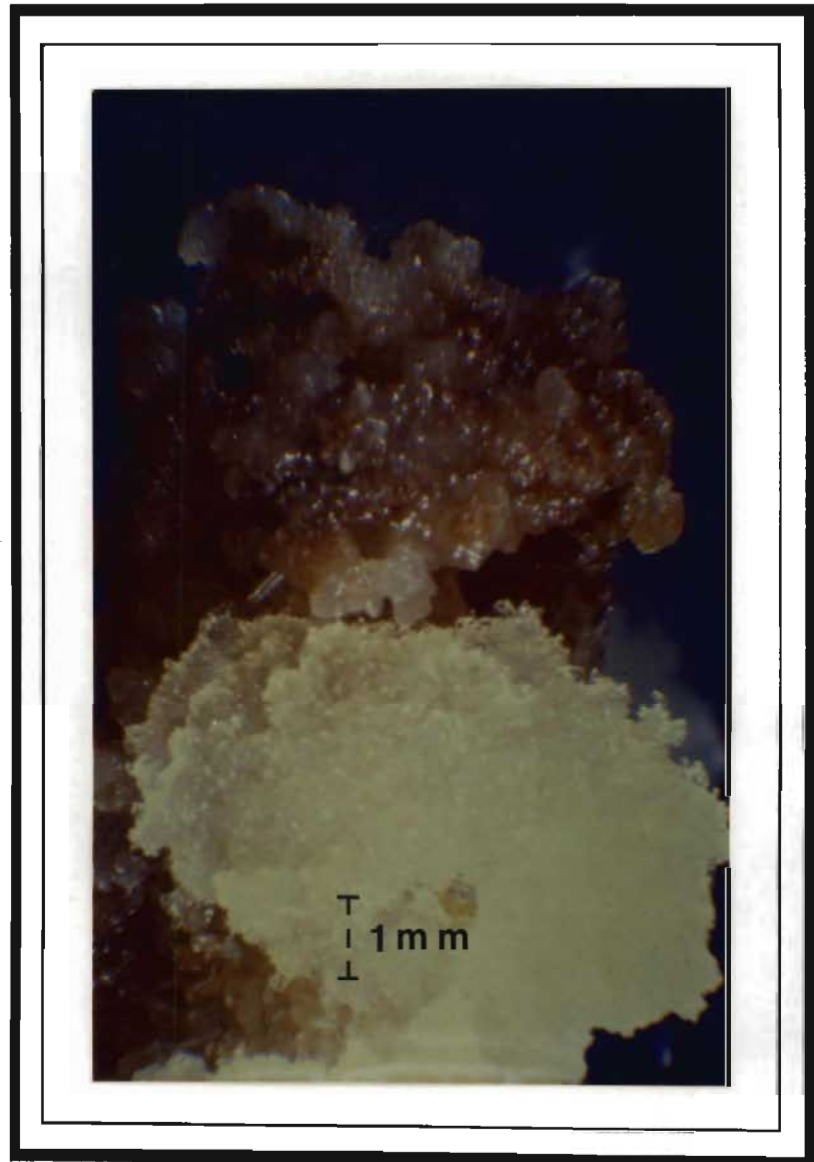
Plate 4.3 A comparison of white leaf callus of *Erythrina caffra* before it was transferred and callus that browned after it had been transferred onto fresh medium.
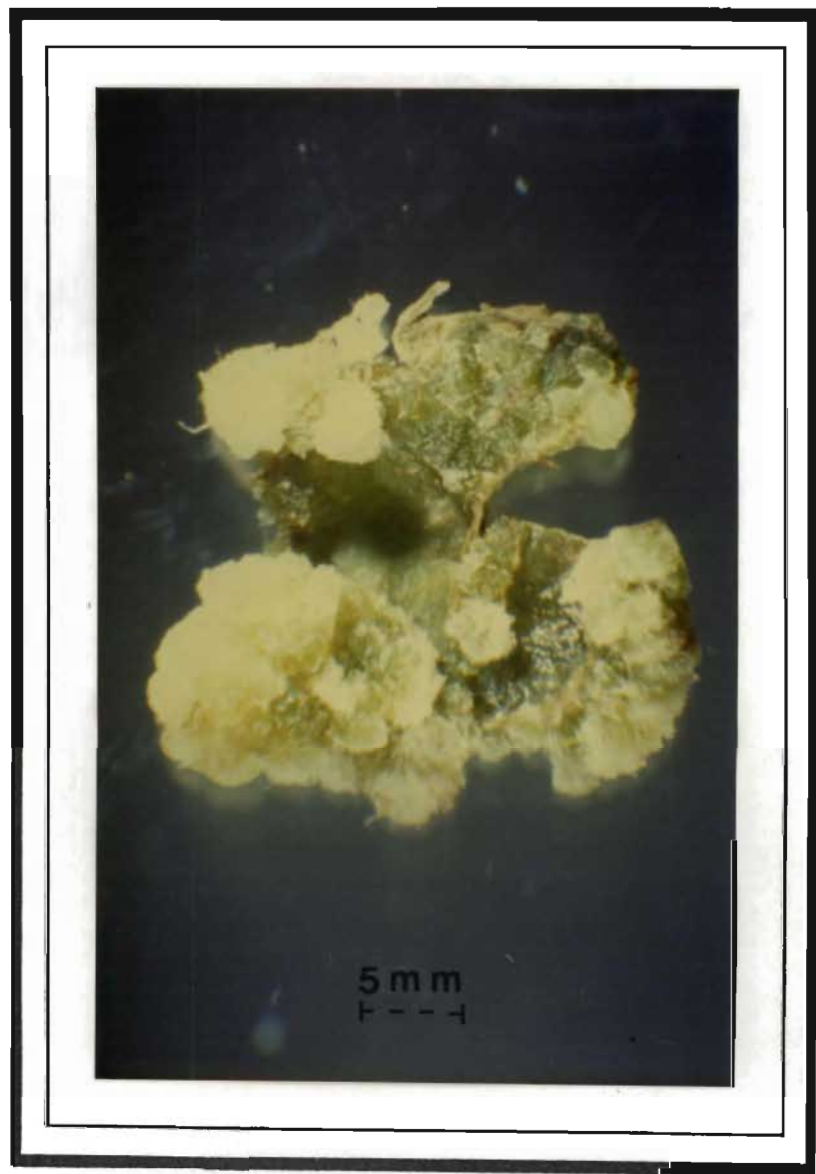
Plate 4.4   Callus developing on the cut surfaces
of a leaf explant of *Erythrina
caffra*.

Plate 4.5   Callus developing from a cotyledonary
explant of *Erythrina caffra*.

Plate 4.6   Callus developing from root   explants
            of *Erythrina caffra*. Callus formation
            at the proximal end   (A)   and on   the
            entire surface of the root (B).

Table 4.3 The quality and mass of callus produced *in vitro* from different organs of *Erythrina caffra* after 60 days of culture.

Explant	Fresh mass (mg)	Friability	Colour
Cotyledon	1133	Hard	White
Root	1423	Soft	Brown
Leaf	1465	Medium	White
Shoot	2209	Soft	White

Stocks of shoot callus were grown on the nutrient medium of MURASHIGE and SKOOG (1962) supplemented with 10 micromoles per litre of BA and 2,4-D respectively.

4.4 EXPERIMENTAL

4.4.1 THE EFFECT OF BA AND 2,4-D ON THE GROWTH OF SHOOT CALLUS

The optimal concentration of BA and 2,4-D in the basal medium for maximum growth of shoot callus was determined by designing a 5 X 5 factorial experiment. Benzyladenine was used at concentrations of 0, 0.1, 1.0, 10 and 100 micromoles per litre in combination with 2,4-D at all combinations of the same concentrations. After addition of the growth regulators the pH of the nutrient medium was adjusted to

5.7. The growth regulators were autoclaved with the nutrient medium. Twenty explants were used for each treatment. Each explant was incubated in a separate vial. The transfer of the explants onto the different media was done over a period of three days. Due to contamination data from a variable number of not less than 10 calli were used in the final analysis. The cultures were incubated under the standard growth conditions described in section 4.2. The fresh mass of the cultures was determined after 40 days of culture.

RESULTS AND DISCUSSION

Significant differences were recorded in the growth of the callus on the different growth regulator combinations (Table 4.4). The analysis of the main effects for BA and 2,4-D showed that the best callus growth was obtained for both BA and 2,4-D at a concentration range of 1 to 10 micromoles per litre (Table 4.5 and 4.6). From the multiple range analysis of the treatment means represented in Table 4.7 it is clear that maximum callus growth was sustained by a range of growth regulator combinations. The results presented in Figure 4.1 suggested that the gradients of growth hormones resulted in an increase in callus growth with an optimum at combinations of BA and 2,4-D concentrations of 1 to 10 micromoles per litre. Thus no single growth regulator combination was found to be optimal for callus growth. For maximum callus growth a

Table 4.4 Analysis of variance of the effect of BA
and 2,4-D on the growth of shoot callus of
Erythrina caffra.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Main effects				
BA	473737	4	118434.2	9.4*
2,4-D	650398	4	162599.7	13.0*
Interaction				
BA X 2,4-D	2685746	16	167859.1	13.4*
Residual	2818065	225	12524.7	
Total	6627945	249		

* Indicate significant differences at a 95 % level of confidence.

Table 4.5   Multiple   range   analysis   of   the   main effects of BA.

Treatment	95% Tukey intervals	Homogenous groups		
Concentration (uM)	Fresh mass (mg)			
10.0	1976.5	*		
1.0	1854.0	*		
0.1	1328.6		*	
1.0	1098.9			*
10.0	763.3			*

*   In different columns indicate significant differences at a 95% level of confidence.

Table 4.6   Multiple range analysis of the   main   effects   of 2,4-D.

Treatment	95% Tukey intervals	Homogenous groups		
Concentration (uM)	Fresh mass (mg)			
100.0	657.8	*		
0.0	705.1	*		
0.1	1474.0		*	
1.0	1966.2			*
10.0	2218.3			*

*   In different columns indicate significant differences at a 95% level of confidence.

Table 4.7 Multiple range analysis of the effect of BA and 2,4-D on the growth of shoot callus of *Erythrina caffra*.

Treatment		Treatment means	Homogeneous groups						
BA	2,4-D (uM)	Fresh mass (mg)							
0.0	0.0	07.6	*						
100.0	100.0	85.9	*						
0.0	100.0	101.4	*						
0.1	0.0	115.2	*	*					
1.0	0.0	140.1	*	*					
1.0	100.0	141.8	*	*					
0.1	100.0	163.4	*	*					
10.0	100.0	167.6	*	*	*				
0.0	0.1	171.0	*	*	*				
100.0	0.0	205.6	*	*	*				
0.0	1.0	232.2	*	*	*				
10.0	0.0	246.8	*	*	*	*			
100.0	10.0	247.2	*	*	*	*	*		
0.1	0.1	250.6	*	*	*	*	*		
100.0	0.1	251.4	*	*	*	*	*		
0.0	10.0	257.3	*	*	*	*	*		
100.0	1.0	311.4		*	*	*	*		
0.1	1.0	358.5			*	*	*	*	
10.0	0.1	365.4			*	*	*	*	
1.0	0.1	437.4				*	*	*	*
1.0	10.0	445.2					*	*	*
1.0	1.0	521.2						*	*
10.0	1.0	543.7						*	*
1.0	10.0	614.4							*
10.0	10.0	655.2							*

* In different columns indicate significant differences at a 95% level of confidence.

Figure 4.1 The effect of BA and 2,4-D on the growth of callus derived from shoot tissue of *Erythrina caffra*.

FRESH MASS (mg)

3500

3000

2500

2000

1500

1000

500

0

LSD

0   0.1   1   10   100

BA

CONCENTRATION (uM)

2,4-D

0

0.1

1

10

100

broad range of growth regulator concentrations could be used between 1 to 10 micromoles per litre with respect to the auxin and cytokinin. Relatively large differences between treatment means could not always be declared significantly different from each other (Table 4.7). A factor which probably contributed to the lack of detecting significant differences between treatment means was the large variation that existed between the fresh mass of calli within a treatment.

4.4.2 THE EFFECT OF DIFFERENT AUXINS AND CYTOKININS ON THE GROWTH OF SHOOT CALLUS

Growth regulators other than BA and 2,4-D could be more stimulatory to the growth of the shoot callus of *Erythrina caffra*. To determine which auxin and cytokinin was optimal for the growth of shoot callus BA and kinetin (Kin) in combination with 2,4-D, alpha-naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and indoleacetic acid (IAA) was used. After considering the results of the previous experiment it was decided to use the cytokinins at a concentration of 10 micromoles per litre and the auxins at 5 micromoles per litre. Each of these eight combinations of an auxin with a cytokinin was added to the basal nutrient medium. The IAA was filter sterilised and added to the nutrient medium after it had been autoclaved and had reached a temperature of 60 $^\circ$C. The other growth regulators were autoclaved with the nutrient medium. Twenty explants in

separate vials were used for each treatment. Data from a variable number of not less than 10 calli were used in the statistical analysis. The cultures were incubated under the standard growth conditions described in section 4.2. The fresh mass of the cultures was determined after 40 days of culture.

RESULTS AND DISCUSSION

It is clear from the results that the different combinations of growth regulators had the same stimulatory effect on the growth of the shoot callus (Tables 4.8 and 4.9). No differences were noted in the friability or colour of the calli grown on the different combinations of growth regulators. The calli were friable and white to light yellow in colour. Benzyladenine and 2,4-D at concentrations of 10 and 5 micromoles per litre respectively was subsequently used to obtain fast growing shoot callus.

4.4.3 OPTIMAL GROWTH REGULATOR CONCENTRATIONS FOR THE GROWTH OF CALLUS DERIVED FROM DIFFERENT ORGANS OF *ERYTHRINA CAFFRA*

After the concentrations of growth regulators supporting the best growth of shoot callus had been determined a set of experiments were conducted with a narrower range of BA and 2,4-D concentrations. The basal medium as described in

Table 4.8 Analysis of variance of the effect of different
          auxins and cytokinins on the growth of shoot
          callus of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatments	31562	7	4508.8	0.56
Residual	869572	108	8051.6	
Total	901134	115		

Table 4.9 Multiple range analysis of the effect of
          different auxins and cytokinins on the growth
          of shoot callus of *Erythrina caffra*.

Treatment Combinations of Growth regulators	Treatment means Fresh mass (mg)	Homogeneous groups
BA 10 2,4-D 5 (uM)	493.4	*
BA 10 NAA 5	514.6	*
Kin 10 IBA 5	539.2	*
BA 10 IAA 5	578.4	*
Kin 10 2,4-D 5	594.5	*
BA 10 IBA 5	603.2	*
Kin 10 NAA 5	625.6	*
Kin 10 IAA 5	637.1	*

* In different columns indicate significant differences at
   a 95% level of confidence.

section 4.2 was used. Explants were taken from plant material as described in section 4.2. The explants used were from root, shoot, leaf and cotyledonary material. The concentrations of growth regulators used were 1, 5 and 10 micromoles per litre for 2,4-D in all combinations with BA at 5, 10, 20 and 40 micromoles per litre. A separate experiment was conducted for each plant organ used. Twenty replicates of one explant each were used. The cultures were incubated under the conditions described in section 4.2. After 30 days of culture the fresh mass of the cultures were determined and analysed as described in section 4.3.

RESULTS AND DISCUSSION

Significant differences were observed in the growth of shoot callus between the different treatments (Table 4.10). At a concentration of 40 micromoles per litre BA inhibited the growth of shoot callus considerably. However, callus growth was stimulated at concentrations of BA 5 to 20 micromoles per litre (Table 4.11). According to the analysis of the main effects for 2,4-D no significant differences were obtained in the growth of callus at any of the concentrations of 2,4-D used (Table 4.12). It was clear that a range of growth regulator combinations can be used to obtain good callus growth (Table 4.13 ; Figure 4.2). The most likely combinations were BA:2,4-D 10:1, 10:5 and 5:5 micromoles per litre.

Table 4.10 Analysis of variance of the effect of BA
            and 2,4-D on the growth of shoot callus of
            Erythrina caffra.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial				
fresh mass	36732	1	36732	0.6
Main effects				
BA	1967354	3	655784.2	11.6*
2,4-D	398567	2	199283.5	3.5*
Interaction				
BA X 2,4-D	2841636	6	473606.0	8.3*
Residual	2667605	47	56575.6	
Total	7901894	59		

* Indicate significant differences at a 95% level of confidence.

Table 4.11 Multiple range analysis of the main effects of BA.

Treatment	95% Tukey intervals	Homogeneous groups		
Concentration (uM)	Fresh mass (mg)			
40	2030.0	*		
20	2457.3		*	
5	2566.7		*	
10	2729.0		*	

* In different columns indicate significant differences at a 95% level of confidence.

Table 4.12 Multiple range analysis of the main effects of 2,4-D.

Treatment	95% Tukey intervals	Homogeneous groups
Concentration (uM)	Fresh mass (mg)	
10	2349.3	*
5	2452.0	*
1	2460.8	*

* In different columns indicate significant differences at a 95% level of confidence.

Table 4.13 Multiple range analysis of the effect of BA and 2,4-D on the growth of shoot callus of *Erythrina caffra*.

Treatment		Treatment mean	Homogeneous groups					
BA	2,4 D (uM)	Fresh mass (mg)						
40	5	1969.2	*					
40	10	1976.4	*					
40	1	2145.3	*	*				
20	10	2289.8		*				
5	1	2350.4		*	*			
20	5	2503.0			*	*		
10	10	2550.2			*	*	*	
20	1	2577.8				*	*	
5	10	2581.0				*	*	
10	1	2735.2				*	*	*
5	5	2769.2					*	*
10	5	2902.6						*

* In different columns indicate significant differences at a 95% level of confidence.

Figure 4.2 The effect of BA and 2,4-D on the growth of callus derived from shoot tissue of *Erythrina caffra*.
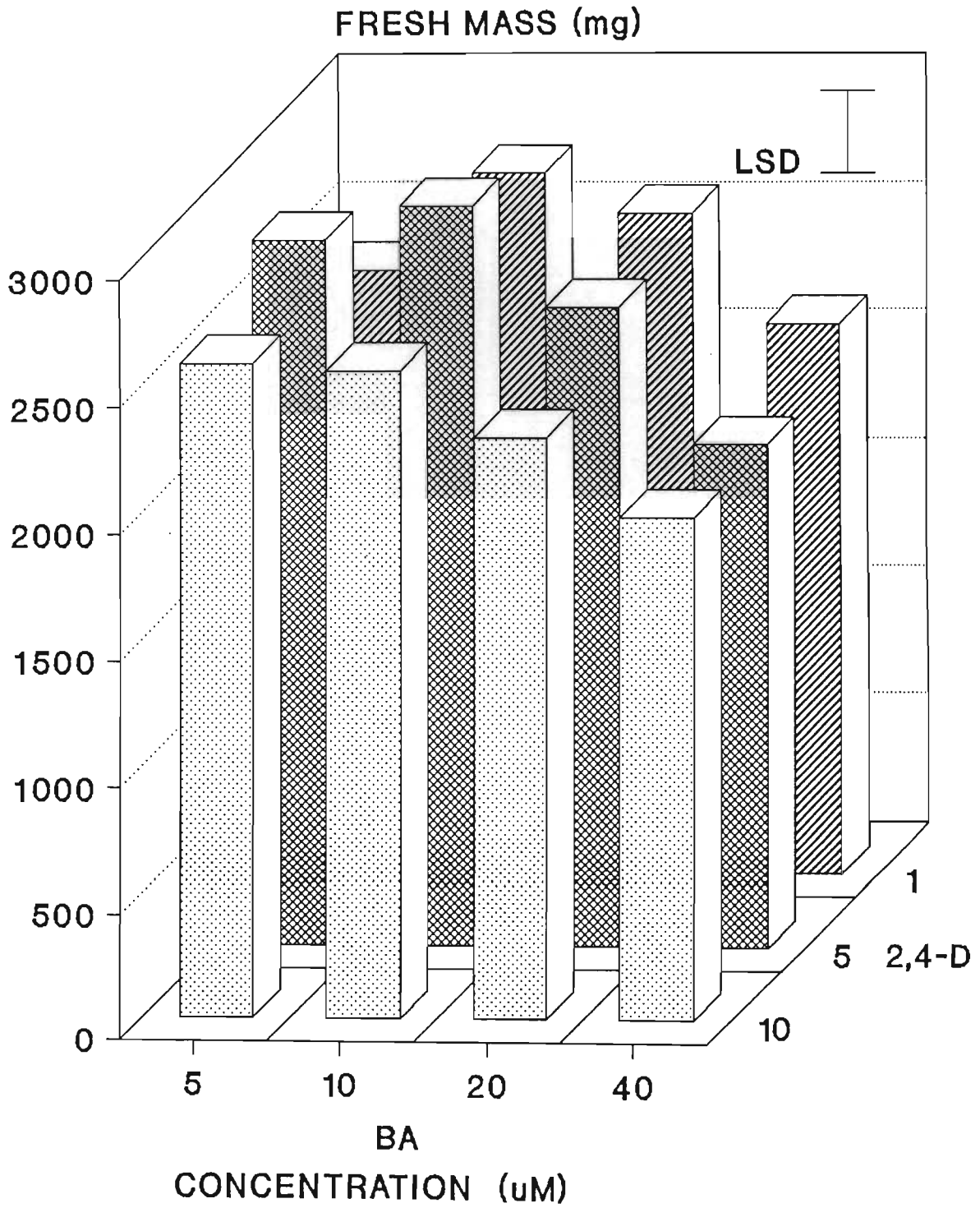
Significant differences in the growth of leaf callus between the different BA and 2,4-D combinations were observed (Table 4.14). Benzyladenine inhibited the growth of leaf callus at concentrations of 20 and 40 micromoles per litre (Table 4.15). However, 2,4-D was inhibitory to callus growth at a concentration of 10 micromoles per litre (Table 4.16). It was obvious from the analysis of the treatment means that the effect of the growth regulators was much more prominent on the growth of leaf callus than on shoot callus (Table 4.17 and Figure 4.3). The leaf callus seemed to be more sensitive to changes in the growth regulator concentration than shoot callus. A much steeper gradient in the growth of the callus as a reaction to the growth regulators was observed for the leaf callus (Figure 4.3). However, the optimal growth regulator combinations to obtain maximum growth of leaf callus with BA:2,4-D 10:5, 10:1, 5:1 were in the same range as for the best growth of shoot callus.

As with the shoot and leaf callus significant differences were detected between the main effects of the two types of growth regulators for cotyledonary callus (Table 4.18). As with leaf callus BA stimulated the growth of cotyledonary callus at concentrations 5 and 10 micromoles per litre (Table 4.19). From the main effects of 2,4-D it was apparent that a concentration of 1 micromole per litre was suboptimal and 10 micromoles per litre 2,4-D became inhibitory to growth (Table 4.20). The auxin 2,4-D was highly stimulatory to callus growth at a concentration of 5

Table 4.14 Analysis of variance of the effect of BA and 2,4-D on the growth of leaf callus of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	57428	1	57428	1.9
Main effects				
BA	2497021	3	832340.3	13.2*
2,4-D	465413	2	232706.5	3.7*
Interaction				
BA X 2,4-D	3217406	6	536234.3	8.5*
Residual	2963635	47	63056.1	
Total	10200812	59		

* Indicate significant differences at a 95% level of confidence.

Table 4.15 Multiple range analysis of the main effects of BA.

Treatment	95% Tukey intervals	Homogeneous groups	
Concentration (uM)	Fresh mass (mg)		
40	892.0	*	
20	1065.7	*	
5	1265.0		*
10	1303.7		*

* In different columns indicate significant differences at a 95% level of confidence.

Table 4.16 Multiple range analysis of the main effects of 2,4-D.

Treatment	95% Tukey intervals	Homogeneous groups	
Concentration (uM)	Fresh mass (mg)		
10	859.0	*	
5	1170.8		*
1	1281.8		*

* In different columns indicate significant differences at a 95% level of confidence.

Table 4.17 Multiple range analysis of the effect of BA and 2,4-D on the growth of leaf callus of *Erythrina caffra*.

Treatment		Treatment mean	Homogeneous groups				
BA	2,4 D (uM)	Fresh mass (mg)					
40	10	720.2	*				
40	5	860.4	*	*			
5	10	888.3	*	*			
40	1	907.8	*	*			
20	10	970.4	*	*			
10	10	1002.0	*	*	*		
20	5	1043.2		*	*		
20	1	1184.8			*	*	*
5	5	1355.0				*	*
10	5	1425.2					*
10	1	1484.2					*
5	1	1552.6					*

* In different columns indicate significant differences at a 95% level of confidence.

Figure 4.3 The effect of BA and 2,4-D on the growth of callus derived from leaf tissue of *Erythrina caffra*.
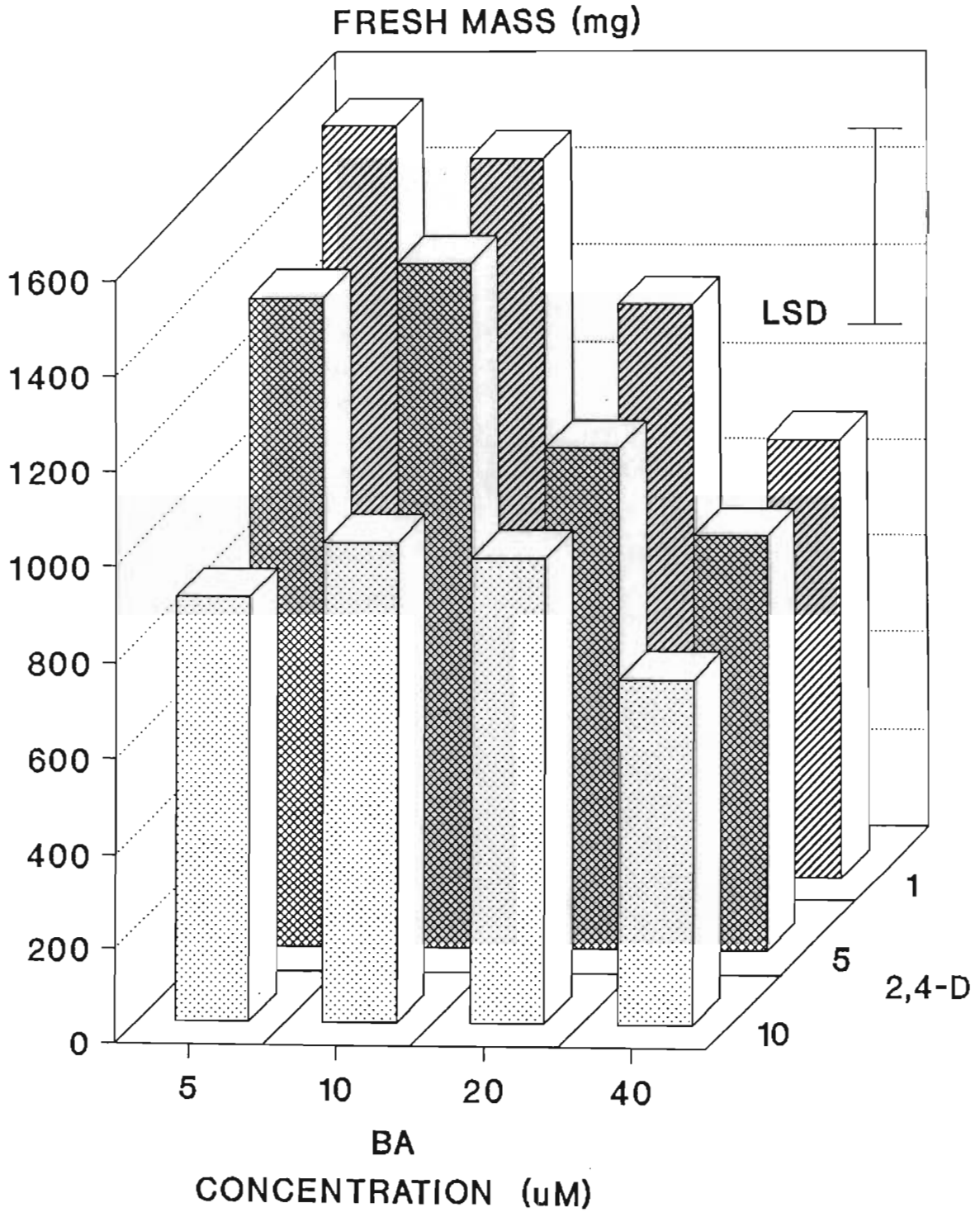
Table 4.18 Analysis of variance of the effect of BA
and 2,4-D on the growth of cotyledonary callus
of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial				
fresh mass	75906	1	75906	1.0
Main effects				
BA	3394006	3	1131335	14.4*
2,4 D	688806	2	344403	4.4*
Interaction				
BA X 2,4 D	5110766	6	851894	10.8*
Residual	3696254	47	78643	
Total	12971753	59		

* Indicate significant differences at a 95% level of confidence.

Table 4.19 Multiple range analysis of the main effects of BA.

Treatment	95% Tukey intervals	Homogeneous groups
Concentration (uM)	Fresh mass (mg)	
40	603.7	*
20	825.3	*
5	1108.7	*
10	1358.7	*

* In different columns indicate significant differences at a
  95% level of confidence.

Table 4.20 Multiple range analysis of the main effects of
            2,4-D.

Treatment	95% Tukey intervals	Homogeneous groups
Concentration (uM)	Fresh mass (mg)	
10	883.7	*
1	952.3	*
5	1232.5	*

* In different columns indicate significant differences at a
  95% level of confidence.

micromoles per litre in combination with BA at 5 and 10 micromoles per litre (Table 4.21 and Figure 4.4). A combinatiuon of BA and 2,4-D at concentrations of 10 and 1 micromoles per litre respectively was just as effective in stimulating callus growth. As with leaf callus the cotyledonary callus was very sensitive to the higher concentrations of BA and an even steeper slope could be observed in the growth of the callus with a change in the concentration of the growth regulators applied (Figure 4.4).

In contrast with the shoot, leaf and cotyledonary callus no differences were detected in the response of root callus to BA (Tables 4.22 and 23). However, significant differences in the growth of root callus were observed with the 2,4-D treatments (Table 4.22). As with the cotyledonary callus the analysis of the main effects for 2,4-D indicated that optimal growth for callus was obtained at a concentration of 5 micromoles per litre 2,4-D (Table 4.24). There was no clear indication as to the best treatment for optimal callus growth (Table 4.25). From the graphic representation of the treatment means in Figure 4.5 it can be seen that higher fresh mass values were obtained with 2,4-D at 5 micromoles per litre in combination with BA at 5 and 10 micromoles per litre. It is noticeable that 2,4-D at 1 micromole per litre in all combinations with BA resulted in a substantial decrease in callus growth.

In summary, callus derived from different organs reacted differently to growth regulators. Callus derived from leaf

Table 4.21 Multiple range analysis of the effect of BA and 2,4-D on the growth of cotyledonary callus of *Erythrina caffra*.

Treatment		Treatment mean	Homogeneous groups		
BA	2,4-D (uM)	Fresh mass (mg)			
40	10	590.8	*		
40	1	591.8	*		
40	5	630.6	*		
5	1	634.6	*		
20	10	774.6	*		
10	10	977.4	*	*	
20	1	1089.2	*	*	
5	10	1192.0	*	*	
20	5	1192.0	*	*	
10	1	1493.0		*	*
5	5	1499.2		*	*
10	5	1606.6			*

* In different columns indicate significant differences at a 95% level of confidence.

Figure 4.4 The effect of BA and 2,4-D on the growth of callus derived from cotyledonary tissue of *Erythrina caffra*.
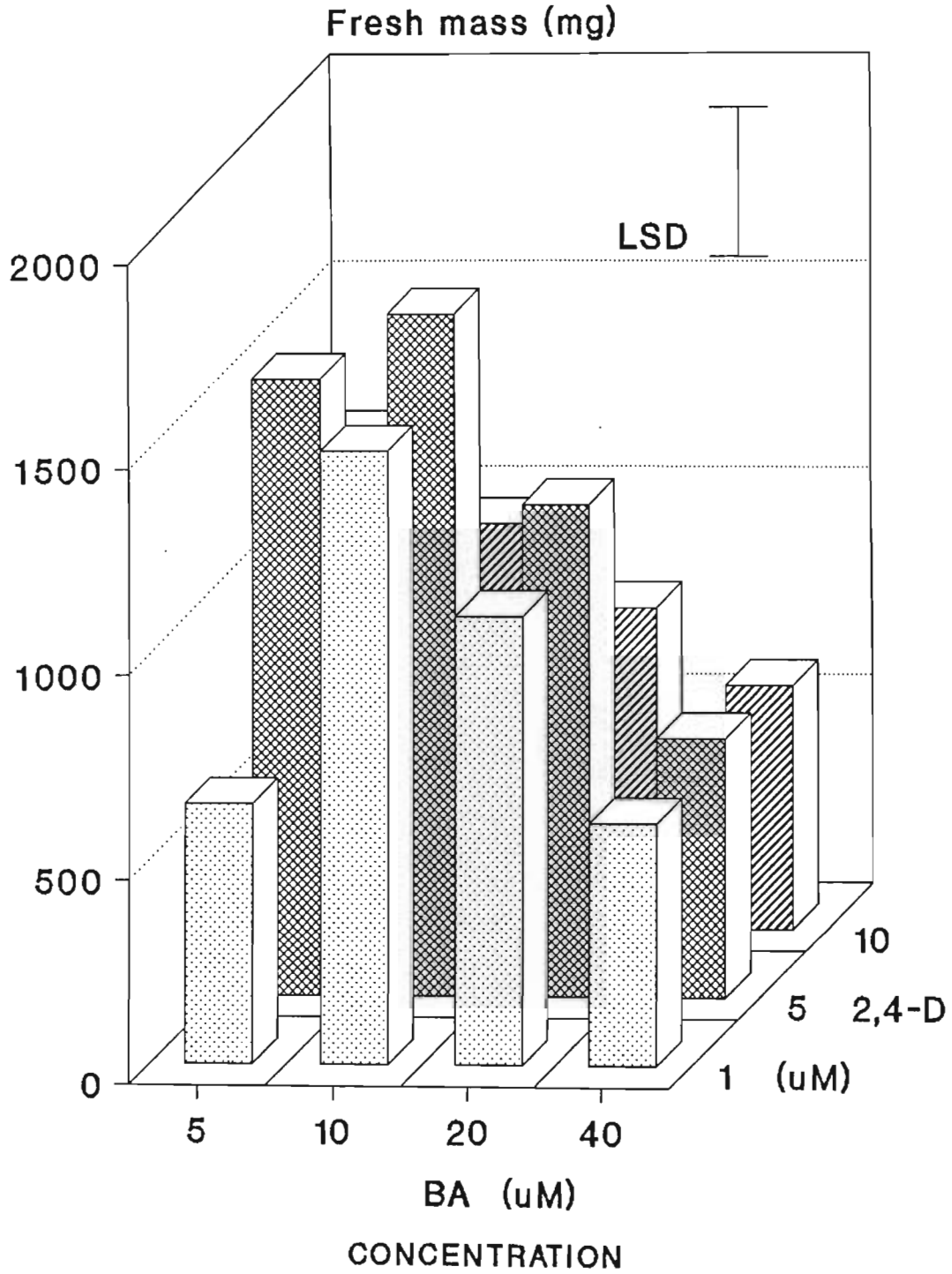
Table 4.22 Analysis of variance of the effect of BA and 2,4-D on the growth of root callus of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	1184	1	1184	< 0.1
Main effects				
BA	142864	3	47621	0.3
2,4-D	2228639	2	1114319	7.6*
Interaction				
BA X 2,4-D	245378	6	408964	2.8*
Residual	6868652	47	146141	
Total	11695114	59		

* Indicate significant differences at a 95% level of confidence.

Table 4.23 Multiple range analysis of the main effects of

BA.

Treatment	95% Tukey intervals	Homogeneous groups
Concentration (uM)	Fresh mass (mg)	
40	878.2	*
20	910.6	*
5	983.1	*
10	994.7	*

* In different columns indicate significant differences at

a 95% level of confidence.

Table 4.24 Multiple range analysis of the main effects of

2,4-D.

Treatment	95% Tukey intervals	Homogeneous groups
Concentration (uM)	Fresh mass (mg)	
1	804.0	*
10	806.7	*
5	1214.1	*

* In different columns indicate significant differences at a

95% level of confidence.

Table 4.25 Multiple range analysis of the effect of BA and 2,4-D on the growth of root callus of *Erythrina caffra*.

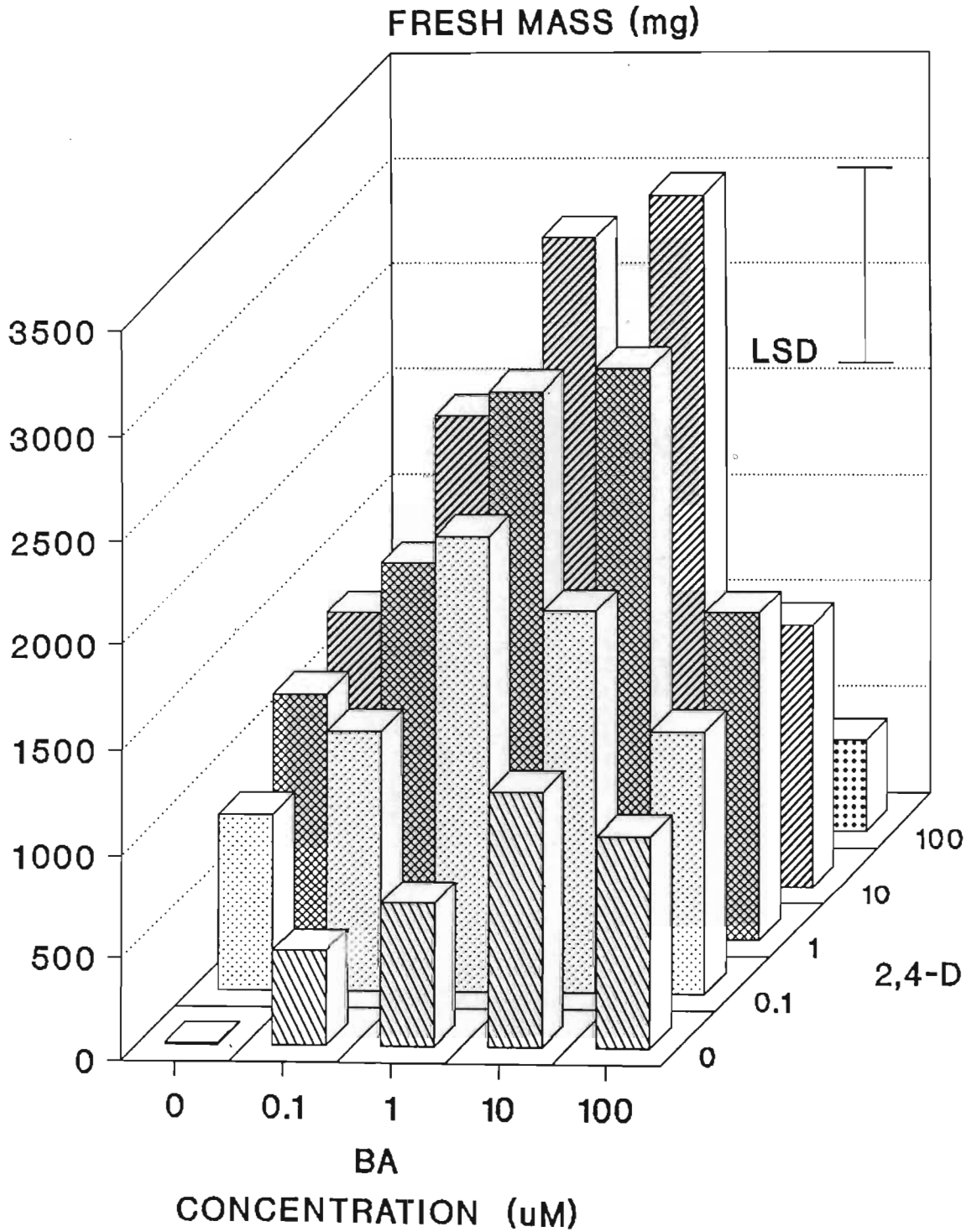| Treatment		Treatment mean	Homogeneous groups
BA	2,4-D (uM)	Fresh mass (mg)	
40	1	394.8	*
10	1	656.6	* *
20	1	669.6	* *
5	1	752.4	* *
40	10	825.0	* *
5	10	979.2	* *
40	5	993.8	* *
20	10	1069.0	* *
10	10	1095.2	* *
20	5	1136.2	* *
5	5	1313.6	*
10	5	1414.0	*

* In different columns indicate significant differences at a 95% level of confidence.

Figure 4.5 The effect of BA and 2,4-D on the
growth of callus derived from root
tissue of *Erythrina caffra*.
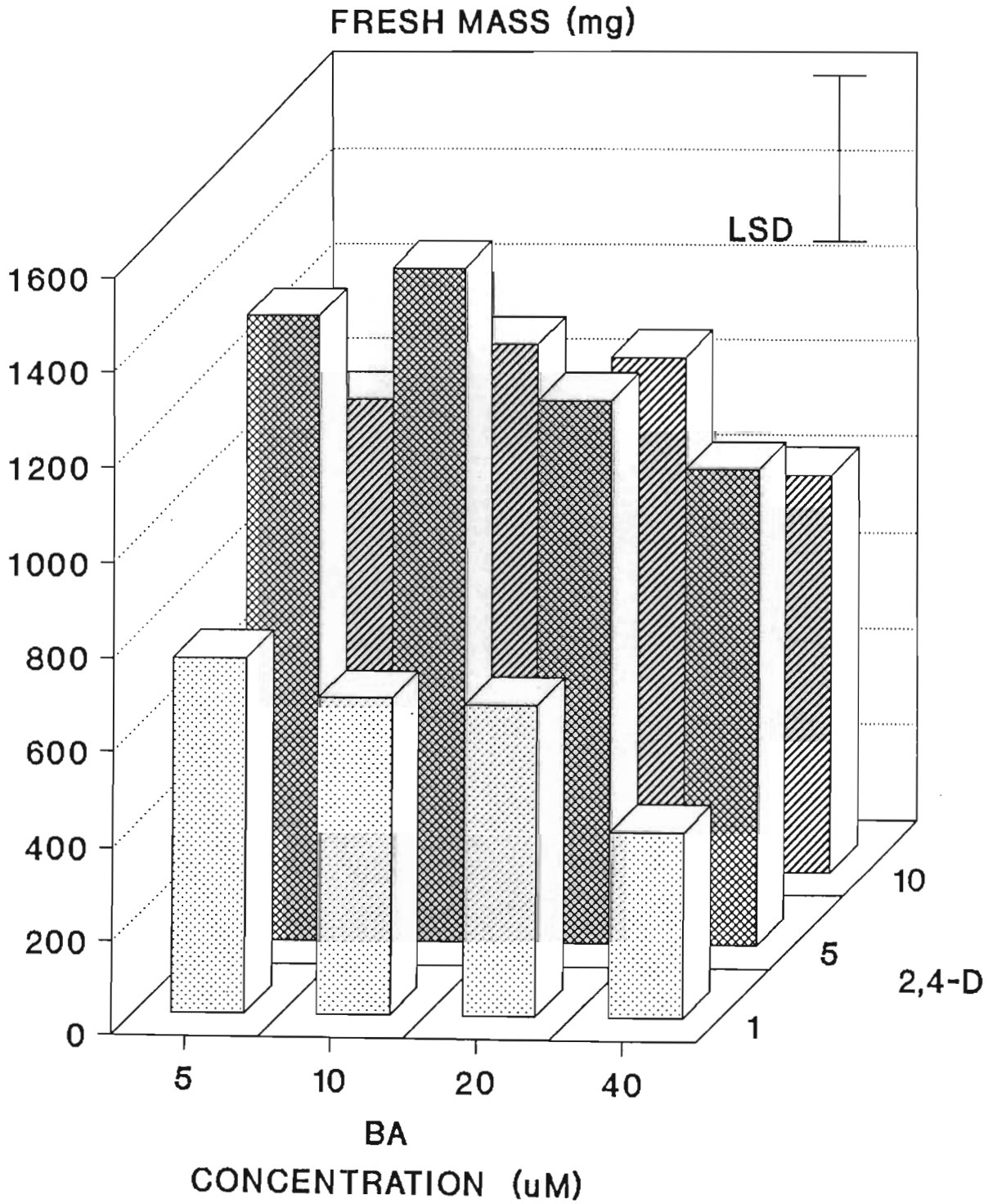
and cotyledonary material was more sensitive to changes in the concentration of the growth regulators used than shoot or root callus. Benzyladenine decreased the growth of leaf and cotyledonary callus at 20 micromoles per litre. Growth of shoot callus was retarded only at 40 micromoles per litre. Root callus grew equally well on concentrations of BA from 1 to 40 micromoles per litre.

The growth of root, leaf and cotyledonary callus was inhibited at 10 micromoles per litre 2,4-D. In contrast 2,4-D had no adverse effect on the growth of shoot callus in this experiment. The callus from the different organs had one point in common. The best growth was within the same range of growth regulator concentrations, e.g. BA 5 to 10 micromoles per litre and 2,4-D 1 to 5 micromoles per litre. With these results the nutrient medium for the growth of shoot callus i.e. EC medium was formulated so as to consist of the basal medium as described in section 4.2 supplemented with 10 micromoles per litre BA and 5 micromoles per litre 2,4-D.

4.4.4 THE GROWTH OF CALLUS FROM DIFFERENT PLANTS AND ORGANS OF *ERYTHRINA CAFFRA* ON EC MEDIUM

It was concluded from the previous experiments that callus from different explants grew almost equally well on the same combination of growth regulators. To verify this, callus from different organs and from different mother plants were

grown on EC medium. Seven seedlings were obtained from seeds collected from seven different *Erythrina caffra* trees. The seeds were treated and germinated as described in Chapter 2, section 2.7. Root, shoot, leaf and cotyledonary explants were obtained from each of the seven seedlings and sterilised as before. The explants were grown on the EC medium until sufficient callus was obtained to conduct the experiment. Callus from each culture was subcultured onto fresh EC medium and incubated under the standard conditions for 30 days. After 30 days the the fresh mass of the calli was determined.

RESULTS AND DISCUSSION

Significant differences were observed in the plant and explant main effects (Table 4.26). Analysis of the explant main effects indicates that callus from leaf tissue did not grow as well as callus from other organs (Table 4.27). From the analysis of the plant main effects it is evident that callus from the same organ obtained from different plants grew at different rates (Table 4.28). Little difference was observed in the production of cotyledonary derived callus from different plants (Table 4.29). However, substantial differences were noted between plants in the growth of callus derived from leaves, roots and shoots. It was also evident that there was a differential effect in the growth of callus for organs between plants (Table 4.30). The significant interaction shown in Table 4.26 implies

Table 4.26   Analysis of variance of the effect of explants
             from different plants and organs on the growth
             of callus of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	215126	1	215126.1	1.6
Main effects				
Plant	15829546	6	2638257.6	19.6*
Explant	12887089	3	4295696.3	31.9*
Interaction				
Plant X Explant	22257425	18	1236523.6	13.8*
Residual	15058827	112	134454.8	
Total	66511521	139		

* Indicate significant differences at a 95% level of
confidence.

Table 4.27 Multiple range analysis of the main effects of
explants.

Treatment	95% Tukey intervals	Homogeneous groups	
Explant	Fresh mass (mg)		
Leaf	1089.3	*	
Cotyledon	1730.6		*
Root	1823.6		*
Shoot	1919.1		*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.28 Multiple range analysis of the main effects of
plants.

Treatment	95% Tukey intervals	Homogeneous groups	
Plant	Fresh mass (mg)		
4	960.3	*	
3	1473.3		*
6	1991.3		* *
1	2011.5		* *
5	2059.4		* *
7	2320.2		*
2	2460.3		*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.29   Multiple range analysis of the effect of explants
            from different plants and   organs on the growth
            of callus of *Erythrina caffra*. Treatments are
            arranged according to the type of explant.

Treatment	Treatment mean	Homogeneous groups
Leaf	Fresh mass (mg)	
Plant 5	450.0	*
Plant 2	749.4	* *
Plant 4	817.2	* *
Plant 6	1165.6	* * *
Plant 3	1181.2	* * *
Plant 1	1628.2	* *
Plant 7	1928.1	*
Cotyledon		
Plant 4	360.6	*
Plant 7	1556.8	*
Plant 6	1718.4	*
Plant 3	1783.2	*
Plant 1	1887.8	*
Plant 5	2395.4	*
Plant 2	2408.6	*

Table 4.29 continued.

Treatment	Treatment mean	Homogeneous groups	
Root	**Fresh mass (mg)**		
Plant 4	1003.2	*	
Plant 3	1106.0	*	
Plant 6	1428.7	*	
Plant 1	1914.4		*
Plant 7	2323.0		*
Plant 2	2411.5		*
Plant 5	2578.4		*
Shoot			
Plant 3	902.0	*	
Plant 4	1201.6	*	
Plant 1	1561.2	*	*
Plant 6	2132.8		*
Plant 5	2266.0		* *
Plant 7	2312.6		* *
Plant 2	3030.4		*

* In different columns indicate significant differences at
   a 95% level of confidence.

Table 4.30 Multiple range analysis of the effect of explants
from different plants and organs on the growth
of callus of *Erythrina caffra*. Treatments are
arranged according to plants.

Treatment	Treatment mean	Homogeneous groups	
Plant 1	Fresh mass (mg)		
Leaf	1628.2	*	
Cotyledon	1887.8	*	
Root	1914.4	*	
Shoot	1561.6	*	
Plant 2			
Leaf	749.4	*	
Cotyledon	2408.6		*
Root	2411.5		*
Shoot	3030.4		*
Plant 3			
Shoot	902.0	*	
Root	1106.0		*
Leaf	1181.2		*
Cotyledon	1783.4		*
Plant 4			
Cotyledon	360.6	*	
Leaf	817.2	*	*
Root	1003.2	*	*
Shoot	1201.6		*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.30 continued.

Treatment	Treatment mean	Homogeneous groups	
Plant 5			
Leaf	450.0	*	
Shoot	2266.0		*
Cotyledon	2395.4		*
Root	2578.4		*
Plant 6			
Leaf	1165.6	*	
Root	1428.7	*	*
Cotyledon	1718.4	*	*
Shoot	2132.8		*
Plant 7			
Cotyledon	1556.8	*	
Leaf	1928.1	*	*
Shoot	2312.6		*
Root	2323.0		*

* In different columns indicate significant differences at a 95% level of confidence.

that the potential for callus production of different organs was a function of the organ as well as the parent plant from which the organ was obtained. It is also clear that the potential to produce callus is not the same for the different organs between different plants (Table 4.29 and 4.30). This suggests that large variation of callus growth in experiments can be reduced to some extent by using callus derived from one plant only.

Limited induction and growth of callus mainly from the actively dividing cambial region of plants was achieved before the discovery of plant hormones (GAUTHERET, 1983). However, the discovery of auxin by WENT (1926) gave new impetus to the induction and growth of callus from plant material. The unlimited growth of callus on an auxin enriched medium was demonstrated by many researchers (WHITE, 1939) and soon it was established that callus required an auxin for growth *in vitro*. With the discovery of cytokinins (MILLER, SKOOG, VON SALTZA and STRONG, 1955) it was soon demonstrated to be an essential factor for the division of cells. Today it is well established that the correct concentration and ratio of auxin to cytokinin can induce and sustain the growth of callus or plants *in vitro* (SKOOG and MILLER, 1957). From the voluminous literature on the effect of growth regulators on *in vitro* cultured plant material it can be concluded that for the induction, establishment and indefinite growth of callus from sources other than cambium an auxin is required.

Of the known auxins 2,4-D is the most effective and most commonly used for the growth of undifferentiated callus. This is due to the characteristic of 2,4-D to inhibit organogenesis in favour of callus formation (MURASHIGE, 1974 ; GRESSHOFF, 1980 ; LEE and RAO, 1980 ; RODRIGUEZ, REVILLA, PEREZ, CEBRANES and TAMES, 1987). However, at low concentrations 2,4-D can induce embryogenesis and initiated morphogenesis in legumes (LIAU and BOLL, 1970 ; GAMBORG and WETTER, 1975 ; OSWALD, SMITH and PHILLIPS, 1977). In general 2,4-D, NAA, IAA and IBA have similar effects with respect to callus growth. This was concluded by LEE and RAO (1980) after making observations on calli from *Eugenia grandis* Wight, *Bauhinia blakeana* Dunn. and *Cassia fistula* L. Exceptions were however, noted as with *Pterocarpus indicus* Wild. (LEE and RAO, 1980) and *Ceratonia siliqua* L. (MARTINS-LÁUCOU and RODRIGUEZ-BARRUECO, 1981). *Pisum sativum* L. cultures were stimulated by 2,4-D and produced substantially more callus than with other auxins (GANTOTTI and KARTHA, 1986). It was reported that 2,4-D was the only auxin on which *Acer pseudoplatanus* L. cultures could be grown (SIMPKINS, COLLIN and STREET, 1970). In many *in vitro* cultures callus as well as roots formed if a cytokinin was not added to the medium (YEOMAN and MACLEOD, 1977).

Cytokinins are routinely used in some nutrient media in combination with auxins for the establishment and growth of callus (SKOOG and MILLER, 1957 ; MURASHIGE and SKOOG, 1962 ; MURASHIGE, 1974). Of the cytokinins BA and kinetin are the

most commonly used. Cytokinins were not always found to be essential in the establishment and growth of callus but appears to fulfill a stimulatory role as in *Pisum sativum* cultures (GANTOTTI and KARTHA, 1986).

In this study it was found that explants from all organs of *Erythrina caffra* produced callus on combinations of BA or kinetin with NAA, 2,4-D, IAA or IBA. With *Erythrina caffra* tissue callus was also formed in the absence of an auxin or cytokinin. However, numerous cases have been reported where callus was only established on a nutrient medium in the presence of an auxin as well as a cytokinin. Woody plants such as *Vitis vinifera* produced callus only on a medium supplemented with 2,4-D and zeatin (JONA and VALLANIA, 1980). *Pterocarpus indicus* formed callus only in the presence of IAA or NAA and kinetin (LEE and RAO, 1980). Callus from *Papaver somniferum* L. could only be established on a medium containing an auxin and a cytokinin (ILAHI and JABEEN, 1987). Similar results were found with the legume *Stylosanthes scabra* Vog. which formed callus on kinetin and 2,4-D (GODWIN, GORDON and CAMERON, 1987).

Although in many cases callus did not appear to be absolutely dependent on specific growth regulators it has been observed that callus growth could be different with different growth regulators. *Stylosanthes scabra* explants did not produce any callus with kinetin alone in the medium. On replacing kinetin with BA callus was formed (GODWIN, GORDON, and CAMERON, 1987). In another study it was found

that *Prosopis juliflora* callus grew much slower on an auxin plus kinetin than in the presence of BA (WAINWRIGHT and ENGLAND, 1987). Benzyladenine was reported to induce less necrosis in callus of *Juglans regia* L. than kinetin (RODRIGUEZ, REVILLA, PEREZ, CEBRANES and TAMES, 1987). In the present study callus of *Erythrina caffra* from different origin reacted similarly with respect to fresh mass increase to the different growth regulators. No generalisations or extrapolations from related species can usually be made in order to predict the behaviour of a plant towards growth regulators *in vitro*. Each plant and cultivar, and even different clones from the same plant, have their own growth regulator requirements.

The growth potential of the various types of explants obtained from *Erythrina caffra* was found to be different on the same nutrient medium. Callus from leaf explants grew much less than the calli which originated from hypocotyl or shoot material. Such differences in the growth of callus was also noted for the legume *Bauhinia blakeana* Dunn. where cotyledonary callus grew substantially better than hypocotyl and stem callus on the same medium (LEE and RAO, 1980). *Ceratonia siliqua* hypocotyl callus responded better to the nutrient medium than cotyledonary or root callus (MARTINS-LAUCOU and RODRIGUEZ-BARRUECO, 1981). Variability in the growth of callus from different organs was also noted for *Pinus banksiana* Lamb. (CHALUPA, DURZAN and VITHAYASAI, 1976). These observations are indicative of the physiological differences between organs and the difference

in hormonal and nutritional requirements between explants from different organs. This is also clear from the observation that hypocotyl and cotyledonary callus from *Ceratonia siliqua* grew better on a combination of BA and an auxin while a maximum amount of root callus was produced when kinetin was the cytokinin used. Hypocotyl callus produced more callus with NAA in the medium while 2,4-D was optimal for the growth of callus derived from root material (MARTINS-LAUCOU and RODRIGUEZ-BARRUECO, 1981). Another observation indicating the dissimilarity between organs was the variation in callus morphology associated with explants from different organs. Callus derived from stem explants of *Feijoa sellowiana* Berg. were fluffy in appearance while callus from immature leaves was nodulated, green and morphogenic on the same medium (BHOJWANI, MULLINS and COHEN, 1987). A similar type of fluffy callus was produced in the present study by root cultures of *Erythrina caffra*. It has also been found that callus from different organs could react similarly on a specific medium. An example of this was the callus from *Arabidopsis thalliana* L. Heynh. derived from root, shoot, cotyledonary and hypocotyl tissue which all grew equally well on a nutrient medium supplemented with 2,4-D (ACEDO, 1986). Similar observations were made for *Hevea braziliensis* callus derived from leaf, stem and cotyledonary tissue which grew similarly on the same auxin (PARANJOTHY, 1987).

Callus from the different organs of *Erythrina caffra* did not differ much in appearance, apart from the fact that leaf

and cotyledonary callus were harder than shoot callus and root callus in most cases was fluffy in appearance. The callus on root, shoot, leaf and cotyledonary tissue were white or creamy coloured or colourless. It was only after the first subculture that leaf callus turned brown while root, shoot and cotyledonary callus remained white. For all the organs callus on the explant, irrespective of the growth regulator concentration used, was hard and not suitable for the establishment of suspension cultures. However, upon repeated subculture and selection of friable callus, callus lines could be established which were so friable that they became difficult to handle it with a pair of forceps. Occasionally this callus reverted back to a less friable callus and had to be reselected for friability. It was, however, more expedient to select appropriate callus lines than to attempt to devise a specific nutrient medium for friable callus. The selection of strains of callus differing in friability has been achieved for species such as *Daucus carota* (BLAKELY and STEWARD, 1964). Apart from selecting friable callus the nutrient medium could be adapted to improve friability. This was achieved by altering the growth regulator composition or growth regulator concentration (BLAKELY and STEWARD, 1964 ; RODRIGUEZ, REVILLA, PEREZ, CEBRANES and TAMES, 1987), increasing the incubation period (HEDTRICH, 1977), increasing the sucrose concentration (PHILLIPS and COLLINS, 1979), repeated subculturing of callus (LEE and RAO, 1980), omitting organic substances such as vitamin B_{12} and folic acid from the medium (REINERT and WHITE, 1956) or by enriching the nutrient medium with

organic substances such as coconut milk or casein hydrolysate (HENDERSON, DURREL and BONNER, 1952).

As mentioned before the variability in the growth of the callus in this study was large. From the literature it is clear that this variability associated with callus cultures has already been noted during the early years of tissue culture. In an extensive study on tobacco callus CAPLIN (1947) found large differences in the growth between calli from the same origin. After a period of growth the difference in the final mass of callus from similar size explants was a ratio of 10:1 with the smallest increase in mass of 28 times and the largest increase of 238 times. It was also observed that the increase in variability in fresh mass between explants increased dramatically with the age of the culture (CAPLIN, 1947) and with an increase in the number of subcultures (PHILLIPS and COLLINS, 1979). CAPLIN (1947) also observed that explants with a smaller initial mass had a higher relative growth rate of callus than larger explants. It was concluded that the large differences in growth could be attributed mainly to inherent differences in the explants expressed in the different growth rates of the calli. These results were supported by HEBLE, NARAYANASWAMY and CHADHA (1974) who observed the development of variant cell lines in cultures of *Plumbago zeylanica* L. callus which differed in growth potential, morphology and pigmentation. Callus cultures from *Prunus mahaleb* L. grew at different rates and were green and compact or friable and yellow to white (HEDTRICH, 1977).

WAINWRIGHT and ENGLAND (1987) observed large differences in the growth of callus from different clones of *Prosopis juliflora*. Similar results were obtained from cell strains of *Haplopappus gracilis* and *Daucus carota* which differed in chromosome compliments from each other (BLAKELY and STEWARD, 1964). It is well established that cytological changes takes place in somatic cells *in vitro* which leads to the development of mainly polyploidy and aneuploidy (DAMATO, 1977 ; PARTANEN, 1963). MURASHIGE (1974) concluded that callus with uniform parenchyma cells was rarely witnessed and could only be found in callus from *Agave* and *Rosa* species.

4.4.5 THE EFFECT OF DIFFERENT NUTRIENT MEDIA ON THE GROWTH OF CALLUS FROM DIFFERENT ORGANS OF *ERYTHRINA CAFFRA*

In the previous experiments conducted callus from *Erythrina caffra* had only been grown on the salts of MURASHIGE and SKOOG (1962). To determine whether callus growth could be improved on other nutrient media callus from leaf, shoot and cotyledonary tissue were grown on the salts and vitamins of MURASHIGE and SKOOG (1962) (MS), the medium of SCHENK and HILDEBRANDT (1972) (SH) and the B5 medium of GAMBORG, MILLER and OJIMA, (1968) (Table 4.31). In these two nutrient media the iron source was replaced with NaFE.EDTA to supply the same molar concentration of iron as in the original media. The media were supplemented with BA at a

Table 4.31 Comparison of the nutrient composition of the
modified nutrient media of MURASHIGE and
SKOOG (1962), SCHENK and HILDEBRANDT (1972)
and GAMBORG, MILLER and OJIMA (1968).

Nutrient	Nutrient medium		
	MS	SH	B5
Macro nutrients	mM	mM	mM
NO_3	40.0	25.0	25.0
NH_4	20.0	2.5	2.0
Total N	60.0	27.5	27.0
K	21.5	25.0	25.0
P	1.5	2.5	1.0
Ca	3.0	1.5	1.0
Mg	1.5	1.5	1.0
Cl	6.0	3.0	2.0
S	1.730	1.619	2.112
Na	0.202	0.111	1.102
Micro nutrients	uM	uM	uM
Fe	100.0	55.0	50.0
B	100.0	80.0	50.0
Mn	100.0	60.0	60.0
Zn	30.0	3.5	7.0
I	5.0	6.0	4.5
Mo	1.0	0.4	1.0
Cu	0.1	0.8	0.1
Co	0.1	0.4	0.1
Organic substances	uM	uM	uM
Meso-inositol	555.0	5550.0	555.0
Nicotinic acid	4.0	40.0	8.0
Pyridoxine.HCl	2.5	2.5	5.0
Thiamine.HCl	0.3	15.0	30.0

MS = MURASHIGE and SKOOG (1962).

SH = SCHENK and HILDEBRANDT (1972).

B5 = GAMBORG, MILLER and OJIMA (1968).

concentration of 10 micromoles per litre and 2,4-D at 5 micromoles per litre. Three per cent sucrose and 0.01 % meso-inositol were added to the media after which it was sterilised at 121 $^\circ$C. The medium was solidified with 0.2 % Gelrite.

RESULTS AND DISCUSSION

Large differences exist in the concentration of the nutrients in the three nutrient media used. The SH and B5 media contain about half the amount of nitrogen, calcium, chlorine, iron, boron and manganese and about 10 % of the ammonium and zinc present in MS medium. The SH medium has more phosphate, copper, cobalt, iodine and nicotinic acid than the other two media. The B5 medium has the lowest nutrient concentrations of the three media except for the higher levels of pyridoxine, thiamine sodium and sulphur (Table 4.31). Despite these differences in the concentrations of the nutrients in the three media no difference was observed in the growth of callus on the three nutrient media (Tables 4.32 and 4.33). This suggests that callus cultures of *Erythrina caffra* from different organs can tolerate a large concentration range of nutrients without affecting their growth. However, differences in the growth of callus from different organs was detected. Substantially more callus was produced with the callus derived from shoots than from leaves or cotyledons irrespective of the nutrient used (Tables 4.34 and 4.35).

Table 4.32   Analysis of variance of the effect of different
              nutrient media on the growth of callus derived
              from different organs of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	10849966	1	10849966.0	35.3*
Main effects				
Medium	5084840	2	2542420.0	2.9
Explant	63520725	2	31760362.5	103.3*
Interaction				
Medium X Explant	15196260	4	3799065.0	12.4*
Residual	46736148	152	307475.6	
Total	137405702	161		

* Indicate significant differences at a 95% level of
confidence.

Table 4.33 Multiple range analysis of the main effects of
nutrient media.

Treatment	95% Tukey intervals	Homogeneous groups
Nutrient medium	Fresh mass (mg)	
MS	1845.8	*
B5	2480.0	*
SH	2857.2	*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.34 Multiple range analysis of the main effects of
the plant organs.

Treatment	95% Tukey intervals		Homogeneous groups
Explant	Fresh mass (mg)		
Cotyledon	1029.4	*	
Leaf	2020.6		*
Shoot	4133.0		*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.35   Multiple range analysis of the treatment means
of the effect of different nutrient media on
the growth of callus derived from different
organs of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups	
Cotyledon	Fresh mass (mg)		
MS	856.4	*	
B5	1251.8	*	
SH	1266.0	*	
Leaf			
MS	1685.6	*	*
B5	1760.2	*	*
SH	2616.8	*	*
Shoot			
MS	2921.8		* *
SH	3257.2		* *
B5	3620.2		*

* In different columns indicate significant differences at
a 95% level of confidence.

This suggests that the growth rate of callus was to a large extent determined by the origin of the callus rather than the nutrient medium used.

The inorganic salts, vitamins and carbon source in a nutrient medium are on the average not as critical as the growth regulators for the growth of callus. Some nutrient medium formulations had been developed and successfully used on a wide variety of plants such as those of MURASHIGE and SKOOG (1962), the B5 medium (GAMBORG and WETTER, 1972) and the medium of SCHENK and HILDEBRANDT (1972). From the more than a thousand nutrient formulations tabulated by GEORGE, PUTTOCK and GEORGE (1987) it is clear that different plants and cultivars each perform optimally *in vitro* on its own specific nutrient medium. However, most of these formulations have with minor modifications been based on the three nutrient media listed above. It is possible to obtain good growth on some of the broad spectrum media by merely diluting the nutrient medium. It was found for instance that *Pisum sativum* callus grew better on 1/3 and 1/2 MS than on full strength MS or 1/2 B5 medium (JACOBSEN, INGENSIEP, HERLT and KAUL, 1980). *Prosopis juliflora* was found to grow better on 1/2 strength than full strength MS medium (WAINRIGHT and ENGLAND, 1987). Full strength modified medium of LINSMAIER and SKOOG (1965) proved to be superior to a 1/2 or 1.5 strength medium in supporting growth of *Pinus banksiana* Lamb. callus (CHALUPA and DURZAN, 1976). In most cases near optimal growth of a culture could be obtained by merely selecting one of the existing

nutrient media withn which good growth can be obtained.
JORDAN (1987) found that the nutrient medium of LLOYD and
MCCOWAN (1980), devised for woody plants, sustained growth
of *Sapium sebiferum* L. Roxb. cultures better than the MS
and SH media. He also found that out of four nutrient media
evaluated *Hevea braziliensis* (Muell.) Arg. callus grew best
on the medium of MURASHIGE and SKOOG (1962).

A nutrient medium can be optimised by modifying an existing
nutrient medium. For example suspension cultures of
Haplopappus gracilis L. could only be grown on MS medium
when the micronutrients of this medium was reduced to 10 %
of the original concentration (ERIKSSON, 1965). PARANJOTHY
(1987) optimised the nutrient medium for callus from *Hevea
braziliensis* by doubling the phosphate and reducing the
the nitrogen content of the MS medium by 50 %. Modification
of existing media by PHILLIPS and COLLINS (1979) to obtain
the PCL2 medium was highly beneficial for the growth of red
clover callus which did not grow on MS, B5 and SH nutrient
media. An effective medium was developed for the growth of
callus from *Phaseolus vulgaris* by merely combining the salts
of the medium of MURASHIGE and SKOOG (1962) with the organic
constituents of WHITE'S (1942) medium (LIAU and BOLL, 1970).
After evaluating a large number of nutrient media being used
for the culture of woody species McCOWN and SELLMER (1987)
concluded that in comparison with herbaceous plants woody
plants are frequently cultured on nutrient media with a
lower concentration of total salts. The source and
concentration of nitrogen and calcium was also found to

be more critical for woody species. The ionic strength of MS, SH and B5 media are 171.1, 105.1 and 95.6 milli equivalents respectively. The ionic strength of SH is about 60 % and B5 about 50 % the ionic strength of MS medium. Even with these large differences in the ionic strength between the three media no differences were found in the growth of callus from *Erythrina caffra*. Considering the fact that MS medium is a medium with a high ionic strength it is clear that the ionic strength of the nutrient medium was not critical for callus initiation and growth of *Erythrina caffra*.

4.4.6 THE EFFECT OF NITROGEN ON THE GROWTH OF CALLUS OF *ERYTHRINA CAFFRA*.

Although no significant difference was found in the growth of callus between different nutrient media it was observed that the growth of callus was not as good on the medium of MURASHIGE and SKOOG (1962) than on the other nutrient media. One of the large differences in the nutrient composition between the media of MURASHIGE and SKOOG (MS) (1962), SCHENK and HILDEBRANDT (SH) (1972) and the B5 (GAMBORG,MILLER and OJIMA, 1968) medium is the high concentration of nitrogen and ammonium in the medium of MURASHIGE and SKOOG (1962) (Table 4.36). The MS medium contains nearly twice as much nitrate and more than double the amount of ammonium than the SH and B5 media. An

Table 4.36 The nitrogen content in the nutrient media
of MURASHIGE and SKOOG (1962) (MS), GAMBORG,
MILLER and OJIMA (1968) (B5) and SCHENK and
HILDEBRANDT (1972) (SH). Concentrations are in
millimoles per litre.

MEDIUM	NO_3^-	NH_4^+	TOTAL N	$NH_4^+:NO_3^-$
MS	40	20	60	1:2
SH	25	0	25	0:25
B5	25	2	27	1:12.5

experiment was conducted to determine the effect of the
ratio of ammonium to nitrate on the growth of shoot callus.
The following ratios of ammonium:nitrate were used, 1:0,
0:1, 1:1, 1:2, 1:4, 1:8, 2:1, 4:1 and 8:1 in EC medium
without its normal nitrogen component. In the formulation of
the treatments the total amount of nitrogen was kept
constant at 60 millimoles per litre which is the total
concentration of nitrogen in the nutrient medium of
MURASHIGE and SKOOG (1962). Chloride and potassium ions were
also involved in the formulation of the nitrogen treatments.
The potassium ion concentration was kept constant at 61.4
millimoles per litre. However, the concentration of all the
ions could not be kept constant and large fluctuations
occurred in the concentration of the chloride ions (Table
4.37). It must be pointed out that a false impression
might be created in that with keeping the ammonium ion

Table 4.37   Formulation   of   the   nitrogen   treatments   for

Experiment 4.4.6.

Ratio	Concentration of compound in medium (mM)				
$NH_4 : NO_3$	NH_4NO_3	KNO_3	NH_4Cl	KCl	Cl^-
1:0	-	-	60	60	126
0:1	-	60	-	-	6
1:1	30	-	-	60	66
1:2	20	20	-	40	46
1:4	12	36	-	24	30
1:8	-	53.4	6.6	6.6	19.2
2:1	20	-	20	60	86
4:1	12	-	36	60	102
8:1	6.6	-	53.4	60	113.4

Total K^+ was kept constant at 61.3 millimoles.

Total N was kept constant at 60.0 millimoles.

Apart from the above mentioned Cl^- concentrations an extra 6 millimoles Cl^- from other sources in the medium was added to the totals in the table.

Table 4.38   The total amount of nitrate and ammonium ions in the basal nutrient medium at different ratios of the two ions.

Ratio	Molarity of ions in 1 l nutrient medium (mM)		
$NH_4^+:NO_3^-$	NH_4^+	NO_3^-	Total N
1:0	60.0	0.0	60.0
1:1	30.0	30.0	60.0
1:2	20.0	40.0	60.0
1:4	12.0	48.0	60.0
1:8	6.7	53.4	60.0
0:1	0.0	60.0	60.0
2:1	40.0	20.0	60.0
4:1	48.0	12.0	60.0
8:1	53.3	6.7	60.0

in the ratios mentioned, that the total amount of ammonium in the medium was kept constant and that the amount of nitrate increased up to eight times in some of the media and *vice versa*. This is not correct as can be seen from Table 4.38. Keeping one ion constant in the ratio effectively resulted in a gradual decrease in the total amount of that ion in the medium. There was a nearly five times decrease of ammonium in the medium between the ammonium:nitrate ratios 1:1 to 1:8. For the same ratios the amount of nitrogen was less than double the amount in MS medium. This suggests that between the ratios of ammonium:nitrate of 1:1 to 1:8 there was a relatively large

decrease in the amount of ammonium and a relatively small increase in the amount of nitrogen. The opposite was true for the ratios of ammonium:nitrate of 1:1 to 8:1.

RESULTS AND DISCUSSION

Significant differences in callus growth were detected between the different treatments (Table 4.39). From the analysis of the treatment means (Table 4.40) and graphic representation of the results (Figure 4.6) it can be seen that callus growth was severely retarded with only ammonium in the medium. With the addition of nitrate to the ammonium in the medium a substantial increase in the growth of callus was observed. Approximately doubling the amount of nitrate from 30 to 53.3 millimoles per litre had no additional stimulatory effect on the growth of the callus. In conjunction with Table 4.38 these results also suggests that callus growth was not affected by a stepwise decrease of ammonium from 30 to 6.7 millimoles per litre together with a stepwise increase of nitrate from 30 to 53.3 millimoles per litre.

From the results in Table 4.40 and Figure 4.7 it is clear that the addition of an equal number of moles of ammonium to the medium with nitrate as the sole nitrogen source (ammonium:nitrate 0:1 to 1:1) a substantial increase in callus mass was observed. However, at the ratios where more ammonium than nitrate were present in the medium i.e.

Table 4.39 Analysis of variance of the effect of ammonium and nitrate on the growth of shoot callus of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	4404631	1	4404631.0	23.7*
Treatment	69582998	8	8697874.7	46.9*
Residual	14831173	80	185389.6	
Total	88818802	89		

* Indicate significant differences at a 95% level of confidence.

Table 4.40   Multiple range analysis of the effect of ammonium
and nitrate on the growth of shoot callus of
Erythrina caffra.

Treatment	95% Tukey intervals	Homogeneous groups			
$NH_4:NO_3$	Fresh mass (mg)				
1:0	406.2	*			
8:1	1024.6		*		
2:1	2216.7			*	
4:1	2452.5			*	
0:1	2585.6				*
1:8	2580.2				*
1:2	3302.7					*
1:4	3460.4					*
1:1	3468.5					*

*   In different columns indicate significant differences at
a 95% level of confidence.

Figure 4.6   The effect of   decreasing concentrations
of ammonium and increasing concentrations
of nitrate on the growth of shoot derived
callus of *Erythrina caffra*.
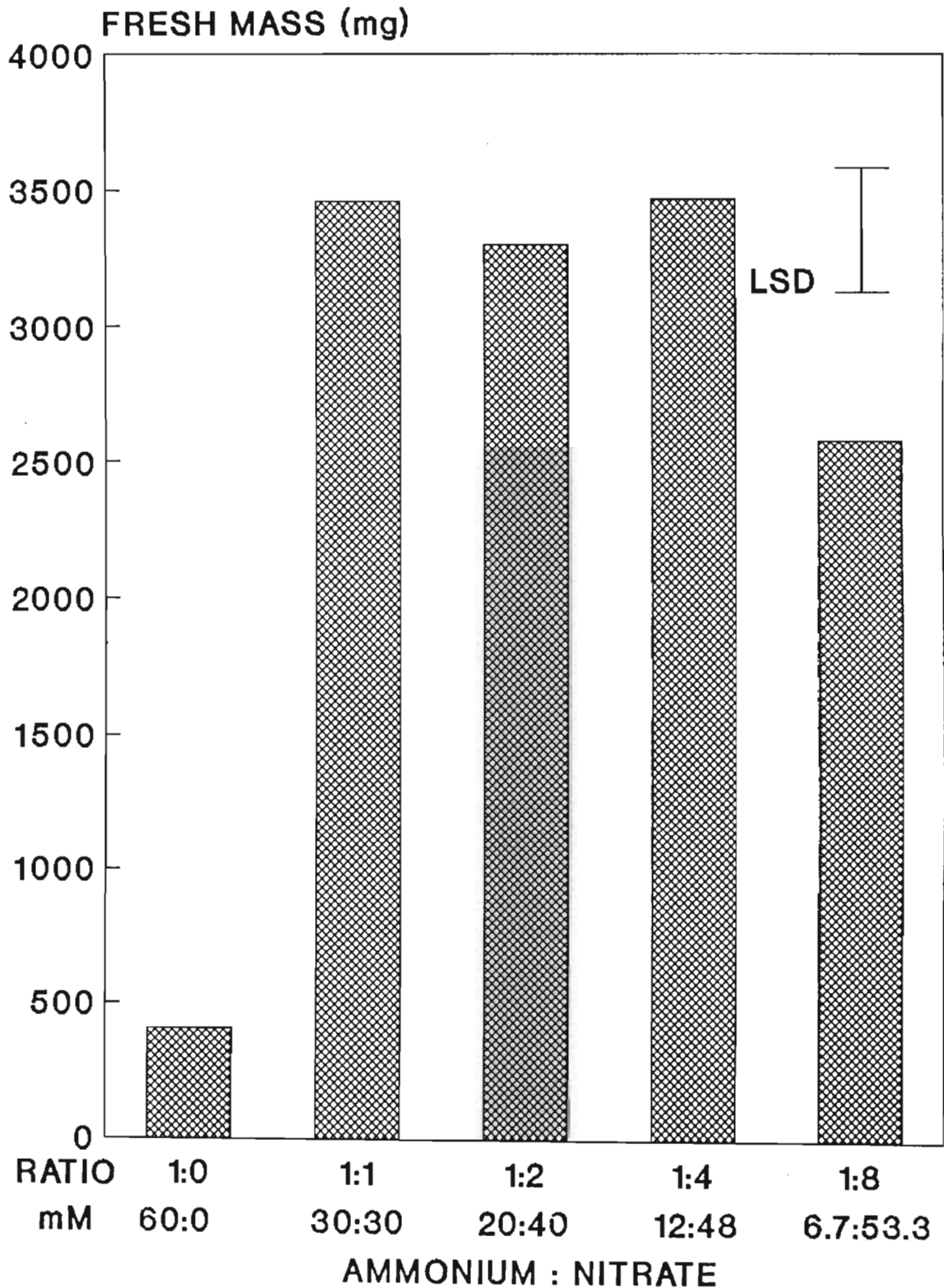
Figure 4.7 The effect of increasing concentrations
of ammonium and decreasing concentrations
of nitrate on the growth of shoot derived
callus of *Erythrina caffra*.
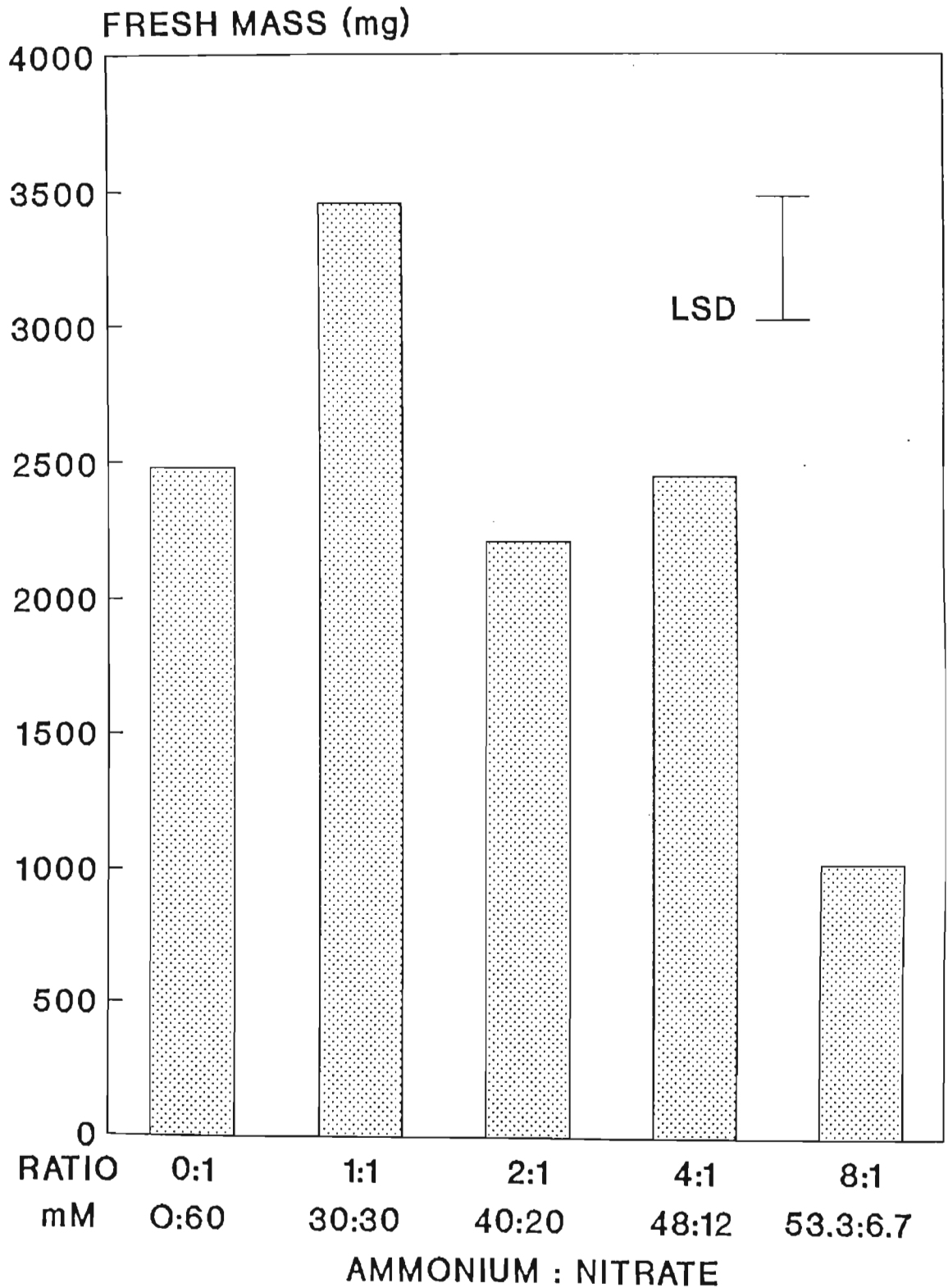
2:1 to 8:1 a significant decrease in callus growth occurred. The decrease in callus growth could have been caused by either the ratio of ammonium to nitrate *per se*; by the absolute increase in ammonium; or by the decrease of nitrate in the medium. It seems as if the increase of ammonium above 30 millimoles per litre or higher molar concentration of ammonium than nitrate reduced the growth of callus. It is therefore clear that callus of *Erythrina caffra* could be grown satisfactorily on the EC medium with the original concentrations of 20 millimoles per litre ammonium and 40 millimoles per litre nitrate.

Both the type and the amount of nitrogen is of great importance in the growth of cultures *in vitro*. It is well established that nitrogen is the primary nutrient limiting the growth of intact plants and that the enzymes involved in nitrogen assimilation is substrate inducible (STEWARD and RHODES, 1977). Earlier results indicated that as with *Erythrina caffra* callus cultures callus growth could not be sustained on a nutrient medium with ammonium as the sole source of nitrogen. However, callus cultures could be grown on nutrient media with nitrate as the only source of nitrogen (NASH and DAVIES, 1972 ; BEHREND and MATELES, 1975 ; JESSUP and FOWLER, 1976). Similar observations were made with callus cultures of *Erythrina caffra*. It has been suggested that the reason for this effect is the pH dependent regulation of nitrate and ammonium uptake by *in vitro* cultures (ROSE and MARTIN, 1975). Ammonium is utilised optimally at a neutral pH and nitrate at a low

pH. Not only does the pH affect the uptake of nitrate and ammonium but the absorption of these ions changes the pH of the nutrient medium itself. The uptake of ammonium from the medium causes a decrease of the pH. In contrast the pH was increased with the uptake of nitrate (GAMBORG and SHYLUK, 1970 ; EEUWENS, 1976). This is as a result of the culture maintaining electric neutrality between the cells and the medium (SIMPKINS and STREET, 1970). It is thus clear that with the uptake of nitrate from the medium the resultant pH makes conditions unfavourable for the continued uptake of nitrate but favoured the uptake of ammonium and *vice versa*. By buffering the pH of the medium with organic acids so as to favour the uptake of ammonium GAMBORG and SHYLUK (1970) demonstrated that cell cultures of *Glycine max*, *Triticum monococcum* L. and *Linum usitatissimum* L. could be grown on ammonium as the sole nitrogen source. These results were supported by studies with *Daucus carota* L. (WETHEREL and DOUGALL, 1976), *Nicotiana tabacum* L., *Lycopersicon esculentum* Mill. (BEHREND and MATELES, 1975) and *Manihot esculenta* Crantz. (PARKE, 1978). The addition of small amounts of ammonium to nutrient media containing nitrate was found to be stimulatory to growth in suspension cultures (WETHERALL, 1978). However, ammonium in the presence of nitrate could also be inhibitory to the growth of cultures as was found with *Helianthus tuberosus* L. (NITSCH and NITSCH, 1956) and *Haplopappus gracilis* L. callus cultures (SARGENT and KING, 1974). Ammonium added to the nitrate containing nutrient medium of callus cultures of *Erythrina caffra* stimulated callus growth at low

concentrations. Ammonium at concentrations lower than the nitrate concentration in combination with nitrate was suitable for the growth of the cultures without any adverse effects being observed. However, the cultures could grow equally well on media from which ammonium was omitted.

4.4.7 THE EFFECT OF SUCROSE ON THE GROWTH OF CALLUS FROM DIFFERENT ORGANS OF *ERYTHRINA CAFFRA*

The effect of sucrose on the growth of callus derived from leaf, shoot and cotyledonary explants was investigated. Sucrose was added at concentrations of 1, 2, 3, 4 and 5 % to the EC medium. The medium was then autoclaved at 121 ^{o}C for 20 minutes. Callus derived from leaf, shoot and cotyledonary explants grown on EC medium were then placed on the medium and incubated under the standard conditions described before. The initial fresh mass of the calli was determined before it was placed on the medium and the final fresh mass of the calli was recorded after 30 days of incubation.

RESULTS AND DISCUSSION

Substantial differences were obtained for the different treatments based on callus fresh mass (Table 4.41). It is clear from the sucrose main effects that callus grew significaantly better on the medium with 3 % sucrose than on the media with the other concentrations of sucrose (Table

Table 4.41 Analysis of variance of the effect of sucrose on
the growth of callus derived from different
organs of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial				
fresh mass	480146	1	480146	21.6*
Main effects				
Organ	3960268	2	1980134	89.1*
Sucrose	4037432	4	1009358	45.4*
Interaction				
Sucrose X Organ	1419782	8	177348	8.0*
Residual	333435	15	22229	
Total	10230065	30		

* Indicate significant differences at a 95% level of
confidence.

4.42). The growth of shoot callus was superior to the growth of callus derived from root and cotyledonary material irrespective of the sucrose concentration in the nutrient medium (Table 4.43). Considering the effect of sucrose on the growth of cotyledonary callus it can be seen that the cotyledonary callus did not grow well on the media with 1 % and 2 % sucrose. It grew the best on 3 % sucrose. At higher concentrations of sucrose there was a gradual but significant decrease in growth. The growth of leaf and shoot callus followed a similar trend with maximum callus being produced at 3 % sucrose (Table 4.44 and Figure 4.8). The significant interaction found in the analysis of variance in Table 4.41 was caused by a small overlap of the fresh mass value of cotyledonary callus with the leaf value at 2 % sucrose. It was thus not interpreted as being caused by a difference in the metabolism of the different types of calli. Since the growth of callus derived from different organs of *Erythrina caffra* was optimal with 3 % sucrose in the nutrient medium this amount of sucrose was routinely used in EC medium.

The most effective sources of energy and carbon skeletons for non-photosynthesising callus and suspension cultures are sucrose, glucose or fructose at concentrations ranging from 2 % to 3 %. However, in most instances sucrose was found to be superior to glucose and fructose (OJIMA and OHIRA, 1978). The type or concentration of the carbon source preferred by *in vitro* cultures is not determined by the species only but can vary between clones from the same

Table 4.42   Multiple range analysis of the main effects of
             sucrose.

Treatment	95% Tukey intervals	Homogeneous groups		
Concentration (%)	Fresh mass (mg)			
1	718.3	*		
2	894.3	*	*	
5	962.8	*	*	
4	1037.5		*	
3	1781.5			*

*   In different columns indicate significant differences at
    a 95% level of confidence.

Table 4.43   Multiple range analysis of the main effects of
             the plant organs.

Treatment	95% Tukey intervals	Homogenous groups	
Explant	Fresh mass (mg)		
Cotyledon	786.4	*	
Leaf	859.3	*	
Shoot	1591.0		*

*   In different columns indicate significant differences at
    a 95% level of confidence.

Table 4.44 Multiple range analysis of the effect of sucrose
on the growth of callus derived from different
organs of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups			
Organ and sucrose	Fresh mass (mg)				
Cotyledon					
Sucrose (%)					
1	553.5	*			
5	617.0	*	*		
2	656.5	*	*		
4	851.4		*		
3	1254.3			*	
- - - - - - - - - - -					
Leaf					
Sucrose (%)					
1	335.2	*			
2	652.0		*		
5	956.5			*	
4	1049.5			*	*
3	1303.0				*

* In different columns indicate significant differences at
a 95% level of confidence.

Table 4.44 Continued.

Treatment	Treatment mean	Homogeneous groups
Organ and sucrose	Fresh mass (mg)	
Shoot		
Sucrose (%)		
1	1163.2	*
5	1222.1	*
2	1305.0	*
4	1477.0	*
3	2787.6	*

* In different columns indicate significant differences at

   a 95% level of confidence.

Figure 4.8   The effect of different concentrations of
            sucrose on the growth of callus derived
            from cotyledonary,   shoot   and   leaf
            explants of *Erythrina caffra*.
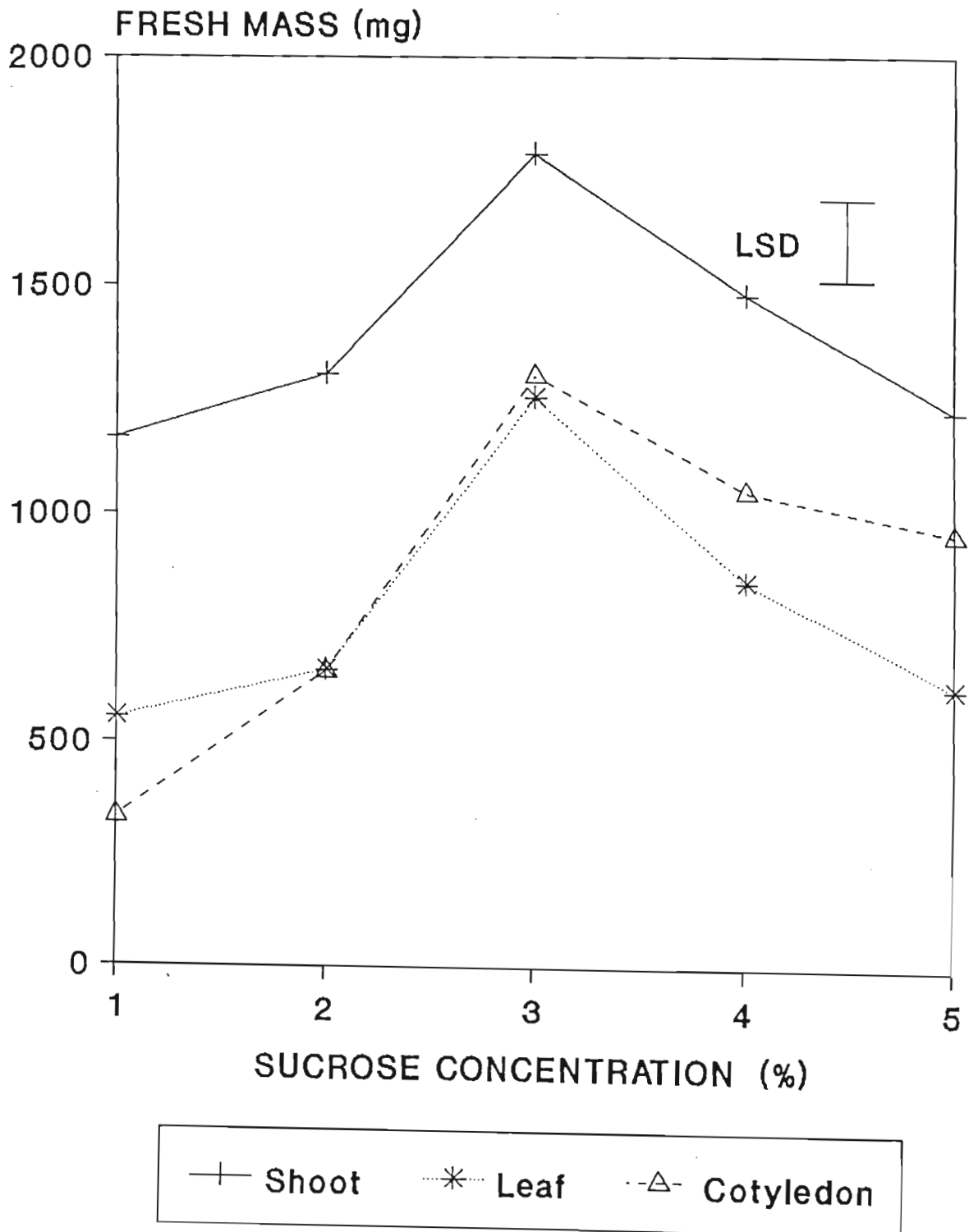
species (DAMIANO, CURIR and COSMI, 1987). This makes it difficult to generalise within a species about the optimal concentration of carbohydrates for a culture *in vitro*. Depending on the species, exceptions have been reported where growth of callus or suspension cultures were supported on other carbon sources. Glucose was for example preferred to sucrose by eight *Alnus* species (CREMIERE, SBAY and PRATT, 1987) and could replace sucrose in suspension cultures of *Acer pseudoplatanus* (SIMPKINS, COLLIN and STREET, 1970). Fructose was as good as sucrose for the culture of *Acer pseudoplatanus* (SIMPKINS, COLLIN and STREET, 1970) and even better than sucrose for the growth of *Castanea sativa* Mill. cultivars (CHAUVIN and SALESSES, 1980). Callus cultures of *Cucumis sativus* L. cultivar GY-3 (GROSS, PHARR and LOCY, 1981) and suspension cultures of *Acer pseudoplatanus* (SIMPKINS, COLLIN and STREET, 1970) was supported by galactose which was just as effective as sucrose as a carbon source. A suspension of *Acer pseudoplatanus* cells could utilise mannose (WRIGHT and NORTHCOTE, 1972) while maltose supported the growth of callus from *Pinus* species (KONAR, 1974), a selected suspension culture of *Glycine max* cv. Mandarin (LIMBERG, CRESS and LARK, 1979), suspension cultures of *Acer pseudoplatanus* (SIMPKINS, COLLIN and STREET, 1970) and cultures of two *Acer* species (MATHES, MORSELLI and MARVIN, 1973). Other sugars which sustained the growth of callus and suspension cultures were cellobiose or trehalose for *Acer* species, Paul's scarlet rose and members of the *Rosaceae* (WRIGHT and NORTHCOTE, 1972 ; MATHES, MORSELLI and

MARVIN, 1973 ; COFFIN, TAPER and CHONG, 1976), lactose
(HESS, LEIPOLDT and ILLG, 1979 ; MITCHELL, JOHNSON and
WHITTLE, 1980), melibiose for *Cucumis sativus* and *Saccharum
officinarum* L. (NICKELL and MARETZKI, 1970 ; GROSS, PHARR
and LOCY, 1981). Raffinose sustained growth of *Cucumis
sativus*, *Acer pseudoplatanus*, *Nicotiana tabacum* and *Hevea
braziliensis* cultures (WRIGHT and NORTHCOTE, 1972 ; THORPE
and LAISHLEY, 1974 ; GROSS, PHARR and LOCY, 1981 ;
PARANJOTHY, 1987). *Daucus carota* L. and *Cucumis sativus* can
be grown on stachyose (VERMA and DOUGALL, 1977 ; GROSS,
PHARR and LOCY, 1981). Ribose can be used for the growth of
sugar cane and tomato suspension and callus cultures
(NICKELL and MARETZKI, 1970 ; ALLSOP, LOCY and OCKLE, 1981).
Starch was found to support the growth of sugar cane
(NICKELL and MARETZKI, 1970). It was suggested that the
cultures were able to hydrolyse the starch in the suspension
medium and then utilised the resultant sugars. The sugar
alcohol sorbitol was utilised by the Pumula apple cultivar
to the same extent as sucrose (CHONG AND TAPER, 1974) and
also by several other genera of the *Rosaceae* (COFFIN, TAPER
and CHONG, 1976). Meso-inositol is required by many
cultures but it was neither essential nor could it be used
as the sole source of carbon (MURASHIGE and SKOOG, 1962 ;
WOLTER and SKOOG, 1966). However, a callus culture of
Fraxinus pennsylvanica Marsh. could utilise mannitol as the
only carbon source (WOLTER and SKOOG, 1966). Glycerol could
sustain the growth of *in vitro* cultured callus and cells of
Nicotiana tabacum, *Daucus carota*, *Cannabis sativa* L. and an
Ipomoea species but only when the cells were mutants or

selected to grow on glycerol (CHALEF and PARSONS, 1978 ; JONES and VELIKY, 1980). However, some suspension cultures grew better on sucrose than on other sources of carbon. Many of the cultures which grew on a carbon source other than sucrose were selected lines or mutants. Many of the sugars and sugar alcohols detailed before are not essential for the growth of the cultures. Many of them seem to be essential for the growth of protoplast cultures. This can be deducted from the formulation of protoplast culture nutrient media (KAO and MICHAYLUK, 1975 ; DAVID and DAVID, 1979 ; LARKIN, 1982 ; CARLBERG, GLIMELIUS and ERIKSSON, 1983).

Some callus and suspension cultures grown *in vitro* seem to utilise sucrose at a very specific concentration. It was observed that maximal growth was achieved by cultures of *Zea mays* L. at 2 % sucrose in the nutrient medium with a sharp decline in growth at 1 % and 4 % sucrose (STRAUS and LA RUE, 1954). Similar observations were made with cultures of *Syringa vulgaris* L. which grew optimally at a sucrose concentration of 3.5 % with a sharp decline in growth at 3 % and 4 % sucrose. (PIERIK, STEEGMANS, ELIAS, STIEKEMA and VAN DER VELDE, 1988). PHILLIPS and COLLINS (1979) found similar trends with red clover and concluded that sucrose at a concentration of 2.5 % stimulated callus growth substantially more than at 1.5 % or 3.5 %. Similar observations were made on the callus cultures of *Erythrina caffra* in this study where a sharp decline in callus growth occurred at sub-optimal sucrose concentrations. However, *in*

vitro cultures can grow well on a wide concentration range of sucrose. It was reported that *Eucalyptus gunnii* Hook. grew optimally on 1 % to 4 % sucrose (DAMIANO, CURIR and COSMI, 1987) and maximum growth was obtained with *in vitro* cultures of various *Magnolia* hybrids with 0.5 % to 2 % sucrose (BIEDERMAN, 1987).

4.4.8 THE EFFECT OF TEMPERATURE ON THE GROWTH OF SHOOT CALLUS OF *ERYTHRINA CAFFRA*

Ambient temperatures of 17 ^{o}C to 32 ^{o}C are normally considered as optimal for the growth of plant cultures *in vitro* (GEORGE and SHERRINGTON, 1987). Shoot derived callus of *Erythrina caffra* was incubated at different temperatures to determine to what extent temperature would affect its growth. The fresh mass of the callus explants was determined after which the calli were placed onto EC medium. The nutrient medium was prepared as described before. The cultures were incubated at 15, 20, 25 and 30 ^{o}C under a quantum flux (400-700 nm) of 50 µE m^{-2} sec^{-1}. After 20 days of culture the fresh mass of the calli was determined. The experimemt was a completely randomised design and analysed accordingly. The initial fresh mass was used as a covariate.

RESULTS AND DISCUSSION

Temperature had a significant effect on the growth of the callus from *Erythrina caffra* (Table 4.45). Callus growth was severely inhibited at 15 ^{o}C and 30 ^{o}C (Table 4.46). The temperature for maximum callus growth was 25 ^{o}C. From Figure 4.9 it is clear that callus growth increased with an increase in temperature up to 25 ^{o}C whereafter it declined.

As mentioned before most *in vitro* cultures are maintained at a temperature of 17 ^{o}C to 30 ^{o}C. However, most plants grow well in tissue culture at 25 ^{o}C (GEORGE and SHERRINGTON, 1987). The optimum temperature for callus growth *in vitro* seems to be a characteristic of the plant. For example *Picea abies* callus grew the best at 25 ^{o}C with a substantial decrease in growth at 20 ^{o}C and 30 ^{o}C CHALUPA and DURZAN, 1973). Similar results have been reported for *Pinus banksiana* Lamb. callus cultures (CHALUPA, 1987). Callus from *Hevea braziliensis* grew optimally at a high temperature of 30 ^{o}C (WILSON and STREET, 1975) and *Castanea* species produced increasing amounts of callus with an increase in temperature from 11 ^{o}C to 33 ^{o}C (CHAUVIN and SALESSES, 1988). Callus from *Prosopis alba* Chev. also grew better at a higher temperature of 30 ^{o}C than at 22 ^{o}C (JORDAN, 1987). The best temperature for the growth of *Syringa vulgaris* L. callus was much lower at 21 ^{o}C with higher temperatures inhibitory to callus growth (PIERIK, STEEGMANS, ELIAS, STIEKEMA and VAN DER VELDE, 1988). Callus from plants which

Table 4.45 Analysis of variance of the effect of temperature

on the growth of shoot callus of *Erythrina*

caffra.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Covariate				
Initial fresh mass	1034383	1	1034383.0	17.2*
Temperature	120762036	3	40254012.0	671.0*
Residual	2099659	35	59990.3	
Total	1238096078	39		

* Indicate significant differences at a 95% level of

confidence.

Table 4.46 Multiple range analysis of the effect of

temperature on the growth of shoot callus of

Erythrina caffra.

Treatment	95% Tukey intervals	Homogeneous groups		
Temperature (^{O}C)	Fresh mass (mg)			
15	184.8	*		
30	1722.8		*	
20	2899.6			*
25	4959.2			*

* In different columns indicate significant differences at

a 95% level of confidence.

Figure 4.9 The   effect of temperature on the growth
           of callus derived from shoot explants of
           Erythrina caffra.
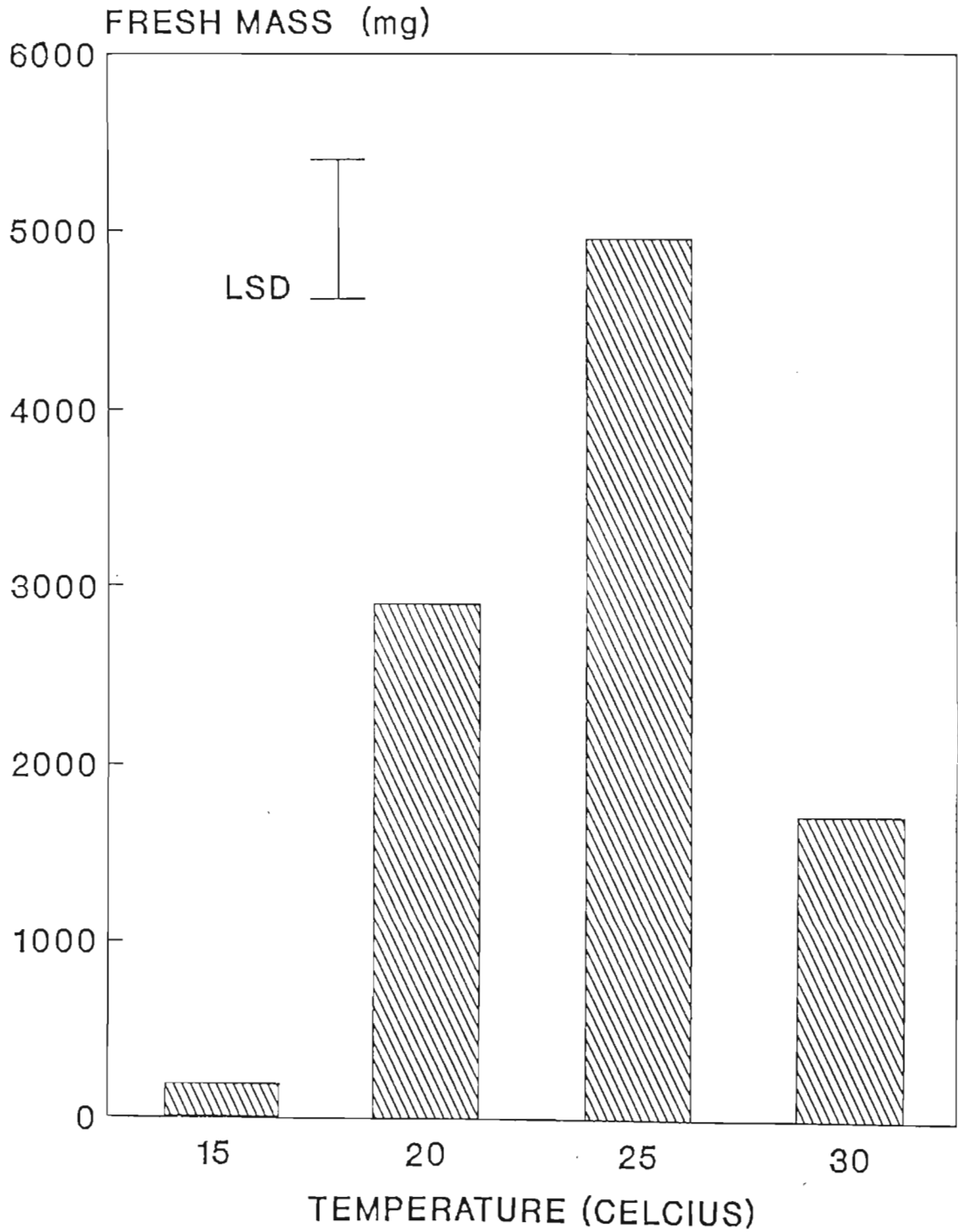
grow in sub-arctic environments such as members of the genera *Picea*, *Pinus* and *Betula* normally grew optimally at lower temperatures of 22 $^{\circ}$C to 25 $^{\circ}$C (CHALUPA, 1987). Similar observations were made for the callus growth of *Vitis vinifera* L. which increased in growth with a decrease in temperature from 27 $^{\circ}$C to 22 $^{\circ}$C (JONA and VALLANIA, 1980). In some cases it was found that alternating temperatures were more beneficial to callus growth than constant temperatures. Examples of such callus cultures include *Alnus glutinosa* Gaertn., *Betula pendula* Roth., *Salix alba* L. (CHALUPA, 1983), *Castanea sativa* (VIEITEZ and VIEITEZ, 1983) and *Quercus robur* L. (CHALUPA, 1984). It thus seems clear that the optimal temperature for the growth of callus is species specific and also related to the natural habitat of the plant.

4.5 CONCLUSIONS

Different types of cytokinins and auxins had similar effects on the induction and growth of callus derived from *Erythrina caffra* shoots. Callus derived from shoot tissue grew optimally within the concentration range of 5 to 10 micromoles per litre for the cytokinins BA and Kinetin and 1 to 5 micromoles per litre for the auxins 2,4-D, NAA, IBA and IAA used. Differences were observed in the growth rate of callus from different organs and from similar organs from different plants. This suggests that with *Erythrina caffra* the physiological differences between plants and

between organs within a plant is of greater importance for the growth of callus than the type of growth regulator. The growth of callus from different organs was not affected by different nutrient media. They grew equally well on MS, SH and B5 medium despite large differences in the concentration of nutrients of the three media. This suggests that callus from *Erythrina caffra* can tolerate a wide concentration range of nutrients without affecting its growth. However, the source of nitrogen and the ammonium:nitrate ratio in the nutrient medium is critical. Shoot callus grew well on a nutrient medium with nitrate only or supplemented with ammonium up to a 1:1 ratio. More ammonium than nitrate in the nutrient medium was detrimental to the growth of the callus. Callus derived from different organs were very sensitive to the concentration of sucrose in the nutrient medium. Three percent sucrose was optimal for the growth of callus from all organs. The growth of callus was significantly affected by temperature. The optimal temperature for callus growth was 25 °C.

It can be concluded that callus derived from root, shoot, leaf and cotyledonary tissue of *Erythrina caffra* could be grown satisfactorily on EC medium containing the micro- and macro nutrients and vitamins of MURASHIGE and SKOOG (1962). These components were supplemented with 3 % sucrose, 10 micromoles per litre of BA and 5 micromoles per litre of 2,4-D for optimum growth of callus.

CHAPTER 5

SUSPENSION CULTURE OF SHOOT TISSUE OF

ERYTHRINA CAFFRA.

5.1 INTRODUCTION

Suspension cultures are commonly seen as a progression from

a callus culture to the dispersion of callus into a liquid

medium with the final step the formulation of an optimal

nutrient medium and physical environmental conditions which

promotes cell separation. This is however, an over

simplification of the situation. Cell separation is enhanced

to a degree by the nutrient medium but KING (1980) stated

that the actual origin of a particular type of growth *in

vitro* is linked to a physiological event taking place during

the early phases of explant exposure to the nutrient medium.

The frequency of this event is governed by the explant

rather than the nutrient medium. As with the morphogenic

response of an explant callus friability is a characteristic

of the explant. This is a feature of the metabolic state of

the explant not initially determined by the chemical and

physical environment *in vitro*. One of the most important

features of a suspension culture is to obtain a friable

callus culture for successful cell separation. One way to

obtain a friable callus is by selection of a friable cell line in a callus supporting nutrient medium. It has been determined that friable cell lines have a specific set of characteristics. KING (1980) found that friable cell lines from several members of the Solanaceae have characteristics such as homogeneous cell type, isodiametric cells, highly cytoplasmic cells, distinct nuclei and prominent starch grains in the cells and limited cytodifferentiation with no apparent morphogenic capacity. The cultures were also auxotophic for several metabolites and became easily auxotrophic to growth regulators. They show growth promotion by elevated carbon dioxide levels and were susceptible to ethylene. The cultures in most cases had abnormal ploidy levels. Although all of these characteristics would not be found in friable cell lines from all plant families many would probably be a general feature of friable callus. As with callus the cells in a cell suspension consists of a highly heterogeneous population of cells which could lead to the development of new cell lines in the cell·suspension.

Initially suspension cultures were maintained in batch culture systems but the uneven growth and difficulty in controlling the metabolic behaviour of these suspension cultures led to the development of chemostat and turbidostat systems for plant suspension cultures. (MARTIN, 1980 ; FOWLER, 1982). Much more control can be exercised over the growth and physiological condition of a suspension culture in chemostat and turbidostat systems since the cells are continually subjected to an unchanged nutrient medium.

These systems were reviewed in detail by KING (1980), KING and STREET (1977), MARTIN (1980) and FOWLER (1982).

5.1.2 GROWTH PHASES OF A CELL SUSPENSION CULTURE

As with microbial cultures different phases in the growth of a plant cell suspension culture can be distinguished. The growth phases characterise the different stages of growth of a suspension culture and are correlated with the onset and culmination of physiological and biochemical events in the cells. Different growth phases can be distinguished in batch culture. The ideal growth curve and the different growth phases of a plant cell suspension culture is illustrated in Figure 5.1 (KING and STREET, 1977).

5.1.2.1 THE LAG PHASE

Upon the transfer of a cell culture in its exponential growth phase to a fresh medium in a batch culture it enters the lag phase (Figure 5.1). This is a period of no cell division or cell growth. However, a number of metabolic changes are initiated which seem to prepare the cells for cell division. Energy rich molecules such as ATP and NADPH are being produced. It was reported that at the onset of the lag phase the activity of the glycolytic pathway and the pentose phosphate pathway increased with a concomitant

Figure 5.1 The ideal sigmoidal growth curve and the different growth phases for plant cell suspension cultures (STREET, 1977).
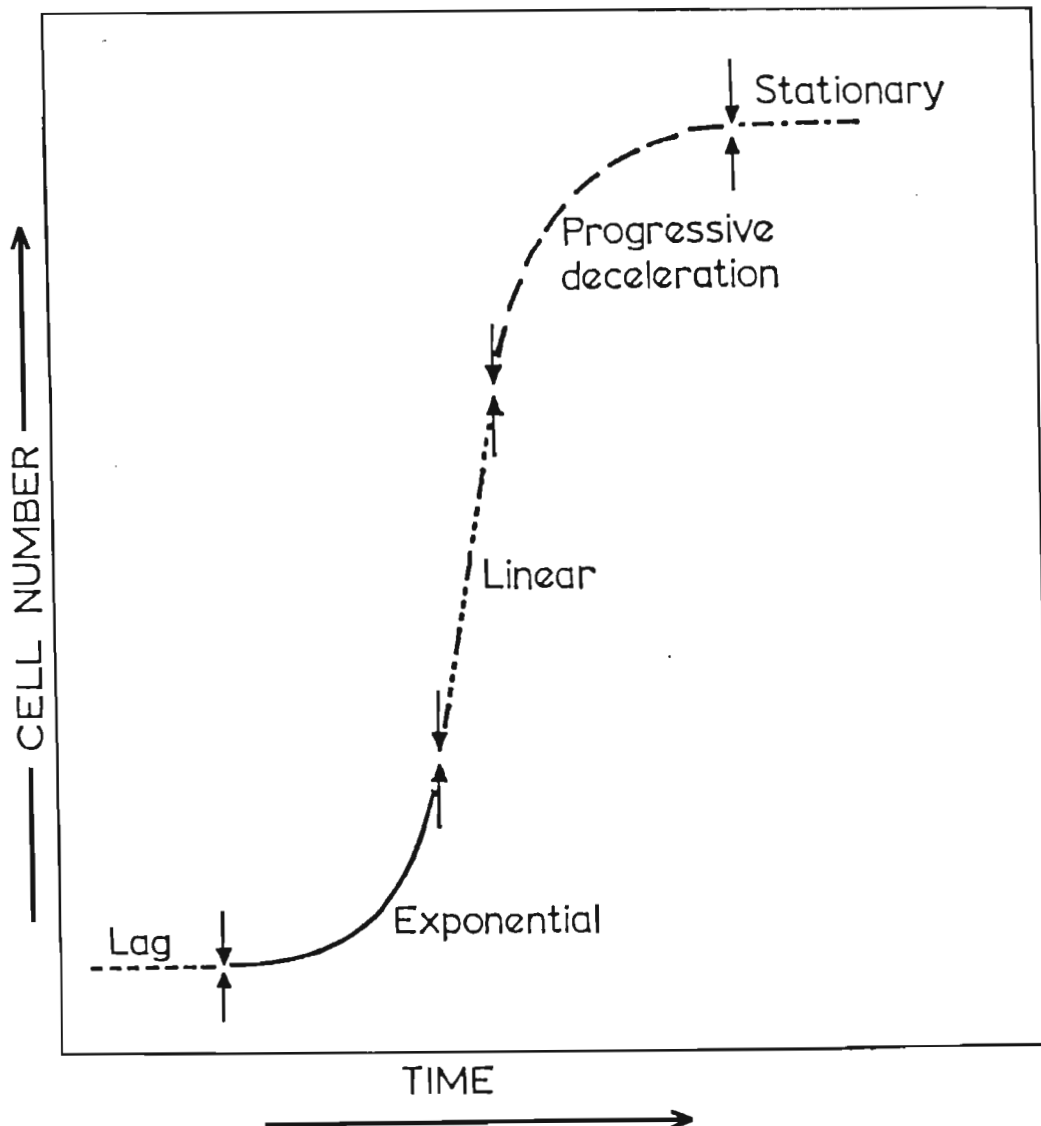
increase in the levels of ATP and NADPH (FOWLER, 1971 ;
SHIMIZU, CLIFTON, KOMAMINE and FOWLER, 1977). Increases in
the RNA content of cell suspension cultures in the lag
phase has been observed (KANAMORI, ASHIHARA and KOMAMINE,
1979). Polysome formation was stimulated (BEVAN and
NORTHCOTE, 1981a) and the translation capacity of the
ribosomes was found to be more efficient (VERMA and MARCUS,
1974). These activities in the cells are indicative of the
preparation of the cells for protein synthesis. Consequently
it has been established that the rate of total protein
synthesis increased during the lag phase (VERMA and MARCUS,
1974).

5.1.2.2 THE CELL DIVISION PHASE

During the cell division phase a decrease in cell size
as well as an increase in cell number and especially
isodiametric cells was reported to occur. These events were
accompanied by increases in the nucleic acid content,
nitrogen levels and respiration rate of the cells (LINDSAY
and YEOMAN, 1985). The cell division phase illustrated in
Figure 5.1 (KING and STREET, 1977) can be subdivided into
an initial exponential phase of fast cell division with an
exponential increase in the cell number, cell mass and cell
volume followed by a linear phase with a linear increase in
the growth parameters.

5.1.2.3 THE STATIONARY PHASE

During the stationary phase a cessation of cell division was observed (Figure 5.1) which coincided with a decline in RNA synthesis and a decline in the respiration rate. The activities of the key enzymes of the pentose phosphate pathway also decreased (KHAVKIN and VARAKINA, 1981). These metabolic changes was followed by the structural organisation of the cells to permit the initiation of meristematic regions and the production and accumulation of secondary products (THOMAS and STREET, 1970 ; KING, 1980 ; LINDSEY and YEOMAN, 1983). An increase in total protein was observed during the stationary phase which was the result of an increase of several enzymes involved in the biosynthesis of secondary products such as polyphenol oxidase (FORREST, 1969), phenylalanineammonia lyase, B-1,3-glucan synthetase (HADDON and NORTHCOTE, 1976 ; WESTCOTT and HENSHAW, 1976) and cinnamic acid hydrolase (EBEL, SCHALLER-HECKELER, KNOBLOCH, WELLMAN, GRISEBACH and HAHLBROCK, 1974). Cell aggregation was observed to decrease during the stationary phase (KING, 1980). Hence, it is clear that during each growth phase in a batch culture the cells are in a different state of metabolism and organisation.

5.2 STANDARD INCUBATION CONDITIONS FOR THE CELL SUSPENSION CULTURE

The cell suspensions were incubated in standard 250 millilitre Erlenmeyer flasks with a tubular side arm. The flasks were stoppered with cotton wool and capped with tin foil. The suspension cultures were incubated on an orbital shaker at 60 rpm. The cell suspensions were kept at standard conditions of 25 $^{\circ}$C and a quantum flux density of 50 to 100 μE m^{-2} sec^{-1}. Stock suspension cultures were subcultured every 10 days and grown under these conditions.

5.3 ESTABLISHMENT OF A STOCK CULTURE OF SHOOT CALLUS

Callus derived from shoot tissue of *Erythrina caffra* was used to establish a suspension culture. A friable callus was obtained through selection. It was found that callus lines grown on the EC medium described in Chapter 4 produced friable callus which retained its friability on this medium for several subcultures. The nutrient medium initially used for the suspension culture was the EC medium without the gelling agent. As the medium was modified through experimentation the stock suspension culture medium was adjusted to improve the growth of the cell suspension culture. A stock culture was initiated by inoculating a volume of 100 millilitres of medium with 4 to 5 grammes of friable callus. The suspension was incubated in the

standard culture flask under the environmental conditions as described in section 5.2. After the establishment of a suspension culture the clumps of callus and large aggregates were separated from the small aggregates by differential sedimentation of the aggregates in a measuring cylinder. The small aggregates were decanted and diluted with three aliquots of fresh medium and cultured as described in section 5.2.

5.4 PREPARATION OF THE INOCULUM

The preparation of the inoculum to be used in experiments was done as follows. Thirty millilitre aliquots of a 100 millilitre stock culture in its exponential growth phase were vacuum filtered aseptically and washed three times in three aliquots of 30 millilitres of sterile distilled water. The inoculum was then transferred aseptically to a standard culture flask containing 100 millilitres experimental suspension medium. The suspension culture was incubated under the standard environmental conditions or under the experimental conditions described.

In some of the experiments three replicates were used per treatment provided that an adequate volume of cell suspension stock could be raised to use one stock culture for all the treatments in the experiment. In cases where sufficient amounts of suspension culture could not be obtained for an experiment the experiment was repeated three

times with different stock cultures. The treatment means and standard errors of the means of the three experiments were then used to establish trends in the growth of the suspension cultures.

5.5 SAMPLING OF THE SUSPENSION CULTURE

Suspension cultures were sampled by swirling the suspension culture flask three times in one direction and once in the opposite direction. While the cells were in suspension a sample of 1 to 5 millilitres were taken from the suspension with a spring loaded pipette. Pipette tips were cut to a final orifice diameter of 3 to 4 millimetres to prevent blocking of the tip by large aggregates. The sample was transferred to a vial for further processing. Sampling was done under aseptic conditions.

5.6 PARAMETERS USED FOR THE DETERMINATION OF SUSPENSION CULTURE GROWTH

The following parameters were determined for the suspension cultures: viability, aggregate density, settled cell volume, cell fresh and dry mass and aggregate size. The viability of a suspension culture was determined with fluorescein diacetate (Sigma) according to the method of WIDHOLM (1972). A stock solution of one gramme per litre fluorescein diacetate was made up in analar grade acetone (Merck) and

stored in the dark at 10 $^{\circ}$C. One drop of stock solution was added to a final volume of 10 to 20 millilitres of suspension in a 30 millilitre vial. Fluorescein diacetate is a non-fluorescing, non-polar molecule which can move freely across cell membranes. Fluorescein, the product of esterase activity is polar and cannot move freely across membranes (LARKIN, 1976). The fluorescing fluorescein accumulates in the cell and is a measure of metabolic activity and membrane integrity of the cell. Brightly fluorescing cells were considered to be alive (Plate 5.1 and 5.2). Fluorescence was obtained with an ultra violet mercury lamp and the proper barrier and excitation filters as prescribed by Zeiss.

The aggregate density was determined by taking a one millilitre sample of the suspension as was described before. The suspension was diluted 10 to 20 times with distilled water and fluorescein diacetate was added to the sample. The sample was swirled and left for 5 minutes for the reaction to take place. The cell suspension was then swirled and with the aggregates and cells properly suspended a volume of one millilitre suspension was pipetted with a Pasteur pipette onto a Sedgewick counting cell (Graticules Ltd.). Six hundred of the thousand 1 mm^2 units on the counting cell were counted. The live and total number of aggregates were counted. The procedure was repeated three times for each sample. The mean of three counts was used to determine the cell density and viability. Only a small fraction of the total suspension consisted of single cells.

Plate 5.1   Green fluorescing cells   of   a   shoot
cell suspension culture of *Erythrina*
caffra. The cells were stained with
fluorescein diacetate.
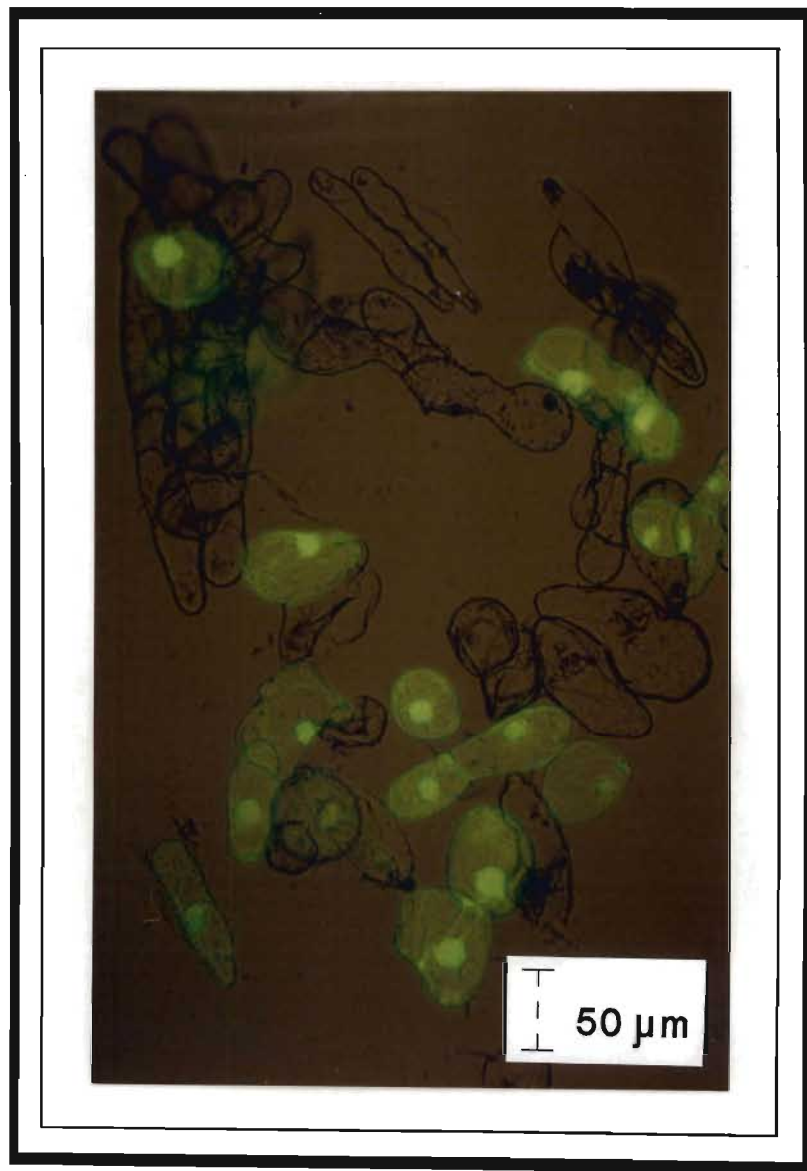
Plate 5.2   A shoot cell   suspension   culture   of
            Erythrina   caffra   stained   with
            fluorescein   diacetate.   The   green
            fluorescing   cells   are   alive   and   the
            non-fluorescing cells are dead.

These cells were mainly non-dividing cells in the stationary growth phase transferred from the stock suspension culture. Since these made no contribution to the growth of the suspension culture they were not counted. Only aggregates were counted to determine the growth of the suspension culture. An aggregate was defined as a group of five or more cells adhering to each other. The diameter of the aggregates was determined as the mean of two diameter readings taken perpendicular to each other with an eyepiece micrometer. Aggregates were then classified according to their mean diameter as less than 0.25, 0.26 to 0.5 , 0.51 to 1.0 and larger than 1 millimetre in diameter.

The settled cell volume was determined using flasks fitted with a side arm attached to each culture flask. The culture flask was swirled and the side arm filled with the suspension. After 5 minutes the length of the settled volume of cells was determined. The length was related to the volume of the side arm. This procedure was repeated until three readings were obtained within at most 5 millimetres from each other. The mean and standard error of the values were determined.

The fresh mass was determined by combining three 2 millilitre aliquots of a suspension sampled as described before. The sample was vacuum filtered on Whatman No 1 filter paper. The filter paper with the sample was then placed on paper toweling. The cells were removed from the filter paper and weighed. The dry mass of a culture was

determined by washing the cultures with 100 millilitres of acetone followed by a wash in 50 millilitres ethyl ether. The sample was weighed after the ethyl ether had evaporated from the cells. This procedure was followed to determine the dry mass of the cells since the acetone dry mass was used with the determination of protein and t-PA inhibitor.

In some instances it was found from the initial measurement of a parameter that large differences occurred in the initial values of the parameter between treatments. To correct the final values for this discrepancy the growth efficiency for the parameter was determined. The formula used for the calculation of the growth efficiency was :

GROWTH EFFICIENCY = (F - I) / I

Where I = Initial value of the parameter

F = Final value of the parameter.

Any parameter used to determine the growth of a suspension culture was measured at the onset of the experiment and at various times as outlined when applicable.

5.7 ANALYSIS OF DATA

Experiments with replications were analysed as described in Chapter 4. However, for experiments that were repeated at different times the mean and the standard error for the data was determined. This was also done for experiments where the result was a function of time such as the determination of the growth curve. The standard error of the means was included in the graphs as a measure of the variability. Correlation coefficients were determined between the different parameters used to construct the growth curve for the suspension culture.

5.8 EXPERIMENTAL

Before describing the experiments it must be mentioned that the cell suspension did not grow under the culture conditions used initially. By trial and error a large number of preliminary experiments were done. These included determination of the effect of different nutrient media, addition of organic factors to the medium, altering the growth regulator quality and quantity, different temperature and light regimes, different explant material and shaking speeds. No results were obtained from most of these experiments until the critical factor responsible for the early death of the cell suspension cultures was determined. These preliminary experiments will not be discussed here. Attention is only given to experiments conducted after the

establishment of a viable suspension culture. The suspension cultures used in the experiments were obtained from shoot callus of *Erythrina caffra*.

5.8.1 THE EFFECT OF SHAKING SPEED ON THE GROWTH AND VIABILITY OF SUSPENSION CULTURES

A shoot suspension culture of *Erythrina caffra* was prepared as described in sections 5.2 to 5.4. Thirty millilitre aliquots of the cell suspension from one stock solution were mixed with 60 millitres fresh EC medium in 500 millilitre Erlenmeyer flasks. The cell suspension cultures were incubated at 60, 90 and 120 rpm under the standard incubation conditions. Three replications were used per treatment. After 14 days of culture the viability, aggregate density and aggregate diameter were determined. The experiment was a complete randomised design and the data was analysed accordingly.

RESULTS AND DISCUSSION

The shaking speed had a significant effect on the viability and the growth of the suspension cultures. The viability of the cultured cells decreased drastically with an increase in the shaking speed from 60 to 120 rpm (Figure 5.2). This decrease in viability could be due to the mechanical damage afflicted to the cells resulting from shearing stresses at

Figure 5.2 The effect of the shaking speed on the viability of shoot cell suspension cultures of *Erythrina caffra* after 14 days of culture.
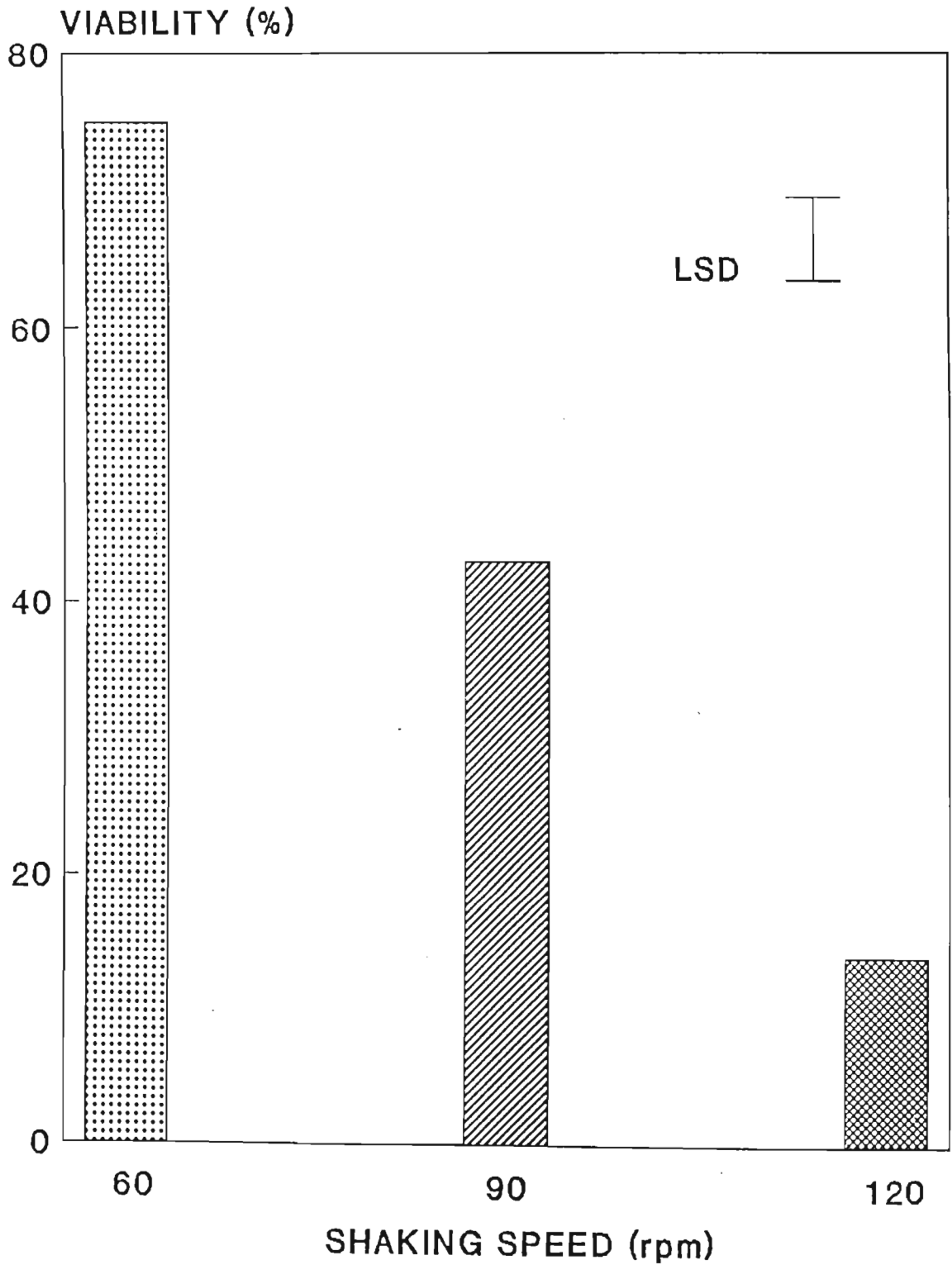
higher shaking speeds. Such damage could lead to the rupture of young cells and probably also the leaking of essential metabolites from the cells. The destructive effect of the high shake speed on the growth of the cell suspensions is evident from Figure 5.3. The fresh mass and aggregate density of the cell suspension culture were substantially lower at a shake speed of 120 rpm than at 60 or 90 rpm.

A decrease in the shake speed can result in the formation of undesirable large aggregates. The ultimate goal of a suspension culture is the production of a suspension with the smallest possible aggregate size. This will ensure that each cell is subjected optimally to the environmental conditions and has an equal opportunity with respect to the uptake of nutrients and other substances from the nutrient medium. With the shoot cell suspension culture of *Erythrina caffra* a large number of small aggregates formed at a shaking speed of 120 rpm and less aggregates of larger size were formed than at 60 or 90 rpm (Figure 5.4). The absence of aggregates larger than 0.5 millimetres in diameter at a shake speed of 120 rpm suggests that the rate of cell separation from the aggregates was so high that the growing aggregates could not attain a larger size. The size distribution of aggregates was very similar at shaking speeds of 60 and 90 rpm. However, more aggregates in the 0.25 to 0.5 millimetre diameter range were observed at 60 rpm than at 90 rpm. Even at a low shaking speed of 60 rpm most aggregates were in the smaller size classes of up to 0.5 millimetre. The decrease in aggregate size with an

Figure 5.3 The effect of shaking speed on the fresh mass and aggregate density of shoot cell suspension cultures of *Erythrina caffra* after 14 days of incubation.
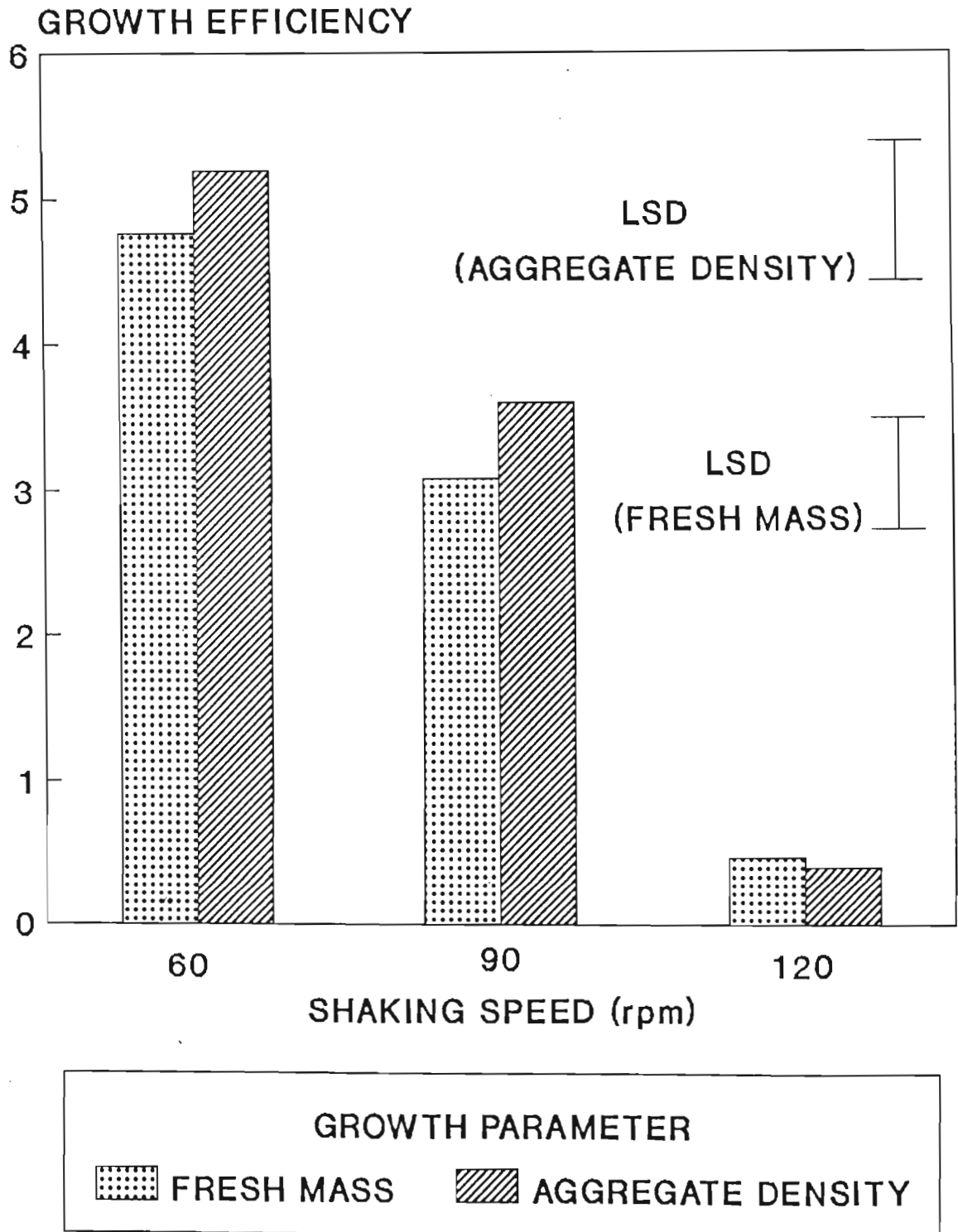
Figure 5.4 The effect of shaking speed on the aggregate size of shoot cell suspension cultures of *Erythrina caffra* after 14 days of incubation.
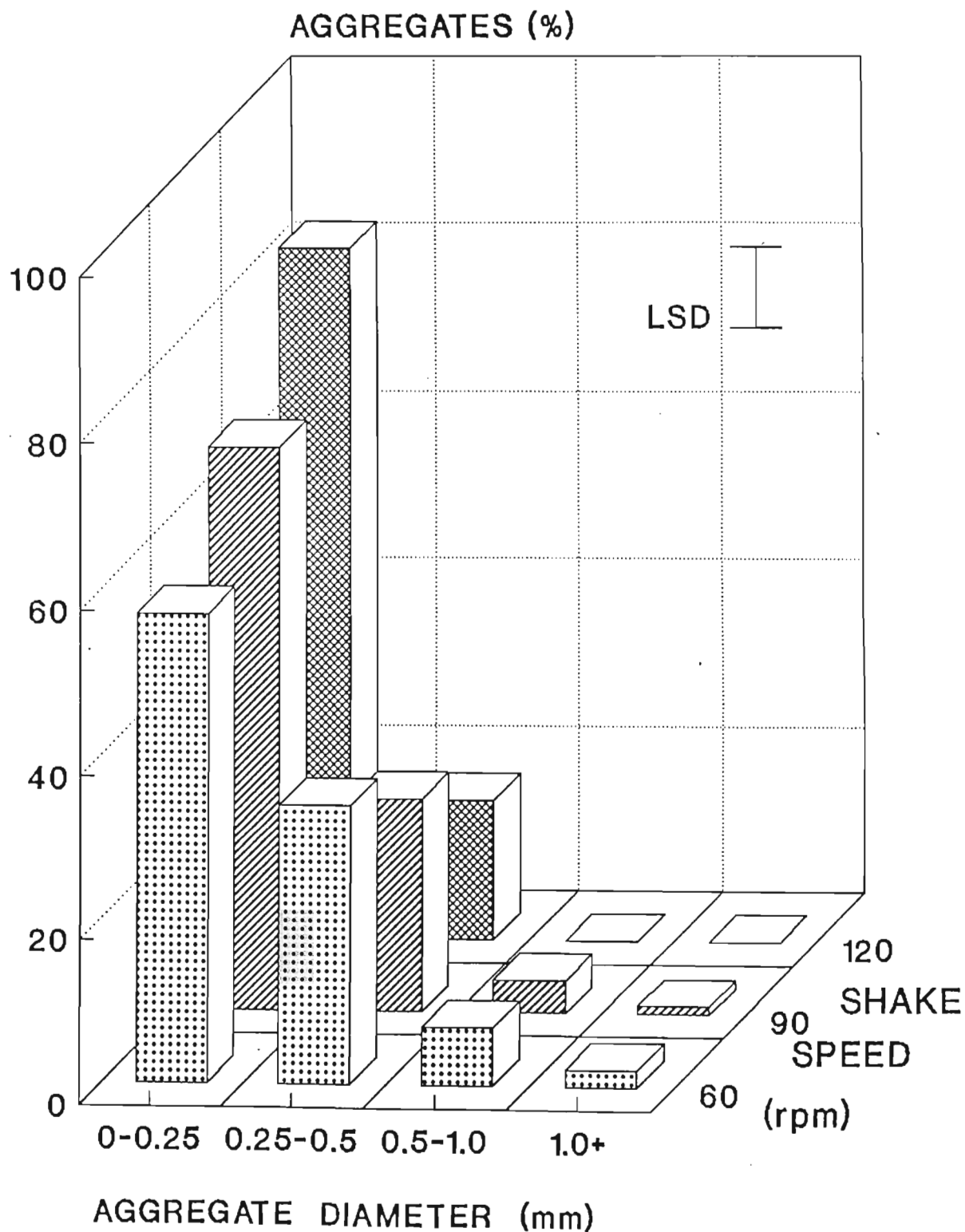
increase in shaking speed is an indication that more cells were separated from the mother aggregate by the shearing forces created in the liquid medium. However, the small aggregates at 120 rpm could also be a result of a the rupturing of newly formed cells on the original aggregate at a stage when the cells were more vulnerable to mechanical damage. This prevents the aggregate from increasing in size. It is clear that a viable suspension culture with a relatively large number of small aggregates could be established at a relatively low shaking speed of 60 rpm.

5.8.2 THE EFFECT OF GROWTH REGULATORS ON THE GROWTH OF SUSPENSION CULTURES

The growth regulators in the EC suspension medium was replaced with the combinations of BA 10 and 2,4-D 5 ; BA 2 and 2,4-D 1, and BA 1 and 2,4-D 0.5 micromoles per litre. Thirty millilitres of a stock of the shoot cell suspension culture was mixed with 60 millilitres of the treatment solution in a 500 millilitre Erlenmeyer flask. The suspension culture was incubated for 14 days at the standard conditions on a shaker at 60 rpm. The experiment was repeated three times and the mean of the three replications used to determine the trend. The aggregate density and viability were determined.

RESULTS AND DISCUSSION

Although a cell suspension could be maintained on the EC
medium at the lower shaking speed of 60 rpm the growth rate
of the suspension culture was not satisfactory. The growth
regulators applied had a significant effect on the growth
of the suspension cultures (Figure 5.5). The best growth was
obtained by a concentration of growth regulators ten times
lower than the concentration used for the growth of callus
i.e. 1 micromole per litre BA and 0.5 micromole per litre
2,4-D. The reason for this behaviour in cell growth is not
clear. It is possible however, that the cells in a callus
in contact with the solid nutrient medium metabolise growth
regulators absorbed from the medium to such an extent that
a gradient of growth regulators is created between the cells
at the nutrient interface and distant cells. Only the cells
which receive the optimal concentration of growth regulators
to stimulate cell division will divide. There are
indications that the actively dividing cells are at the
surface of a callus with the other cells in the callus in a
quiescent stage (AITCHISON, MACLEOD and YEOMAN, 1977).

5.8.3 CHARACTERISATION OF THE SUSPENSION CULTURE

The inoculum was prepared from a stock solution as described
in section 5.4 and transferred to the EC suspension medium
containing 1 micromole per litre BA and 0.5 micromole per
litre 2,4-D. The experiment was replicated three times. The

Figure 5.5 The effect of BA and 2,4-D on the
fresh mass of shoot cell suspension
cultures of *Erythrina caffra* after 14
days of incubation.

GROWTH EFFICIENCY
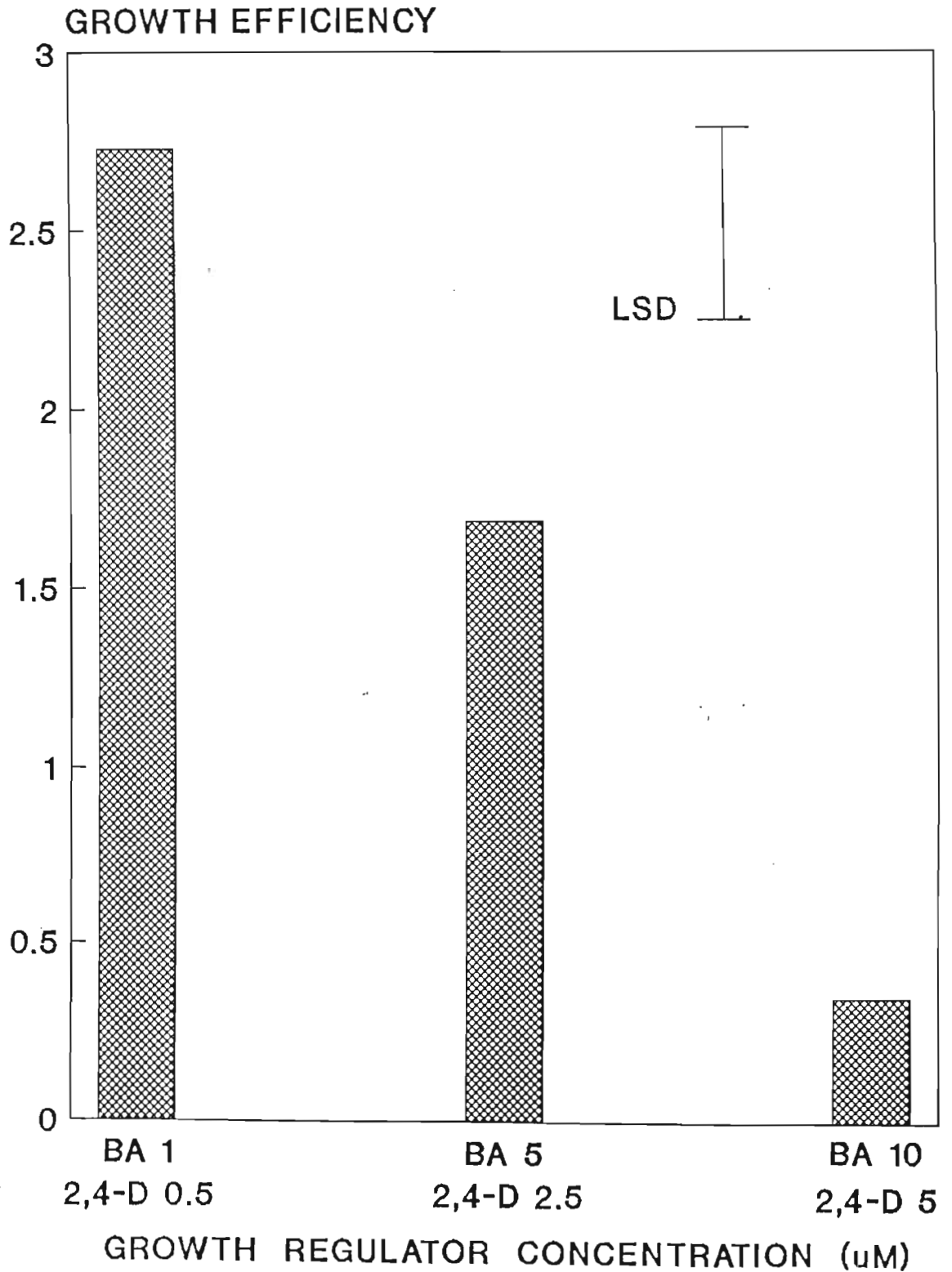
suspension was incubated at 60 rpm and the standard culture conditions described in section 5.2. The following parameters were determined at the onset of the experiment and every second day thereafter: aggregate density, viability, fresh mass, dry mass, pH, conductivity and settled cell volume.

RESULTS AND DISCUSSION

With the successful establishment of a suspension culture it became necessary to establish a growth curve for the culture. It was necessary to determine which parameters would estimate the growth of the suspension most accurately. The most commonly used parameter to establish a growth curve is cell density (Figure 5.6) According to the growth curve the aggregate suspension was in the lag phase for the first two days after inoculation. From the third day to approximately the sixth day the suspension culture entered the exponential growth phase with a rapid increase in cell division. For the next two days the culture was in the linear phase of growth. After eight days of incubation the culture reached a phase of progressive deceleration. Cell division gradually decreased until the suspension was in the stationary growth phase after about ten days of incubation.

The use of the fresh and dry mass to construct the growth curve (Figure 5.7) resulted in a curve very similar to that obtained with other parameters such as the settled cell

Figure 5.6 Growth curves of shoot cell suspension cultures of *Erythrina caffra* based on the settled cell volume and aggregate density. The cell suspension cultures were incubated over a period of 12 days.
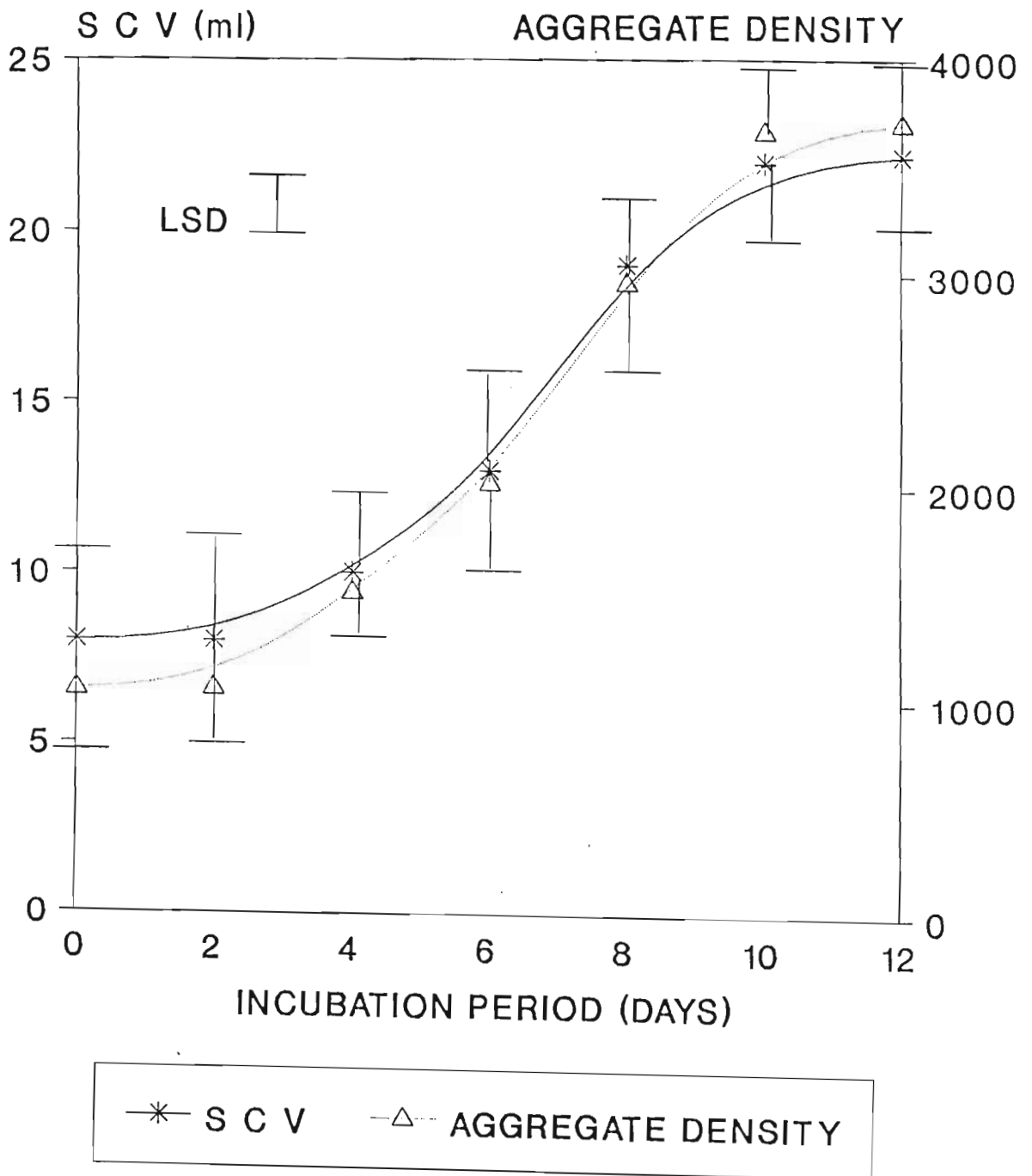
Figure 5.7 Growth curves of shoot cell suspension cultures of *Erythrina caffra* based on the fresh and dry mass of the cultures. The cell suspension cultures were incubated over a period of 12 days.
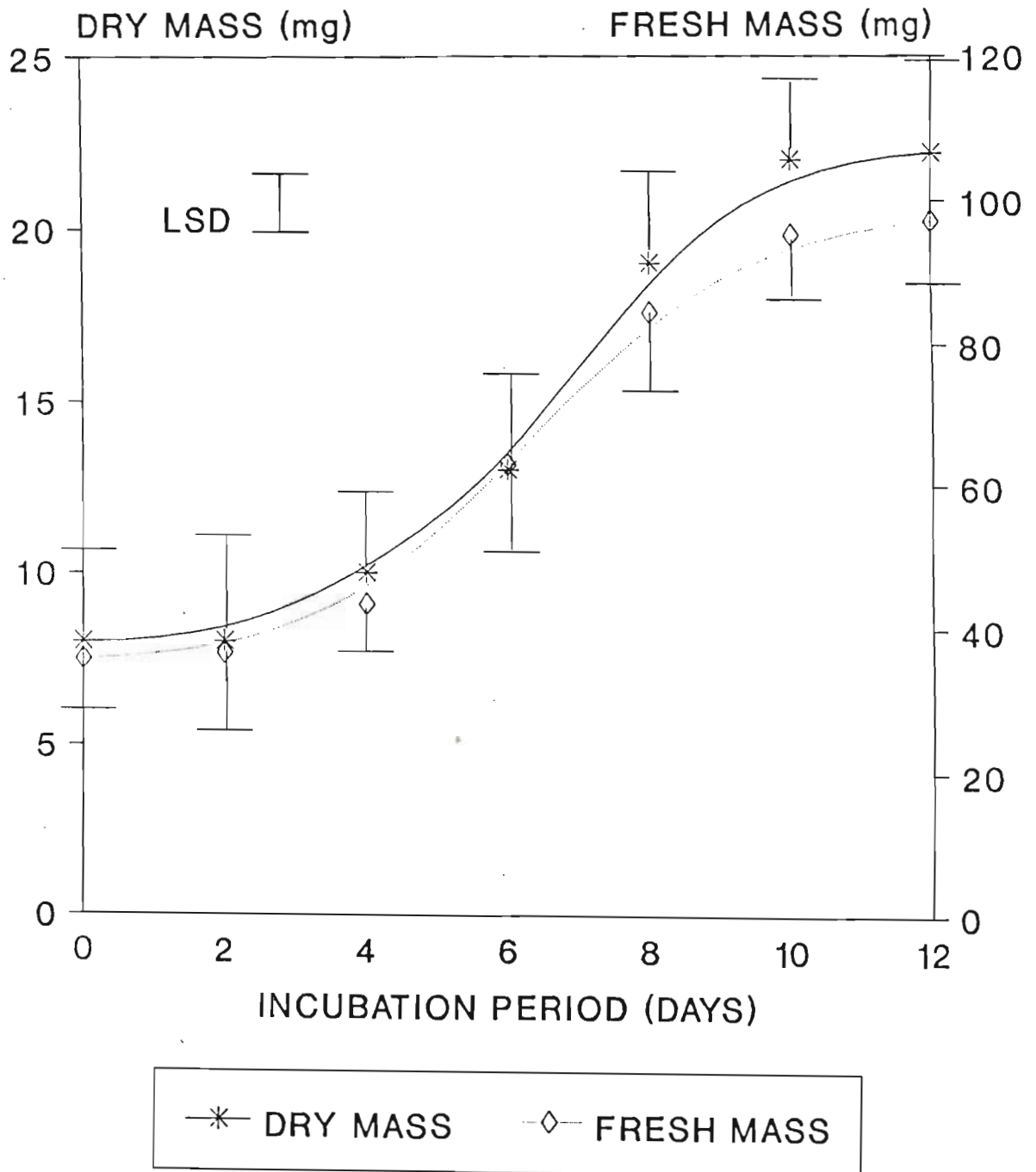
volume (SCV). There was a good correlation between the increase of the fresh and dry mass of the cell suspension cultures (r=0.90). A small increase in the fresh mass relative to the dry mass was observed at the onset and during the stationary phase. This increase in the water content of the cells was not significant but was an indication of the enlargement of the cells and probably induced vacuolation.

The growth curve based on the settled cell volume (Figure 5.6) had a similar appearance to the growth curve based on aggregate density with the exception that the exponential phase and the onset of the stationary phase was not well defined.

The conductivity of the suspension culture was measured to ascertain whether it could be a good measure of the growth of the suspension culture. From the results (Figure 5.8) it is clear that an inverse relationship existed between the growth of the suspension culture based on the aggregate density and the conductivity of the nutrient medium. The exponential phase was not well defined by the determination of conductivity. However, the linear and stationary phases of growth was better defined. The use of conductivity to determine the growth of cell suspension cultures was first proposed by HAHLBROCK, EBEL and OAKS (1974) for *Glycine max*. Good correlations between growth and conductivity were later determined for cell suspension cultures of other species such as *Petroselinum hortense* Hoffm. and

Figure 5.8 Growth curves of shoot cell suspension cultures of *Erythrina caffra* based on the conductivity of the aqueous phase and the aggregate density of the cell cultures. The cell suspension cultures were incubated over a period of 12 days.
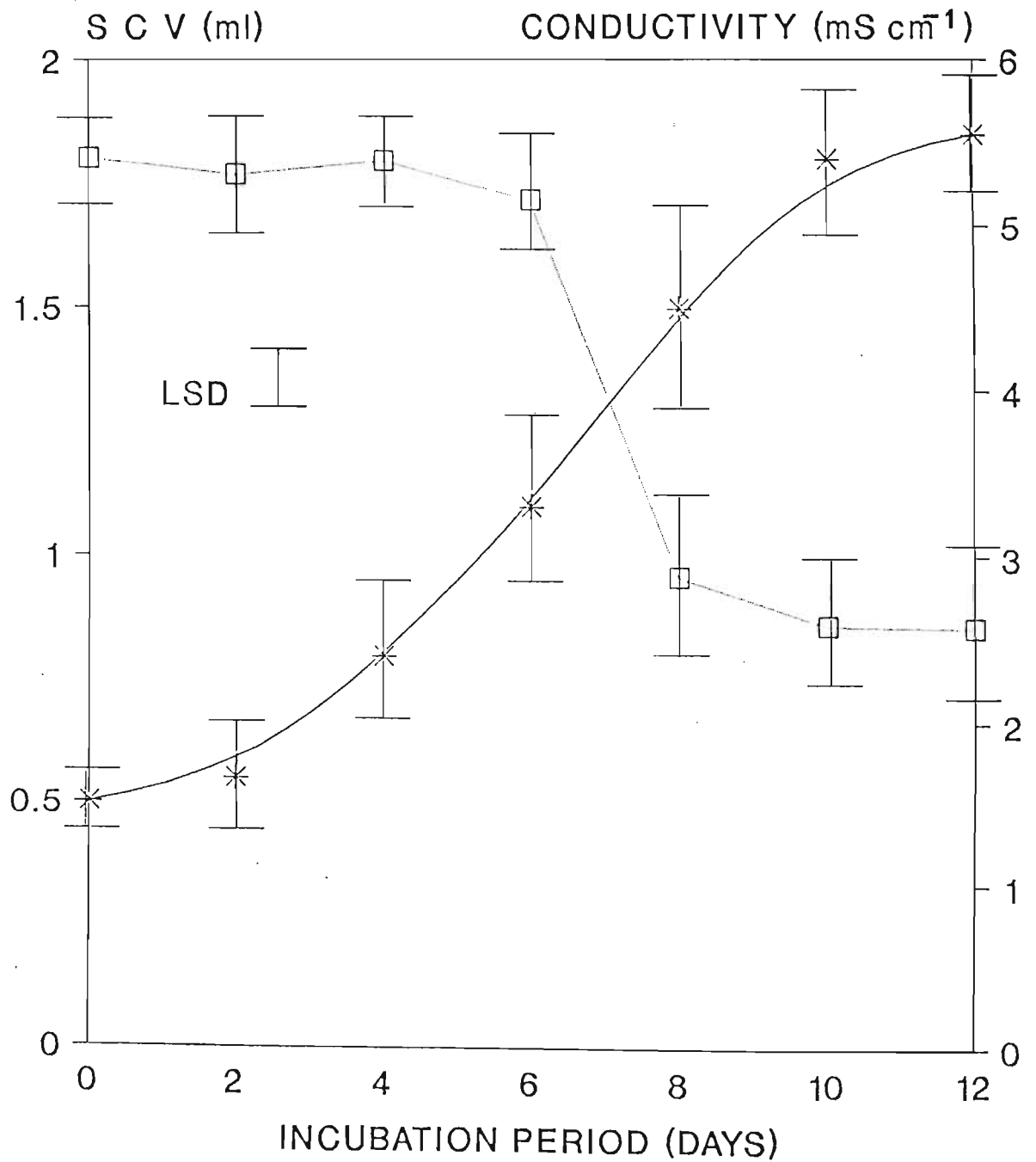
Glycine max L. (HAHLBROCK and KUHLEN, 1972), *Happlopappus gracilis*, (HAHLBROCK, 1975). However, a good correlation between the growth of suspension cultures and conductivity could not be obtained for *Cicer arietinum* and *Acer psuedoplatanus* (HAHLBROCK, 1975). The conductivity of the suspension culture is regulated by the depletion of nutrients from the aquaeous phase and the release of organic and inorganic ions from the cells. The entire content of dead cells will be released in the aquaeous phase with a resultant increase in the conductivity. Since it is not clear yet to what extent the release of ions from cells affect the conductivity of the culture it is a parameter that should be used with caution.

The change in the pH during the growth of the suspension culture was also determined. This was not done to correlate growth with pH but merely to observe the change in pH during the growth of the culture. A decrease in the pH was observed from the onset of the incubation period up to the fourth day of growth when it reached a minimum value (Figure 5.9). After the fourth day the pH increased until by the sixth day to nearly its original value. It then stabilised at about pH 5.0 for the duration of the experiment.

From the literature it was evident that the fluctuation in the pH of a suspension culture was largely determined by the uptake of ammonium and nitrate by the cells from the nutrient medium. The pH will then stabilise at a value

Figure 5.9 The change in the pH of shoot cell suspension cultures of *Erythrina caffra* over a period of 12 days.
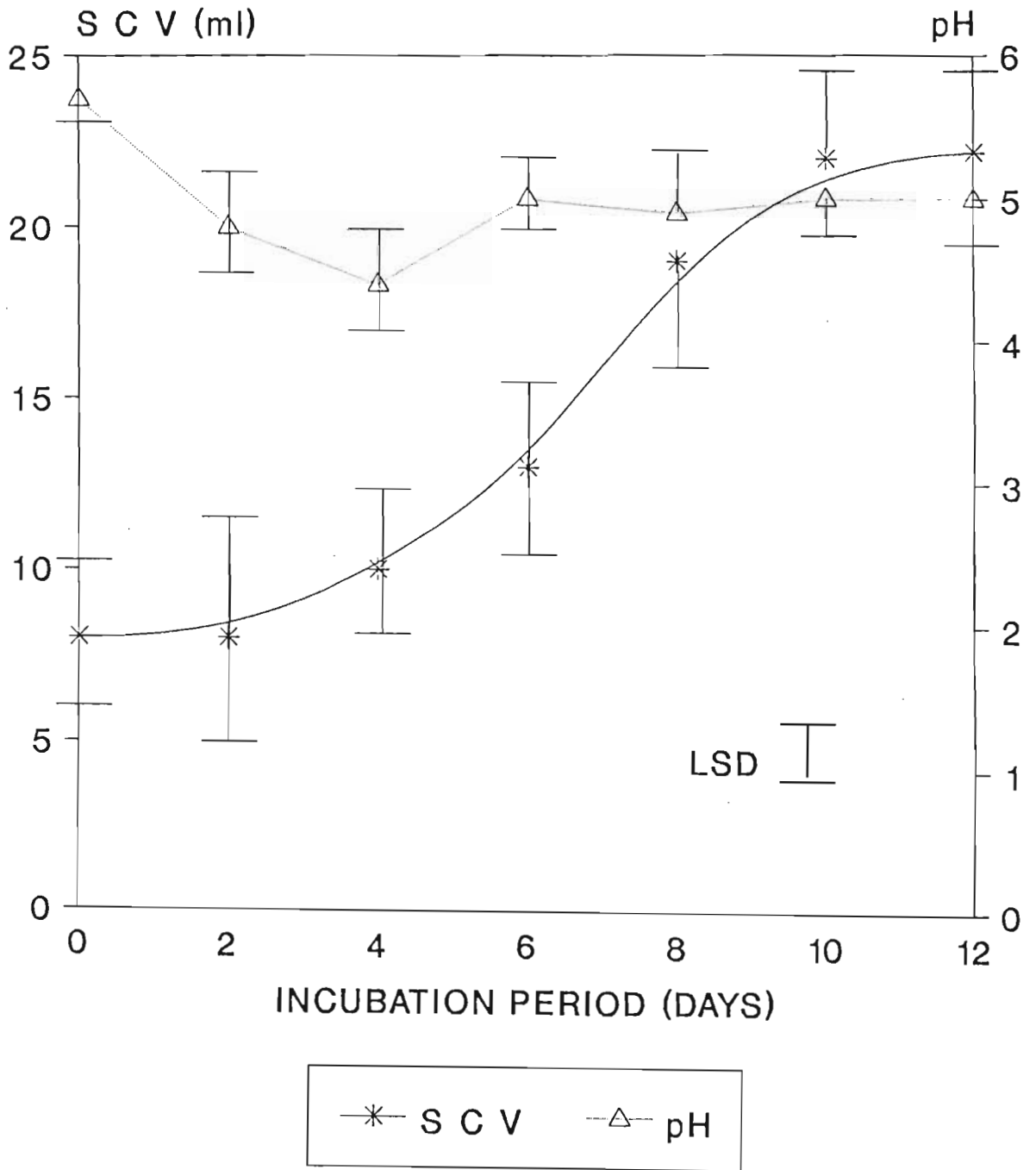
characteristic to the specific culture. Ammonium is preferentially absorbed by suspension cultures which results in a decrease of the pH. This happens to restore the electric neutrality between the cells and the nutrient medium. However, it also resulted in the diminished uptake of ammonium since the absorption of ammonium was positively related to to the pH of the medium. The decrease in pH together with the decrease in ammonium uptake resulted in an increased absorption of nitrate. The consequence of the uptake of nitrate was an increase in the pH since the cells had to restore the electric neutrality. If it is assumed that the same is true for the suspension culture of *Erythrina caffra* then it could be assumed that the stages of active cell division coincided with the uptake of at least ammonium and nitrate. The sharp decrease in the conductivity after the sixth day which coincided with the stabilisation of the pH at day six was an indication of a large increase in the uptake of presumably non-nitrogenous nutrients. However, from the observations on *Petroselinum hortense* and *Glycine max* it seemed as if the changes in pH was also dependent on the species being cultured. With only a small increase in the pH of the suspension culture of *Petroselinum hortense* in the lag phase the pH of the same nutrient medium with *Glycine max* cells changed drastically from the lag phase up to the stationary phase (HAHLBROCK and KUHLEN, 1972).

The correlation coefficients were determined between the different parameters used to measure the growth of the cell

suspension culture. This was done to establish whether parameters which could be determined without affecting the volume or culture conditions of the cell suspension culture would be reliable to determine the growth of the cell suspension. From the results in Table 5.1 it is evident that

Table 5.1 Correlation coefficients between the parameters used for the estimation of the growth of shoot cell suspension cultures of *Erythrina caffra*.

Parameter	Conductivity	SCV	Dry mass	Fresh mass
Density	-0.89	0.86	0.95	0.93
Fresh mass	-0.87	0.85	0.90	
Dry mass	-0.87	0.84		
SCV	-0.73			

Density = Aggregate density (Aggregates per ml).

SCV = Settled cell volume.

a high negative correlation with a correlation coefficient higher than -0.86 was found between the conductivity of the nutrient medium and the aggregate density, fresh mass and dry mass. A correlation coefficient of higher than 0.83 was observed between the settled cell volume and the aggregate density, fresh mass and dry mass. The measurement of the settled cell volume with a sidearm fixed to the culture vial is the only method to measure the growth of the cell suspension without affecting the suspension culture at all.

Parameters such as cell density and fresh mass or dry mass are thus recommended to quantify the growth of a suspension. To estimate growth by means of cell density is a tedious process since a large number of counts have to be done to obtain an accurate estimate of the cell density (KING and STREET, 1977). The advantage of using cell density as a measure of growth is the small volume of suspension removed from the cell suspension culture. With the shoot cell suspension culture of *Erythrina caffra* an aliquot of 1 millilitre was satisfactory for the determination of the aggrgate density. Although it is a simple and easy procedure to determine the fresh or dry mass of a sample from a suspension it was reported by KING and STREET (1977) that a large sample was needed to obtain a reasonably accurate fresh mass. With the *Erythrina caffra* shoot cell suspension culture it was found that an accurate estimate of the fresh or dry mass could only be obtained with a 4 to 5 millilitre sample of the suspension culture. It was also found that a sample representative of the suspension could only be obtained if the volume at the end of the incubation period was not less than 60 % of the initial culture volume. Extracting 5 millilitres suspension at a time from a 100 millilitre suspension it implies that the suspension culture could be sampled only eight times. This restricts the number of samples that could be taken and it changes the volume of the suspension drastically. At a constant shaking speed a decrease in the volume of the suspension culture of *Erythrina caffra* increased the movement of the cells in suspension drastically. This caused

larger abrasive stresses on the moving cells which resulted in incubation conditions different from the initial conditions. Thus if a large number of samples has to be taken from a suspension culture mass is not the parameter of choice for the determination of growth since frequent sampling of the culture brings about drastic changes in the initial culturing conditions of the suspension. In contrast with the above mentioned the use of conductivity or settled cell volume is much more appealing since the suspension culture is left intact after the parameter has been determined. With the measurement of conductivity the suspension culture is opened and the conductivity determined with an aseptic probe dedicated for this purpose. Contamination can however, not be ruled out. Settled cell volume is determined in the side arm attached to the culture flask. Since it is done without opening the culture the possibility of contamination is reduced and the disturbance of the suspension culture is negligible. The precision of the method can be increased by attaching graduated glass tubes as side arms to the culture flasks.

Growth curves of plant cell suspension cultures generally follow the trend of the ideal sigmoidal growth curve illustrated in Figure 5.1. Examples of such curves are the growth curves in Figure 5.10 of suspension cultures of (A) Paul's scarlet rose (NASH and DAVIES, 1972), (B) *Acer psuedoplatanus* (GOULD, BAYLISS and STREET, 1974), (C) *Nicotiana tabacum* (MORRIS and FOWLER, 1981), (D) *Nicotiana tabacum* (IKEDA, MATSUMOTO and NOGUCHI, 1976) and (E)

Figure 5.10 Growth curves of cell suspension cultures of Paul's scarlet rose (A), *Acer pseudo-platanus* (B), *Nicotiana tabacum* (C, D), *Phaseolus vulgaris* (E) and a synchronised cell suspension culture of *Acer pseudoplatanus* (F).
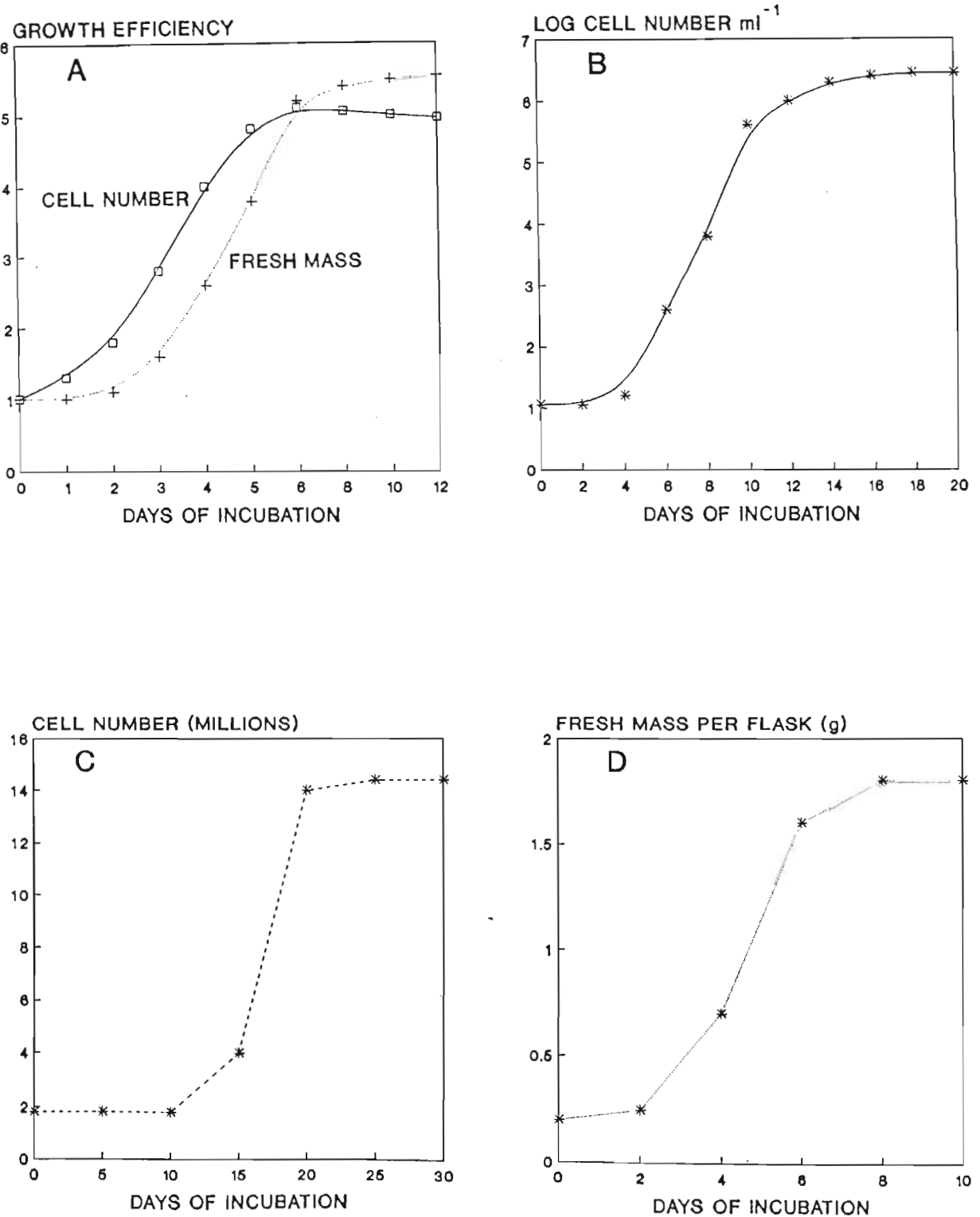
Figure 5.10 Continued.
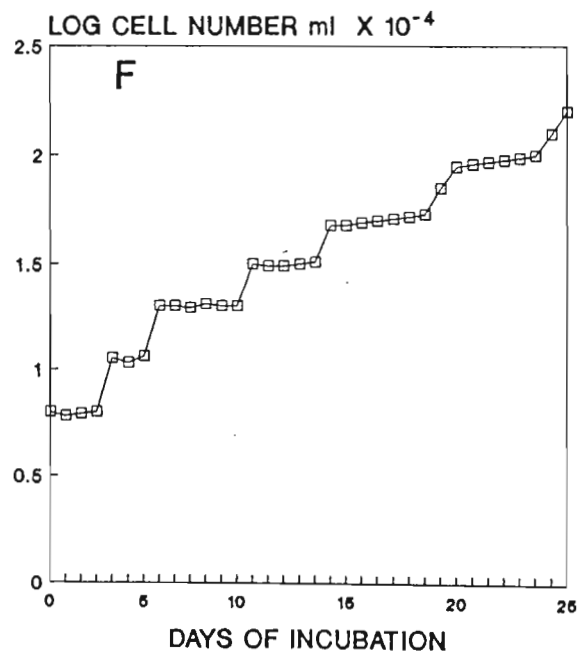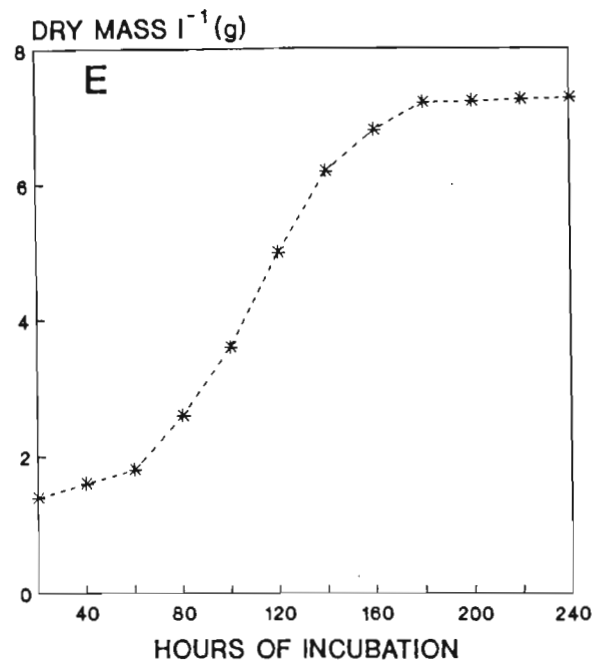
Phaseolus vulgaris (VELIKY and MARTIN, 1970). It is clear that the growth curves resemble the ideal growth curve although the function and shape of the curve can deviate significantly from the ideal. The growth curve of the same species can change drastically when the cell suspension is being subjected to a different culture environment. *Nicotiana tabacum* cell suspension cultures for example in Figure 5.10 (C) and (D) had a very similar growth curve but the growth was approximately double in the case of (C) than in (D). It is also clear from Figure 5.10 (A) and (D) that very similar growmh curves were obtained with different parameters such as fresh mass, dry mass and cell number. The linear phase of the growth curve is in many cases not a straight line but approach the sigmoidal curve. The deviation from linearity could be minimized with a log or natural log transformation of the data (Figure 5.10 (B) and (E)). A characteristic of the cell suspension cultures with sigmoidal growth curves is the asynchronous cell division. Suspensions with synchronous cell division have staggered growth curves (Figure 5.10 (F)) which differs dramatically from the sigmoidal curve. It is ideal to have a synchronous dividing cell suspension since all the cells will be in the same physiological stage of growth and inferences about the metabolic behaviour of the culture will be more accurate than with an asynchronous dividing culture. It is however, recommended to use more than one parameter to obtain a good estimation of the growth of a suspension culture. It was clear from the results obtained with the *Erythrina caffra* suspension culture that a satisfactorily

representation of the growth of the suspension culture could be obtained with the use of the settled cell volume.

5.8.4 GROWTH PHASE AND CELL MORPHOLOGY

Distinct differences in the size and shape of the cells in suspension were observed in the different growth phases. A suspension culture was initially established using a suspension in an active dividing phase. Most of the cells in the lag phase thus looked similar to cells in the exponential and linear growth phases (Plate 5.3). These cells were relatively small and isodiametric (Plate 5.4). However, since cell division was not synchronised cells in an early to late stage of senescence which looked similar to cells in the stationary phase were also present in the cultures (Plate 5.5). These cells were elongated, filamentous and larger than the isodiametric cells. During the exponential phase mainly isodiametric cells divided to from aggregates. Within a suspension culture mainly one pattern of cell division is found. The most common pattern of cell division which occurred in the suspension culture of *Erythrina caffra* was the division of isodiametric cells which proliferated to form aggregates. The formation of septated filamentous cells and aggregates from these cells comprised approximately 15 % of the cell division activity in the exponential and linear growth phases. The formation of aggregates from isodiametric cells were also reported for suspension cultures of *Rubus* and *Glycine max* (STREET and

Plate 5.3 A shoot cell suspension culture of *Erythrina caffra* in the lag phase with non-dividing filamentous cells (F) and aggregates of isodiametric cells (I).
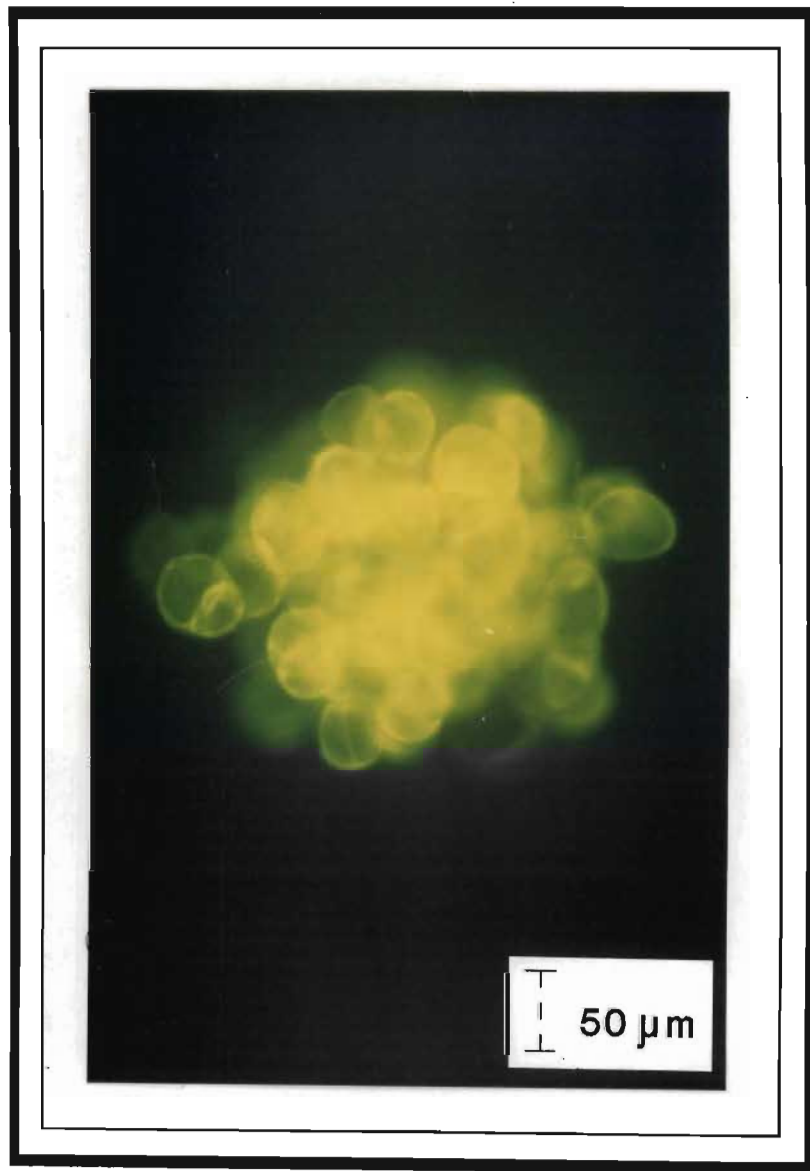
Plate 5.4 A typical aggregate of small, viable, isodiametric cells of a shoot cell suspension culture of *Erythrina caffra* which occur in the exponential and linear growth phases.

Plate 5.5   Filamentous cells of a shoot cell suspen-
            sion culture of *Erythrina caffra* in the
            stationary growth phase.

HENSHAW, 1963 ; GAMBORG, MILLER and OJIMA, 1968). The daughter cells resulting from the division of isodiametric cells were of equal size or smaller than the mother cell. This was an indication of a degree of differentiation in the culture.

Different patterns of cell division took place in a small number of the filamentous cells. Some of the filamentous cells divided by forming several cell walls in the mother cell resulting in a septated cell (Plate 5.6). No further cell division or enlargement took place before all the septums had been formed. In the first pattern of division most of the resultant daughter cells enlarged by expanding mainly at a right angle to the axis of the original long cell. This created the impression of a budding cell. The first division of a daughter cell took place by the formation of a cell wall at the base of the protrusion. These resulting isodiametric cells divided further to form an aggregate. The second pattern of division of the filamentous cells was the terminal division of the daughter cells. One or two daughter cells at both or only one end of the filamentous cell would enlarge. The enlarged cells divided several times resulting in a dumbbell-shaped aggregate or an embryo-like structure (Plate 5.7). The structure with an aggregate at one end of the septated cell did not develop into an embryo but was indicative of physiological polarity in these filamentous cells. The inconsistency of polarity was demonstrated by cell division which occurred at both ends or at any cell in the septated

Plate 5.6 A filamentous cell of a shoot cell suspen-
          sion of *Erythrina caffra*. Cell walls
          developed in the brightly fluorescing
          areas.

Plate 5.7   A   filamentous septated cell   of   a   shoot
cell   suspension   of   *Erythrina*   *caffra*
dividing   actively   at   one   end   to   form   an
aggregate.

filament. It should be possible to induce consistent polarity and hence organised growth in these cultures with a change of the nutrient medium.

The late linear phase and the onset of the stationary phase was characterised by a decrease in cell division and the onset of cell enlargement. Cell enlargement manifested mainly as the unidirectional elongation of isodiametric cells to form filamentous cells (Plate 5.4). These filamentous cells further enlarged to form giant cells (Plate 5.8). However, these giant cells represented less than 5 % of the total number of cells in a suspension culture. Giant cells were formed by filamentous or isodiametric cells (Plate 5.9). With the enlargement of cells a larger difference should be detected between the fresh and dry mass of the cultures. Although an increase in the fresh mass of the cultures was observed (Figure 5.5) relative to the dry mass it was not significant. This was probably the result of the unsynchronised growth resulting in a mixed population of cells in different growth stages with the largest proportion of cells in one specific growth stage. During all the growth phases starch granules were observed in the cells (Plate 5.9). Starch grains occurred in the cells mainly during the cell enlargement phase with only a few grains visible during the stages of active cell division.

The patterns of cell division in the shoot cell suspension cultures of *Erythrina caffra* was very similar to those
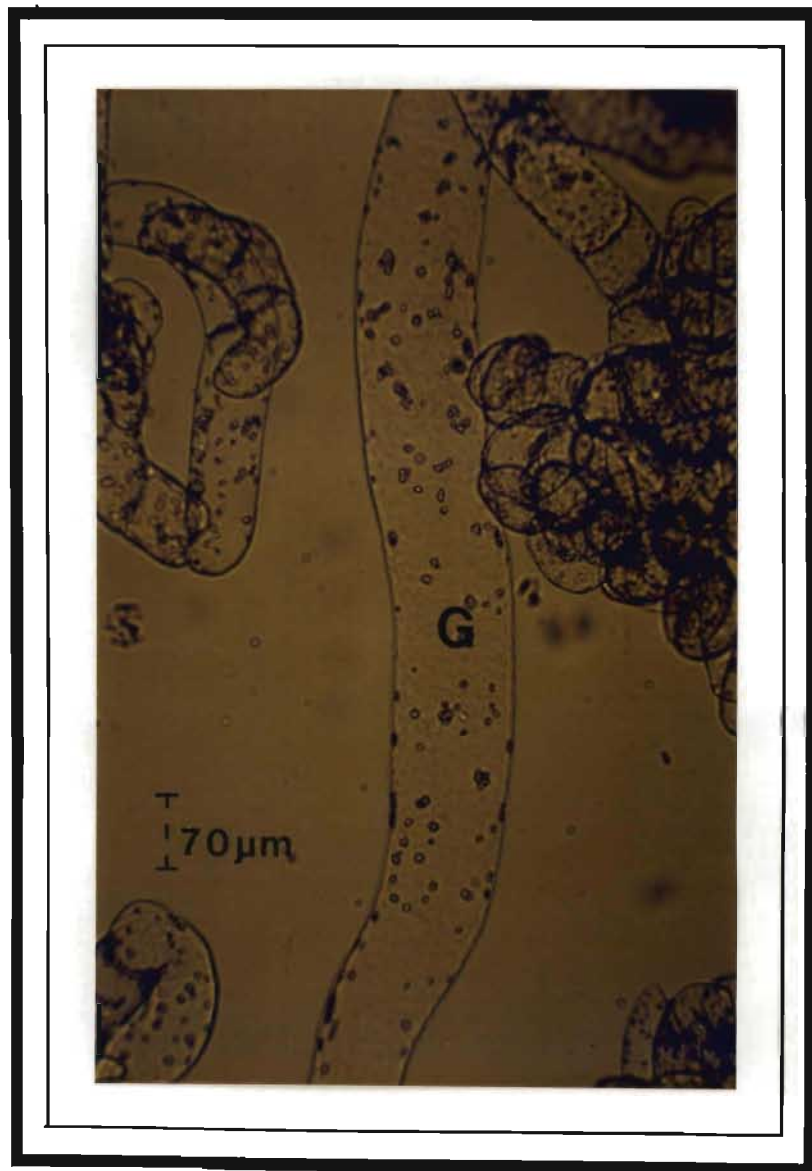
Plate 5.8   A giant filamentous cell (G) in a shoot
            cell suspension culture of *Erythrina*
            caffra. Note the difference in size
            between the giant cell, the smaller
            generally occurring filamentous cells and
            the isodiametric cells.
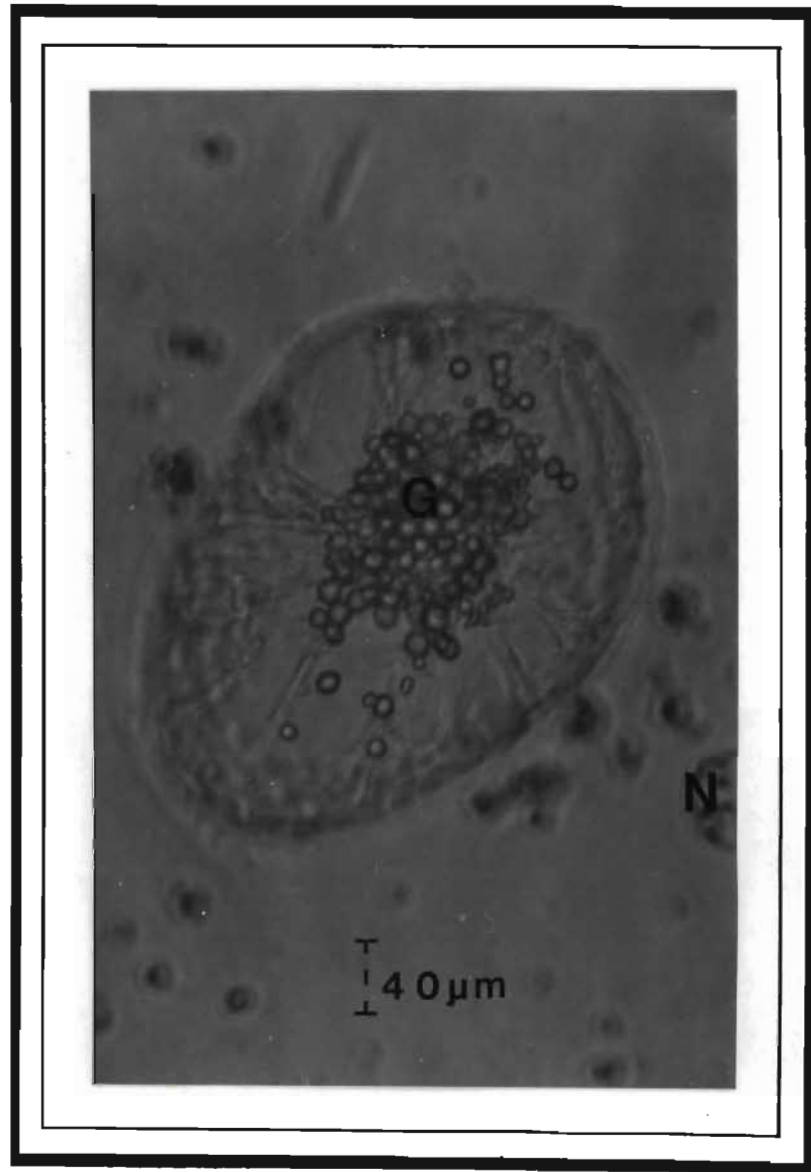
Plate 5.9   A giant (G) and normal size   (N)   isodia-
metric   cell   in   a   shoot   cell   suspension
culture of *Erythrina caffra*.

reported for a hypocotyl cell suspension culture of *Phaseolus vulgaris* cv. Contender. As with *Erythrina caffra* septated filamentous cells, dumbbell shaped and embryo-like structures formed during the cell division phase. The most prominent type of cell found in the stationary phase was the filamentous type (LIAU and BOLL, 1971). The formation of giant cells were also observed in these hypocotyl cell suspension cultures. However, the shape of the giant cells were mainly isodiametric or slipper shaped. In contrast with the giant cells formed by the shoot cell suspension culture of *Erythrina caffra* the giant cells in the suspension culture of *Phaseolus vulgaris* were able to divide to form aggregates (LIAU and BOLL, 1971). The division of giant cells was reported for suspension cultures of *Daucus carota* and *Nicotiana tabacum*. However, in these two cultures the giant cells divided more frequently than other cells (JONES, HILDEBRANDT, RIKER and WU, 1960 ; STREET and HENSHAW, 1963).

The main cell shapes most frequently reported for batch cultures are isodiametric and oval shaped cells (NICKELL, 1956 ; BERGMANN, 1960 ; ERIKSSON, 1965 ; GAMBORG, MILLER and OJIMA, 1968 ; LIAU and BOLL, 1971), elongated cells (MUIR, HILDEBRANDT and RIKER, 1958 ; HENSHAW, JHA and MEHTA, 1966 ; SIMPKINS, COLLIN and STREET, 1970 ; LIAU and BOLL, 1971) and various shapes of giant cells (STEWARD, MAPES and SMITH, 1958 ; STREET and HENSHAW, 1963 ; GAMBORG, 1966 ; LIAU and BOLL, 1971). These three cell types do not occur simultaneously in most suspension cultures. However, as with suspension cultures of *Phaseolus vulgaris* all three cell

types were found in shoot cell suspension cultures of *Erythrina caffra*. At this point in time the large number of cell types in suspension cultures seems to be unique to *Erythrina caffra* and *Phaseolus vulgaris*.

CHAPTER 6

THE OCCURRENCE AND DISTRIBUTION OF t-PA

INHIBITOR IN *ERYTHRINA CAFFRA* PLANTS

6.1 THE t-PA INHIBITOR CONTENT OF DIFFERENT ORGANS

OF *ERYTHRINA CAFFRA*

The t-PA inhibitor used for medical purposes is currently extracted from seeds of *Erythrina caffra*. However, it has not yet been established whether the inhibitor occurs in larger quantities in other parts of the plant. Knowledge of the distribution of the t-PA inhibitor in the plant can be indicative of the function of this protein and may reveal information with respect to its site of synthesis.

EXPERIMENTAL

To establish whether t-PA inhibitor occurs in other parts of the plant than the seed, protein was extracted from root, shoot, leaf, living bark and cotyledonary material. Seeds were collected from the collection site described in Chapter 2. The cotyledons used were from green seeds which had reached their maximum length of 11 to 12 centimetres. The

seeds were stored at -20 ^{0}C until used for extraction. The root, shoot, leaf and living green bark material was obtained from greenhouse grown plants (Chapter 2). Throughout the studies care was taken to use only plant material free from diseases and insect damage. The plant material obtained from the greenhouse was processed immediately after collection. Protein was extracted from the material and determined as described in Chapter 2, sections 2.5 and 2.6 respectively. The protein extracts were assayed for t-PA inhibitor using the ELISA technique described in Chapter 3.

RESULTS AND DISCUSSION

From the results presented in Table 6.1 it is clear that significant differences were recorded for the protein content of different organs of *Erythrina caffra*. Cotyledonary material yielded substantially more protein than the other organs (Table 6.2). Based on the dry mass and protein content of the plant material significant differences were found in the t-PA inhibitor content of the different organs (Table 6.3 and 6.5). Substantially more t-PA inhibitor was found in the cotyledons than in any other part of the plant. The leaf material contained nearly double the amount of t-PA inhibitor than the other vegetative organs (Table 6.4). However, when expressed as a measure of total protein little difference was observed between the t-PA inhibitor content of the vegetative organs analysed (Table 6.6). As is the case for the t-PA

Table 6.1 Analysis of variance of the protein content of

different organs of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	74257.3	4	18564.3	80.8*
Residual	4596.4	20	229.8	
Total	78853.8	24		

* Indicate significant differences at a 95% level of confidence.

Table 6.2 Multiple range analysis of the protein content

of different organs of *Erythrina caffra*.

Organ analysed	Treatment mean	Homogeneous groups	
	Protein (mg g^{-1} DM)		
Cotyledon	151.4	*	
Leaf	23.9		*
Root	15.5		*
Bark	6.3		*
Shoot	5.6		*

* In different columns indicate significant differences at a

95% level of confidence. DM — Dry mass.

Table 6.3 Analysis of variance of the t-PA inhibitor content
          of different organs of *Erythrina caffra* expressed
          in terms of units of dry mass.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	640830.1	4	160207.5	97.9*
Residual	32723.1	20	1636.2	
Total	673553.2	24		

* Indicate significant differences at a 95% level of confidence.

Table 6.4 Multiple range analysis of the t-PA inhibitor
          content of different organs of *Erythrina caffra*.

Organ analysed	Treatment mean	Homogeneous groups
	t-PA inhibitor (µg g^{-1} DM)	
Cotyledon	402.2	*
Leaf	13.3	*
Bark	5.3	*
Root	4.6	*
Shoot	1.8	*

* In different columns indicate significant differences at a
  95% level of confidence. DM — Dry mass.

Table 6.5   Analysis of variance of the t-PA inhibitor content

of different organs of *Erythrina   caffra*   expressed

in terms of units of protein.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	825976.0	4	206494.0	139.0*
Residual	29711.4	20	1485.6	
Total	855687.4	24		

* Indicate significant differences at a 95% level of confidence.

Table 6.6   Multiple   range   analysis of   the t-PA inhibitor

content of different organs of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups	
Organ analysed	t-PA inhibitor (μg g^{-1} protein)		
Cotyledon	2662.3	*	
Leaf	43.0		*
Bark	36.5		*
Shoot	32.1		*
Root	23.6		*

* In different columns indicate significant differences at a

95% level of confidence.

inhibitor of *Erythrina caffra*, trypsin inhibitors were found to be more abundant in the cotyledons than in the roots, stems or leaves of *Dolichos lablab* and *Faba vulgaris* (AMBE and SOHONIE, 1956). Mature roots, stems and leaves of *Faba vulgaris* and *Dolichos lablab* contained 4 %, 5 % and 10 % of the trypsin inhibitor in the cotyledons of the plants concerned (AMBE and SOHONIE, 1956). In contrast, high levels of trypsin inhibitor activity was reported for the roots of *Psophocarpus tetragonolobus* (POULTER, 1982). The vegetative organs of *Erythrina caffra* yielded relatively low concentrations of t-PA inhibitor, being less than 2 % expressed in units of total protein and less than 0.5 % when expressed in units of dry mass of the inhibitor concentration in the cotyledons.

The amount or enzymic activity of trypsin inhibitors in cotyledons varies considerably between species. Trypsin inhibitors in similar amounts of cotyledonary tissue of *Glycine max*, *Dolichos lablab* and *Phaseolus aureus* inhibited 17, 117 and 160 milligrammes trypsin respectively (HSIEN-MING and CHENG-WU, 1965). Most of the assays for trypsin and other proteinase inhibitors were enzyme inhibition assays. The amount of trypsin inhibitor in the tissue examined was reported in units of enzyme inhibited and not in mass units. This makes it difficult to compare any quantitative data based on mass, as was obtained with the ELISA for t-PA, with these other observations. Hence, little information is available on the relative mass of proteinase inhibitors in plants. However, it has been established that trypsin

inhibitors represent about 6 % of the protein in seeds of *Glycine max* and 10 % of the protein in seeds of *Hordeum vulgare* (MIKOLA and KIRSI, 1972). Over and above the other chymotrypsin and trypsin inhibitors which had been isolated from the seeds of *Erythrina caffra* (JOUBERT, 1982b) the t-PA inhibitor represents approximately 0.27 % of the total protein in the cotyledons of mature seed. At a first glance this figure seems low. However, the t-PA inhibitor is not the only proteinase inhibitor in the seeds of *Erythrina caffra*. Two protein fractions DE1 and DE2 which inhibited chymotrypsin and one fraction DE4 which was an inhibitor of trypsin were isolated from seeds of *Erythrina caffra* (JOUBERT, 1982b). The inhibitory activity of these proteinase inhibitors in the seeds of *Erythrina caffra* was reported to be lower than the t-PA inhibitor activity of the DE3 protein fraction. The inhibitory activities of the protein fractions which inhibited mainly chymotrypsin (DE1, DE2), trypsin (DE4) and t-PA (DE3) were 7 220, 9 230, 10 410 units per milligramme and 69 490 international units respectively (JOUBERT, MERRIFIELD and DOWDLE, 1987). These reported inhibitory activities of the inhibitors does not imply that the inhibitors are present in the seeds in ratios based on the inhibitory activities since it is not clear at this point in time whether the inhibition of t-PA in the chromatographed protein fractions is brought about by one or more types of inhibitor molecules.

6.2 THE t-PA INHIBITOR CONTENT OF DEVELOPING SEEDS OF *ERYTHRINA CAFFRA*.

EXPERIMENTAL

Pods of different sizes were collected from one tree at the collection site described in Chapter 2, section 2.1. The seeds were removed from the pods at the day of collection and temporarily stored at 4 °C. The size of the smaller seeds was determined with an eyepiece micrometer attached to a microscope. Seeds longer than 5 millimetres were measured with a vernier caliper. The seeds were classified into classes with a 1 millimetre interval. The cotyledons of the seeds were homogenised and defatted as described in Chapter 2, section 2.4. Protein was extracted from the seeds and determined according to the methods described in Chapter 2, sections 2.5 and 2.6. The t-PA content of the seeds was determined with the ELISA described in Chapter 3. The experiment was repeated three times over a three year period by collecting seeds from the same tree each year. The means and standard errors were determined for the t-PA inhibitor and protein content of the cotyledons for each class of seeds.

RESULTS AND DISCUSSION

The t-PA inhibitor content of the seeds was low in seeds

Figure 6.1   The t-PA inhibitor content of cotyledons during the growth and development of seeds of *Erythrina caffra*. Seed length was used as measure of seed development. The t-PA inhibitor content of the seeds was expressed in units of dry mass (DM) and protein.
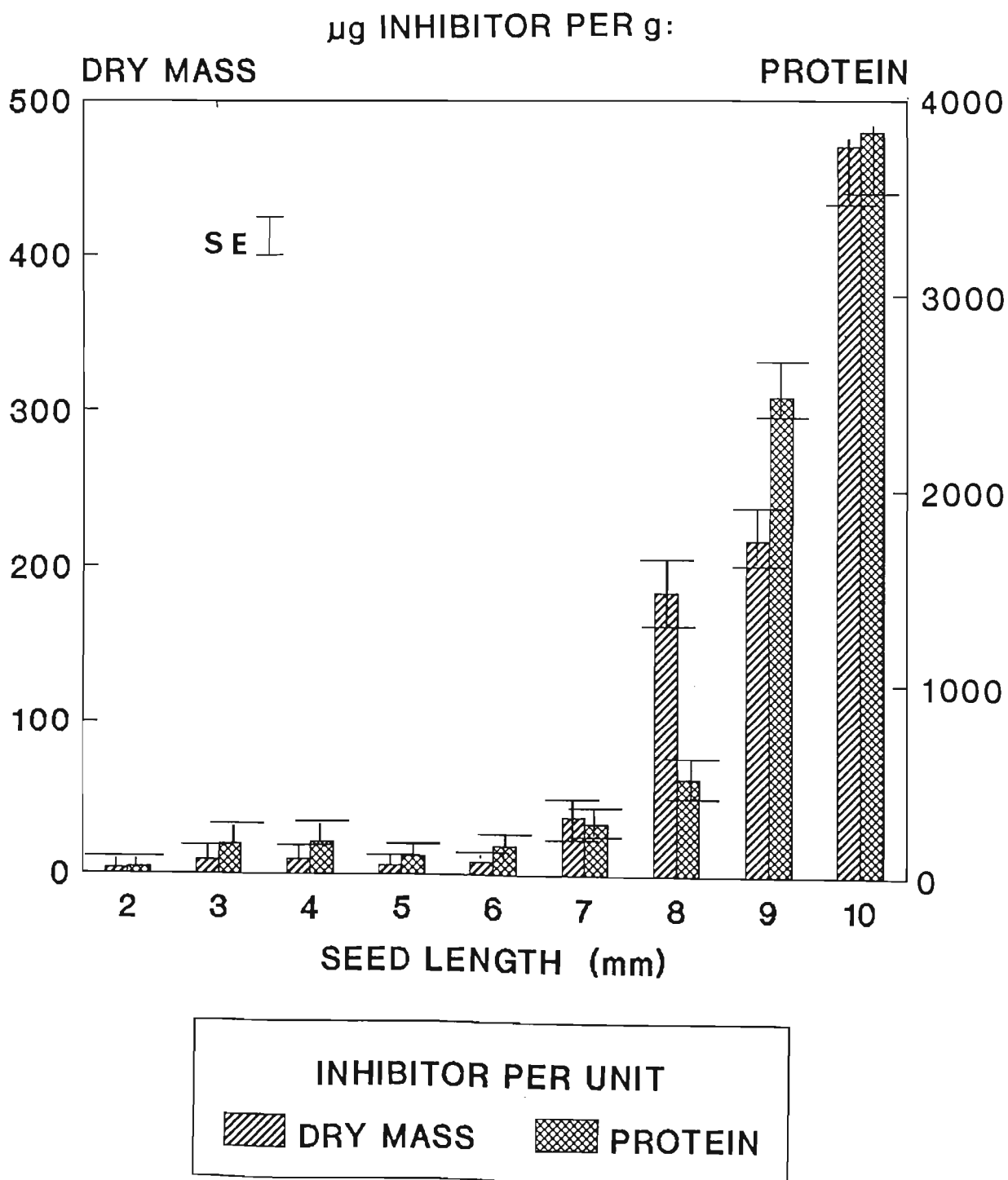
Figure 6.2 The protein and t-PA inhibitor content of cotyledons during the growth and development of seeds of *Erythrina caffra*. Seed length was used as measure of their development. The t-PA inhibitor and protein content of the seeds were expressed in units of dry mass (DM).
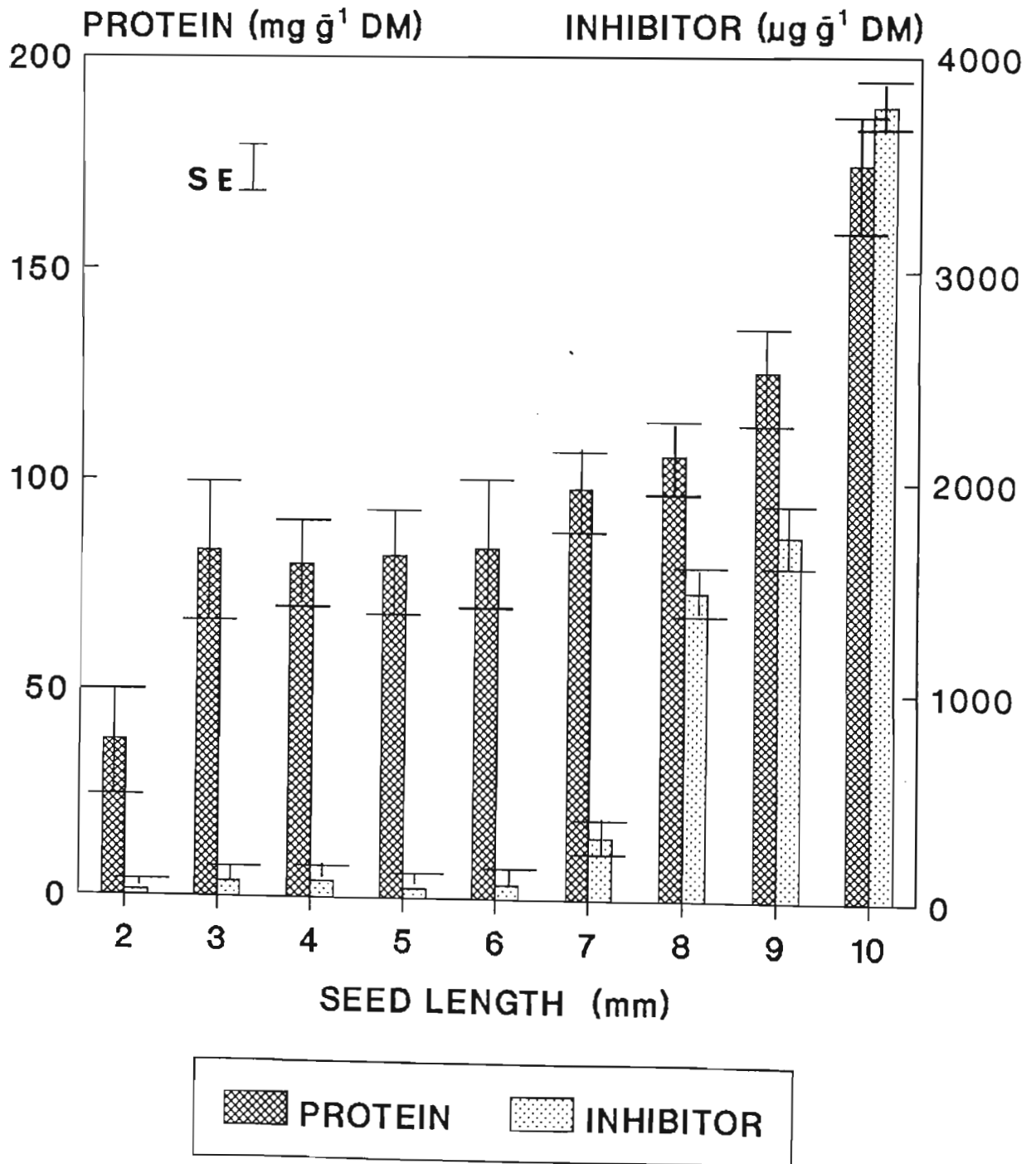
which developed up to a length of 6 millimetres. After this stage of seed development, up to the time when the seeds reached maturity, the t-PA inhibitor content of the seeds increased exponentially to levels significantly higher than detected during the early stages of seed development (Figure 6.1). The observation that trypsin inhibitors were synthesised from the onset of seed development was made by ORF, MIES and HYMOWITZ (1977) for seeds of six different cultivars of *Glycine max*. During the growth and development of the seeds the total protein content of the seeds followed a trend similar to that for the t-PA inhibitor (Figure 6.2). During the early stages of seed development protein accumulated at a relatively low rate in the cotyledons. During the later stage of seed development the protein content of the seed increased substantially. These findings on *Erythrina caffra* are in agreement with the observations that storage protein start to accumulate in seeds of *Vicia faba* from the time when they reached a length of 5 millimetres until the mature stage is reached (MILLERD, SIMON and STERN, 1971). Storage proteins in legume seeds accumulate in small quantities during the early phase of seed development with a rapid increase during the cell expansion phase of the cotyledons (FLINN and PATE, 1968 ; BRIARTY, COULT and BOULTER, 1969 ; SMITH, 1973). It is clear that t-PA inhibitor is present in the cotyledons from the onset of seed development. Similar patterns of increase in storage protein and t-PA inhibitor suggests that the inhibitor behaves like the true storage proteins in its accumulation in the cotyledons.

Storage proteins of legume seeds accumulate mainly in protein bodies in the cells of the cotyledons (DURE, 1975). If the function of trypsin inhibitors is that of a storage protein it would be expected to accumulate along with the other storage proteins in the protein bodies. However, with the exception of lipid containing spherosomes KUNITZ and BOWMAN-BIRK trypsin inhibitors were reported to be ubiquitous intracellularly in cotyledonary tissue of all *Glycine max* cultivars investigated. In some cultivars the inhibitors were even more abundant in the cytoplasm than in the protein bodies (HORISBERGER and TACCHINI-VONLANTEN, 1983a, 1983b). The association of trypsin inhibitors with the cytoplasm rather than with the protein bodies in cotyledonary tissue was also reported for *Vigna radiata* (CHRISPEELS and BAUMGARTNER, 1978). These observations suggest that trypsin inhibitors are not related in function to the true storage protein in the cotyledons. It was suggested that the widespread distribution of trypsin inhibitors in the cell indicates a protective role for trypsin and related inhibitors. This is achieved by preventing the proteolytic degradation of organelles and cytosolic proteins (CHRISPEELS and BAUMGARTNER, 1978).

It was reported that proteinase inhibitors do occur mainly in the vacuoles of the leaves of *Lycopersicon esculentum* (SHUMWAY, RANCOUR and RYAN, 1970 ; SHUMWAY, YANG and RYAN, 1976 ; WALKER-SIMMONDS and RYAN, 1977b). However, the cotyledons and the leaves have different functions and it is

possible that trypsin inhibitors do not have the same function in different organs. There is substantial evidence for the suggestion that the trypsin and other proteinase inhibitors in the leaves act as a defense against attacks by chewing insects and fungal infection (RYAN, 1974b).

6.3 THE t-PA INHIBITOR CONTENT OF COTYLEDONS OF *ERYTHRINA CAFFRA* DURING GERMINATION AND SEEDLING GROWTH.

EXPERIMENTAL

Seeds of *Erythrina caffra* were sterilised, scarified and imbibed as described in Chapter 2, section 2.7. The seeds were placed on sterilised, moist Whatman No 1 filter paper in glass jars. They were then incubated under aseptic conditions at 27 $^{\circ}$C in the light. The period of incubation started at onset of the imbibition process. Three seeds were removed every two hours during the first 48 hours of incubation and then every second day thereafter until the cotyledons were shed from the seedlings on the 28th day of incubation. The fresh mass of the cotyledons was determined after they had been removed from the germination jars. The cotyledons were frozen with liquid nitrogen and stored at -20 $^{\circ}$C until further use. Protein was extracted from the cotyledons as described in Chapter 2, section 2.5. The protein and t-PA inhibitor content of the cotyledons were determined as described in Chapter 2 section 2.6 and

Chapter 3 respectively. The experiment was repeated three times and the mean values of the three experiments were recorded. The mean protein and t-PA inhibitor content was determined for each period of seed incubation. Germination was taken as having occurred when the radicle became visible. Subsequent development of the seedling is defined as seedling growth.

RESULTS AND DISCUSSION

During the two day period of imbibition and germination a relatively small decrease in the protein content of the cotyledons was observed (Figure 6.3). However, during the period of seedling development the protein content of the cotyledons decreased rapidly and reached a minimum value at day 22 when the cotyledons were shed from the seedlings (Figure 6.5). The total protein content of legume seeds was reported to decrease from the onset of germination as for *Vigna radiata* or to fluctuate during the initial period of germination and decrease during seedling development thereafter as with *Phaseolus vulgaris* seeds (PUSZTAI, 1972). However, it was observed that the protein content of the cotyledons of *Vicia faba* increased during the final stage of germination (BHATTY, 1977).

The t-PA inhibitor content of the cotyledons of *Erythrina caffra* decreased substantially during the period of germination and continued to decline rapidly up to day 14

Figure 6.3 The effect of imbibition and germination of seeds of *Erythrina caffra* on the protein and t-PA inhibitor content of the cotyledons. The t-PA inhibitor and protein content of the seeds were expressed in units of dry mass (DM).
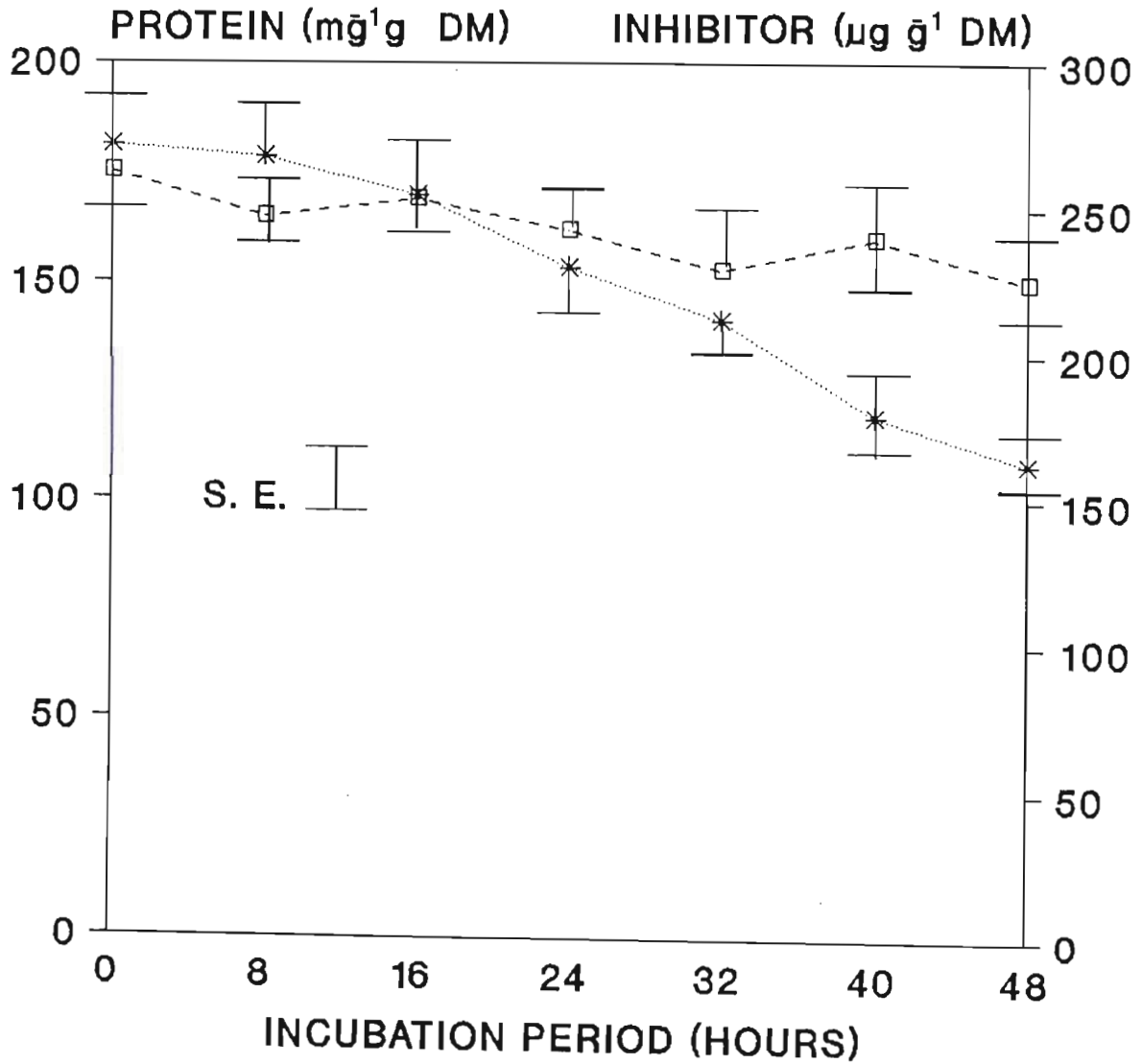
Figure 6.4 The effect of imbibition and germination of seeds of *Erythrina caffra* on the protein and t-PA inhibitor content of the cotyledons. The t-PA inhibitor and protein content of the cotyledons were expressed in units of protein and dry mass (DM) respectively.
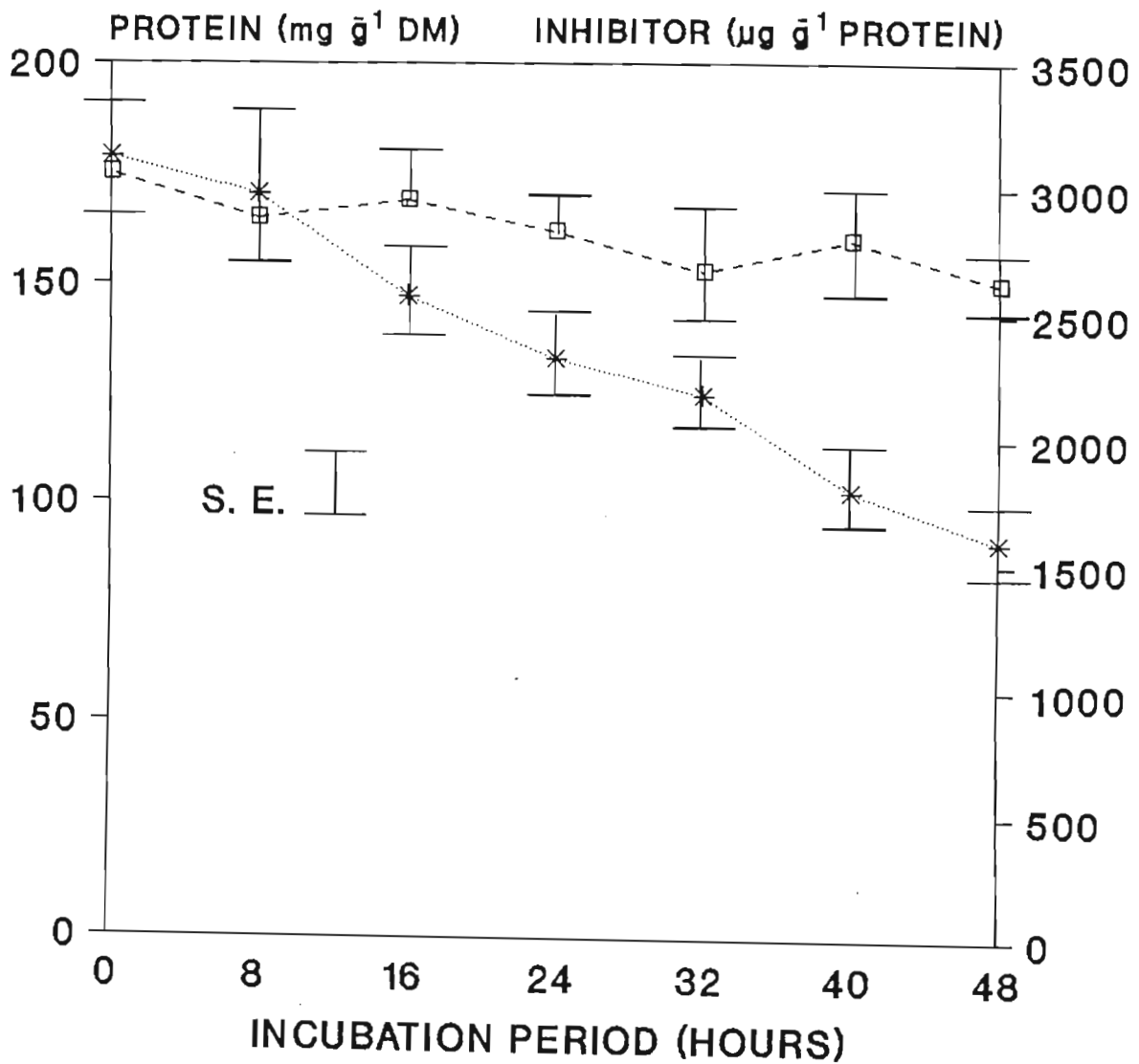
(Figure 6.3 and 6.4). Beyond this stage of seedling growth the t-PA inhibitor content of the cotyledons stabilised at a low level until the cotyledons were shed from the seedlings (Figure 6.5 and 6.6). As with *Erythrina caffra* a decrease of trypsin inhibitors was observed during the germination of seeds of *Vigna radiata* (BAUMGARTNER and CHRISPEELS, 1976 ; CHRISPEELS and BAUMGARTNER, 1978) and several cultivars of *Glycine max* (ORF, MIES and HYMOWITZ, 1977). However, the trypsin inhibitor content of seeds of *Phaseolus vulgaris* cultivars and *Vicia faba* increased during the initial phase of germination and decreased drastically during the later stage of germination and the early stage of seedling development (PUSZTAI, 1972 ; BHATTY, 1977). Similar observations were made for non-leguminous plants such as *Oryza sativa* (HORIGUCHI and KITAGISHI, 1971) and *Lactuca sativa* (SHAIN and MAIER, 1968). It is clear that the trypsin inhibitor content of the cotyledons change during germination and that it is lower at the end of the germination period. The period of imbibition and germination is not very well defined in the literature. It can be the period as defined for *Erythrina caffra* or include the period of seedling growth up to the shedding of the cotyledons by the seedling. This different descriptions of the germination process makes it difficult to compare the observations of different authors regarding the behaviour of trypsin inhibitors during germination.

The simultaneous decrease in the t-PA inhibitor and the total protein in the cotyledons during germination may

Figure 6.5   The protein and t-PA inhibitor content of the cotyledons of *Erythrina caffra* from the onset of imbibition until they were shed by the seedlings. The t-PA inhibitor and protein content were expressed in units of dry mass (DM).
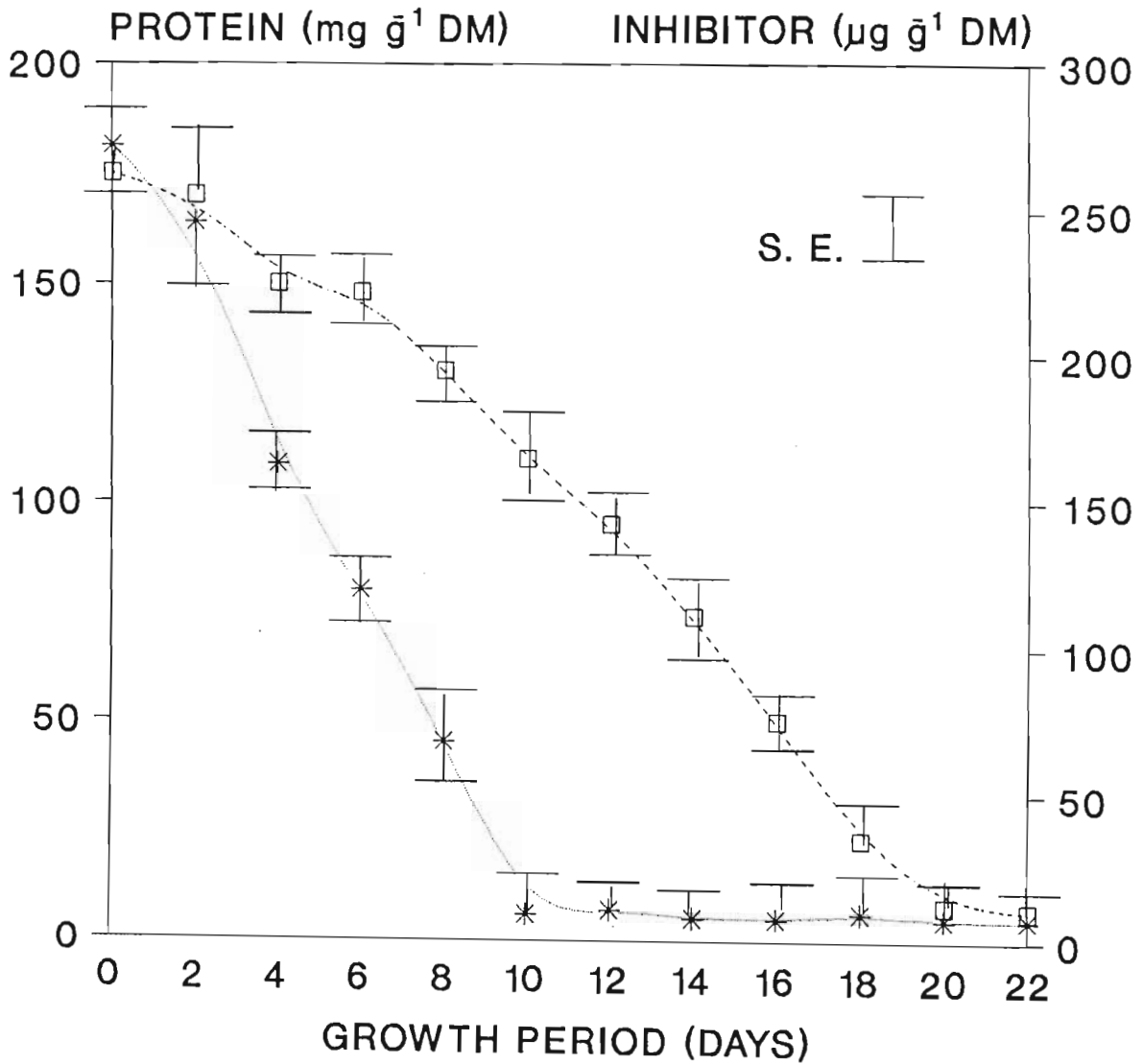
Figure 6.6 The effect of the growth of *Erythrina caffra* seedlings on the protein and t-PA inhibitor content of the cotyledons of *Erythrina caffra* from the onset of imbibition until they were shed by the seedlings. The t-PA inhibitor and protein content of the cotyledons were expressed in units of protein and dry mass (DM) respectively.
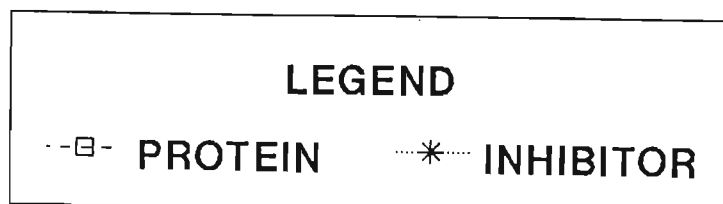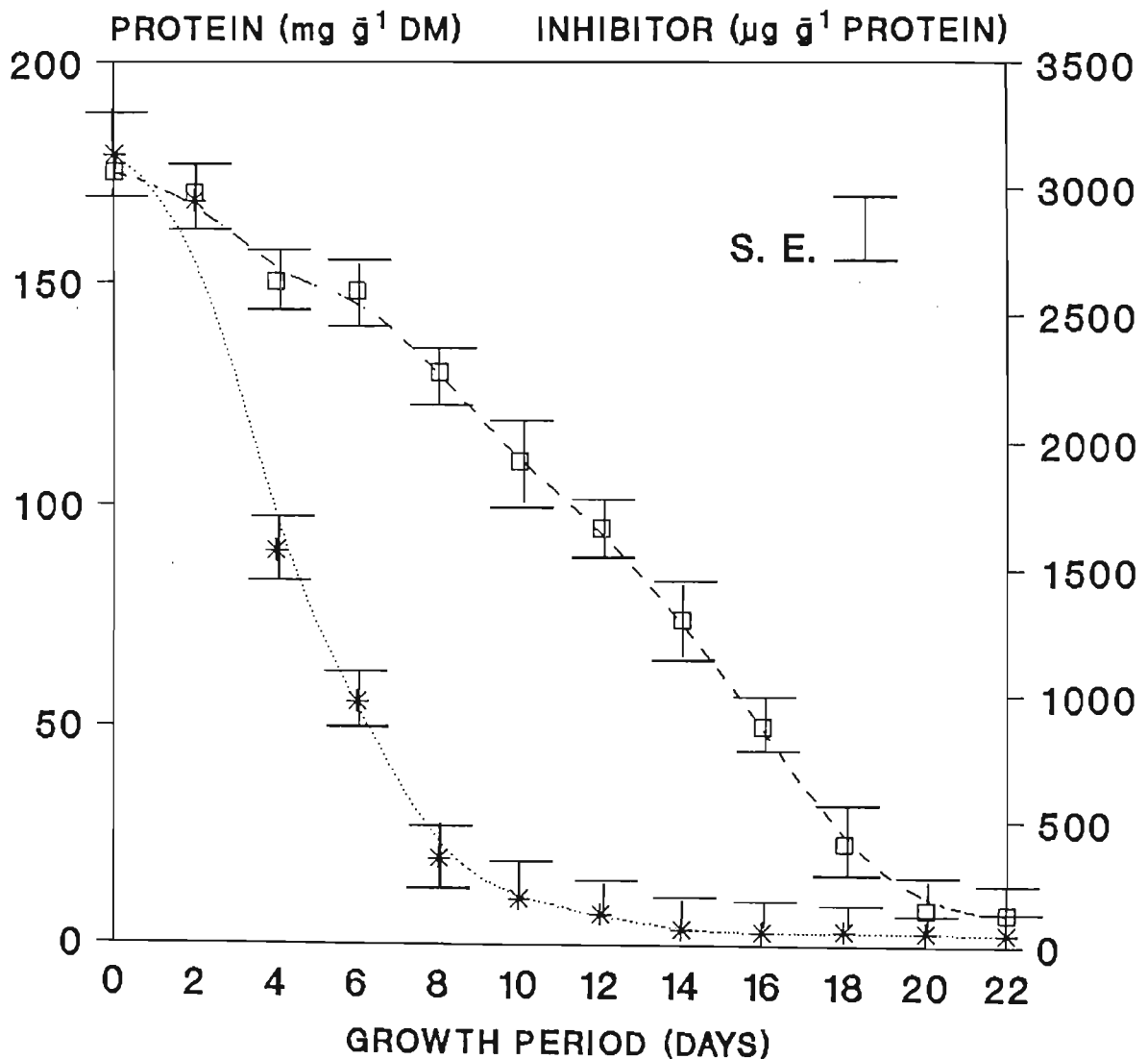
indicate that the inhibitor is degraded by endogenous proteinases and utilised by the growing embryo and seedling as happens for the storage proteins. It is well established that the storage proteins of the legumes are rich in asparagine, glutamine, arginine and proline (HIGGINS, 1984). This means that amino acids such as tryptophan and especially the sulphur containing amino acids cysteine and methionine are underrepresented in the main storage proteins of the seeds. A property of the inhibitors of trypsin and chymotrypsin and the t-PA inhibitor of *Erythrina caffra* is the relatively large number of cysteine residues in these molecules (VOGEL, TRAUTSCHOLD and WERLE, 1968 ; JOUBERT and DOWDLE, 1987). It was postulated that a function of these inhibitors can be to serve as a source of sulphur-containing amino acids for the developing embryo (RYAN, 1973).

It has been suggested that trypsin and related inhibitors inhibit the activity of endogenous proteinases. In a process of degradation of the proteinase inhibitor during germination the inhibited proteinases are released from this proteinase inhibitor to become active. However, it is clear from kinetic studies that the catabolism of trypsin inhibitors during germination and the simultaneous appearance of proteinase activity in both mung bean and scots pine are not causally related (BAUMGARTNER and CHRISPEELS, 1976 ; CHRISPEELS and BOULTER, 1975 ; CHRISPEELS and BAUMGARTNER, 1978 ; SALMIA, 1980). Thus it seems as if the function of trypsin inhibitors during germination is not

associated with their proteinase inhibiting activity.

The function of the trypsin and other proteinase inhibitors in seeds are obscure. If these proteinase inhibitors are essential to the development of the embryo, seeds will not germinate in the absence of the inhibitor. However, ORF and HYMOWITZ (1979) found seeds of cultivars of *Glycine max* lacking trypsin inhibitor activity. HYMOWITZ (1980) concluded that trypsin inhibitors are not essential for the structure or metabolism of the wild and cultivated soybean.

Can it thus be speculated that the proteinase inhibitors have an non-essential function in the cotyledons such as being a portion of the storage proteins ?. This does not seem likely since recent studies indicated that proteinase inhibitors in cotyledonary tissue can undergo specific degradation, different from the non-specific degradation of the true reserve proteins (YOSHIKAWA, KIYOHARA, IWASAKI and YOSHIDA, 1979 ; LORENSEN, PREVOSTO and WILSON, 1981 ; HARTL, TAN-WILSON and WILSON, 1986; WILSON and CHEN, 1983 ; MADDEN, TAN-WILSON and WILSON, 1985). The occurrence of a large number of trypsin inhibitors and iso-inhibitors in seeds (VOGEL, TRAUTSCHOLD and WERLE, 1968 ; CHEN and MITCHELL, 1973 ; RYAN, 1981) as well as the disappearance of these inhibitors and iso-inhibitors at different times and the appearance of iso-inhibitors during germination (PUSZTAI, 1972 ; FREED and RYAN, 1978 ; TAN-WILSON and WILSON, 1982 ; UDUPA and PATTABIRAMAN, 1987) is an indication that the function of trypsin inhibitors during germination is

probably more complex than being a source of nitrogen or sulphur to the seedling. ORF, MIES and HYMOWITZ (1977) suggested that new bands of trypsin inhibitors which appear during germination was a modification of the original inhibitors and not the result of the synthesis of new inhibitors. A limitation of the ELISA for t-PA and ELISA's in general is that the slightest change in the conformation of the t-PA inhibitor molecule due to limited degradation will lead to a change in the epitope for the antibodies raised against the native molecule. This will result in a negative immunoassay indicating that the inhibitor is not present in the tissue used. The suggested disappearance of the t-PA inhibitor during germination does not rule out the possibility that the inhibitor was just slightly degraded by hydrolytic enzymes *in vivo*. The modification of the molecule through hydrolysis can change its antigenic properties. This can result in the lack of detecting the modified molecule although it may still be present in the tissue in a slightly modified form.

6.4 THE EFFECT OF PROTEIN INDUCING COMPOUNDS ON THE t-PA INHIBITOR CONTENT OF LEAVES OF *ERYTHRINA CAFFRA*

EXPERIMENTAL

Several compounds which have been reported to induce the

synthesis of proteins were used to determine whether these chemicals would increase the t-PA inhibitor content of excised leaves of *Erythrina caffra*. The following substances were used spermine (0.1 mM) ; spermidine (0.1 mM) ; 1-aminocyclopropane-1-carboxylic acid (ACC) (0.1 mM) ; phytic acid (1 mM) ; polyethylene-glycol chitin (1 mgl^{-1}). Cell wall hydrolysates of *Erythrina caffra*, *Ecklonia maxima* and *Lycopersicon esculentum* were used at concentrations of 0.01, 0.1 and 1 grammes per litre. The control treatment consisted of distilled autoclaved water. The cell wall hydrolysates were prepared as described in Chapter 2, sections 2.2 and 2.3. The other compounds were made up to the proper concentrations in distilled autoclaved water. Flat bottomed vials (25 mm X 50 mm) were filled with the treatment solutions.

Fully expanded leaves of *Erythrina caffra* plants grown in a greenhouse were used. The leaves were disease free and had no visible signs of insect damage. The petioles of the leaves were cut at the base of the petiole with a sharp razor blade and were immediately immersed in distilled water. In the laboratory the petioles of the leaves were immediately randomly placed in each of the vials filled with treatment solution. The leaves were incubated at 25 $^{\circ}$C under a quantum flux density (400-700 nm) of 50 to 60 umoles m^{-2} sec.$^{-1}$ After 48 hours of incubation the leaves were dried with paper toweling and their fresh mass determined. Immediately after weighing the leaves were frozen with liquid nitrogen and stored at -20 $^{\circ}$C. The leaf material

was pulverised in liquid nitrogen with a mortar in pestle. The chlorophyll was extracted with acetone using the defatting procedure described in Chapter 2, section 2.4. The protein extraction and the determination of total protein and t-PA inhibitor was done as described in Chapter 2, sections 2.5 and 2.6 and Chapter 3. Three replications were used per treatment and three to four leaves were used per replication. The experiment was a complete random design and analysed accordingly.

RESULTS AND DISCUSSION

The t-PA inhibitor content of the cell extract was not significantly increased by any of the treatments (Tables 6.7 and 6.9). The t-PA inhibitor content of the leaves was slightly decreased by all the protein inducing compounds (Tables 6.8 and 6.10). No significant differences were observed in the total protein content of the leaves for all the treatments (Table 6.11 and 6.12). Various substances have been reported to induce the accumulation of proteins or increase the activity of enzymes in plants. For example the phenylalanine ammonia lyase activity in *Pisum sativum* was increased by spermine, spermidine, synthetic polyamines and various natural and synthetic polypeptides (HADWIGER, JAFRI, VON BROEMSEN and EDDY, 1974). Phenylalanine ammonia lyase activity was also increased by glucans from the mycelial cell walls of *Phytophthora megasperma* Drechs f. sp. glycinea (previously *Phytophthora megasperma* Drechs var.

Table 6.7 Analysis of variance of the effect of protein
inducing compounds on the t-PA inhibitor content of
excised leaves of *Erythrina caffra* expressed in
terms of units protein.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	525.3	8	65.7	1.2
Residual	963.0	17	56.6	
Total	1488.3	23		

Table 6.8 Multiple range analysis of the effect of
protein inducing compounds on the t-PA inhibitor
content of excised leaves of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups
Protein inducing compound	t-PA inhibitor content of leaf ($\mu g\ g^{-1}$ protein)	
Phytic acid	24.3	*
ACC	28.9	*
Spermine	57.0	*
Spermidine	59.8	*
Ethylene glycol chitin	65.9	*
Cell wall hydrolysates of:		
Ecklonia maxima	56.5	*
Erythrina caffra	62.5	*
Lycopersicon esculentum	63.2	*
Control	67.3	*

* In different columns indicate significant differences at a
95% level of confidence.

Table 6.9 Analysis of variance of the effect of protein inducing compounds on the t-PA inhibitor content of excised leaves of *Erythrina caffra* expressed in terms of units of fresh mass.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	25.1	8	3.1	1.0
Residual	52.5	17	3.1	
Total	125828	23		

Table 6.10 Multiple range analysis of the effect of protein inducing compounds on the t-PA inhibitor content of excised leaves of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups
Protein inducing compound	t-PA inhibitor content of leaf (μg g^{-1} FM)	
Phytic acid	0.7	*
ACC	0.9	*
Spermine	1.7	*
Spermidine	1.8	*
Ethylene glycol chitin	1.8	*
Cell wall hydrolysates of:		
Ecklonia maxima	1.9	*
Lycopersicon esculentum	1.8	*
Erythrina caffra	2.0	*
Control	2.1	*

* In different columns indicate significant differences at a 95% level of confidence. FM = Fresh mass.

Table 6.11 Analysis of variance of the effect of different
protein inducing compounds on the protein content
of excised leaves of *Erythrina caffra*.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	384.9	8	48.1	1.2
Residual	684.5	17	40.3	
Total	1090.4	23		

Table 6.12 Multiple range analysis of the effect of protein
inducing compounds on the protein content of excised
leaves of *Erythrina caffra*.

Treatment	Treatment mean	Homogeneous groups
Protein inducing compound	Protein content of leaf (mg g^{-1} FM)	
Ethylene glycol chitin	13.6	*
Phytic acid	14.5	*
Spermine	14.9	*
Spermidine	15.0	*
ACC	15.5	*
Control	15.6	*
Cell wall hydrolysates of:		
Lycopersicon esculentum	14.2	*
Erythrina caffra	16.0	*
Ecklonia maxima	16.8	*

* In different columns indicate significant differences at a
95% level of confidence. FM = Fresh mass.

sojae A. A. Hildb.). Cell wall fragments of several fungi also increased the activity of other enzymes. For example the chitinase activity in *Pisum sativum* and *Cucumis melo* L. cv. cantaloupe was increased by a pure hyphal cell wall extract of *Fusarium solani* f. sp. *phaseoli* and *Colletotrichum lagenarium* (Pers.) E. and H. respectively (DATEMA, WESSELS and VAN DEN ENDE, 1977 ; HADWIGER and BECKMAN, 1980). It was reported that purified mycelial cell walls of *Phytophthora megasperma* Drechs f. sp. glycinea increase the flavanone synthetase activity of *Glycine max* (ZAHRINGER, EBEL and GRIESEBACH, 1978). Pectic fragments originating from the hydrolytic activity of endopolygalacturonase from *Rhizopus stolonifer* (Ehr. and Fries) on the cell walls of *Ricinus communis* L. resulted in a large increase in the casbene synthetase activity of the plants (LEE and WEST, 1981).

Pathogenesis-related proteins were observed to appear in *Nicotiana tabacum* when the plants were treated with salicylic acid, ethephon, benzoic acid, phytic acid, polyacrylic acid, ACC and various other substances (VAN LOON, 1983). Since it was reported that proteinase inhibitors accumulate during the fungal infection of plants (PENG and BLACK, 1976) the possibility exists that some of the substances which introduce pathogenesis-related proteins may also induce the accumulation of t-PA inhibitor in *Erythrina caffra*. The effect of some of the mentioned compounds such as polyethyleneglycol chitin, chitosan, spermine, spermidine and ACC were determined with excised

leaves of *Erythrina caffra* to establish whether they could increase the t-PA inhibitor content of the cells. However, none of these compounds increased the t-PA inhibitor content of the leaves. These protein inducing compounds therefore do not seem to cause a general increase in proteins but to be very specific in their action.

GREEN and RYAN (1972) reported on a proteinase inhibitor inducing factor (PIIF) which was released by the leaves of *Lycopersicon esculentum* and *Solanum tuberosum* upon insect damage or mechanical wounding. This factor resulted in a large increase in the proteinase inhibitor concentration of the affected leaves. The proteinase inhibitor in tomato suspension cultures was also induced by chitosan, ethylene glycol chitin and a trigalacturonic acid (WALKER-SIMMONDS and RYAN, 1986). An increase in the trypsin inhibitor content of the leaves of *Medicago sativa* L. was reported when the leaves were damaged or tomato PIIF supplied to the leaves (BROWN and RYAN, 1984). The tomato PIIF was produced by the heat hydrolysis of tomato leaf cell walls (RYAN, 1974a). The addition of hydrolysed cell wall extracts from *Lycopersicon esculentum*, *Ecklonia maxima* and *Erythrina caffra* to excised leaves of *Erythrina caffra* did not increase the t-PA inhibitor level of the leaves. The cause of this inability of *Erythrina caffra* leaves to react to the elicitor stimulus is not clear. It was found that the effect of the tomato PIIF on tomato plants was both light and temperature dependent. Proteinase inhibitor did not accumulate in darkness or below 20 $^{\circ}$C. The maximum response

was at a light intensity of 600 foot candles. In the light
the reaction of the tomato plants towards the PIIF was
temperature dependent (GREEN and RYAN, 1973). The leaves of
Erythrina caffra were incubated in fluorescent light with
photosynthetic active radiation (400-700 nm) of 50 to
60 μmoles m^{-2} sec^{-1} which is lower than the the light
intensity used for tomato plants by GREEN and RYAN (1973).
This suggests that the light intensity could be the limiting
factor although no response was obtained by GREEN and RYAN
(1973) in darkness. The incubation temperature of the
leaves of 25 ^{o}C could be below the optimal temperature for
the induction of t-PA inhibitor since 36 ^{o}C was the optimal
temperature for the accumulation of tomato proteinase
inhibitor. However, the minimum temperature for the
induction of the proteinase inhibitor in tomato leaves was
20 ^{o}C (GREEN and RYAN, 1973). It was reported that all
plants do not react similarly to *Lycopersicon*
esculentum or *Solanum tuberosum* on the tomato PIIF or on
possible endogenous inducers of proteinase inhibitors. The
effect of tomato PIIF and endogenous PIIF was determined
for 23 species which covered ten plant families. Seven
species did not respond to the treatment. Six members of
the Leguminosae were used in the experiment and in four of
them the proteinase inhibitory activity of the leaves was
not increased with the treatment (WALKER-SIMMONDS and RYAN,
1977a). This also seems to be true for *Erythrina caffra*.
It is not clear why some plants with proteinase inhibitors
do not accumulate these inhibitors when treated with
hydrolysed cell wall fragments.

6.5 THE OCCURRENCE OF t-PA INHIBITOR IN MEMBERS OF THE GENUS *ERYTHRINA*

EXPERIMENTAL

Fully expanded young leaves of several members of the genus *Erythrina* was collected at the residence of dr. I. A. WHITTON in Durban. Care was taken to collect only leaves without signs of insect damage. The leaves were collected on the same day and stored at 10 ^{o}C. Within five hours after collection the leaf material was rinsed with tap water, dried and weighed. The leaves were then frozen in liquid nitrogen and stored at -20 ^{o}C. The leaves were pulverised in liquid nitrogen with a mortar and pestle. Chlorophyll was extracted from the leaf material with cold acetone. Protein was extracted from the leaves and the protein content of the material was determined as described in Chapter 2, section 2.5 and 2.6. The material was assayed for t-PA inhibitor with the ELISA described in Chapter 3. Since only one tree per species was available no statistical analysis was done on the data.

RESULTS AND DISCUSSION

The number of species sampled represent 22 % of the total number of species of the genus *Erythrina*. This excludes subspecies, varieties and different forms of the genus. Comparing the total protein in the leaves of the different

species with the t-PA content of the leaves it is clear that there is very little correlation between the protein content and the t-PA inhibitor in the leaves (Figure 6.7). The correlation coefficient for the t-PA inhibitor and the protein content of the different species was very low with a value of 0.196. The t-PA content of the leaves of the different species based on the protein content followed a trend similar to that of the t-PA inhibitor content based on dry mass (Figure 6.8).

When arranged in the different taxonomic sections it is clear from Table 6.13 that all the species from the different taxonomic sections contain t-PA inhibitor in their leaves. The species can be grouped according to their t-PA inhibitor content. The first group of 7 species has a low level of t-PA inhibitor. The second group of 16 species has a higher concentration of inhibitor and the last group of 5 species yielded substantially higher levels of t-PA inhibitor than the first two groups (Figure 6.7). The species with the highest levels of t-PA inhibitor are *Erythrina senegalensis*, *E. humeana*, *E. caffra* and the two members of the hybrid *E. coddii*. There does not seem to be a relationship between the t-PA inhibitor content of the species and either their taxonomic classification or their geographical distribution. The relatively high levels of t-PA inhibitor in *Erythrina coddii* indicates the possibility of increasing the levels of t-PA inhibitor through hybridisation.

Based on the species distribution data of KRUKOFF (1982) the species sampled represent the following percentages of the

Figure 6.7 The protein and t-PA inhibitor content of the leaves of various members of the genus *Erythrina*. The t-PA inhibitor and protein content were expressed in units of dry mass (DM).
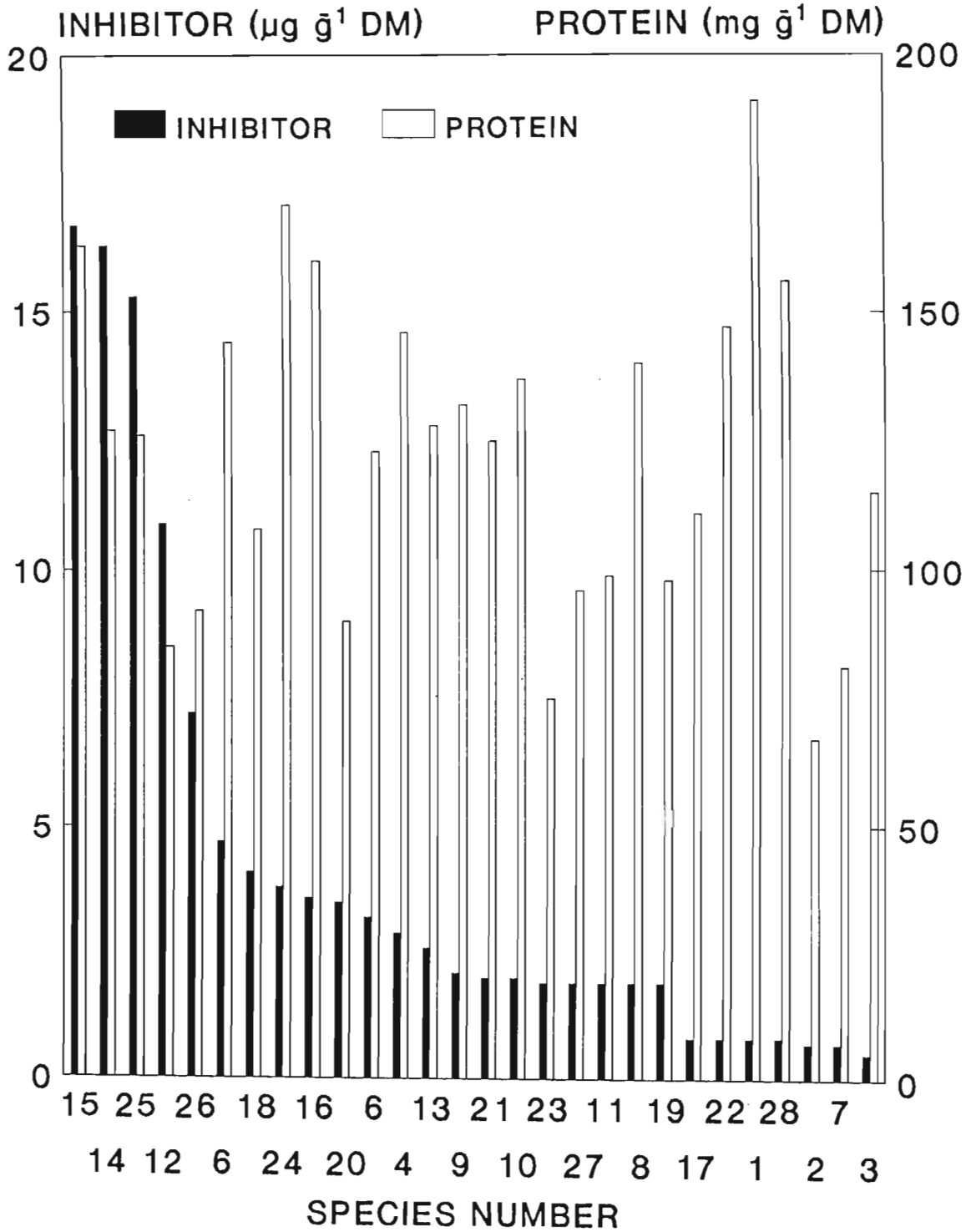
Figure 6.8 The t-PA inhibitor content of the leaves of various members of the genus *Erythrina*. The t-PA inhibitor content is expressed in units of protein and dry mass (DM).
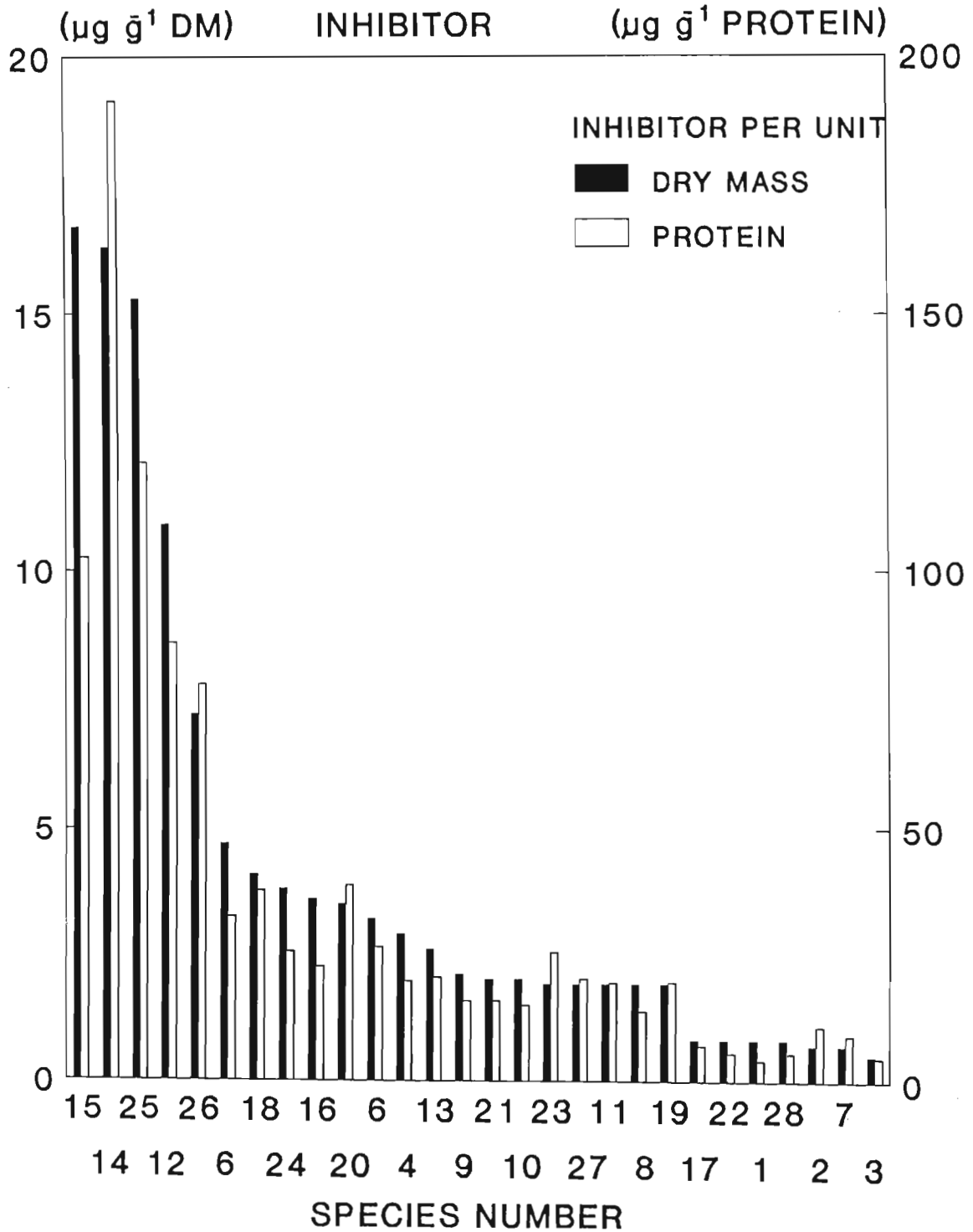
Table 6.13   The occurrence of t-PA inhibitor in leaf material of
members of the genus *Erythrina*.

Identifi-cation number	Taxanomic section	Species	Geographical distribution	t-PA Inhibitor ($\mu g\ g^{-1}$)	
				DM	PROTEIN
1	Cristae-galli	*E. crista-galli* L.	S. Am.	0.8	4.1
2	Cristae-galli	*E. falcata* Benth.	S. Am.	0.7	10.8
3	Micropteryx	*E. dominguezii* Hass.	S. Am.	0.5	4.7
4	Micropteryx	*E. poeppigiana* Cook	S. Am.	2.9	19.7
5	Erythrina	*E. flabelliformis* Ker.	C. Am.	3.2	26.4
6	Erythrina	*E. americana* Mill.	C. Am.	4.7	32.5
7	Erythrina	*E. lanceolata* Stand.	C. Am.	0.7	9.0
8	Erythrina	*E. berteroana* Urban.	S. Am.	1.9	13.7
9	Erythrina	*E. coralloides* M.& S.	Mexico	2.1	15.8
10	Corallodendra	*E. pallida* Britt.	S. Am.	2.0	14.9
11	Caffrae	*E. lysistemon* Hutch.	T. Afr.	1.9	19.3
12	Caffrae	*E. caffra* Thunb.	T. Afr.	10.9	85.8
13	Humeanae	*E. zeyheri* Harv.	T. Afr.	2.6	20.4
14	Humeanae	*E. humeana* Spreng.	S. Afr.	15.3	121.0
15	Macrocymbium	*E. senegalensis* DC.	T. Afr.	16.7	102.6
16	Chirocalyx	*E. livingstoniana* Bak.	T. Afr.	3.6	22.5
17	Chirocalyx	*E. abyssinica* Lam.	T. Afr.	0.8	7.0

Table 6.13   Continued

Identification number	Taxonomic section	Species	Geographical distribution	t-PA Inhibitor ($\mu g\ g^{-1}$)	
				DM	PROTEIN
18	Chirocalyx	*E. decora* Harms	T. Afr.	4.1	37.7
19	Chirocalyx	*E. sigmoidea* Pobeguin	T. Afr.	1.9	19.5
20	Chirocalyx	*E. latissima* E. Meyer	T. Afr.	3.5	38.8
21	Erythraster	*E. perrieri* Vieg.	T. Afr.	2.0	15.8
22	Erythraster	*E. burana* Chiov.	T. Afr.	0.8	5.5
23	Erythraster	*E. burttii* Baker	T. Afr.	1.9	25.4
24	Erythraster	*E. melanacantha* Taub.	T. Afr.	3.8	22.5
		Hybrids			
25		*E. coddii* Bar. & Kru.	Mel.	16.3	191.0
26		*E. coddii* Bar. & Kru.	Emp.	7.2	77.9
27		*E. johnsoniae* Fr.-Henn.	S.Afr.	1.9	20.1
28		*E. sykesii* Bar. & Kru.	Aus.	0.8	5.0

T. Afr. = Tropical Africa.   S. Afr. = Southern Africa.

S. Am. = South America.   C. Am. = Central America.

Mel.   = Melmoth (Republic of South Africa).

Emp.   = Empangeni (Republic of South Africa).

Aus.   = Australia.

total number of species indigenous to the following countries: South America (28%), Central America (12%), Mexico (3%), Tropical Africa (42%) and South Africa (80%). No species were available from the West Indies, Continental Asia and the Pacific Islands. Seed from a hybrid was obtained from Australia. From the results it is clear that t-PA inhibitor is present in all the species irrespective of their geographical distribution. It seems as if the t-PA inhibitor is a substance common to the genus *Erythrina*. The possible occurrence in members from genera closely related to *Erythrina* has not yet been investigated. It will be of taxonomic value to determine whether all the members of the genus *Erythrina* contain t-PA inhibitor and whether the inhibitor is also present in closely related genera.

CHAPTER   7

THE   OCCURRENCE   OF   t-PA   INHIBITOR   IN   *IN   VITRO*

CULTURES   OF   *ERYTHRINA   CAFFRA*

7.1   THE   OCCURRENCE   OF   t-PA   INHIBITOR   IN   CALLUS
CULTURES   OF   *ERYTHRINA   CAFFRA*

EXPERIMENTAL

Callus cultures from root, shoot, leaf, embryo and cotyledonary material of *Erythrina caffra* were maintained *in vitro* as described in Chapter 4. The dry mass was determined for three replicates of actively growing calli. Protein was extracted and determined from the calli as described in Chapter 2, sections 2.5 and 2.6 respectively. The t-PA inhibitor content of the samples were determined as described in Chapter 3. The experiment was a complete randomised design.

RESULTS AND DISCUSSION

No significant difference could be detected between the protein or t-PA inhibitor content of the calli from different organs (Table 7.1; 7.3; 7.5). The t-PA inhibitor was present in very small amounts in the callus derived from different organs (Table 7.4; 7.6). Compared with the concentration of t-PA inhibitor in intact organs the t-PA inhibitor content of the calli was approximately ten times lower than the concentration in roots, shoots, leaves and living green bark. The t-PA inhibitor content of cotyledonary material was more than 400 times higher on a dry mass basis and more than 2000 times higher based on protein content than the t-PA inhibitor content of the calli. It is thus clear that very low concentrations of t-PA inhibitor is present in callus cultures. The callus cultures will only be of any practical importance as a source of t-PA inhibitor if the level of t-PA inhibitor can be increased drastically.

There was no significant difference in the protein content of the calli from different organs (Table 7.2). In comparison with Table 6.2 the protein content of the calli was similar to the protein content of intact shoots and living green bark. However, it was more than four times lower than the protein content of intact leaves and roots and nearly 40 times lower than the protein levels in cotyledonary tissue.

Table 7.1 Analysis of variance of the protein content of callus derived from different parts of *Erythrina caffra* plants. Protein content is expressed as a unit of dry mass.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	2.67	4	0.667	0.81
Residual	16.57	20	0.829	
Total	19.24	24		

Table 7.2 Multiple range analysis of the protein content of callus derived from different parts of *Erythrina caffra* plants. Protein content is expressed as a unit of dry mass.

Treatment	95% Tukey intervals	Homogeneous groups
Origin of callus	Protein (mg g^{-1} DM)	
Leaf	3.9	*
Cotyledon	3.9	*
Shoot	3.8	*
Embryo	3.7	*
Root	3.5	*

* In different columns indicate significant differences at a 95% level of confidence. DM = Dry mass.

Table 7.3   Analysis of variance of the t-PA inhibitor
             content of callus derived from different parts
             of *Erythrina caffra* plants.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	3.17	4	0.79	0.82
Residual	19.36	20	0.97	
Total	22.53	24		

Table 7.4   Multiple range analysis of the t-PA inhibitor
             content of callus derived from different parts
             of *Erythrina caffra* plants. Inhibitor content
             is expressed as a unit of dry mass.

Treatment	95% Tukey intervals	Homogeneous groups
Origin of callus	Inhibitor (μg g^{-1} DM)	
Shoot	1.3	*
Root	1.1	*
Cotyledon	1.0	*
Leaf	0.9	*
Embryo	0.9	*

* In different columns indicate significant differences at
   a 95% level of confidence. DM - Dry mass.

Table 7.5   Analysis of variance of the t-PA inhibitor content of callus derived from different parts of *Erythrina caffra* plants. Inhibitor content is expressed as a unit of protein.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Treatment	3.24	4	0.81	0.91
Residual	17.85	20	0.892	
Total	21.09	24		

Table 7.6   Multiple range analysis of the t-PA inhibitor content of callus derived from different parts of *Erythrina   caffra*   plants.

Treatment	95% Tukey intervals	Homogeneous groups
Origin of callus	Inhibitor ($\mu g\ g^{-1}$ protein)	
Shoot	5.1	*
Root	4.6	*
Cotyledon	3.8	*
Leaf	3.3	*
Testa	3.2	*

*   In different columns indicate significant differences at a 95% level of confidence.   DM - Dry mass.

Reports on the occurrence of proteinase inhibitors *in vitro* are scarce in the literature. A proteinase inhibitor of *Nicotiana tabacum* plants accumulated in a callus culture. The inhibitor content of the callus increased steadily during the growth of the culture and attained its highest concentration in callus in a phase of reduced growth (WONG, KUO and RYAN, 1975). However, when the callus tissue had adapted to the new nutritional conditions after subculturing the inhibitor content of the callus decreased. It was suggested that the inhibitor was degraded and used for the establishment of new growth. Total protein remained constant in callus until the growth rate decreased. Thereafter the protein content of the cells also decreased. Inhibitor preferentially accumulated while the total protein decreased. The accumulation and utilisation of the inhibitor was found to be growth dependent which is unusual for vegetative plant tissue (WONG, KUO and RYAN, 1975).

7.2 THE OCCURRENCE OF t-PA INHIBITOR IN SUSPENSION CULTURES OF *ERYTHRINA CAFFRA*

Suspension cultures of shoot derived callus was prepared as described in Chapter 5. The nutrient medium used was the same as described for the stock solution in Chapter 5. A 100 millilitre aliquot of suspension culture in the exponential growth phase was decanted into a sterile measuring cylinder and left to settle out. The spent stock solution was

pipetted off and the cells washed with 50 millilitres fresh culture medium. The procedure was repeated three times with fresh medium. The cells were suspended in 100 millilitres fresh stock medium as described in Chapter 5 and decanted into a 500 millilitre Erlenmeyer flasks with side arms. The suspension cultures were incubated at a quantum flux (400-700 nm) of 5-10 μmole m^{-2} sec^{-1}, 25 ^{o}C and shaken at 60 rpm. The growth of the suspension was recorded every day by using the side arm as described in Chapter 5. Ten millilitres of the suspension culture was aseptically removed at each growth phase as described in Chapter 5. The cells were vacuum filtered and washed with 50 millilitres of distilled water. After filtration the fresh and dry mass was determined for each sample. The cells were immediately frozen in liquid nitrogen and stored at -20 ^{o}C. Protein was extracted from the cells as described in Chapter 2. The total protein and t-PA inhibitor content of the samples were determined as described in Chapters 2 and 3 respectively. The extractions were done with three different cultures at different times. The means and the standard errors were determined for the results.

RESULTS AND DISCUSSION

It was clear from the results in Figure 7.1 and 7.2 that no substantial differences were found in the t-PA inhibitor and protein content of the different growth phases of the suspension cultures. There was slightly less t-PA

Figure 7.1 The protein content of shoot cell suspension cultures of *Erythrina caffra* at the different growth phases of the culture after 12 days of incubation. The settled cell volume (SCV) was used as a measure of growth.
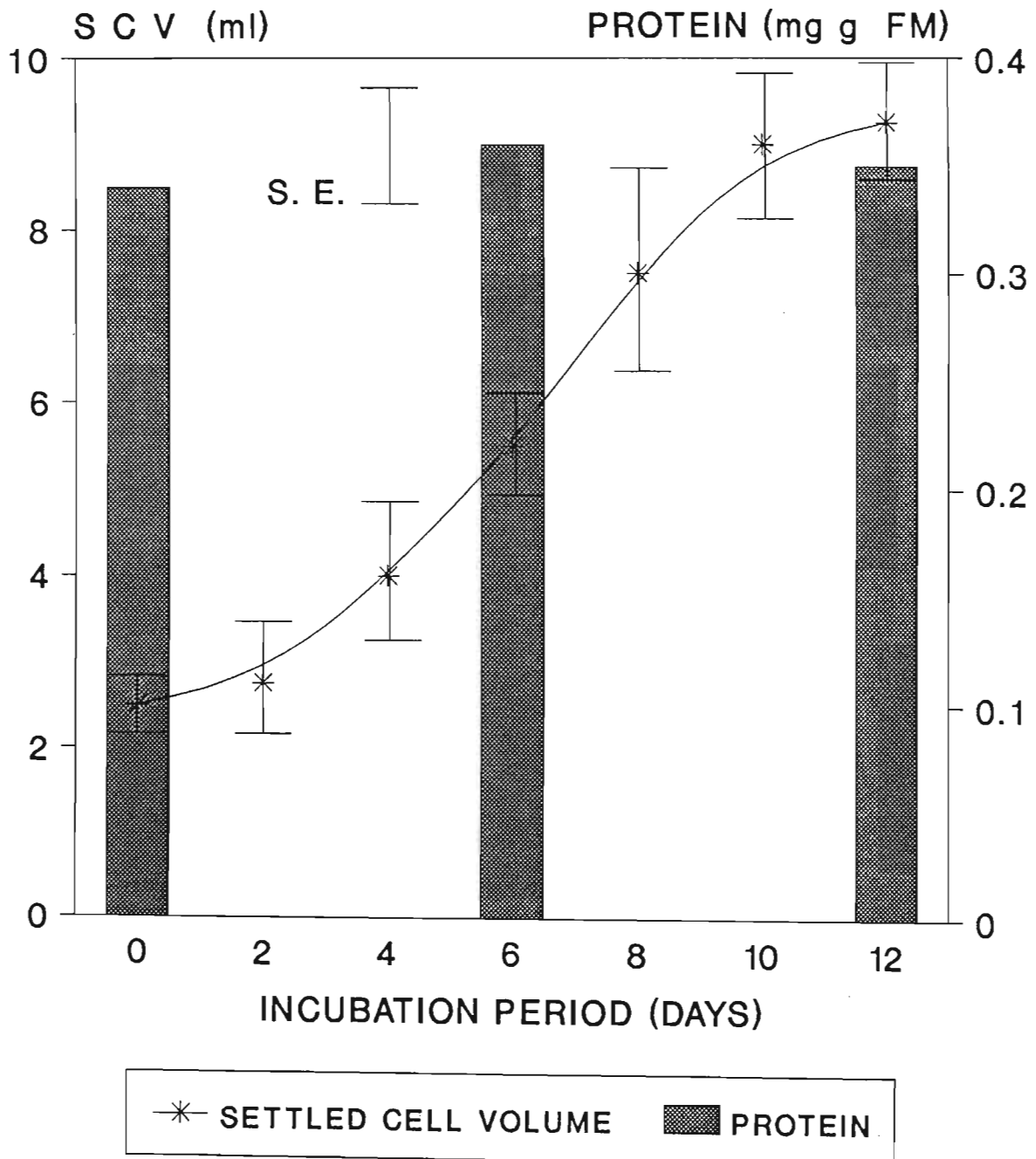
Figure 7.2   The t-PA inhibitor content of shoot cell
             suspension cultures of *Erythrina caffra*
             at the different growth phases of the
             culture after 12 days of incubation.
             Settled cell volume (SCV) was used as a
             measure of growth.
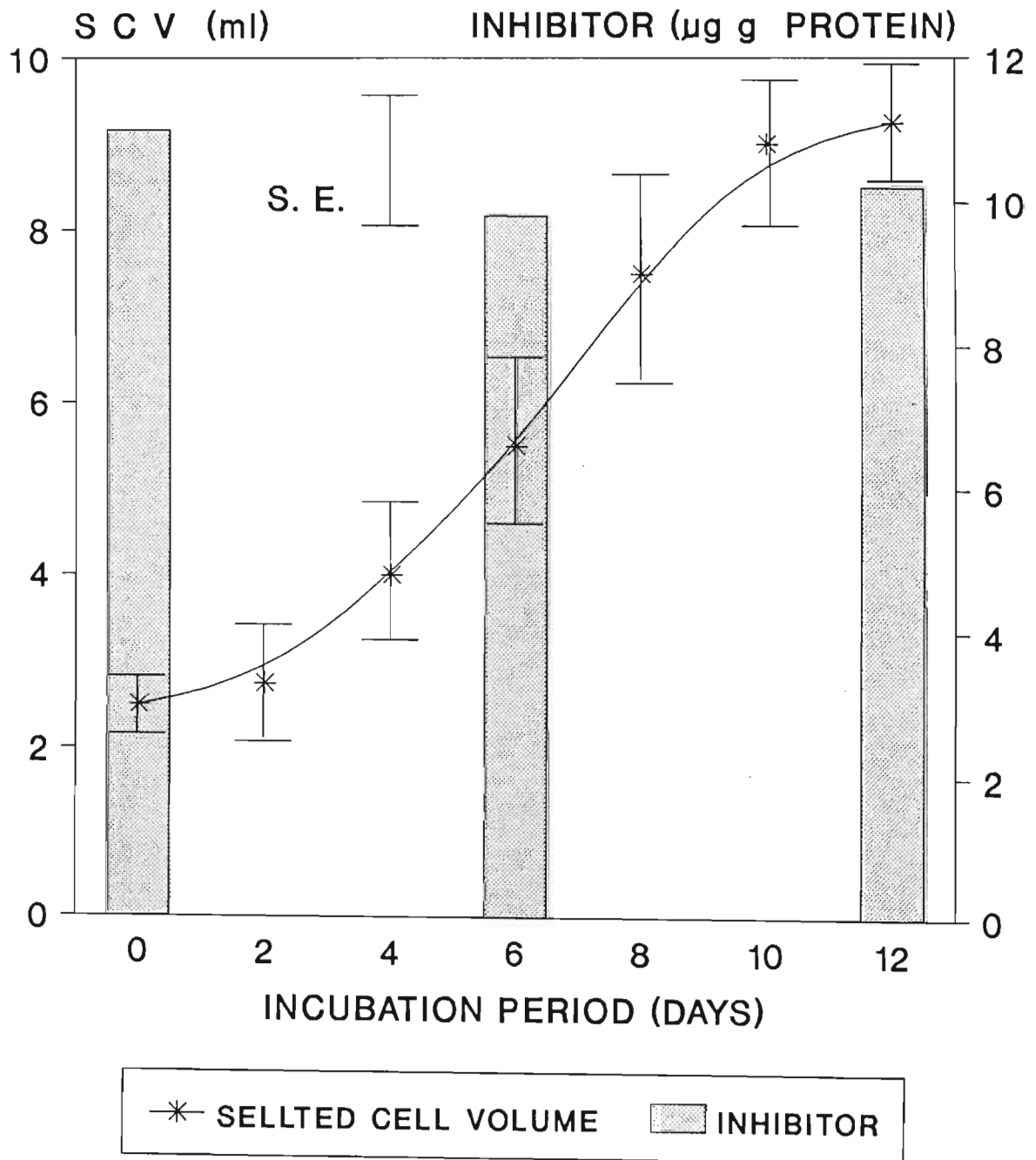
inhibitor in the linear growth phase than in the lag or stationary phases. It is possible that the t-PA inhibitor is preferentially degraded and utilised in some other manner during the phase of active cell division. A decrease in the levels of a proteinase inhibitor in *Nicotiana tabacum* callus cultures was reported by WONG, KUO and RYAN (1975). They suggested that the proteinase inhibitor was recycled during the phase of active cell division.

Plants which contain proteinase inhibitors *in vivo* seem to accumulate the inhibitors in suspension cultures. A proteinase inhibitor was reported to accumulate in a cell suspension culture of *Nicotiana tabacum* (WONG, KUO and RYAN, 1975). Trypsin inhibitors were found in suspension cultures of *Daucus carota* L. (CARLBERG, JONSSON, BERGENSTRAHLE and SODERHALL, 1987) and a *Lycopersicon esculentum* X *Lycopersicon peruvianum* L hybrid (WALKER-SIMMONDS and RYAN, 1986). Proteinase inhibitors can even accumulate in higher quantities *in vitro* than *in vivo*. This was reported for a plasmin inhibitor which accumulated in higher quantities in a root cell suspension culture of *Scopolia japonica* than in the plant (SAKATO, TANAKA and MISAWA, 1975).

The growth phase of the suspension culture which affects the physiological condition of the cells in the culture has a pronounced effect on the production of proteinase inhibitors. This could not be clearly demonstrated with the suspension culture of *Erythrina caffra*. However, the

levels of a plasmin inhibitor in cell suspension cultures of *Scopolia japonica* and a proteinase inhibitor in callus cultures of *Nicotiana tabacum* increased during the stationary growth phase of the cultures (MISAWA, TANAKA, CHIYO and MUKAI, 1975 ; WONG, KUO and RYAN, 1975). It was reported that a trypsin inhibitor in the suspension culture of the *Lycopersicon esculentum* hybrid increased substantially with an increase in the age of the culture (WALKER-SIMMONDS and RYAN, 1986). With the increase in inhibitor a concomitant decrease in total protein was observed (WONG, KUO and RYAN, 1975). This indicates that the proteinase inhibitors are probably involved in a specific physiological process in cell cultures. A significant observation was the appearance of an uronic acid-rich polysaccharide in the suspension medium prior to the accumulation of a proteinase inhibitor in a tomato cell suspension culture. The addition of the partially purified polysaccharide to the suspension culture induced the accumulation of the proteinase inhibitor (WALKER-SIMMONDS and RYAN, 1986). It thus seems that the accumulation of proteinase inhibitors in the stationary phase is the consequence of the release of a specific inducing compound by the cells. The proteinase inhibitor inducing compound does not seem to be unique to *in vitro* cultures. It seems to be a pectic breakdown product linked with a defense mechanism in plants. A pectic fragment containing a high percentage of polygalacturonic acid was reported to induce the accumulation of a proteinase inhibitor in tomato plants. This pectic fragment was released by the plant when it was

mechanically damaged (RYAN, 1988). It is not clear which factors in suspension cultures trigger the breakdown of the cell wall to produce the inhibitor inducing compound. Whether the proteinase inhibitor has a specific function *in vitro* or whether it is synthesised as a result of the non-specific production of the elicitor are aspects which need clarification.

Differentiation and organised growth affect the pattern of the accumulation of proteinase inhibitors in suspension cultures. This was demonstrated by suspension cultures of *Daucus carota* which produced a trypsin inhibitor *in vitro* which was released into the culture medium by embryogenic cells but not by undifferentiated cells. The mechanism by which the trypsin inhibitor is released has not been clarified. However, it was not a result of cell lysis or the type of growth regulator used (CARLBERG, JONSSON, BERGENSTRAHLE and SODERHALL, 1987). Organised growth in suspension cultures does not seem to affect the level of proteinase inhibitors. It was demonstrated that isolated embryos of *Daucus carota* had the same trypsin inhibitory activity as undifferentiated cell aggregates (CARLBERG, JONSSON, BERGENSTRAHLE and SODERHALL, 1987). A BOWMAN-BIRK trypsin inhibitor was isolated from plant tissue as well as undifferentiated and partly differentiated callus culture of *Glycine max* cv. Amsoy 71. The KUNITZ trypsin inhibitor which was found in the intact plant tissue was not detected in the callus cultures. It was suggested that apart from other factors differentiation may cause the

differential metabolism of the two inhibitors (TAN-WILSON, HARTL, DELFEL and WILSON, 1985).

The chemical composition of the nutrient medium was found to affect the accumulation of proteinase inhibitors *in vitro*. This was illustrated by the decrease in the plasmin inhibitor activity from *Scopolia japonica* with an increase in the concentration of auxin and cytokinin. The decrease was not associated with an increase in growth. Thus, the regulators as such seemed to have caused a decrease in the activity of the inhibitor (MISAWA, TANAKA, CHIYO and MUKAI, 1975). Organic nitrogen sources such as casein hydrolysate and yeast extract improved the growth of the culture but resulted in a decrease in the plasmin inhibitory activity of the suspension culture. Reducing the ammonium nitrogen by half and increasing the nitrate nitrogen four times increased growth and promoted the formation of the inhibitor (MISAWA, TANAKA, CHIYO and MUKAI, 1975). The sucrose concentration in the nutrient medium was found to affect the accumulation of a proteinase inhibitor in *Lycopersicon esculentum* suspension cultures. The inhibitor accumulated in the suspension cultured cells when the sucrose was reduced from 1 % to 0.1 % (WALKER-SIMMONDS and RYAN, 1986).

It is clear that the metabolism of proteinase inhibitors in *in vitro* cultured tissue is affected by the environment in which the culture grows. It is also influenced by the physiological condition of the cells resulting from the

growth phase or stage of differentiation of the cells. An aspect which needs attention is the physiological significance of proteinase inhibitors in *in vitro* cultures.

7.3 THE EFFECT OF CYSTEINE AND SULPHATE ON THE t-PA INHIBITOR CONTENT OF SHOOT CELL SUSPENSION CULTURES OF *ERYTHRINA CAFFRA*

EXPERIMENTAL

The basal nutrient medium used for these experiments were the cell suspension medium described in Chapter 5. Two experiments were conducted. In the first the sulphate concentration in the basal medium was adjusted to 0.173 ; 0.346 ; 1.73 ; 8.65 and 17.3 millimoles per litre respectively. This corresponds to 0.1 ; 0.2 ; 1 ; 5 and 10 times the concentration of sulphate in the original basal medium. The sulphate levels in the basal medium was regulated by decreasing the original magnesium sulphate concentration or by supplementing the nutrient media with sodium sulphate. The concentration of magnesium was kept constant at 1.73 millimoles per litre in the treatment solutions by supplementing the nutrient media with magnesium carbonate.

In the second experiment one set of treatments consisted of 0.173 ; 1.73 and 8.65 millimoles per litre of sulphate in the basal medium. In another set of three

treatments the same concentrations of sulphate were used in the suspension culture but the nutrient media were supplemented with 0.1 millimoles per litre of L-cysteine.

For each treatment a volume of 100 millilitres of stock cell suspension in the exponential growth phase was decanted into a sterile 100 millilitre measuring cylinder. The cells were washed with the treatment solution as described in section 7.2. The washed cells were suspended in 100 millilitres of treatment solution in 500 millilitre Erlenmeyer flasks fitted with a side arm. The suspension cultures were incubated under the conditions described in section 7.1. Twenty millilitres of the cell suspension were harvested after seven days incubation. The rest of the culture was maintained to determine the effect of sulphate and cysteine on growth. The cell suspensions were washed under vacuum filtration as described before and the fresh mass determined. The cells were then frozen in liquid nitrogen and stored at -20 ^{o}C. The protein extraction and the determination of total protein and t-PA inhibitor was done as described in Chapter 2, sections 2.5 and 2.6 and Chapter 3 respectively. The experiment was repeated three times and the means and standard deviations determined for each treatment.

RESULTS AND DISCUSSION

The different sulphate treatments had no significant

effect on the total protein content of the suspension cultures at concentrations higher than 0.346 millimoles per litre (Figure 7.3). At 0.173 millimoles per litre sulphate the protein content of the suspension cultures was significantly lower than at the higher sulphate levels. The t-PA inhibitor content of the cell suspensions was significantly higher at a sulphate concentration of 17.3 millimoles per litre (Figure 7.3). There was no significant difference between the t-PA inhibitor content of the cells in the basal medium (1.73 mM) and in nutrient media with sulphate lower than 17.3 millimoles per litre. The optimal concentration of sulphate for the increase of the t-PA inhibitor *in vitro* was in the range of 17.3 millimoles per litre. This is ten times the sulphate concentration in the basal nutrient medium. The growth of the suspension culture was affected by the different concentrations of sulphate in the medium. A decrease in growth occurred at the concentrations of 0.173 and 17.3 millimoles per litre sulphate respectively (Table 7.7). The viability of the cells at any sulphate treatment was not different from the control (Table 7.7). Thus, sulphate at ten times and 0.1 times the concentration in the culture medium decreased cell growth. The relatively high viability of the cells at 17.3 millimoles per litre sulphate indicated that the reduction in the growth of the cell suspension culture was not a result of toxic levels of sulphate but a reduction in cell growth.

The addition of L-cysteine to the basal medium which

Figure 7.3 The effect of sulphate on the protein
and t-PA inhibitor content of shoot cell
suspension cultures of *Erythrina caffra*
after 7 days of incubation.

PROTEIN (mg $\bar{g}^1$ FM)   INHIBITOR (μg $\bar{g}^1$ PROTEIN)

SULPHATE IN NUTRIENT MEDIUM (mM)

PROTEIN   INHIBITOR
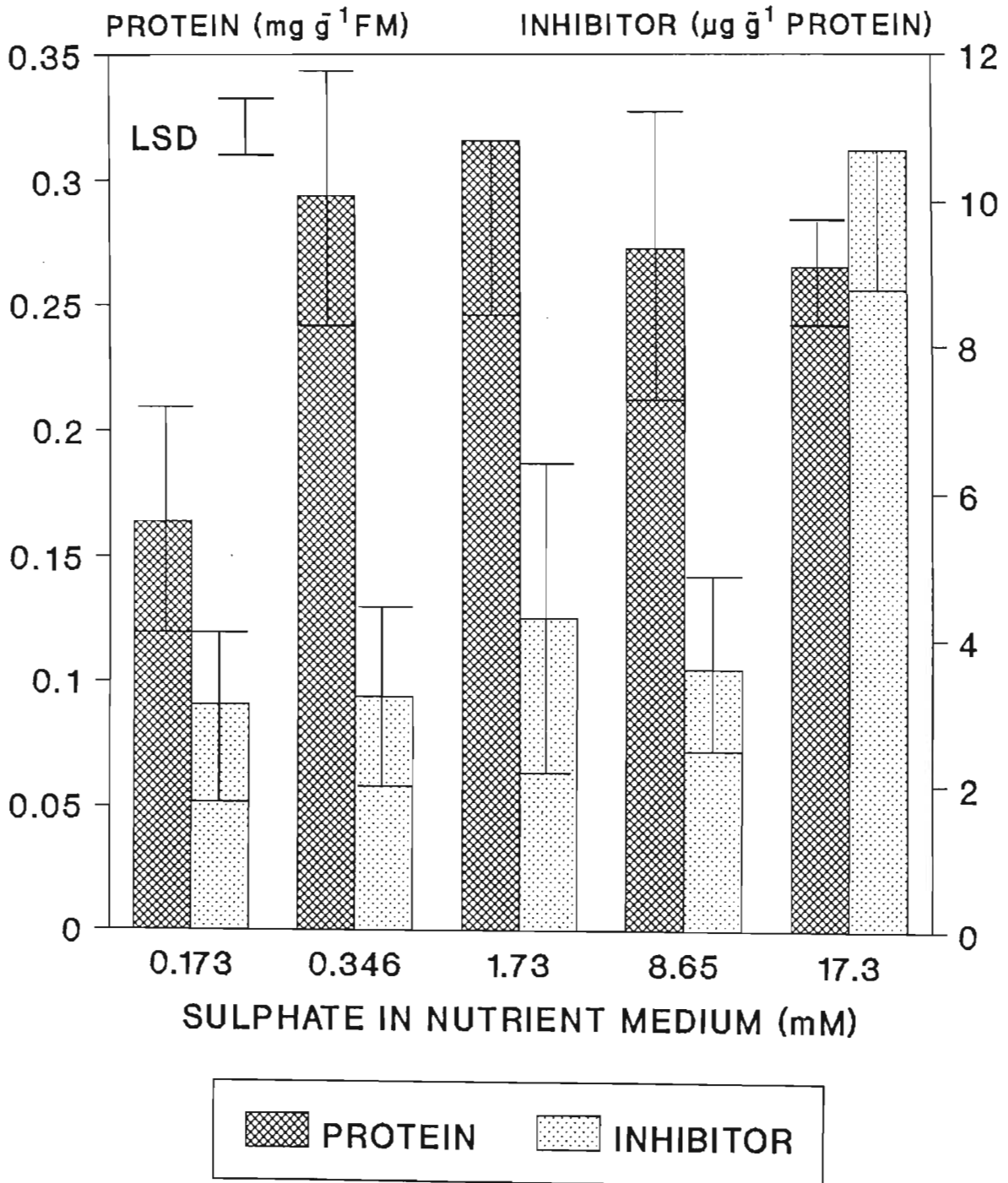
Table 7.7 The effect of sulphate on the viability and growth efficiency of shoot cell suspension cultures of *Erythrina caffra* after 14 days of culture.

Sulphate (mM)	Growth efficiency	Viability of cells (%)
0.173	1.7 ± 0.9	74 ± 23
0.346	3.1 ± 1.4	70 ± 25
1.73	3.2 ± 1.7	68 ± 18
8.56	3.4 ± 1.7	77 ± 19
17.3	1.6 ± 1.0	62 ± 21

Table 7.8 The effect of sulphate and L-cysteine on the growth efficiency and viability of shoot cell suspension cultures of *Erythrina caffra* after 14 days of culture.

Sulphate (mM)	L-cysteine (mM)	Growth efficiency	Viability of cells (%)
0.173	0.0	1.8 ± 1.2	72 ± 32
0.173	0.1	1.2 ± 0.8	37 ± 17
1.73	0.0	3.6 ± 1.8	68 ± 26
1.73	0.1	1.4 ± 0.7	31 ± 14
8.56	0.0	3.9 ± 1.1	70 ± 17
8.56	0.1	1.2 ± 0.8	29 ± 11

contained 0.173 millimoles per litre sulphate increased the total protein of the cell cultures significantly. At the higher concentrations of sulphate the addition of L-cysteine to the suspension culture had no beneficial effect on the protein content of the cell suspension cultures (Figure 7.4). The increase in the accumulation of protein in the cell suspension cultures with the addition of cysteine to the nutrient medium containing 0.173 millimoles per litre sulphate, suggests that sulphate is a limiting nutrient at this concentration. However, the addition of L-cysteine to the suspension medium at all the levels of sulphate used, decreased the growth of the cell suspension (Table 7.8). The addition of L-cysteine to the cell suspension cultures decreased the t-PA inhibitor content of the cells irrespective of the sulphate concentration in the suspension medium (Figure 7.5). A negative effect of cysteine on the growth and production of proteinase inhibitors of suspension cultures was reported for *Scopolia japonica* L. The addition of amino acids in the form of yeast extract, casein hydrolysate or NZ-amine to suspension cultures of *Scopolia japonica* was followed by a decrease in the plasmin inhibitor content of the cells (MISAWA, TANAKA, CHIYO and MUKAI, 1975). Cysteine added to suspension cultures of *Erythrina caffra* had an inhibitory effect on the growth of the cultures and decreased the viability of the cell suspension at all the cysteine and sulphate combinations (Table 7.8). The decrease in growth as well as the t-PA inhibitor content of the cell suspension culture with the addition of cysteine to the culture medium suggests

Figure 7.4 The effect of sulphate with and without
cysteine on the protein content of
shoot cell suspension cultures of
Erythrina caffra after 7 days of
incubation.

PROTEIN (mg ḡ^1 FM)

SULPHATE IN NUTRIENT MEDIUM (mM)

NUTRIENT MEDIUM WITH

SULPHATE   SULPHATE + CYSTEINE
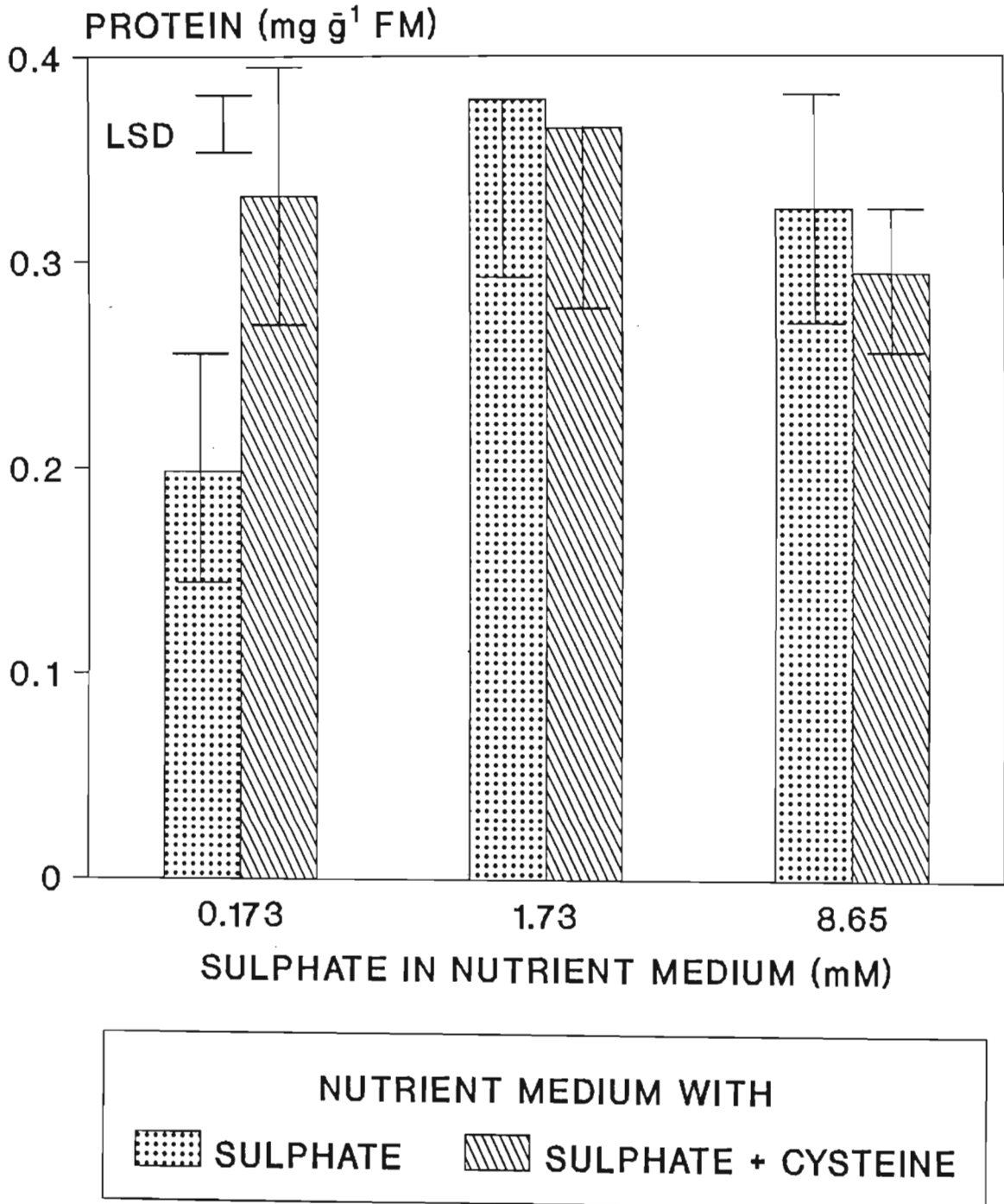
Figure 7.5   The effect of sulphate with and without
             cysteine   on   the   t-PA inhibitor content
             of shoot cell   suspension cultures of
             Erythrina   caffra   after   7   days   of
             incubation.

INHIBITOR (μg $\bar{g}^1$ PROTEIN)

SULPHATE IN NUTRIENT MEDIUM (mM)

NUTRIENT MEDIUM WITH

SULPHATE   SULPHATE + CYSTEINE
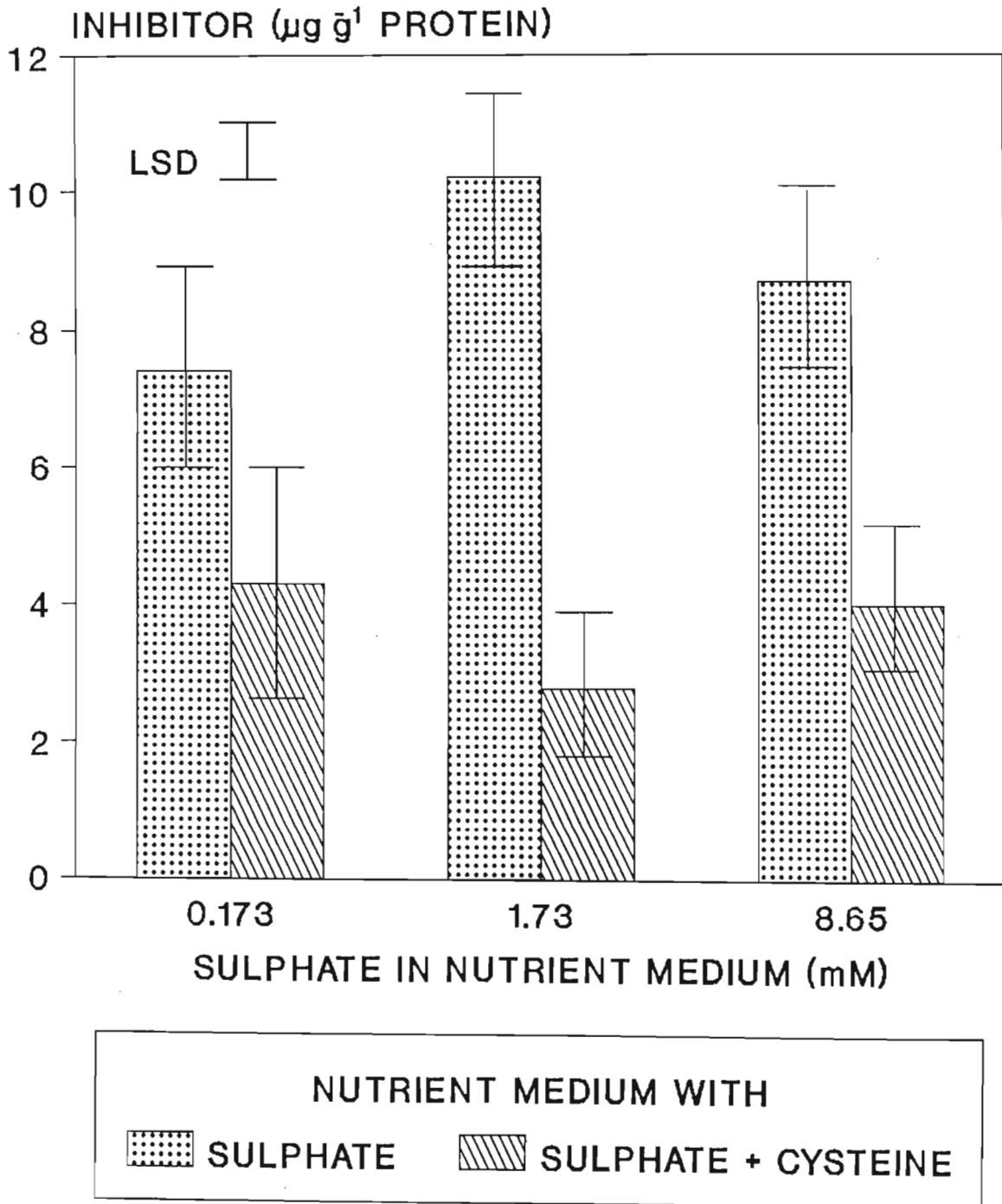
that a link exists between the metabolism of cysteine, t-PA inhibitor and the growth of the cells. The decrease in the growth of the cell suspension at 0.173 and 17.3 millimoles per litre sulphate without a reduction in the t-PA inhibitor levels suggests that cell growth is not directly related to the t-PA inhibitor content of the cells. Thus it seems as if cysteine affects the synthesis of t-PA inhibitor and cell growth separately. The addition of sulphur containing amino acids to suspension cultures should be done with caution. It has been reported that these amino acids can be detrimental to the growth of suspension cultures. The addition of cysteine to a root suspension culture of *Lycopersicon esculentum* inhibited the growth of the cultures (STREET, HUGHES and LEWIS, 1960). The omission of cysteine from an amino acid mixture supplement increased the growth of *Oryza sativa* L. (FURUHASHI and YATAZAWA, 1970) and *Saccharum officinarum* L. (NICKELL and MARETZKI, 1969). It thus seems as if cysteine added as a single amino acid or as part of an amino acid mixture is detrimental to the growth of suspension cultures. The inhibition of growth by sulphur containing amino acids is ascribed to retarded sulphate absorption and the depression of the key enzyme in sulphur assimilation, adenosine triphosphate sulphurylase (REUVENY and FILNER, 1977).

The the regulatory effect of sulphur on the synthesis of sulphur-rich proteins has not yet been reported for *in vitro cultures*. However, it has been demonstrated with leguminous seeds. It has been reported that the sulphur-rich alpha- and

gamma-conglobulin levels of the seeds of *Lupinus angustifolius* L. decreased when they were subjected to low concentrations of sulphur. The beta-conglobulin fraction which has a low cysteine content did not decrease in the seeds. No difference was found in the total protein content of the seeds (BLADGROVE, GILLESPIE and RANDALL, 1976). Legumin, one of the major storage proteins in legumes, contains substantially more sulphur than the other major storage protein vicilin (JACKSON, BOULTER and THURMAN, 1969). It was observed that a reduction in the synthesis of legumin took place in seeds of *Pisum sativum* during a sulphur induced deficiency. However, the level of vicilin increased in the sulphur deficient seeds. The major sulphur regulatory effect was reported to be on the level of the legumin mRNA (RANDALL, THOMSON and SCHROEDER, 1979). It is clear from these reports that the level of proteins rich in sulphur can be increased with an increase in the sulphur content of the plant above deficient levels. It is clear from the results observed with *Erythrina caffra* cell suspension cultures that sulphur do play a regulatory role in the synthesis of sulphur-rich proteins and this aspect needs more attention *in vitro* cultures.

The t-PA inhibitor and the other trypsin and chymotrypsin inhibitors in *Erythrina caffra* are proteins with a relatively high sulphur content in the form of cysteine residues (JOUBERT, 1982b). It was thus anticipated that as for intact plants the sulphur status of the cells in suspension would affect the amount of sulphur-rich proteins

in the cell suspension cultures. The results with the cell suspension culture of *Erythrina caffra* clearly indicated that t-PA inhibitor can be increased at a high concentration of sulphate in the nutrient medium but not by the addition of sulphur containing amino acids such as cysteine.

7.4 THE EFFECT OF PROTEIN INDUCING COMPOUNDS ON THE t-PA INHIBITOR CONTENT OF SHOOT CELL SUSPENSION CULTURES OF *ERYTHRINA CAFFRA*

EXPERIMENTAL

The protein inducing substances used with leaves in an effort to increase the levels of t-PA in leaves were also used to determine whether these chemicals would increase the t-PA inhibitor content of shoot cell suspension cultures. The protein inducing substances added to the basal nutrient medium were spermine (0.1 mM) ; spermidine (0.1 mM) ; ACC (0.1 mM) ; phytic acid (1 mM) ; chitin (1 mg l^{-1}) and ethylene glycol chitin (1 mg l^{-1}). These compounds were dissolved in distilled water and added to the basal suspension medium defined in Chapter 5. Cell wall hydrolysates of *Erythrina caffra*, *Ecklonia maxima* and *Lycopersicon esculentum* were added to the nutrient medium to obtain a final concentration of 0.1 milligrammes per litre. The cell wall hydrolysates were prepared as described in Chapter 2, section 2.2. A control treatment was included

in the experiment which consisted of the basal suspension medium.

For each treatment a volume of 30 millilitres of stock cell suspension in the exponential growth phase was decanted into a 50 millilitre measuring cylinder. The cells were washed with the treatment solution as described in section 7.2. The washed cells were suspended in 30 millilitres of treatment solution in 100 millilitre Erlenmeyer flasks. The suspension medium used was the basal nutrient medium described in Chapter 5. The suspension cultures were incubated under the conditions described in Chapter 7 section 7.1. The cells were harvested after an incubation period of 48 hours. The cell suspensions were washed under vacuum filtration as described before and the fresh mass determined. The cells were then frozen in liquid nitrogen and stored at -20 $^{\circ}$C. Protein extraction and the determination of total protein and t-PA inhibitor was done as described in Chapters 2 and 3. The experiments were replicated three times and the treatment means and standard errors determined.

RESULTS AND DISCUSSION

It is clear from the results that the t-PA inhibitor content of the cell suspension based on the fresh mass or total protein content was not significantly increased by any of the treatments (Table 7.9). The total protein of the

Table 7.9   The effect of protein inducing compounds on the
            protein and t-PA inhibitor content of shoot cell
            suspension cultures of *Erythrina caffra*.

Protein inducing compound	Protein content of suspension (mg g^{-1} FM)	Inhibitor content of suspension (µg g^{-1} protein)
Control	2.3 ± 0.5	0.40 ± 0.11
Spermine	3.0 ± 0.7	0.38 ± 0.10
Spermidine	2.9 ± 0.9	0.42 ± 0.08
Phytic acid	1.8 ± 0.4	0.39 ± 0.07
ACC	1.9 ± 0.6	0.29 ± 0.11
Ethylene glycol chitin	2.0 ± 0.8	0.29 ± 0.09
Cell wall hydrolysates of:		
Lycopersicon esculentum	1.8 ± 0.6	0.31 ± 0.12
Erythrina caffra	2.2 ± 0.9	0.37 ± 0.09
Ecklonia maxima	2.1 ± 0.5	0.32 ± 0.11

FM = Fresh mass.

cells for all the treatments was lower than the control value. Most of the substances used decreased the total protein and t-PA inhibitor levels of the cells.

Only a few cases have been reported in the literature on the occurrence of proteinase inhibitors in suspension cultures and the induction of these inhibitors by elicitors. Suspension cultures of *Medicago sativa*, *Lycopersicon esculentum* and *Nicotiana tabacum* were found to accumulate proteinase inhibitors when ethylene glycol chitin, chitosan or hydrolysed cell walls of tomato or of the mother plant was added to the suspension medium (WALKER-SIMMONDS and RYAN, 1986). The activity of enzymes in plants and plant cell suspension cultures was increased by various chemically unrelated compounds. For example the phenylalanine ammonia lyase activity in *Pisum sativum* was increased by spermine, spermidine, synthetic polyamines and various natural and synthetic polypeptides (HADWIGER, JAFRI, VON BROEMSEN and EDDY, 1976). Phenylalanine ammonia lyase activity was also increased by glucans from the mycelial cell walls of *Phytophthora megasperma* Drechs f. sp. glycinea (previously *Phytophthora megasperma* Drechs var. sojae A. A. Hildb.). Cell wall fragments of several fungi increased the activity of other enzymes. For example the chitinase activity in *Pisum sativum* and *Cucumis melo* L. cv. Cantaloup was increased by a pure hyphal cell wall extraction of *Fusarium solani* f. sp. phaseoli and *Colletotrichum lagenarium* (Pers.) E. and H. respectively (DATEMA, WESSELS and VAN DEN ENDE, 1977; HADWIGER and BECKMAN, 1980). In a similar way

purified mycelial cell walls of *Phytophthora megasperma* Drechs f. sp. glycinea increased the flavanone synthetase activity of *Glycine max* (ZAHRINGER, EBEL and GRIESEBACH, 1978). Pectic fragments resulting from the hydrolytic activity of endopolygalacturonase from *Rhizopus stolonifer* (Ehr. and Fries) on the cell walls of *Ricinus communis* L. resulted in a large increase in the casbene synthetase activity of the plants (LEE and WEST, 1981).

Pathogenesis-related proteins were observed to appear in *Nicotiana tabacum* when the plants were treated with salicylic acid, ethephon, benzoic acid, phytic acid, polyacrylic acid, ACC and various other substances (VAN LOON, 1983). The effect of some of the mentioned compounds such as ethylene glycol chitin, chitosan, spermine, spermidine and ACC on cell suspension cultures of *Erythrina caffra* were determined to establish whether it would induce the t-PA inhibitor content of the cells. None of these compounds increased the t-PA inhibitor content of the cell suspension culture.

GREEN and RYAN (1972) reported on a proteinase inhibitor inducing factor (PIIF) which was released by the leaves of *Lycopersicon esculentum* and *Solanum tuberosum* upon insect damage or mechanical wounding. This factor resulted in a large increase in the proteinase inhibitor concentration of the injured leaves. The proteinase inhibitor in tomato was also induced by chitosan, ethylene glycol chitin and a trigalacturonic acid (WALKER-SIMMONDS and RYAN, 1986). A

similar increase in the trypsin inhibitor content of the leaves of *Medicago sativa* L. was reported when the leaves were damaged or tomato PIIF supplied to the leaves (BROWN and RYAN, 1984). The tomato PIIF was produced by the heat hydrolysis of tomato leaf cell walls (RYAN, 1974a). However, the addition of hydrolysed cell wall extracts from *Lycopersicon esculentum*, *Ecklonia maxima* and *Erythrina caffra* to shoot cell suspension cultures of *Erythrina caffra* did not increase the t-PA inhibitor concentration of the cells. The cause of this inability of *Erythrina caffra* cell suspension to react to the elicitor stimulus is not clear. It was found that the effect of the tomato PIIF on tomato plants was light and temperature dependent. No proteinase inhibitor accumulated in darkness, a low light intensity or below 20 $^{\circ}$C. The maximum response was at a light intensity of 600 foot candles. In the light the plants' reaction towards the PIIF was temperature dependent (GREEN and RYAN, 1973). The *Erythrina caffra* suspension cultures were incubated at a lower light intensity than was used by GREEN and RYAN (1973). However, leaves treated with the cell wall extracts (Chapter 6) were kept at a high light intensity without any increase in the t-PA inhibitor. This suggests that the light intensity was not the limiting factor. The incubation temperature of the suspension culture of 25 $^{\circ}$C could be below the optimal temperature for the induction of t-PA inhibitor since 36 $^{\circ}$C was the optimal temperature for the accumulation of tomato proteinase inhibitor. However, it is unlikely that a suspension culture will survive such a high temperature. All plants do not

react like *Lycopersicon esculentum* or *Solanum tuberosum* on the tomato PIIF or on possible endogenous inducers of proteinase inhibitory activity. The effect of tomato PIIF and endogenous PIIF was determined for 23 species which covered ten plant families. Seven species did not respond to the treatment. Six members of the Leguminosae were used in the experiment and four of them did not respond to the treatment (WALKER-SIMMONDS and RYAN, 1977b). This also seems to be true for the t-PA inhibitor of *Erythrina caffra*. The effect of cell wall hydrolysates as elicitors of the trypsin and chymotrypsin inhibitors in *Erythrina caffra* has not been determined. It is thus possible that the synthesis of these inhibitors may be affected by cell wall components. With the limited knowledge available it is not clear what role an inhibitor of t-PA could possibly play in the defense mechanism of a plant. From an evolutionary point of view it is possible that the t-PA inhibitor has not developed as part of the defense mechanism of the plant. This would partially explain why the synthesis of the t-PA inhibitor is not affected by molecules involved in the defense of plants.

CHAPTER 8

CONCLUSIONS

Callus from different organs of *Erythrina caffra* Thunb. was successfully cultured *in vitro* on a defined nutrient medium. It was clear that the growth of shoot callus was not affected by different cytokinins and auxins. Callus grew optimally within the same concentration range for the cytokinins and for the auxins used. However, differences were observed in the growth of callus from different organs with the best growth from shoot derived callus. Differences were found in the growth rate of callus from the same organs on different plants. These observations suggest that with *Erythrina caffra* the physiological differences between organs and plants are of greater importance in the growth of callus than the type of growth regulator used.

The growth of callus from different organs of *Erythrina caffra* was not affected by different nutrient media which have large differences in the concentration of the macro and micro nutrients as well as the vitamin complement of the nutrient media. However, the source of nitrogen and the relative amounts of ammonium and nitrate in the nutrient medium was critical to the growth of the callus. Although ammonium stimulated callus growth, higher concentrations of ammonium than nitrate seems to be inhibitory to the growth

of *Erythrina caffra* callus. Another nutrient critical to the growth of callus from different organs was sucrose. Sucrose is the best source of carbon for most *in vitro* cultures. However, the concentration of this carbohydrate is critical to the growth of these cultures. With *Erythrina caffra* the optimal sucrose concentration was 3 % irrespective of the origin of the callus.

The growth of callus was affected greatly by temperature. The *Erythrina caffra* shoot callus did not grow well at temperatures lower or higher than 25 °C. It should be mentioned that the trees grow naturally in a subtropical environment with summer temperatures higher than 30°C. However, the callus did not grow well at this temperature. The optimal temperature for the best growth was at 25 °C.

A critical factor for the establishment and growth of cell suspension cultures of *Erythrina caffra* was a low shake speed of 60 rpm. A low shake speed can result in the formation of large cell aggregates. However, a relatively large proportion of small aggregates was obtained from the cell suspension cultures. In comparison with callus cultures cell suspension cultures could not tolerate the relatively high concentrations of growth regulators used in the gelled nutrient medium for callus growth. The suspension cultures proliferated on a ten times lower concentration of growth regulators than the callus cultures. Apart from the growth regulators both the callus and suspension cultures grew well on the same nutrient medium.

Some advantages and disadvantages of the enzyme-linked immunosorbent assay should be mentioned. With an ELISA a specific molecule can be detected very accurately in minute quantities. A fairly crude plant extract requiring minimal purification or concentration can be used in the assay. With the antibodies available the exact extracellular and intracellular location of a molecule can be determined with immunocytochemical techniques. However, a major disadvantage of the use of antibodies is that the slightest change in the conformation of the molecule can affect the antigenicity of the molecule to varying degrees. This will result in a negative assay indicating the disappearance of the molecule even though the molecule is still present in a slightly different form.

The t-PA inhibitor in *Erythrina caffra* is mainly concentrated in the seed. Although it does occur in all parts of the plant the concentration is very low when compared with the level in seeds. During seed germination and subsequent seedling development the accumulation and disappearance of the t-PA inhibitor followed a pattern similar to that of the storage proteins. These observations suggest that since t-PA inhibitor behaves like a storage protein that one of the functions of the t-PA inhibitor can be that of a storage protein. However, limited proteolysis of the molecule can change the antigenicity of the molecule which will result in a negative response in the immunoassay. This implies that although a decrease in the t-PA inhibitor content was observed the t-PA inhibitor could still be present in a modified form in the tissue. Recent reports on

this matter indicated that trypsin inhibitors are partially and specifically hydrolysed by proteinases in seeds. This suggests that these inhibitors have a function in seeds other than that of a storage protein. A study of the electrophoretic patterns of the t-PA inhibitor and related trypsin and chymotrypsin inhibitors during germination and seedling growth will be of great value to determine the physiological significance of the inhibitors.

A matter that needs attention is the evolution and function of a proteinase inhibitor which do not only inhibit trypsin and chymotrypsin but also tissue plasminogen activator, a proteolytic enzyme in the blood plasma which is very unlikely to have been involved in the evolutionary development of the t-PA inhibitor. Various functions have been proposed for trypsin inhibitors such as a storage protein with the special function of providing sulphur compounds to the developing seedling. It was suggested that trypsin inhibitors could also play a protective role against attacks by chewing insects and fungal infections (GARCIA-OLMEDO, SALCEDO, SANCHEZ-MONGE, GOMEZ, ROYO and CARBONERO, 1987). However, not enough evidence is available at present to prove it beyond any doubt. A possible explanation for the inhibition of t-PA by the t-PA inhibitor in *Erythrina caffra* and plasmin by the plasmin inhibitor in *Scopolia japonica* (SAKATO, TANAKA, and MISAWA, 1975) that has not yet been investigated, is the occurrence of proteolytic or other enzymes in these plants with a molecular conformation very similar to that of t-PA and plasmin respectively. The inhibitors with the plant enzymes as their primary target

may well coincidentally inhibit the plasma proteinases.

A more direct link of proteinase inhibitors as a defense mechanism against insects and fungi is presented by the systemic response of plants to chewing insects and fungi. Mechanical lesions caused by insects and fungal infections, induced through a messenger molecule the synthesis of proteinase inhibitors in leaf material (RYAN, 1984 ; PENG and BLACK, 1976). However, a similar response of t-PA inhibitor to the messenger molecule in the hydrolysates of cell walls could not be induced in leaf material of *Erythrina caffra*. Literature indicated that the induction of proteinase inhibitor synthesis by cell wall hydrolysates does not occur in all plants. It is however, possible that the t-PA inhibitor has not evolved as part of the defense mechanism of *Erythrina* species. Further investigations into the effect of the cell wall hydrolysates on the other trypsin and chymotrypsin inhibitors in *Erythrina caffra* should be conducted to determine their role in the defense mechanism of the plant.

An aspect of interest is the intracellular location of t-PA inhibitor. Very few reports are available on the inter- and intracellular distribution of proteinase inhibitors. The reports indicate a difference in the spatial distribution of proteinase inhibitors in seeds and leaves. It seems as if the metabolism of the inhibitors is related to the function of the organ in which it is produced. A study in this respect will be valuable to make inferences about the function and metabolism of the inhibitor.

The inhibitor of t-PA is not metabolised in the same way as other proteinase inhibitors *in vitro* cultures. Proteinase inhibitors of members of the Solanaceae accumulate *in vitro* during the stationary growth phase (WALKER-SIMMONDS and RYAN, 1986). This was not observed with the t-PA inhibitor of *Erythrina caffra*. The accumulation of proteinase inhibitors during the stage of cell senescence was preceded by the appearance of an uronic-rich polysaccharide which induced the synthesis of the proteinase inhibitors. The synthesis of proteinase inhibitors *in vivo* and *in vitro* were also induced by a galacturonic acid rich polysaccharide involved in the defense mechanism of the plants. This molecule which is a breakdown product of the plant cell wall did not induce the synthesis of the t-PA inhibitor. It is concluded that the t-PA inhibitor does not accumulate like other proteinase inhibitors *in vitro* because the synthesis of the t-PA inhibitor is not affected by breakdown products of cell walls. Proteinase inhibitors are metabolised differently during the growth phases of *in vitro* cultured cells. It was suggested that trypsin inhibitors are not essential for the growth of *Glycine max* (ORF and HYMOWTIZ, 1979). This raises the question about the function of proteinase inhibitors and whether they are essential for the growth and development of *in vitro* cultures.

The occurrence of t-PA inhibitor in low quantities in cell suspension cultures of *Erythrina caffra* does not make *in vitro* cultures a viable alternative source of this protein. The production of t-PA inhibitor in a suspension culture does however, have the benefit of a reliable and

continuous source of the inhibitor. An additional benefit is that pure white protein is obtained from *in vitro* cultured cells in contrast to the undesirable pink coloured protein which is obtained from seed due to pigments in the seed coat. Suspension cultures also creates the potential to study the effect of environmental factors on the regulation of the expression of inhibitor genes and inhibitor synthesis. The existing knowledge of the amino acid sequence of the inhibitor creates the possibility to obtain transgenic cells with multiple copies of the gene which codes for the t-PA inhibitor. The yield of t-PA inhibitor may be substantially increased *in vitro* in this way. A practical solution may well be to use transgenic bacterium suspension cultures for the production of t-PA inhibitor since bacteria grow at a much faster rate than plant cells *in vitro* and will provide more inhibitor than a plant suspension culture.

It is clear from this study that the largest amount of t-PA inhibitor is present in the seeds of *Erythrina caffra*. The seeds are still the cheapest and richest known source of t-PA inhibitor and compared to other parts of the plant the most convenient to collect and store. The removal of seeds from the environment can have a detrimental effect on the regeneration of the species and on the food chain in the ecosystem since the seeds are severely predated by bruchid beetles. More than one beetle utilise a seed at a time and a seed can be utilised more than once by the beetles. The beetles are a source of food for predators higher up in the food chain such as birds. Considering that a metric tonne

of *Erythrina caffra* contains in the order of 5 000 000 seeds it can be envisaged what effect it can have on the regeneration of the species as well as on the food chain if only one tonne of seeds is collected each year. The maximum amount of seed which can be harvested without upsetting the balance in the biotic environment should be determined through an impact study.

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