6. INVOLVEMENT OF IAA OXIDASE IN BUD OUTGROWTH

6. INVOLVEMENT OF IAA OXIDASE IN EPIPHYLLOUS BUD OUTGROWTH

6.1 INTRODUCTION

6.1A. Plant growth hormones and enzymes during development:

While progressive development is manifest by form changes and associated alterations in structural element, functional activity of newly formed organs becomes possible only through biochemical mechanisms. Thus, enzyme involved in a function will attain maximum activity in appropriate spatial configurations prior to or simultaneously with functional maturation. Since all enzymes are proteins, the synthesis of enzymes must be linked to the synthesis of specific proteins necessary for the initiation of functionally significant structures. The genetic control of enzyme synthesis so well documented in microorganisms and in eukaryotes indicates that absnece or presence of an enzyme at a given site or time may provide information on differential gene activity during progressive development (Raghavan,1976).

It is now a well documented fact that hormones influence the enzyme status or level in a plant (Datko and Maclachlan,1968; Kasamo and Yamaki, 1974; Varner and Ho, 1976; Barendse, 1983; Koukkari and Warde,1985), through qualitative alteration in the mRNA molecules (Sen, 1984). Ho (1979) suggested that hormones can also modify existing enzyme systems in the target tissue, in order to increase their metabolic efficiency to meet the

physiological demands. Hormones can thus, also act as inducers of differentiation in target tissue (Ho, 1979; Wareing 1982a). However, the biochemical mechanisms underlying any stimuli induced morphogenetic events are still unknown (Halperin,1986). Thus, an attempt was made to detect any change in selected enzymes inducing/ during bud outgrowth from isolated notches.

6.1B Phenolic metabolism during plant growth:

Phenolic metabolism has been shown to intimately associated with growth. The occurrance of phenolic compounds in the vacuoles (Harborne, 1984) is the main factor causing varied physiological roles of these phenolics in relation to plant growth and development (Kefeli and Kutacek,1977; Sembdner et al., 1980). Their accumulation causes cessation of growth (Nash and Davies 1972; Lam and Street,1977; Heyser and Mpft, 1980; Patel et al., 1984; Muhitch and Fletcher,1985). Their influence is mediated through hormonal action, as they are known to impinge upon IAA oxidase activity and thus regulating IAA metabolism. (Lee and Skoog, 1965; Tomaszewski and Thimann, 1966; Haissig, 1974; Wolf et al., 1976; Schneider and Wightman, 1978). Numerous reports indicate that monophenols and m-diphenols act as cofactors or activators while o- and p-diphenols and polyphenols inhibit IAA oxidase activity or induce a lag period (Lee and Skoog, 1965; Stonier and Yoneda, 1967; Yoneda and Stonier, 1967; Janssen,1970; Van der Mast, 1970; Imbert and Wilson,1972; Runkova et al.,1972; Hamilton et al.,1976;

Machackova and Zmrhal, 1976; Robert et al.,1976). Thus, o- and p-diphenols and poly-phenols act as growth promoters as they inhibit IAA oxidase activity whereas monophenols and m-diphenols are cofactors for IAA decarboxylation and so growth inhibitors (Stafford, 1974; Bearder,1980; Sen,1984). Many workers have demonstrated the occurrance of high molecular weight-auxin protectors from tobacco (Phipps,1965), Pharbitis (Stonier and Yang,1971,1973; Stonier and Yoneda,1967). These are extremely high molecular weight substances (Mato and Vieitez, 1986) which are partly proteinaceous (Novak and Galston, 1971) and polymers of o-dihydroxyphenylpropanoids (Griffin and Stonier,1975) with an active site of o-dihydroxyphenol (Stonier and Yang, 1971; 1973).

Apart from acting as inhibitors/activators of IAA oxidase these phenoles are themselves very susceptible to enzymatic oxidation due to the specific phenolase enzymes (Butt, 1979; Duke and Vaughn,1982; Harborne, 1984). Earlier, Houck and Rieseberg (1983) observed a strong stimulatory effect of p-coumaric acid (PCA) on the epiphyllous bud activation in case of B.calycinum. These authors suggested that the stimulatory effect of PCA on the bud growth is due to the activation of IAA oxidase enzyme, although no data was given on the actual enzyme activity. In this context, the following sequence was hypothesized in the resumption of growth of dormant epiphyllous buds in K.mortagei.

(1) Removal of leaf from the plant causes stress in/near the meristematic region of epiphyllous buds.

(2) This stress triggers ethylene biosynthesis.

(3) Increased ethylene triggers induction of IAA oxidase.

(4) Augmented IAA oxidase level brings down the endogenous IAA content, thereby reducing auxin/cytokinin ratio favourable for resumption of bud growth.

Evidence in favour of the first two events have been presented in chapter 3 and 4. In the present chapter evidence for events 3 and 4 are elaborated.

Because of the availability of numerous epiphyllous buds and their rapid and synchronous manner of development, they provide an excellent experimental material for studies on biochemical and molecular analyses of underlying physiological processes. A large number of factors have been found to trigger growth of these dormant buds. The detachment of leaves from mother plant has been found to break the dormant state of buds in most of the Kalanchoe species (Resende,1959). In Bryophyllum diagremontianum and B.tubiflorum, the activation of buds on attached leaves is under photoperiodic control (Heide, 1965), long day being stimulatory. However, bud activation is possible also in short days under high temperature regimes in B.tubiflorum and with kinetin treatment in B.diagremontianum (Heide, 1965).

Earlier, Loeb (1915) demonstrated that in B.calycinum leaf detachment of injury promoted the dormant buds to develop

into plantlets. This was presumed to be due to a block in the flow of an inhibitory substance away from the notches in the leaf. Later on, this inhibiting substance was assumed to be a hormone (Loeb,1917).

In recent past, it was suggested that the trigger of bud outgrowth could be brought about by a decrease in the auxin/cytokinin ratio within the buds as the exogenously applied auxin suppressed the growth and cytokinin treatment stimulated the bud growth in vitro (Vardar and Acarer, 1957; Heide, 1965; Yazgan and Vardar,1977; Henson and Wareing, 1977; Karpoff,1982; Houck and Riesebergs, 1983). Thus, the knowledge on the biochemical mechanism that could decrease auxin/cytokinin ratio in or around the buds prior to their growth could provide a fundamental support to this idea. This chapter outlines the changes in IAA oxidase levels in the dormant buds during their induction and course of outgrowth. The enzyme IAA oxidase has been implicated to reduce the endogenous IAA level in various systems (Galston and Hillman, 1961; Lee and Skoog,1965; Lee, 1972; Thimann, 1972; Gaspar et al., 1975; Wolter and Gorden, 1975).

Anatomical studies made during the ontogenesis of epiphyllous buds indicated that eventhough the buds are present on young leaf located on the first node from the plant apex, they attain capability to grow only when the subtending leaf attains the 4th node position. Therefore, in first part of

this chapter, changes in endogenous levels of IAA and IAA oxidase in the leaves located on different nodal positions in a plant are presented in order to relate their levels with the changes in the ability of epiphyllous buds to grow. In the second part, changes in the level of IAA oxidase were studied in the excised epiphyllous buds that were induced to grow in vitro. Two groups of inhibitory compounds - one of protein synthesis and the other of IAA oxidase activity, were found to suppress the growth of the buds in vitro. Changes in IAA oxidase activity in these two treatments would provide a clue to establish a cause and effect relationship between the changes in IAA oxidase activity and the reactivation of bud outgrowth. Further, de novo synthesis of IAA oxidase was confirmed using L- (^{14}C)-serine during the course of bud outgrowth.

6.2 MATERIALS AND METHODS

6.2A Experimental material :

The fourth nodal leaves from clonal stock of Kalanchoe mortagei plants were taken and washed thoroughly as mentioned earlier. For all biochemical analysis during bud development, leaf notches measuring 1 cm^2 were excised from the middle portion of the leaves. They were incubated on filter paper soaked with dist water (or otherwise mentioned at respective places). All test solutions including dist water were adjusted to pH 5.8 ± 0.1 before use. The cultures were kept in a culture room at 25 ± 2 °C with 15 hr photoperiod of 10.6 Wm^{-2} from 40 W fluorescent tubes

(philips India Ltd.). Some notches (20-30) were removed every 24 hr (or otherwise mentioned) and the bud meristematic zone measuring 6 mm^2 (approximately) were dissected out precisely with a sharp scalpel from the notch region. Care was taken to avoid any damage to the actual meristematic point. Such meristematic points were used for all biochemical investigations.

6.2B. Enzyme extraction

The meristems weighing 0.5 gm were homogenized in 2.5 ml cold 0.2 M borate buffer (pH 9.0), with insoluble PVP (2 %, w/v) and a pinch of glass powder in chilled pestle and mortar. The homogenate was left on a magnetic stirrer (30 min). Cell debris from the slurry was removed by centrifugation at 15,000 g (15 min). The resultant supernatant was mixed with 3 volumes of chilled acetone and kept on ice (30 min). The precipitated proteins were pelleted by centrifugation at 20,000 g for 30 min and vacuum dried to remove traces of acetone. The resulting precipitated proteins were dissolved in respective buffers and used as an enzyme source. All these steps were carried out at 0-10 °C temperature.

6.2B.1 IAA oxidase assay

The enzyme IAA oxidase was assayed using the modified procedure of Gordon and Weber (1951). The reaction mixture consisted of 0.5 ml each of partially purified enzyme extract, 0.2 M citrate phosphate buffer (pH 3.0), manganous chloride

(1.0 mM), 2,4-dichlorophenol (1.0 mM) and IAA (1.14 mM). The mixture was incubated in dark at 30 °C (30 min). After incubation, 5 ml Salkowski reagent (1 ml 0.5 mM $FeCl_3$ added to 50 ml of 35% perchloric acid) was added to the reaction mixture and mixed thoroughly and left for 25 min at room temperature. The absorption of the resulting solution was measured at 530 nm wavelength. The unit of IAA oxidase activity was defined as the amount of enzyme necessary for oxidizing 1 μg IAA. Specific activity of the enzyme was calculated as unit activity h^{-1} mg^{-1} progein.

To study the changes in IAA oxidase levels during leaf development, leaves from 1-6 nodes of six month old plants were excised and washed thoroughly. Immediately, bud meristem measuring approximately 6 mm^2 were dissected from the middle notches and used for enzyme assay as mentioned earlier.

6.2B.2. Peroxidase assay

Peroxidase enzyme was analyzed according to Shannon et al. (1966). The assay mixture contained 2.8 ml of o-dianisidine buffer (1.6 ml of 0.5 % o-dianisidine in HCl (1 N) + 4.8 ml Na-acetate buffer (0.6 M, pH 5.4) + 43.6 ml dist water), 0.1 ml H_2O_2 (1 %, v/v) and 0.1 ml of enzyme extract. The absorption of resulting solution was recorded spectrophotometrically (Shimadzu, Japan) at 460 nm at every 30 sec interval upto total 90 sec. The amount of enzyme required to change the absorption by 0.001 per min per mg protein was taken as unit enzyme activity.

6.2B.3. Polyphenol oxidase (PPO) assay

PPO was assayed according to the method described by Stafford and Galston (1970). The assay system included chlorogenic acid (0.1 mM), EDTA (1.0 mM) and enzyme extract in phosphate buffer (100 mM, pH 6.0). The absorption of resulting solution was recorded at 326 nm at every 30 sec interval upto total 120 sec. The unit activity was the amount of enzyme necessary to increase the absorption by 0.001 under the assay conditions. The specific activity was the unit activity per mg protein.

Protein quantification:

The protein content in the enzyme source was estimated by the dye binding method (Bradford, 1976) using BSA as standard.

6.2C. Extraction and quantification of IAA during leaf development:

IAA was extracted and quantified fluorimetrically (Stoessl and Venis, 1970) in the developing leaves. 15-20 gm frozen tissue (middle part of the devained leaves) was homogenized in 80 % (v/v) cold methanol (1:10, w/v) as suggested by Morgan and Durham (1983) using butylated hydroxytoluene (1 mg/ml) (Iino et al., 1980), glass powder in chilled pestle and mortar.

The slurry was kept on magnetic stirrer for 12 hr, vacuum filtered and reextracted for further 12 hr using same solvent.

The combined filterate was centrifuged (10,000 g; 15 min) to remove cell debris. The methanolic supernatant was reduced to aqueous phase under vacuum (37 °C), and left overnight in freezer. The frozen extract was thawed and mixed with $(NH_4)_2SO_4$ (0.76 gm/ml) and kept on magnetic stirrer (1.0 hr). The precipitated proteins were removed by centrifugation (10,000 g; 15 min). The clear supernatant was adjusted to pH 2.5 (with conc H_2SO_4) and extracted thrice with diethyl ether. The combined ether phase was reduced partly and stored overnight in deep freezer to remove traces of water. The ether phase was then taken to dryness and the residue was redissolved in absolute ethanol. The total IAA in this extract was determined fluorometrically.

Briefly, small aliquote of sample was taken to dryness in test tube. The sample was then derivatized with a mixture of trifluoroacetic acid and acetic anhydride (1:1, v/v) at 0-10 °C temperature. After 15 min the reaction was stopped with chilled Na_2CO_3 (5% w/v). The fluorescence of resulting solution was measured at 500 nm using 450 nm as excitation wavelength (Systronics, India). Correction for background fluorescence was obtained by adding Na_2CO_3 to the sample piror to derivatizing mixture. The total IAA in the extract was calculated from reference line prepared using indole-3-acetic acid as standard. The value was corrected for loses during purification, by running the same procedure using authentic IAA (Sigma).

6.2D. Extraction and quantification of phenols:

Phenolic compounds were extracted by homogenizing sampled and dried (50 mg) notch explants (6 mm^2) in chilled mortar with cold methanol (80 %, v/v) and a pinch of glass powder. The homogenate was centrifuged at 8000 g (10 min). The supernatant was collected and the residual pellet was reextracted (twice) with same solvent. The supernatants of all three extractions were pooled and final volume was made to 25 ml with same solvent.

The total phenolics in the pooled extract was quantified using the method of Swain and Hillis (1959). An appropriate aliquote was diluted to 7.0 ml with dist water and 0.5 ml of Folin-phenol reagent was added and mixed thoroughly. After an incubation (3 min), saturated Na_2CO_3 (1 ml, 33 % w/v in water) was added, vortexed and left at room termperature (60 min). The absorption of resulting solution was read at 725 nm and total phenols were calculated using chlorogenic acid as standard.

6.2E. <u>In vivo</u> labeling of IAA oxidase during bud outgrowth:

Total 65-70 notch explants measuring (0.25 cm^2) were induced for bud outgrowth by keeping on filterpaper soaked with dist water, cycloheximide (10 mM), BAP (1 μM) and pyrocatechol (0.1 mM) each containing (^{14}C)serine (105 mCi/mmol) at 13.5 μCi/ml in petridish. After incubation (12 hr) the explants were taken out and washed thoroughly with dist water containing cold serine.

6.2F. Qualitative analysis of IAA oxidase:

The labeled explants were homogenized in 0.2 M borate buffer (pH 9.0) and left on stirrer for 30 min, followed by centrifugation at 15,000 g (15 min). The clear supernatant was mixed with three volumes of cold acetone and left on ice (30 min). The precipitated proteins were pelleted by centrifugation at 20,000 g (20 min). The resultant pellet was vacuum dried to remove traces of acetone and later on dissolved in Tris-HCl buffer (0.5 M; pH 6.8). Total 60 µg of cleared and partially purified protein samples were applied to nondenatured polyacrylamide (8 % or 10-18 %) vertical slab gel (PAG) (0.5 mm thick). Proteins were separated with Tris-glycine buffer 0.25 M, pH 8.3 at 150 volts and 25 mAmp current (for approximately 3-4 hr). These all analytical steps were conducted in cold (0-10 °C).

The separated proteins were stained for IAA oxidase using the modified method as described by Hoyle (1977).

IAA oxidase staining solution -

A : Fast Blue BB salt (10 mg) dissolved in ethanol (2.5 ml);

B : A mixture of 2 µM each of p-coumaric acid, IAA and H_2O_2 dissolved with few drops of ethanol in dist water.

C : 2 M Na-acetate buffer (pH 3.8).

The working staining solution was prepared by mixing solutions, A, B and C in the ratio of 1:2:1.

The isozymes (A-C) of IAA oxidase detected, were cut from the gel and solubilized individually in scientilation fluid (10 ml) using H_2O_2 (few drops) and the radioactivity counts were taken on scientilation counter (LKB, Sweden). Similarly, aliquotes were taken from earliest clear supernatants, and protein samples for radioactivity counts to calculate uptake and incorporation of (^{14}C)serine in soluble proteins.

6.2C. Statistical analysis:

Various parameters were subjected to student's test for any correlation between them, as described by Redei (1982).

6.3. RESULTS

6.3A. Changes in IAA content during leaf development:

Compared to the leaves of other nodes, the first nodal leaves contained the maximum amount of IAA (Fig. 6.1a). With the progressive maturity of the leaf, the IAA levels declined rapidly upto the 4th nodal leaf position which registered (0.31 µg) almost eight times less than that of the first nodal leaf. In the leaves of subsequent lower nodes (towards the base of the plant) the IAA levels maintained almost the same value as that of the 4th nodal leaf.

6.3B. Kinetic analysis of IAA oxidase:

For kinetic analysis of IAA oxidase, the partially purified enzyme source (acetone precipitation) was assayed at various pH (2-10) and over a range of substrate (IAA, 0.27-1.5 mM) and enzyme concentrations (10-50 %). The optimum activity of the enzyme (20 %, w/v) was found at pH 3.0 (Fig. 6.1b). The K_m value for IAA as calculated from Lineweaver-Burk double reciprocal plot is 0.66 mM (Fig. 6.1c).

6.3C: Changes in IAA oxidase levels during leaf ontogenesis:

In the very young leaves of first node, the activity of IAA oxidase enzyme was minimum (Fig. 6.1a). During leaf meturation the enzyme activity displayed a progressive increasing trend with rapid rise upto 3rd nodal leaves and thereafter the pace declined. The enzyme activity in the 4th nodal leaves, in which the epiphyllous buds are physiologically mature to germinate on isolation, was 3.6 times higher than the youngest leaves.

6.3D. IAA oxidase activity during epiphyllous bud outgrowth:

In the dormant buds (on attached leaves - 0 hr) the IAA oxidase activity was low (Fig. 6.2). When these buds were dissected with their subtending notches from leaves and incubated in dist water, the enzyme activity rapidly increased till 24 hr incubation registering almost 6 fold rise (Fig. 6.2). Visible appearance of bud, on the other hand, was noted only on the 4th day. As the growth process progressed, the enzyme activity

began to decline upto 72 hr and later on it stabilized around the initial value of the dormant buds after attaining a slight rise on the 4th day.

Epiphyllous buds, in notch explants, incubated in BAP also showed growth. A slight stimulation over control was observed in the germinating buds. However, notches incubated in BAP (1 μM) displayed only 2 fold increase in IAA oxidase activity upto 24 hr. This is in contrast to the six fold increase observed in the control. In the presence of pyrocatechol and cycloheximide, the enzyme activity did not increase and remained at the same level as that of 0 hr value throughout the period of incubation (Fig. 6.2); during this period and thereafter none of the buds showed growth.

6.3b. Qualitative analysis of IAA oxidase during epiphyllous bud outgrowth:

On 10-18 % gradient nondenaturing PAG, dormant epiphyllous buds showed a very faint band for IAA oxidase. The isolated notches incubated in dist water for 12 hr displayed a very intense band (Fig. 6.3a). However, on 8 % nondenaturing PAG a total of three isozymes namely A, B and C of IAA oxidase were noted with B as the major band (Fig. 6.3b). On the other hand, buds incubated in pyrocatechol and BAP displayed more stain intensity in band C than B and A. Cycloheximide treatment to the buds caused very slight staining intensity of all the three

isozymes suggesting de novo synthesis of all the isozymes.

To confirm this de novo synthesis of IAA oxidase during the activation of bud growth, the enzymes were pulse labelled with (^{14}C)serine for 12 hr immediately after excision of notches from the leaves. In control, almost 13 % of total incorporation of proteins was noted only in IAA oxidase with most of the incorporation in bands B and A. Cycloheximide treatment showed only 5 % incorporation almost 61.5 % less than that of the control. Treatment with pyrocatechol and BAP, exhibited almost 10 and 7 % incorporation of (^{14}C)serine in total IAA oxidase with more relative abundance in isozyme C (Table 6.1).

6.3F. Changes in Polyphenol oxidase (PPO) activity:

After an initial slight rise, the PPO activity showed declining trend upto day 3 preceding the actual day of bud appearance (Fig. 6.4). On day-1 the PPO activity found was slightly higher than day-0 value. With further development, the PPO activity declined rapidly, the activity being 2.8 times lower compared to activity in dormant buds.

Afterwards PPO registered increasing trend again, however the increase being very slight on the day of appearance of buds from isolated notches.

6.3G. Changes in peroxidase activity during bud outgrowth:

Dormant buds exhibited very high activity of peroxidase. However, the activity continued to decrease during the process

of bud outgrowth (Fig. 6.5). On day-3 the enzyme activity registered was almost 30 times lower than the activity in dormant buds. After attaining this lowest activity, the peroxidase showed increasing trend registering 5.3 fold rise on the day of bud appearance, compared to the day-3 activity. On subsequent days the activity continued to decline again.

6.3H. Changes in phenolic contents during bud outgrowth:

The dormant epiphyllous bud contains 13.67 mg phenolics per gm dry weight. The phenolic content declined on very first day of incubation. However, it began to increase afterward and attained maximum value on day-3 prior to visual appearance of bud outgrowth (Fig. 6.6).

6.4. DISCUSSION

The physiological and biochemical events occurring during the early stages of in vitro development in higher plant systems are not well documented (Nato *et al.*, 1981). Nevertheless, the activation of cell leading to differential gene action, includes the cellular events of replication, transcription and translation (Thorpe and Binnidi, 1981). This causes a shift in metabolism leading to changes in structural and enzymatic proteins. In fact, there is an apparent need for the synthesis of specific proteins, e.g. in Douglas for (Hasegava *et al.*, 1979; Yasuda *et al.*, 1980). Since, these changes precede organized development, then as Bonner (1965) pointed out, such changes, in which new

enzymes originally absent are synthesized or which are present show increased synthesis and/or activity, *a priori* must be cause rather than a result of differentiation.

The greatest amount of IAA in the first nodal leaves (youngest leaves) and its gradual decline with leaf maturity indicates the differential requirement of IAA during leaf development. As such leaves are known to contain auxin, particularly, the young leaves being the site of auxin synthesis (Moore, 1969; Schneider and Wightman, 1974, 1978). Further, the rapid decline in IAA content with the progressive leaf age denotes the role of IAA in the growth of leaf which on attainment of maturity shows decline in auxin levels (Sheldrake,1973; Wareing and Phillips, 1982; Degroote and Muir,1977).

A continuous increase in IAA oxidase activity during leaf development in present studies is in agreement with the observation of Galston and Hillman (1961) that the IAA oxidase activity shows increasing gradient from actively growing (young) to nongrowing (old) tissues, being highest in the mature ones. Further, a significant negative correlation was established between the IAA content and IAA oxidase levels in the leaves ($r = -0.93$ and $p < 0.01$). Similar, conclusions were made for these two parameters during abscission in bean seedlings (Jain *et al.*, 1969), light induced hook opening of pea seedlings (De Greef *et al.*,1977), in apple callus (Epstein and Lavee,1975) and olive leaf (Epstein and Lavee, 1977).

Very low IAA oxidase activity in the dormant buds and several fold induction as early as 4 hr (Fig. 6.2b) after incubation indicates its role in controlling epiphyllous bud outgrowth. Many workers have reported consistant increase in IAA oxidase during ripening of fruits and suggested that oxidative degradation may represent a mechanism for the inactivation of endogenous IAA in the fruit and thus promoting ripening (Frenkel,1972, 1975; Frenkel and Haard, 1973).

A very quick augmentation as early as 4 hr and upto 12 hr in IAA oxidase activity (Fig. 6.2b) and then marked decline in the activity coinciding with the bud appearance, implies the differential requirement of IAA for growth of primordia than for their induction. Similar, conclusions were drawn for initiation and growth of root primordia (Druart et al., 1982; Moncousin and Gaspar,1983). Jasdanwala et al. (1977) have also demonstrated the diacritical demand of IAA in developing cotton hairs during elongation phase and secondary thickening and demonstrated the role of IAA oxidase in these events. The rapid decline in the IAA oxidase activity during the later stages of bud appearance seems to be due to the accumulation of phenolic inhibitors. A direct negative correlation ($r = -0.75$; $p < 0.1$) was obtained between the IAA oxidase level and total phenolic content during the course of epiphyllous but outgrowth, implying inhibitory effects on IAA oxidase activity (Wolf et al.,1976, Reynolds, 1978; Bearder,1980).

To clarify whether the increase in IAA oxidase activity is the cause/consequence of the bud growth process, three sets of check were performed.

In the first check, effect of exogenous cytokinin on the level of IAA oxidase was examined. It was hypothesized that the function of increased activity of IAA oxidase is to degrade endogenous inhibitory IAA levels so as to reduce the auxin/cytokinin ratio favourable for bud growth. The decrease in auxin/cytokinin ratio within/around the buds can also be brought about by the exogenous cytokinin. In this situation, one would not expect any increase in IAA oxidase in the buds undergoing growth. As expected, there was an extremely small increase in the activity of IAA oxidase (Fig. 6.2b) in the induced buds in the presence of BAP (1 μM). Similarly, in tobacco callus also, increased levels of kinetin caused a decrease in the specific activity of IAA oxidase with almost 60 % decline in cytoplasmic fraction (Lee, 1974). Similarly low IAA oxidase activity was reported in kinetin grown cells of sycamore (Weston et al.,1978).

In a second set of control, the application of pyrocatechol caused inhibition of epiphyllous bud growth. Pyrocatechol (1,2-dihydroxybenzene) is a specific inhibitor of IAA oxidase (Gaspar et al.,1964). As such diphenols are known for their inhibitory effects on IAA oxidase activity (Schneider and Wightman, 1974). None of the cultured buds (in pyrocatechol,0.1 mM)

could show any increase in the activity of the enzyme (Fig. 6.2b).

In a third set of control, endogenous protein synthesis was inhibited by cyclo heximide. As a consequence, the buds cultured in cycloheximide (10 µM) failed to show any growth. No increase in IAA oxidase activity was noted in the buds treated with cycloheximide in the present studies (Fig.6.2b). The result along with in vivo labelling of enzyme with (^{14}C)serine implies that de novo synthesis of IAA oxidase is necessary for bud outgrowth. Earlier work has shown that the formation of IAA oxidase was inhibited by cycloheximide suggesting a requirement of protein synthesis in tobacco callus (Lee, 1971, 1972) for organ formation.

These results clearly show that the epiphyllous buds achieve the competance for growth when there is an increase in IAA oxidase activity with concomitant decrease in their IAA content. A diphenol pyrocatechol, the inhibitor of IAA oxidase suppresses the growth of buds and the inhibitor of protein synthesis inhibits induction of bud growth per se as well as the rise in the activity of IAA oxidase.

Earlier, based on the stimulatory effects of PCA (p-coumaric acid) on the growth of epiphyllous buds of B.calycinum, Houck and Riesberg (1983) suggested the possible involvement of IAA oxidase in the reduction of auxin/cytokinin ratio to favour bud growth. At this juncture, it is necessary to mention that in

B.diagremontianum bud growth was associated with lower rather than higher extractable cytokinins levels (Henson and Wareing, 1977). This is in line with the presumption that isolation of leaf (stress) induces de novo synthesis of IAA oxidase which brings down the inhibitory levels of auxin to an optimum auxin/cytokinin ratio without any appreciable changes in cytokinin levels (Henson and Wareing,1977). In this context, water stress enhanced IAA oxidase activity in etiolated pea seedlings and tomato leaves has been reported (Darbyshire,1971 a and b). Sembdner et al.(1980) also emphasised the role of nutritional conditions and physical factors in regulating the IAA oxidase activity, either directly or by influencing hormonal and other chemical effectors.

Through (^{14}C)serine labelling, it was noted that 17 % of the total incorporation attributed to proteins. Of the total incorporation in proteins, 13 % alone was found in IAA oxidase thus confirming the hypothesis that IAA oxidase is being de novo synthesized which in turn induces the bud growth by lowering the IAA levels.

Further, it was also noted that isozyme B comprised almost half of the total incorporation in total IAA oxidase, and therefore could be the main source for IAA decarboxylation in the leaf notches. In this context, there are reports of increased IAA oxidase isozymes during fruit ripening, bringing down the supraoptimal auxin level thereby promoting ripening (Frenkel,1975; Frenkel and Haard,1973).

Earlier studies (chapter 2 and 3) implicated the role of ethylene in inducing epiphyllous bud outgrowth in K.mortagei. It seems that, stress induced ethylene triggers the synthesis of IAA oxidase, thereby stimulating the bud growth. Recently, Broglie et al (1985) have isolated, characterized several chitinase DNA clones and demonstrated the induction of enzyme chitinase by ethylene with transcriptional control in bean leaves. Similarly, Grierson et al. (1986) also reported ethylene induced accumulation of new mRNAs for polygalacturonase in tomato fruit during mature green stage and implicated its role in fruit ripening.

The slightly higher level of PPO enzyme in the early phase of bud development, may be producing enough quantity of inhibitor o-diphenol (Duke and Vaughn,1982) of IAA oxidase to sustain bud growth during later stages of actual bud appearance in the notches. In this regard, Bassuk et al. (1981) have reported involvement of PPO in the synthesis of phenolic cofactors which promote rooting by acting as auxin protectors (Balasimha and Subramonian,1983). Earlier, Griffin and Stonier (1975) had also reported the involvement of PPO in auxin protector production in the developing coffee fruits.

It has been also demonstrated that PPO catalyzes ortho-hydroxylation of p-coumaric acid to caffiec acid (Butt,1979; Duke and Vaughn,1982) which is a potent inhibitor of IAA oxidase (Bearder,1980). This reaction has been suggested to occur in vivo also (Halliwell,1975). From these, it is quite apparent that PPO

has its effect on bud outgrowth process by controlling IAA oxidase levels through its ability to catalyse the formation of o-diphenols and polyphenol. Partially purified PPO has been reported to form aldehydes using amino acids and diphenols (Srivastava, 1986), thus regulating the metabolism of phenols which can be synthesized rapidly and at rates which would appear to impose plant metabolic capacity (Creasy, 1987).

Peroxidase - an enzyme regulating numerous facets of growth and differentiation (Thorpe and Gaspar,1978; Epstein and Lamport, 1984; Lamport,1986), due to its effect on endogenous auxin including lignification (Barlow,1982; Ricard and Job 1974; Gaspar et al.,1985; Goldberg et al.,1983; Sembdner et al.,1980). Normal developmental process demand lignification for xylem formation. Further, growth processes also include cell wall changes where lignin plays an important role mainly through rigidification (Taiz,1984). It is likely that peroxidases are responsible for the assembly of polymers (lignin, polysaccharides and proteins) in the cell wall and are therefore, involved in the differentiation process (Epstein and Lamport,1984).

The high levels of peroxidase in dormant buds could be due to the inhibitory levels of auxin, which is known to maintain and promote peroxidase activity (Richard et al.,1976). In a germinating pollen of lily, the peroxidase activity was found distributed only throughout the older region and not

within the growing tip or region immediate subjacent to it (Dashek et al.,1979).

No correlation was observed between the IAA oxidase level and peroxidase activity during the course of bud development, nullifying the possibility of peroxidative degradation of IAA. Similar conclusions were drawn by Raa (1971) for any role of peroxidase in IAA degradation. The differential effects of ethrel on IAA oxidase and peroxidases in Trigonella seedlings indicated that two enzymes are functionally different (Megha and Laloraya, 1978).However, there are reports of cationic peroxidase which serve as IAA oxidases (Gove and Hoyle,1975; Richard et al.,1972; Nakajima and Yamazaki,1979; Hoyle 1977; Rawal and Mehta, 1982; Brooks, 1986) even in vivo (Boyer et al.1979). But many workers have reported the seperation of IAA oxidase which were devoid of any peroxidase activity (Segeira and Mineo,1966; Bryant and Lane, 1979; Talwar et al.,1985).

In a photosynthetically active metabolism in a normal leaf such a high level of this enzymatic system could be involved mainly in regulating the inhibitory levels of H_2O_2 (Goldberg et al. 1987) through oxidation of various substrates (Scandalios,1974; Brennan et al., 1979; Thaker et al.,1986) including diamines and polyamines (Hazell and Murray, 1982).

The possibility of lower peroxidative activity during the bud growth process could be due to the pool of peroxidase in transit from cytoplasm to the cell wall (Askerlund et al.,1987).

since peroxidase readily binds to the membranes by adsorption. Thus, catalyzing lignification and taking care of any possibility of pathogens' entry (Gaspar et al.,1985) thus exhibiting very low cytoplasmic activity.

The optimum activity of IAA oxidase found at pH 3.0 is in sharp contrast to earlier reports of this enzyme from young leaves of Impatiens (Kanwar and Nanda, 1986) and cotton fibre (Rama Rao et al., 1982), where the maximum activity was reported at near neutral pH. In present studies the K_m of partially purified enzyme is 0.66 mM as calculated from Lineweaver-Burk double reciprocal plot (Lehninger,1975). On the other hand Talwar et al.,(1985) reported Km value of 0.78 of purified IAA oxidase (non-peroxidative) from mung bean cotyledons. Thus, this study indicates that the enzyme IAA oxidase of K.mortasei is unique than other plants so far reported in having a very acidic pH optima for its activity and still of greater affinity towards the substrate-IAA.

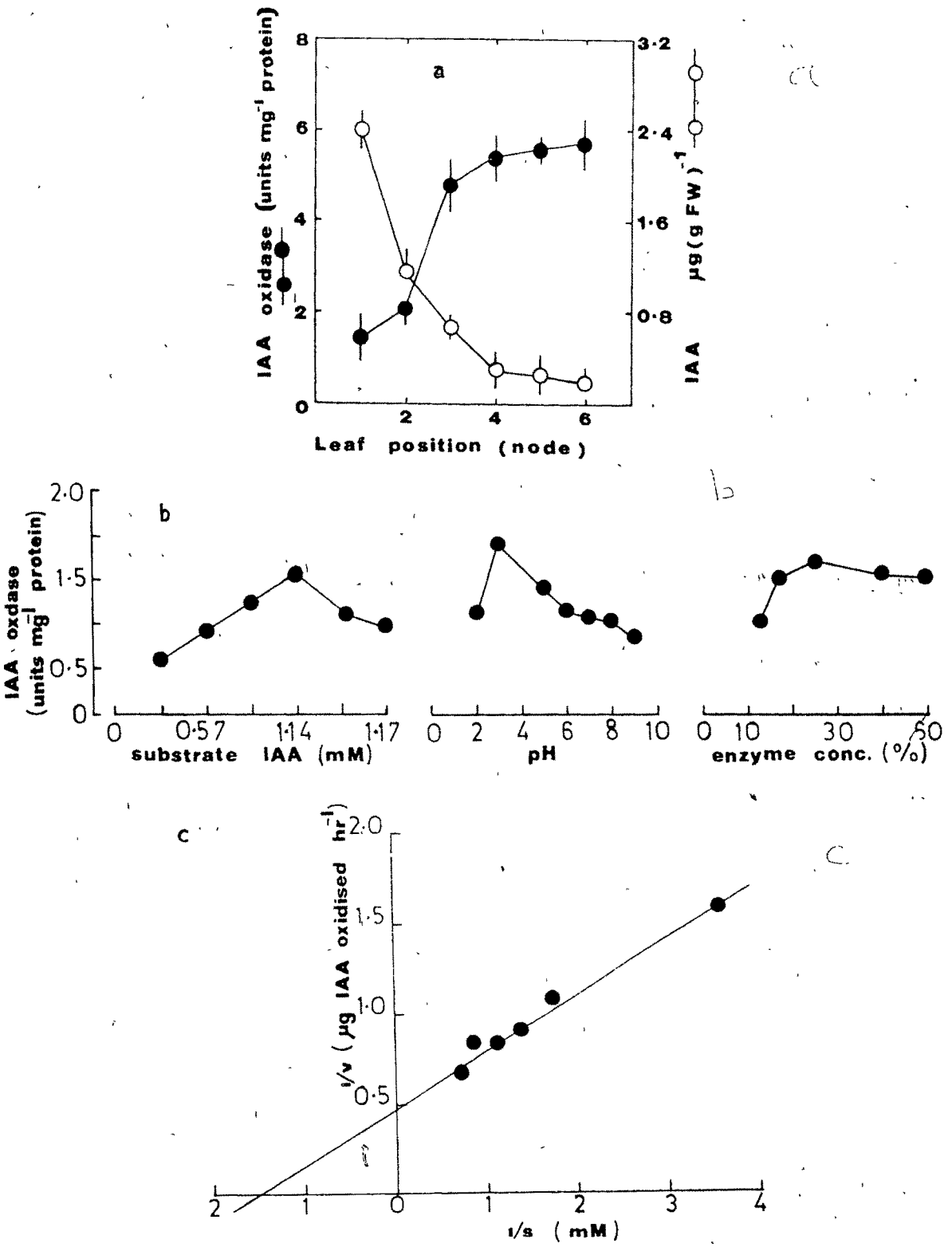
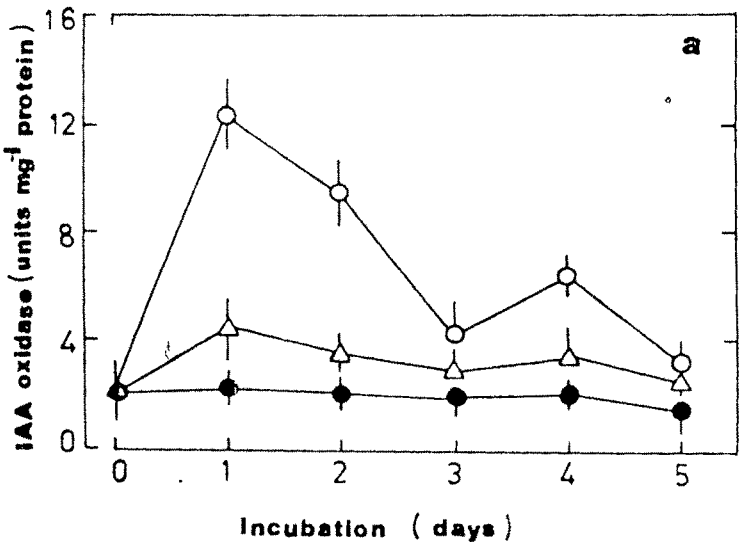


Fig. 6.1. (a) Changes in endogenous IAA and IAA oxidase in leaves at various stages of leaf development.
(b) & (c) Enzyme kinetics of IAA oxidase.



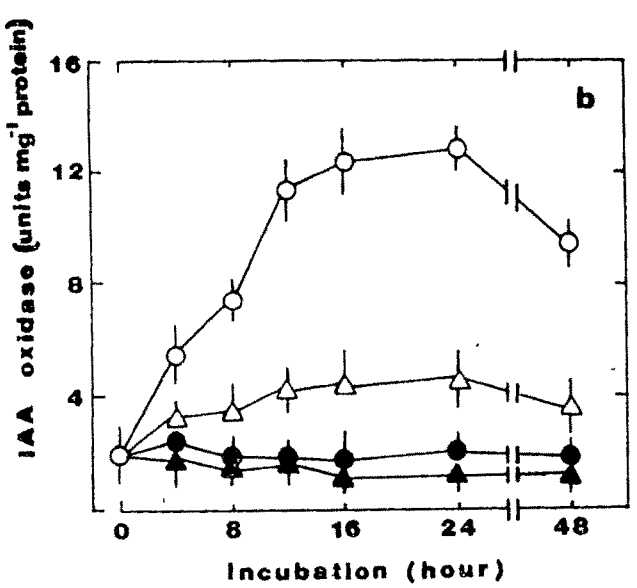


Fig. 6.2. a & b. Changes in IAA oxidase levels during the outgrowth of dormant epiphyllous buds from notches incubated in dist water (○), BAP (△), CYCLO (●) and pyro-catchol (▲).

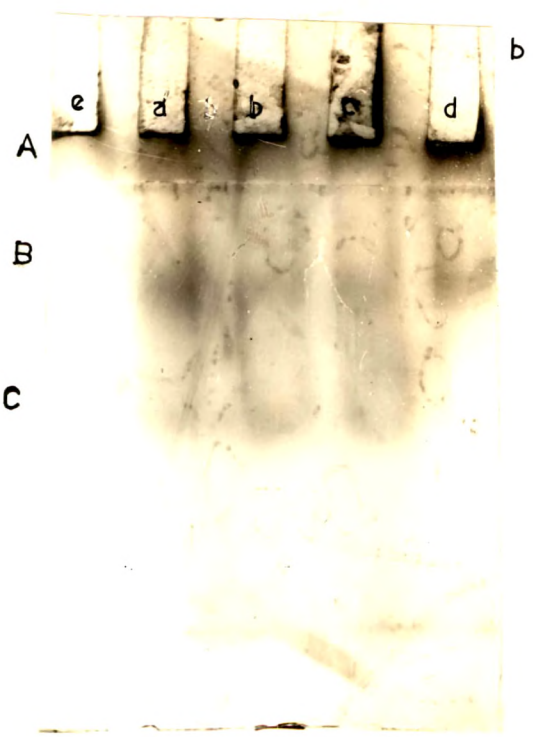
Fig. 6.3. Qualitative analysis of IAA oxidase during bud outgrowth.

(a) Induction of IAA oxidase during very early stages of bud outgrowth.

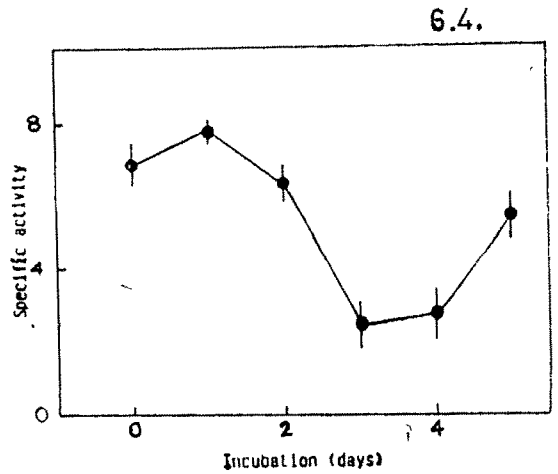
(b) Effect of inhibitors on the pattern of IAA oxidase isozyme after 12 hr of incubation.

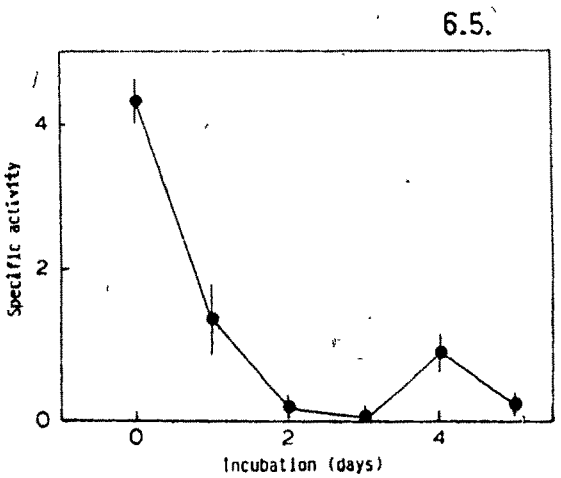
a - dist water control
b - pyrocatechol
c - BAP
d - cycloheximide
e - 0 hr control.

a



b





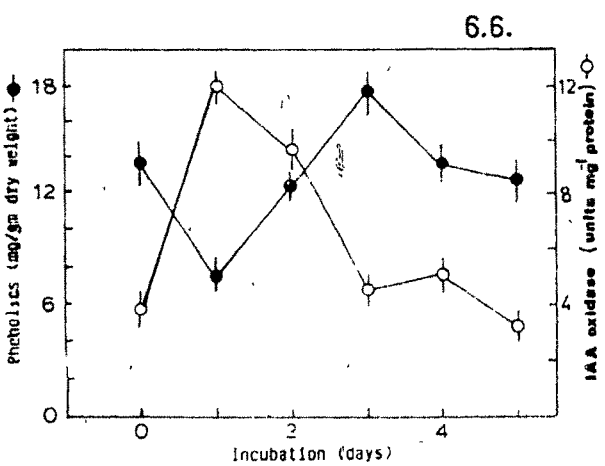


Fig. 6.4. Changes in polyphenol oxidase activity in developing buds.

Fig. 6.5. Peroxidase activity during bud outgrowth.

Fig. 6.6. Changes in phenolic content in comparison to IAA oxidase levels during bud outgrowth.

Table 6.1 Incorporation of (^{14}C)serine in IAA oxidase:

Treatment	% incorporation			
	Band-A	Band-B	Band-C	Total
Control (water)	4.89	5.88	1.93	12.74
Cyclohexamide	2.49	1.58	1.28	05.37
Pyrocatechol	3.62	3.01	2.89	09.52
Benzyladenine	2.95	1.31	2.98	07.25