

STUDIES ON ORGANOGENESIS FROM EXPLANTS OF
BEGONIA ERYTHROPHYLLA

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ABSTRACT

Caulogenesis and rhizogenesis were studied in cultured explants of *Begonia erythrophylla*. The patterns of development were compared to each other and to explants cultured on non-organogenic media. Initially morphological and histological observations were carried out using both light and scanning electron microscopy. Shoots and roots were formed from cells of epidermal origin and were often associated with glandular hairs. Deposition of starch was correlated to organogenesis, with starch grains localized in the superficial layers of the explant, prior to the formation of meristematic regions. Once formed, meristematic regions showed high levels of enzymatic activity compared to the surrounding tissues.

Media transfer experiments were conducted between root inducing medium (RIM) and shoot inducing medium (SIM), shoot inducing medium and a basal medium containing no growth regulators (BM), and RIM and BM. It was found that explants became determined for shoot production after 7 days, while they were determined for root production after 3 days on RIM before transfer to BM. Explants were found to be weakly canalized for rhizogenesis for the first 2 days after determination, thereafter they became strongly canalized. Explants were strongly canalized for caulogenesis once determined. Culture on BM prior to exposure to an organogenic medium resulted in the gradual loss of competence with time, but exposure for 2 days to either SIM or RIM resulted in explants becoming competent to respond to opposite inductive medium.

Using one and two-dimensional gel electrophoresis, both unlabelled protein and protein labelled *in vivo* with [³⁵S]-methionine were extracted from organogenic and non-organogenic explants. Silver-stained gels and fluorographs were analyzed to identify different sets of polypeptides associated with development *in vitro*. Overall the levels of 59 silver-stained and 29 labelled polypeptides were altered. It was shown that rhizogenesis and caulogenesis in petiole sections of *B. erythrophylla* are associated with both quantitative and qualitative changes in the expression of polypeptides. Most of these changes occur at, or after, the point of organ determination. Some of those associated with caulogenesis were also observed when leaf discs were cultured on SIM. Tissues of mature plants were examined for the presence of these polypeptides.

The process of caulogenesis in petiole explants can be further divided by the use of chemical inhibitors of shoot formation. Additions of sorbitol, ribose, acetylsalicylic acid or tri-iodobenzoic acid to SIM, respectively, inhibits shoot formation without killing the explants. These inhibitors are only effective if explants are exposed prior to certain stages of development, after which they fail to inhibit shoot formation. The effects of these inhibitors on the pattern polypeptide changes associated with culture on SIM was determined.

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| 1D | one dimensional |
| 2D | two dimensional |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ASA | acetyl, salicylic acid |
| BA | benzyladenine |
| BM | basal medium |
| CHAPS | cholamidopropyl dimethylhydroxypropanesulphonate |
| CIM | callus inducing medium |
| dd | distilled water |
| DTT | dithiothreitol |
| IEF | isoelectric focusing |
| MM | maintenance medium |
| NAA | naphthaleneacetic acid |
| NBT | nitroblue tetrazolium |
| PDA | piperazine di-acrylamide |
| PMS | phenazine methosulphate |
| PMSF | phenylmethylsulphonyl fluoride |
| PPO | 2,5 diphenyloxazole |
| RIB | ribose |
| RIM | root inducing medium |
| S.E.M. | scanning electron microscope |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SIM | shoot inducing medium |
| SORB | sorbitol |
| TEMED | N,N,N',N'-tetramethylethylenedimine |
| TIBA | 2,3,5-triiodobenzoic acid |
| T.S. | transverse section |

CHAPTER I

INTRODUCTION

The ability of shoots, roots and flowers to arise unexpectedly or adventitiously is a widespread natural phenomenon, which is essentially being exploited in plant tissue culture (Flick *et al.*, 1983).

The regenerative capacities of many cultivated and ornamental plants are known in horticultural practice and are frequently used in vegetative propagation and preservation of valuable individuals. To increase the rate of propagation, considerable research has been carried out to find suitable conditions for cultivation, the time suitable for collection of cuttings and the optimal concentration of growth regulators required for the induction of organogenesis. Tissue culture techniques have increased the regenerative capacity of many plant species by allowing the cultivation of isolated cells or tissues under sterile conditions, providing optimal nutritional requirements, regulating the external conditions of cultivation (i.e. temperature, light intensity, photoperiod and aeration) and controlling growth and developmental processes with growth substances. Few techniques provide such a wide basis for study of important biological problems as that of plant cell culture.

(1) THE REGENERATIVE CAPACITY OF EXPLANTS

Plant cells are capable of differentiating and developing into various organs or whole plants. Culture can produce callus tissue, induce organogenesis or somatic embryogenesis. The ability of apparently differentiated cells to change their fate and differentiate to form various organs, or a whole plant, poses several questions. One of the most important of these is, are all cells within a plant developmentally flexible? Thomas and Wernicke (1979) suggested most cells have lost the ability to form new plants and only cells from which meristems arise are developmentally flexible. However, Meins and Binns (1979) pointed out, there is a distinction between stability and permanence. This distinction is extremely important in understanding plant development.

Permanent genomic changes induced during normal development are very rare and to my knowledge only occur in animals. Such changes involve the total or partial elimination of the genome as occurs in erythrocytes and in somatic cells of the nematode worm *Ascaris lumbricoides* (Maclean and Hall, 1987).

Almost universally the whole genome is retained in the nuclei of differentiated plant cells, but there is real difficulty in determining to what extent it remains totally available for transcription. Other genomic modifications could take place during the development of plant cells, which affect the responsiveness of the genome. These modifications come under the categories of relatively stable changes, such as DNA methylation, which can sometimes be overcome by DNA replication during cell division, or readily reversible changes such as chromatin condensation, histone acetylation-deacetylation, association of regulatory molecules and short term adoption of novel three-dimensional structures, for example, supercoiling (Maclean and Hall, 1987). Such mechanisms, although affecting the expression of genes, can be reversed so that even inactive gene sequences can be reactivated, or at least give rise, following DNA replication and mitosis, to such activated sequences (Maclean and Hall, 1987).

Many specialized cell types from higher plant species retain the capacity to form complete, fertile plants (Reinert and Bajaj, 1977), i.e. the cells are totipotent, this and the fact that some heritable habituation has been shown to be the result of epigenetic changes rather than from permanent alterations of the cell genome (Meins and Binns, 1979), suggests the regulatory mechanisms involved in the development of plant cells are to some extent reversible.

This leads us to the second question, if all cells within an explant contain the genetic information to undergo any form of morphogenesis, what changes within the cells or associated tissues are required to enable them to do so? Christianson and Warnick (1988) have divided the process of *in vitro* morphogenesis into three developmental events, competence, induction and determination. Competence is the ability of cells to respond to an inductive stimulus. It is much like the concept of dedifferentiation proposed by Gautheret (1966), although unlike dedifferentiation, competence does not imply proliferation of unorganized callus tissue. Once competent tissues are able to respond to the growth regulator balance or other stimuli in the culture medium and undergo induction. Induction is the process by which a stimulus results in a specific developmental fate, or in other words it is a process seen by its end result, a change in the fate of a cell or group of cells. This change in fate is determination, or more specifically, determination is the singling out of one fate from among a number for which a cell, or a group of cells, may be competent. It is important to note that competence and determination are only concepts, they cannot be measured by direct inspection of the tissue, only demonstrated by means of experimental manipulation (Meins and Binns, 1979).

Several studies have been carried out temporally defining the stages of competence and determination and the conditions required by explants to attain these developmental stages (Christianson and Warnick, 1983; Flinn *et al.*, 1988; Coleman and Ernst, 1990; Attfield and Evans, 1991b). With explants that require the production of callus before organogenesis, the state of competence has been shown to vary in both stability and duration among genotypes (Christianson and Warnick, 1983; Coleman and Ernst, 1990). Genotypes of *Convolvulus arvensis*, that were recalcitrant to *in vitro* manipulation, could be induced to form organs if first exposed for brief periods to callus inducing medium (Christianson and Warnick, 1985). This suggests that explants, which initially lack competence to respond to inductive stimuli, can gain competence if exposed to the correct balance of growth regulators.

In *Pinus strobus* cotyledons, competence for caulogenesis is associated with the presence of 3 and 4 cell clusters and high lipid reserves within cotyledons. Loss of both during normal maturation of cotyledons was associated with the loss of competence (Flinn *et al.*, 1988; 1989). It would therefore appear that the presence of a normal complement of chromosomes within cells of the explant may not be all that is required for competence, but in some bulky explants competence may also involve factors at the tissue level. Henshaw *et al.* (1982) stated that there is little doubt that competence and determination exist in plants, but it is not clear whether these are properties of individual cells or groups of cells. Further studies are required to define biochemical, physiological and molecular events that occur during the attainment or loss of competence.

(2) EXPRESSION OF MORPHOGENESIS

Despite our ability to grow large numbers of shoots, roots or embryos from somatic or gametic tissues we still have a poor understanding of why these processes occur (Ammirato, 1986). Little is known about why cells become embryogenic or organogenic and the underlying mechanisms of embryo and organ formation. The processes of embryogenesis and organogenesis often appear to be mutually exclusive, with cells forming either shoots, roots, flowers or embryos (Henshaw *et al.*, 1982). The propensity to form either organs or embryos seems to some extent to depend upon the species in question. For example, tobacco usually form organs with embryos forming only rarely, while in carrot, embryos form more readily than organs (Ammirato, 1986).

The distinction between the two processes rests mainly on morphological and anatomical evidence. In somatic embryogenesis a new individual is formed from a single cell and shows no vascular connection to the parent tissue (Haccius, 1978). Somatic embryos, like zygotic embryos, are bipolar structures consisting of a basic plant, where the shoot and root are connected by a solid procambium, which ultimately generates vascular tissue. Organogenesis on the other hand involves the production of a unipolar structure rather than a bipolar one. Shoot formation involves the production of a shoot primordium, which subsequently develops into a leafy shoot. As the development of the shoot continues procambial strands which connect the young shoot to the maternal tissue are formed. Shoots often form independently of roots, with the shoot itself subsequently producing root primordia from which roots establish.

(3) FACTORS AFFECTING MORPHOGENESIS

Several factors have emerged that appear to influence the ability of cells in culture to undergo morphogenesis and subsequently to modify their development. These factors can be divided into three major groups, namely, chemical effects, which essentially involve the composition of the medium, physical effects due to culture environment and explant effects.

3.1 Explant effects

Although generally all cells within a plant may be considered to be of the same genotype, there are often pronounced differences in ability to regenerate in culture from cell to cell and organ to organ. Van Den Ende *et al.* (1984a,b) studied the capacity for differentiation of epidermal tissues isolated from vegetative and flower stalks of tobacco plants. They demonstrated the decisive importance of the ontogenetic state of the maternal plant for explant development. Explants from vegetative parts regenerated shoot buds or roots, whereas epidermal cells from flower stalks differentiated flower buds. Even the position of the explant along the axis of the plant has been shown to effect organogenesis (Tran Thanh Van, 1980).

Tissue age can also significantly effect its ability to respond to morphogenic stimuli. Often meristematic, embryonic and reproductive tissues have a greater propensity for growth and morphogenesis in culture than mature tissue. These differences are most noticeable in cereals that have traditionally been among the most difficult plants to culture (Vasil, 1985). In maize immature embryos produce both shoots and somatic embryos more readily than do mature embryos (Green and Phillips, 1975). Selection of young radiata pine cotyledons results in high shoot productivity (Aitken-Christie *et al.*, 1985), however, in English ivy (*Hedera helix*), which progresses through a clear-cut juvenile-adult phase transition, only cultures initiated from adult material can produce somatic embryos (Banks, 1979), therefore there is no absolute rule concerning tissue age.

It has been recognized from the earliest studies that one of the most influential factors in determining the morphogenic response is genotype. Species and cultivar dependent regeneration has been reported (Ammirato, 1986). In some instances certain genotypes within a species or cultivar often regenerate plants more readily than others (Ammirato, 1986). It is now well accepted that genetic factors can contribute to the relative responsiveness of plant tissues in culture (Raquin, 1982). These genetic factors appear not only to act in an all or none fashion, but can also affect the timing of the morphogenic response and the sensitivity of the explant to experimental manipulation (Christianson and Warnick, 1983 and 1984)

3.2 Chemical effects

The chemical composition or physical make-up of the nutrient medium can have significant effects on the initiation and development of shoots. For example, one of the major components of the medium affecting morphogenesis is the source and concentration of nitrogen. A high level of nitrogen, particularly in a reduced form, appears to be beneficial to caulogenesis (Flick *et al.*, 1983). Generally speaking there is no single medium that is suitable for every species or type of culture and any study of organogenesis usually involves the optimization of the medium (Brown and Thorpe, 1986). Several reviews have been published on the subject of medium composition, for example, Murashige, 1974; Gamborg *et al.*, 1976.

As well as the actual nutritional composition of the medium, the overall osmotic concentration can also exert significant effects on morphogenesis. Brown *et al.* (1979) demonstrated an osmotic requirement for shoot formation in tobacco callus. Therefore, when developing a tissue culture medium, the osmotic effects of the medium as well as the nutritional aspects must be considered.

3.3 Physical effects

Both light and photoperiod can have strong morphogenic effects on plant development in culture. Pillai and Hildebrandt, (1969) showed that in *Pelargonium zonale* a photoperiod of 16 hours light and 8 hours dark, in contrast to continuous lighting, was essential for shoot regeneration. Photoperiod has also been shown to play a role in flower formation (Kerbaudy, 1984).

As well as duration, the quality of light can also affect the regenerative potential of explants. Shoot regeneration on callus derived from Douglas fir (*Pseudotsuga menziesii*) embryos can be enhanced by culture under red light (660nm) (Kadkade and Johri, 1978). This effect is transient and only increases shoot number if explants are exposed during the early stages of bud initiation and development.

Temperature is also of great importance in *Begonia*, maximum induction of shoot buds occurs at temperatures between 15 and 24°C. Culture at temperatures above 25°C drastically inhibits the number of buds formed per explant for *B. x cheimantha* (Fonnesbech, 1974a and b).

The way in which cultures are grown can also have significant effects on morphogenesis. Cultures can be grown on solid or liquid media, stationary or agitated, in sealed, or tightly stoppered cultures vessels with closures that permit gas exchange. All these factors affect the response of explants (Murashige, 1979). As with medium composition the optimum culture conditions for a particular species are important and are usually determined by empirical methods (Brown and Thorpe, 1986).

(4) A CLOSER LOOK AT ORGANOGENESIS

4.1 Histological aspects

Organogenesis can occur in two ways (Hicks, 1980), either directly or indirectly. If the explant is induced to form an unorganized mass of callus, giving rise to meristematic centers which then develop into organs, organogenesis is said to occur indirectly (Hicks, 1980). Indirect organogenesis occurs in many systems and can be induced both from callus remaining attached to the original explant (Monacelli *et al.*, 1988; Christianson and Warnick, 1988; Attfield and Evans, 1991a) or from cultures of callus (Banks, 1979; Howarth and Peterson, 1983). When there is no intervening callus production and meristematic centers form from cells of the original explant,

organogenesis is said to be direct (Hicks, 1980). Many examples of direct organogenesis have been reported (Pramanik and Datta, 1986; Wright *et al.*, 1986; Reynolds, 1989).

Irrespective of the path of organogenesis some generalizations can be made about the histological events involved in organogenesis, by comparing the formation of shoots from callus to those formed directly from the explant. One of the most thoroughly studied indirect systems is that of shoot formation from tobacco callus (Thorpe and Murashige, 1970; Ross *et al.*, 1973; Maeda and Thorpe, 1979).

Caulogenesis begins with the formation of cell files around the inoculum with tracheary elements forming between the files and the inoculum (Maeda and Thorpe, 1979). After 7 days in culture localized active cell division occurs near these tracheary elements from which meristemoids begin to arise after further culture. Shoot primordia develop from these meristemoids arising mainly from the lower half of the callus in contact with the medium. Leafy vegetative shoots are observed after 18 to 21 days in culture.

More recently several studies have been carried out on shoot formation from leaf discs of tobacco (Gupta *et al.*, 1966; Xu and Liu, 1980; Attfield and Evans, 1991a). Shoot primordia occur all-round the cut edge of the leaf disc and at any point at which wounding occurs (Attfield and Evans, 1991a). Callus nodules formed by division of palisade and bundle sheath cells center on the vascular tissue and spread out from each vein (Attfield and Evans, 1991a). These nodules contain tracheids like those found when tobacco callus is exposed to a shoot inducing medium. Meristemoids develop in the regions of the nodule forming tracheids, as observed in shoot forming tobacco callus. These meristemoids develop into shoot primordia. In this system, despite callus remaining attached to the original explant, the pattern of caulogenesis is very similar to that observed when callus alone is used as an explant. In other indirect systems the basic pattern of shoot formation is essentially similar to that observed in the above systems (Handro *et al.*, 1973; Sharma and Bhojwani, 1990).

With direct organogenesis shoot buds can arise directly from cells of deep or superficial origin. Reynolds (1989) observed that in *Solanum carolinense*, shoot buds arose from the inner cortex and external phloem of stem explants. In other studies organ primordia also formed from either the external phloem or in close proximity to the vascular bundles (Sterling, 1951; Bonnett and Torrey, 1966). Organ primordia can also arise from the superficial layers of explants, with epidermal or subepidermal layers giving rise to organs (Tran Thanh Van, 1973a & b; Chlyah, 1974; Villalobos *et al.*, 1985; Von Arnold and Gronroos, 1986). In *Pinus radiata* cotyledons, cultured on shoot-inducing medium, cell division initially occurs randomly throughout the explant,

but by day 3 of culture mitotic activity occurs mainly in the epidermis and subepidermal parenchyma cells in contact with the medium (Villalobos *et al.*, 1985). Organized structures originating from single dividing epidermal cells, are detected after day 3, from which promeristemoids consisting of six to eight cells developed by day 5. With continued culture the promeristemoids increase in size to become meristemoids giving the explant a nodular appearance. By day 21 leaf primordia can be observed.

In all the examples referred to above, irrespective of whether organogenesis was direct or indirect, or from superficial or deep tissues, there are certain common features. In all instances meristematic regions were formed that contained small, thin walled cells, which were tightly packed together, with densely staining nuclei and cytoplasm. Cells giving rise to the initial meristematic cells were also, in all examples, not initially meristematic, having vacuoles, chloroplasts and being weakly stained. Therefore in all examples, although arising from cells of completely different origin, the development of meristematic regions that give rise to shoot primordia is a consistent feature of caulogenesis.

A further similarity between many systems was the initial accumulation of starch in cells surrounding the meristematic regions and its subsequent decline during the development of the meristemoid (Thorpe and Murashige, 1970; Mangat *et al.*, 1990). Although starch accumulation has not been shown to be essential for the formation of meristematic structures in all systems, (Von Arnold, 1987) some form of stored energy reserve appears to be required. This may take the form of lipids or storage proteins, the lack of which has been shown to inhibit shoot production in *Pinus strobus* cotyledons (Flinn *et al.*, 1989).

In *Torenia* epidermal peels formation of the meristematic center or meristemoid has been studied in fine detail. Chlyah (1974) found that cell division began in a single cell and was followed by division of adjacent cells. The original dividing cells became the center of meristematic activity dividing rapidly, with cells peripheral to them dividing more slowly. This resulted in formation of the meristematic center. Meristemoids may therefore be formed from one or more cells. There has been considerable debate about the single or multicellular origin of shoots formed *in vitro*. Norris *et al.* (1983) argued for a multicellular origin of *de novo* shoots while the original work of Steward (1958a and b) showed that a single cell is capable of giving rise to a complete shoot. It has been suggested that in tomato cotyledons the origin of shoots from a single cell is not likely, since rarely observed single activated cells never developed in to shoots (Monacelli *et al.*, 1988). Irrespective of whether the meristemoid is made up of cells that originate from one or

many cells of the explant, the formation of a meristematic center is a common feature of the shoot forming process.

Having said this it is also important to note that meristemoids not only appear to be common to shoot formation, but also to other forms of organogenesis such as root formation. In tobacco thin layers under root inducing conditions, meristematic structures similar to those that are observed in the aforementioned systems develop from subepidermal cells (Altamura *et al.*, 1989), these meristemoids develop into root primordia and finally roots. In leaf discs of *Lycopersicon esculentum* Mill (Coleman and Greyson, 1977) roots also arise from similar structures.

This brings to light the description of the meristemoid as developmentally plastic and the stage of development at which meristematic structures become committed to a morphogenic pathway. Waddington (1966) makes the observation that determination, or the decision to take a particular morphogenic pathway, necessarily precedes the actual morphogenesis. Many workers have described the meristemoid as a developmentally plastic undetermined structure (Bonnett and Torrey, 1966; Ross *et al.*, 1973; Thorpe, 1978, 1980), but the question has been put forward what does a meristemoid actually represent developmentally (Flinn *et al.*, 1988)? Flinn *et al.* have suggested that in *Pinus strobus* cotyledons, groups of three to four cells that occur prior to the formation of promeristemoids may be determined, while in other systems more advanced structures have been deemed determined (Villalobos *et al.*, 1985). Again this relates back to the problem of whether determination occurs at the cell or tissue level and highlights the fact that determination is not a phenomenon that can be observed. At present there seems no clear-cut stage during the development of the meristemoid that defines a structure committed to a developmental pathway. What really is required is a proven molecular marker of the transition to the determined state to distinguish the stage at which meristematic structures do in fact become determined.

4.2 Physiological aspects

A further topic of controversy has been the involvement of cell isolation in the induction of organogenesis. Two schools of thought have developed regarding the physiological requirements for organogenesis. Firstly, it has been suggested that physical and/or physiological isolation of a cell is required for it to express its developmental potential (Steward *et al.*, 1958b, 1964). This isolation of a cell is thought to remove the repressive (or differentiation-inductive) effects of neighboring cells and tissues. These repressive effects may be in the form of chemical or physical

restrictions. Cells from which the correlative influences of the surrounding tissue have been removed are then capable of active growth and the expression of their genetic potential if supplied with essential nutrients (Brown and Thorpe, 1986). There are precedents for suggesting association of morphogenetic phase change with both physical and physiological separation. For example, when single cells of a gametophyte fern are artificially isolated from surrounding differentiating tissues, their pattern of determination is altered to produce a new gametophyte (Ito, 1960). An absolute requirement for physical isolation is unlikely, although cannot be ruled out in light of ultrastructural studies (Halperin and Wetherell, 1964).

The auxin-controlled property of callus friability depends on wall characteristics, which produce rapid separation of cells (Evans *et al.*, 1981). It has been suggested that changes in wall characteristic may be involved in the redetermination of cells for indirect embryogenesis (Williams and Maheswaran, 1986). This is also applicable to indirect organogenesis. Some evidence for plasmodesmatal cellular communication may indirectly support a requirement for cell isolation (Brown and Thorpe, 1986). For example, enhancement of somatic embryogenesis has been associated with severance of plasmodesmata by plasmolysis (Wetherell, 1984).

There are certainly grounds for believing that disruption of normal tissue influences can be involved in organogenesis and it is in this region that the first school of thought overlaps with the second. Skoog and Tsui (1948) proposed that both organ formation and subsequent development, depend upon quantitative changes in the amounts and the interactions between nutrients and growth regulators essential for the growth of cells. In short, the pattern of development is regulated by the relative supplies of these substances at particular sites within the explant. Ross and Thorpe (1973) showed that the position within tobacco callus at which shoots form can be altered by inverting the tissue at different times during culture, supporting the view that physiological gradients may determine the site in the tissue at which meristemoids are initiated. In several studies shoots only form from regions of the explant in close contact with the media (Sterling, 1951; Villalobos *et al.*, 1985) and in others, shoots and roots form at different poles of the explant (Sharma and Bhojwani, 1990). Geneve (1991) has shown that the polarity of the root forming response in English ivy can be overcome by tissue inversion, although wounding was also a critical factor. These studies also suggest that the site of organogenesis may be a function of gradients within the tissue.

Explanting is itself a disruption of tissue interrelationships that can alter the physiological gradients existing within the explant prior to excision. In particular, the internal balance of growth regulators within an explant can be altered. For example, in

members of the genus *Bryophyllum* adventitious buds are produced on the edges of leaf blades. These marginal buds are in general only produced when leaves are detached from the plant. If the leaf remains attached to the plant axillary buds develop, but the formation of adventitious buds is inhibited. Henson and Waring (1977) demonstrated that the initiation of shoot buds on leaf margins was due to a decrease in the auxin/cytokinin ratio within the leaf, resulting from its isolation from the source of IAA, the shoot apex. These findings emphasize the importance of the growth regulator balance and organogenesis in tissue explants.

Shortly after the discovery of cytokinins, Skoog and Miller (1957) demonstrated that in tobacco callus, a high cytokinin to auxin ratio induces the formation of shoots, while a low ratio induces root formation. Their study provided the first evidence of controlled organogenesis in cultured tissue resulting from the interaction of auxin and cytokinin levels. These basic effects of growth regulator levels have since been confirmed in many studies.

Recently a great deal of information has been obtained from studies on crown gall tumors, which supports the suggestion of Skoog that quantitative interaction of growth regulators is a major factor involved in the initiation of organogenesis. The synthesis of growth regulators is essential for the success of *Agrobacterium tumefaciens* and *A. rhizogenes* as plant pathogens. Genes for the synthesis of auxin and cytokinin are located within the region of DNA transferred to the plant during transformation (T-DNA) (Klee and Estelle, 1991). Within the T-DNA three loci have been detected controlling tumor morphology. They effect tumor size (*tml*), root production (*tmr*) and shoot production (*tms*) (Akiyoshi *et al.*, 1983). Locus *tmr* contains genes for IAA synthesis, locus *tms* a gene for cytokinin synthesis (Klee and Estelle, 1991), and *tml*, which does not code for growth regulator synthesis, but may be involved in alteration of hormone signal transduction (Tinland *et al.*, 1989). A further locus *tzs* located outside the T-DNA also contains a gene for cytokinin synthesis and may be involved in bacterial host range (Beatty *et al.*, 1986). These genes are active in transformed plant tissue and the excess production of growth regulators leads to the characteristic crown gall (Weiler and Schroder, 1987). Analysis of transgenic plants with altered auxin and cytokinin levels has confirmed the complex interactions between growth regulators. Shoots, roots or callus can be induced to form by selectively transferring different types of genes into plants and the effects of these genes can be modified by exogenous supply of the appropriate growth regulator (Inze *et al.*, 1984; Akiyoshi *et al.*, 1983; Brown and Thorpe, 1986).

Thus regulation of organogenesis in cell cultures seems surprisingly simple and is realized in principle on the balance of two growth regulators, i.e. cytokinin and auxin. In spite of this, all groups of growth regulators have been immunochemically detected in crown gall tissues, i.e. gibberellins, ABA, IAA, isopentenyladenosine and zeatin (Sebanek *et al.*, 1991). Ethylene is also produced in crown gall and many "auxin" effects are, in reality, ethylene effects (Klee and Estelle, 1991).

Therefore, the relationships between individual growth regulators is likely to be much more complex than a simple auxin/cytokinin interaction. It has been suggested that other growth regulators also play a role in organized development (Thorpe, 1980; Brown and Thorpe, 1986). For example, abscisic acid (ABA), a naturally occurring growth inhibitor, has been shown to promote shoot development in explants of *Ipomea batatas* (Yamaguchi and Nakajima, 1973) and in protoplast-derived callus of *Solanum tuberosum* (Shepard, 1980). ABA also induces shoot formation on internode segments of tobacco cultured on media containing ABA alone or in combination with NAA, or NAA and zeatin combinations that do not normally induce the formation of shoots. ABA can therefore shift explants requirements for exogenous plant growth regulators in promoting caulogenesis (Ammirato, 1986).

Other bacterial genes known to affect plant morphology include *rolA*, *rolB*, and *rolC* from *A. rhizogenes* (Spena *et al.*, 1987). The actual function of these genes is not known, but they do not code for growth regulators synthesis (Spano *et al.*, 1988). It has been suggested that they act by altering the normal growth regulator signal transduction pathway, making transformed cells more sensitive to auxin (Spano *et al.*, 1988). While hormones produce many major effects on developmental processes, some cells do not respond to alteration in hormone levels. For example, over production of auxin or cytokinin in maturing *Petunia* embryos has no effect on their development (Klee and Estelle, 1991), thus perception, as well as the synthesis of hormones is important and may be developmentally controlled.

Recently it has been recognized that exposure to a sequence of media containing different balances of growth regulators may be required to induce organogenesis (Christianson and Warnick, 1985). This suggests that some explants may not respond to a single growth regulator balance, but may require different growth regulator ratios at different stages during the process of organogenesis. The role of growth regulators is therefore not as simple as the original observations of Skoog and Miller (1957).

Considerable research has been carried out to elucidate the mechanisms of action of traditional plant hormones and growth regulators and to identify other signaling molecules. Oligosaccharides are among the more interesting candidates for consideration as signaling molecules, as it would appear that they have multiple roles in regulating both plant defense and developmental processes (Ryan and Farmer, 1991). Oligosaccharides have for many years been implicated as elicitors of plant defense responses (Darvill and Albersheim, 1984). More recently it has been found that plant cell wall fragments, produced by partial acid hydrolysis of cell walls, influence flowering and vegetative growth in *Lemna gibba* (Gollin and Darvill, 1984). These fragments can also cause changes in the morphological characteristics of tobacco thin layer cultures. Depending on the composition of the medium, the fragments have been shown to cause cells to form vegetative shoots rather than flowers and *vice versa* (Tran Thanh Van *et al.*, 1985). These cell wall derived fragments can also influence root and flower formation, either inhibiting root formation, enhancing root formation in a polar manner, or enhancing tissue growth in a polar manner leading to flower development, depending upon the auxin and cytokinin concentration in the medium (Eberhard *et al.*, 1989; Mohnen *et al.*, 1990). The effects cannot be explained by fragments influencing the auxin or cytokinin concentrations, because changing the concentrations does not cause the same effect as the fragments. From these results it appears that traditional growth regulators such as auxins and cytokinins may not be the only regulatory molecules involved in organogenesis, but other signaling molecules may be able to modulate their effects.

4.3 Biochemical aspects

Very few biochemical studies have been carried out on organ forming tissues (Thorpe, 1980; Brown and Thorpe, 1986). Again the most thoroughly investigated systems to date are tobacco callus and cotyledons of *Pinus radiata*. The major common feature of organogenesis in these two systems is the high demand for energy required during the process of shoot formation. In shoot-forming tobacco callus, starch accumulates prior to the formation of meristemoids and shoot primordia, with a continuous supply of free sugars in the medium required during the process of organogenesis (Thorpe, 1974). In *Pinus radiata* cotyledons, starch and stored lipids are present in the explant at the time of excision and like shoot forming tobacco callus the utilization of these stored reserves during caulogenesis has been shown to occur (Yeung *et al.*, 1981; Biondi and Thorpe, 1982), although the utilization of stored lipids may for the most part be a response to wounding (Thorpe, 1990). In both systems these stored reserves act as a readily available source of energy for the shoot forming process.

The high demand for energy during caulogenesis is reflected in the increased respiration rate in shoot forming tobacco callus and increased activities in the glycolytic and pentose phosphate pathways (Ross and Thorpe, 1973; Thorpe and Laishley, 1973). Such tissues have also been shown to contain higher levels of total adenosine phosphates and NAD⁺ (Brown and Thorpe, 1980b). In *P. radiata* cotyledons, respiration also increases (Thorpe, 1990) and increased staining for the enzymes ATPase and succinate dehydrogenase occurs in the shoot forming areas of cotyledons (Patel and Thorpe, 1984), pointing to such activity being localized mainly in the shoot forming regions of the explant.

Not only is there a requirement for energy, but also for the reducing power required for general biosynthesis. Plumb-Dhindsa *et al.* (1979) demonstrated that there is a rapid decline in NADPH levels and a build up of NADP⁺ during shoot organogenesis in tobacco callus. They suggested that this reflected a greater need for the reducing power of NADPH during organogenesis.

Studies carried out on nitrogen assimilation and amino acid metabolism have also indicated that nitrogen-metabolism is important in the organogenic process. Shifts in amino acid pool sizes, higher nitrate reductase activity and increased activity of enzymes in the shikimate pathway have been observed in shoot forming tobacco callus (Brown and Thorpe, 1980b; Beaudion-Eagan and Thorpe, 1983). In *P. radiata* cotyledons the synthesis of glutamine strongly increases prior to differentiation and amino acid synthesis is enhanced under shoot forming conditions (Thorpe, 1990).

Such requirements are not unexpected, as nitrogen containing molecules such as proteins are vital to cell and tissue growth.

Polyamines have been implicated in many plant growth and developmental processes (Evans and Malmberg, 1989). Several studies have correlated an increase in polyamine levels with cell division and a drop in polyamine levels during any subsequent lessening of metabolic activity (Walker *et al.*, 1985; Evans and Malmberg, 1989). Polyamine levels have also been shown to increase during the process of organogenesis. Jarvis *et al.* (1985) found that increased levels of polyamines during root initiation and early growth were not only correlated with, but may be essential for these processes. Treatment of cuttings with IBA increased the levels of putrescine, spermidine and spermine in hypocotyls prior to primordia development. Application of exogenous spermine increased the numbers of roots formed while application of methylglyoxal-bis(guanylhydrazone), which inhibits the conversion of putrescine to spermidine and spermine, raised putrescine levels and stopped the IBA-induced rise in spermidine and spermine, with no root induction and/or growth. Similar observations on polyamine levels have been made during *de novo* root, bud and flower formation in thin layers of tobacco (Torrighiani *et al.*, 1987; 1989). Increased arginine carboxylase activity, an enzyme involved in putrescine biosynthesis, has also been reported with exposure of *Pinus radiata* cotyledons to bud forming conditions (Thorpe, 1990). Little is known about how polyamines function in higher plant physiology. Some authors have postulated that polyamines, or other such compounds, may be a type of plant growth regulator or hormonal second messenger. Alternatively, it has been suggested that as polyamines are important in cell division, they might influence patterns of cell division and the form of the plant (Evans and Malmberg, 1989). Polyamines could also have no causal role and increased levels may simply be the consequence of another event.

The information available shows that the transition of non-organogenic tissue to organogenic tissue results in major changes in explant metabolism and that as Brown and Thorpe (1986) stated, a great deal more work is required on the metabolic aspects of *de novo* organogenesis.

4.4 Molecular aspects

It has been shown that developmental events associated with organogenesis can occur very early in the culture cycle (Christianson and Warnick, 1988). These events include the attainment or loss of competence and the induction of a determined fate. Such events occur prior to the formation of differentiated structures and the identification of specific biochemical and anatomical changes associated with such events has proved difficult.

Considerable interest has been generated concerning the biochemical and molecular changes associated with somatic embryogenesis, with many studies identifying stage specific polypeptides associated with embryogenesis (Stirn and Jacobsen; Choi *et al.*, 1987; Hahne *et al.*, 1988; Racusen and Schiavone, 1988). Yet it is only recently that studies began to appear identifying changes in gene expression during adventive organogenesis. Because of this, very little is known about the coordinate changes in gene expression accompanying meristem initiation and shoot/bud development during organogenesis.

Total protein synthesis has been shown to increase before bud initiation (Hasegawa *et al.*, 1979; Thorpe, 1980; Tanimoto and Harada, 1983). Hasegawa *et al.* (1979) used 1D SDS-PAGE to identify two putative markers for adventitious bud formation in cultured Douglas fir cotyledons. The appearance of new polypeptide bands on 1-D SDS-PAGE gels has also been correlated with caulogenic treatment of *Pinus ponderosa* cotyledons (Ellis and Judd, 1987) and in *Torenia* stem segments (Tanimoto and Harada, 1983). In addition to these studies a group of polypeptides correlated well with organogenesis in cultured melon cotyledons (Leshem and Sussex, 1990). This study was of particular interest as the polypeptide markers could also be used to distinguish between the processes of shoot and root formation.

Because of the low resolution of 1D SDS-PAGE several studies have been carried out using high resolution 2D PAGE to analyze protein synthesis and/or accumulation during adventitious shoot formation (Reynolds, 1990; Stabel *et al.*, 1990; Coleman and Ernst, 1991; Renaudin *et al.*, 1991). In two of these studies only quantitative changes in polypeptide patterns were observed during meristem initiation and bud development. Stabel *et al.* (1990) found that in embryos of *Picea abies*, that were induced to form shoot buds from subepidermal cells by pulse treatment with cytokinin, no new polypeptides were synthesized as a result of bud formation. They suggested that many gene products that are induced by cytokinin treatment of mature tissue explants may already be active in the germinating embryo and that treatment resulted in the maintenance of the pattern of protein expression typical of the

germination phase in untreated embryos, rather than specific polypeptides synthesized in response to shoot inducing conditions.

In a study of *Petunia hybrida* callus (Renaudin *et al.*, 1991), induced to form shoot buds, only quantitative changes were also found. In this system the presence of polypeptides associated with the germinating embryo would appear unlikely. Renaudin *et al.* proposed that the lack of significant markers during the formation of meristemoids in *Petunia* callus, suggests that any metabolic and cytological changes that occur, do not involve major changes in the protein composition of the callus. They suggested that regulatory events rather than large changes in gene expression would account for the inductive effects of cytokinin on shoot meristem initiation.

Despite these results, Reynolds (1990) observed both quantitative and qualitative changes in the polypeptide pattern of tissue undergoing caulogenesis. In this study, stem sections of *Solanum carolinense* L. were cultured on callus inducing or shoot inducing media. As well as a number of quantitative differences, two polypeptides putatively specific for caulogenesis and two callus-specific polypeptides were detected. In this system organogenesis may not only result in the regulation of genes already expressed in the tissue, but in the induction of specific gene products.

(5) AN INTRODUCTION TO BEGONIA

Begonias are important as ornamental plants throughout the world. They are used as garden and potted plants and over 200 species have been introduced by commercial growers. Over 2000 species of begonia have been recorded most of which are perennial herbs. Begonia species are distributed from Central and South America to Asia and Africa, in both tropical and subtropical regions (Takayama, 1990).

Generally, Begonias are easy to propagate vegetatively via leaf cuttings, stem cuttings, top cuttings or by splitting, although some varieties appear to have poor regenerative properties (Bowes and Curtis, 1991). Leaf cuttings appear to be the method of choice in the genus *Begonia*, for many species are able to form both adventitious buds and roots on detached leaves (Heide, 1964, Bowes and Curtis, 1991). Even attached leaves often bear many leaf-like epiphyllous appendages on their surfaces (Maier and Sattler, 1977; Sattler and Maier, 1977). Regeneration of shoot buds on detached leaves can be greatly enhanced by the application of growth regulators, while the growth conditions of parent plants can also greatly influence the ability of cuttings to regenerate, with both temperature and light being important

(Heide, 1965). Heide (1967) suggested that the budding ability of *Begonia x cheimantha* leaf cuttings appears related to a lowering of the internal auxin/cytokinin ratio, which occurs under short-day conditions. In contrast to this study Welander (1977) found that cultured petiole sections of *Begonia x hiemalis* formed organs more readily from long-day treated stock plants.

Although a non-sterile micro-propagation technique using leaf explants has been used successfully to propagate many begonia lines in the British national begonia collection, which is a significant step for the conservation of rare and endangered Begonias, conventional propagation techniques of begonia species can be problematic (Takayama, 1990). It is often difficult to produce large numbers of genetically homogeneous plants and the use of cuttings can spread many diseases to which Begonia are susceptible (Takayama, 1990). Begonias are susceptible to many pathogens including viruses such as cucumber mosaic, powdery mildew, species of *Phytophthora*, *Botrytis cinerea* and many bacterial blights. Tumors also can be induced by the aphid transmitted *Corynebacterium fascians*. Water borne nematodes also damage plants (Takayama, 1990).

The use of cell culture techniques for the propagation of begonia plants has largely arisen because of the potential for the technique to overcome many of these problems associated with conventional culture. Disease-free plants can be obtained with new varieties and/or hybrids easily multiplied. *In vitro* techniques for plant improvement, such as cell fusion, are also being applied (Takayama, 1990).

In general begonia species respond well to culture, with roots, shoots, flowers and callus induced from some species/cultivars (Takayama, 1990) although as previously mentioned, temperature during the process of shoot induction appears critical for a maximal response in some cultivars (Fonnesbech, 1974a and b).

5.1 Anatomical Features of the Begoniaceae

Summarized from Metcalfe and Chalk (1950)

5.1.1 The leaf

Hairs, include the following types. (A) Non-capitate: these are always multicellular, including uniseriate, multiseriate and various other types. (B) Capitate: these vary greatly with the few or many-celled head being spherical, ellipsoidal, club- or hammer-shaped; stalks vary in length. Multicellular, easily detached, shortly stalked pearl glands also occur. The *epidermis*, on both surfaces usually consist of large, thin-walled cells; frequently papillose; red cell sap is common in the lower epidermal cells. The *cuticle* is thin and granular. One or more layers of large-celled hypoderm occur

beneath one or both surfaces. *Stomata*, are not numerous and are confined to the lower surface. Each pair of guard cells is surrounded by 3-6 subsidiary cells, often arranged in two rings which may be solitary or in definite groups. The *mesophyll* generally includes one layer of palisade tissue or more rarely parenchyma. Palisade cells are short and conical or elongated with concertina-like foldings of the lateral walls. Air-filled spaces between the epidermis and the palisade cells give a silvery sheen to the leaves of some species. Stone cells, and similar mechanical elements, occur frequently in the mesophyll or around the veins. Ground tissue around the larger veins often contains strongly developed collenchyma.

The petiole exhibits vascular bundles arranged in a circle in some species but others include medullary strands as well. The outer part of the petiole is often collenchymatous.

5.1.2 The stem

The *epidermis* consists of one to four layers with cork arising in or immediately below the epidermis. The *cortex* is made up of 2 cell types, collenchymatous cells make up the outer part of the cortex with the inner part composed of thin-walled tissue containing crystals and chlorophyll; sometimes with red cell sap. *Vascular bundles* appear in transverse sections, as a single ring, occasionally accompanied by others in the cortex, or more frequently in the pith. Bundles of the main ring are generally isolated but sometimes form an almost closed ring. Tissue between bundles can consist of elongated cells with lignified walls and slit-shaped pits in shrubby species, with the corresponding tissue composed of radially elongated or isodiametric, thin-walled cells in the root-climbers. The xylem is made up of vessels in radial rows, and progressively increase in diameter toward the exterior of the stem. Vessels are surrounded by parenchyma. Vessels have scalariform bordered pits where in contact with each other, and simple, circular perforation, and/or scalariform plates in the end walls.

5.2 *Begonia erythrophylla (erthrophylla)*

Otherwise known as the beefsteak begonia, has kidney-shaped leaves that are shiny and green on top and red underneath. It has pale green veining on both sides of the leaf and bristly hairs on the margins. Pretty pale pink flowers appear on tall panicles during spring. Probably of garden origin the plant is also known as *B. x erythrophylla* J. Neuman [*B. x Feistii* Hort. ex L.H. Bailey].

(6) THE OBJECTIVES OF THIS STUDY

The purpose of this study was primarily the identification of molecular markers associated with specific stages in the process of organogenesis in *B. erythrophylla*. Particular emphasis was placed on finding molecular markers of events taking place prior to the differentiation of organ primordia. *B. erythrophylla* was chosen for this study because *Begonia* species are well known to readily produce adventitious shoots and because very little work has been carried out on aspects of organogenesis in *Begonia*, not specifically related to micropropagation. Furthermore, such a study may provide an interesting comparison to the numerous studies carried out on members of the Solanaceae and various conifers.

Because no previous studies were known to have been carried out specifically on organogenesis in petiole sections of *B. erythrophylla*, the initial part of this study comprised general observations on the morphological, anatomical, physiological and to some extent biochemical events, associated with organogenesis. In this portion of the study particular emphasis was placed on the developmental stages involved in *in vitro* organogenesis and so was not intended to be a highly detailed study of individual events, but rather to provide an outline of the changes taking place during the process of organogenesis so that they may be correlated to changes observed at the molecular level.

Molecular studies involved inspection of the polypeptides profiles/patterns of both accumulated (silver/Coomassie stained) and *in vivo* labelled polypeptides, separated by both one and two-dimensional PAGE. The aim was to compare the processes of caulogenesis and rhizogenesis to assess the similarities and differences of the two processes, in the hope of finding stage specific markers for each. Care was taken to include several controls in the study to try to eliminate as many possible causes for changes observed not related specifically to the organogenic process. These non-organogenic controls included, callus forming explants (to distinguish changes associated with induced random cell division), culture on non-inductive basal medium, (to distinguish general responses to the culture process unrelated to growth regulators) and to separate the response of exposure to growth regulators from the process of caulogenesis, explants were cultured on shoot inducing medium without sucrose (no shoot induction). The aim was also to determine if polypeptides associated with organogenesis were present in developed organs of donor plants.

The aforementioned portion of this study was intended to be essentially descriptive, out-lining events involved in organogenesis and finding molecular markers for them. The rest of this study was primarily involved with caulogenesis and aimed to further divide the process by the use of inhibitory chemicals, as described by

Christianson and Warnick (1984) and to determine which, if any, of the molecular markers of caulogenesis were affected by these chemicals.

The final aim of this study was to ascertain if the molecular markers associated with caulogenesis were unique to *in vitro* petioles or if they were also expressed in leaf tissue exposed to the same shoot forming conditions.

6.1 SUMMARY OF THE AIMS OF THIS STUDY

- (1) To establish developmental stages in the processes of caulogenesis and rhizogenesis, by observing morphological, anatomical, physiological and biochemical changes associated with organogenesis.
- (2) To identify molecular markers for the developmental stages observed.
- (3) To determine if these markers are present in organs of the donor plant.
- (4) To further subdivide the process of caulogenesis by using chemical inhibitors.
- (5) To ascertain the effects of these inhibitors on the markers identified for caulogenesis.
- (6) To ascertain if the markers associated with caulogenesis are associated with caulogenesis in other tissues of the donor plant.

CHAPTER II

MATERIALS AND METHODS

(1) TISSUE CULTURE

1.1 Culture media

Basal medium was made up of Murashige and Skoog (1962) salts plus, inositol, nicotinic acid, pyridoxine HCL, thiamine HCL, glycine, folic acid and biotin, at 100, 5, 0.5, 0.5, 2, 0.5, 0.05mg/liter respectively, 30g/liter sucrose and 0.7% Davis agar. Shoot inducing medium (SIM) contained 0.1mg/liter NAA and 1mg/liter BA. Root inducing medium (RIM) contained 1.0mg/liter NAA and 0.05mg liter BA. Callus inducing medium (CIM) contained 0.2mg/liter NAA, 0.1mg/liter BA and 0.2mg/litre 2.4.D. Maintenance medium (MM) consisted of half strength Murashige and Skoog salts and 0.7% agar, no organics were included in this medium. If inhibitors were to be included in a medium they were added prior to autoclaving. All media were adjusted to pH 5.8 prior to autoclaving.

1.2 Plant material (potted plants)

Plants were derived from leaf-cuttings of a single parent plant and were maintained in a growth room under cool-white fluorescent lamps supplemented with tungsten bulbs for a 16 hour day at 25°C and 8 hours dark, at 15°C. The plants were watered twice weekly and no additional nutrients were supplied.

1.3 Surface sterilization of plant material

Petiole or leaf pieces were washed in distilled water, soaked for 30 seconds in 70% ethanol (v/v), washed again in distilled water and then soaked with occasional agitation for 10 minutes in a solution of 2% sodium hypochlorite (diluted from a commercial bleach containing 5% w/v NaOCl) to which was added 1 drop of Tween 20 for every 200mls of bleach solution. The tissue pieces were then washed at least 3 times with sterile distilled water.

1.4 Plant material (*in vitro*)

Initially plants grown *in vitro* were derived from adventitious shoots induced by culture of petiole sections from potted plants on a shoot inducing medium consisting of basal medium to which 0.5mg/l BA, 0.01mg/l NAA and 100mg/liter adenine sulfate were added. These *in vitro* plants were then used to establish more plants in culture (refer results section). Plants established *in vitro* were grown in 250ml polycarbonate screw capped tissue culture vessels, each containing approximately 50mls of maintenance medium. Once established plants were subcultured every 8 weeks. Subculturing involved transfer of the first 2cm of the stem, consisting of the shoot apex, 2 or 3 expanded leaves and numerous adventitious roots to fresh maintenance medium. Plants were cultured on maintenance medium for 4 to 6 months, after leafy shoots were initiated on SIM, before being used as a source of experimental material. After a further 6 months plants were established out of culture or discarded. Plants were maintained in a tissue culture room under continuous lighting, provided by cool-white fluorescent lamps supplemented with tungsten bulbs. The light intensity was approximately $100\mu\text{E m}^{-2} \text{sec}^{-1}$.

1.5 Culture procedures; petiole sections

Leaves from the 4th, 5th and 6th, expanded leaves were excised, complete with petiole, from *in vitro* grown plants. The lamina were removed and the petioles were cut into 5mm sections. These sections were placed vertically, with the basipetal surface in contact with the medium, unless stated otherwise. Sections from different positions within the petiole and from petioles of other *in vitro* plants, were placed on petri dishes containing medium in a random manner. Five or ten sections were placed on each petri dish.

1.6 Culture procedures; leaf discs

Leaf tissue excised from the leaves of potted plants was surface sterilized and 1cm discs were cut from between the veins, using a 1cm cork borer. Leaves of approximately the same age were used as a source of tissue, having a diameter of about 8cm. Discs were placed with the red pigmented lower surface in contact with medium.

1.7 Media transfer experiments

All basic media transfer experiments were carried out as stated. With the RIM to SIM/SIM to RIM experiments, all explants were transferred to BM after 22 days in culture to allow organ development. All media transfer experiments were replicated at least twice with most replicated 3 times.

1.8 Scoring organ number

Shoot number was scored with the aid of a stereomicroscope after 6 weeks in culture. Leafy shoots were counted unless otherwise stated. Due to the high density of shoots with some treatments, which made counting difficult, shoot number was determined in a destructive manner. Shoots were dissected from the explant as they were scored. Roots were easily scored without dissection from the explant. A treatment was considered to organogenic if a high percentage of explants produced organs and the mean value of organs per explant was greater than one.

(2) HISTOLOGY AND PREPARATION FOR THE S.E.M.

2.1 Thin sections

2.1.1 Fixation

Petiole explants were cut into 1 to 2 mm sections and fixed at room temperature for 3 to 4 hours under water vacuum in 2.5% glutaraldehyde, in a 0.075 M sodium phosphate buffer pH 7.2.

2.1.2 Dehydration

Tissue was dehydrated for 2 hours in an ethanol series for 20 minutes, in each of 10, 20, 40, 60, 80% grades, with 2 x 30 minute changes in absolute ethanol. Specimens were also re-evacuated at a lower surface tension, in 80 and 100% alcohol changes, in order to remove any air adhering to hairs on the surface of the tissue.

2.1.3 Infiltration

Tissue was infiltrated in mixtures of 25, 50, 75 and 100% (x2) LR White acrylic resin (London Resin Company) dissolved in ethanol, for 2 days each at room temperature.

2.1.4 Polymerization

Polymerization of the resin was carried out at 4°C using the LR White cold curing procedure. Flat-embedding caps were filled with LR White acrylic resin to which the cold-cure additive had been added (1 drop of cold cure additive to 12mls of resin), tissue sections were then quickly positioned in the resin. The cap was then filled to overflowing and covered with a piece of parafilm taking care that no air was trapped under the parafilm. The cap was then covered with a small piece of heavy glass and placed in a refrigerator at 4°C overnight.

2.1.5 Microtomy

2 or 4µm transverse sections of petiole explants were obtained with a Reichert rotocut 2000 EX equipped with glass knives.

2.1.6 Staining

Thin sections were stained with Methylene blue-azure A (Warmke *et al.*, 1976) and mounted in immersion oil. Handsections were cut as for histochemical analysis and were stained with 0.05% toluidine blue in 0.075M phosphate buffer pH 7.2.

2.1.7 Photomicrography

Bright-field micrographs were recorded on Kodak Ektar 25 film using a Zeiss IM35 photomicroscope

2.2 **Preparation of explants for the S.E.M.**

Detailed observations of explants were made using a Cambridge Stereoscan 250 Mk2 scanning electron microscope, operated at 8-20kV, equipped with a Robinson backscatter detector.

Explants, fixed and dehydrated as for thin sections, were freeze-dried (Robards, 1978) and affixed to aluminium stubs with double sided sticky tape. Specimens for S.E.M. were routinely coated with a 5-10nm layer of gold-palladium using an Edwards model coater.

(3) HISTOCHEMISTRY

3.1 Sections

All histochemical examinations were carried out on fresh unfixated tissue. Thin sections (30-40 μm) were cut by hand, by placing the petiole explant between 2 polystyrene blocks, submerging it in cold Tris-HCL or sodium phosphate buffer and cutting with a razor blade.

3.2 Starch

Starch grains were detected by floating specimens in a drop of iodine-potassium iodide and zinc-chloride reagent (Jensen, 1962).

3.3 Enzymes were localized using the following procedures

3.3.1 ATPase activity was demonstrated by the method of Wachstein *et al* (1960). Sections were incubated for 30 minutes at 37°C in the following solution, 25mg ATP in 40mls of Tris-HCL buffer (pH 7.2), 3mls of 2% lead nitrate and 5mls of 2.5% magnesium sulphate. After incubation the sections were washed with distilled water, treated with 1% ammonium sulphide for 1 minute and then washed with distilled water and wet mounted.

3.3.2 Acid Phosphatase activity was demonstrated by the method of Burstone (1958). Sections were incubated for 30-60 minutes at 37°C in the following solution, 50mls of 0.1M acetate buffer (pH 5.5), to which 20mg sodium naphthol AS-MX phosphate in 1ml N,N-dimethylformamide and 50mg of coupling reagent Fast blue B was added. After incubation sections were washed and water mounted.

3.3.3 Succinate dehydrogenase activity was demonstrated by the method of Nachlas *et al.* (1957). Sections were incubated for 30-60 minutes at room temperature in a solution consisting of, 0.05M phosphate buffer (pH 7.6), 0.05M sodium succinate and 0.5mg/ml nitro-blue tetrazolium (NBT). After incubation sections were washed and water mounted.

3.3.4 Peroxidase activity was demonstrated by the method of Lojda *et al.* (1979). Sections were incubated for 30 -60 minutes at room temperature in a solution consisting of 10mg of 3,3-diaminobenzidine tetrahydrochloride in 10mls of 0.1M Tris-HCl (pH 7.2) to which 10ml of freshly prepared 0.2% hydrogen peroxide was added. After incubation sections were washed and water mounted.

3.3.5 Glucose-6-phosphatase activity was demonstrated by the method of Shaykh and Roberts (1974). Sections were incubated for 30-60 minutes at 37°C in the following solution, 40ml of 0.1M Tris-malate buffer (pH 6.6) containing 26mg of glucose-6-phosphate and 1ml of 2% lead nitrate. After incubation the sections were washed with distilled water, treated with 1% ammonium sulphide for 1 minute and then washed with distilled water and wet mounted.

3.3.6 Glucose-6-phosphate dehydrogenase activity was demonstrated by the method of Gahan (1984). Sections were incubated for 30-60 minutes at 30°C in the following solution, 100ml of 0.1M Tris-HCL (pH 7.5) containing 40mg of glucose-6-phosphate, 15mg of NADP+, 20mg NBT, 4mg of PMS and 1ml of 1M magnesium chloride. After incubation sections were washed and water mounted.

Controls. For all of the above enzymes control incubations were carried out consisting of incubation without substrate, and the incubation of heat killed tissue.

3.4 Photomicrography

Bright-field micrographs were recorded on Agfa APX 25 film while dark-field were recorded on Kodak Ektar 25, using a Zeiss IM35 photomicroscope.

(4) DETERMINATION OF STARCH CONTENT

The starch content of explants was estimated using the anthrone procedure (McCready *et al.*, 1950). Tissue was ground to a fine power using a mortar and pestle pre-chilled in liquid nitrogen. One gram of the powder was weighed into a 10ml centrifuge tube, mixed with 1ml of distilled water and centrifuged for 15 minutes at 15300 rpm.

The supernatant was discarded, and the pellet extracted 5x with 10ml of hot, 80% ethanol. After the final extraction, the pellet was suspended in 2mls of distilled water and cooled in an ice bath. 2.6mls of perchloric acid (52%) was added while vortex mixing and the sample was left for 15 minutes. 4mls of distilled water was then added

and the sample was centrifuged as before. The supernatant was decanted into a 25ml volumetric flask.

1ml of distilled water was added to the residue in the centrifuge tube and it was placed back in the ice bath. A further 1.3mls of perchloric acid was added and the sample treated as before, except only 2mls of water were added.

The extract in the volumetric flask was then made up to 25mls with distilled water. Aliquots of the extract were then diluted to give a final concentration of 25 to 100ug of starch per 5mls. 1ml of the diluted extract was added to 2mls of anthrone reagent (2 grams of anthrone in 1 liter of cold 95% sulphuric acid) in a test tube on ice. After adding anthrone to all the samples for which the starch level was to be estimated, the tubes were mixed and heated at 100°C for 5.5 minutes, cooled in a water bath, and then read at 630nm in Beckman DB spectrophotometer. A factor of 0.9 was used to convert glucose to starch.

(5) DETERMINATION OF PROTEIN CONTENT

The protein content of cultured explants was determined using the original Bradford method (Bradford, 1976) and the method as modified by Ramagli and Rodriguez (1985) for use with protein samples solubilized in 2D extraction buffers.

The amount of soluble protein extracted from cultured explants using 0.1M Tris-HCL (pH 7.2) buffer was determined using the original Bradford method. Protein extracted using the SDS extraction buffer was also determined using the unmodified Bradford method. In this case interference by SDS was avoided by protein pellets being redissolved in phosphate buffer after acetone precipitation rather than the SDS sample buffer. This avoided SDS interfering with the assay.

The protein content of extracts for 2D-electrophoresis was assayed using the modified Bradford assay (Ramagli and Rodriguez, 1985). This method enabled an accurate estimate of the protein concentration of these extracts without interference from ampholytes and without precipitation of the protein. It was found necessary, however, to assay the protein concentration prior to the addition of protamine sulphate and urea to the extract, as both interfered even with the modified assay. For this reason aliquots of each extraction were maintained without adding these chemicals for the protein content to be assayed. The protein content of every extract used for 2D-electrophoresis was assayed.

(6) IN VIVO LABELLING, DETERMINATION OF RADIOACTIVITY AND PROTEIN SYNTHESIS

In vivo labelling was carried out by incubating explants in the appropriate liquid medium to which 100 μ C/ml [35 S]-methionine (Amersham) had been added. 200 to 300mg of tissue was incubated per milliliter of medium for 3 hours prior to protein extraction.

To determine the total uptake of [35 S]-methionine 2 μ l aliquots of protein extract were added to 5ml of scintillation cocktail in a standard 20-ml low-potassium glass scintillation vial and counted for 60 seconds in Pharmacia Wallac 1410 Liquid Scintillation Counter. Three aliquots of each extract were counted and the mean value calculated.

To measure the level of [35 S]-methionine incorporation a modified method of the filter disc method of Mans and Novelli (1961) was employed. Disks of Whatman No. 3MM chromatography paper, 2.3cm in diameter, were numbered and mounted on straight pins. Two microliters of labelled extract was spotted onto each disc and allowed to dry in a fume hood. The discs were then immersed in an ice-cold solution of 10% TCA and 0.1M methionine for 1 hour. Discs were then resuspended twice for 15 minutes each in 5% TCA. Discs were then collected and resuspended in an ether-ethanol mixture (70/30 v/v) and incubated at 37 $^{\circ}$ C for 30 minutes for extraction of the TCA and lipid material. The disks were then washed with the ether-ethanol mixture and then suspended in ethyl ether for 15 minutes at room temperature to remove the alcohol and water. After recovery, the pins were removed and the discs air dried. Each disc was placed in a standard 20-ml glass counting vial, and 5ml of scintillation mixture was added [0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis-2(5-phenyloxazolyl)benzene in toluene] and the sample counted as above. Again 3 aliquots of each extract were counted and a mean count per extract obtained.

(7) ONE-DIMENSIONAL SODIUM DODECYLSULPHATE GEL ELECTROPHORESIS (SDS-PAGE)

7.1 Apparatus

Both a Biorad Protean II chamber and a chamber constructed following the design of Hames (1990) were employed for 1D SDS-PAGE electrophoresis. Power was supplied with Hoeffer P5500xT power pack. Gels for use with the Protean II chamber (160 x 200 x 1.5/1mm) were cast on the stand-alone casting device (Bio-

Rad). Gels for the other apparatus (120 x 160 x 1.5mm) were cast between glass plates sealed with 1% agarose.

7.2 Reagents

Reagents were from the following sources: acrylamide, SDS, Triton X-100, ampholytes 4-8, glycerol, b-mercaptoethanol (BDH): tetramethylethylenediamine (TEMED), ammonium persulphate, N,N'-methylene bisacrylamide (BIS) (Bio-Rad): glycine, Tris-HCL, urea, Nonidet P-40 cholamidopropyltrimethylhydroxypropanesulphonate (CHAPS), ampholytes 3-10, protamine sulphate, phenylmethyl sulphonyl fluoride (PMSF) (Sigma).

7.3 Procedure

7.3.1 Sample preparation

Tissue samples (300-500mg) were collected and transferred to a pre-chilled mortar, frozen in liquid nitrogen and ground to a fine powder. The powder was transferred to pre-chilled 1.5ml Eppendorf tubes and sample buffer was added at a ratio of 0.5 to 1 (v/w). Two extraction procedures were employed. Firstly total protein was extracted using the procedure of Chen and Luthe (1987). In this procedure extraction buffer consisting of 62.5 mM Tris-HCL (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) b-mercaptoethanol, 0.001% (w/v) bromophenol blue, and 1mM phenylmethyl sulfonyl fluoride (PMSF) was added and the sample was intermittently vortex mixed for 5 minutes. Samples were then boiled for 5 minutes and centrifuged at 15 300g for 15 minutes. The supernatant was decanted in a new Eppendorf tube and protein was precipitated by incubation with 4 volumes of cold acetone for 30 minutes. After centrifugation as above, the resulting pellet was vacuum-dried, and resuspended in SDS-PAGE sample buffer. This protein extract was layered on gels. Because the sample buffer contained SDS and b-mercaptoethanol, essentially all proteins were solubilized. With the second extraction procedure protein was extracted as per 2D-electrophoresis. Prior to running, a 5x concentration of the above extraction buffer, minus the PMSF was added at a ratio of 1 part buffer to 4 parts sample (Mayer *et al.*, 1987) and the sample mixed at room temperature for 5 minutes.

7.3.2 Gel preparation and running conditions of SDS-PAGE gels

Uniform 12%, 15% and 18%T gels were prepared as per Hames (1990). Gels were overlaid gently with distilled water and allowed to polymerize for 1 to 2 hours after which time the top of the gels were extensively washed with distilled water. The surface of the gels were dried from the side by suction with a syringe. The stacking gel was then overlaid and the comb inserted. Stacking gels were allowed to polymerize for 30 minutes prior to use. Two gels were prepared and run together using the Protean II, while a single gel was run using the home made apparatus. For Coomassie staining 200x160 x1.5mm gels were employed. For fluorography the gel thickness was reduced to 1mm to facilitate crack free drying. Electrophoresis was conducted using the Laemmli buffer system (Laemmli, 1970). Gels were run at 40mA/gel constant current during the stacking phase after which the current was reduced to 25mA/gel constant current and run until the tracking dye just reached the end of the gel. No cooling was employed.

7.3.3 Coomassie staining procedures

Following electrophoresis the gels were stained with a solution containing 0.2% (w/v) Coomassie blue R250, 45% (v/v) methanol and 10% (v/v) glacial acetic acid for 30 minutes with gentle agitation. Gels were then destained in a solution containing 5% methanol and 10% glacial acetic acid. Background was destained completely by agitation overnight in fresh destain. A 2cm length of yarn was used to absorb stain which diffuses from the gel. Gels were photographed using a Polaroid MP40 camera with Polaroid 665 film providing both a negative and a positive print. A Kodak Y2 filter was used.

7.3.4 Fluorography

At the end of the run, gels were either stained in Coomassie blue as above, or fixed in 500mls of ethanol/acetic acid/distilled water solution (40/10/50) for 1 hour on an orbital shaker and then transferred to glacial acetic acid for 5 minutes. Gels were then soaked in 20% (w/v) 2,5-diphenyloxazole (PPO) (Sigma) in acetic acid for 1.5 hours (Skinner and Griswold, 1983). Gels were transferred to distilled water for 10 minutes x3 after which time they were placed in a gel softening solution (3% glycerol, 30% methanol, 67% distilled water) (Hames, 1990) overnight. Gels were then dried under vacuum onto whatman number 1 filter-paper using a Bio-Rad vacuum gel dryer. Once dried, gels were exposed to pre-flashed (Appendix 1) Kodak X-OMAT X-ray film at -80°C for 3 to 7 days. Film was processed for 5 min at 20°C in KODAK GBX developer with intermittent agitation, fixed for 10 minutes, followed by washing for 15 minutes under running water.

(8) 2-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

8.1 Apparatus

The isoelectric focusing (IEF) separation was performed in the Protean II chamber (Bio-Rad) using the Protean II Multi-cell 2-D conversion kit (Bio-Rad); the Protean II chamber was also employed for SDS-PAGE electrophoresis. Power was supplied with a Model 3000/300xi (Bio-Rad) power pack for IEF separation and a Hoeffer 5500xT power pack for the SDS-PAGE separation. The gels (160 x200 x1.5/1mm) were cast on the stand-alone casting device (Bio-Rad).

8.2 Reagents

Reagents were from the following sources: acrylamide, SDS, Triton X-100, ampholytes 4-8, glycerol (BDH): tetramethylethylenediamine (TEMED), ammonium persulphate, piperazine di-acrylamide (PDA) (Bio-Rad): glycine, Tris-HCl, urea, Nonidet P-40 cholamidopropyl dimethylhydroxypropanesulphonate (CHAPS) ampholytes 3-10 (Sigma): DTT (Calbiochem):

8.3 Procedure

8.3.1 Preparation of IEF gels

Isoelectric focusing gel solution was prepared as per Hochstrasser *et al.* (1988a) (Appendix 2) except that PDA was utilized as a cross-linking agent instead of N,N'-methylene bisacrylamide (Hochstrasser *et al.*, 1988b) and ampholytes 3-10 and 4-8 were used in place of the 3.5-10 and 5-7 used in their procedure. The gel solution was filtered through a 0.22µm nitrocellulose membrane, degassed and kept as 1ml aliquots at -80°C; no degassing prior to use was necessary after this treatment. 2µl of 20% ammonium persulfate per milliliter was added to start polymerization (Mayer *et al.*, 1987). No TEMED was added.

The gels were polymerized in glass capillary tubes (Bio-Rad) 1.5mm internal diameter, 180mm in length. Tubes were filled to 145mm with a Bio-Rad tube gel loading needle. No overlay was used with gels allowed to polymerize for at least 3 hours before use. Before use, the top and bottom, of the gels were rinsed with distilled water to remove any unpolymerized gel mix. Gels had a final length of approximately 140mm.

8.3.2 Sample preparation

Tissue samples were ground with a mortar and pestle pre-chilled in liquid nitrogen. The powder was weighed in a pre-chilled Eppendorf tube and extraction buffer was added. 2D-MH extraction buffer (Mayer *et al.*, 1987) with the 2% Triton X-100 replaced with 1% Nonidet P-40 and 1% CHAPS was used for protein extraction. The buffer to sample ratio was 0.5 to 1 (v/w). The extract was incubated on ice with 1-2 mg of protamine sulfate per milliliter for 15 minutes with intermittent vortex mixing. After centrifugation for 15 minutes at 1°C (15300 rpm), the supernatant was transferred to a new Eppendorf tube, and urea added to a final concentration of 9M. After five minutes vortex mixing at room temperature, the sample was divided into 70ul aliquots and stored at -80°C until required.

8.3.3 IEF separation conditions and gel loading

The anolyte contained phosphoric acid, 25mMol; the catholyte contained sodium hydroxide, 50mMol (Duncan & Hershey, 1984). The samples were loaded on the basic side of the capillary tubes approximately 50ug of protein was loaded per gel for silver staining while 150 000 to 200 000 C.P.M. was loaded for fluorography. Samples were overlaid with 3ul of extraction buffer with out urea and then the tube was filled with catholyte. The isoelectric focusing was performed at room temperature with the voltage increased in steps, 20 minutes each, 200, 400, and 900 volts, and run up to 18000 volt/hours (Mayer *et al.*, 1987).

8.3.4 IEF gel removal and storage

To remove the gels from the tubes, a 5ml syringe filled with water was connected to the electrophoresis tube via a short piece of tubing, and the gel slowly forced out by pressure on the syringe. Gels were rinsed with 200ul of transfer solution (Hochstrasser *et al.*, 1988a) (Appendix 3) then sealed in a plastic tube containing 500ul of transfer solution. Gels were then immediately transferred to a -80°C freezer without prior equalibration.

8.3.5 Preparation of SDS-PAGE gels

Uniform 15% T gels were prepared as per Hochstrasser *et al.* (1988b) using PDA as a cross-linker in place of BIS to reduce background staining of the gel matrix during silver staining. Gels were overlaid gently with distilled water and allowed to polymerize for 1 to 2 hours after which time the top of the gels were extensively washed with distilled water. The surface of the gels were dried from the side by suction with a syringe just prior to the second-dimension sample overlay. Stacking gels were not employed. Two gels were prepared and run together. For silver staining

200x160 x1.5mm gels were employed. For fluorography the gel thickness was reduced to 1mm to facilitate crack free drying.

8.3.6 Transfer and second-dimension separation.

IEF gels were thawed out for 10 to 15 minutes at room temperature. They were then immediately layered on top of the second-dimension gel. Gels were easily slipped between the glass plates on 1.5mm thick gels, however, care was required when layering the IEF gels on 1mm thick second-dimension gels. The IEF gels were not sealed with any agarose solution or filter paper and adhered well to the top of the second-dimension gels provided it was well dried. Once assembled into the gel apparatus the gels were gently overlaid with running buffer. The top reservoir was then filled and the gels run immediately. Top and bottom running buffers were as described by Hochstrasser *et al.* (1988a) (Appendix 4). The gels were run at 25mA/gel constant current generally for 5 hours. The voltage increased from 80 to 100 volts to between 250 and 300 volts. No cooling was employed.

8.3.7 Silver staining procedures

At the end of the run gels were removed from the glass plates and fixed in 500mls of ethanol/acetic acid/distilled water solution (40/10/50) for 1 hour on an orbital shaker. Gels were then either washed 4 times for 5 minutes each with 500 mls of distilled water, or the fixing solution was replaced and the gels stored for staining later. Once gels had been washed they were then soaked individually for 30 minutes in 100ml/liter glutaraldehyde solution. Extensive washes with deionized water were then performed to entirely remove the glutaraldehyde (4x 15 minutes followed by an overnight wash with 1 liter of distilled water per gel). The gels were then stained individually for 15 minutes in an ammoniacal silver nitrate solution (Appendix 5). After staining, the gels were washed with distilled water for 5 minutes x4. The image was then developed in a citric acid and formaldehyde solution (0.1g citric acid and 1ml formaldehyde in 1 liter of deionized water (Hochstrasser *et al.*, 1988a) until a slight background stain appeared (8 to 10 minutes). The development process was stopped with an acetic acid/distilled water solution (5/95) for 10 minutes after which time gels were immediately photographed using a Polaroid MP40 camera with Polaroid 665 film providing both a negative and a positive print.

8.3.8 Fluorography

At the end of the run gels were fixed as per silver staining and then transferred to glacial acetic acid for 5 minutes. Gels were then treated as per one-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis except that once dried, gels were exposed to pre-flashed (Appendix 1) Kodak X-OMAT X-ray film at -80°C for 5 to 16 days.

8.4 Comparison of gels/fluorographs

Visual comparison of photographs of silver stained gels, with prominent spots as reference points was utilized to identify differentially expressed polypeptides. Black and white photographic prints (30x34cm) were made of each gel on Ilford Multigrade paper (G2). Initially, 6 Day 0 gels from 3 separate extractions were compared, and a base pattern of spots established, consisting of spots only present in all 3 extractions. Comparison of this base pattern to the patterns observed with various treatments was carried out in a pair-wise manner. Spots were only deemed to be differentially expressed if the difference was present in at least 2 replicate experiments, with 2 gels normally compared from each experiment. Individual experiments were extracted and isoelectric focused independently. A Day 0 extract was run with each batch of IEF gels and compared to the base pattern to determine if there was any major variation between isoelectric focussing runs.

If extracts from one treatment were to be compared to one another, extracts were isoelectric focused together to allow more accurate comparison. For example, when SIM was compared to RIM a typical run would consist of protein extracts from, DAY 0, SIM 3, RIM 3, SIM 7, RIM 7, SIM 14, RIM 14, and BM 14.

Fluorographs were compared in the same manner as silver stained gels, except, the fluorographs were either compared directly on a light box, or positive prints obtained by contact printing onto AGFA LITEX RD 821 RC direct positive photographic paper were compared.

9 Cytokinin activity

Cytokinin activity was assayed using excised Radish cotyledons (Letham, 1971).

CHAPTER III

RESULTS

(1) ESTABLISHMENT OF PLANTS *IN VITRO*

As the aim of this study was to find markers of shoot organogenesis, minimal genetic and environmental variation between plants used as a source of explant material was a desirable feature. For this reason it was decided to establish large numbers of clonally derived *B. erythrophylla* plants in culture. The use of clonally derived plants maintained in culture, would not only reduce genetic variation between explants, but it also would avoid any artifacts induced by the surface sterilization of plant tissue and minimize any environmentally induced variation.

Initially glasshouse grown *B. erythrophylla* plants (PLATE 1A) were propagated via leaf cuttings, which when placed in water formed roots and then shoots close to the root petiole junction (PLATES 1B & 1C). Leaf discs and petiole sections excised from these plants produced shoots when surface sterilized and exposed to a basal medium supplemented with 0.1mg/l NAA and 0.5mg/l BA. Shoot primordia were clearly visible on both explant types after 21 days in culture, with well developed leafy shoots present after 6 weeks (PLATES 2A & 2B). Although both types of explant produced shoots, petiole sections gave a more uniform response with more shoots per explant. For this reason petiole sections were chosen further study.

Despite the presence of many shoots per petiole section after six weeks in culture, only 2 or 3 plantlets large enough to be easily excised were produced with continued culture on shoot inducing medium. These plantlets were typical of *B. erythrophylla*, consisting of a leafy stem ending in a shoot apex. The base of the stem, which was attached to the original explant, was covered with adventitious roots. Greater numbers of large plantlets were produced if explants were divided after 4 to 6 weeks culture on shoot inducing medium. Clumps of shoots attached to a small amount of explant tissue were excised and transferred to basal medium which allowed plantlets to develop more successfully.

Individual plantlets were usually large enough to be subcultured after a total of 10 to 14 weeks in culture. Plantlets were transferred to half strength Murashige and Skoog salts with no organics and 0.7% agar. This medium, termed maintenance medium (MM) promoted further root development and subsequent plantlet growth (PLATE 4E). Organics were omitted from the maintenance medium to allow as normal a physiology in the *in vitro* plants as possible and to simplify the propagation procedure.

Using this procedure over one hundred plants derived clonally from a single parent plant were established in culture. Plants could be maintained *in vitro* indefinitely by subculturing the first 2 centimeters of the stem, consisting of the shoot apex, adventitious roots and 2 or 3 well developed leaves to MM. Subculturing was also achieved by transferring to MM, micro-cuttings (PLATE 4F) consisting of the shoot apex with a few expanded leaves. Establishment and growth of micro-cuttings was considerably more rapid than the apex alone.

Plants were maintained *in vitro* for 4 to 6 months (2 or 3 subcultures) before use as parent plants for experimentation to overcome any possible conditioning effects from the regeneration procedure. Petiole sections from plants established *in vitro* did not show any predisposition to form shoots spontaneously without exposure to the shoot inducing medium. Petiole sections cultured on basal medium without growth regulators failed to produce any organs, with explants gradually becoming senescent.

Although the medium used to establish *B. erythrophylla* plants in culture induced shoots, it was not necessarily optimal. For this reason and to develop root and callus inducing media, the effect of growth regulator balance on organogenesis was investigated.

(2) THE INFLUENCE OF GROWTH REGULATORS

The effect of exogenously supplied NAA and BA was determined in experiments where various concentrations of NAA (0/0.01/0.1/1 mg/l) were combined with various concentrations of BA (0/0.1/0.5/1/2/4 mg/l) respectively (FIGURES 1A & 1B). The survival rate of explants was very low in the absence of both growth regulators, with most explants gradually turning chlorotic after 14 days in culture. BA alone, increased the survival rate of explants with increasing concentration, but in combination with NAA this BA effect could not be discerned since most explants survived. NAA alone, also increased survival rate with increasing concentration.

Shoot number was strongly influenced by the concentration of BA, but only slightly by the lowest concentration of NAA. NAA yielded no shoots in the absence of BA, but in the presence of BA shoot number was proportional to the BA concentration. The lowest NAA concentration was without significant effect on shoot number, but there was a small tendency towards an increase in the number of shoots produced at 0.1mg/l NAA. At 1mg/l NAA few shoots were formed at BA concentrations below 0.5mg/l. The optimum concentration of NAA for shoot production was 0.1mg/l with 1mg/l BA. This growth regulator balance resulted in the

formation of many healthy shoots (PLATE 3D). Higher concentrations of BA gave similar numbers of shoots, but they appeared stunted (PLATE 4B).

Root number increased with increasing NAA concentration. BA alone gave no roots at concentrations above 1 mg/l. Below this concentration small numbers of roots formed, but they did not develop from the petiole explants, but from the bases of newly formed shoots and so were not scored. In the combined series, increasing the concentration of NAA increased the number of roots per explant. BA had a slight promotional effect on root number at 0.05 mg/l, but at greater concentrations root formation was suppressed proportional to the concentration of BA. At BA concentrations of 2 mg/l or greater, roots were suppressed even at 1 mg/l NAA.

There was a clear interaction between the 2 growth regulators in both root and shoot formation. Shoot formation was favored by 1-4mg/l BA. At concentrations of BA less than 0.5mg/l root formation was favored. With the lower BA concentrations the number of roots depended upon the NAA concentration.

The external appearance of roots was also affected by the presence of BA in the medium. Roots produced on media without BA appeared long and thin with little branching and were only produced at the cut surface of the explant in direct contact with the medium. Addition of 0.05 mg/l BA induced the formation of roots from the sides of the explant over the entire lower half. Roots were also larger, with more branching and had many root hairs. Higher concentrations of BA only served to induce shoots and reduce root number without altering the appearance of the roots.

A concentration of 1 mg/l NAA and 0.05mg/l BA resulted in large numbers of roots forming with almost complete suppression of shoot induction (FIGURE 1B). After six weeks a few small shoots were occasionally seen, but these originated from the junction of newly formed roots and the explant, rather than arising spontaneously from original explant tissue.

None of the BA/NAA combinations tested resulted in the formation of callus alone. Intermediate concentrations of BA and NAA resulted in a mixture of shoots and roots, with only a small amount of callus forming at the base of the explant after prolonged culture. Compact callus could, however, be induced by the addition of 0.2mg/l 2,4-D to a medium containing 0.2mg/l NAA and 0.1mg/l BA (PLATE 4D). Callus formed not only from the lower cut surface of the explant, but also as discrete clumps over the rest of the explant (PLATE 4C). Initially this callus inducing medium (CIM) formed only compact calli, however, with prolonged (more than 6 weeks) culture, stunted, root like structures, sometimes formed from within the clumps of compact callus.

From this point four basic media were chosen for further experimentation. Shoot inducing medium (SIM) containing 1 mg/l BA and 0.1mg/l NAA, root inducing

medium, (RIM) containing 1mg/l NAA and 0.05mg/l BA and callus inducing medium (CIM) containing 0.2mg/l 2.4.D, 0.2mg/l NAA and 0.1mg/l BA. Basal medium (BM), containing no growth regulators was also used as a non-growing control.

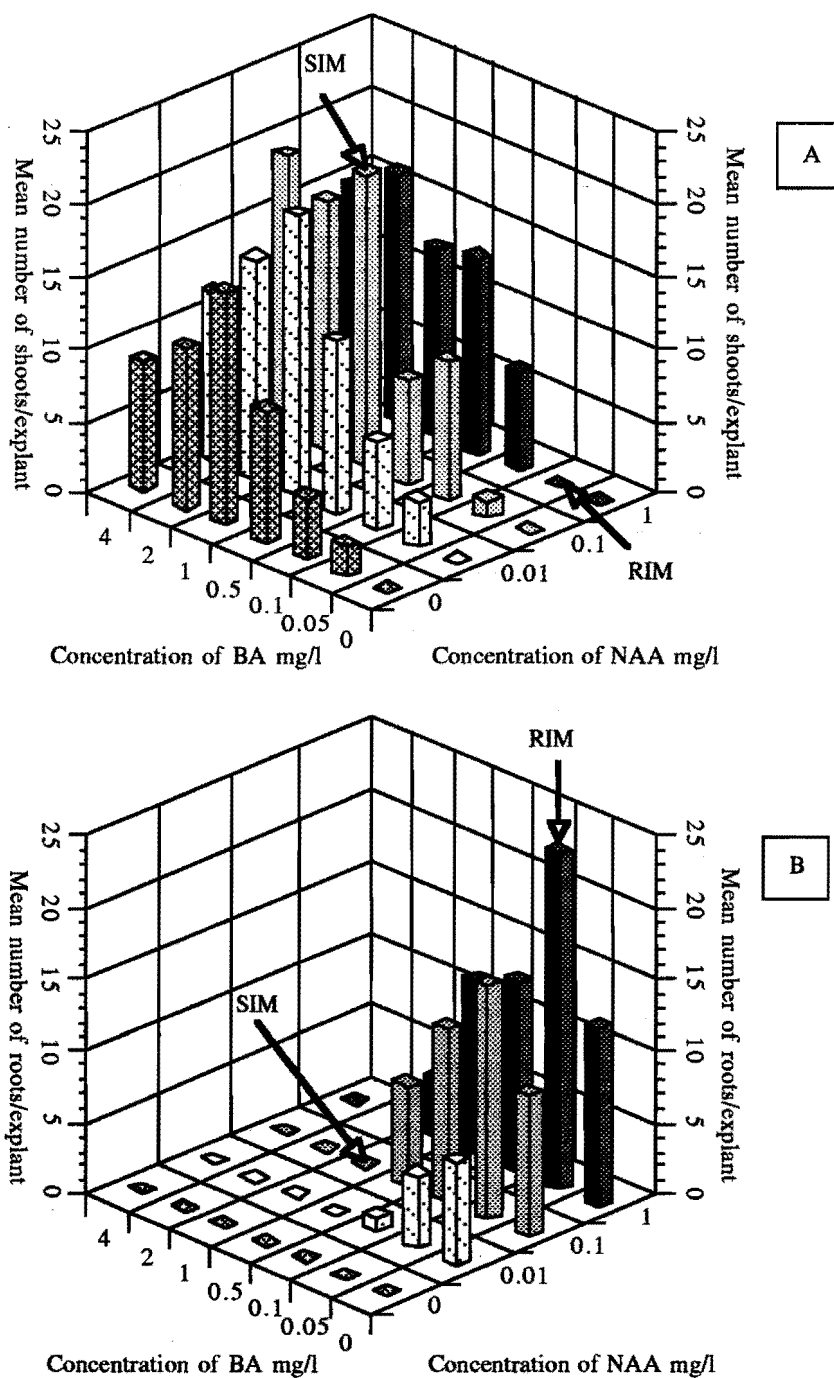


FIGURE 1 [A]- The influence of growth regulator balance on the number of shoots per explant.

[B]- The influence of growth regulator balance on the number of roots per explant.
 Mean value $n=30$. Explants were scored after a total of 6 weeks in culture.

A

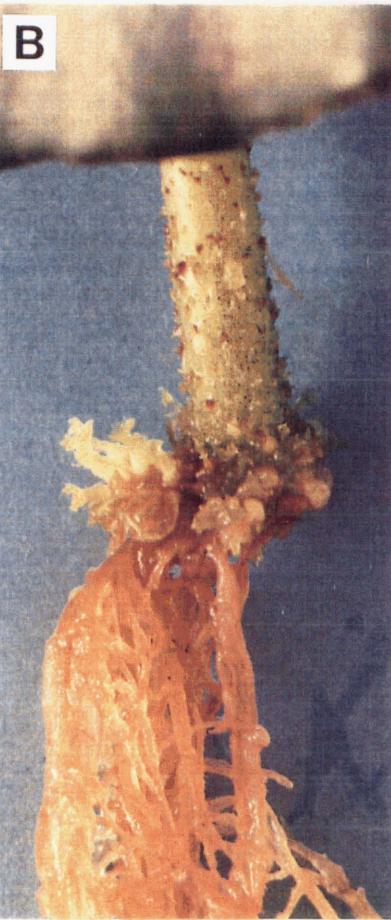
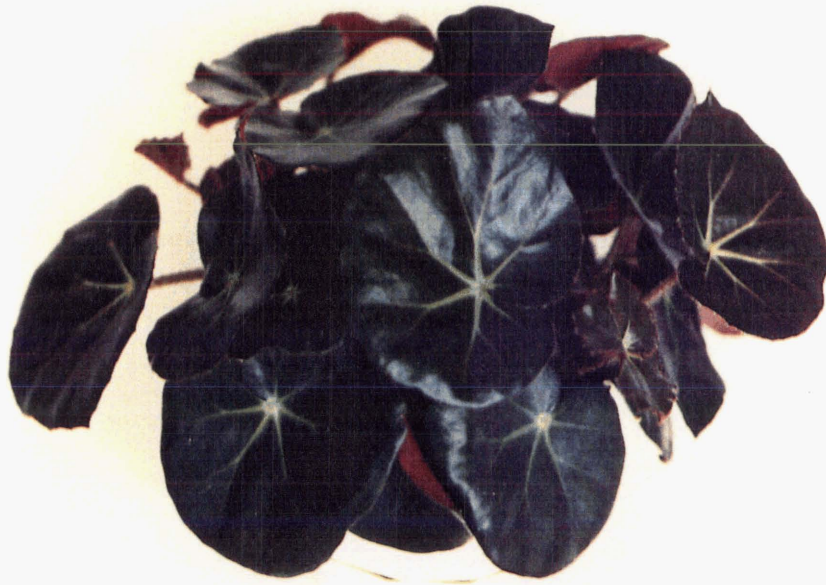


PLATE 1: Propagation of *B.erythrophylla* via leaf-cuttings

[A]- *B.erythrophylla* plant propagated from a leaf-cutting.

[B]- Leaf-cutting with several shoot buds, 5 weeks after excision.

[C]- Leaf-cutting after 7 weeks with shoots forming at the root/cutting interface.

(3) THE INFLUENCE OF SUCROSE CONCENTRATION ON SHOOT FORMATION

The concentration of sucrose in the shoot inducing medium had a marked effect on the ability of explants to form shoots. Complete removal of sucrose from SIM resulted in only occasional shoots forming. Addition of sucrose up to a concentration of 3% increased the number of shoots forming per explant to a maximal level, although 2 and 3% sucrose showed little difference in the number of shoots formed (TABLE 1). Sucrose at concentrations above 3% resulted in a gradual decline in shoot number, with concentrations of 9% or greater resulting high explant mortality and only occasional shoot formation (TABLE 1).

SIM containing three percent sucrose was therefore considered optimal for shoot induction. SIM without sucrose (SIM(-)) was thought to be useful as a control, for explants could be exposed to a growth regulator balance favoring shoot formation without producing shoots. This would enable the isolation of effects induced by exposure to SIM growth regulators not associated with shoot formation. A further advantage with SIM(-) as a control, was explants senesced more slowly than with culture on BM.

| | Percentage sucrose in SIM | | | | | | |
|-------------------------------|---------------------------|----------------|-----------------|-----------------|---------------|----------------|----|
| | 0 | 1 | 2 | 3 | 6 | 9 | 12 |
| Number of shoots per explant. | 0.1 | 9.7 +/- 3.6 | 19.4 +/- 2.9 | 21.3 +/- 2.4 | 8.4 +/-3.1 | 0.3 +/- 0.1 | 0 |

TABLE [1] The influence of sucrose concentration on the number of shoots formed per explant. Mean plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

(4) **POLARITY AND RESPONSIVE REGIONS FOR
REGENERATION IN CULTURED PETIOLE SECTIONS**

4.1 The polarity of the organogenic response

Preliminary studies placing petiole sections either horizontally or vertically in contact with SIM, showed that a more uniform shoot forming response was obtained if explants were cultured vertically, with a cut surface in contact with the medium. When petiole sections were cultured vertically on SIM, shoot buds developed evenly over the entire surface of the explant, with no apparent polarity towards either cut surface (PLATE 3C). The orientation of the basipetal surface of the petiole explant had no effect on the response. Culture with either the basipetal surface in contact with the medium or in the reverse orientation caused no polarity, with shoots uniformly distributed over the entire explant. If an explant was placed horizontally shoots still formed over the entire explant, but those on the lower surface in contact with the medium failed to expand properly and were more difficult to count.

Petiole sections with a cut surface exposed to RIM, responded in a polar manner. Culture with the basipetal surface in contact with the medium resulted in root primordia forming only on the lower half of the explant (PLATE 3F), with the upper half devoid of any development. Culture with the acropetal surface in contact with the medium destroyed this polarity, with root primordia forming over most of the surface of the explant (PLATE 4A), although a greater density of primordia were often observed closer to the cut surfaces. To maintain consistency all future experiments were carried out with the basipetal surface of the explant in contact with the inductive medium.

4.2 Explant Size

A mean explant length of approximately 5mm was optimal for both the organogenic response and explant survival. The use of smaller explants resulted in fewer explants surviving, probably due to tissue damage upon excision. Larger explants were more difficult to handle and often resulted in the upper portion of the explant failing to respond to SIM.

4.3 Position of the petiole on the parent plant

Petiole sections responded to an organogenic stimulus irrespective of the position of the petiole from which they were excised. The position of the petiole did, however, affect the number of shoots produced per explant, as the diameter of the explant was dependent on the position of the petiole from the shoot apex. The 1st, 2nd and 3rd expanded leaves all had progressively larger diameter petioles. Older petioles had greater diameters and produced more shoots per section. The 4th, 5th and 6th developed leaves all tended to have similar diameter petioles and so produced similar numbers of shoots. Only petioles from these leaves were used for experimentation.

4.4 Explant position within the petiole

The position of the explant within the petiole prior to excision, had little affect on the explants ability to respond to the organogenic stimulus. Explants removed from the basipetal portion of the petiole, responded as well to the organogenic stimulus as petiole explants taken from the acropetal portion. This response was the same for both RIM and SIM. A slight reduction in the number of organs formed on acropetal sections was occasionally observed, if a petiole showed a substantial reduction in diameter from top to bottom.

(5) INCREASING THE NUMBER OF PLANTS *IN VITRO*

As large amounts of tissue would be required for future experiments, the establishment of large numbers of plants *in vitro* was required. Plants already established *in vitro* were used as sources of explants to further increase the number of plants in culture. As only the 4th, 5th and 6th expanded leaves were used as sources of explants, removal of only 3 leaves allowed the donor plant to continue to grow rapidly.

Increasing the number of plants in culture using *in vitro* petiole sections was carried out in a similar manner to the initial establishment of plants in culture. The only differences were, no surface sterilization was required and SIM was used for shoot induction. Approximately six months were required from the first excision of the explant, to the attainment of an *in vitro* plant of sufficient size for experimental use (PLATE 4E).

Once *in vitro* plants had reached a suitable size they were maintained in culture for a further 6 months. During this time they were used as a source of explants for experimentation. After 6 months they were either destroyed or transplanted out of culture. The culture cycle is summarized in Figure 2. A continuous supply of plants at the correct stage for experimentation was maintained by establishing batches of plants at various stages in the culture cycle.

Transplanting *in vitro* grown *B.erythrophylla* plants out of culture was relatively easy. Plants were simply removed from the culture vessel and any senescent leaves and the majority of adhering agar removed. Plants were then potted out in a 50% mixture of potting mix and sand and covered with the bottom half of the culture container in which they were grown. After 1 week the bottom of the culture container was raised 1 cm and supported at this height to allow air movement. After a further 1 to 2 weeks the culture container was removed. Plants responded well to this method of transfer with at least 75% surviving. Once established out of culture *in vitro* propagated plants grew rapidly.

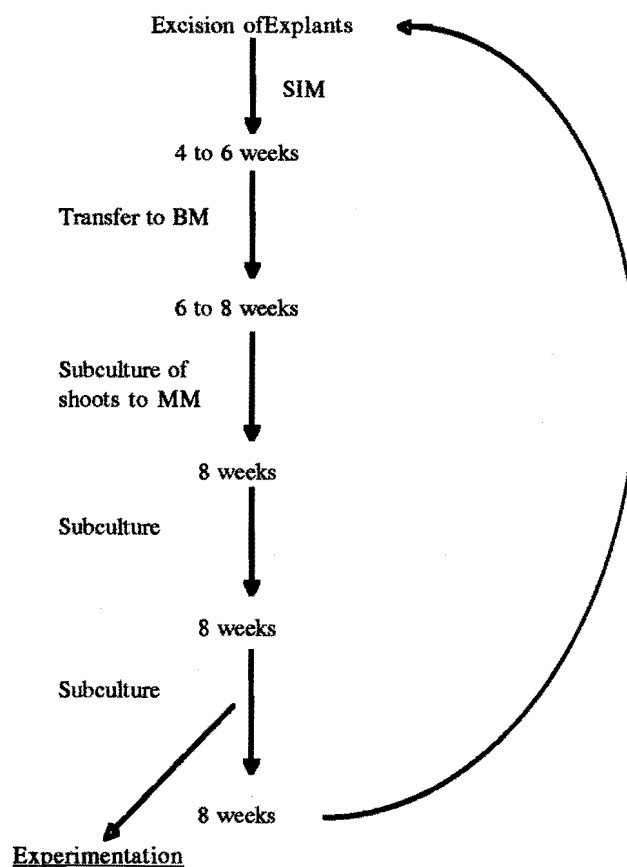
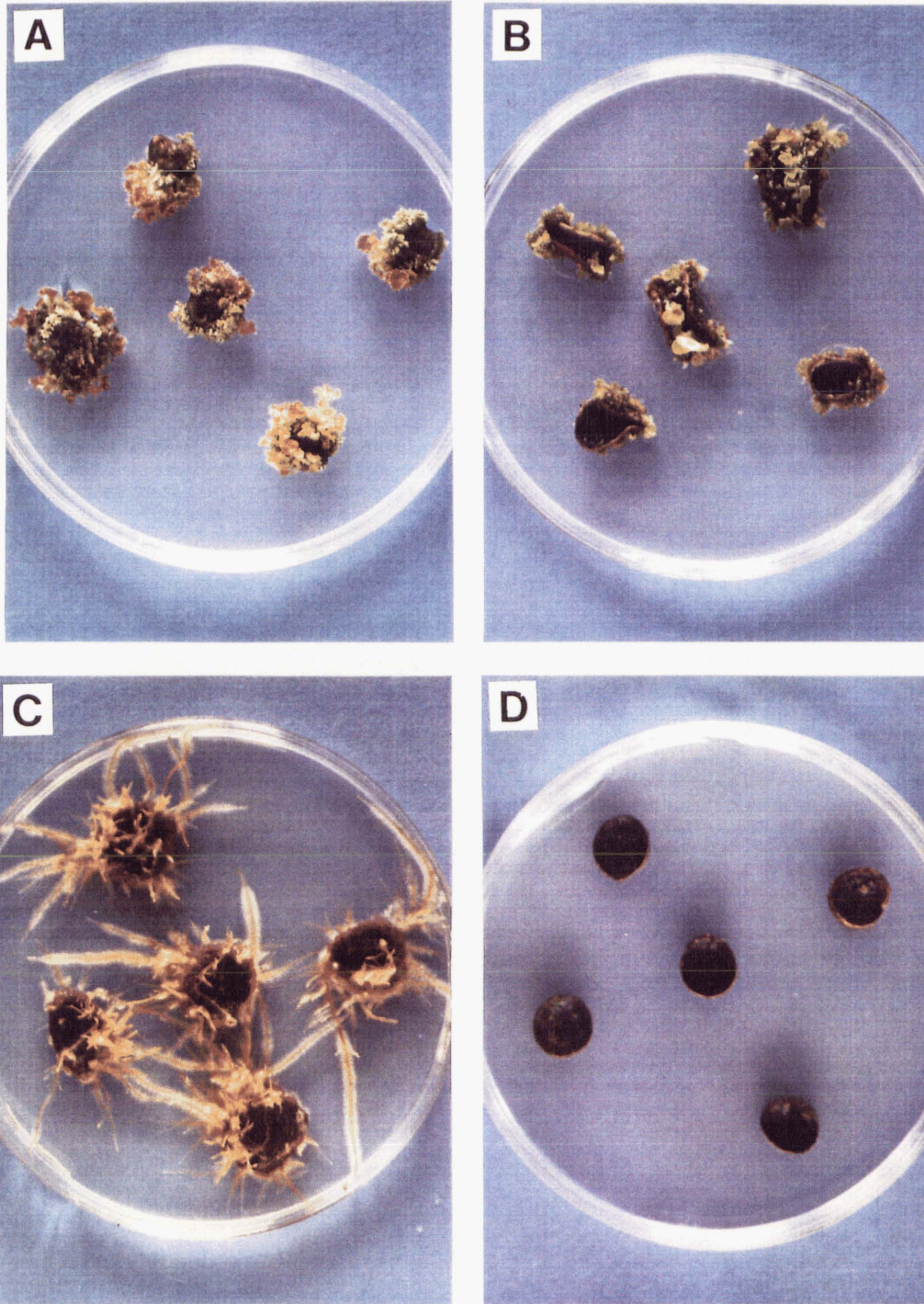


FIGURE [2] Outline of the culture cycle for maintaining *B.erythrophylla* plants *in vitro*.

Once suitable for experimentation plants are maintained in culture for 6 months by subculturing to MM every 8 weeks.



- PLATE 2:** [A]- Petiole sections from potted plants with numerous leafy shoots after 6 weeks on a shoot inducing medium.
- [B]- Leaf discs from potted plants with numerous leafy shoots after 6 weeks on SIM.
- [C]- Leaf discs from potted plants with well developed roots after 6 weeks on RIM.
- [D]- Leaf discs from potted plants showing no development after 6 weeks on basal medium.

PLATE 3: Surface views of *B. erythrophylla* petiole explants

[A]- Petiole at the time of excision. (mh) multicellular hair.
(gh) glandular hair (x12.5).

[B]- Explant cultured for 14 days on Basal medium (x10).

[C]- Explant cultured for 21 days on Shoot Inducing Medium (SIM).
Note shoot buds distributed over the entire surface of the explant
(x7).

[D]- Leafy shoots cover the explants after 6 weeks culture on SIM.

[E]- Explant cultured for 14 days on Root Inducing Medium (RIM).
Note swellings on the basal half of the explant only (x7).

[F]- Explant cultured for 21 days on RIM.

[G]- Well developed roots after 6 weeks culture on RIM.

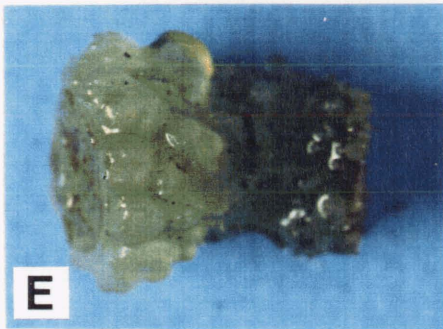
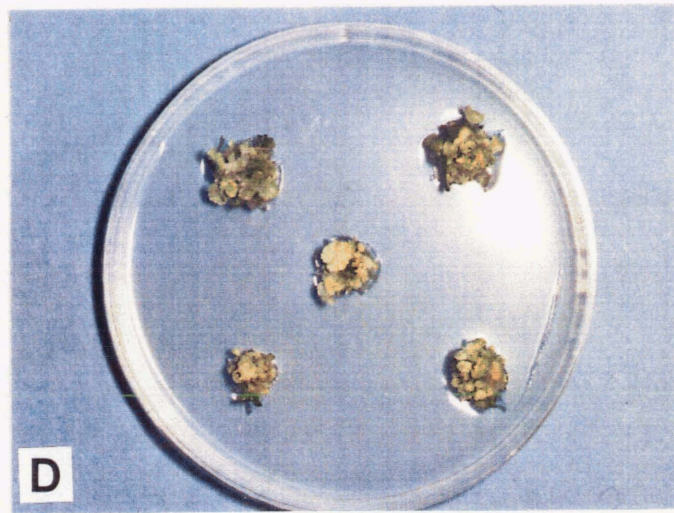
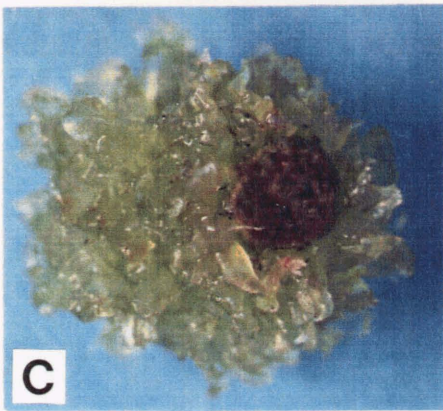
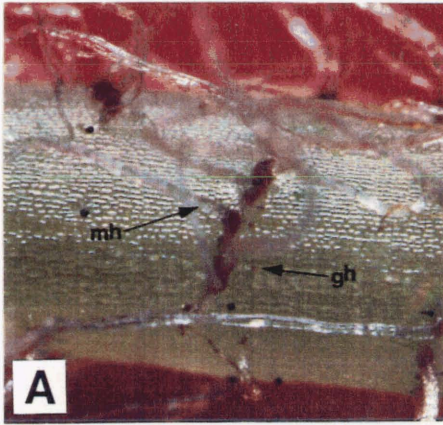


PLATE 4: Surface views of *B. erythrophylla* petiole explants

[A]- Explant cultured for 21 days on RIM with the acropetal cut surface in contact with the medium. Note primordia over the entire surface of the explant (x12.5).

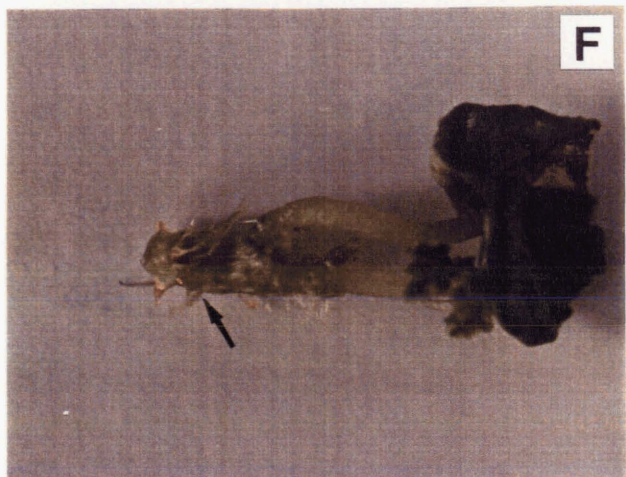
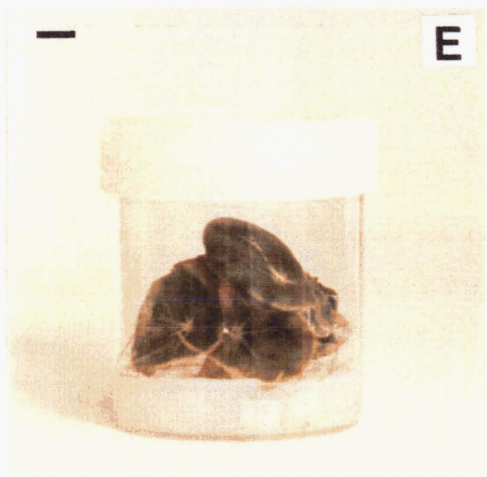
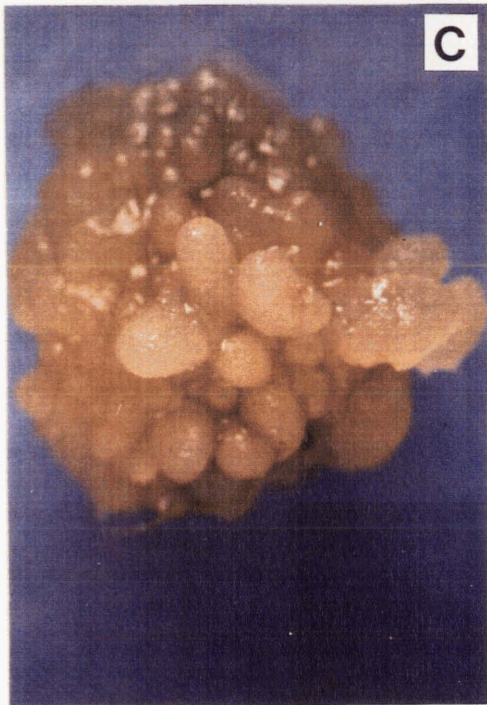
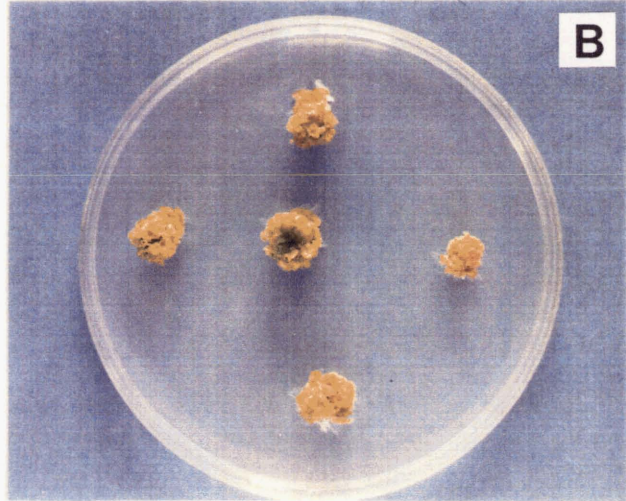
[B]- Explants cultured for 6 weeks on BA4 medium. Note the stunted appearance of the shoots.

[C]- A closer view of the upper half of an explant cultured for 6 weeks on Callus Inducing Medium (CIM) (x12.5). Note the nodular appearance of the callus in this region

[D]- Explant cultured for 5 weeks on Callus Inducing Medium (CIM) (x3).

[E]- *B.erythrophylla* plant 28 weeks after the initial excision of the initial explant. Bar equals 1cm.

[F]- Micro-cutting consisting of the shoot apex (out of view), the youngest developed leaves and adventitious roots (at arrow) (x2)



(6) MORPHOLOGICAL AND HISTOLOGICAL OBSERVATIONS ON ORGANOGENESIS

6.1 The explant at the time of excision

In surface view, the epidermal cells were generally hexagonal with highly variable dimensions. Two types of hairs were present: (1) widely spaced, long, multicellular hairs, the bases of which were often pigmented (PLATE 3A), and (2) numerous, small, multicellular glandular hairs, attached to the epidermis by small basal glandular hair cells (PLATES 3A & 5A). No other surface structures were observed at the time of excision.

Transverse sectioning of the petiole revealed anatomy typical of the Begoniaceae. Petioles consisted of a single layered epidermis, with underlying corner thickened collenchyma cells, followed by a cortex, consisting of equal sized, highly vacuolated cells, with interspersed discrete vascular bundles (PLATE 7A). Epidermal, collenchyma and cortical cells, all showed only slight cytoplasmic staining, reflecting a low RNA/protein content, related to their low level of activity. No plastids were visible at the light microscope level. In contrast to the large highly vacuolate epidermal cells, the basal glandular hair cells were small, with a high nucleoplasmic ratio and a denser cytoplasm .

6.2 Culture on Basal Medium (BM)

Culture on basal medium containing no growth regulators resulted in no development of the petiole explant, either in surface view or in transverse section (PLATES 3B & 10B). Explants remained green on BM for up to 14 days after which they rapidly turned chlorotic. Except for a few random anticlinal divisions, no cell divisions in the epidermis or the underlying collenchyma cells were apparent after 14 days in culture.

6.3 Culture on SIM without sucrose (SIM-)

Culture on SIM medium without sucrose, like culture on BM, resulted in no visible development of the explant in surface view. Explants cultured on SIM(-) remained green for longer than those cultured on BM, with many still green after 28 days in culture. Sections taken after 14 days culture showed no shoot primordia and only rarely were regions of meristematic activity observed.

6.4 Culture on Shoot Inducing Medium (SIM)

The first meristematic activity observed when explants were cultured on SIM occurred after 3 to 4 days culture. Periclinal cell divisions were observed in the epidermal and sometimes the immediately underlying collenchyma cell layers after 72 hours (PLATE 7B). Seldom were periclinal cell divisions observed prior to 72 hours. After 4 days culture, multiple periclinal, followed by anticlinal divisions of epidermal cells had occurred, with many underlying collenchyma cells also dividing periclinally (PLATE 7C). Cell division took place in localized zones throughout the epidermis of the explant with no polarity toward either cut surface. These localized areas appeared as clusters of densely staining, small, mitotically active cells, with prominent nuclei and were often, but not always associated with glandular hairs (PLATE 7D).

Continued cell division in these regions resulted in the formation of small discrete zones of highly cytoplasmic dividing cells (PLATES 7E & 7F). Periclinal followed by anticlinal divisions in these epidermal regions, with occasional divisions in the underlying cells, provided the bulk of cells for the developing primordia. After at least seven days culture, these zones developed into meristematic domes (PLATE 8A). The domes appeared continuous with the epidermis with no epidermal rupture and appeared as bulges on the surface of the explant (PLATES 5B & 5C). No meristematic activity was observed within the ground or vascular tissues of the explant at this stage of culture. By day 9 the domes had enlarged and often were enclosed by a distinct tunica (PLATE 8B). Sometimes a vascular trace was associated with the developing domes. Cells within the domes stained deeply with Methylene blue-azure A, indicating a higher content of proteins compared with the rest of the explant. Both epidermal and collenchyma cells next to and often directly beneath these domes, had by this time divided several times, giving rise to medium size cells with little cytoplasmic staining (PLATE 8B). This cell division often resulted in the progressive projection of many domes away from the initial explant tissue.

By day ten foliar primordia were observed developing from the domes (PLATE 8C). Well developed foliar primordia were present after 14 days in culture, giving the domes a nodular appearance in surface view (PLATE 5D). Although some divisions had occurred in cortical cells surrounding the vascular bundles by day 14, the vascular bundles themselves showed no meristematic activity associated with culture on SIM (PLATE 10C). By day 18, numerous apical domes enclosed by developing leaves were present on the explant (PLATE 5E & 8D) and meristemoids more deeply embedded in the explant were often observed (PLATE 8E). These meristemoids appeared as discrete structures, with distinct boundaries, surrounded by files of cells derived from multiple periclinal divisions of the original epidermal cells (PLATE 8E). Cells within the meristemoids stained deeply for protein and had prominent nuclei, where as the cell files surrounding the meristemoids were highly vacuolated and stained only lightly. Meristemoids, like meristematic domes, usually formed beneath glandular hairs (PLATE 8E).

By day 24 of culture the most advanced shoots had well organized apical meristems, with leaf primordia developing from the shoot apex. The apex had the oblique orientation typical of *Begonia*. (PLATE 8F). Most shoots contained some vascular tissue, but remained isolated from the vascular bundles of the initial explant. Externally the shoots had a characteristic bud like appearance (PLATES 5F & 6B), with the surface of the explant almost completely covered with many shoot buds (PLATE 3C). With continued culture the foilar primordia expanded (PLATE 6A) giving rise to leafy shoots after 6 to 7 weeks of culture.

6.5 Culture on Root Inducing Medium (RIM)

The initial stages of rhizogenesis appeared similar to those of caulogenesis. The first cell divisions were associated with the epidermal layer and were observed after 3 days of culture. By day 4, multiple periclinal and anticlinal divisions of epidermal and epidermally derived cells had given rise to regions of meristematic activity (PLATE 9A). Like those formed upon exposure to SIM, meristematic regions induced by RIM, consisted of small, densely stained cells, with prominent nuclei. Unlike culture on SIM where these regions were present throughout the tissue, meristematic regions were mostly confined to the lower half of the explant on RIM. These meristematic regions were often associated with glandular hairs. Continued cell division in these regions gave rise to small protruding domes after 5 to 7 days culture (PLATE 9B). Domes were often observed in pairs and continued to enlarge until distinct root primordia were observed after 14 days (PLATES 9C & 3E). By day 18, typical adventitious root primordia were observed (PLATES 6C & 9D).

Root primordia only developed from the superficial layers of the explant. No adventitious roots of perivascular origin were observed and no connection to the vascular tissue had taken place by day 24 of culture (PLATE 9E). In surface view clearly identifiable roots were present (PLATES 3F & 6D).

In undamaged explants little internal disruption of the original explant occurred, although some cell division in the cortical and vascular tissues did take place with prolonged culture. Many explants did, however, show regions of random cell division (PLATE 10A). Most of these regions were devoid of meristematic structures and consisted of large, highly vacuolate, dividing cells, similar to wound callus. The epidermal and collenchyma layers above these regions often appeared disrupted, containing collapsed cells. This localized damage, or wounding of explants, probably occurred during the initial excision and subsequent handling of the petiole sections. Such regions were also observed in explants cultured on SIM.

6.6 Culture on Callus Inducing Medium (CIM)

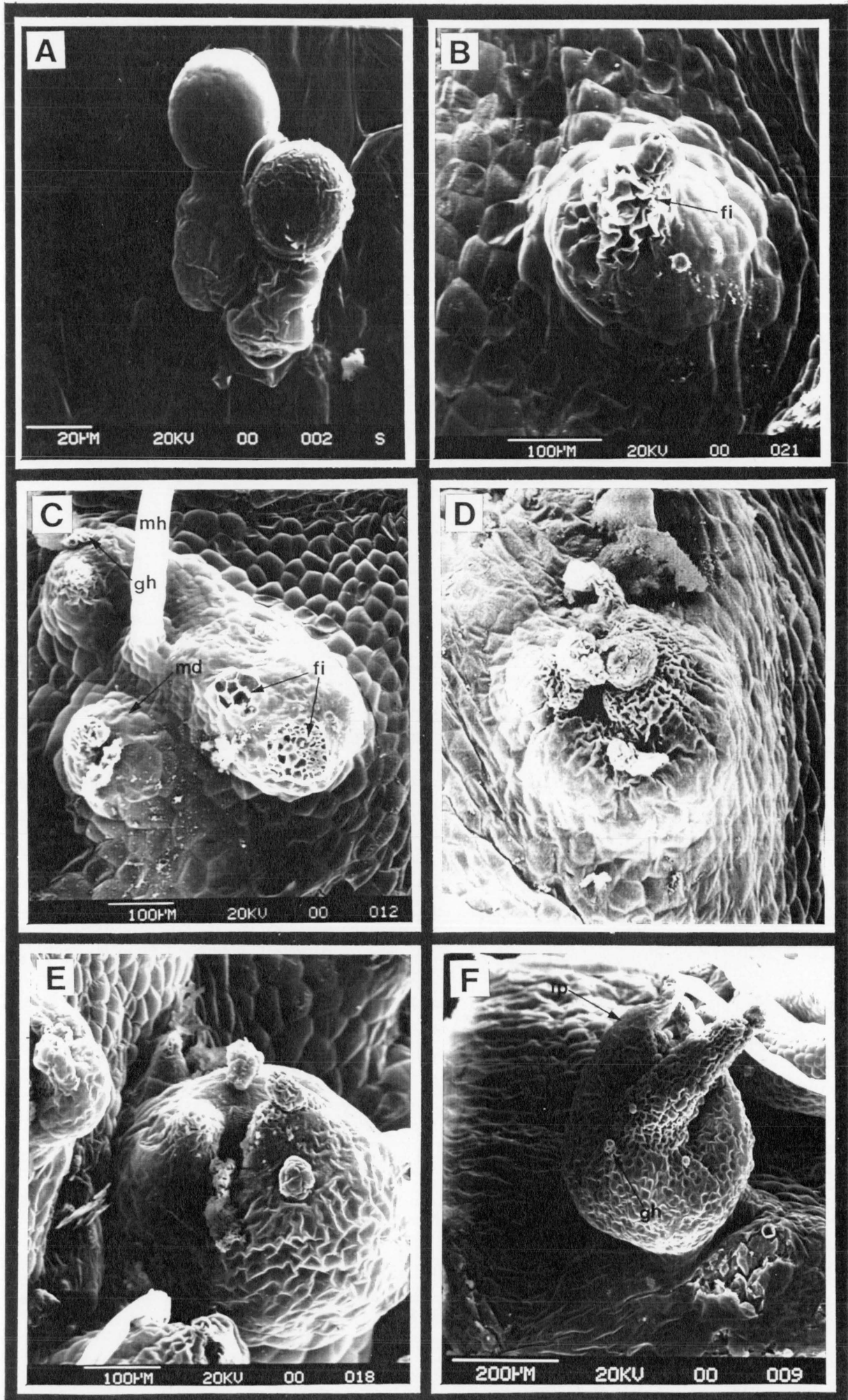
When explants were cultured on callus inducing medium, they developed compact clumps of callus polarized towards the basal end of the petiole section (PLATE 4C). Cell divisions occurred in all tissues of the explant, including the vascular bundles, at the basal cut surface in contact with CIM. Away from the cut surface, divisions also occurred throughout the explant, but they were most pronounced in the superficial layers showing signs of wounding (PLATE 10D). Small humps of tissue, like those seen in wounded regions of explants cultured on organogenic media, were easily detectable after 21 days on CIM. These humps continued to expand, growing away from the explant, with a narrow connection to the original tissue (PLATE 10E). These outgrowths gave the explant a nodular appearance (PLATE 4C).

6.7 General observations on organogenesis in *B.erythrophylla*

Both rhizogenesis and caulogenesis were not preceded by callus formation. The target cells for both inductive media appeared primarily to be in the epidermal layer. Target cells were often closely associated with basal glandular hairs. The internal tissues of the explant, including the vascular bundles, showed no meristematic activity associated with organogenesis and only minimal cell division occurred unless in response to wounding. Wounding resulted in the division of the collenchyma and cortical tissues giving rise to large highly vacuolated callus like cells.

PLATE 5: S.E.M. views of *B. erythrophylla* petiole explants

- [A]- Glandular hairs on the petiole surface at the time of excision.
- [B]- Dome of meristematic cells surrounded by cells with very few divisions after 11 days culture on SIM. Note, early foliar initial (fi).
- [C]- Cluster of meristematic domes (md) surrounding a multicellular hair (14 days). One dome has two damaged foliar initials (fi) while another shows the remnants of a glandular hair (gh).
- [D]- A meristematic dome with two intact foliar initials (14 days).
- [E]- Primordia appear bud like after 18 days culture on SIM.
- [F]- Bud primordium with well developed foliar primordia (fp) after 24 days in culture. Newly formed glandular hairs (gh) are present and the epidermis of the explant is continuous with the primordium.



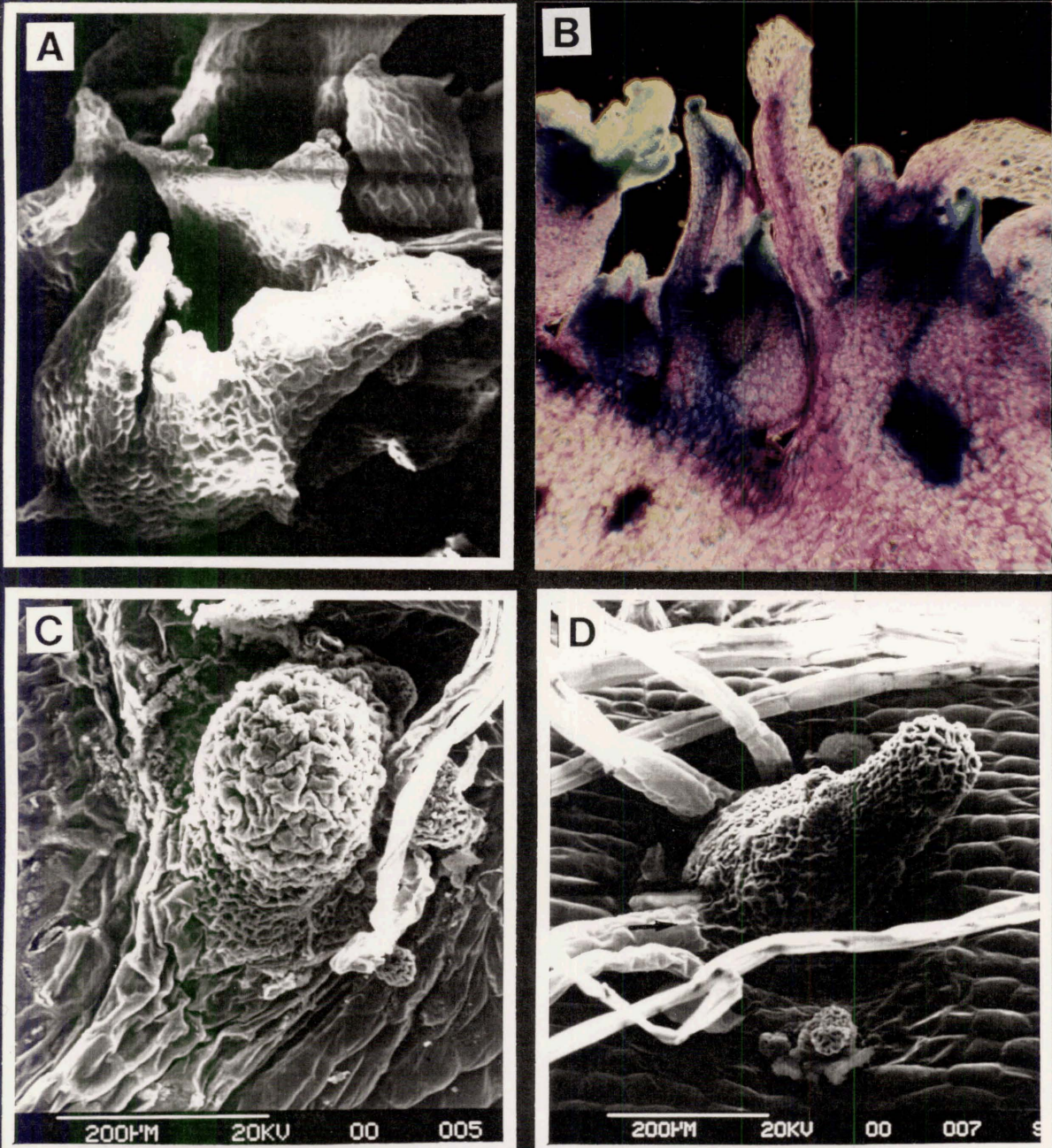


PLATE 6: [A]- Shoot bud with expanding foliar primordia (28 days on SIM).

[B]- Hand-section showing the apical region of a primordium enveloped by expanding foliar primordia (28 days on SIM).

[C]- Root primordium formed after 18 days culture on Root Inducing Medium (RIM).

[D]- Root primordium after 21 days on RIM. Note the multicellular hair of the original explant (arrow) remains continuous with lower portion of the primordium.

PLATE 7: Shoot formation in cultured petiole sections of *B. erythrophylla*
Transverse sections through petiole explants

- [A]- Transverse section through the petiole at day 0. (e) epidermis; (c) collenchyma; (cx) cortex; (vb) vascular bundles (x30).
- [B]- Early epidermal division at day 3 (x120).
- [C]- Meristematic cells derived from multiple divisions of epidermal cells at day 4 (x120).
- [D]- Numerous divisions in the epidermis at the base of a glandular hair (gh) and the underlying collenchyma tissue at day 4 (x120).
- [E]- Meristematic regions formed beneath glandular hairs (gh) on either side of a multicellular hair (mh) at day 5 (x75).
- [F]- A closer view a meristematic region, showing the small densely stained cells with prominent nuclei (x300).

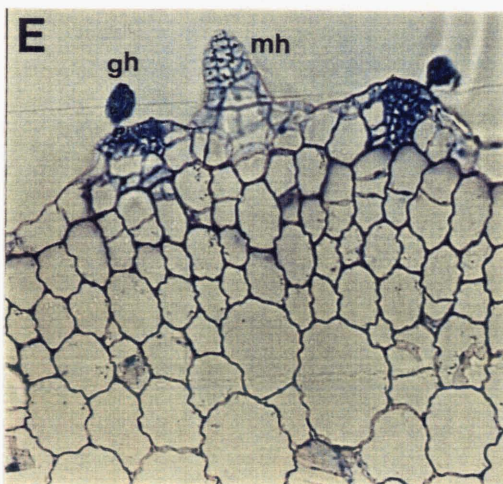
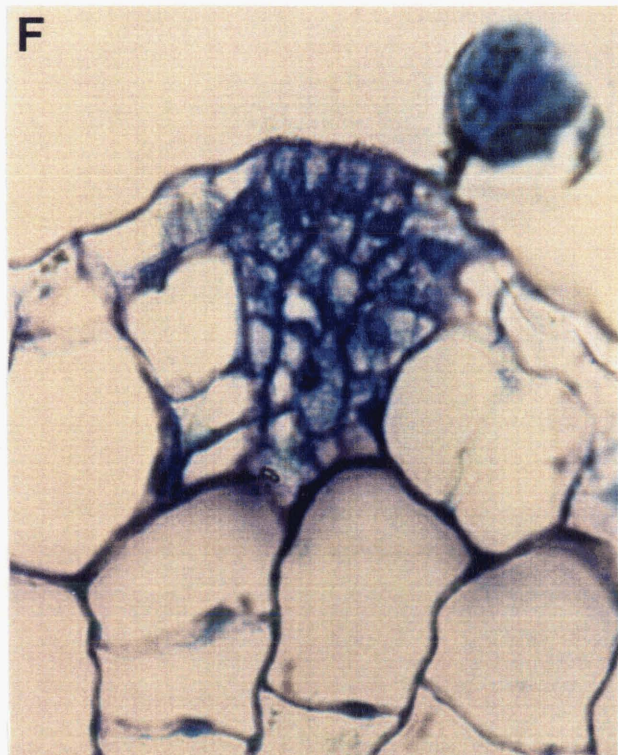
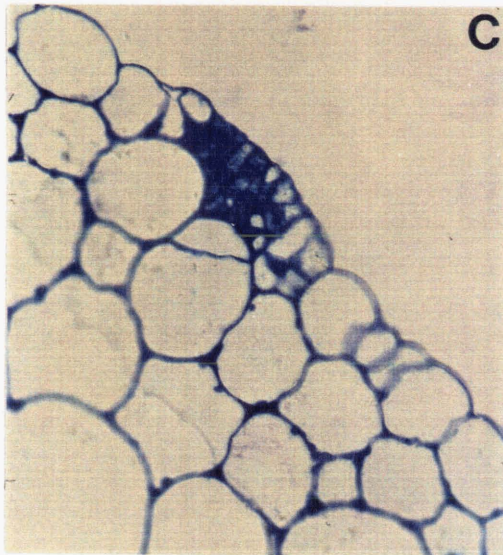
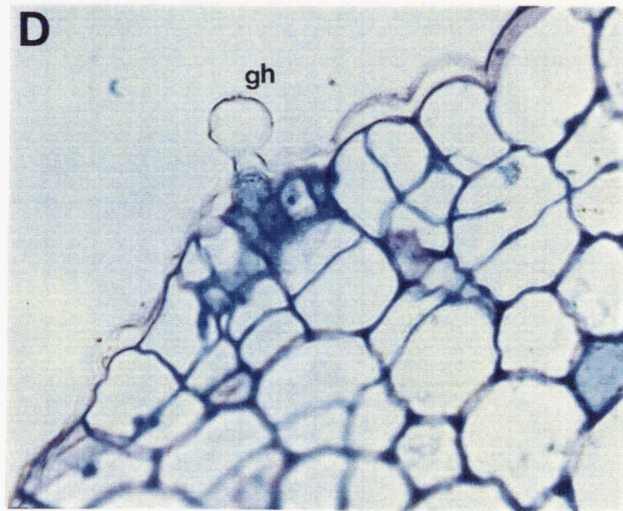
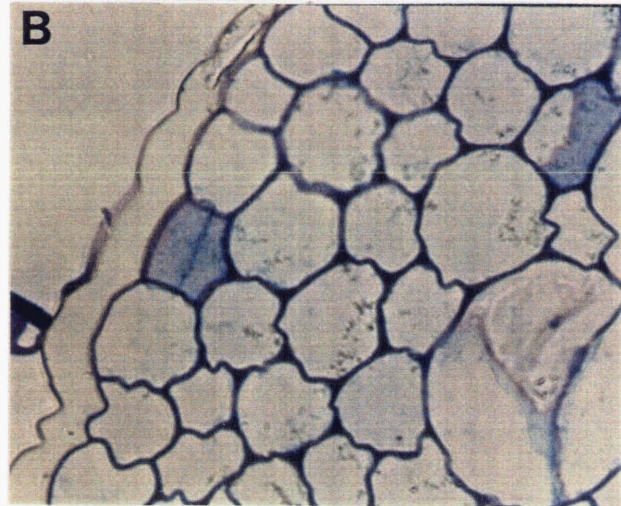
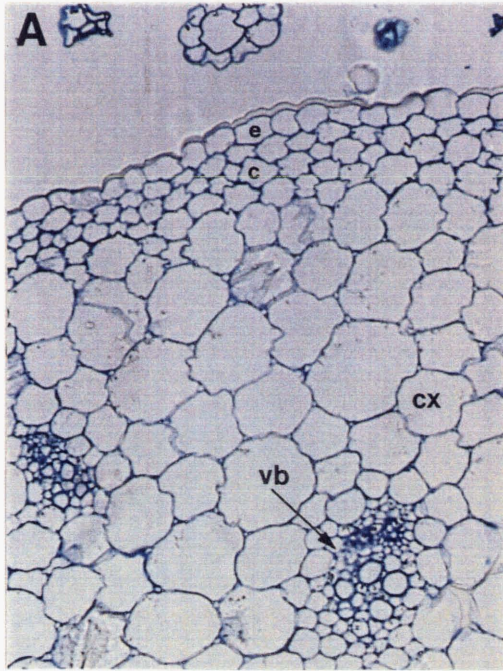


PLATE 8: Shoot formation in cultured petiole sections of *B. erythrophylla*
Transverse sections through petiole explants

[A]- Meristematic dome (md) at the base of a glandular hair (not visible in this section) at day 7 (x120). Note the lack of a tunica at this stage of development.

[B]- Dome shaped bud meristem with a tunica (tu) covering a central mass of small, meristematic cells at day 9 (x75).

Note the developing vascular tissue and the division of the underlying collenchyma and cortical cells pushing the bud away from the initial explant .

[C]- Foliar primordia (fp) are prominent after 14 days (x75)

[D]- The apex enclosed by a foliar primordium after 18 days (x120).

[E]- As well as meristematic domes, meristemoids partly surrounded by files of dividing cells are often observed (18 days). Note the distinct boundaries (at arrowhead) that separate the meristemoid from the surrounding tissue and the association of the meristemoid with a glandular hair (gh) (x120).

[F]- Well organized shoot primordium after 24 days in culture. Note the foliar primordia and the oblique orientation of the apex/leaf primordia typical of *Begonia* (x25).

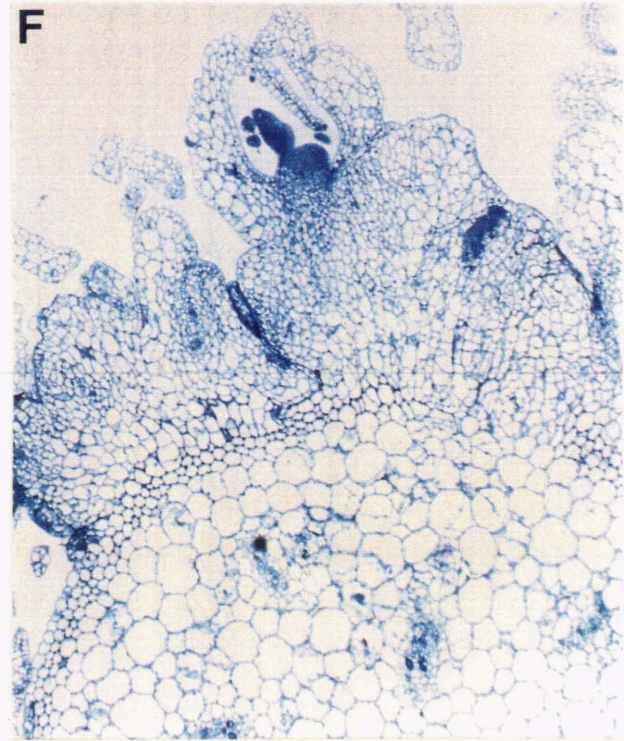
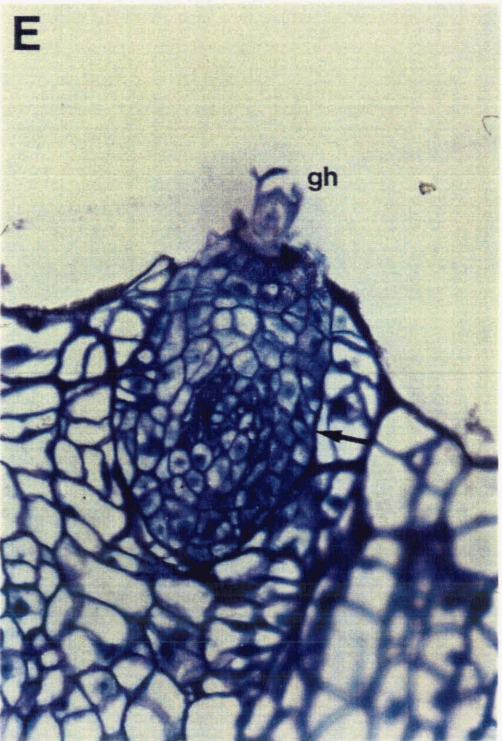
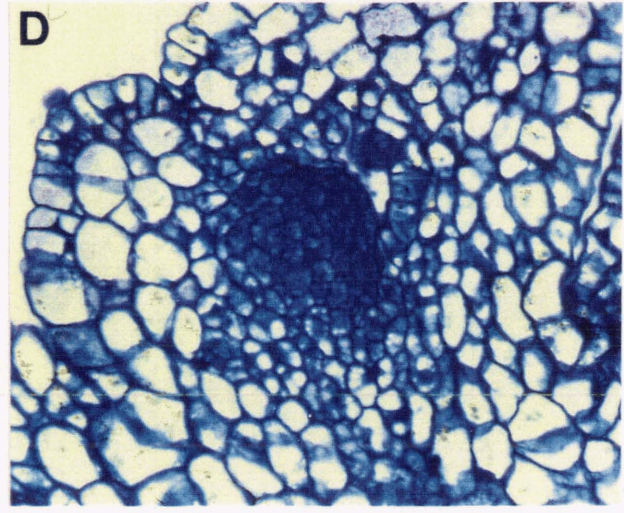
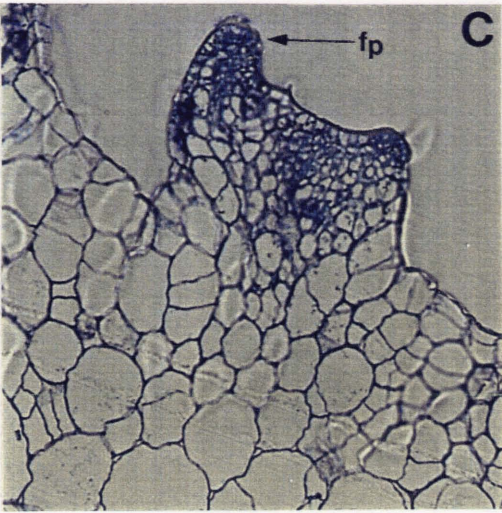
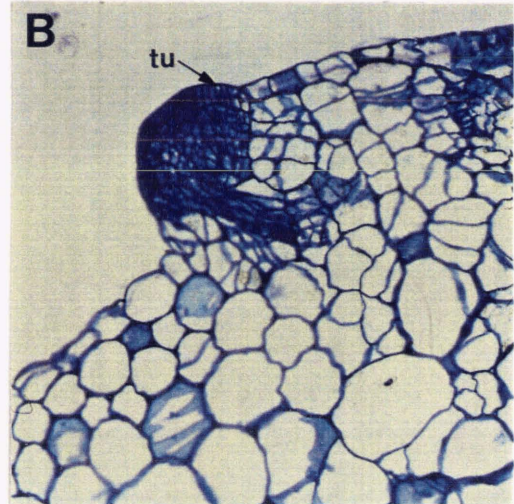
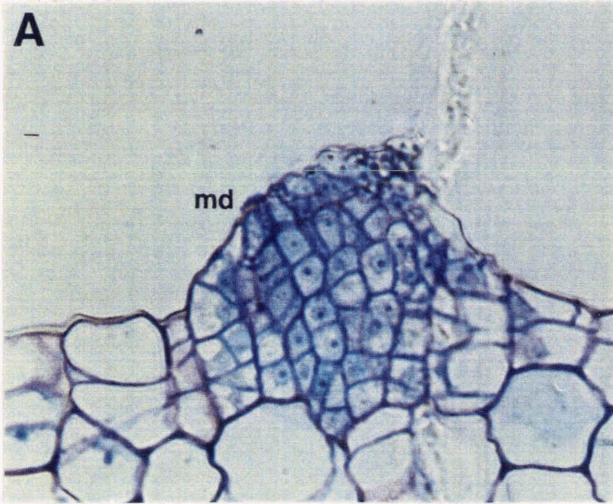


PLATE 9: Root formation in cultured petiole sections of *B. erythrophylla*
Transverse sections through petiole explants

- [A]- Divisions of epidermal and underlying collenchyma cells after 4 days culture on Root Inducing Medium (RIM) (x120).
- [B]- Small meristematic protrusions of epidermal origin (5 days) (x120).
- [C]- Root primordia often occur in pairs and show signs of vascular development after 14 days (x120).
- [D]- The developing root cap (rc) becomes prominent after 18 days (x75).
- [E]- Adventitious root after 24 days. Note the central cylinder and the minimal disruption of the cortical (cx) and collenchyma (c) layers. The epidermis of the original explant is still continuous over most of the explant (arrow) and the remnants of the glandular hair, beneath which the primordia formed, can be seen (gh) (x40).

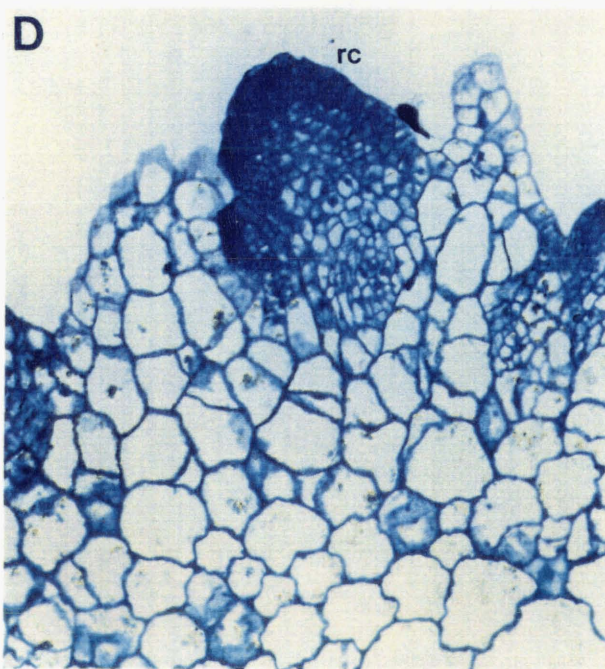
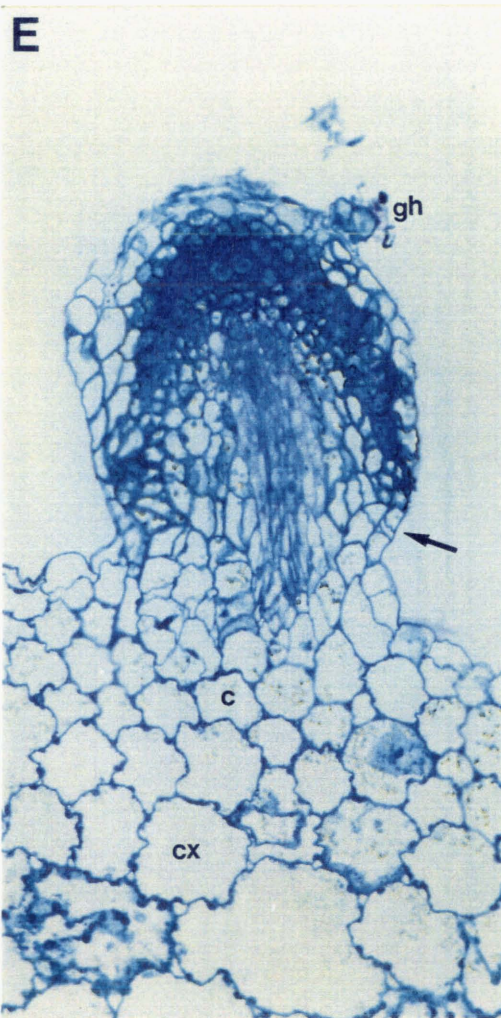
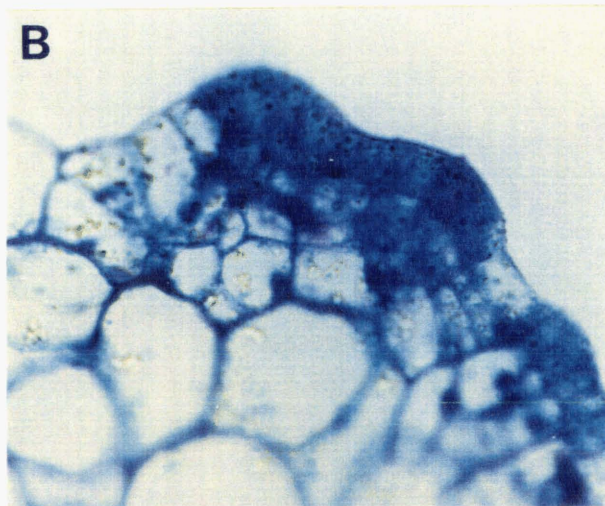
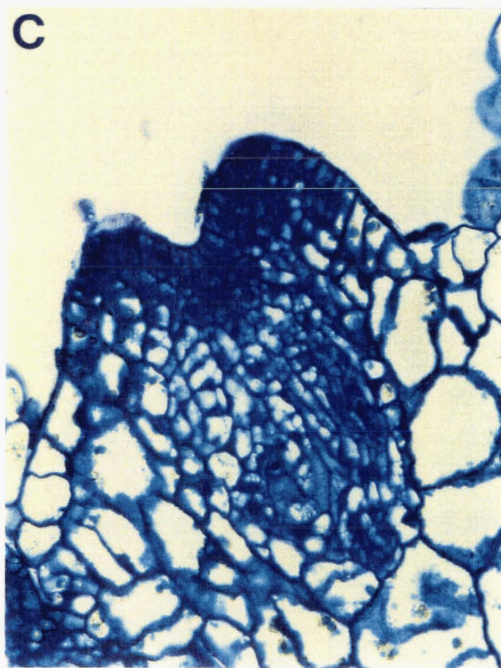
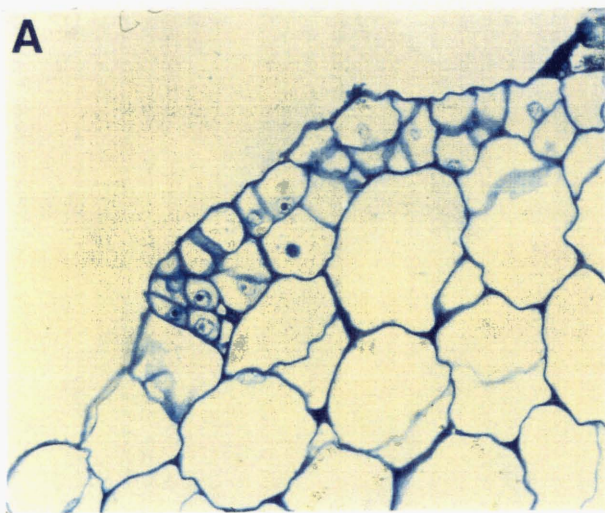


PLATE 10: Cultured petiole sections of *B. erythrophylla*

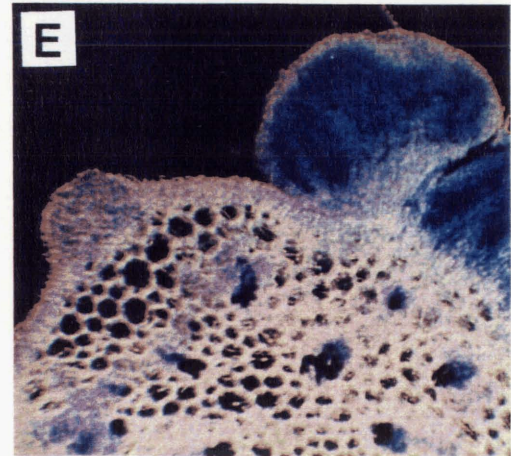
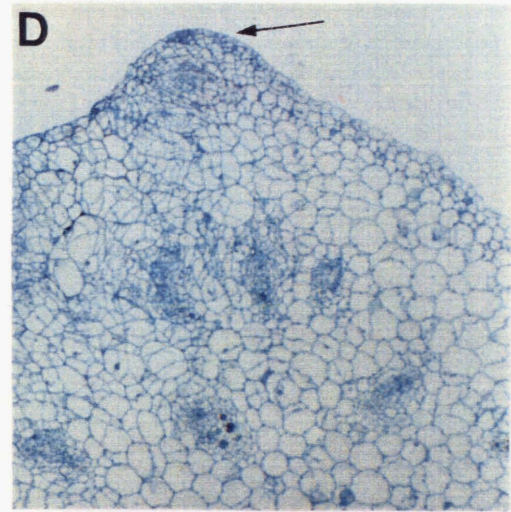
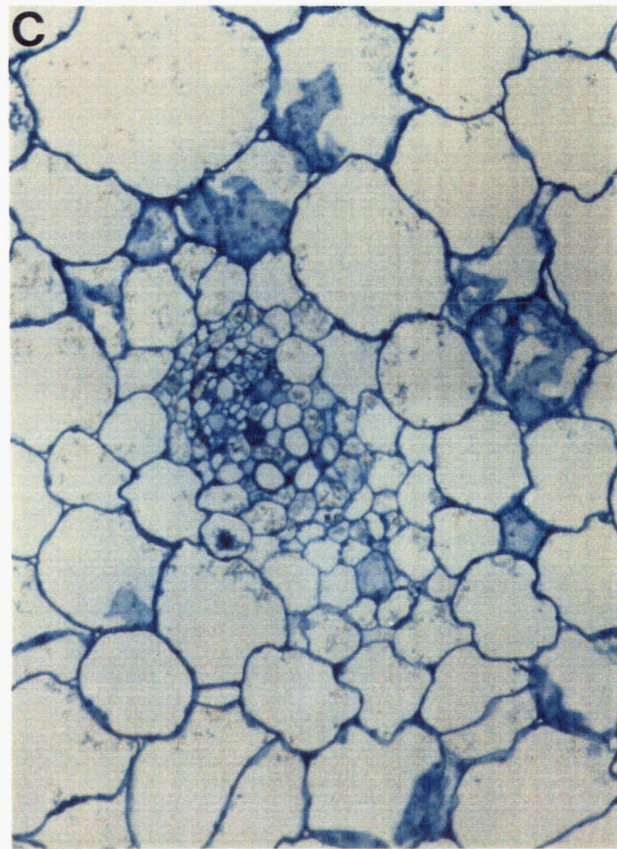
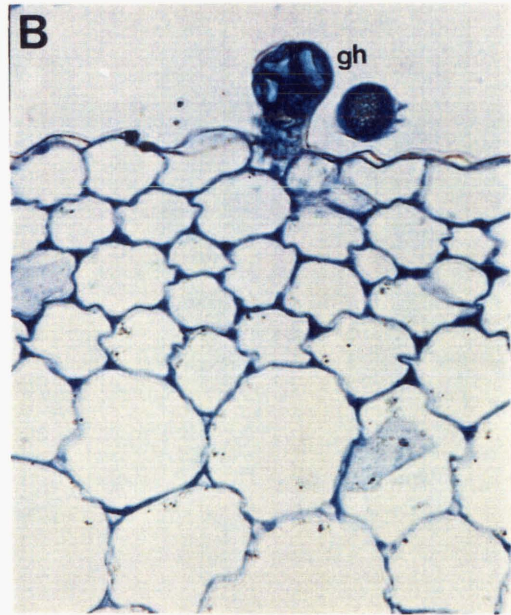
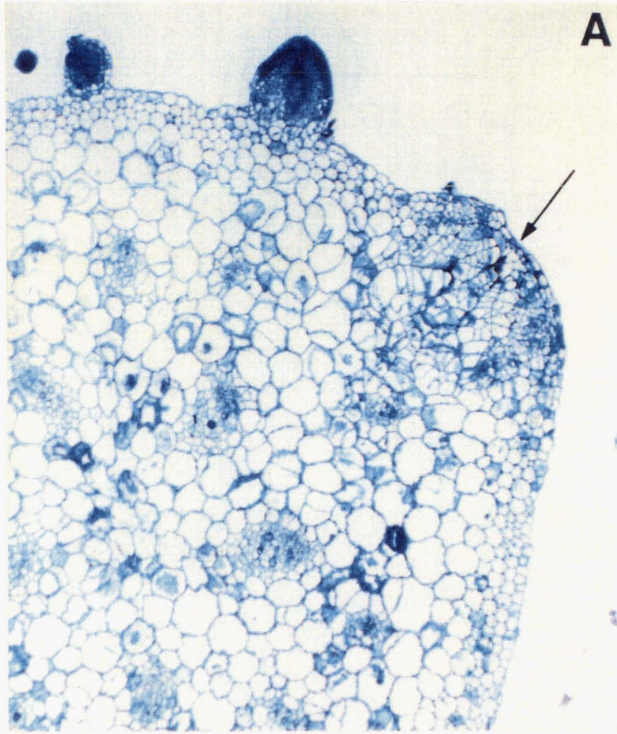
[A]- T.S. showing random cell division in the cortex, and localized random divisions of collenchyma and epidermal cells as a result of wounding during explant excision (21 days, RIM) (x25).
Note the collapsed epidermal cells (at arrow) and the root primordia not associated with the wounded region of the explant.

[B]- Culture on basal medium resulted in no meristematic regions forming beneath glandular hairs (gh). T.S. day 14 (x120).

[C]- Vascular bundle showing no meristematic activity associated with organogenesis after 18 days on RIM T.S. (x75).

[D]- T.S. showing random cell divisions throughout the explant after 21 days culture on CIM. Note the superficial swelling at a wound site (arrow) (x25).

[E]- Transverse hand section through the upper portion of an explant cultured for 5 weeks on CIM. Note the localized production of callus, with clumps of compact callus growing out from the original explant (x25).



6.8 The effect of removal of the epidermis on shoot formation.

To further support the epidermal origin of shoot primordia, both epidermal peels and the remaining peeled cylinder of petiole tissue were cultured on SIM or basal media respectively. Culture on BM caused the rapid senescence of both epidermal peels and the remaining tissue cylinder, with both types of explant turning brown within 14 days of excision. Culture of both explant types on SIM for 21 days, resulted in no detectable surface development of the explants, but neither type of explant appeared to become senescent. Epidermal peels remained opaque (peels were brown by this time on BM), while tissue cylinders were green, except for the uppermost portion, which appeared to have dried out. Of particular interest was the observation that if only one half of an explant was peeled, after 21 days culture on SIM, the peeled half of the explant failed to produce shoots while the unpeeled half produced shoots as normal (PLATE 11A).

Although no development of either the epidermal peels or the peeled tissue cylinder was observed after 21 days, continued culture on SIM induced development in both types of explant. The peeled petiole sections showed a noticeable increase in diameter after several weeks in culture, with callusing of the lower half of the explant (PLATE 11B). By 8 weeks, sections had increased substantially in size with fresh weight increasing by at least 20-fold (data not presented). Sections were covered with small surface swellings, with many producing roots from the basal region of the section. Stunted root-like structures were also present over much of the surface of the section, with only occasional stunted shoot primordia observed (PLATE 11C).

The peeled petiole sections were therefore capable of growth in the absence of the epidermis when cultured on SIM, but initially this involved mainly the production of callus. Interestingly, the peeled petiole sections produced roots and root-like structures with prolonged culture on SIM with only occasional shoots.

The epidermal peels had by 8 weeks developed large surface swellings (PLATE 11D). These swellings developed from the basal region of multicellular hairs. Initially the swellings appeared as small green structures, but with prolonged culture they turned yellow/green and increased in size. Some swellings produced malformed shoot primordia but these did not develop further. It was apparent that epidermal peels were capable of development in the absence of the bulk of the explant, although culture on SIM failed to induce shoot production. The failure of epidermal explants to produce shoots may have simply been the result of SIM not being a suitable medium for the culture of isolated epidermal peels. SIM was only capable of inducing shoot formation in *B. erythrophylla* petiole sections if regions of the explant retained an intact epidermis.

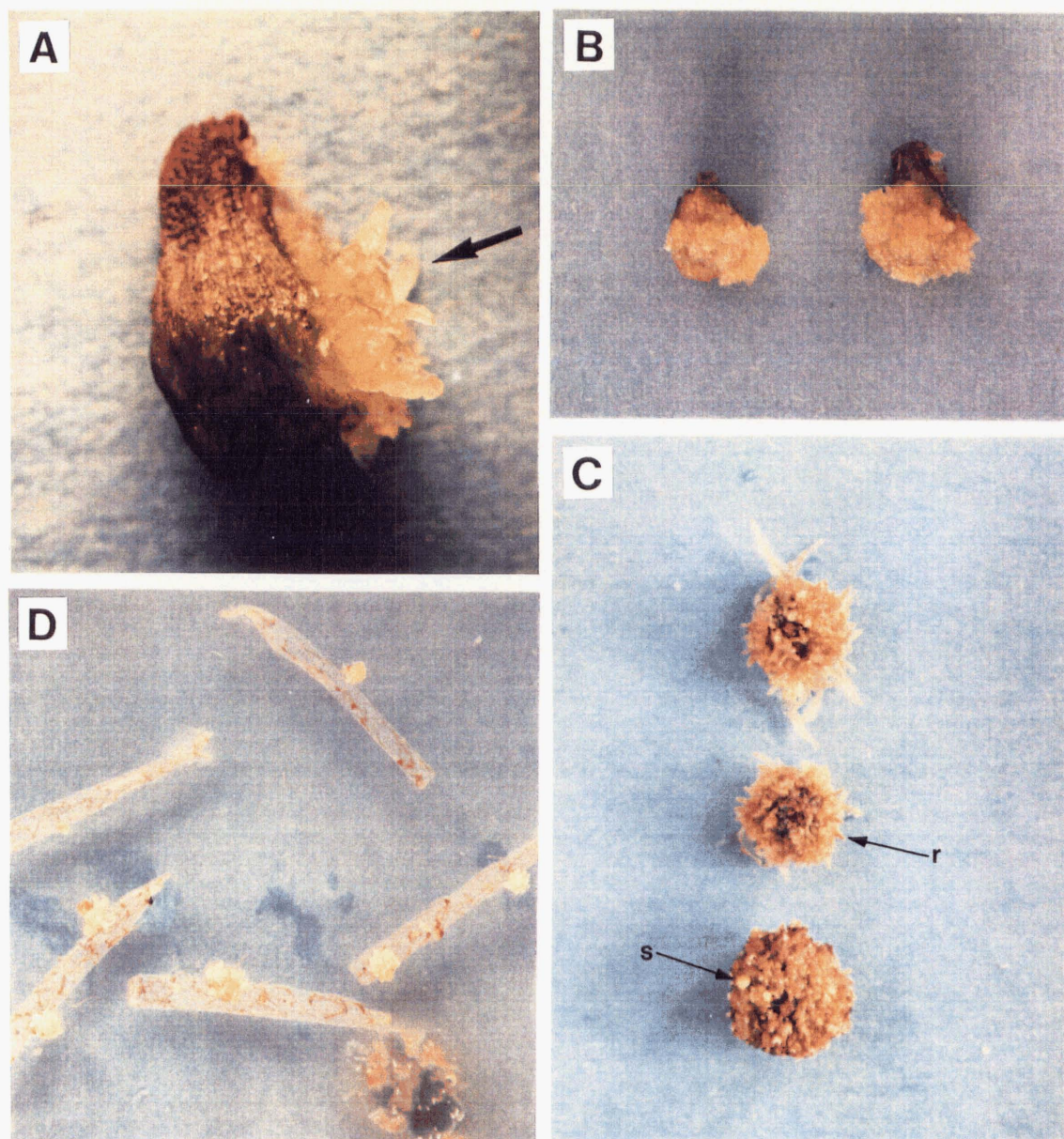


PLATE 11: [A]- Petiole section from which half the epidermis has been peeled (21 days, SIM). Note the production of shoots is only from the unpeeled side of the explant (arrow) (x10).

[B]- Peeled explants after 5 weeks on SIM. Only callus is produced, mostly from the lower half of the explant (x4).

[C]- Peeled explants after 8 weeks on SIM. Note the root and root like structures produced (r), with only a few stunted shoots (s) (x4).

[D]- Nodular structures with small deformed shoot primordia form from epidermal peels (8 weeks, SIM) (x2).

6.9 Histological examination of starch distribution in the explant during organogenesis

6.9.1 The explant at the time of excision

At the time of excision (Day 0), thin sections of petiole explants were stained with iodide/iodine reagent and examined under the light microscope. Most cells were devoid of starch, except for some cortical cells surrounding the vascular bundles and cells of the vascular bundles themselves (PLATE 12A). Starch grains were completely absent from cells of the collenchyma and the epidermal layers, except for stomatal guard cells, which contained small numbers of starch grains most easily seen in epidermal peels (PLATE 12B).

6.9.2 Culture on shoot inducing medium

Examination of iodine-stained sections of petiole explants cultured on SIM, showed an accumulation of starch grains in cortical cells previously without starch, within 24 to 48 hours of culture (PLATE 12C). Initially starch accumulation occurred mainly in the cortical cells with some deposition in the collenchyma layers, but with only very light deposition in some epidermal cells. After 72 hours culture on SIM, starch grains were evenly distributed throughout cells of the cortex. Starch deposition had also become heavy in the collenchyma cell layers and in regions of the epidermis (PLATE 12D), with starch grains particularly prominent in epidermal cells adjacent to glandular hairs (PLATE 12E). These epidermal cells eventually became meristematic. Prior to cell division a significant deposition of starch accumulated in the collenchyma cells immediately below these starch-enriched epidermal cells. After 4 to 5 days of culture on SIM, accumulation of starch localized mainly around these zones of dividing cells (PLATE 12F). The accumulation of starch continued in localized regions throughout the epidermal tissue and eventually most of the epidermis became meristematic. These zones of increased mitotic activity in the epidermal layer resulted in the formation of shoot-bud primordia.

During the formation of meristematic domes starch was present in the surrounding tissue (PLATE 13A), but the meristematic cells making up the domes had few starch grains. With development of the dome into a shoot primordium the starch grains in the surrounding cells also disappeared (PLATES 13B & C).

6.93 Culture on root inducing medium

Petiole explants exposed to RIM showed a less pronounced accumulation of starch grains, but the general pattern of starch accumulation was similar to that observed under shoot forming conditions. Starch grains initially accumulated in cortical cells with few present in the collenchyma and epidermal layers (PLATE 13D), but with continued culture grains became progressively more prominent in collenchyma and epidermal cells (PLATE 13F). Localized starch accumulation was observed in the superficial layers of the explant prior to the formation of meristematic regions (PLATE 14A). As with shoot formation starch grains accumulated in cells surrounding the meristematic region, but with development of a root primordium these grains disappeared (PLATE 14 C).

Under shoot forming conditions the accumulation of starch in the superficial layers takes place throughout the explant and is unaffected by explant orientation. Under root forming conditions with basipetal orientation accumulation of starch only occurs in the lower half of the explant. Small starch grains still accumulate to some extent in the cortical cells of the upper half of the explant (PLATE 14 D), but no localization in the superficial regions occurs and no meristematic zones are formed. In contrast, if the orientation of the explant is reversed, starch accumulation in the superficial layers occurs in both halves of the explant (PLATE 13E).

6.9 4 Culture on non-organogenic media

When iodine-stained thin sections of explants cultured for 4 days on three non-organ forming media, BM, SIM without sucrose, and RIM without sucrose, were examined, no accumulation of starch grains was observed (PLATE 14B). Culture on CIM medium for 4 days showed some starch accumulation, both general and localized, but it appeared less than that observed with culture on SIM.

PLATE 12: Iodine/potassium iodide-stained cross-sections of *B. erythrophylla* petiole explants

- [A]- Section through the explant, at the time of excision (0 days), showing few starch grains (x40).
- [B]- Dorsal view of epidermal peel at day 0, showing starch grains in the guard cells (arrow) (x100).
- [C]- Section of explant cultured for 48 hours on SIM, illustrating starch accumulation mainly in the cortical cells (x40).
- [D]- Section of explant cultured for 72 hours on SIM. Starch is evenly distributed throughout the cortex and deposition is heavy in collenchyma and epidermal cells (x40).
- [E]- Starch deposition is particularly heavy in epidermal cells associated with glandular hairs (gh) (72 hours) (x40).
- [F]- Section of explant cultured for 4 days on SIM, showing the localized build-up of starch in the collenchyma tissue (arrow) beneath dividing epidermal cells (x40).

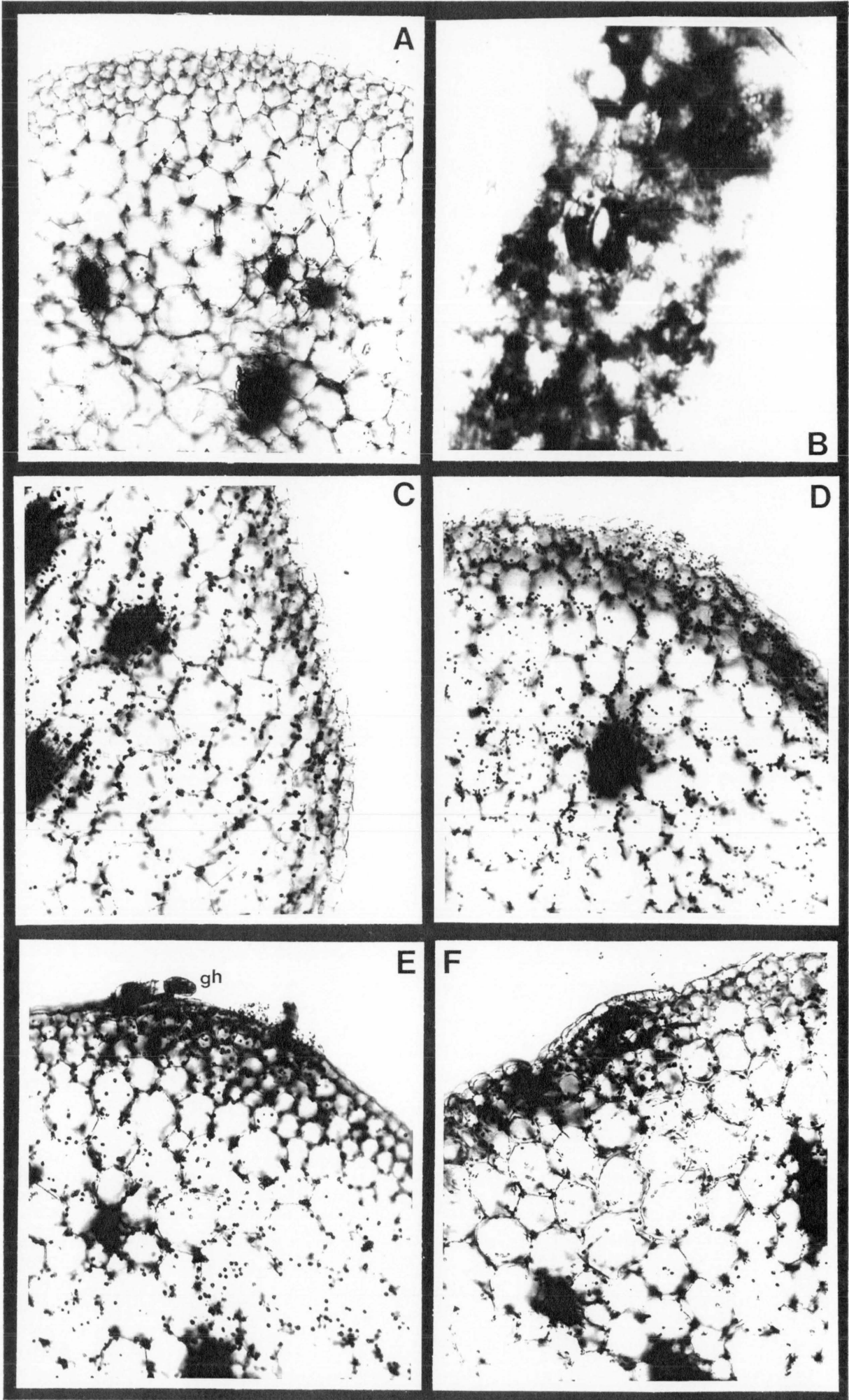


PLATE 13: Iodine/potassium iodide-stained cross-sections of *B. erythrophylla* petiole explants

- [A]- Starch grains surrounding a developing meristematic dome (arrow) after 7 days on SIM (x40).
- [B]- Starch gradually disappears from the cells surrounding the bud primordium (14 days) (x40).
- [C]- Starch eventually disappears from cells of the older, developing, shoot-buds and the surrounding tissue (21 days) (x40).
- [D]- Section of explant cultured for 48 hours on RIM, showing a similar pattern of starch accumulation in the basal half of the explant, as seen with culture on SIM (x40).
- [E]- Section of an explant cultured with the acropetal cut surface in contact with RIM, rather than the basipetal surface (48 hours). Starch accumulation also occurs in the upper half of the explant (x40).
- [F]- Starch accumulates more heavily in the collenchyma and epidermal cells with prolonged culture on RIM (72 hours); x40.

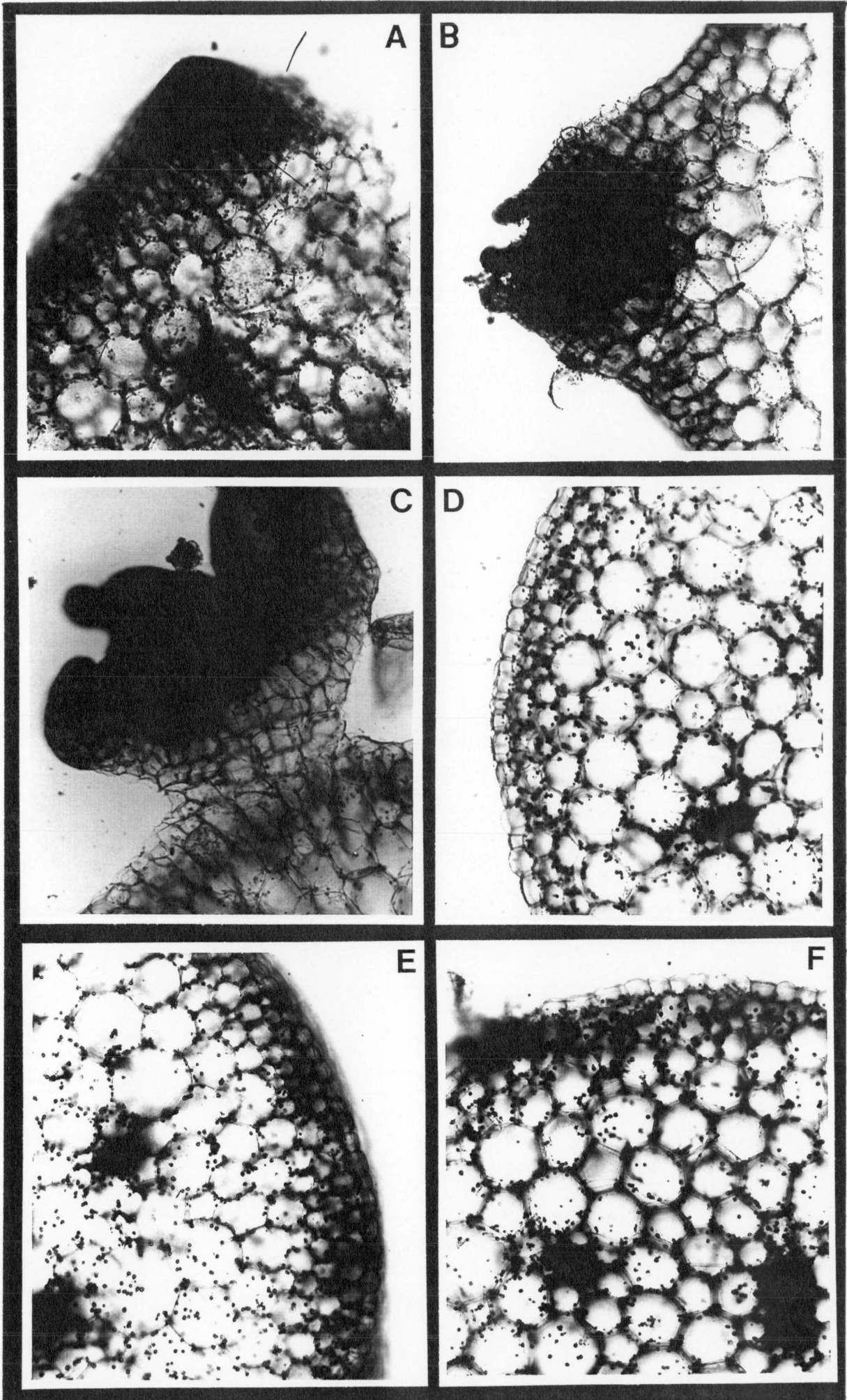


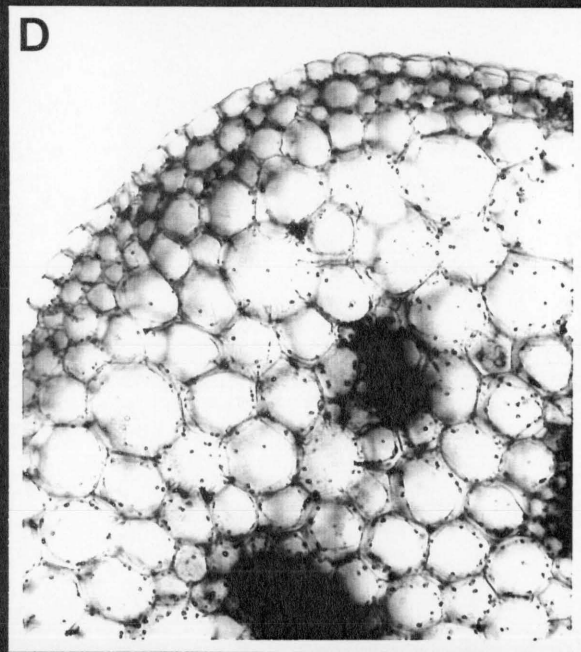
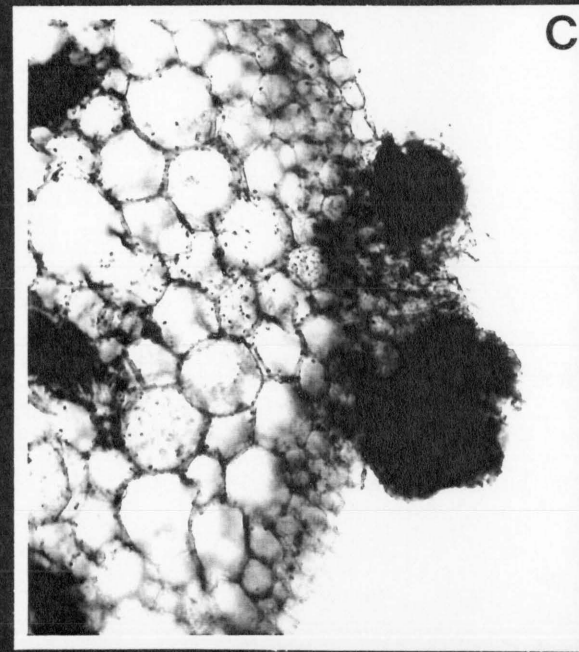
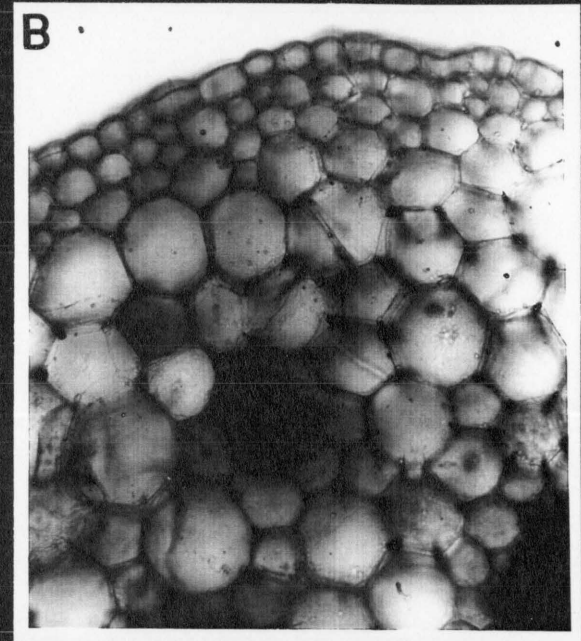
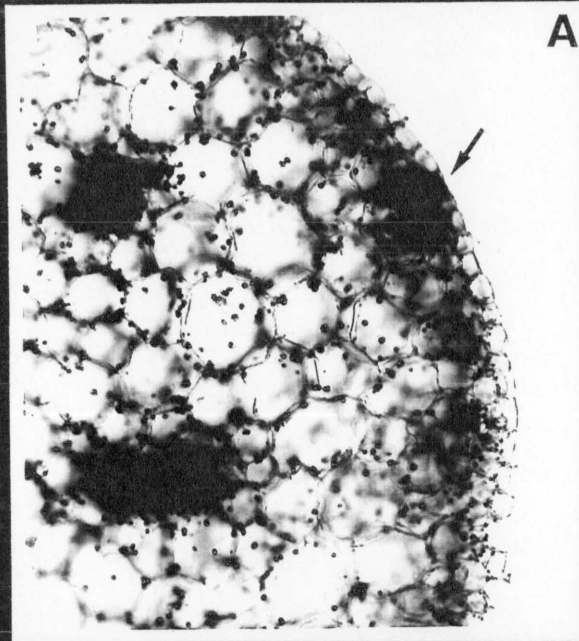
PLATE 14: Iodine/potassium iodide-stained cross-sections of *B. erythrophylla* petiole explants

[A]- Transverse section of an explant cultured for 4 days on RIM, showing localized build-up of starch in the collenchyma tissue (arrow) prior to any cell division (x40).

[B]- Transverse section of an explant cultured for 4 days on basal medium, showing no starch accumulation (x40).

[C]- Transverse section showing the loss of starch grains from cells of the explant surrounding the developing root primordia (18 days,RIM) (x40).

[D]- Transverse section through the upper half of an explant cultured with normal orientation (basipetal cut surface in contact with the medium) for 4 days on RIM, showing no localized starch accumulation (x40).



(7) DETERMINATION OF STARCH CONTENT

Biochemical analysis of the starch content of *B. erythrophylla* explants cultured on SIM, RIM, SIM(-), or BM, respectively, supported the histochemical observations (FIGURE 3). Petiole explants cultured on either SIM without sucrose or BM containing sucrose, but without growth regulators, accumulated little starch throughout the experimental period. Culture on either SIM or RIM respectively, resulted in the rapid accumulation of starch. Starch levels continued to accumulate for 7 to 10 days on both media, after which time the levels declined rapidly. The level of starch accumulation was considerably greater with culture on SIM than with culture on RIM. Culture on SIM also resulted in a more rapid decline in the level of starch than observed with culture on RIM (FIGURE 3).

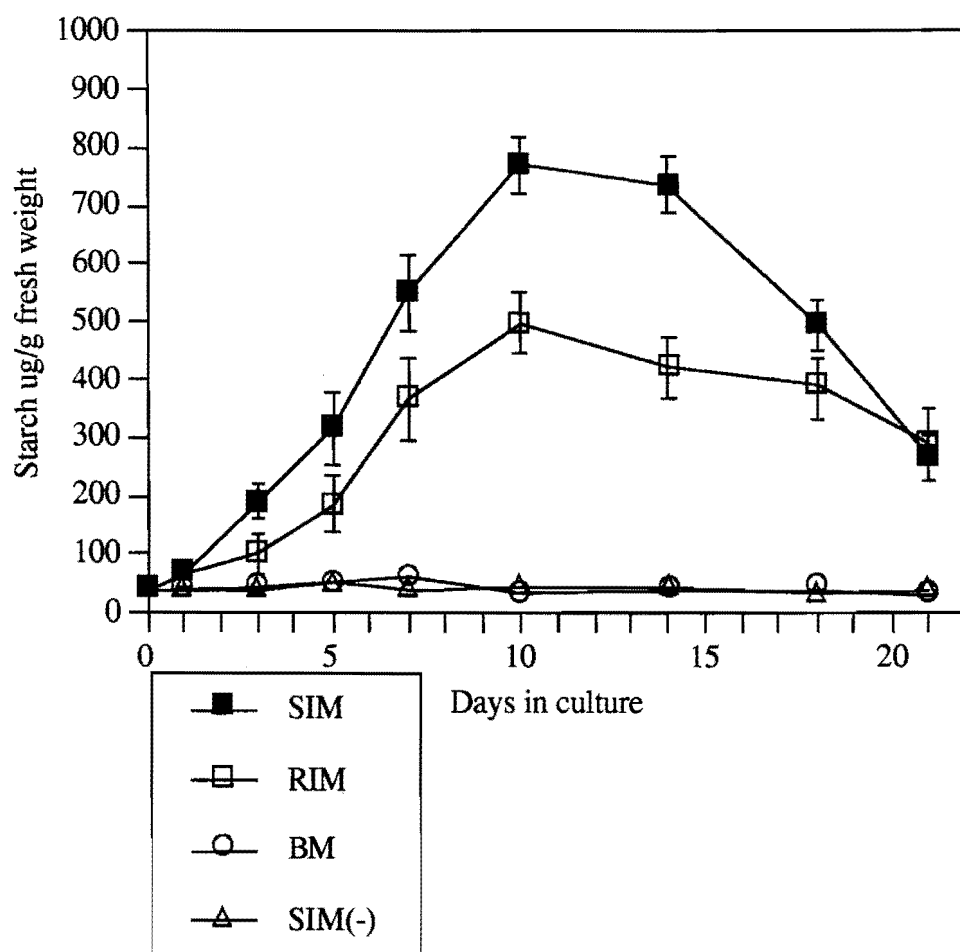


FIGURE [3] Changes in the starch content of *Begonia erythrophylla* petiole explants cultured for 21 days.

Mean plus or minus 1 standard error, n=30.

(8) HISTOCHEMICAL LOCALIZATION OF ENZYME ACTIVITY

Culture of *B. erythrophylla* petiole explants on either root or shoot inducing media resulted in increased metabolic activity localized specifically in the organogenic regions of the explant. This increased activity was reflected in localized increases in the activity of several enzymes involved in general metabolism.

8.1 Culture on shoot inducing media (SIM)

8.1.1 Acid phosphatase

Acid phosphatase activity was most intense in the vascular bundles and surrounding cells in the initial explant (PLATE 15A). Some staining of the cell walls and cytoplasm was also evident in epidermal cells associated with glandular hairs. After 5 days on SIM the areas of future shoot formation beneath glandular hairs could easily be distinguished by the strong localization of acid phosphatase activity compared to the surrounding epidermal tissue (PLATE 15B). The intensity continued to increase with the development of meristematic domes (PLATE 15C). Vascular bundles also showed increased acid phosphatase activity with further culture on SIM (PLATE 15C).

As the meristematic domes developed into shoot primordia a distinct zonation of acid phosphatase activity became apparent within the dome, with the strongest activity detected in the region giving rise to the shoot apex. By day 10 to 12 of culture clearly identifiable shoot primordia were apparent, with acid phosphatase activity strongly localized in the developing shoot apex and in the emerging foliar primordia (PLATE 15D). By day 18 to 21, strong acid phosphatase activity was apparent throughout the shoot bud, with activity most intense in the apical region (PLATE 15E). Activity throughout the rest of the explant was still confined mainly to the vascular bundles.

8.1.2 Peroxidase

Peroxidase activity was localized mainly in the vascular bundles at the time of excision of the petiole explant. The epidermal and cortical cells showed little activity, as did the majority of collenchyma cells. Occasional collenchyma cells with strong peroxidase activity were detected, but these cells appeared to be randomly distributed throughout the collenchyma layer (PLATE 15F). After 3 days on SIM, some epidermal and collenchyma cells showed strong peroxidase activity. This activity was mainly associated with dividing cells. By day 5, meristematic regions could be clearly

identified by the presence of strong peroxidase activity. Peroxidase localization occurred mainly in the cell walls, but was also detected as granular deposits in the cytoplasm, particularly in the meristematic cells. With continued culture localized peroxidase activity was observed in the cells making up the meristematic dome (PLATE 16A). As the dome developed into a shoot bud, peroxidase activity was localized mainly in the shoot apex.

8.1.3 Succinate dehydrogenase

Succinate dehydrogenase (SDH) of all the enzymes examined was most specifically localized in the regions associated with shoot formation. At the time of excision from the explant little SDH activity was detected. Only the vascular bundles and some epidermal cells showed slight activity (PLATE 16B). After 5 days in culture areas of localized activity could be detected in the superficial layers of the explant, but these were very faint. By day seven, activity was strongly localized in both the vascular bundles of the explant and in the regions forming meristematic domes. The rest of the explant was essentially without any activity. As the meristematic domes developed into shoot primordia, SDH activity continued to be strongly localized only in the organogenic center (PLATE 16C) and latter in the meristematic region of the shoot bud.

8.1.4 Glucose-6-phosphatase, Glucose-6-phosphate dehydrogenase and ATPase

As with the other enzymes glucose-6-phosphatase (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH) and ATPase were localized only faintly in the vascular bundles at day 0. During culture on SIM, the activity of all three enzymes increased in superficial areas of the explant where meristematic activity occurred (PLATE 16D). The level of activity increased with the size of the meristematic region (PLATE 16E). Once shoot buds had developed, enzyme activity was localized mainly in the tip of the shoot apex with little detected in the rest of the shoot bud (PLATE 16F).

8.2 Culture on basal medium

Culture on basal medium resulted in no increase in enzyme activity. After 14 days culture, enzyme activity was only detected faintly in the vascular bundles of the explant. No localized activity in the superficial layers of the explant was observed.

8.3 Culture on root inducing medium.

Generally the pattern of enzyme localization in explants cultured on RIM paralleled that observed with culture on SIM. All six enzymes localized in regions of the explant giving rise to meristematic zones that subsequently formed root primordia. It was only as root primordia became progressively more developed that enzyme activity became localized in specific regions of the developing organ. All six enzymes were localized mainly in the rapidly dividing cells of the root cap and in the central cylinder.

8.4 Controls

Explants heated to denature their enzymes prior to enzyme localization showed no enzymatic activity. Sections only stained slightly yellow when incubated in the solutions for the localization of peroxidase, G-6-P, and ATPase, respectively. Localization of these enzymes was in the form of dark brown, or black deposits so this caused no problem.

Incubation of sections without substrate resulted in faint peroxidase, acid phosphatase, G-6-P and ATPase activity detected in the vascular bundles. SDH and G-6-PDH lacked detectable activity in the absence of substrate.

PLATE 15: Histochemical localization of enzyme activity in cultured petiole explants of *B. erythrophylla*

- [A]- Transverse section of the petiole at day 0. Note acid phosphatase activity only in the vascular bundles and glandular hairs (x30).
- [B]- Transverse section of an explant after 5 days on SIM. Note acid phosphatase activity in the superficial layers of the explant, particularly intense beneath glandular hairs (arrow) (x25).
- [C]- Transverse section of an explant after 10 days on SIM, showing most acid phosphatase activity is localized in the meristematic regions and vascular bundles (x30).
- [D]- Transverse section of an explant showing acid phosphatase activity after 14 days on SIM. Note the activity is strongest in the apical region of the dome and in the tip of the developing foliar primordia (x30).
- [E]- Transverse section of an explant after 21 days on SIM. Note acid phosphatase activity is mostly localized in the apical region of the shoot bud (x30).
- [F]- Transverse section of the petiole at day 0 showing peroxidase activity mostly in the vascular bundles and in some randomly distributed collenchyma cells (x30).

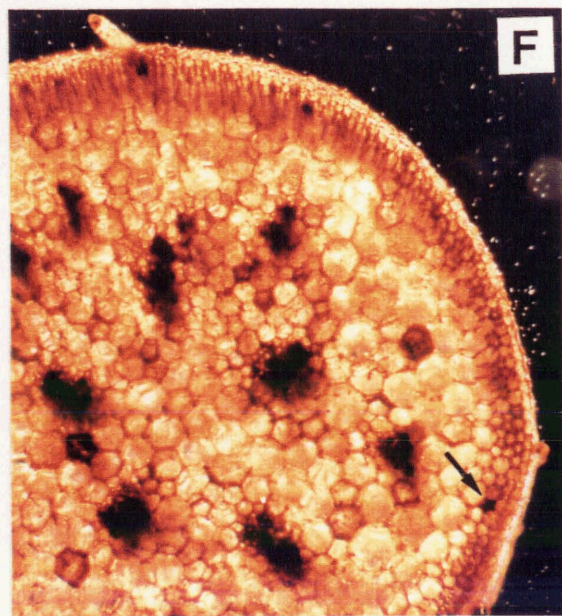
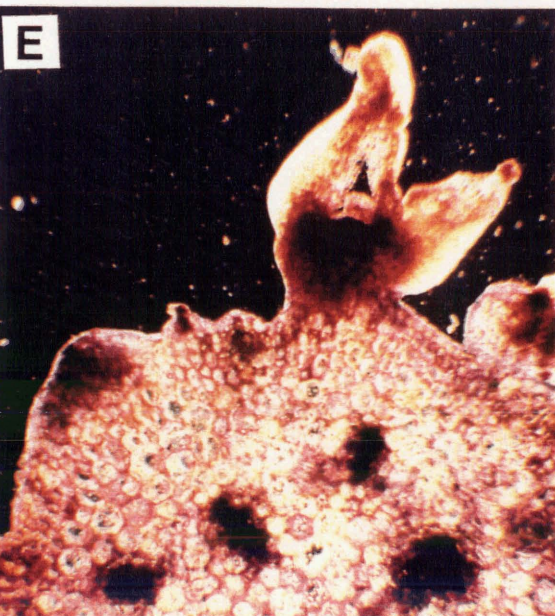
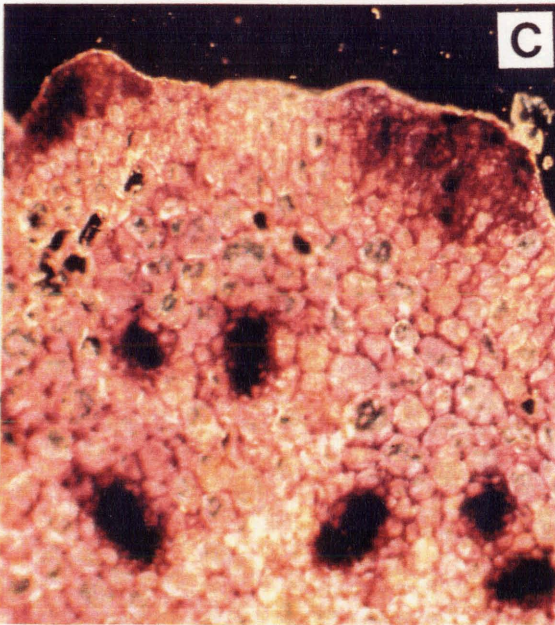
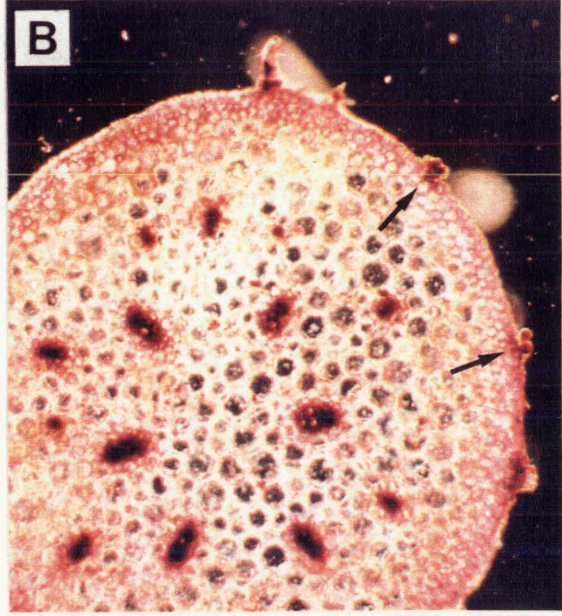
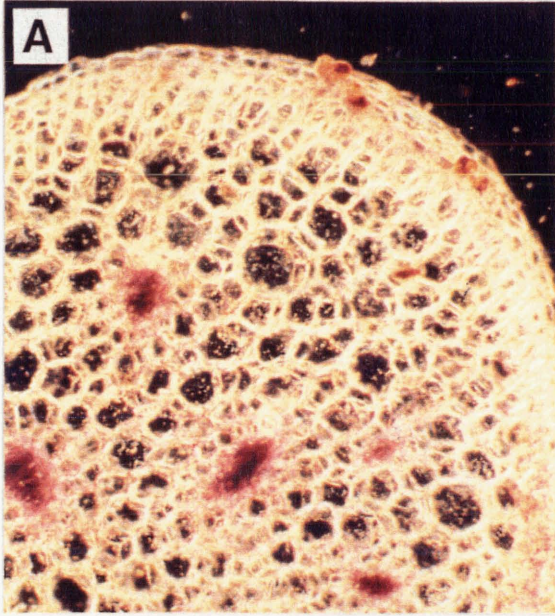
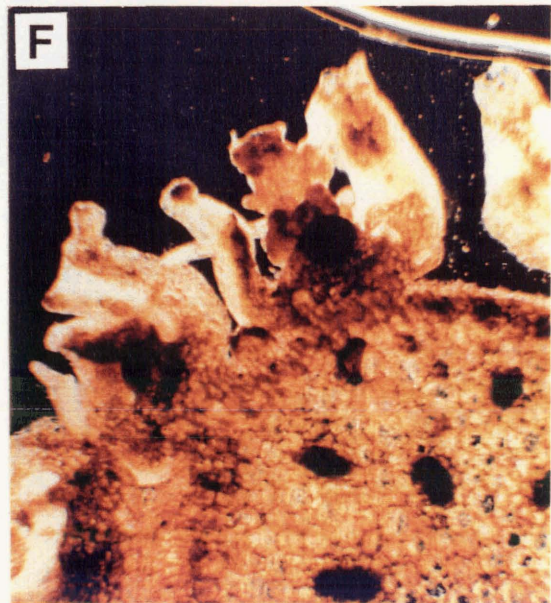
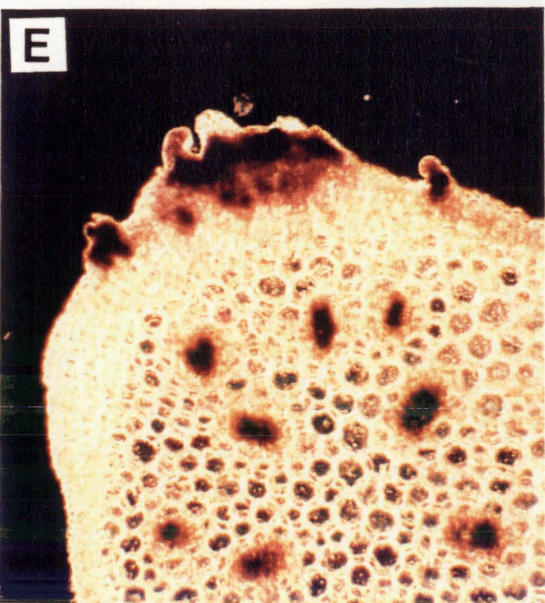
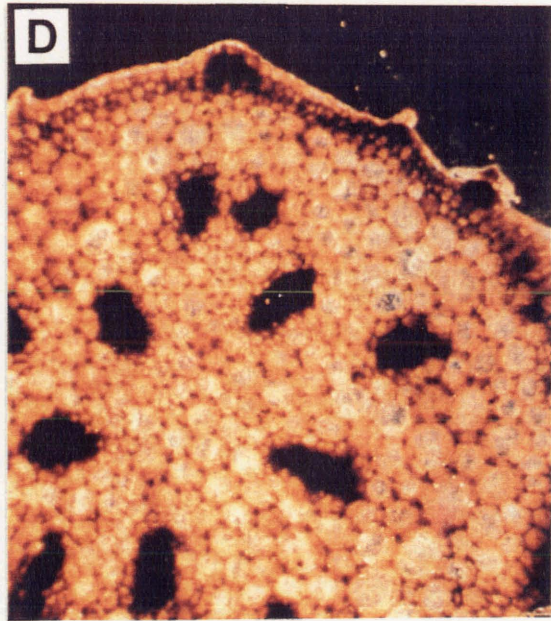
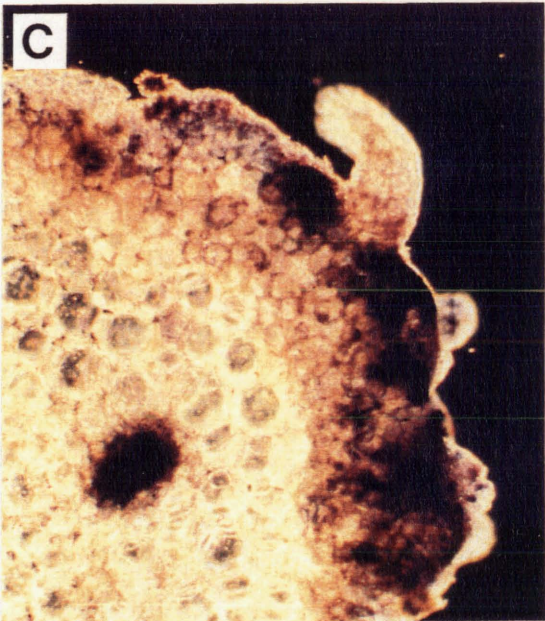
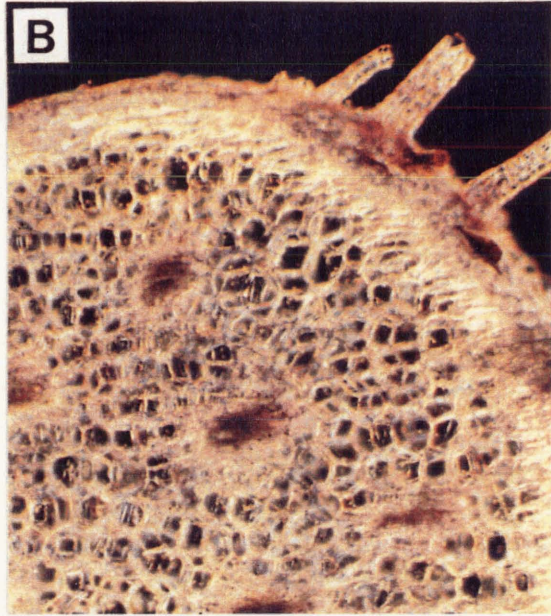
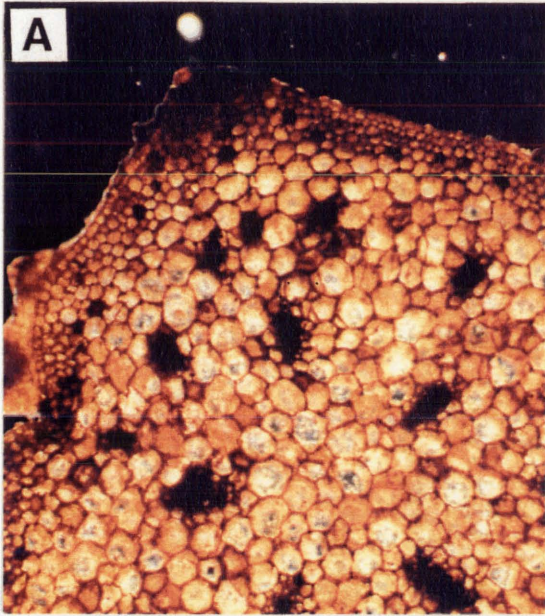


PLATE 16: Histochemical localization of enzyme activity in cultured petiole explants of *B. erythrophylla*

- [A]- Transverse section of the petiole after 7 days on SIM. Note intense peroxidase activity in the meristematic regions (x30).
- [B]- Transverse section of the petiole showing succinate dehydrogenase activity localized mainly in cells of the vascular bundles at day 0 (x30).
- [C]- Transverse section of an explant after 10 days on SIM, showing succinate dehydrogenase activity localized in the vascular bundles and only the meristematic regions in the superficial layers of the explant (x30).
- [D]- Transverse section of an explant after 7 days on SIM, showing ATPase activity associated with the meristematic domes (x30).
- [E]- Transverse section of an explant after 10 days on SIM. Note glucose-6-phosphate dehydrogenase activity associated with the meristematic regions (x30).
- [F]- Transverse section of an explant after 21 days on SIM. Note glucose-6-phosphatase activity is localized mainly the apical region of the shoot bud (x30).



(9) FRESH AND DRY WEIGHT CHANGES ASSOCIATED WITH ORGANOGENESIS

Culture of *B. erythrophylla* petiole sections on an organogenic medium (SIM or RIM) resulted in little change in the fresh weight of explants for the first five days of culture (FIGURE 4A). After this time, fresh weight increased gradually until day 16, when fresh weight began to increase exponentially. As little callus formed in the first 21 days of culture on either medium, this increase in growth was mainly attributed to the formation and development of organogenic structures.

Although the general pattern of fresh weight increase was the same for both SIM and RIM, culture on SIM resulted in a greater increase in fresh weight per explant than culture on RIM (FIGURE 4A). After 21 days explants cultured on SIM weighed approximately 50% more than those cultured on RIM. With culture on CIM explants responded similarly to those cultured on RIM, having a similar mean fresh weight after 21 days. Culture on basal medium did not induce any increase in the fresh weight of explants.

The dry weight response essentially paralleled that of FW, except the dry weight increase was first observed earlier (day 3) and the increase on all media except BM was more dramatic (FIGURE 4B). SIM induced the greatest increase in dry weight, followed by RIM and CIM. Culture on basal medium resulted in no increase in dry weight with a slight decline noticeable after 10 days in culture.

Perhaps of more interest than changes in the mean fresh and dry weight per explant was the change in the dry weight to fresh weight ratio, best expressed as percentage fresh weight (FIGURE 5). At the time of excision from the parent plant petiole sections of *B. erythrophylla* had very high water contents whether from plants grown in soil or in culture. At day zero explants taken from *B. erythrophylla* plants grown *in vitro* had a mean fresh weight of 16 mg/explant. Over ninety seven percent of the fresh weight was water, with explants only having a mean dry weight of 0.49 mg/explant (2.9% of fresh weight). With 5 days culture on SIM the mean percentage dry weight of explants had increased to nearly 5% and continued to increase to a maximum of over 7 percent after 16 days culture. By day 21 the percentage had dropped slightly to just under 6%.

Culture on RIM showed a similar trend although the total increase was less pronounced, with the maximum percentage dry weight being just under 5% at day 16. Culture on CIM resulted in a smaller increase than culture on either of the organogenic media. Basal medium caused a decline in the dry weight to fresh weight ratio after a slight increase.

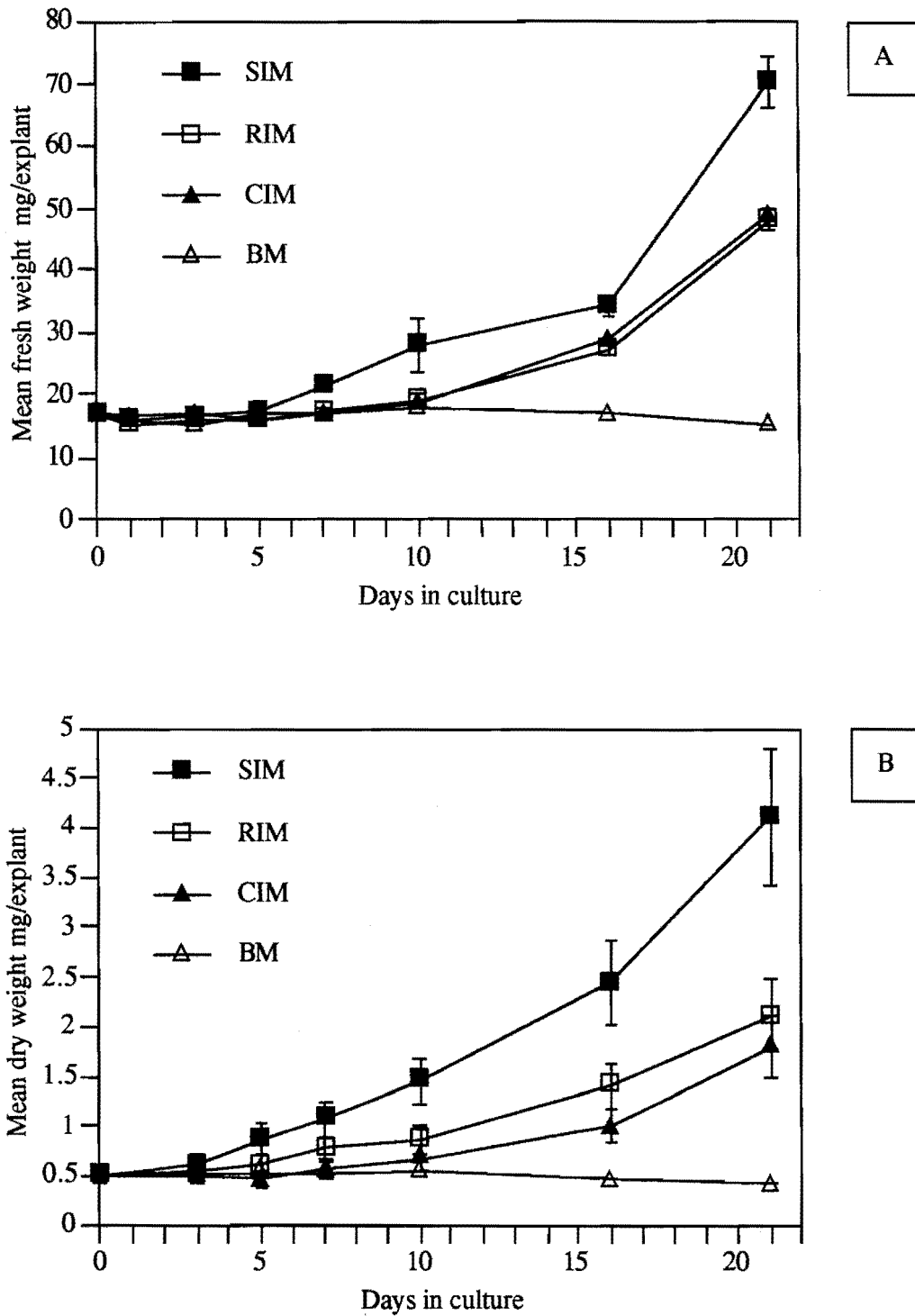


FIGURE 4 [A]- Fresh weight of petiole sections cultured on organogenic and non-organogenic media. Mean value plus or minus 1 standard error, n=30.

[B]- Dry weight of petioles sections cultured on organogenic and non-organogenic media.. Mean value plus or minus 1 standard error, n=30.

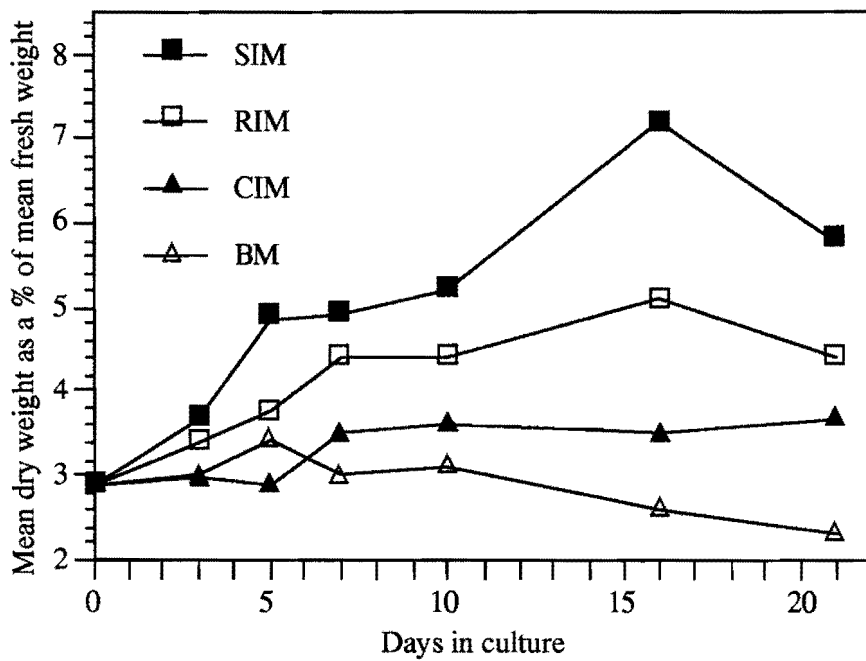


FIGURE [5] Dry weight/fresh weight ratio changes, expressed as percentage fresh weight, with culture on organogenic and non-organogenic media.

(10) **THE EFFECT OF ORGANOGENESIS ON THE PROTEIN COMPOSITION OF PETIOLE EXPLANTS**

10.1 The protein content of explants exposed to organogenic or non-organogenic conditions.

Exposure of explants to an organogenic medium resulted in a rapid increase in the protein content of explants (FIGURE 6B). Culture on either SIM or RIM resulted in at least a 2 fold increase the protein content within 7 days of culture, after which time the content increased only slightly. Culture on CIM also resulted in an increase in protein content, but the increase was less than that observed under organogenic conditions. SIM caused a slightly higher increase in protein content than RIM, despite protein being extracted only from the lower, responsive half of the explant on RIM. Culture on BM caused a slight initial increase in protein content, followed by a gradual decline after seven days in culture.

As different extraction procedures were used during this study, the effects of extraction procedure on the amount of protein extracted was determined (FIGURE 6A). A non-detergent extraction procedure using TRIS-HCl (pH 6.8) resulted in only low levels of soluble proteins being extracted when compared to two detergent based systems. The Triton X-100/CHAPS buffer, used when extracting protein for 2D electrophoresis, extracted much higher levels of protein than TRIS-HCL, but a buffer containing SDS (Chen and Luthe, 1987) extracted the highest concentration of protein.

All the extraction buffers tested displayed the same pattern, of protein content, under shoot forming conditions. A rapid increase in protein after day 3 and a leveling off after day 7. This initial increase in protein content coincided with the formation of meristematic zones and the resultant increased enzyme activity. The protein content of explant tissue increased little after day 7 as the fresh weight of explants began to rise.

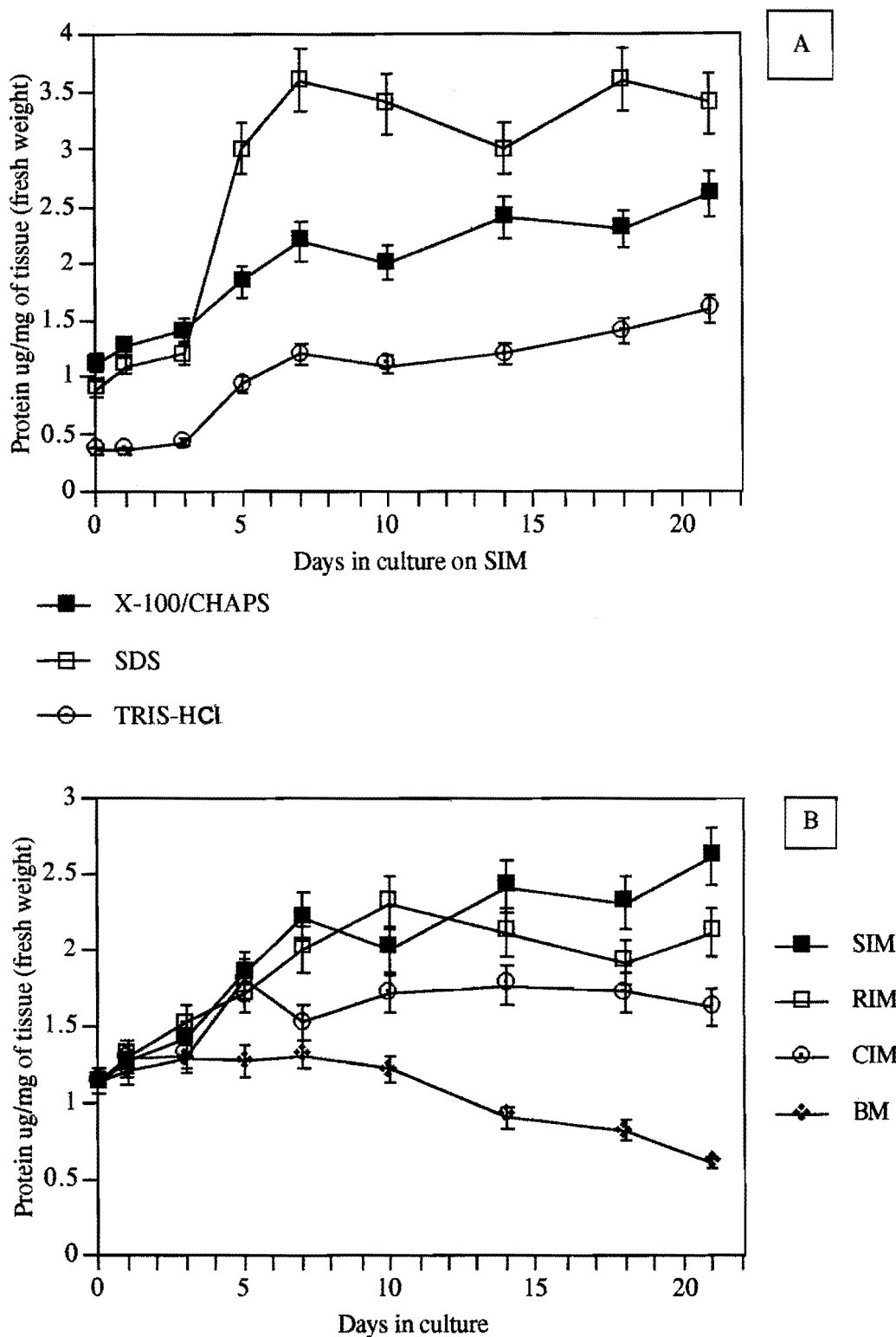


FIGURE 6 [A]- The effect of extraction buffer on the amount of protein extracted from explants cultured for various lengths of time on SIM.

[B]- Changes in the protein content of explants cultured for various lengths of time on organogenic and non-organogenic media. Protein was extracted using the X-100/CHAPS extraction buffer. Mean value plus or minus 1 standard error, n=6 with values pooled from 2 separate extractions.

10.2 The effect of organogenesis on protein synthesis

Figures 7A and 7B show the total amount of [^{35}S]-methionine both taken up and incorporated into the protein fraction with culture on SIM or RIM respectively, and Table (2) with culture for 14 days on various control media. It appeared that there was a direct relationship between the length of time in culture on SIM, RIM or CIM and the amount of [^{35}S]-methionine taken up by the explant. Progressively less label was taken up by explants with increasing time in culture. On organogenic media the percentage incorporation also dropped within the first three days of culture, after which the level increased until day 7 when it began to drop again. Callusing medium (CIM) showed a slightly lower level of uptake and incorporation than organogenic media after 14 days culture (Table 2).

Of particular interest were the controls. Culture on BM did not show the same decline in [^{35}S]-methionine uptake as did SIM, RIM, and CIM, although incorporation was less than observed on these media. SIM(-) gave an unusual response with a considerable increase in the uptake of [^{35}S]-methionine compared to day 0, although incorporation was again less than observed on organogenic media (TABLE 2).

| | Culture media | | | | | |
|---|---------------|--------|-------|-------|-------|-------|
| | BM | SIM(-) | CIM | BA8 | RIM | SIM |
| Protein ug/mg fresh weight | 0.91 | 1.12 | 1.66 | 2.22 | 2.11 | 2.34 |
| Uptake, ^{35}S counts per minute/mg of tissue (FW) | 44547 | 68302 | 22543 | 25954 | 23495 | 25690 |
| Incorporation, ^{35}S counts per minute/mg of tissue | 5087 | 5745 | 5987 | 8109 | 7749 | 8230 |

TABLE 2 The mean protein content, uptake of [^{35}S]-methionine and percentage of [^{35}S]-methionine taken up incorporated into protein extracted from explants exposed for 14 days to various media. n=6 with values pooled from 2 separate extractions.

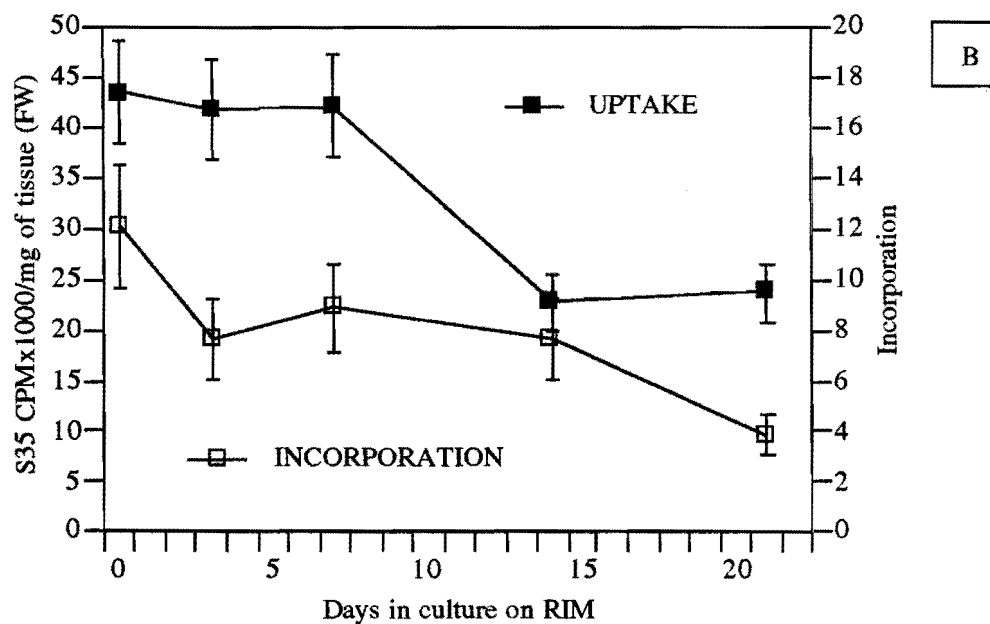
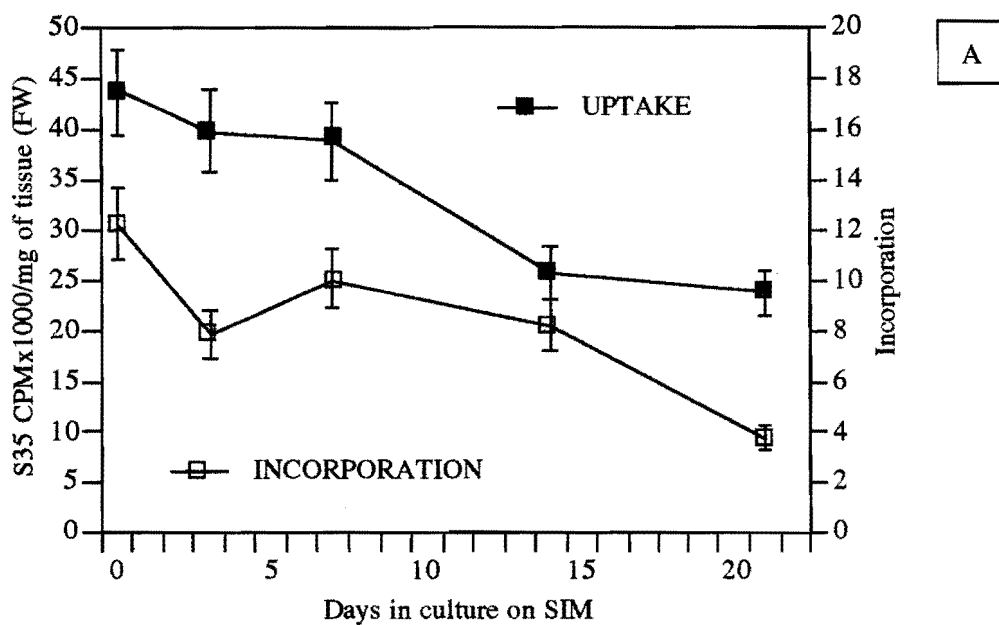


FIGURE 7 [A]- Uptake of [^{35}S]-methionine, and protein synthesis in petiole sections of *B. erythrophylla* cultured for up to 21 days on SIM. Mean value plus or minus 1 standard error, $n=6$ with values pooled from 2 separate extractions.

[B]- Uptake of [^{35}S]-methionine, and protein synthesis in petiole sections of *B. erythrophylla* cultured for up to 21 days on RIM. Mean value plus or minus 1 standard error, $n=6$ with values pooled from 2 separate extractions.

10.3 1D SDS-PAGE analysis of polypeptide changes associated with organogenesis. (Accumulation)

As exposure of petiole sections to either SIM or RIM resulted in a rapid increase in the protein content of the exposed tissue, the question was asked was the increase general, with all polypeptides present at day 0 increasing to an equal extent, or was the increase due to the accumulation of polypeptides specific to organogenesis? One dimensional SDS PAGE separation of polypeptides was carried out on protein extracted from explants by both the method of Mayers *et al.* (1987) for 2D sample preparation and Chen and Luthe's (1987) method for the extraction of total proteins using SDS PAGE sample buffer as the extraction buffer. Both extraction procedures gave similar results, although Chen and Luthe's method for total protein extraction resulted in considerably better gel resolution.

Gradient gels were initially run to determine the molecular weight ranges where differences in the protein profiles were observed. These gels had poor reproducibility because of inconsistencies in the gradients formed in different gels, due to the use of too large a gradient former. For this reason trial gels were run containing various concentrations of acrylamide (10, 12.5, 15 and 18%), to determine the gel concentration required to give optimum resolution of bands in the molecular weight range where polypeptides patterns differed. Fifteen and eighteen percent gels were found to give the best resolution.

Initially quantitative gels were run using protein samples extracted from equal amounts of tissue. Samples were extracted and run over the time course of shoot induction and development. These gels showed general increases in the intensity of all bands present in the petiole tissue at the time of excision from the parent plant (day 0) (PLATE 17A).

Because of the low level of protein in day 0 samples, qualitative gels, where an equal amount of protein was loaded were required to determine if any new bands were induced under shoot forming conditions. Qualitative gels mainly revealed changes in the intensity of polypeptide bands present at day 0 (PLATE 18B). Most of these changes were the result of the culture procedure and occurred when explants were cultured on any medium including basal. A 29kd and a 49kd band both increased in abundance with culture, while a 55kd band decreased. Culture on CIM resulted in a reproducible increase in a single band that appeared specific to this medium (PLATE 18A). This 18.5 to 19kd band was prominent after 21 days culture on CIM while being detected at a lesser level at day zero or with culture on organogenic media.

As well as revealing culture induced changes in band intensity, two low molecular weight bands (18 and 16.5kd) appeared consistently at reasonable levels only under shoot forming conditions. These 2 bands only resolved clearly using the SDS extraction procedure (Chen & Luthé, 1987) with samples run on 18% gels (PLATE 17B). These bands were undetectable (using coomassie blue) in the initial explant, in leaf tissue, or in explants cultured on BM for 21 days and were only faintly detectable in some protein extracts from explants cultured on RIM for more than 14 days (PLATE 17B). Both the 18 and 16.5kd bands were detectable at day 7 of culture and increased in intensity during culture on SIM (PLATE 17B).

Except for the bands detected with culture on SIM and the single band with increased intensity on CIM, no significant changes were detected as a result of culture on growth regulator containing media. For this reason it was decided to see if any changes in the protein profile of newly synthesized polypeptides were detectable using 1D-PAGE and to carry-out further separations using 2D-PAGE to see if any marker polypeptides could be detected using this higher resolution technique.

10.4 1D SDS-PAGE analysis polypeptide changes associated with organogenesis. (Synthesis)

The pattern of newly synthesized [³⁵S]-methionine labelled polypeptides extracted from explants exposed to SIM, RIM, CIM, SIM(-) or BM respectively, for various lengths of time, was analyzed on 15% SDS-PAGE gels. No differences other than those detectable when gels were stained with Coomassie blue were observed (PLATE 18B). The 18.5 to 19kd band that increased in abundance with Coomassie staining had a greater level of synthesis on CIM than on any of the other media. Changes induced by culture observed with Coomassie staining also showed the same trends with respect to synthesis, with the 29 and 49kd bands showing increased synthesis, while the 55kd band had a reduced level. Neither the 18 or the 16.5kd bands observed at high levels on SIM were clearly detectable, probable due to the use of a 15% gel, which resulted in poorer resolution in this region than the 18% gel used with Coomassie blue staining. The use of 18% gels was precluded by severe cracking upon drying.

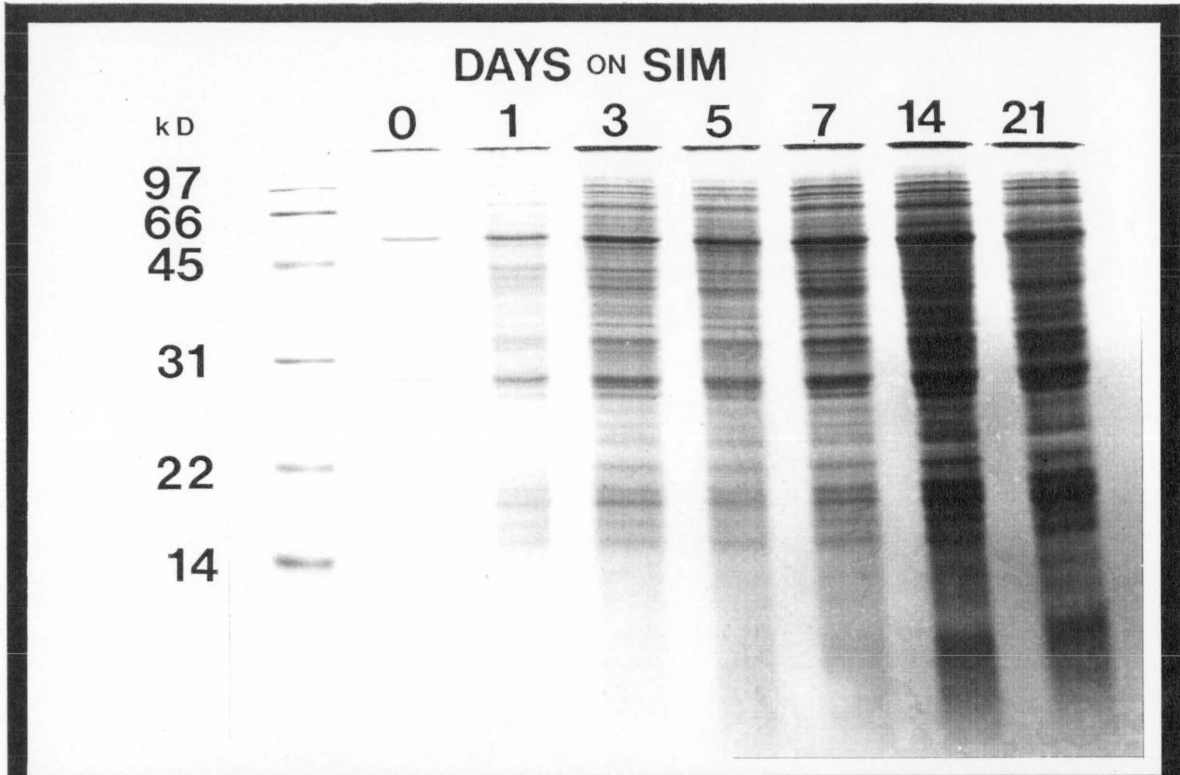


PLATE 17: [A]- Coomassie-stained SDS-PAGE gel (18% T) of protein extracted from petiole explants cultured on SIM for up to 21 days. Protein from equal weights of tissue was loaded per lane. Note the general increase in the intensity of bands present at day 0.

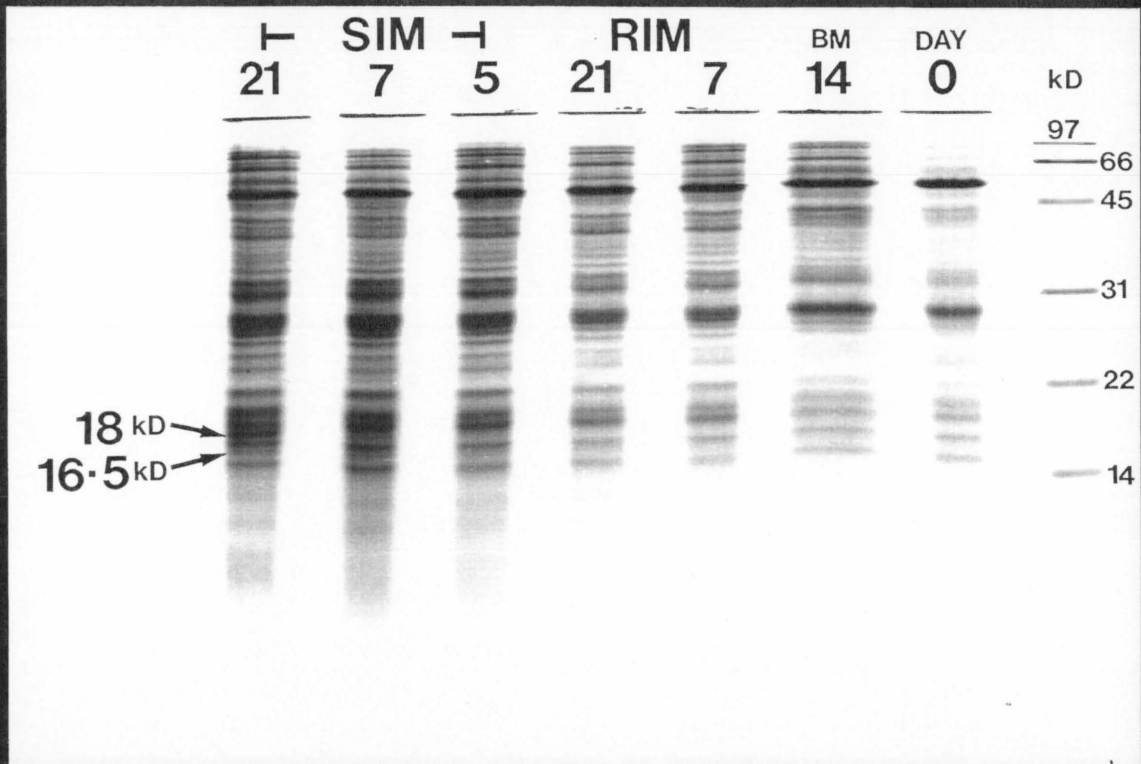
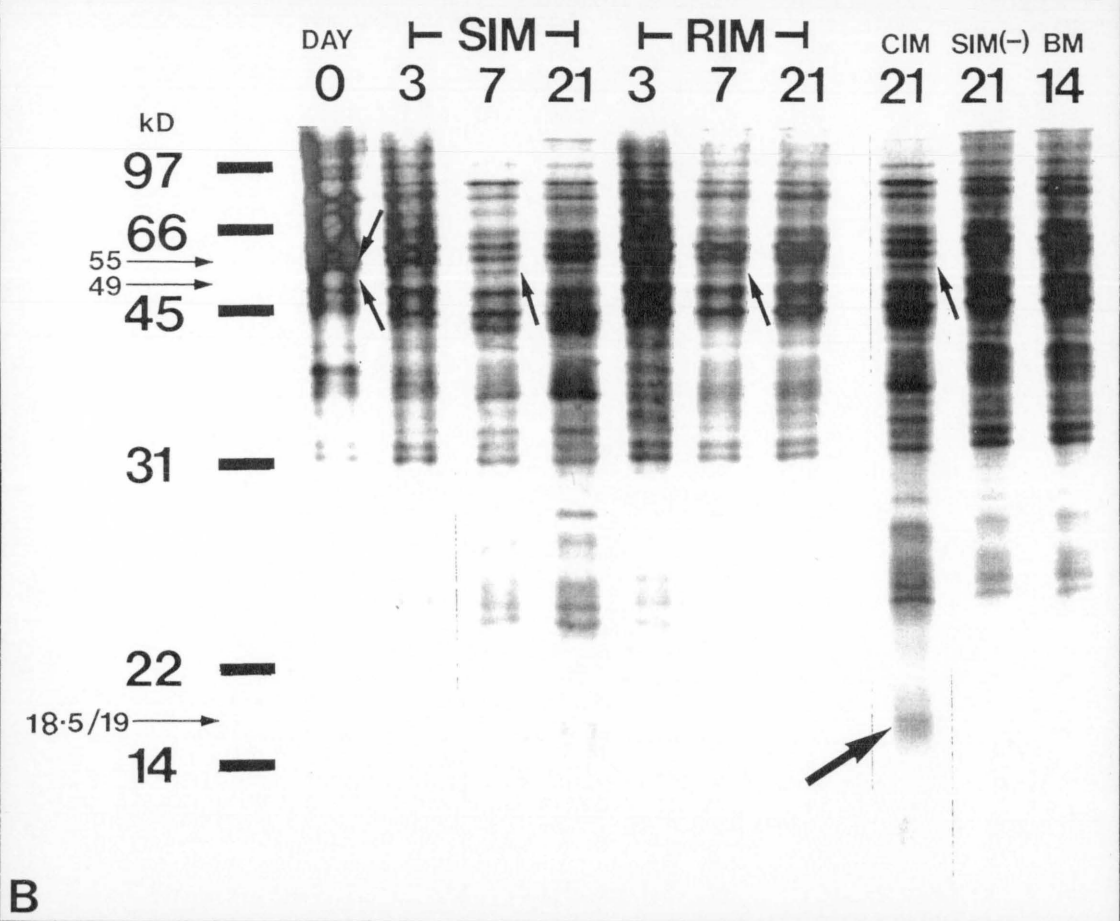
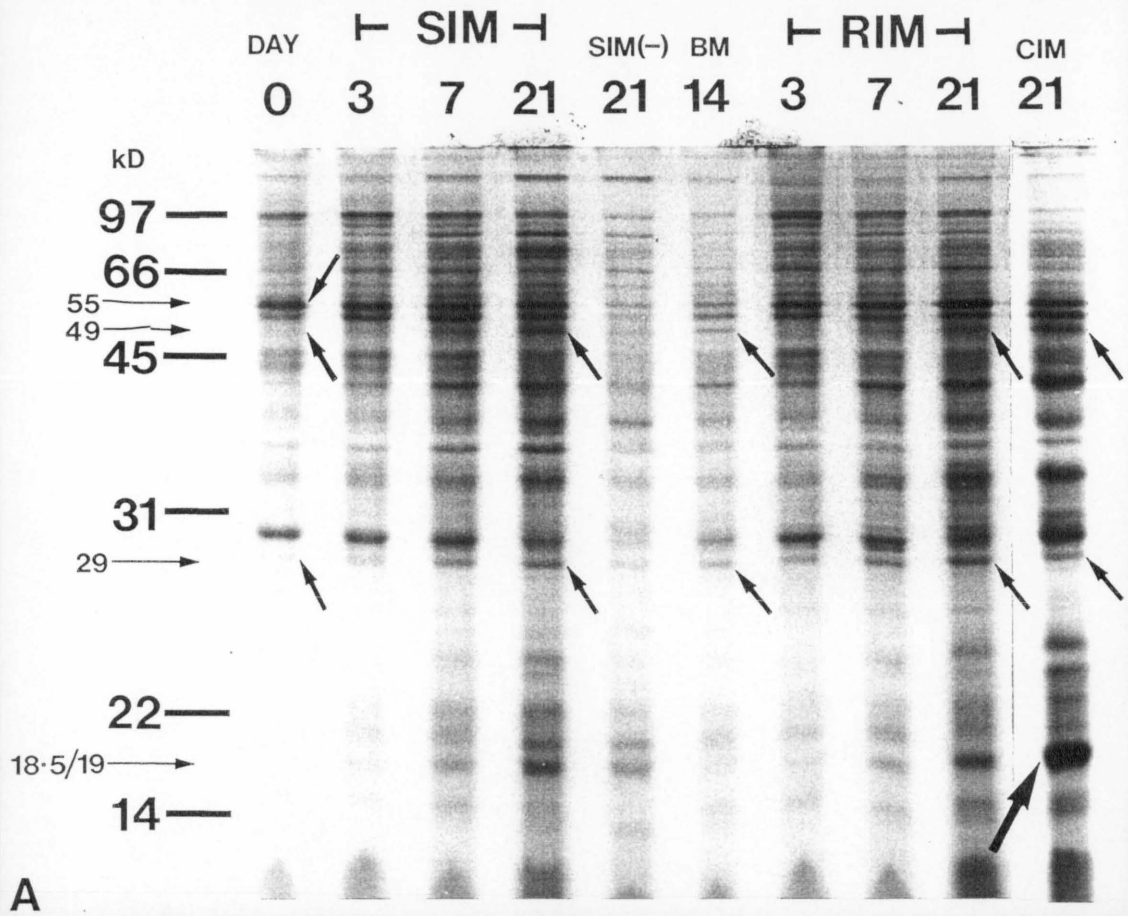


PLATE 17: [B]- Coomassie-stained SDS-PAGE gel (18% T) of protein extracted from petiole explants cultured for up to 21 days on shoot-inducing medium (SIM), or root-inducing medium (RIM), and for 14 days on basal medium, respectively. Equal amounts of protein were loaded per lane. Arrows indicate bands which accumulated primarily with culture on SIM.

PLATE 18: [A]- Coomassie-stained SDS-PAGE gel (15% T) of protein extracted from petiole explants cultured for up to 21 days on shoot-inducing medium (SIM), or root-inducing medium (RIM), and for 14 days on basal medium, 21 days on shoot-inducing medium without sucrose (SIM(-)), and 21 days on callus-inducing medium (CIM), respectively. Arrows indicate culture induced changes in the intensity of bands present at day 0. The large arrow indicates a band which increases greatly in abundance with culture on CIM.

[B]- Fluorograph of an SDS-PAGE separation (15% T) of protein extracted from petiole explants cultured for up to 21 days on shoot-inducing medium (SIM), or root-inducing medium (RIM), and for 14 days on basal medium, 21 days on shoot-inducing medium without sucrose (SIM(-)), and 21 days on callus-inducing medium (CIM), respectively. Arrows indicate culture induced changes in the intensity of bands present at day 0. The large arrow indicates a band which increases greatly in abundance with culture on CIM. Note the similarity to the coomassie-stained gel above



10.5 Changes in the pattern of polypeptides accumulating upon culture of petiole explants, as determined by 2-Dimensional Polyacrylamide Gel Electrophoresis.

Total proteins extracted from petiole tissue at the time of excision from the parent plant (day 0) were analyzed by 2D-electrophoresis. More than 700 polypeptides were detected in the pI range from 7.5 to 4.5, but only 450 of these polypeptides separated consistently between independent extractions and IEF/SDS gel separations, having similar IEF, molecular weight positions and similar relative intensities. Polypeptides were detected across the entire pH and molecular weight range of the gels. Once a base pattern of polypeptides was established, the effects of various inductive and non-inductive media on the pattern of polypeptides observed at day 0 were determined.

Initially protein extracts from explants exposed to SIM, RIM, CIM or BM respectively, for 14 days, were compared to the day zero pattern and to each other (PLATES 19B, 22A, 22B, & 25B), to establish polypeptide changes associated with the culture process (present on BM), rather than growth regulator induced callus formation or organogenesis. A careful inspection of gels led to the identification of 35 spots present at day 0, the intensity of which varied significantly either during culture on a given medium or when extracts from the 4 media were compared. A further 24 spots were detected that were absent at day 0, but were induced by culture on 1 or more of the media mentioned above. A total of 474 polypeptides were reproducibly detected combining all 4 media effects. Changes in the pattern of polypeptides observed at day 0 were divided into 6 major groups with several subgroups (TABLE 3).

(1) Group 1- polypeptides were visible at an approximately constant level throughout the culture period irrespective of the media to which explants were exposed. This group comprised 87.6% of observed polypeptides and was presumed to represent the products of basic metabolism or <<housekeeping gene>> products.

(2) Group 2- polypeptides decreased in abundance during culture, irrespective of the medium to which explants were exposed. This group included a continuous spectrum of polypeptides, ranging from those that completely disappeared with 3 days culture, to those that remained at very low levels after 42 days on SIM or RIM. This group comprised 4.2% of observed polypeptides and may represent the products of genes concerned primarily with petiole physiology or autotrophic growth.

(3) Group 3- comprised 8 newly detected polypeptides all of which were induced by culture on all 4 media and remained present for at least 14 days. A further 3 polypeptides present at day 0, increased greatly when cultured on any of the 4 media. This group of polypeptides was termed culture induced and may be the result of metabolic changes caused by explant excision and tissue culture. These polypeptides comprised 2.3% of the total number of polypeptides detected.

(4) Group 4- polypeptides were present at day 0, but changed in abundance as a result of culture on one or more of the growth regulator containing media (SIM, RIM or CIM). No changes in the abundance of polypeptides in this group were detected with culture on BM. Polypeptides in this group were divided into 4 subgroups.

- A- polypeptides whose abundance changed as a result of exposure to all 3 media.
- B- polypeptides changing only on SIM
- C- polypeptides changing permanently only on RIM
- D- polypeptides changing only on CIM

These polypeptides comprise 1.9% of the total number of polypeptides detected.

Polypeptides in this group may represent gene products that accumulate at different levels under different inductive conditions.

(5) Group 5- polypeptides comprised 0.6% of the total number detected. These polypeptides were present at day 0 and permanently increased in abundance only when exposed to RIM, CIM or BM respectively.

(6) Group 6- polypeptides were absent from the initial explants and were not induced by culture on basal medium. They appeared during culture and in most cases remaining present through the remainder of the culture period. These polypeptides were divided into 3 subgroups.

- A- polypeptides induced by all three media.
- B- polypeptides induced at significant levels by SIM only.
- C- polypeptides appearing during culture on SIM, RIM or CIM respectively, but remaining permanently only on RIM or CIM, while disappearing with prolonged culture on SIM.

This group includes a continuous spectrum of appearance times, from day 3 to day 14 of culture. These polypeptides comprised 3.4% of the total number detected and are presumed to represent the products of genes associated with organogenesis and/or callus formation.

Table 3 summarizes the changes in the polypeptide pattern associated with culture on SIM, RIM, CIM or BM, for the first 21 days of culture.

As the major objective of this study was to find markers specific to caulogenesis, only polypeptides falling into Groups 4, 5, and 6 were intensively studied over the time course of organogenesis. Of greatest interest were polypeptides appearing only under organogenic conditions. Polypeptides induced by culture are, however, indicated on PLATE 25B, while group 2 polypeptides are indicated on PLATE 19A.

10.5.1 Newly detected polypeptides associated primarily with shoot formation/development

Culture on SIM induced 11 polypeptides undetectable at day 0 and virtually absent with culture on RIM or CIM (TAB 3; PLATES 18A, 18B, 22A & 22B). None of these polypeptides could be detected prior to day 7 of culture.

Four polypeptides, 376, 377, 378 and 401, were detected at low levels after 7 days on SIM (PLATE 21A). Although categorized as shoot only, 3 of these polypeptides, 376, 377 and 378, were occasionally detected at trace levels in explants cultured on RIM. The appearance of these polypeptides on RIM was not consistent between extractions and as the levels on RIM were very low in comparison to SIM, the polypeptides were classified as SIM only. Their appearance on RIM may have been due to the formation of occasional shoot primordia. These four polypeptides increased greatly in abundance from days 7 to day 21 when explants were cultured on SIM (PLATES 21A, 22A, 23A).

All the polypeptides in this group were only detected after the formation of meristematic structures. Polypeptides 376, 377, 378 and 401 were first detected at the point where explants no longer required SIM to form shoots (day 7 on SIM), at which time the most advanced meristematic structures were meristematic domes without any specialization and so could not be defined as shoot primordia. These polypeptides increased dramatically in abundance as the number of meristematic regions no longer requiring exposure to SIM for shoot formation increased (see section explants requirements for SIM). This makes them good markers for this phase of the caulogenic process. As shoot induction/determination in *B. erythrophylla* is not a synchronous process, such an increase would be expected as an increasing number of meristemoids form with time. After 42 days culture on SIM these polypeptides were less abundant (PLATE 24A), possibly reflecting a decreased requirement during plantlet growth and development. With 42 days culture on RIM trace levels of some SIM only polypeptides were clearly detected (PLATE 24B), most probably due to the appearance of small numbers of shoot primordia that develop with prolonged culture on RIM. The appearance of these polypeptides at this time further supports their SIM only categorization.

A further 7 polypeptides, 329, 330, 348, 364, 390, 391, and 402, were first detected after 14 days culture (PLATE 22A). Polypeptide 348 once detected did not increase in concentration with further culture on SIM (PLATE 23A). Polypeptides 329, 330, 391 and 402 all increased in abundance with continued culture (PLATE 23A), while polypeptide 364 appeared transient, as it was detected only after 14 days culture on SIM and disappeared with further culture (PLATE 23A). Polypeptide 390 was only just detectable at trace levels after 14 days and could only be positively identified after 21 days on SIM (PLATES 22A & 23A).

As these SIM only polypeptides were first detected after 14 days culture, at which time the most advanced meristematic domes were becoming specialized, with the formation of foliar primordia. These polypeptides may represent the expression of genes involved in the induction and possibly development of specialized structures making up the shoot apex, and may not be involved in shoot induction/determination.

10.5.2 Newly detected polypeptides associated with root formation/development

No RIM only polypeptides were clearly detected during the first 21 days of culture on RIM, despite extensive analysis. A single 18kd polypeptide, 450, was detected after 42 days, only on RIM (PLATE 24B).

10.5.3 Newly detected polypeptides induced by culture on SIM, RIM or CIM

Five polypeptides induced by culture on media containing growth regulators and sucrose were detected. Of particular interest in this group were 2 polypeptides 382 and 383. Both were strongly detected in explants cultured on RIM or CIM after only three days (TAB 3; PLATE 20B). Culture on SIM also induced these polypeptides, after three days, but at slightly lower levels (PLATE 20A). By day seven, both polypeptides were still abundant on RIM or CIM, but had reduced levels on SIM (TAB 3; PLATES 21A & 21B). By day 14, both were undetectable on SIM and remained so with prolonged culture (PLATE 21A), while still being detectable on RIM and CIM (PLATES 19B & 22B). These 2 transient polypeptides are of particular interest as they may reflect the point of divergence in the 2 organogenic pathways. As these 2 polypeptides disappear, new SIM only polypeptides are detected which increase in abundance in parallel with increasing shoot determination.

Three other polypeptides 108, 388 and 389 were all induced by culture on SIM, RIM or CIM respectively (PLATE 19B). These polypeptides may represent gene products involved in culture induced cell division.

10.5.4 Changes to polypeptides detected at day 0 induced by SIM, RIM or CIM Respectively

As well as several newly accumulated polypeptides, culture on any of the above media resulted in a number of changes in the abundance of polypeptides detected a day 0. Culture on SIM, RIM or CIM, resulted in the decline of 2 polypeptides, 266 and 267, which did not decline with culture on BM (PLATE 19A). The loss of these 2 polypeptides occurred rapidly (day 3).

Two polypeptides, 288 and 366, present at day 0, increased strongly in abundance after 14 days on SIM, while another, 324, increased after 21 days on SIM (PLATES 22A & 23A). A further polypeptide 343 initially declined with culture on SIM or RIM (TABLE 3). On RIM the decline was permanent, while on SIM the polypeptide increased too greater than the original level by day 14 (PLATES 22B & 22A). This polypeptide may represent a gene product not initially required on either SIM or RIM, but required latter during shoot development.

Although no newly detected RIM only polypeptides were observed, 2 polypeptides that increased in abundance on RIM were detected. Polypeptides 358 and 359 were abundant after 3 days culture on either RIM or SIM. With prolonged culture both increased in abundance on RIM while reducing in abundance on SIM (PLATES 23A & 23B). Polypeptide 343 was also present at elevated levels after 42 days on RIM.

One polypeptide, 223, was found to increase in abundance only when explants were cultured on CIM (PLATE 19B). Several other polypeptides that changed in abundance with culture were those of group five, 341, 381, and 384. These 3 polypeptides increased permanently on RIM, CIM, or BM but only transiently on SIM. After 7 days on SIM all 3 of these polypeptides declined in abundance and by day 21 were just detectable (PLATES 23A & 23B).

10.5.5 Polypeptide changes associated with culture on SIM(-)

As a further control protein was extracted from explants cultured for 14 days on SIM(-). Culture on this medium failed to induce any of the changes associated with caulogenesis (PLATE 25A). All of the culture induced polypeptides were detected.

PLATE 19: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) petiole tissue from *in vitro* plants, i.e. DAY 0. Numbered arrows indicate polypeptides which declined with culture on any medium. Bold arrows indicate polypeptides which only declined with culture on SIM, RIM or BM respectively.

(B) explants cultured for 14 days on callus inducing medium (CIM). Numbered arrows indicate polypeptides which are only detected with culture on SIM, RIM or CIM respectively.

The bold arrow indicates a polypeptide which increased in abundance with culture on CIM only.

Circles, diamonds and squares indicate the positions of polypeptides identified on the opposite gel. Triangles indicate the absence of polypeptides associated with caulogenesis

Arrow heads indicate reference polypeptides for comparison of gels.

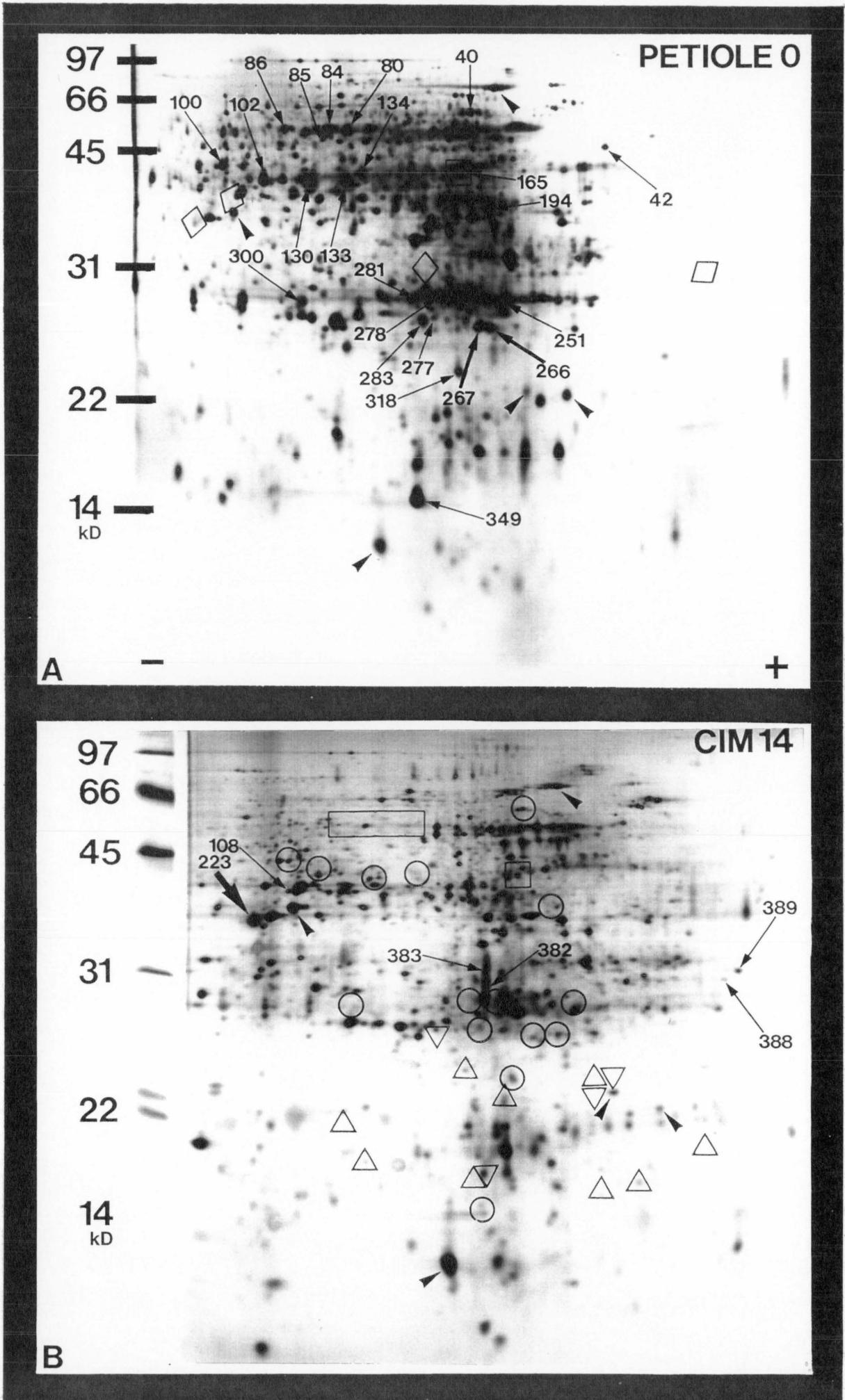


PLATE 20: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 3 days on SIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

(B) explants cultured for 3 days on RIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

Arrow heads indicate reference polypeptides for comparison of gels.

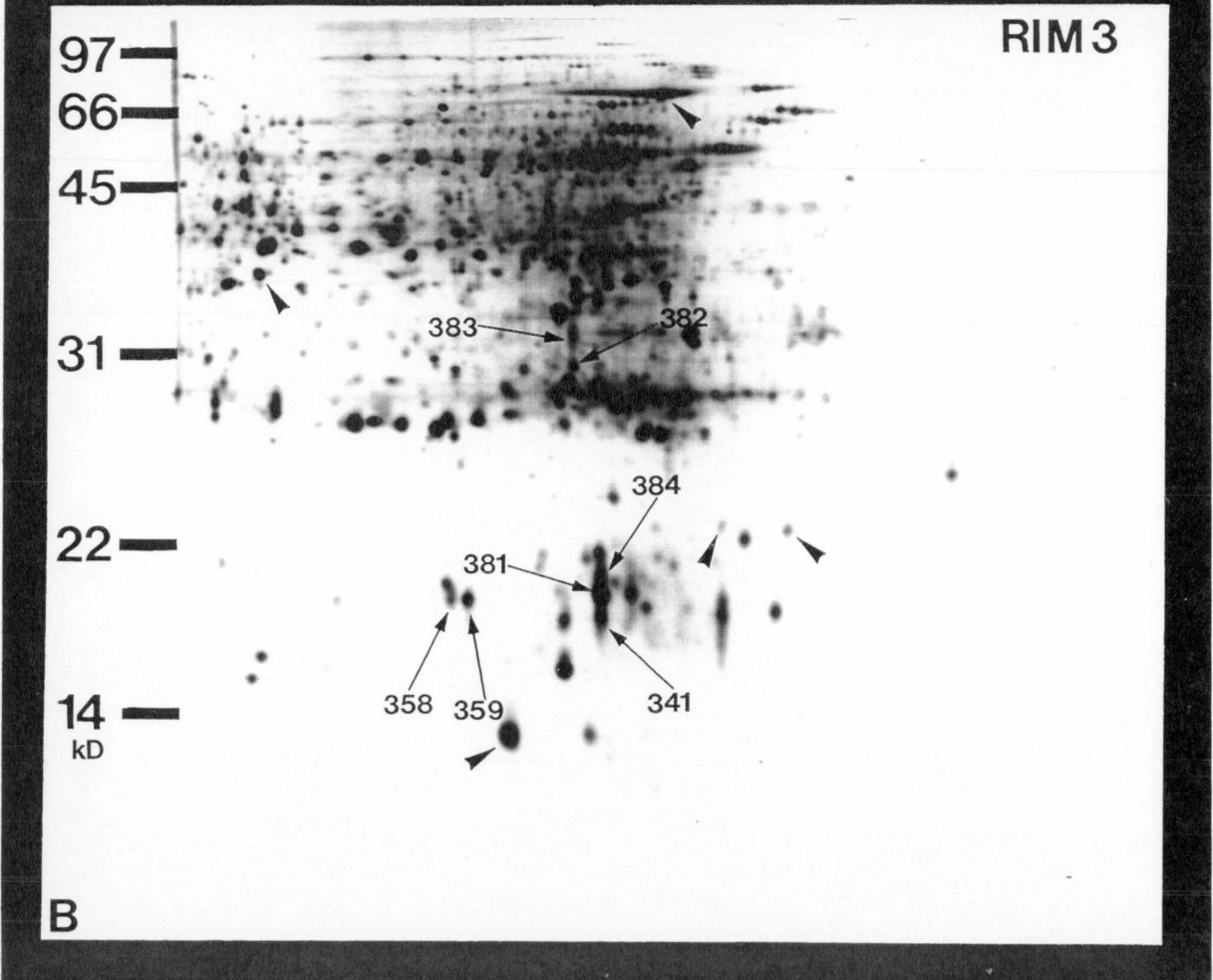
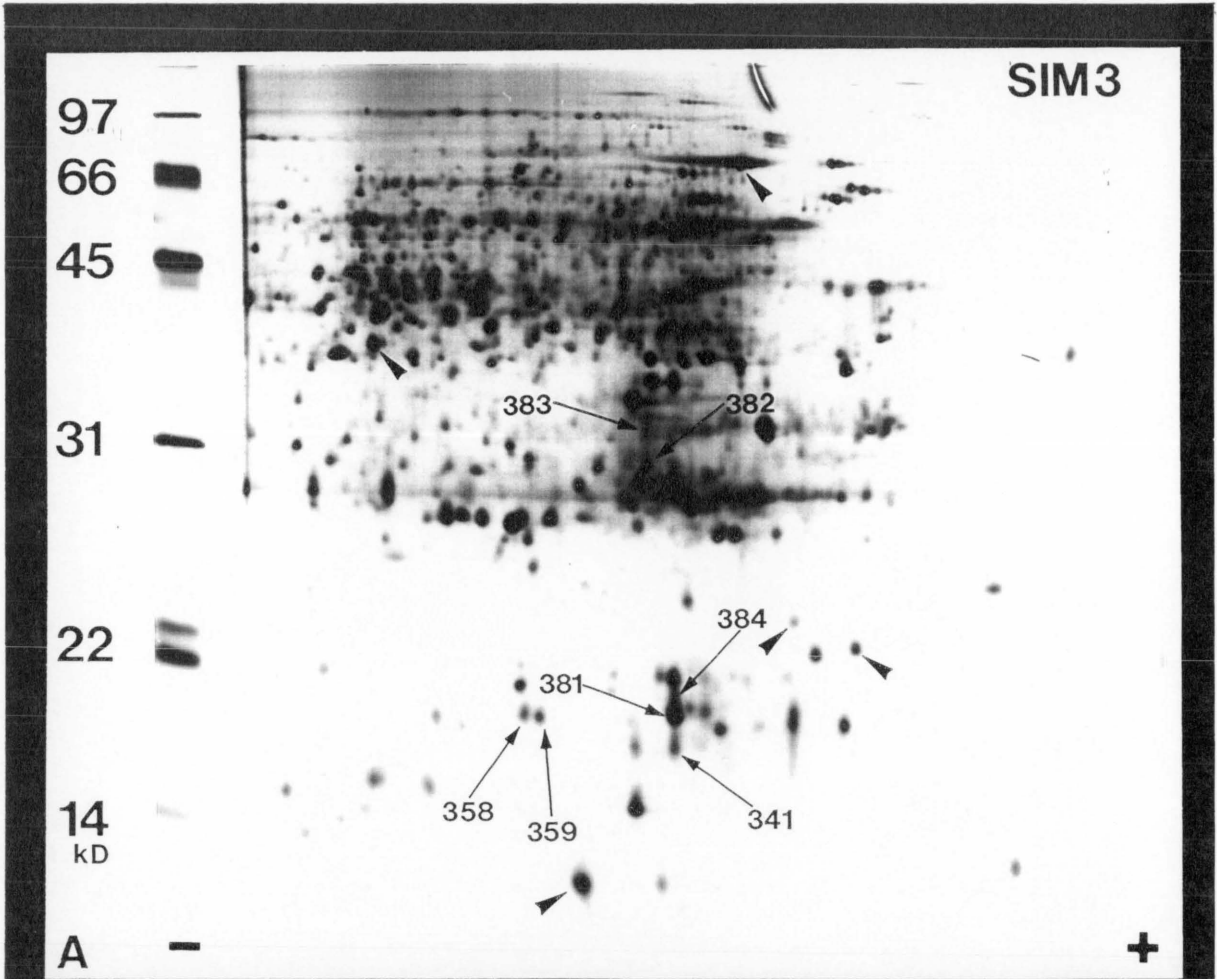


PLATE 21: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 7 days on SIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

(B) explants cultured for 7 days on RIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

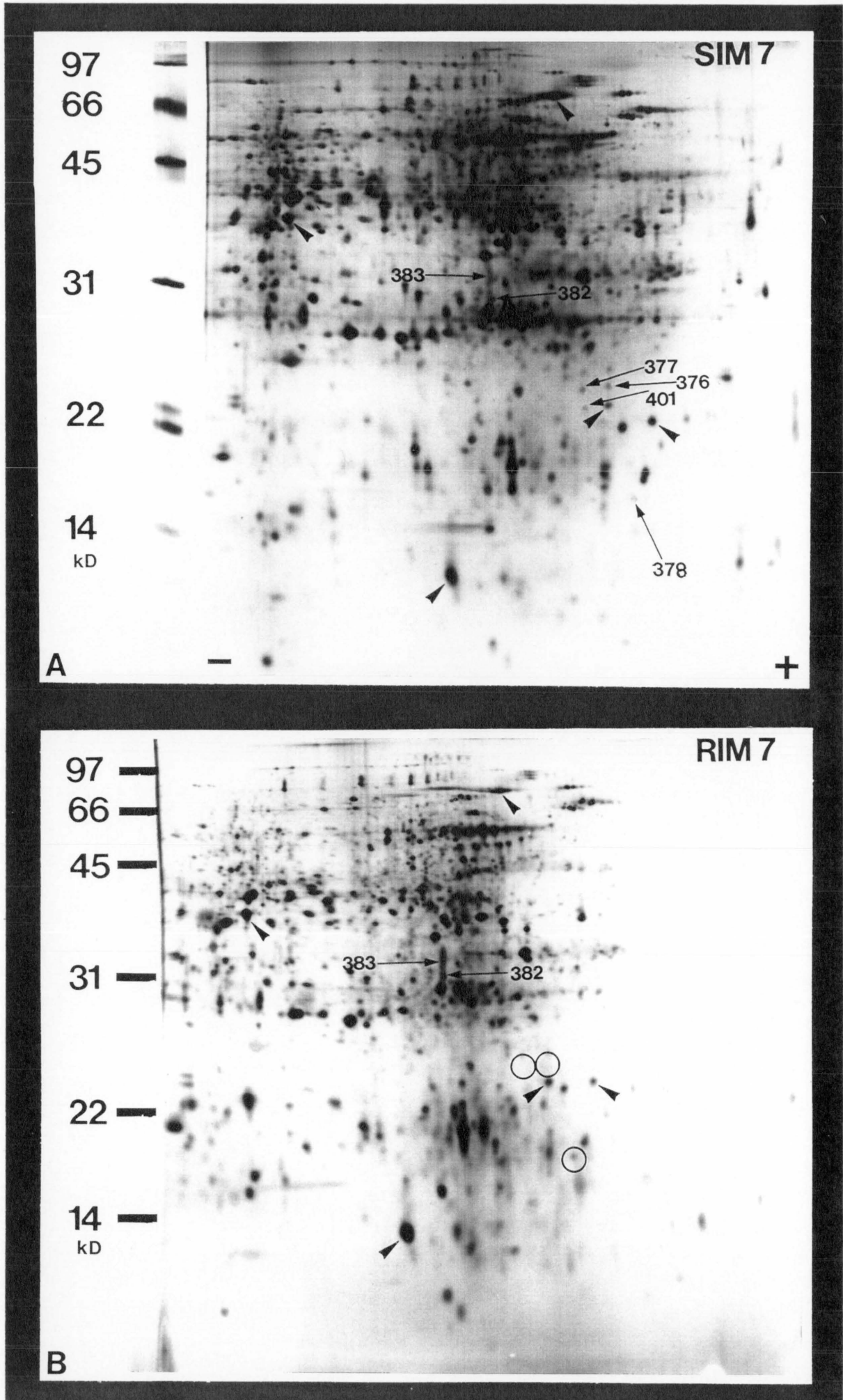


PLATE 22: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 14 days on SIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

(B) explants cultured for 14 days on RIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

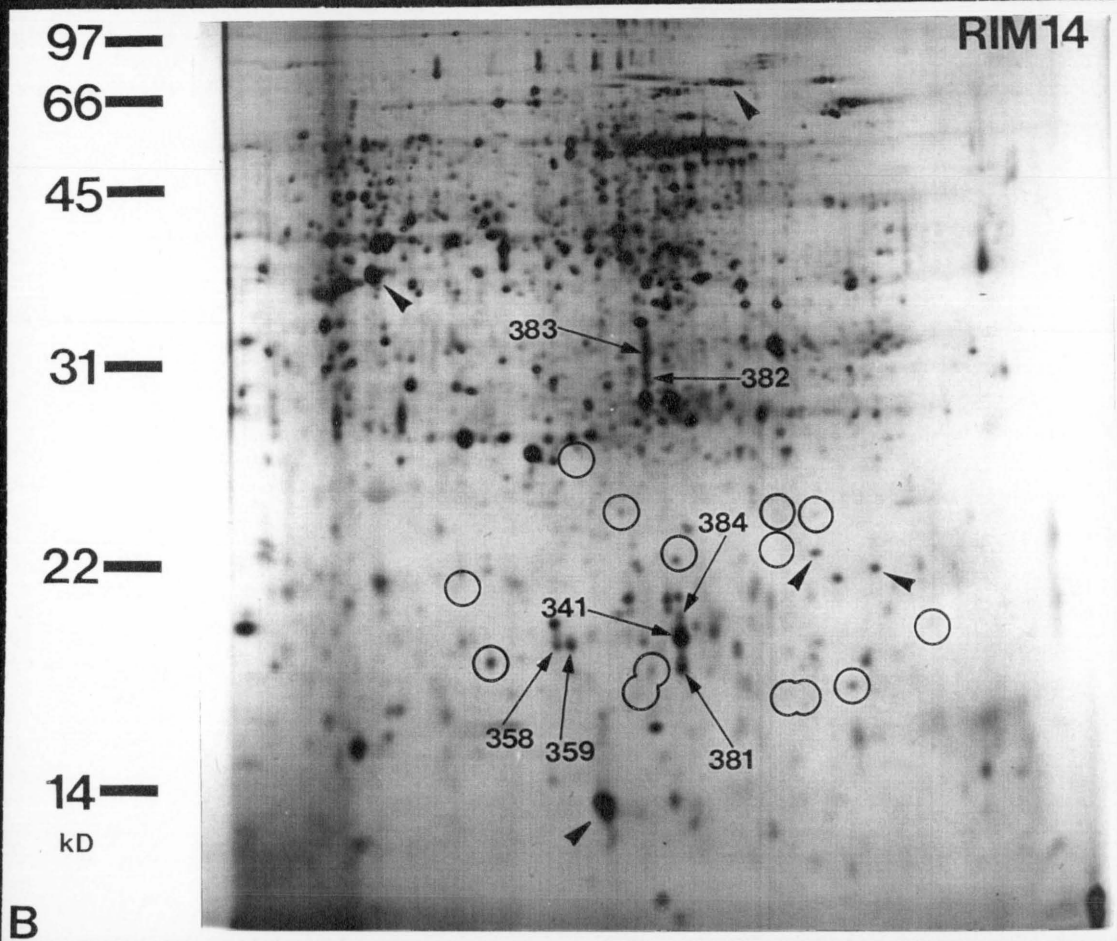
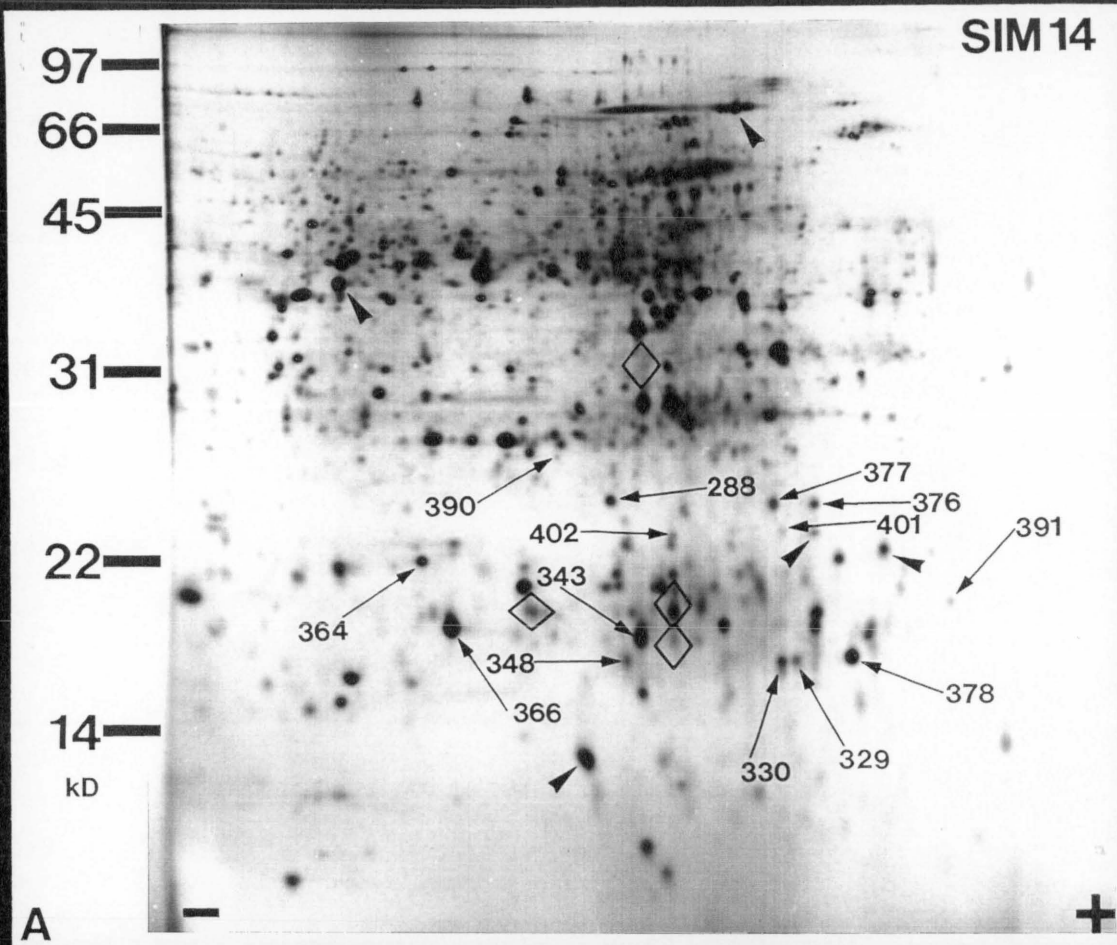


PLATE 23: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 21 days on SIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

(B) explants cultured for 21 days on RIM. Numbered arrows indicate polypeptides not detected at day 0, or those which increased or decreased in abundance.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

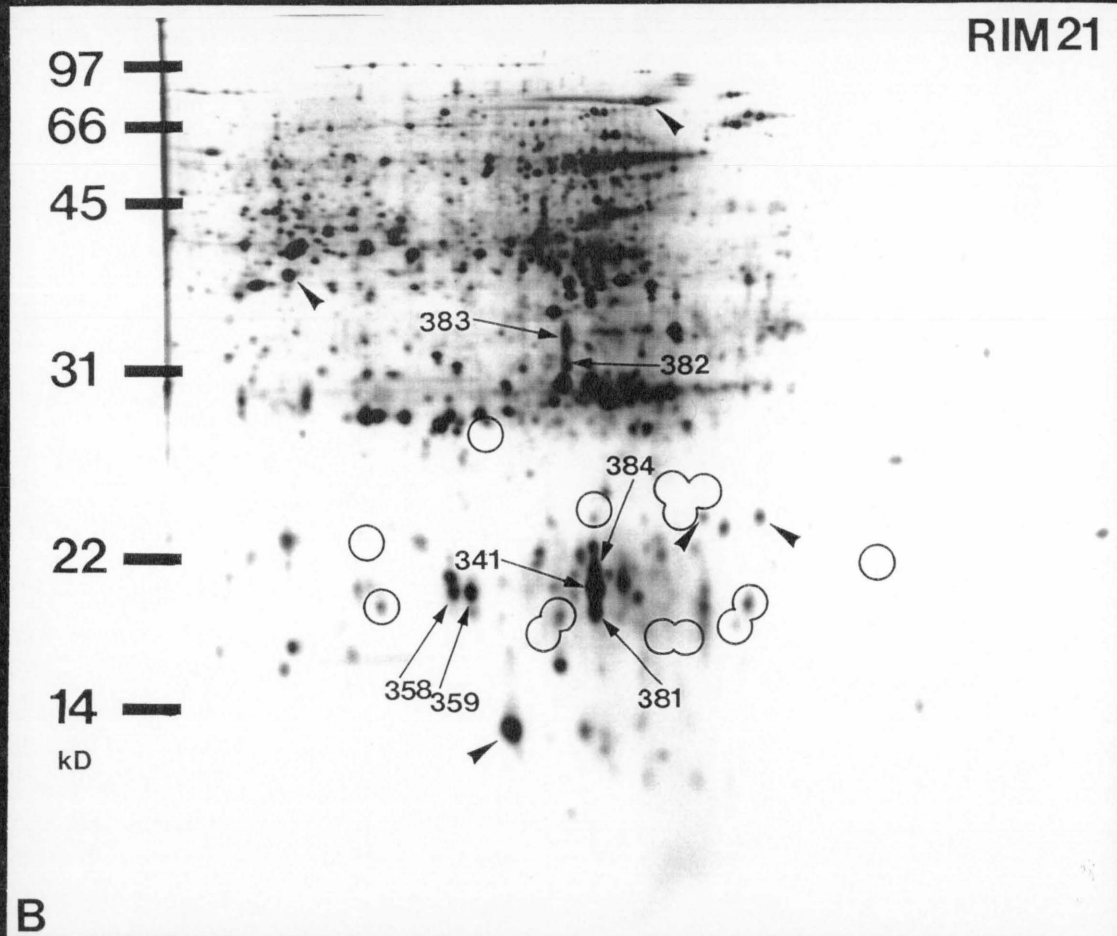
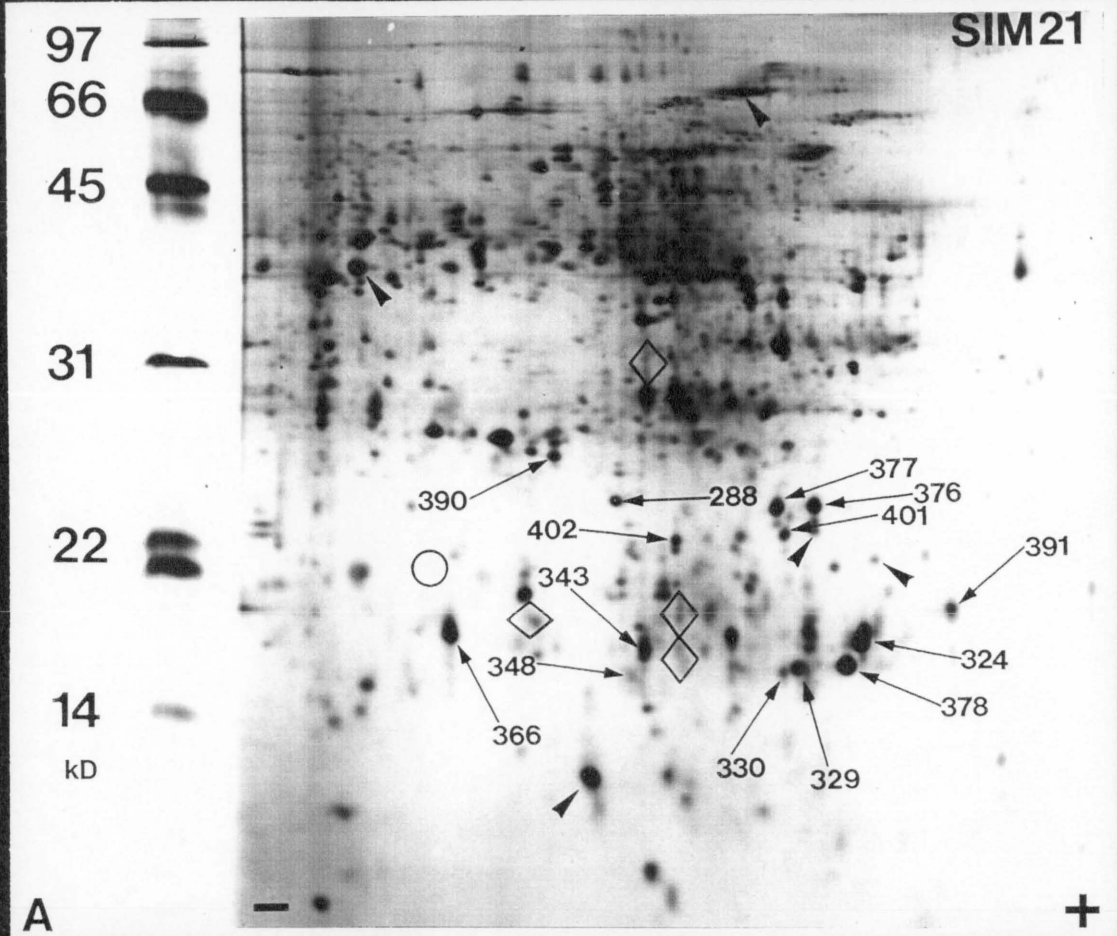


PLATE 24: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 42 days on SIM. Numbered arrows indicate polypeptides associated with caulogenesis.

(B) explants cultured for 42 days on RIM. Numbered arrows indicate polypeptides associated with caulogenesis or rhizogenesis (450).

Arrow heads indicate reference polypeptides for comparison of gels.

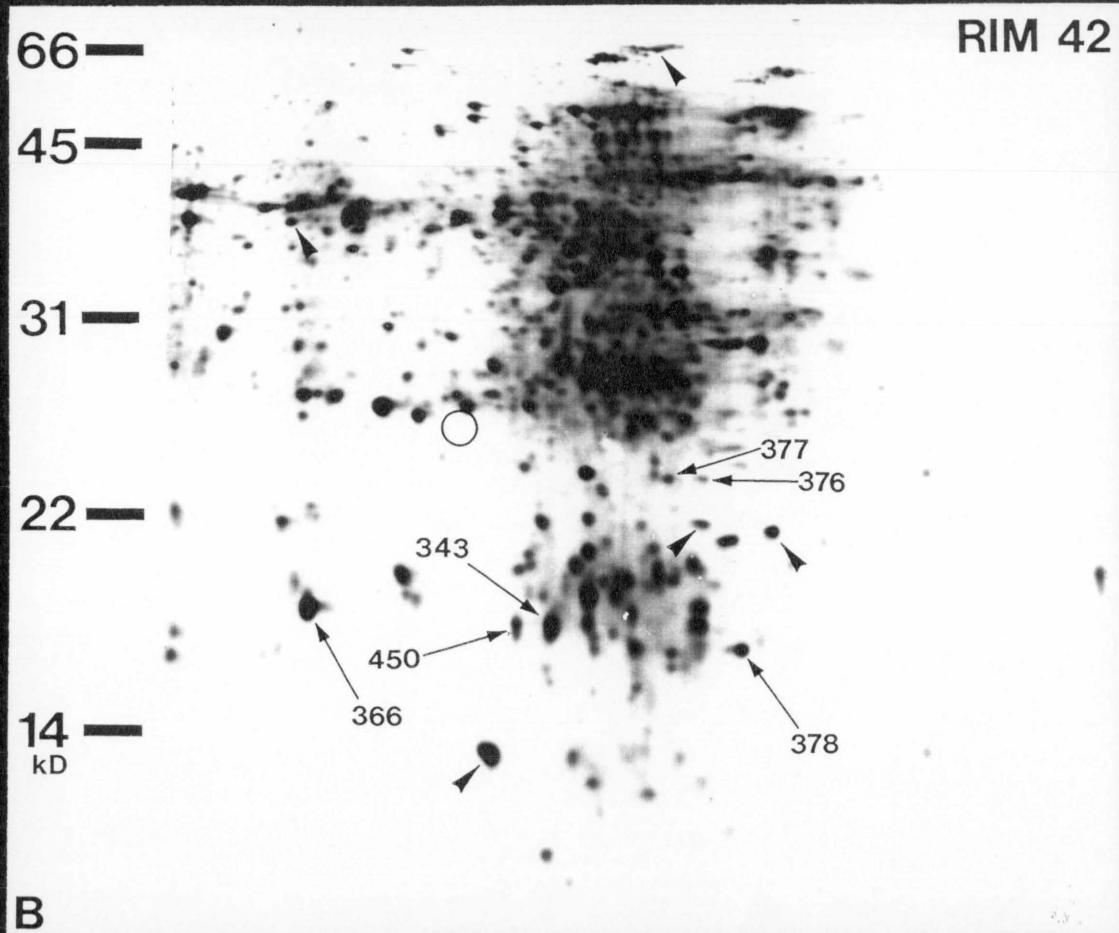
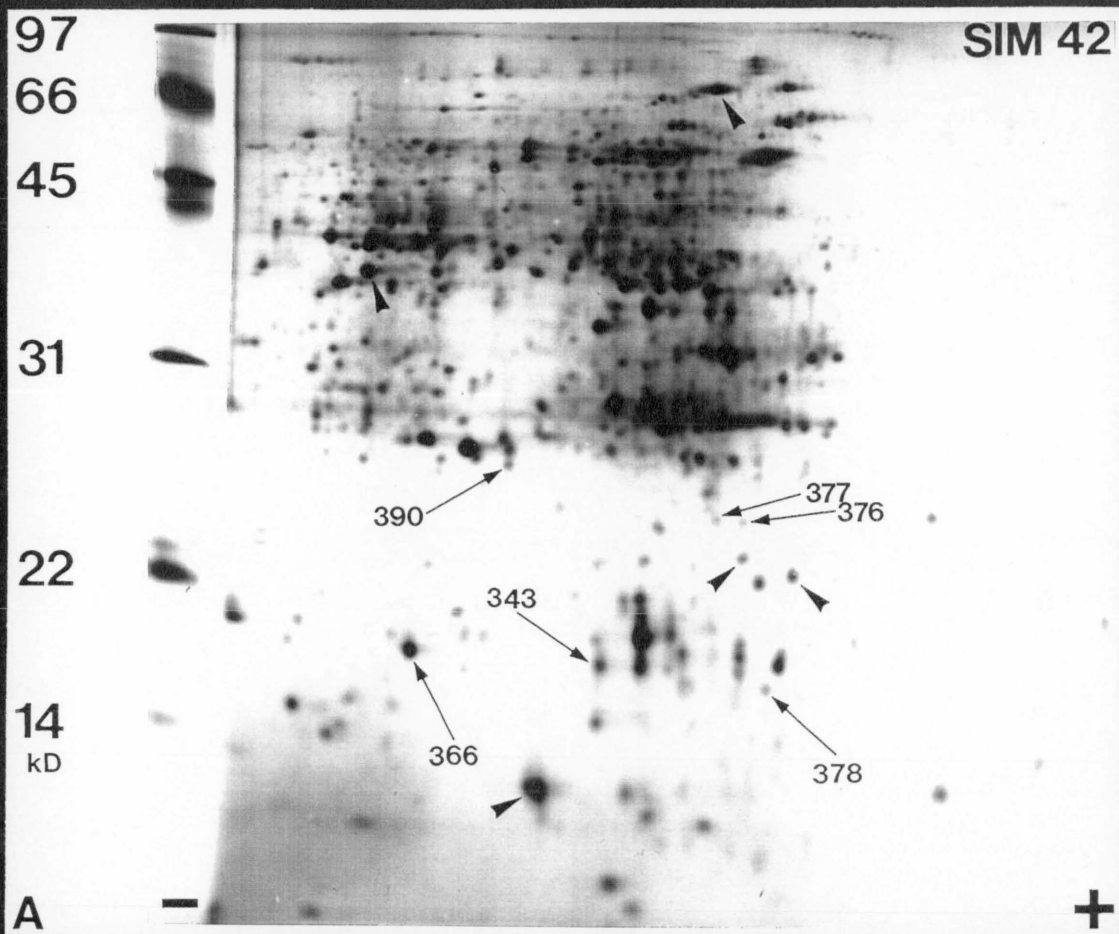


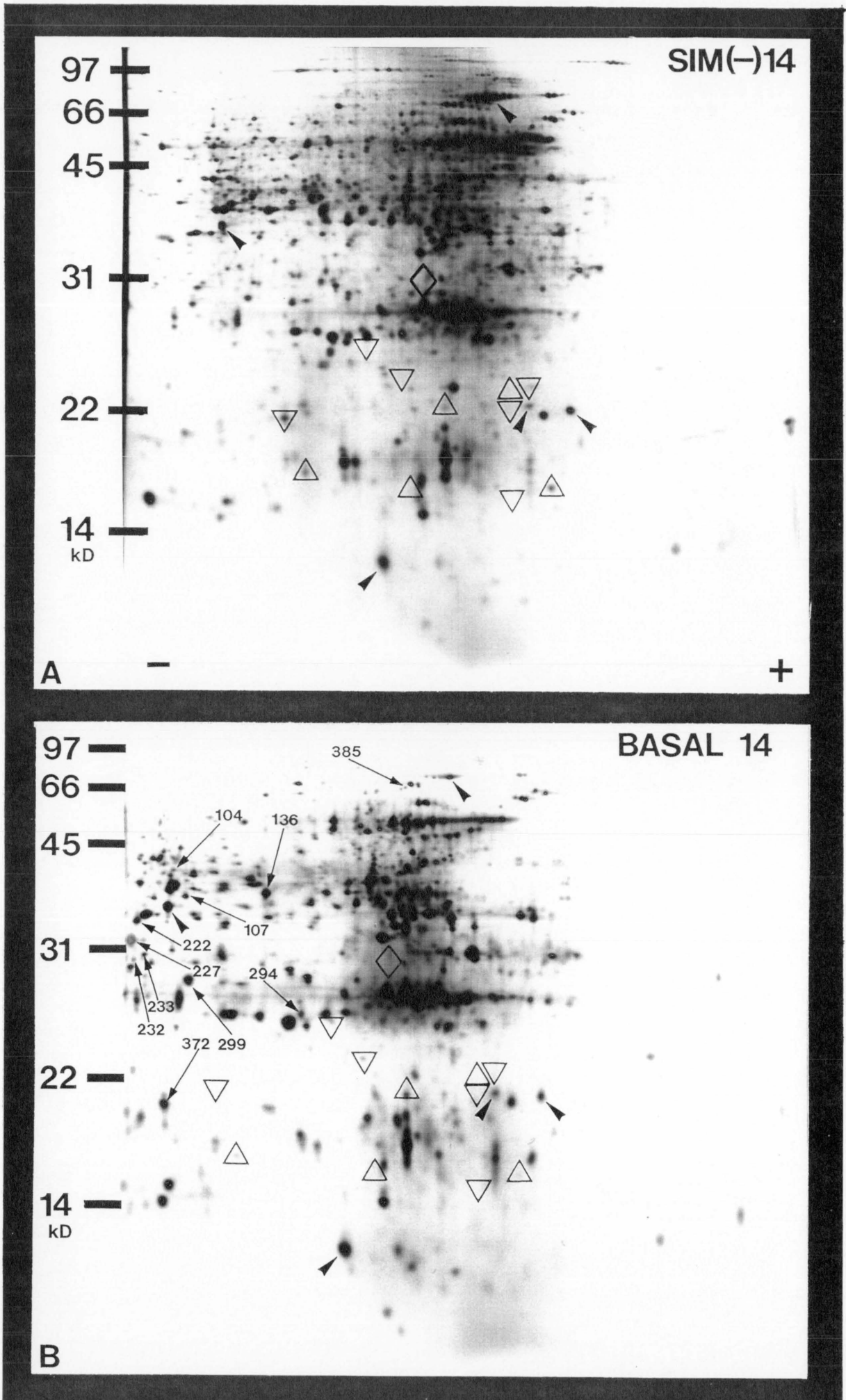
PLATE 25: Silver-stained two-dimensional PAGE gel of protein extracted from

(A) explants cultured for 14 days on SIM(-).

(B) explants cultured for 14 days on BM. Numbered arrows indicate polypeptides not detected at day 0, which appeared with culture on all media

Open symbols indicate the absence/or presence at reduced levels of polypeptides associated caulogenesis.

Arrow heads indicate reference polypeptides for comparison of gels.



KEY (**BOLD**) change or polypeptide first detected at this time.
(UNDERLINED) polypeptide undetectable.
(+) polypeptide increased in abundance.
(-) polypeptide decreased in abundance.
(outline) polypeptide shows a different response on SIM while retaining the earlier response on the other media (+) increases in abundance, (-) decreases in abundance or disappears.
(N/A) not applicable.
(N/AN) not analyzed.

TABLE [3] Summary of changes in the pattern of silver stained polypeptides observed with culture on SIM, RIM, CIM or BM for various lengths of time.

| Behavior | Day 3 | Day 7 | Day 14 | Day 21 | Total number of proteins in group |
|--|--|---|--|--|-----------------------------------|
| (1) Basic metabolism | N/A | N/A | N/A | N/A | 415 |
| (2) Petiole physiology. Decline or <u>disappear</u> on all culture media | 40, 42, <u>80</u> , 84, 85, 86, 100, 102, 130, 133, 134, 165, 194, <u>277</u> , <u>278, 281</u> , 283, <u>300</u> , 318, 349 | N/AN | 40, 42, <u>80</u> , 84, 85, 86, 100, 102, 130, 133, 134, 165, 194, <u>277</u> , <u>278, 281</u> , 283, <u>300</u> , 318, 349 | N/AN | 20 |
| (3) Culture induced (Polypeptides not detected at day 0, but induced upon culture on all media). <i>(*Polypeptides present at day 0 which increased upon culture)</i> | 104, 222, 372, 385 | 104, 222, 227, 232, 233, 294, 372, 385 <i>107, 136, 299</i> | 104, 222, 227, 232, 233, 294, 372, 385 <i>107, 136, 299</i> | 104,222, 227, 232, 233, 294, 372, 385 <i>107, 136, 299</i> | 8 (3)* |
| (4) Changes to polypeptides detected at day 0, not induced by basal medium | | | | | |
| A- SIM, RIM & CIM | 266-, 267-, | 266-, 267-, | 266-, 267-, | 266-, 267-, | 2 |
| B- SIM only | | | 288+, 366+, | 288+, 366+, 324+ | 3 |
| C- Permanent on RIM but transient on SIM | 358+, 359+, 343- | 358+, 359+,343- | 358-, 359- 343+ | 358-, 359- 343+ | 3 |
| D- CIM only | N/AN | N/AN | 223+ | N/AN | 1 |
| (5) Changes to polypeptides detected at day 0, induced permanently by RIM, CIM and BM, but transient on SIM. | 381+, 384+, 341+. | 381+, 384+, 341+. | 381-, 384-, 341- | 381-, 384-, 341- | 3 |
| (6) Polypeptides not detected at day 0, and not induced by basal medium | | | | | |
| A- RIM, SIM & CIM | | 108, | 108, 388, 389 | 108, 388, 389 | 3 |
| B- SIM only | | 376, 377, 378, 401 | 329, 330, 348, 364, 376+, 377+, 378+, 390, 391,401, 402 | 329+, 330+, 348, 376+, 377+, 378+, 390+, 391+, 401+, 402+ | 11 |
| C- Permanent on RIM and CIM but transient on SIM | 382, 383 | 382+, 383+ | 382-, 383- | 382-, 383- | 2 |

10.6 2-Dimensional analysis of *in vivo* protein synthesis associated with culture

When tissue was labelled *in vivo* with [³⁵S]-methionine, the general pattern of the resulting fluorographs was very similar to the silver-stained gels (PLATES 29A & 29B), although the number of polypeptides resolved was somewhat less. Analysis of [³⁵S]-methionine labelled polypeptides revealed more than 450 spots, of which 351 separated consistently. A base pattern of 334 polypeptides consistently detected in day 0 petiole tissue was established and the effects of culture analyzed as per silver staining.

Despite equal amounts of radioactivity being loaded onto the first dimension IEF gels and X-ray film being pre-flashed to give a linear response during fluorography, resolution between different treatments and in particular between high and low molecular weight polypeptides on the same gel, varied somewhat. Middle and high molecular weight polypeptides resolved best after 5 to 7 days fluorography, with little background observed, but with this length of exposure many low molecular weight polypeptides were detected very faintly. Low molecular weight polypeptides, particularly on day zero gels, resolved best after 14 days exposure, but with this length of exposure the higher molecular weight polypeptides became obscured by high background. For this reason each gel was fluorographed twice, for 5 or 7 days depending on the treatment and for 14 or 16 days. Only low molecular weight differences that showed the same trend with both exposure times were recorded.

In all 29 *in vivo* labelled polypeptides were identified that were either newly synthesized with culture, or had greatly altered levels of synthesis compared to day 0 (TABLE 4). 18 of these polypeptides were identical to those identified in silver stained gels, while the remainder appeared only after *in vivo* labelling (TABLE 4). A distribution pattern similar to that observed in the silver stained gels emerged with polypeptides found over almost the whole molecular weight and charge range.

In general, individual polypeptides detected by both silver-staining and *in vivo* labelling could be classified the same (TABLE 4). Only one polypeptide, 343, fell into a different sub-group, with *in vivo* labelling, moving from group 4C to 4A. No polypeptides falling into group 4, sub-group A, were detected, but a further sub-group, Rim only, was required to accommodate polypeptides present at day 0 that had increased levels of synthesis on RIM, as no such polypeptides were detected by silver staining. It was also necessary to add 2 new sub-groups to group 6. SIM+ and RIM+. Polypeptides that were synthesized at low levels on RIM but at high levels on SIM were termed SIM+, with those synthesized at high levels on RIM termed RIM+.

Two polypeptides, 376 and 377, while being labelled SIM only with silver staining were classified as SIM plus with *in vivo* labelling as they were synthesized at low levels with culture on RIM (PLATE 29B). Both polypeptides had greatly increased levels of synthesis with continued culture on SIM (PLATES 28A, 29A & 30A), but showed no increased synthesis with culture on RIM (PLATES 29B & 30B). Two other polypeptides, 299 and 227, both had elevated levels of synthesis with culture on all media, but the level of synthesis on RIM was much greater than observed on other media (PLATES 30B & 30A).

Eight polypeptide changes detected only by *in vivo* labelling, all fell into groups 4 and 6 and were associated with either shoot or root formation. In group 4, polypeptide, A, increased greatly with culture on RIM. This polypeptide showed increased synthesis after 7 days in culture with levels progressively increasing over the time course of the experiments (PLATES 28B, 29B & 30B).

In group six, a single polypeptide, F, was synthesized after 14 days culture under shoot forming conditions only (PLATE 29A). All the other polypeptides detected by *in vivo* labelling were synthesized on both SIM and RIM, but at greatly different levels.

Unlike with silver staining where no polypeptide changes associated with early culture on RIM were observed, *in vivo* labeling detected 2 polypeptides, J and P, with enhanced synthesis on RIM at day 3 of culture (PLATE 27B). One polypeptide, N, showed a greater level of synthesis on RIM at day 5 (PLATE 27B, 28B & 30B). This polypeptide was first detected at the point where explants no longer required exposure to RIM to go on and form roots and increased in its level of synthesis as more roots were determined. Polypeptides J and P showed increased synthesis with culture on RIM after only 3 days, but there was no further increase with prolonged culture (PLATE 30B).

Three polypeptides first detected after 3 days in culture showed differential levels of synthesis on SIM or RIM after 14 days. Polypeptide G increased greatly with culture on SIM, while polypeptides H and R increased with culture on RIM (PLATES 29A & 29B).

No polypeptides unique to CIM were detected, although polypeptide 379 had a higher level of synthesis on CIM than on other media (PLATE 26B).

Five, of the 8 group 3, polypeptides induced by culture and detected by silver staining were identified by *in vivo* labeling (PLATE 31A). These polypeptides were not synthesized at day zero and appeared with culture on all media.

All the other changes associated with organogenesis paralleled those observed with silver staining, such as reduced synthesis of several 55kd polypeptides. Culture on SIM(-) failed to induce the synthesis of polypeptides associated with shoot formation, but polypeptide 383 was synthesized at a reasonable level (PLATE 31B).

PLATE 26: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) petiole tissue from *in vitro* plants, i.e. DAY 0. Arrows indicate polypeptides which showed reduced synthesis with culture on all media.

(B) explants cultured for 14 days on callus inducing medium (CIM). Polypeptide 108 was synthesized with with culture on SIM, RIM or CIM respectively. Polypeptide 379 showed increased synthesis with culture on CIM only.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling. Arrow heads indicate reference polypeptides for comparison of gels.

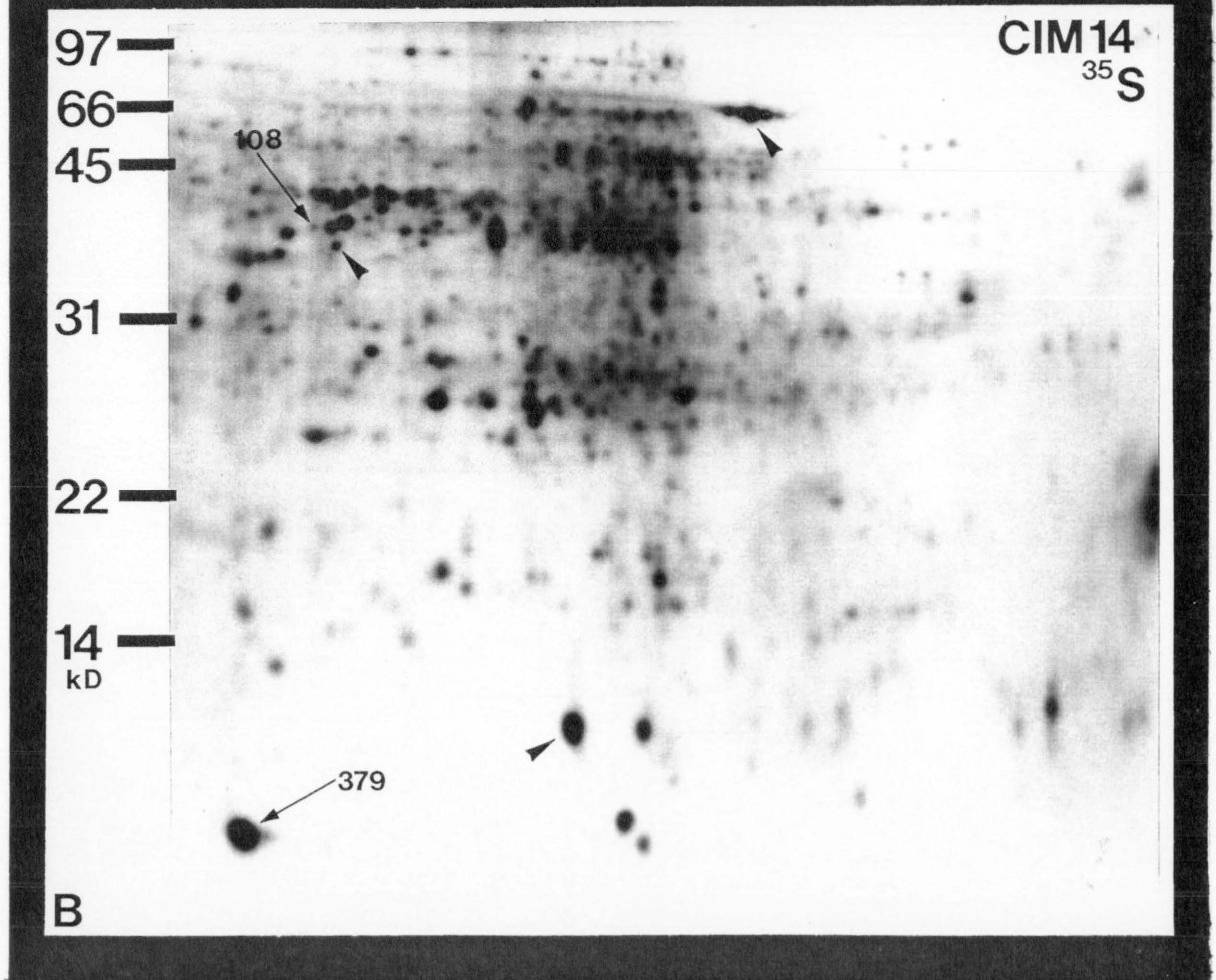
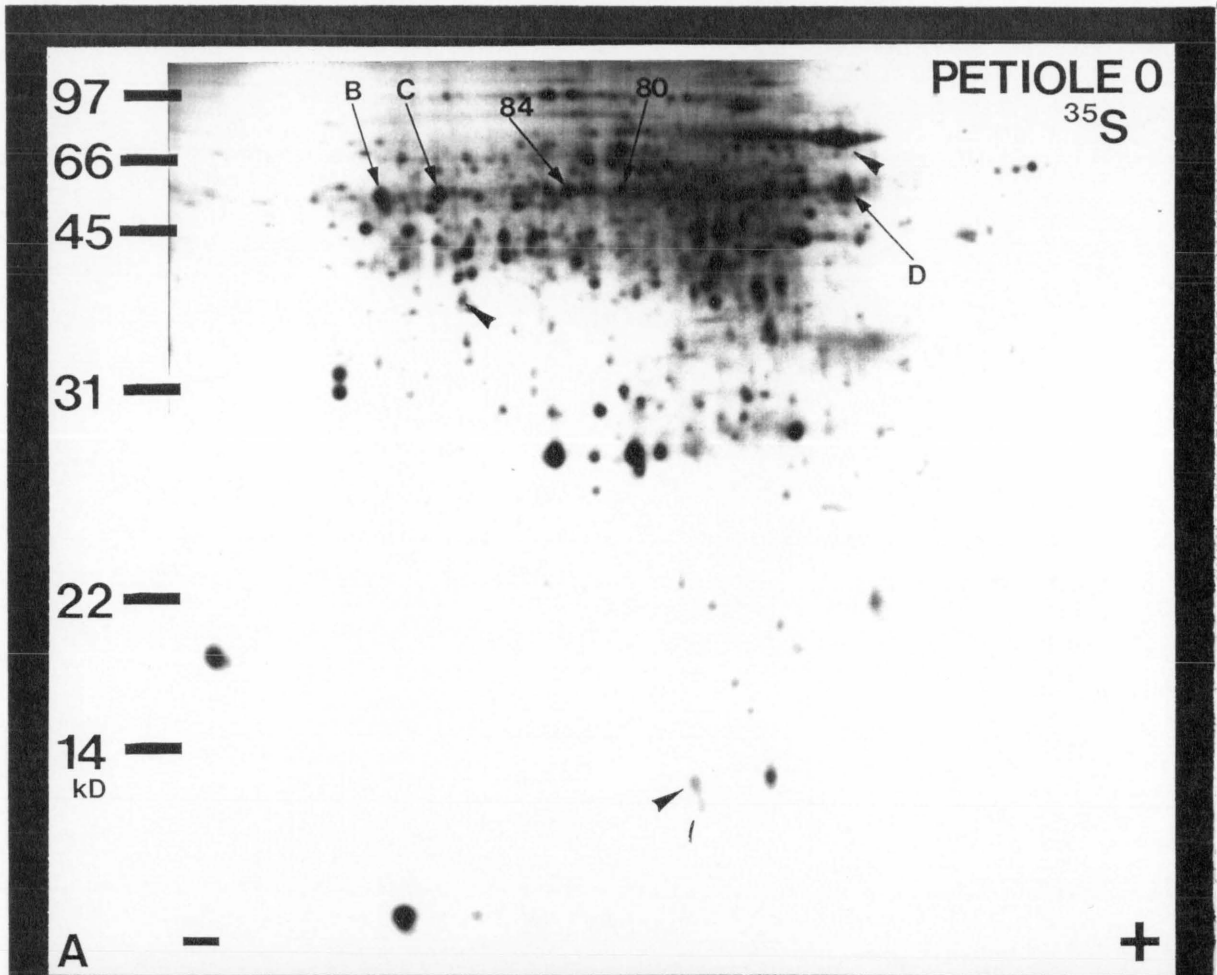


PLATE 27: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 3 days on SIM. Arrow indicates a polypeptide not synthesized at day 0

(B) explants cultured for 3 days on RIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

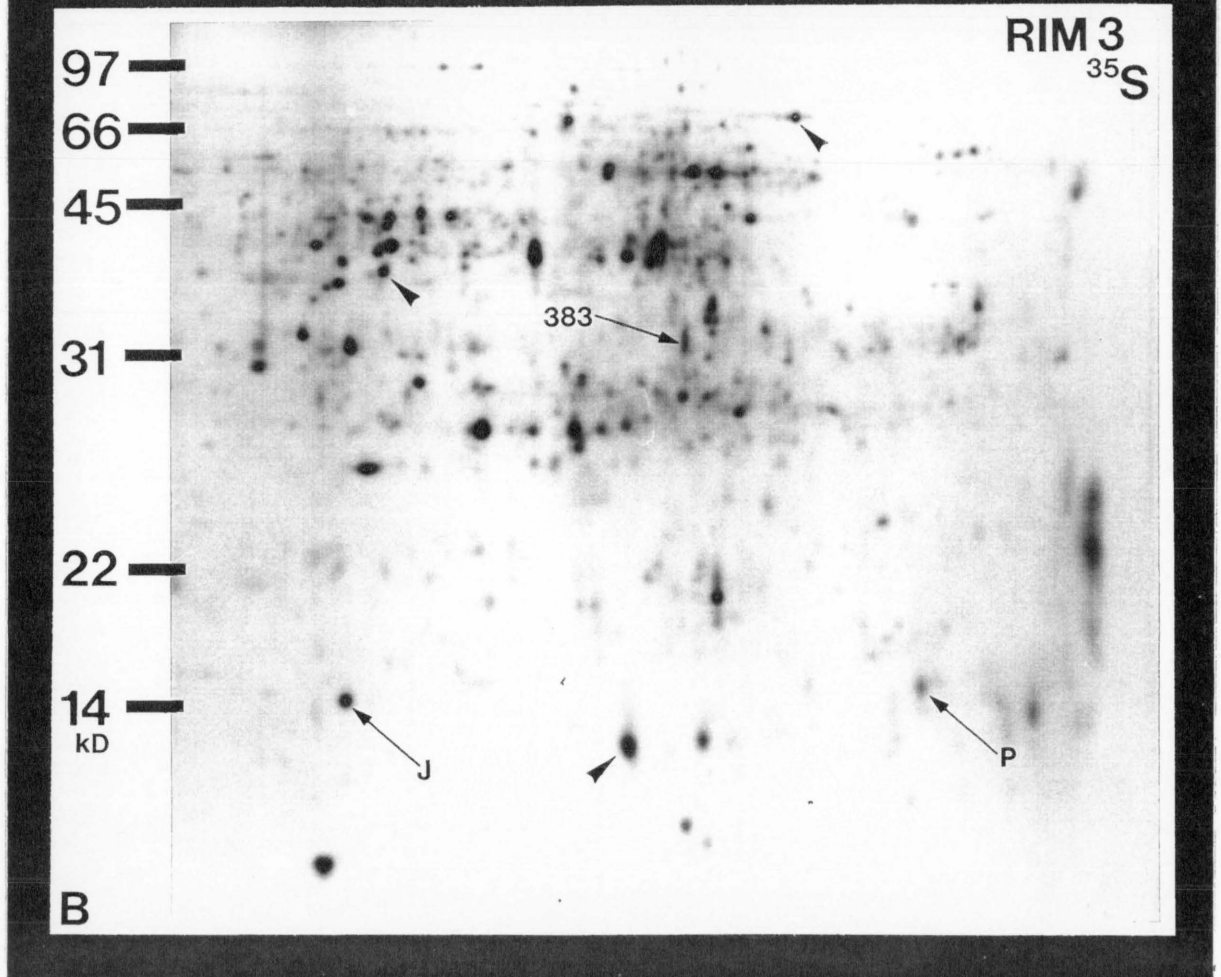
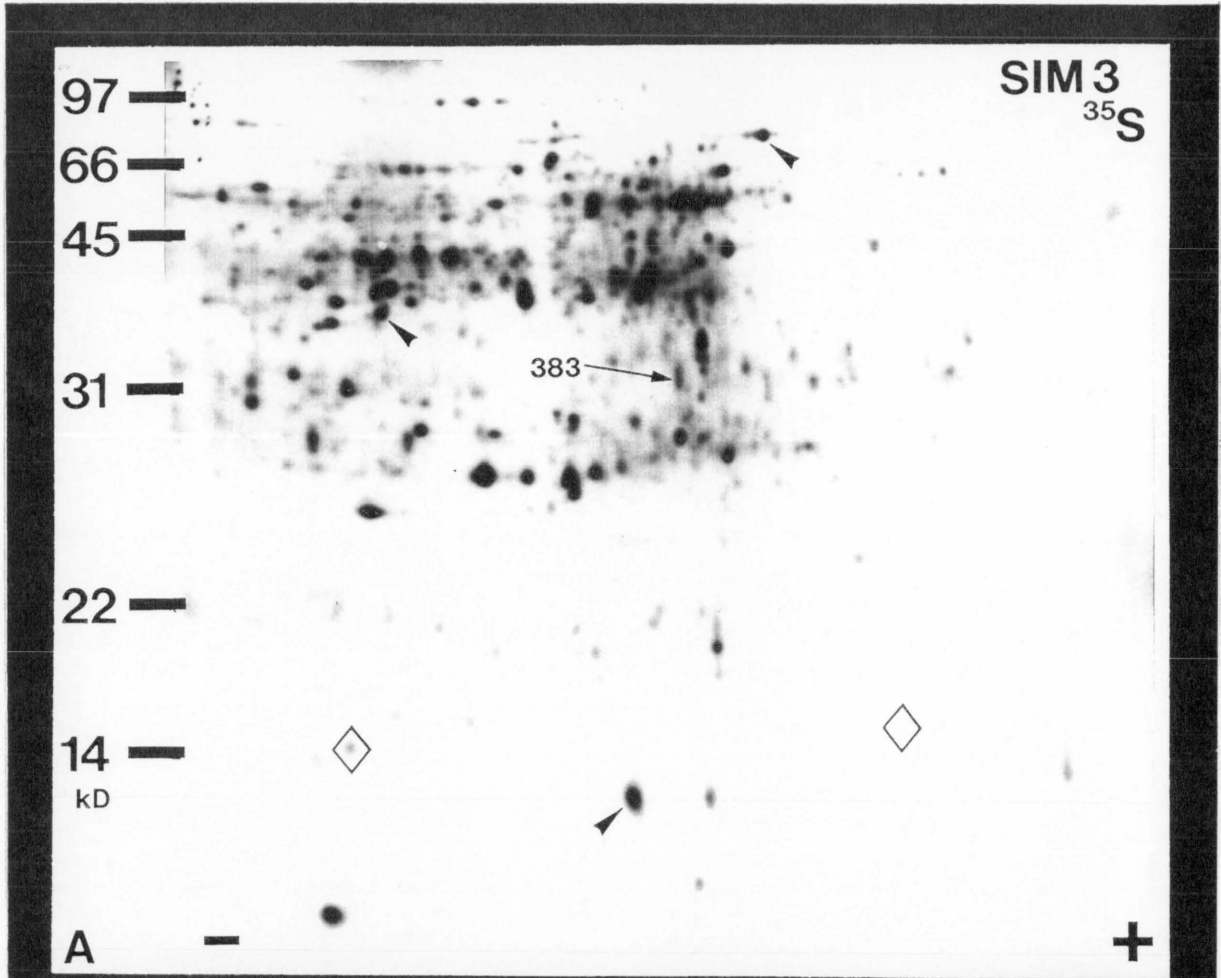


PLATE 28: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 7 days on SIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

(B) explants cultured for 7 days on RIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

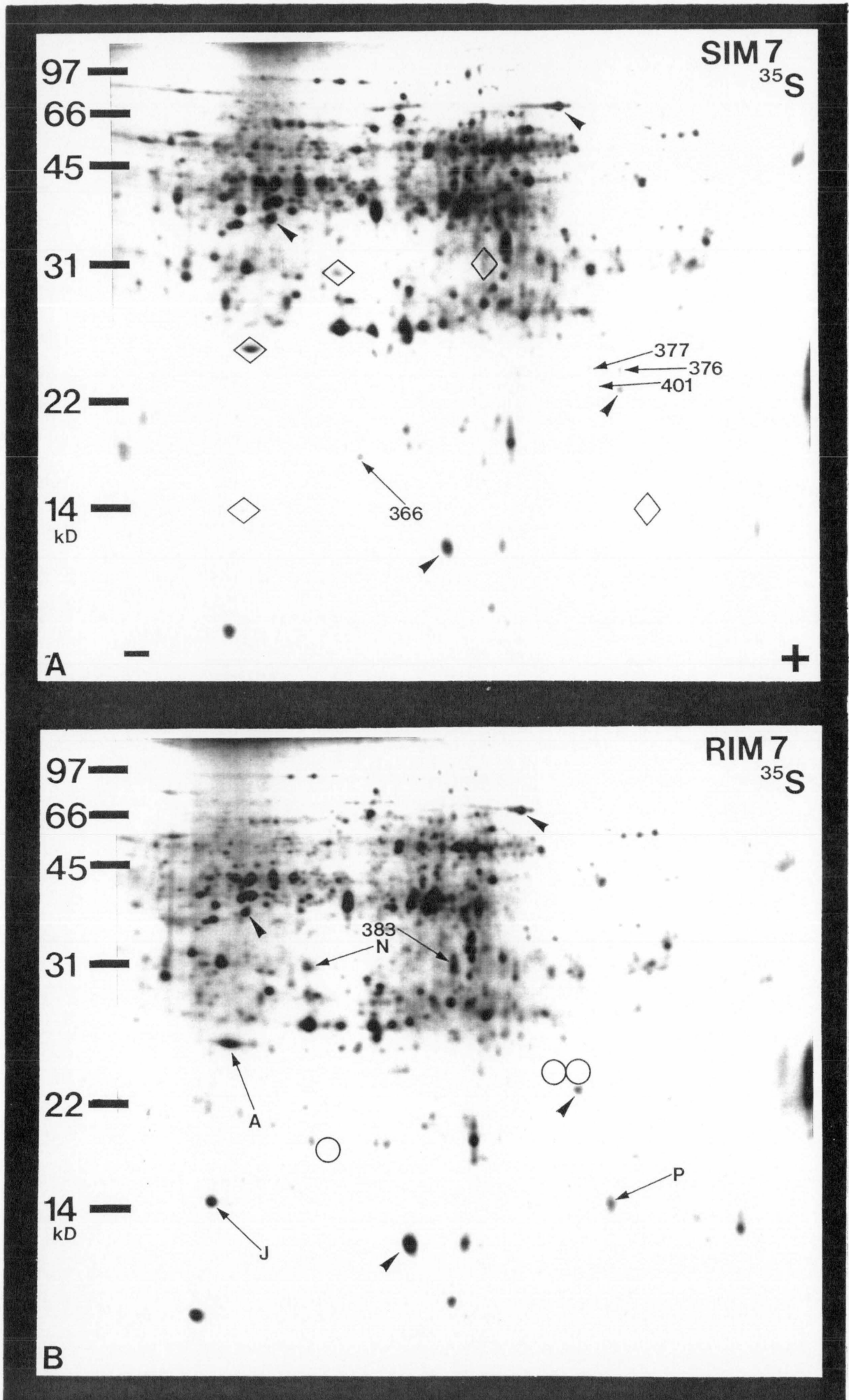


PLATE 29: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 14 days on SIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

(B) explants cultured for 14 days on RIM. Arrows indicate polypeptides not detected at day 0, or those with increased or decreased levels of synthesis.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

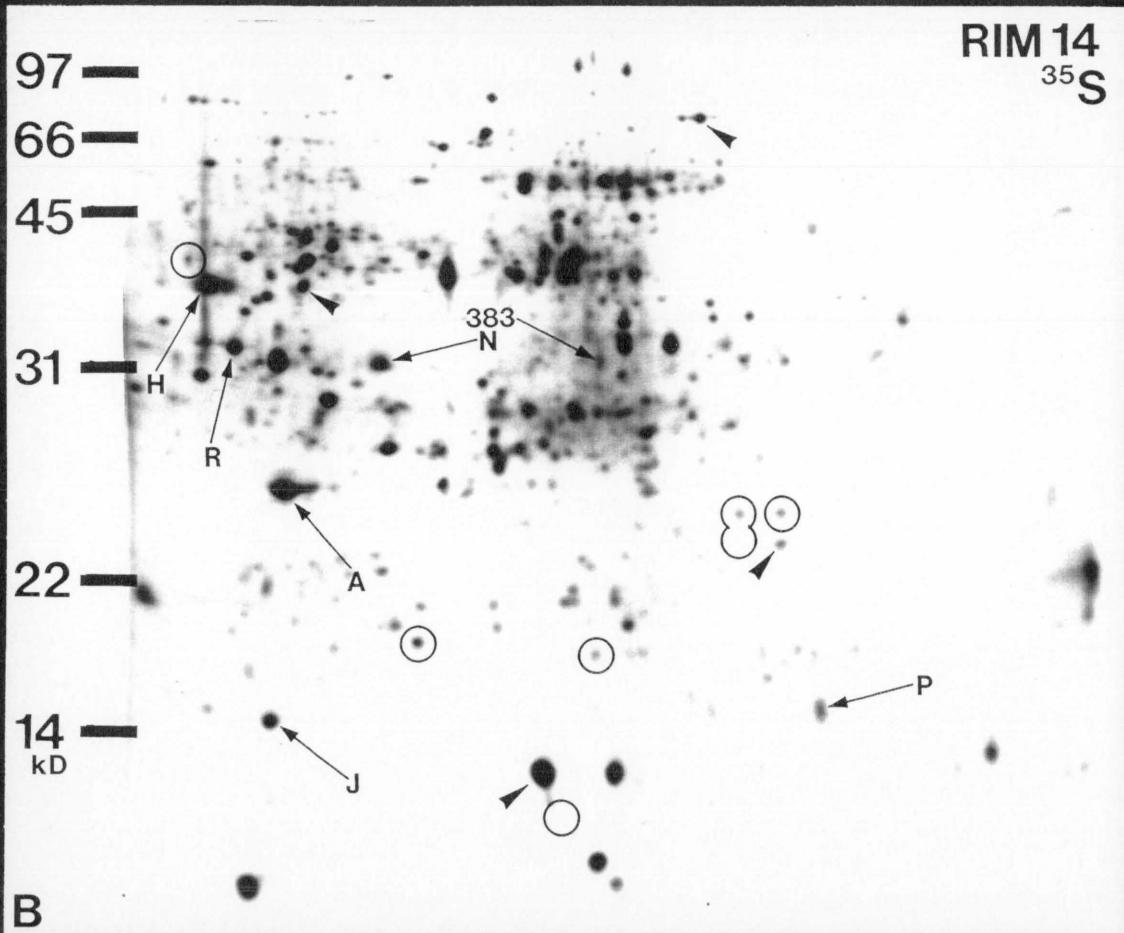
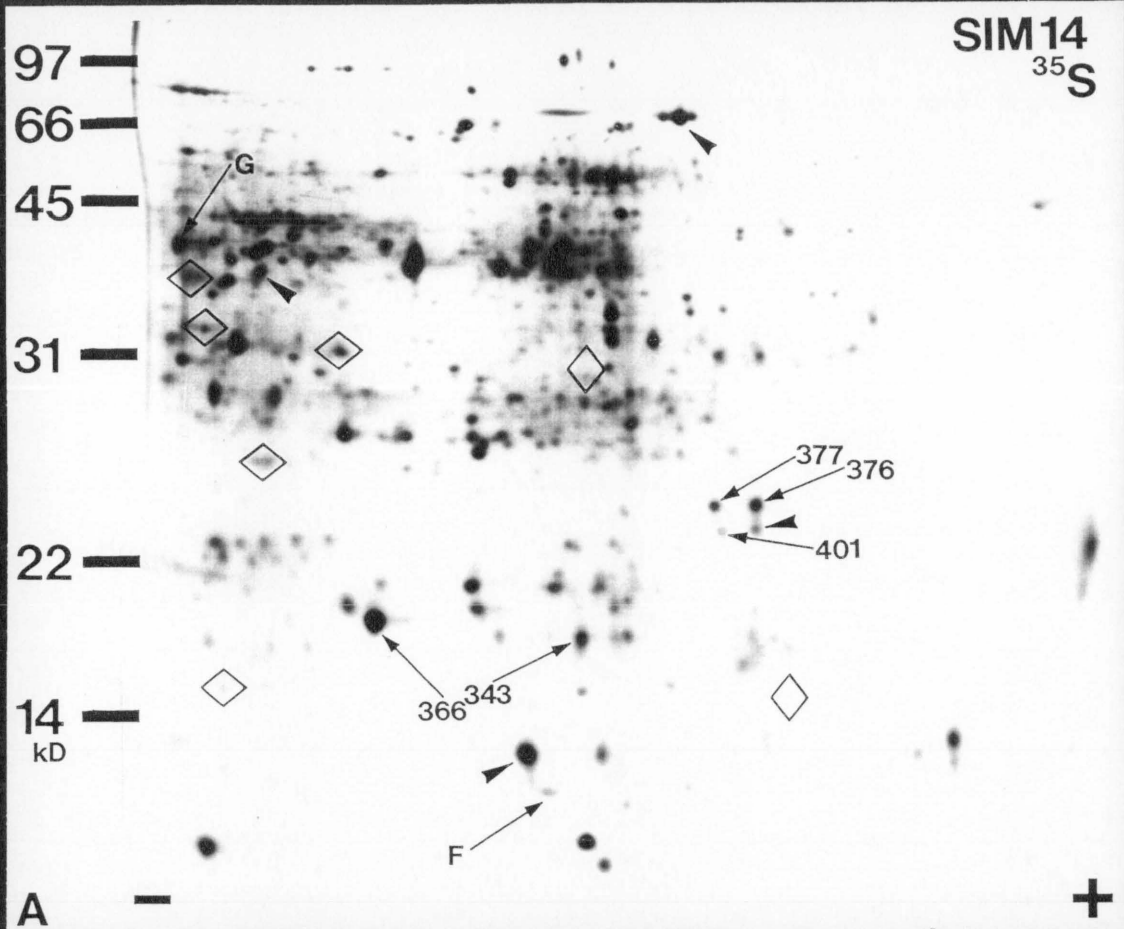


PLATE 30: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 21 days on SIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

(B) explants cultured for 21 days on RIM. Arrows indicate polypeptides not synthesized at day 0, or those with increased or decreased levels of synthesis.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling.

Open symbols indicate the positions of polypeptides identified on the opposite gel.

Arrow heads indicate reference polypeptides for comparison of gels.

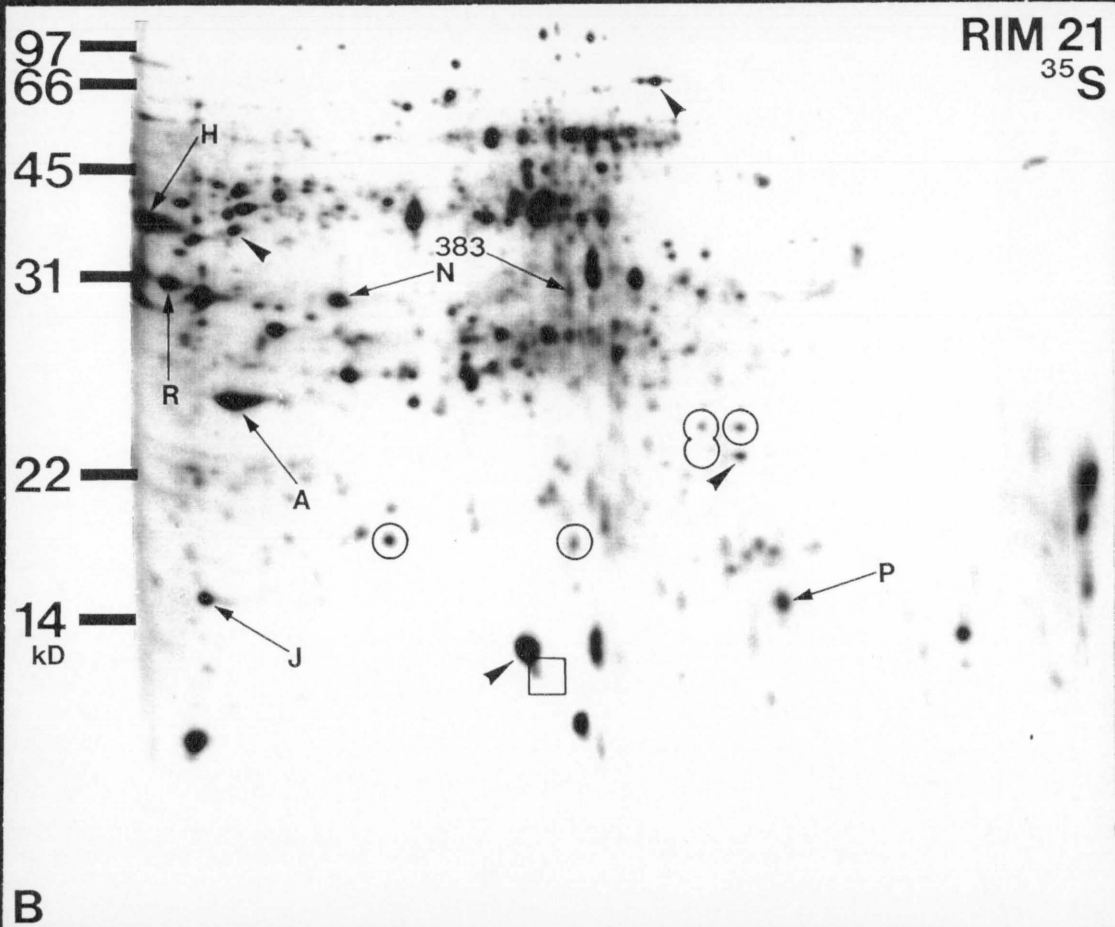
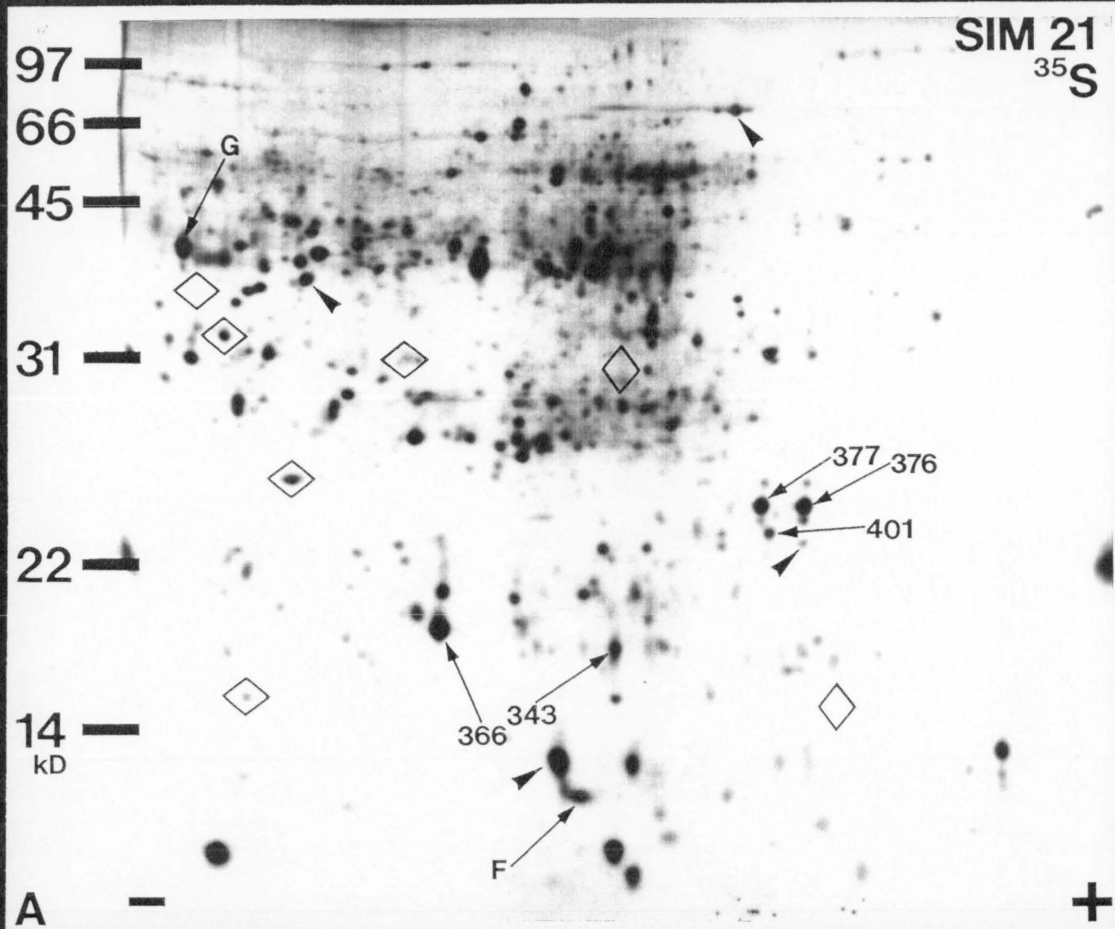


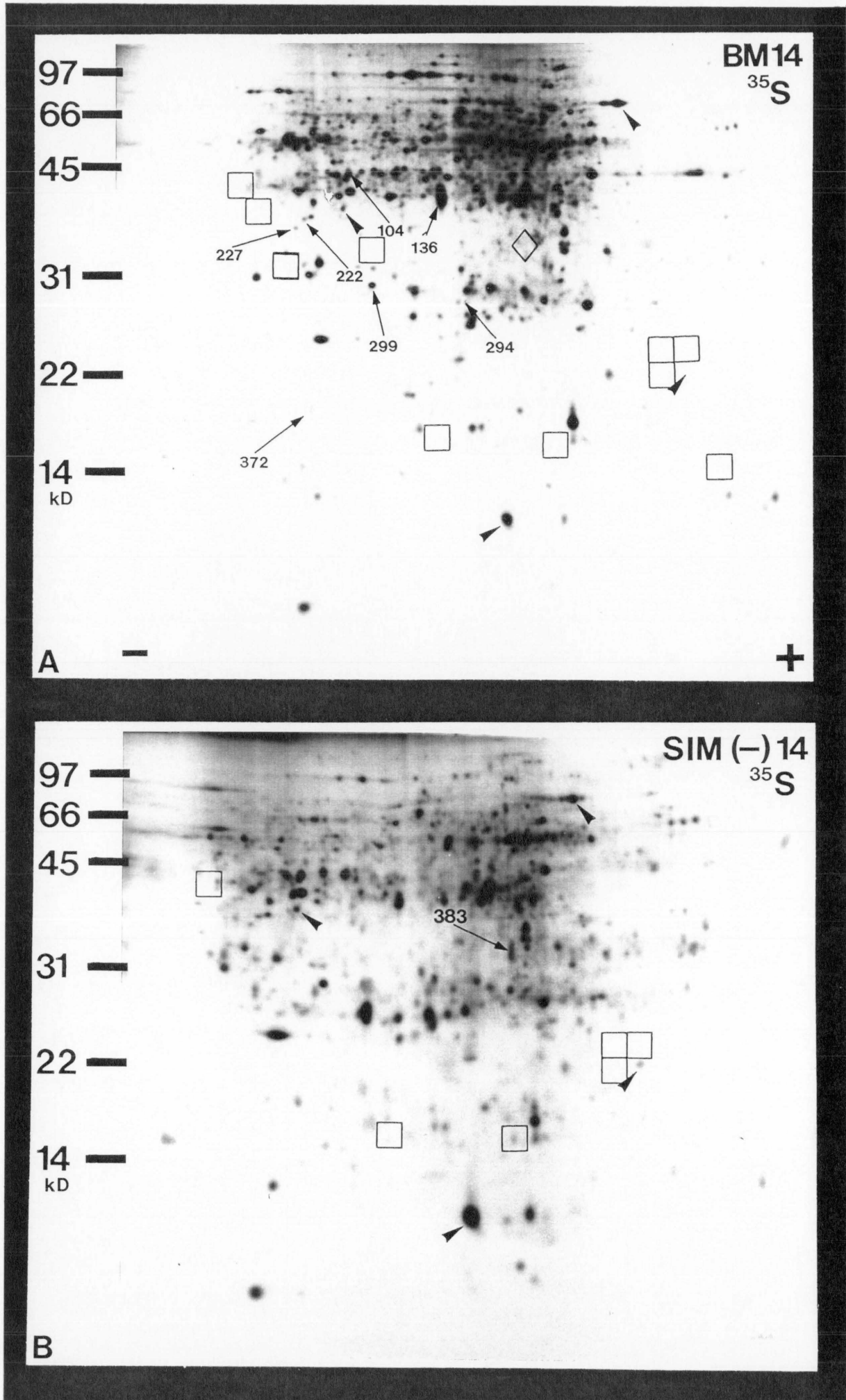
PLATE 31: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 14 days on BM. Arrows indicate polypeptides not synthesized at day 0, which were synthesized with culture on all media.

(B) explants cultured for 14 days on SIM(-).

Open symbols indicate the absence of polypeptides associated with organogenesis.

Arrow heads indicate reference polypeptides for comparison of gels.



KEY (**BOLD**) change or polypeptide first detected at this time.
(UNDERLINED) polypeptide undetectable.
(+) polypeptide had an increased level of synthesis.
(-) polypeptide had a decreased level of synthesis.
(outline) polypeptide shows a different response on SIM while retaining the earlier response on the other media (+) increased synthesis, (-) decrease synthesis or disappears.
(N/A) not applicable.
(N/AN) not analyzed.
(*) first detected on day 5 gels
(@) greater level with culture on RIM

TABLE [4] Summary of changes in the pattern of *in vivo* labelled polypeptides observed with culture on SIM, RIM, CIM or BM for various lengths of time.

| Behavior | Day 3 | Day 7 | Day 14 | Day 21 | Total number of proteins in group |
|---|-------|----------|---------------------------------------|------------------|-----------------------------------|
| (1) Basic metabolism | N/A | N/A | N/A | N/A | 322 |
| (2) Petiole physiology. Decline or <u>disappear</u> on all culture media | N/AN | N/AN | B, C, 80, 84, D | N/AN | 5 |
| (3) Culture induced (Polypeptides not synthesized at day 0, but induced upon culture on all media). (*Polypeptides present at day 0 which increased upon culture) | N/AN | N/AN | 104, 222, 294, 372, 227@ 136, 299@ | N/AN | 5 (2)* |
| (4) Changes to polypeptides synthesized at day 0, not induced by basal medium | | | | | |
| A- SIM only | | 366+ | 366+, 343+ | 366+, 343+ | 2 |
| B- RIM only | | A+ | A+ | A+ | 1 |
| D- CIM only | N/AN | N/AN | 379+ | N/AN | 1 |
| (5) Changes to polypeptides synthesized at day 0, induced permanently by RIM CIM and BM, but transient on SIM | 381+ | 381+ | 381- | 381- | 1 |
| (6) Polypeptides not synthesized at day 0 and not induced by basal media | | | | | |
| A- RIM, SIM & CIM | | 108 | 108 | 108 | 1 |
| B- SIM only | | 401 | 401+, F | 401+, F+ | 2 |
| - SIM+ | | 376, 377 | 376+, 377+, G+ | 376+, 377+, G+ | 3 |
| -RIM+ | J, P | J, P, N* | H+, J, R+, P, N+ | H+, J, R+, P, N+ | 5 |
| C- Permanent on RIM and CIM but transient on SIM | 383 | 383- | 383- | 383- | 1 |

10.7 2-Dimensional analysis of protein extracted from tissues of *B. erythrophylla* for polypeptides associated with organogenesis

A distinction must be made between polypeptides that differ between tissues forming roots, shoots or callus because of their developmental program and those that simply reflect their different anatomies and physiologies. To see if any of the SIM induced polypeptides were due to the differentiated state of the shoot forming explant, protein was extracted from tissues of the parent plant and the polypeptide composition analyzed using silver-stained gels. Leaves, roots and the shoot apices from several *in vitro* plants were analyzed. Protein extracted from complete immature flowers harvested from potted plants, was also analyzed.

10.7.1 The shoot apex

The polypeptide pattern observed in the shoot apex was very similar to that found in day 0 petioles (PLATE 32A). Only one of the group 6, SIM only, polypeptides was clearly detected, 391, with a second, 330, faintly detected on some gels. None of the changes in existing polypeptides associated with shoot, root or callus induction were detected. Some quantitative changes in polypeptides associated with petiole physiology were also detected (TABLE 5), as were slight changes in the levels of polypeptides associated with basic metabolism (data not presented).

10.7.2 Leaf tissue

Leaf tissue showed a somewhat different polypeptide pattern than that of petiole tissue (PLATE 33A). In particular, polypeptides 86, 84, 80, 76 and several polypeptides present in small amounts in petiole tissue (all with a molecular weight of approximately 55kd), were present in large quantities in leaf tissue, so much so that they merged into a single large spot. This spot probably represented the large subunit of Rubisco. Polypeptide 200 associated with basic metabolism in the petiole was also very prominent in leaf tissue, as was the polypeptide 293. A further polypeptide, 452, was reliably detected in petiole tissue, but was present in large amounts in the leaf. Only one group 6, SIM only, polypeptide, 390, which required 21 days culture, on SIM, before being clearly resolved, was detected (PLATE 33A). This polypeptide may be associated with leaf structure and/or function.

10.7.3 Root tissue

Roots harvested from *in vitro* plants had a very low protein content and so only half the amount of protein normally loaded onto the first dimension gel could be separated. Concentration of the protein sample was not carried out as the methods available may have modified the charge on the polypeptides present and so alter the 2D gel pattern. As a smaller amount of protein was separated, only a general analysis of the most prominent polypeptides was carried out, although particular attention was paid to the detection of SIM only polypeptides. No SIM only polypeptides were detected in root tissue (PLATE 32B).

Polypeptide 343 was, however, abundant in root tissue. This polypeptide declined from day 0 to day 21 under root forming conditions, while under shoot forming conditions it declined only temporarily and then increased too greater than the original level detected at day 0. But after 42 days on RIM, 343 had also increased greatly in abundance. Polypeptide 343 appeared to be required at high levels in mature tissues and developing shoots. Polypeptide 450 detected after 42 days on RIM was also present at a high level in root tissue. A further polypeptide, 451, was only detected clearly in the root, where it was present in considerable abundance. This polypeptide was faintly detectable after 21 days on RIM, but its detection was not reliable between extractions so it was not recorded as RIM only. Polypeptides 450 and 451 were never detected with culture on any of the other media or in any other plant tissue and so were most likely specific to the developed root.

10.7.4 Flower buds

Flower buds from potted plants displayed a somewhat different pattern of polypeptides compared to *in vitro* petioles, although the reference polypeptides were clearly detectable (PLATE 33B). Flower buds contained several specific polypeptides and displayed large quantitative differences in many of the polypeptides common to the petiole. As the aim of this analysis was to determine if polypeptides associated with shoot formation were present in flower buds this data is not presented. None of the polypeptides associated with shoot formation were detected in the flower buds (PLATE 33B), with those induced by culture also absent.

Table 5 summarizes the presence or absence of polypeptides associated with organogenesis in B.erythrophylla tissues.

PLATE 32: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) Shoot apices of *in vitro* *B. erythrophylla* plants.

(B) Roots of *in vitro* *B. erythrophylla* plants.

Numbered arrows indicate tissue specific polypeptides, or those present at very high levels in the particular tissue.

Circles indicate the absence/presence of polypeptides associated with caulogenesis. Diamonds indicate the absence or reduced levels of polypeptides predominant in root tissue.

Arrow heads indicate reference proteins for comparison of gels.

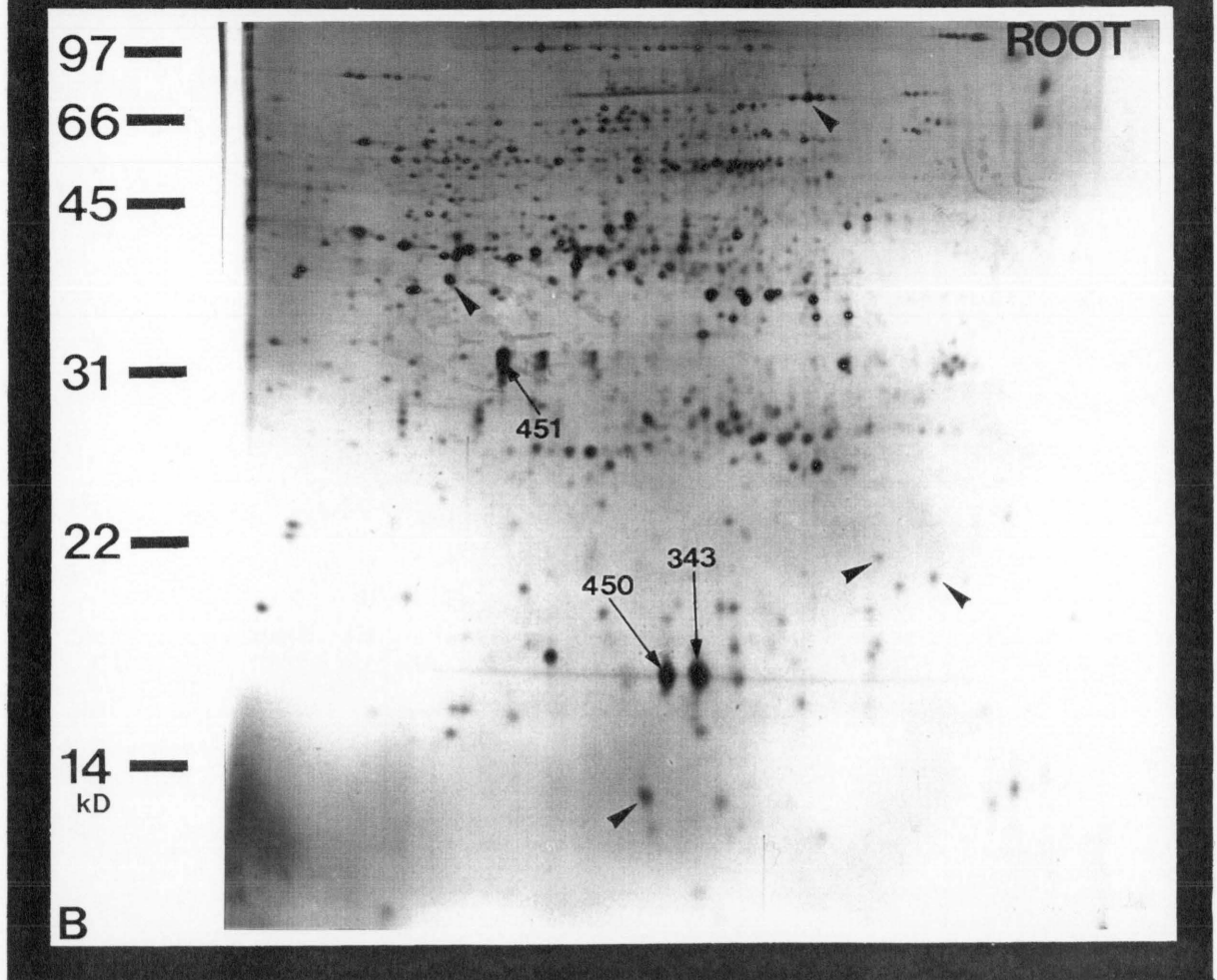
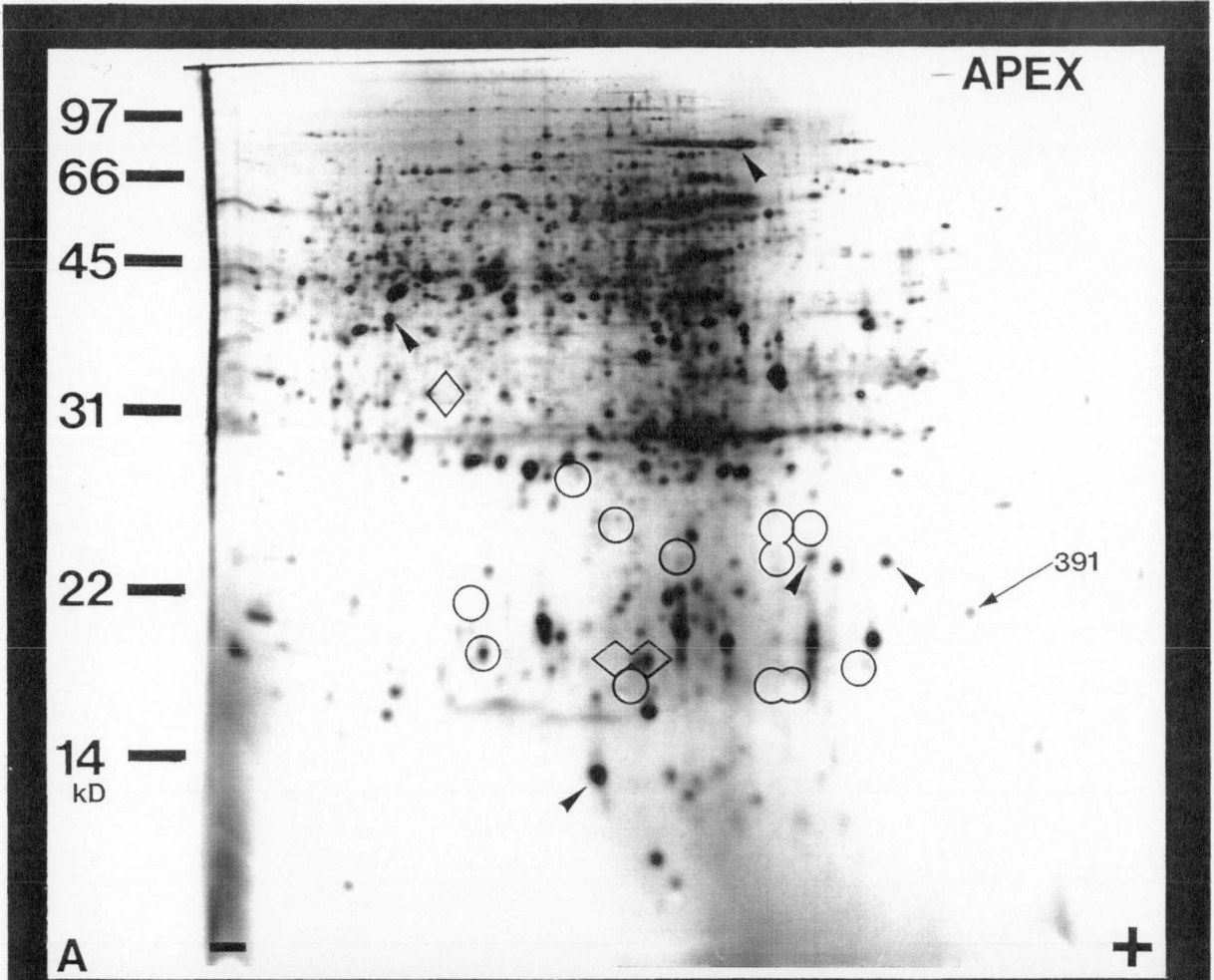


PLATE 33: Silver-stained two-dimensional PAGE gels of protein extracted from

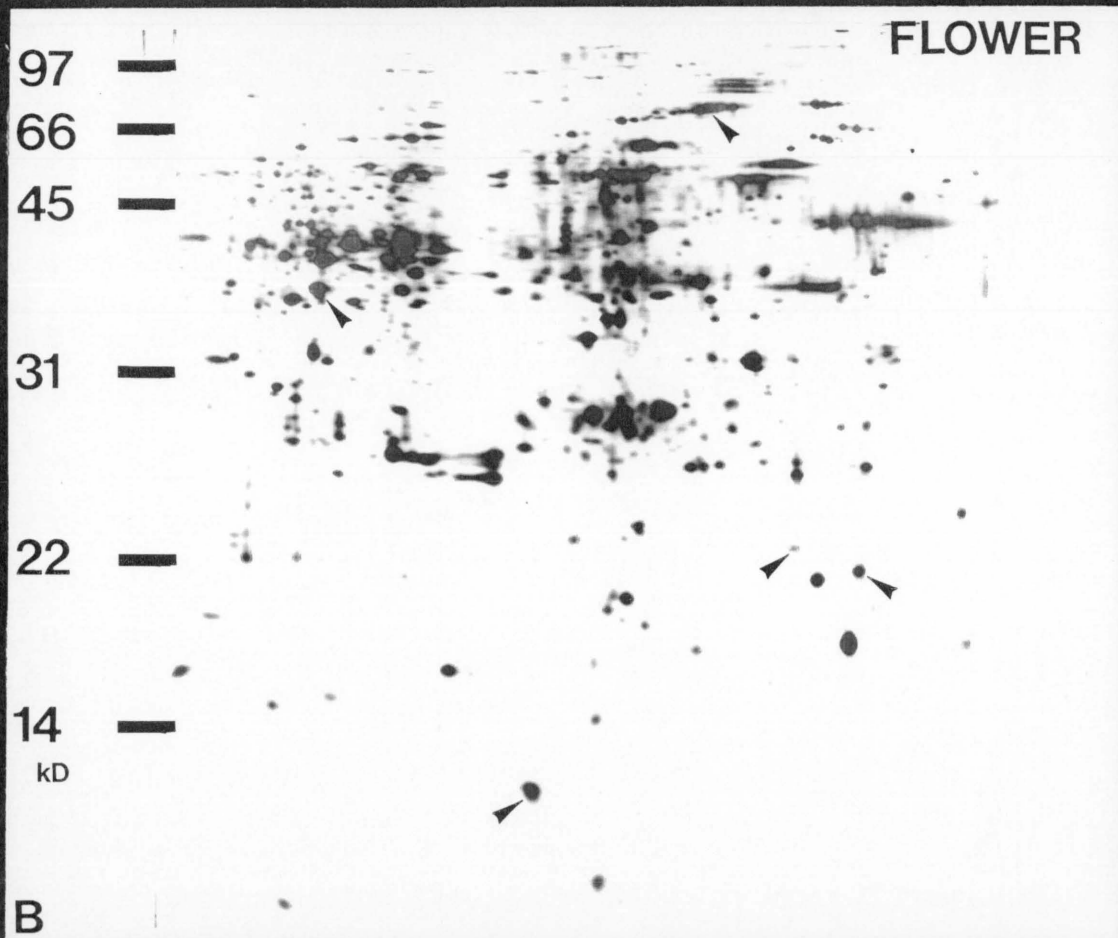
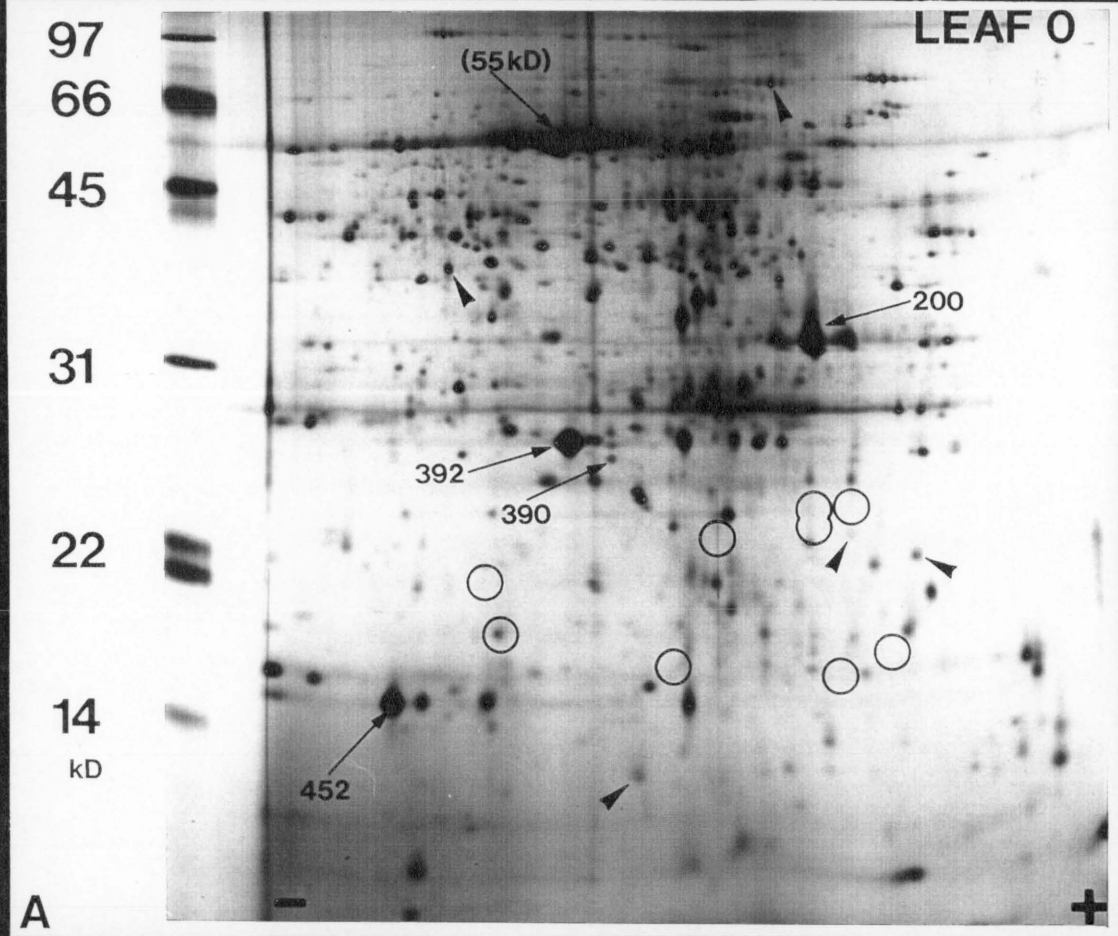
(A) Leaf tissue of *in vitro* *B. erythrophylla* plants.

(B) Immature flowers of potted *B. erythrophylla* plants.

Numbered arrows indicate tissue specific polypeptides, or those present at very high levels in the particular tissue.

Circles indicate the absence/presence of polypeptides associated with caulogenesis

Arrow heads indicate reference proteins for comparison of gels.



| Designation | Mr (kDa) | Total protein SIM | [³⁵ S] Methionine SIM | Total protein Leaves | Total protein Shoot apex | Total protein Roots | Total protein Flower bud | Total protein RIM | [³⁵ S] Methionine SIM |
|-------------|----------|-------------------|-----------------------------------|----------------------|--------------------------|---------------------|--------------------------|-------------------|-----------------------------------|
| 40 | 59 | - | NI | ND | - | | | - | NI |
| 42 | 55 | - | NI | ++++ | | | | - | NI |
| 80 | 55 | ND | - | ++++ | - | | | ND | - |
| 84 | 55 | - | - | ++++ | | | | - | - |
| 85 | 54 | - | NI | ++++ | - | | | - | NI |
| 86 | 55 | - | NI | ++++ | - | | | - | NI |
| 100 | 43 | - | NI | ND | = | | | - | NI |
| 102 | 41 | - | NI | - | = | | | - | NI |
| 104 | 41.5 | N+ | N+ | ND | ND | ND | ND | N+ | N+ |
| 107 | 35.5 | + | NI | ND | ND | ND | ND | + | NI |
| 108 | 40 | N+ | N+ | ND | ND | N | ND | N+ | N+ |
| 130 | 41 | - | = | - | = | | | - | = |
| 133 | 40 | - | = | - | - | | | - | = |
| 134 | 41 | - | = | - | - | | | - | = |
| 136 | 39 | + | + | - | = | | | + | + |
| 165 | 41 | - | NI | = | = | | | - | NI |
| 194 | 36.5 | - | NI | = | = | | | - | NI |
| 222 | 35 | N+ | N+ | ND | ? | ND | ND | N+ | N+ |
| 223 | 35.5 | = | = | ND | = | | | = | = |
| 227 | 32 | N+ | N+ | ND | ? | ND | ND | N+ | N+ |
| 232 | 30.5 | N+ | N | ND | ? | ND | ND | N+ | N |
| 233 | 31 | N+ | N | ND | ND | ND | ND | N+ | N |
| 266 | 27 | - | NI | = | = | | | - | NI |
| 267 | 27 | - | NI | = | = | | | - | NI |
| 277 | 28 | ND | ND | = | - | | | ND | ND |
| 278 | 29 | ND | NI | ND | = | | | ND | NI |
| 281 | 29 | ND | NI | = | - | | | ND | NI |
| 283 | 28 | - | NI | = | - | | | - | NI |
| 288 | 25 | + | = | ND | = | | | = | = |
| 294 | 28 | N+ | N+ | ND | ND | ND | ND | N+ | N+ |
| 299 | 30 | + | + | = | = | | | + | + |
| 300 | 29 | ND | ND | NI | - | | | ND | ND |
| 318 | 24.5 | - | NI | = | = | | | - | NI |
| 324 | 18.5 | N+ | ND | ND | ND | ND | ND | N | ND |
| 329 | 16 | N+ | ND | ND | ND | ND | ND | ND | ND |
| 330 | 16 | N+ | ND | ND | FD* | ND | ND | ND | ND |
| 341 | 19.5 | +/- | +/- | = | = | | | + | + |
| 343 | 18 | -/+ | -/+ | - | = | ++ | | - | - |
| 348 | 16.5 | N+ | NI | ND | ND | ND | ND | ND | NI |
| 349 | 16 | - | = | - | - | | | - | = |
| 358 | 19.5 | +/- | ? | ND | + | | | + | ? |
| 359 | 19.5 | +/- | ? | ND | + | | | + | ? |
| 364 | 22 | N+ | N+ | ND | ND | ND | ND | ND | ND |
| 366 | 18.5 | +++ | +++ | NI | = | | | = | = |
| 372 | 20.5 | N | N | ND | ND | | | N | N |
| 376 | 24 | N+ | N+ | ND | ND | ND | ND | ND | FD |
| 377 | 24 | N+ | N+ | ND | ND | ND | ND | ND | FD |
| 378 | 16 | N+ | NI | ND | ND | ND | ND | N | NI |
| 381 | 16 | +/- | +/- | = | + | | | + | + |
| 382 | 30 | N+/- | ? | ND | FD | | | N+/- | ? |
| 383 | 31.5 | N+/- | N+/- | ND | ND | | | N+/- | N+/- |
| 384 | 21 | +/- | = | = | = | | | + | = |
| 385 | 66 | N+ | ND | ND | ND | ND | ND | N+ | ND |
| 388 | 31 | N | ND | ND | ND | ND | ND | N | ND |
| 389 | 32 | N | ND | ND | ND | ND | ND | N | ND |
| 390 | 26.5 | N+ | ND | N* | ND | ND | ND | ND | ND |
| 391 | 21 | N+ | ND | ND | N* | ND | ND | ND | ND |
| 401 | 23 | N+ | N+ | ND | ND | ND | ND | ND | ND |
| 402 | 22.5 | N+ | ND | ND | ND | ND | ND | ND | ND |
| 450 | 18 | ND | ND | ND | ND | N* | ND | N | ND |
| 451 | 31.5 | ND | ND | ND | ND | N* | ND | N | ND |
| 452 | 14.5 | ND | ND | N* | ND | ND | ND | ND | ND |
| A | 25 | ND | = | | | | | ND | + |
| B | 55 | ND | - | | | | | ND | - |
| C | 55 | ND | - | | | | | ND | - |
| D | 55 | ND | - | | | | | ND | - |
| F | 10.5 | ND | N+ | | | | | ND | ND |
| G | 40.5 | ND | N+ | | | | | ND | FD |
| H | 37 | ND | FD | | | | | ND | N+ |
| J | 14.5 | ND | FD | | | | | ND | N |
| N | 31 | ND | FD | | | | | ND | N+ |
| P | 14.5 | ND | FD | | | | | ND | N |
| R | 32 | ND | FD | | | | | ND | N+ |

TABLE 5 Summary of both silver-stained and *in vivo* labelled polypeptides associated with caulogenesis and rhizogenesis and their occurrence in tissues of the whole plant. ND not detectable; N not detected at day 0; + increases or level greater than day 0 petiole; - decreases or level less than day 0 petiole; = same as day 0 petiole; NI not identifiable; FD faintly detected; ? inconsistent results; +++ greatly increased compared to day 0 petiole; * tissue specific; Bold marker for organogenesis; If no symbol is present, spots were not analyzed due to unequal protein being loaded onto the IEF gel or non-tissue culture material making accurate comparison difficult.

(11) MEDIA TRANSFER EXPERIMENTS

11.1 Explants requirements for inductive media

To determine the duration of exposure to the inductive medium explants require to develop organs upon transfer to a non-inductive medium, reciprocal transfer experiments were carried out. These experiments involved transferring explants from an inductive medium (SIM or RIM) to a non-inductive medium, able to sustain growth of developing organs (BM) and *vice versa*.

11.1.1 Caulogenesis

Culture for 7 days on SIM before transfer to BM was the minimum requirement for shoot induction, with most surviving explants producing at least one shoot (FIGURES 8A & 8B). Prolonging the period of culture on SIM increased the number of shoots formed per explant up to a maximum after 18 to 21 days on SIM (FIGURE 8A). Further culture on SIM caused little change in the number of shoots subsequently produced, but shoots produced on explants transferred to BM after 21 days on SIM, were better developed than those of explants cultured for 6 weeks on SIM alone. Transfer prior to day 7 resulted in only occasional shoot formation, although culture for five days did result in the formation of a few nodular structures that failed to develop further. Although transfer to BM after 7 or 10 days on SIM resulted in the production of leafy shoots, the shoots formed were poorly developed compared to those formed after longer exposures to SIM (PLATE 34A).

To find if SIM was needed from the beginning of culture or whether it was necessary only at a critical exposure time, experiments involving delayed exposure to SIM were conducted by pre-culturing explants on basal medium (BM) prior to exposure to SIM. Culture on basal medium for up to 3 days before transfer to SIM did not effect the number of shoots produced per explant or the percentage of explants forming shoots (FIGURES 8A & 8B). Further culture on BM resulted in reduced shoot numbers with complete loss of competence after more than seven days culture on BM (FIGURE 8A). The percentage of explants surviving after six weeks of culture was also greatly reduced by pre-culture for more than 5 days on BM (FIGURE 8B).

11.1.2 Rhizogenesis

As with SIM, media transfer experiments were carried out to find the critical time of exposure to RIM for root induction. Transfer from BM to RIM showed an almost identical effect on rhizogenesis as observed with caulogenesis (FIGURES 9A & 9B). Explants could be cultured on BM for up to 3 days with no effect on root number or percentage explants forming roots, but prolonged culture caused a rapid decline in root number with few roots produced from explants pre-cultured for 7 or more days on BM. As with transfer from BM to SIM, the number of explants surviving after 6 weeks in culture was reduced by pre-culture for more than five days on BM (FIGURE 9B).

In the reciprocal experiment, culture for 3 days on RIM before transfer to BM was the minimum time required for a low level of root formation (FIGURE 9A). Maximal root induction occurred after 10 days culture on RIM, with further culture resulting in little increase in root number. Unlike the stunted shoots produced after a minimal exposure to SIM, roots produced after only 3 days exposure to RIM grew well and appeared normal, often with many fine root hairs.

Although the percentage of explants surviving after 6 weeks in culture was increased greatly with 2 or more days on RIM before transfer to BM, the percentage survival did not appear to directly influence the percentage of explants forming roots. After 2 days on RIM greater than 70% of explants survived, but less than 30% formed roots. The same trend was observed with transfer from SIM to BM.

11.2 The strength of determination

Although explants no longer require exposure to SIM to go on to produce small numbers of shoots after 7 days in culture, the question arises whether growth-regulator containing media could still influence the formation of shoots at this point?

Further media transfer experiments were conducted where explants were transferred from either SIM to RIM or RIM to SIM at various times over a 21 day period. After 22 days all explants were transferred to BM to allow organ development.

11.2.1 SIM to RIM

A similar pattern of shoot formation was observed when explants were transferred from SIM to RIM, as that seen upon transfer from SIM to BM (FIGURE 10A). Seven days on SIM still resulted in small numbers of shoots forming per explant, despite 15 days culture on RIM before transfer to BM. The number of shoots produced per explant also increased with longer culture on SIM. Transfer from SIM to RIM prior to day 7 resulted in only the occasional shoot forming. Transfer from SIM to RIM and then to BM rather than to BM directly, appeared to have a beneficial effect on shoot development. Shoots produced were much larger and greener than those produced upon transfer from SIM to BM.

Root induction showed a reciprocal pattern. One days exposure to SIM resulted in a slight reduction in root number compared to culture on RIM alone, but after that culture for up to 7 days on SIM resulted in no further reduction in the number of roots produced. Transfer after day 7 showed a steady decline in root number with reduced time on RIM and increased shoot number. Culture on SIM did not appear to inhibit the ability of explants to respond to RIM, with roots of explant origin still, produced even after 15 days culture on SIM. Reduced time on RIM did, however, result in smaller numbers of roots forming.

11.2.2 RIM to SIM

As with caulogenesis the pattern of root formation upon transfer from RIM to SIM was similar to that produced upon transfer from RIM to BM, but fewer roots were produced (FIGURE 10B). Transfer from RIM to SIM appeared to suppress the development of many roots that would have developed upon direct transfer to BM, rather than to SIM and then to BM. Three days on RIM before transfer to SIM resulted in very few roots forming. Five days were required to induce a reasonable number of roots and 18 days to give a near maximal response.

Caulogenesis was dependent upon the time of exposure to SIM and was apparently independent of RIM. Culture for up to 3 days on RIM did not affect shoot number with a maximal response obtained. Further culture on RIM resulted in a rapid decline in shoot number as increased culture time on RIM resulted in reduced culture time on SIM. Culture on RIM appeared not to effect the ability of the explant to respond to SIM. Unlike with pre-culture on BM, a few healthy shoots, of explant origin, still formed after 14 days pre-culture on RIM (7 days on SIM).

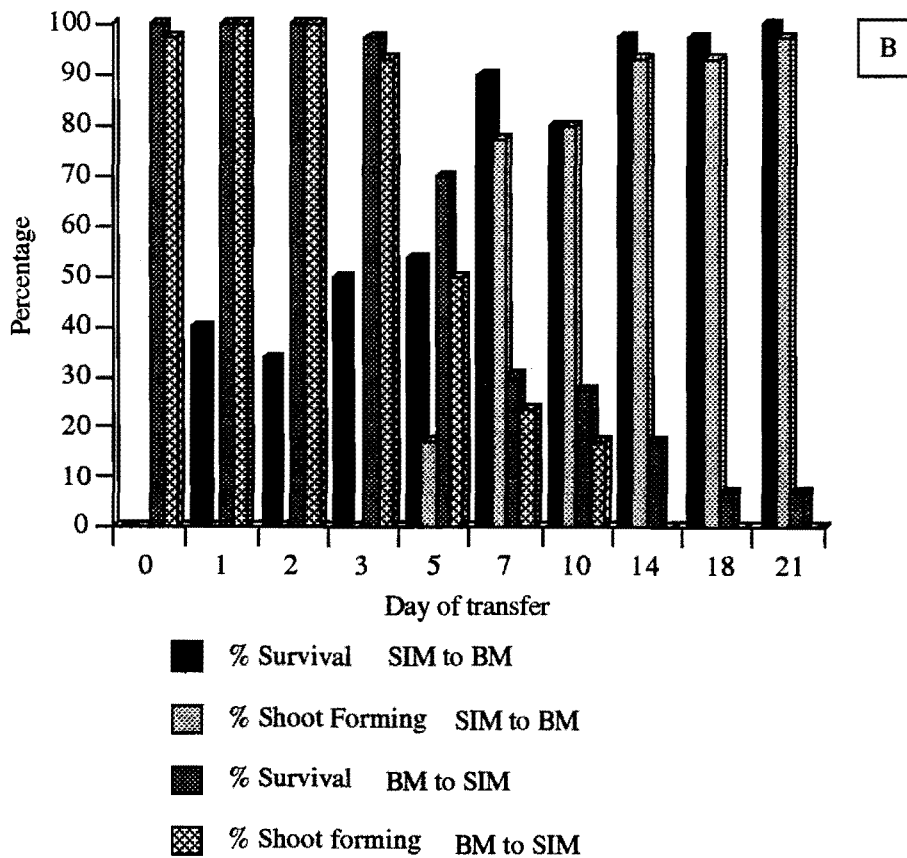
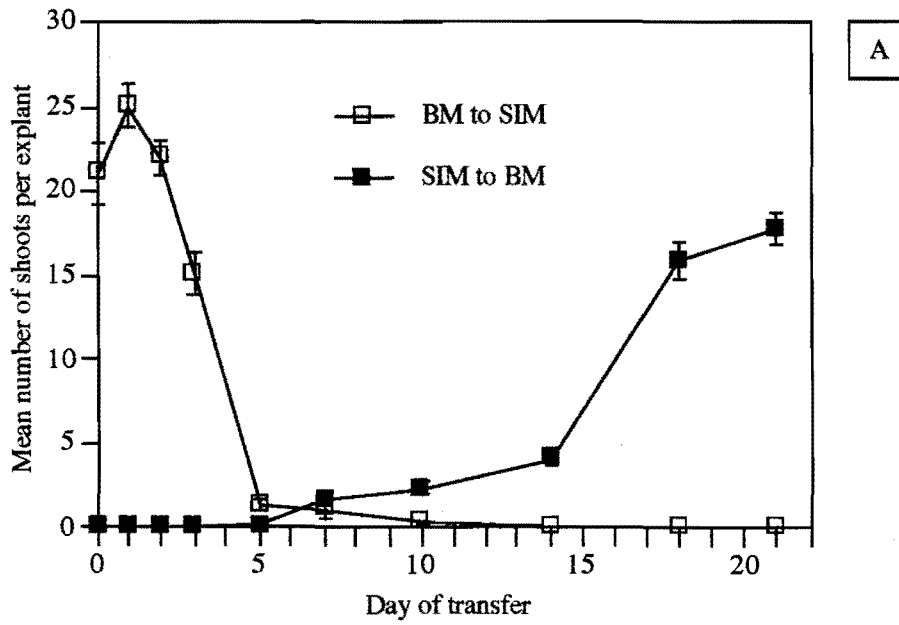


FIGURE 8 [A]- Changes in the number of shoots produced after transfer from SIM to BM and BM to SIM, respectively. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

[B]- The percentage of explants surviving and the percentage forming shoots after transfer from SIM to BM and BM to SIM

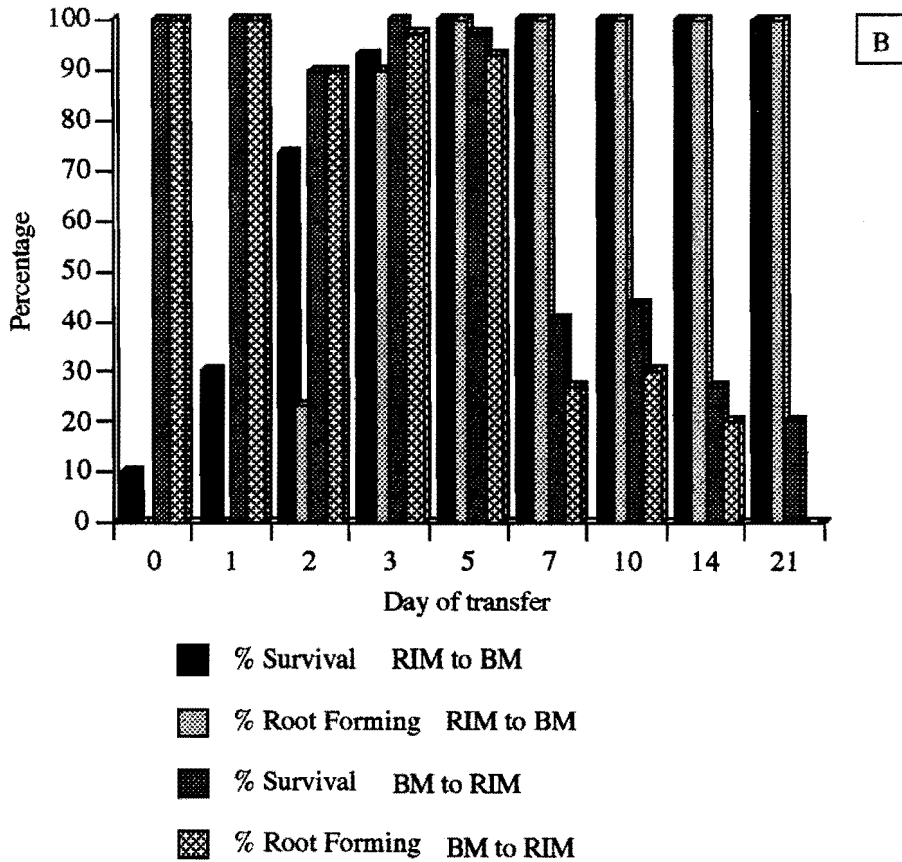
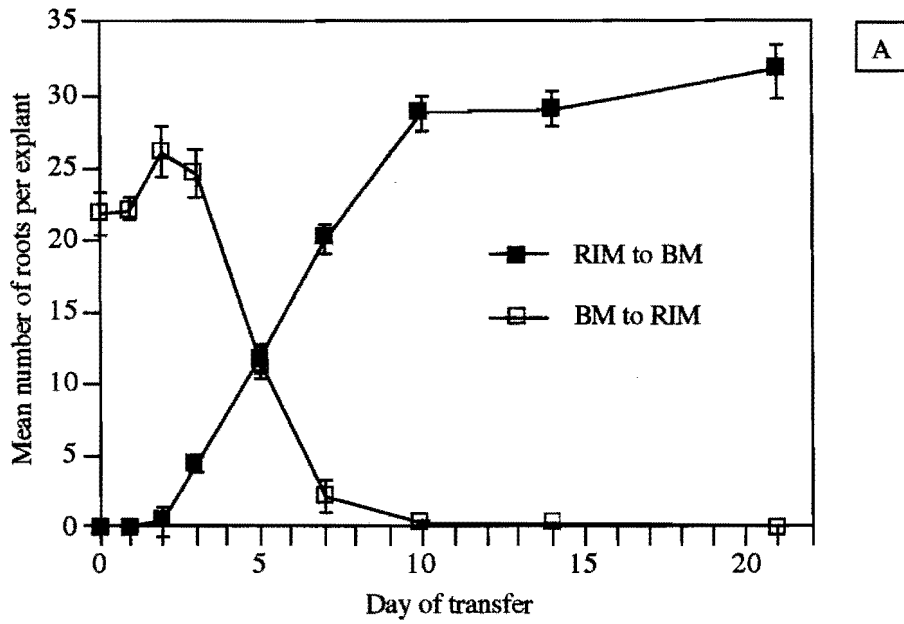


FIGURE 9 [A]- Changes in the number of roots produced after transfer from RIM to BM and BM to RIM, respectively. Mean value plus or minus 1 standard error, n=30. Roots were scored after a total of 6 weeks in culture.

[B]- The percentage of explants surviving and the percentage forming roots after transfer from RIM to BM and BM to RIM.

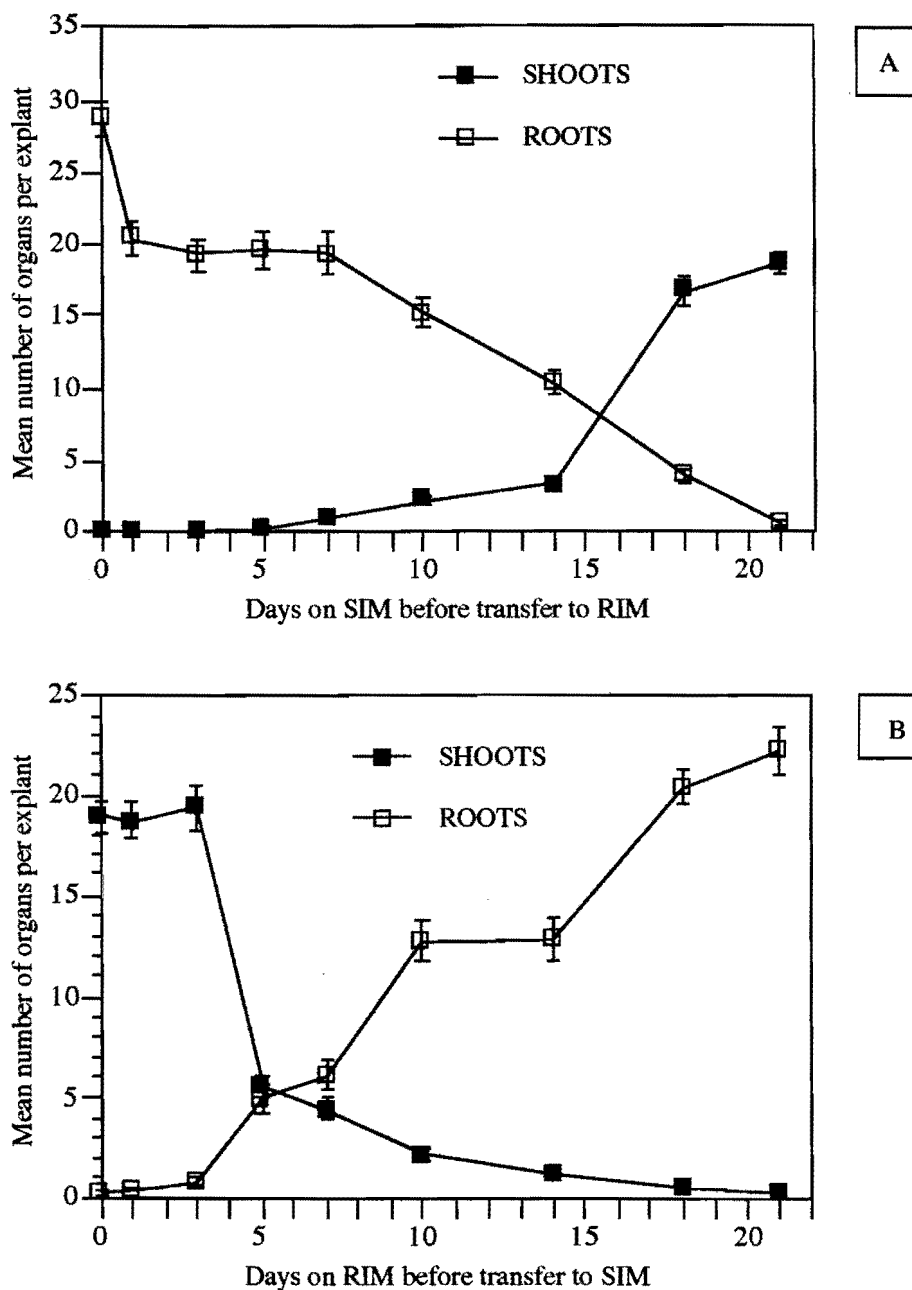


FIGURE 10 [A]- Changes in the number of shoots and roots produced after transfer from SIM to RIM, respectively. Mean value plus or minus 1 standard error, $n=30$. Explants were all transferred to basal medium after 22 days. Organs were scored after a total of 6 weeks in culture.

[B]- Changes in the number of shoots and roots produced after transfer from RIM to SIM, respectively. $n=30$ plus or minus 1 SEM.

11.3 Do explants require a specific inductive medium prior to the first cell divisions?

From the media transfer experiments carried out to this point it has been shown that small numbers of roots could be induced if explants were exposed for three days to RIM, while a 7 day exposure to SIM was required for shoots to form upon transfer to BM.

As the first cell divisions on either medium were observed after 3 days culture, the requirement for RIM was apparently rapidly lost once cell division had begun. This posed a further question, did the explants require the inductive medium prior to the first cell division or only at the point at which cell division occurs and for a short period after? To determine which was the case, further media transfer experiments were carried out. Explants were exposed to BM or RIM for 1, 2 or 3 days prior to transfer to SIM and then to BM, to determine if exposure to SIM was required prior to day 3. The same procedure was carried out for RIM, with explants exposed to BM or SIM before transfer to RIM and then BM.

BM which contained no growth regulators had no inductive effect on either rhizogenesis or caulogenesis. Pre-culture on BM did not reduce the time required on either inductive media (TABLES 7 & 9).

Pre-culture on RIM for 24 to 48 hours reduced the time required on SIM for minimal determination to 6 or 5 days respectively. Further pre-culture caused no further reduction on the time required on SIM (TABLE 6).

Pre-culture on SIM for 24 or 48 hours also reduced the time required on RIM for a minimal root forming response, to 48 and 24 hours respectively (TABLE 8). From these results it was apparent that for both organogenic processes the first 24 and possibly 48 hours of culture, were independent of the growth regulator balance of the inductive medium, but exposure to growth regulators was required, as BM alone had no inductive potential.

Days on SIM before transfer to BM

| DAYS ON RIM | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 |
|-------------|---|---|---|------|------|-------------|-------------|-------------|------|------|------|
| 0 E1 | 0 | 0 | 0 | 0 | 0.05 | 0.15 | 0.85 | <u>1.57</u> | 2.30 | 5.00 | 20.9 |
| E2 | 0 | 0 | 0 | 0 | 0.15 | 0.10 | 0.70 | <u>1.90</u> | 2.80 | 5.25 | 17.5 |
| 1 | 0 | 0 | 0 | 0.05 | 0.10 | 0.50 | <u>1.30</u> | 1.70 | 2.40 | 5.45 | 22.1 |
| | 0 | 0 | 0 | 0 | 0 | 0.75 | <u>1.75</u> | 2.45 | 3.65 | 5.25 | 18.2 |
| 2 | 0 | 0 | 0 | 0.05 | 0 | <u>1.10</u> | 1.10 | 1.30 | 2.70 | 4.80 | 19.8 |
| | 0 | 0 | 0 | 0 | 0.30 | 0.95 | <u>1.55</u> | 1.90 | 3.75 | 4.35 | 17.8 |
| 3 | 0 | 0 | 0 | 0 | 0.50 | 0 | <u>1.70</u> | 2.05 | 2.45 | 3.60 | 16.9 |
| | 0 | 0 | 0 | 0 | 0.07 | 0.85 | 0.90 | <u>1.10</u> | 2.10 | 3.40 | 20.2 |

TABLE 6 Mean numbers of shoots produced from explants exposed to RIM, then SIM before transfer to basal medium. E1 and E2 represent independent experiments, n=15 per expt. Shoots were scored after a total of 6 weeks in culture.

Days on SIM before transfer to BM

| DAYS ON BM | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 |
|------------|---|---|---|------|------|------|------|-------------|-------------|------|------|
| 0 E1 | 0 | 0 | 0 | 0 | 0.05 | 0.15 | 0.85 | <u>1.55</u> | 2.30 | 5.00 | 20.9 |
| E2 | 0 | 0 | 0 | 0 | 0.15 | 0.10 | 0.70 | <u>1.90</u> | 2.80 | 5.25 | 17.5 |
| 1 | 0 | 0 | 0 | 0 | 0.05 | 0.70 | 0.60 | <u>1.70</u> | 2.70 | 4.50 | 21.2 |
| | 0 | 0 | 0 | 0 | 0.15 | 0 | 0.55 | <u>1.95</u> | 2.75 | 5.25 | 19.1 |
| 2 | 0 | 0 | 0 | 0.05 | 0 | 0 | 0.95 | <u>1.20</u> | 1.75 | 3.90 | 17.9 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0.65 | <u>1.65</u> | 3.65 | 3.65 | 18.4 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0.70 | <u>1.95</u> | 2.80 | 4.35 | 13.6 |
| | 0 | 0 | 0 | 0 | 0.05 | 0 | 0.50 | 0.95 | <u>1.70</u> | 2.30 | 13.3 |

TABLE 7 Mean numbers of shoots produced from explants exposed to BM, then SIM before transfer to basal medium. E1 and E2 represent independent experiments, n=15 per expt. Shoots were scored after a total of 6 weeks in culture.

Days on RIM before transfer to BM

| DAYS ON SIM | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 |
|-------------|------|-------------|------|-------------|------|------|------|------|------|------|------|
| 0 E1 | 0 | 0 | 0.50 | <u>4.30</u> | 6.10 | 11.7 | 14.1 | 19.8 | 25.1 | 23.6 | 26.1 |
| E2 | 0 | 0 | 0.17 | <u>3.65</u> | 6.50 | 12.1 | 13.2 | 20.1 | 21.2 | 25.7 | 28.3 |
| 1 | 0.20 | <u>1.50</u> | 4.1 | 4.00 | 5.10 | 10.8 | 11.7 | 20.1 | 19.8 | 20.4 | 21.2 |
| | 0 | <u>1.75</u> | 4.35 | 4.45 | 6.60 | 11.9 | 11.6 | 17.2 | 23.4 | 21.6 | 21.2 |
| 2 | 0.35 | <u>1.20</u> | 3.70 | 4.70 | 6.10 | 11.2 | 12.2 | 18.2 | 18.5 | 21.2 | 21.1 |
| | 0.95 | <u>2.75</u> | 5.45 | 5.25 | 5.45 | 10.8 | 14.7 | 16.1 | 19.5 | 22.8 | 23.5 |
| 3 | 0.85 | <u>2.80</u> | 2.75 | 3.65 | 4.75 | 9.75 | 10.6 | 11.2 | 18.2 | 19.2 | 17.9 |
| | 0.85 | <u>2.75</u> | 2.75 | 4.00 | 4.90 | 9.90 | 9.90 | 12.2 | 17.9 | 19.2 | 19.4 |

TABLE 8 Mean numbers of roots produced from explants exposed to SIM, then RIM before transfer to basal medium. E1 and E2 represent independent experiments, n=15 per expt. Roots were scored after a total of 6 weeks in culture.

Days on RIM before transfer to BM

| DAYS ON BM | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 |
|------------|------|-------------|-------------|-------------|------|------|------|------|------|------|------|
| 0 | 0 | 0 | 0.50 | <u>4.30</u> | 6.10 | 11.7 | 14.1 | 19.8 | 25.1 | 23.6 | 26.1 |
| | 0 | 0 | 0.17 | <u>3.65</u> | 6.50 | 12.1 | 13.2 | 20.1 | 21.2 | 25.7 | 28.3 |
| 1 | 0.20 | 0 | <u>4.10</u> | 4.00 | 5.10 | 10.4 | 10.8 | 20.4 | 19.1 | 19.4 | 24.1 |
| | 0.55 | 0.95 | <u>4.10</u> | 4.35 | 5.25 | 13.1 | 14.2 | 18.8 | 17.2 | 20.1 | 19.3 |
| 2 | 0.35 | <u>1.20</u> | 3.70 | 4.70 | 6.10 | 11.2 | 12.2 | 18.2 | 18.5 | 21.2 | 29.1 |
| | 0.85 | <u>1.65</u> | 4.45 | 5.25 | 5.45 | 12.2 | 13.7 | 16.9 | 18.2 | 19.8 | 15.1 |
| 3 | 0.95 | <u>1.95</u> | 2.75 | 3.65 | 4.35 | 10.8 | 10.1 | 14.1 | 18.2 | 17.6 | 17.9 |
| | 0.95 | <u>1.50</u> | 1.90 | 4.20 | 4.90 | 9.70 | 9.90 | 12.7 | 17.1 | 17.9 | 19.4 |

TABLE 9 Mean numbers of roots produced from explants exposed to BM, then RIM before transfer to basal medium. E1 and E2 represent independent experiments, n=15 per expt. Roots were scored after a total of 6 weeks in culture.

11.4 The effect of BA concentration on time required on shoot inducing media

Continuous culture on a medium containing 0.5 mg/l BA (half that in SIM) resulted in the formation few shoots (FIGURE 1a), suggesting that the ratio of auxin to cytokinin in the medium was suboptimal. Continuous culture on media containing 4 mg/liter BA or above, resulted in the formation of large numbers of shoot buds, but shoots formed appeared stunted (PLATE 4B). SIM, containing 1mg/liter BA, allowed both the induction and development of large numbers of shoots.

To determine if the concentration of BA in SIM could effect the time explants required on the shoot inducing media, further media transfer experiments were carried out. Explants exposed for various lengths of time to SIM containing an increased or decreased concentration of BA, were transferred to BM to allow shoot development. Like continuous culture, culture on 0.5mg/l BA for 7 days or more resulted in the formation of small numbers shoots irrespective of the time of transfer to BM (FIGURE 11A).

If explants were exposed to either SIM, BA 4 (mg/l BA) or BA 8 (8 mg/l BA) for 18 days or more, similar numbers of shoots were produced (FIGURE 11A). The time required for a maximal shoot forming response therefore not greatly altered by increasing the concentration of BA in SIM, but the minimum time required for shoots to form was reduced. Culture for 4 to 5 days on either BA 4 or BA 8 induced a mean value of greater than 1 shoot per explant (FIGURE 11A). Culture on either BA 4 or BA 8 also resulted in a 4-fold increase in the number of shoots determined by day 10 of culture (FIGURE 11A; PLATE 34A).

On SIM greater than 70% of shoots formed required continued exposure to the inductive medium for 15 to 21 days, with less than 20% requiring exposure for 8 to 14 days. Culture on BA 4 medium increased the percentage of shoots requiring exposure for 8 to 14 days to greater than 40%, while culture on BA 8 increased this to greater than 50 % (FIGURE 11B). Both the minimum time required on the inductive medium and the number of shoots forming after a given exposure time (between days 5 and 21), was dependent upon the concentration of BA in the shoot inducing medium. SIM, although the optimal single medium for both induction and development, did not have the greatest inductive potential during the early stages of culture. It required at least a four fold increase in the BA concentration of SIM for a maximal response.

Exposure to BA8, although reducing the time required for shoot formation and causing an increase in the number of shoots formed after brief exposures to the inductive medium, did not speed up the development of shoot primordia. Both in surface view and histologically no difference was observed between explants cultured

on SIM or BA8 for 7 or 14 days. This posed 2 questions, was the higher concentration of BA in the medium speeding up the expression of genes involved in organogenesis, or alternatively, did the increased concentration of BA in the medium simply increase the amount of BA taken up by the explant in a given length of time?

Explants exposed to BA8 for 5 days may have accumulated enough BA within the tissue to allow shoots to form, while explants exposed to SIM require 7 to 10 days to achieve the same level. To ascertain which of the above hypotheses was correct, 2D gels were run comparing the polypeptides patterns of explants exposed to BA8 medium, to those exposed to SIM. It was also attempted to compare the amount of cytokinin activity in explants exposed to SIM, to that in explants exposed to BA8 medium.

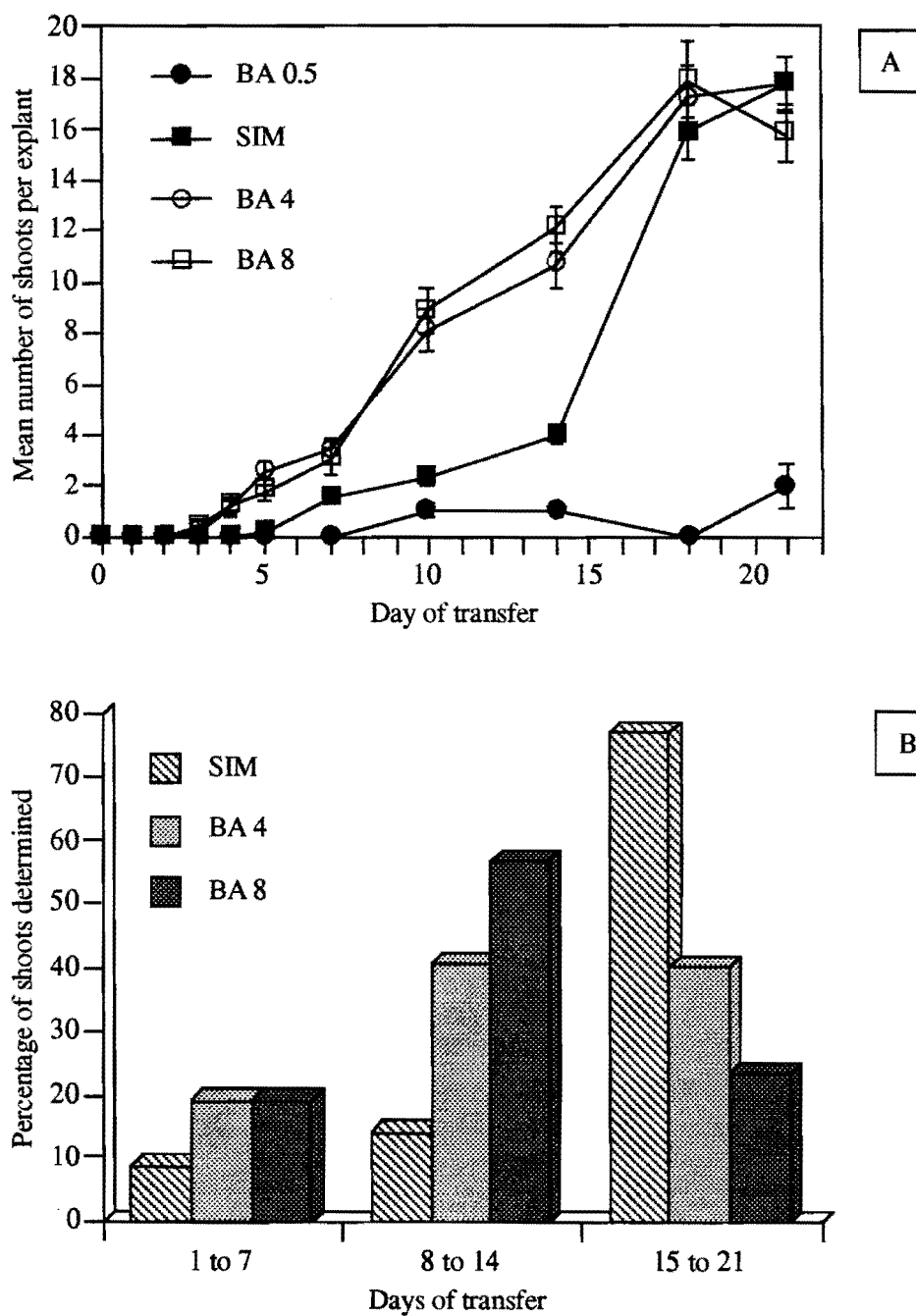


FIGURE 11 [A]- The effect of BA concentration on the number of shoots produced after transfer from BA containing media to BM. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

[B]- The effect of BA concentration on percentage of shoots determined after various lengths of time on various shoot inducing media.

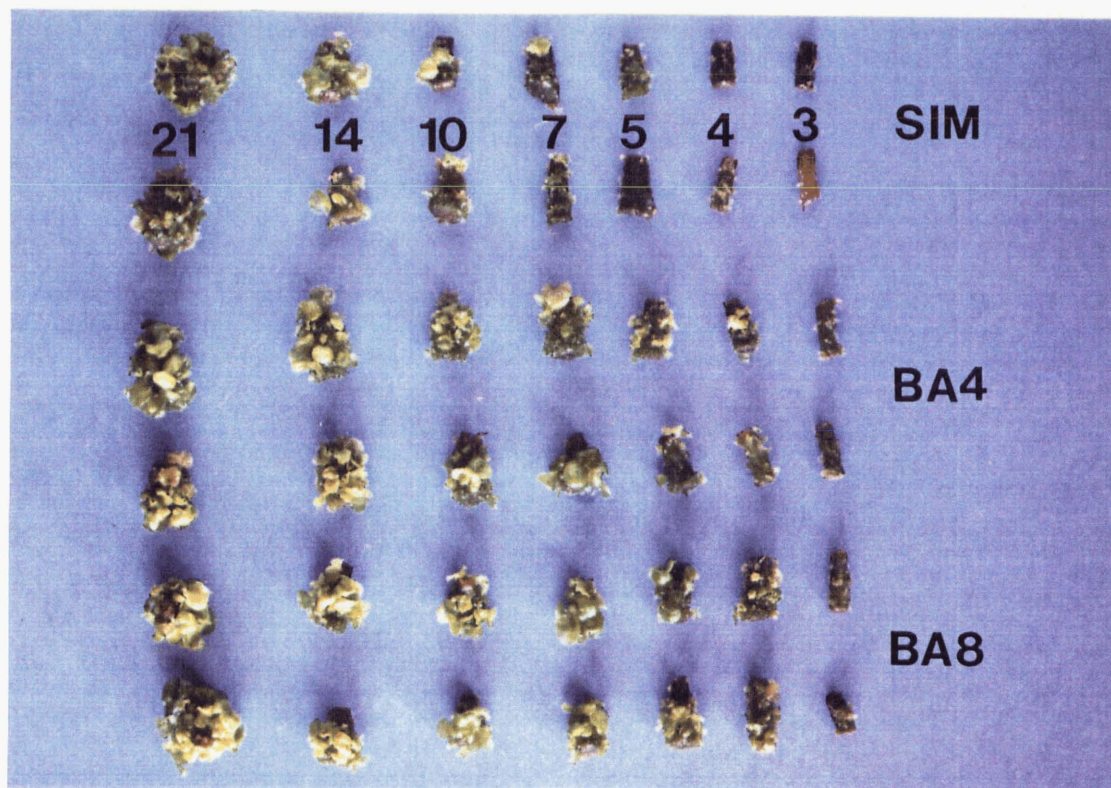


PLATE 34: [A]- The effect of BA concentration on the time required on shoot inducing medium for explants to develop shoots upon transfer to basal medium. SIM (1mg/l BA), BA4 (4mg/l BA) and BA8 (8mg/l BA). Explants after a total of 6 weeks in culture. Note 7 days on SIM is required for minimal determination, while 4 to 5 days on either BA4 or BA8.

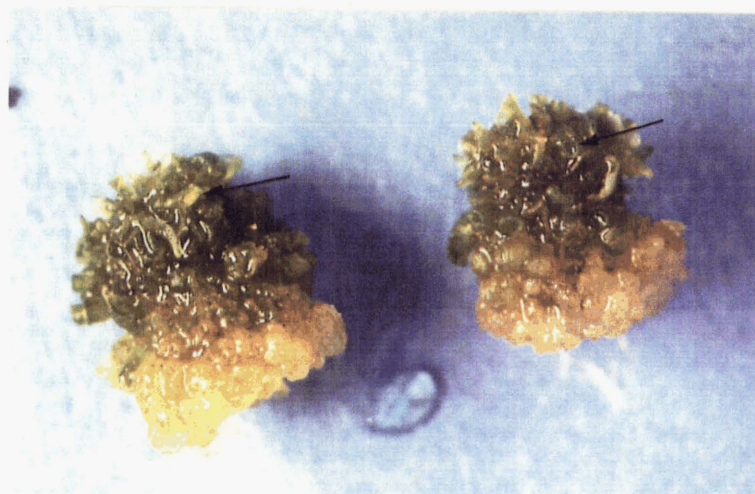


PLATE 34: [B]- Explants 6 weeks after transfer from SIM (14 days exposure) to SIM plus TIBA (4 μ m). Note shoots either fail to develop leaves, or those that do, produce abnormal leaves (arrows).

11.6 The effect of BA concentration on accumulated polypeptides associated with shoot formation

To determine if exposure to a higher concentration of BA effected the 2D pattern of polypeptides detected upon exposure to SIM. Gels were run separating protein extracted from explants after 5, 7 or 10 days exposure to either SIM or BA8. Both total protein and newly synthesized polypeptides were analyzed. If exposure to BA8 effected the shoot forming process at the level of gene expression, the markers of caulogenesis detected on SIM should have been detected earlier on BA8. Those polypeptides that accumulated as the number of shoots no longer requiring SIM to develop increased, should also have had elevated levels on BA8 compared to SIM, if the higher concentration of BA was effective directly at the gene level.

Five days culture on BA8 failed to induce any of the SIM only polypeptides detected after 7 days culture on SIM. In addition, neither 7 or 10 days culture on BA8 caused any increase in the levels of the group 6, SIM only, polypeptides (PLATES 35A & 35B). From these results it would appear that increasing the concentration of BA in the shoot inducing medium did not effect the timing or the level of accumulation of the SIM only subgroup of polypeptides. Furthermore no changes in the polypeptide pattern expressed upon exposure to SIM were detected with exposure to BA8, suggesting that the effect of increasing the BA concentration is not at the gene level.

11.7 The effect of BA concentration on the amount of cytokinin activity in explants exposed to SIM or BA8

The level of cytokinin activity was so low in extracts from explants exposed to either SIM or BA8, that the bioassay used was not sensitive enough to detect any significant activity in the amount of tissue available. A more sensitive assay is required.

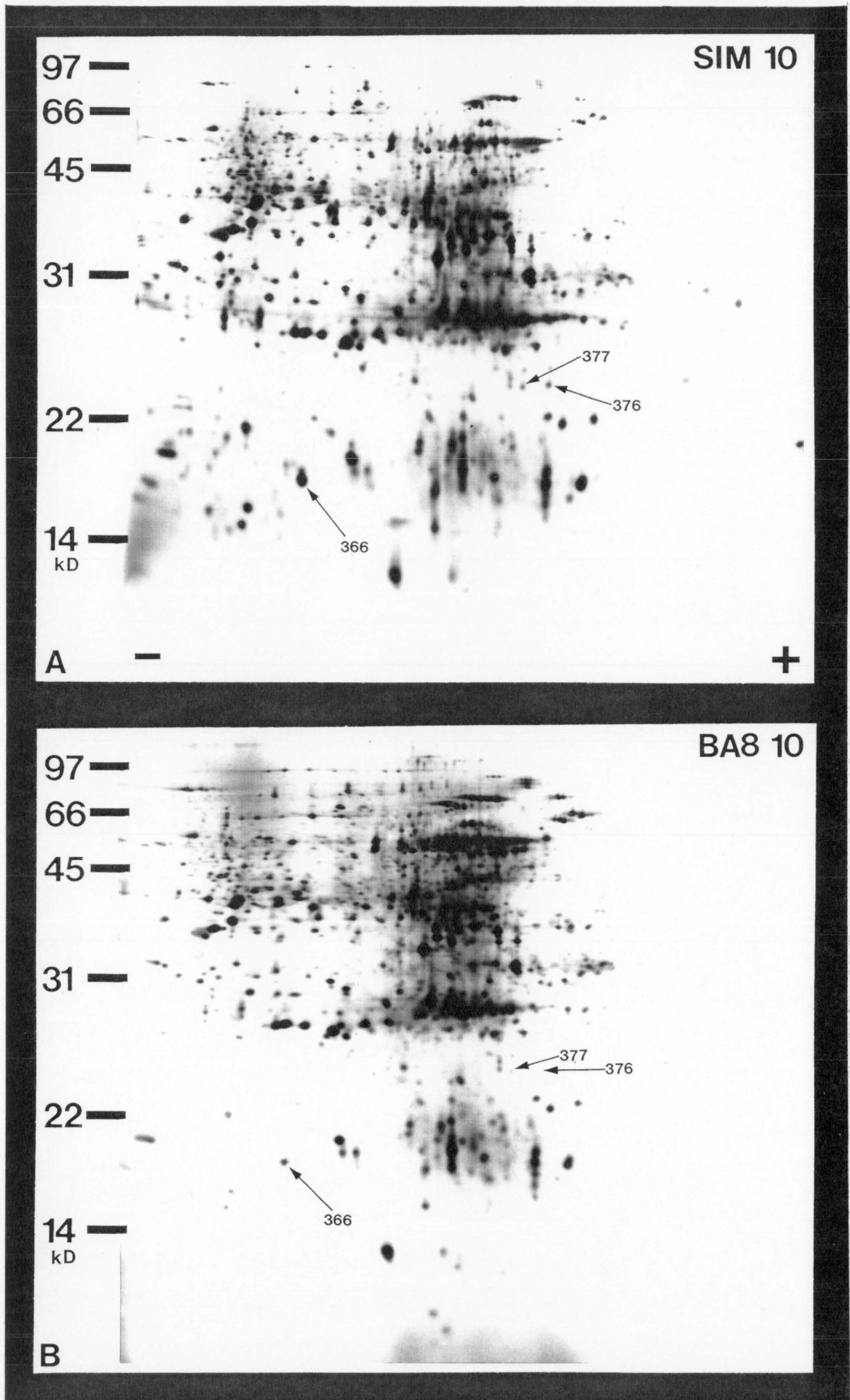
PLATE 35: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 10 days on SIM.

(B) explants cultured for 10 days on BA8.

Numbered arrows indicate polypeptides associated with caulogenesis.
Note that none of the polypeptides increase in abundance with culture
on BA8

Arrow heads indicate reference polypeptides for comparison of gels.



11.7 The strength of determination after exposure to BA8

As only 4 to 5 days on BA8 were required for explants to form small numbers of shoots, again the question arises as to whether exposure to RIM could still influence the formation of shoots at this point? Unlike with 7 days exposure to SIM where explants formed shoots even after exposure to RIM, exposure to BA8 for less than 7 days before transfer to RIM resulted in very few shoots forming. Less than 30% of shoots formed if exposed to RIM prior to day 15, with about 70% requiring 15 to 21 days on BA8 prior to transfer to RIM (FIGURE 12). Although a high concentration of BA shortens the time required on the shoot inducing medium before transfer to BM, BA8 has little effect if explants are transferred to RIM prior to day seven.

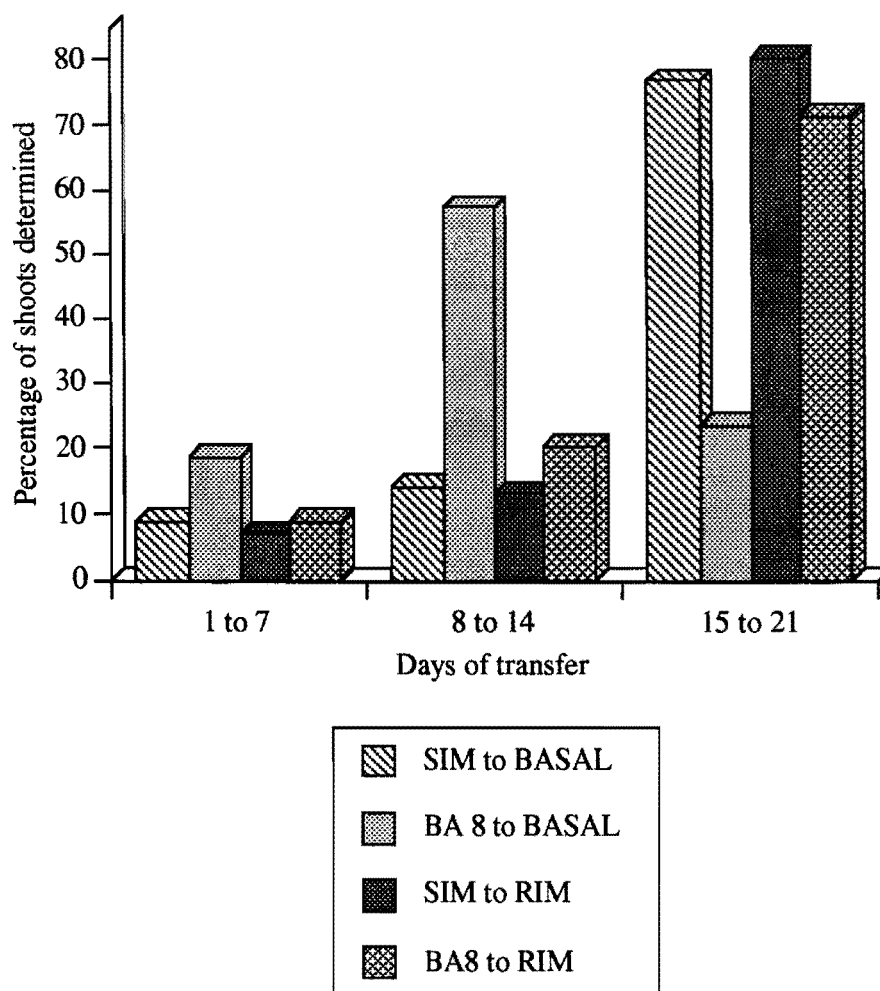


FIGURE 12- The effect of BA concentration on the percentage of shoots strongly determined after various lengths of time on shoot inducing media. n=30. Shoots were scored after a total of 6 weeks in culture.

(12) THE EFFECT OF INHIBITORS ON SHOOT FORMATION

12.1 Sorbitol

12.1.1 Sensitivity to sorbitol

Sorbitol at a concentration of 30g/liter, in addition to the 30g/liter sucrose in SIM, completely inhibited shoot formation (PLATE 36C) with only small amounts of basal callus produced. Lower concentrations of sorbitol had little effect on shoot production, but did cause stunting of developing shoots at concentrations greater than 5g/liter (PLATES 36A & 36B). In surface view explants cultured on SIM plus sorbitol (30g/l), for 21 days, were essentially devoid of any shoot like structures except for an occasional nodular structure that failed to develop further (PLATE 36D). Histological examination rarely showed an apex-like structure, but small meristematic regions could be detected beneath some glandular hairs, with random cell divisions occurring throughout the explant (PLATE 38A). With prolonged culture explants became senescent .

12.2.2 Stage specificity of sorbitol sensitivity

The sensitivity of *B. erythrophylla* petiole sections to sorbitol was localized in time by media-transfer experiments in which explants were moved to, or from, sorbitol containing medium after various lengths of time. The results of such an experiment are shown in Figures 13A and B. If explants were moved to SIM containing sorbitol on day 3 of culture or later, they went on to produce large numbers of shoots. The longer they were cultured on SIM without sorbitol prior to transfer, the larger the number of shoots that were produced, up to a maximum value after seven days. Transfer after this time had no effect on the number of shoots produced, the only result being a stunting of shoot growth and in some explants, pigmentation of the developing leaves. Transfer prior to day 3 resulted in few shoots forming. The mean number of shoots per explant was less than 1 if explants were transferred to SIM plus sorbitol before day 3 of culture (FIGURE 13A), but it should be noted that a high percentage of explants produced a single stunted shoot if cultured on SIM for as little as 24 hours (FIGURE 13B). Transfer from SIM containing sorbitol to SIM alone resulted in normal numbers of shoots formed up to day 5, after which the number of shoots produced per explant steadily declined, with shoots becoming progressively less healthy with continued culture (FIGURE 13A).

Sorbitol appeared to affect an event or events that occurred prior to the formation of meristematic regions. Once these regions had formed the effects of exposure to sorbitol became progressively less with time. The most significant histological event observed prior to day 3 of culture on SIM was the accumulation of starch within tissues of the explant.

12.1.3 Histological and biochemical examination of the occurrence of starch in sorbitol treated tissue

Hand sections of explants cultured on SIM plus sorbitol showed a reduction in starch grain accumulation compared to those observed when cultured on SIM alone. In the presence of sorbitol starch grains were all but absent from the epidermal and collenchyma cells of explants cultured for 4 days, with only a slight increase in the amount of starch observed in the cortical cells. Only rarely were regions of localized accumulation in the epidermal/collenchyma layers observed. Biochemical analysis of total extractable starch as determined by the anthrone procedure, showed a reduction in the amount of starch accumulated in the explant if sorbitol was added to SIM (TABLE 10).

| | Days in culture | | | |
|--|-----------------|-----|-----|-----|
| | 0 | 3 | 7 | 14 |
| Starch $\mu\text{g}/\text{mg}$ (FW). On SIM | 43 | 197 | 553 | 741 |
| On SIM plus sorbitol | 43 | 82 | 102 | 97 |

TABLE 10- Changes in the starch content of *B. erythrophylla* petiole explants cultured on SIM or SIM plus sorbitol (30g/l). Mean value, n=10, combined from 2 separate extractions.

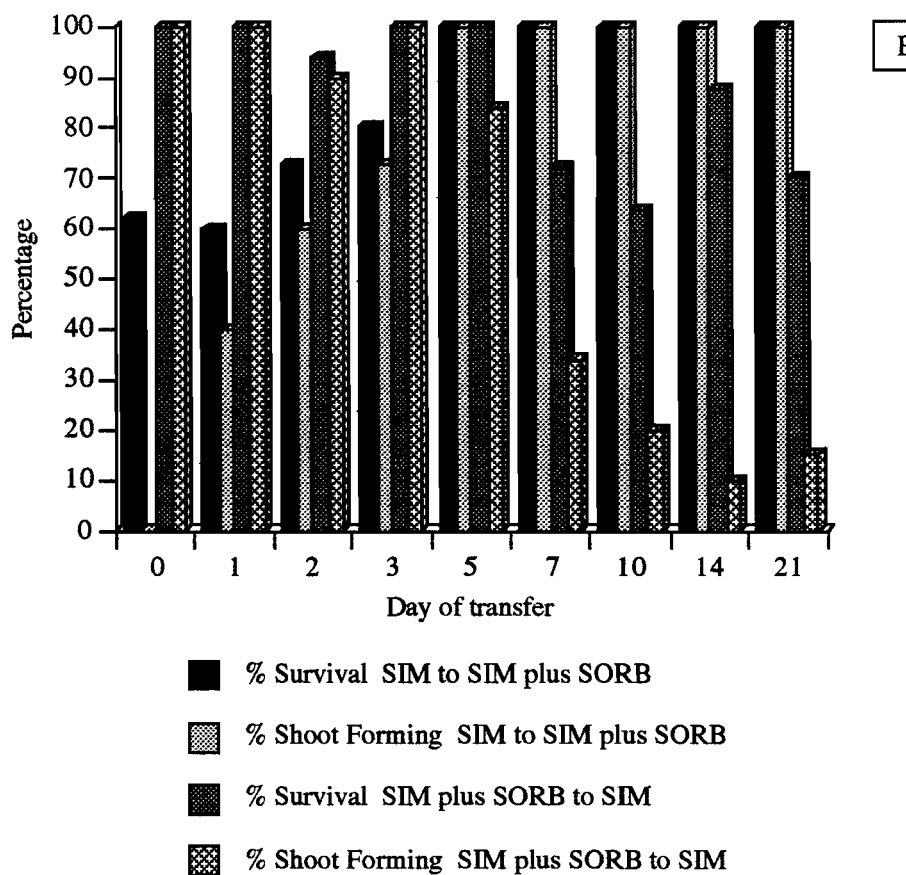
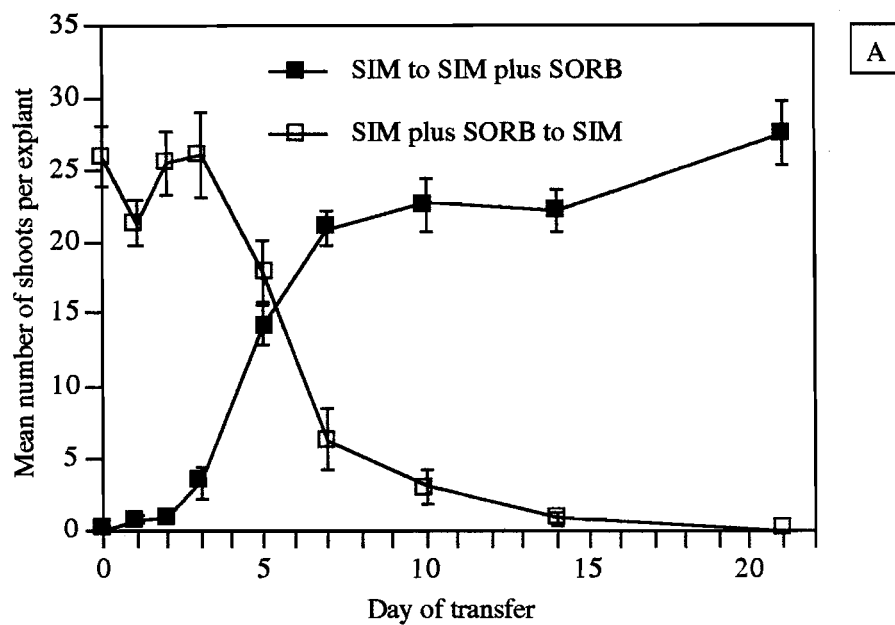


FIGURE 13 [A]- Changes in the number of shoots produced after transfer from SIM to SIM plus sorbitol and from SIM plus sorbitol to SIM alone. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

[B]- The percentage of explants surviving and the percentage forming shoots after transfer from SIM to SIM plus sorbitol and SIM plus sorbitol to SIM alone.

12.2 RIBOSE

12.2.1 Sensitivity to Ribose

The addition of 20g/liter ribose to SIM resulted in the suppression of shoot formation (PLATE 36G). Lower concentrations of ribose did not inhibit shoot formation but as with sorbitol, the shoots produced appeared stunted (PLATES 36E & 36F). Concentrations greater than 20g/liter resulted in significant necrosis of the explant. As with the sorbitol treatment, after 21 days in culture, explants were devoid of shoots when viewed externally (PLATE 36H). The only surface development observed was an occasional nodular structure. Most explants cultured on SIM plus 20g/liter ribose remained green for more than 21 days, but prolonged culture resulted in the gradual senescence of many explants. No basal callus was formed with culture on SIM plus ribose, suggesting ribose may be a more general inhibitor. Histologically explants appeared similar to those cultured on SIM plus sorbitol.

12.2.2 Stage specificity of ribose sensitivity

Transfer of explants to SIM plus ribose (20g/liter) before day 7 of culture inhibited shoot formation. Transfer to SIM plus ribose prior to day 3 resulted in explants almost completely devoid of development. Transfer after 3 or 5 days, although not inducing leafy shoots, did allow the development of a few nodular structures. Transfer on day 7 resulted in small numbers of leafy shoots produced per explant. After day 7 shoot number progressively increased with time on SIM alone (FIGURE 14A). The response of explants upon transfer from SIM to SIM plus ribose was uniform. Greater than 90 percent of explants transferred on day 7 survived after six weeks in culture with greater than seventy percent having at least 1 shoot per explant (FIGURE 14B).

As observed with sorbitol, prolonged culture on SIM plus ribose resulted in the loss of the ability of explants to produce shoots when transferred to SIM alone (FIGURES 14A & 14B). Culture for five days or less had little effect on shoot number, but further culture caused a rapid decline in the number of shoots produced. The percentage of explants surviving after six weeks in culture also declined greatly after 21 days on SIM plus ribose (FIGURE 14B).

12.2.3 Histological examination of the occurrence of starch in ribose treated tissue

Culture on SIM plus ribose although not completely suppressing the accumulation of starch did appear to reduce the level of accumulation observed in hand sections. Sections of explants cultured for 4 days on SIM plus ribose showed prominent starch grains in the cortical, collenchyma and epidermal cell layers, but there appeared to be fewer grains than with culture on SIM alone. Some localized areas of starch deposition were also observed, but these were harder to find and less pronounced than those observed upon culture on SIM alone.

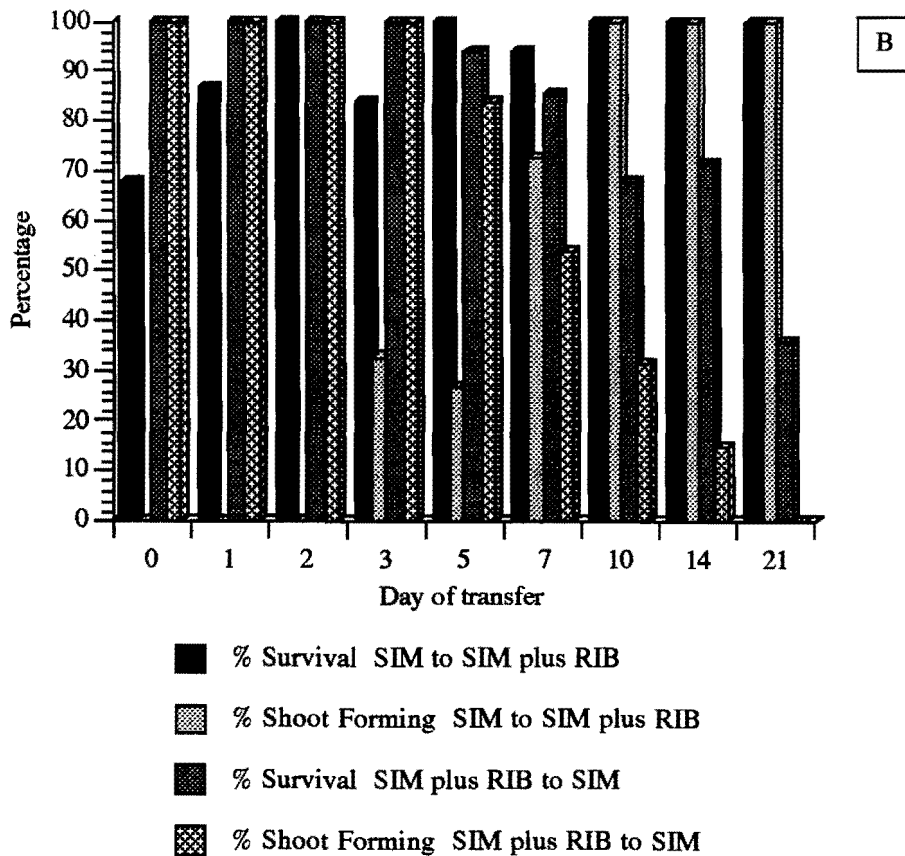
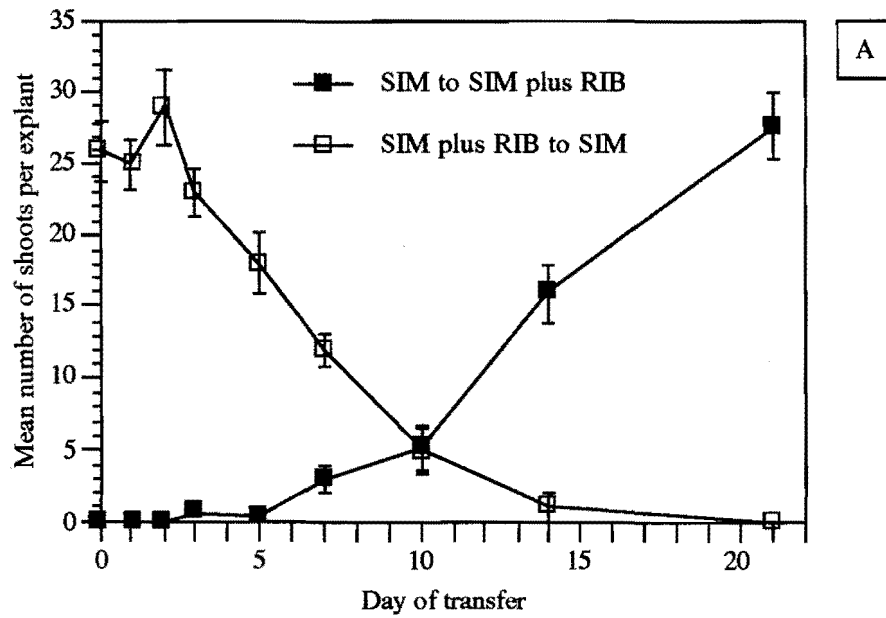


FIGURE 14 [A]- Changes in the number of shoots produced after transfer from SIM to SIM plus ribose, and from SIM plus ribose to SIM alone. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

[B]- The percentage of explants surviving and the percentage forming shoots after transfer from SIM to SIM plus ribose and SIM plus ribose to SIM alone.

12.3 2,3,5-Triiodobenzoic acid (TIBA)

12.3.1 Sensitivity to TIBA

TIBA at a concentration of 4 μ m resulted in complete inhibition of shoot production (PLATE 37C). Explants exposed to lower concentrations of TIBA although showing few shoot primordia after 21 days in culture had developed leafy shoots by 6 weeks. The number and degree of development of shoots formed, was dependent upon the concentration of TIBA to which the explants were exposed. At 2 μ m TIBA shoots formed only at the extreme top of explants, while at lower concentrations shoots formed progressively lower down the explant (PLATES 37A & 37B). Concentrations of TIBA above 6 μ m resulted in death of explants within 14 days. Despite 4 μ m TIBA completely inhibiting shoot production, explants did show some differentiation. In surface view dome-like structures were present (PLATE 37C). These domes appeared smooth and were more rounded than shoot primordia, which were more nodular with distinct protrusions. The domes increased in size with prolonged culture, causing considerable expansion of the explant.

Hand-sections showed the domed structures were derived from the outer tissue layers (PLATE 38D) and as they enlarged they grew away from the explant to which they were attached by a narrow base of explant tissue (PLATE 38E). The outer most cells of the domes stained strongly with toluidine blue. TIBA induced domes in no way resembled shoot primordia, but appeared similar to the compact callus clumps produced on CIM.

12.3.2 Stage specificity of TIBA sensitivity

The stage at which explants were sensitive to TIBA was more difficult to define than with the other inhibitors. TIBA not only inhibited shoot production at 4 μ m, but it also appeared to effect the structure of developing leaves if shoot primordia were transferred to SIM plus TIBA (4 μ m). Transfer from SIM to SIM plus TIBA prior to day 14 suppressed the production of leafy shoots. Even after 21 days pre-culture on SIM, by which time most shoots are determined, exposure of explants to TIBA strongly suppressed leafy shoot production with few normally developed shoots produced per explant (FIGURE 15A). Shoot buds with severely deformed leaf primordia or unexpanded leaves were, however, observed (PLATE 34B).

As normal leafy shoots could not be scored, it was decided to score shoot buds with leaf-like structures to estimate the stage specificity of TIBA. Exposure to SIM plus TIBA prior to day 5 resulted in only domes like those induced by exposure to SIM plus TIBA being produced. At least 5 days exposure to SIM before transfer to SIM plus TIBA was required before small numbers of buds were detected (FIGURE 15A).

In the reciprocal transfer experiments it was apparent that exposure to TIBA had rapid permanent effects on the ability of explants to respond SIM. The number of shoots per explant and the percentage of explants forming shoots, declined rapidly with exposure to TIBA for more than 24 hours (FIGURES 15A & B). No shoots, only basal callus and domes were produced after 7 days exposure to SIM plus TIBA, prior to transfer to SIM alone. Exposure to TIBA briefly or permanently had little effect on explant survival (FIGURE 15B).

12.3.3 Histological examination of starch in TIBA treated tissue

Addition of TIBA to SIM did not inhibit the appearance of starch grains in exposed explants. After 4 days culture on SIM plus TIBA, starch grains were detected throughout the explant with a similar distribution to that seen with exposure to SIM alone. Some localized accumulation of starch grains in epidermal and collenchyma layers was also observed. TIBA would appear to divert the process of shoot production to the formation of domed structures. The formation of these domed structures, like shoot formation, may require some localized deposition of starch grains.

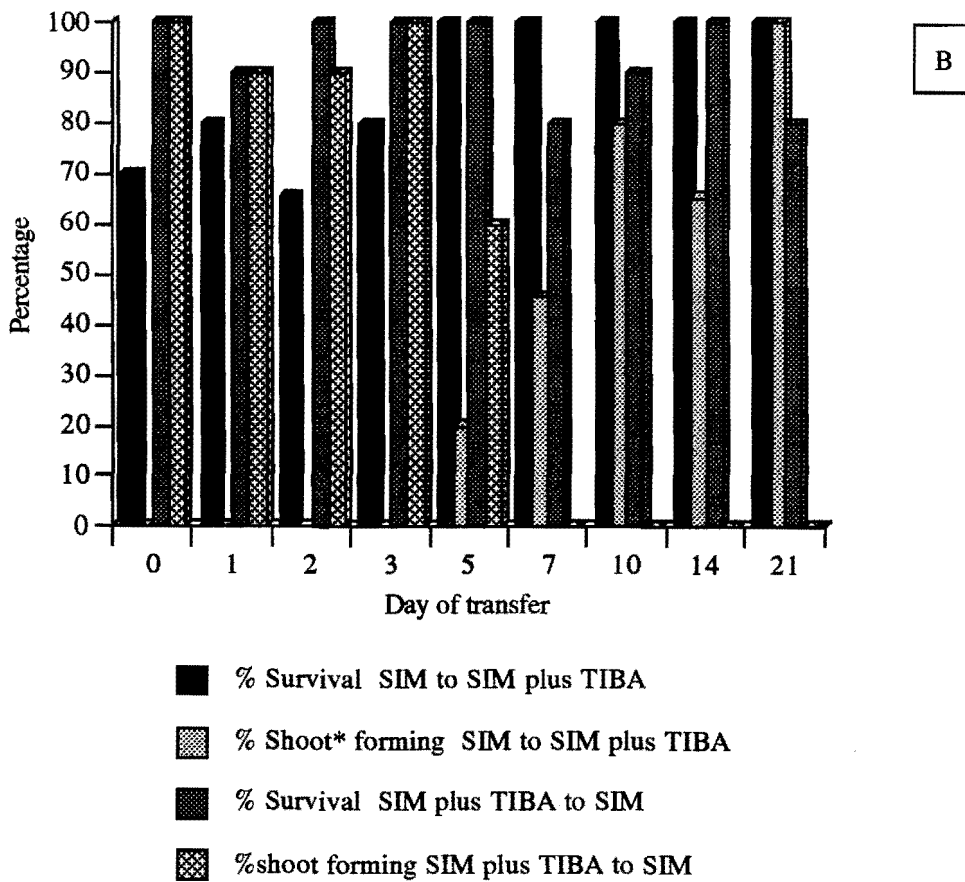
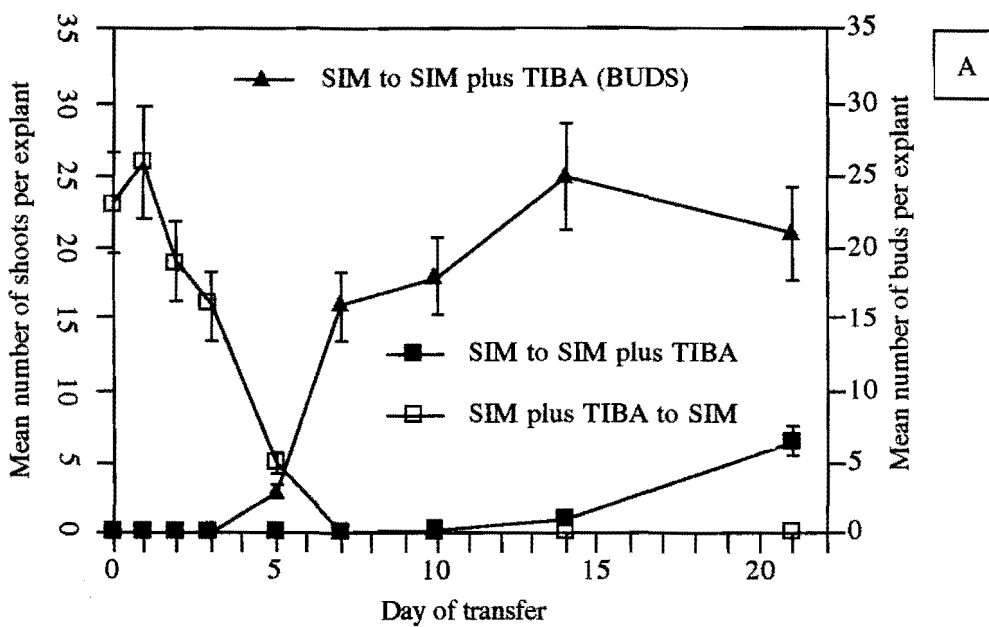


FIGURE 15 [A]- Changes in the number of shoots produced after transfer from SIM to SIM plus TIBA and from SIM plus TIBA to SIM alone. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture. * shoots with normal, expanded leaves.

[B]- The percentage of explants surviving and the percentage forming shoots after transfer from SIM to SIM plus TIBA and SIM plus TIBA to SIM alone.

12.4 ACETYL SALICYLIC ACID

12.4.1 Sensitivity to Acetyl Salicylic Acid

Acetyl salicylic acid (ASA) was a potent inhibitor of shoot formation, showing a substantial reduction in the number of shoots per explant at 10^{-6}M and complete inhibition at $5 \times 10^{-6}\text{M}$ (PLATES 37A & 37B). Higher concentrations of ASA resulted in tissue death and lower concentrations in incomplete inhibition of shoot production (PLATES 37E & 37F). Addition of ASA at $5 \times 10^{-6}\text{M}$ to SIM resulted in complete inhibition of shoot formation while the tissue remained green and healthy with no obvious shoot primordia, although some nodular structures were observed after 21 days in culture. After 6 weeks a high percentage of explants survived, with many showing considerable callus production at the base of the explant in contact with the medium. Examination of sections of ASA ($5 \times 10^{-6}\text{M}$) treated tissue, after 21 days of culture, failed to detect any apex-like structures, but more advanced regions of meristematic activity were observed than seen with culture on either SIM plus sorbitol or ribose (PLATE 38C). Even a few meristematic domes were observed (PLATE 38B).

12.4.2 Stage specificity of ASA sensitivity

ASA was only effective as an inhibitor of shoot formation if explants were exposed prior to day 10 of culture (FIGURE 16A). If explants were moved from SIM to SIM plus ASA ($5 \times 10^{-6}\text{M}$) after 7 days they went on to produce a mean value of greater than one shoot per explant (FIGURE 16A). Despite this, the shoots formed were stunted and less than 50% of explants actually formed shoots (FIGURE 16B). Ten days on SIM before transfer to SIM plus ASA was required to give a definite shoot forming response with all explants producing at least a single healthy shoot (FIGURE 16B). Further culture on SIM before transfer to SIM plus ASA increased the mean number of shoots per explant (FIGURE 16A). Transfer from SIM to SIM plus ASA after 21 days culture, resulted in almost a 300% increase in shoot number compared to transfer after 14 days (FIGURE 16A).

Unlike the effects of both sorbitol and ribose that could be overcome if explants were transferred to SIM early enough, the effects of ASA, like those of TIBA, could not be completely reversed by transfer back to SIM. Transfer from SIM plus ASA to SIM after 7 days resulted in no shoots, but callus production at the basal cut surface of the explant. Transfer at day 7 resulted in callus production with the occasional stunted shoot. The shorter the exposure to ASA the greater the number of shoots produced, but even a three day exposure to ASA resulted a big reduction in

shoot number (FIGURE 16A) and in drastic stunting of the shoots produced. ASA was one of the more interesting inhibitors studied as it is had little effect on explant survival (FIGURE 16B) and allowed callusing, while specifically inhibiting shoot formation.

12.4.3 Histological examination of the occurrence of starch in ASA treated tissue

Hand sections through explants cultured on SIM plus ASA and stained with iodine showed a general accumulation of starch throughout the explant after 4 days of culture. Levels appeared somewhat less than when cultured on SIM alone and fewer areas of localized starch accumulation were observed in the epidermal and collenchyma regions, but ASA had only a minimal effect on the initial accumulation of starch as observed with culture on SIM alone.

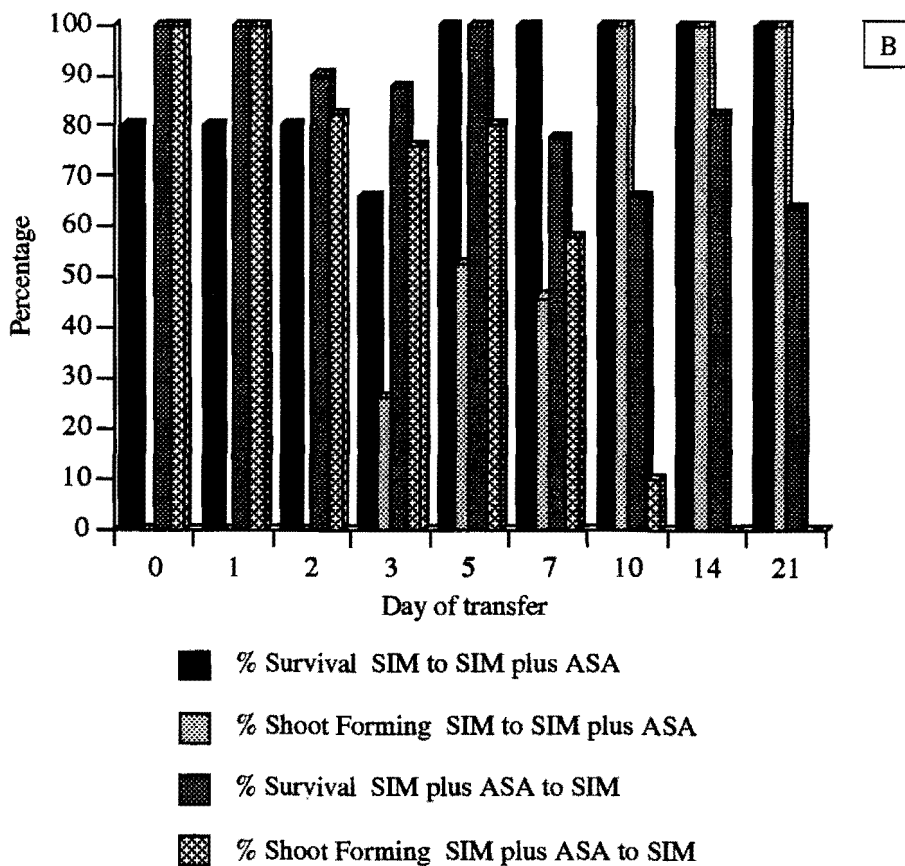
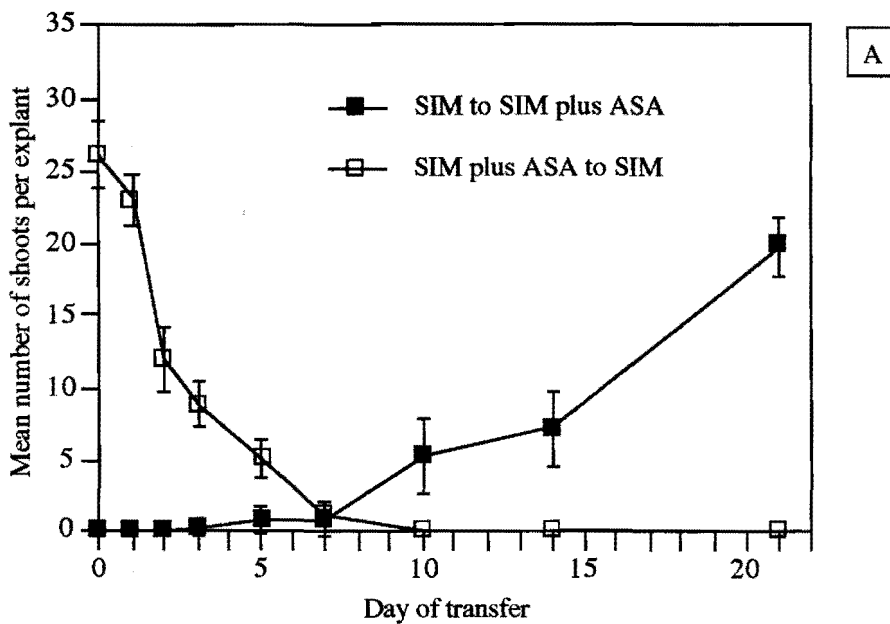


FIGURE 16 [A]- Changes in the number of shoots produced after transfer from SIM to SIM plus ASA and from SIM plus ASA to SIM alone. Mean value plus or minus 1 standard error, n=30. Shoots were scored after a total of 6 weeks in culture.

[B]- The percentage of explants surviving and the percentage forming shoots after transfer from SIM to SIM plus ASA and SIM plus ASA to SIM alone.

PLATE 36: The effect of inhibitors of caulogenesis on cultured petiole explants of *B. erythrophylla*

[A]- Explants after 6 weeks culture on SIM plus 10g/l sorbitol.
Note the stunted shoots.

[B]- Explants after 6 weeks culture on SIM plus 20g/l sorbitol.

[C]- Explants after 6 weeks culture on SIM plus 30g/l sorbitol.
Note little basal callus production.

[D]- Explant after 21 days on SIM plus 30g/l sorbitol.
Note the explant is still green with no shoots evident.

[E]- Explants after 6 weeks culture on SIM plus 5g/l ribose.

[F]- Explants after 6 weeks culture on SIM plus 10g/l ribose.
Note the stunted shoots.

[G]- Explants after 6 weeks culture on SIM plus 20g/l ribose.
Note no basal callus production.

[H]- Explant after 21 days on SIM plus 20g/l ribose.
Note the explant is still green with only the cut surfaces browning. No shoots are evident.

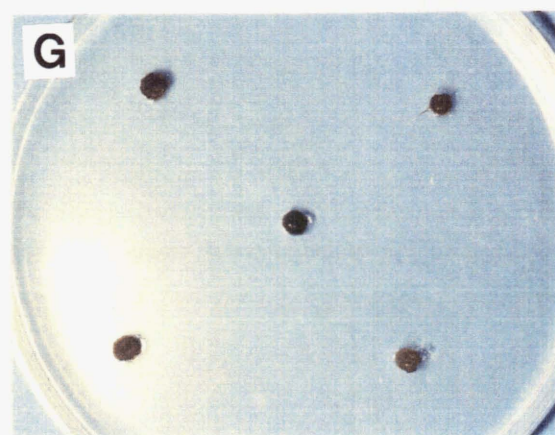
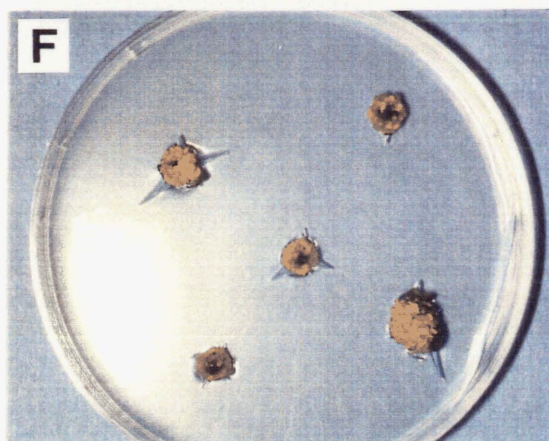
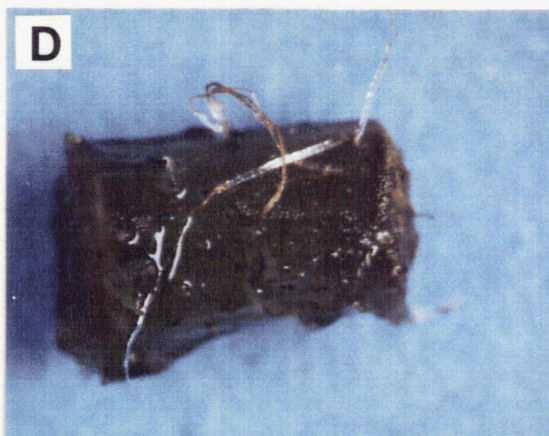
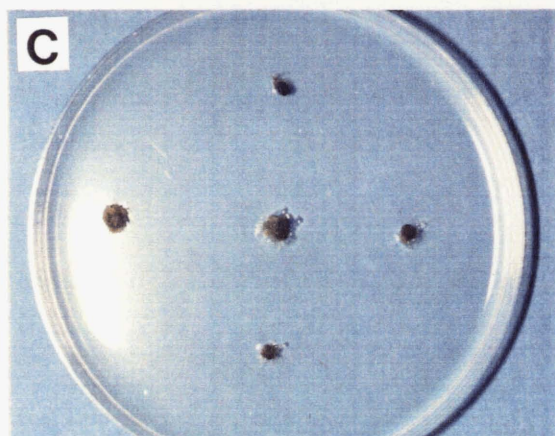
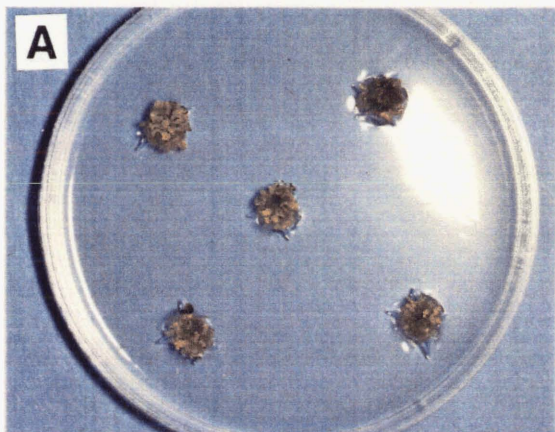


PLATE 37: The effect of inhibitors of caulogenesis on cultured petiole explants of *B. erythrophylla*

- [A]- Explant after 6 weeks culture on SIM plus 1 μ m TIBA.
Note callus formation at the base of the explant and shoots at the top.
- [B]- Explant after 6 weeks culture on SIM plus 2 μ m TIBA.
Note only stunted shoots are produced at the top of the explant.
- [C]- Explant after 6 weeks culture on SIM plus 4 μ m TIBA.
Note only small domed structures are produced.
- [D]- Explant after 6 weeks culture on SIM plus 6 μ m TIBA.
- [E]- Explant after 6 weeks culture on SIM plus 10⁻⁷M ASA.
- [F]- Explant after 6 weeks culture on SIM plus 5x10⁻⁷M ASA.
- [G]- Explant after 6 weeks culture on SIM plus 10⁻⁶M ASA.
Note the stunted shoots.
- [H]- Explant after 6 weeks on SIM plus 5x10⁻⁶M ASA.
Note basal callus is still produced while no shoots are evident.

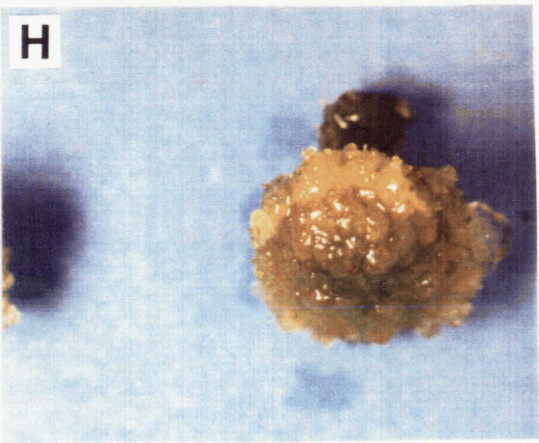
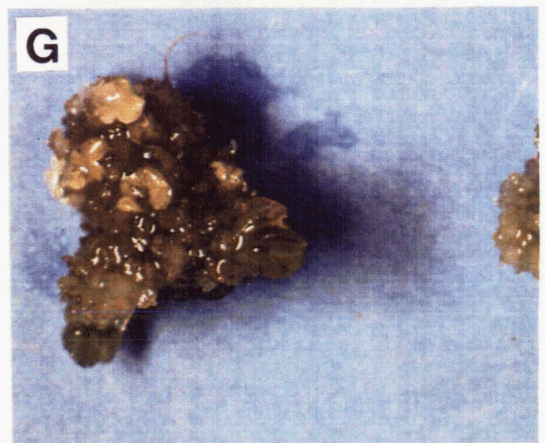
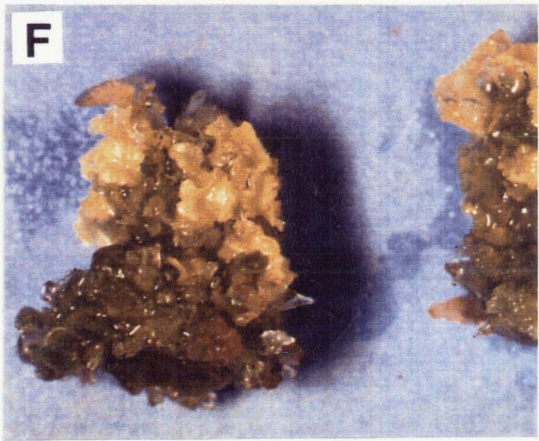
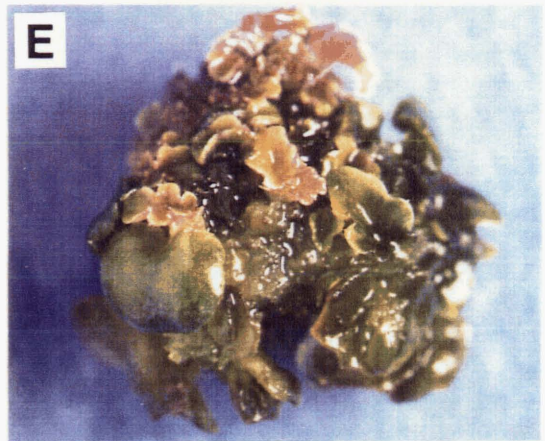
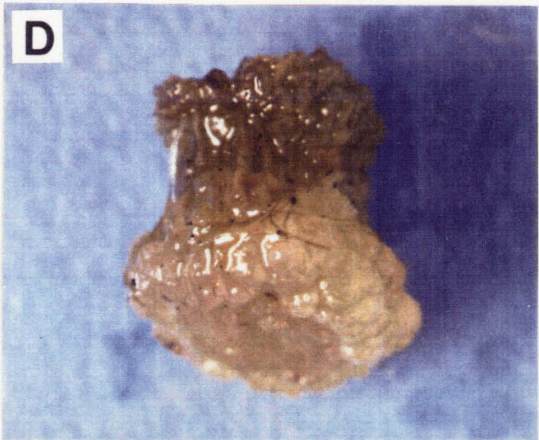
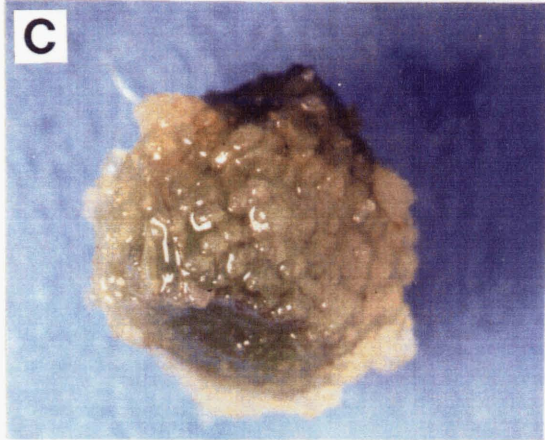
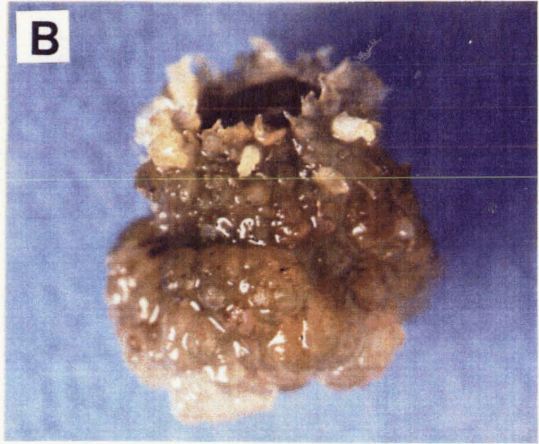
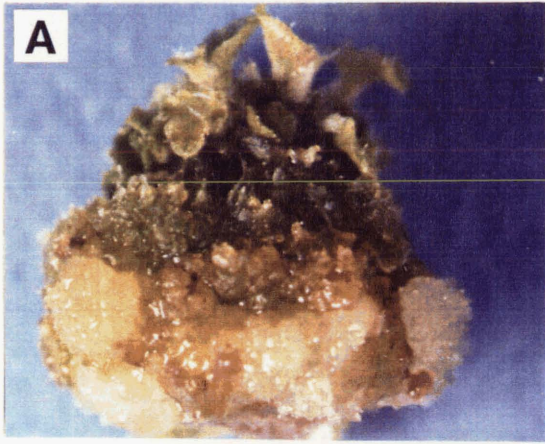
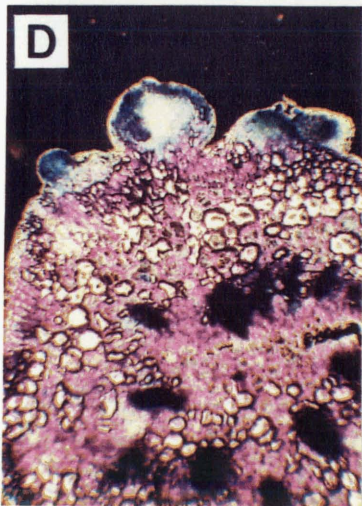
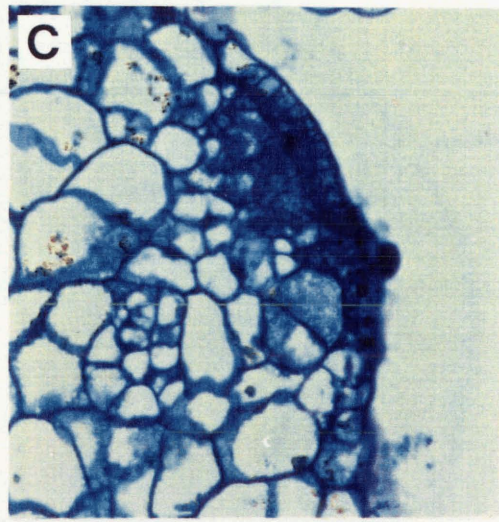
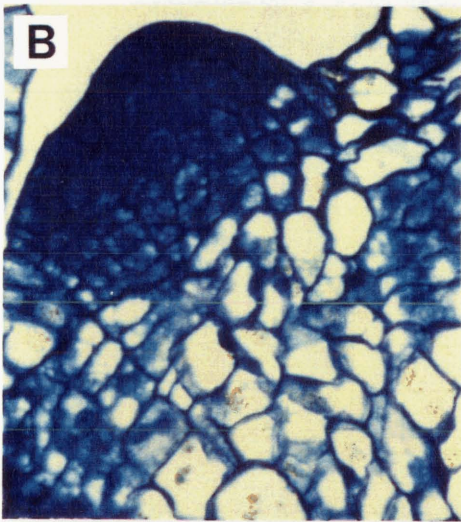
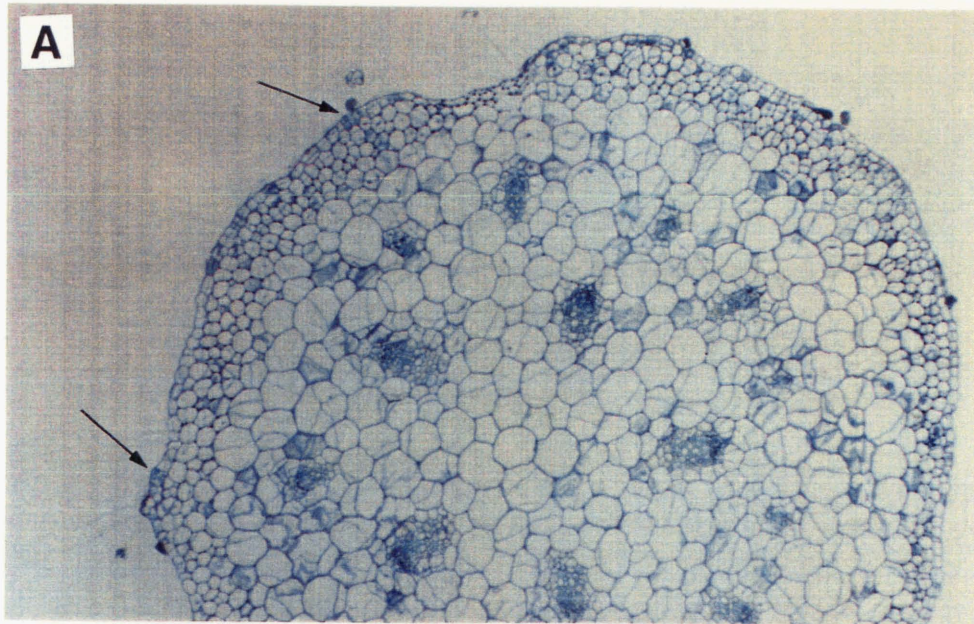


PLATE 38: The histological effects of inhibitors of caulogenesis on cultured petiole explants of *B. erythrophylla*

- [A]-** Transverse section through a petiole explant cultured for 14 days on SIM plus 30g/l sorbitol. Note the random cell divisions throughout the explant and the small meristematic regions (arrows) associated with glandular hairs (x25).
- [B]-** Transverse section through the upper half of a petiole explant cultured for 14 days on SIM plus 5×10^{-6} M ASA, showing a well developed meristematic dome. Note meristematic domes were generally the most advanced structures observed (x120).
- [C]-** Transverse section through the lower half of a petiole explant cultured for 14 days on SIM plus 5×10^{-6} M ASA, showing a meristematic region of epidermal origin (x120).
- [D]-** Transverse handsection through a petiole explant cultured for 21 days on SIM plus 4 μ m TIBA. Note the domes of cells produced from the superficial layers of the explant (x17).
- [E]-** Transverse handsection through a petiole explant cultured for 6 weeks on SIM plus 4 μ m TIBA. Note the domes have enlarged, and are growing away from the explant to which they are attached by a narrow bridge of tissue. The outer dividing cells stain most intensely. No shoots are evident (x17).



(13) **THE EFFECT OF INHIBITORS ON PROTEIN SYNTHESIS AND ACCUMULATION**

Explants exposed to SIM plus sorbitol or ribose respectively, showed similar levels of total protein and protein synthesis after 14 days in culture (TABLE 11). Both the protein content and the level of protein synthesis of explants cultured on either medium was lower than that measured with culture on SIM alone, but compared to day 0 tissue, the protein content had increased by about 50%, with the level of protein synthesis remaining about the same (TABLE 11).

Culture on SIM plus TIBA or ASA respectively, resulted in a higher level of total protein than observed with culture on SIM plus ribose or SIM plus sorbitol, but the levels were slightly less than observed with culture on SIM alone (TABLE 11). Explants cultured on SIM plus ASA had a slightly higher level of protein synthesis than those cultured on SIM alone, while those on SIM plus TIBA, had a slightly lower level (TABLE 11).

Although inhibiting shoot formation, none of the above inhibitors suppressed protein synthesis.

| | Culture medium | | | | |
|--|----------------|-------|-------|-------|-------|
| | SIM | RIB | ASA | TIBA | SORB |
| Protein ug/mg fresh weight. | 2.34 | 1.44 | 1.94 | 2.10 | 1.52 |
| Uptake, ³⁵ S counts per minute/mg fresh weight. | 25690 | 20910 | 20133 | 23169 | 20550 |
| Incorporation, ³⁵ S counts per minute/mg (FW). | 8230 | 5587 | 9230 | 7405 | 5717 |

TABLE 11 The mean protein content, uptake of [³⁵S]-methionine, and percentage of [³⁵S]-methionine incorporated into protein, from explants exposed for 14 days to inhibitory media. n=6 with values pooled from 2 separate extractions.

(14) **THE EFFECT OF INHIBITORS OF SHOOT FORMATION ON THE PATTERN OF POLYPEPTIDES OBSERVED UNDER SHOOT FORMING CONDITIONS**

14.1 2D-Electrophoresis: Protein accumulation in the presence of inhibitors of shoot formation

Inhibition of shoot formation by the inclusion of inhibitory substances in SIM resulted in pronounced changes in the pattern of polypeptides normally detected after culture on SIM alone. All four inhibitors effected the pattern of polypeptides to a greater or lesser extent. Ribose, ASA and sorbitol primarily effected the expression of group 4, 5 and 6 polypeptides, with few major effects on group 1 polypeptides. In contrast, TIBA not only effected polypeptides associated with organogenesis, but also induced major changes in the abundance of polypeptides associated with basic metabolism. Table 12 summarizes the changes in the pattern of silver stained polypeptides observed after 14 days culture on SIM, resulting from the addition of inhibitors.

14.1.1 The effects of ribose or sorbitol

Both ribose and sorbitol had very similar effects on the polypeptide pattern associated with shoot induction and development. The most marked effects of both inhibitors were on the SIM only polypeptides of group 6. Both ribose and sorbitol completely inhibited the accumulation of 10 of the 11 SIM only polypeptides (PLATES 41A & 41B). Only polypeptide number 378 from this subgroup was detected, but at a reduced level (PLATES 41A & 41B). Seven of the polypeptides in this subgroup were first detected after 14 days culture on SIM and as the effects of both ribose and sorbitol appear to occur prior to day 7, the absence of these polypeptides might have been expected. On SIM, polypeptides 376, 377, 378 and 401 of this subgroup were first detected at the point of minimal determination (day 7) and increased in abundance with prolonged culture. The absence of polypeptides 376, 377 and 401 supports the timing of inhibition with both inhibitors suppressing the accumulation of polypeptides normally detected after 7 days on SIM. The absence of polypeptides 376, 377 and 401 further suggests that they are associated with a caulogeneic event as they do not accumulate with culture on medium containing all the requirements for shoot formation, but on which shoot formation has been suppressed chemically. The presence of polypeptide 378 suggests that it may be the result of culture on medium containing SIM growth regulators and not the result of shoot

induction. This is further supported by its presence in explants cultured on SIM(-), where shoot formation is suppressed by the absence of sucrose. The level of accumulation of 378 on these media is, however, very low and incomplete inhibition cannot be ruled out.

Polypeptides 108, 388 and 389 that were detected when explants were exposed to either SIM, RIM or CIM were unaffected by addition of ribose or sorbitol to SIM. These polypeptides were detected in explants cultured on any medium promoting cell division. A few poorly developed meristematic regions were observed with culture on both SIM plus ribose or sorbitol. Neither inhibitor completely stopped cell division, therefore polypeptides associated with cell division may not, at least initially, have been inhibited by either sorbitol or ribose.

Two other polypeptides in group 6, 382 and 383, that would normally have disappeared after 14 days on SIM, remained prominent after 14 days culture on SIM plus ribose or sorbitol respectively (PLATES 39A & 39B). These polypeptides were induced by day 3 of culture on SIM, RIM or CIM respectively, but normally would have begun to decline by day 7 on SIM. Addition of either sorbitol or ribose to SIM resulted in the inhibition of events that would normally have taken place after 2 or 5 days culture respectively. As both polypeptides were still detected at day 7, the event/events leading to their decline was most probably inhibited and resulted in their increased abundance at day 14 in the presence of these inhibitors. The retention of these 2 polypeptides, at increased levels, further supports their loss as an important event during caulogenesis and as neither inhibitor stopped the accumulation of these 2 polypeptides, supports the view that the inhibitory effects of sorbitol and ribose effect only some aspects of organogenesis.

Addition of ribose or sorbitol also affected changes induced by SIM to existing polypeptides. Polypeptides 288 and 366 that increased upon exposure to SIM alone, failed to do so if ribose or sorbitol was added to SIM. Furthermore, the transient accumulation of five polypeptides (358, 359, 381, 384, 341) was prolonged by addition of ribose or sorbitol (PLATES 39A & 39B).

As well as the above effects on changes associated with shoot organogenesis, ribose and sorbitol effected the accumulation of two group one polypeptides. Polypeptide, 324, was completely absent on ribose treated tissue, while polypeptide, 272, increased greatly when cultured on either SIM plus sorbitol or ribose.

14.1.2 The effect of ASA

Like both ribose and sorbitol, explants were only sensitive to ASA if exposed prior to day 7. ASA also inhibited the induction/accumulation of most the SIM only group 4 polypeptides (PLATE 40B). Only polypeptide 378 was clearly detectable, with, 376 and 377 faintly detectable on some gels (PLATE 40B). Again this suggests that most group 6, SIM only, polypeptides are involved in the specialization of the shoot primordia rather than in the induction/determination phase. The faint detection of polypeptides 376 and 377 on some gels was most probably the result of the formation of the occasional shoot primordium, which fails to develop, as the levels of both polypeptides were very low. Yet as the exact timing of ASA inhibition cannot be clearly distinguished between days 7 or 10, it is possible that ASA allows the low level expression of SIM only polypeptides detected detected prior to day 10 on SIM.

Unlike both sorbitol and ribose, ASA did not inhibit the loss of the 2 transient polypeptides (382 and 383) on SIM (PLATE 40A). ASA also failed to completely inhibit the increase of the 2 polypeptides that increased in abundance only on SIM (PLATE 40B). Both these increases were inhibited by both ribose and sorbitol but still increased slightly on SIM plus ASA. Five polypeptides, 381, 384, 341, 356 and 359 that increased only temporarily on SIM alone and then decreased, were retained at high levels on SIM plus ASA. SIM plus ASA had no significant effect on group 1 polypeptides with similar levels of expression as seen with culture on SIM for 14 days.

ASA would appear to be a more specific inhibitor of caulogenesis than sorbitol or ribose, allowing some events associated with the early stages of caulogenesis to occur while inhibiting those involved in the latter stages.

14.1.3 The effect of TIBA

Unlike both ribose, sorbitol and ASA, culture on SIM plus TIBA had marked effects on the group 1 polypeptides. One of the most significant effects was a large increase in the abundance of polypeptide 272 (PLATE 40A), to the extent that it stained negatively (the center of the spot failed to stain black). Numerous other polypeptides in this region also appeared to increase greatly in abundance but their proximity to each other made definite identification of each polypeptide impossible. The molecular weights of these polypeptides were all similar and coincided with a large increase in 28kd band observed in a 1D analysis of TIBA exposed explants (Data not presented). TIBA like ribose also caused the loss of polypeptide 324, which was clearly detectable with culture on SIM, RIM, SIM plus sorbitol or SIM plus ASA respectively.

As with culture on the other inhibitory media the SIM only polypeptide 378 could also be detected. All other SIM only polypeptides were not reliably detected (PLATE 40A). Polypeptides which increased transiently on SIM remained at high levels with the addition of TIBA. Particularly prominent were the polypeptides 382 and 383 both of which continued to increase in abundance to very high levels (PLATE 40A). All the general changes associated with culture were induced by culture on SIM plus TIBA. TIBA had the greatest effect on the polypeptide pattern normally detected after 14 days on SIM alone.

PLATE 39: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) explants cultured for 14 days on SIM plus 30g/l sorbitol.

(B) explants cultured for 14 days on SIM plus 20/l ribose.

Arrows indicate polypeptides which are normally absent, or present at much lower levels after 14 days culture on SIM alone.

Open symbols indicate the presence/absence of polypeptides normally detected after 14 days culture on SIM alone.

Arrow heads indicate reference polypeptides for comparison of gels.

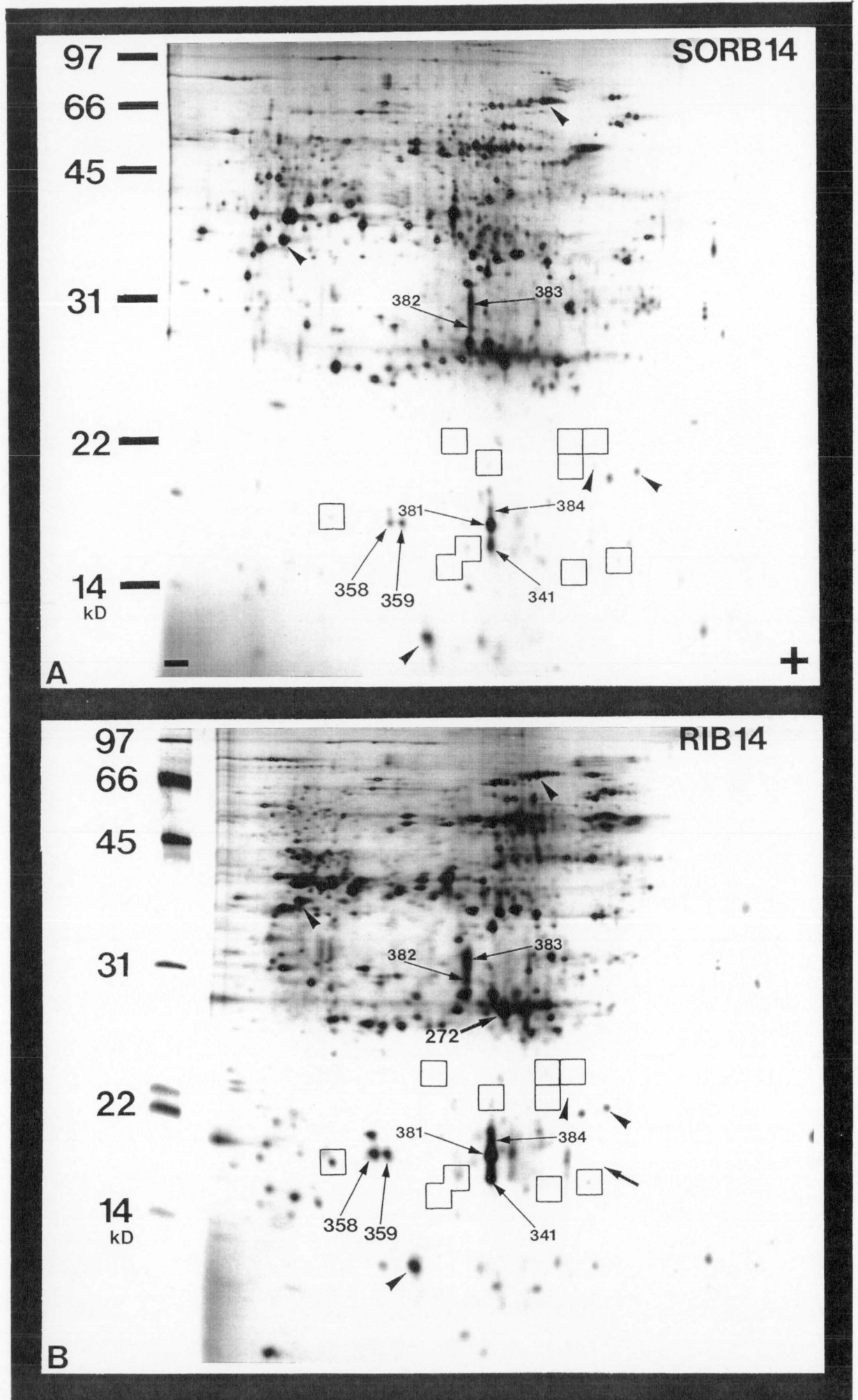


PLATE 40: Silver-stained two-dimensional PAGE gels of protein extracted from

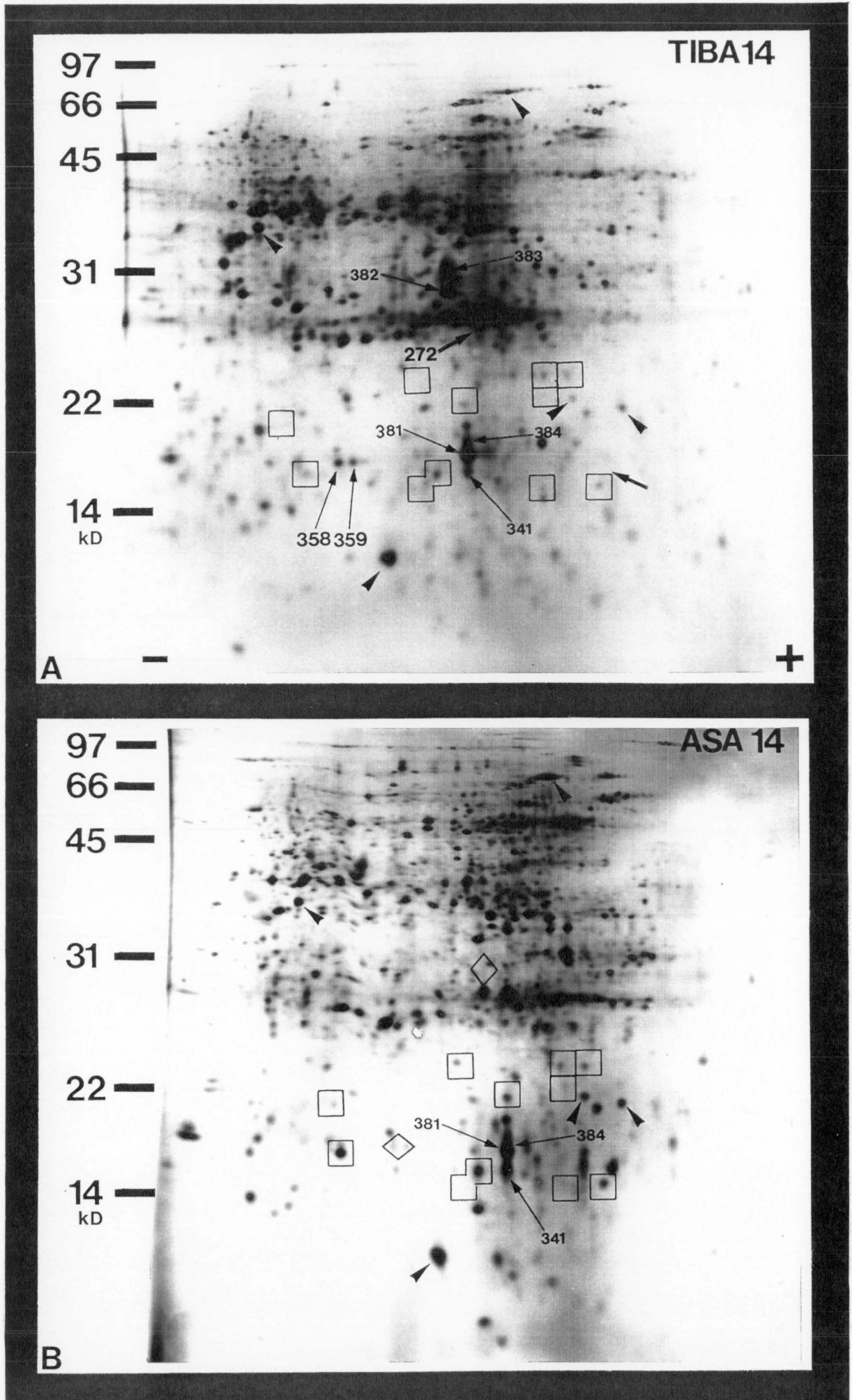
(A) explants cultured for 14 days on SIM plus 4 μ m TIBA.

(B) explants cultured for 14 days on SIM plus 5 $\times 10^{-6}$ M ASA.

Arrows indicate polypeptides which are normally absent, or present at much lower levels after 14 days culture on SIM alone.

Open symbols indicate the presence/absence of polypeptides normally detected after 14 days culture on SIM alone.

Arrow heads indicate reference polypeptides for comparison of gels.



| Behavior | RIB 14 | ASA 14 | TIBA 14 | SORB 14 | SIM 14 | SIM(-) 14 |
|--|------------------|------------------|-------------------|------------------|--|------------------|
| (1) Basic metabolism affected by addition of inhibitor. | 272+, <u>324</u> | None | 272++, <u>324</u> | 272+ | N/A | <u>324</u> |
| (4) Changes to polypeptides detected at day 0, not induced by basal medium | | | | | | |
| A- SIM, RIM & CIM | 266-, 267- | 266-, 267- | 266-, 267- | 266-, 267- | 266-, 267- | No decline |
| B- SIM only | | 288+, 366+ | 288+ | | 288+, 366+ | |
| C- Permanent on RIM and CIM but transient on SIM | 358+, 359+ | | 358+, 359+ | 358, 359 | 343+ | 358+, 359+, 343? |
| (5) Changes to polypeptides detected at day 0, induced permanently by RIM, CIM and BM, but transient on SIM. | 381+, 384+, 341+ | 381+, 384+, 341+ | 381+, 384+, 341+ | 381+, 384+, 341+ | | 381+, 384+, 341+ |
| (6) Polypeptides not detected at day 0, and not induced by basal media | | | | | | |
| A- RIM, SIM & CIM | 108, 388, 389 | 108, 388, 389 | 108, 388, 389 | 108, 388, 389 | 108, 388, 389 | 108, 388, 389 |
| B- SIM only | 378 | 376, 377, 378 | 377, 378 | 378 | 329, 330, 348, 364, 376, 377, 378, 390, 391, 401, 402. | 378 |
| C- Permanent on RIM and CIM but transient on SIM. | 382, 383 | | 382, 383 | 382, 383 | | 382, 383 |

KEY (-) decreased from day 0 level
 (+) increased from day 0 level
 (++) very large increase
 (*ITALIC*) faintly detectable on some gels
 (underlined) no longer detectable
 (?) effect could not be determined
 (N/A) not applicable.

TABLE [11] Summary of the changes in the pattern of silver stained polypeptides observed after 14 days culture on SIM, resulting from the addition of the inhibitors Ribose (RIB), TIBA, ASA, or Sorbitol (SORB) to SIM, or the omission of sucrose from SIM, (SIM(-)).

14.2 2D-Electrophoresis: *in vivo* protein synthesis in the presence of inhibitors of shoot formation

The effects of inhibitors on the pattern of polypeptide synthesis normally observed after 14 days on SIM essentially paralleled those observed with silver staining (TABLES 12 & 13). The inhibitors primarily suppressed the synthesis of polypeptides specifically associated with shoot formation and those present at day zero that increased in response to the shoot forming stimulus (PLATES 41A, 41B, 42A & 42B).

Polypeptides transiently expressed with culture on SIM retained significant levels of synthesis in the presence of inhibitors, with some having greatly increased levels of synthesis. Polypeptide A, which increased in synthesis only on RIM was also synthesized at high levels in the presence of RIB, SORB or ASA, while remaining at a low level in the presence of TIBA (PLATES 41A, 41B, 42A & 42B). Only 2 SIM induced changes in synthesis occurred with culture on SIM plus ASA, polypeptide 343 showed a higher level of synthesis than in the presence of the other inhibitors, while polypeptide 383 had a lower level of synthesis in the presence of ASA than with the other inhibitors.

TIBA was the only inhibitor to induce the synthesis of a specific polypeptide. With culture on SIM plus TIBA, a 12 kd polypeptide (T) was detected that was not detectable in any of the other treatments (PLATE 42A).

PLATE 41: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 14 days on SIM plus 30g/l sorbitol.

(B) explants cultured for 14 days on SIM plus 20/l ribose.

Arrows indicate polypeptides which are not normally synthesized, or synthesized at much lower levels after 14 days culture on SIM alone.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling.

Open symbols indicate the presence/absence of polypeptides normally synthesized after 14 days culture on SIM alone.

Arrow heads indicate reference polypeptides for comparison of gels.

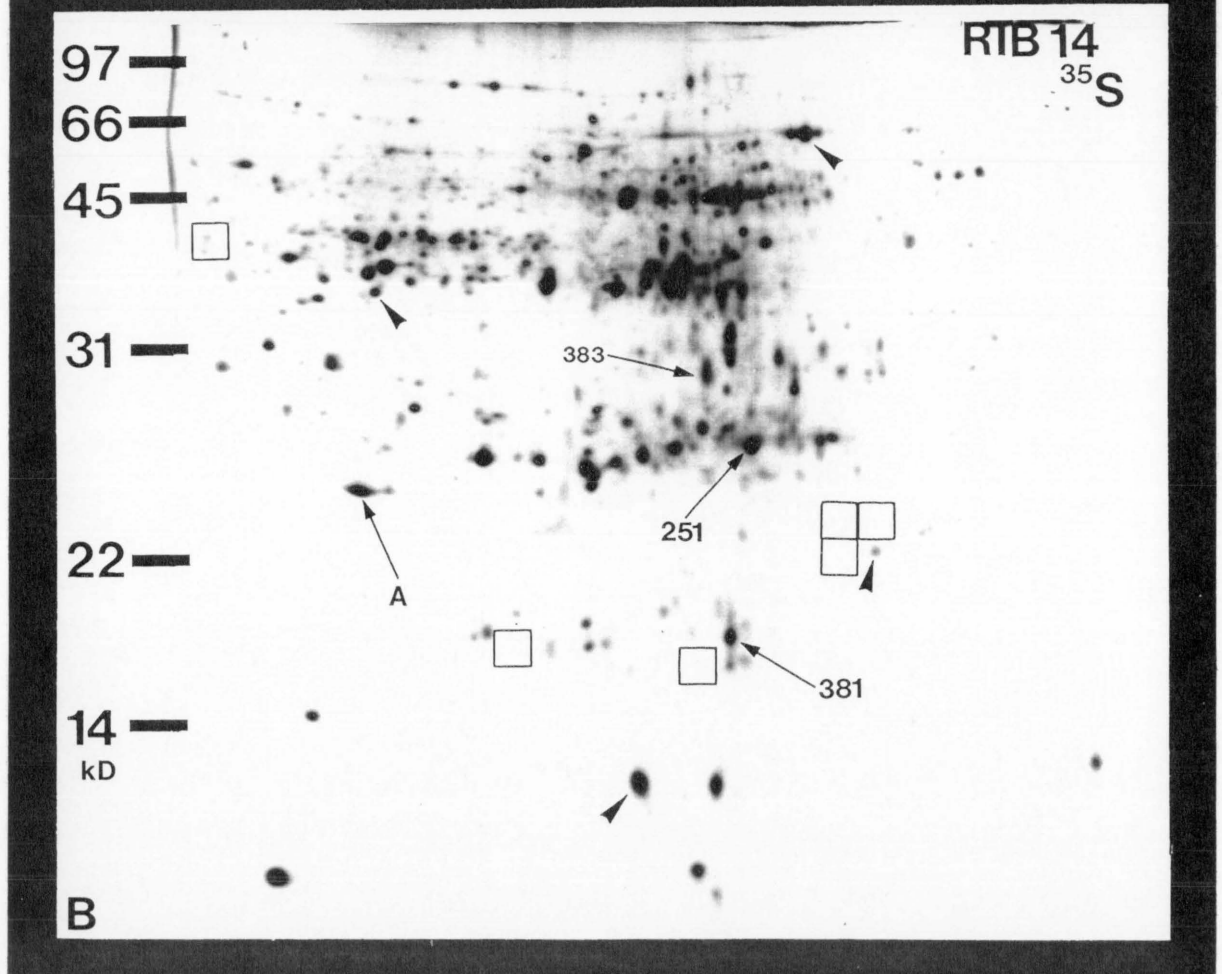
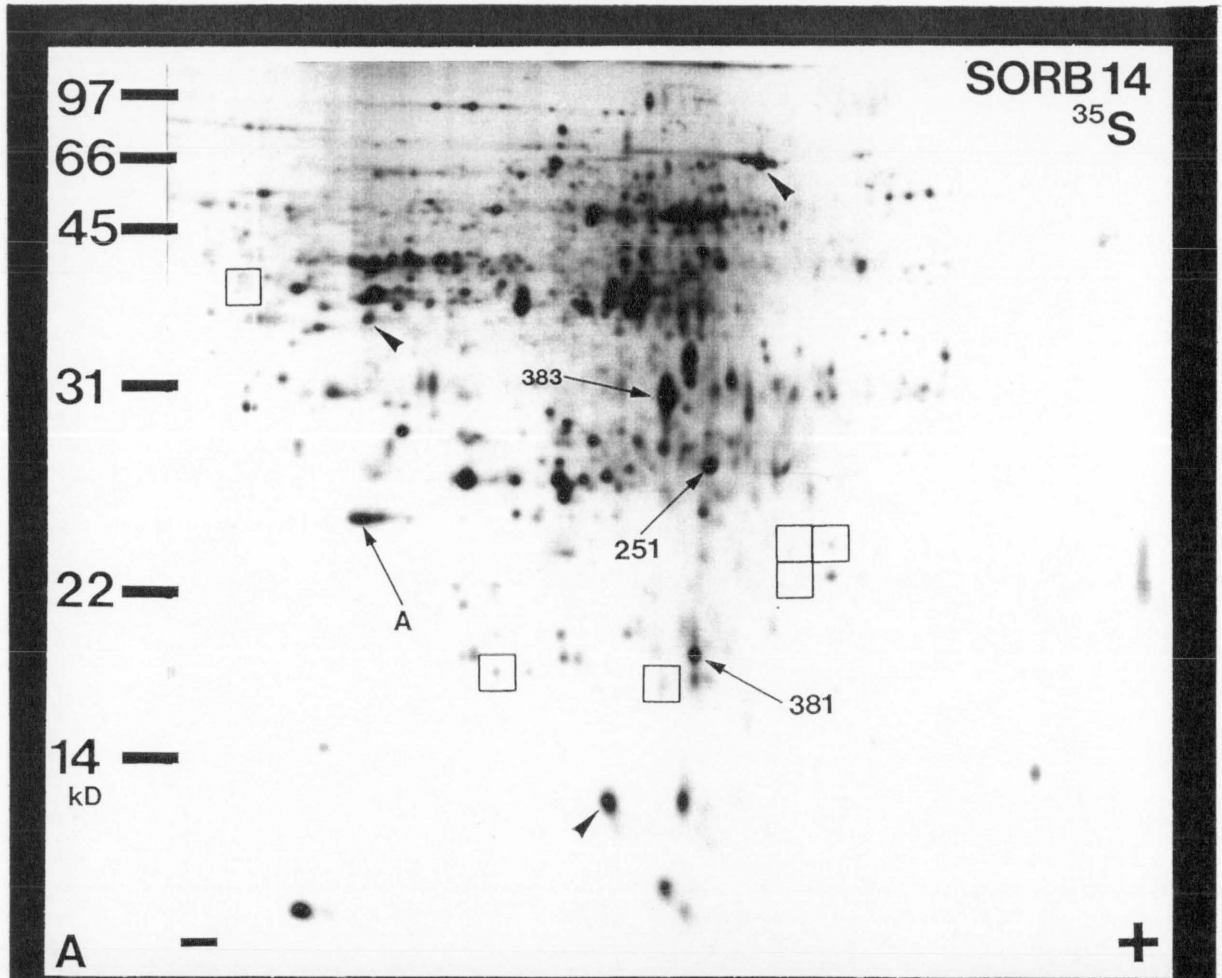


PLATE 42: Fluorographs of two-dimensional PAGE separations of *in vivo* labelled protein extracted from

(A) explants cultured for 14 days on SIM plus 4 μ M TIBA.

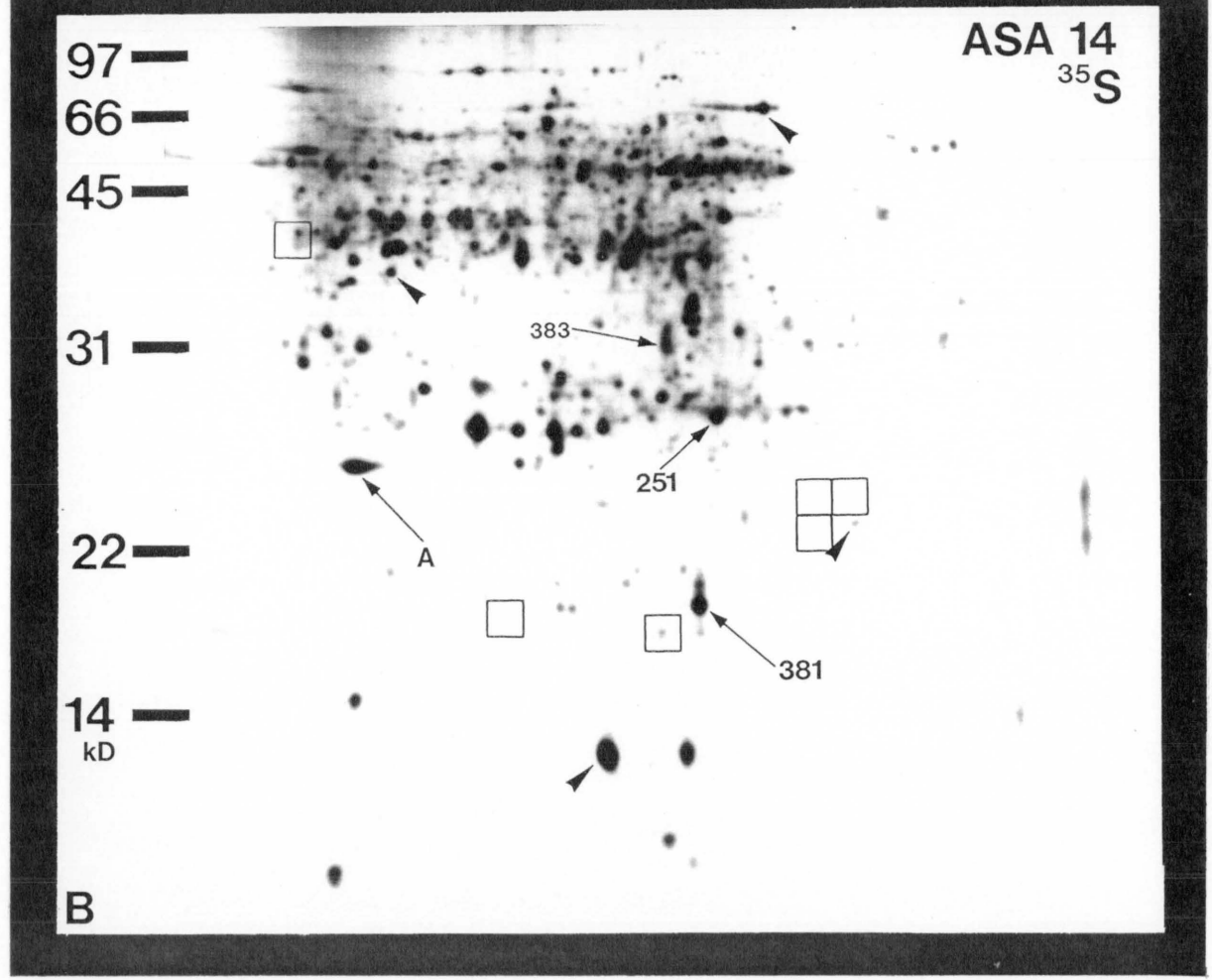
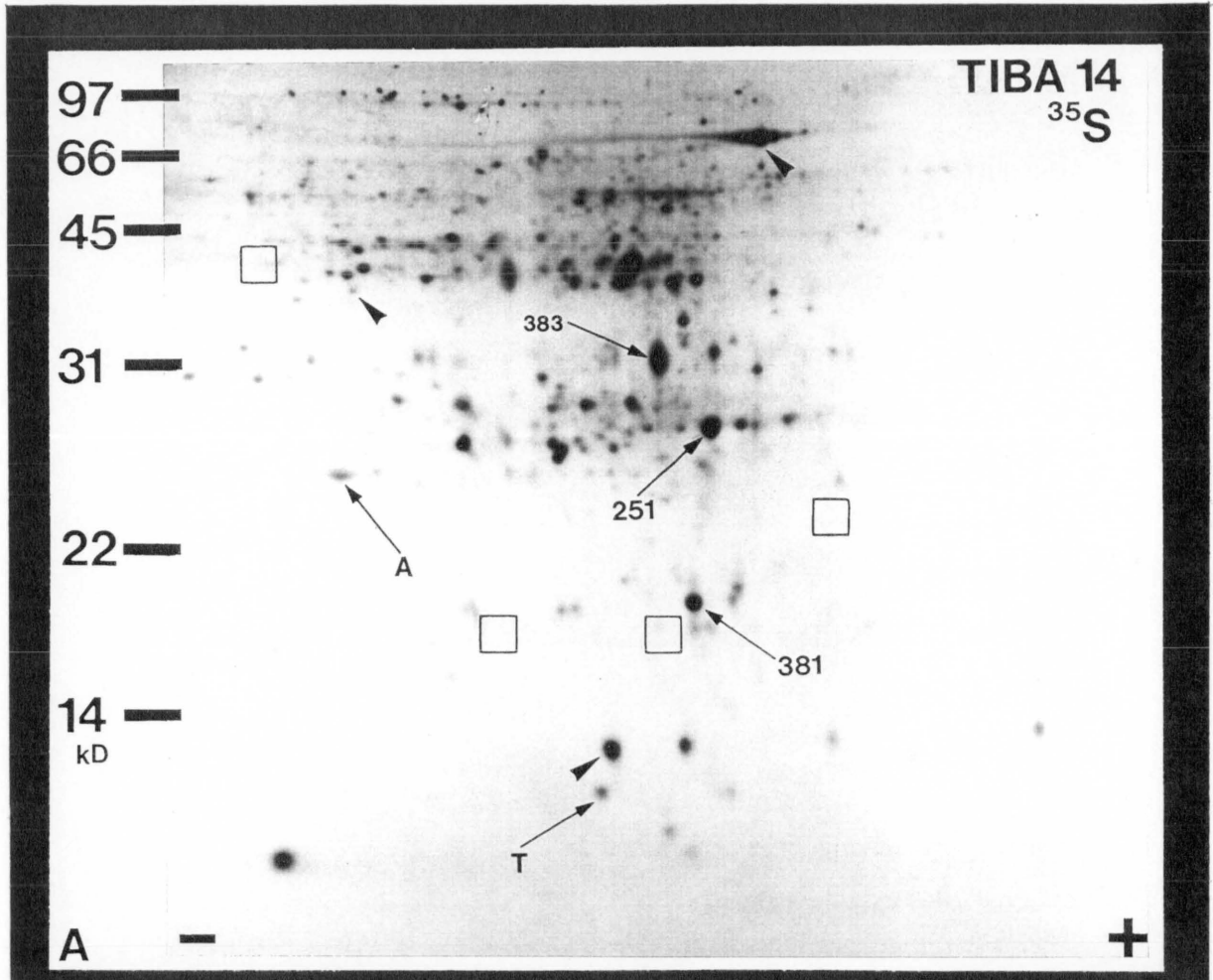
(B) explants cultured for 14 days on SIM plus 5 $\times 10^{-6}$ M ASA.

Arrows indicate polypeptides which are not normally synthesized, or synthesized at much lower levels after 14 days culture on SIM alone.

Numbered arrows indicate polypeptides detected by silver-staining, while letters indicate polypeptides only detected with *in vivo* labelling. Polypeptide T is only synthesized with culture on SIM plus TIBA.

Open symbols indicate the presence/absence of polypeptides normally synthesized after 14 days culture on SIM alone.

Arrow heads indicate reference polypeptides for comparison of gels.



| Behavior | RIB 14 | ASA 14 | TIBA 14 | SORB 14 | -SIM 14 | SIM(-) 14 |
|---|--------|------------|---------|---------|----------------|-----------|
| (1) Basic metabolism affected by addition of inhibitor. | 251+ | 251+ | 251+ | 251+ | N/A | 251+ |
| (4) Changes to polypeptides synthesized at day 0, not induced by basal medium | | | | | | |
| A- SIM only | | 343+ | | | 366+, 343+ | 343? |
| B- RIM only | A+ | A+ | | A+ | | A+ |
| (5) Changes to polypeptides synthesized at day 0, induced permanently by RIM, CIM and BM, but transient on SIM. | 381+ | 381+ | 381+ | 381+ | | 381+ |
| (6) Polypeptides not synthesized at day 0, and not induced by basal media | | | | | | |
| A- RIM, SIM & CIM | 108 | 108 | 108 | 108 | 108 | 108 |
| B- SIM only | | | | | 401+, F+ | |
| - SIM+ | | | | | 376+, 377+, G+ | |
| C- Permanent on RIM and CIM but transient on SIM. | 383+ | <i>383</i> | 383++ | 383++ | | 383+ |

KEY (-) decreased from day 0 level
 (+) increased from day 0 level
 (++) very large increase
 (*ITALIC*) faintly detectable
 (?) effect could not be determined
 (N/A) not applicable.

TABLE [12] Summary of the changes in the pattern of *in vivo* labelled polypeptide observed after 14 days culture on SIM, resulting from the addition of the inhibitors Ribose (RIB), TIBA, ASA, or Sorbitol (SORB) to SIM, or the omission of sucrose from SIM (SIM(-)).

(15) **SHOOT FORMATION FROM LEAF DISCS OF**
B. ERYTHROPHYLLA

To determine if newly detected polypeptides and changes in the abundance of existing polypeptides under shoot forming conditions were specific to petiole tissue, or were a general response of *B.erythrophylla* tissue to shoot formation, leaf discs from *in vitro* plants were cultured on SIM. These discs did not respond to the inductive medium. Leaf discs rapidly became senescent after excision from the leaf. After 24 hours large regions adjacent to the cut surface turned brown and this necrosis rapidly spread throughout the leaf disc. Increasing the size of discs failed to substantially increase their survival.

As leaf discs from potted plants were known to survive and produce shoots in culture, it was decided to analyze the pattern of polypeptides obtained when leaf discs obtained from potted plants were cultured on SIM, RIM or BM respectively. Before this was carried out some preliminary observation were under taken to determine if the response of leaf discs to SIM was similar to that of petiole sections

15.1 External observations of leaf disc development on SIM

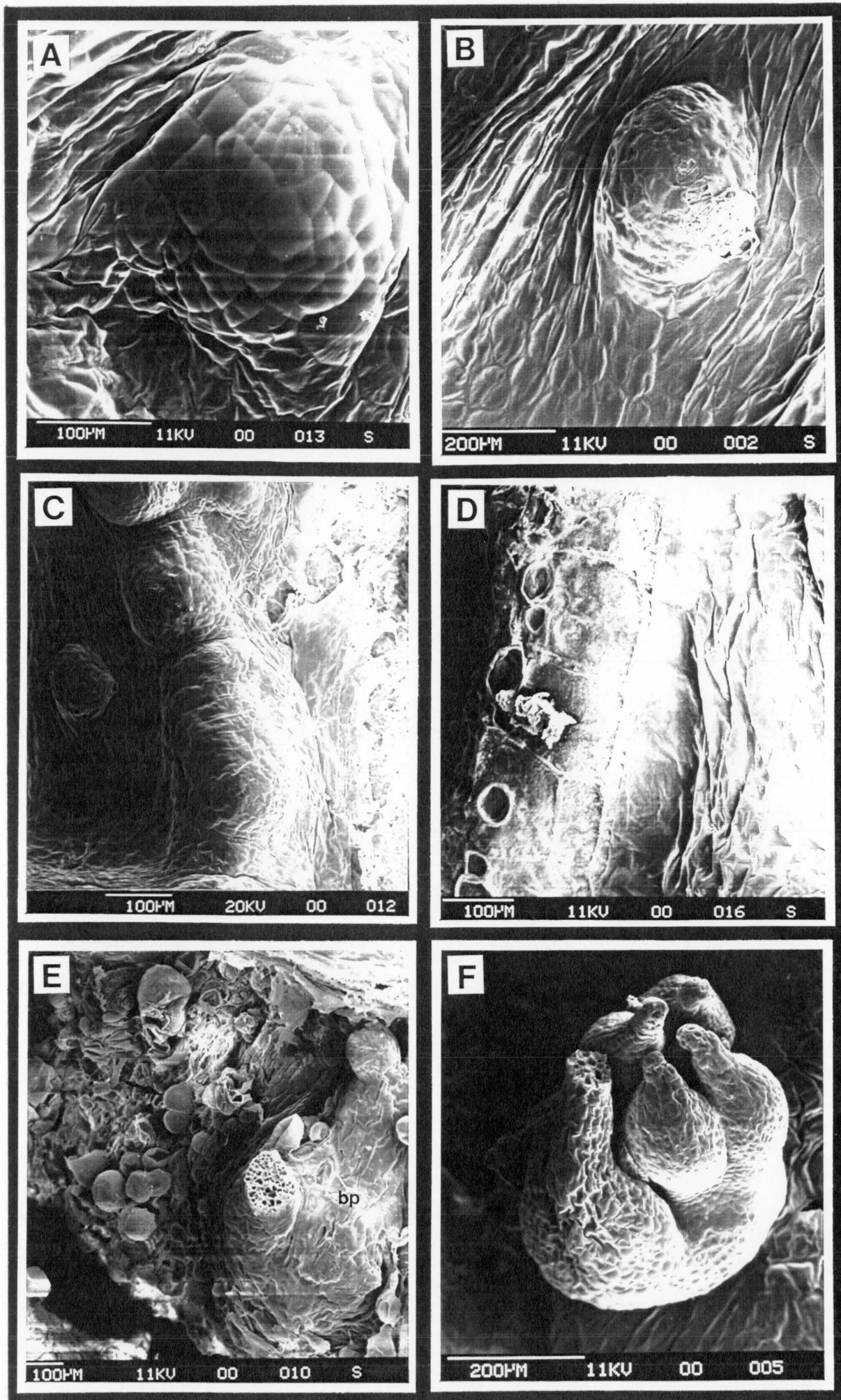
Leaf discs showed a similar time course for shoot formation as *in vitro* petiole sections. Regions of cell division in the epidermis were readily detectable after 7 days culture (PLATE 43A) and appeared as distinct domes after 10 days (PLATE 43B). Well developed bud primordia were observed after 21 days (PLATE 43F), with leafy shoots present after 6 to 8 weeks (PLATE 2A). Shoots developed from both the lamina surface of the disc and from cells within the cut surface of the disc, where callus also formed (PLATE 43E).

With culture on basal medium explants rapidly became senescent after more than 14 days culture (PLATE 2D). Cells at the cut surface of the disc collapsed with culture on BM (PLATE 43D) rather than forming a raised ridge around the cut surface as seen with culture on SIM (PLATE 43 C).

Leaf discs cultured on RIM readily produced roots. These formed mainly from the cut surface of the explant (PLATE 2C).

PLATE 43: S.E.M. views of cultured leaf discs of *B. erythrophylla*

- [A]- A swelling, resulting from multiple epidermal cell divisions, on the surface of a leaf disc cultured for 7 days on SIM.
- [B]- Meristematic dome surround by cells with few divisions after 10 days on SIM.
- [C]- The cut edge of a leaf disc showing the development of a ridge at the cut edge and a meristematic dome just inside the ridge (SIM, Day 10).
- [D]- The cut edge of a leaf disc cultured for 10 days on basal medium. Note the collapse of cells at the cut surface and the lack of the ridge seen with culture on SIM.
- [E]- Inside the cut edge of a leaf disc cultured for 18 days on SIM. Note the production of callus cells (arrow) and the formation of bud primordia (bp) within the cut surface.
- [F]- Shoot bud forming for the lamina surface after 28 days on SIM.



15.2 The effects of SIM on the fresh and dry weights of cultured leaf discs

After a rapid initial loss of nearly 50% of the original fresh weight within the first 3 days of culture (FIGURE 17A), the fresh and dry weight responses of leaf discs cultured on SIM were essentially the same as seen in petiole sections (FIGURES 17A & 17B). A gradual increase in fresh weight was paralleled by a more dramatic increase in dry weight. After 21 days culture on SIM the mean fresh weight of leaf discs was only slightly greater than that at the time of excision due to the initial loss in weight. Dry weight on the other hand had increased to more than 4 times that at day 0. Culture on basal medium caused no significant increase in either fresh or dry weights after the initial loss in fresh weight.

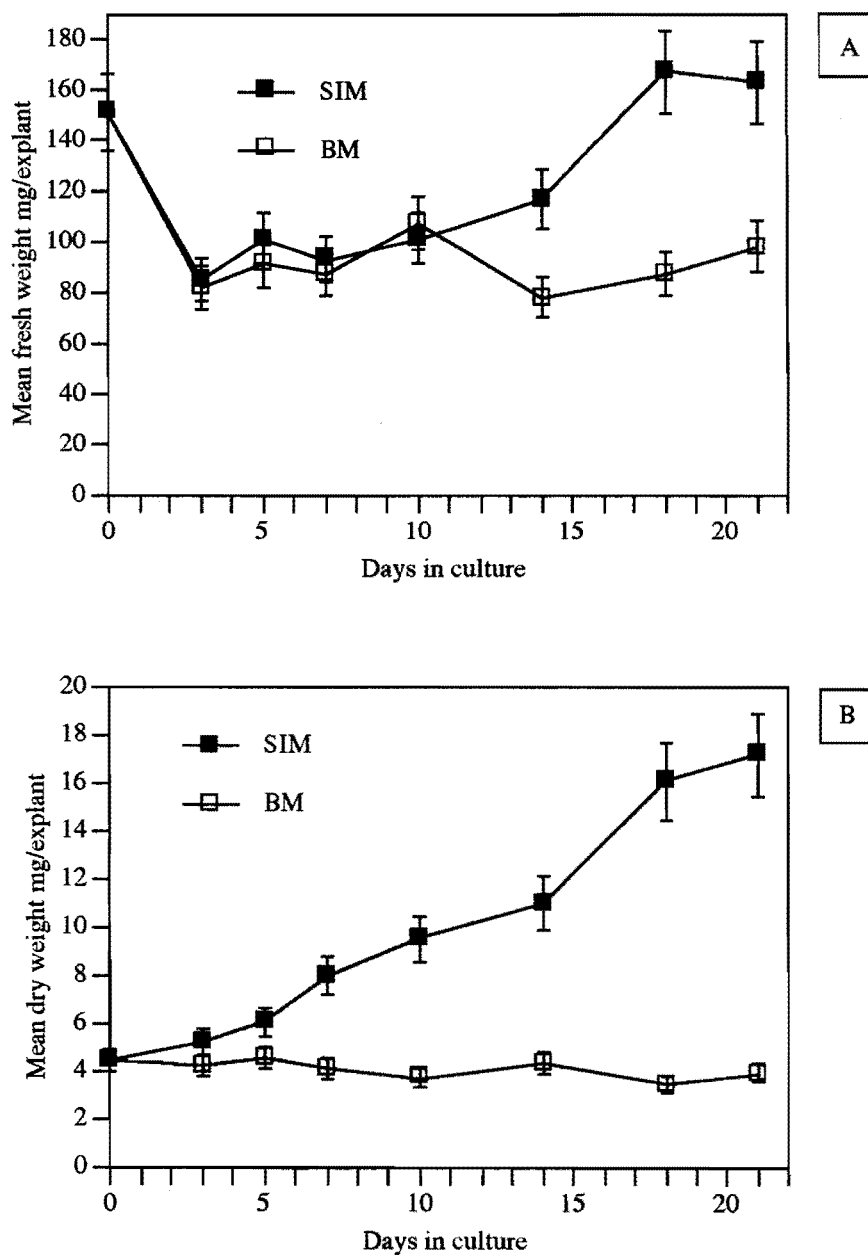


FIGURE 17 [A]- Fresh weight of leaf discs cultured on organogenic and non-organogenic media. Mean value plus or minus 1 standard error, n=30.

[B]- Dry weight of leaf discs cultured on organogenic and non-organogenic media. Mean value plus or minus 1 standard error, n=30.

15.3 The effect of SIM on the protein content of cultured leaf discs

At the time of excision the protein content of leaf discs was four fold greater than *in vitro* petiole tissue (FIGURE 18). Culture on BM or SIM resulted in a rapid increase in the protein content of discs within the first 3 days of culture. After this initial increase the protein content of discs exposed to BM gradually declined, while the content of those exposed to SIM continued to increase until day 7, after which the level declined slightly.

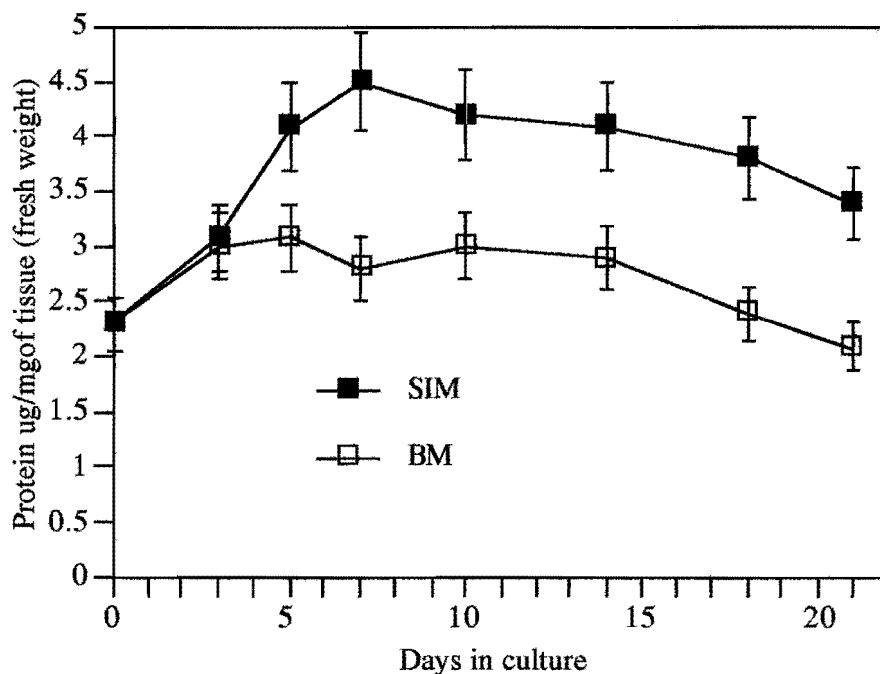


FIGURE 18 Changes in the protein content of leaf discs cultured for various lengths of time on organogenic and non-organogenic media. Protein was extracted using the X-100/CHAPS extraction buffer. Mean value plus or minus 1 standard error, n=6 pooled from 2 separate extractions.

15.4 Leaf discs requirements for SIM

Media transfer experiments were carried out using leaf discs to ascertain if determination of leaf discs cultured on SIM occurred at the same time as *in vitro* petiole sections.

Transfer from SIM to BM prior to day 5 of culture resulted in no shoot formation, with explants gradually becoming senescent (FIGURES 19A & 19B). Transfer after 5 or more days on SIM resulted in the formation of shoots. In general leaf discs responded less uniformly than *in vitro* petiole sections, with many discs not responding to the inductive media while others produced large numbers of shoots. Although there was a trend of increasing shoot number with time on SIM, it was not as clearly defined as that observed with *in vitro* petiole sections.

Pre-culture of leaf discs on BM before transfer to SIM showed the same trend as petiole sections, with a rapid decline in the shoot forming response and increased senescence with increased pre-culture on BM.

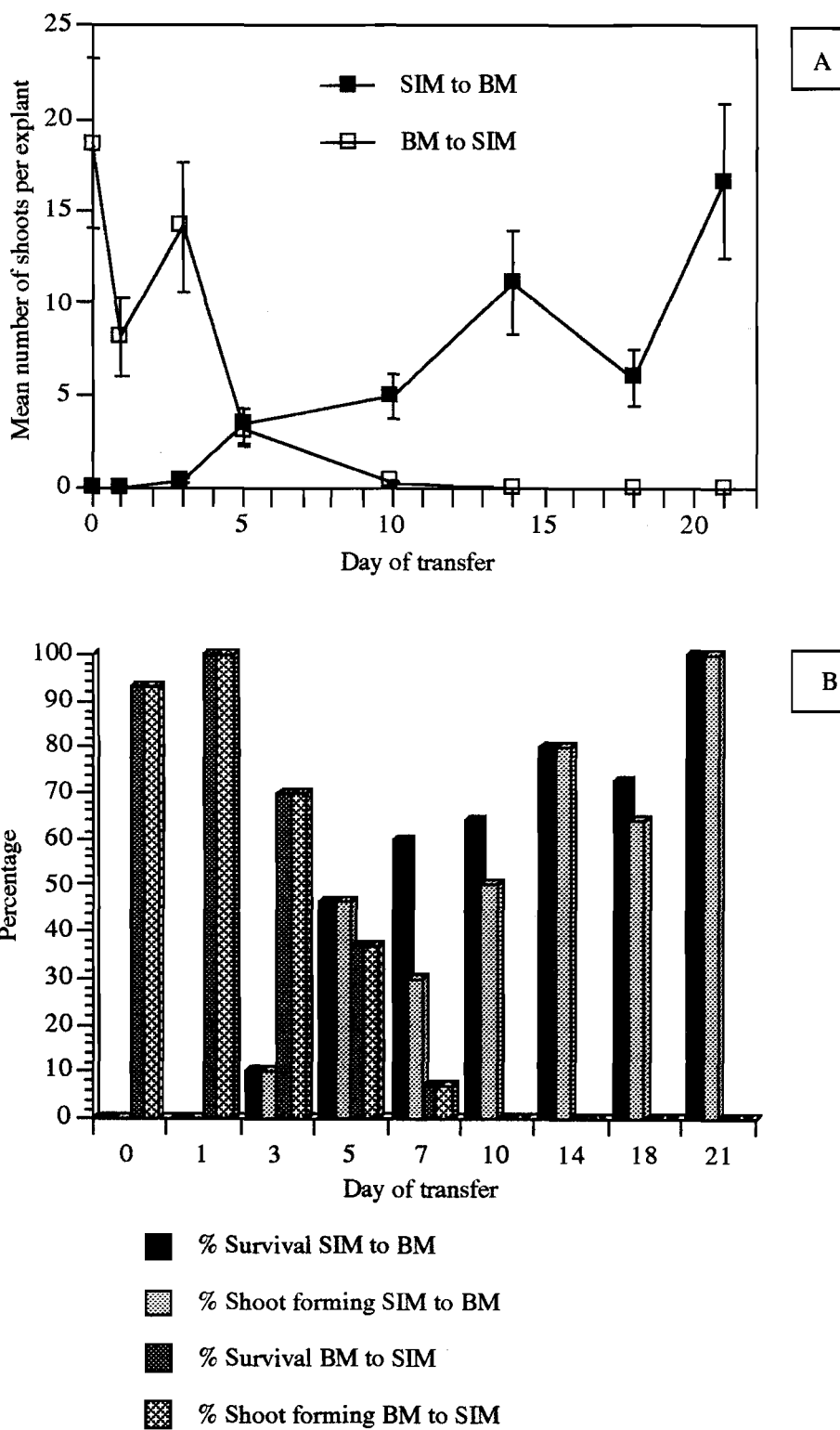


FIGURE 19 [A]- Changes in the number of shoots produced after transfer of leaf discs from SIM to BM and BM to SIM. Mean value plus or minus 1 standard error, $n=30$. Shoots were scored after a total of 6 weeks in culture.

[B]- The percentage of leaf explants surviving, and the percentage forming shoots after transfer from SIM to BM and BM to SIM.

15.5 2D-Electrophoresis of protein extracted from cultured leaf discs

Both the leaves from *in vitro* grown plants and those of potted plants showed a similar pattern of polypeptides (PLATES 33A & 44A). Culture on BM for 14 days resulted in several major changes to this pattern. The most prominent change was the almost complete loss of the large 55kd spot thought to represent Rubisco (PLATE 44B). Three polypeptides 381, 384 and 341 increased in abundance with culture on BM as they had with culture on petiole sections. No SIM only polypeptides or SIM induced changes were detectable when leaf discs were cultured on BM.

Culture of leaf discs on SIM for 14 days also caused the loss of the major 55 kd spot, but the 3 polypeptides that increased strongly with culture on BM were present at much lower levels (PLATE 45A). These 3 polypeptides showed the same response when petiole sections were cultured on SIM. Numerous other polypeptides detected in petiole explants but absent or detected at low levels in leaf tissue, appeared with culture on both SIM and BM.

Two group 6, SIM only, polypeptides (376, 377) were consistently detected at low levels in leaf discs cultured on SIM. Also detected was a pronounced increase in polypeptides 366 and 288, which increased in petiole sections only with culture on SIM. Polypeptide 343 associated with culture on SIM in petioles, also increased in abundance with culture of leaf discs on SIM. Over all the pattern of polypeptides detected when leaf discs were cultured on SIM for 14 days resembled that of petioles cultured on SIM. Culture of leaf discs on RIM failed to induce the above SIM only polypeptides (PLATE 45B).

PLATE 44: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) leaf discs from potted plants, i.e. day 0.

(B) leaf discs cultured for 14 days on BM.

Open symbols indicate the absence of polypeptides associated with
caulogenesis

Arrow heads indicate reference polypeptides for comparison of gels.

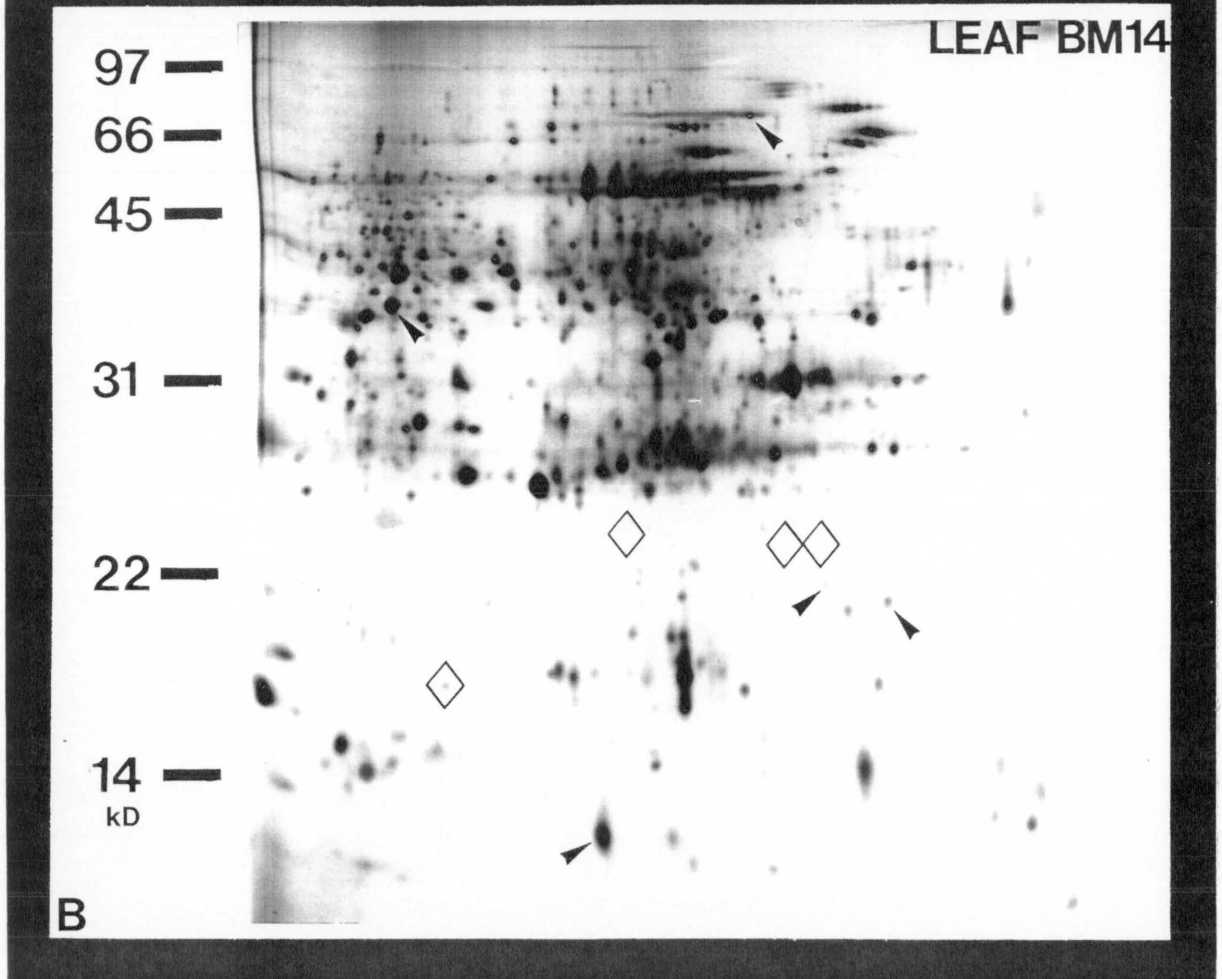
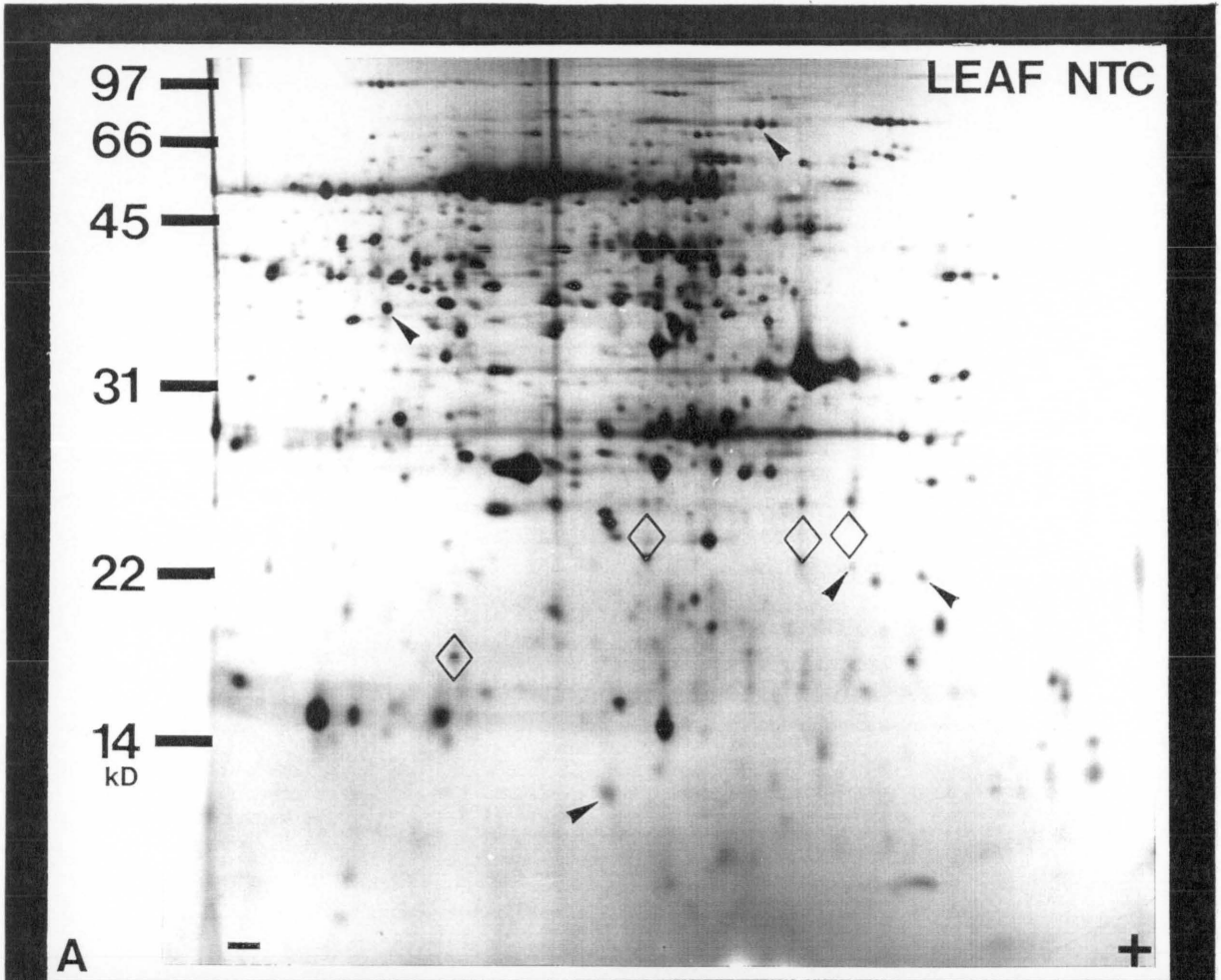


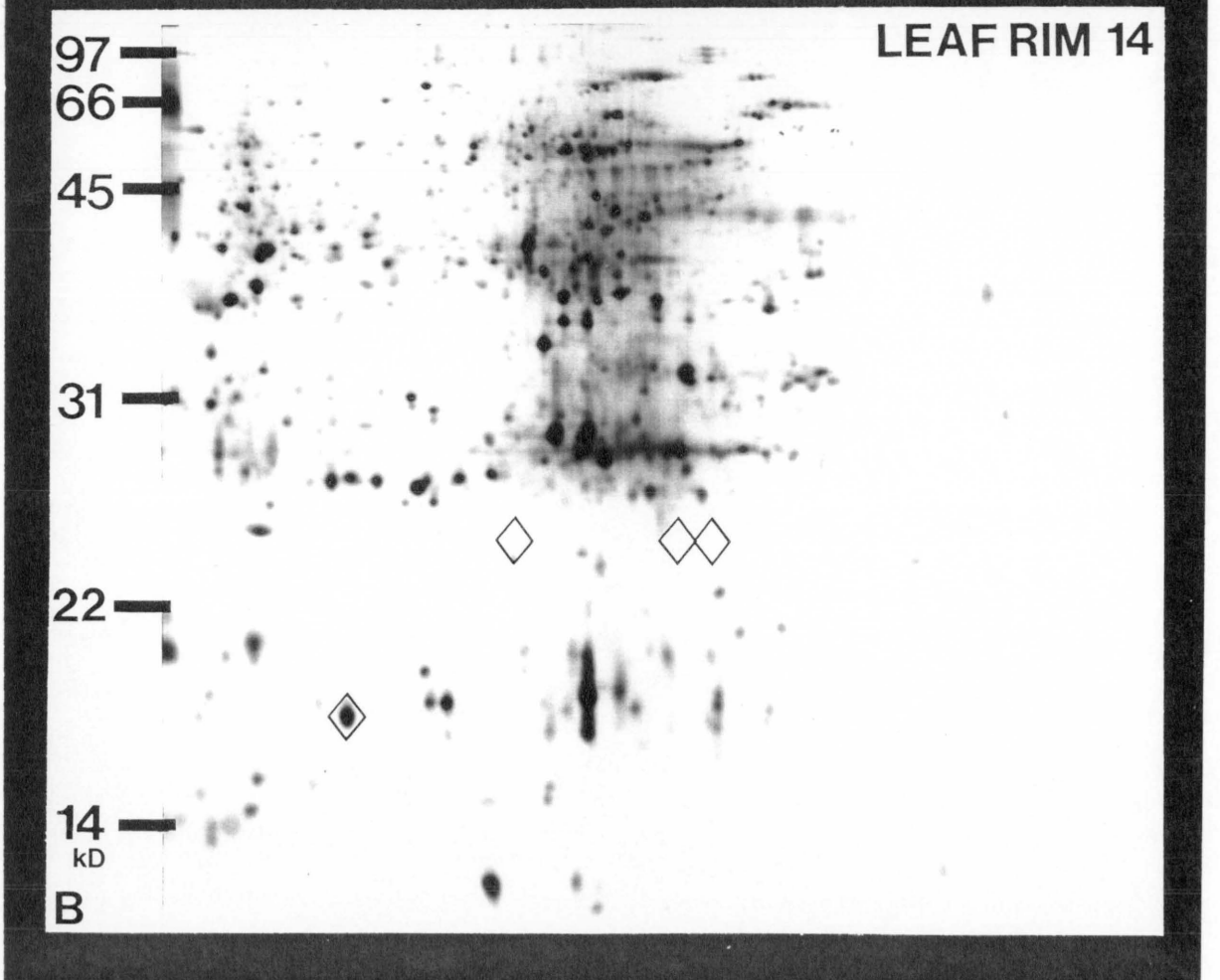
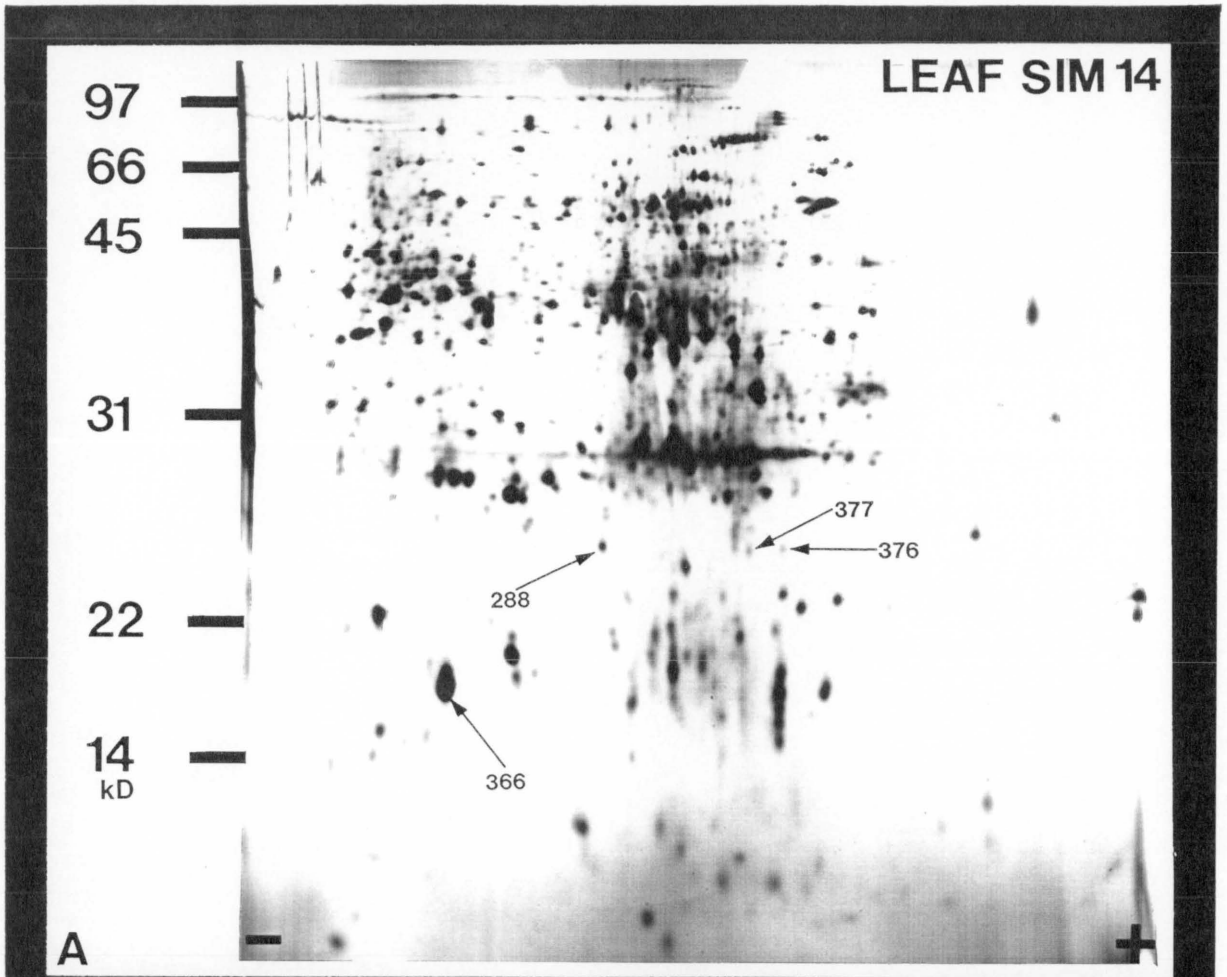
PLATE 45: Silver-stained two-dimensional PAGE gels of protein extracted from

(A) leaf discs cultured for 14 days on SIM. Arrows indicate polypeptides associated with caulogenesis

(B) leaf discs cultured for 14 days on RIM.

Open symbols indicate the absence/or presence at reduced levels of polypeptides associated with caulogenesis

Arrow heads indicate reference polypeptides for comparison of gels.



CHAPTER IV

DISCUSSION

(1) THE INFLUENCE OF GROWTH REGULATORS

Numerous begonia species and hybrids have been successfully cultured (Takayama, 1990), but to my knowledge there are few reports of the tissue culture of *B. erythrophylla* (Ramachandra and Khatamian, 1989; Tsimafyeyeva, *et al.*, 1990).

Most of the tissue culture work carried out on *Begonia* species has been orientated towards the propagation of plants rather than the study of the organogenic process. With such studies the aim is primarily to establish procedures for the reliable multiplication of a particular species, with little importance placed on the absolute specificity of the organogenic response. Often, media used for propagation result in the formation of a mixture of shoots and roots (Welander, 1977).

The ability of *Begonia* leaf-cuttings to form shoots has been shown to be dependent upon the lighting conditions under which the parent plants have been grown (Heide, 1967). Day length influences the internal balance of growth regulators with short days lowering the internal auxin/cytokinin ratio of leaf cuttings of *B. x cheimantha*. Heide (1967) suggested that the increased budding potential under short day conditions may be the result of the lowering of the internal auxin to cytokinin ratio. In contrast to the above study, Welander (1977) found that in *B. x hiemalis*, long-day treatments increased the organogenic response of petiole sections in culture.

As this study was on the process of organogenesis, it was critical that the form of organogenesis induced by a specific medium, be it SIM or RIM, resulted predominantly in the formation of either roots or shoots. As *in vitro* *B. erythrophylla* plants, unlike the potted plants used to establish them in culture, were grown under continuous light, it was necessary to establish media which induced a maximal shoot forming response with a minimal root forming response and *vice versa*.

Development of media, which form only shoots or roots was straight forward. A concentration of 0.1mg/l NAA and 1mg/l BA induces shoots only (SIM), while 1mg/l NAA and 0.05mg/l BA induces roots only (RIM). These concentrations are similar to those observed for organogenesis in other *Begonia* species (Takayama, 1990). Prolonged culture on either medium eventually results in the formation of the opposite organs, but these are not formed spontaneously from the original explant. On SIM, roots form from the bases of shoots, while on RIM, shoots form from the junction of the newly formed roots and the original explant. These organs probably

form because of a decline in the concentration of growth regulators in the medium or because of the synthesis of endogenous growth regulators in the newly developed organs. It is generally accepted that cytokinins are formed in the roots from where they are translocated into the stem (Salisbury and Ross, 1978) and auxins in the shoot/leaf (Salisbury and Ross, 1978). Leaf cuttings of *B. erythrophylla* placed only in water, first produce roots and then form adventitious shoots. This observation further suggests that developed roots are able to induce shoot formation in *B. erythrophylla* in the absence of exogenous cytokinin.

Little callus forms on explants within the first 3 weeks of culture on any of the combinations of BA and NAA tested. Prolonged culture on most media induces the formation of callus at the basal cut surface of explants, but in all the combinations of NAA and BA tested organs also form. Intermediate concentrations of auxin and cytokinin, which often promote callus formation, cause a mixture of shoots and roots. Similar observations have been made with other *Begonia* species (Welandar, 1977).

Few examples of callus specific media have been reported for *Begonia* (Takayama, 1990) and in those that have, 2,4-D was added to the culture medium (Takayama, 1990). Addition of 2,4-D induces callus formation in *B. erythrophylla* while suppressing organogenesis. The callus forms not only from the bottom cut surface of the explant, but also from the sides of the explant, possibly in response to wounding during the initial explant excision. The identification of a callus inducing medium is useful, because it enables explants under different organogenic conditions to be compared to explants undergoing unorganized cell division in the form of callus.

(2) POLARITY AND RESPONSIVE REGIONS

Regeneration of shoots from *B. erythrophylla* petiole sections shows no polarity. Polar shoot regeneration has been observed in other tissue culture systems (Monacelli *et al.*, 1988; Leshem, 1989), although proliferation throughout the explant also occurs (Von Arnold and Gronroos, 1986). Lack of polarity suggests that certain cells distributed throughout the epidermal layer of an explant are capable of responding to the shoot forming stimulus and that this stimulus, namely the internal concentration of growth regulators, is distributed throughout the tissue in a uniform manner.

A high cytokinin to auxin ratio is primarily responsible for shoot induction (Skoog and Miller, 1957) and non-polar transport of cytokinins has been reported in petioles (Fox and Weis, 1965; Veen and Jacobs, 1969). Non-polar transportation of BA to responsive cells throughout the petiole section appears the most likely explanation for the uniform response of explants to SIM.

Unlike shoot formation, root formation is a polar phenomenon. Basipetal orientation of explants results in root formation only from the lower half of the petiole sections, while with the opposite (acropetal) orientation roots form over the entire explant. This implies that, like shoot formation, cells able to respond to the root inducing stimulus are distributed throughout the epidermis, but explant orientation affects the distribution of the inductive stimulus.

A very similar response was observed when debladed petioles of *Hedera helix* L (English Ivy) were cultured on a NAA containing medium (Geneve, 1991). In this instance the polarity of root formation was explained in terms of basipetal transport of auxin. High levels of radio-labelled NAA accumulated at the basal cut surface of petioles irrespective of orientation. Basipetal orientation of petiole sections resulted in no NAA accumulation at the upper cut surface, with roots only formed at the basal cut surface. Acropetal orientation also resulted in the accumulation of NAA at the top cut surface of the petiole, which was in contact with the medium. This caused the formation of roots at both ends of the explant. It is easy to envisage a similar distribution of NAA in *B. erythrophylla* petiole sections, although NAA distribution studies need to be carried out to confirm this.

It is important that the polarity of an organogenic response is known and is considered when designing experiments to study organogenesis, especially if one of the objectives of the study is to look for polypeptide differences between explants undergoing rhizogenesis or caulogenesis. If, as in *B. erythrophylla*, caulogenesis is non-polar and rhizogenesis is polar, extraction of protein from entire explants exposed to RIM will result in dilution of polypeptides associated with rhizogenesis and make comparison with SIM exposed explants less accurate. In this study, as I wished all explants to be cultured basipetally to standardize culture conditions, only the basal portion of explants exposed to RIM were extracted for biochemical/molecular analysis. Thus avoiding, as much as possible, the effects of polarity.

(3) THE ADVANTAGES OF *IN VITRO* PLANTS FOR STUDIES ON MORPHOGENESIS

Both age and genotype have been shown to influence regenerative potential (Jain *et al.*, 1988; Niederwieser and Staden, 1990). The use of young clonally derived *B. erythrophylla* plants completely overcomes these problems, providing petiole tissue that is uniformly responsive to organogenic stimuli. As organogenesis in *B. erythrophylla* is direct, somaclonal variation often induced when regeneration occurs via an indirect pathway (Scowcroft and Larkin, 1982) is likely to be minimal. Such tissue is particularly suitable for molecular studies. The minimization of genetic variation should enable experimentally induced changes in gene expression to be more easily isolated, in the absence of genetic differences between explants.

The use of *in vitro* plants also excludes the possibility of environmental variation affecting regenerative potential, for plants are grown under identical conditions that are easily controlled. Possible artifacts induced by surface sterilization of plant tissues are also avoided. The only major problem associated with the *in vitro* approach is that plants grown in culture are small and large numbers are required to carry out many experiments. This involves both considerable space in the culture room and many hours of work maintaining stocks of plants in culture. In my opinion the experimental merits outweigh the costs, as uniformity of plants and no requirement for surface sterilization, increases the reliability and speed with which experiments can be set up.

(4) MORPHOLOGICAL AND HISTOLOGICAL OBSERVATIONS

While it is more common to induce organogenesis indirectly from callus (Banks, 1979, Christianson and Warnick, 1983; Cheng and Raghavan, 1985), numerous examples of direct regeneration of shoots and roots from cultured explants have been reported (Pramanik and Datta, 1986; Wright *et al.*, 1986; Reynolds, 1989). Despite this, very few studies have been carried out describing the basic anatomy and morphology of direct adventive organogenesis *in vitro*. In studies that have been carried out, organ primordia tend to form from two general areas of the explant, either in close proximity to the vascular tissue (Sterling, 1951; Bonnett and Torrey, 1966; Reynolds, 1989) or, in some systems, the epidermis or sub-epidermal layers have been identified as the origin of meristemoids (Tran Thanh Van, 1973b; Chlyah, 1974; Von Arnold and Gronroos, 1986). Often caulogenesis and rhizogenesis do not

both occur directly in the same explant (Attfield and Evans, 1991a). Shoot and roots may develop from different cell types.

Begonia species provide the added interest that, in both *B. erythrophylla* petiole sections and *B. rex* thin layers (Chlyah and Tran Thanh Van, 1984), both shoots and roots are directly formed from cells of epidermal origin. In *B. erythrophylla*, shoot buds are produced evenly around the entire surface of the petiole section, provided the epidermis is intact. Removal of part of the epidermis results in shoots only forming from the regions of the explant retaining the epidermis. Shoots only arise from the unwounded areas of the explant suggesting that the superficial layers are critical for the shoot forming response and that wounding, which has been found to increase shoot production in some systems (Bajaj, 1972; Attfield and Evans, 1991a), is not a direct factor in shoot formation in *B. erythrophylla*.

Culture of epidermal peels on SIM does not induce shoot formation, as seen in *B. rex* (Chlyah and Tran Thanh Van, 1984), but primordia like structures do develop. This suggests that SIM may be optimal for shoot formation in *B. erythrophylla* petiole sections, but not in epidermal peels. The concentration of growth regulators to which epidermal cells are exposed in an intact petiole section, is most probably different to that which cells of an epidermal peel are exposed. Direct contact of epidermal peels with SIM may inhibit shoot formation, just as high BA concentrations inhibit shoot formation in petiole sections. Alternatively, the internal tissues of the explant may have correlative influences on the epidermis, as has been observed in other thin layer systems (Chlyah, 1974).

The ability of epidermal cells to give rise to differentiated structures has been the subject of considerable research (Walker, 1978; Walker and Bruck, 1985; Bruck and Walker, 1985; Bruck and Walker, 1986). Walker and Bruck (1985) showed that an intact mature epidermis precluded formation of approach grafts in stems of five angiospermous species. They suggested that the lack of callus formation from epidermal cells during grafting may indicate a high degree of determination compared to parenchymatous cells, which divide periclinally to form callus during grafting. They did, however, admit that under special circumstances, epidermal cells may become less differentiated or less determined, as Tran Thanh Van (1973b) had demonstrated the totipotent nature of the epidermis of *Nicotiana tabacum* L by its ability to produce floral buds. Chlyah and Tran Thanh Van (1984) have also clearly shown that epidermal cells in thin layers of *B. rex* are capable of forming shoots.

In both *B. erythrophylla* petiole sections and *B. rex* thin layers, the pattern of shoot development is very similar. The first periclinal cell divisions associated with shoot formation are observed in epidermal cells. Walker and Bruck (1985) stated that, although anticlinal divisions occur throughout the intact epidermis during the course of

normal development, periclinal divisions occur only occasionally. If divisions do occur, they suggested that they occur in epidermal cells neighboring a wound and that such periclinal divisions may push a derivative cell into an underlying cell layer so that non-epidermal cell redifferentiation occurs.

This would appear unlikely in *B. erythrophylla*, as either partial or complete removal of the epidermis inhibits shoot formation while allowing callus formation. This points to a more direct role for the epidermis in the formation of shoots from *B. erythrophylla* petiole sections.

In both *B. erythrophylla* and *B. rex* (Chlyah and Tran Thanh Van, 1984), the vast majority of epidermal cells responding to the shoot inducing medium were associated with glandular hairs. This specificity has been shown in a number of cultivars of *B. rex* (Prevot, 1948; Bigot and Chlyah, 1970). A few shoot primordia showed no association with glandular hairs, but it cannot be ruled out that these may have evolved from epidermal cells about to develop into glandular hairs. Thorpe (1980) suggests that the *de novo* formation of organs in tissue culture is directly related to previously differentiated cells or tissues in the explant. In other words, certain cell types within an explant may be more responsive than others. Attfield and Evans (1991a) proposed that the state of differentiation of individual cell populations within the explant may influence their response to growth regulators and that some cells within the explant may be considered to be less differentiated than others. For example, vascular parenchyma cells are less differentiated than epidermal cells that are less specialized than mesophyll cells.

It is interesting to note that structures resembling glandular hairs have been implicated as sites of somatic embryogenesis on immature zygotic embryos of *Trifolium repens*. (Williams and Maheswaran, 1986). It is possible that some epidermal cells, particularly those associated with glandular hairs, may be more amenable to organogenic/embryogenic stimuli than others. In other words, such cells may be more weakly committed to a differentiated state. The presence of such cells in the initial explant may do away with the requirement for dedifferentiation in the form of callus production.

It has been suggested (Williams and Maheswaran, 1986) that direct embryogenesis in culture may proceed from cells, which are already determined for embryogenic development prior to explanting. Such cells have been termed pre-embryogenic determined cells (PEDC's) (Evans *et. al*, 1981). These cells require growth regulators or favorable conditions to allow release into cell division and expression of embryogenesis. Indirect embryogenesis requires redetermination of differentiated cells, thus a dedifferentiation step (callus formation) is required followed by the development of the embryogenically determined state. These induced

embryogenically determined cells (IEDC's) require growth regulators for both dedifferentiation and for determination of the embryogenic state.

Such a theory has been further extended to account for the pattern of adventitious root formation in English ivy (Geneve, 1991). Adventitious roots developing on debladed petiole cuttings, form directly from phloem parenchyma cells if exposed to NAA immediately after excision. If cuttings are pre-cultured on basal medium prior to transfer to NAA containing medium, rooting takes place indirectly from the outer tissue layers. Geneve (1991) proposed that "competent root forming cells" (CRFC) were involved in direct root formation, with cells only requiring exposure to NAA to promote root formation. If this competence is lost by pre-culture on basal medium, these cells fail to form roots when exposed to NAA, but cells in the outer layers of the explant are first induced to become competent and then induced to form roots. These cells were termed induced competent root forming cells (ICRFC). The two terms are analogous to the PEDC's and IEDC's used to describe the cells involved in direct and indirect embryogenesis, except Geneve (1991) considered that the concept of competency better agrees with the scheme for organ formation proposed by Christianson and Warnick (1988) and the use of "determined" better describes the latter stages of root primordia development as proposed by Lovell and White (1986). I also think that competency is a better term as it refers to the ability of the tissue to respond to the inductive medium rather than a fixed cell fate.

It is doubtful that competent cells exist in *B. erythrophylla* petioles at the time of excision, as three days are required on an inductive media before cell division occurs. It is more likely that weakly committed cells, more amenable to outside influences, exist in the epidermal layer of the explant at the time of excision and that these cells may be associated with glandular hairs. As both roots and shoots originate from the same epidermal regions, such cells are most likely not predisposed for a particular organogenic pathway, but may be amenable to the influence of any inductive stimuli. Formation of organs from these cells may simply be the result of sidetracking epidermal cells destined to become epidermal hairs. Alternatively, cells associated with a newly formed glandular hair may become more responsive to inductive stimuli during the formation of the hair than the surrounding epidermal cells.

Given the apparent totipotent nature of plant cells (Meins and Binns, 1979) it cannot be ruled out that even strongly committed cells can attain a responsive state without the need for cell division if provided with the correct conditions. Fukuda and Komamine (1980) demonstrated that isolated mesophyll cells can be induced to change form without cell division and become fully specialized xylem cells. Such transdifferentiation although rare also occurs in animals. Exposure of isolated striated muscle cells of the jellyfish *Podocoryne carnea* to collagenase enzyme causes the

cells to disaggregate. When the enzyme is withdrawn the cells reagregate into a complex, made up of many cell types, without cell division taking place (Schmid and Alder, 1984). Even specialized cells can therefore change fates without cell division, providing they are released from the correlative influences of the maternal tissue. It is interesting to note that in both the examples given for transdifferentiation, cells are first isolated. In large tissue sections where direct organogenesis occurs, the initial isolation of cells from the maternal tissue appears unlikely, although cell wall changes cannot be ruled out. The presence in the original explant of less committed cells, able to respond to external stimuli, may be more likely.

In *B. erythrophylla*, once the initial cell divisions have taken place in the epidermis, the pattern of both shoot and root formation is similar to that seen in *B. rex* thin layers (Chlyah and Tran Thanh Van, 1984). Exposure to either SIM or RIM results in further cell division, initially confined to the epidermis, which gives rise to typical meristematic cells as described by Brown and Thorpe (1986). These meristematic cells under the influence of the inductive medium rapidly divide, forming meristematic domes protruding from the surface of the explant.

With culture on SIM, once determined, meristematic domes rapidly differentiate forming a single layered tunica followed by foliar primordia. By day 24, a well developed shoot apex is present from which further foliar primordia are developing. The epidermis remains unruptured during the formation of meristematic domes and remains continuous with the developing organ, further supporting its role in shoot formation. Shoot primordia formed from *B. erythrophylla* petiole sections appear much the same as those arising from epidermal peels of *B. rex* (Chlyah and Tran Thanh Van, 1975; Chlyah and Tran Thanh Van, 1984).

Not only meristematic domes protruding from the surface of the explant are formed with culture on SIM, but with continued culture, meristematic structures very similar to those observed in epicotyl explants of white spruce (Rumary *et al.*, 1985) develop. These meristemoids are surrounded by files of dividing cells rather than protruding from the surface of the tissue. Meristemoids are, however, still associated with glandular hairs and still contain cells of epidermal origin.

It cannot be definitely stated that meristematic domes or meristemoids are derived from a single epidermal cell, for often many cells in the same epidermal region divide. Therefore, meristematic regions and hence shoot primordia, may be derived from the division of one or more epidermal cells.

Unlike in many other systems where roots arise from the internal tissues of the explant, in *B. erythrophylla*, roots also arise from cells of epidermal origin. The initial pattern of rhizogenesis is similar to that of caulogenesis. Meristematic regions of

epidermal origin develop into root primordia, with minimal epidermal disruption. The process of root formation also resembles that observed in *B. rex* thin layers.

It must, however, be noted that when leaf fragments of *B. rex* are cultured on a root inducing medium, roots originate from the inner tissues of the explant and form only from epidermal cells using the thin layer system (Chlyah and Tran Thanh Van, 1984). This suggests that the breakdown of tissue correlations is required to induce roots of epidermal origin to form from *B. rex* leaf explants. This does not appear necessary for *B. erythrophylla* petiole sections. There seems no predisposition of cells of the inner tissues for root formation as is seen in both *B. rex* leaf fragments (Chlyah and Tran Thanh Van, 1984) and petioles of English ivy (Geneve, 1991). The epidermal layer appears equally capable of giving rise to roots or shoots providing the explant is given the correct stimulus.

Shoot primordia formed from *B. erythrophylla* petiole sections also appear similar to those seen on leaf-petiole cuttings of *Begonia x Hiemalis* (Mikkelsen and Sink, 1978). In the above study of organogenesis it was concluded that shoots arose from epidermal cells, but adventitious roots were derived from internal parenchymal cells rather than epidermal cells. This difference may be the result of a different type of cell being more responsive to the root forming stimulus in this hybrid, or under the totally different conditions used in their study. The retention of the leaf may also influence the internal physiology of the petiole. It also must be considered that as *in vitro* *B. erythrophylla* plants were used as a source of explants in this study, petioles were relatively young and the strong tissue correlations associated with mature organs may not have been established.

(5) THE IMPORTANCE OF STARCH ACCUMULATION

The accumulation of starch in tissue exposed to shoot forming conditions has been reported in a number of studies (Thorpe and Murashige, 1970; Maeda and Thorpe, 1979; Thorpe *et al.*, 1986; Mangat *et al.*, 1990), as has starch accumulation under root forming conditions (Coleman and Greyson, 1977). In both forms of organogenesis deposition of starch occurs prior to the formation of meristemoids and often prior to any observable cell division.

In *B. erythrophylla*, heavy localized starch accumulation in epidermal and collenchyma cells occurs prior to any cell division under both shoot and root forming conditions. This pattern of starch accumulation prior to organ initiation may be common to *Begonia* species, a recent study has shown that localized starch accumulation occurs prior to shoot initiation in stem sections of *B. rex* (Mangat *et al.*, 1990).

The general pattern of starch deposition and utilization under shoot forming conditions in *B. rex* is very similar to that observed in *B. erythrophylla*. Starch accumulation initially takes place in the cortical cells close to the vascular bundles, with starch grains progressively appearing in cortical cells further from the vascular bundles and in the collenchyma and epidermal cell layers. Intense localized accumulation of starch eventually occurs in the superficial layers of the explant, denoting epidermal regions about to become meristematic. In both systems the total starch content of explants initially rises, but with the formation of meristematic regions and the development of shoot primordia, the starch content steadily decreases, especially in cells of the developing shoot buds. In *B. erythrophylla*, these findings can be further extended to include root formation. With culture on RIM, starch also appears prior to the formation of meristematic regions and subsequently disappears during root development.

Thorpe and Murashige (1970) found a similar pattern of starch accumulation during shoot formation in tobacco callus cultures. They suggested that starch is used during both organ initiation and the subsequent developmental stages of the shoot primordia. Accumulation of starch is the first visible sign of organogenesis in *B. erythrophylla*. Its accumulation is correlated to both forms of organogenesis, for without its accumulation neither shoots or roots form.

Organogenesis is a high energy requiring process. This has been demonstrated by increased respiration and enzymatic activity in tobacco callus under shoot inducing conditions (Ross and Thorpe, 1973; Thorpe and Laishley, 1973) and the strong localized enzyme activity in meristemoids formed when conifer cotyledons are induced to produce shoots (Patel and Berlyn, 1982; Patel and Thorpe, 1984).

Increased enzymatic activity in organ forming regions of *B. erythrophylla* petiole sections has been shown in this study. In particular, ATPase and succinate dehydrogenase activity is high in organogenic regions, suggesting high energy requirements.

Starch deposition has distinct advantages as a source of energy for developing tissues. Firstly, large amounts of starch can be deposited in the regions associated with organogenesis, providing a readily available source of energy without direct osmotic effects. Secondly, as Thorpe and Murashige (1970) have suggested, starch degradation results in the formation of glycolytic intermediates, the subsequent oxidative catabolism of which yields high amounts of ATP. This ATP is immediately available for cellular metabolism.

Thorpe and Murashige (1970) also noted that the addition of gibberellic acid (GA₃) to the shoot inducing medium suppressed shoot formation in tobacco callus and that addition of GA₃ reduced the level of starch in the callus. They concluded that a certain concentration of starch was required to allow organ initiation and that GA₃ prevented this concentration being reached by stimulating α -amylase synthesis, with resultant starch hydrolysis. This lack of starch correlated with the subsequent inhibition of shoot formation.

Heide (1969) discovered that stimulation of bud formation by BA and stimulation of root formation by NAA on detached petioles of *Begonia x cheimanthus* leaves, could be inhibited by exposure to GA₃. This inhibition was only effective if explants were exposed prior to the formation of bud primordia. The effect of GA₃ on starch synthesis was not analyzed in Heide's study, but the inhibition of starch accumulation may also be found here.

In both *B. erythrophylla* and *B. rex* (Mangat *et al.*, 1990), removal of sucrose from the inductive medium inhibits both starch accumulation and organ formation, even if another carbohydrate with similar osmotic potential is substituted. Mangat *et al.* (1990) found that culture on a medium containing 3% mannitol in place of sucrose inhibited shoot production and transfer back to a shoot inducing medium containing only sucrose restored starch accumulation with subsequent shoot formation. They suggested that "the causative role of starch in organogenesis" is reflected in these results.

Although some tissue cultures are capable of metabolizing these sugar alcohols, (Chong and Taper, 1972) some are not (Thorpe, 1974), therefore such experiments do not necessarily suggest starch has a causative role in organogenesis, for explants may not be able to metabolize the carbohydrate provided, although osmotic conditions may be the same. It should also be noted that Mangat *et al.* (1990) used 3% mannitol as an osmotic replacement for 3% sucrose. 3% mannitol has

roughly the same osmotic potential as 6% sucrose not 3% as stated (Mangat *et al.*, 1990).

The exact role of starch in organogenesis remains unclear, but it can be said that in *B. erythrophylla*, accumulation of starch is required for initiation of both shoot and root formation. Meristematic regions do not form without localized starch accumulation.

(6) HISTOCHEMICAL OBSERVATIONS

The transition from differentiated to meristematic tissue capable of forming organs, involves pronounced changes in the metabolism of induced cells (Brown and Thorpe, 1986). In *B. erythrophylla*, the cells responding to organogenic stimuli are located in the epidermal layer of the explant. Since only a few cell layers are directly involved in organ initiation "quantitative determinations using biochemical techniques may not yield meaningful data because of dilution effects" (Patel and Thorpe, 1984).

For this reason histochemical localization of several enzymes previously shown to be associated with the process of shoot initiation (Patel and Berlyn, 1982; Patel and Thorpe, 1984) was undertaken to try to gain some insight into the metabolic state of explants during organogenesis. The aim was to see if changes in enzyme activity could be related to the anatomical changes observed during organogenesis and to see if shoot and root formation differed in the pattern of enzyme localization.

Before discussing the results obtained, it must be pointed out that *B. erythrophylla* petioles were difficult to process for thin sectioning due to highly vacuolated, thin walled cortical cells and the presence of numerous large multicellular hairs, which made infiltration difficult. Embedding in wax resulted in considerable cell disruption and the loss of a large part of the cell contents. Resin embedding was also difficult, requiring a long infiltration procedure after which no enzyme activity remained. Incubation of thick tissue sections in the enzyme localization solution, followed by block processing of tissue, resulted in poor if any localization of enzyme activity due to leaching of the reaction products during the embedding procedure. For this reason, thin hand sections were used for enzyme localization. This has the advantage that enzymes are more readily detectable (Gahan, 1984), but it does limit the resolution obtainable, allowing only a more general analysis of enzyme distribution within the tissue. For these reasons detection of enzyme activity in single cells prior to cell division was not possible.

At the time of excision from the petiole, only the vascular bundles of explants showed any significant enzyme activity. In particular, acid phosphatase and peroxidase were strongly localized in these regions. Strong acid phosphatase activity has also been reported in the vascular bundles of *P. radiata* cotyledons (Patel and Thorpe, 1984), while strong localization of peroxidase activity in the vascular bundles is to some extent expected in light of reports of its role in lignification of xylem (Goldberg *et al.*, 1983). High ATPase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activity was also found in the vascular bundles, suggesting higher metabolic activity therein. Succinate dehydrogenase (SDH) was only detected at low levels. As SDH has been shown to be localized mainly in the mitochondria (Avers and King, 1960; Sedar and Rosa, 1961), low SDH activity may reflect a low level of respiration in the petiole at the time of excision. Cortical, collenchyma and epidermal cells also showed little activity at day 0, for any of the enzymes tested, again suggesting low metabolic activity.

Culture on either SIM or RIM for 3 days caused only a slight increase in enzyme activity in the vascular bundles and in dividing epidermal cells. It was only with the formation of meristematic zones, after 4 to 5 days in culture, that strong localized ATPase, acid phosphatase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and peroxidase activity was detected in the epidermal regions. SDH was only strongly localized after 6 to 7 days. Enzyme activity continued to remain localized in the meristematic regions during the development of shoot buds. A similar pattern of enzyme activity associated with the formation of meristemoids under shoot forming conditions has been observed in conifers (Patel and Berlyn, 1982; Patel and Thorpe, 1984).

In *B. erythrophylla*, both shoot and root formation occur in the superficial layers of the explant. Culture on either SIM or RIM results in the same initial pattern of enzyme localization in the meristematic regions. No differences in the pattern of distribution of any of the enzymes studied under shoot forming or root forming conditions were detected at the level of resolution employed in this study. Only once distinct organ primordia are formed are differences in the distribution of enzyme activity observed. These differences are probably due to differentiation of the tissue. Zonal distribution of enzyme activity has been reported for both shoot (Fosket and Miksche, 1966) and root apices (Shaykh and Roberts, 1974).

The inability to distinguish histochemically between meristematic regions formed under shoot inducing conditions and those formed under root inducing conditions, may be a reflection of the initial similarity and possible plasticity of the early stages of organ formation, as described by Bonnett and Torrey (1966), or it may be due to the low resolution of the procedure used.

As previously mentioned, during the formation of meristemoids under shoot forming conditions there is a high requirement for both energy and reducing potential (Thorpe and Laishley, 1973). In *B. erythrophylla* petiole sections, once cell division is initiated, the regions of dividing cells rapidly shift from a metabolically inactive state to state of high metabolic activity. On non-organogenic media, as no meristematic regions are formed, no metabolic shift is observed. As the formation of a meristematic region is common to both shoot and root formation it follows that, at least initially, the metabolic requirements for both processes may be similar, but in depth biochemical studies comparing the two organogenic processes are required to ascertain if this is so.

Recent advances in non-invasive high resolution NMR may provide the means to study metabolic changes associated with organogenesis in a more precise manner, as metabolic changes can be followed in the same specimen. Already a few studies have been carried out on plant metabolism using this technique (Roberts, 1984; Martin, 1985; Vogel, 1987; Thorpe *et al.*, 1989).

Despite the initial similarity between the pattern of enzyme localization during both rhizogenesis and caulogenesis different isozymes may be specific to each form of organogenesis (Thorpe *et al.*, 1978; Coppens and Dewitte, 1990). Although not presented in this thesis, isozyme analysis of peroxidase, malate dehydrogenase, succinate dehydrogenase, esterase and acid phosphatase have been carried out. Both peroxidase and malate dehydrogenase have isozymes specific to organogenic tissue, with some differences between caulogenesis and rhizogenesis observed. Therefore despite the two processes having a similar pattern of enzyme distribution, they may have specific isozyme requirements.

MEDIA TRANSFER EXPERIMENTS

Before discussing the media transfer experiments it is important to define some of the terminology used in the context of this study.

Determination. In developmental biology a cell is considered to be determined when it has committed towards a specialized fate, but the overt demonstration and realization of that fate has not yet become apparent (Maclean and Hall, 1987). This specialization is attained by differentiation, so determination should precede differentiation, although this is not always so (Maclean and Hall, 1987). The timing of determination is only detectable by experimental intervention, mostly involving media transfer experiments. Determination may be gradual, with cells becoming progressively committed to specific fates, or it may occur rapidly. For the purpose of this study, determination is defined as the stage at which explants no longer require the organogenic stimulus for cells within the explant to continue on a pathway of organogenesis.

Competence. Is the capacity of cells to react to developmental signals (Meins and Binns, 1979). It can be inherent or induced (Geneve, 1991). In this study competence is defined as, the point at which cells within the explant are capable of cell division giving rise to meristematic regions under the influence of organogenic stimuli. Competence is not absolute, at any one time only a portion of cells within the explant may be competent.

7.1 Explants requirements for SIM

Explants require a minimum of 7 days on SIM prior to transfer to BM before any shoots are produced (the point of minimal determination). As in several other systems (Christianson and Warnick, 1983; Flinn *et al.*, 1987; Attfield and Evans, 1991b) the process of shoot determination is not synchronous, a further 11 to 14 days on SIM is required for a maximal shoot forming response (the point of maximal determination).

In *B. erythrophylla*, the time required for minimal shoot determination is not fixed and can be manipulated by altering the medium composition. Exposure to a higher concentration of BA reduces the time required for minimal determination by up to three days and greatly increases the number of shoots determined after short

exposures to the inductive medium. Studies have been carried out which suggest that when an explant is exposed to a sequence of media, the growth regulator composition of the first medium can have effects carried over to the second medium. (Halperin, 1986; Renaudin *et al.*, 1990).

Renaudin *et al.* (1990) proposed two main hypotheses to account for carry-over effects, which are not mutually exclusive and can be modified to apply to the effects of BA concentration in *B. erythrophylla*. Firstly, exogenous growth regulators could be carried over in the explant during transfer from one medium to the other. The extent of the carry-over would be dependent on the concentration during culture and on the duration of this culture. Exogenous growth regulators could also modulate the size of the endogenous growth regulator pools. Accordingly, the actual levels of growth regulators in the explant at the time of transfer may influence the response of the explant on the second medium. Secondly, culture on a shoot inducing medium containing high concentrations of BA, may induce more rapid initial shoot induction in responsive cells of the explant

Exposure of *B. erythrophylla* petiole sections to high concentrations of BA does not appear to alter the rate at which tissue differentiation occurs, at least at the light microscope level. Explants after 5 days culture on BA8 appear to have the same level of development as those cultured on SIM. Culture on BA8 also had no effect on the pattern of polypeptides that developed with culture on SIM, suggesting that increasing the concentration of BA did not affect the pattern of gene expression exhibited with culture on SIM. This points to a high concentration of BA in the medium, possibly having indirect effects on the shoot forming process, although the presence of changes in gene expression not detectable at the level of resolution employed cannot be ruled out.

When pulsed exposure to an organogenic medium is used for shoot induction, the concentration of BA to which the explant is exposed has been shown to affect the number of shoots produced (Goldfarb *et al.*, 1991). In this study Douglas-fir cotyledons were pulsed with BA in liquid medium for 2 hours and then placed on a solid medium without growth regulators to allow shoot formation. Increasing the concentration of BA in the liquid medium increased the number of shoots produced up to a maximum value. It was proposed that increasing the concentration of BA in the medium provided optimal conditions for shoot induction within the explants.

For *B. erythrophylla* petiole sections, the first hypothesis may be more probable. A high concentration of BA in the shoot inducing medium may simply allow a greater internal concentration of BA to build up within the tissue before transfer to basal medium. This may result in explants more rapidly attaining an internal auxin to cytokinin ratio favoring shoot formation and possibly retaining it for longer in the

absence of exogenous BA. A high internal concentration of BA may also support the induction of larger numbers of shoots. The uptake of a high concentration of BA would be particularly important if at the time of excision, the endogenous level of auxin within explants was high. The possibility of endogenous growth regulators affecting organogenesis will be discussed latter.

Bioassays were carried out to determine if there was any difference in the concentration of cytokinin activity in petiole explants cultured on media containing different concentrations of cytokinin. The level of cytokinin present in the explants was so low that the assay used was not sensitive enough to detect any significant difference between the treatments. A more sensitive radioactive assay, or preferably an immuno-assay, as employed by Van Der Krieken *et al.* (1991), is required to ascertain if the concentration of BA in the tissue is related to the time of exposure and the concentration of BA in the inductive medium.

This difference in the apparent timing of determination when explants are exposed to 2 different inductive media highlights one of the problems encountered when studying organogenesis in multicellular explants. The results obtained from this type of experiment must be interpreted with some care as the experimental conditions can to some extent dictate the results. The term determination was originally coined for use in the developmental biology of animals. In this case transfer experiments were carried out by transferring a cell or a small group of cells from one environment to another. Such small groups of cells were exposed directly to the new environmental stimuli. In contrast, when comparatively large explants are transferred from one set of morphogenic conditions to another, the new set of conditions may not be immediately apparent at the site of the responsive cells. Exogenous hormones could be carried-over in the explant during transfer to the non-inductive medium, where they could continue to influence the regenerating cells within the explant for sometime. Thus, such transfer experiments involving large explants may not reflect absolutely the anatomical stage of development at which determination at the cellular level occurs, but may simply reflect the attainment of a relatively stable internal balance of growth regulators.

It must also be considered that the timing of determination for caulogenesis is estimated by counting the number of leafy shoots. Exposure to SIM may be required for the early stages of development of the shoot primordia, as well as determination, making the two events difficult to distinguish. In *B. erythrophylla*, a requirement for BA during the early stages of shoot development would appear likely. Shoots that are produced after 7 or 10 days exposure to SIM, although having leaves, are small and stunted in appearance compared to those observed after 14 days or longer on SIM. This suggests that SIM is required for both the induction of the shoot primordia and

the early stages of development. Therefore, stage of development at which determination occurs is difficult to ascertain with certainty.

7.2 Explants requirements for RIM

Explants only require exposure to RIM for 3 or 4 days before producing a small number of roots upon transfer to BM. At this point few cell divisions have occurred in the explant, suggesting that root determination may occur almost immediately after cell division takes place and prior to the formation of large meristematic structures. It is interesting to note the difference in the time required for root determination compared to shoot determination, as both caulogenesis and rhizogenesis are direct processes occurring in the same region of the explant.

In tobacco leaf discs, rhizogenesis is direct while caulogenesis is indirect. Roots form from bundle sheath and vein parenchyma cells, with shoots arising from callus (Attfield and Evans, 1991a). Root determination in this system occurs after only 1 day on RIM, while shoot determination requires 6 days. In the tobacco system, root formation occurs at low levels in the absence of exogenous auxin. The addition of auxin is thought to exploit a potential already in the leaf, possibly by supplementing low levels of endogenous auxin (Attfield and Evans, 1991b). Pre-culture of explants on basal medium results in the loss of their ability to form roots, while reducing the time required on SIM for shoot determination. Changes are therefore occurring, which benefit shoot initiation and adversely affect root initiation (Attfield and Evans, 1991b). These changes were thought to be in the level of endogenous growth regulators in the explant. A similar suggestion of high endogenous auxin levels adversely affecting shoot formation in tomato stem explants has been proposed by Cassels (1979).

In *B. erythrophylla*, a high internal concentration of auxin is also a possible cause for the reduced time required on RIM, but there are complicating factors. Explants cultured on basal medium do not form roots and at least 3 days on RIM are required for root formation. This may be explained in two ways, (1) although the internal concentration of auxin is high, the level is not great enough to induce root formation without exposure to RIM, (2) Explants are not capable of responding to the endogenous auxin. It is interesting to note that if explants are exposed to SIM for less than 3 days they occasionally produce small numbers of roots and no shoots. This may mean that exposure to an organogenic media is required to elicit a response required before rhizogenesis can take place, or in other words the explant may not be competent to respond to any organogenic stimuli, endogenous or otherwise.

7.3 The strength of determination

As discussed, transfer of an explant from SIM/RIM to BM does not provide conclusive evidence that meristematic cells within the explant are strongly committed, at the developmental stage observed, when explants no longer require exposure to the organogenic medium to form organs. BM has no inductive effects on *B. erythrophylla* petiole sections. RIM on the other hand induces roots. Exposure to RIM may to some extent overcome problems associated with the timing of determination in large explants. The internal growth regulator balance may be destabilized more rapidly, exposing cells or groups of cells to a different inductive stimulus.

If groups of cells within the explant are determined and strongly canalized for shoot formation they will not have their fate altered by transfer from SIM to RIM (Christianson and Warnick, 1983), as has been demonstrated in tomato where organ primordia are initiated, which can be canalized either toward carpel or stamen formation (Sawhney and Greyson, 1979). If cells within the explant are not strongly committed to caulogenesis after seven days on SIM, one would expect that no shoots would be produced upon transfer to RIM. Furthermore, if determination is a multi step process involving a weak commitment toward organogenesis, followed by a progressive canalization to either root or shoot formation, one would expect root formation only, up to and past the time of determination for shoot production as measured by transfer from SIM to BM (Christianson and Warnick, 1983). Such transdetermination is widely recognized because of its dramatic occurrence in *Drosophila* imaginal disc cells, where a cell or cells within a particular location are known to exist in a state of commitment to become one type of cell on differentiation, but they are then found to develop into something quite different (Strub, 1977). It is possible that directed transdetermination may occur in plants, root meristem cells have been shown to produce both shoots and roots (Colijn *et al.*, 1979).

As observed with *convolvulus* leaf discs (Christianson and Warnick, 1983), in *B. erythrophylla*, explants are in fact strongly canalized for caulogenesis after determination. Explants after 7 days exposure to SIM go on to form shoots even after transfer to RIM, suggesting that determined meristematic cells within the explant are no longer competent to respond to RIM. Exposure to BA8 and then transfer to RIM showed that shoots are not strongly canalized with less than seven days exposure to shoot inducing conditions, even if the concentration of BA in the medium was high. In contrast to transfer directly to BM, the percentage of shoots determined, prior to day 15 with culture on BA8, is not much greater than observed with culture on SIM.

The effects of BA8 on shoot development could not be ascertained as shoots forming after 7 or more days exposure to BA8 and then transferred to RIM, are significantly better developed than those formed when transferred directly to BM. Either RIM itself, or the formation of roots that occur upon transfer to RIM and their subsequent influence on the explant, may promote shoot development.

These results further support the view that exposure to BA8 does not have a direct effect on shoot development, but has an indirect effect, possibly by altering the growth regulator concentration in the explant. If shoots are strongly canalized for shoot formation after 4 to 5 days on BA8, one would expect a similar response with transfer to RIM as upon transfer to BM. This does not occur, with only a slight effect on determination observed.

One problem with this type of experiment could be the polarity of the root forming response. The upper portion of the explant may not be exposed to a strong root forming stimulus and so allow some meristematic domes to escape and develop shoots. This may account for the few shoots that form with transfer from BA8 to RIM prior to day 7. It must, however, be pointed out that after 7 days on SIM, shoots not only form from the top half of an explant, but also from the lower half where roots also form. Meristematic domes in this region of the explant would be exposed to the influences of RIM and so must not be responsive to the root inducing stimulus.

Unlike caulogenesis, where transfer from SIM to RIM has no effect on the timing of determination, transfer from RIM to SIM increases the time required on RIM for roots to form upon transfer. Instead of determination occurring after 3 days on RIM, 5 days are required to give reasonable numbers of roots per explant upon transfer to SIM. Therefore, transfer to SIM has adverse effects on root formation at days 3 and 4. These results suggest that cells within the explant are not strongly canalized until after 5 days on RIM. A similar occurrence was observed with tobacco leaf discs (Atfield and Evans, 1991b), where the time required for root determination was increased from 1 to 3 days. In both *B. erythrophylla* petiole sections and tobacco leaf discs a brief exposure to RIM, allows explants to form roots upon transfer to BM, but does not result in a change in the competence of the responding cells with less than 5 days exposure to the root inducing stimulus. These cells remain able to be influenced by exposure to an alternative inductive stimulus.

Bonnett and Torrey (1966) proposed that meristemoids formed from *Convolvulus arvensis* roots cultured *in vitro* were initially plastic and were capable of giving rise to shoot or root primordia. Villalobos *et al.* (1985) stated that in *Pinus radiata* cotyledons exposed to shoot forming medium, meristemoids were determined, while less developed meristematic structures made up of 6 to 8 meristematic cells termed promeristemoids, were developmentally plastic. These promeristemoids

disorganized and degenerated if removed from the shoot inducing medium. Flinn *et al.* (1987) observed that *Pinus strobus* cotyledons exposed to shoot inducing conditions produced 4 cell clusters, which were even less complex than promeristemoids and were correlated with the acquisition of shoot determination. Such evidence suggests that determination may occur at a very early stage preceding meristemoid formation, but as previously discussed the simple media transfer experiments carried out in these studies do not necessarily accurately reflect determination at a cellular level. One cannot rule out Bonnett and Torrey's proposal that meristemoids are initially developmentally plastic.

It is not known if the small meristematic regions that are observed in *B. erythrophylla* petiole sections after 4 days on RIM go on to form shoots upon transfer to SIM, or if they simply stop dividing. As explants were transferred to BM after 22 days for organ development and no shoots were subsequently formed, we can assume that these regions are at least permanently inhibited from forming roots. The same applies to the meristematic regions which fail to form shoots with transfer to RIM after 7 days on SIM. It is also evident that meristematic regions associated with both root and shoot formation need to be made up of many cells before undergoing a change in competence, enabling them to form roots/shoots even with exposure to the opposite inductive stimulus.

Although the times required for explants to be strongly determined for rhizogenesis or caulogenesis are closer than those required simply for determination. The processes of rhizogenesis and caulogenesis, despite occurring from the same region of the explant, still appear to require different lengths of time to be strongly determined.

As previously discussed, the initial endogenous growth regulator balance of the explant may favor root production and so more time on SIM may be required to overcome this. Also, the processes of determination and development are difficult to separate, with shoot formation explants may require extra time on SIM to allow even strongly determined meristematic domes to develop into shoot primordia capable of developing leaves upon transfer to BM. Roots, which are somewhat less complex structures, once determined, do not require RIM for further development. Alternatively, as the formation of roots or shoots may involve the expression of two different developmental pathways, determination for caulogenesis may occur at a later stage than for rhizogenesis.

7.4 Medium requirements during the lag phase

Shoot formation in both *Convolvulus arvensis* and tobacco leaf discs is preceded by callus formation (Christianson and Warnick, 1983; Attfield and Evans, 1991b). During the formation of callus, explants acquire "competence" i.e. the ability to respond to organogenic induction (Christianson and Warnick, 1985). This process is formally analogous to the commonly held concept of the dedifferentiation of the explant (Christianson and Warnick, 1985).

Callus formation is not a prerequisite for either root or shoot formation in *B. erythrophylla*, with organs forming directly from cells of the explant. Unlike shoot formation, root formation from tobacco leaf discs is direct. Explants are determined for rhizogenesis after one day of exposure to RIM, with 3 days required for a strongly canalized response (Attfield and Evans, 1991b). Rhizogenesis in this system appears to require no interval to gain competence. With *B. erythrophylla* petiole sections 3 days on RIM are required for explants to become determined, with 5 required for strong determination. Furthermore, there is a 2 day lag period prior to the first observed cell divisions, whether explants are exposed to RIM or SIM. If explants are competent to respond to the inductive medium at the time of excision one might expect a more rapid response.

For at least 24 hours of this lag phase explants may be exposed to either SIM or RIM with no reduction in the total time required for minimal determination of either type of organ. It would appear that during the initial part of this lag phase, explants are independent of the specific inductive properties of the medium, with both SIM and RIM capable of fulfilling the others role. After this initial period cell division begins and the inductive properties of the medium take effect.

Christianson and Warnick (1985) suggested that in *Convolvulus* leaf discs the processes of rhizogenesis and caulogenesis are independent and do not involve common developmentally plastic "meristemoids" like those observed in *Convolvulus* root segments (Bonnett and Torrey, 1966). They also proposed that there was no general state of competence for organogenesis, which under the influence of the growth regulator balance is directed toward the formation of shoots or roots. The fact that during the lag phase, both SIM and RIM are capable of fulfilling the others role, suggests that in *B. erythrophylla* petiole sections a general state of competence may exist. This could reflect a fundamental difference between the processes of direct and indirect organogenesis.

Although exposure to either SIM or RIM during the lag phase induces a response, exposure to BM does not. Exposure to basal medium only results in the gradual loss of the explants ability to respond to either SIM or RIM. Pre-culture on basal medium has been shown to result in the gradual loss of competence in several other systems (Christianson and Warnick, 1983; Flinn *et al.*, 1988; Attfield and Evans, 1991b).

During the first 48 hours of culture the only histological change observed within cultured explants is the accumulation of starch grains. This accumulation occurs with culture on either organogenic medium. As previously discussed, because organogenesis is an energy intensive process, a readily available energy reserve is required. Starch is not the only energy reserve involved in organogenesis, the maintenance of lipid and possibly protein reserves has been implicated in the process of shoot formation from *Pinus strobus* cotyledons (Flinn *et al.*, 1988). In this system, when cotyledons were cultured on basal medium, lipid reserves that are present at high concentrations in the initial explant, rapidly declined. This decline, as well as ultrastructural changes associated with the maturation of cells on basal medium, was associated with loss of competence to respond to the shoot forming medium. Depletion of storage reserves has also been implicated in the loss of competence of *Pinus radiata* cotyledons to respond caulogenically to BA (Aitken-Christie *et al.*, 1985).

It is conceivable that competence in *B. erythrophylla* may at least in part be a function of the ability of the explant to provide a readily available source of energy, possibly in the form of starch grains. The induction process may not occur in the absence of this stored energy reserve. As very little starch is present in the explant at the time of excision, the attainment of competence during the lag phase may in part be the result of the build up of starch within the tissue. This idea is further supported by the fact that starch does not accumulate with culture on basal medium, which does not induce a competent state. The inhibition of starch accumulation and caulogenesis by the addition of sorbitol to SIM also supports an early requirement for starch. Even if a small amount of starch accumulates as a result of a brief exposure to SIM, sorbitol is no longer a completely effective inhibitor of caulogenesis.

(8) THE EFFECT OF ORGANOGENESIS ON THE PROTEIN COMPOSITION OF EXPLANTS

8.1 1D SDS-PAGE analysis of polypeptide changes associated with organogenesis

This study was initiated to explore whether the regeneration of shoots and roots, that can be predictably induced from *B. erythrophylla* petiole explants by manipulation of the culture media composition, is correlated with molecular events that might be used as markers for the subsequent morphological developments. The aim was also to tie these changes to experimentally defined stages in the organogenic processes.

Storage proteins have been used as markers for somatic embryogenesis in *Brassica napus* (Crouch and Bopp, 1984) and *Trifolium* (McGee *et al.*, 1989), while other polypeptides have been used to identify the time of bud regeneration in detached pine cotyledons (Villalobos *et al.*, 1984) and as markers to outline stages in axillary bud development in pea (Stafstrom and Sussex, 1988). The stage specific expression of polypeptides in cultured melon cotyledons can also be used to distinguish between the processes of rhizogenesis and caulogenesis, prior to the formation of an identifiable structure (Leshem and Sussex, 1990).

In *B. erythrophylla*, although some changes in the pattern of polypeptides, separated by 1D SDS-PAGE, associated with shoot formation from petiole sections are detectable, these changes are subtle and are often hard to clearly resolve. The 18 and 16.5kD polypeptides, identified by Coomassie blue staining, provide the best markers for discriminating between the shoot and root forming processes. Both polypeptides are first clearly detected after 7 days culture on SIM and increase in abundance with time in culture. The expression of these polypeptides is correlated with the loss of the explants requirements for SIM. No earlier changes specifically associated with shoot formation were detected and no changes associated specifically with root formation were detected.

Because the responsive region of a *B. erythrophylla* petiole section makes up only a very small portion of the total explant and initially the processes of caulogenesis and rhizogenesis are similar, the detection of few clearly identifiable differences by a low resolution technique like 1D SDS-PAGE is not unexpected. The most clearly defined changes observed with culture are those reflecting changes in the general metabolism of the tissue as a result of the excision and culture of explants. These changes most probably occur throughout the explant and so are readily detectable by 1D SDS-PAGE.

One polypeptide present at the the time of excision (day 0) increases in abundance with culture on callus inducing medium. Such polypeptides have been detected with culture on callus inducing media in other systems (Chen and Luthe, 1987; Hahne *et al.*, 1988) and provide a means of discriminating between organogenesis and random cell division in the form of callus.

From these observations it follows that to identify clearly those molecular events that reflect localized organogenesis, as occurs in *B. erythrophylla*, a much higher resolution technique such as 2-dimensional electrophoresis is required. A similar conclusion was arrived at by Leshem and Sussex (1990) in their study of shoot and root organogenesis in cultured melon cotyledons. Despite finding early quantitative differences between the two forms of organogenesis, they also concluded that higher resolution than that afforded by 1D SDS-PAGE was required to identify qualitative differences between the two processes.

8.2 2D PAGE analysis of polypeptide changes associated with organogenesis

The assignment of polypeptides in different samplings of 2D gels and fluorographs to categories of temporal and spatial expression, based on qualitative and quantitative evaluations, involves considerable subjective judgement. This is irrespective of whether the analysis is carried out by computer, or by visual pair wise comparison as in this study. Variation between sample extracts, 1st and 2nd dimension electrophoresis and staining or fluorography, no matter how stringently a protocol is adhered to, results in subtle variation between gels (Dunbar, 1987).

The procedures used in the 2D separation of polypeptides in this study, combined many of the latest modifications to O'Farrell's original protocol (O'Farrell, 1975), which have been shown to increase gel resolution and reproducibility (Duncan and Hershey, 1984; Mayer *et al.*, 1987; Hochstrasser *et al.*, 1988a; Hochstrasser *et al.*, 1988b). Despite this, many inconclusive changes in polypeptide pattern were observed as a result of the various explant treatments. Such changes failed to appear in every protein sample extracted and despite their possible significance they were not recorded in this thesis. Only clearly defined and consistently reproducible changes in the polypeptide pattern are presented.

Having said this, the pattern of many major polypeptides present in the tissue at day 0 was surprisingly consistent, both within and between gels, irrespective of the treatment of explants from which protein was extracted. On both silver-stained gels

and the fluorographs taken from gels on which labelled polypeptides were separated, reference spots were detected that were readily identifiable. This greatly simplified gel comparison.

Although a great deal of work has been carried out investigating biochemical and molecular changes associated with somatic embryogenesis, very few studies have been carried out concerning adventive organogenesis. There is a distinct lack of information concerning the direct formation of both shoots and roots independently from the same explant. *B. erythrophylla* petiole sections provide a good system for the comparison of shoot and root formation at the molecular level, as both shoots and roots originate from the same region of the explant. This enables the two organogenic processes to be analyzed without the added complication of initial metabolic differences between target cells, due to cells of different origin and type responding to the different inductive stimuli. The absence of callus during the early stages of organogenesis reduces the possible swamping out of any changes associated with organogenesis by callus production and minimizes the chance of somaclonal variation. Both are considered undesirable when studying organogenesis (Hicks, 1980).

The aim of the 2D analysis was primarily to define polypeptides suitable for use as markers of the stages of shoot organogenesis, outlined by the earlier histological and physiological work. Comparison of the shoot and root forming processes was the secondary objective. Developmentally specific polypeptides *in vitro* are defined as "polypeptides that must be undetectable in tissues prior to culture, they must appear in protein samples before there are any overt signs of differentiation and they must be more prominently expressed in protein gels from one pathway compared to another" (Reynolds, 1990).

8.2.1 Polypeptides associated with organogenesis

Three polypeptides (376, 377, 401) detected in this study may be classified as putatively shoot specific, while 3 may be classified root specific (J, P, N). The shoot specific polypeptides were of particular interest. These three polypeptides are first detectable after 7 days culture on SIM and all increase in both abundance and in the level of synthesis from day seven to twenty one of culture. Polypeptides 376 and 377 are synthesized at low levels with culture on RIM, but the level of synthesis does not increase with time in culture. The presence of low levels of these polypeptides with culture on RIM may be the result of a low level requirement for them under root forming conditions. Their presence could also be the result of the formation of an occasional shoot primordia. The fact that the level of synthesis did not increase with prolonged culture on RIM suggests that they do not play a significant role in root organogenesis. None of these three polypeptides associated with caulogenesis are

detectable with culture on CIM, BM or SIM(-). Their absence on SIM(-) rules out the possibility that they are induced by high concentrations of cytokinin and supports a role in caulogenesis.

When these polypeptides first appear the most advanced structures present on explants are domes of meristematic cells. These domes show no shoot specific differentiation, with no tunica or foliar primordia present. Therefore the appearance of these polypeptides precedes the formation of even the most rudimentary shoot primordia.

The detection of these polypeptides coincides with the loss of the explants requirement for SIM. Minimal determination occurs after seven days exposure to SIM, at the precise time these polypeptides first become detectable. The appearance of these polypeptides at this time may purely be coincidence, but it should be noted that all three polypeptides increase in both abundance and in the level of synthesis from the onset of determination at day 7, to day 21, when maximal determination occurs. This increase could either be due to increased synthesis of these polypeptides during the development of shoot primordia, or as the process of shoot induction in *B. erythrophylla* is not synchronous, increasing levels of these polypeptides may reflect a progressive increase in the number of meristematic structures becoming determined.

After 21 days the number of shoots produced per explant does not increase greatly with prolonged culture on SIM. Silver-stained gels of explants cultured on SIM for 42 days show only trace levels of polypeptides 376, 377 and 401. A requirement for large amounts of these polypeptides in developing shoots would not appear to be essential, although dilution effects caused by the increased fresh weight of explants and reduced protein content cannot be ruled out.

None of the three polypeptides are detectable by silver-staining in leaf or root tissue, or even in the apex of *in vitro* plants. They are also absent from flower buds taken from potted plants. The absence of these polypeptides from the shoot apex further supports the transient nature of their expression. If these polypeptides are associated with the development and/or growth of the shoot apex, one would expect them to be detectable in the apices of *in vitro* plants. These polypeptides may be involved in the transition of weakly, or undetermined meristematic structures into those strongly determined to produce shoot primordia.

Stage specificity of polypeptides, possibly involved in the formation of organized structures *in vitro*, has been observed in carrot embryogenesis, (Choi *et al.*, 1987). Choi *et al.* detected several proteins associated with somatic embryogenesis in carrot cell suspensions. These proteins increased in abundance as embryos formed, but upon germination of the embryos to form seedlings, the proteins apparently

disappeared during the differentiation of leaves petioles and roots. Reproductive tissue from mature plants also lacked detectable amounts of these proteins.

The other developmentally specific polypeptides detectable in *B. erythrophylla* explants (J,P and N) are associated with rhizogenesis and are only detectable after *in vivo* labelling with [³⁵S]-methionine. The inability to detect these polypeptides by silver-staining could be because, although capable of incorporating [³⁵S]-methionine, the polypeptides may not accumulate in the tissue to a level that can be observed in total soluble protein samples, even using a sensitive silver-stain. These polypeptides are synthesized at low levels on SIM but at much greater levels on RIM. Two of these polypeptides (J and P) show differential synthesis after just 3 days on RIM, while the other (N) requires 5 to 7 days before the difference is evident. Determination for root formation occurs weakly after only 3 days on RIM, with explants only being strongly canilized after 5 days. Only polypeptide N shows increased synthesis as more roots become strongly committed. As mentioned polypeptides J and P show differential synthesis at day 3, but as earlier *in vivo* labelling was not carried out and as the levels of synthesis of these polypeptides did not increase significantly with continued culture, it cannot be ascertained if they are involved in the induction process or if the increased synthesis is simply the result of exposure to RIM.

Christianson and Warnick (1988) stated that when *Convolvulus* leaf discs were cultured on RIM, changes to the pattern of *in vitro* synthesized polypeptides, specific to rhizogenesis, occurred only after determination. *B. erythrophylla* shows a similar trend with the majority of polypeptides associated with either rhizogenesis or caulogenesis only appearing after explants are strongly determined. The exact nature of these polypeptides, which mark the progression from medium dependent induction, to medium independent differentiation, is unknown. They may be directly or indirectly involved in the determination process. Irrespective of this, their detection enables us to discriminate between these two phases in both organogenic processes.

Two other polypeptides (H and G), also only observed by *in vivo* labelling with [³⁵S]-methionine, are differentially synthesized in root and shoot primordia, respectively. These two polypeptides are synthesized at low levels after 3 days culture on either SIM or RIM, while being absent with culture on BM or CIM. As organ primordia develop (day 14), the level of synthesis of polypeptide G increases only on SIM, while on RIM, only the level of polypeptide H increases. Both continued to increase on their respective media throughout the time course of this study.

These polypeptides may represent gene products, which are initially synthesized in response to organogenesis and become differentially synthesized in developing organs. Both are initially detected at similar levels at day 3 when cell division has just been initiated. During the formation of meristematic regions the level of synthesis of these polypeptides remains constant, with differential synthesis only apparent once organ primordia have formed.

In a study of spatially and stage-specific polypeptides in protein extracts from single embryos of the domesticated carrot, Racusen and Schiavone (1988) discovered that nine radio-labelled polypeptides were asymmetrically distributed into apical or basal halves of sectioned torpedo embryos. They theorized that, since polarity is the pivotal morphological event which signals the conversion to organized growth, spatially polarized gene expression may have given rise to the distribution of these polypeptides, which might then serve as molecular determinants of the ensuing polarized morphology. They also proposed that these protein differences may simply represent fundamental biochemical differences between the cell types in the apical and basal regions of the embryo. Because their analysis was carried out on torpedo embryos, which are well developed, independent separations of proteins in the apical and basal regions of much earlier stages are required to ascertain if such differences in distribution have causal significance. One would have to go back well into the globular stage to ascertain a causal role, as it is towards the end of the globular stage of proembryo development that the structural differentiation of its cells begin.

Differential synthesis of polypeptides G and H in *B. erythrophylla* petiole sections, under shoot or root forming conditions, is only clearly detectable after the formation of differentiated structures, with the differences becoming more pronounced as the primordia develop. This suggests that Racusen and Schiavone's latter hypothesis may be correct for these polypeptides. The failure to detect differential expression of these polypeptides prior to the formation of differentiated primordia does not, however, completely rule out the possibility that it does occur, just that the difference is too small to be detected by visual comparison of gels.

A further 4 polypeptides (329, 330, 348, 402) detected only by silver-staining and one detected only by *in vivo* labelling (F), may be associated with the development of shoot primordia. The failure to detect the above polypeptides by *in vivo* labeling could be due to (1) the polypeptides being synthesized at undetectable levels, but turning over slowly and so accumulating; or (2) they may have a low methionine content and so incorporate little [³⁵S]-methionine making them undetectable. All five polypeptides only appear with culture on SIM and are first detected after 10 to 14 days in culture. By this time meristematic domes have begun to differentiate, domes often have a distinct tunica, with the most advanced having foliar

primordia. These five polypeptides may be involved in the initiation and/or development of structures associated with the specialization of meristematic domes and their development into shoot primordia.

If involved in differentiation of a determined meristematic dome rather than the induction phase of organogenesis, one would expect that these polypeptides would be detected in the apex of *in vitro* plants, as this is where specialized organs associated with the shoot are initiated. None of these polypeptides could be detected in the shoot apex. The failure to detect them could be explained in three ways, either the detection technique was not sensitive enough to detect the small amounts present in the apex, or conversely, the polypeptides may be involved in the induction/determination process, but they are not present at high enough levels to be detected prior to day 14. Finally, these polypeptides may be involved in the initial organization on the shoot apex, but disappear once the structure is formed.

A further polypeptide 391 detected after 14 days on SIM was also present in the shoot apex, while being absent from protein extracted from leaf tissue, root tissue, or flower buds. This polypeptide may have a specific role in the developed shoot apex. Polypeptide 390, detected with culture on SIM, is definitely associated with a developed structure. This polypeptide was only positively identified after 21 days on SIM. At this time leaf primordia are prominent, with further primordia arising from the shoot apex. This polypeptide was also detected in leaf tissue and faintly in some extracts of apical proteins, suggesting a leaf specific nature. Tissue, and in particular leaf specific polypeptides, have been detected in other plants (Jernstedt, 1985). The detection of this polypeptide provides a useful marker for the appearance of leaf primordia.

Although not developmentally specific, in the sense that they are present in abundance under shoot forming conditions only, two other polypeptides not present at day 0, are interesting as markers of shoot induction. Polypeptides 382 and 383 are both detectable by silver-staining, while only 383 is clearly identified with *in vivo* labelling. Both polypeptides were first observed after 3 days culture on either SIM, RIM or CIM. They remain strongly detectable with continued culture on CIM, while on SIM, the levels of both polypeptides decline rapidly after day 3, being undetectable by silver-staining after more than 7 days. On RIM, both polypeptides decline slightly, but are still detectable by silver staining after 21 days in culture, while 383 is still faintly synthesized.

In cultured melon cotyledons the disappearance of a group of low molecular weight polypeptides more rapidly under shoot forming, than under root forming conditions, can be used to distinguish between the two organogenic processes early in culture (Leshem and Sussex, 1990). These polypeptides were thought to be storage

proteins and the difference in the timing of their loss was thought to be the result of differential utilization under different organogenic conditions.

Such a role for the two polypeptides detected in *B. erythrophylla* petiole explants would be unlikely as neither is synthesized at day zero, or with culture on BM. The synthesis of storage proteins in a petiole, which unlike a cotyledon is not a storage organ, would serve little useful purpose in the intact plant. The temporal appearance of these polypeptides under shoot forming conditions may represent a transient requirement for a new gene product during the initial stages of caulogenesis, which is lost, or at least reduced to undetectable levels, as meristematic regions form and are determined for shoot formation. Under callus forming conditions the polypeptides may be required permanently, while on RIM their loss may be more gradual, possibly due to a longer requirement under root forming conditions. It is also possible that despite protein being extracted from only the lower half of explants exposed to RIM, the polar nature of the root forming response may have resulted in explants only partially responding to the root forming stimulus. Non-responsive areas may be induced to synthesize the polypeptides but may not be exposed to a strong enough root forming stimulus to induce root formation and so synthesis continues in these regions.

Under either root or shoot forming conditions, both polypeptides are detected at the greatest level prior to the formation of any meristematic structures. It is tempting to consider that these polypeptides may be involved in the transition of certain cells of the initial explant from a differentiated to an undifferentiated state, in which they are able to respond to the organogenic stimulus. The continued detection of these polypeptides at high levels with culture on CIM may reflect the maintenance of the dedifferentiated state. This is only speculation, all that can definitely be said from this study is that both polypeptides provide good markers of an event that occurs early in the organogenic process prior to the formation of meristematic cells and that the rapid loss of both polypeptides during the formation of meristematic regions is characteristic of caulogenesis.

Three other polypeptides are also induced by culture on SIM, RIM or CIM, but these remained at similar levels throughout the culture period examined. These polypeptides most probably represented gene products required during general culture induced cell division and/or callus formation. Polypeptides common to organogenic/embryogenic and callusing media have been detected in several other studies (Chen and Luthe, 1987; Hahne *et al.*, 1988; Racusen and Schiavone, 1988; Reynolds, 1990).

All the other changes induced by organogenesis involve quantitative changes in the levels of polypeptides present in the petiole at the time of excision (day 0). Such changes are by far the most commonly observed, with many studies of organogenesis, embryogenesis and flower induction finding only such quantitative changes (Araki & Komeda, 1990; Stabel *et al.*, 1990; Renaudin *et al.*, 1991). In *B. erythrophylla* petiole sections such changes probably represent differences in the metabolism of shoots and roots, as the majority are detected only after primordia are formed, although regulatory roles may, as previously discussed, be possible.

One polypeptide (366) detected in this study shows a greatly increased level of synthesis after 14 days or more on SIM. Several other polypeptides show transient changes on SIM, which are more permanent on RIM. Such changes are interesting for not only do they provide markers for the shoot and root forming processes, but they show that during the initial stages of organogenesis changes common to both organogenic pathways are induced. Once meristematic structures are determined and differentiation begins to take place, the requirements of the organ-forming tissues rapidly diverge.

Evidence of specialized gene expression has already been demonstrated in the form of leaf and apex specific polypeptides. Evidence for root specialization was also detected. Two polypeptides (450,451) are only clearly detected in mature roots and after prolonged culture on RIM. Polypeptide 450 is a major spot detected in root tissue from *in vitro* plants and is not detected in any other tissue. Root caps of mature roots often secrete "slime" a mucopolysaccharide (Christianson and Warnick, 1988), the production of such a protein is indicative of mature roots. The polypeptides detected in both mature culture induced roots and in roots taken from *in vitro* plants, may represent specialized proteins required in the root. Although not the objective of this study, the appearance of proteins associated only with mature organs may be of interest in the study of the later stages of developmental gene expression.

8.2.2 Culture induced polypeptides

Again, although not the objective of this study, the induction of newly synthesized polypeptides in response to tissue culture alone and not the result of exposure to growth regulators is interesting. Tran Thanh Van *et al.* (1990) also found such a class of polypeptides were detectable by silver-staining upon culture of tobacco thin layers. In this study of *B. erythrophylla*, as well as the accumulation of these polypeptides it has been shown that many are synthesized at detectable levels in response to culture. These polypeptides may be related to excision shock (Theillet *et al.*, 1982) and/or they may be a reaction to the *in vitro* culture conditions (salts, pH).

Exposure to sucrose can be ruled out as a cause in *B. erythrophylla*, as culture on SIM(-) also induces these polypeptides.

Interestingly, one polypeptide induced by culture was synthesized at a higher level after seven days culture on RIM. Tran Thanh Van *et al.* (1990) found the opposite response with two polypeptides in this class declining with culture on root forming media. The possible role of such polypeptides needs further investigation.

(9) INHIBITION OF THE SHOOT FORMING PROCESS

The induction and development of organs is a complex process possibly involving several stages (Christianson and Warnick, 1988). In order to understand the process of *de novo* organogenesis a technique is required to selectively halt the process, at as many stages as can be identified, in order to ascertain the degree of commitment to differentiation. It has been demonstrated that temperature sensitive variant cell lines can be isolated from carrot cell cultures that undergo normal embryo development at a permissive temperature, but are arrested at specific developmental stages if exposed to a restrictive temperature (Breton, and Sung, 1982; Schnall *et al.*, 1991). This method of arresting the developmental process involves the isolation of mutants, which can be a difficult process.

Christianson and Warnick (1984), in an alternative approach, utilized the inhibitors tri-iodobenzoic acid (TIBA), sorbitol, ribose, ammonium ion, and acetylsalicylic acid (ASA) to determine several distinct stages in the process of shoot formation in *Convolvulus* leaf discs, but since then few such studies have been undertaken. Four of the above chemical inhibitors were used to determine stages in the process of shoot formation in *B. erythrophylla* petiole sections.

9.1 The effects and timing of inhibitors

9.1.1 Sorbitol

Sorbitol, in the presence of sucrose, inhibits shoot formation in *Convolvulus* leaf discs (Christianson and Warnick, 1984). A similar inhibition is seen in *B. erythrophylla*, although the concentration of sorbitol required and the timing of inhibition is quite different. Thirty grams per liter of sorbitol in addition to the three percent sucrose in SIM is required to give complete inhibition of shoot production. Sorbitol is only an effective inhibitor if explants are transferred from SIM to SIM plus

sorbitol prior to day three of culture. This means that sorbitol is only effective prior to the onset of cell division. Transfer from SIM to SIM plus sorbitol once cell division has begun only results in a reduction in shoot number and some stunting of the shoots formed. The effects of sorbitol are not initially detrimental. Exposure to SIM plus sorbitol prior to transfer to SIM prolongs the time explants can be maintained in culture before inducing shoots. Pre-culture on basal medium results in a more rapid loss of the ability to respond to SIM. This maintenance ability may be due to the presence of cytokinin in the medium, which is known to delay senescence (Salisbury and Ross, 1978).

With culture on SIM alone, the most significant histological observation prior to day three is the accumulation of starch in the explant and in particular, heavy deposition in the regions of the explant associated with the formation of the meristematic zones. Addition of sorbitol to SIM reduces the total amount of starch accumulating with few regions of localized starch deposition in the superficial layers of the explant and only minimal meristematic activity. Interference by hexose analogues in carbohydrate metabolism is known in fungi (Moore, 1981) and Berliner and Martindale (1981) have suggested that mannitol may suppress starch synthesis in *Cosmarium botrytis* cells. As already discussed, failure to accumulate starch results in the suppression of shoot formation. Addition of sorbitol to SIM, although allowing a small amount of starch to accumulate randomly throughout the tissue, appears to inhibit the localized accumulation of starch and therefore may inhibit shoot initiation in this manner.

Sorbitol is also an osmotic agent and a high osmotic potential has been shown to inhibit shoot formation (Brown *et al.*, 1979; Hammersley-Straw and Thorpe, 1988). Inhibition of shoot formation in *B. erythrophylla* requires a high concentration of sorbitol be added to SIM. Addition of 30 grams per liter sorbitol to the 30 grams per liter sucrose already in SIM, gives an osmotic strength roughly equal to 90 grams of sucrose. This concentration of sucrose greatly reduces the number of shoots forming if explants are cultured on SIM containing 9% sucrose.

If explants are transferred from SIM alone to SIM plus sorbitol after less than seven days, normal numbers of shoots, albeit slightly stunted, develop. This suggests that if sorbitol is acting as an osmoticum it is only effective during the early stages of shoot formation. Brown and Thorpe (1980a) have demonstrated that changes in water relation components are among the earliest events observed in shoot forming tobacco callus. Such an event, occurring prior to the initiation of meristematic regions, may be osmotically inhibited at a lower level than that required to inhibit the development of meristematic regions once established. Meristematic cells have a much higher internal concentration of solutes than most other cells, which may make them more resistant to

osmotic effects. To determine whether sorbitol suppresses the synthesis of starch, effects starch localization, acts as an osmoticum, or has other unknown effects requires further investigation.

9.1.2 Ribose

An active pentose phosphate pathway is one important component of the energy requirements for shoot formation in tobacco callus (Thorpe and Laishley, 1973). Histochemical studies suggest that it may also be required in *B. erythrophylla*, as glucose-6-phosphate dehydrogenase activity is strongly localized in the developing meristematic regions of the explant.

Christianson and Warnick (1984) found that the addition of either ribose, or two other intermediates in the pathway, 6-phosphogluconate and erythrose, inhibited shoot formation in *Convolvulus* leaf discs. These intermediates in the pentose phosphate pathway have all been shown to stop the flow of carbon through the pathway (Pontremoli and Grazi, 1969).

Addition of 20g/l ribose to SIM inhibits the formation of shoots from *B. erythrophylla* explants, if they are transferred from SIM to SIM plus ribose prior to day 7 of culture. The key histogenic events associated with shoot formation occur prior to day 7, for at day 7 the most advanced meristematic domes are strongly committed and by day 9 to 10 show signs of specialization. With 7 or more days culture on SIM prior to transfer to SIM plus ribose, the number of shoots produced per explant rapidly increases as more meristematic domes reach a strongly determined state. This suggests that ribose does not inhibit determined meristematic domes.

In tobacco callus, cultured on a shoot inducing medium, the activities of both the PPP and the EMP pathways increase during the formation of meristemoids and then decline somewhat as primordia develop (Thorpe and Laishly, 1973). This suggests that compared to meristemoid formation, during the development and growth of the shoot primordia, there is a reduced requirement for the products of these pathways. If ribose does inhibit the pentose phosphate pathway and high demands for the products of this pathway occur mostly during the formation of meristematic regions, these demands may not be met on SIM plus ribose, with the result that little meristematic activity is observed. Once meristematic regions have reached a significant size, after 7 or more days on SIM, their continued development may require less PPP activity and so the level of activity in the presence of ribose may be sufficient to allow continued development. Alternatively, developed meristematic structures may be able to overcome the effects of ribose in an unknown manner.

Other inhibitory effects for ribose are also possible, particularly as ribose not only inhibits shoot production, but also the production of basal callus. Again further studies are required to determine the exact biochemical nature of ribose inhibition.

9.1.3 TIBA

Shoot formation from leaf explants of *Convolvulus* and tobacco callus can be inhibited by the inclusion of TIBA in the shoot inducing medium (Murashige, 1965, Christianson and Warnick, 1984). Shoot formation from *B. erythrophylla* petiole explants can also be inhibited by the addition of TIBA to SIM. The response of plant tissue to TIBA is often associated with an inhibition of polar auxin transport (Hay, 1956; Niedergang-Kamien and Leopold, 1957), although at high concentrations TIBA can function as an anti-auxin (Thompson *et al.*, 1973). The mode by which TIBA inhibits shoot formation is not known, although Christianson and Warnick (1984) suggest TIBA may act as an inhibitor of polar auxin transport and so alter the balance of growth regulators in SIM governing caulogenesis. This appears feasible as the addition of TIBA to tissue culture media can simulate caulogenesis in explants with a high internal concentration of auxin, purportedly by inhibiting polar auxin transport (Cassells, 1979; Cambeceades, 1991).

TIBA, although inhibiting shoot formation in *B.erythrophylla* petiole sections, does not inhibit development of the explant. Domes of cells, which bear no resemblance to shoot primordia, develop from the superficial layers of the explant. Thus addition of TIBA to SIM may result in an alternate developmental pathway, rather than just inhibiting the formation of shoot primordia as do sorbitol and ribose. This supports the effect of the addition of TIBA to SIM being a modification of the growth regulator balance within the explant, normally brought about by exposure to SIM alone.

Unlike both ribose and sorbitol, the effects of a brief exposure too which can be overcome, the effects of exposure to SIM plus TIBA are rapid and irreversible. Exposure to SIM plus TIBA for 5 days results in almost complete inhibition of the ability of explants to form shoots upon transfer to SIM alone. Furthermore, with prolonged culture on SIM plus TIBA explants do not gradually become senescent, as observed with pre-culture on SIM plus either sorbitol or ribose, but upon transfer to SIM form basal callus and a few TIBA induced domes of callus like cells. Therefore, TIBA can permanently change the physiology of the explant so that it is not capable of responding to the shoot forming stimulus, without harmful effects on the explant. This suggests that TIBA may be more specific in inhibiting caulogenesis than either ribose or sorbitol.

Unlike *Convolvulus* leaf discs, where the inhibitory effects of TIBA only occurs during a brief period, *B. erythrophylla* petiole sections show a continuous spectrum of sensitivity to TIBA. The degree of sensitivity depends on the stage of development of the shoot primordia at the time of exposure. Transfer from SIM alone to SIM plus TIBA prior to day 7, results in the formation of structures like those observed when explants are cultured on SIM plus TIBA from day zero. These nodular structures appear similar to the compact calli observed with culture on CIM. Transfer to SIM plus TIBA before day 7 may divert the uncommitted meristematic regions from the pathway of shoot formation, resulting in the production of callus like domes. Such a diversion could be the result of changes in the internal growth regulator balance of the tissue brought about by TIBA. Along these lines, it is interesting to note that TIBA does not inhibit the localized accumulation of starch to the same extent as sorbitol, thus this early event in the process of organogenesis is not affected by TIBA.

With transfer to SIM plus TIBA at or after day 7, shoot buds with deformed leaves and leaf initials are formed. TIBA not only appears to inhibit the formation of shoot primordia, but can also affect the development of strongly committed primordia. Transfer after 14 or 21 days on SIM resulted in very few leafy shoots forming. Therefore, exposure of even well developed shoots to SIM plus TIBA can significantly alter the appearance and development of leaf primordia.

The effect of TIBA on leaf morphogenesis from axillary buds of pea plants has recently been investigated (Gould *et al* , 1991). In pea, addition of TIBA to the culture medium, on which single node explants were cultured, resulted in abnormal foliar development. The axillary buds of pea plants bear up to 6 leaf primordia (Gould *et al* , 1987), of these, only the four youngest leaf primordia were effected by TIBA, the oldest two were already determined structures when the explants were taken.

Meristematic domes, arising from *B. erythrophylla* petiole sections cultured on SIM, are determined after 7 days on SIM, but at this time they showed no sign of leaf primordia. Leaf primordia developing from these domes appear susceptible to the effects of TIBA. This points to a developmental window where the growth centers for leaf formation are receptive to chemical signals which will ultimately determine leaf form. Exposure to TIBA during this developmental window alters the normal pattern of leaf development, only strongly determined leaf primordia are not affected by exposure to TIBA. This may be why transfer from SIM to BM after 21 days results in better shoot development than continued culture on SIM and why the shoots produced from explants cultured on BA8 medium are stunted. The inductive stimulus, which promotes bud development may be inhibitory to leaf development. TIBA is a useful inhibitor not only inhibiting the formation of shoot primordia, but also influencing the development of leaves arising from these primordia.

The effects of TIBA demonstrate that although meristematic domes are determined for caulogenesis after 7 days on SIM, foliar primordia arising from these domes are not initially strongly determined and are responsive to other inductive, stimuli until they develop to a stage where they are determined and no longer competent to respond to TIBA. Therefore shoot formation may involve the initial determination of a shoot meristem followed by the separate determination of organs arising from the meristem.

9.1.4 Acetyl salicylic acid (ASA)

Both acetyl salicylic acid and salicylic acid have a number of biological effects in both humans and plants. They have been reported to induce the process of floral initiation as well as inducing the appearance of stress related polypeptides (Kumar and Nanda, 1981)

Acetyl salicylic acid has been shown to be a potent inhibitor of shoot regeneration from *Convolvulus* leaf discs (Christianson and Warnick, 1984). ASA is also an effective inhibitor of shoot formation from *B. erythrophylla* petiole sections. Culture on SIM plus ASA (5×10^{-6} M) rapidly inhibits the explants ability to form shoots, while still allowing callus formation from the base of the explant. ASA unlike ribose or sorbitol does not appear to inhibit a metabolic process essential for callus formation, but a process or event specifically associated with the formation of shoots. SIM plus ASA also fails to induce in any alternative development of the explant, as seen with exposure to SIM plus TIBA.

Culture on SIM plus ASA does not inhibit localized accumulation of starch and allows the formation of better developed meristematic regions than the other inhibitors. These observations, combined with the fact that 7 to 10 days exposure to SIM is required before explants are able to form shoots upon transfer to SIM plus ASA, suggests, that ASA may allow the early stages of caulogenesis to proceed in at least some parts of the explant. It seems that addition of ASA to SIM may affect a process/event, specific to caulogenesis, that occurs at or just prior to determination. Thus, ASA is a useful stage specific inhibitor with the potential to isolate the early events associated with induction from those of the determined meristematic dome.

9.2 Subdividing the process of caulogenesis

Christianson and Warnick (1984) used shifts to and from permissive to non-permissive media to identify stage-specific inhibitors of the process of shoot induction in *Convolvulus* leaf discs. In this study it has been shown that the process of caulogenesis in *B. erythrophylla* can also be inhibited using four of the inhibitors used by Christianson and Warnick (1984). The order of sensitivity to these inhibitors, in *Convolvulus*, showed a general pattern between genotypes, with all four inhibitors effective during the induction phase. Sensitivity to salicylates preceding TIBA sensitivity, while sorbitol sensitivity fell between TIBA sensitivity and determination (Christianson and Warnick, 1984). The sequence of sensitivity in *B. erythrophylla* was not only completely different to that observed in *Convolvulus*, but sorbitol was only effective as an inhibitor if explants were exposed prior to cell division taking place and sensitivity to ASA was only lost at or just after the point of determination.

These differences were not unexpected as Christianson and Warnick (1984) did find some variation in timing between genotypes and when comparing the two systems, not only are totally unrelated species being compared, but direct caulogenesis is being compared to indirect caulogenesis. Irrespective of such differences and the lack of knowledge regarding the possible modes of inhibition, the use of chemical inhibitors does enable the process of caulogenesis in *B. erythrophylla*, to be further subdivided and provide a means of isolating events occurring during the early stages of caulogenesis.

9.3 The effect of inhibition on the pattern of SIM induced polypeptides

Despite the apparent stage specific nature of the inhibitors used to suppress shoot formation, the polypeptide patterns observed upon chemical inhibition of caulogenesis were similar. Addition of sorbitol, ribose or TIBA to SIM, completely suppressed the synthesis of newly detected polypeptides associated with both the determination of meristematic domes for caulogenesis and the development of shoot primordia. Increases in existing polypeptides associated with caulogenesis were also inhibited. To some extent this was to be expected as these inhibitors are effective early in the culture process (prior to day 7) and most of the changes in the polypeptide pattern associated with caulogenesis occur at, or after, day seven. Only culture on SIM plus ASA showed the presence of polypeptides that may be associated with

determination for caulogenesis and these could only be detected, at trace levels, with silver staining.

SIM plus ASA, which allows the formation of some meristematic regions, appears to be inhibitory around day 7 of culture, the point at which these SIM only polypeptides are beginning to appear. ASA may allow the early events associated with shoot formation to occur, while inhibiting those taking place at or just prior to the onset of shoot determination. This is particularly evident when polypeptides 382 and 383 are considered. Under normal shoot forming conditions both polypeptides decline after 3 days on SIM. Addition of either sorbitol, ribose or TIBA to SIM, results not in a decrease in the level of these two polypeptides, but in an increase. Thus, the switching off of the synthesis of these polypeptides does not occur in the presence of these inhibitors.

With the addition of ASA to SIM both polypeptides, although not completely suppressed, showed little increase in abundance when compared to the other three inhibitors. ASA does not appear to completely inhibit this initial critical switch in the shoot forming pathway, but is effective only later. ASA also fails to inhibit the increased synthesis of SIM+ polypeptide G to the same extent as the other three inhibitors. ASA is a useful inhibitor for studies on the early stages of shoot induction as it does not appear to totally inhibit the molecular events associated with this phase of organogenesis.

The other three inhibitors, despite their different effects on explant morphology, essentially have similar effects at the molecular level. This is interesting for although sorbitol and ribose allow little cell division to occur, TIBA allows considerable development of the explant. Addition of TIBA to SIM results in the formation of domes of callus like cells. Polypeptides 382 and 383 accumulate with culture on CIM, although the level of synthesis of 383 is not high compared to that observed with culture on SIM plus TIBA. The continued synthesis of this polypeptide may represent a similarity between the cells of the compact callus and those of the TIBA induced nodules.

Culture on SIM plus either sorbitol or ribose results in little cell division and only occasionally do meristematic regions form. The decline in the level of polypeptides 382 and 383 occurs during the formation of meristematic structures and the failure of these structures to form, under inhibitory conditions, appears not to trigger the reduction in synthesis of these polypeptides, instead the levels continue to rise.

These results support the earlier observation that the down-regulation of these two polypeptides is the result of an event that takes place during the early stages of caulogenesis and more specifically, that this event takes place during and is dependent

upon, the formation of meristematic regions. As both sorbitol and ribose inhibit the formation of such regions, albeit possibly in different ways, their effects on the molecular events leading to caulogenesis are similar if not identical. TIBA also inhibits the formation of meristematic regions associated with shoot formation, not by suppressing cell division, but by inducing the formation of domes of callus-like cells rather than meristematic regions. Again, despite caulogenesis being suppressed in a completely different manner, the effects on shoot formation at the molecular level appear similar.

It is also interesting that polypeptide 383 is still synthesized at a high level after 14 days on SIM plus sorbitol or ribose, despite little cell division taking place in the explants and the complete loss of competence. Although both ribose and sorbitol may inhibit biochemical or genetic processes required for development, in general, they still allow polypeptides associated with the early stages of culture on inductive media to occur and be maintained for a considerable length of time.

The absence or greatly reduced levels of polypeptides normally detected under shoot forming conditions, in the presence of inhibitors effective prior to day 7, supports their association with caulogenesis.

10 SHOOT FORMATION FROM LEAF DISCS

The isolation of polypeptide markers associated with phases of caulogenesis in petiole sections will enable more detailed studies to be carried out on the genetic events associated with this process. These markers could be considered even more useful if they were common to caulogenesis in other tissues of the parent plant. For this reason, the last part of this study was carried out to determine if the markers for caulogenesis in petiole sections were also present in leaf discs exposed to SIM.

10.1 General culture responses

Overall, leaf discs excised from potted plants respond to culture on SIM in much the same way as *in vitro* petiole sections. Although histological examination of the origin of shoots from leaf discs was not carried, external observations using a scanning electron microscope revealed that shoots arose both from the undamaged tissue of the leaf disc and from the cut surfaces from which callus forms. Whether shoots at the cut surface arise indirectly from callus, or directly from cells of the

explant is not known, but externally the development of shoot buds from undamaged regions closely parallels that observed with *in vitro* petiole sections.

Leaf discs are less responsive to culture than *in vitro* petiole sections, with many discs failing to form shoots and the numbers of shoots formed varying greatly between discs. This is probably due to differences in the position of discs within the leaf, leaf age (although leaves of the same size were used) and the less stringent control of environmental conditions afforded by growth of plants out of the culture environment. As plants were clonally derived from leaf-cuttings, genotype effects would be unlikely.

After day 3 of culture the changes in fresh weight, dry weight and protein content of leaf discs exposed to either SIM or BM essentially parallel those observed in petiole sections. Culture on BM resulted in no increases, while with culture on SIM there is a gradual increase in fresh weight, dry weight and the protein content of discs. The increases are less pronounced than those observed with petiole sections, possibly due to a smaller percentage of the tissue being responsive to the inductive stimulus, or to a less uniform exposure of the tissue to the culture medium. Petiole sections were exposed with the basal cut surface of the explant in contact with the medium. This would allow transport of nutrients and growth regulators up the vascular cylinder giving a uniform distribution throughout the explant. Distribution would most likely be less uniform with leaf discs, due to the cut surface of the explant not necessarily being in contact with the medium and a less regular organization of the explant tissue. In a study on petunia leaf discs, the size and cut surface area of discs has been shown to have a significant effect on the number and distribution of shoots formed (Beck and Camper, 1991).

One major difference between leaf discs and petiole sections is the substantial loss in fresh weight by leaf discs, immediately upon culture. Petiole sections show only a very small loss. This drop in fresh weight is due to water loss as there was no decline in the dry weight of explants. The loss stabilizes after 3 days in culture after which time the fresh weight increases on SIM, while with culture on basal media no increase in fresh weight is observed. *B. erythrophylla* tissue whether it be leaf or petiole has a high initial water content and this rapid loss in fresh weight is most probably due to water loss from the cut surface of the tissue. Such a large water loss may be the reason leaf discs excised from *in vitro* plants fail to respond to culture.

Leaves of *in vitro* plants often have not developed the specialized water retention abilities of potted plants (Pasqualetto, 1990). Although even large (1cm) leaf discs from *in vitro* plants did not survive when cultured, if large strips of leaf tissue were placed on SIM they survived and produced shoots. Therefore leaf tissue of *in vitro* plants is responsive to SIM, but this ability is not observed if discs fail to

survive excision. Broad strips of leaf tissue have a much smaller cut surface area to total tissue area ratio, than leaf discs and so water loss would presumably be less. Leaf discs from potted plants tend to be thicker than those from *in vitro* plants and this may serve to reduce water loss.

This rapid loss of fresh weight within the first 3 days of culture, induced an apparent increase in the protein content of leaf tissue exposed to either SIM or BM. This is simply because as protein content is expressed as ug protein per mg of tissue fresh weight, a greater dry weight of tissue is extracted to make up for the water loss. Thus, it appears that there is a very large and rapid increase in protein content with culture on either SIM or BM.

10.2 Media transfer experiments

Leaf discs require 5 days on SIM before transfer to BM for minimal determination. The numbers of shoots determined after 5 days increases with time in culture, as with petiole sections. The time on SIM required for minimal determination is 2 days less for leaf discs than for petioles. This shortening of the time required for minimal determination may reflect differences in the ability of the tissue to respond to the organogenic stimuli. Epidermal cells of the leaf may be more responsive to SIM than epidermal cells of the petiole. It also must be considered that petiole sections become determined after 4 or 5 days exposure to BA8 medium and that this reduction in the time for determination may simply be the result of a greater concentration of BA being taken up by the tissue. A difference in the internal growth regulator balance of petiole and leaf tissue, may simply mean that an internal growth regulator balance favouring shoot formation is achieved in leaf tissue after only 5 days on SIM, while petioles require longer. This is quite possible as potted plants were not grown under 24 hours of daylight as were *in vitro* plants and, as already discussed, the internal ratio of auxin to cytokinin may be more favorable to caulogenesis under these conditions.

As the objective of this section of work was to determine if the polypeptide changes associated with shoot formation in the petiole are common to shoot formation in leaf discs, a slight difference in the timing of determination should not have affected the results obtained, as protein was extracted well after the point of determination in both systems.

10.3 2-Dimensional electrophoresis

10.3.1 General changes induced by culture

The major change to the protein pattern observed upon culture of leaf discs on either SIM or BM, was the decrease in intensity of several 55-kD polypeptides. Although synthesis studies were not carried out, such a massive decline in the intensity of these polypeptides most likely corresponds to a decline in their synthesis upon culture. One of these polypeptides probably represents the large subunit of Rubisco, while at least some of the other polypeptides may be chloroplastic polypeptides. Charge modification of Rubisco during protein extraction could account for the large number of spots observed. Francis *et al.* (1985) found that appearance of multiple spots on 2D-gels closely associated with Rubisco, were the result of degradation that occurred with some extraction procedures.

The disappearance of Rubisco and several related polypeptides has also been observed when thin layers from tobacco leaves are cultured (Tran Thanh Van *et al.*, 1990). Several polypeptides also increased in intensity during the period in which Rubisco declined. Culture of *B. erythrophylla* leaf discs also results in the appearance of previously undetectable polypeptides and increased the relative abundance of many polypeptides present at low levels at day zero. Tran Thanh Van (1990) suggested that these polypeptides probably corresponded to "house keeping proteins", whose synthesis stayed relatively stable, but whose relative abundance in the cells increased by virtue of the large decrease in the 55-kD spots. Thus, most of the changes observed with culture probably corresponded to the loss of polypeptides involved in specialized functions of the leaf, such as photosynthetic activity. Such a hypothesis also would fit the observations made with *B. erythrophylla* leaf discs.

The levels of several 55-kD polypeptides also decline in *B. erythrophylla* petiole sections as a general response to culture, but in the petiole these polypeptides are less abundant than in the leaf, reflecting a less active role in photosynthesis. As by far the greatest portion of the protein extracted from leaf discs at the time of excision (day 0) is made up of 55-kD polypeptides and equal concentrations of protein were loaded on each gel, far more polypeptides were detectable in the petiole than in the leaf at the time of excision. With culture and the decline in abundance of the 55-kD polypeptides, the general pattern of polypeptides observed after culture of leaf discs changes to resemble that of the petiole. This suggests that in both the petiole and the leaf, most of the polypeptides detected are simply "house keeping" and that with the loss of specialized polypeptides in the leaf, they increase to levels similar to those observed in the petiole.

Culture of leaf discs also resulted in the appearance of many of the culture induced polypeptides observed with culture of the petiole sections. Most of these polypeptides therefore appear not to be tissue specific and are a general response of *B. erythrophylla* tissue to culture.

10.3.2 Polypeptides associated with caulogenesis in leaf discs

Although not all the polypeptide changes associated with shoot formation in petiole sections were detected in leaf discs cultured on SIM, the 4 most prominent markers were detected. Two group 6, SIM only polypeptides (376 and 377), which provided good markers of determination for caulogenesis, were detected in leaf discs cultured for 14 days on SIM, as were increases in the levels of polypeptides 288 and 366, which were general markers for the formation of shoot primordia in petiole explants. Polypeptide 366 showed an even greater increase in abundance in leaf discs than in petiole sections. Polypeptides 382 and 383 were also absent with culture of leaf discs on SIM, while being detectable in leaf discs cultured on RIM, further supporting their disappearance as being important for caulogenesis.

The polypeptides correlated to caulogenesis that were detected in the leaf discs were those most strongly detected in petiole. The other markers of caulogenesis were not detectable, probably due the reduced shoot forming response of leaf discs. Compared to petiole sections, leaf discs produce fewer shoots from a greater weight of tissue, so shoot specific polypeptides would be expected to have a proportionately lower abundance making many undetectable. The enhanced expression of polypeptide 366 in the leaf discs cultured on SIM, while other polypeptides associated with shoot formation are expressed at lower levels, casts some doubt on its direct role in shoot formation. It may be the result of a general tissue response elicited only in shoot forming tissue, but further work is required to ascertain its role.

Culture of leaf discs on RIM failed to induce any of the changes associated with shoot formation, but the most prominent markers of root formation, namely, the retention of polypeptides 382 and 383 were observed. These results suggest that at least some polypeptide markers of shoot formation detected in this study are not tissue specific, but are expressed in response to caulogenesis in both leaf and petiole tissues of *B. erythrophylla*.

GENERAL DISCUSSION AND CONCLUSIONS

Christianson and Warnick (1985) divided the organogenic process into 3 phases. A dedifferentiation phase in which cells become competent to respond to an inductive stimulus. An induction phase in which competent cells are induced into a particular organogenic pathway into which they become progressively committed. Once cells are committed to one organogenic pathway and no longer require the inductive stimulus they are considered determined. After determination is achieved the third phase differentiation occurs, during which a cell or group of cells develop into a specialized organ.

The processes of caulogenesis and rhizogenesis in *B. erythrophylla* can also be divided into three phases all of which overlap due to the asynchronicity of the processes. The phases and events occurring during organogenesis in *B. erythrophylla* petiole explants are summarized in figures (FIGURES 20A & 20B).

As organogenesis in *B. erythrophylla* is direct, dedifferentiation in the form of callus production as described by Christianson and Warnick (1985) is not applicable. The first phase of organogenesis is a lag phase of 2 days in which no cell division takes place. During this phase some epidermal cells become responsive to organogenic media.

Markers of the lag phase of organogenesis.

- (1) The appearance and localized accumulation of starch in collenchyma and epidermal cells of the explant
- (2) The sensitivity of caulogenesis to sorbitol
- (3) The appearance of polypeptides 382 and 383.
- (4) A decline in the abundance/synthesis of polypeptides associated with petiole function

The continued commitment of a group of cells to integrated development depends on suppression of any tendency on the part of individual cells to act independently. Loss of this integrated control is possibly the factor permitting a single cell or group of cells within an explant to respond to morphogenic stimuli.

With culture upon the correct medium, changes take place within the explant that result in some cells gaining the ability to respond to an inductive stimulus or

stimuli. These changes may involve interrelated events at both the cell and tissue level. A change in the phenotype, or in other words a change in the state of competence, of cells within an explant may be required in order for them to become "target cells" able to respond to organogenic stimuli. Such a change may enable cells to perceive inductive stimuli, i.e. growth regulators, and thus become responsive. A change in phenotype may involve switching off genes involved in specialized cellular functions and switching on and/or off others involved in growth regulator perception.

Certain physiological/metabolic constraints within the explant as a whole may also have to be met to allow "target cells" to respond. Metabolic constraints may simply involve supplying "target cells" with a readily and rapidly accessible supply of energy, possibly in the form of starch. Any inhibitory factors present within the explant maintaining cells in an unresponsive state, or predisposing them to a particular developmental pathway, may also have to be overcome. Target cells must also be exposed to the inductive stimulus, which may involve transport within the explant.

If "target cells" inhibited by the correlative influences of surrounding tissue are present in an explant at the time of excision, once these influences are removed, only the physiological/metabolic constraints may have to be overcome. If, however, no target cells are present, both a change in the phenotype of cells and physiological changes within the tissue may be required before explants are competent to respond to induction.

B. erythrophylla petiole sections take approximately 48 hours to become competent to respond to an inductive stimulus, although the process is probably not synchronous. Competence is a general phenomenon, which can be achieved by exposure to either SIM or RIM. Once achieved, certain epidermal cells are capable of responding to either organogenic medium. From this study it could not be ascertained whether target cells exist in the explant at the time of excision, or if they are induced. It can, however, be stated that epidermal cells associated with glandular hairs are more amenable to organogenic stimuli than other cells of the explant, including the majority of epidermal cells. This suggests that such cells may be less strongly committed than other epidermal cells.

The localized accumulation of starch is correlated to the formation of meristematic regions irrespective of the type of organogenesis. This means that at least one metabolic constraint, at the tissue level, must be overcome for competence to be achieved. The importance of starch accumulation is emphasized by the inhibition of the ability of explants to respond to SIM in the presence of 30g/l sorbitol. Sorbitol is only an effective inhibitor of caulogenesis if explants are exposed during the lag phase, for once starch has accumulated and cell division has begun, sorbitol is no

longer effective as an inhibitor of caulogenesis. Addition of sorbitol to SIM provides an effective means of separating the lag and induction phases of caulogenesis.

During the first 3 days of culture the pattern of both accumulated and synthesized polypeptides observed at day 0 undergoes many changes. Most of these are either culture induced or occur as a result of culture on either organogenic medium. These changes include the synthesis of polypeptides not synthesized at day 0 and reduced synthesis of polypeptides associated with petiole physiology. Changes specific to either caulogenesis or rhizogenesis are only clearly detected with further culture, suggesting that during the lag phase that effects of both media on explants are similar.

Of greatest interest during this phase were 2 polypeptides (382 and 383), which were detectable at similar levels after 3 days culture on either SIM or RIM, but which rapidly disappeared with further culture on SIM, while remaining detectable with culture on RIM. The decline of these 2 polypeptides clearly marks the induction phase of caulogenesis and can be used to distinguish between the 2 organogenic pathways prior to development of organ primordia. Chemical inhibition of caulogenesis at the onset of the induction phase or shortly after, results in an increase in the levels of these polypeptides rather than a decline. This indicates that the down regulation of their synthesis is linked to the induction phase of the caulogenic process.

Markers of explants ability to respond to the organogenic media i.e. the transition to the inductive phase.

- (1) Cell division in the epidermal regions where starch has accumulated.
- (2) The loss of sorbitol sensitivity.

Once competence is achieved the induction phase begins. This phase primarily involves the formation of meristematic regions in the superficial layers of the explant.

During this phase explants must be exposed to a specific inductive medium for a particular organ to form, but despite this requirement many changes that occur within the explant are common to both caulogenesis and rhizogenesis. During the inductive phase localized meristematic regions with high metabolic activity are formed on both organogenic media. This increased metabolic activity is reflected in increased enzymatic activity common to both forms of organogenesis, as well as a rapid increase in the protein content of the organogenic tissues. Most of this increase is due to a general increase in the level of synthesis of polypeptides present at the time of excision. Such increases probably reflect increased expression of "house keeping

genes," including enzymes involved in general metabolic functions. Along with the increased protein content, the overall starch content of the tissue continues to rise resulting in an increase in the dry weight of explants.

The shoot forming response shows no polarity, but root formation can occur in a polar manner depending on explant orientation. Although cells capable of responding to RIM occur throughout the epidermal layer of the explant, their exposure to the root forming stimulus can be governed by physiological constraints within the explant. This emphasizes the regulatory effects of the explant as a whole.

Markers associated with the induction phase.

- (1) The appearance of meristematic cells and the formation of meristematic regions in the superficial layers of the explant.
- (2) Increased enzymatic activity localized in the meristematic regions.
- (3) An increase in the dry weight, fresh weight ratio
- (4) A rapid increase in the protein content of the explant
- (5) The loss of sensitivity to ribose during caulogenesis.
- (6) Differential levels of synthesis of polypeptides 383 and 382 with exposure to SIM or RIM (CIM).

The induction phase is rapid. Once competence is achieved, 24 hours on RIM are required for minimal root determination while 48 to 120 hours, depending on the concentration of BA in the shoot inducing medium, are required for minimal shoot determination. Despite explants no-longer requiring exposure to the inductive medium once determined, further culture is required for explants to be determined strongly enough not to be influenced by other inductive conditions. Explants exposed to RIM are strongly determined into the pathway of root formation after 5 days on RIM, with some meristematic regions no-longer competent to respond to SIM. With culture on SIM or BA8 explants can be transferred to RIM and still form shoots after 7 Days, although the point at which the meristematic regions change their state of competence is not clear.

The time explants require on an inductive media for determination is difficult to ascertain in bulky explants by simply transferring to basal medium, for the concentration of growth regulators in the inductive medium can effect the apparent timing of determination without any observable developmental or molecular effects. Exposure to an alternative inductive stimulus is perhaps a better method of ascertaining the stage of development when explants no longer require exposure to an inductive medium to form organs and when the meristematic regions from which these organs form, are no longer easily influenced by an alternative stimulus. Such experiments may give a truer picture of the stage at which meristematic regions become determined.

One of the most interesting findings of this study is the detection of polypeptides not synthesized at the time of excision, which appear when explants are strongly determined. These polypeptides are differentially synthesized, with one class expressed primarily in explants forming roots and the other in explants forming shoots. Both classes increase in abundance as the number of strongly determined organs increases.

The synthesis of these polypeptides under shoot forming conditions does not occur if the shoot forming process is chemically inhibited, prior to determination, further supporting a specific role. These polypeptides provide good markers for the transition from the growth regulator dependent inductive phase, to the growth regulator independent stage of differentiation and may prove useful for further studies of the determination process.

Markers associated the loss of explants requirements for the inductive media i.e. the transition from induction to differentiation.

- (1) A rapid increase in fresh weight.
- (2) The differential synthesis of polypeptides associated with either shoot or root formation.

Once strongly determined, meristematic structures rapidly become specialized both at the structural and molecular level. The meristematic regions organize to form distinct primordia and as the primordia develop, polypeptides associated with mature organs begin to appear and increase in concentration with time. The fresh weight, dry weight, starch and protein contents all decline, as explants move from the induction phase to the development of induced primordia.

Markers associated with differentiation

- (1) The appearance of polypeptides preferentially expressed in developed/developing organs.
- (2) Appearance of specialized structures such as foliar primordia.
- (3) A rapid increase in fresh weight, accompanied by a decline in the dry weight, fresh weight ratio.

From this study it is apparent that the process of organogenesis can be divided into distinct phases by combining information obtained from histological, physiological and biochemical studies and that polypeptides can be used as molecular markers to distinguish between these phases. When the timing of these molecular markers associated with organogenesis and in particular shoot formation is considered, a pattern emerges. Most of the changes are first observed at the transition stages, when explants move from one phase of organogenesis to the next, suggesting that it is at these points that the greatest changes in gene expression take place.

The nature of these polypeptides is unknown at this time, they may represent polypeptides synthesized in response to culture on different media, although the apparent transient nature of the expression, of many of them, would make this unlikely. They also could reflect metabolic differences between meristematic regions forming in response to the different inductive stimuli, for example isozymes specific to particular form of organogenesis. The possibility of a regulatory role must also be considered. Numerous regulatory proteins have been identified in bacterial operons, most being gene repressors, but some are capable of inducing gene activity. In *Drosophila* regulatory proteins are involved in the puffing of polytene chromosomes in response to both heat shock (Parker & Topol, 1984) and hormonal synthesis. For example the hormone ecdysone binds within target cells to a specific receptor protein, that then associates with specific chromatin sites and activates puffing (Ashburner *et al.*, 1974). Recently, genes encoding a family of auxin binding proteins (ABPs) have been isolated from maize (Klee & Estelle, 1991) and Arabidopsis (Inohara *et al.*, 1989; Tillmann *et al.*, 1989), but whether these proteins are receptors remains to be determined. Several models for coordinate regulation of gene expression by plant growth regulators, involving regulatory proteins, have been formulated (Lazarus, 1991), but to date the role of regulatory proteins involved in the response of plant tissue to growth regulator remains inconclusive.

The results of this study further emphasise both the complexity and the integrated nature of plant morphogenesis in bulky explants. Regulation may be at both the cell level and at the tissue level.

The ability of explants to respond to an inductive stimulus may depend on one or several factors, the presence of target cells sensitive to the inductive stimulus or of cells able to become target cells, physiological constraints such as the transport of the inductive stimulus (growth regulators) to the target cells, metabolic constraints such as a supply of energy and overcoming the correlative effects of the surrounding tissues, which may simply involve a change in the internal growth regulator balance. Physiological, metabolic and correlative effects are particularly important when studying morphogenesis in bulky explants, because the regions of the explant responding to an organogenic stimuli make up only a small portion of the overall tissue mass.

Future Work

Recent advances in the field of preparative high resolution two-dimensional gel electrophoresis have been documented for obtaining highly purified preparations of low abundance proteins (Hochstrasser *et al.*, 1990). Yields are such that comprehensive amino acid sequencing and antibody production are feasible. With the production of antibodies specific to polypeptides found to be correlated to organogenesis, it could be determined if these polypeptides are synthesized/accumulated specifically in the regions of the explant undergoing morphogenesis and so further support a direct role for these polypeptides in organogenic processes. Sequencing of the polypeptides would allow the construction of probes, which could be used to screen genomic/cDNA libraries to identify specific clones, which could subsequently be used to analyze genes and gene products associated with regeneration through the organogenic pathway. Such future studies may allow us too more fully characterize the complexities of gene expression during *in vitro* organogenesis.

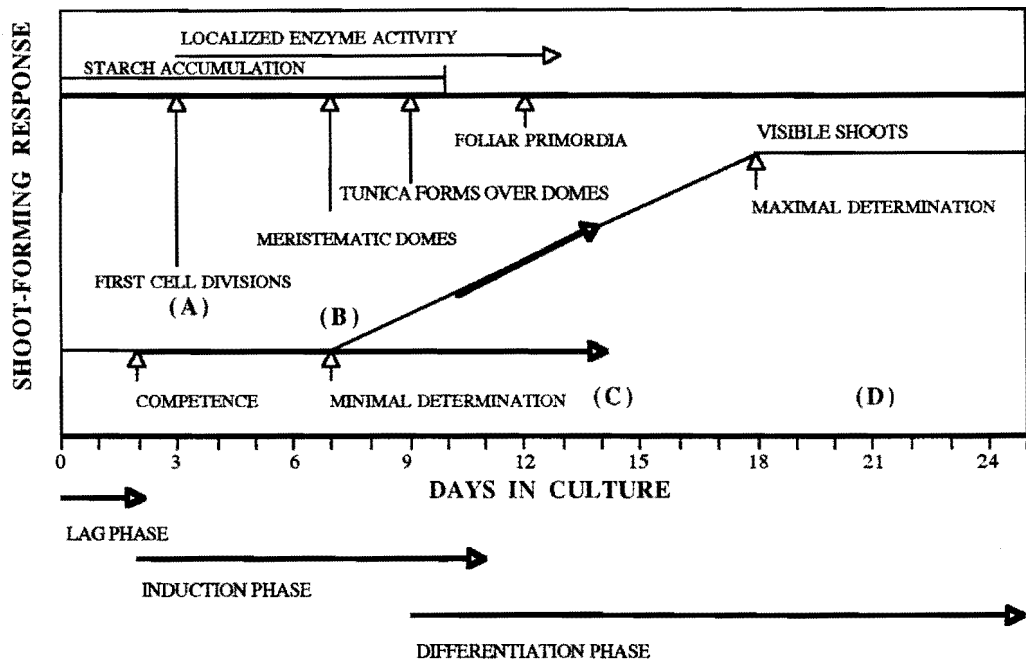


FIGURE 20 [A]: Schematic representation of the developmental phases and events leading to shoot formation. (A) changes in the pattern of polypeptides common to both caulogenesis and rhizogenesis. (B) the synthesis of polypeptides correlated to shoot determination. (C) the synthesis of polypeptides correlated with differentiation. (D) tissue specific polypeptides are detected.

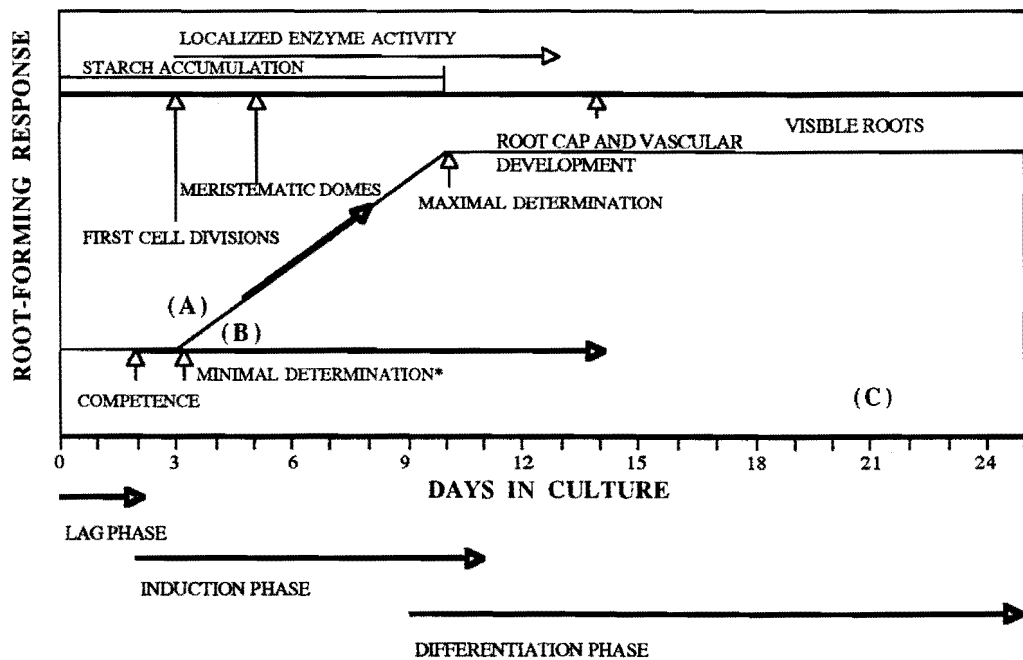


FIGURE 20 [B]: Schematic representation of the developmental phases and events leading to root formation. (A) changes in the pattern of polypeptides common to both caulogenesis and rhizogenesis. (B) differential synthesis of a polypeptide correlated to root determination. *Note roots were only strongly determined after 5 days on RIM. (C) with further culture organ-specific polypeptides are detected.

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APPENDICES

Appendix 1

Preflashing for fluorography

Preflashing was carried out to achieve about a 0.15 increase in film density (Kodak technical information M3-508, Autoradiography of Macroscopic Specimens). The exposure was made at a distance of 6 feet using an AGFA 183 electronic flash, which was covered with a Kodak No 22 gelatin filter, a 3.0 neutral density filter, and two layers of Whatman No1 filter-paper. Film was preflashed against a yellow card.

Appendix 2

Isoelectric focussing gel solution

| | | Final volume |
|-------------------------|-------|--------------|
| Urea | 10g | |
| Acrylamide/PDA (30/0.8) | 2.5ml | |
| dd H ₂ O | 7ml | |
| Ampholytes 4-8 | 0.2ml | >10ml |
| Ampholytes 3.5-10 | 0.8ml | |
| CHAPS* | 0.3g | |
| Nonidet P-40* | 0.1ml | |

*added as a combined solution

(0.3g CHAPS, 0.1ml Nonidet P-40, 0.9ml dd H₂O)

Appendix 3

Transfer solution

| | | |
|---------------------------------|------|-------|
| SDS (100g/liter) | 40ml | |
| Tris-HCL (0.5 mol/liter. pH6.8) | 20ml | |
| Bromophenol blue (0.5g/liter) | 8ml | |
| dd H ₂ O | 72ml | 140ml |

Appendix 4

Electrolyte buffers

| | | |
|-----------------------------------|--------|----------|
| Glycine | 57.6g | |
| Tris base | 12g | |
| SDS | 2 | |
| lower buffer only* (Sodium azide) | (0.4g) | |
| dd H ₂ O | | 2 liters |

*The lower buffer was reused for up to 6 months, while the upper buffer was discarded after each run.

Appendix 5

Ammoniacal silver nitrate

6 grams of silver nitrate was dissolved in 30ml of distilled water. 6mls of this solution was slowly mixed into a solution containing, 30.6ml of 0.36% sodium hydroxide and 2.28ml of ammonium hydroxide. To this, 110mls of distilled ethanol was slowly added to this solution with constant stirring.