2. ANATOMICAL STUDIES OF BUD OUTGROWTH

2. Anatomical studies during epiphyllous bud outgrowth.

2.1. Introduction.

2.2. Materials and Methods.

2.2A. Experimental Materials.

2.2B. Histochemical studies.

2.2C. Cytological measurements.

2.3. Results

2.4. Discussion

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2.1 INTRODUCTION

The genus Bryophyllum, now referred as Kalanchoe (Willis, 1973), is being routinely used to demonstrate vegetative propagation occurring through foliar propagules. The origin and early developmental studies on these buds of B. calycinum were made by Howe (1931), Naylor (1932), Yarbrough (1932) and Karpoff (1982). These buds are also found to be a favourite experimental material for physiological studies (Siranval, 1956; Vardar and Acarer,1957; Heide, 1965; Purohit et al.,1969; Purohit and Nanda,1971; Karpoff, 1982; Houck and Riesseberg,1983). The factors controlling the post-initiation development have also been studied (Nanda et al., 1968; 1970; Nanda and Jain, 1972). Out of the four most commonly used species of Bryophyllum, in B.tubiflorum and B. diagremontianum the plantlets develop on intact leaves under longday photoperiods. However, treatment with cytokinin, to the intact leaves, can substitute for long day requirement (Heide 1965; Yazgan and Vardar,1977). Similarly in B. calycinum, detachment of the leaf from plant or cytokinin application to the attached leaf causes the epiphyllous bud outgrowth (Heide,1965). On the other hand, in K.mortagei, removal of the leaf from parent plant induces

growth of these epiphyllous buds. On account of this trigger and other advantages mentioned in chapter I, the epiphyllous bud outgrowth in K.mortagei was selected for studies on developmental anatomy. In this chapter results on structures of dormant and reactivated epiphyllous buds and cytochemical changes in total protein and insoluble polysaccharides during the course of bud activation and subsequent growth are described.

2.2 MATERIALS AND METHODS

2.2.A Experimental material:

The uppermost notches (located at the extreme leaf tip) of the 4th nodal leaves were used (as the response was optimum in them). In order to induce the growth of dormant epiphyllous buds, the excised leaves were kept in an upright position with their petioles dipped, in an Erlenmeyer flask (50 ml capacity) filled with dist water. They were incubated in the culture room at 16 hr photoperiod and 25 ± 1°C temperature.

2.2.B Histological studies:

The topmost notches were removed at every 24 hr interval upto total 120 hr from the experimental leaves incubated as above, and fixed immediately in FAA (formalin : glacial acetic acid : 50 % alcohol, 5 : 5 : 90) and Carnoy's fixatives

(Jensen, 1962). Materials were dehydrated in graded series of tertiary butyl alcohol, infiltrated, embedded in paraffin (56-58 °C) and 10 µm thin sections were cut in median longitudinal plane.

Insoluble polysaccharides were localized by periodic acid-Schiff's (PAS) reaction (Jensen,1962) and total protein by mercuric bromophenol blue reaction (Mazia et al., 1953).

2.2.C Cytological measurements:

Length and breadth of the dormant and reactivated buds were measured using ocular micrometer fitted on a microscope (Leitz Dialux 22).

2.3 RESULTS

The development of epiphyllous buds in Kalanchoe occurs from well defined primordia (Naylor,1932; Yarbrough, 1932; Vardar and Acarer, 1957; Karpoff,1982) embedded in the notches located on crenate margin of the leaves. The factors like long day, cytokinin, auxin etc. responsible for controlling the development of these epiphyllous buds have been studied in B.calycinum and B.diagremontianum (Heide, 1965; Henson and Wareing, 1977; Yazgan and Vardar, 1977; Houck and Riseberg,1983). In K.mortagei outgrowth of buds occurred in the detached leaves. It takes about 3-4 days for the visual appearance of the buds in the notches (Fig.1.1a).

When the notch region of leaf was sectioned in median longitudinal plane, the meristematic cells of bud can be seen (Fig.1.1b). The epiphyllous buds possessed a distinct vascular connection traversing the mesophyll tissue.

The leaf has a single layered epidermis. The hypodermis is made up of closely packed thin walled single layered cells, most of which are filled with PAS and protein positive stain. Unlike, most of dicot leaves, the mesophyll tissue is not differentiated into palisade and spongy parenchyma. Instead, only one type of chlorenchymatous cells comprise the mesophyll tissue. These cells contain abundant starch grains.

At the crenate hump, in between two notches of leaves, are located groups of cells. Each group of cells has subtending Vasculature separating it from the mesophyll tissue (Fig. 1.1 f). The cells in this group are small, closely packed and possess prominent nuclei. During the course of leaf maturation, this group of cells develops intense purple pigment. Yarbrough (1932) has also reported occurrence of such cells in B.calycinum and considered as an anamoly in the general growth. Development of epiphyllous buds was found to occur quite normally even in the absence of these cells this excludes a possibility of having their role in the outgrowth of epiphyllous buds. Based upon the progressive development of purple pigments and their distinct connection with the rest of the mesophyll tissue, it seems that they may probably play a role in exclusion of harmful substances.

In a laboratory conditions, conspicuous shoots which can be seen by naked eye, arise from epiphyllous primordia. Abundant starch grains were observed in the mesophyll cells surrounding the epiphyllous primordial region during early stages (0-2 days excision of leaf) of bud outgrowth (Fig. 1.1 b). However, with further development of the bud, the starch grains from surrounding mesophyll cells get depleted (Fig. 1.1 c).

Throughout the bud outgrowth process, there is steady increase in the staining intensity of total proteins in developing buds (Fig. 1.1 d, e). Intensity of protein stain as well as PAS positive stain in the leaf hypodermal cells gradually decreased as the bud development progressed.

There was continuous augmentation in the height and width of the growing bud. The average height and width of the dormant epiphyllous bud was 81 and 41 μ respectively (Table 1.1). As the bud development advanced further, the length and breadth increased to 358 and 211 μ on 4th day.

2.4 DISCUSSION

In *K. mortagei*, the appearance of buds was observed only from detached leaves. Growth of buds on the attached leaves (those which are still attached to the mother plant) is scarce and occur naturally only if the leaf tip comes in contact with the soil. Similar mode of bud growth has been

reported in B. calycinum (Beals, 1923; Howe, 1931; Naylor, 1932; Yarbrough, 1932) and in B. diagremontianum (Varder and Acarer, 1957; Heide, 1965).

While examining general histology of bud growth in various plants, Beals (1923) found that the meristematic tissue in the leaf notch was derived from the phloem of vasculature. Howe (1931) contradicted this and suggested that the young plantlets developed from meristematic groups of cells which were present right from the beginning in the young leaves. During leaf ontogenesis, the meristematic patches of cells get organised into distinct meristem. This observation was later on confirmed and further extended by Naylor (1932) who suggested that primordia of both apices - root and shoot, are present in the notches of mature leaves.

In present studies, the visible appearance of shoots from ventral side of leaf notch was observed on about 3-4 day of incubation, the timing varying slightly from plant to plant. Later on, the roots (1-3 in number) appeared from the base of the shoot on 6-9 day of isolation of leaf. Thus in K.mortagei the shoot buds always appeared first followed by roots. However, in B. calycinum, Yarbrough (1932) reported that the roots initiated first (in about 4 days) and shortly afterwards shoot made their appearance. Similar pattern was

observed with *in vitro* studies on bud growth in *B.calycinum* from the apical notch on White's medium (Mohan Ram,1963). However, working with same plant Karpoff (1982) demonstrated emergence of shoot first followed by roots.

Leaf position on plant and plant age also influenced the reactivation of epiphyllous bud growth (Fig. 3.3 a,b). During vegetative phase, the occurrance of bud growth in the leaves located on 4th and subsequent lower nodes (i.e. towards the plant base) indicates that during leaf ontogeny the meristem undergoes maturation process, at the end of which they attained the ability to grow into a complete plantlet. This is substantiated by earlier anantomical studies reported in *B.calycinum* (Naylor, 1932; Yarbrough, 1932). But maturation of bud is followed by dormancy in the plant under present study (*K.mortagei*). However, in case of other species *B.diagremontianum*, *B. tubiflorum*) though the buds develop on the intact plant into a shoot bearing two pairs of leaves, its further growth is arrested. It is only when they get detached that the shoots develop further into complete plantlets (see Chapter III for the review).

During the reproductive phase (bolting) of plant unlike the vegetative stage the bud growth was observed on all the leaves (Fig. 3.3 b). Bolting is a sign of a change in the plant development from vegetative to the flowering phase (Wareing,1977). Probably, by the time the plant has completed bolting process, all the leaves along with primordia in their notches might have

achieved complete maturity and thus exhibiting bud growth in all the leaves tested for the response. In support of the above view it is now known that cytokinin accumulation induces flowering in Pharibitis (Ogawa and King, 1979; 1980); Wolffia (Venkataraman et al., 1970); Lemna (Gupta and Maheshwari,1970); Arabidopsis (Michniewicz and Kamienska, 1965); Sinapis (Bernier, et al.,1977) etc. probably through its role in assimilate transport to the stem apex (Tanimoto and Harada,1984; Vince-Prue, 1985). So the upper leaves might be having comparatively higher cytokinin levels thereby bringing down the auxin/cytokinin ratio to an optimum for epiphyllous bud outgrowth.

Morphology and nomenclature of epiphyllous buds:

Due to the ability of notch meristems to give rise to complete plantlet with root and shoot axis, it was interpreted either as a bud (Howe,1931) or foliar embryo (Naylor,1932). The term foliar embryo is still used occassionally in the literature (Yarbrough, 1932; Mohan Ram, 1963; Karpoff,1982; Houck and Rieseberg, 1983). However, according to the classical definition an embryo always originates from a single cell and has no direct vascular connection with the mother plant (Maheshwari,1950). The epiphyllous bud meristems of Kalanchoe possesses a distinct vascular connection with the main vasculature. Ontogenetically, this bud originates from a group of meristematic cells of leaf primorida (Naylor,1932; Yarbrough,1932)

and not from a single cell. Therefore, it has been proposed that these primordia should not be termed as foliar embryo, rather leaf margin meristem or more appropriately, epiphyllous bud would be the correct nomenclature of it (Jasrai et al.,1987).

Presence of abundant starch grains in the mesophyll cells surrounding the epiphyllous bud during dormancy and subsequent early stages of growth (upto 2 days after excision of leaf) indicates its role in the process. Earlier histochemical examination of shoot forming tobacco tissue revealed accumulation of starch in specific loci from which shoot primordia developed (Thorpe and Murashige,1970). In other in vivo organogenetic processes also starch accumulation has been a conspicuous feature; for example, inouction of floral parts in cauliflower (Sadik and Ozbun,1967) and growth of potato buds (Marinos, 1967). In the present study depletion of starch grains from the surrounding mesophyll cells during later stages (3 days onward) reflects active growth and nutritional requirement for bud growth. Likewise accumulation and later on disappearance of starch have been correlated with the bud forming process in tobacco callus (Thorpe, 1980), embryo of Pinus coulteri (Patel and Berlyn,1985) and Picia abies (Von Arnold,1987) and cotyledons of Pinus radiata (Patel and Thorpe, 1984). The physiological significance of starch accumulation prior to and dissappearance during organogenesis which reflects high energy requirements for the said process is well documented (Ross et al.,1973; Thorpe and Meier, 1974).

This has been proved with (^{14}C)sucrose feeding of tobacco calli (Thorpe et al.,1986) and ultrastructural studies in Datura innoxia (Brossard-Chriqui and Iskander,1980). As a source of energy, starch possess a distinct advantage for its degradation results in high yields of glucose-1-phosphate and its subsequent catabolism into ATP through glycolytic substrate phosphorylation and followed by oxidative phosphorylation, without the expenditure of existing high energy phosphates (Mehta,1980).

Progressive increase in total protein content in the induced buds indicates a continuous synthesis of proteins during the process. This is in constrast to the reports that detachment of leaves causes senescence resulting in loss of total soluble protein (Peterson et al.,1973; Wittenbach,1978). However, depletion of protein from hypodermal cells with accompanying accumulation in growing buds indicates rapid turnover necessary for sustained growth of growing primordia (Dice and Goldberg,1975; Huffaker,1982).

In present studies the continuous increase in average height and width of the bud indicates active growth through cell expansion (Fletcher and Dale,1974; Nagao and Rubinstein,1976). Wardlaw and Mortimer (1970) has reported increase in bud length as early as 6 hr in Pisum after decapitation. However, the possibility of rapid mitotic activity cannot be ruled out during the spurt in bud growth. Several investigators have found

increased cell divisions within 24-48 hrs after removal of the apex (Naylor,1958; Ali and Fletcher,1970; Peterson and Fletcher,1973; Tobin et al.,1974; Couot-Castelier,1979). In this context, Cicer arietinum lateral buds treated with BAP (1 mM) exhibited increased cell division even within one hr, whereas increased elongation occurred two hr after treatment (Usciati et al.,1972). However, Yeang and Hillman (1981) through anatomical studies suggested that following decaptitation growth by cell extension can be detected after a lag of 3-4 hr. Later on Hillman (1984) concluded that bud development proceeds in two phases- an initial release from inhibition involving cell expansion for very short period of a few hours, followed by cell division in the period of 12-48 hr (Rubinstein and Nagao,1976; Couot-Castelier,1978).

From these studies it is quite apparent that the bud meristems are being laid down during the ontogensis of the leaf. Once the leaf is matured, the buds undergo dormancy till the onset of favourable conditions which is the isolation of leaf from the plant as in the present case. Further, during the course of bud growth following induction, protein and carbohydrates from the surrounding leafy tissue (hypodermis and mesophyll etc.) are being channalized to the growing bud meristem - a process analogous to the germinating seeds (Ashton, 1976; Mayer and Poljakoff-Mayber,1982).

Fig.1a. Reactivation of very first buds in the uppermost apical notches of the leaf.

Fig. b-f. Median longitudinal sections of leaf passing through notch portion of Kalanchoe mortagei. CP, Cell Patch; DB, dormant bud; RB, regenerating bud; SG, starch grain; V, vascular connection.

Fig. b,c. Localization of total insoluble polysaccharides.

Fig. b. Dormant bud (x 125). Note the starch grains in the mesophyll cells surrounding the bud.

Fig. c. Regenerating bud after 4 days of incubation (x 312.5).

Fig. d,e. Localization of total proteins.

Fig. d. Regenerating bud after 2 days of incubation (x 312.5).

Fig. e. Bud after 5 days of incubation (x 312.5).

Fig. f. Section in nonnotch region showing peculiar cell patch having vascular connections (x 787.5).

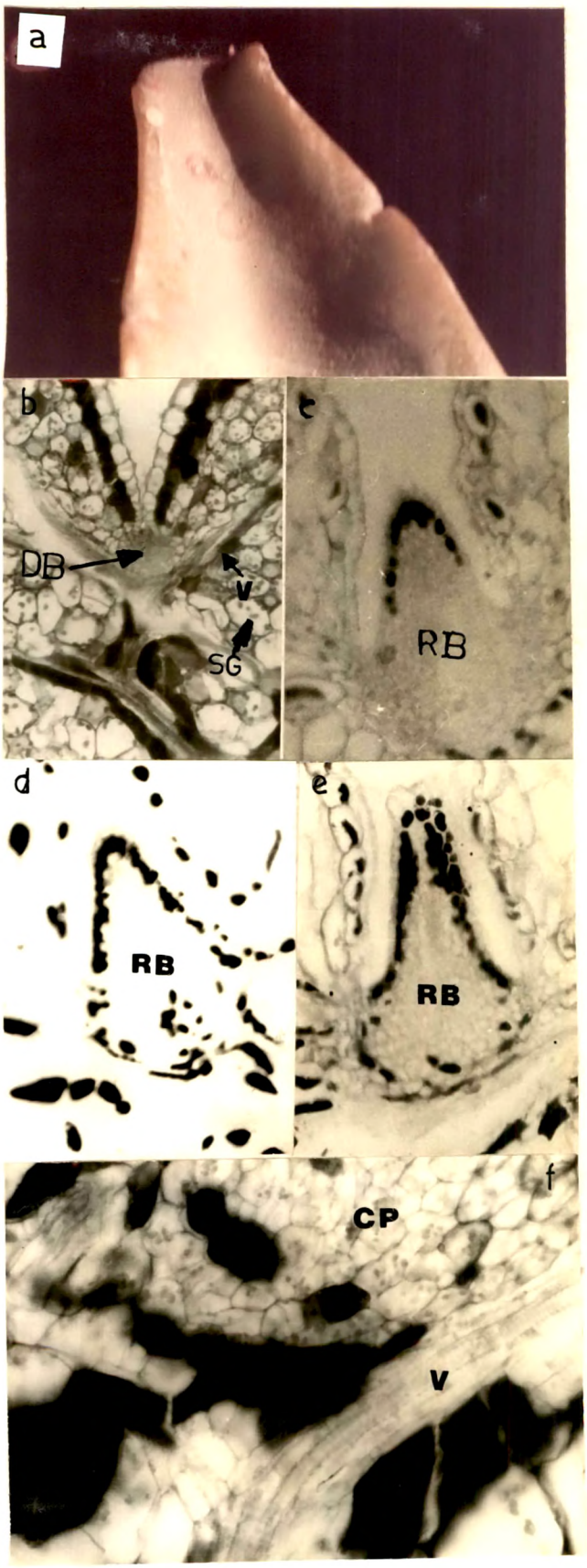
a
b
DB
V
SG
c
RB
d
RB
e
RB
f
CP
V

Table - 1.1. Changes in length and breadth of epiphyllous buds of *Kalanchoe mortagei* during outgrowth.

Days of incubation	Bud length (Micron)	Bud breadth (Micron)
0	81.40 ± 1.84	48.80 ± 1.02
1	146.52 ± 2.46	81.48 ± 3.20
2	244.20 ± 6.72	126.80 ± 2.96
3	325.60 ± 4.04	162.80 ± 2.78
4	358.16 ± 4.84	211.64 ± 2.46
5	520.96 ± 3.68	221.84 ± 4.26

The figures in parentheses are the standard errors