HISTOCHEMICAL STUDIES ON RESERVE MOBILIZATION IN WINGED BEAN (*Psophocarpus tetragonolobus* (L.) DC) SEEDS DURING GERMINATION

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CERTIFICATE

This is to certify that the thesis entitled **"Histochemical Studies on Reserve Mobilization in Winged Bean** (*Psophocarpus tetragonolobus* (L.) DC) **Seeds During Germination"** submitted by **Khaleel Kurunthrayil Manha** in part fulfilment of **Doctor of Philosophy** in Botany, University of Calicut, is a bonafide record of research work undertaken by him in this department under my supervision during the period 1991-1998 and that no part of it has been submitted before for the award of any degree.

Dr. NABEESA SALIM.

DECLARATION

I hereby declare that the thesis entitled **Histochemical Studies on Reserve Mobilisation in Winged Bean (*Psophocarpus tetragonolobus* (L.) DC) Seeds During Germination** submitted by me for the degree of **Doctor of Philosophy** in the Faculty of Science of the University of Calicut has not been submitted for the award of any degree.

Calicut University Campus
20 - 10 - 1998

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CONTENTS

GENESIS	i
INTRODUCTION	1
REVIEW OF LITERATURE	11
MATERIALS AND METHODS	49
RESULTS	59
DISCUSSION	93
CONCLUSION	128
REFERENCES	133

GENESIS

GENESIS

Research in seed reserve mobilization has been characterised in recent years by increased recognition on quantitative and qualitative variations in the cotyledonary/endosperm reserves. Similarly investigations to describe the movement of reserve materials from the cotyledons in to the developing embryonic axis have been carried out during recent years, in particular, studies on transport phenomenon in plants is mainly described focussing on apoplastic and symplastic movements of water and solutes.

The botanical literature abounds with papers on reserve mobilization during seed germination as well as mechanism of metabolite transport in plants. However, ultra structural studies on cotyledons of dicotyledonous plants are so meagre. More than 300 years ago, Grew (1682) briefly described the structure of cotyledon. Ontogenetic and structural aspects of cotyledons in the embryogenesis are studied only by very few (Esau, 1954, 1965; Philip, 1972; Swami and Krishnamurthy, 1980 and Krishnamurthy, 1994). But the pattern of procambialization and its bearing on the vasculature of cotyledons during seed germination and seedling growth are almost neglected by anatomists. To the best of the authors knowledge, investigations by Smith and Flinn (1967), Philip (1974) are the available references on vasculature of cotyledons during germination. These studies revealed some developmental and structural aspects of vascularisation in

cotyledons of dicots and the relationship between vasculature differentiation and reserve mobilization is described by Smith (1974).

Studies on reserve mobilisation in winged bean *(Psophocarpus tetragonolobus)* (Anonymous, 1975: Kamaladevi *et al.* 1989: Nabeesa – Salim and Harikumar (1994) showed that the cotyledons of seeds after 10 days of seedling growth are left with considerable amount of protein, carbohydrate and total biomass. Since the mobilisation of reserves from the cotyledon in winged bean is a slow process, the seedlings are found to be surviving for several days under Petri dish condition supplied only with water

During germination it is observed that a pair of cotyledonary shoots develop precociously in seedlings where the original plumule is damaged or ill-grown. So it appears that the cotyledonary reserve mobilization can be hastened by excising the plumule to force or induce the lateral shoots at the axils of cotyledons. So the reserve mobilization pattern in the cotyledons of the plumule excised seedling is investigated in comparison with the normal (intact) seedlings, during a period of ten days of germination.

In addition to the above aspects morphological and anatomical studies of procambial strands of cotyledon also is included. Histochemical localization of metabolites in the cotyledons of dry seed and germinating seedlings may help to pin point the localisation of metabolites and the tissues involved in this process.

Dicotyledonous seeds with hypogeal germination are not at all studied so far on vascular differentiation in relation to germination.

More over in winged bean seeds, characterised by hypogeal germination are having very conspicuous cotyledons which are the source of metabolites for prolonged and profused seedling growth superimposed with a slow rate of reserve mobilizations during germination and early seedling growth. In this dissertation a new approach has been made and a new orientation given to the study of reserve mobilisation in connection with vascular differentiation.

INTRODUCTION

INTRODUCTION

Winged bean (*Psophocarpus tetragonolobus* (L.) DC), a tropical legume, is a promising source of oil and protein and is listed as one of the under exploited crop by National Academy of Sciences (Anonymous, 1975) of United States of America. Winged bean is largely grown in Papua New Guinea, South East Asia and West Africa and very recently it has been introduced to some parts of India. It is unique among the other leguminous crops because all parts of the plant are edible.

Winged bean belongs to the tribe Phaseolae of the sub family Fabaceae of Leguminosae. The winged bean (*Psophocarpus tetragonolobus)* so named because of its wing like flanges on its pod, is a climbing or twining perennial but usually grown as an annual. It has also come to be known in different parts of the world by other names such as four angled bean, four cornered bean, Goa bean, Manila bean, princess bean and Asparagus bean (Purseglove, 1968; Masefield, 1973). The tubers, young pods, seeds, leaves, flowers and shoot are rich in protein, amino acids, oils, vitamins and minerals (Claydon, 1978).

The best time for planting winged bean is June-July. After sowing, seedling will emerge from the ground in 5-10 days. Flowering usually commences after 50-60 days of sowing. The leaf contain 15% protein and high amount of vitamins (Anonymous, 1975). The

extensive root system with unusual amount of nodulation may help the plant to grow in nitrogen-poor soils, reflecting its ability to obtain fixed nitrogen via its root nodules. Tubers are formed from the fibrous roots of certain varieties. Tubers contain 10-12% crude protein on fresh weight basis, an amount exceeding that of any other tuber crop. Five to six days after anthesis pod formation become visible. About 60 days duration is enough for the pods to mature and dry (Anonymous, 1975; Pospisil *et al.*, 1971; Data and Pratt, 1980). Variation in pod colour, shape and wing characteristics are seen widely in winged bean (Khan, 1978).

The non endospermous seeds of winged bean are rich in protein, oil, minerals and vitamins (Bailey, 1968; Watson, 1971; Pospisil *et al.*, 1971). The amino acid composition of protein have been studied in detail (Cerny *et al.*, 1971).

According to Kadam *et al.* (1981), the seeds contain 25 – 45 % carbohydrates. However, controversial reports are available on the presence of starch. Garcia and Palmer (1980) determined starch contents in mature seeds of five cultivars of winged bean and reported that there was no starch. It has been suggested that during the early stages of development of winged bean the seeds contain starch. In contrast, Sajjan and Wankhede (1981) reported 36.5% starch in defatted flour of mature seed. Hildebrand *et al.* (1981), studied sugar and starch contents of 21 cultivars of winged bean and reported a mean value of 3.4% for starch and 7% for sugars.

Numerous studies have been undertaken to elucidate the occurrence of different types of proteins in winged bean (Blagrove, 1978; Kortt, 1979; de Lumen and Salamat, 1980; Sathe and Salunkhe, 1981; Chan and deLumen 1982; Tan and Wong, 1982; Hafez and Mohmed, 1983). According to Kute *et al.* (1984), like other legumes winged bean seeds contain trypsin inhibitor activity which is found to increase as the seed maturation is advanced and progresses until complete maturity.

The seed proteins of winged bean has been investigated and many proteins have been characterised and reported a series of papers by Kortt *et al.* (1985), Kortt, (1986), Kortt and Caldwell, (1985), Kortt *et al.* (1989), Caldwell *et al.* (1990).

Kortt *et al.* (1985), isolated and characterized the seed lectins of winged bean. Isolation and properties of lectins from the tuberous roots of winged bean was reported by Kortt and Caldwell (1986). According to these authors the molecular and physico-chemical properties of tuber lectins were essentially identical to that of corresponding seed lectins. According to Kortt (1986), albumin fraction of winged bean seeds is composed of a single polypeptide designated as winged bean albumin-1, which is a crystalline protein. Studies on amino acid sequence of this crystalline seed albumin (WBA-1) showed that this single polypeptide chain consists of 175 amino acid residues (Kortt *et al.*,1989a). The primary structure of trypsin inhibitors of winged bean has been elucidated by Caldwell *et al.*(1990) and Kortt *et al.* (1991).

Winged bean seeds shows hypogeal type of germination. The seeds of which is found to be an ideal material to study the reserve mobilization during germination since, it is a large seeded legume. The cotyledons are bulky and conspicuous even after 10 days of seedling growth.

The percentage of germination in winged bean seeds is very low and this is attributed to their hard seed coat (Csizinsky, 1980). Scarification of the hard coated seeds has been achieved by immersion in concentrated H_2SO_4 or in boiling water or exposing them to very low temperature by immersion in liquid nitrogen (Phipps, 1973; Mayer and Shain, 1974; Csizinsky, 1980). On the basis of the seed dormancy distribution in winged bean (Variety PT2), three types of seeds i.e. imbibing, delayed imbibing and hard, have been identified in this laboratory (Nabeesa *et al.*, 1988).

In this laboratory a few aspects of seed germination have also been undertaken on winged bean seeds (Kamaladevi and Madhusudanan, 1989; Kamaladevi *et al.*, 1989; 1990; Madhusudanan and Padmakumar, 1990; Nabeesa and Harikumar, 1990; 1994; Nabeesa-Salim and Lalitha, 1997). All these studies revealed that reserve mobilization during seed germination is very slow and even after 10 days of seedling growth under Petri dish condition, more than 70-80% of the dry matter is retained in the cotyledons.

The development of laterals at the cotyledonary axils of winged bean was induced by epicotyl excision and the pattern of growth and the establishment of plants under field conditions were studied by

Madhusudanan and Padmakumar, (1990). However, the change in reserve mobilization influenced by epicotyl excision was not analysed so far.

A comparative study of salinity tolerance of winged bean and soybean (Weil and Khalil, 1986) suggested that winged bean have atleast as great a potential as some soybean varieties for adaptation to saline-sodic soils.

Regeneration of winged bean plants *in vitro* studies are reported from epicotyl segments (Mehta and Mohan Ram, 1981; Tran Thanh Van *et al.*, 1986; Venketeswaran *et al.*, 1992). The first report of plantlet regeneration from callus tissue derived from the leaf of a grain yielding legume came from the winged bean (Gregory *et al.*, 1980).

Secretion of six specific proline rich protein in suspension culture of winged bean was studied by Esaka and Hayakawa (1995) and the presence of these proteins are attributed to salt adaptation of these variety. Ahmed *et al.*(1996) reported the regeneration of somatic embryos from callus cultures derived from leaf explants of winged bean.

Reserve mobilization and metabolic changes associated with seed germination have been the objectives of several studies and this subject has been reviewed by many (Bewley and Black, 1983, 1985, 1994; Khan, 1984; Mayer and Poljakoff-Mayber, 1989). Germination is characterized by the hydrolysis of reserves, including lipids, proteins and carbohydrates of the storage tissues. The products such as sugars and amino acids are subsequently translocated to the embryonic axis for

synthesizing cellular constituents required for growth and differentiation.

The commitment of cotyledons to the storage and mobilization of reserves is absolute. Cotyledons fail to develop stomates and so display no useful photosynthetic activity as demonstrated with $^{14}CO_2$ for the cotyledons of Pea. In Pea with cotyledons adapted completely for storage, the early growth rate of the axis depends upon the size of the cotyledons (Murray, 1984).

The most striking change which can be observed after the onset of germination is seedling growth. During growth an increase in size and dry weight of the hypocotyl occur and it is accompanied by an almost similar loss of dry weight in the cotyledons. The increase in weight of the other parts of the embryo ie. epicotyl, plumule and radicle begins later and takes place at slower rate. Studies on reserve mobilization (Mayer and Poljakoff –Mayber, 1989) showed that various substances such as soluble sugars, insoluble polysaccharides, soluble protein and.nitrogen as well as nucleic acid, phosphorus move out of the cotyledons and are transferred to other parts of the growing embryo.

The changes in storage materials during germination are the result of the activity of many hydrolytic enzymes. These enzymes are either present in the dry seed or very rapidly become active as the seed imbibes water and many are synthesised *de novo*. Generally enzymes breaking down starch, proteins, hemicellulose, polyphosphates, lipids and other storage materials, rise in activity fairly rapidly as germination proceeds. The enzymes are not necessarily produced in the same cells

in which the storage materials are located. More over signalling system exist which regulate the production of enzymes and the interaction between different parts of the seed – embryonic axis and cotyledons, endosperm, embryo and aleurone layer – depending on the seed.

Smith (1974) carried out some interesting work on reserve hydrolysis in 500 species of legumes and revealed that there are eight basic patterns of hydrolysis of reserves from the cotyledon. According to him mobilization begins around vascular strands and at adaxial side of cotyledon in winged bean, the only example found so far. Mobilization of the reserves in the storage parenchyma is initiated around the vasculature and adaxial side of the cotyledon and proceeds inwards (Smith, 1974). There appears to be correlation between the break down of the reserves and changes in DNA and RNA content of the cells (Smith and Flinn, 1967).

A net loss of metabolites and mineral nutrients during seedling establishment has been explained and interpreted in terms of hormonal regulation and sink-source metabolism of reserves (Bewley and Black, 1983, 1985, 1994; Copeland and McDonald, 1995) and some ecological explanations are also available in many references cited by Mayer and Poljakoff- Mayber (1989). But the pattern and control of mobilization in dicotyledonous plants is not well understood.

Studies on differentiation of the primary vascular tissues in seeds (embryogenesis) and seedlings (germination) are very few and that available are dealing with the course of vasculature in the well formed seedlings rather than their ontogeny (Bisalputra and Esau,1961; Swamy

and Parameswaran, 1962; Philip, 1972, 1974; Swamy and Krishnamurthy, 1980). Since cotyledons are found to be the least investigated seed/seedling part in the present investigation, the vascular differentiation and reserve mobilisation patterns combined together are proposed to study by histochemical and biochemical methods.

Translocation of mineral ions from the roots to other parts and photosynthates and organic products from leaves to various parts of plant bodies, the direction of phloem and xylem tissues and their specific roles in translocation and the strategies and mechanisms of transport are fairly documented in plant physiology (Anderson and Beardel, 1991; Salisbury and Ross, 1992; Canny, 1995; Hopkins, 1995; Taiz and Zeiger, 1991; Raman, 1997). Nevertheless the pattern of translocation and tissue differentiation occurring in cotyledons during germination is poorly understood and authentic investigations have not been done in this aspect.

Factors influencing translocation such as light, temperature, concentration gradient and role of mineral ions, radicals and growth regulators are found to enhance or inhibit the rate of translocation (Hillman, 1984).

As the seedling develops by utilizing the reserves from storage tissue like cotyledon/endosperm, the function of epigeous cotyledons of some angiosperm shifts to photosynthesis (Marshall and Kozlowskii, 1975). Despite the lack of photosynthesis, hypogeous cotyledons acting as powerful sources of metabolites during seed germination are not investigated for their function as powerful source during seedling

growth. Comparatively little attention has been paid to the nature of reserve mobilisation and sink-source relationship of the embryonic axis and cotyledons of the seeds which are charactersied by hypogeal germination. So the present study is proposed to undertake various aspects of reserve mobilisation.

A perusal of literature shows that in recent years relatively large amount of research has been carried out in the field of seed germination and reserve mobilisation. Similarly solute transport and phloem translocation are also well documented. However, a comprehensive correlation of reserve mobilisation and the path, influx and efflux of metabolites in the cotyledons and embryonic axis is not yet drawn. More over, the vascular differentiation of cotyledons is incomplete since only procambial strands are formed during embryogenesis. This process is expected to undergo further differentiation possibly during early phases of seed germination.

It is evident that in epigeal type of germination the cotyledons are differentiated and transformed in to a first pair of leaves in many plants where the reserve mobilisation may be superimposed with photosynthetic activity. Generally in hypogeal type of germination particularly in legumes the cotyledons undergo differentiation to enable the translocation of degraded reserve metabolites to the growing axis. Comprehensive studies are not done so far to elucidate the correlation between reserve mobilisation and vascular differentiation in the cotyledons of dicotyledonous or monocotyledonous plants. So the present study is also aimed to correlate the vasculature developments of cotyledons of winged bean (hypogeal type of germination) and the mode

of translocation of reserves during seedling growth. As mentioned earlier, the studies on winged bean seeds in this laboratory revealed that majority of the dry matter of cotyledons are retained even after seedling growth for ten days. The forcing of cotyledonary axillary bud development by excising the original plumule may stimulate or trigger the reserve mobilisation from the cotyledons. So the effect of plumule excision on the metabolite degradation and mobilisation also is included in the present investigation.

Histochemical analysis are carried out to obtain more visual observation for fundamental understanding of general metabolism and functioning at cellular/tissue level. Histochemistry concerns the study of chemistry of tissues and enables the study of morphological /anatomical investigations as well as localisation of metabolites by using various specific staining procedures. Biochemical analyses of proteins, carbohydrates and lipids are included in this study to elucidate the quantitative changes of these reserves during seed germination and seedling growth.

In order to find out the effect of stress due to mineral deficiency on the vascular differentiation of cotyledons, a comparison of vasculature development in the cotyledons of winged bean seedlings grown in garden soil (control) and in acid washed sand (experimental) also is included in this investigation.

REVIEW OF LITERATURE

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I. RESERVE MOBILISATION DURING GERMINATION

Recently the process of seed germination has been defined by Bewley (1997) and according to him, germination commences with the uptake of water by the dry seed (imbibition) and is completed when a part of the embryo usually the radicle extends to penetrate the structure that surrounds it. Subsequent events including the mobilization of the major storage reserves are associated with seedling growth.

Imbibition of water is followed by a general activation of seed metabolism. Increased respiration is one of the earliest biochemical events to be detected in imbibed seeds. This is followed closely by the release of hydrolytic enzymes that digest and mobilise the stored reserves and renew cell division and cell enlargement in the embryonic axis (Bewley and Black, 1985).

The mobilization of the stored reserves in seeds has been studied extensively in cereal grains and legumes (Bewley and Black, 1983,1985; Mayer and Poljakoff –Mayber, 1989; Copeland and McDonald, 1995). The pathways of breakdown of carbohydrate reserves in cereals, starting with enzymes and the regulation of these enzymes activated by hormones are well documented in botanical literature.

In non-endospermic seeds such as legumes (peas, beans) the initial stages of radicle elongation appear to depend on reserves stored in the tissue of the radicle itself and later, carbon reserves are mobilized from the cotyledons and transported to the elongating axis (Copeland and McDonald, 1995; Hopkins, 1995).

Generally in dicots, legumes in particular, the hormonal regulation of reserve degradation is not as clear as monocots. Additionally, the role of hormones in dicot seed germination has been debated. Some investigators believe that dicot seed germination is mediated by the growing embryonic axis. As the axis continues to grow it incorporates breakdown products into the synthesis of axis compounds. The effect of embryonic axis on reserve mobilization during germination has been shown in many seeds.

An excellent review by Murray (1984) includes many aspects of embryonic axis-cotyledon interactions. The regulatory role of the axis in controlling the enzyme activities of cotyledons are emphasised. The axis is found to influence the mobilization of reserves in the cotyledon by transmitting some chemical stimuli. Nevertheless, Halmer and Bewley (1982) stated that the degree to which the axis controls the cotyledonary reserves of carbohydrates is uncertain.

Redistribution of minerals and organic nutrients from the cotyledons of seeds in plants is essential to support the early growth and development of the seedlings. Previous studies with legumes like *Phaseolus vulgaris* (Bukovac and Riga, 1962), and *Pisum sativum*

(Guardiola and Sutcliffe, 1972; Ferguson and Bellard, 1976; Collins and Sutcliffe, 1977) have shown that along with cotyledonary organic reserves, certain macro nutrients are transferred with great efficiency to the developing seedlings and their mobilisation is linked closely with the loss of dry matter from cotyledons. These authors also suggested that the degree of translocation of mineral nutrients from cotyledons may be related to their respective mobilities in phloem. Hocking (1980) suggested that in *Lupinus albus* and *Lupinus angustifolius*, the transport of dry matter, Nitrogen, Potassium, Phosphorus, Sulphur, Calcium, Magnesium, Sodium, Iron, Manganese and Copper from cotyledons of seeds to the seedling axis showed difference in their rate of transport. Nitrogen, Potassium, Phosphorus and Sulphur showed more efficiency followed by other elements and Calcium showed the least efficiency in mobility.

The important interaction between the axis tissues and the cotyledons of the germinating pea was suggested by Varner *et al.* (1962). He concluded that some beneficial factor from the axis tissues was moving into the cotyledons. According to Simon and Meany (1965), root shoot axis of *Phaseolus* is related to the reserve materials in the cotyledons in much the same way as in the embryo of barley to its adhering endosperm. In both, most of the reserve material is consumed for dry weight increase rather than respiration and in both dry weight increase begins quite suddenly some time after the start of germination.

Effect of embryonic axis removal during seed germination has been investigated in many plants like black gram (Morohashi, 1982), *Phaseolus* (Davis, 1983) *Pisum sativum* (Monerri *et al.*, 1986). All these

studies showed that α amylase activity and starch degradation are under the control of the axis.

The initial restriction on transport of solutes from the cotyledons to the radicle is the time taken to complete the differentiation of functional phloem cells. (Murray, 1984). This process requires about 13 hours or more in Pea (Smith and Flinn, 1967). According to Murray *et al.* (1979), an increase in the dry matter content of the radicle accompanies expansion growth leading to emergence.

Enzymes for the hydrolysis and transformation of sucrose are absent from the cotyledons but are present in the axis, to which sugar is presumably transported (Bewley and Black, 1985). Control by the embryonic axis of the breakdown of storage proteins in cotyledons of germinating seeds of *Citrus limon* has been studied by Garcia - Agustin *et al.* (1991).

The importance of the effect of embryonic axis on reserve mobilization during germination was emphasized by Pretorius *et al.* (1998) according to whom the soaking injury is more pronounced in the embryonic axis than in the cotyledon in *Phaseolus vulgaris*.

The degradation of storage proteins during seed germination has been studied for a long time, the starting point being the establishment of protein degradation and the detection of proteolytic enzymes in seeds. Since then, histochemical and cytological investigations on the characteristics of the process of protein degradation which leads

eventually to free amino acids have been carried out (Ashton, 1976, Muntz *et al.*, 1985)

Protein degradation as well as synthesis were analysed in peanut (Cherry, 1962) and reported that in cotyledon, the protein content slightly decreased after the first five days of germination, followed by a rapid decrease in content between six to nine days. By 10 days of germination about 70% of the protein was depleted with little loss thereafter. The depletion of total and cytoplasmic protein (debris free) roughly paralleled each other.

A histochemical study in *Pisum arvense* using ninhydrin-schiff's reagent revealed that initially the protein bodies in the storage parenchyma cells of cotyledon were up to 3μ in diameter (Smith and Flinn, 1967). The inner cells appeared to contain more of them per unit volume than do the outer cells. During the first two days of germination these protein bodies appeared to enlarge atleast twice their original diameter and subsequently to fuse together in groups to form larger aggregate bodies. These may be 25 μ or more in diameter and they stain more intensely than did the original bodies. The staining changes from red-purple to definite red. This change in intensity was due to the colour reaction with small peptides rather than with large proteins. Small, very deeply staining bodies become apparent in the protein bodies after coalescence. Ultimately the large aggregate bodies disintegrated and formed small irregular bodies which finally disappear. At the tissue level, the protein first disappears from the peripheral cells and those in the basal region of the cotyledons. This was because of greater rate and

earlier initiation of lysis. Break down initiated in the outer cells and they spreads inwards. Complete disappearance from outer region occurred after five days. After 12 days the storage parenchyma was almost completely free of protein and only the nuclear protein showed staining (Smith and Flinn, 1967).

According to Chin *et al.* (1972), total protein declined in the cotyledons of *Pisum sativum* attached to the embryonic axis but not in the detatched ones. Concurrent with the decline in protein level in the intact cotyledons there was an increased capacity to incorporate exogeneously supplied amino acid in to protein. The result suggest that the axis component may regulate the availability of messenger RNA in the cotyledons during germination.

Some interesting work on reserve hydrolysis in legume seeds has been carried out histochemically by Smith (1974), whose exhaustive studies of 500 legume species revealed that as far as the specific region on the cotyledon there are eight basic patterns of hydrolysis of reserves from the cotyledons. *Psophocarpus tetragonolobus* is found to be unique in that the mobilization of reserves begins around vascular strands and from the adaxial side of cotyledon, compared to other species studied.

Chrispeels *et al.* (1976) elucidated the regulation of reserve protein metabolism in the cotyledons of mung bean seedling. These authors reported that the endopeptidases responsible for reserve protein break down are synthesized *de novo* and become associated with the protein bodies. From ultra structural evidence it is shown that vesicles

that originate from the rough endoplasmic reticulum may mediate the transport of enzyme from its site of synthesis to the protein bodies.

During seed germination the protein bodies show the presence of proteolytic and hydrolytic activities in mung bean. Low level of acid protease activities were demonstrated in protein bodies. Similarly acid phosphatase activities have been reported in germinating seeds (Chrispeels *et al.*, 1976).

According to Vozzo (1978), in *Quercus alba* embryos, cytoplasmic contents of the epidermis and the immediate lower layers gave the most intense staining for proteins in cotyledons of ungerminated acorns. There was a little positive staining elsewhere in the cotyledons.

According to Gillespie and Blagrove (1978a, 1978b), winged bean seeds contain proteins predominantly of the 7S and 2S type which was resolved by cellulose acetate electrophoresis into three fractions namely Psophocarpin A,B and C, which can be isolated by isoelectric precipitation. Psophocarpin A and C constitute the seed storage globulins. Psophocarpin A, a minor component, is essentially a single protein comparatively rich in sulphur containing amino acids. Psophocarpin C, which constitute more than 60% of the seed protein, is composed of 7S and 2S globulins.

A review of the literature on mobilization of seed storage proteins (Ashton, 1976) reveals that ungerminated and germinating seeds contain storage protein bodies constituting several subunits and several proteolytic enzymes atleast one such enzyme is present in the protein body. Pattern of protein bodies degradation in the cotyledons of several

seeds like *Pisum arvense, Glycine max, Arachis hypogea, Vicia faba* etc. are systematically described by this author.

During germination the protein bodies which are apparently vesicles derived from endoplasmic reticulum and later deposited with proteins are broken down and protein bodies become empty (Ashton, 1976; Pernollet, 1978). According to Ashton (1976) the protein degradation during germination involves a multiple enzyme system and it appears that specific control mechanism may be present for the storage proteins hydrolytic enzymes to prevent their degradation of structural and functional proteins.

Wang *et al.* (1979) studied the changes of RNA, DNA and protein in cotyledons and embryonic axis of germinating soybean seeds. In the first two days, there was less low molecular weight proteins than was in subsequent stages. The small protein molecules increased after the 4^{th} day of germination. The soybean seed has a high content of RNA. This may be due to the presence of abundant protein bodies in the cells of the cotyledons, and protein bodies contain ribonucleic acid.

According to Quail (1979), protein bodies of seed contain reserve protein in a dense matrix enclosed by a single limiting membrane. As germination proceeds the matrix proteins disappear and the protein bodies enlarge or coalesce eventually forming the tonoplast bounded central vacuole. This author also suggested that, protein bodies are defined as transient organelle and they contain non-storage proteins also.

Okezie and Martin (1980) analysed the chemical composition of dry seeds and fresh leaves of winged bean varieties grown in US and

Puerto Rico and found that the seeds contain high levels of protein ranging from 31.56-41.35% in the undehulled and 35.66 to 50.92% in the dehulled. Protein in seed hulls ranges from 8.38 to 13.49%.

Although many hydrolytic changes of storage protein are observed in germinating seeds, usually there is a little change in the total nitrogen content during germination. In the place of the protein, broken down amino acids and amides appear. Part of the amino acids are oxidatively deaminated and the carbon skeleton enders various respiratory cycles and the ammonia formed by deamination is detoxicated by amide formation and chief amides are glutamine and asparagine (Lea and Joy, 1983).

Ryan (1981) studied the proteinase inhibitors in graminae and leguminosae showed that natural proteinase inhibitors are often found as major components of the cytoplasm and secretions and intercellular fluids of seeds and tubers.

The regulatory role of embryonic axis on reserve mobilisation of seeds during germination is a well documented aspect of seed physiology. Protein degradation is found to be affected by embryonic axis control. Removal of embryonic axis from the seeds resulted in a reduction of protein degradation in *Cucumis striata* during germination (Davies and Chapman, 1979b). Later, Davies and slack (1981) studied the control of food mobilization in seeds of dicotyledonous plants. According to these authors two alternative hypotheses can be used to explain how the embryo or embryonic axis controls food mobilization in seeds of many dicotyledons plants. The first involves the production of a hormonal stimulus by the axis which initiates the development of optimal rates of

development of optimal rates of hydrolytic enzyme activity in the storage organs. The second involves the continual operation of a source – sink relationship between the storage organ and axis during germination and early seedling development.

A comparative study on the storage proteins and anti-nutritional factors of seeds of winged bean from five south east Asian countries have been made by Kortt (1983). The results revealed marked variations in the electrophoretic patterns which could be exploited by plant breeders to improve nutritional quality with respect to sulphur containing amino acids. All the varieties showed trypsin and chymotrypsin inhibitory activities and hemagglutinating activities.

In soybean, endopeptidase activity was analysed (Bond and Bowles, 1983) and two distinct endopeptidase activities were identified in both the embryonic axis and the cotyledons during germination. One activity is characteristic of a neutral alkaline metallo endopeptidase and the other of an acidic carboxyl endopeptidase. Neither activity was membrane bound.

During germination there is considerable interconversion of amino acids, necessitated by other metabolic events, the requirement of the transport system and differences between amino acid composition of reserves compared with new cytoplasmic protein (Lea and Joy, 1983). According to these authors, amino acids released from storage protein can contribute directly to protein synthesis in growing parts, but labelling studies have shown that considerable interconversion and metabolism of amino acid carbon occurs in storage tissue during germination. Even

during the period of massive protein break down in the cotyledons some amino acids were reincorporated into the insoluble fraction. Amino acids may contribute large amounts of substrate carbon to the respiratory system, and even to sugar synthesis in gluconeogenic seeds such as castor bean.

Bewley and Black (1983) suggested that protein, nitrogen and starch reserves in the cotyledons of *Vigna sesquipedalis* appeared to be hydrolysed simultaneously, although *Vigna unguiculata* cotyledons, starch remains in cells from which all protein and fat have been mobilized.

Characteristics of seed storage proteins have been systematically described by Bewley and Black (1985). According to these authors, seed storage proteins are usually deposited within special cellular organelles called protein bodies. These range in diameter from 0.1 –25 μ m and are surrounded, atleast during development, by a single membrane. Some protein bodies are simple in that they consists of a protein matrix surrounded by a limiting membrane. Inclusions frequently occur, however particularly crystalloids and globoids and more rarely, druse (calcium oxalate) crystals. The crystalloids are water insoluble proteinaceous inclusions embedded in the soluble protein matrix. In caster bean the crystalloid is an insoluble 11S protein, and matrix is made up of 2S and 7S albumins. Globoids are non crystalline, globular structures and are the most commonly occurring inclusion in protein bodies. Globoids are the site of deposition of phytin the potassium, magnesium and calcium salts of phytic acid.

According to Bewley and Black (1985) protein synthesis is essential for germination to be completed and for the radicle to emerge. Newly formed RNA is needed for protein synthesis, DNA synthesis in most seeds occur only after germination and is an integral part of growth of the axis. 80S ribosomes associated with m RNA is necessary to commence protein synthesis in cytoplasm. They also found that, in the 6 days following the start of imbibition, seed germinates and the seedling growth ensues. During this time, the amount of storage protein in the cotyledon falls by about 75%, a change that is accompanied by a rise in activity of extractable proteinase (vicilin peptido hydrolase). The enzymatic break down of stored protein leads at first to an accumulation of free amino acids in the cotyledon but these decrease after about 3 days as they or their products are transported into the growing axis.

From winged bean a major seed albumin is isolated and characterized by Kortt (1986). He observed that the albumin fraction of the seeds of winged bean contains the anti nutritional factors and several other proteins that are significant components of the total seed protein.

Ramachandra and Monteiro (1986) using micro kjeldahl analysis of seeds of 25 varieties of winged bean revealed that crude protein content ranged from 29.92 to 43.80%. Amino acid analysis of the seed protein of 10 of the varieties showed that although the sulphur containing amino acids were deficient, all other essential amino acids were present in adequate amounts. Solubility fractionation of the proteins of two of the varieties revealed that albumins and globulins were the major storage proteins while the electrophoresis indicated the presence of several polypeptide compounds in the albumins and globulins.

Isolation and characterization of the lectins from the seeds of *Psophocarpus scandens* were done by Kortt (1988). There were two distinct groups of lectins. The lectins absorbed by melibiose Bio-Gel P 150 yield two distinct components (lectins B_1 and B_2) on gel filtration on Ultrogel AcA 44. Both were glycoproteins composed of two non-covalently bound subunits with isoelectric points much lower than the corresponding melibiose adsorbed lectins from *Psophocarpus tetragonolobus.*

The current view on the process of storage protein degradation and enzymes responsible for their mobilization have been extensively reviewed by Shutov and Vaintraub (1987). According to these authors, seeds contain proteins such as legumin like (11S) and viciline like (7S) proteins and these are localized in protein bodies. Proteases degrade the higher molecular weight storage proteins to peptides and these are further hydrolysed by amino peptidases to form free amino acids.

Mayer and Poljakoff-Mayber (1989) suggested that in legumes, break down of storage proteins in the cotyledons is accompanied by the appearance of new proteins in other parts of the seedling. Seeds contain a variety of proteolytic enzymes some of which are present in the dry seeds while others appear during germination. The proteolytic enzymes can be divided into proteinases and peptidases depending on the size of the molecule which is attacked. Removal of axis only had very little effect on trypsin and aminopeptidase activities . Therefore it was suggested that the axis does not affect protein break down directly, but creates a sink for the break down products- the aminoacids. The enzymes responsible for proteolytic break down initially present in the

protein bodies may be synthesized or activated in the protein bodies themselves. However, during germination the storage protein vicilin is not broken down until an additional endopeptidase is synthesized *de novo* in the cytoplasm and transported to the protein bodies. According to Mayer and Poljakoff- Mayber (1989),Dry seed contain very little free amino acids. The growth of the embryo in the germinating seed is dependant on a supply of amino acids for its protein synthesis.

Kortt *et al.* (1989) studied the amino acid sequence of a crystaline seed albumin (WBA – 1) from winged bean. The protein consists of a single polypeptide chain of 175 amino acid residues, with one disulfide bond, corresponding to a molecular mass of 19333 Da. WBA –1 was found to be homologous with the Kunitz – type seed trypsin inhibitors.

A preliminary study on the effect of cotyledonary axillary bud development induced by plumule excision on acid phosphatase activity during seed germination in winged bean seeds showed powerful acid phosphatase activity. During seed growth, the forcing of axillary branch resulted in an enhanced activity of both fructose 1,6 bisphosphatase and β glycerophosphatase in the cotyledons (Nabeesa and Harikumar, 1990).

Hussain *et al.* (1990) studied the protease activity in cotyledonary tissue of the winged bean. After 7 days of germination, the proteinase activity showed an increase during germination. Caldwell *et al* (1990) elucidated the primary sequence of trypsin inhibitor to (WBT_1-2), which is a single polypeptide chain containing 182 amino acids. The primary structure of the trypsin inhibitor-2a from *Psophocarpus tetragonolobus*

seeds showed that this trypsin inhibitor consists of a single polypeptide chain of 180 amino acids (Kortt *et al.*, 1991).

The control of embryo axis on the break down and mobilisation of storage proteins in cotyledons of germinating seeds have been studied by many investigators in different plants. Garcia-Agustin *et al.* (1991), reported that in *Citrus limon*, the protein bodies disappear in the first six days after the onset of imbibition before the emergence of radicle. Cytokinin released by the radicle may control the endopeptidase activity in *Citrus limon*. (Guardiola and Sutcliffe, 1971; Yomo and Varner, 1973; Kern and Chrispeels, 1978; Minamikawa, 1979; Morohashi, 1982; Davies and Chapman, 1979, 1980).

Studies on reserve mobilisation during germination of *Tagetes minata* L. showed that in germinating seeds albumin increased, while globulins decreased. Lipid composition remained constant except for a slight decline in linolic acid in the germinating seeds (Drewes and VanStaden, 1991).

The ultrastructural evidence on the origin of protein bodies in rough endoplasmic reticulum of developing cotyledons of soybean was analysed by Zheng *et al.* (1992). As per their hypothesis during early stages, vacuoles in the cells are filled with proteinacious materials and turn into protein bodies.

Nabeesa Salim and Harikumar (1994) studied germination and reserve mobilization in winged bean. Protein hydrolysis and translocation were found to be very slow and only about 5% was mobilized upto 7^{th} day of germination.

Utilization of storage proteins by excised radicle/hypocotyl during *in vitro* germination of embryo axes of cotton seeds was studied by Vigil and Fang (1994). A previous analysis of the fine structure of radicles from dry seeds revealed an abundance of protein and lipid reserves in the cortical parenchyma. In this study, the hypothesis that utilization of stored protein is involved in radicle elongation and protrusion was tested.

Islam *et al.* (1995) Investigated biochemical changes in germinating seeds of winged bean. In this plant total dry weight of cotyledons decreased while those of embryonic axis increased on germination. Total nitrogen and protein nitrogen underwent a gradual increase up to day 6 followed by a progressive decrease at day 15. Levels of non protein nitrogens as well as total free amino acid showed 2 and 3 fold increase respectively during the period between 0 and 15 days after germination. The protease activity towards haemoglobin were relatively low in resting seeds of winged bean, increased steadily with the onset of germination and reached peaks at day 12 and then declined.

Purification and characterization of an acidic protease in germinating winged bean seeds showed that two major classes of proteases are shown to occur in germinating winged bean seeds by assaying extracts at pH 8 and pH 5 with ^{14}C gelatin as substrate (Usha and Singh, 1996). At pH 8, the activities profile of the enzyme shows a steady rise throughout the period of germination, where as the activity at the acidic pH is very low up to day 5 and then increases sharply reaching a peak on day 11 followed by an equally sharp decline. The winged bean acidic protease (WbAp) has been purified to apparent homogenity as attested by a single protein band on both PAGE and SDS / PAGE. WbAp

is a monomeric enzyme with a molecular mass of 3S k Da and a pH optimum of 6. It is a protease that does not belong to the papain family and not has tightly bound Ca^{2+} as shown by 45 Ca^{2+} exchange studies.

Bewley (1997) suggested that all of the components necessary for the resumption of protein synthesis upon imbibition are present within the cells of mature dry embryos, although polysomes are absent. However, within minutes of rehydration there is a decline in the number of single ribosomes as they become recruited into polysomal protein synthesizing complexes.

During germination, carbohydrates, the principal reserve materials, are degraded in the cotyledon or endosperm and the products are translocated to the developing axis. Considerable number of reference are available on the starch mobilizing metabolism in legumes(Bewley and Black, 1983, 1985; Mayer and Poljakoff-Mayber, 1989).

The embryonic axis is known to influence the mobilization of starch reserves through the development of amylase activity in the cotyledons of dicot seeds. In the absence of the axis the amylase activity is declined, remained in unaltered or even increased in various cultures of peas (Morohashi and Ueno, 1980) and beans (Morohashi, 1982). Plant growth substances like gibberellins or osmotic stress conditions were shown to be responsible for the effect of cereal embryo and hydrolase development. An assessment of the control of breakdown of food reserves in germinating dicotyledonous seeds was done by Chapman and Davies (1983). According to these authors the axis act as a sink for the

products of enzyme action and in the absence of the axis the products accumulate causing feed back inhibition.

Smith and Flinn (1967) made histochemical analysis of the cotyledon of *Pisum arvense* during germination and found that initially starch is present in all the cells except those of the epidermis and possibly the potential conducting cells of the procambium. It is present in large quantities in the storage parenchyma, particularly in the inner zone. The hypodermis and procambium contain only very small grains which disappear within the first three days. The pattern of degradation of starch in the storage parenchyma is similar to that of protein degradation which begins at the periphery of the cotyledon and proceeds in a wave towards the centre. During the first seven days the outer storage cells became depleted of their starch and after 12 days no starch remains in the central core. During the first two days small starch grains, mostly in the size range 3 to 15μ appear in the storage cells. They become aggregated round the nucleus, they have disappeared from the outer region of the cotyledon by day 5 and from the central core by day 7.

Smith (1974) noted the appearance of new, small starch granules in the cotyledon parenchyma cells of *Phaseolus vulgaris* during germination. Starch reserves in legume seeds are normally degraded during germination and the process has been documented for a range of species (Bewley and Black, 1978; ~~Preiss~~ A.P Rees, 1980).

The development of new, small starch granules in germinating cotyledons of *Phaseolus vulgaris* and *Vicia faba* described by Briarty and Pearce (1982), showed that axis removal inhibits starch synthesis in

Phaseolus vulgaris. When the embryo axis was removed early in germination (after 5 h and 16 h of hydration in *Phaseolus vulgaris* and *Vicia faba* respectively) starch metabolism appears to be affected. In *Phaseolus vulgaris* the number of small granules per cell at day 7 is reduced by this treatment from 700 to just over 400, while in *Vicia faba* there appears an increase in their number.

According to Kadam *et al.* (1981), the seeds contain 25-45% carbohydrates. However controversial reports are available on the presence of starch. The carbohydrate composition of winged bean has been studied by Sajjan and Wankhede (1981). They found that the seed contain about 42.2% total carbohydrate of which starch alone accounts for 36.2%. The total monosaccharides constituted 2.7% and were identified as glucose (1.17%) and fructose (1.5%); oligosaccharides amounts to only 0.61% and were identified as sucrose, raffinose, stachyose and verbascose on a dry defatted basis.

Kute *et al.* (1984) analysed three cultivars of winged bean for starch, sugar and trypsin inhibitor activity at different stages of seed maturity. The starch content ranged from 5.66 – 6.22% where as sugar content varied from 4.40-6.53% in the matured seeds. Starch content increased as the seed maturation progressed followed by a decrease upto complete maturity.

According to Murray (1984), sucrose is the predominant form of transport carbohydrate in the majority of plants. The supply of sucrose to phloem cells in cotyledons is derived initially from the reserves of free sugars which are often substantiated as in the cotyledons of wrinkle

seeded pea cultivars. This supply of sucrose for export can be maintained through lipid break down or polysaccharide break down. In pea cotyledons, the initial break down of starch must involve starch phosphorylase alone, since β amylase can act only on soluble substrates and α amylase is absent.

Studies on the changes in cell wall polysaccharides in relation to seedling development and the mobilization of reserves in the cotyledons of *Lupinus angustifolius*, have shown that some 22% of the dry weight of the cotyledons of resting seeds has been shown to be non – starch polysaccharide material comprising the massively thick cell walls of the storage mesophyll cells (Crashaw and Reid, 1984). On hydrolysis this material released galactose (76%) arabinose (13%), xylose (4%) uronic acid (7%) and traces of glucose showing absence of cellulose from the walls. Starch, which was not present in the dry seed, appeared transitionally following germination.

A critical review of the literature on starch metabolism during seed germination was made by Bewley and Black (1985). According to these authors starch is the carbohydrate most commonly found in seeds. It is stored in two related forms, amylose and amylopectin. Sucrose, the sugar translocated from the mother plant to the seed is the substrate for starch formation. First it is converted to fructose and UDPGI (Uridine diphospho glucose) by sucrose synthetase. Glucose molecule formed by UDPGI unite to form starch molecule. In soybean a substantial increase in starch content was observed in the cotyledons during development followed by a decline so that in the mature seed little starch remains.

This is in the later stages of development to provide carbon skeleton for fat and protein synthesis.

Studies of starch mobilization and the activity of starch mobilizing enzymes in germinating legume seeds revealed that, the mode and rate of phosphorolytic or amylolytic activity and nature and fate of hydrolysed products differ widely from species to species (Halmer, 1985; Monerri *et al.*, 1986).

The two shoot system formed by the excision of epicotyl in winged bean seedlings (Kamaladevi *et al.*, 1989) showed lowered starch level in particular in the laterals and the radicle. The pair of laterals had significantly higher sugar content than the control plumule, without alteration in the distribution among the component sugars. Epicotyl excision also resulted in the accumulation of dry matter and sugar in the radicle. Although seedling decapitation resulted in increased investment of dry matter in the growing parts, the greater part of dry solids and starch and a major part of sugar were retained in the massive cotyledons.

Mayer and Poljakoff – Mayber (1989) concluded that most of the enzymes involved in the break down and interconversion of carbohydrates become active during germination, most by *de novo* synthesis, some by activation or release. In many seeds disappearance of lipids is accompanied by the appearance of carbohydrates. This reaction proceeds as follows: the fatty acids undergo β oxidation. The acetyl CoA formed is converted to malate via glyoxylate cycle. The malate thus formed is converted to carbohydrate by a number of reactions. These reactions occur in the cotyledons, glyoxysomes, mitochondria and

cytoplasm. In *Tagetes minuta,* a transient accumulation of starch synthesised from free sugars formed as a result of glycoxylate pathway from lipid reserves was reported (Drewes and VanSteden, 1991).

Karunagaran and Rao (1991) found that in horse gram (*Macrotyloma uniflorum*), starch and total soluble carbohydrates declined while the reducing sugars increased in the cotyledons during the 4^{th} day of germination. Starch mobilizing enzymes ie, α amylase, β amylase, maltase and pullulanase were active where as starch phosphorylase declined from the beginning. The effect of the axis on α amylase development was not replaced by gibberellic acid or benzyladenine. The α amylase activities and the reducing sugar content of intact cotyledons increased to the same extent from day 0 to day 4. Starch phosphorylase activity declined from the beginning, all the other enzymes necessary for the degradation of starch to glucose appear to be functional in the cotyledons of horse gram during germination. The pattern of decrease in weight, starch and total soluble sugars in cotyledons indicate the translocation of soluble products to the axis for respiration.

The changes in carbohydrate during germination of lentil have been studied by Vidal-Valverde *et al.* (1992). In germinated lentils (*Lens culinaris* and *L. medicus*) the amount of total soluble sugars decreased (from 4.3% to 2% and from 5.3% to 2.2% respectively). Glucose not present in raw seeds was relatively high (0..6% and 0.7%) Fructose increased and sucrose decreased slightly. The oligosaccharides of the raffinose family disappeared from germinated seeds. Total starch decreased considerably in germinated lentils in comparison to dry seeds.

Germination pattern and food reserve mobilization in winged bean showed that comparatively very small amount of starch was present in winged bean seeds. But during germination starch content was increasing gradually. The enhancement of starch content was significant up top 5th day of germination and later only marginal increase was observed (Nabeesa-Salim and Harikumar, 1994). According to these authors considerable amount of ethanol soluble sugar content was present in dry and imbibed seeds of winged bean. However during germination, sugar distribution was reduced significantly and as seedling growth advanced, the sugar content was more or less the same up to 7 days.

The effect of germination on carbohydrate contents, trypsin inhibitors and protein digestibility (*in vitro)* of some local varieties of cow pea (*Vigna unguiculata*) was studied by Issa *et al.* (1994). Germination of three cow pea cultivars decreased seed starch and non reducing sugar contents but increased reducing sugars. Sucrose content was highest after 2 days of germination, while protein digestibility was slightly improved.

Islam *et al.* (1995) have studied the biochemical changes in germinating seeds of winged bean. Total dry weight of cotyledons decreased while these of embryonic axis increased with germination. Total soluble carbohydrate decreased from 0 to 9 days of germination and then declined.

Sugar import and metabolism during seed development in legume seeds was studied by Weber et al (1997). During seed development, cell division is followed by elongation, differentiation and storage. In legume

this sequence of events has been found to spread in a wave like manner, creating a developmental gradient across the cotyledons. All these processes including storage activities appeared to be subject to metabolic control. Sucrose is imported during seed development and a sucrose breakdown pathway mediated by cell wall invertase operate in the seed coat during early development. The resulting high hexose state is associated with growth and mitotic activity. Invertases are regarded as a control element in the changing carbohydrate status of seeds. Sucrose metabolism is controlled by a cycle of synthesis and break down involving sucrose phosphate synthase and sucrose synthase respectively.

The lipids which occur in special organelles referred to as lipid bodies or spherosoms are broken down into fatty acids and glycerol during germination . The initial break down of lipids is by lipase activity. Several lipase are reported to be active in germinating seeds (Muto and Beevers, 1974).

Lipid containing seeds such as soybean, castor bean and groundnut have been investigated and in these seeds, the disappearance of lipid is accompanied by the appearance of carbohydrates, since the acetyl CoA formed by β oxidation of fatty acids is converted to carbohydrates either by glyoxylate cycle or reversal of glycolysis, (Mayer and Poljakoff - Mayber 1989).

According to Vozzo and Young (1975), in *Quercus nigra* during germination lipids shifted from cotyledon to the embryo axis, and the total lipid content diminished. Cotyledon from dormant specimens were filled with coalesced lipid droplets. These droplets separated during

stratification and were more plentiful in germinated than stratified embryo axis.

Vozzo (1978), by histochemical analysis found that during germination phospholipids were concentrated in the procambium, cotyledon, embryo axis, shoot apex, periphery and ground meristem in ungerminated acorns of *Quercus alba.* A cuticle layer was evident. All cells that stained positive for phospholipids contained large dense droplets. The droplets were found in groups of one, two or three per cell but nearly filled the cell in all cases. Positive phospholipid reactions were localised heavily along the ground meristem, apical meristem, procambium and peripheral zone while cotyledonary cells were almost depleted of stored phospholipids in sections of germinated embryo.

According to Bewley and Black (1983,1985), Mayer and Poljakoff-Mayber (1989), the reserve fats and oils found within the cells of storage organs in seeds generally confined to organelles called lipid bodies. The origin and development of these bodies have been the subject of considerable controversy over the years, but their origin is found to be endoplasmic reticulum. When fat filled vesicle reaches a critical size it may bud off completely. Triglycerides are major storage lipid in seeds. Their initial hydrolysis is by lipases, which convert lipid into fatty acid and glycerol. Glycerol enters glycolytic pathway after its phosphorylation and oxidation to the triose phosphates to yield pyruvate then oxidise in citric acid cycle. Mobilization of stored lipid in the castor bean endosperm begins on about the third day after imbibition, digestion takes further 4 days. Glycerol may be converted into sucrose for the translocation.. There is an alkaline lipase associated with the oil storage

bodies that increase in activity considerably with the increase in fat mobilization. The enzyme might be *de novo* synthesized for it is absent from the dry seed.

According to Mayer and Poljakoff-Mayber (1989) the break down products of hydrolysis of lipids accumulates in the seed and are present if at all in small amounts. The fatty acids produced further by β oxidation, while the glycerol which is formed becomes part of the general carbohydrate pool present in the seed and as such becomes available for various processes including respiration.

Reserve mobilization during germination of *Tagetes minuta* was studied by Drewes and VanStaden (1991). Changes in the lipid complement of the seeds are determined by examining fatty acid levels in the neutral glyco and phospholipid fractions at different stages of germination and thermo inhibition. No linolenic acid was ever detected, but plamitic stearic oleic and linoleic acid were present with no marked difference between fatty acid levels in the different lipid fractions. There was a decrease in linolate in the phospholipid fraction, 24 and 48 hours after the start of incubation at 25°C.

Garcia-Agustin *et al.* (1991) studied lipid mobilization in *Citrus* cotyledons during germination. The lipases were found in extracts of cotyledons. One with optimal activity at pH5 (acid lipase) and the other with pH optimum between 7.5 to 8 (alkaline lipase). Both increase activity peak at 16^{th} day of germination. Most acid lipase activity was recovered in the fat layer obtained from crude extracts. The rate of lipid break down was lower in excised cotyledons when compared with intact

ones. However, substantial hydrolysis of lipids occur in the absence of the axis. In excised cotyledons lipase activities were markedly reduced indicating that the presence of axis was necessary for maximum enzyme formation. The appearance of new lipoxygenases in the cotyledons after germination and evidence for expression of a major new lipoxygenases gene have been reported in soybean by Kato *et al.*, (1992).

In winged bean Islam *et al.* (1995) studied biochemical changes during germination. According to these authors fat content increased slightly at day 3 followed by a gradual decrease throughout the experimental period. Oil depleted rapidly and degraded into glycerol and fatty acids. These fatty acids give rise to soluble carbohydrates via the glyoxylate cycle followed reverse glycolysis.

Singh *et al.* (1995) reported composition of fatty acids in winged bean seed oil. The oil content ranged from 14.5 to 22.7% in different varieties of winged bean. Oleic acid 41.4% and linoleic acid 29.7% were main constituents and contributed above 70%, however the total unsaturated fatty acid ranged from 66.8 to 78.9%; palmitic acid 10.9%; behenic acid 6.7% and stearic acid 3.5% were the major saturated fatty acids. Oil can be stored for longer periods being low in linolenic acid. Toxic parinaric acid is absent. For a long time it has been assumed that lipid mobilization is initiated by the liberation of free fatty acids from the storage lipids and these fatty acids are subsequently transported to peroxisomes and these may undergo β oxidation (Mayer and Poljakoff-Mayber, 1989).

A specific lipid body lipoxygenase induced during early stages of seed germination was recently reported by Feussner *et al.* (1995). It appears to translocate to the lipid storage organelles to oxygenate the storage lipids. This oxygenation was recently suggested to be initiation of the mobilizing cascade of the storage lipids (Feussner *et al.*, 1997). These authors have reported the structural elucidation of oxygenated storage lipid in cucumber cotyledons during germination. The enzyme lipoxygenase was localised at the lipid storage organelles and oxygenates their storage triacyle glycerol. Isolation of this lipid body lipoxygenase from the cucumber seedlings have been done and found that it is capable of oxygenating *in vitro* di and trilinolein to the corresponding mono-,di- and tri hydro peroxy derivatives. This enzyme play an important role during the germination process of plants. To investigate the *in vivo* activity of this enzyme during germination, lipid bodies were isolated from cucumber seedlings at different stages of germination and the triglycerols were analysed for oxygenated derivatives by a combination of high pressure liquid chromatography, gas chromatography/ mass spectrometry and nuclear magnetic resonance spectroscopy. During germination the amount of oxygenated lipids increased strongly, reaching a maximum after 72 h and declining afterwards. The specific oxygenation of the storage lipids may initiate their mobilization as a carbon energy source for the growing seedlings.

II. VASCULATURE OF COTYLEDONS

Studies on vascular anatomy of cotyledons in dicotyledonous plants are very scanty. Esau (1940) reported that in *Daucus carota*, Three vascular bundles enter into each cotyledon. The median bundle of these

three traces consists of a strand of exarch xylem which is continuous with the protoxylem of the root and is accompanied laterally by two phloem strands. In this strand centripetal differentiation can be followed for some distance into the cotyledon. In contrast with this each of the lateral cotyledonary trace is collateral with external phloem and endarch xylem on the inside .

Bailey (1956) studied different types of vascularisation of the cotyledonary node in the dicotyledonous plants. The most common type of cotyledonary node in the 99 dicotyledonous families that have been investigated, is the unilacunar node with two traces. According to him the double nature of the cotyledonary traces has phylogenetic importance.

Wardlow (1965) observed a zone of cells which extends into the apical dome above the insertion of the youngest leaf primordium and this is the procambium. Vascular differentiation involves the formation of procambial cells and different views are existing regarding the origin of procambium in shoot apices. Extensive reviews are available on the vascular tissue development in the shoot (Esau, 1965; Wardlow, 1965; Cutter 1978; Shininger, 1979; Fahn 1982) and with root (Esau, 1965 ; Torrey, 1965; Cutter, 1978; Shininger, 1979; Fahn, 1982).

The complex structure of the transition region between root and shoot is apparently suggested by Esau (1961). The differentiation in that region where the two opposite trends meet should be intermediate between the two. The extension of the transition region into one or more internodes above the cotyledon in seedlings, in which the germination is hypogeal, may be explained by the extended influence of the root apex on

the basal internodes of the stem as a result of the retarded growth of the hypocotyl in such seedlings.

According to Smith and Flinn (1967), who made histochemical studies of the cotyledons of *Pisum arvense,* the cotyledon has a complex, reticulate vascular system. Differentiation of the conducting elements from the procambium appears to begin about 12hrs and to be completed 48hrs after the commencement of imbibition. Differentiation of phloem precedes that of xylem. The relationship between the timing of vascular differentiation and various physiological events in the cotyledon is discussed by these authors. The cells of adaxial and abaxial sides of the cotyledons contain abundant starch grains and protein bodies. According to them, the mobilisation of reserves in the storage parenchyma in *Pisum arvense* is initiated at the periphery of the cotyledons and proceeds inwards. There appears to be a correlation between the break down of the reserves and changes in DNA and RNA contents of the cells

Polarised light microscopic study of phloem differentiation in embryo of *Chenopodium album* was made by Bisalputra and Esau (1961). In both cleared material and paraffin sections differentiating sieve elements are detected by the double refraction of their walls as seen in polarized light. Differentiation begins in the lower part of the cotyledons proceeds basipetally in to the hypocotyl and acropetally in the cotyledons. In the mature embryo the differentiating protophloem appears in the form of net works in the cotyledons; thus the cotyledonary venation system is initiated during embryogeny. The protophloem of the cotyledons is continuous with that of the hypocotyl, and differentiating sieve elements

are found approximately 100μ from the root apex. Electron microscopic observation revealed that sieve element of the mature embryo are only partly differentiated. They still have nuclei. Differentiation of the secondary wall in protoxylem does not occur until after germination and their appearance in polarized light is distinct from that of the protophloem.

According to Esau (1965) in cotyledons, phloem is differentiated early in germination. Connection is existing from the cotyledons to the embryonic axis. She also suggested that phloem is the main vascular channel for transport of nutrients to the seedling axis. Bukovac and Riga (1962), Guardiola and Sutcliffe (1972) showed that the extent to which a mineral is retrieved from the cotyledon is clearly related to its mobility in phloem.

The vascular connection between bud and stem was studied by Esau (1965). According to her the cause of the procambial development in bud traces depends on the character of the bud. An axillary bud that is initiated close to the apical meristem soon develops into a branch and become connected with the vascular system of the stem by branch traces which are equivalent to leaf traces in their developmental relation to the bundles in the stem. A bud, the development of which is delayed (apical dominance) is initially separated from the vascular cylinder of the stem by vacuolated parenchyma. When a bud of this kind begins to grow either spontaneously or after removal of the shoot tip (the source of apical dominance), it establishes a direct vascular connection with the nearest

vascular strands in the central cylinder through the vacuolated parenchyma.

Shininger (1979) reviewed the control of vascular development in plants. According to him, the process of vascularization involves first the production of a population of cells in which vascular differentiation can be induced. The process is viewed as a continuum of developmental events involving a first identifiable step to distinguish the potential vascular cells from the non-vascular tissue by differential parenchymatisation. The non parenchyma cells can be considered to be in the pathway to vascular development but are not obligated to that fate because removal of exogenous stimuli (auxin, sucrose, etc.) allows them to divert to the parenchyma state.

The pattern of procambialisation in the developing embryo and its bearing of vasculature of the seedling was investigated in *Catharanthus roseus* (Philip, 1972, 1974). According to him with the organisation of the apical and sub - apical groups of initials in the cotyledonary primordium, the vertical extension is accomplished. During this process the central row of derivatives of the subapical initial mature into procambium. The longitudinal course of differentiation of procambium strictly conforms to the acropetal pattern. This procambium refers to the later midrib region of the cotyledon and it is superimposed over the pairs of phloic procambium of the subjascent hypocotylary region. A group of sub marginal initials also becomes differentiated and the cotyledon attains breadth. The ontogenetic order of maturation of protophloem within the procambial system of the embryo and young seedling is perfectly correlated with the time of appearance of the procambium in the

cotyledon, the root and the hypocotyl, that is first in the cotyledon and root and subsequently in the hypocotyl. Because of the acropetal method of development of xylem in the cotyledon, a large number of primary xylem elements during successive stages of ontogeny are seen to differentiate at the base of the cotyledon.

Swamy and Krishnamurthy (1980) reviewed the relationship between the procambium and vascular cambium and suggested that procambium is not a pre-requisite for the formation of vascular cambium. And described the ontogeny of vascular cambium in dicotyledonous plants. According to them, procambium and vascular cambium are different and vascular cambium is not directly originated from procambium. Cambium is found to be originated from parenchyma. The procambium is to be converted into parenchyma which in turn give rise to the cambium.

According to Larson (1982), Iqbal and Ghouse (1990) all cells of the central core of the developing embryo never become transformed in to the procambium. This will be evident after comparing the cytology and biochemistry of cells of the central core with the characteristics of the procambium that differentiates from it.

Vascular differentiation is directly related to hormonal status of plants (Naylor, 1984). According to him terminal meristem and leaf primordia determine the pattern of the vascular structure to be found and since young leaves and apices are sources of auxins and the main plant hormone involved in vascular bundle induction and differentiation is auxin.

According to Fahn (1982) the structure of transition between the conducting systems of root and stem are given in detail, but the transition between the conducting system of the root and the conducting system serving the cotyledons and shoot above them are not studied so far.

Based on the study of serial section of seedlings in which primary vascular tissue is developed, it was concluded that separate strands of phloem twist and that their orientation is inverted during their passage through the hypocotyl and into that part of the stem above the cotyledons (Fahn, 1982). According to him, however, these conclusion have not been confirmed by more recent ontogenetic studies

Krishnamurthy (1994) suggested that procambialisation of the embryo is noticed earliest in the first appendicular structure, the cotyledon. The linear derivatives of sub apical groups of initials form the locus of differentiation of the median trace of the cotyledon. Developmental studies on procambialisation of cotyledons, hypocotyl and root show that cotyledonary procambium has no connection with that of root or of the hypocotyl. According to Krishnamurthy (1994) the information is deficient even on differentiation of vascular tissues from procambium of the embryo. The differentiation pattern of phloem and xylem from the procambial cells has been studied only in a few species and it varies widely even within the few taxa studied.

Lateral bud growth and its control by the terminal apex is described in term of apical dominance. The term apical dominance expresses the fact that the shoot's mode of growth is regulated by an intact terminal bud. If apical dominance is lost, by removal of the terminal bud

(decapitation), the axillary buds which were resting, sprout. This process is related to a massive change in gene expression (Hillman, 1984). The sprouting axillary bud quickly adapts its protein pattern to that of terminal bud. Rubinstein and Nagao (1976) stated that in *Pisum sativum* the outgrowth of lateral bud occurs as soon as 6 to 10 hours after decapitation of the apex and the apical dominance is due to in sufficient vascular connection between lateral bud and the stem. According Fletcher and Dale (1974) measurable growth is observed by laterals many hours before the evident vascular connections.

Decapitation of young tissues or cotyledons (Sebanek, 1972) revealed the lateral buds outgrowth in peas. However, Peterson and Fletcher (1975) found that the cotyledons are essential for the bud outgrowth in soybean and 1% sucrose could act as a substitute at least for a short period.

Apical dominance was correlated in the nutrients such as nitrogen, phosphorous and potassium distribution in *Phaseolus vulgaris* (Phillips, 1968). According to him the theory and evidence that hormone directed nutrient transport may play a role in correlative bud inhibition ie, an axillary bud does not grow when the main shoot apex is present, because it is starved for either mineral nutrients or carbohydrate.

Metabolite movements in plants in relation to axillary bud growth and vascular development are not studied so far in detail. However, carbohydrate movement in pea plants in relation to axillary bud growth and vascular development was studied by Wardlaw, (1968), Wardlaw and Mortimer (1970). According to these authors, the link between the

axillary bud and the stem is poorly developed, so rapid movement of carbohydrate is restricted. Nevertheless, the growth of lateral buds in intact pea plants was not primarily inhibited by the incomplete vascular connection between the stem and the bud. But it is likely that vascular development is a consequence of increased bud growth which results in a suitable concentration of substrate and hormone levels.

Peterson and Fletcher (1975) studied the lateral buds growth of cotyledonary node of soybean and stated that lateral buds are connected to the vascular system of the plant by functional xylem and phloem, so it is difficult to say why the lateral buds should lack for nutrients or growth regulations any more than the apex, except that the physiological status of the bud is such that it is not growing and therefore does not provide a sink which would result in the import of nutrients.

Studies on early events associated with lateral bud growth in *Pisum sativum* (Nagao and Rubinstein, 1976) revealed that bud out growth after apex removal may be considered as release from inhibitory conditions and the phenomenon may be related to auxin metabolism. Phillips (1975), and Rubinstein and Nagao (1976) are also of the same view.

Shibata *et al.* (1974) found that removal of cotyledons from intact lettuce seedlings retarded gibberellic acid induced hypocotyl elongation. During germination in *Phaseolus vulgaris* the cotyledons exhibited active abscisic acid catabolism and these changes are implicated in the control of apical dominance (Van Onckelen *et al.*, 1981)

Influence of cotyledons in axillary and adventitious shoot production from cotyledonary nodes of *Cucumis sativus* was studied by

Gambley and Dodd (1991) and stated that cotyledons of seedlings play an important role in the growth by supplying organic substrates. Apart from their role in nutrition, cotyledons have also been shown to have a regulatory role in the growth of the seedlings

Morpho histochemical studies of the shoot apex of *Phlox drummondii* have been done by Goyal and Pillai (1986). Histochemical localisation DNA, RNA and total proteins in the cells of shoot apex have been carried out. The plumular apex shows a tunica – corpus organisation. The first evidence of zonation occurs in the shoot apex of a 3 day old seedling. Zonation is well established on the 5th day after seed wetting. The shoot apex showed a single layered tunica covering a lightly stained central mother cell zone, a subjacent pith meristem and a densely stained peripheral zone.

Studies on the laterals from cotyledonary axils in winged bean have been made by Madhusudanan and Padmakumar (1990). Epicotyl excision in field grown winged bean seedlings led to the development and firm establishment of laterals from the cotyledonary axil. Field establishment of laterals took place also from propagules made up of decapitated etiolated seedlings and from etiolated lateral bud out growth from decapitated seedlings.

In *Ginkgo biloba* Tredici and Peter (1992) observed natural regeneration from down ward growing cotyledonary buds. These aggregates of suppressed buds originates from superficial meristems located in the cotyledonary axils of all *Ginkgo* seedlings as part of their normal ontogeny. Within six weeks of germination these buds became

embedded in the cortex of stem and their subsequent growth and development occurs below the surface of the bark. When stimulated by damaging the seedling axis of these embedded cotyledonary buds grows down from the trunk to form a woody rhizome like basal chichi which give rise to aerial shoot and adventitious root.

MATERIALS AND METHODS

Plant material

Seeds of winged bean (*Psophocarpus tetragonolobus* (L.) DC), cultivar 'PT -2', were collected from Kerala Agricultural University, Mannuthy, Thrissur. Seed multiplication was done by planting some seeds in the botanical garden of Calicut University. So freshly harvested seeds were available for the investigation.

Germination Studies

Healthy seeds were surface sterilized with 0.1% (w/v) mercuric chloride solution and washed in distilled water thoroughly before they were put for germination.

Germination studies were carried out in sterilized Petri dishes lined with filter papers and moistened with distilled water. Sterilized seeds were uniformly scarified by making a small incision on the testa, and placed 20 numbers in each Petri dish and kept for germination in dark at room temperature.

General Procedure

1) Plumule excision

Sprouted seedlings were taken on the 5th day and the plumules were excised with sterilized sharp knife above the epicotyl leaving a short stump and these seedlings were again placed in the Petri dishes lined with moistened filter paper in darkness for further development.

Ten intact seedlings were considered as the control and immediately processed for histochemical studies. These seedlings were trimmed in such a way that the cotyledons and the embryonic axis with the plumule stump and radicle were intact. These were fixed in FAA after trimming.

2) Sampling

Sampling of experimental and control were done at an interval of 12, 24, 48 and 72 hours after plumule excision. After 12 hours of excision some seedlings were taken out from control and experimental (the experimental seedlings did not show any visible cotyledonary axillary growth) and were trimmed and fixed in FAA. Similarly control after experimental seedlings were sampled at 24, 48 and 72 hours after plumule excision. Dry seeds were used as the actual control for germinated seeds and these seeds also were trimmed and fixed after decoating for histochemical studies.

I. HISTOCHEMICAL STUDIES

The fixed tissues were washed thoroughly and dehydrated through TBA-Alcohol series. After paraffin infiltration, tissues were embedded in blocks and individual blocks were cut at 10μ using a rotary microtome and the sections were stained for the following studies.

a) Localization of Insoluble Polysaccharides

Insoluble polysaccharides localization was done using periodic acid-Schiff's reagent as described by Berlyn and Miksche (1976). The method available for the localization of the total polysaccharide compliment of the cell is an excellent histochemical procedure. The basis of the reaction is the production of aldehydes by the action of an oxidative agent on the polysaccharides. The aldehydes then react with leucofuchsin (Schiff's reagent) producing highly coloured complexes. The most commonly used oxidant is periodic acid.

After deparaffinisation the slides were placed in 0.5% (w/v) periodic acid solution in distilled water at 24°C temperature for 15 minutes and washed thoroughly in running water. Then the slides were stained in Schiff's reagent for 10 to 15 minutes. The sections were rinsed in water and placed in 2% sodium bi sulphite for 1 - 2 minutes and washed in running tap water for 5 - 10 minutes. The polysaccharides were stained in an intense purplish red or magenta colour. The cytoplasm remained colourless. The structure of the cell wall were seen clearly. Starch reacted with the stain very strongly. The slides were mounted in DPX after dehydration in alcohol and clearing in xylem.

Preparation of Schiff's reagent

Five hundred milligram of basic fuchsin and 0.5 gm of potassium metabisulphite were dissolved in 100ml of 0.15 N HCl; The mixture was shaken for 2 - 3 hours until dye was converted to fuchsin - sulfurous acid. Three hundred milligram of fresh decolourising (activated) charcoal was added and the mixture was shaken for 5 minutes and filtered through filter paper.

b) Total Proteins

Mazia *et al.* (1953) introduced mercuric bromophenol blue method of staining for total proteins. This method was used in the present study as described by Berlyn and Miksche (1976). After deparaffinization the material was immersed for 15 minutes in the bromophenol blue solution and it was rinsed for 20 minutes in 0.5% acetic acid. Material was treated in Sorensen's buffer at pH 6.5 for 3 minutes to form blue colouration. After dehydration and clearing in alcohol and xylene respectively, the sections were mounted in DPX.

Preparation of bromophenol blue stain

Ten grams of mercuric chloride and 100mg of bromophenol blue were dissolved in 100ml of distilled water to obtain the stain.

c) Lipids

Lipids are difficult substances to work histochemically. The organic solvents removes lipid from the tissue (Jensen, 1962). The standard histological procedures are all based on the use of organic

solvents. Thus the fact that lipids are soluble in organic solvents rules out the use of paraffin unless the lipids have been rendered insoluble to organic solvents by fixation.

The easiest, safest and most widely used method of obtaining sections for lipid localization is simply to section the fresh material. Sudan IV stains fats, oils, waxes and free fatty acids blue to black. Lipid localization was done according to Krishnamurthy (1988).

Fresh sections were taken and placed in 50% ethyl alcohol for a few minutes. Sections were stained in a saturated and filtered solution of SudanIV in 70% ethyl alcohol for 5-20 minutes. It is then differentiated in 50% ethyl alcohol for 1 minute and mounted in glycerin-gelatin.

All the prepared slides were observed using Leitz binocular research microscope. Drawings were made wherever necessary using Camera lucida. Photomicrographs were taken by using Leica Camera fitted to Leitz microscope.

II. BIOCHEMICAL STUDIES

Sampling

Sampling was done as mentioned under Histochemical Studies. However, samples of cotyledons, plumule and radicle were separately taken for the analysis.

Methods

(a) Dry weight

The fresh weight of the seed samples were taken in pre-weighed containers. The samples were dried in a hot air oven at 100°C for one hour and kept at 60°C until constant weight was obtained. It gave the dry weight of the tissue and dry weight percentage was calculated on the basis of fresh weight.

(b) Protein

The method of Lowry *et al.* (1951) was followed to estimate the total protein, as modified by Khanna *et al.* (1969) in trichloroacetic acid precipitates which had been specially treated to remove contaminants.

Two hundred mg of the tissue was ground in water. The suspension was mixed with an equal volume of 10% (w/v) trichloroacetic acid to give a final concentration of 5% (w/v) of the precipitant and the precipitate was allowed to flocculate for 30 minutes in an ice bath. The protein precipitate was collected by centrifugation for 20 minutes and the supernatant decanted off. The residue was washed twice with cold 2% trichloroacetic acid (w/v), followed by three washings with 80% (v/v) acetone and then two washings with anhydrous acetone, for removing the pigments. The final residue was suspended in 10 ml 0.1N NaOH and heated in a bath of boiling water for five minutes. The resulting suspention was clarified by centrifugation. The residue was suspended in small volume of 0.1N NaOH and centrifuged and the process repeated. The three extracts were combined,

aliquotes were taken and after suitable dilution colour was developed with Folin-Ciocalteau reagent. Bovine serum albumin fraction V powder, procured from Sigma chemical company, was used as standard.

(c) Ethanol soluble sugars

Preparation of extract: A weighed out quantity of 2g. of tissue were ground with 80% (v/v) ethanol. A steam bath was used to reflux the suspension for four hours and filtered through Buchner funnel under suction. The residue was ground with 80% (v/v) ethanol and again refluxed for 2 hours and filtered as before. The two extracts were combined and evaporated to dryness at 60°C. The residue was taken up in 2ml. water and centrifuged to yield a clear golden yellow solution. This sample was directly used for sugar estimation according to Somogyi (1945). After appropriate dilution, colour development was done by adding Somogyi's alkaline copper reagent and Arsenomolybdate reagent. Optical density was read at 540 nm using Spectronic 21 (Bausch and Lomb) spectrophotometer.

(d) Starch

The method of Pucher *et al.* (1948) described by Whelan (1955) was used to estimate the starch in fresh tissues. One gram of tissue was taken and made a suspension with water. The suspension was placed in a bath of boiling water for 15minutes to gelatinise the starch. After cooling to room temperature, perchloric acid was added to a final concentration of 30% (v/v). The suspension was centrifuged for 15minutes and the supernatent collected by decantation. The residue was

extracted four times by suspending with the aid of a glass rod in 30% (v/v) perchloric acid and the various extracts were combined. Aliquotes of the combined extracts were precipitated with iodine-potassium iodide reagent. The starch iodine complex formed was collected on the centrifuge and washed with alcoholic sodium chloride to remove excess iodine, followed by treatment with alcoholic sodium hydroxide to remove bound iodine and again washed with alcoholic sodium chloride. The residual starch was estimated with phenol sulphuric acid reagent (Montgomery,1957), using soluble starch (E.Merck) as standard.

(d)Lipid

Lipid was estimated gravimetrically. Weighed samples about 2 – 3 g. were ground with pre –chilled diethyl ether. The homogenate was centrifuged and the supernatant was decanted to a pre-weighed container. The residue was again ground and re-extracted in diethyl ether. After centrifugation the supernatant was added to the first supernatant. This process was repeated six times to ensure complete extraction of lipids. The combined extract in the pre-weighed container was kept in a hot air oven at 60°C to evaporate the ether. The container with the lipid was weighed after complete evaporation of ether. The lipid content was calculated as the difference in weight.

III. VASCULATURE OF COTYLEDONS

To study the development and differentiation pattern of the vasculature in the cotyledon at various stages of seedling growth under different nutrient conditions, the following procedure was adapted.

1. Vasculature of cotyledons in dry seeds and young seedlings

In order to study the vascular morphology, microtechnical clearing method was not used because of the bulky nature of cotyledons. So the vascular structure was reconstructed from drawings of serial cross sections of the entire cotyledon and counting the vascular bundles in each cross section. The vasculature of cotyledons of all samples used for histochemical studies, as described earlier, were reconstructed.

2. Vasculature of cotyledons in seedlings grown under different growth media

Seeds of winged bean were planted in two different media, garden soil and acid-washed sand.

Preparation of acid washed sand

River sand was washed thoroughly in running water and refluxed in con.HCl. for six hours .After thorough washing with distilled water the sand was dried and used as growth medium. The last washings were tested for the presence of chloride ions by treating with silver nitrate solution. The pH of the washings was also tested using pH meter to ensure the acid removal.

Cultivation

Twenty plastic pots each filled with garden soil and acid washed sand were used for cultivation. Six winged bean seeds each were planted in these pots. The pots were kept at open atmospheric conditions. Seedlings were watered with distilled water.

Sampling

Cotyledons were collected from the seedlings after germination at intervals of 6, 12 and 18 days. Freehand cross sections of the cotyledons were taken and number of vascular strands were noted after staining the sections with saffranin. Serial section were taken and temporary preparations were made. By observing the sections and counting the number of vascular bundles, the whole vascular system of the cotyledon was reconstructed and drawings were made.

Statistical Analyses of Data

All experiments were repeated a minimum of six times and the mean values are given in tables and figures. Standard deviation and Standard error are also calculated. The values given in tables and figures are the mean ± Standard error. Test of significance was done wherever required using Fisher's 't' test.

RESULTS

RESULTS

HISTOCHEMICAL STUDIES

1. PROTEIN

Dry Seed

a. Cotyledon

Sections of the mature seeds stained with mercuric bromophenol blue were observed to detect the protein content.

The epidermal cells of the cotyledon were very small, (Table 1) round/elongated in shape, partially filled with stained proteins (Plate 3A). Ground tissues of cotyledon showed variation in shape and size from region to region. Towards the abaxial side of the cotyledon, the cells were of different sizes (Table 1) but mostly isodiametric with intercellular spaces. The densely stained proteinaceous masses filling about ¾ part of each cell were located towards the central region of the cell (Plate 3A). The cellular details were not clearly seen because the densely stained protein masks the entire structure.

Towards the adaxial side of the cotyledon, also the epidermal cells were small and rectangular while hypodermal cells were smaller and isodiametric compared to cortex, where cells were bigger in size (Table 1). The densely stained protein mass was confined to the central part of

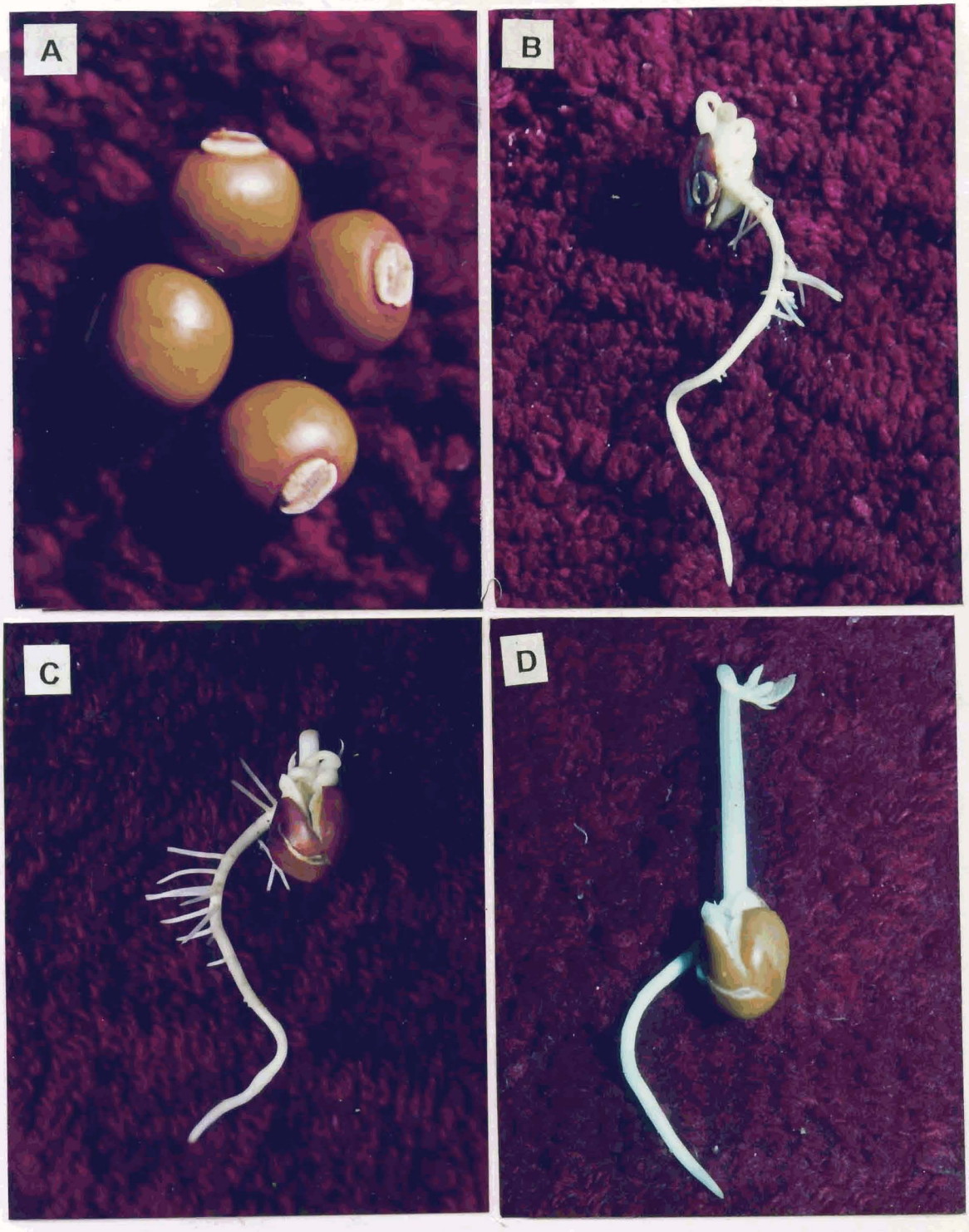


Plate-1. Morphology of winged bean seeds and seedlings

A. dry seeds **B.** germinated seeds (5 days)
C. experimental seedling after one day of plumule excision (5+1)
D. control seedling (5+1)

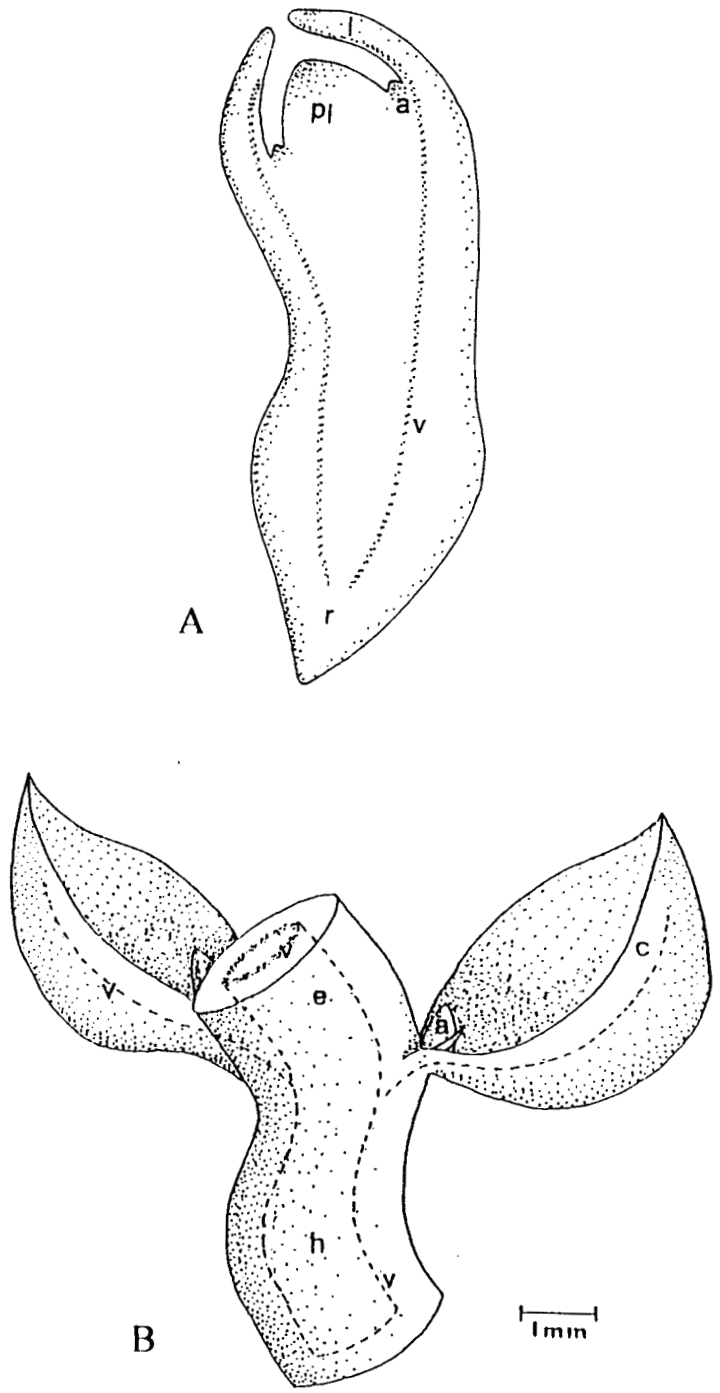


Fig.1. Semi diagramatic drawings of winged bean seeds.
A. Dry seed **B.** Germinated seed after five days

a. axillary bud; **c.** cotyledon; **e.** epicotyl; **h.** hypocotyl; **l.** leaf primordium; **p.** prophyll; **pl.** plumule; **r.** radicle; **v.**vasculature

Table 1. Distribution of cell types and starch grains in winged bean cotyledons during germination and seedling growth

Stage	Tissue type	Average cell area in μm^2	Average number of starch granules/cell	Size of starch granule in μm (diameter)
Dry seed	**Cotyledon**			
	Epidermis	135 ± 15	Nil	Nil
	Hypodermis	360 ± 30	Nil	Nil
	Cortex	1330 ± 110	Nil	Nil
5^{th} day seedling	**Cotyledon**			
	Epidermis	176 ± 20	Nil	Nil
	Hypodermis	803 ± 74	5 ± 1	4 ± 0.2
	Cortex abaxial	1890 ± 150	9 ± 1	4.5 ± 0.3
	Cortex adaxial	2461 ± 188	10 ± 2	4.5 ± 0.2
	Cells near vasculature	530 ± 48	15 ± 3	4 ± 0.1
5 + ½ day	**Cotyledon**			
	Epidermis	180 ± 14	Nil	Nil
		(190 ± 18)	(Nil)	(Nil)
	Hypodermis	706 ± 62	8 ± 1	2 ± 0.1
		(810 ± 38)	(13 ± 2)	(2 ± 0.1)
	Cortex abaxial	2289 ± 192	15 ± 3	4.5 ± 0.3
		(2248 ± 130)	(16 ± 2)	(4.5 ± 0.3)
	Cortex adaxial	2661 ± 151	20 ± 4	4.5 ± 0.2
		(2830 ± 146)	(18 ± 3)	(4.5 ± 0.3)
	Cells near vasculature	1256 ± 108	25 ± 5	4.5 ± 0.3
		(2018 ± 160)	(20 ± 4)	(4.5 ± 0.2)
5 + 1 days	Cotyledon			
	Epidermis	180 ± 12	Nil	Nil
		(184 ± 16)	(Nil)	(Nil)
	Hypodermis	706 ± 55	11 ± 2	6 ± 0.5
		(826 ± 74)	(10 ± 1)	(5.5 ± 0.4)
	Cortex abaxial	1917 ± 88	13 ± 3	7.5 ± 0.6
		(2150 ± 102)	(11 ± 1)	(6 ± 0.5)
	Cortex adaxial	2530 ± 52	20 ± 4	7 ± 0.5
		(2680 ± 130)	(18 ± 2)	(7 ± 0.6)
	Cells near vasculature	1230 ± 98	24 ± 4	7 ± 0.7
		(1620 ± 120)	(20 ± 2)	(7 ± 0.5)

Control values are given in parenthesis

PTO

Table I continued

	Cotyledon			
	Epidermis	190 ± 20	Nil	Nil
		(185 ± 11)	(Nil)	
	Hypodermis	1888 ± 204	8 ± 1	4.5 ± 0.4
		(1765 ± 160)	(10 ± 1)	(4.5 ± 0.4)
5 +2 days	Cortex abaxial	1962 ± 140	29 ± 2	4.5 ± 0.4
		(1820 ± 174)	(25 ±2)	(4.5 ± 0.4)
	Cortex adaxial	2856 ± 210	15 ± 1	5 ± 0.5
		(2908 ± 250)	(12 ± 1)	(5.5 ± 0.5)
	Cells near	2920 ± 170	30 ± 2	4.5 ± 0.4
	vasculature	(3010 ± 226)	(27 ± 3)	(4.5 ± 0.4)
	Cotyledon			
	Epidermis	200 ± 18	Nil	Nil
		(196 ± 18)	(Nil)	(Nil)
	Hypodermis	1152 ± 80	3 ± 1	3 ± 0.2
		(1260 ± 108)	(5 ± 1)	(3 ± 0.2)
5 + 3 day	Cortex abaxial	2050 ± 180	15 ± 1	4.5 ± 0.4
		(2890 ±204)	(18 ± 2)	(3 ± 0.3)
	Cortex adaxial	2652 ± 200	20 ± 2	6 ± 0.5
		(2680 ± 126)	(22 ± 2)	(4 ± 0.3)
	Cells near	2020 ± 98	10 ± 1	4 ± 0.3
	vasculature	(2420 ± 188)	(11 ± 1)	(4 ± 0.4)

Control values are given in parenthesis

the cell occupying almost ¾ part of the cell area. Throughout the cotyledonary cells protein staining intensity was very high (Plate 3B).

The provascular strands consisting of elongated cells with tapering ends in longitudinal sections were observed in the cotyledonary tissue. At certain regions provascular strands appeared as channels in longitudinal section (Plate 3C) while in certain other regions cluster of cells were found in cross sections. The cells of procambium were comparatively small and filled with fully stained protein matrix. In some cells nuclei were present.

b. Embryonic axis

The embryonic axis of the mature seed contained distinct regions of plumule and radicle which consisted of densely stained cells with conspicuous nuclei (Plate 3D, E, F). The cells of the plumular apical meristems were small and filled with protein. The provascular strands can be seen and their staining intensity was higher compared to the ground tissue (Plate 3D). At the plumular tip, the developing primary leaves were seen consisting of isodiametric cells with conspicuous nuclei. The radicle tip showed typical cellular details of root apex (Plate 3F). The radicle showed different distinct tissue zones (Plate 3E), i.e. calyptrogen, dermatogen, periblem and plerome. Provascular region was more stained than the other cells. Cells of the pith region were comparatively larger than the cortical and epidermal cells. Cells were found in longitudinal rows. But cellular contents and structure were masked by the intense staining.

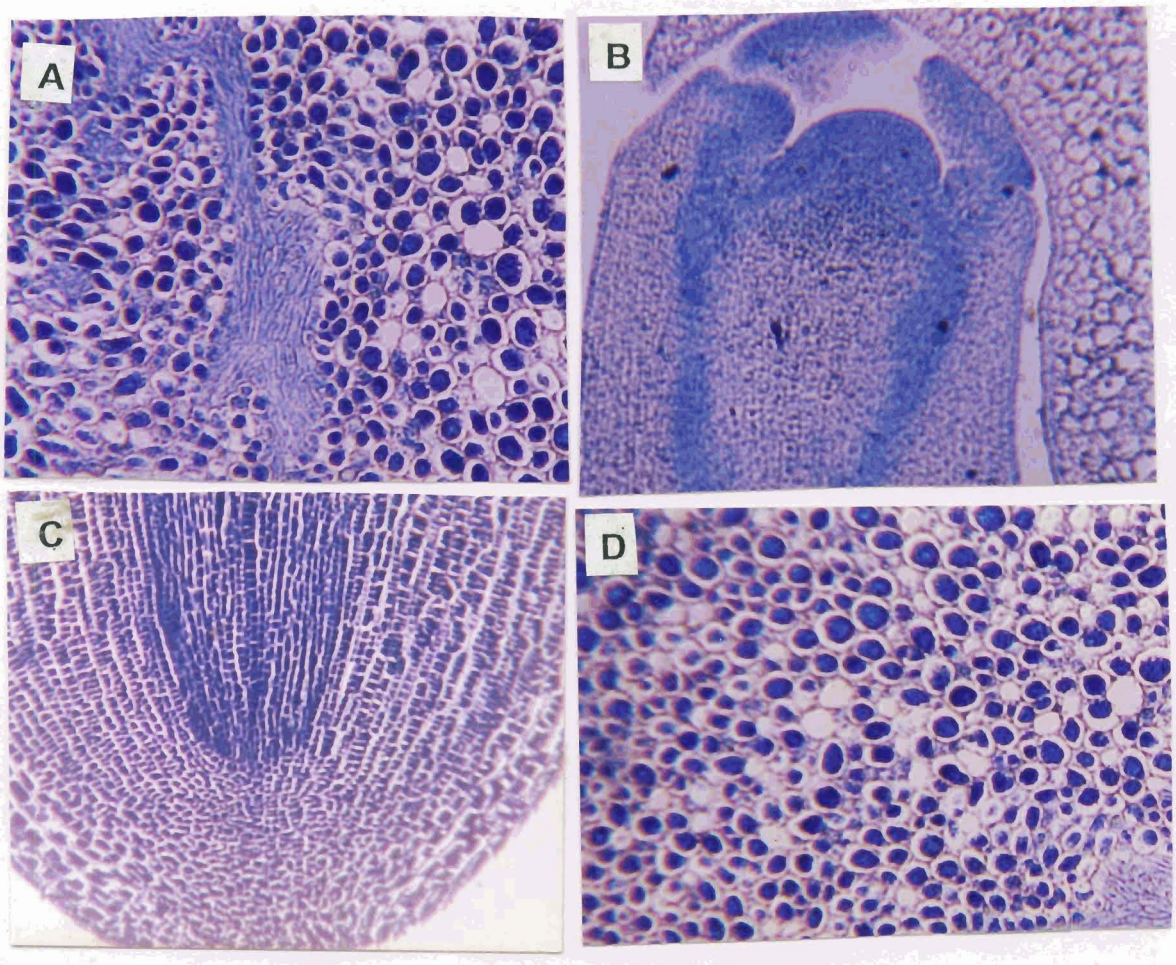


Plate- 3 Protein distribution in dry seeds of winged bean.

A. Abaxial side of cotyledon B. Adaxial side
C. Cotyledonary portion showing vasculature
D. Plumular apex E. Radicle apex
F. Embryonic axis portion showing axillary bud

ax. axillary bud **d** dermatogen **pf** primary leaf primordia. **p** periblem
pl pleurome **v** vasculature

The cells of procambial cells in the plumular as well as radicle region were small thin walled and meristematic, undergoing differentiation into xylem and phloem. Pith cells were parenchymatous elongated in shape and were incompletely filled with densely stained protein masses (Plate 3D, E).

An interesting observation in winged bean seed was the presence of an axillary meristem situated in the axil of each cotyledon (Plate 3F). This meristem consisted of a mass of almost isodiametric undifferentiated cells with lightly stained granular protein. Nuclei and nucleoli of the cells were clearly seen. However, this meristem did not show shoot apical organisation into tunica or corpus (Plate 3F).

5th day seedling

a. Cotyledon

Proteins localized by bromophenol blue stained in the cotyledonary cells showed a granular appearance but the intensity of staining was very dense, whereas the dry seed protein appeared as dense mass. The abaxial side of the cotyledon contained more protein bodies than the adaxial sides and cells were smaller at abaxial side than the adaxial side. The degradation of protein bodies took place first from the adaxial side as well as from the cells around the vascular strands. Generally cells were larger compared to the dry seeds and appearance of the protein content showed variation from region to region (Plate 4A). The abaxial side of the cotyledon had epidermis with rectangular and square shaped cells and densely stained for proteins. The hypodermal cells of the cotyledon were made up of large parenchymatous cells with densely stained protein

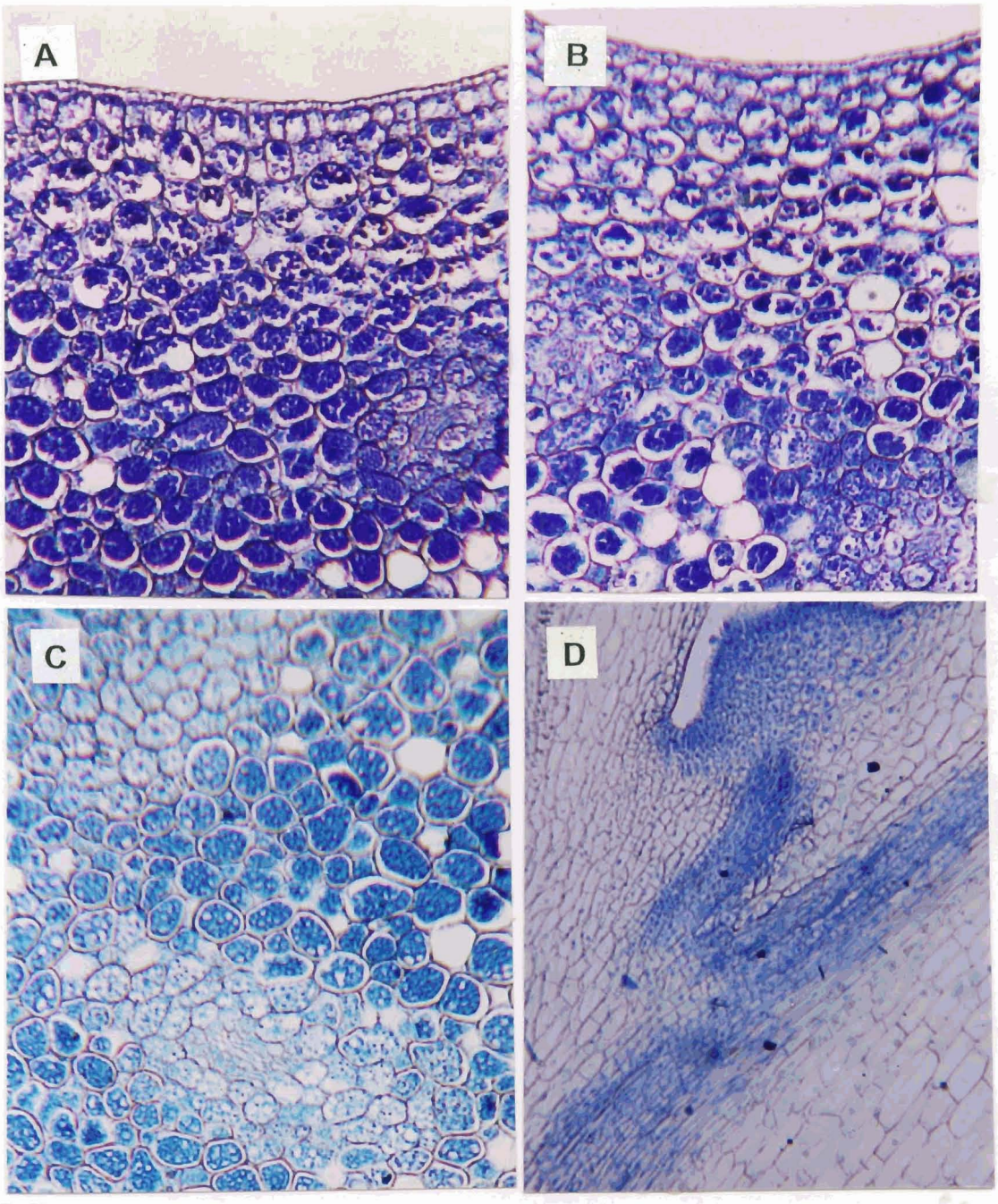


Plate-4 Protein distribution in seedlings of winged bean after 5 days of germination

A. abaxial side of the cotyledon **B.** adaxial side of the cotyledon
C. vasculature of the cotyledon D.Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

bodies which became granular compared to the central cells, where the parenchymatous cells contain more densely stained but less granular protein bodies (Plate 4A).

The adaxial side of the cotyledon consisted of large loosely arranged isodiametric or elongated cells. The density of protein mass which appeared as loosened granules, was less at the adaxial side compared to that of the abaxial side as well as the central region (Plate 4B).

Procambial strands consisting of narrow elongated cells (in longitudinal section) contained feebly stained protein. The cotyledonary cells in the vicinity of these vascular strands showed difference in the distribution of granular protein bodies in comparison with that of distant cells in both longitudinal section and cross section of provascular strand (Plate 4C, D). The protein bodies became comparatively smaller and lesser in number in cotyledonary cells nearest to the procambial cells, while that of the cotyledonary cells away from the vascular strands showed bigger protein granules clustered together (Plate 4B, C). About 75% of the cell area was filled with protein. However, the empty spaces where protein was not localized were observed in both types of cells. In cotyledonary cells near the procambial strands, disintegrating protein granules were found occasionally (Plate 4B, C). Outer envelope of these protein bodies were clearly seen retaining a few smaller granules like protein bodies inside. A gradation of disappearance / dissolution of the protein bodies gradually from the cotyledonary cells can be observed near the procambial strands. Further studies on the embryonic axis was not included in the histochemical studies due to the bulk growth of the

plumule and radicle. However, the development of cotyledonary axillary bud was included because the growth of this bud mainly depends on the plumule excision as a result of apical dominance manifestation.

b) Cotyledonary axillary bud

Even though the plumule excision has been done to accelerate the cotyledonary axillary bud growth, the control itself i.e. the seedling where the excision was done on 5th day, showed initiation of cotyledonary axillary bud development. The axillary meristem which appeared only as a cluster of meristematic cells in the dry seed, showed differentiation into a shoot apex consisting of well formed epidermal layer and meristematic ground cells (Plate 4D). It was composed of almost angular isodiametric cells with conspicuous nuclei and densely stained protein compared to adjacent cotyledonary or axial ground tissue.

An important observation was the development of accessory buds, subtended by the cotyledon and / or central axis (Plate 4D). The accessory buds were made up of highly meristematic cells with dense protein content and conspicuous nucleus.

(5+½) Days' seedling (Experimental after 12 hrs of plumule excision)

a. Cotyledon

In the cross section the abaxial side of the cotyledon showed epidermis consisting of rectangular or square shaped cells with slightly stained protein. The cells of hypodermis and the cortex of the abaxial side contained cells of various size, almost rounded in shape with intercellular

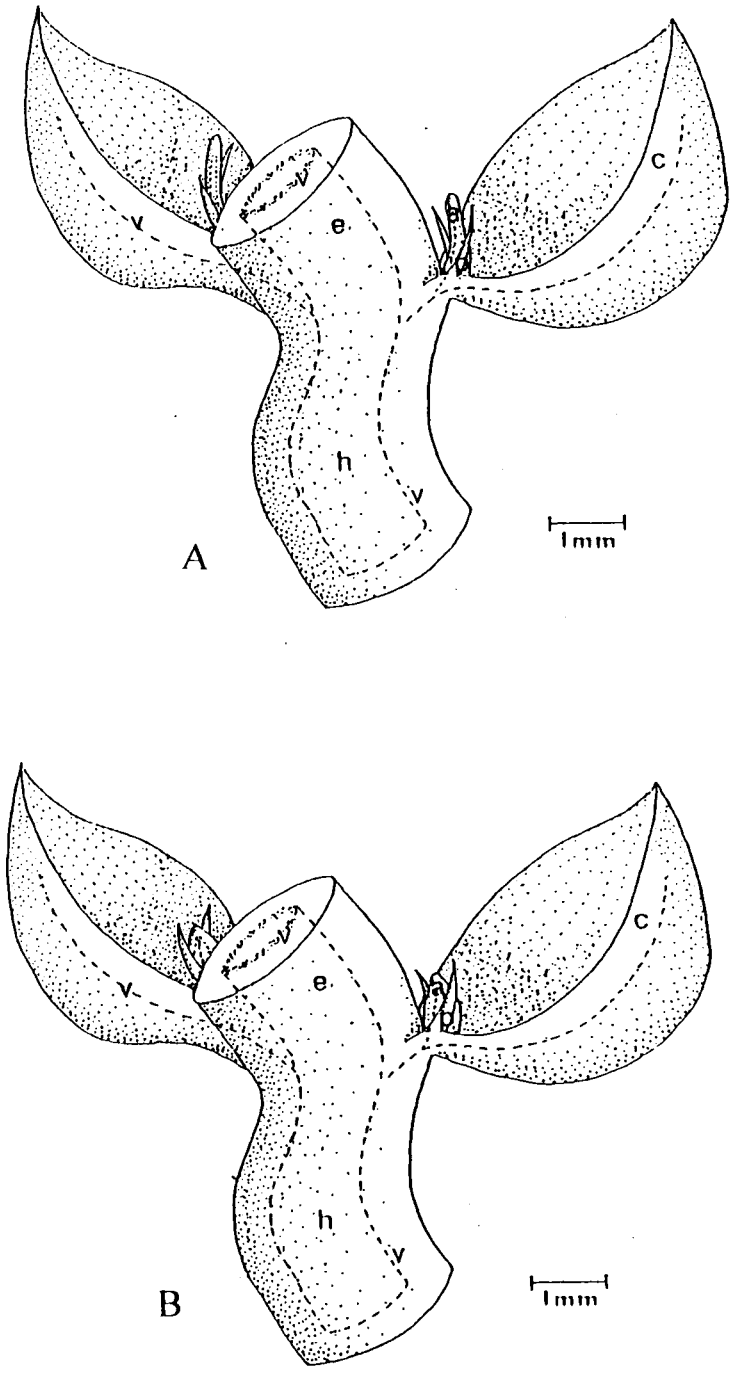


Fig.2. Semi diagramatic drawings of winged bean seedlings.

- **A.** Germinated seeds after ½ day of plumule excision (5 + ½ experimental)
- **B.** Germinated seed after 5½ days (5 + ½ control).

a. axillary bud; **c.** cotyledon; **e.** epicotyl; **h.** hypocotyl; **p.** prophyll; **v.** vasculature.

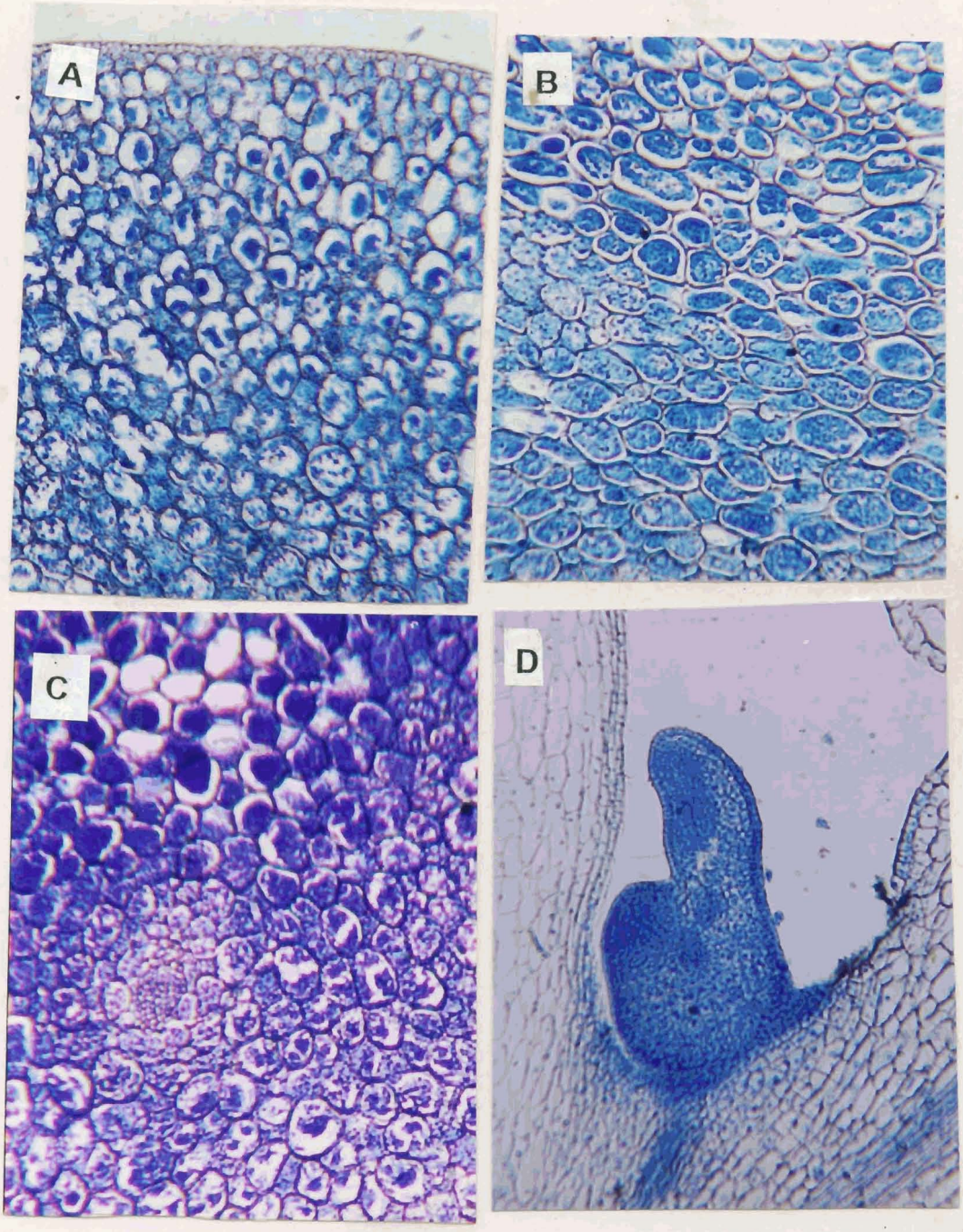


PLATE - 5 Protein distribution in seedlings of winged bean 12 hours after plumule excision (5+½ -experimental)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

spaces. In most of the cells proteins were found scattered and occasionally occur in clumps occupying most area of the cell (Plate 5A).

Epidermis of adaxial side was similar to that of abaxial side consisting of rectangular or square shaped cells with very lightly stained protein. The hypodermis and cortex were not distinguishable as all the cells of this region were irregular in shape and larger than those present in the abaxial side. The staining and distribution pattern of protein present in the cells of the adaxial side was different from that of the abaxial side. (Plate 5A, B) in some cells densely stained protein bodies occurred in clumps and occupied about ¾ area of the cells. While in many cells, the protein bodies appeared as scattered small and granular bodies with slightly lower staining compared to the other cells. Protein hydrolysis was found to be enhanced near the vascular strands compared to that of the cotyledon, on 5th day (Plate 4B, C).

b) Cotyledonary axillary bud

After 12 hrs of plumule excision, the cotyledonary axillary bud showed further development exhibiting differentiated shoot apex and leaf primordia and vascular strands (Plate 5D). The axillary plumule consisted of densely stained cells, the details of which were not seen due to denser staining intensity.

The vascular strands of the embryonic axis were found to be connected to the cotyledonary vascular strand. Cells of the vascular strands were elongated, narrow and with tapering ends. Xylem and phloem were not differentiated (Plate 5D).

(5+½) Days' Seedling (Control after 12 hours of plumule excision)

a. Cotyledon

Generally there was no difference in the anatomy and protein distribution in the cells of cotyledon in the control tissue compared to the experimental. The epidermal cells of the abaxial side of the cotyledon were small elongated rectangular with lightly stained protein (Plate 6A). The hypodermis and cortex of abaxial side were having cells larger than the epidermis which were of varying sizes but all were rounded/angular with intercellular space. About 80 to 90% of the area in most of the cells, were filled with densely stained protein, while in the adaxial side the intensity of protein was slightly lesser compared to abaxial side. (Plate 6B).

The cotyledonary cells showed degradation of protein in the cells near to the vascular strands. The cells of vascular strands were narrow elongated and found in a group of 4 to 6 cells in width and were not differentiated to xylem or phloem (Plate 6C).

b. Cotyledonary axillary bud

The axillary bud with a meristematic apex consisting of small isodiametric cells with conspicuous nuclei and were densely stained for protein. Compared to (5+½) experimental, the development of axillary shoot was not progressed. Accessory buds were not clearly seen in the section. The provascular tissue in the axillary bud was not much differentiated (Plate 6D).

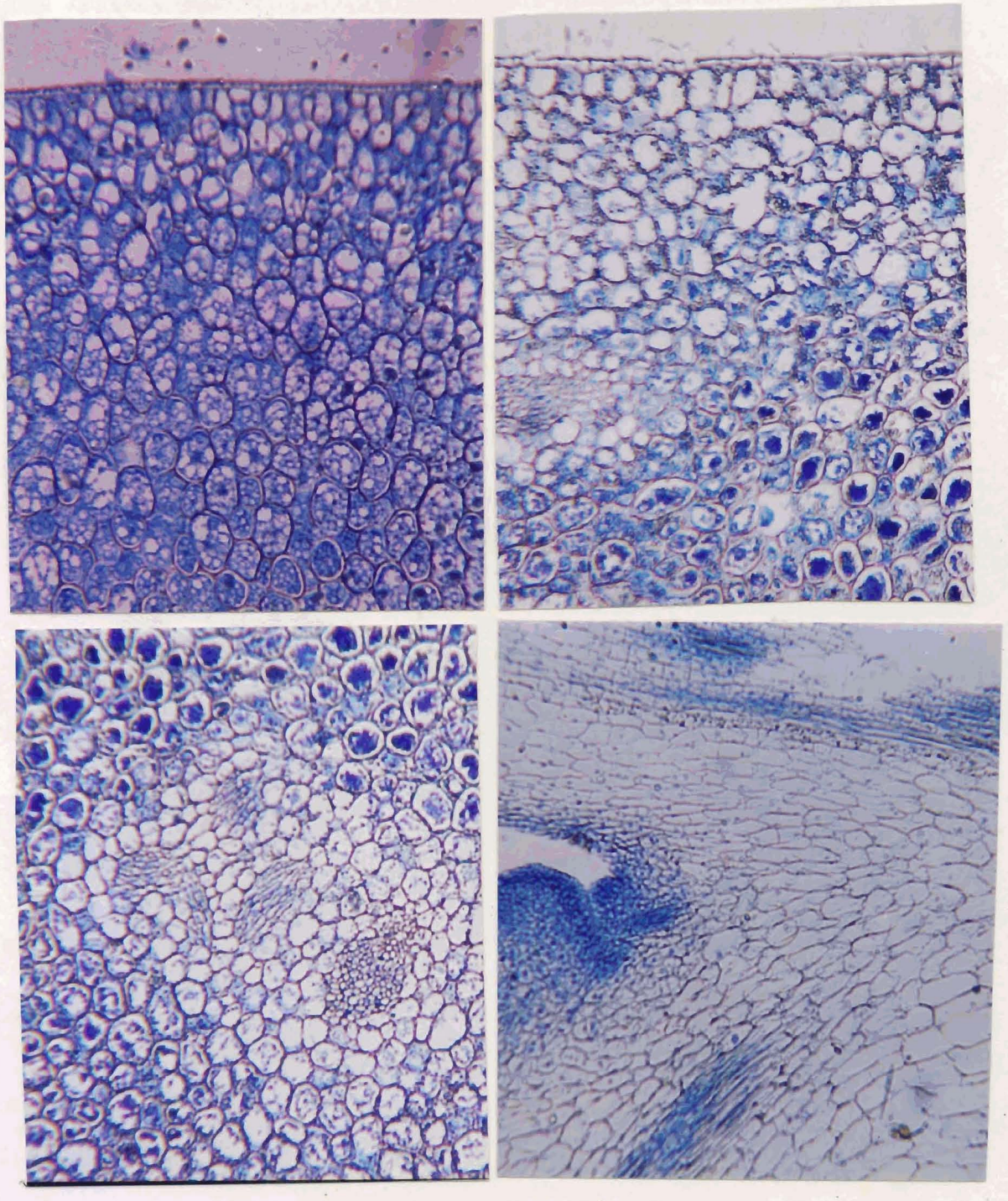


PLATE – 6. Protein distribution in the seedlings of winged bean after 5+½ days of germination (5+½ - control)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

(5+1)Days' seedling (Experimental 24 hours after plumule excision)

a. Cotyledon

Epidermal cells of the abaxial side were rectangular or square shaped and lightly stained for protein. The hypodermis and cortex were composed of cells with different sizes with intercellular spaces and were densely stained for protein. Clumps of protein were found in many of these cells (Plate 7A). Compared to the abaxial side, the adaxial side was having large polygonal and rounded cells with less dense protein mass (Plate 7B). Both abaxial and adaxial cells showed a gradation of protein staining intensity from the central region towards the periphery.

In the section of the cotyledon the procambial strands consisted of elongated (in longitudinal section) and rounded (in cross section) cells. Nuclei were seen in some cells only. The degradation pattern of proteins in the nearest neighbouring and distal cells of cotyledon exhibited considerable variation. Roughly 8-10 cotyledonary cells (diameter) distance around the procambial tissue shows degradation of protein bodies which were restricted to only the nearest cells in the 5^{th} day control cotyledons. At the same time the cells beyond this zone contained clumps of large protein bodies (Plate 7A, B).

Vascular differentiation was progressed in this stage (Plate 7A) when compared to the dry seed/5^{th} day seedling (control) in which only procambial undifferentiated cells were present as described earlier (Plate 3C, 4 B).

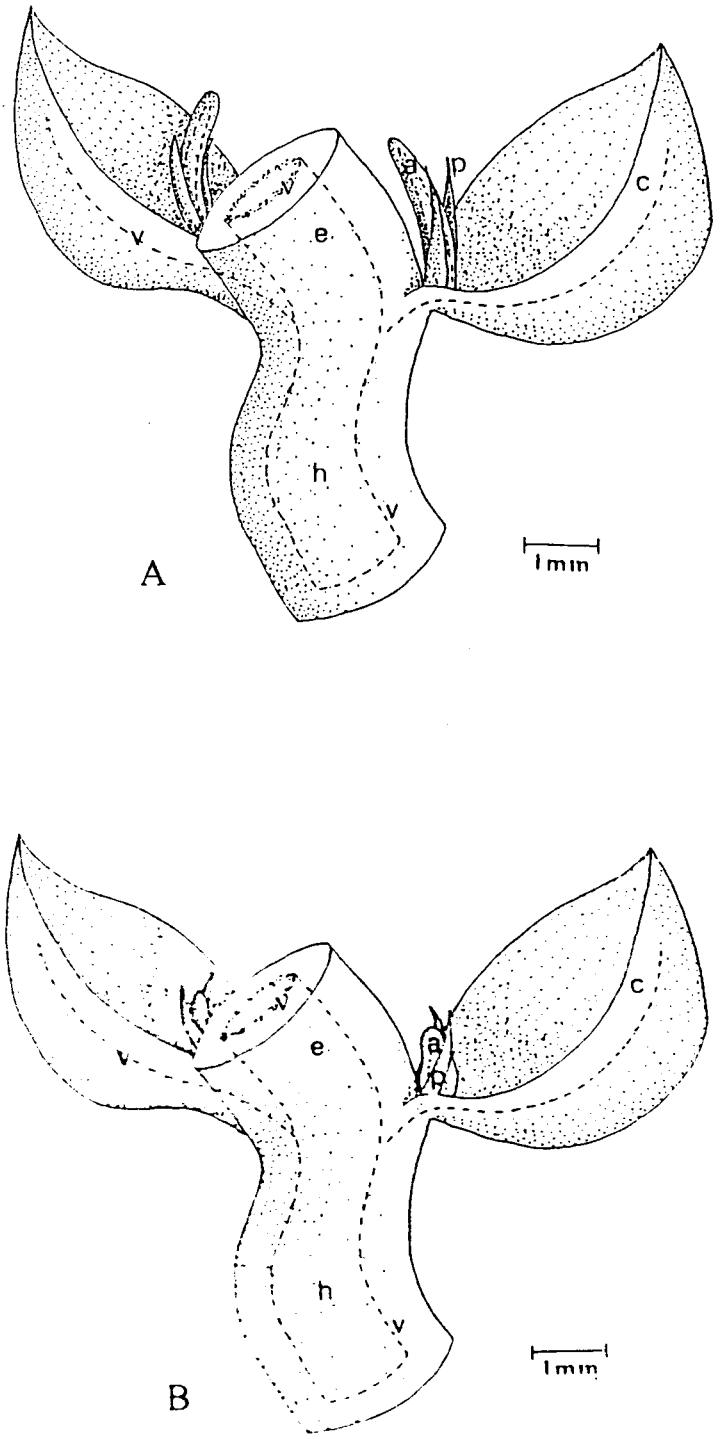


Fig.3. Semi diagramatic drawings of winged bean seeds.

A. Germinated seed after one day of plumule excision (5+1 experimental)

B. Germinated seed after six days (5+1 control)

a. axillary bud; **c.** cotyledon; **e.** epicotyl; **h.** hypocotyl; **p.** prophyll; **v.**vasculature.

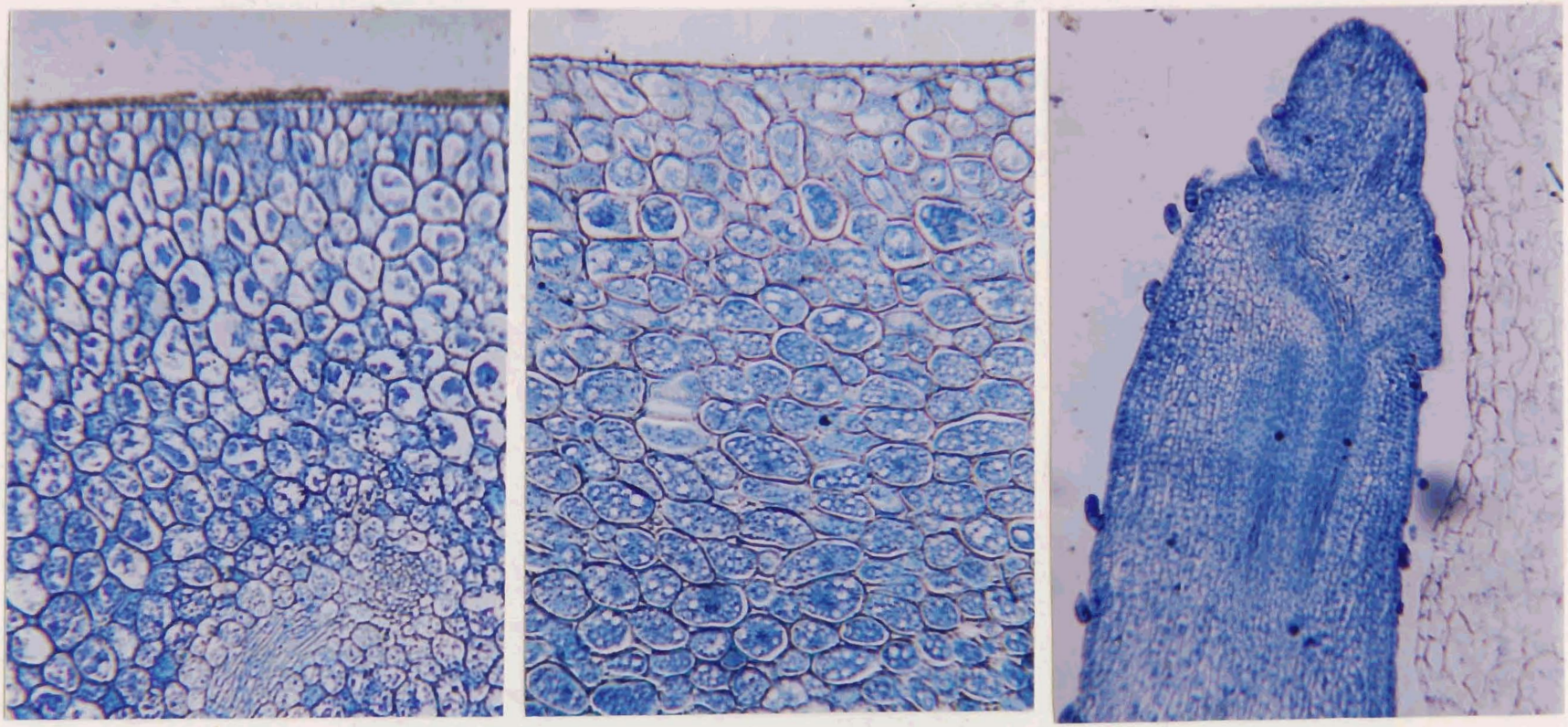


PLATE - 7 Protein distribution in seedlings of winged bean one day after plumule excision (5+1 -experimental)

A. abaxial side of the cotyledon showing vasculature
B. adaxial side of the cotyledon
C. Embryonic axis portion showing axiallary bud

ax. axillary bud **v** vasculature

b. Cotyledonary axillary bud

The axillary buds showed further growth compared to the (5+1/2) day experimental. The apical region of the axillary shoot consists of small isodiametric meristematic cells with nuclei which were densely stained (Plate 7C).

(5+1) Days' seedling (Control 24 hours after plumule excision)

a. Cotyledon

The epidermis consists of rectangular and square shaped cells. Very lightly stained protein mass was found inside the epidermal cells. The cells of hypodermis and cortex were more rounded compared to the previous stages and intercellular spaces were present. In most of the hypodermal cells and outer part of cortical cells of the abaxial side, the protein mass was found to be degraded to form granular structure, while at the central part of the cortex, the cells were having clumps of protein bodies (Plate 8A). Towards the adaxial side also protein masses were degraded in to granular form and were lightly stained (Plate 8B). The epidermis of adaxial side also consists of small rectangular or square shaped cells with very slightly stained protein.

The procambial strands were seen as cross section in the cotyledon (Plate 8C). It was composed of isodiametric small cells which were slightly stained for protein. Nuclei were seen in some cells. Longitudinal section of procambial strand was also found in certain regions, where the cells were elongated and narrow. Procambial cells were not differentiated

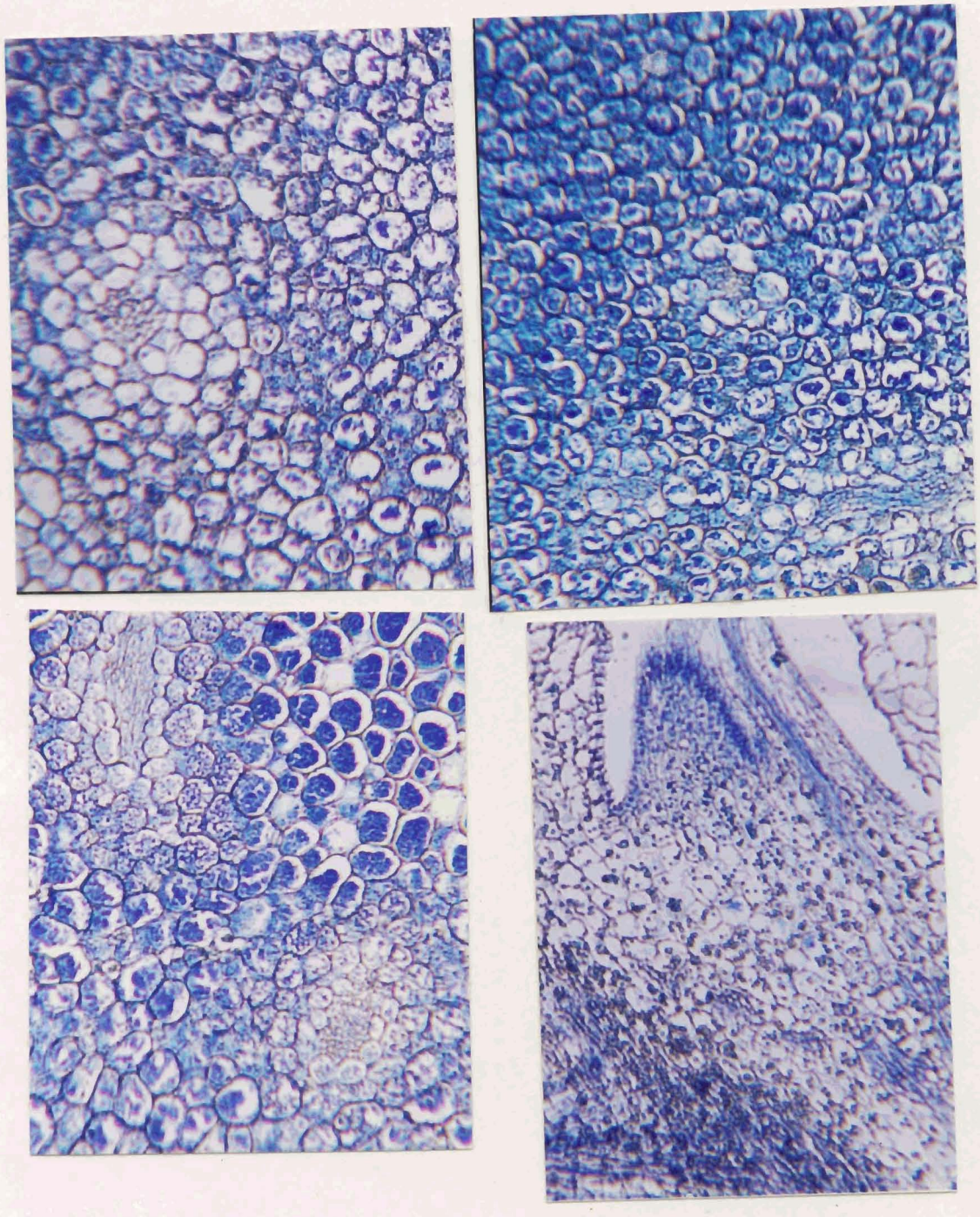


PLATE – 8. Protein distribution in the seedlings of winged bean after 6 days of germination (5+1 - control)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

into xylem or phloem. Degradation of protein was found to be high in the parenchymatous cells nearest to the procambial cells (Plate 8A, B, C).

b. Cotyledonary axillary bud

The cotyledonary axillary bud in the control appeared as the differentiating axillary bud subtended by a prophyll. Vascular differentiation was initiated in this bud (Plate 8D). Prophylls were present in the axillary position near the bud (Plate 8D). At the tip of the axillary plumule isodiametric, densely stained angular cells with prominent nuclei were seen. The rest of the cells were rectangular parenchymatous with prominent nuclei. Vascular strands were seen extending into the axillary buds.

(5+2) Days' Seedling (Experimental 2 days after plumule excision)

a. Cotyledon

The epidermis consisted of rectangular small parenchyma cells lightly stained for protein (Plate 9A). The hypodermis contained protein bodies but comparatively in less amount than that of cortex. Most of the area of the cells of the cortex was filled with granular protein mass. A degradation in protein body was found in many cells (Plate 9A)

In the adaxial side, the small rectangular epidermis contained only negligible amount of protein. The large hypodermal and cortical cells of the adaxial side contained disintegrating protein bodies. The hypodermal cells of the adaxial side consisted of large irregularly shaped parenchyma cells with inter cellular spaces and these cells contain slightly stained protein inside. (Plate 9B)

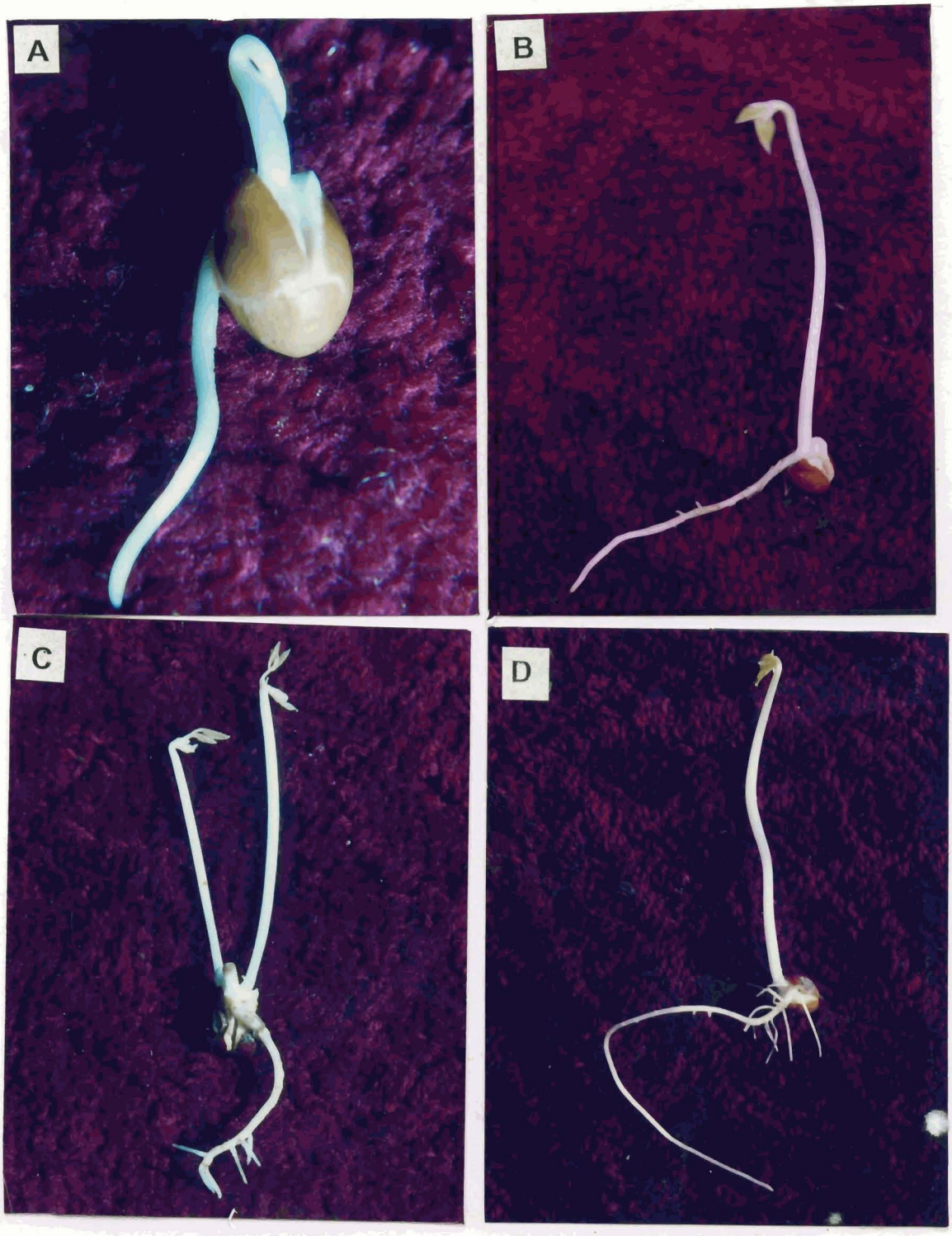
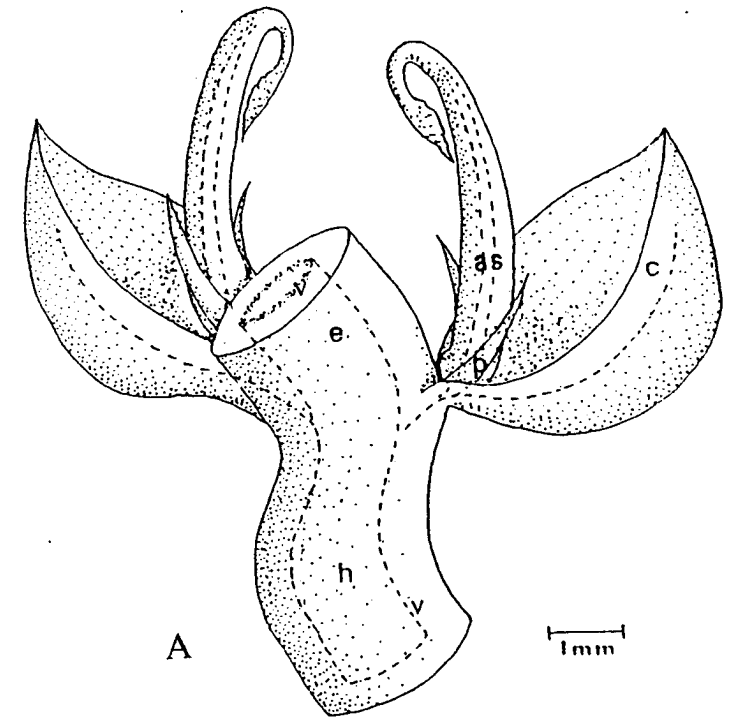


Plate-2. Morphology of winged bean seedlings after plumule excision

A. Experimental seedling after two days of plumule excision (5+2)
B. Control seedling (5+2)
C. Experimental seedling after three days of plumule excision(5+3)
D. Control seedling (5+3)



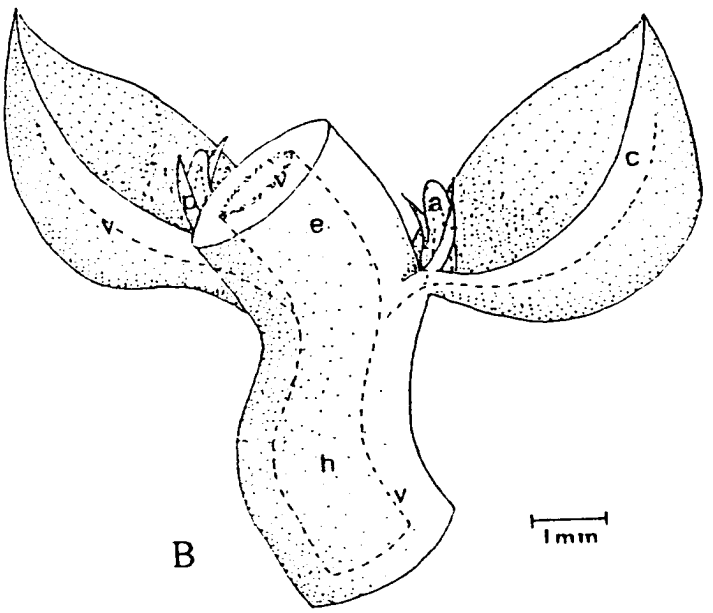


Fig.4. Semi diagramatic drawings of winged bean seeds.

A. Germinated seed after two days of plumule excision (5+2 experimental)

B. Germinated seed after seven days (5+2 control)

a. axillary bud; **c.** cotyledon; **e.** epicotyl; **h.** hypocotyl; **p.** prophyll; **v.**vasculature.

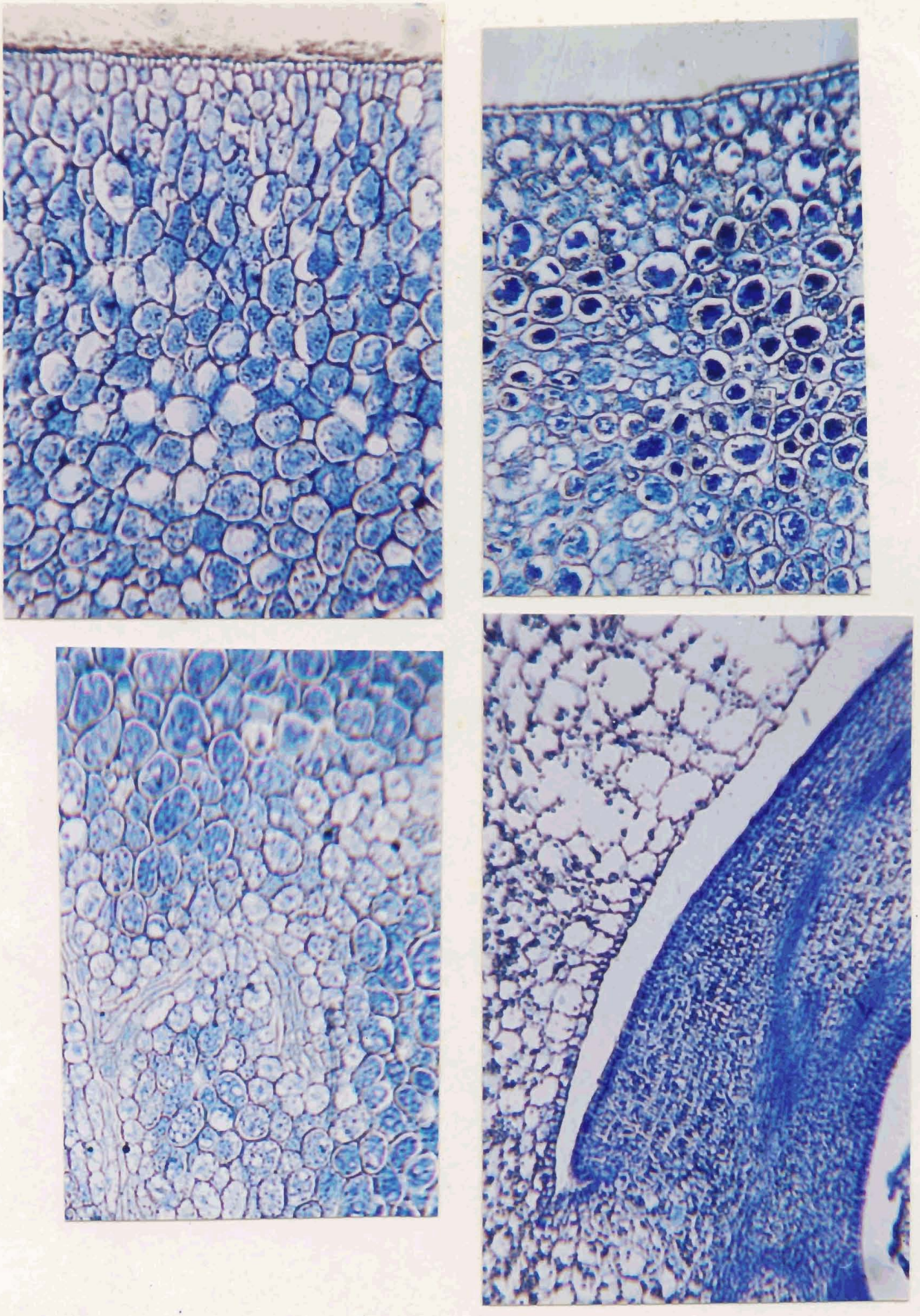


PLATE - 9 Protein distribution in seedlings of winged bean two days after plumule excision (5+2 -experimental)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

The vascular strands were found to be differentiated than the previous stages. The spiral or scalariform thickening of the xylem elements were clearly seen (Plate 9B, C). The vascular strands contained narrow elongated cells with prominent nuclei in some cells. Degradation of protein bodies in the cotyledonary cells become more pronounced and cells nearest to the vasculature were devoid of protein content (Plate 9C).

b. Cotyledonary axillary bud

Two days after plumule excision the axillary shoot were grown up consisting of epidermis, cortex, differentiated vascular strands and pith region. Most of the cells of epidermis, cortex and pith were nucleate. Nucleoli were also seen (Plate 9D).

The accessory bud present in the axil of the axillary bud and main axis was not further developed. The accessory bud consisted of small isodiametric parenchyma cells with prominent nuclei and densely stained for protein. (Plate 9D)

(5+2) Days' Seedling (Control 2 days after plumule excision)

a. Cotyledon

The epidermis of the abaxial side of cotyledon after 7 days of germination showed no difference compared to the previous stages. The hypodermis was with elongated rounded or isodiametric cells with intercellular spaces. The protein bodies in most of these cells were degraded and appeared granular. Many vacuoles were appeared inside the cell. The central region of the cortex contained large cells with granular protein (Plate 10A).

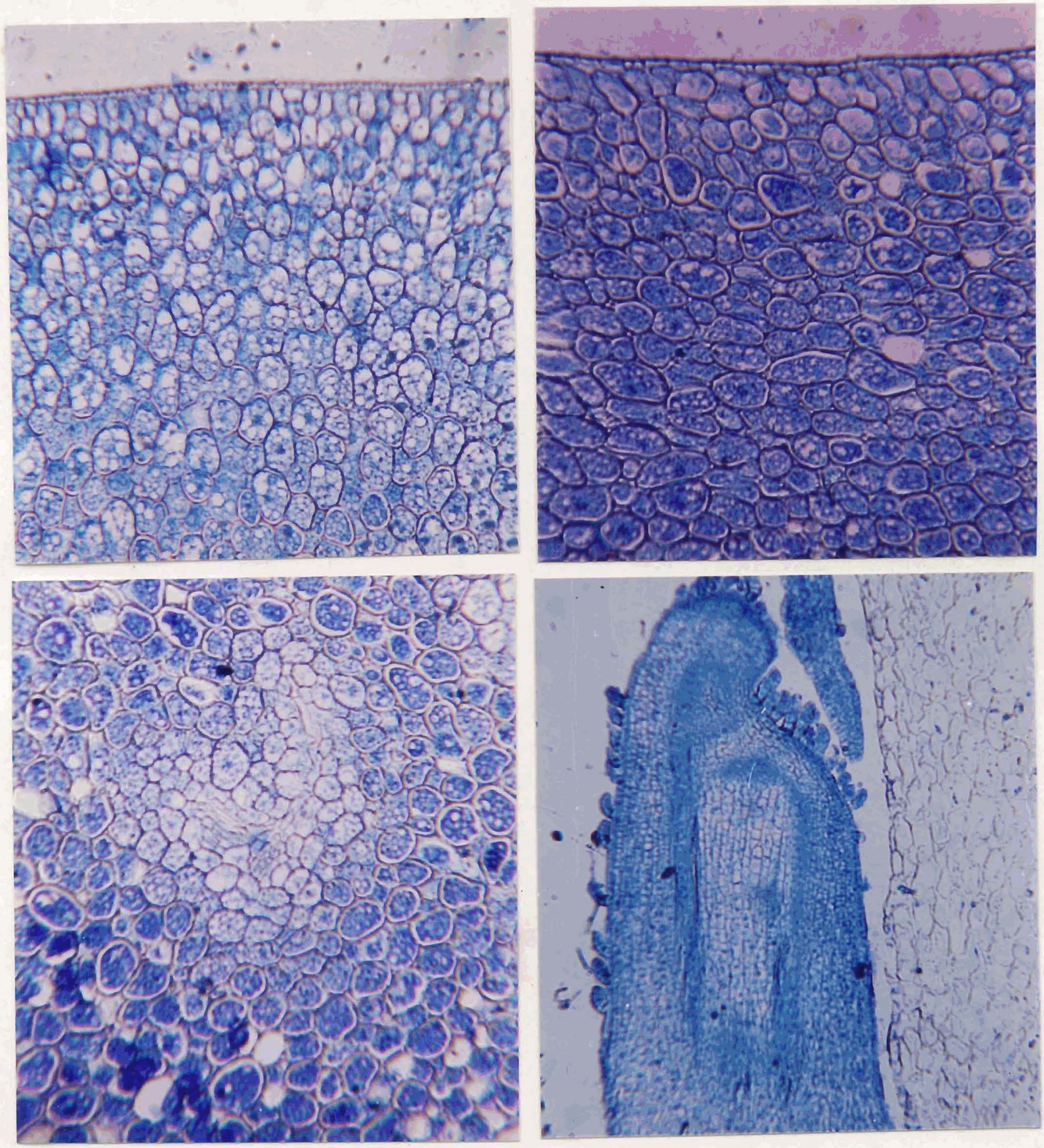


PLATE – 10. Protein distribution in the seedlings of winged bean after 7 days of germination (5+2 - control)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v** vasculature

Towards the adaxial side again degradation of protein bodies was evident. The elongated small epidermis of adaxial side was having very small amount of protein. The hypodermal and cortical cells were large with various shapes and having intercellular spaces showing less amount of protein compared to abaxial cells and were highly vacuolated. The cortical cells showed granular protein and many vacuoles were seen in these cells (Plate 10B).

Vascular strands showed cells with thickened cell walls. The cotyledonary cells nearer to the vascular strands were found to be more and more vacuolated due to the degradation of protein (Plate 10C).

b. Cotyledonary axillary bud

Compared to (5+2) days experimental the axillary buds of the control was not grown up into shoot. It remained as a bud itself. The epidermis of this bud consists of small square shaped thick walled parenchyma cells with large prominent nuclei. Other cells of the bud were also small with large nuclei inside it. Altogether the cells of the bud stains lightly for protein (Plate 10D).

(5+3) Days' Seedling (Experimental 3 days after plumule excision)

a. Cotyledon

The rectangular or square shaped parenchymatous cells of the epidermis contained small amount of protein inside (Plate 11A). The hypodermal cells were parenchymatous almost isodiametric and some were elongated also. Due to the degradation of protein, cells appeared as more vacuolated. In the cortical region the parenchymatous cells were

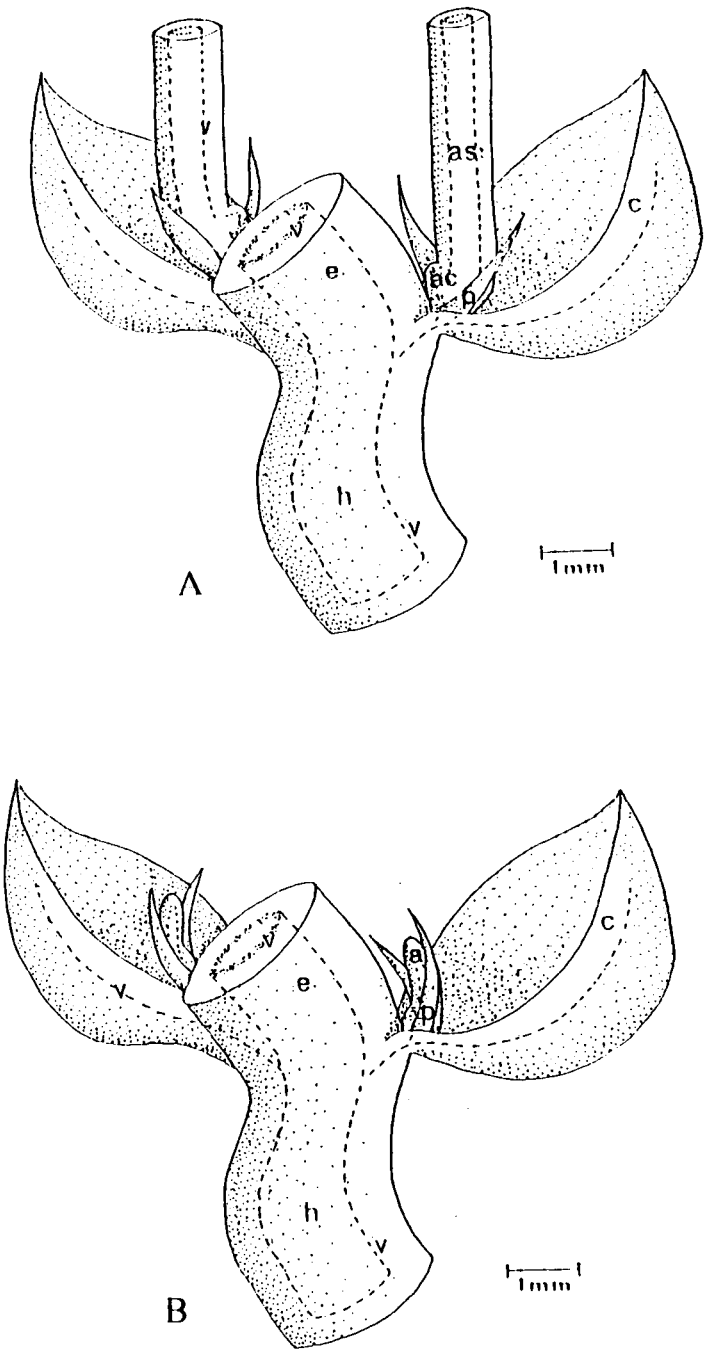


Fig.5. Semi diagramatic drawings of winged bean seeds.

A. Germinated seed after three days of plumule excision (5+3 experimental)

B. Germinated seed after eight days (5+3 control)

a. axillary bud; **ac.** accessory bud; **as.** axillary shoot; **c.** cotyledon; **e.** epicotyl; **h.** hypocotyl; **p.** prophyll; **v.**vasculature.

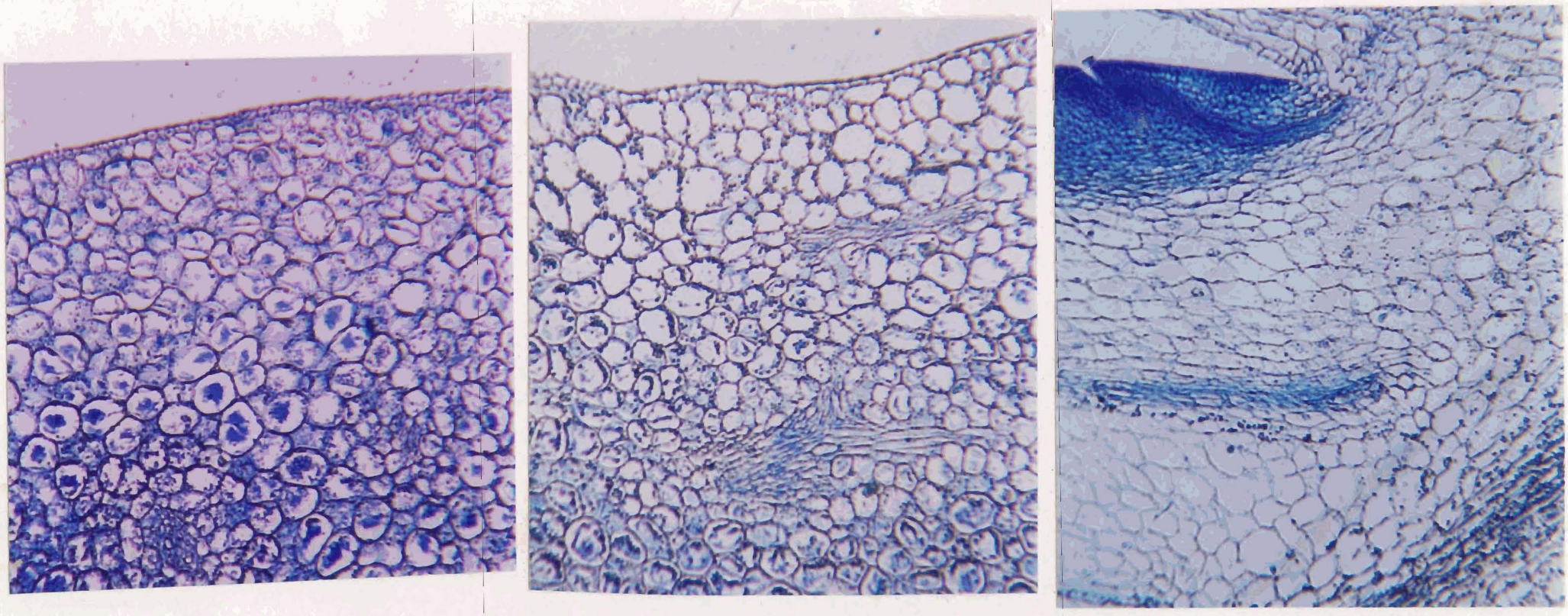


PLATE - 11 Protein distribution in seedlings of winged bean three days after plumule excision (5+3 -experimental)

A. abaxial side of the cotyledon
B. adaxial side of the cotyledon showing vasculature
C. Embryonic axis portion showing axiallary bud

ac accessory bud **ax.** Axillary bud **v** vasculature

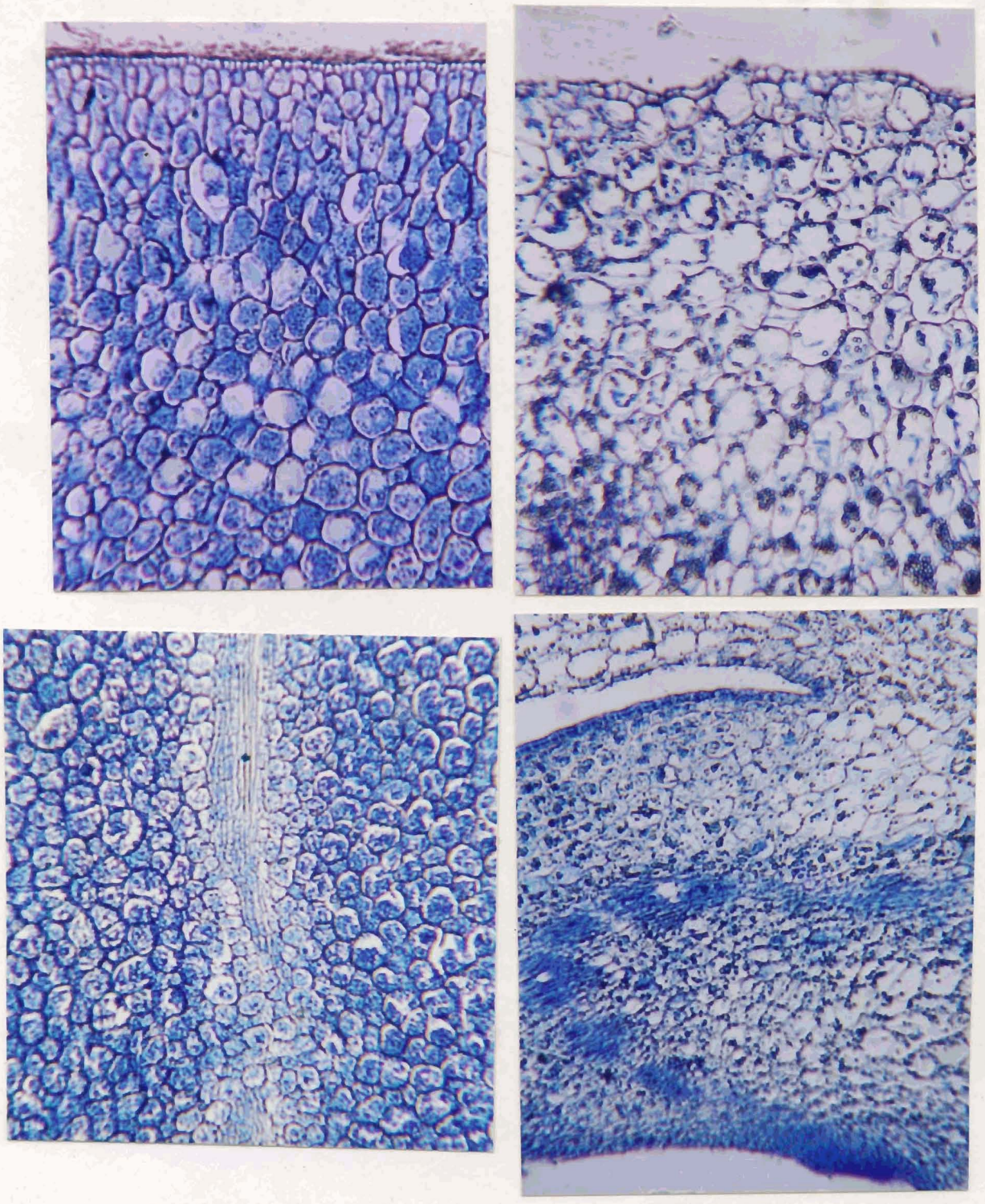


PLATE – 12. Protein distribution in the seedlings of winged bean after 8 days of germination (5+3 - control)

A abaxial side of the cotyledon B. adaxial side of the cotyledon
C vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ac. accessory bud **ax.** axillary bud **v** vasculature

with inter cellular spaces. Some cells of the cortex contained densely stained mass of protein bodies clumped in the cortex while in others degraded protein bodies were scattered inside the cells (Plate 11A). In the abaxial side of the cotyledon protein mass in clumps were seen in some cells towards the central region. But most of the cells were having protein bodies degraded and the cells appeared as highly vacuolated (Plate 11A). Towards the adaxial side the small rectangular epidermal cells and large almost isodiametric or elongated hypodermal cells were mostly devoid of protein (Plate 11B).

Many layers of cotyledonary cells near the vascular strands also showed highly depleted protein content. Vascular strands with many layers of elongated cells in longitudinal section contain slightly stained protein. Protein degradation in the neighbouring parenchymatous cells were more in this stage (Plate 11B).

b. Cotyledonary axillary bud

The axillary bud was well developed into a shoot. Parenchyma cells in the cortex and pith region contained very low amount of protein. A well differentiated vascular strands were seen which was connected to the vasculature of main axis (Plate 11C). Developing accessory bud also was seen in the axil subtended by the main axis and axillary branch.

(5+3) Days' Seedling (Control 3 days after plumule excision)

a. Cotyledon

The epidermis of abaxial region of the cotyledon contained small rectangular or square shaped cells with scanty protein. The hypodermis

and cortex consisted of more or less isodiametric in cross section. Protein degradation was almost uniform through out the cotyledon (Plate 12A). Compared to the abaxial side, adaxial side showed more degradation of protein (Plate 12B). Due to protein degradation many cells appeared as highly vacuolated (Plate 12B). But the central core of cells of the cotyledon still retained a good amount of protein bodies inside it. Many cell layers adjacent to the vascular tissues showed degradation of protein and vacuolation (Plate 12C).

Vascular strands were further developed compared to previous stages. Thickening of the xylem vessels were clearly seen. Protein bodies and nuclei were seen inside some cells of the vascular strands (Plate 12C).

b. **Cotyledonary axillary bud**

The axillary bud was not developed into a shoot system. However, provascular strands were present. The cells of the epidermis, cortex and of axillary buds were small isodiametric and with prominent nuclei. Cells of the pith region were larger than the cortical cells. (Plate 12D).

2. STARCH

Periodic acid-Schiff's reagent specifically stains insoluble polysaccharides. The sections stained with the PAS showed beautifully stained cell walls and starch content (if any) of the cells.

The anatomical details of winged bean seeds and seedlings during germination, seedling growth and the effect of plumule excision were described in the result section of protein histochemistry. However, the

characteristic changes of cell wall and starch contents are described under this section.

Dry seed

a. Cotyledon

Starch granules were not present in the epidermal, hypodermal or cortical cells (Plate 13A, B). Some cells of the hypodermis and cortex particularly in the abaxial side of the cotyledon, showed stained material inside and outside of the cells. The PAS positive stained material of some cells might be due to the surface view of the cell, since the cutting plane might be passed through the surface of cell walls of these cells. This type of surface views of cells could be seen in all sections of cotyledon throughout the study. (Plate 13A). In addition to these stained patches in some cells, slightly stained thin film like materials were observed especially in the abaxial side. An interesting observation was the PAS negative (not in magenta colour) granular substances presumably protein almost filling the lumen of cells. Procambial strands consisting of thin walled elongated cells were devoid of starch grains (Plate 13B).

b. Embryonic axis

Plumule and radicle apices of the embryonic axis consisting of thin walled cells were found to be meristematic and so undifferentiated also. These cells were devoid of starch grains (Plate 13C, D). Root apical organisation and cellular characters were clearly seen in the radicle apex (Plate 13D). Starch was not present in both apices.

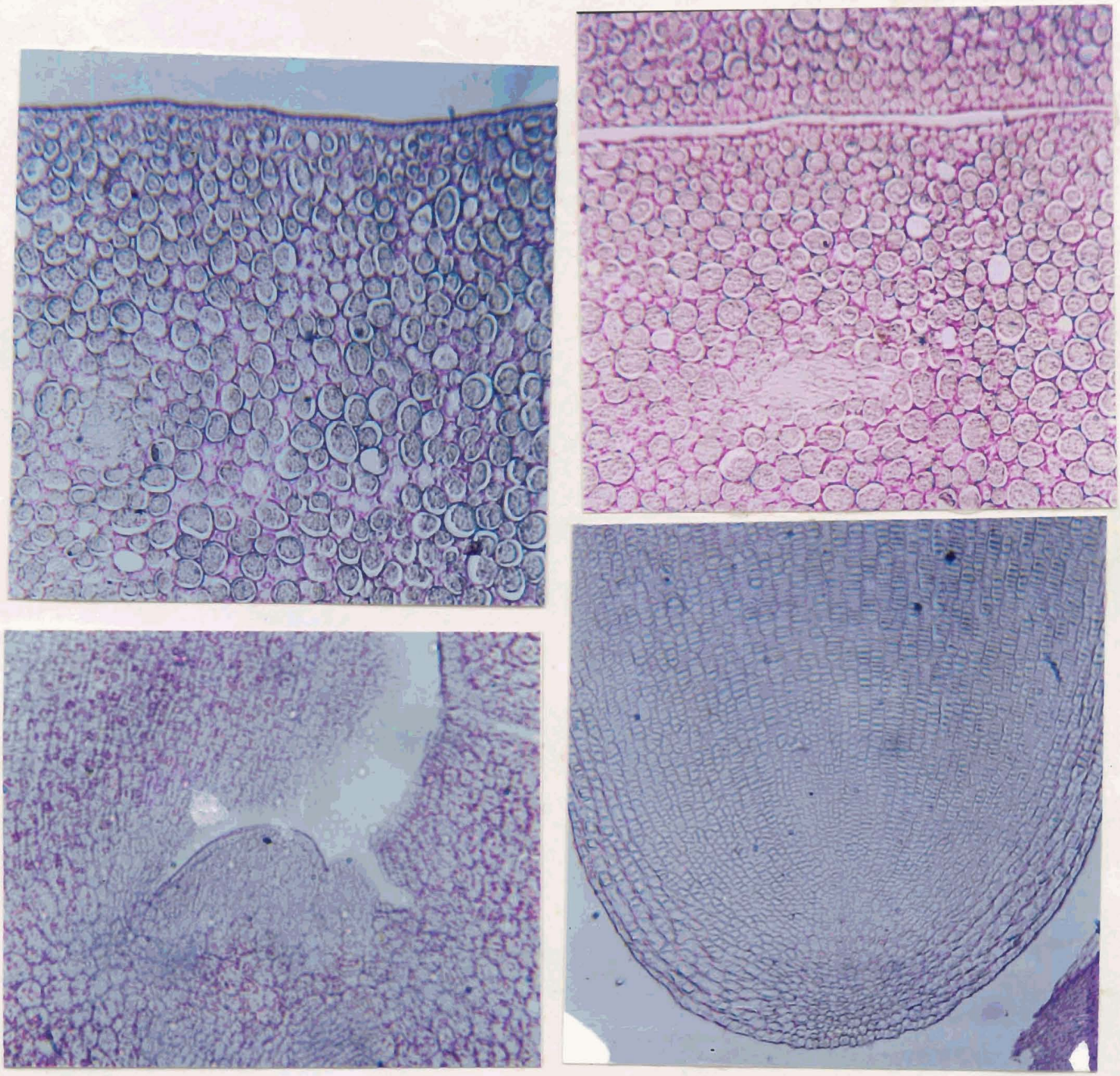


PLATE - 13 Starch distribution in the dry seeds of winged bean.

A abaxial side of the cotyledon B adaxial side of the cotyledon

C portion of cotyledon where lipid was removed before staining with PAS D. plumule apex E. radicle apex

a. plumule apex **d.** dermatogen **p.** plerome **pl.** periblem **v.** vasculature

5th Day Seedling

a. Cotyledon

The cells of the cotyledons, five days after germination were larger in size due to cell enlargement during imbibition compared to the cells of dry seeds (Table 1). The epidermis of abaxial side contained no starch grains inside, while the hypodermal cells were having a few starch granules in each cell, which were stained deeply by periodic acid-Schiffs reagent (Plate 14A). Towards the cortex, cells were having starch granules of same size and more in number as that of hypodermis. But, towards the adaxial side the cortical cells contained more starch grains than that of abaxial side (Table 1; Plate 14B). Procambial strands were seen as patches consisting of thin walled elongated cells with no starch grains (Plate 14C). The neighbouring cells of the procambial strand contained more starch grains than other cells of the cotyledon (Plate 14C, D). The unspecific PAS positive stained thin film like materials are reduced in the cotyledonary sections of the germinated seeds. Similarly PAS negative granular structures also are reduced emptying the lumen of cells and these areas are occupied by starch grains.

b. Cotyledonary axillary bud

Axillary bud was found developed from the axil of main axis and the cotyledon as a small shoot apex constituting a group of thin walled isodiametric and meristematic cells. Starch granules were not observed in these cells. (Plate 14D).

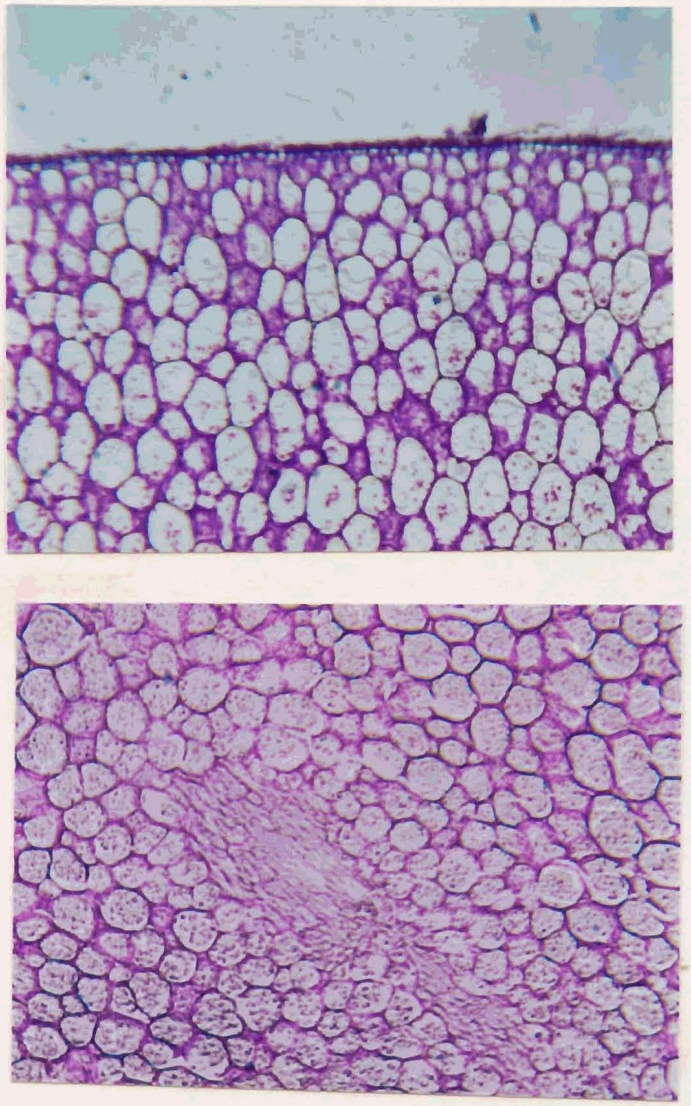


Plate-14 Starch distribution in seedlings of winged bean after 5 days of germination

A. abaxial side of the cotyledon **B.** adaxial side of the cotyledon
C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v.** vasculature

(5+½) Days' seedling (Experimental seedling 12 hours after plumule excision)

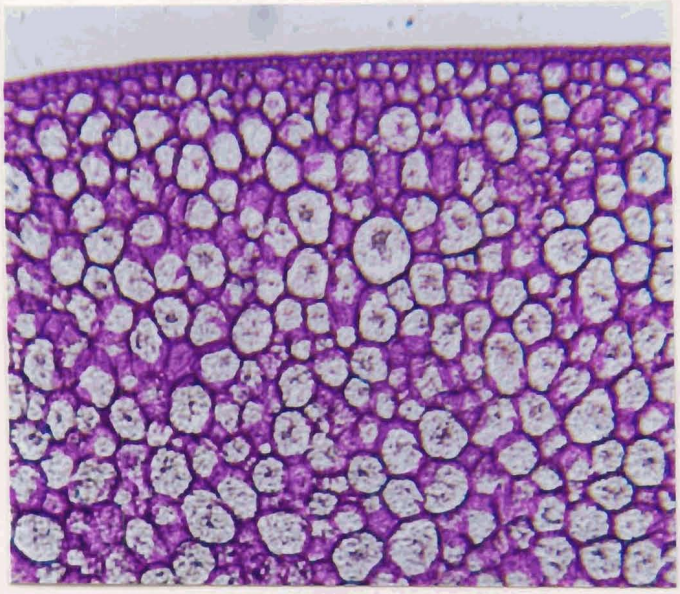
a. Cotyledon

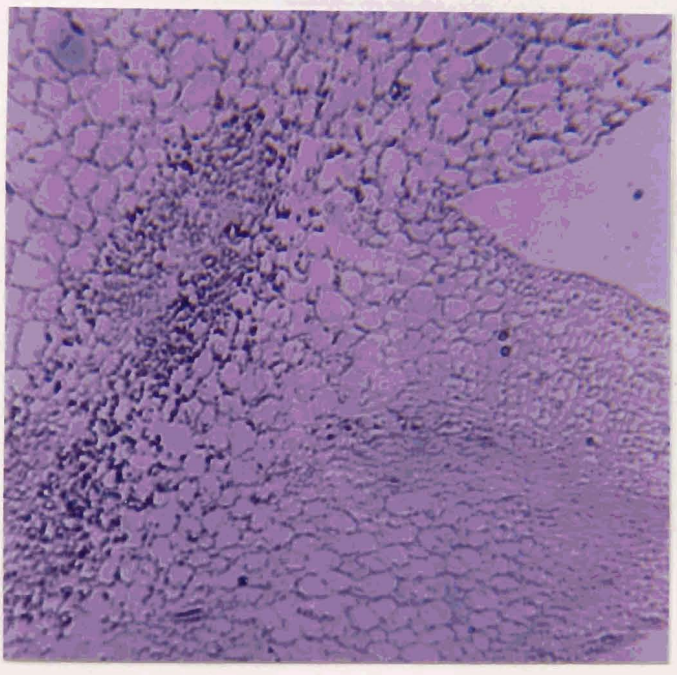
In the abaxial side of the cotyledon, the epidermal cells contained no starch grains. The hypodermal cells were having a few of starch granules (Table 1). The size of the granules were very small. There were many cells which contained stained film like mass. The cortical cells which were larger than the hypodermal cells, contained many small starch granules (Table 1; Plate 15A). Towards the adaxial side the cells contained comparatively large sized starch granules, clumped together in some cells. Number of granules were more in the cells of adaxial side than that of abaxial side (Plate 15B).

The procambial cells in cross sections appeared as small angular or rounded cells. An interesting observation was the accumulation of starch grains in the cotyledonary cells around the vascular strands (Plate 15C).

b. Cotyledonary axillary bud

The axillary bud found in the axil of cotyledons started development. The epidermal cells were without any starch granules inside (Plate 15D). The vascular strands were found to be traversing to this axillary bud which consisted of undifferentiated procambial cells inside which starch granules were absent. But in the cotyledonary cells beneath the axillary bud showed abundance of starch granules in many cells. (Plate 15D).





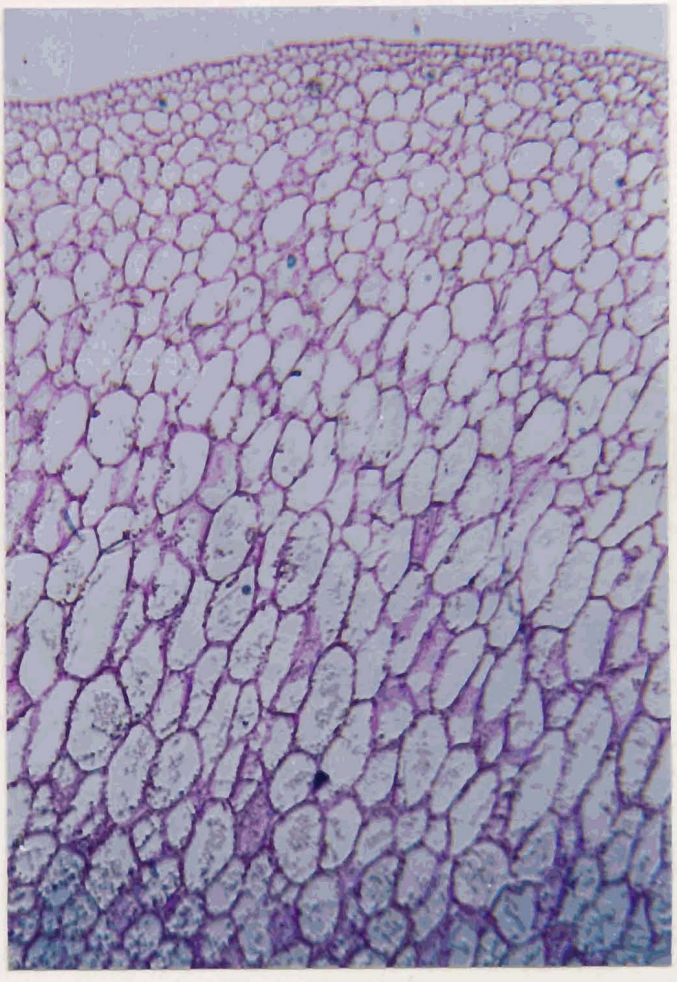


PLATE - 15 Starch distribution in seedlings of winged bean after 12 hours after plumule excision (5+½ experimental)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon
C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v.** vasculature

(5+½)Days' seedling (Control seedling 12 hours after plumule excision)

a. Cotyledon

Epidermis of the abaxial side of cotyledon consisted of cells which were devoid of starch granules but some cells contain a thin film like stained mass(Plate 16A). Hypodermis consisted of cells containing many starch granules (Table 1). Comparatively more starch granules were present in the cotyledons (Table 1). The number of starch granules was found to be less when compared to the experimental. Towards the adaxial side the number of starch granules increased but reduced in size (Table 1; Plate 16B).

Procambial strands in the cotyledon appearing in longitudinal sections as elongated cells showed further differentiation. But starch grains were absent in these cells. (Plate 16C). The cotyledonary cells around the vascular strands contained many starch granules (Plate 16C).

b. Cotyledonary axillary bud

In the axil of the cotyledons developing axillary bud was present in the control also. This bud appear as a group of undifferentiated cells (Plate 16D).

(5+1) Days' Seedling (Experimental seedling one day after plumule excision)

a. Cotyledon

The cotyledon after one day of plumule excision showed the following features. The epidermis in the abaxial and adaxial side consisted of cells without starch granules inside. But some cells

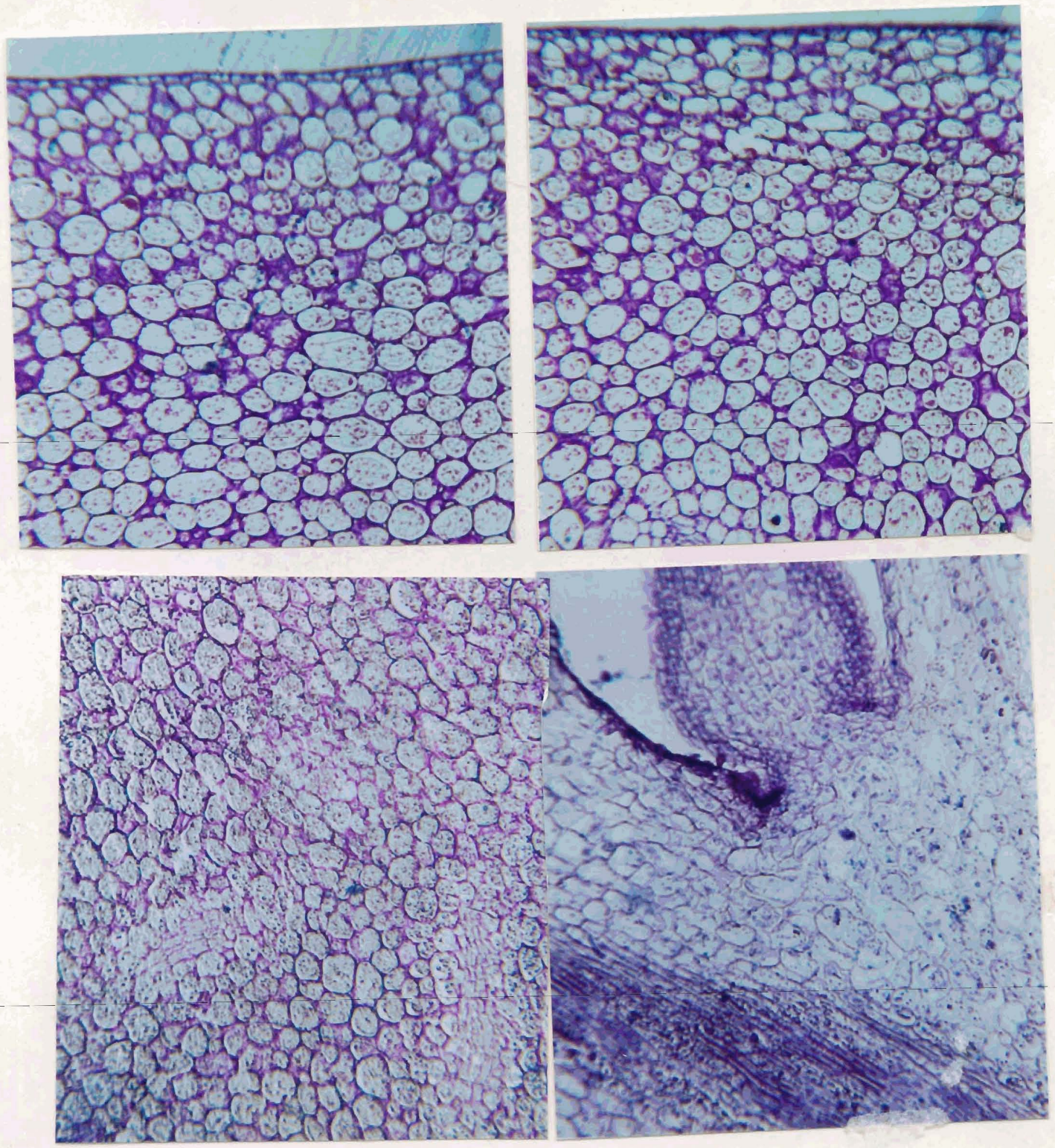


PLATE – 16. Starch distribution in the seedlings of winged bean after 5+½ days of germination (5+½ - control)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v.** vasculature

contained thin film like stained mass (Plate 17A, B). Many starch granules were present in the hypodermal and cortical cells of abaxial side (Table 1). But in the adaxial side the cells contained smaller starch granules compared to the cells of abaxial side (Table 1). The size of starch grains was found to be large particularly in the abaxial cells compared to the previous samples.

Vascular strands were seen as in cross section as well as in longitudinal sections. These cells show cell wall thickening than the previous stage of germination. Near the vascular region the number of starch grains were found to be less than that of the other cells (Plate 17B).

b. Cotyledonary axillary bud

The axillary bud was more developed than the previous experimental. The cortical cells and pith cells contain starch granules which were larger in size. Vascular strands of axillary bud were differentiated further. A prophyll, consisting of colourless small compactly arranged cells without starch granules was present subtending the axillary bud (Plate 17C).

(5+1) Days' Seedling (Control seedling one day after plumule excision)

a. Cotyledon

The epidermis of the abaxial side of the cotyledon contained no starch granules. The cells of the hypodermis contained less starch grains than the cortical cells (Table 1; Plate 18A). In the epidermis of adaxial side, starch granules were absent. The number of starch granules became more towards the adaxial side in all types of tissues (Plate 18B). The

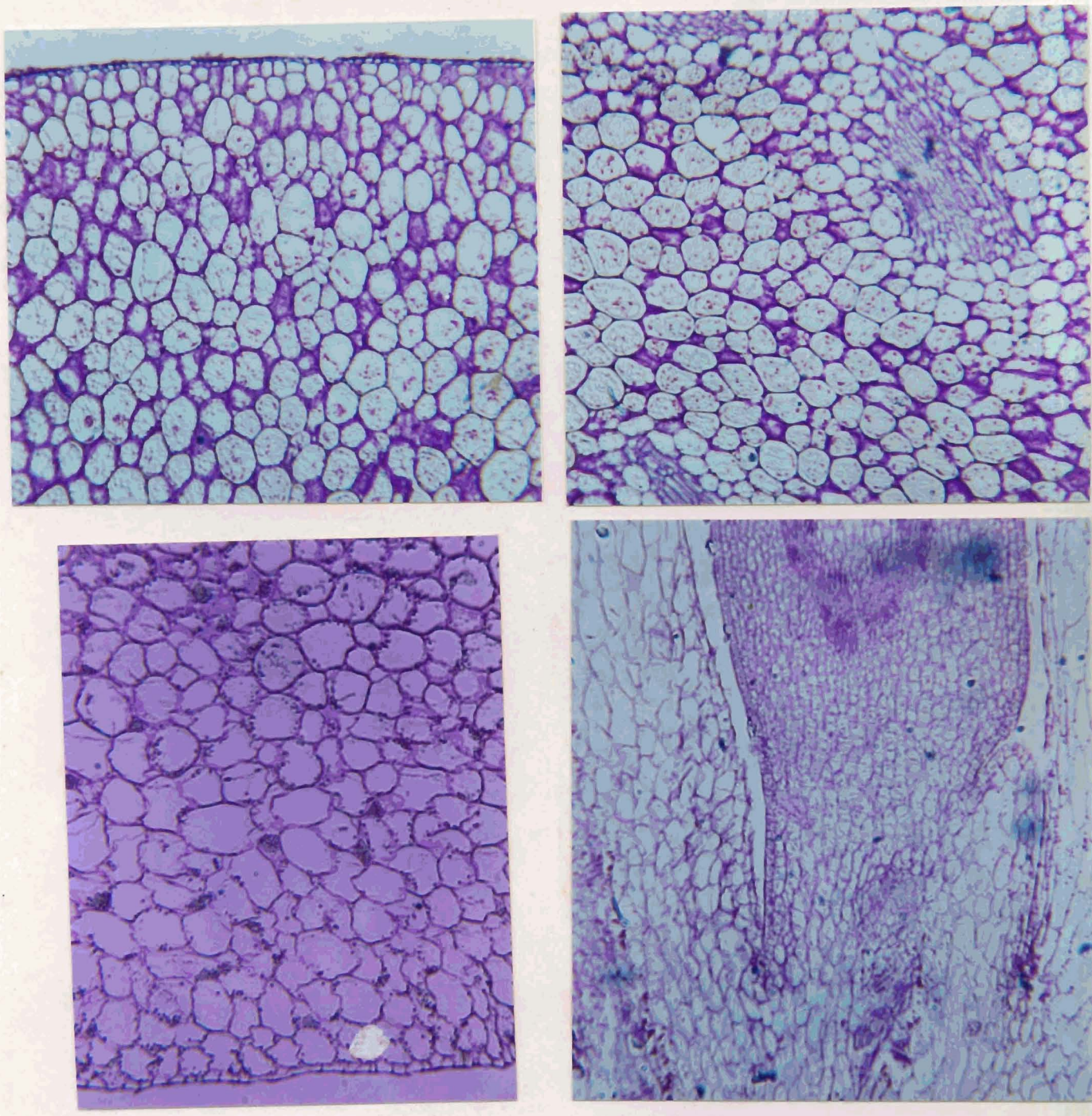


PLATE - 17 Starch distribution in seedlings of winged bean one day after plumule excision (5+1 experimental)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon
C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. axillary bud **v.** vasculature

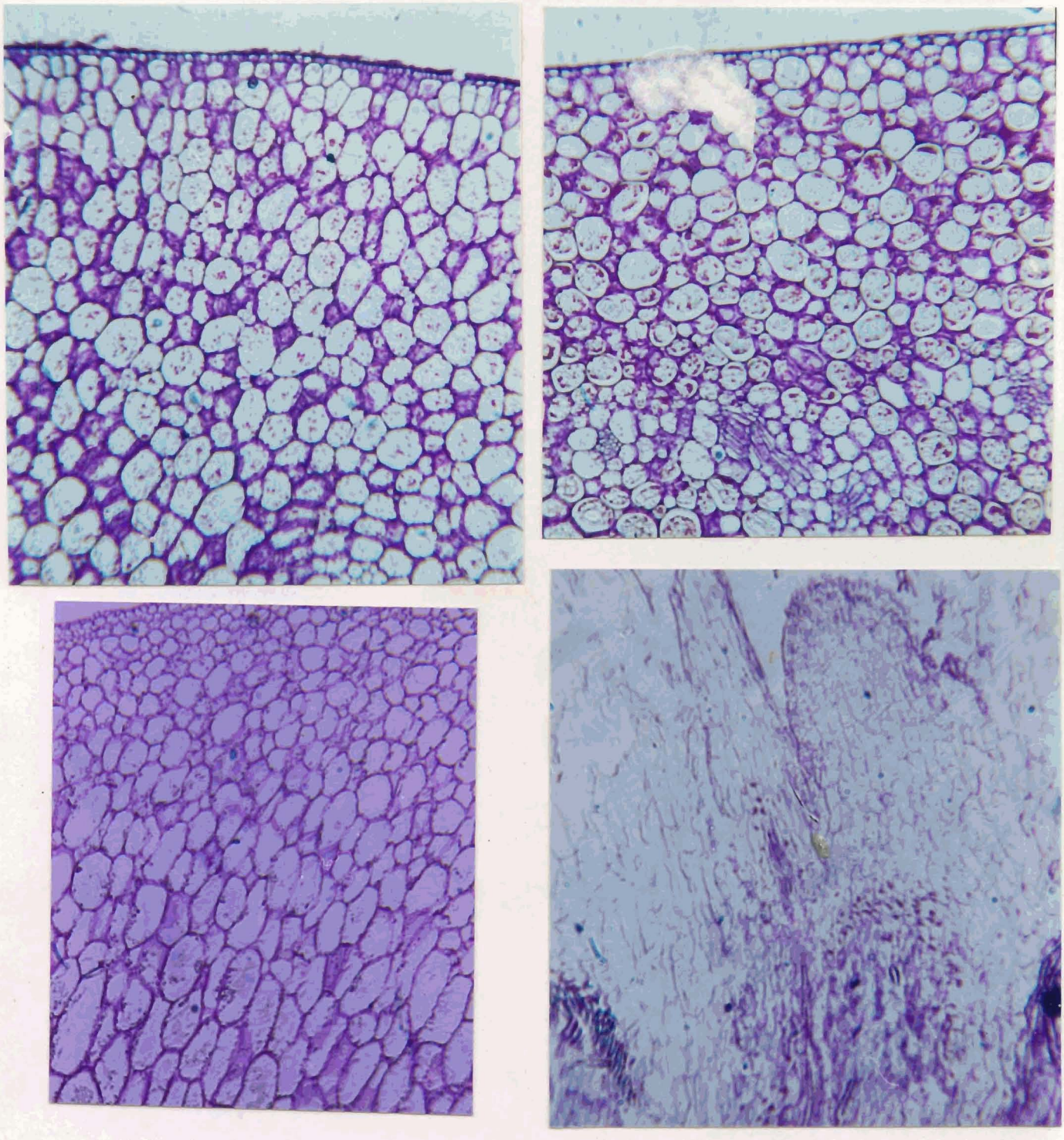


PLATE – 18. Starch distribution in the seedlings of winged bean after 6 days of germination (5+1 - control)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ax. **axillary bud** v. **vasculature**

vascular strands consisted of elongated cells with no starch granules inside. Cells nearer to the vasculature contained less starch granules than other cells (Plate 18C).

b. Cotyledonary axillary bud

The cotyledonary axillary bud appeared as a developing shoot apex consisting of meristematic cells which were devoid of starch grains. The apex was subtended by a prophyll (Plate 18D). Starch granules were seen in the cells beneath the axillary bud.

(5+2) Days' Seedling (Experimental seedling two days after plumule excision)

a. Cotyledon

Outer epidermis of the abaxial part of the cotyledon did not contain any starch granule inside. The hypodermis contained a few starch granules while in the cells of the cortex showed more starch grains which varied in number. (Table 1; Plate 19A). Towards the adaxial side of the cotyledon, the number and size of starch granules became increased. The hypodermis of the adaxial side contained very few starch granules, (Table 1). The cortical and hypodermal cells of the adaxial side contained more starch grains than the abaxial side (Plate 19A, B). The procambial cells of the cotyledon at this stage showed differentiation into xylem and phloem. Xylem vessels were clearly seen with thickened cell walls (Plate 19C). The cotyledonary cells nearer to the vasculature showed no starch granules.

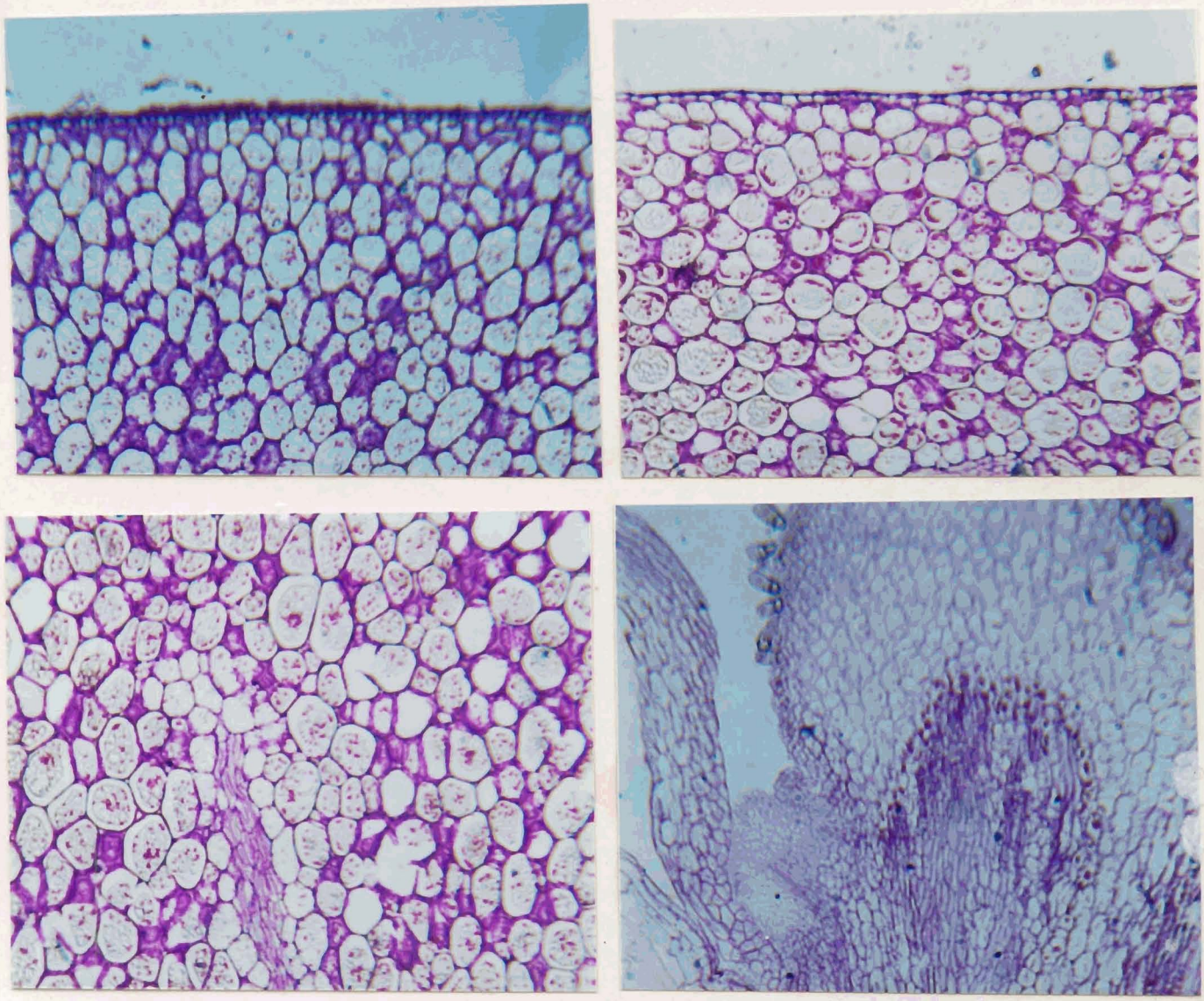


PLATE - 19. Starch distribution in seedlings of winged bean two days after plumule excision (5+2 experimental)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon
C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ac. accessory bud **ax.** axillary bud **v.** vasculature

b. Cotyledonary axillary bud

Axillary bud showed further differentiation into a small shoot with full vascular differentiation. The cells surrounding the vascular cells contained plenty of starch grains (Plate 19D). Accessory bud showed no starch grains. (Plate 19D).

(5+2)Days' Seedling (Control seedling two days after plumule excision)

a. Cotyledon

In the abaxial side of the cotyledon, the epidermal cells were devoid of starch granules. Starch grains were very small in the cells of the hypodermis and cortex. (Plate 20A). Towards the adaxial side the starch grains were more in number and seen clumped together. In the hypodermis very less amount of starch granules were present in the adaxial side (Plate 20B).

The vasculature of the cotyledon of seedling after 7 days of germination (5+2 control) shows differentiated cells which were devoid of starch grains (Plate 20B).

b. Cotyledonary axillary bud

The axillary bud was found to develop in the control also. It consisted of meristematic cells which contained very tiny starch granules in cells near the vascular region (Plate 20C).

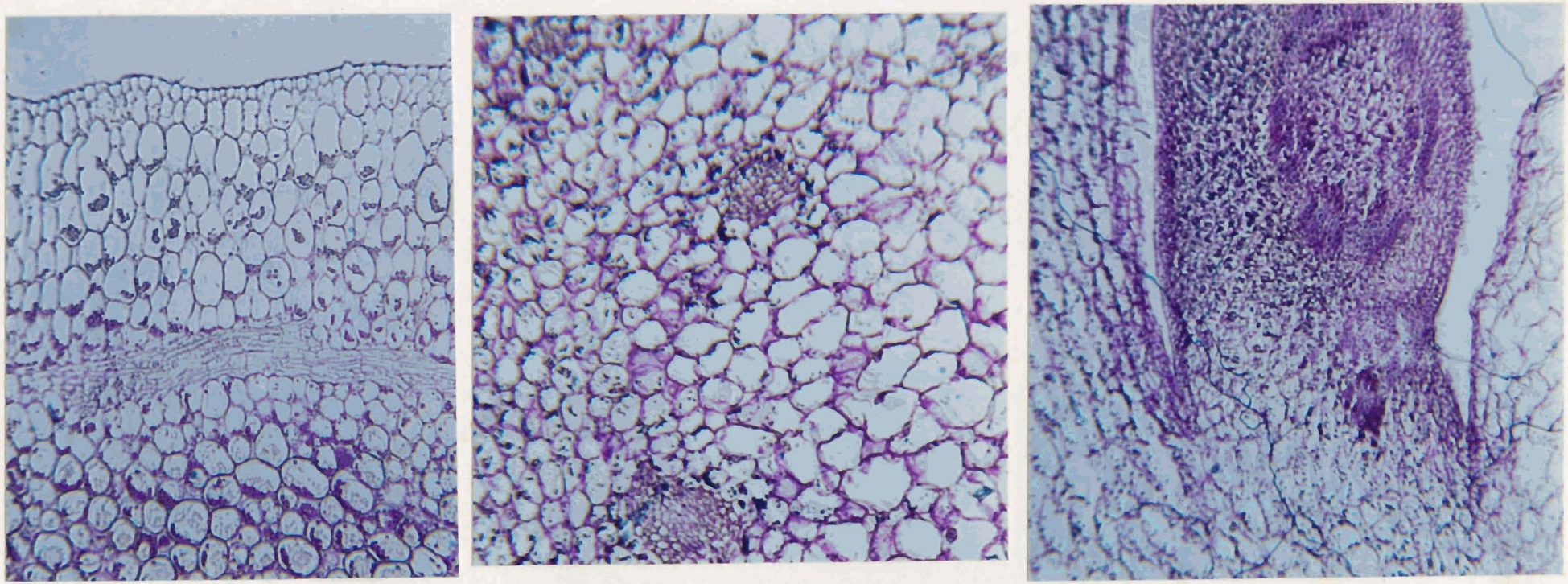


PLATE – 20. Starch distribution in the seedlings of winged bean after 7 days of germination (5+2 - control)

A. abaxial side of the cotyledon
B. adaxial side of the cotyledon showing vasculature
C. Embryonic axis portion showing axiallary bud

(5+3) Days' Seedling (Experimental seedling three days after plumule excision)

a. Cotyledon

After three days of plumule excision the following characteristics were observed in the section of cotyledon. The epidermal cells of the abaxial side were devoid of starch grains (Plate 21A). Only a few small starch grains were present in the cells of hypodermis and cortex in the abaxial side.

Towards the adaxial side many cells contained starch grains in the hypodermis and cortex (Plate 21B). Starch grains were decreased compared to previous stage. The vascular strand composed of many elongated cells of which some were having spiral or reticulate thickening (Plate 21C). Scattered starch grains were localized near the vascular strands.

b. Cotyledonary axillary bud

The cotyledonary axillary bud at this stage (after three days of plumule excision) developed in to a shoot with much vascular system and leaf primordia, the accessory bud also developed further showing leaf primordia (Plate 21D). Starch granules were present in the cortical cells of axillary bud, but accessory bud contained no starch grains.

(5+3) Days' Seedling (Control seedling three days after plumule excision)

a. Cotyledon

In the abaxial side of the cotyledon, the epidermis contained no starch grains. An important observation was that the grain size of the

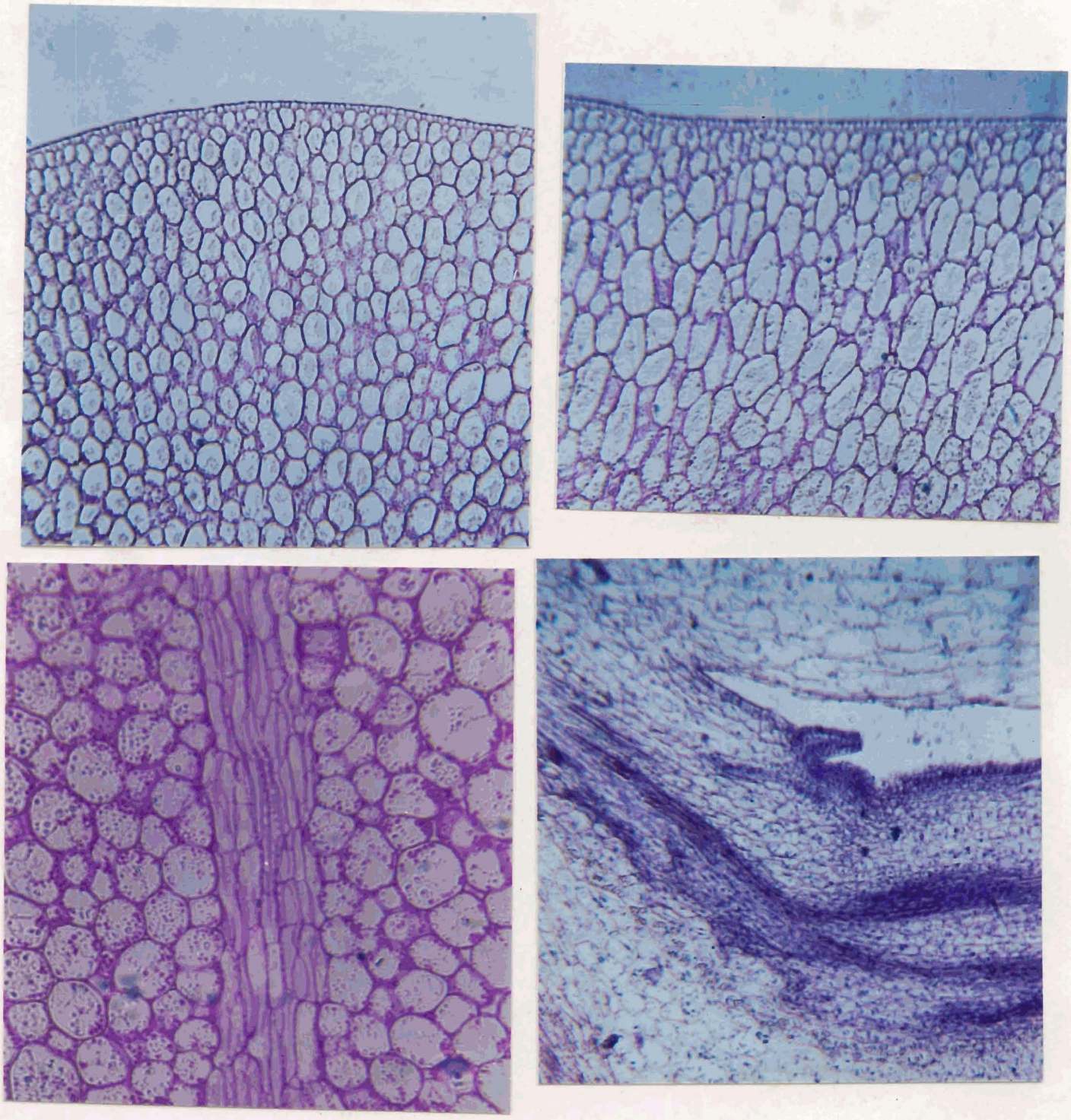


PLATE - 21. Starch distribution in seedlings of winged bean three days after plumule excision (5+3 experimental)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon
C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ac. accessory bud **ax.** axillary bud **v.** vasculature

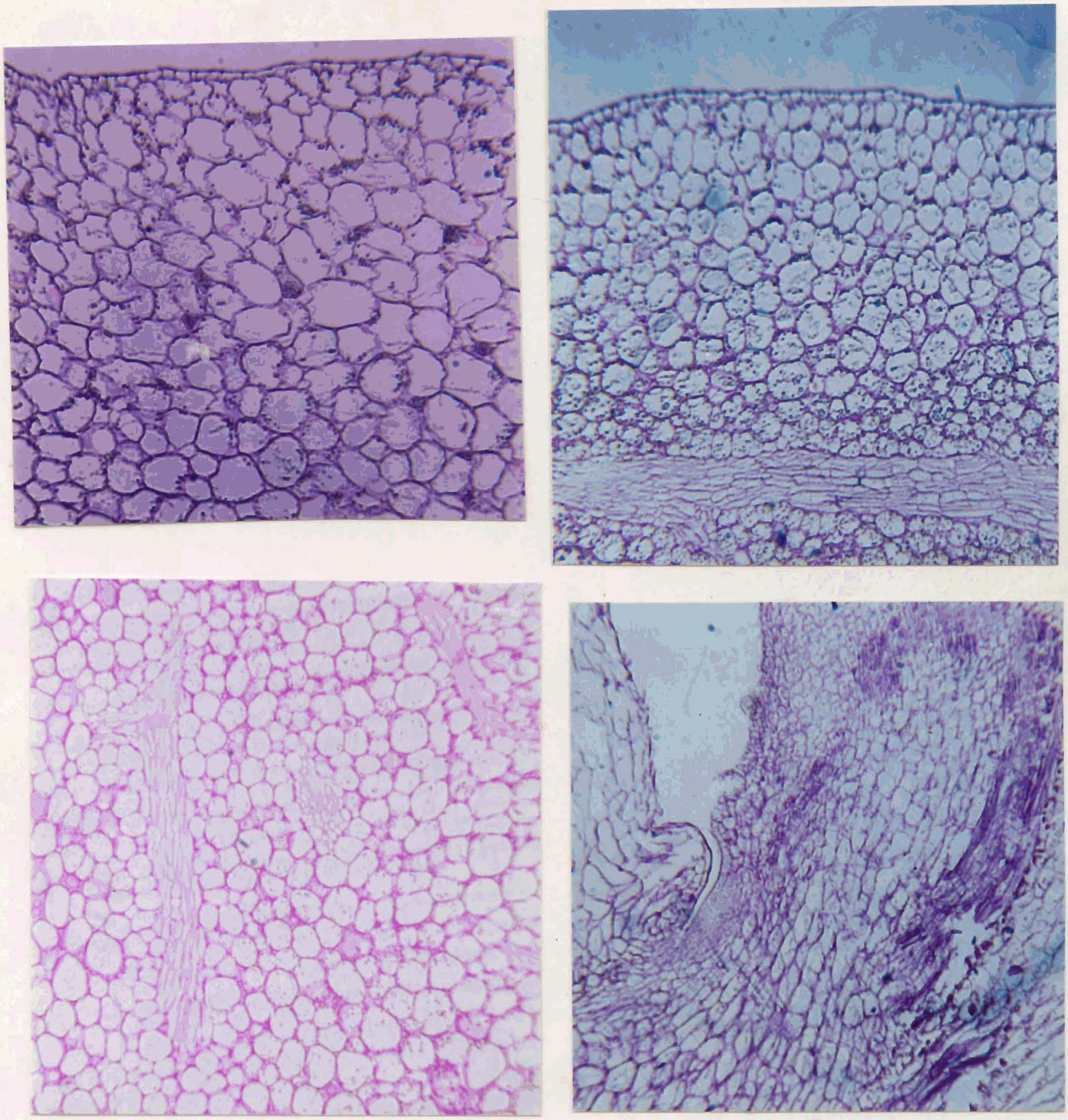


PLATE – 22. Starch distribution in the seedlings of winged bean after 8 days of germination (5+3 - control)

A. abaxial side of the cotyledon B. adaxial side of the cotyledon C. vasculature of the cotyledon D. Embryonic axis portion showing axillary bud

ac. accessory bud **ax.** axillary bud **v.** vasculature

starch became very small and were found in less numbers localized on the deep cortical cells compared to the experimental and previous samples (Plate 22A). Same structural features were found in the adaxial side also (Plate 22A, B). But the number of starch grains were more in adaxial side. Many of the cells were found to be broken in the cortical region of both abaxial and adaxial sides.

The vascular tissue showed differentiation of cells into xylem and phloem with secondary wall thickening. Starch grains were very few in the cells near the vascular strands (Plate 22C).

b. Cotyledonary axillary bud

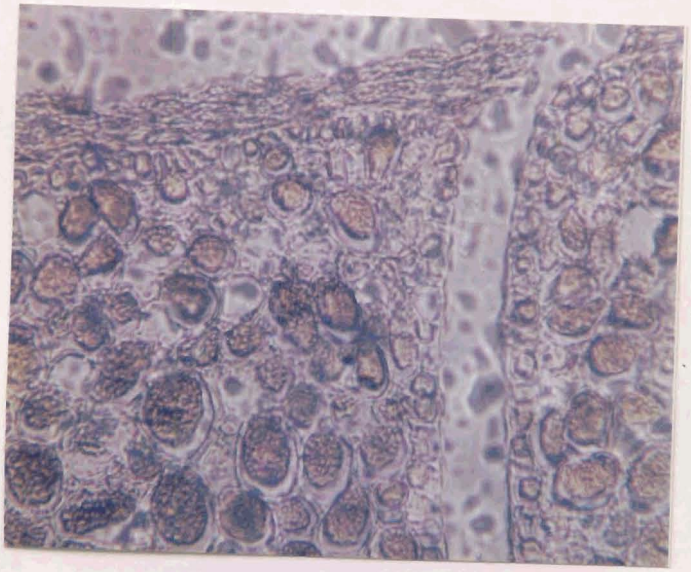
The cotyledonary axillary bud of the control seedling had grown into a small but developed shoot. Accessory bud also was found to be initiated in the axil (Plate 22D).

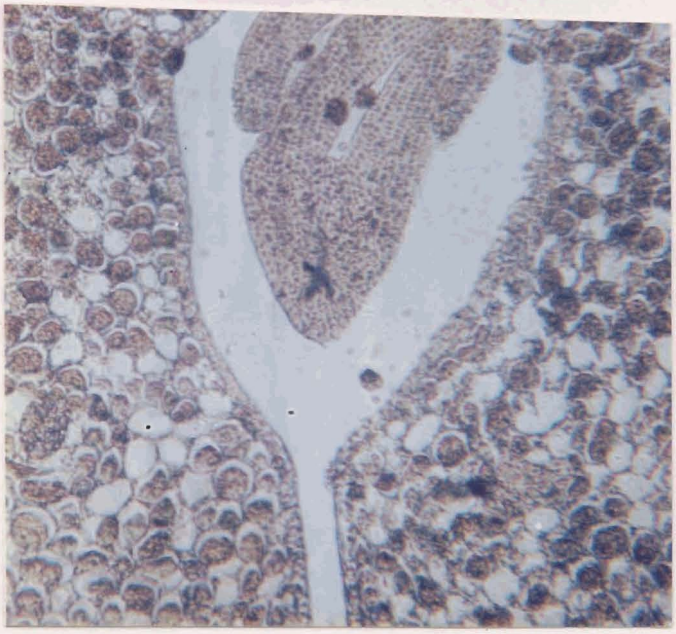
The vascular strands of axillary shoot were differentiated containing thin film of stained substance. The axillary bud was subtended by prophylls. The prophylls did not contained starch granules. Starch granules were not found in the accessory buds also.

3. LIPID

Dry seed

Lipid localisation was done by staining with Sudan IV dye. Since the lipid staining was found to be very feeble in the axis tissues, lipid localisation was done only in the cotyledonary tissues of the experimental and control. Since the cells contained large quantity of protein bodies, the





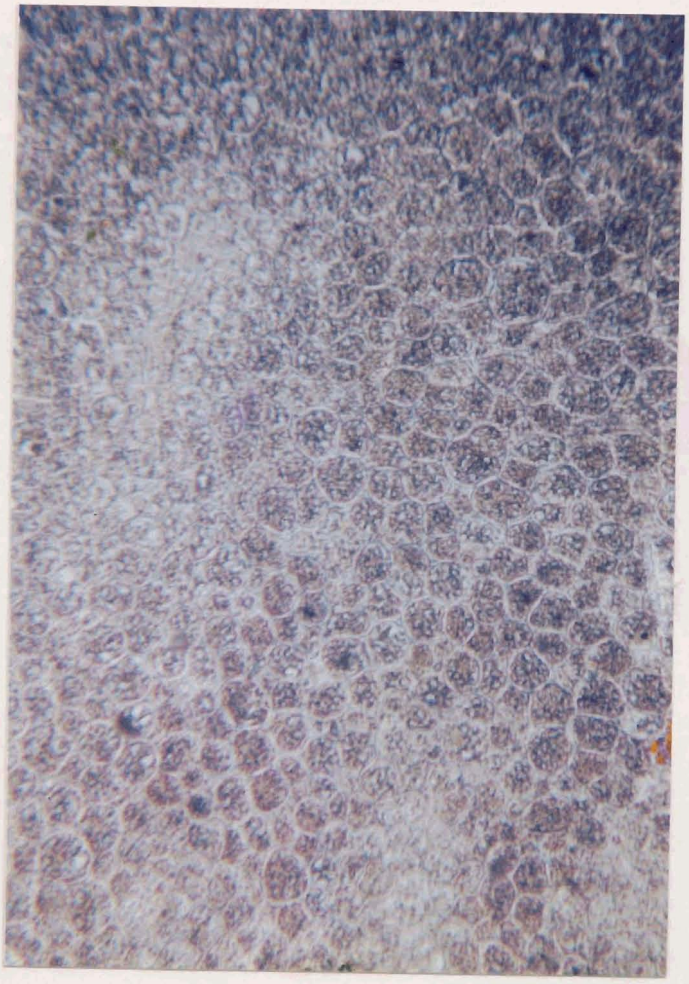


PLATE - 23 Lipid distribution in the dry seeds of winged bean.

A. abaxial and adaxial side of the cotyledon
B. vasculature cross section of the cotyledon
C. vasculature of the cotyledon

stained lipid content was found to be interfered with stained materials occupying most of the area of the cells.

In the epidermis of the abaxial and adaxial sides of cotyledonary cells contained no lipid body. But lipid content was localized in the hypodermis and cortex. The intensity of staining was higher towards the abaxial side compared to the adaxial side (Plate 23A).

The cells of provascular strands were devoid of lipid and the cellular details were not clear because of the feeble staining in cell walls. In the cotyledonary cells near the vascular strands lipid content was comparatively lower than the distant cells (Plate 23B, C).

5th Day Seedling

Five days after germination, the cells of the abaxial side contained very scanty lipid content as it was stained lightly. The lipid content of the abaxial side was more than that present in the cells of adaxial side (Plate 24A, B).

In the cells of provascular strands each lipid bodies were not observed. The cotyledonary cells near to the vascular strands did not contained much lipid bodies compared to the cells away from vascular strand and this may be due to degradation started at these regions (Plate 24C).

(5+1) Days' Seedling (Experimental seedling one day after plumule excision)

The cotyledonary cells after one day of plumule excision contained large quantity of lipid bodies towards the abaxial side. But in the adaxial

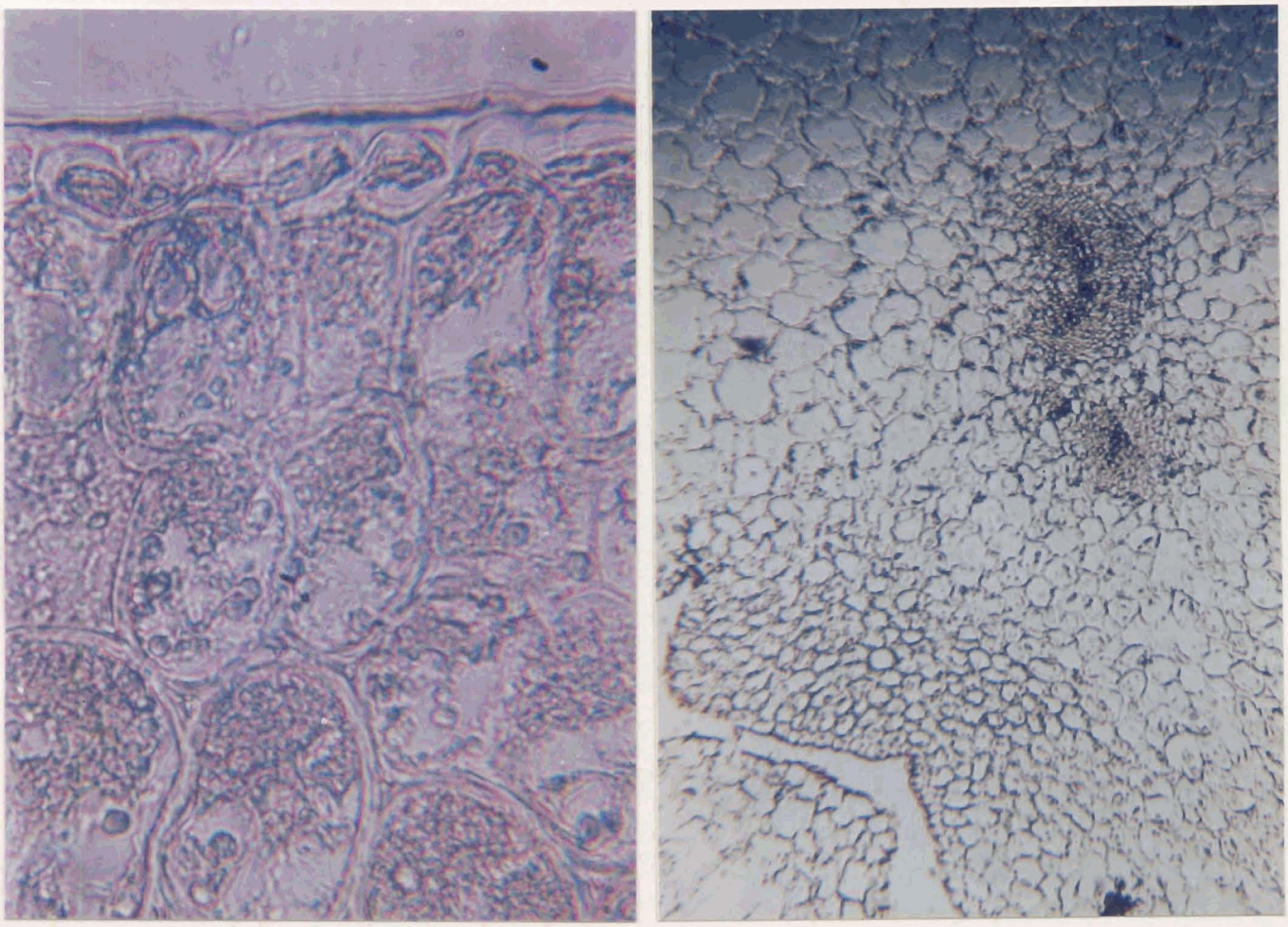


Plate-24 Lipid distribution in seedlings of winged bean after 5 days of germination

A. abaxial side with vasculature of the cotyledon

B. adaxial side of the cotyledon

sides lipid bodies were comparatively less (Plate 25A, B). The cells of the cotyledon near the provascular strand contained low amount of lipid materials compared to the cells away from the vasculature (Plate 25B).

(5+1) Days' Seedling (Control seedling one day after plumule excision)

The cells of the abaxial side contained large amount of lipids compared to the adaxial side where many cells were devoid of lipid bodies (Plate 25C, D).

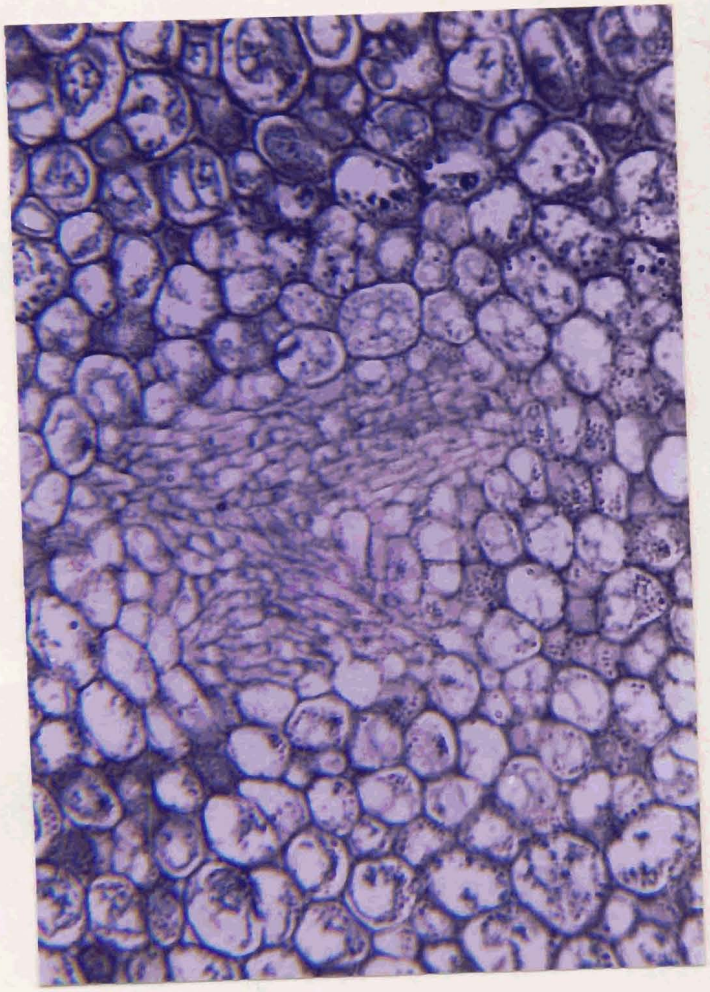
Provascular strands were devoid of lipid bodies. The degradation of lipid material in the cotyledonary cells from many cell width of the nearest cells of vascular strands also was observed (Plate 25D).

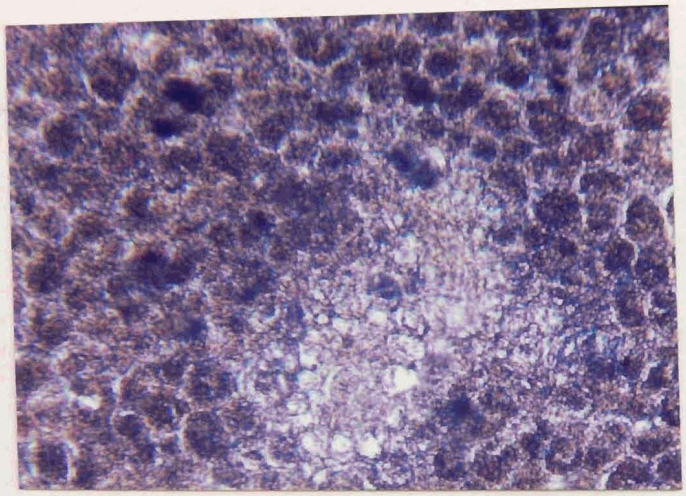
(5+2) Days' Seedling (Experimental two days after plumule excision)

After two days of plumule excision lipid content of all cotyledonary cells were reduced compared to the previous stage (Plate 26A, B). Degradation of lipid in the cotyledonary cells around the vascular strands was more in this stage compared to the previous seedlings (Plate 26B).

(5+2) Days' Seedling (Control-seedling two days after plumule excision)

At the abaxial side more lipid content was present compared to the adaxial side (Plate 26C, D). Some cells were fully devoid of lipid bodies. The degradation of lipid bodies were also increased in the cotyledonary cells around the vascular strands (Plate 26D).





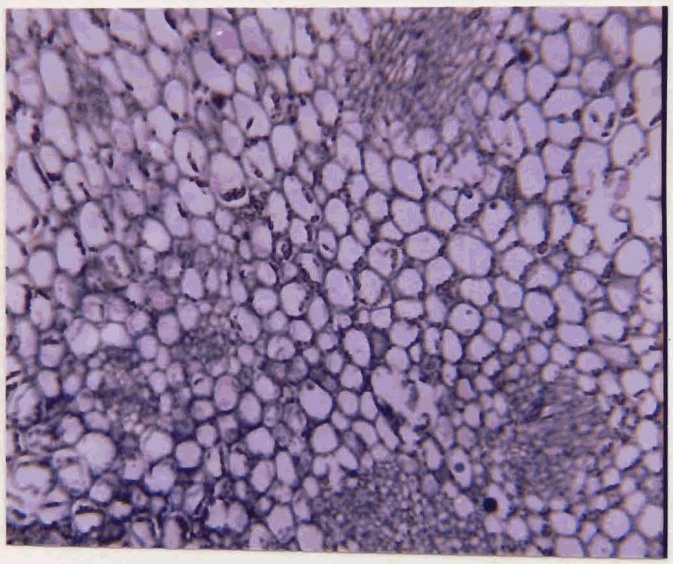


PLATE - 25 Lipid distribution in the seedlings of winged bean one day after plumule excision

A. abaxial side of the cotyledon (5+1 experimental)
B. vasculature of the cotyledon (5+1 experimental)
C. cotyledon of (5+1 control)
D. vasculature of the cotyledon (5+1 control)

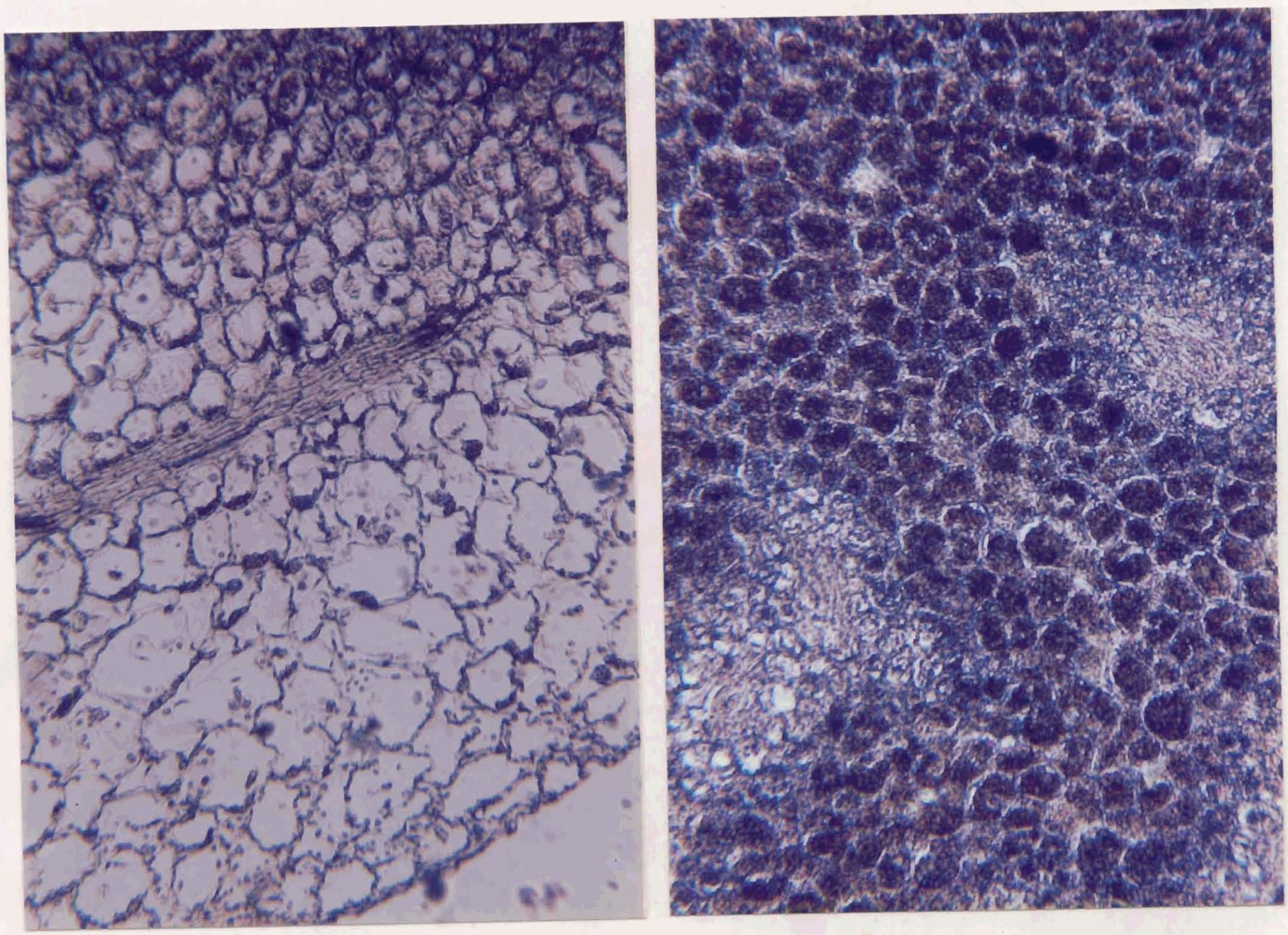


PLATE - 26 Lipid distribution in the seedlings of winged bean two days after plumule excision

A. abaxial side of the cotyledon (5+2 experimental)
B. vasculature of the cotyledon (5+2 experimental)
C. cotyledon of (5+2 control)
D. vasculature of the cotyledon (5+2 control)

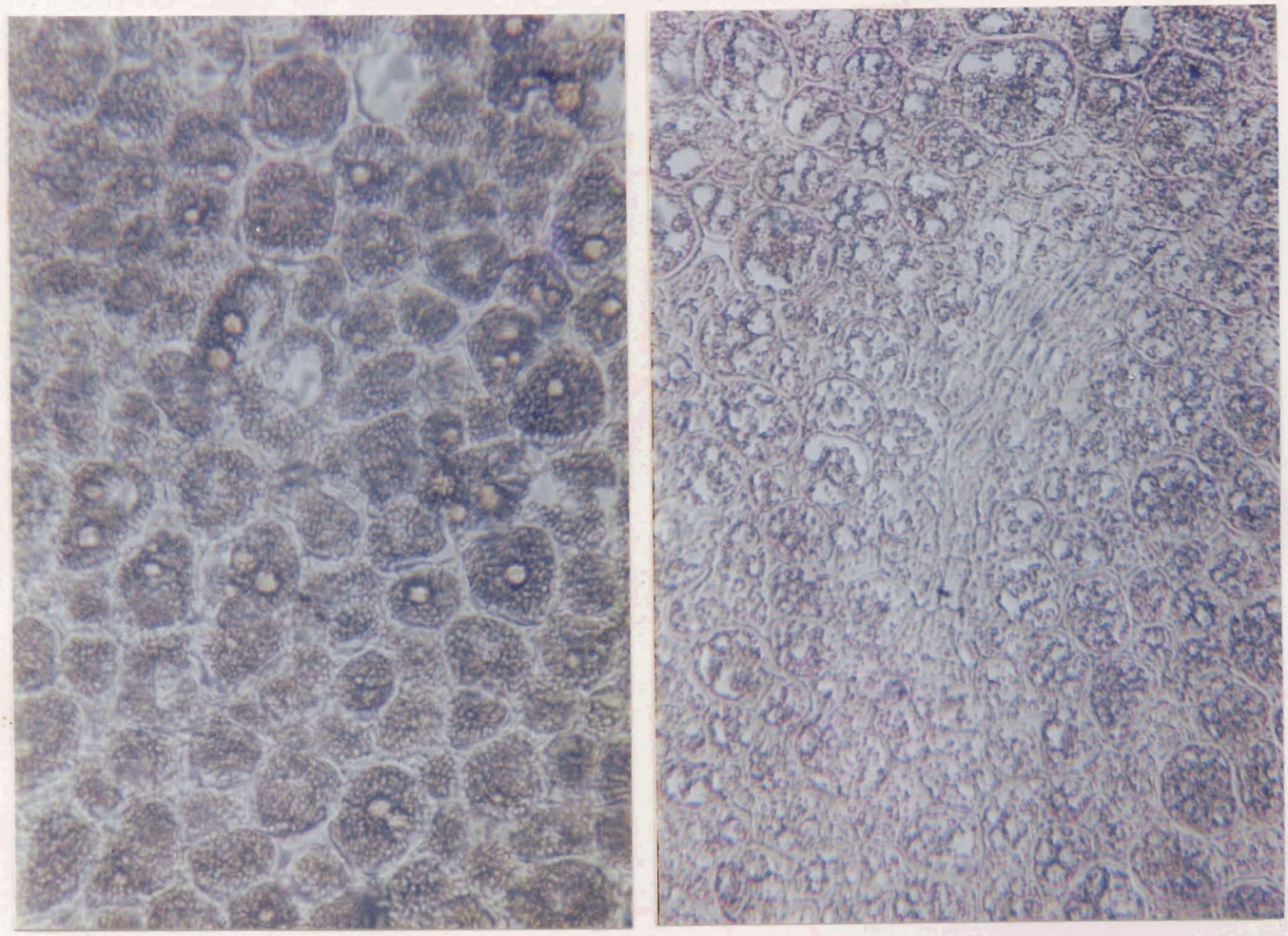


PLATE - 27 Lipid distribution in the seedlings of winged bean three days after plumule excision

A. abaxial side of the cotyledon (5+3 experimental)
B. vasculature of the cotyledon (5+3 experimental)
C. cotyledon of (5+3 control)

(5+3) Days' Seedling (Experimental-seedling three days after plumule excision)

The cotyledon after three days of plumule excision showed a decrease in the content of lipid bodies in all types of cells. The degradation of lipid bodies proceeded from the adaxial side towards abaxial side (Plate 27A). The degradation of lipid content was progressed from the nearest cells of vascular strands towards many cells away from the vascular strands (Plate 27B).

(5+3) Days' Seedling (Control seedling three days after plumule excision)

In the cells of abaxial and adaxial sides of the cotyledons contained very low amount of lipid and lipid bodies were totally absent in many cells (Plate 27C).

The vascular strands were well differentiated. They contained elongated narrow cells in which some cells showed considerable thickening. The cotyledonary cells showed degradation of lipid bodies many cells away from the vascular strands (Plate 27C).

II. BIOCHEMICAL STUDIES

1. Dry weight

Dry seeds of winged bean contained about 90% dry weight (Table 2; Fig. 6). After 5 days of germination, the dry weight percentage of cotyledon was reduced to about one third compared to the dry seed. The plumule and radicle showed more or less same dry weight content and these values are comparatively lower than that of the cotyledon.

TABLE II

Dry weight % and protein content of winged bean seeds during germination and seedling growth after plumule excision

Days of Sampling	Tissues analysed	Dry weight %	Protein mg/g dry weight
0	Dry seed	90.4 ± 1.9	423 ± 11
5 Control	Cotyledon	32.7 ± 10	453 ± 37
	Plumule	7.05 ± 0.46	259 ± 8
	Radicle	6.42 ± 0.51	230 ± 14
5 + 1* Experimental	Cotyledon	29.4 ± 1.9	490 ± 14
	Plumule	--	--
	Radicle	7.31 ± 0.49	278 ± 10
5+1 Control	Cotyledon	29.7 ± 2.1	473 ± 12
	Plumule	--	--
	Radicle	6.92 ± 0.51	246 ± 11
5+2* Experimental	Cotyledon	30.7 ± 2.1	498 ± 19
	Plumule	--	--
	Radicle	7.52 ± 0.51	271 ± 14
5 + 2 Control	Cotyledon	31.75 ± 1.01	468 ± 17
	Plumule	--	--
	Radicle	6.92 ± 0.08	248 ± 12
5 + 3* Experimental	Cotyledon	29.48 ± 1.6	502 ± 29
	Plumule	5.18 ± 0.23	405 ± 23
	Radicle	7.10 ± 0.48	262 ± 12
5 + 3 Control	Cotyledon	30.75 ± 1.11	401 ± 24
	Plumule	6.61 ± 0.40	223 ± 7
	Radicle	6.67 ± 0.48	202 ± 9
5+5* Experimental	Cotyledon	28.8 ±1.1	477 ± 25
	Plumule	5.64 ± 0.74	351 ± 21
	Radicle	7,82 ± 0.69	236 ± 12
5 + 5 Control	Cotyledon	29.3 ± 1.7	392 ± 24
	Plumule	6.10 ± 0.89	217 ± 8
	Radicle	6.37 ± 0.51	195 ± 10

* Plumule excision on 5th day and experimental sampled after 1 day, 2 days and so on.

Values are mean of 5 values ± standard error.

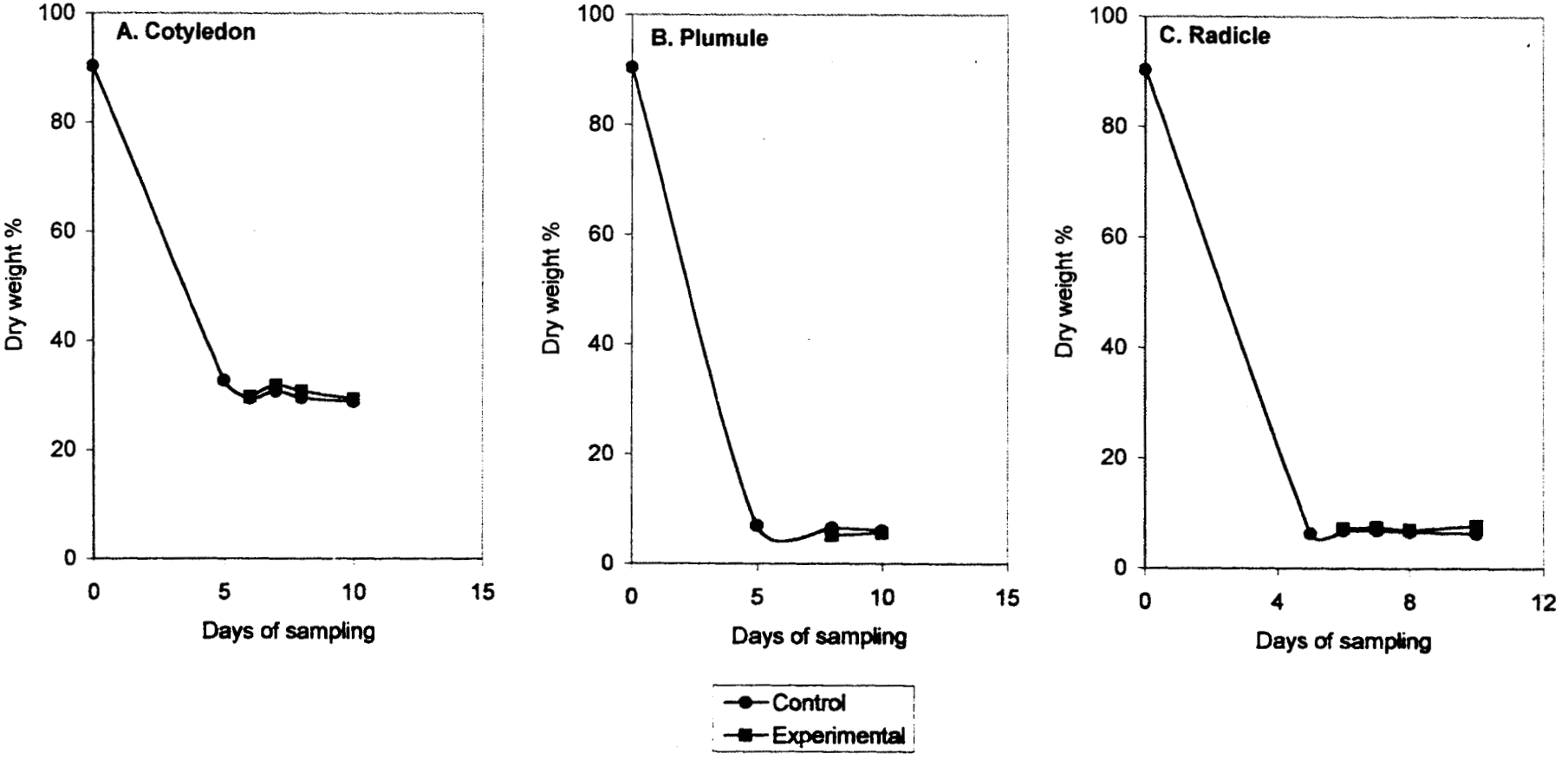


Fig. 6 Dry weight percentage of winged bean cotyledon, plumule and radicle during germination and seedling growth
Control : Intact seedling Experimental : Plumule excised seedling

One day after the excision of plumule (5 + 1-experimental) as well as the control did not show any change in the dry weight of the cotyledon. The radicle showed a slight increase in dry weight compared to the control (One and two days after the plumule excision, axillary plumule was not sampled because sufficient tissue was available only after three days).

After two days of the plumule excision in the experimental samples, the dry weight percentage remained unchanged. The radicle registered a slight increase in dry weight compared to the control of 5^{th} day and 6^{th} day experimental samples.

In the samples after eight days of germination (5+3 experimental), cotyledons not exhibited significant change compared to the earlier samples. The plumule which were developed in the axils of the cotyledon sampled on 3rd day after plumule excision contained only very low dry weight percentage. No change in dry weight was observed when compared to the original control (5^{th} day). However, the dry weight percentage of the plumule was slightly reduced ($P<0.02$) in comparison with the control.

Five days after the excision of plumule (5+5 experimental), Cotyledon, plumule and radicle showed no difference in dry weight compared to the control of the (5+5) day. However, when a comparison is made with the control of 5th day, the radicle exhibited a significant increase ($P<0.01$) in dry weight percentage.

2. Protein content

Seeds of winged bean variety 'PT 2' contained about 42% protein on the basis of dry weight (Table 2; Fig. 7). After 5 days of germination, the protein content was found to be slightly increased in the cotyledons compared to the dry seed. The plumule and radicle of the germinated seeds on 5th day showed more or less similar protein content ie. about 23 - 25% range.

The plumule excision was done on 5th day and the samples after one more day (5+1 experimental) showed increase ($P<0.01$) in protein in the cotyledon and the radicle compared to the 5th day control ($P<0.05$). The radicle showed more protein in the experimental than the control ($P<0.03$). The experimental samples after 2 days of plumule excision (5+2 experimental) contained more protein content in the cotyledons in comparison with the control (5+2). The radicle also showed an increase in protein than the control.

The protein content of cotyledon in the experimental samples after 3 days (5+3 experimental), was almost similar to the previous experimental samples (5+2) which when compared to control (5+3control) the cotyledons registered a significant increase. The axillary plumule sampled on the three days after original plumule excision (5+3 experimental) contained very high protein content compared to the plumule of the control (5+3) as well as the original plumule of fifth day of germination. The protein content of the radicle also was significantly higher in the experimental (5+3) than its control.

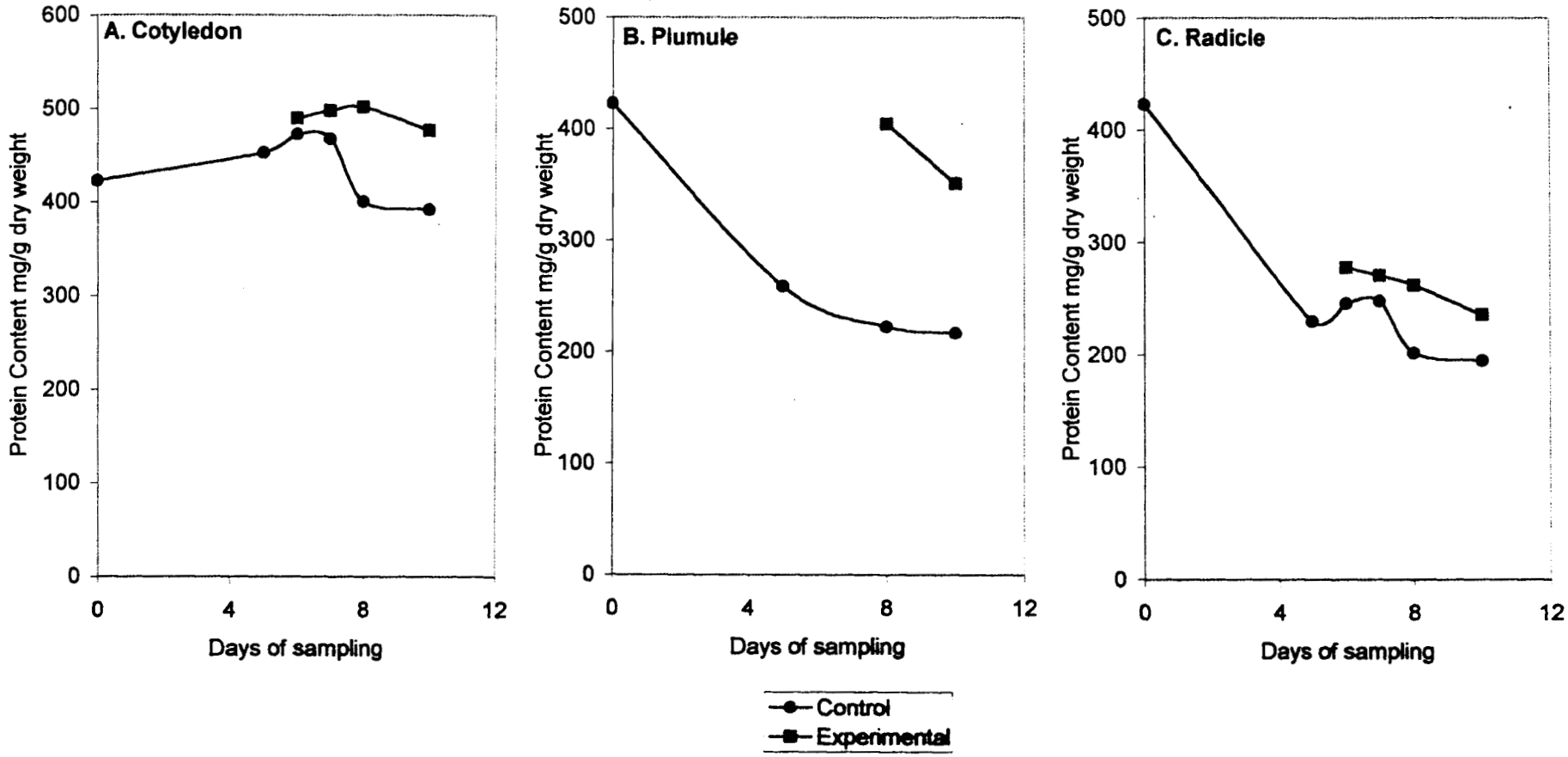


Fig. 7 Protein Content in mg/g dry Weight of Winged bean cotyledon, plumule and radicle during germination

Control : Intact seedling Experimental : Plumule excised seedling

On fifth day after plumule excision (5+5 experimental) the cotyledons showed a reduction in protein compared to the experimental cotyledons of the previous stage (5+3). The plumule showed a reduction in protein compared to the previous experimental sample. At this stage the experimental plumule showed more protein than the control. The protein content of the radicle decreased further ($P<0.01$) and the content was significantly higher than the control.

3. Starch

The variety PT 2 of winged bean seeds contained only very low amount of starch (Table 3; Fig. 8). However, during germination starch content of cotyledon was found to increase about three fold on 5th day. The plumule and radicle also showed comparatively considerable amount of starch.

After one day of plumule excision, the cotyledons sampled as (5+1) experimental, showed no change of starch content in comparison with the control and previous samples. In the radicle also, there occurred no significant change in starch both in the experimental and control.

After two days of plumule excision (5+2 experimental) the starch content of cotyledonary tissue was significantly higher than its control. In comparison with the experimental cotyledons of the previous stage (5+1 experimental), the starch content was very high at this stage. The starch content of the radicle was slightly higher in the experimental than the control.

TABLE III

Starch , Total sugar and Lipid content of winged bean seeds during germination and seedling growth after plumule excision

Days of Sampling	Tissues analysed	Starch mg/g	Total sugar mg/g	Lipid mg/g
0	Dry seed	2.04 ± 0.25	80.1 ± 1.4	227 ±17
5	Cotyledon	6.11 ± 0.22	42.4 ± 1.7	191±13
Control	Plumule	14.01 ± 0.41	71.8 ± 2.02	ND
	Radicle	13.9 ± 0.23	102 ± 6.12	ND
5 + 1*	Cotyledon	7.13 ± 0.27	49.2 ± 3.2	184±17
Experimental	Plumule	--	--	ND
	Radicle	15.4 ± 0.42	126 ± 7.4	ND
5+1	Cotyledon	6.9 ± 0.19	43.7 ± 2.4	178±18
Control	Plumule	--	--	ND
	Radicle	16.7 ± 0.91	131 ± 8.2	ND
5+2*	Cotyledon	13.3 ± 0.42	48.4 ± 2.9	171±13
Experimental	Plumule	--	--	ND
	Radicle	18.6 ± 0.71	108 ± 7.8	ND
5 + 2	Cotyledon	9.82 ± 0.44	40.5 ± 2.9	189±11
Control	Plumule	--	--	ND
	Radicle	16.8 ± 0.90	112 ± 5.4	ND
5 + 3*	Cotyledon	9.4 ± 0.34	41.2 ± 1.8	151 ± 14
Experimental	Plumule	4.22 ± 0.27	64.5 ± 3.8	ND
	Radicle	16.5 ± 0.69	94.4 ± 4.4	ND
5 + 3	Cotyledon	6.2 ± 0.18	40.7 ± 1.2	173±14
control	Plumule	12.14 ± 0.09	71.1 ± 4.9	ND
	Radicle	12.4 ± 0.49	70.2 ± 3.4	ND
5+5*	Cotyledon	8.7 ± 0.26	34.5 ± 1.8	122 ±09
Experimental	Plumule	5.61 ± 0.21	70.4 ± 5.1	ND
	Radicle	13.1 ± 0.38	78.1 ± 2.7	ND
5 + 5	Cotyledon	5.7 ± 0.18	35.1 ± 1.8	145 ± 09
control	Plumule	10.4 ± 0.39	64 ± 2.2	ND
	Radicle	9.5 ± 0.29	62.4 ± 1.9	ND

* plumule excision on 5th day and experimental sampled after 1 day, 2 days and so on.

Values are mean of 5 values ± standard error.

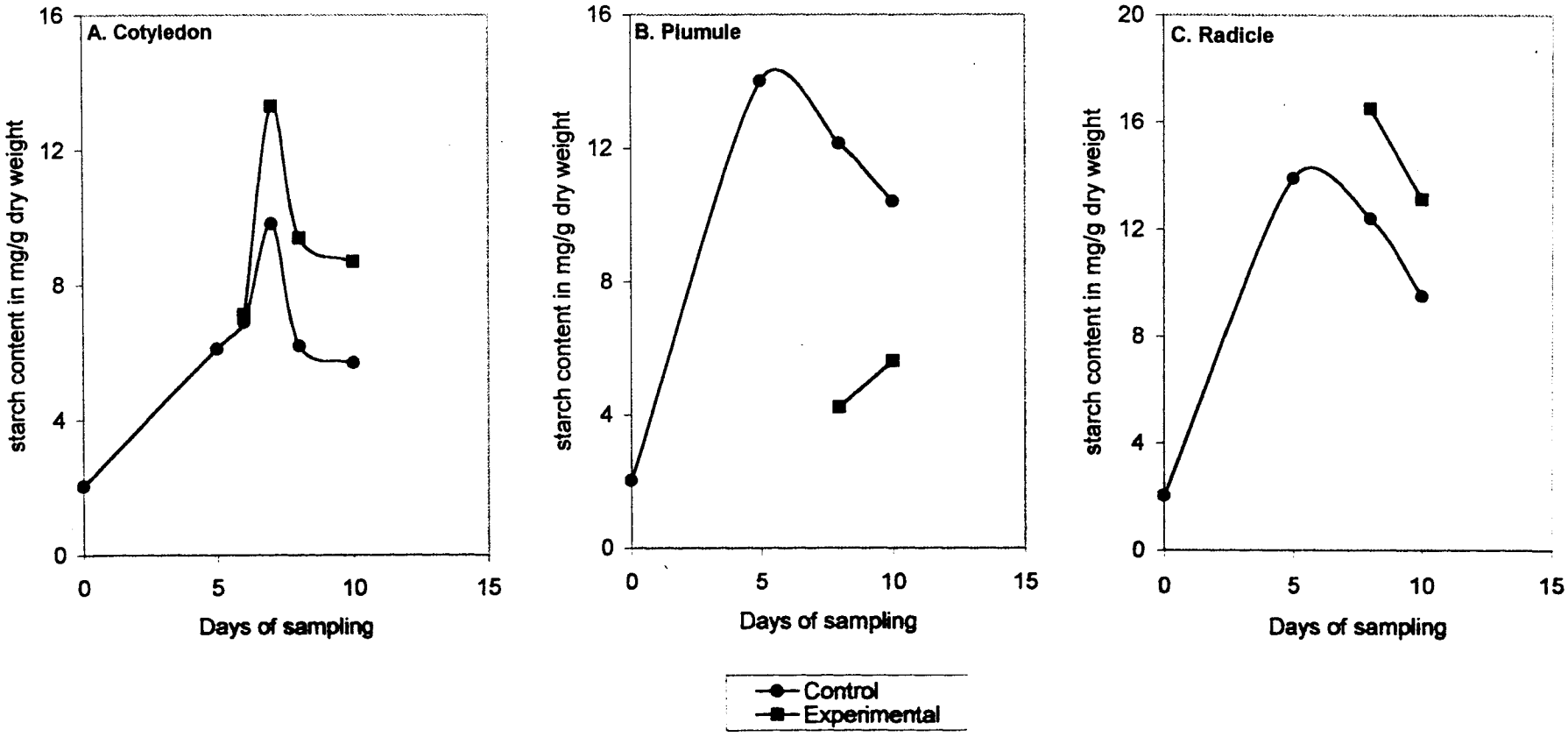


Fig.8. Starch content of winged bean cotyledon, plumule and radicle during germination and seedling growth
Control : Intact seedling Experimental : Plumule excised seedling

Starch content in the cotyledons of the experimental samples on (5+3) day again showed a significant reduction compared to the previous stage. However, the starch content was significantly higher in comparison with its control (5+3). Plumule (axillary) of the experimental of the sample contained only very low amount of starch compared to the corresponding control (5+3). Starch content of the experimental radicle tissue was slightly reduced ($P<0.01$) compared to the previous stage, but in comparison with the corresponding control (5+3), there occurred a significant increase.

The cotyledons of the experimental on 10th day (5+5) contained almost similar quantity of starch compared to the previous stage. However, this was significantly higher than the corresponding control (5+5). The starch content of plumule was about only half in the experimental when compared to that of the control on 10th (5+5) day. In these samples, the radicle showed a reduction in starch content than the previous experimental. While in comparison with the corresponding control (5+5) the starch content of the radicle was significantly higher in the experimental.

4. Total Sugars

Total ethanol soluble carbohydrates of winged bean seeds was about eight percent (Table 3; Fig. 9). During germination, the sugar distribution showed considerable variation among cotyledon, plumule and radicle. The radicle contained more sugar than the plumule and cotyledon on 5th day of germination. Plumule excision on 5th day resulted in an increase in sugar content of the cotyledon ($P<0.01$) and radicle ($P<0.01$) in the

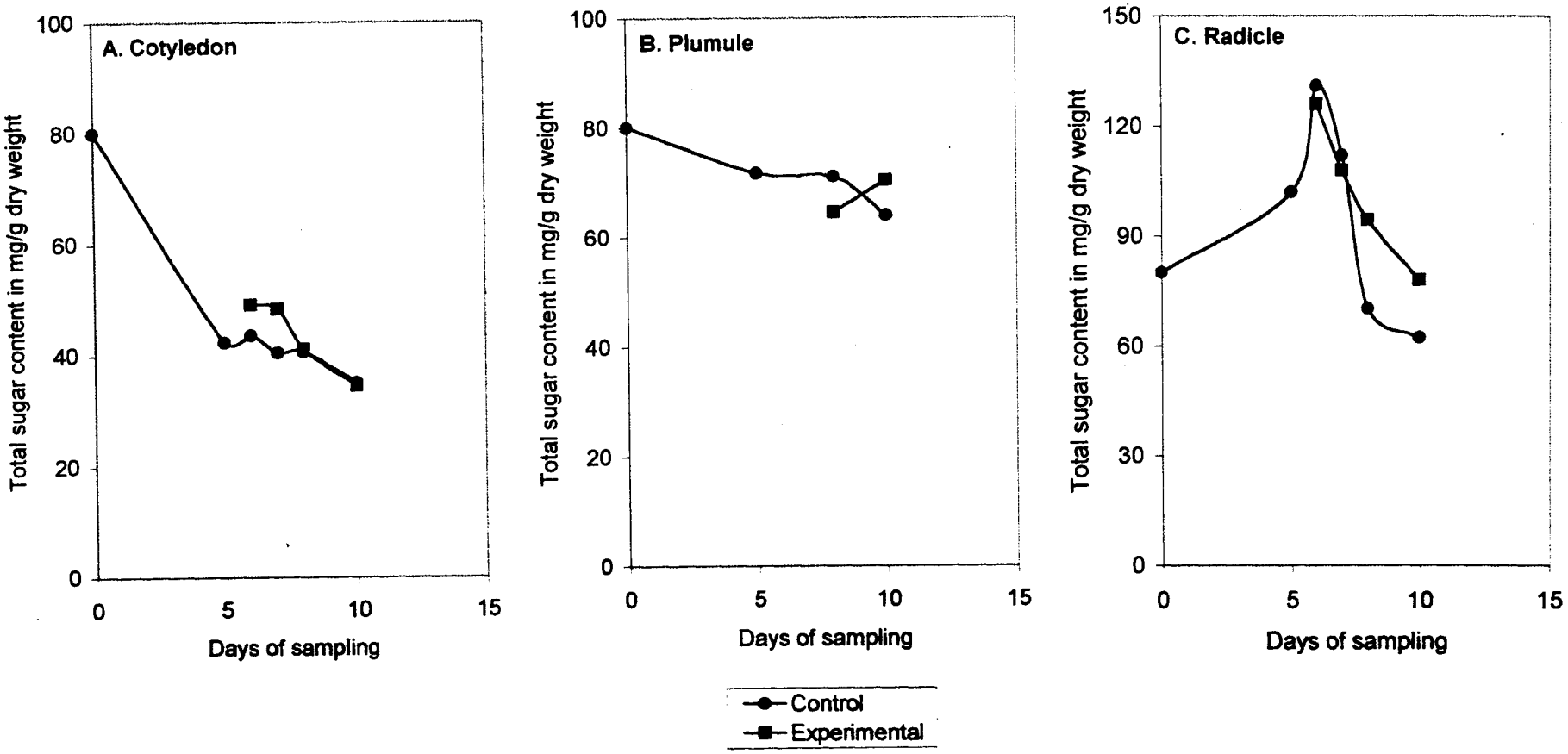


Fig. 9 Total sugar content of winged bean cotyledon, plumule and radicle during germination and seedling growth

Control : Intact seedling Experimental : Plumule excised seedling

experimental samples after one day (5+1 experimental), compared to the previous sample. Compared to the corresponding control (5+1) the total sugar content of cotyledons were more and in the radicle there observed no significant change.

Two days after plumule excision, (5+2 experimental) experimental cotyledons showed more sugars than the control ($P<0.01$). In this stage radicle exhibited no difference in sugar content than the experimental and control. However, the sugar content of radicle (experimental) was comparatively lower than the previous stage experimental (5+1 experimental).

The experimental samples of cotyledons after 3 days of plumule excision (5+3 experimental) registered a significant reduction compared to the previous experimental (5+2 experimental). There was no difference in sugar content of cotyledon between the experimental and control at this stage. Sugar content of axillary plumules (5+3 experimental) at this stage was comparatively similar to the control plumule of 5th day. The experimental radicle contained significantly high sugar content compared to the corresponding control (5+3 control). However, this sugar content was comparatively lower than the previous experimental.

After five days of plumule excision (5+5 experimental) the experimental cotyledons, exhibited further reduction in sugar content when compared to the previous stage, but between the corresponding control (5+3 control) no difference was observed in the sugar content of cotyledons. Sugar content of plumule tissue in this experimental sample

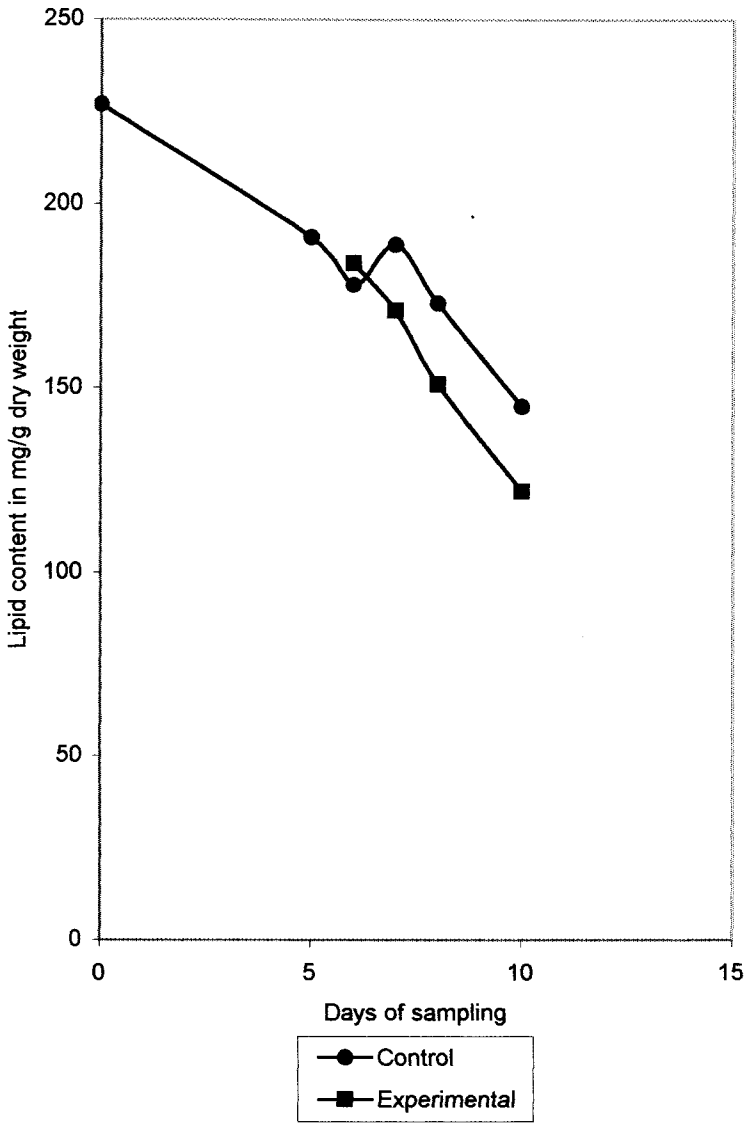


Fig. 10 Lipid content of winged bean cotyledon during germination and seedling growth

was almost same as that of previous experimental and only negligible difference was observed between the experimental and control on 10th day (5+5).

5. Lipids

Dry seeds of winged bean consist of about 22 percent lipid content. After five days of germination, the cotyledons showed a slight reduction of lipid, while measurable (gravimetrically) amount of lipid was absent in the plumule and radicle at this interval. During further growth of seedling both plumule and radicle contained no measurable (gravimetrically) quantity of lipid.

Plumule excision on fifth day resulted in no change in lipid content of cotyledons on 6^{th} day, but the control cotyledon exhibited a negligible reduction. After 2 days (5+2) both in the experimental and control no significant changes occurred. However, experimental cotyledons exhibited a reduction, compared to the previous experimental as well as control, of lipid content after 3 days (5+3) of plumule excision. After 5 days of plumule excision (5+5) also the experimental cotyledon exhibited a reduction ($P<0.02$) in comparison with its control and the previous experimental.

III. VASCULATURE

1. Vascular differentiation of cotyledons during germination

Figures reconstructed from serial cross sections of cotyledons (Fig. 11, 12) showed that cotyledons of dry seed contain three procambial strands in the region that attaches with the embryonic axis. From the

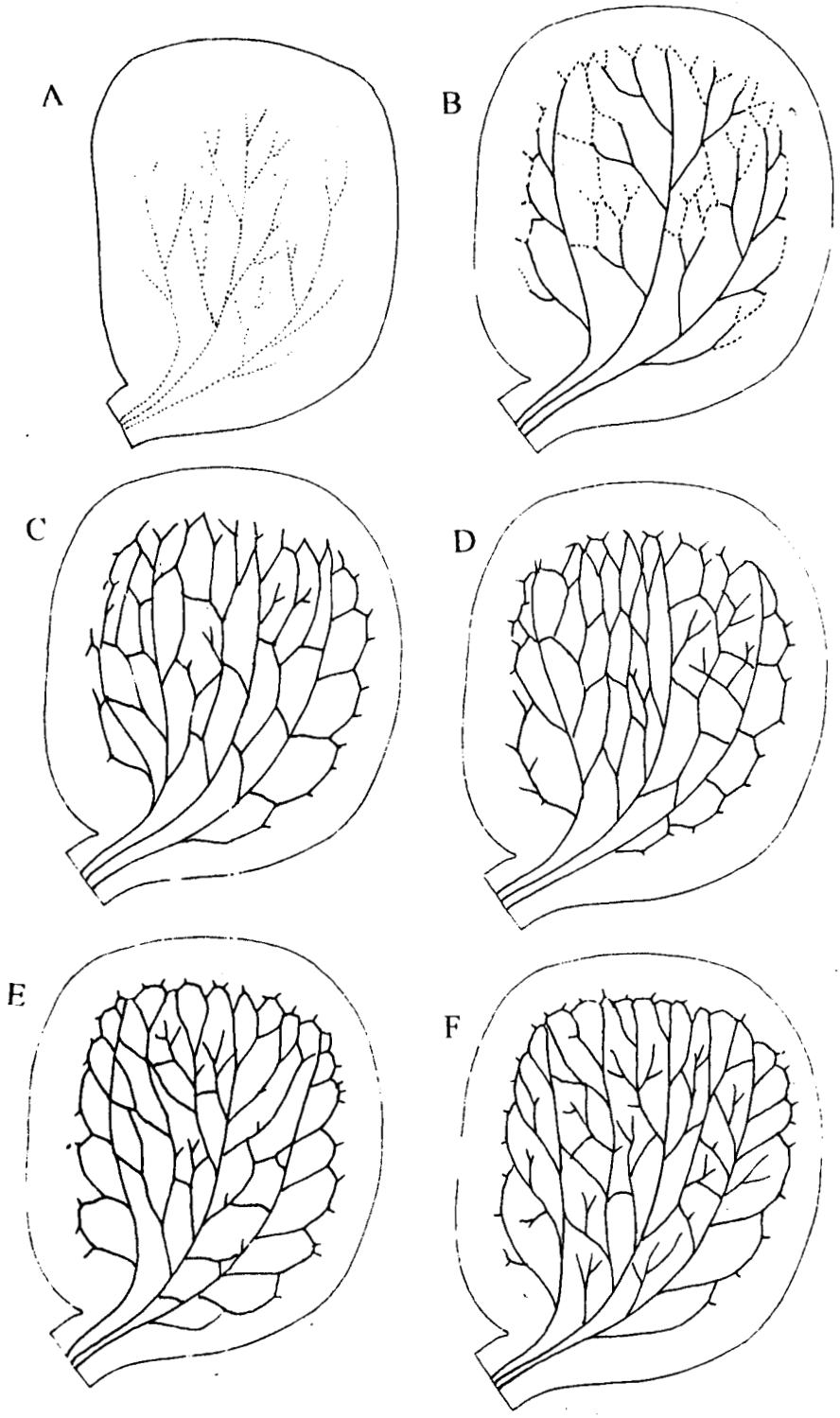


Fig. 11. Semi diagramatic drawings of vasculature in winged bean cotyledons.

A. Dry seed

B. Germinated seedling after 5 days

C. Germinated seedling after ½ day of plumule excision (5 + ½ experimental)

D. Germinated seedling after 5½ days (5 + ½ control).

E. Germinated seed after one day of plumule excision (5+1 experimental)

F. Germinated seed after six days (5+1 control)

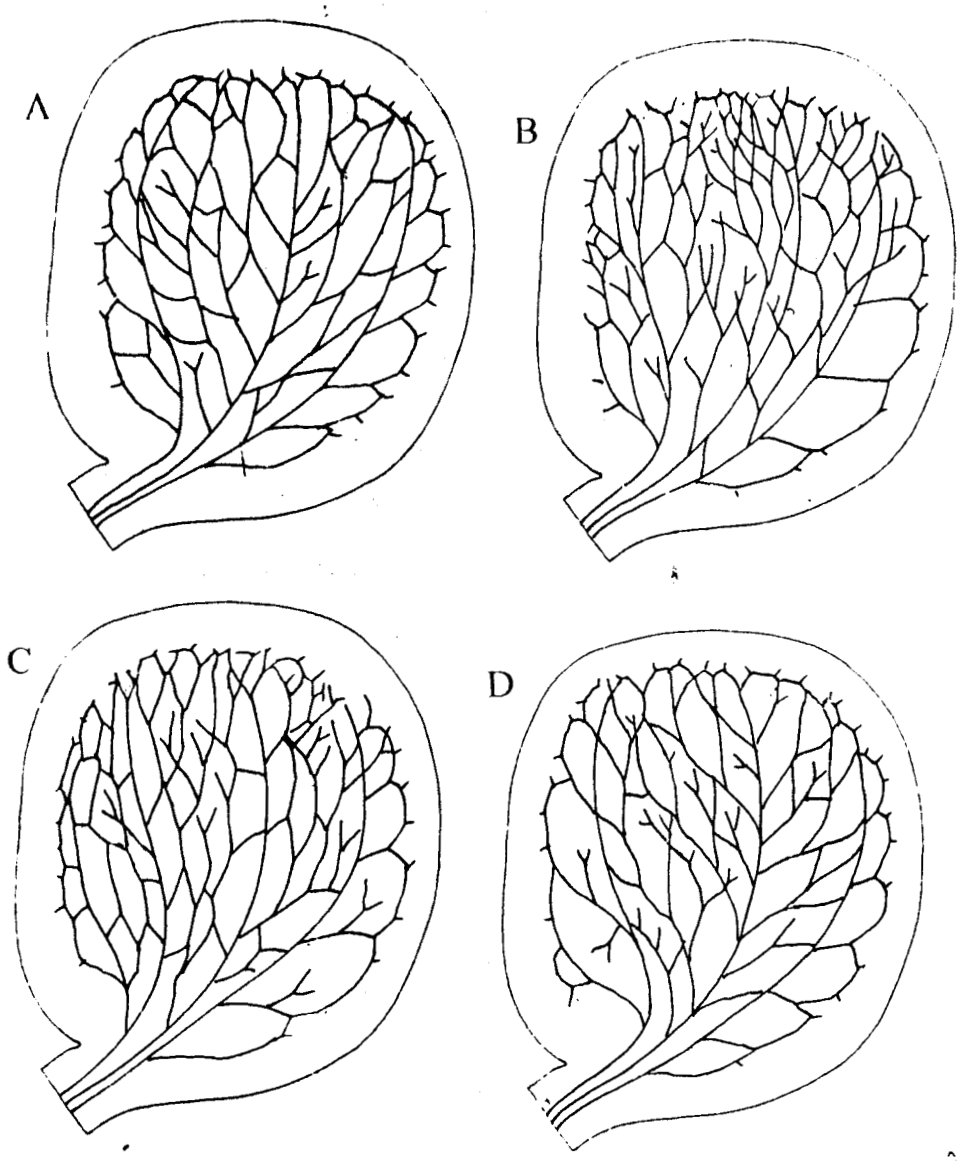


Fig. 12. Semi diagramatic drawings of vasculature in winged bean cotyledons

A. Germinated seedling after two days of plumule excision (5+2 experimental)

B. Germinated seedling after seven days (5+2 control)

C. Germinated seedling after three days of plumule excision (5+3 experimental)

D. Germinated seedling after eight days (5+3 control)

sections stained for protein (Plate 3) it is clear that these procambial strands consists of only thin walled elongated cells.

During germination these vascular strands branched from all the three initial strands and rebranching was increased further during seedling growth to acquire a net work like appearance, similar to the venation of foliage leaves.

In the seedlings when the plumule excision was made there occurred only slight variation in the net work like venation between the experimental and control. Generally it was seen that the branching of vascular strands was more in the control(Fig. 11, 12) than in the experimental. However more branching of vasculature was seen when the seedling growth was advanced upto eight days, both in the experimental and control.

2) Cotyledonary vascular differentiation of seedling grown in garden soil and acid washed sand

a. Control (Dry seed)

As mentioned earlier cotyledons of dry seeds exhibited three provascular strands near the region of attachment to the embryonic axis (Fig. 13).

b. Samples after six days of germination

The provascular strands of the cotyledons get branched gradually from the attachment region to distal end. Both experimental and control

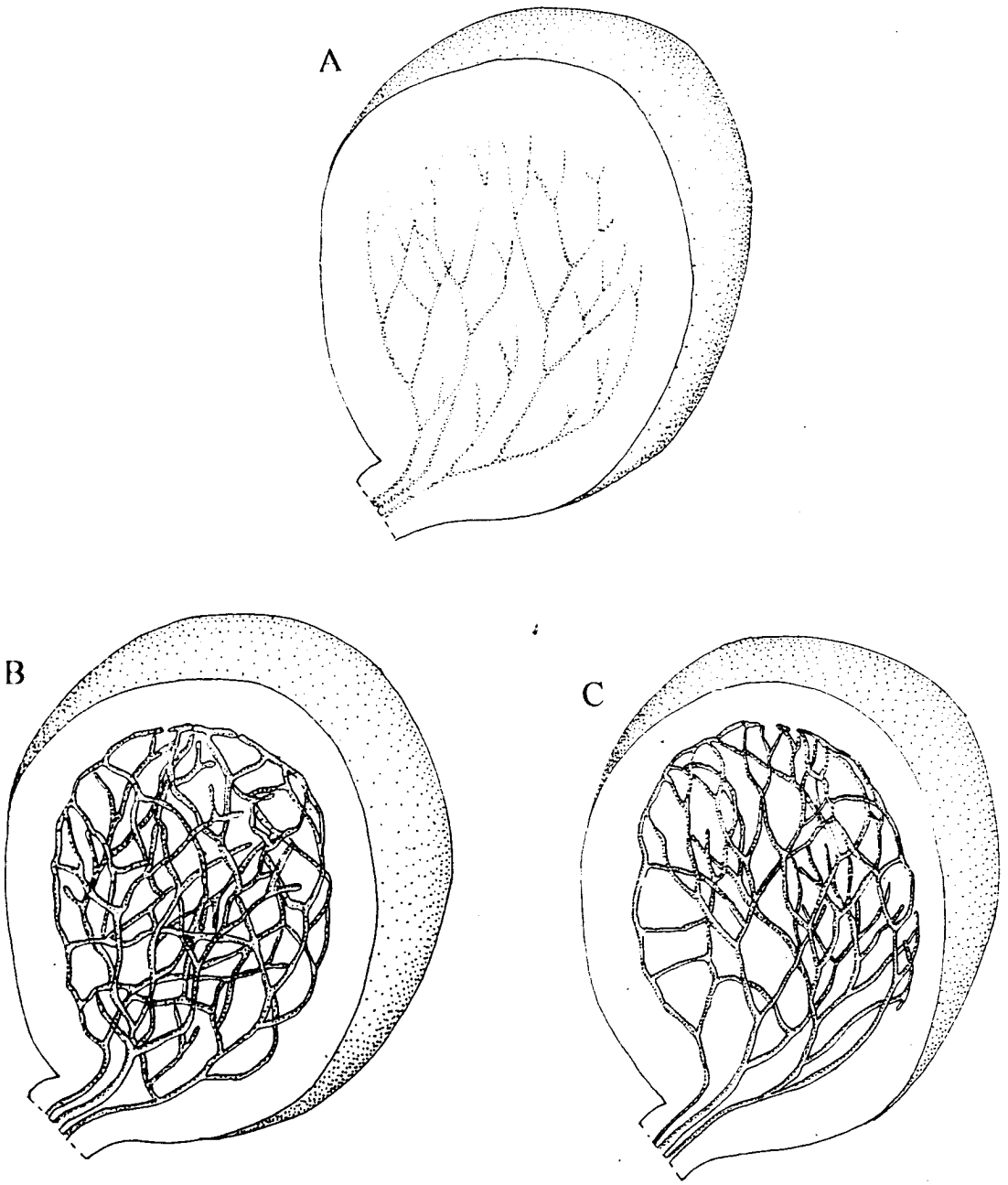


Fig.13. Effect of nutrient stress on vascular differentiation in winged bean. Vascular structure reconstructed from serial cross sections of cotyledon of

A. dry seed. **B.** Six days after germination in garden soil

C. Six days after germination in acid washed sand.

did not show much variation in the pattern of branching of vasculature (Fig. 13).

c. Samples after twelve days of germination

After twelve days the cotyledons of seedlings grown in garden soil exhibited further branching compared to that of the previous stage. Cotyledons of the seedling grown garden soil (control) showed more branching compared to that of the acid washed sand (Experimental) (Fig. 14)

d. Samples after eighteen days of germination

Branching pattern of the vasculature in the cotyledon was similar to the previous stage (Fig. 14).

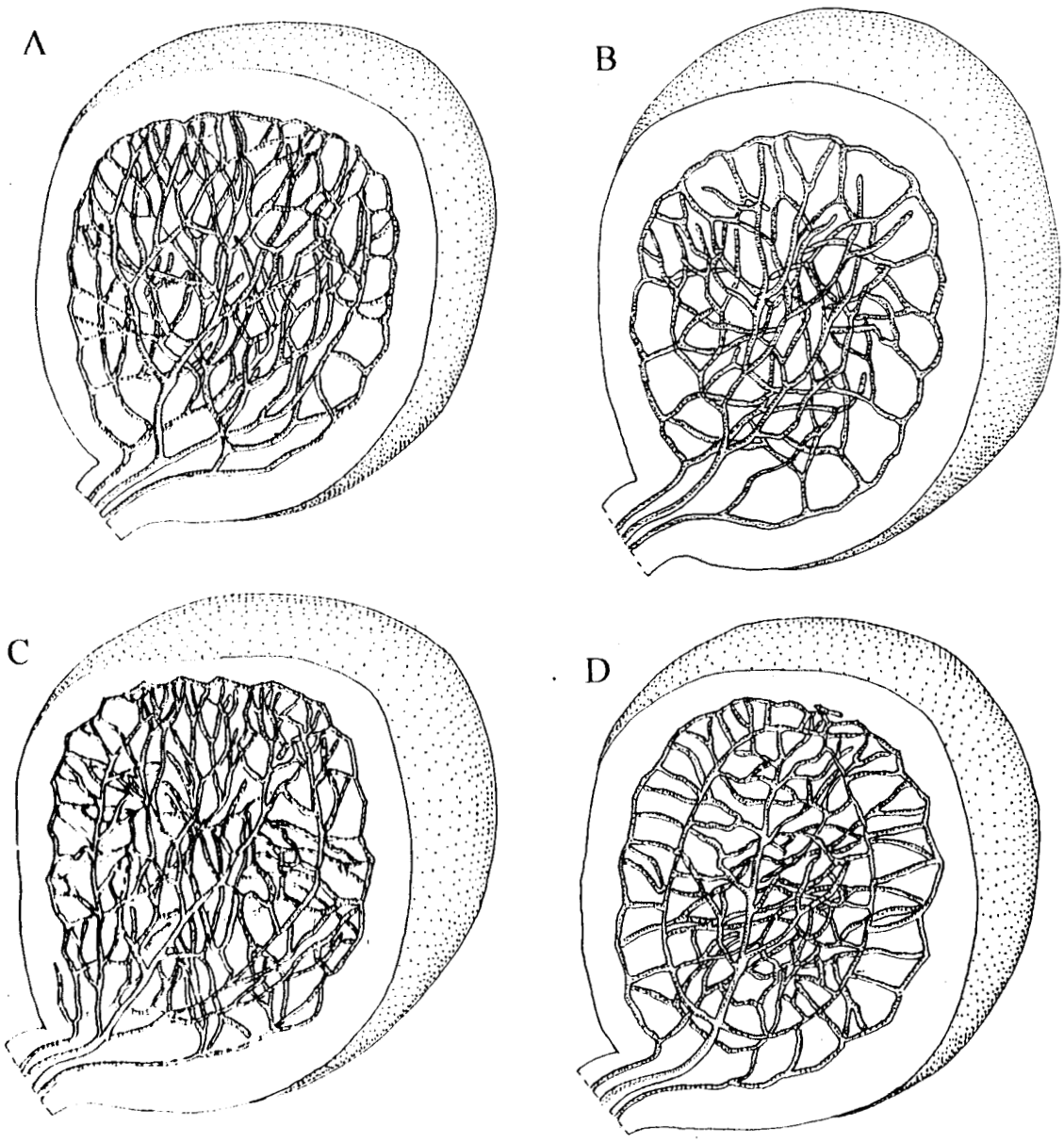


Fig.14. Effect of nutrient stress on vascular differentiation in winged bean. Vascular structure reconstructed from serial cross sections of cotyledon

A. Twelve days after germination in garden soil
B. Twelve days after germination in acid washed sand.
C. 18 days after germination in garden soil
D. 18 days after germination in acid washed sand.

DISCUSSION

DISCUSSION

Since the embryonic axis of seeds requires energy for growth during seed germination, storage compounds must be hydrolysed into soluble forms, translocated from the cotyledon/endosperm to the embryonic axis and transformed to energy bound molecules that can be immediately and easily utilised. In monocots, the hormonal regulation of storage product degradation is well documented (Bewley and Black, 1983, 1985, 1994; Mayer and Poljakoff-Mayber, 1989; Copeland and McDonald, 1995). However, in dicots, the hormonal activation of reserve degradation is never as great as that of cereals.

Many investigators, believe that dicot seed germination is mediated by the growing embryonic axis, which continue to grow and incorporate breakdown products in to synthesis of new compounds. Regulation of reserve mobilisation from cotyledons by the axis (Murray, 1984) reveals that axis tissue functions as sink for reserves mobilised from the cotyledon. The reduced concentration of compounds in the cotyledons, in turn stimulates the hydrolysis of other storage reserves for use by the embryonic axis. This stimulation prove to be too great, and hydrolysed storage products begin to accumulate, a feed back mechanism may be operative that retard further storage reserve hydrolysis (Copeland and McDonald, 1995).

The mobilisation and transfer of nutrients in germinating seeds is through the conductive tissue of the cotyledon to the growing axis and this process is regulated by several factors. The most documented factor is hormones. Oligosaccharides and sugars are consumed in the respiratory metabolism during early phase of germination (Mayer and Poljakoff-Mayber, 1989). Protein comprises the most abundant intrinsic reserve and get hydrolysed after imbibition (Dalling and Bhalla, 1984). Trelease and Doman (1984) suggested that lipid reserves are mobilised immediately after germination ie during seedling growth. According to Copeland and McDonald (1995), the hydrolysis of protein bodies occurs at the centre of the cotyledon and then moves to wards the outside of the cotyledon. Mobilisation of all these reserves leads to considerable reduction in the dry weight of seeds during germination and seedling growth.

Dry weight distribution of winged bean seeds during germination shows a significant reduction on 5^{th} day (Table 2, Fig. 6) indicating a high rate of water uptake during imbibition and germination. In winged bean it has been proved that during germination only very small quantity of dry matter is mobilised from the cotyledons to the growing embryonic axis (Kamaladevi *et al.*, 1989, Nabeesa-Salim and Harikumar, 1994). The plumule excision on 5^{th} day after germination does not show any significant difference in dry matter content in the cotyledons compared to that of the intact seedlings (control) (Fig. 6). Similar results have been reported by Kamaladevi *et al.* (1989) in winged bean. The results clearly show that plumule excision is not affecting the mobilisation adversely. Plumule excision results in a reduction of sink activity, concomitant with

a dry weight increase in the cotyledons compared to intact seeds is not significant revealing a diversion of metabolites utilisation. During later periods of seedling growth after plumule excision (5+5) the radicle shows a significant accumulation of dry matter, but the resultant reduction is not seen in the cotyledons probably by the reserve mobilisation to the cotyledonary axillary buds which grows profusely from the axils of both cotyledons (Plate-2).

Another explanation for the significant increase of biomass in the radicles due to plumule excision can be drawn from the hormonal regulation of reserve degradation in the cotyledons during germination. The plumule excision results in a transient inhibition on axis growth and a reduction in metabolite degradation and translocation. But the immediate development of cotyledonary axillary buds, triggered probably by removal of apical dominance serve as a sink for metabolites. Moreover the axillary shoots produce large quantity of auxins and results in a high reserve degradation as suggested by Sutcliffe (1976). But the axillary buds are only in the differentiating stage, so the sink activity is not so strong, while the radicle shows profuse growth. Hence enhanced reserve mobilisation may be taking place to the radicle resulting in an accumulation of dry weight.

Contradictory to the findings of the present investigation, Sutcliffe (1976) observed that dry matter content of the roots in de-shooted plants was lower than that of intact plants, but total nitrogen, phosphorus and sulphur contents were higher. The distribution of cotyledonary reserves between the shoot and root depends on the relative rates of growth of the

two organs. Dark-grown pea seedlings accumulated higher proportion of the transported reserves in the shoot than light-grown seedlings.

The biomass distribution of the cotyledons of winged bean is not affected by the plumule excision. Nevertheless, the plumule removal influences the pattern or orientation of reserve mobilisation. In other words, the source activity of the cotyledons is ceaselessly taking place irrespective of the difference in the position or directions of sinks.

Protein metabolism of winged bean seeds during germination has been studied by many authors (Kortt, 1986; Kamaladevi *et al.*, 1989; Kortt *et al.,* 1991). Despite the high protein content (about 40%) of winged bean seed reserves, the utilization and mobilisation during germination is very feeble, the variety PT 2 contains 42% of protein in dry seeds and up to 5 days of germination a slight increase in protein content is observed. *De novo* synthesis of protein during germination (Bewley and Black, 1983, 1985; Mayer and Marbach, 1981) may contribute to this increase. The plumule excision resulted in a slight increase of protein in the cotyledons when compared to the respective controls and the increase is continuously observed in all experimental samples with a further increase in all intervals. So it is evident that storage protein utilization and mobilisation in the experimental samples are relatively at reduced rate. It seems that protein degradation might have taken place but the resulting amino acids are used for de *novo* synthesis of proteins which are essential for the morphological changes like apical dominance removal and enhanced radicle growth occurring due to the plumule excision. Direct utilisation of free amino acids formed by proteolytic activities on storage protein, are occurring in germinating

seeds (Quail, 1979; Copeland and McDonald, 1995). So it is clear that storage protein are not catabolized considerably in winged bean presumably due to high lipid content which is found to be reduced significantly during germination (Table-2; Fig. 10). According to Shutov and Vaintraub (1987) during germination proteins undergo quantitative changes (modification). Many storage proteins become more soluble (Tully and Beervers, 1978) and susceptible to the action of more endogenous proteases which are *de novo* synthesised or translocated during germination (Shutov and Vaintraub, 1987). However it seems that changes now register a net gain or loss in the total protein in winged bean and it is found that only very low quantity of protein is utilised during germination (Table-2, Fig. 7).

Eventhough degradation of protein bodies can seen at various grades in the cells around the vasculature (plate-7-10) generally protein metabolism is found to be very slow because the degraded protein granules and small patches are retained in almost all cells even in the cotyledon of seventh and eighth days. In this character winged bean seems to be a unique legume since *Arachis* (Bagley, *et al.*, 1963) *Phaseolus* (Opik,1966) and *Pisum sativum* (Bain and Mercer, 1966) showed protein body coalesce and complete disappearance during germination. The cell population where the protein hydrolysis starts, around the vasculature is generally very low in comparison with the other cells of cotyledons which are almost filled with protein granules, degraded as well as intact.

Protein mobilisation of the cells in the adaxial side is taking place at a faster rate. So most of the cells towards the adaxial side is devoid of

protein content both in control and experimental cotyledons after seven days of germination and two days of plumule excision respectively (plate 9, 10).

In spite of the marked hydrolysis of protein content, many cells near the vasculature in most of the ground cells of the adaxial side shown by histochemical studies, reduction occurred in protein content as estimated by biochemical methods is very low. The probable reason is that small peptides and/ or free amino acids formed after protein hydrolysis in the cotyledonary cells, especially near the vascular strands may be immediately utilised for *de novo* synthesis of many metabolically active proteins, hence net reduction is negligible. *De novo* synthesis of proteins is reported in many seeds during germination (Bewley and Black, 1983; Wilson, 1987: Torrent *et al.*, 1989).

The limited rate of protein mobilisation from the cotyledons of winged bean during germination can be correlated with the vasculature differentiation of the cotyledons. Eventhough cotyledons contain more than 40% proteins (Table 2), protein body hydrolysis is observed only in the cells near the vasculature (Plate 8). Morphologically, cotyledons are very conspicuous but the distribution of vascular strands is very sparse in the cotyledons compared to its size and weight. So quantity of cells where protein degradations are taking place is very low in comparison with the whole cell population of the entire cotyledon. According to Quail (1979), the fate of protein in germinating seeds has not been well documented. However the major storage proteins which constitute 50-70% of the total extractable protein can be histochemically localised. In

agreement with this view, the differences in the protein content studied histochemically and biochemically in winged bean is negligible.

Protein body degradation is clearly seen in the cotyledonary cells of winged bean during germination particularly near the vascular strands (Plate 6, 7). Gradual hydrolysis of protein bodies finally results in the formation of vacuoles. Almost similar observation is reported in *Pisum sativum* cotyledons, the protein bodies clumped together or vacuolated indicating their break down during germination (Ashton, 1976). According to him in *Pisum arvense* cotyledon protein bodies fuse to form large aggregate bodies, In *Glycine max* cotyledons, protein bodies become granular and limiting membrane disappears whereas in pea nut (*Arachis hypogea*) cotyledons protein bodies swell and develop cavities, some assume a loose spongy structure and aggregate in the centre of the cell and then disintegrate into fragments which disappear as germination proceeds. Almost all these characters of protein body degradation can be seen in the proteins of winged bean cotyledonary cells, localised histochemically during germination (Plate 6-10).

Protein hydrolysis during germination of cereal grains has been emphatically described by Dalling and Bhalla (1984) and they suggested that in the early stage of germination, the protein hydrolysis provide amino acids for the synthesis of new proteins. Afterwards hydrolysis of reserve protein results in the production of amino acids to the growing axis and finally the reserve proteins get depleted due to more translocation resulting in ageing or senescence of source tissue i.e. endosperm/cotyledon. Nevertheless the protein degradation of winged bean seed cotyledons is slow even after plumule excision revealing the

limited utilisation of protein reserves for the synthesis of new proteins or for translocation to the growing axis. In other words, protein depletion during germination is comparatively negligible probably due to the protein abundance in winged bean seeds (Table 2; Fig. 7; Plate. 3-10).

The protein content of the axillary plumular shoot developed from the axils of cotyledons, sampled after two days of plumule excision is found to be significantly higher than the control on 5^{th} day. When a comparison is done between the respective controls also, these axillary plumule showed more protein (Table-2, Fig. 7). This abundance of protein in the actively growing plumule is correlated with the high meristematic activity of the shoot apex. The protein content of radicle showed that the plumule excision resulted in a slight difference between the experimental and control. However these changes are not significant. More reduction of protein content occurs in the control so it seems that plumule excision enhances protein synthesis in the radicles, being a comparatively more active sink, radicle receives more degradation products of protein (amino acids) from the cotyledons.

Histochemical localisation of proteins in winged bean clearly indicates that the degradation of protein bodies initially starts in the cotyledonary cells present around the vasculature and spread to the distant cells as seedling growth advances. Protein bodies of seeds contain non-storage enzyme proteins also within the limiting membrane and during germination proteolytic activities are exhibited (Quail, 1979). Chrispeels *et al.* (1976) conclusively demonstrated that in mung bean some proteases present in protein bodies are incapable of degrading endogenous legumin and vicilin. So for mobilisation of these proteins *de*

novo synthesis of some peptidases is essential. So in winged bean it seems that the storage protein present in the protein bodies of cells near the vasculature contain proteolytic enzymes and they become active soon after germination. While the protein bodies of cells away from vasculature may require *de novo* synthesis of proteases and so their degradation is comparatively delayed.

Removal of embryonic axis is reported to affect metabolism of cotyledons in germinating seeds. Embryonic axis excision results in depression of proteolytic enzymes in the cotyledons (Guardiola and Sutlciffe, 1971; Yomo and Varner, 1973; Kern and Chrispeels, 1978; Minamikawa, 1979; Morohashi, 1982) and consequently mobilisation of proteins is slowed down (Kern and Chrispeels, 1978; Chin *et al.*, 1972; Davies and Chapman, 1979a, 1979b and 1980)

Effect of embryonic excision on break down of storage proteins in the cotyledons of *Citrus limon* (Garcia-Agustin *et al.*, 1991) revealed that the proteolytic enzymes, endopeptidases in particular, are inhibited. It is proposed that the cytokinin released by the radicle may control the endopeptidase activity in *Citrus limon* cotyledons.

Accoding to Matile (1982) only endopeptidases and carboxy peptidases are present in protein bodies and these enzymes are not able to complete their break down to free amino acids. Apparently dipeptides or tripeptides may be formed and can be translocated to other regions. In the staining pattern of winged bean it is very evident that protein degradation taking place in the cotyledonay cells around the vasculature,

shows different gradation of hydrolysis and different types of proteinaceous masses or patches also are localised in these cells.

Metabolism of insoluble polysaccharides particularly starch in winged bean seeds is controversial among many investigators (Kortt, 1986; Kute *et al.*, 1984; Kamaladevi *et al.*, 1989), probably, due to varietal difference. In the present study the variety PT2 contains very low amount of starch in dry seeds. But during germination the starch content increased significantly and upto 10 days of seedling growth, even after plumule excision the starch content do not show any significant quantitative change (Table 2, Fig. 8).

The plumule contains considerable quantity of starch (Table 2). But a pair of cotyledonary axillary shoots developed as a result of apical dominance removal by excising the original plumule, exhibits comparatively low amount of starch. It is probable that the original plumule developed from the embryonic axis which contains considerable amount of starch in ungerminated condition is a characteristic of many other seeds (Smith, 1974; Murray, 1984). But the cotyledonary axillary plumule originated from the rudimentary axillary buds occurring in the form of group of meristematic cells in the dry seed, are very potentially growing tissue and in which only some starch accumulation is observed. An interesting observation is that these axillary plumules showed uniform distribution of total sugars as that of the original plumule during all stages of germination irrespective of the plumule excision (Table 3, Fig. 9). On the other hand, the radicle of the original seedling containing almost similar quantity of starch, as that of the plumule, showed further increase

upto 7 days of germination but soon after plumule excision, starch content of radicle increased compared to the control radicle (Table 2).

The starch accumulation in the radicle of plumule excised seedlings may be due to the enhanced translocation of sugars from the cotyledons and their immediate conversion into starch. Nevertheless the stimulated growth of radicle exhibited morphologically (Plate 2) as well as dry weight increase (Table 2, Fig. 6) further reveals the enhanced translocation of soluble carbohydrate to the radicle due to plumule excision. The distribution of whole carbohydrates (sugars) also shows almost the same pattern (Fig. 9) of distribution in the radicle tissue.

Both total sugar content and starch in the cotyledons indicate that the mobilisation or inter conversion of these metabolites are rather very low and the slight decrease in these contents during later stages (8-10 days) of germination is partially due to lack of ageing or senescence of cotyledons which characterizes the seedling growth in many plants (Dalling and Bhalla, 1984).

Eventhough starch mobilisation and metabolism during germination varies from plant to plant, Marbach and Mayer (1976) and Garcia-Luis and Guardiola (1978) reported that sugars are not accumulated in the cotyledons. In winged bean cotyledons the starch is synthesised from lipid by gluconeogenesis. Notwithstanding the enzymes are not accumulated. Starch grains localised in cotyledons shows that the cotyledons of five days old seedlings contained starch grains. The plumule excision is affecting starch grain localisation considerably. At all intervals, the experimental cotyledons contained more starch grains than

the control. Generally the adaxial side particularly near the attachment to the axis showed large amount of starch grains. The translocation of sugars formed in the cotyledons are curtailed as a result of plumule excision and they are converted into starch and getting accumulated.

According to Monerri *et al.* (1986) starch hydrolysis and mobilisation is reduced when the embryonic axis is removed in *Pisum sativum* seeds and this may at least in part, be due to resynthesis of starch from the products of starch hydrolysis already occurred. Plumule excision in winged bean exhibited an increase in starch grain accumulation in the cotyledonary cells of the experimental compared to the control and the same trend was observed throughout the experiments.

Both biochemical (Fig. 8) and histochemical (Plate 13) studies on starch in winged bean confirmed that starch content is very low in dry seeds. This observation is in agreement with the reports by Kute *et al.* (1984) and Kamaladevi *et al.* (1989) However during germination starch is increased in the cotyledons (Fig. 8). Starch grains are localised histochemically in the cotyledons of 5 day old seedlings (Plate 14). Only a few studies exist on starch degradation in legumes during seed germination (Bewley and Black, 1983). Contrary to the commonly accepted view that the process of germination is associated with the extensive degradation of stored starch, an actual synthesis of starch was reported in *Phaseolus vulgaris* (Smith, 1974; Briarty and pearse, 1982).

In microstructure studies of the winged bean Saio *et al.* (1983) found starch grains in the cotyledons of some varieties. Ravindran and Palmer (1984) reported that a particular cultivar of winged bean

contained little or no starch. The present study also confirms the absence of starch grains in the cotyledons (Plate 13) of dry seeds of winged bean.

The best histochemical reaction for starch is Periodic acid-Schiff's reagent staining. In winged bean cotyledons unexpected staining reaction is encountered in some cells. Densely stained patches (Plate 13) covering some cells and less intense stained material filling the lumen of some other cells are observed in the dry seed cotyledons. These got reduced gradually with progress in germination (Plate 14-22). The treatment of the fixed sections of dry seeds with a mixture of ether and alcohol (1:1) resulted in a partial reduction of the unspecific staining with PAS, suggesting that lipid is being stained. The unspecific staining material is lipids escaping the processing of the tissue for histochemical studies. From the studies on lipid localisation, these materials are found to be Sudan IV positive. According to Jensen (1962) lipid staining is normally done in fresh tissues, but in the method of Gurr (1965) formaldehyde fixed tissue are found to be specifically stained for lipids. Lipids are known to interfere in the PAS reaction for polysaccharides (Hale, 1957; Jensen, 1962). Stored lipids are degraded and utilized during germination and this will account for the progressive decrease of unspecific staining in the cotyledonary cells during seedling growth.

According to Meir and Reid (1982) and Reid (1985) non starch polysaccharides also may contribute to cotyledonary reserve material in legumes. The cotyledons of seeds of certain lupin species have massive thickened cell walls, which actively participate in seedling development. However, in winged bean the cotyledonary cell wall thickness is not

altered during germination in spite of the cell enlargement following water uptake.

The appearance of starch grains (Plate 14-22) and increased starch content (Fig. 8) in the cotyledons of winged bean seeds during germination may be attributed to gluconeogenesis from lipid reserves. Acetyl CoA of lipid origin is used for carbohydrates synthesis *via* glyoxylate cycle during germination in fat rich seeds (Beevers, 1980). Sucrose is generated through intermediate function of hexose phosphate by reversal of glycolytic reactions. The sucrose synthesized in the cotyledons is transported into phloem or converted into starch and stored in the plastids. Starch synthesis during germination, presumably from lipids, has been reported in jojoba (Moreau and Huang, 1977) in soybean cotyledons (Adams *et al.* 1980) and castor been endosperm (Reibach and Benedict, 1982). Another potential source of carbohydrates during legume seed germination, inclusive presumably of winged bean is the carbohydrate prosthetic group of storage proteins (Lamport, 1980).

Phosphatase activity is constitutive of dry seeds of winged bean and an increase in acid phosphtase activity occurs during germination (Kamaladevi *et al.*, 1989; Nabeesa and Harikumar, 1990). The increase in fructose 1, 6- bisphosphatase activity fit in with the concept of the gluconeogenetic reactions in winged bean cotyledons during germination. The activity of fructose 1, 6- bisphosphatase increases several fold in castor bean at the time of lipid degradation (ApRees, 1980; Bewley and Black, 1983).

The elaboration of starch in the cotyledons of winged bean during germination may serve as a temporary sink for soluble degradation products from reserve metabolites mainly lipids, thereby limiting the build up of sugars that may inhibit further gluconeogenesis reactions. The transformation of sugars to starch during germination would also serve to maintain osmoticum of the cells.

A slight reduction of the starch (Plate 21,22 Fig. 8) during later stage of germination indicate that this transient storage of starch reserves act as a source of the sugar for further development. The plumule excisions resulted in the slight depletion of starch also reveals that a pair of potentially growing cotyledonary axillary plumules (Plate 2) and vigorously differentiating root system are utilising the starch formed during early period of germination.

A temporary oversupply of sugars from lipids *via* glyoxylate cycle resulting in an accumulation of transient starch levels in *Tagetes minuta* has been reported by Drewes and Van Staden (1991). According to these authors lipid degradation products never directly involved in respiration, but sugars or starch are synthesized from the storage lipids. The results observed in winged bean seed are agreeable with this finding.

Starch reserves in legume seeds are normally degraded during germination and this process has been documented for a range of species (Bewley and Black, 1983, 1985). But, Briarty and Pearce, (1982;), Preiss, 1980; Smith, 1974) noted appearance of starch granules in the cotyledon of *Phaseolus vulgaris* during germination. Webster and Leopold (1977) and Adams *et al.* (1980) also reported similar results in *Glycine max*.

In contrast to the large size of the starch grains formed during seed development, grains synthesized during germination are smaller having the mean volume 20 μm^2 and 50μm^2 in *Phaseolus* and *Vicia* respectively are found grouped together around the nucleus. In winged bean cotyledons the average size of starch grains in the cotyledons of control seedling is smaller (Table 1) and during seedling growth the starch grains are enlarged .

It is reported that the cell wall polysaccharides constitute quantitatively important reserves of *Lupinus angustifolius* and this is almost equal to protein (Crawshaw and Reid, 1984). Matheson and Saini, 1977) suggested that monosaccharide residues are lost from the cotyledonary cell walls and it seems likely that the loss of these monosaccharides permit cell wall expansion during germination. Nevertheless in winged bean the periodic acid- Schiff's reagent staining does not show any lose of cell wall thickness during germination inspite of cell enlargement and cell wall expansion occurred during imbibition and germination (Table 1). Two proteins named as expansins have been isolated from growing cucumber hypocotyl walls. These expansins can enhance wall elongation without altering the molecular mass of polysaccharides without hydrolysing pectins or hemicelluloses (McQueen-Manson and Cosgrove, 1995). In winged bean seed the cell expansion occurs without effecting the cell wall thickness and expansins may play a role in the cell enlargement during imbibition and germination.

Histochemical localisation of lipid also exhibit almost similar pattern of protein degradation. However, fat is found to be the first seed

reserve to undergo fast hydrolysis and utilisation in winged bean particularly during seedling growth as confirmed by analytical data (Table 3, Fig. 10). It seems that lipid degradation by lipases activity and further hydrolysis by β oxidation also mainly confined to the cotyledonary ground cells located near the vasculature.

Starch content of cotyledonary cells, when histochemically localised shows their occurrence only in germinating seeds (Plate 16) and a concomitant loss of lipid can also be observed indicating the starch formation from the sugars by gluconeogenetic reactions.

Distribution of starch granules in almost all cells of hypodermis and cortical or ground cells of cotyledons indicates the synthesis and/or translocation of sugars presumably of lipid origin and if not metabolised is converted into starch to maintain the osmoticum of the cells. As mentioned earlier the adaxial side of the cotyledon show more reserves hydrolysis and hence, more starch grains are localised in the adaxial cells in comparison with abaxial side.

As growth is advanced in seedlings to 7^{th} day the starch grains of cotyledonary cells become enlarged and occasionally appear as clumped together. But afterwards the starch grains disappear, This transient occurrence of the starch in cotyledons during germination and seedling growth in winged bean confirms the possibility of gluconeogenesis.

As mentioned earlier starch grains are localised in the cotyledonary cells during germination. After plumule excision, it is observed that starch granules of the experimental samples after 12h of plumule excision are accumulated in the cotyledonary cells around the vasculature

(Plate 15). This accumulation is not much elaborated in the control (Plate 16). After one day of plumule excision, this type of accumulation of starch is not evident (Plate 17, 18). It seems that the plumule excision results in an immediate accumulation of translocating carbohydrate (sugars) near the vasculature of the cotyledons and these sugars may be converted in to starch and get accumulated in the cotyledonary cells around the vasculature. Afterwards the grains may be degraded and mobilized to the actively growing axillary shoot.

Lipid content of dry seeds of winged bean shows a gradual reduction during germination. The plumule excision does not show any effect on lipid content of cotyledons for two days. But afterwards there is a significant reduction. When a comparison is made between the plumule excised seedlings and control, the latter contains more lipid indicating a reduction in the utilisation/mobilization of lipids from the cotyledons. A similar observation is reported in *Quercus alba* when the lipid content of the seeds is found to be utilized during germination and the concentration of phospholipids in the cotyledons are reduced considerably (Vozzo, 1978).

In seeds which are rich in lipid reserves, the hydrolytic products of lipids are mobilized for utilization as carbon and energy source in germinating seeds and seedlings. Winged bean seeds contain 22% of lipid (Table 2 Fig. 10) and significant reduction occurs during germination and seedling growth.

Recently, lipid mobilization during seed germination of cucumber seeds has been reported by Feussner *et al.* (1997) and according to these

authors, a special lipoxygenase is expressed in the cotyledons. This enzyme is localized at the lipid storage organelles and play an important role during germination. This specific oxygenation reaction may initiate the lipid mobilization as source of carbon and energy for seedlings. By histochemical and biochemical analyses in winged bean, lipid is found to be the initial storage material to undergo hydrolysis. So the enzyme lipoxygenase reported by Feussner *et al.* (1997) may be very active during germination in winged bean.

As reported earlier (Kamaladevi *et al.*, 1989) investigations of seed reserves during early seedling growth in winged bean is very slow. The plumule excision on 5^{th} day of germination, resulted in a stimulated development of a pair of cotyledonary axillary buds, also seems to influence the reserve mobilization to a limited extent.

Two peculiar characters of winged bean seeds that is the slow rate and small quantity of reserve mobilisation and potential growth of cotyledonary axillary buds can be correlated to the reserve mobilisation to some extent. The forcing of axillary bud growth by excising the original plumule is expected to induce reserve mobilisation compared to the normal seedlings.

Nevertheless, both histochemical and biochemical data of the pattern of protein, carbohydrate and lipid reserves show only slight difference between the intact (control) seedling and plumule excised seedlings (experimental). The contents of these reserves and their distribution among various seedling parts ie. cotyledon, plumule and radicle are interpreted under other sections. The effect of plumule

excision can be explained in terms of hormonal regulation of reserve mobilization as well as on the basis of source- sink relationship and also by the physiological basis of apical dominance.

The effect of plumule excision on the growth and development of winged bean seedling can be explained in terms of the apical dominance. Following the formation of the embryo, the apical region influences the development of lateral structures and this continues throughout the life of the plant (Hillman, 1984). If the apical dominance is lost by removal of the terminal bud, the resting axillary buds sprout. This sprouting is related to a massive change in gene expression. The sprouting axillary bud quickly adapts its protein pattern to that in a terminal bud (Mohr and Schopfer, 1995)

In winged bean two rudimentary cotyledonary axillary buds are present in the mature seeds (Fig. 1) and when plumule is decapitated these buds develop. Zajaczkowski *et al.* (1984) stated that when the apical bud is removed, the auxin wave moving down from the apex disappears. Then the auxin wave in the intact shoot (rudimentary axillary bud) closest to the point of decapitation is affected most strongly by its activity. Similar correlative interaction between the decapitated plumule and cotyledonary buds results in apical dominance (Phillips, 1975; Hillman, 1984).

The apical bud is able to exert a dominant influence that suppresses cell division and enlargement in the axillary buds. The most widely accepted theory holds that the stream of auxin flowing out of the shoot apex towards the base of the plant is thought to maintain an inhibitory

concentration of auxin at the axillary bud. Removal of this auxin supply by decapitations, reduces the supply of auxin in the region of the axillary bud and thereby relieves the bud from inhibition (Hopkins, 1995).

According to Phillips (1975) and Hillman (1984) auxin induces axillary bud growth inhibition as well as long distance transport of metabolites. In the present investigation it is evident that the plumule, very active site of auxin synthesis is excised on 5^{th} day after germination which results in the development of cotyledonary axillary bud. An active metabolic change is expected in the cotyledon since a pair of cotyledonary shoots are developed from each seed (Fig. 5) However, the reserve mobilization pattern during germination shows only slight variation in the seedlings, where plumule excision is done. It is probable that the plumule excision results in the removal of an active area of auxin synthesis (Phillips, 1975; zajaczkowski *et al*; 1984) also inhibited long distant nutrient transport from the cotyledons to the growing plumule (Hillman, 1984), hence metabolites are retained in the cotyledons. Apart from this the plumule is a very active sink of the seedlings and the removal of which causes an inhibition of the source activity of the cotyledons.

Effect of forcing or induction of cotyledonary axillary buds growth resulting in an active mobilization of cotyledon reserves is anticipated in winged bean. But contradictory to this, reserve mobilization is not enhanced significantly. A probable reason for this observation may be that, plumule excision cause an accumulation of degraded metabolites in the cotyledon. So a feed back inhibition is occurring in the cotyledon (Copeland and McDonald, 1995).

Cotyledonary axillary buds developed in the seedlings after plumule excision show vascular connections with cotyledon and main axis (Fig. 3 Plate 7). A similar observation was reported in Pea in which bud growth following release from correlation inhibition coincided with establishment of xylem continuity between the bud and stem (Sorokin and Thimann; 1964).

Rubinstein and Nagao (1976) suggested that bud growth can be detected many hour before increased vascular inter connections become manifested. Similar condition is observed in winged bean also because the cotyledonary axillary buds are present in the mature seeds but, the vasculature and connections are developed only after apical dominance is removed by plumule excision.

In species such as *Glycine max* and *Phaseolus vulgaris* inhibited buds appear to possess functional xylem and phloem connections with the stem (Peterson and Fletcher, 1973, 1975; Hall and Hillman, 1975; Yeang, 1980). Vascular elements connecting the stem with the primary leaf axillary bud and first infoliate leaf bud (Mullins, 1970) have been detected in *Phaseolus*. Nevertheless, in winged bean cotyledonary axillary bud, the vascular differentiation occurs only after two days of plumule excision. However, all these studies are on axillary buds of foliage leaves not in cotyledons. So the vasculature differentiation in winged bean cannot be directly correlated with *Phaseolus* or *Glycine*. In these plants vascular connections of inhibited axillary buds are found to be connected with the main stem and buds are occurring as rudimentary structure in the axils of leaves. In contrast, in winged bean axillary buds are subtended by the cotyledon where the vasculature is undifferentiated

on one side and on the other by embryonic axis, where the vasculature is undergoing differentiation. In spite of the occurrence of the cotyledonary axillary buds in the mature seeds, the vascular connection are developed only after apical dominance removal after germination.

Eventhough the ontogenetic aspects of vascular strands of cotyledons from seeds to seedlings are not traced in the present study, their morphological development is found to be related with seedling growth. The procambial strands of dry seeds constituting many elongated meristematic cells, show further development into multicellular differentiated vascular strands in the seedling. Cotyledonary procambial cells differentiation of *Catharanthus roseus* seeds showed that xylem and phloem elements are formed during seedling development (Philip, 1974). In winged bean it is clear that during seedling growth, the vascular strands of cotyledons are getting differentiated into phloem and xylem with characteristic wall thickening, which are observed in the sections stained for protein and insoluble carbohydrates. In *Pisum arvense,* cotyledonary vascular differentiation and reserve mobilisation are related each other during seed germination (Smith and Flinn, 1967).

While microscopic observations of histochemically localized metabolites of winged bean seedlings is done, the vascular anatomy also is involved in the investigation. However, the detailed study of the vascular structure of seedlings, cotyledon, epicotyl, and hypocotyl, is not coming in the purview of this study because of many reasons.

Studies on the original differentiation of vascular tissues in seedlings are very few and those that are available, describe only their

structure but not their developmental pattern in relation to reserve mobilization. Another reason is that the ontogeny of vascular differentiation is a topic that requires careful study with reference to longitudinal and transverse directions of differentiation of the procambium, protoxylem and protophloem and also to understand the interrelationship of the seedling structures – root, hypocotyl, epicotyl and cotyledon.

According to Philip (1972) during embryogenesis in *Catharanthus roseus* the procambium is formed earliest in the cotyledon and roots and these are directly related to apical meristem and the formation of the procambium in the hypocotyl is not only located but also is not related to apical meristem. Philip (1974) has pointed out that in seedlings procambial system and the initial xylem tissue of the hypocotyl do not show continuity with the vasculature of the cotyledons above or the root below. However, this ontogenetic discontinuity of the early vascular tissues of the cotyledons and hypocotyl become bridged up at the time of the formation of metaxylem.

Under these circumstances, in the present study it is not easy to correlate the ontogenetic differentiation of vasculature and mobilization of metabolites from the cotyledons to the growing axis of winged bean seeds. Nevertheless, the vasculature is included in this investigation and the distribution of vascular tissue in the cotyledon is correlated with the histochemical localisation of metabolites at different stages of germination.

Bisalputra and Esau (1961) showed that phloem is the first vascular tissue identifiable in the ontogeny. However, pattern of vascular differentiation in germinating seeds and seedlings are not described by these authors. Procambial strands of the mature (dry) seeds in winged bean show their incomplete development and distribution in the cotyledons (Fig 11). These strands are connected to the embryonic axis also. During imbibition and germination procambium undergoes differentiation and different views are existing on this topic (Philip, 1974; Krishnamurthy, 1994).

In the present investigation of histochemical and biochemical aspects of reserve mobilization, the seedlings of five days after germination is included. So procambial differentiation into xylem and phloem occurs to a considerable extent in these seedlings. However, the branching of vascular strands, to form a net work of strands, is important from the point of view of reserve mobilization from the cotyledons.

Figures 11 and 12 show the vascular strand distributions of the intact and plumule excised seedling at specific intervals. In the cotyledons, the vascular strands spread during germination (Fig. 11, 12) but towards the abaxial side of the cotyledons, vascular connections are not reaching the boundary of the cotyledon. In sections stained for protein and starch (Plate 4, 20) this character is clearly seen. The timing of vascular differentiation in relation to physiological events of germination in the cotyledons is of particular interest. In *Pisum arvense* after 24-32 hours of socking, a continuous conducting system through the cotyledon was laid down (Smith and Flinn, 1967).

Vascular tissue development and reserve material distribution are found to be interrelated. Development of phloem cells of the vascular strands is more important because of its involvement in metabolite translocation from the cotyledons to the axis whereas water imbibition takes place through all surface area of the seed particularly through xylem to reach with cotyledons. According to Hocking (1980), cotyledonary phloem is known to differentiate early in germination in *Lupins* seeds. In winged bean cotyledons, procambial cells are differentiated during early phase of seed germination. Number and size of cell layers are increased in cotyledonary tissue (Plate 9) in order to translocate more metabolites to radicle and plumule. However, cell types like sieve elements, companion cells, tracheid etc., cannot be distinguished because anatomical stains specific to these cells are not employed in the present study. Moreover, the intensity of histochemical staining of metabolites inhibited the cellular structure of the vascular strands (Plate 24). Almost similar characters are shown by the vasculature of plumule excised seedling probably because the plumule excision is seemed to affect reserve mobilization only to a limited extent.

An interesting observation is the difference in the pattern of reserve protein, starch and lipid in the ground tissue of cotyledons owing to the presence of vascular strands. Degradation and mobilization of the reserve starts first from the nearest cells to the vascular strands (Plate 9). This character is shown throughout the period of germination and seedling growth. Reserve degradation spreads from the nearest cells to the next as germination process progresses as well as reserve mobilization increases during further seedling growth. In contrast to this, mobilisation of

reserves in storage tissues of *Pisum arvense* seeds is initiated at the periphery of the cotyledon and proceed inwards (Smith and Flinn, 1967). Contradictory to this, in *Phaseolus vulgaris* degradation of seed reserves begins in the cells farthest from the vascular bundles and from the epidermis and after four days the central regions are devoid of reserves while the cells around the vascular strands are still packed with reserves (Opik, 1966). Similarly in *Arachis hypogea* cotyledons, Bagley *et al.* (1963) reported that the break down of reserves were delayed in the cells around the vascular bundles.

It has been observed that distribution of cotyledonary reserves between the shoot and root depends on the relative growth rate of the two organs, (Sutcliffe, 1976). Vascular strand distributions of cotyledons in winged bean seedlings were also analysed to elucidate a correlation of vascular tissue development and cotyledonary reserve mobilization under different condition of growth.

Studies on vascular differentiation of winged bean seeds germinated in Petri dishes under darkness revealed that the procambial strands constituting thin walled elongated cells are distributed only to limited extent (Fig 11). When the seedling growth is advanced to five days, cell multiplication and expansion are observed in the procambial strands (Plate 4). It seems that further branching and extension of the procambial strands occurs throughout the cotyledons to form a network like appearance analogous to the venation of foliage leaves. In this context it is worth quoting the reference of Grew (1682). More than 300 years back Grew (1682) published a beautiful diagram to illustrate the vascular strands of cotyledons. He described the vasculature as ".....

throughout the parenchyma run the branched vessels, which in the lobes make the seminal root; in the radicle and plumule, the wood of the root and stalk. In all of them, distributed as hath been formerly showed...." Afterwards, the botanical literature abounds with progress where various aspects of cotyledons have been explained. However, only a few authentic studies are available to elucidate the ontogeny of vascular differentiation during embryogenesis and seedlings development (Philip, 1972, 1974; Krishnamurthy, 1994). In spite of the abundance of reports on seed reserve mobilization, only one or two references are available dealing with the structure and role of cotyledons in reserve mobilization (Smith and Flinn, 1967; Smith, 1974).

According to Smith and Flinn (1967), differentiation of the vascular tissue from the procambium was first observed 12 to 17 hours after the commencement of soaking. The first detectable differentiation were those of protophloem of the main vein. In the present study the vascular differentiation from the procambial cells which occur as thin walled elongated cells in mature seed (Plate 3) is almost occur in the next sample that is seedling of fifth day after germination. In pea seeds, Smith and Flinn (1967), reported that the time taken for complete differentiation of vascular strands difficult to determine precisely but strands which are apparently fully differentiated were observed at the distal end at 42 to 48 hours.

Eventhough the vascular differentiation is found to occur in the cotyledons of fifth day old seedling in winged bean further advancement and distribution in side the cotyledons give a picture of their presence and effect on the surrounding ground tissue where the reserve are localised

(Plate 4 - 12). So the histochemical study enables to pin point the region of reserves hydrolysis.

Storage proteins localised by bromophenol blue staining in the cotyledons of dry seeds showed densely stained grains of various sizes and their distribution in the cells varies from region to region of cotyledons. Generally the cells of abaxial and adaxial side of the cotyledons contained densely stained protein masses in the dry seed (Plate 3). When the seeds are germinated the cells of cotyledons enlarge and protein content spreads in the expanded area and their intensity of staining is slightly reduced. In some cells near the vasculature the protein mass is degraded in to many small granules. However, a meticulous quantitative counting of the distribution of protein grains is difficult in histochemically stained sections. Quail (1989) identified three types of specialised storage organelles in which storage proteins are located. According to him the dry seed proteins are defined as "transient organelle" and during seed germination, these protein bodies enlarge and coalesce and eventually the contents are hydrolysed and become vacuoles.

In germinated seeds protein and lipid content of cotyledonary ground tissue shows hydrolysis first in the nearest cells of vasculature (Plate 8, 24) whereas in dry seeds all ground cells are compactly filled with protein and lipid bodies (Plate 3, 23). By an exhaustive study in 500 legume species (Smith, 1974) revealed eight specific patterns of hydrolysis of reserves from the cotyledons. According to him *Vicia faba* and *Glycine max* show mobilization starts around the vascular strands of adaxial side of cotyledon and mobilization begins at the centre of cotyledon

in *Phaseolus vulgaris, Vigna sinensis* and *Dolichos lablab. Psophocarpus tetragonolobus* is found to be the only legume in which hydrolysis of reserves begins around the vascular strands at the adaxial side of the cotyledon. In the present study it is confirmed that in winged bean, the reserve moblisation starts in the cells around the vascular strands and spreads to the whole vasculature of both adaxial and abaxial sides of cotyledon.

In spite of considerable vascular differentiation in the cotyledons, the reserve moblisation during germination is found to be very slow. A comparative study by planting the seedlings in garden soil and acid washed sand showed that the seedlings grown in acid washed sand suffered from nutrient stress and survived only for one month (Data not given). The cotyledonary vascular differentiation of these seedlings was retarded compared to that of the plants grown in garden soil (Figs. 13, 14). So it seems that the vasculature development of cotyledons during seedling growth depends on the nutritional level of the seedlings. A meticulous accounting of the effect of nutrient stress on vascular differentiation is difficult by this study. However, it is evident that reserve moblisation and vascular differentiation are related to the seedling vigour which in turn depends on the nutrient status of the growth medium.

Investigations on transport in pea *(Pisum sativum)* seedlings to describe the control of the mobilization of materials from cotyledons into the developing axis show that distribution of cotyledonary reserves between the shoot and root depends on the relative growth rate of the two organs. Dark-grown seedlings accumulated more transported reserves in

the shoot than did the light grown seedlings and growth of shoot increased markedly while the root did not show any difference (Guardiola and Sutcliffe, 1971, 1972; Guardiola, 1973;Garcia – Luis and Guardiola, 1975).

During early growth, seedlings are more or less dependent on endogenous reserves. These are mobilized at a rate determined by the growth of the axis and distributed between shoot and root according to the need of each organ *via* the phloem.

Sutcliffe (1976) suggested that the movement of solutes from the cotyledons or endosperm (source) of germinating seed into the shoot or root (sink) of the developing axis could be described using an electrical anology. The cotyledons, the source is represented by a capacitor which is related to its volume. This source in germinating seed acquires solutes as a result of breakdown of reserve materials and release of soluble molecules. The amount of an individual solute transported to an individual sink will be related to the solute potential difference between the two solutions and a resistance is represented by the connecting phloem. So according to Sutcliffe (1976), it appears that removal of one of the sinks (root or shoot) should not directly affect the rate of solute movement to the other sink. Guardiola (1973) showed that the removal of the shoot from a week old pea seedlings did not affect the movement of potassium into the roots suggesting that there is little or no competition between shoot and root for this nutrient.

Redistribution of reserve material is profoundly affected by growth hormones. Davies and Wareing (1965), found an increased transport of

phosphorus towards the tip of a decapitated bean seedling treated with IAA and this effect was detected within a few hours. However, Kinetin and Gibberellic acid showed no effect on transport of phosphorus in French bean plants (Seth and Wareing, 1967).

In a review (Ilan and Gepstain, 1981) suggested that cytokinins emanating from the embryonic axis acts as regulators of food reserves breakdown in germinating dicotyledonous seeds. It provides a mechanism, which appears to be similar to the control of mobilization of endosperm reserves in cereals (Bewley and Black, 1983; Mayer and Poljakoff-Mayber, 1989). However, contradictory to this view Halmer and Bewley (1982) concluded that for most of the legumes, the embryonic axis is not required for the initiation of starch degradation.

Studies on exogenously applied plant hormones to excised storage organs indicated that hormones control the reserve mobilization in germinating seeds. Cytokinins have been shown to increase protein break down in cotyledons of squash and to replace the axis requirement (Penner and Ashton, 1967; Sze and Ashton, 1971; Tsay and Ashton, 1974). However, gibberellic acid and indol acetic acid are apparently not involved in the control of protease in squash (Penner and Ashton, 1967). Nevertheless, convincing evidences cannot be drawn solely from the studies of exogenous applications of hormones to prove the role in mobilisations (Davies and Slack, 1981). However, Chapman and Davies (1983) agreed with the influence of cytokinin in reserve mobilization and further studies are required to substantiate the control that takes place in intact system.

Karunagaran and Rao (1991) showed that in *Macrotyloma uniflorum* translocation of soluble sugars and amylase activity are controlled by the embryonic axis during germination. The axial control of α amylase activity and accumulation of sugars is explained in sink-source relationship. Similar situations are also reported in peas (Morohashi *et al.* 1980) and in mung bean (Morohashi, 1982).

A review of Davies and Slack (1981), revised the current position with regard to mobilization and enzyme production of a wide range of materials. According to these authors the embryonic axis produce an hormonal stimulus, which initiates the development of optimal rates of hydrolytic enzyme activity in the storage organs. These authors also suggested the continuous operation of source- sink relationship between the storage organs (source) and axis (sink) during germination and early seedling development.

The concept of metabolic control of cotyledonary reserves by the embryonic axis contradicted by a few authors (Bryant and Haezycki, 1976 and Ford *et al.*, 1976) who suggested that when the excised cotyledons are incubated under optimal conditions, hydrolytic activities are developed which are comparable to those produced in intact seeds.

According to Chapman and Davis, there is no direct evidence for a growth substance moving from the embryonic axis in to the cotyledonary tissue and if at all such a substance there the origin of such that (cotyledon or axis) has not been established (Chapman and Davies, 1983). Cotyledons of seedlings play an important role in the growth by

supplying organic substrate. Apart from their role in nutrition, cotyledons have also been shown to have a regulatory role in the growth of seedlings (Gambley and Dodd 1991). Similar findings are reported by Shibata *et al.* (1974) who found that removal of cotyledon from intact lettuce seedlings retarded gibberellin induced hypocotyl elongation. Van Onckelen *et al.* (1981) suggested that during germination in *Phaseolus vulgaris*, the cotyledon showed active abscisic catabolism as were implicated in the control of apical dominance.

The control of the breakdown of food reserves in germinating dicotyledonous seeds has been discussed and assessed by Chapman and Davies (1983). These authors suggested that the most reasonable hypothesis to explain the control of reserve mobilization in germinating seeds is the 'source-sink' hypothesis which quite simply suggests that hydrolytic enzyme activities automatically increase in cotyledons during germination and subsequent early seedling growth, and are regulated by feed back effects mediated by reserve breakdown products. In the presence of the axis (sink) breakdown products are automatically removed and reserve breakdown can continue and in the absence of such a sink reserve breakdown is curtailed. Investigations on cucumber seeds (Davies and Chapman, 1979 a, 1979 b, 1980; Davies and Slack, 1981; Davies *et al.*, 1981) clearly showed that the cotyledon-axis relationship in reserve mobilization can be explained by source-sink concept.

In the present study it is clear that the reserve mobilisation during seed germination in winged bean is not solely controlled by embryonic axis because the plumule excision results only slight changes of metabolite translocation. The development of cotyledonary axillary buds

indicates the involvement of apical dominance since the occurrence of rudimentary buds in the axils. So the most reasonable hypothesis to explain the control of reserve mobilisation is the source sink concept. The plumule excision exhibited its immediate effect on the development of a pair of cotyledonary axillary branches. So, the source activity of the cotyledon is not interrupted considerably. This is evident from the negligible difference in reserve mobilisation between the plumule excision and intact seedlings. Nevertheless, the reserve mobilisation during germination in winged bean buds is found to be controlled by source-sink relationship super imposed by apical dominance.

CONCLUSION

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Reserve mobilization and vascular differentiation of winged bean seeds during germination, studied histochemically and biochemically reveal that, winged bean seeds consist of a pair of conspicuous cotyledons with a comparatively small embryonic axis and the pattern of germination is hypogeal. Reserve materials of winged bean, variety 'PT 2' seeds consists mainly of protein 40%; lipid 20%; starch 3%; soluble sugars 7% and dry weight 90%. Histochemical studies of dry seeds prove that the cotyledonary cells are almost filled with protein bodies thereby masking cellular structures. During germination the protein bodies undergo degradation especially in the cotyledonary cells around the vascular strands and the rate of degradation spreads retrogressively to the cells away from the vasculature. The cells of the adaxial side of cotyledon show more degradation of protein compared to the abaxial side. Even after eight days of germination only very few layers of cells near the vasculature show complete or partial mobilization of protein. Similarly a few layers in the adaxial side of cotyledone are devoid of protein mass. Distribution of protein grains in the cotyledons is not significant when the plumule excision is done. Biochemical analysis of protein results in a slight increase of protein in the germinated seeds compared to the dry seeds. However protein mobilized during germination and after plumule excision are found to be very low, agreeing with the biochemical data.

Starch grains are not seen localized by PAS in dry seeds and only about 2.3% is present in biochemical estimations. However during germination, starch grains are synthesised probably from lipids by gluconeogenesis. During seedling growth and plumule excision starch is utilized, so a reduction is observed. Immediately after plumule excision, starch grains are found to be localised near the vasculature probably due to synthesis from accumulated soluble sugars after plumule excision. Lipid degradation is found to be faster than protein and carbohydrate. The reduction of lipid is progressively increased during the seedling growth. Starch synthesis of cotyledonary cells during germination is presumably from lipids by gluconeogenesis.

Plumule excision on 5^{th} day after germination exhibits the development of a pair of axillary buds from the axils of each cotyledons. These buds are observed in the dry seed as a rudimentary axillary bud in the axils of both cotyledons. Even though the plumule excision results in the development of a pair of axillary shoots and a concomitant faster growth of the radicle, the metabolism of reserve mobilization is not much changed in terms of metabolites such as protein, carbohydrate and lipid distribution in the cotyledons. However starch synthesis from lipid content and degradation of storage protein for *de novo* synthesis of new enzymes are found occurring during germination and seedling growth and after plumule excision, so the reserve mobilisation is slightly changed.

Effect of plumule excision on reserve mobilization can be interpreted by a number of physiological phenomena. An established concept of plant growth regulators (hormones) is the control of

embryonic axis in inducing hormones like GA in cereals and an almost similar effect is shown by exogenously applied cytokinin in legumes. In the present study, the plumule excision may result in an accumulation of cytokinin in the roots due to lack of translocation. So root growth is enhanced. It is one of the morphological results of plumule excision observed in winged bean. Another morphological difference is the development of two cotyledonary axillary buds into fully developed axillary shoots and this character is a typical example of apical dominance. Plant growth regulators mainly involved in apical dominance is IAA and plumule excision results in the removal of IAA synthesis, so the apical dominance is removed and the rudimentary cotyledonary axillary buds get triggered to develop. These explanations however do not show any effect of plumule excision on reserve mobilization. The axillary bud growth as well as induced plumule growth occurring after plumule excision may cause an enhancement in reserve metabolism because these seedlings are solely depending on the cotyledonary reserves alone as they grow in Petri dishes supplied only with water. However quantitative changes occurring during seedling growth after plumule excision do not show much significant change except in lipid which is found to be as the primary reserve mobilised during the initial metabolic activities of germination.

Reserve mobilization during seed germination and the control of embryonic axis on the hydrolytic process have been explained in terms of source-sink relationship. The plumule excision results in the removal of an active sink of the seedling, thereby cutting short the reserve flow from the cotyledons. So the hydrolytic products of the reserves in the

cotyledons may accumulate itself or can mobilize to the other sink i.e., the radicle. The accumulation of hydrolysed products in the cotyledon may cause a feedback inhibition to regulate the hydrolytic process. As a result the reserve mobilisation may be retarded. So source activities are reduced.

On the other hand, the plumule excision resulting in the development of a pair of cotyledonary axillary plumule can change the source-sink relationship. Instead of the removed plumule which cause the reduction of the sink activity, after a period of two days a pair of equally potential shoot become functional and act as a potential sink. Consequently a parallel source activity occurs. However, the flux of mobilization is comparatively very low though significant. The possible reason for this may be the presence of the conspicous cotyledons with very high biomass constituting reserves. In other words only comparatively low amount of dry matter is mobilized to the growing sinks from the cotyledons. So the effect of plumule excision is not resulting in a drastic change of the reserve mobilisation.

In winged bean seeds vascular differentiation and reserve mobilization are found to be related to each other. The procambial strands present in the dry seed get differentiated into phloem and xylem during early stages of germination. It is clearly seen, histochemically that the vascular strands are distributed as a network throughout the cotyledon similar to the venation of foliage leaf.

The protein degradation of cotyledonary cells, during germination is observed in the cells around the vasculature. Similar pattern of

hydrolysis and mobilizations occur in lipid also. Starch grains are found to accumulate near the vasculature. Nevertheless the vasculature differentiation and the quantity of reserve mobilization can be correlated only when a quantitative analysis of both are done. It is found that since the cotyledon is very bulky it contain very high quantity of reserves and, major part of them are retained in the cotyledons after germination and plumule excision.

The vasculature network is found to be elaborated only to a limited extent because in the sections of cotyledons their distribution is sparse. So the hydrolysis of lipid and protein in the cells near the vasculature contributes to a very low amount of reserves compared to the total reserves present in the conspicuous cotyledons. Nutrient stress during seedling growth causes a slight retardation in the differentiation of vasculature in winged bean.

By the present study however it is not possible to delineate the ultra structural aspects of vascular differentiation and the mode and flux of reserve mobilizations from the cotyledons to the growing embryonic axis because of the limitations of methodology and overlapping of two different aspects of the vascular anatomy and the mobilization of reserves. However, the vascular differentiation of the cotyledons *vis-à-vis,* the reserve mobilization of seeds is equally important as far as the process of germination is concerned.

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