# 5. QUALITATIVE AND QUANTITATIVE STUDIES OF PROTEINS

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# QUALITATIVE AND QUANTITATIVE PROTEIN CHANGES DURING EPIPHYLLOUS BUD OUTGROWTH

## 5.1. INTRODUCTION

### 5.1A. Apical meristem and plant development:

In the early stages of the development of embryo all the cells undergo divisions, but during the further course of embryogeny cell division becomes restricted to special parts of the embryo. Such typical tissues remain embryonic in nature exhibiting very little differentiation and the cells retain the ability to divide. These embryonic tissues are called meristems. The cells of meristem continue to divide indefinitely and as a result new cells are continuously added to the plant body (Fahn, 1982).

Apical meristems initiated in the embryo of higher plants, function directly and indirectly through the formation of other meristems to produce the entire body of the plant. The indeterminate pattern of growth of higher plants by the continuous activity of the apical meristem leads to the steady accretion of mature tissue in the older parts of the plant body, while the meristematic activity is maintained at the shoot and root apices. The overall structure and organisation of the constituent units of the shoot are established during the meristematic phase of development at which stage the primordia of the various units are quite small - of the order of few

millimeters or so (Wareing, 1982a). However, the cellular differentiation in plant appears to depend more on the position that a cell occupies in the plant than on the clonal lineage of the cell (Sussex, 1985). But for the formation of regular and heritable pattern at any level of organisation the cell requires positional signals whose nature, mode of transmission and origin is still obscure. Moreover, once the cell is beckoned, it can embark on differential gene expression resulting in cell specialization (Sachs, 1978). Walker and Bruck (1985), from the series of experiments using the epidermal layer of citrus advocated the involvement of morphogens - other than the major phytohormones.

5.13. Problems in studies on molecular aspects of plant development:

The installation and subsequent organisation of meristematic function in localized groups of cells at specific sites is a major event in establishing the pattern of continuous and sequential pattern of plant development. Still very little is known of the molecular and genetic events underlying the developmental pathway of meristem formation or how exactly these events are related to observable changes in cellular and tissue structures (Meinke and Sussex, 1979; Sanchez-Martinez et al., 1986). One of the major reasons responsible for paucity of such knowledge is the lack of appropriate experimental system (Thorpe, 1978; Mehta, 1980; Tran Thanh Van and Trinh, 1986).

Shoot meristems of most of the angiospermic plants are very small of the order of 40-80 μ (Shah and Umnikrishnan, 1969; Shah and Raju, 1975; Shah and Vasudeva Rao, 1977). So that it is difficult to get sufficient experimental tissue for molecular analysis. Besides their small size, number of meristems per plant are limited by the number of nodes per plant. Further, the meristems of different parts of a plant as well as of different plants are not highly synchronous in their development. Moreover, the multiplicity of correlation between organs, tissues and cells in intact plants render the studies on morphogenetic pattern leading to differentiation, difficult to locate precisely the recognition sites as well as the cells (Tran Thanh Van and Trinh, 1986). Since the demonstration of Steward et al. (1958) and Reinert (1959) of induced somatic embryogenesis from isolated explants and subsequently from cultured cells as well as presentation of induction of shoot and root meristems on tobacco pith explant by manipulating auxin/cytokinin ratio by Skoog and Miller (1957), these systems have emerged as alternative systems for information on such fundamental studies on plant development (Nomura and Komamine, 1986). But, such systems suffer from the common drawback of artificial triggers, where a stimulation for morphogenetic differentiation with the initial reverse process - from differentiation to dedifferentiation (Sanchez de Jimenez and Fernandez, 1983) seems to be an external source. Apart from this, there is an intervention of callus phase in between giving more heterogeneity to the system.

and nonsynchronous response (Thorpe, 1978; Mehta, 1980). The use of thin cell layers as an experimental system has been emphasized due to the direct formation of wide range of morphogenetic patterns from homogenous differentiated cells (Tran Thanh Van, 1973 a and b; 1981). However, such superficial explants suffer from their own problems. They include the exacting conditions required to grow the donor plants and very large number of explants and meticulous handling of explants required to carry out investigations (Thorpe and Biondi, 1981) including the lack of synchrony between the developing meristemoids (Hicks, 1980).

5.1C. Model system for molecular studies on plant development:

In search of a model system to study molecular events of plant shoot meristems, one sets following criteria (Thorpe, 1979).

1) easy accessibility
2) availability in large quantity
3) synchronous and rapid growth in vivo and as well as in vitro
4) precise location.

In this regard, the epiphyllous bud meristems of Kalanchoe spp. fulfills all the above criteria. Earlier, Hicks (1980) in his review on patterns in organ development conclusively suggested the use of simpler systems such as meristems or organ primordia for studies on the determination in plant development.

5.1D. Plant developmental studies and gene expression:

It is well documented that plant development involves a progressive differentiation of tissues and organs giving rise to a wide range of different types of cells (Wareing and Phillips, 1982). Further, the process of development involves a controlled gene expression, whereby right genes are expressed in the right cells in sequential manner as earlier proposed by Haldane (1932) is now well accepted (Grierson and Smith, 1982). But, for establishing and maintaining specific developmental status in higher plants a differential gene expression is required. For example, an anther has at least 11000 diverse mRNAs that are absent from the polysomes of other organ systems, with the root having atleast 7000 organ - specific mRNAs. Further, both transcriptional and post - transcriptional processes are known to regulate the sequence composition and prevalence distribution of each developmental - specific mRNA set (Goldberg, 1986). At the same time, the end product of gene expression are biologically active proteins (Nover, 1982). Thus different types of specialized cells or tissues in a multicellular organism exhibit different protein complements in spite of the fact that all have the same genetic constitution. This reflects that various kinds of differentiated cells differ qualitatively and quantitatively in proteins including enzymes (Scandalios, 1983).

Using various techniques, extensive changes in the mRNA population during differentiation of many different animal cell types have been reported. Comparatively, variation in gene

expression during higher plant development has only been studied very recently (Vaillant et al., 1983; Mainke, 1986). De Vries et al. (1982, 1983) studied the changes in abundant mRNA population in pea seedlings and showed the presence of small number of shoot specific mRNAs. Similarly, Kamalay and Goldberg (1980) demonstrated that the gene expression is highly regulated in different but fully differentiated organs of the tobacco plant. Analysis of embryogenesis has shown large scale changes in the concentration of mRNAs during development in cotton seed (Dure and Chlan, 1981; Dure and Galau, 1981), soybean (Goldberg et al., 1981) and Phaseolus vulgaris (Misra and Bewley, 1985). Gene expression has also been studied, likewise, during leaf development in various plants like barley (Viro and Kloppstech, 1980), wheat (Dean and Leech, 1982), oats (Taylor and Mackender, 1977), maize (Mayfield and Taylor, 1984; Martineau and Taylor, 1985) and Lolium (Cugham et al., 1987). Changes in specific proteins synthesized has been reported in elongating hypocotyl of soybean (Zurfluh and Guilfoyle, 1980, 1982), in tobacco leaves induction of pathogenesis - related proteins (Matsuoka and Ohashi, 1986) and during germination of fern (Onoclea) spores (Huckaby and Miller, 1984).

Due to the more complicated morphological structure of shoot than that of root - apices (Barlow, 1982; Coleman and Thorpe, 1985), the later is the widely used experimental material for studies on molecular mechanisms involved in plant development (Rohm and Werner, 1987; Vaillant et al., 1983; Feldman and Gildow,

1984; Mohan Ram, 1980; Vartanian et al., 1987; Dhindsa et al., 1987). However, except for few studies on floral transition of vegetative apex (Stiles and Davies, 1976; Pierard et al., 1980) no comparable studies are available for meristem initiation and development, in spite of the occurrance of characteristic developmental plasticity of apical meristems (Meinke, 1986).

Based upon these reasonings and the well documentation of central role of proteins in determining the specific morphogenetic structure in plants, an attempt was made to examine the quantitative and qualitative composition of proteins in the meristems of epiphyllous buds during their resting - dormant stage. Further any change(s) in such composition was also traced when they are reactivated to form new plantlets in vitro by culturing them on simple medium.

## 5.2. MATERIALS AND METHODS

### 5.2A. Extraction and quantification of nucleic acids:

The method described by Cherry (1962) was followed for extraction and quantification of DNA. A known weight (600 mg) of sampled notches were homogenized in cold 80 % methanol (1:2 w/v). The insoluble material was sedimented by centrifugation (8000 g; 10 min). The sedimented material was subjected to reextraction (two times) with 0.2 M $HClO_4$ (8.0 ml) and followed by ethanol (8.0 ml). The residue was then extracted with 10 ml ethanol : ether (2:1) at 50 °C (30 min). The leftout residue

after centrifugation (10,000 g) was incubated with 5 ml of $HClO_4$ ( 5 %, v/v) at 70 °C (40 min) in waterbath. The $HClO_4$ hydrolysate was kept in refrigerator overnight. Absorption difference at 260 and 290 nm of the cleared supernatant was used to find out total nucleic acid using similarly treated Torula Yeast RNA (Sigma) as standard.

Total DNA content was determined using diphenylamine (DPA) reaction (Burton, 1956).

Briefly, an aliquote of nucleic acid extract supernatant was diluted to 2.0 ml with $HClO_4$ (0.5 N) and mixed with 4.0 ml of DPA reagent. The mixture was incubated overnight (12 hr) in waterbath (30 °C). The absorbancy of the coloured solution was measured at 600 nm and compared with the standard DNA (Calf thymas) (Sigma) to calculate total DNA content.

The total RNA content was determined by substracting total DNA content from total nucleic acid.

Preparation of DPA reagent

1.5 gm of purified DPA was dissolved in glacial acetic acid (100 ml). Concentrated $H_2SO_4$ (1.5 ml) was added to the mixture and stored in dark. At the time of use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent. required.

5.2B. Changes in RNase activity during bud outgrowth:

To understand the decline in total RNA content after an early increase, changes in RNase activity during the course of bud outgrowth was studied. The extraction of the enzyme was done as described in Chapter VI.

The enzyme RNase ↑ was assayed by a modified procedure of McDonald (1955). The reaction mixture comprised 1.8 ml Na - acetate buffer (10 mM, pH 3.0) containing yeast RNA (2 mg) and enzyme extract (0.2 ml). The reaction mixture was incubated at 37 °C (60 min) and afterwards reaction was stopped by adding 8 volumes of chilled ethanol. After overnight storage (5 °C), the reaction mixture was filtered through Whatman No. 1 filter paper. Absorption difference, of the resulted filterate, at 260 and 280 nm was used to calculate the activity of RNase after deducting the blank values. An enzyme unit was defined as the amount of enzyme required to increase the absorption by 0.1 per mg protein per hr. Protein quantification in enzyme sample was done by dye binding method (Bradford, 1976).

5.2C. Extraction and quantification of total soluble proteins:

The extraction of soluble proteins was done as reported earlier (Jasrai et al., 1985).

Briefly, sampled notches (500 mg) were homogenized in 0.1 N NaOH (2.0 ml) with a pinch of neutral glass powder and insoluble PVP (2 %, w/w) and kept on magnetic stirrer (15 min).

The resulting slurry was centrifuged at 10,000 g (10 min). Equal volume of cold TCA (18 % w/v) was added to the clear supernatant and tubes were kept on ice (30 min). The precipitated proteins were pelleted by centrifugation for 15 min (12,000 g). The resulted protein pellet was washed twice with water saturated diethyl ether and dissolved in 0.1 N NaOH (0.75 ml). The protein in the resultant solution was assayed by modified lowry's procedure (Hartree, 1972) using BSA as standard. All these steps were conducted at 0-10 °C temperature.

5.2D. Qualitative analysis of soluble proteins during epiphyllous bud outgrowth:

5.2D.1. Extraction of proteins:

During the preliminary studies on protein analysis in the present investigations, extensive proteolytic degradation in the leaf protein samples was observed. The detailed analysis of protease was done to avoid this artifact; details are given in section - 5.2G. of this chapter. Number of extraction procedures were tried to select the one in which minimum or no protease artifacts were observed. Finally, the procedure given by Hari (1981) was found to be satisfactory with slight modifications.

Briefly, sampled notches (500 mg) were homogenized in cold extraction buffer (2.5 ml) with a pinch of glass powder and insoluble PVP (2 % w/w). The extraction buffer contained $K_2CO_3$ (5 mM), urea (9.5 M), dithiothreiotol (0.5 %), NP-40 detergent

(2 % v/v), L-lysine (0.05 %) and Ampholines (pH range 3.5 to 10, 5 to 7 and 9 to 11; 2 % v/v each) dissolved in borate buffer (0.2 M, pH 9.0).

The homogenate was left on magnetic stirrer (30 min) and centrifuged at 10,000 g (15 min). The resulting supernatant was mixed with three volumes of chilled acetone (7.5 ml) and kept on ice (30 min). The precipitated proteins were collected by centrifugation at 20,000 g (20 min), and washed with cold acetone (90 % v/v) containing mercaptoethanol (0.1 M). The proteins were then vaccum dried to remove traces of acetone and dissolved in extraction buffer and distributed as different aliquotes and stored in refrigerator till further use.

5.2D.2. Isoelectric focusing (IEF) of extracted proteins

IEF was done in a horizontal polyacrylamide slab gel (0.5 mm thick) on LKB 2117 Multiphore - II Electrophoresis unit (LKB, Sweden). The composition of stock solutions for gel preparation are given as follows (LKB Application Note 320, 1981)-

a) Acrylamide solution - Acrylamide (22.2 gm) and methylene-bisacrylamide (1.4 gm) were dissolved in dist water to a final volume to 100 ml, filtered and stored in brown coloured bottle.
b) Ammonium persulfate solution (AMPS) - AMPS (400 mg) was dissolved in dist water and final volume made to 1.0 ml. Always prepared freshly.

c) Composition of the gel solution -

| | |
|---|---|
| Solution a | 1.8 ml |
| Urea | 5.4 gm |
| Nonidet P-40 | 0.2 ml |
| Temed | 10.0 µl |
| Ampholine | |
| 3.5 to 10.0 pH | 0.5 ml |
| 5 to 7.0 pH | 0.25 ml |
| 9 to 11 pH | 0.25 ml |

The given ingradients were mixed thoroughly and final volume was made to 10 ml with dist water.

d) Anodic solution - 0.1 ml of O-phosphoric acid was diluted to 10 ml with dist water.

e) Cathodic solution - 0.2 ml of ethylene diamine diluted to 10 ml with dist water.

10 µl of AMPS solution (b) was added to the deaerated gel solution (C) prior to pouring in gel mould. Gel mould was prepared by keeping teflon spacers (0.5 mm thick) on the three sides, between two glass plates, which were then sealed by agarose (1 % w/v). The gel solution in the mould was allowed to polymerize (about 60 min) at room temperature.

The gel plate was then kept on the cooling plate (10 °C) of the multiphore electrophoresis unit, with a few drops of kerosene in between the two surfaces. Care was taken to remove any air bubble trapped in between the glass plate and cooling

plate. Each absorbant electrode strip, soaked with respective electrode solution, was blotted with tissue paper and applied to the corresponding electrode sites on the gel.

The protein samples (corresponding to 100 µg each) dissolved in extraction buffer were applied to the cathodic side of the gel using application pieces (0.5 x 1 cm) cut from Whatman filter paper (No. 42). The two platinum electrodes were lowered on the two electrode strips and electrical circuit was completed by giving connections to constant power supply. The current supplied for the run was as follows:

| Volt (V) | Current (mA) | Power (W) | Time (Min) | Comment |
|---|---|---|---|---|
| 600 | 250 | 10 | 30 | Prefocussing |
| 600 | 250 | 10 | 30 | Focussing with application pieces, containing protein sample. |
| 1200 | 250 | 15 | 180 | Focussing of proteins |

After prefocussing (30 min) the application pieces loaded with protein sample were applied to the gel and focussing was done (30 min). Afterwards application pieces were removed and focussing was continued (180 min). Temperature (10 °C) was maintained during the run using circulating water.

The composition of staining and destaining solutions are given below:

(i) Staining stock solutions -

(A) Coomassie brilliant blue G-250 (CBB G-250) (300 mg) was dissolved in 90 ml of methanol and diluted to 100 ml with dist water.

(B) $CuSO_4 \cdot 5H_2O$ (1.0gm ) was dissolved in 80 ml of dist water and diluted to 100 ml with glacial acetic acid.

ii) Staining solution -

Solution A and B were mixed in equal proportion just at the time of use.

iii) Destaining solution -

$CuSO_4 \cdot 5H_2O$ (1.0 gm) was dissolved in dist water (130 ml) and diluted to 200 ml with glacial acetic acid (20 ml) and methanol (50 ml).

iv) Preserving solution -

Methanol (70 %, v/v) and glycerol (10% v/v) in dist water.

The separated proteins were fixed with cold TCA (20 %, w/v) and localized with CBB G-250 stain (solution-ii) for overnight. The nonspecific stain was removed with three changes of destaining solution-iii, at every 60 min interval . The destained gel was stored in solution iv. till their scanning. The separated proteins were densitometrically scanned at 600 nm on UV-240 spectrophotometer (Shimadzu, Japan).

The pH gradient of the gel was measured using a surface pH microelectrode (LKB, Sweden) at same temperature (10 °C), before the gel was fixed.

5.2E. Determination of time for de novo synthesis of proteins:

Isolated notches (measuring 1 $cm^2$ from middle part of the 4th nodal leaves) were incubated in the presence of transcriptional and translational inhibitors - actinomycin-D (ACTD) and cycloheximide (CYCLO) respectively at 1-100 $\mu M$ concentrations. After incubation of 14 days, bud outgrowth response was noted and calculated as percent bud outgrowth.

To determine the period during which new proteins are synthesiszed that are involved in the process of bud outgrowth, isolated dormant buds incubated in the induction medium (dist water) were transferred at every 4 hr intervals to the inhibitory medium (cycloheximide) and observed for bud outgrowth.

5.2F. Determination of in vivo rate of protein synthesis during bud outgrowth:

Notch explants measuring 0.5 $cm^2$ were induced for bud outgrowth by keeping on filter paper soaked with dist water in petridish. Every 12 hr interval (upto total 84 hr) explants were transferred to medium (dist water) containing ($^{14}C$)serine (specific activity 108 mCi/mmol) at 1.0 mCi/ml concentration. After incubation of 3 hr the labelled explants were taken out and washed thoroughly (three times) with dist water containing cold serine and divided into two sets.

One set of labelled notches was homogenized in scientillation fluid directly to determine the uptake of ($^{14}C$)serine.

The scientillation fluid consisted of naphthalene (15 gm), PPO, (1.0 gm) and POPOP (0.05 gm) dissolved in dioxane (250 ml). Another set of labelled notches were homogenized as per the procedure employed for protein extraction and quantification, to estimate the incorporation in total soluble proteins. The aliquotes of the supernatant of both the sets were used for radioactivity counts on scientillation counter (LKB, Sweden).

5.2G. Analysis of protease and its partial characterisation:

5.2G.1. Assay system

Eventhough the protein extraction was performed at subzero temperature, non-reproducibility of the electrophoretic separation of protein was a problem. This prompted to undertake detail analysis of protease of K. mortagei. The modified protease assay system (Laskowski, 1955) contained 0.5 ml each of enzyme extract, 0.2 M phosphate buffer (pH 8 or 3) and ovalbumin (2 % w/v) as the substrate. The reaction was carried out in water-bath (47 °C temperature) (60 min). The reaction was terminated by the addition of 05 ml of chilled trichloroacetic acid (18 % w/v). The mixture was kept on ice (30 min) to precipitate proteins. The precipitated proteins were discarded after centrifugation (10000 g) for 15 min and the amount of liberated aromatic amino acids in the supernatant was determined by modified Lowry's procedure (Hartree, 1972). Control reaction did not include the substrate ovalbumin (which was added after the termination of the reaction). The unit activity of the

enzyme protease was defined as the enzyme protein equivalent to release 10 µg tyrosine per hr in the assay conditions. The specific activity is the unit activity per mg protein.

5.2G.2. Characterisation of Protease

The effect of pH on proteolytic activity was studied using various buffers. They are : 0.2 M KCl - HCl (for pH 1.0 to 2.0), 0.1 M citrate Phosphate (3.0 to 7.0), 0.2 M Phosphate-phosphate (pH 8.0) and 0.2 M Glycine - NaOH (9.0 to 10.0). To study the effect of temperature on protease activity, the assay was carried out with 0.2 M Phosphate buffer (pH 8.0) at 27°, 37°, 47°, 57° and 67 °C.

To characterise the nature of protease, the following inhibitors in the range 0.1 to 10 mM were used. Ethylene diamine tetra acetic acid (EDTA), p-hydroxymercuribenzoate (PHMB) and phenylmethane sulfonyl fluoride (PMSF). Before initiating the reaction, the enzyme extract was incubated in the presence of inhibitor (2.5 µl/ml) for 30 min.

5.3. RESULTS

5.3A. Quantitative analysis of nucleic acids:

During the process of epiphyllous bud outgrowth, total RNA content displayed maximum accumulation till day-1 after isolation of the notches from the leaves, demonstrating about 1.3 fold enhancement over 0 hr value (Fig. 5.1)a with further developments, the RNA content remained same throughout. On the day of visual

appearance of buds, the RNA content was approximately the same as that of the day-0 content. The constancy of RNA content with the initial rise indicates the possibility of identical rates of synthesis and degradation with rapid turnover.

Total DNA content during bud outgrowth displayed continuous decreasing trend (Fig. 5.1)$_a$. On the day of leaf detachment (day-0) in the dormant buds the DNA content registered was 188 $\mu g\ gm^{-1}$ fresh weight. With subsequent progress in the process, the DNA content showed decline which was rapid upto day-2. On the day of bud appearance the DNA content registered was only 100 $\mu g\ gm^{-1}$ fresh weight-almost 45 % less than that of the dormant buds. The continuous decline in DNA levels implies the rapid turnover of DNA probably due to the active growth in absence of any nutrient medium.

5.3B. Changes in Ribo nuclease (RNase):

During the course of bud development, the RNase activity displayed increasing trend upto day-2 after leaf isolation (Fig. 5.1b). The activity registered on day-2 was higher than that of the day-0 activity. Thereafter the RNase displayed rapid and sharp decline. On day-4, the activity noted was about 3.1 fold lower than that of peak value on day-2 of leaf isolation. With further advance in the bud release process, the RNase activity enhanced again.

5.3C. Protease and its characterisation:

Soluble protein content in a Kalanchoe mortagei leaf was found to be extremely low (0.12 mg $gm^{-1}$ fresh weight). For biochemical (qualitative) analysis, it is mandatory that protein is maintained to a certain minimum concentration. Buffer extractable proteins were, therefore, concentrated by $(NH_4)_2SO_4$ precipitation. Desalting of precipitated proteins was performed by dialysis. On overnight dialysis in refrigerator most of the proteins (93 %) were found to be lost (Table 5.1). This indicated a strong proteolylic activity occurring even at low temperature. So partial characterisation of protease was done.

5.3C.1. Partial characterisation of Protease

The proteolytic activity displayed diphasic pattern over the pH range from acidic to basic (2 to 9) (Fig. 5.2a). In the acidic side, the peak value of protease was noted at pH 3.0, whereas on alkaline range the activity was maximum at pH 8.0. On the other hand at pH 9.0 there was very low proteolytic activity. No precipitation was observed during the pH studies, at any of the pH tested.

From the range of temperature regime (27 to 67 °C) tested at pH 8.0, the maximum activity was observed at 47 °C temperature. It was also found that even at low temperature (27 °C) there was a proteolytic activity (Fig. 5.2b).

To determine the nature of protease, further characterisation was accomplished using various specific inhibitors. The proteolytic

activity at pH 8.0 was found to be completely inhibited by PHMB at 10 mM concentration (Table 5.2). On the other hand, at pH 3.0, PHMB caused 50 % inhibition, however, when the concentration was raised to 50 mM, the protease activity was completely repressed.

The other protease inhibitor, PMSF failed to suppress the protease at both the pH optima. However, in the presence of EDTA (10 mM) there was a partial inhibition of proteolytic activity; the reduction in the activity being 54 and 57 % at pH 3.0 and 8.0 respectively. It appears that there are two protease in Kalanchoe leaf with pH optima 3.0 and 8.0. These enzymes are seem to be of sulfhydryl type. Subsequently for all biochemical analysis involving protein quantitative and qualitative analyser, PHMB was incorporated in the extraction and subsequent steps.

5.3C.2. Changes in proteases during bud outgrowth

Proteases were also analysed during bud development. Proteolytic activity demonstrated a decline after attaining a significant initial rise (Fig. 53a). On day-1 the protease activity registered was 1.6 fold higher compared to the day-0 value (dormant buds). Further development in the bud outgrowth process caused pronounced but gradual decrease in the protease activity. On the day of bud appearance (4th) the activity found was 4.3 times lower than that of peak value. But with further progress in bud development the protease exhibited increasing trend.

5.3D. Quantitative analysis of soluble proteins:

Quantitative analysis of soluble protein content was followed during the course of epiphyllous bud outgrowth. On day-0 the total protein content was found to be 0.12 mg $gm^{-1}$ fresh weight of notch explants (Fig. 5.3b) with the progress of bud development process, the protein content exhibited continuous increasing trend. Till day-3 after leaf isolation, a rapid accumulation of protein content was noted. On the day 4th of visual appearance of buds, the protein content recorded was 0.32 mg $gm^{-1}$ fresh weight which is almost 2.6 times higher than that of day-0 value. After 5 days of notch incubation (24 hr after visible bud appearance), the total protein content registered was 0.37 mg $gm^{-1}$ fresh weight. This continuous augmentation of protein content indicates that during bud development, there is continuous and rapid synthesis of soluble proteins.

5.3E. Qualitative analysis of total soluble proteins:

Marked changes in the protein pattern of notch region ($N_1$) and nonnotch part ($N_2$) were observed (Fig. 5.4)[a]. For better comparison, each separated protein band was numbered serially starting from the cathodic to anodic end. In all total (47 and 37 bands were noted in $N_1$ and $N_2$ samples respectively (Fig. 5.4)[a]. Most of the changes in protein pattern were observed in the pH range 5.3 to 10.0. In the pH range 7.0 to 9.2 the proteins from $N_2$ displayed dense staining when compared to $N_1$, the prominent being those numbered 26 to 36 proteins. On the other

hand, proteins numbered 8-13 (in the pH range 5.5 to 6.0) exhibited more dense staining in $N_1$ sample than that of $N_2$ proteins.

More specifically, band numbered 37-41 in the pH range 9.2 to 9.7 were exclusively present in $N_1$ protein sample, being absent in $N_2$ samples.

During the course of epiphyllous bud outgrowth from isolated notches, proteins were fractioned by IEF technique at every 24 hr interval upto total 120 hr.

After 24 hr incubation of isolated notches, the number of proteins found was only 12 (Fig. 5.4)b. The prominent one mostly in the central part of the gel (pH range 5.3 to 7.2) were those numbered 7-10, 12 and 17. In the extreme cathodic side of the gel, the major bands were 3, 5 and 6. Some minor faint bands were noted in few away anodic side, namely 35 and 42. In the pH range 7.5 to 8.0 many new bands without clear separation were observed. Only one of the 6 unique proteins of day 0 notches was found to be present after 1 day of incubation.

With further advancement in the bud development process total 16 and 11 proteins were registered on 2nd and 3rd day respectively. On day-2, four new bands reappeared in the pH range 7.5 and 10. They were 25, 30, 33 and 41. On 3rd day of explant incubation, the protein numbered 7 showed intense staining. The proteins numbered 11-15 also showed enhanced staining intensity.

On the 4th day of leaf explant incubation, concomitant with the visual appearance of buds, total 22 proteins were seen. The major bands were in the pH range 5.3 to 8.2. The most prominant being 7-11, 13-15, 25 and 26. On the extreme anodic side, there were six clear but faint protein bands (numbered 35-42) all of which except numbered 35 were not found on 3rd day of incubation.

5.3F. Determination of the time of de novo synthesis of proteins:

Application of CYCLO and ACTD inhibited the development of dormant buds in isolated notches (Fig. 5.5a). Both CYCLO and ACTD exhibited complete inhibition of bud outgrowth at 100 μM concentration.

In later studies with pulse application of protein synthesis inhibitor CYCLO it was judged that the bud outgrowth specific proteins are being synthesized during the first 12 hr of isolation (Fig. 5.5b), and subsequent culture of the notch explants. Hence, the proteins were extracted and fractioned from notches incubated for 6 and 12 hr.

On day-0 there were 42 protein bands, but after 6 hrs of incubation, total 23 major proteins were identified (Fig. 5.6). Thus after isolation and subsequent incubation of notches, the number of protein bands was reduced to almost half. However, still the unique proteins bands (37-41) were retained in the

6 hr incubated notches. But proteins numbered 37-39 were found more prominent compared to the rest of the unique proteins. The acidic proteins (13 and 14) were more prominant with high staining intensity. The protein (numbered 7) at pH 5.0 was more conspicuous compared to day-0 protein pattern.

On subsequent interval, protein namely 8-10 (pH 5 to 6) 19 and 30 exhibited prominent staining comparatively. On the other hand, bands 13 and 14, which were more pronounced at 6 hr interval, represented proportionally faint staining. Same was the case with those proteins numbered 38 and 39 (pH 9.02 to 10.0).

5.3G. Determination of *in vivo* rate of protein synthesis:

Uptake of ($^{14}$C)serine by the notch explants registered a biphasic trend (Fig. 5.7). An increase in the uptake was noted uptp 24 hr of isolation, followed by a slow decline upto 60 hr incubation. Subsequently, the uptake of ($^{14}$C)serine showed increasing trend upto 72 hr followed by decline again.

A rapid increase in the incorporation of ($^{14}$C)serine in proteins was registered upto first 12 Hrs of incubation (Fig. 5.7). With further advances in the bud growth, the incorporation declined upto 36 hr. During successive stages, the incorporation began to increase. A very rapid rate of incorporation was achieved between 36 to 60 hr incubation period. Eventhough, uptake and incorporation of ($^{14}$C)serine displayed decline during the bud outgrowth process, the protein accumulation continued with high rate throughout the development.

## 5.4. DISCUSSION

It is now known that plant differentiation and development result from changes in gene expression. The visible differences in structure of various cell types or tissues and their specific and significant metabolic characteristics result from the possession of unique patterns of proteins and the enzymes (Trevawas, 1982b) the concentrations of which are controlled at transcriptional or posttranscriptional levels (Wareing, 1982a).

During bud outgrowth from isolated notches of K. mortagei a continuous decline in DNA content was observed. This is in contrast to those studies that DNA synthesis takes place prior to differentiation and that hormones affect this synthesis (Roberts, 1976). However, the maximum amount of DNA observed in dormant epiphyllous bud of K. mortagei could be due to two possibilities: Firstly, it is known that most of the cells of leaf are highly differentiated and moreover such cells are found to be polyploid (Fosket, 1968; Torrey, 1961). Such polyploid cells could have contributed in the DNA analysis of epiphyllous dormant buds, their extreme small size at this stage would have inevitably included some amount of mesophyll cells. Secondly, the cells in the dormant meristems in the notches, probably are not spared to divide further due to their arrest at the early mitotic phase $G_1$. In support of the latter possibility, it has been shown that in the inhibited buds of pea, group of cells were characterized by 2C DNA and subsequently on activation (decapitation) they underwent cell division and exhibited decreased DNA/

histone ratio (Dwividi and Naylor, 1968).

In pea, it has been proved that RNA synthesis is required for the induced growth of lateral buds (Sharpe and Schaeffer, 1970). Inhibition of bud outgrowth by actinomycin-D in the present study supports the role of RNA synthesis for the induction of bud outgrowth. Our data show that a slight rise in total RNA content was taking place during day-1 of isolation and thereafter almost the same amount was maintained throughout the process. This slight decrease in RNA content on day-2 and maintainence of same levels on subsequent days indicates rapid turnover of RNA fractions.

A decrease in the amount of RNA can only be observed if RNA breakdown has taken place due to RNase activity. RNase activity shows a clearcut correlation with growth and differentiation (Vetter, 1974). This is true in the sense that RNAse activity found to be usually low in the young meristematic cells and with cell age (maturity) gradually increases (Pilet and Braun, 1970; Trebal et al., 1979).

A slow increase noted in RNase activity with the lag period (24 hr) followed by a decline and subsequent rise again indicates its putative role in active turnover and differential requirement of RNA fractions for bud outgrowth and its subsequent development (Dove, 1971). The physiological significance of such an enzyme which is very sensitive to stress conditions (Lauriere, 1983) could be the selection of specific RNAs which

are needed at particular stages during bud development as concluded by Farkas (1982) in addition to the probable regulation of RNA at transcriptional level (Jacobson, 1977). Apart from all these possibilities, wound induced RNase activity also can not be ruled out with the probable involvement of ethylene and/or ABA which are known to be synthesized in injured cells. Stimulating effect of ethylene on the induction of wound RNase in turnip root tissue has been reported (Sacher et al., 1979). Similarly, ABA induced rise in RNase activity has been found in Rheo leaves (De Leo and Sacher, 1970), Avena (Wyen et al., 1972).

Proteolysis plays critical roles in various cellular processes such as cell differentiation including activation or inactivation of specific enzymes (North, 1982). A considerable interest in the molecular basis of ontogenetical processes has led to the realization that protease are intricately involved in plant metabolism (Storey and Wagner, 1986). In fact, in higher plant cells, intracellular protein breakdown is a fundamental process which has important implications in biochemical regulation and physiological events (Davies, 1982; Lauriere, 1983; Canut et al., 1985). However, in plant tissues, proteolytic analysis has centered mostly on the role of proteases in mobilizing reserve proteins, mainly during seed germination and subsequent seedling development (Ashton, 1976; Ryan and Walker-Simmons, 1981; Mikkonen, 1986) and in leaf tissues during senescence (Sabater, 1984).

Very high activity of protease (alkaline) in the early

stages of bud growth indicates rapid changes in proteins for the sustained and active growth in the notches. In this regard Carlberg et al., (1984) have shown that protease activity during differentiation increases and this seems to be a general phenomenon (North, 1982; Huffaker and Peterson, 1974). The proteolytic enzymes are found to be present in leaves in relatively inactive form (Davies, 1982). An increase in protease activity was noted in bean leaves (Racusen and Foote, 1970); and its developmental role was suggested in the control of selective protein levels. Jameel et al. (1984) has correlated the loss of isocitratelyase activity with the appearance of proteinase in flax seeds during germination. Such a mechanism in turn may also be supplying metabolised product (reduced nitrogen) to the developing points (Ragster and Chrispeels, 1979) in the excised notches.

The decline in the proteolytic activity observed in the later stages coinciding with bud appearance could be due to the presence of protease inhibitor which are abundant in higher plants (Ryan, 1973), and known to accumulate even during the growth of tobacco callus (Wong et al., 1975).

Relatively no change in the acidic protease during the course of bud development indicates the differential role of the two proteases. Earlier, Ragster and Chrispeels (1979) have also reported two proteases; one occurring in young leaf during expansion and maturation and other one during leaf senescence. Similar differential roles and activities were ascribed to proteinases during germination in kidney bean (Mikkonen, 1986).

Both the transcriptional and translational inhibitors completely inhibited the bud growth in Kalanchoe mortagei, indicating the necessity of de novo synthesis of proteins for bud outgrowth.

A continuous increase in total soluble protein content during the course of bud growth indicates active growth of the primordia. In fact, substantial increase in the rate of protein synthesis has been reported to accompany the activation of growth of quiscent cells in a many plant systems (Byrne and Setterfield, 1977; Cella et al., 1976; Kahl and Wielgat, 1976; Ramagopal et al., 1977, Sparkuhl et al., 1976; Verma and Marcus, 1974; Fosket, 1981). The substantial increase in total protein content reflects the active growth of the epiphyllous bud upon isolation. This is in contrast to the reports that in detached leaves and leaf discs there is a large decrease in total protein content (Martin and Thimann, 1972; Thomas, 1978; Davies, 1982; Lamattina et al., 1987) and stress (Cooke et al., 1979a and b).

In present studies, time course experiments using cycloheximide suggested that the new protein(s) necessary for the induction of growth are being synthesized within first 12 hr of incubation. Sung and Okimoto (1981) reported the synthesis of embryogenic marker proteins in cells within 4 hr after the onset of embryogenesis, appearing several days prior to detectable morphological changes. The present studies clearly indicates that even though the visible appearance of buds occur on 4th day, the synthesis of new protein(s) mandatory for

growth induction are being synthesized in first 12 hr of isolation. However, direct demonstration of de novo synthesized proteins by 2-dimensional gel separation followed by fluorography in the present material posed several technical problems. The foremost being extremely low content of protein per gm fresh weight of tissue coupled with high protease activity distributed over a wide pH range between 2 and 9. Phenolics and organic acids of tissues also rendered electrophoretic separation difficult (Hari, 1981; Berger et al., 1985). However, the qualitative analysis of soluble proteins using IEF technique was possible.

Comparison of urea extracted protein profiles of notch and nonnotch region revealed that the notch region having dormant bud primordia harbours few unique proteins (37-41) which were not seen in nonnotch i.e. mesophyll region of leaf. This is in confirmation with many earlier observations e.g. specific proteins are known to occur in different floral parts of tomato (Sawhney et al., 1985); root nodules in soybean (Legocki and Verma, 1980), pathogenesis related proteins (Pierpoint, 1986; Parent et al., 1985; Antoniw et al., 1983; Matsuoka and Ohashi, 1986). Recently, Choi et al.,(1987) reported the occurrance of 50 kD protein which was correlated with embryogenic ability in cultures of carrot including cereals.

These particular proteins (37-41) specific to only notch region termed as notch specific proteins. The presence of such specific proteins could be due to the attainment of certain physiological state-fulfledged primordia in the exact notch

region. Recently, the comparison of three different cell types in the root of pea have shown that a few meristematic abundant mRNA were lacking in both elongating and mature stages. Therefore, they were regarded as meristematic-stage specific and suggested that they code for proteins involved in cell division (Vaillant et al., 1983). The other possibility could be the differential net protein synthesis in the two sites (Huckaby and Miller, 1984) of the leaf. Proteins in nonnotch mesophyll region namely 22, 23, and 26-28 displayed higher content which may be the major proteins involved in the photosynthetic metabolism (Link et al., 1979; Viro and Kloppstech, 1980; De Vries et al., 1983) and partly therefore of Rubisco protein (Des Frances et al., 1985).

During the course of epiphyllous bud outgrowth gradual disappearance of many proteins and reappearance of new proteins reflects their active turnover during the process. However, possibility of senescence induced slight changes also cannot be ruled out as already shown for senescence induced modifications in protein patterns (Dhindsa et al., 1986). Earlier, during gradual ageing of pea leaves, a decline in certain proteins with increase in others has been reported (Malik and Berrie, 1977). Similarly, slight changes in protein profile of apple seeds during stratification (Eichholtz et al., 1983), spore germination and rhizoid differentiation in Onoclea fern (Huckaby and Miller, 1984), cold acclimation and desiccation stress in wheat and rye (Cloutier, 1983), alfalfa (Mohapatra et al., 1987a and b) and

induction of frost tolerance in bromegrass cultured cells (Robertson et al., 1987) and heat shock in various systems (Cooper and He, 1987; Necchi et al., 1987).

The maximum uptake of ($^{14}$C)serine by the explant was noted upto first 24 hr of isolation and subsequent incubation. However, the incorporation of labelled serine into TCA precipitable protein was maximum till first 12 hr of incubation. This coincides exactly with the enhancement and peak attainment of IAA oxidase in the epiphyllous buds (chapter VI), and with the knowledge using cycloheximide that new proteins are synthesized within first 12 hr of isolation of the leaf. With further advances in the bud growth process, while the uptake and incorporation of ($^{14}$C)serine reduced with rapid pace in the latter, the protein accumulation continued throughout growth. Similar patterns of protein accumulation with low rate of incorporation was reported during cytodifferentiation of tracheary element (Simpson and Torrey, 1977) in pea cortical explants. This low rate of incorporation and continuous accumulation of proteins indicates very low degradation which was substantiated by proteolytic studies. However, with subsequent progress in bud growth process, a continuous accumulation of protein including rapid incorporation (protein synthesis) was noted.

Surprisingly, even though the importance of correlative inhibition has been realized long back in agricultural and ecological interests (Tucker, 1979) and many factors have been

shown to affect the process, a sequence of events between the induction and bud growth has not yet been understood (Rubinstein and Nagao, 1976; Hillman, 1984; Tamas, 1987). The main reasons for the lack of such information are already discussed in the introduction of this chapter. It seems that the studies of molecular mechanisms involving identification and characterisation of proteins synthesized at various stages of budgrowth, induction and subsequent development as reported here using simple experimental system of epiphyllous buds of Kalanchoe mortagei, may help in understanding the process of correlative inhibition and thus ultimately plant development.

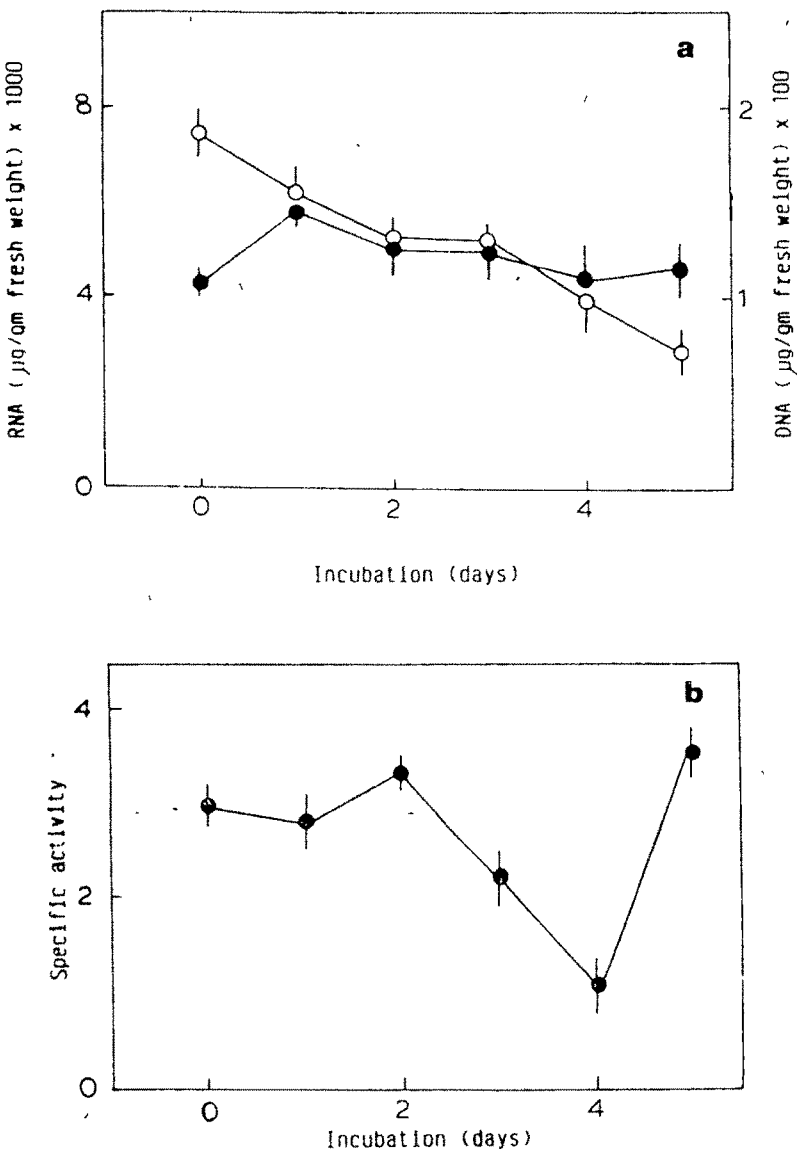


Fig. 5.1. Changes in RNA ( ○ ) and DNA ( ● ) content (a) and RNase enzyme (b) during the course of epiphyllous bud outgrowth.

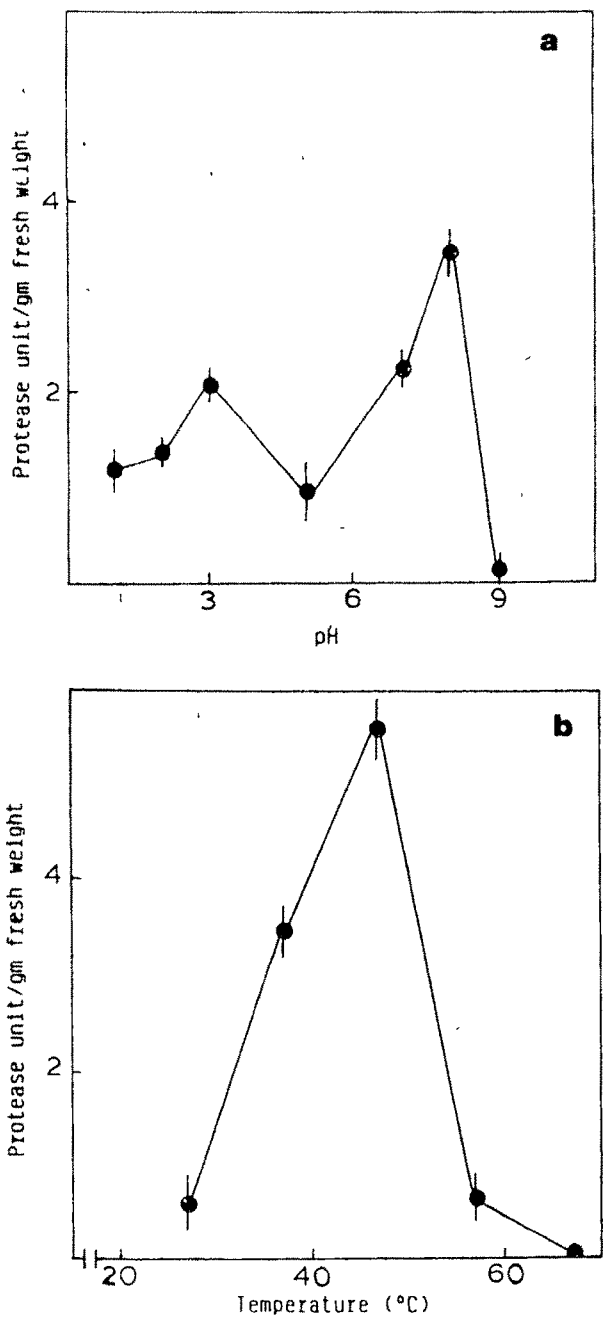


Fig. 5.2. Effect of pH (a) and temperature (b) on proteolytic activity from Kalanchoe mortagei leaf.

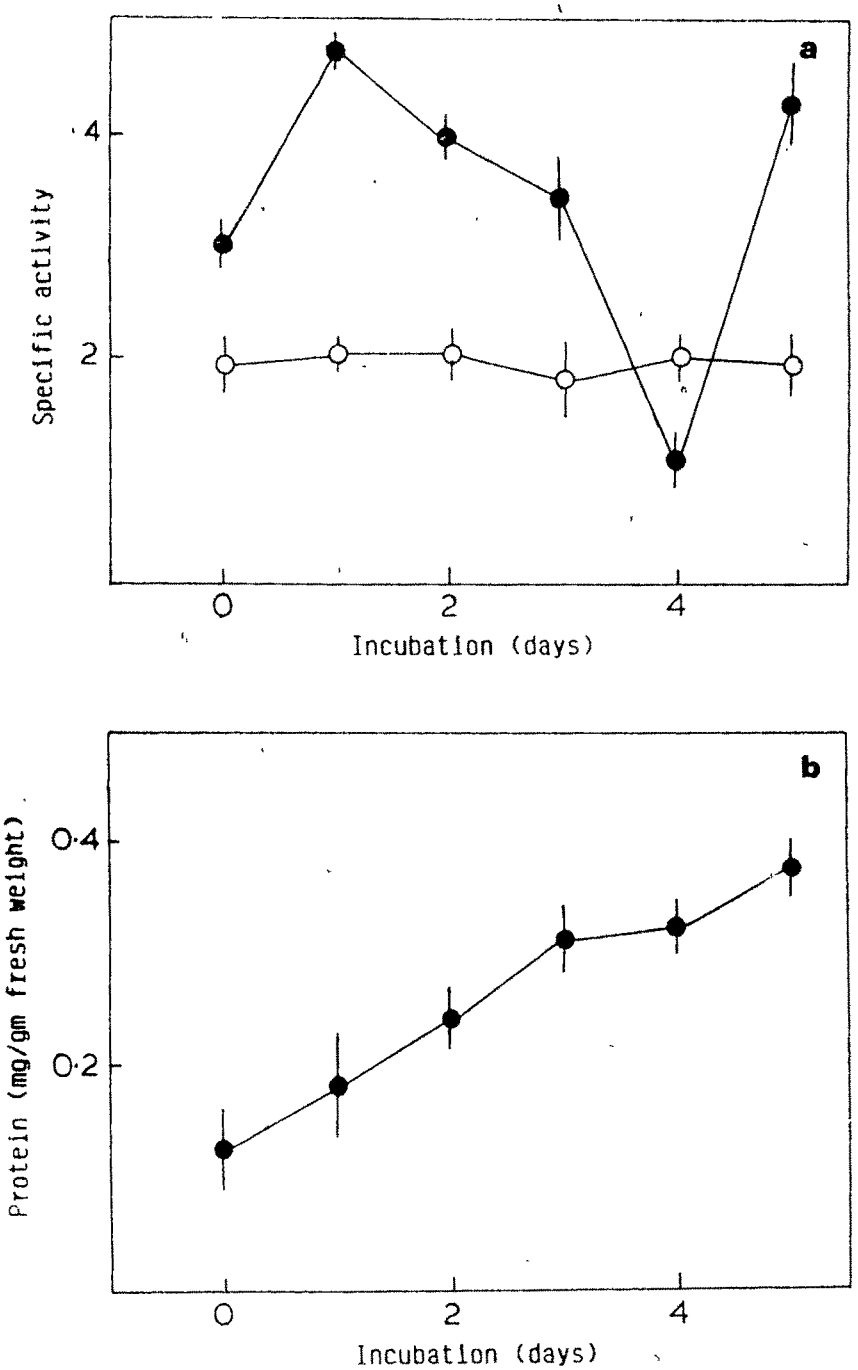


Fig. 5.3. Progressive changes in acidic (○) and alkaline (●) protease activity (a) and total soluble protein content (b) during bud outgrowth.

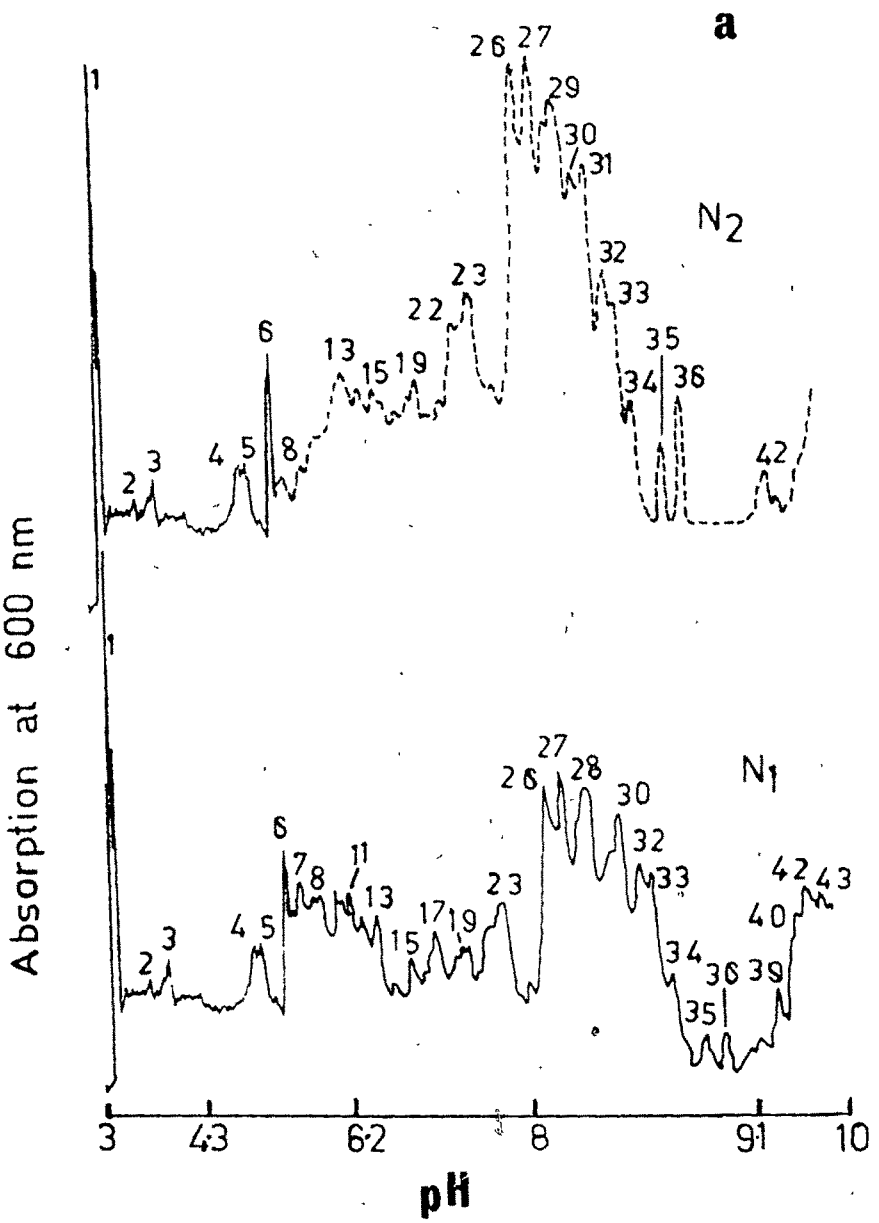


Fig. 5.4. Densitometric scan of soluble proteins.
a) Comparison of notch ($N_1$) and nonnotch ($N_2$) part of a leaf.
Note the notch specific proteins in the extreme right.of the scan.

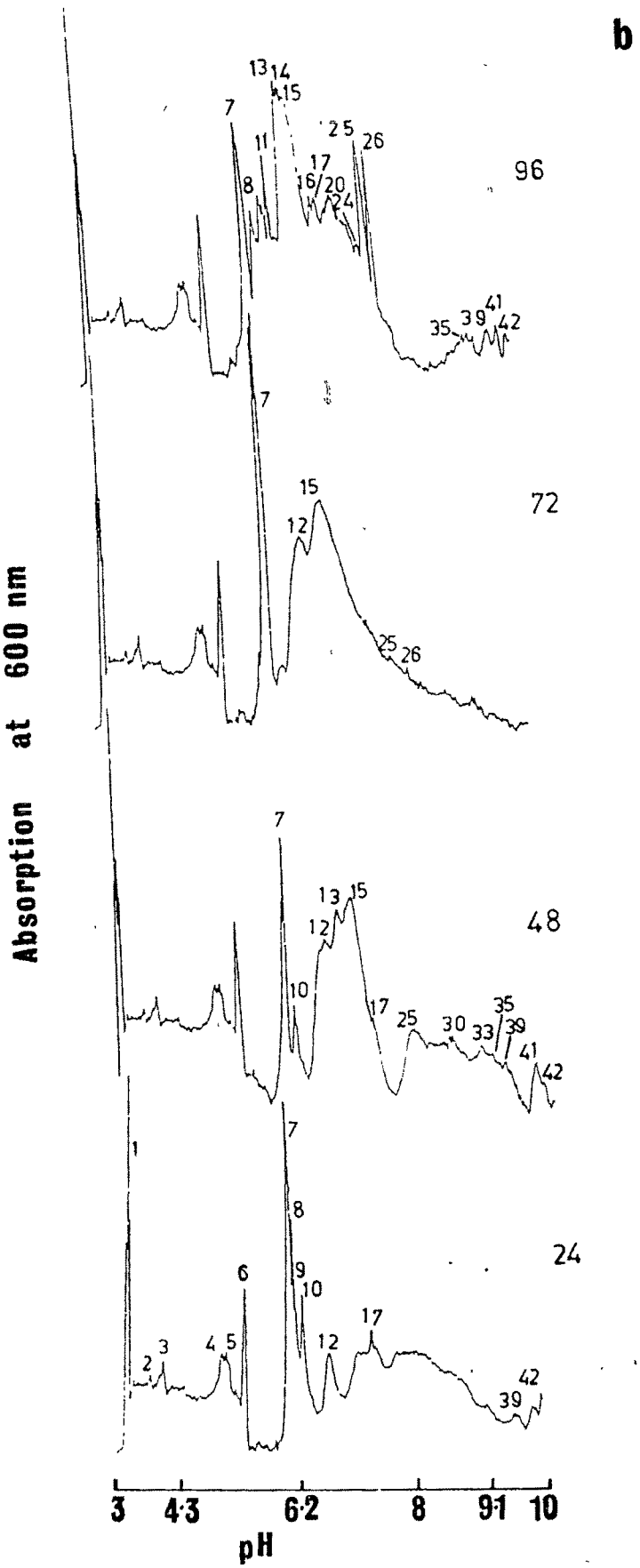


Fig. 5.4. Densitometric scan of soluble proteins.
(b) Continuous changes in protein profile during the course of bud outgrowth.

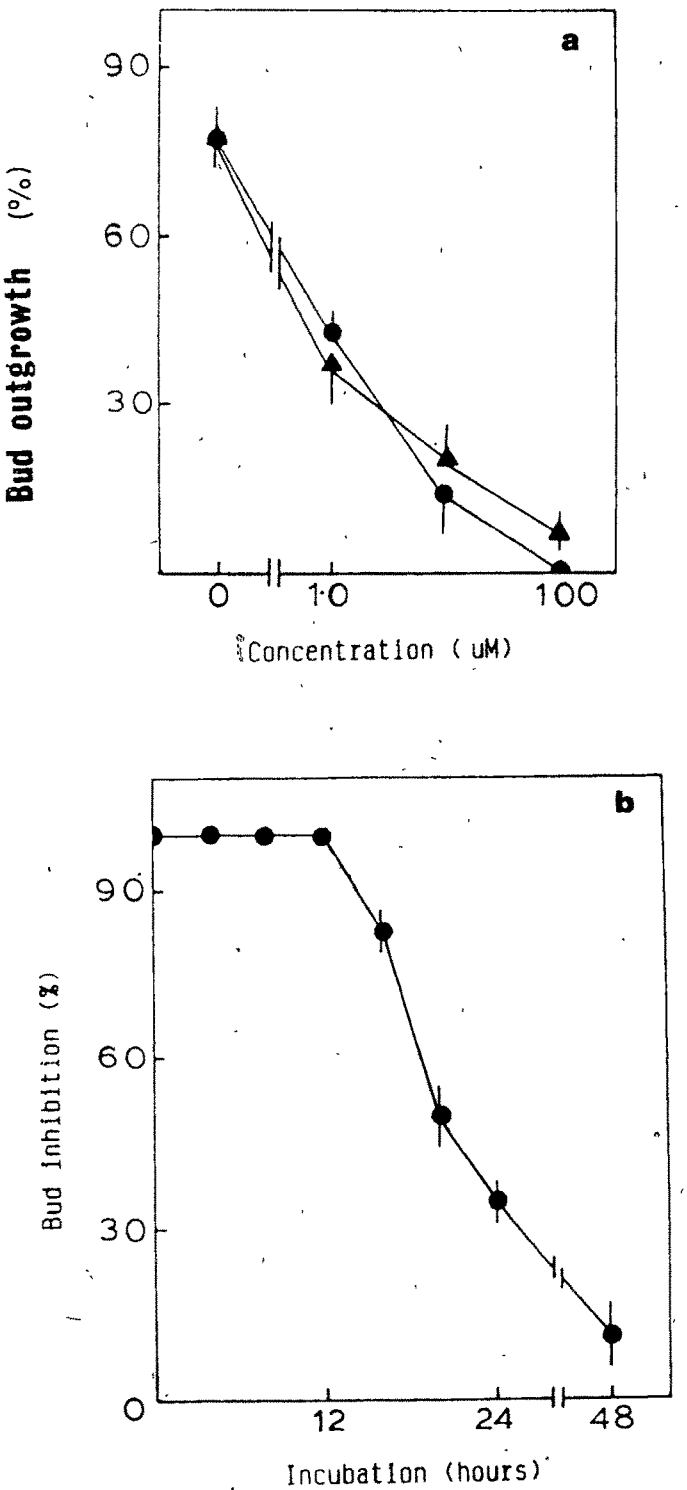


Fig. 5.5(a) Effect of cycloheximide (●) and actinomycin-D (▲) on bud outgrowth response.

(b) Effect of pulse application of cycloheximide on bud growth. Note the inhibition of bud growth upto first 12 hr of isolation and subsequent incubation in cycloheximide.

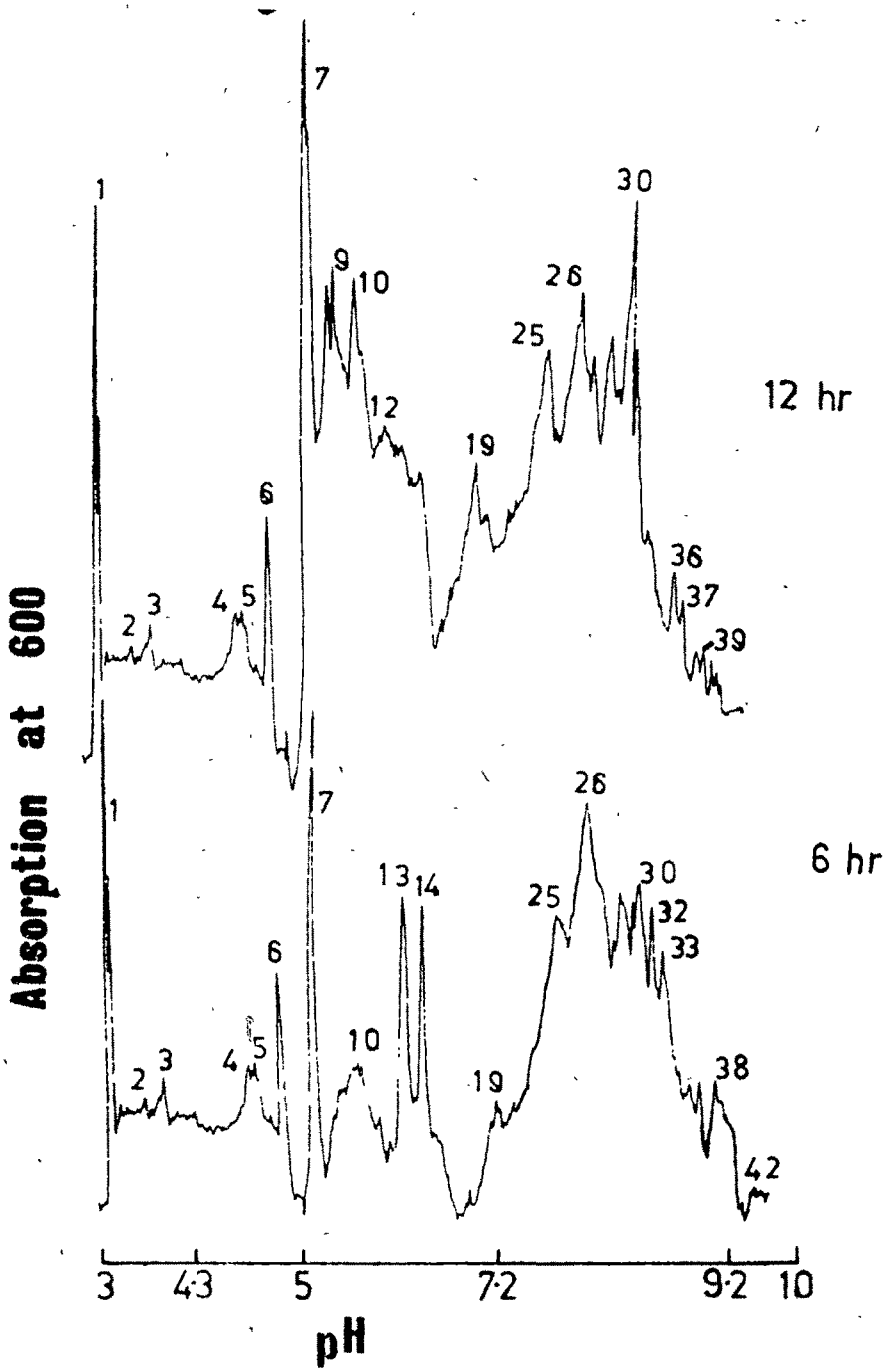


Fig. 5.6. Changes in protein profile of soluble proteins from reactivated dormant buds at 6 and 12 hr interval.

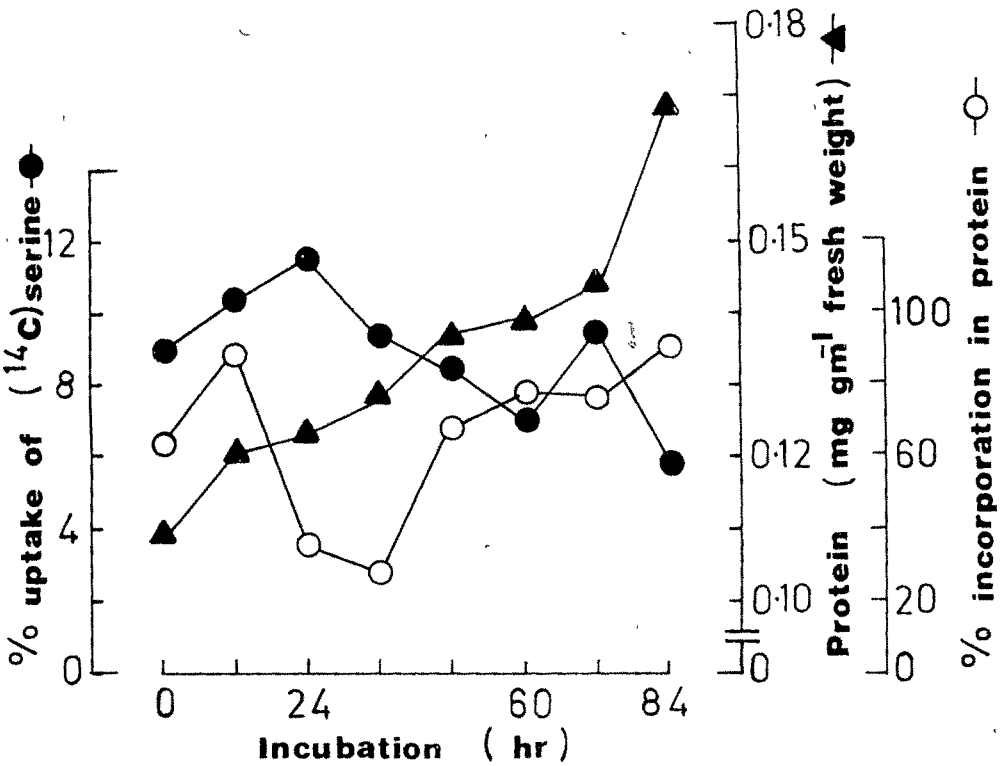


Fig. 5.7. Changes in ($^{14}$C)serine uptake and incorporation in soluble proteins and accumulation of proteins during reactivation and subsequent growth of dormant buds.

Table 5.1 Soluble Protein loss during dialysis after $(NH_4)_2SO_4$ precipitation

| | Protein concentration (mg/gm fresh weight)* | % protein loss |
|---|---|---|
| Before precipitation | 0.135 | - |
| After precipitation and dialysis | 0.009 | 93.33 |

* Extracted in citrate-phosphate buffer pH 7.6 and quantified by modified Lowry's procedure (Hartree, 1972).

Table - 5-2. Effect of inhibitors on proteolytic activity of crude enzyme from *Kalanchoe mortagei* leaf.

| Control/Inhibitor | Concentration used (mM) | Unit protease activity at pH | |
|---|---|---|---|
| | | 3.0 | 8.0 |
| Control | - | 2.42 | 4.14 |
| EDTA | 0.1 | 1.86 | 2.60 |
| | 10.0 | 2.06 | 2.33 |
| Alcohol Control | 25.0 | 1.28 | 1.92 |
| PMSF | 0.1 | 1.28 | 1.92 |
| | 1.0 | 0.96 | 1.92 |
| | 10.0 | 0.96 | 1.27 |
| NaOH Control | 1.0 | 1.28 | 0.64 |
| PHMB | 0.1 | 1.28 | 0.64 |
| | 1.0 | 0.64 | 0.64 |
| | 10.0 | 0.64 | 0.64 |
| | 50.0 | 0 | 0 |