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City University of New York PH.D. 1984

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ISOPEROXIDASES CORRELATED WITH
CYTODIFFERENTIATION AND
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EPIDERMAL EXPLANTS OF TOBACCO

by

LOU ELLEN KAY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1984

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Isoperoxidases Correlated with Cytodifferentiation and Organogenesis in Tobacco Epidermal Explants

By: Lou Ellen Kay

Advisor: Professor Dominick V. Basile

We have examined the isoperoxidase composition correlated with the histology of the organogenetic system of tobacco epidermal explants. These explants can be induced to produce vegetative buds, floral buds, or callus reproducibly and have been found in this study to have a wide range of isoperoxidases, some of which occur only in specific organ-forming tissues at very specific times. The occurrence of these can be correlated with specific stages as seen in the histological data. Six of these isoperoxidases could be correlated with unregulated cell division, as seen in callus formation. Four isoperoxidases were correlated with various aspects of wound healing, and another two were associated with cessation or repression of cell division and differentiation. An additional eight isoperoxidases could be correlated with specific stages of bud formation and development. One of these was found only while vegetative buds were initiated; another two were present all during the formation and development of the vegetative buds; and a fourth was only evident as the vegetative buds developed. A floral-bud-specific isoperoxidase was also seen; its presence correlated with stamen development. These correlations are the most extensive ever reported from a study of isoperoxidases and organogenesis. This was probably due to two features. First, a more exhaustive extraction method was used that yielded three fractions: soluble, ionically bound, and covalently bound, rather than the usual single soluble fraction. The ionically bound fraction contained many of the most interesting isoperoxidase bands. This is the first report of the potential importance of this fraction as related to organogenesis. The second methodological advantage of this study was electrophoresis (horizontal slab) utilizing acidically buffered polyacrylamide gels, a system that allowed simultaneous resolution of both anodic and cathodic isoperoxidases. Most other studies have used alkaline buffered electrophoresis methods. In the course of this study both acidic and basic buffers were used. The differences in the results obtained were striking. Only two-thirds as many isoperoxidase bands were observed in the alkaline gels as compared to the acidically buffered gels. This appeared to be due to the pH optima of most isoperoxidases being at about 4.5, causing those with narrow ranges of pH-tolerance not to show any activity at alkaline pHs. It is suggested on the basis of the methodological findings of this study that standards be proposed for studies of isoperoxidases, especially those correlated with organogenesis, so that the results of different studies can be compared accurately.

ACKNOWLEDGEMENTS

I would like to thank many people for their contributions to this dissertation. First and foremost, I am deeply grateful to my major professor, Dr. Dominick V. Basile, for his judicious views as to how scientific research should be carried out and his appreciation of the importance of writing well. Dr. Charles T. Gregg, of Los Alamos National Laboratory (LANL), was a great aid in writing - a ready and willing reference for science, wording, grammar, and punctuation. Several others helped by proofreading: Dr. Paul M. Kraemer of LANL, Ms. Susan Atchley of LANL, Mr. Ross D. Kay - my dear father, and Dr. Vincent P. Gutschick - my ever helpful husband. I would also like to thank Ms. C. Jan Richardson and Ms. Eloise A. Margiotta, both of LANL, for their technical help and advice. Ms. Monica H. Fink has my deep appreciation for her flawless typing, lasting patience and good humor, and useful advice about graphics.

The financial support by Associated Western Universities (AWU) and provision of supplies and laboratory space by LANL for much of the research of this dissertation is gratefully acknowledged. I would specifically like to thank Dr. Donald I. Walker and Ms. Madeleine Franco of AWU, and Drs. Donald F. Petersen, Thomas W. Whaley, and Ronald A. Walters of LANL.

Finally, I would like to thank: the members of my advisory committee, Drs. Thomas E. Jensen and Jack G. Valdovinos, for their assistance; Dr. Irwin M. Rosenblum for blazing the way and being a stimulating colleague; my parents, Ross D. and Grace E. Kay, for their gifts of love of learning and life; and lastly, my husband, Vincent P. Gutschick, for all his invaluable aid, scientific stimulation, and loving support.

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INTRODUCTION

Although plant development has been an active area of investigation for a long time, relatively little has been learned about the cytochemical and enzymatic changes that are correlated with organ initiation and development. There are, of course, many different biochemical processes or metabolic pathways that might be investigated. In selecting one that might be pivotal in organogenesis several criteria were deemed important. The system should be involved in phytohormone regulation. It should be responsive to phytohormones or other known morphogenetic stimuli, and it should be involved in cellular events that ultimately regulate plant form: cell division and its cessation and cell elongation and its cessation. Peroxidase (EC. 1.11.1.7; donor: H_2O_2 oxidoreductase), an iron-porphyrin containing enzyme utilizing hydrogen peroxide as the electron acceptor and a variety of substances as the electron donor, appears to be an ideal subject to study. The number and variety of functions suggested by *in vitro* data imply that peroxidase is crucial in organogenesis and morphogenesis. It seems to be involved in auxin and ethylene metabolism. It responds to all five classes of phytohormones and to phytochrome, and it is implicated in rigidification of cell walls, correlated with the cessation of cell elongation. Peroxidase is intriguing additionally by its existence in many isoenzyme forms. These isoperoxidases are genetically controlled, are found in specific organs, tissues, and subcellular locations, and have various physico-chemical specifications, implying that the different isoenzymes are involved in different and specific chemical reactions.

Peroxidase - Responses and Functions

One of the most well established functions of peroxidase with regard to development is as an indoleacetic acid (IAA) oxidase. This function has been known since the early 1950's (Goldacre, 1951; Galston, Bonner, and Baker, 1953). Most IAA in plants is covalently linked to compounds such as amides or esters, which protect it from peroxidation. It is suggested that this IAA acts as a reserve form of hormone. When the linkage is broken the IAA functions as a hormone and then may be destroyed by peroxidase (Cohen and Bandurski, 1978). Thus peroxidase is one means of regulating the levels of free, active IAA in the plant. Interestingly, in sugarbeet callus that does not require exogenous auxin for growth, the number and activity of the isoperoxidases is far lower than in normal auxin-requiring callus (Kevers *et al.* 1981), suggesting that normal callus cells may make enough auxin for growth, but it is usually degraded by the peroxidase present, thus resulting in insufficient IAA.

There is *in vitro* evidence that peroxidase may be involved with the synthesis of auxin and ethylene. Horseradish peroxidase when added to a solution containing tryptophan, Mn^{++}, and pyridoxal-5-phosphate produces indole-3-acetamide, which possesses auxin activity, and a small amount of IAA (Riddle and Mazelis, 1964). Ethylene is produced when horseradish peroxidase is added to a solution of methional (β-methylthiopropionaldehyde), sulfite, resorcinol, and oxygen and Mn^{++}, or hydrogen peroxide (Yang, 1967). The addition of IAA to an *in vitro* system stimulates the formation of ethylene via peroxidase (Mapson and Wardale, 1972). This mimics the situation in intact plants, where IAA application also causes increased ethylene production.

Peroxidase parameters respond to exogenous auxin. Indoleacetic acid application causes changes in the peroxidase localization pattern in onion root cell walls (DeJong, 1967). Increasing the IAA levels in the media in Vanda seedling culture causes an increase in peroxidase activity (Alvarez and King, 1969). When tobacco pith tissue is treated with IAA, there are specific isoperoxidases whose presence is induced, and others that are repressed. Actinomycin D inhibits both induction and repression, showing that the isoperoxidases are expressed de novo (Galston, Lavee, and Siegel, 1968). The application of 2,4-dichlorophenoxyacetic acid (2,4-D) to tobacco tissue has the same effect as IAA, causing some isoperoxidases to increase in activity and others to decrease (Lee, 1972).

Ethylene tends to increase peroxidase activity levels in plant tissues. This is found in sweet potato root slices (Imaseki, 1970), petiole, stem, and leaf blade tissue of cotton plants (Herrero and Hall, 1960; Morgan and Fowler, 1972), in tobacco leaf abscission zone (Henry, Valdovinos, and Jensen, 1974), in pith tissue of intact tobacco plants plants (Adams and Galston, 1974), and in pea stem tissue (Ridge and Osborne, 1970a; Catalfamo et al., 1978). Ethylene application induces changes in the isoperoxidase pattern in tobacco leaves (van Loon, 1977) and suppresses development of a single isoperoxidase in tobacco pith after excision (Adams and Galston, 1974). Haard and Marshall (1976) found its application to sweet potato root caused an increase in the peroxidase activity in the soluble and ionically bound enzyme fractions, but not in the covalently bound fraction. On the other hand, Osborne, Ridge, and Sargent (1972) found ethylene treatment of pea internodes increased peroxidase activity in all three extraction fractions: soluble, ionically bound, and covalently bound.[1]

Cytokinin at high concentrations (5 μM) decreased peroxidase activity in tobacco callus, while lower levels (0.2 μM) increased the activity (Lee, 1971a). In the embryonic axis of lentils cytokinin (10 μM) promoted the activity of four isoperoxidases and repressed that of one (Gaspar, Khan, and Fries, 1973).

Gibberellic acid (GA) has a very interesting relationship with peroxidase. Application of GA to tissue segments or callus of normal plants may cause either an increase or decrease in peroxidase activity (Glasziou, Gayler, and Waldron, 1968; Lee, 1971b). Dwarf plants of pea and corn, however, generally have much higher peroxidase activity levels than normal plants. If GA is applied to dwarf plants they grow to a normal stature and have the lower peroxidase activity levels equivalent to those of normal plants. The isoperoxidase pattern of the GA-treated dwarfs resembles that of normal plants rather than that of untreated dwarfs (McCune, 1961; McCune and Galston, 1959). Fry (1979) demonstrated that GA may effect cell elongation by restricting

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[1] Soluble fraction refers to those isoperoxidases that are soluble in H_2O or low ionic strength buffers; it includes peroxidase found in the cytoplasm and in the cell wall. Ionically bound isoperoxidases are soluble in a high salt buffer, usually 1M NaCl, and are considered to be bound to the cell wall. Covalently bound isoperoxidases have linkages to the cell wall that can only be broken by enzymes, e.g., cellulase and pectinase. Protoplast isoperoxidases are those soluble ones found in the cytoplasm.

secretion of peroxidase from the cytoplasm to the cell wall, thus preventing the cell wall from becoming rigid before maximum cell elongation has occurred.

Abscisic acid application to lentil embryonic axis inhibited peroxidase production (Gaspar, Khan, and Fries, 1973). On the other hand, in potato tuber slices it induced a specific isoperoxidase which appeared to be involved in suberization (Cottle and Kolattukudy, 1982).

The phytochrome system, which responds to red and far red light, can control peroxidase activity by regulating phenolic production. Monophenols and m-diphenols stimulate peroxidase activity while o-diphenols inhibit it (Galston, Lavee, and Siegel, 1968; Yang, 1968). Penel and Greppin (1979) have also found that it can effect activity in an <u>in vitro</u> spinach-extract system; far red light increases peroxidase activity and red light decreases it. The same effect is found in intact plants, both in the leaves exposed to the light and in leaves not exposed, but on the same plant (Karege, Penel, and Greppin, 1982).

Peroxidase appears to be involved in plant cell wall rigidification via several routes. Siegel (1953) was the first to suggest that peroxidase was involved in lignification. Higuchi (1957) and Freudenberg's (1959) <u>in vitro</u> work proved that peroxidase can catalyse the oxidative polymerization of monomeric precursors to form lignin complexes. Several histological studies (Gagnon, 1968; Hepler, Rice, and Terranova, 1972; Harkin and Obst, 1973; Goldberg, Catesson, and Czaninski, 1983) confirmed its involvement by showing that peroxidase is found in the correct tissue at the time required for lignification. Gibson and Liu (1981) demonstrated that all four isoperoxidases found in pea stem cell walls are capable of oxidizing eugenol, a lignin precursor. Peroxidase is also known to be responsible for localized lignification in response to pathogen invasion (Vance and Sherwood, 1976) and in wound healing (Fleuriet and Deloire, 1982). Fukuda and Komamine (1982), using an elegant system of tracheary element differentiation in suspension cultures, found that the activity of the ionically bound peroxidase fraction peaked just before lignification and the activity of the covalently bound fraction peaked during active lignin synthesis. Whitmore (1976) found the ionically bound peroxidase of Pinus seedlings was the most efficient in binding ^{14}C ferulic acid to carboxymethylcellulose. Similarly, Cline (1976) found the ionically bound Pinus peroxidase to be the most efficient in binding ^{14}C coniferyl alcohol to cellulose. Mäder and coworkers demonstrated that two groups (G_I and G_{II}) of isoperoxidases in tobacco cell walls had high efficiencies in polymerizing cumaryl and coniferyl alcohol into lignin-like substances (Mäder, Nessel, and Bopp, 1977). The third isoperoxidase group (G_{III}) found in the walls was shown to be efficient in producing hydrogen peroxide, which is essential for lignin production (Mäder, Ungemach, and Schloss, 1980). Halliwell's data (1978) confirmed this ability of peroxidase to produce hydrogen peroxide.

Fry (1979) has proposed there are two additional ways in which peroxidase is instrumental in cell wall rigidification. Peroxidase can catalyse the cross linkage of feruloyl side chains found on some soluble polysaccharides. This crosslinkage causes gel formation. Additionally, peroxidase can catalyse the conversion of soluble phenols to hydrophobic quinones or polymers. This could lower the effective concentration of cell wall water and permit more cell wall rigidity by the formation of additional hydrogen-bonds between adjacent matrix polymers. Thus, peroxidase-induced cell wall rigidification could occur where there is no lignification.

Studies That Show Peroxidase to be of Interest Developmentally

Van Fleet (1947, 1959) was the first to draw attention to peroxidase as being important in plant development. He noted it was possible to identify pre-meristematic areas that would form secondary roots or leaf primodia by the localized increases in peroxidase activity. He also noted that the pattern of peroxidase localization in tissues shifted with maturation. His observation of the localized occurrence of peroxidase in specific tissues of the root have been duplicated by many others, some of whom have made additional interesting observations on the cellular level. Avers and Grimm (1959) found intense peroxidase activity in festucoid grass epidermal root hair initials. The other epidermal cells had lower peroxidase activity. Jensen (1955) found the cells near the root tip that would form the endodermis were clearly delineated by their high peroxidase activity although the endodermis would not be distinct for several more centimeters. Goff (1975) found in onion root that the staining of peroxidase in the meristematic region was mainly cytoplasmic, while that in the more mature areas was generally in the cell walls.

Changes in peroxidase activity are found as an organ or tissue ages. Lavee and Galston (1968) found tobacco pith tissue had increasing peroxidase activity with increasing age up to a point (about two thirds of the way down the stem) and then the peroxidase activity decreased. Birecka, Catalfamo, and Urban (1976) also noted an increase in peroxidase activity with increasing age of sweet potato tissues. The intracellular location of the activity also changed. In young sweet potato leaves the largest fraction of peroxidase activity was in the ionically bound fraction, whereas in old leaves most activity was in the protoplast fraction.

Differences in peroxidase activity levels have been correlated with several other phenomena. Karege, Penel, and Greppin (1982) found an increase in peroxidase activity that was correlated with the induction of flowering in spinach plants. On the other hand, Hilgenberg, Baumann, and Knab (1978) found a decrease in peroxidase activity in the liverwort, Marchantia polymorpha L., associated with sexual and asexual reproduction. Alvarez and King (1969) observed high peroxidase activity during the formation of roots and vascular tissue in Vanda seedlings. Quoirin, Boxus, and Gaspar (1974) noted that the buds from those Prunus species that root easily in culture have greater activity in their anodic peroxidases than do species that root with difficulty. Thorpe and Gaspar (1978) found the highest activity per unit protein in shoot-forming tobacco callus occurred just before the appearance of the shoot primordia. Stebbins (Gupta and Stebbins, 1969) suggested that the high sustained peroxidase activity in hooded barley was associated with its longer period of meristematic activity, as opposed to that found in awned barley. Goff (1975) found cytoplasmic staining of peroxidase in onion root tips most intense in the meristematic regions. Vanden Born (1963) and Ramaiah, Durzan, and Mia (1971) observed that in conifers peroxidase activity was associated with rapidly dividing cells or cells that were going to divide. Arnison and Boll (1976a) found, on the contrary, that peroxidase activity (per unit protein) was at a minimum during cell division in bush bean cell suspension culture. Ridge and Osborne (1970b) demonstrated that the ethylene-induced cessation of cell elongation in etiolated pea was strongly correlated with an increased level of cell wall-bound peroxidase activity. Gardiner and Cleland (1974) showed a very dramatic increase in the cell wall peroxidase activity per unit cell wall weight that coincided with the cessation of cell elongation. Bartošová, Macháčková, and Zmrhal (1982) found an increase in total peroxidase activity accompanied the cessation of elongation in wheat. Cunningham, et al., (1975) examined six near-isogenic lines of Triticale that varied in height. They found plant height was negatively correlated only with peroxidase activity in the internodes. One additional intriguing correlation with peroxidase activity was described by Mathan and Cole (1964). They studied the effects of the "lanceolate" allele in the tomato on peroxidase activity. The lowest peroxidase activity in the shoot was found in the normal plant. In plants with one copy of the allele, the leaves were reduced to a simple lanceolate shape, and the peroxidase activity was higher than that of the normal. The homozygous lanceolate had the highest peroxidase activity level, and very reduced leaves (and no flowers). Although not stated, it appears that the lanceolate allele inhibits lateral meristem growth.

Isoperoxidases

Isoperoxidase existence was first demonstrated in the early 1950's when horseradish peroxidase was shown electrophoretically to consist of at least four isoenzyme forms (Jermyn 1952; Wood and Balls, 1955). Since then the many plant species studied have been found to have multiple forms of peroxidase (Scandalios and Sorenson, 1976). Not only do these isoperoxidases have different size and charge distributions (pI < 4 to pI > 11, Marklund et al., 1974) as indicated by electrophoresis and chromatography, but they also vary in: protein structure (Stephan and van Huystee, 1981), substrate specificities (Gibson and Liu, 1978; Marklund et al., 1974), substrate concentration for maximal activity (Evans, 1970), pH optima with different substrates (Evans, 1970; Kay, Shannon, and Lew, 1967), product pattern with some substrates (Marklund et al., 1974), optimal cofactor concentration (Shinshi and Noguchi, 1975), hydrogen peroxide concentration requirements for optimal activity (Shinshi and Noguchi, 1975), inhibitor susceptibility (Kawashima and Uritani, 1965; Kay, Shannon, and Lew, 1967), optimal temperature (Kovács, Fejér, and Dévay, 1978), reaction to environmental factors such as ionic strength (Marklund et al., 1974), heat stability (Gordon, 1968), molecular weights (Gibson and Liu, 1978), and carbohydrate composition (Shannon, Kay, and Lew, 1966). Isoperoxidases are under genetic control in the intact plant (Garcia, Pérez de la Vega, and Benito, 1982; Houston and Hood, 1982; van den Berg, Wijsman, and Bianchi, 1983) and in tissue culture (Fieldes, Deal, and Tyson, 1981).

Studies That Show Isoperoxidases to be of Interest Developmentally

Isoperoxidases occur in distinctive banding patterns in each plant organ and tissue. This has been observed in: barley seedlings (Upadhya and Yee, 1968), maize (Scandalios, 1964), wheat (Bartosova *et al.*, 1982), petunia (van den Berg and Wijsman, 1981), pea (Siegel and Galston, 1967), peanut seedlings (Thomas and Neucere, 1974), tobacco (Mäder, Meyer, and Bopp, 1975), potato (Borchert, 1974), and tomato (Evans and Alldridge, 1965). The banding patterns change during development, both in the isoperoxidase pattern found (Siegel and Galston, 1967; Alvarez and King, 1969; Chen, Towill, and Loewenberg, 1970; Conklin and Smith, 1971; Thomas and Neucere, 1974; Mäder, 1976; Fielding and Hall, 1978; and van den Berg and Wijsman, 1981), and in the intracellular location of the isoperoxidases (Gordon, 1968; Mäder *et al.*, 1975). The isoperoxidase pattern does not seem to change much in roots though (Smith *et al.*, 1970; van den Berg and Wijsman, 1981), which is interesting in view of their phylogenetic developmental stability. Changes in isoperoxidase pattern also occur as the plant goes from a vegetative to a flowering state. Renaldo, Bailey, and Nagel (1981) observed this in *Narcissus*. Sawhney, Basra, and Kohli (1981) noted the appearance of two new isoperoxidases in the plant apex of *Amaranthus viridus* L. upon the induction of flowering. Jaiswal and Kumar (1980) found two isoperoxidases in the floral primordia of *Coccinia indica* that were not present in the vegetative primordia. Koul and Bhargava (1983) found several isoperoxidase bands that appeared at specific times during floral bud development. Kahlem (1975) examined thirty-four plant species and found twenty-six that had specific anodic peroxidases which occurred in the stamens or "male" flowers but not in the leaves. By using histoimmunology he was able to locate these specifically in the microspore and tapetum tissues in *Mercurialis annua* L. (Kahlem, 1976).

Benvenuto *et al.* (1983) found evidence that the hairy root plasmid may influence the expression of isoperoxidases. The leaves of plants regenerated from tissue infected with *Agrobacterium rhizogenes* (which causes extensive adventitious root proliferation) had a group of four isoperoxidase bands that were normally found only in roots.

There are some other interesting whole plant - isoperoxidase phenomena. Long day treatment of *Citrus* and *Poncirus* plants caused greater stem area growth, total linear growth, and number of branches. This was correlated with the finding of one additional isoperoxidase (Warner and Upadhya, 1968). When tobacco plants were exposed to varying photoperiods (6, 12, and 18 hours/day) the pattern of isoperoxidases found was different for each regime (De Jong, 1973). In the same study it was found that the temperature at which the plants were grown had the same sort of effect. Those plants, raised at 10/15°C, 20/25°C, and 30/35°C (night/day), had very different banding patterns from one another. Broyer, Chapelle, and Gaspar (1979) found that repeated rubbing of *Bryonia dioica* plants caused inhibition of internode elongation (a thigmomorphogenetic response) and the appearance of a new isoperoxidase. Treatment with lithium prevented the growth inhibition and suppressed the development of the new isoperoxidase. Van Lear and Smith (1968) found that pine seedlings grown in sand without nitrogen fertilizer had three isoperoxidases in their leaves, while those grown with nitrogen had only two isoperoxidases. Lastly, Gordon and Alldridge (1967) noted that in cells involved in the healing process, individual isoperoxidases developed at specific times after wounding.

Work with tissue culture has yielded similarly interesting results. Arnison and Boll (1976b) found that in suspension cultures of bush bean one isoperoxidase, D2, was only present when the cells were entering the division phase. Nash and Davies (1975) found that in suspension cultures of Paul's scarlet rose the different growth stages in the culture cycle had differences in the isoperoxidase pattern present. They suggested the period of highest peroxidase activity, during the exponential phase, was related to cell expansion. Arnison and Boll in an earlier study (1974) found differences between calli derived from different organs of the same bean seedling. Bassiri and Carlson (1979) in a parallel study with tobacco found slightly different results. The differences in patterns between the original explants were lost by the second transfer and the only remaining differences reflecting the tissue origin were between those of vegetative origin and floral origin. Interestingly, the rate of callus growth did not seem to effect the isoperoxidase pattern. Rawal and Mehta (1982) observed differences in haploid tobacco calli when the calli were in callus maintainance, shoot forming, or root forming conditions. Verma and van Huystee (1970) found differences in peanut suspension culture based on the size (and the amount of differentiation) of the clumps. As the clump size increased, so did the number of isoperoxidases. Wochok and Burleson (1974) noted in wild carrot suspension culture that the number of isoperoxidases increased as proembryoids developed into embryoids, and then decreased as they matured into plantlets. Bajaj, Bopp, and Bajaj (1973) described the changes in isoperoxidase pattern found in shoot-forming Sinapis alba callus as being comparable to those in seedlings. Mäder (1975) found that shoot differentiation in callus actually consisted of two independent processes. There was an inhibition of growth in non-differentiating cells, which correlated with a reduction in activity of the fast migrating anodic isoperoxidases, and there was meristemoid formation, which correlated with a sharp rise in the activity of the other isoperoxidases. As in intact plants, temperature seemed to effect peroxidase in tissue culture. Using suspension cultures of tobacco grown at different temperatures (13°, 25°, and 35°C), De Jong et al. (1968) found the pattern of isoperoxidases secreted into the media was different at each temperature. The peroxidase activity for the intact cells was highest at 13°C and lowest at 35°C. McCown et al. (1970) obtained different results in a slightly more complicated experiment. They raised a winter-hardy strain of Dianthus in callus culture under four sets of conditions: light-warm (25°C), dark-warm, light-cold (0-5°C), and dark-cold. The callus in light-warm conditions had seven heavy-staining bands, dark-warm had five bands, light-cold had four bands, and dark-cold had seven bands, but five of them were very faintly stained.

The above references indicate that isoperoxidases may play an important role in differentiation and development. If their role in organogenesis is to be investigated further, one should seek a well defined organogenesis system as the experimental tool.

Epidermal Thin Layer Technique

There are many systems that have been used to study organogenesis, but most of them have one or more severe drawbacks for use in a study of metabolic events. The most commonly used tissue culture system, organogenesis from callus, involves a very small percentage of the cells, and these tend not to be synchronous. Additionally, the ploidy of most calli tends to fluctuate (Sheridan, 1975). Tran Thanh Van (1973b) has, however, developed a very elegant system using small "epidermal" tissue fragments from Nicotiana tabacum L., cv. Wisconsin 38. It can produce floral buds, vegetative buds, roots, or callus. (However, I was unable routinely to get roots.) The initial tissue is smaller and more uniform than any comparable organ-producing explant. It produces only one organ type at a time, making systematic studies easier and eliminating complications due to other developing tissues. As opposed to most other systems, the thin layers produce the organogenetic meristems directly, without intermediary callus formation. Because of the small size of the explant, it is presumably relatively free of large pools of endogenous phytohormones, as well as hormonal, nutritional, and environmental gradients. (A polar gradient is evident in floral bud formation, in which organogenesis occurs mainly at the basal end of the explant.) The smallness of the pool of endogenous phytohormones makes the thin layers very responsive to the addition of exogenous substances. Another advantage is that the organogenesis is complete within two weeks, whereas in other systems it takes several weeks to months before organogenesis occurs.

Tran Thanh Van and coworkers Gaspar and Thorpe used starch gel electrophoresis to examine this organogenesis system for changes in the soluble isoperoxidases (Gaspar, Thorpe, and Tran Thanh Van, 1977; Thorpe, Tran Thanh Van, and Gaspar, 1978). They found the quantitative and qualitative patterns of the isoperoxidases differed both between organ types induced in their time of appearance and time of peak activity. Additionally, there were distinct differences between the four organogenetic regimes and the peroxidase activity per unit protein.

The literature reviewed suggested that isoperoxidases other than soluble, i.e. ionically and covalently bound ones may also have important roles in development and differentiation. Accordingly, it was judged worthwhile to initiate a study that would overlap and extend Gaspar, Thorpe, and Tran Thanh Van's pioneering study. It was decided to use polyacrylamide gels rather than starch gels since polyacrylamide gives greater sensitivity and resolution (Fredrick, 1964), and because a buffer of pH 8 or greater must be used for separation of isoperoxidases on starch gels (Siegel and Galston, 1967). Isoelectric focusing, which might have been used, has the disadvantage of poor resolution of cationic isoperoxidases (McLellan and Robinson, 1983). Lee (1973) found pH 4.5 to be optimal for peroxidase staining so gel buffers of both acidic and basic pHs were used to compare pH effects. A horizontal slab polyacrylamide electrophoresis unit was modified so that samples could be placed in the center, allowing simultaneous separation of cathodic and anoidic isoperoxidases. A concomitant study of the histological changes occuring was carried out as Nash and Davies (1975) pointed out that the changes in individual isoenzymes in relation to development had not been examined. The previous histological studies of this organogenesis system (Dien and Tran Thanh Van, 1974; Tran Thanh Van and Dien, 1975) examined different time points than those used in this study, so it was deemed necessary to do an independent histological study.

MATERIALS AND METHODS

Tissue Culture

A modification of the procedure developed by Tran Thanh Van was used (Tran Thanh Van 1973a, 1977; Tran Thanh Van, Chlyah, and Chlyah, 1974). (Phytohormone and light levels were amended.) Culture material, consisting of the epidermis and underlying cortex was taken from the basal portion of the flowering branches of vigorously growing tobacco, Nicotiana tabacum L., cv. Wisconsin 38. The terminal fruit of these plants was green, but full-sized. The use of such a specific stage allowed uniformity of physiological state. The tobacco plants were grown in greenhouse conditions and fertilized weekly with a Murashige and Skoog (1962) macro- and micronutrient solution. The branches were excised, washed gently using Ivory soap, rinsed, then surface sterilized for three minutes in a 25% (v/v) solution of commercial bleach (5.25% sodium hypochlorite). They were immediately rinsed in three changes of sterile deionized water. Then the 4 x 10 mm epidermal thin layers were removed aseptically and placed cortex side down on the medium in disposable plastic petri dishes. The dishes were sealed with "Parafilm M" and placed in an appropriate environment. (See Table 1.)

The culture media (modified Murashige and Skoog, 1962) all included the following substances per liter: 1.65 g NH_4NO_3, 1.90 g KNO_3, 0.44 g $CaCl_2 \cdot 2H_2O$, 0.37 g $MgSO_4 \cdot 7H_2O$, 0.17 g KH_2PO_4, 6.2 mg H_3BO_3, 22.3 mg $MnSO_4 \cdot 4H_2O$, 8.6 mg $ZnSO_4 \cdot H_2O$, 0.83 mg KI, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 0.025 mg $CoCl_2 \cdot 6H_2O$, 3.72 mg Na_2H_2EDTA (ethylenediaminetetraacetic acid), 2.78 mg $FeSO_4 \cdot 7H_2O$, 100 mg myoinositol, 0.1 mg thiamine•HCl, and 10 g agar. The microelements were made up as a 100X stock solution and the Fe(II)EDTA as a 200X stock solution. The pH was adjusted to 5.1 using 0.05 N NaOH, and the medium was autoclaved for 15 minutes. See Table 1 for specific additions to each medium.

Table 1. Variables for differential organ induction in tobacco epidermal thin layers.

Culture Medium	Floral Buds	Vegetative Buds	Callus
Auxin	10^{-6} M IAA	2×10^{-6} M IAA	10^{-5} M IBA
Cytokinin	10^{-6} M Kin	2×10^{-5} M BA	10^{-7} M Kin
Sugar(s)			
sucrose	8.33×10^{-2} M	8.76×10^{-2} M	7.30×10^{-2} M
glucose	8.33×10^{-2} M		
Culture Conditions			
Light levels*	6 watts/m^2	15 watts/m^2	Total darkness
Photo-period	16 hr/day	Continuous	----

IAA = indole-3-acetic acid, IBA = indolebutyric acid, kin = 6-furfurylaminopurine, BA = benzyladenine.

*Light was provided by a combination of fluorescent and incandescent lamps, measured in the visible spectrum.

Tissue Harvest

The tissues (whole thin layers for vegetative buds and callus, the basal 20% of the thin layers for floral buds) were blotted dry, frozen in liquid nitrogen, lyophilized, and then stored at -23°C until used.

Extraction of Three Peroxidase Fractions

All of the following steps were carried out at 4°C unless otherwise indicated. The lyophilized tissue was weighed and then ground with a prechilled mortar and pestle in 20 times its weight (1 volume) in a buffer (van Kammen, 1967) containing 0.05 M Tris·HCl [tris(hydroxymethyl)aminomethane], 0.5 M sucrose, 0.01 M $MgCl_2$, and 0.006 M 2-mercaptoethanol, pH 7.2. The slurry was centrifuged for 10 minutes at 10,000 rpm; the resultant supernatant fluid was the soluble fraction. The pellet was then washed twice with two to four volumes of 1% Triton X-100, and six times in two to four volumes of distilled deionized water, each time followed by a 10 minute centrifugation at 10,000 rpm. (The Triton X-100 washings had no unique isoperoxidases.) To obtain the ionically bound fraction the pellet was washed in one half volume of 1 M NaCl three times, being centrifuged after each wash. The combined supernatant fluids were the ionically bound fraction. The pellet again went through a series of washes in two to four volumes of solution. The washes were: two in 0.5 M $NaHCO_3$, six in 1 M NaCl, and three in H_2O. The slurries were centrifuged after each wash. The pellet was then incubated overnight at 25°C in 0.1 M sodium acetate buffer, pH 5.5, containing 0.5% (w/v) cellulase (ICN Pharmaceuticals) and 2.5% pectinase (ICN Pharmaceuticals). This step was repeated. The combined supernatant fluids after centrifugation were the covalently bound fraction. All three fractions were dialyzed in 2 l of 0.025 M borate buffer, pH 8.0, for at least five hours. Dialysis was especially necessary to get good electrophoretic resolution of the ionically bound fraction.

Protein Determination

The protein concentration of the three fractions was determined by the Bio-Rad method (a modification of the Bradford method, Bradford, 1976) using the dialysis fluid as a control. Bovine serum albumin and horseradish peroxidase (Sigma, P-8000) were used as the protein standards. Three to five replicates were used for protein determination, as well as for all the remaining procedures.

Peroxidase Activity Determination

Peroxidase activity was determined using a modification of the method of Gahagan, Holm, and Abeles (1968). A 0.01 ml sample was added to a solution containing 1 ml 100 mM KPO_4 buffer, pH 7, 1 ml 8 mM H_2O_2, and 1 ml freshly made 100 mM pyrogallol. The absorbance at 430 nm was read every 15 seconds for 60 seconds. The change in absorbance was used to calculate the activity. Horseradish peroxidase (Sigma P-8000) was used as the standard.

Electrophoresis

Thin layer horizontal slab polyacrylamide gel electrophoresis was performed using an LKB 2117 Multiphor apparatus. The gel was 2 mm thick, 11.5 cm wide, and 25 cm long. The samples were placed in slots, 1.5 mm deep, 1 mm wide, and 6.3 mm long, which were in the middle of the gel, perpendicular to its long axis. The slot-former was hand crafted. Filter paper wicks (eight layers thick) were used to conduct the current from the buffer tanks to the gel. During the 30 minute preelectrophoresis and the actual electrophoresis of the samples, the water flowing in the cooling plate supporting the gel was at 10^{o}C. Samples of 10 µl were applied and bromophenol blue was used as the tracking dye. Acidic and basic gels were used, both consisting of 7.5% acrylamide. The acidic gels were made with acetate buffer, pH 4.5, final acetate ion concentration 0.025 M, the basic with borate buffer, pH 8.0, final borate ion concentration 0.025 M. The buffer tanks were filled with the same buffer as that of the gel being used. The acrylamide, Bis (N,N'-methylene-bis-acrylamide), TEMED (N,N,N',N'-tetramethylethylenediamine), and ammonium persulfate were of electrophoretic purity, from Bio-Rad.

Isoperoxidase Staining

The gels were immersed in a freshly prepared solution consisting of equal parts of 100 mg o-dianisidine dissolved in 100 ml 95% ethanol and 100 ml acetate buffer, pH 4.5, added just before using. This acetate buffer was made from 0.88 M sodium acetate and 0.62 M acetic acid. After a 30 minute period the o-dianisidine solution was drained off and replaced with a 0.03% H_2O_2 solution until the bands were clearly visible, usually 2 to 5 minutes. The banding pattern was recorded immediately, and then again after 30 minutes when the fainter bands became evident.

Duplicate gels were stained using guaiacol. The gels were immersed in a solution containing 100 ml of 1% guaiacol in 28.5% ethanol, and 100 ml of the acetate buffer used for o-dianisidine staining for 30 minutes. The gels were then drained and reimmersed in 0.03% H_2O_2 until the bands emerged. The results were recorded immediately as this stain faded rapidly. With both stains the banding pattern was recorded manually. The graphs in this dissertation were drawn to about a 1 to 1 scale, the bromophenol blue dye front was plotted as being 10 cm from the origin.

The above techniques were found to be the best after testing several other methods. Staining with 2,6-dimethoxyphenol was tried, but it gave fewer bands than o-dianisidine. Sequential staining with o-dianisidine and guaiacol revealed fainter bands and gave better resolution than the simultaneous application of o-dianisidine or guaiacol and H_2O_2. This may be due to a pH effect, especially with the borate gels. Lee (1973) found pH 4.5 to be optimal. Various H_2O_2 concentrations were tried, but this factor did not seem critical, although at very low concentrations the faint bands did not become visible. The concentration of ethanol in the guaiacol stain was not very critical either, although at high concentrations the faint bands did not appear. Although o-dianisidine was a more sensitive stain (7 bands/sample vs. 4 with guaiacol), guaiacol was used in addition to insure that isoperoxidases with differing substrate specificites would be detected. Fleuriet and Deloire (1982) and McLellan and Robinson (1983) also found more isoperoxidases stained with o-dianisidine than with guaiacol.

Histology

The tissues were fixed for 4 hours under vacuum in either 3% glutaraldehyde or 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2-7.4 at 4°C. They were then rinsed, also at 4°C, in 0.1 M phosphate buffer until all traces of glutaraldehyde were gone, usually 5 or 6 10 minute rinses. They were stored in 4°C phosphate buffer until the dehydration step. Dehydration was done at room temperature (20°C or more) via a modified procedure (Rosenblum, 1981) of Lin, Falk, and Stocking (1977), which was itself a modification of Postek and Tucker's procedure (1976). The phosphate buffer was replaced with acidified 2,2'-dimethoxypropane (DMP, acidified with 3 drops of 0.1 N HCl per 25 ml DMP). The DMP was changed twice at 5 minute intervals, then replaced with a 1:1 solution of acetone and DMP. After 5 minutes that solution was replaced with pure acetone, which was changed once after 5 minutes. Five minutes later the acetone was replaced with a 1:1 acetone, tertiary butyl alcohol (TBA) mixture. Fifteen minutes later the mixture was removed and TBA added; the TBA was changed twice after 15 minute intervals. Then after a 15 minute period, the tissue and TBA were placed in a paraffin oven. Pellets of paraffin were added over a several hour period. The vial remained in the oven for 24 hours or more to allow the TBA to evaporate. The paraffin was replaced twice; then the tissue was embedded. The tissue was sectioned 15 μ thick using a rotary microtome, then mounted on slides. The slides were stained for 5 minutes in fresh 0.05% toluidine blue, rinsed briefly in water three times, blotted gently with lint-free paper, then placed on a slide warmer to dry for at least 24 hours. The slide was then immersed in xylene for 5 minutes to dissolve the paraffin, covered with a coverslip, using a resin mounting medium, and dried on a slide warmer.

Photography

The developing thin layer tissues were photographed with a Topcon Super D camera, equipped with bellows and a reversed 58 mm lens. Tri-X film was used; a flash provided the light. Photomicrographs of the histological sections were taken with a Zeiss microscope equipped with a 35 mm camera.

RESULTS

Differences among the various organogenetic regimes were evident at the macroscopic level after six days in culture (Figures 1, 4, and 7). The thin layer tissues in vegetative bud-inducing conditions were swollen uniformly, the ones in floral bud-inducing conditions were enlarged at the basal end, and the ones in callus-inducing conditions showed evidence of callus formation as small areas of new white tissue. By the twelfth day both types of buds became visible; the vegetative buds were scattered over the entire epidermal surface while the floral buds were all located at the swollen basal end of the thin layer.

To determine when the period of organ induction was completed, the thin layer tissues were transferred from the standard organ inducing media to media lacking phytohormones at 24 hour intervals after inoculation. Normal organ induction in both vegetative and floral bud inducing conditions was complete after six days.

Histological Studies

To understand further the events during organ induction and formation, histological studies at the light microscope level were performed. The fresh thin layer tissue was composed of three distinct zones of different cell types (see Figure 1b). Starting from the outer surface, there was an epidermal zone that contained some stomata and supported some hairs. The second zone was a palisade chlorenchyma, one cell layer thick. These cells were two to three times longer than wide, with the

Figure 1. Photographs of vegetative bud-forming tissues, days zero, two, four, and six. a) Fresh thin layer tissue, X 7.7. b) Longitudinal section of fresh thin layer tissue, X 108. The three cell zones are indicated by brackets. c) Explant, day two, X 7.7. d) Longitudinal section of the explant, day two, X 108. Note the shortened cells (arrow) resulting from the anticlinal cell divisions in the third cell zone. e) Explant, day four, X 7.7. f) Longitudinal section of explant, day four, X 67. Observe that the plane of cell divisions in the third cell zone had changed to become periclinal. g) Explant, day six, X 7.7. Notice the swollen appearance of the thin layer. h) Longitudinal section of explant, day six, X 67. Note the area of cells that had divided rapidly (arrow) in the second cell zone.

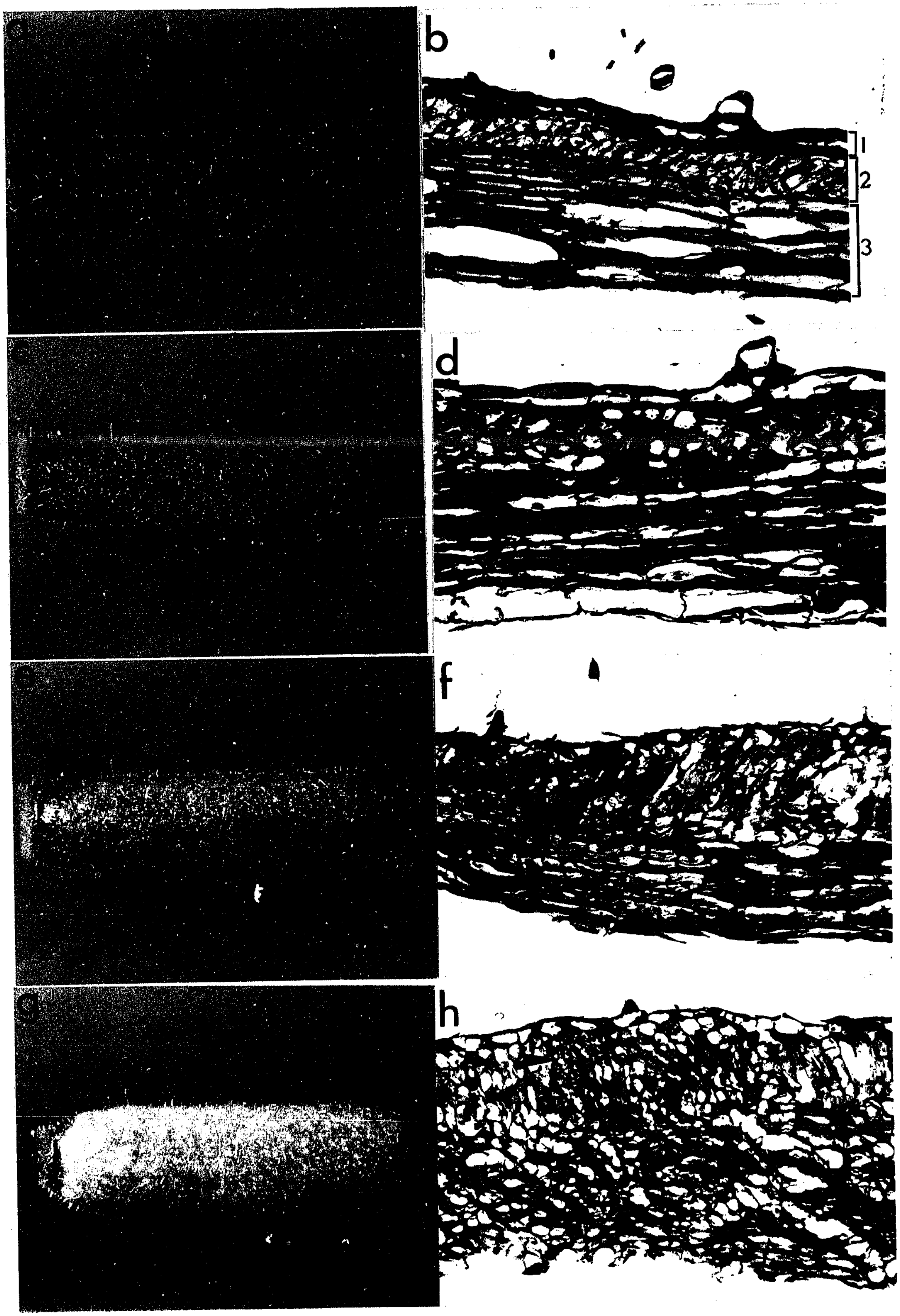
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Figure 2. Photographs of vegetative bud-forming tissues, days eight, ten, twelve, and fourteen. a) Explant, day eight, X 7.7. b) Longitudinal section of explant, day eight, X 42. Observe the meristematic area (arrow). c) Explant, day ten, X 7.7. d) Cross section of explant, day ten, X 42. Note the protuberance of the meristematic area. The sharp distinction between the zones has been obliterated by the numerous cell divisions. e) Explant, day twelve, X 7.7. The buds (arrows) are now visible. f) Cross section of explant, day twelve, X 42, with a bud. g) Explant, day fourteen, X 7.7. h) Cross section of explant, day fourteen, X 42.

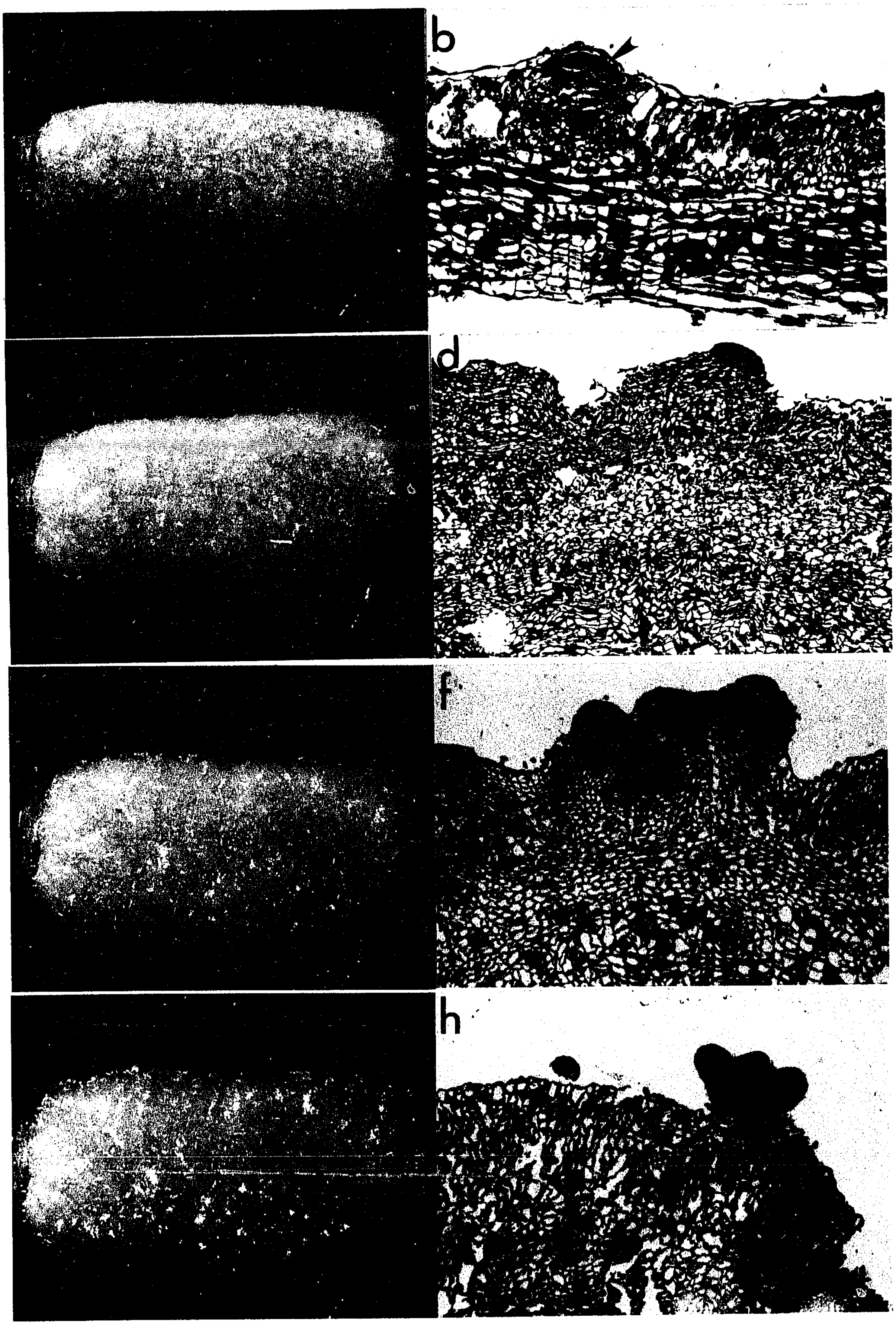
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Figure 3. Photographs of vegetative bud-forming tissues, days sixteen, eighteen, and twenty. a) Explant, day sixteen, X 6.3. b) Cross section of explant, day sixteen, X 42. Note the procambium (arrow). c) Explant, day eighteen, X 4.3. d) Cross section of explant, day eighteen, X 42, showing the procambium development (arrow). e) Explant, day twenty, X 4.1. f) Cross section of explant, day twenty, X 42. There are tracheary elements in the developing leaf. Note the cross section of a developing leaf.

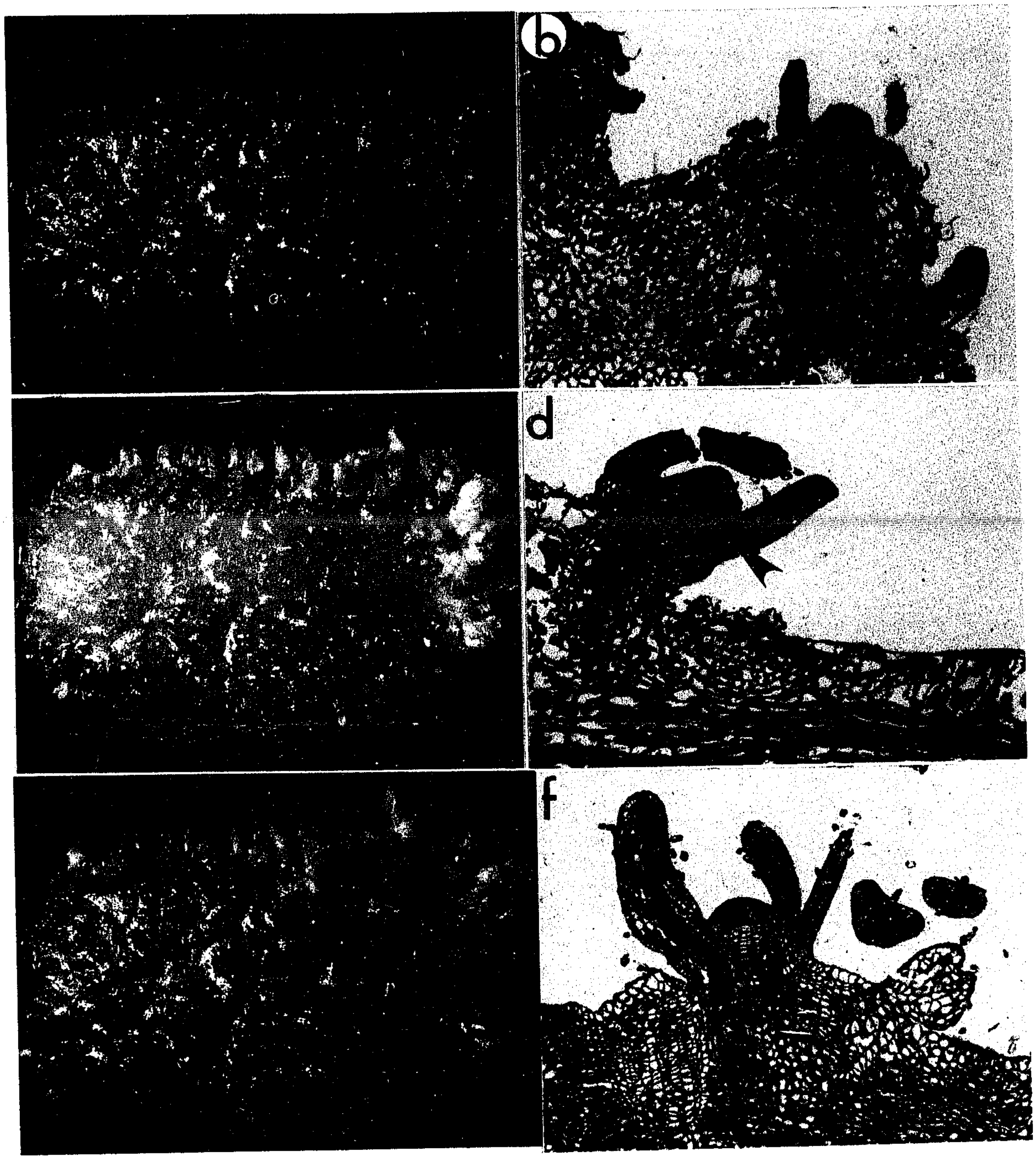
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Figure 4. Photographs of floral bud-forming tissues, days zero, two, four, and six. a) Fresh thin layer tissue, X 7.7. b) Longitudinal section of fresh thin layer tissue, X 108. The three cell zones are indicated by brackets. c) Explant, day two, X 7.7. d) Longitudinal section of explant, day two, X 108. Notice the shorter cells in the third cell zone resulting from anticlinal cell divisions (arrow). e) Explant, day four, X 7.7. f) Longitudinal section of explant, day four, X 67. The plane of cell divisions in the third zone had changed to become periclinal. There is also an area of small cells (area of divisions) in the second cell layer. g) Explant, day six, X 7.7. The basal end is swollen. h) Longitudinal section of explant, day six, X 42. Note the area of cells that had divided rapidly at the basal end of the thin layer tissue in cell zone two.

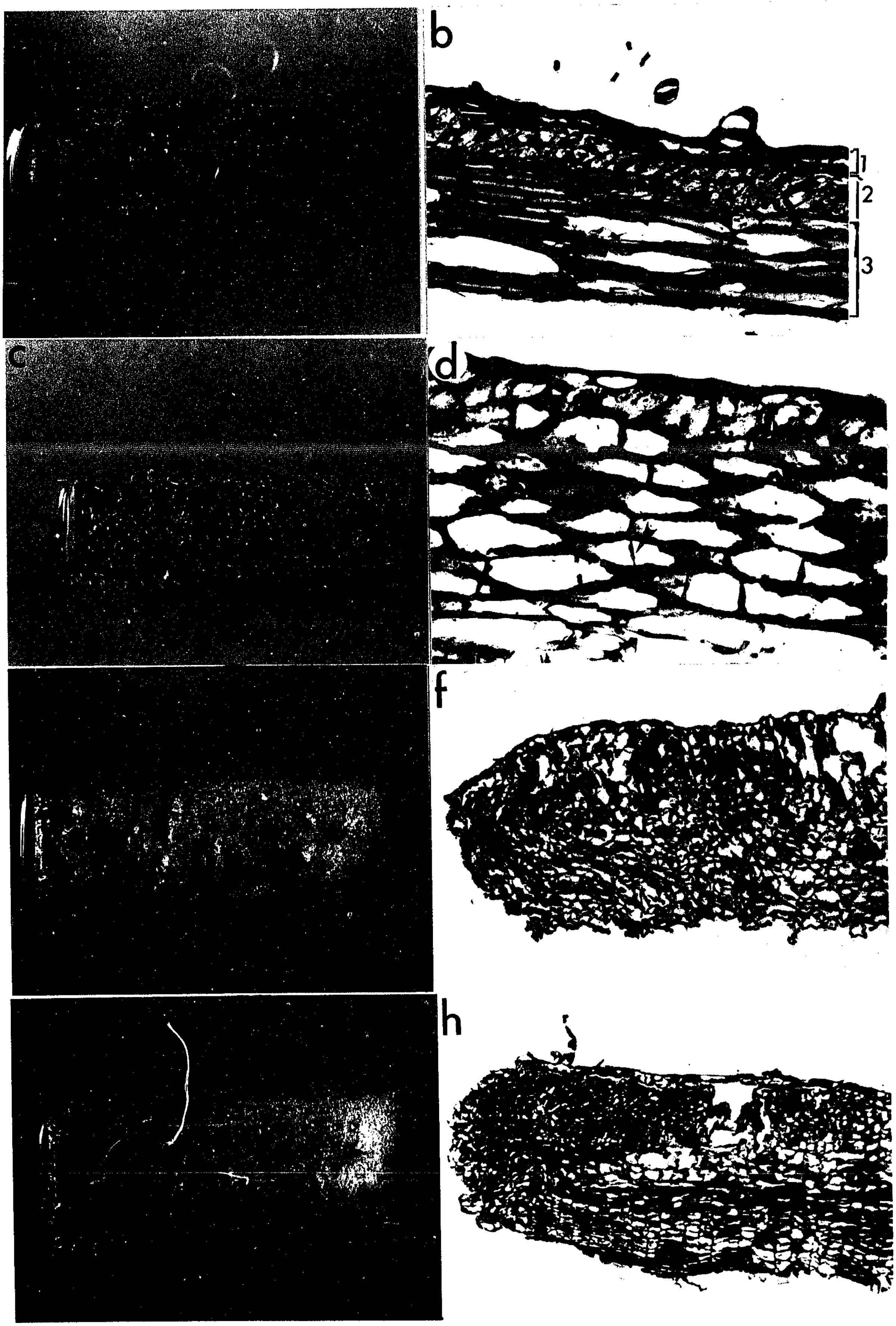
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Figure 5. Photographs of floral bud-forming tissues, days eight, ten, twelve, and fourteen. a) Explant, day eight, X 6.3. b) Cross section of explant, day eight, X 42. Notice the organized meristem (arrow). c) Explant, day ten, X 5.6. d) Cross section of explant, day ten, X 42. e) Explant, day twelve, X 5.6. Note the emerging floral buds at the basal end of the thin layer. f) Longitudinal section of explant, day twelve, X 42, showing the developing sepals (S). g) Explant, day fourteen, X 5.6. h) Cross section of explant, day fourteen, X 42. The sepals (S) and stamens (St) are well defined.

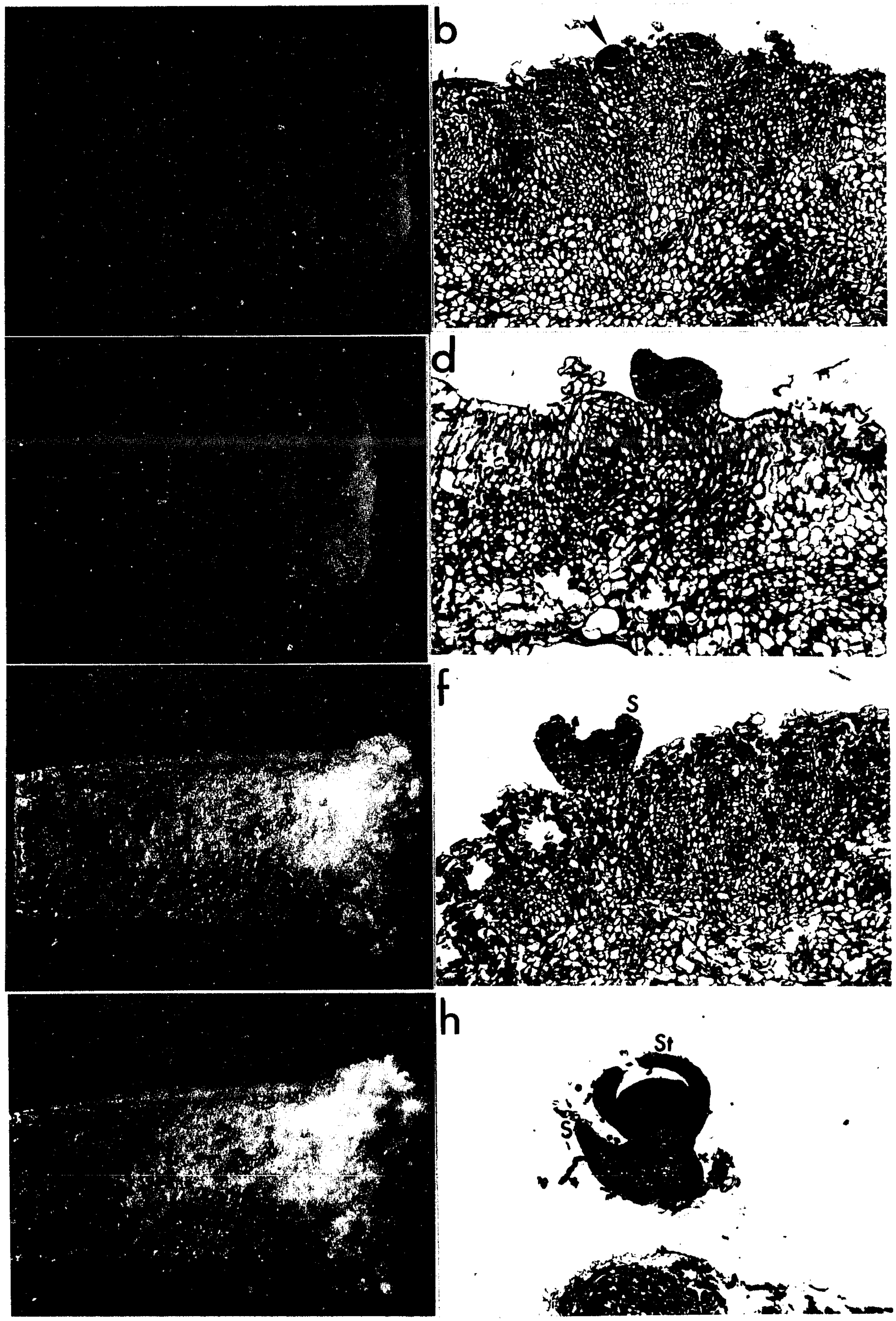
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Figure 6. Photographs of floral bud-forming tissues, days sixteen, eighteen, and twenty. a) Explant, day sixteen, X 5.6. b) Cross section of explant, day sixteen, X 42. Note the sepals (S), stamens (St), procambium (Pr), primordia where the petals will arise (arrow), and the bud elongation. c) Explant, day eighteen, X 5.0. d) Cross section of explant, day eighteen, X 42. Observe the sepals (S), stamens (St), and carpels (C). e) Explant, day twenty, X 5.0. f) Cross section of explant, day twenty, X 42, showing the sepals (S), emerging petal (P), elongating stamens (St), and carpels (C).

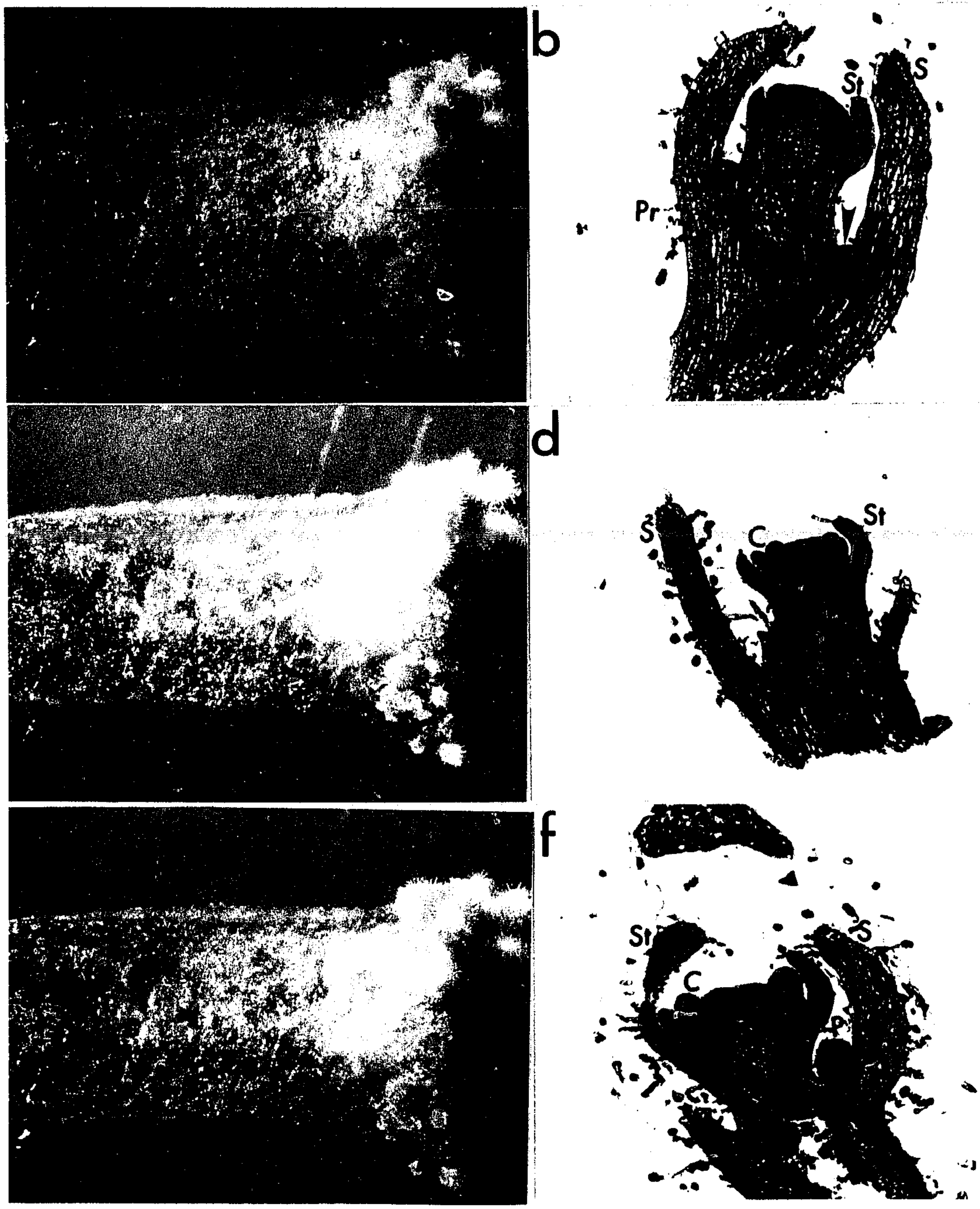
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Figure 7. Photographs of callus-forming tissues, days zero, two, four, and six. a) Fresh thin layer tissue, X 7.7. b) Longitudinal section of fresh thin layer tissue, X 108. The three cell zones are indicated by brackets. c) Explant, day two, X 7.7. d) Longitudinal section of explant, day two, X 108. Note the shortened cells (arrow) in the third cell zone resulting from anticlinal cell divisions. e) Explant, day four, X 7.7. f) Longitudinal section of explant, day four, X 108. The plane of cell divisions in the third cell zone changed to periclinal. g) Explant, day six, X 7.7. Notice a few whitish areas where callus growth had erupted through the epidermis. h) Cross section of explant, day six, X 67. There is an area of cell division in zone two (arrow).

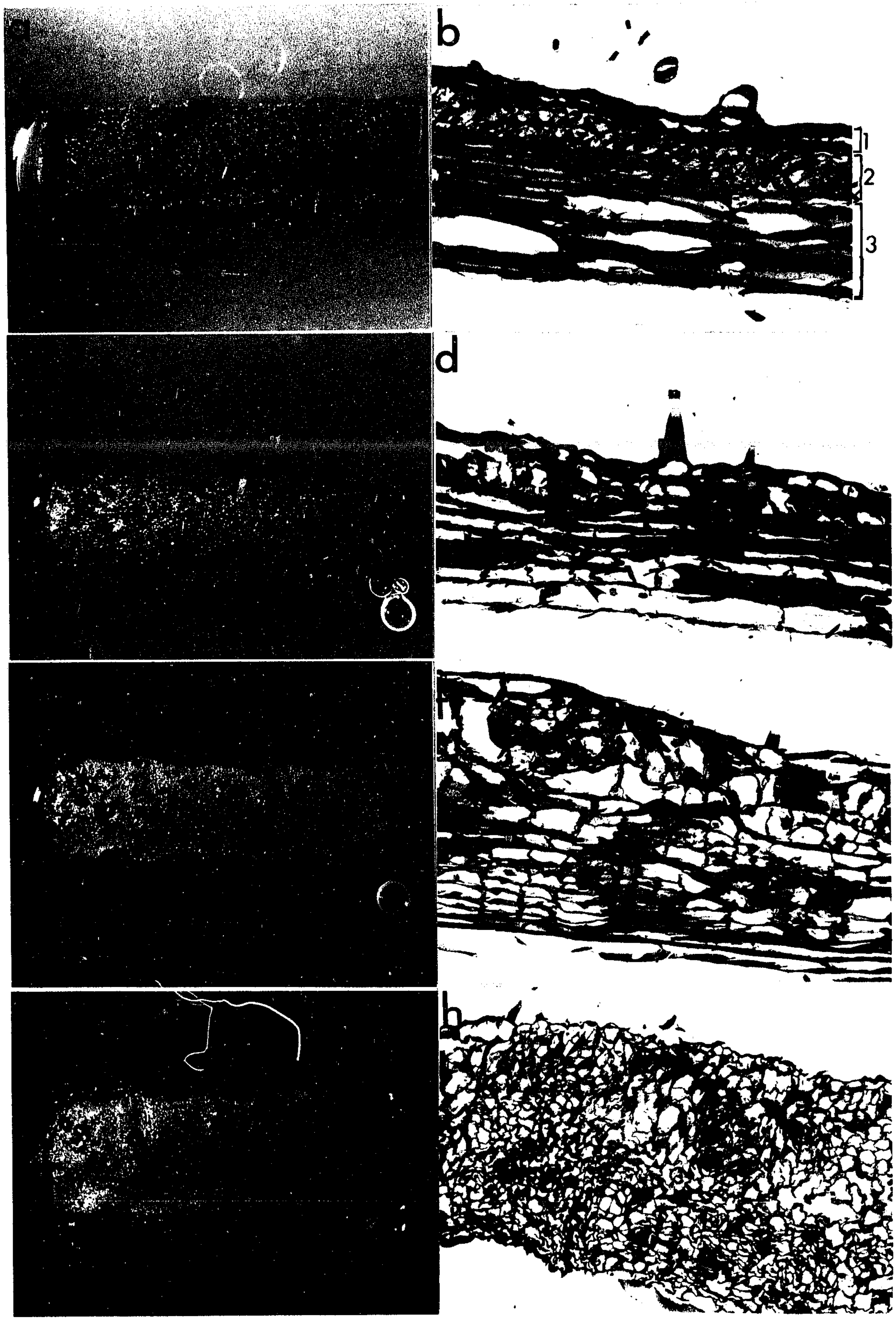
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Figure 8. Photographs of callus-forming tissues, days eight, ten, twelve, and fourteen. a) Explant, day eight, X 7.7. b) Cross section of explant, day eight, X 67. There are tracheary elements (TE) and numerous small cells resulting from cell divisions in the second cell zone. c) Explant, day ten, X 7.7. d) Longitudinal section of explant, day ten, X 67. e) Explant, day twelve, X 7.7. f) Longitudinal section of explant, day twelve, X 42. g) Explant, day fourteen, X 6.3. h) Cross section of explant, day fourteen, X 42. Note the tracheary element (TE) cluster (in the upper portion of cell zone three), a mass of small dividing cells (M) above the tracheary element cluster, and large, vacuolated "callus" cells at the surface (V).

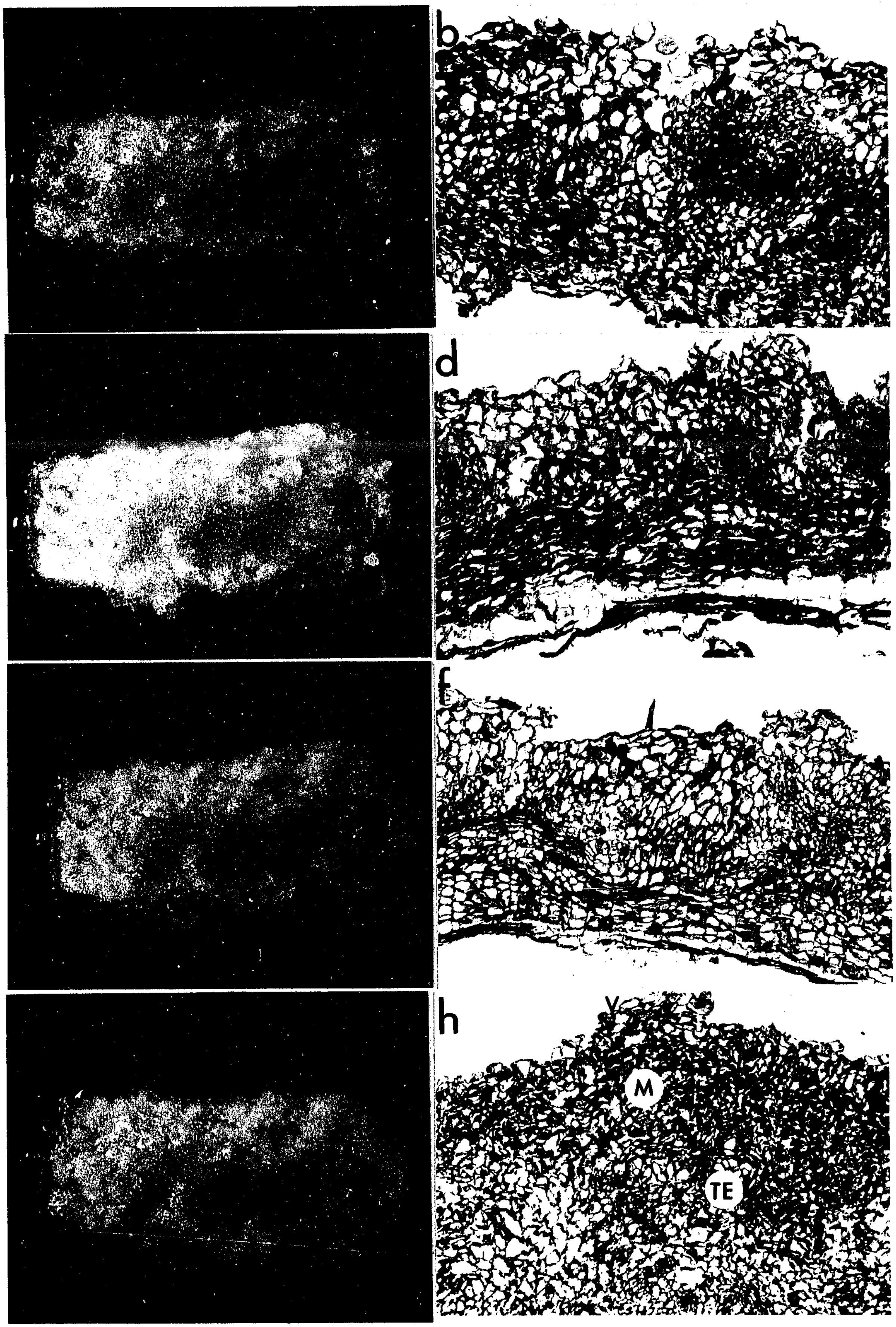
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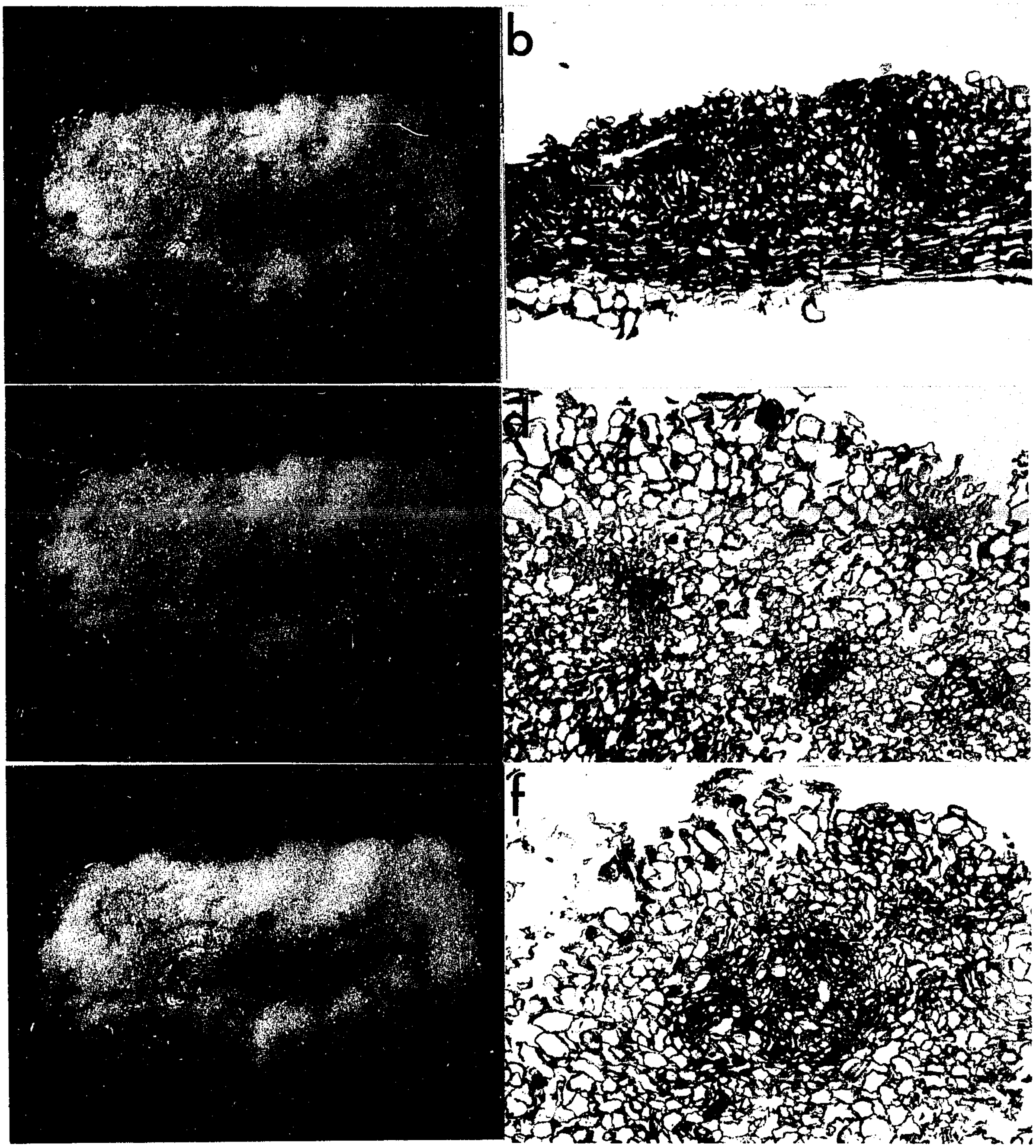
Figure 9. Photographs of callus-forming tissues, days sixteen, eighteen, and twenty. a) Explant, day sixteen, X 5.6. b) Longitudinal section of explant, day sixteen, X 42. c) Explant, day eighteen, X 5.0. d) Cross section of explant, day eighteen, X 42. e) Explant, day twenty, X 4.1. f) Cross section of explant, day twenty, X 42.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long axis at about a sixty degree angle to the long axis of the floral branch from which it came. The third zone was up to six cells thick. The long axis of the cells was parallel to the long axis of the floral branch. The uppermost cells (one layer thick) were about three times longer than wide, and contained some chloroplasts. The remaining cells averaged eight to ten times longer than wide and were characterized by their length and the very numerous pits in their cell walls.

The changes observed during the induction and development of vegetative buds were as follows. After two days in culture (Figure 1d), cell divisions had occurred in the third zone of cells, dividing the very long cells into shorter units. Most of these cell divisions were in the uppermost cells. At four days in culture (Figure 1f), the cell divisions in the third cell zone had continued, but the plane of the divisions had changed to periclinal so that downward files of cells were obvious. The cell surface in contact with the culture medium began to show evidence, via differential staining, of lignification. Additionally, there were discrete areas of cell divisions in the second cell zone (chlorenchyma). By day six in culture, when organ induction was complete, there were numerous areas of small cells in the second zone (Figure 1h). There was evidence of some continued periclinal cell divisions on the third zone. The bottom of the thin layer showed evidence that lignification had occurred over this entire surface. After eight days in culture (Figure 2b), the areas of cell divisions in the second zone had produced true meristematic areas, with small non-vacuolate cells. There were some scattered tracheary elements at the bases of these meristematic areas. (Some of these tracheary elements had formed in small chains that appeared to be going toward the surface.) By the tenth day in culture, the upper surface of the thin layer was lumpy due to the masses of meristematic cells in the second cell zone (Figure 2d). There were some trachery elements near these protuberances. At the stage of day twelve (Figure 2f), the leafy buds were apparent above the epidermis. Leaf growth appeared to be via the primary leaf meristem. By the fourteenth day (Figure 2h) some of the tracheary elements were growing toward the leaf buds. Growth in the entire thin layer seemed limited to that occurring in the buds. At the stage of the sixteenth day (Figure 3b), the leaves were growing mainly by apical growth, but the lateral marginal meristems were beginning to show some activity. Procambium tissue in the leaf was beginning to form. Leaves of eighteen day cultures (Figure 3d) showed lateral growth, development of the procambium and cell expansion. The twenty day old cultures (Figure 3f) showed a continuation of the above leaf growth, with maturation of the vascular tissue.

The initial changes observed in floral bud forming tissue were similar to those noted in vegetative bud forming tissue. At two days in culture cell divisions in the third cell zone had divided the very long cells into much shorter cells (Figure 4d). By the fourth day the plane of cell divisions in the third cell zone had changed to periclinal (Figure 4f), thus producing files of cells. It also became apparent that there were discrete areas of cell division in the second zone of cells (the palisade chlorenchyma). These areas of cell division were at the basal end of the thin layer tissue. There were indications that lignification of the "underside" of the thin layer had begun. Tissues from the sixth day in culture revealed that cell divisions had continued rapidly in some areas in the second cell zone (Figure 4h). There were masses of small cells, including some cells that had differentiated into tracheary elements. The third cell zone also contained tracheary elements as well as more extensive files of cells that indicated continued cell divisions. The underside of the tissue had extensive lignification. By the eighth day in culture the entire bottom of the thin layer was lignified. More tracheary elements had formed, in chain-like groups in the third cell zone, and in circular clusters in the midst of the masses of small dividing cells in the second cell zone. The first organized meristem was seen at this stage (Figure 5b). Tissues ten days old showed a very high number of tracheary elements, usually in cell zone two, and usually in semi-circular clusters in the midst of small dividing cells. The meristems were usually above clusters of tracheary elements, but were never observed to have vascular connections with them (Figure 5d). By the twelfth day (Figure 5f), when floral buds were visible to the naked eye, the meristems had grown above the surface of the thin layer tissue and generally had developing sepals. By the fourteenth day, the procambium was evident in the buds, the buds were elongated, and the stamens had started to develop (Figure 5h). Meristems were evident at the adaxial base of the sepals; the petals arose from these later (day twenty). The sixteen-day-old tissue had buds that were much elongated (Figure 6b), raising the buds above the surface of the thin layer tissue. The number of tracheary elements in the whorls below the buds was greatly increased; the semi-circular strands were often six tracheary elements thick. By the eighteenth day, some of the tracheary element "semi-circles" had become filled with lignified cells, most of them tracheary elements. In the buds (Figure 6d), primordia for the carpels had begun to form. Carpel development progressed by day twenty (Figure 6f). Additionally, the stamens had begun to elongate and the petal primordia had begun to develop at the adaxial base of the sepals.

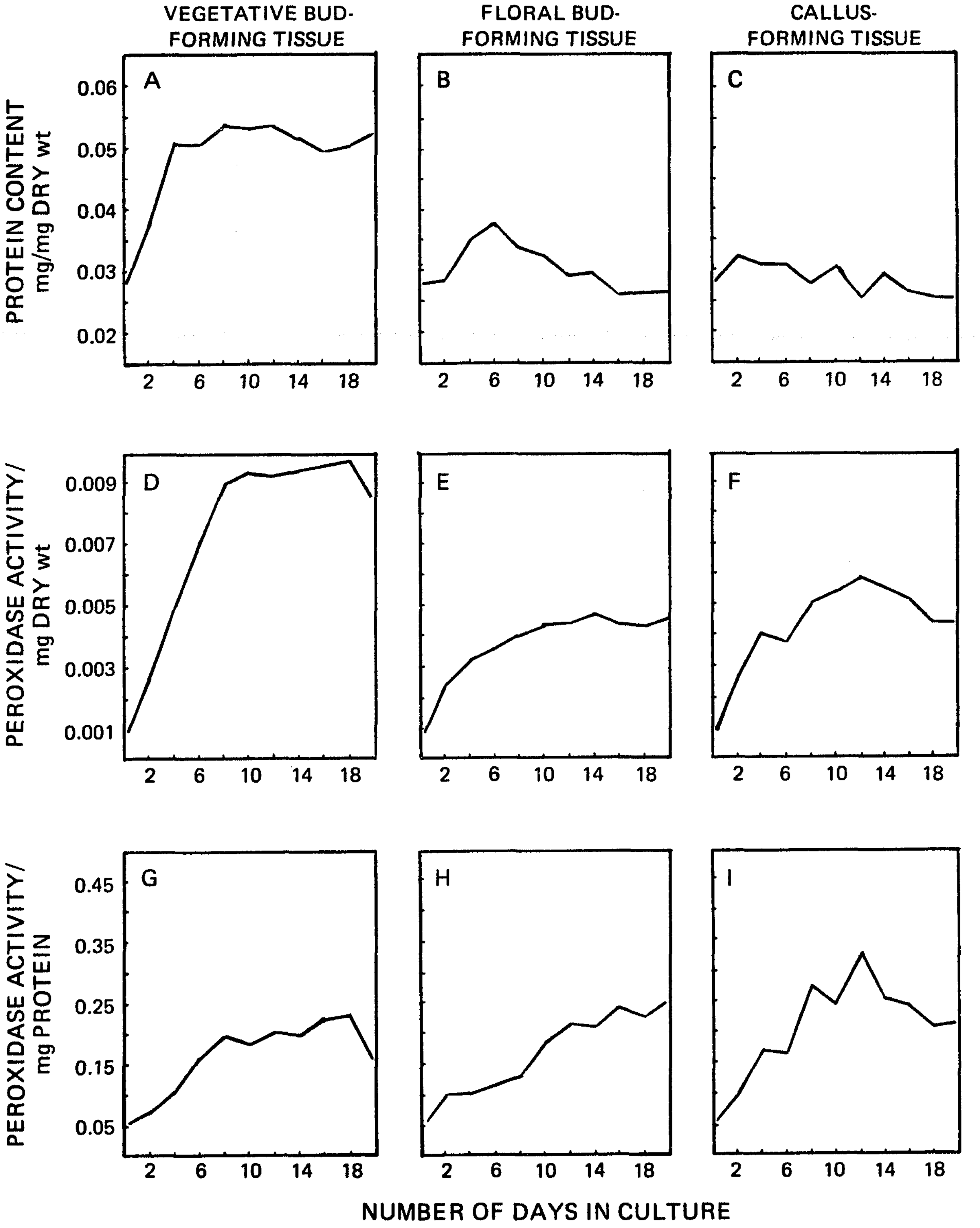
The formation of floral buds in this regeneration system was abnormal in its developmental pattern. The parts developed in the following sequence: sepals, stamens, carpels, and then petals. Dien and Tran Thanh Van (1974) found the petals developed before the carpels, but still after the stamens. The normal whole plant sequence is: sepals, petals, stamens, and then carpels. One can speculate and ask if the sequence seen in the tissue culture system was the same as that of some ancestor, or perhaps somehow petal development was specifically repressed?

The changes observed in callus-forming tissue were initially like those in the bud-forming tissues. Tissue in culture two days (Figure 7d) had anticlinal cell divisions evident in zone three, dividing the characteristically long cells into shorter units. By day four in culture, the plane of cell division had changed to periclinal, producing files of cells (Figure 7f). Six-day-old tissue revealed continued cell divisions in zone three (Figure 7h) as well as some differentiation of tracheary elements there. There was some lignification at the base of this layer, the surface of which was in contact with the medium. There were also small areas of dividing cells in zone two, some of which broke through the epidermis. Some of the areas of dividing cells contained scattered tracheary elements. Examination of eight-day-old tissue (Figure 8b) revealed that most cell divisions were then occurring in the second cell zone (chlorenchyma). Lignification of the bottom surface of the thin layer had progressed, and clusters of tracheary elements were found in the third zone. In ten-day-old tissue, chains of tracheary elements were found in the upper portion of the third cell zone (Figure 8d). By day twelve (Figure 8f), it appeared that these chains of tracheary elements were the basement layer of the cell division complexes. Above the tracheary element chains were clusters of small cells presumed to be dividing. Above these were large vacuolated "callus" cells. The fourteen-day-old tissue (Figure 8h) continued the trend seen on day twelve, except the files of cells present in the third zone of cells had started to expand and become vacuolated. After day fourteen, the growth of the callus tissue was fairly uniform (Figures 9b, 9d, and 9f). There was some increase in the size of the chains of tracheary elements.

Protein Content and Peroxidase Activity Level

The developing thin layers were assayed for changes in their protein content and peroxidase activity levels (Figure 10). The early divergence between protein levels demonstrated that there are very early differences between the organogenetic regimes. The protein content in tissue induced to form vegetative buds rose very rapidly in the first four days, while that in the tissue induced to form floral buds increased only slightly the first two days and then rapidly until day six (when induction was completed). The protein level in callus-forming tissue fluctuated within a fairly small range over the entire twenty day period examined. The protein level in the vegetative bud-forming tissue continued to increase, slowly with fluctuations, until day twelve; it decreased slightly until day sixteen, and then exhibited another slight

Figure 10. Protein content and peroxidase activity of the soluble extraction fraction vs. time. A-C) Protein content expressed as mg of protein per mg dry weight. D-F) Relative peroxidase activity expressed as change in absorbance per unit time per mg dry weight. G-I) Relative peroxidase activity expressed as change in absorbance per unit time per mg protein.


VEGETATIVE BUD-FORMING TISSUE
FLORAL BUD-FORMING TISSUE
CALLUS-FORMING TISSUE
PROTEIN CONTENT mg/mg DRY wt
0.06
0.05
0.04
0.03
0.02
A
B
C
PEROXIDASE ACTIVITY/ mg DRY wt
0.009
0.007
0.005
0.003
0.001
D
E
F
PEROXIDASE ACTIVITY/ mg PROTEIN
0.45
0.35
0.25
0.15
0.05
G
H
I
2
6
10
14
18
NUMBER OF DAYS IN CULTURE

increase. The tissue induced to form floral buds showed a third trend, the protein content decreased during bud formation, and then increased slightly, from day sixteen to day twenty during bud development.

The peroxidase activity per unit dry weight also demonstrated differences between the organogenetic regimes. The peroxidase activity of the tissue induced to form vegetative buds increased rapidly until day eight, then it increased slowly until day eighteen, after which it dropped. The peroxidase activity of the tissue induced to form floral buds rose slowly until day fourteen, then plateaued with a slight fluctuation the remainder of the time. The callus-forming tissue had an increase in peroxidase activity until day twelve, and then a gradual decrease in activity. If, on the other hand, the peroxidase activity was computed per unit protein, the differences between the organogenetic regimes were reduced. The activity of the tissue induced to form vegetative buds rose slowly until day eight, then it increased slowly, with fluctuations, until day eighteen when it began to decrease. The tissue induced to form floral buds had a continual, although fluctuating, increase in peroxidase activity the whole twenty day period observed. The peroxidase activity of the callus-forming tissue rose, with fluctuations, until day twelve, then decreased until day twenty.

Isoperoxidase Occurrence

The isoperoxidase patterns (zymograms) are presented in Figures 11 through 16. Figures 11, 12, and 13 are those from gels using acetate buffer, pH 4.5, while Figures 14, 15, and 16 are those from gels using borate buffer, pH 8.0. In analyzing the data present in these figures

Figure 11. Isoperoxidase banding patterns in acetate gels of the soluble extraction fraction. Note the unique presence of C2 in floral bud-forming tissue and C1 in callus-forming tissue. C16 developed only in vegetative bud- and callus-forming tissues. C9, A1, and A8 all appeared earlier in callus-forming tissue than in vegetative or floral bud-forming tissues. The band representations are shown in order of decreasing intensities: ▬ ▤ ▭ ▭ ⬚

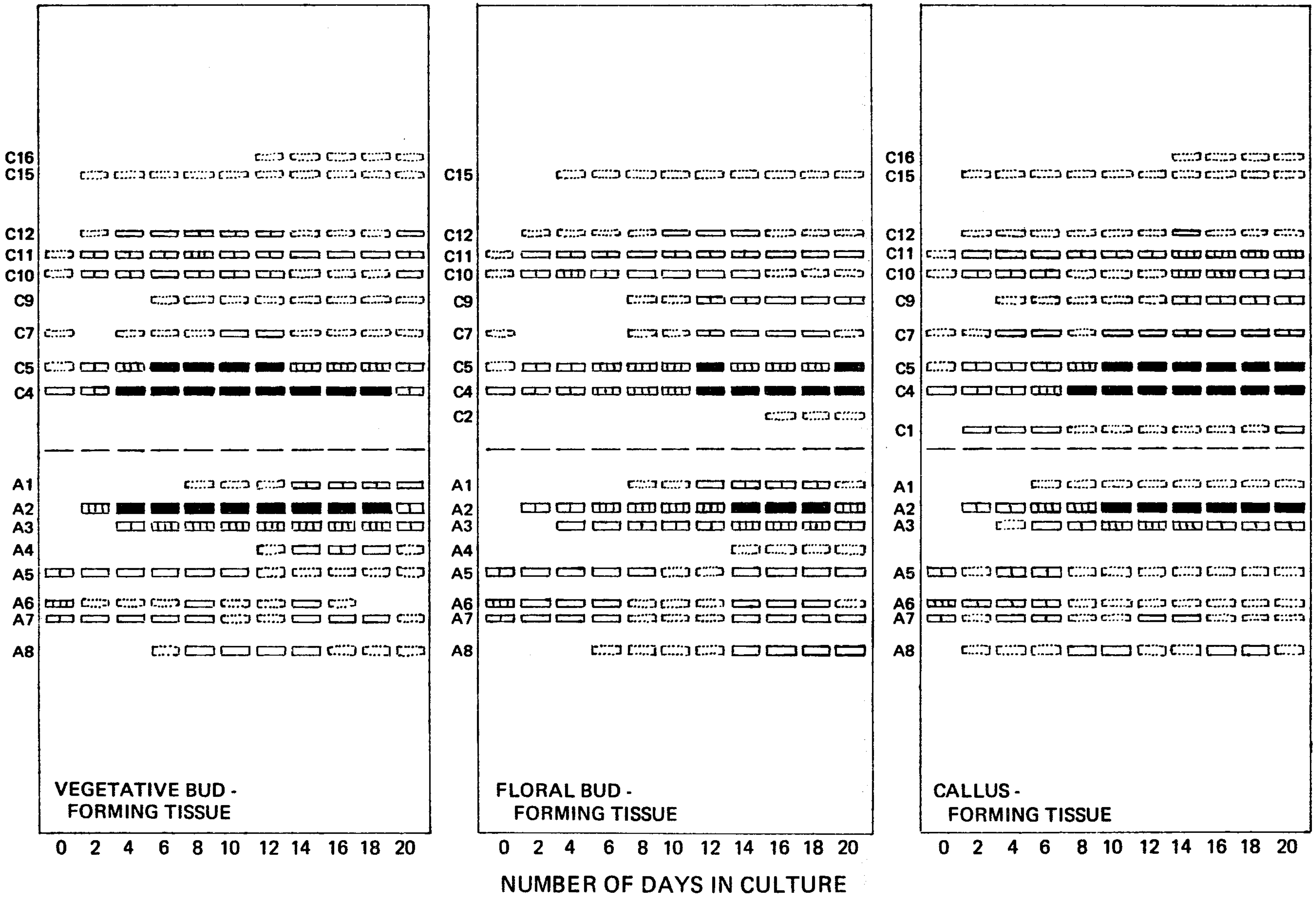

C16
C15
C12
C11
C10
C9
C7
C5
C4
C2
C1
A1
A2
A3
A4
A5
A6
A7
A8
VEGETATIVE BUD - FORMING TISSUE
FLORAL BUD - FORMING TISSUE
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

Figure 12. Isoperoxidase banding patterns in acetate gels of the ionically bound extraction fraction. Note the unique presence of C17 in the vegetative bud-forming tissue, and of C2 in callus-forming tissue. C7, C6, and A7 all were found in fresh thin layers. C7 reappeared in vegetative bud-forming tissue, although it appeared continuously in floral bud-forming tissue. C6 reappeared in vegetative bud-forming tissue, and A7 reappeared in callus-forming tissue. C16, C13, C12, and C8 all appeared first in vegetative or floral bud-forming tissues. C1 and A5 were interesting in their disappearance on vegetative bud-forming tissue. Both were continuously present in callus-forming tissue, and A5 was also present in floral bud-forming tissue.

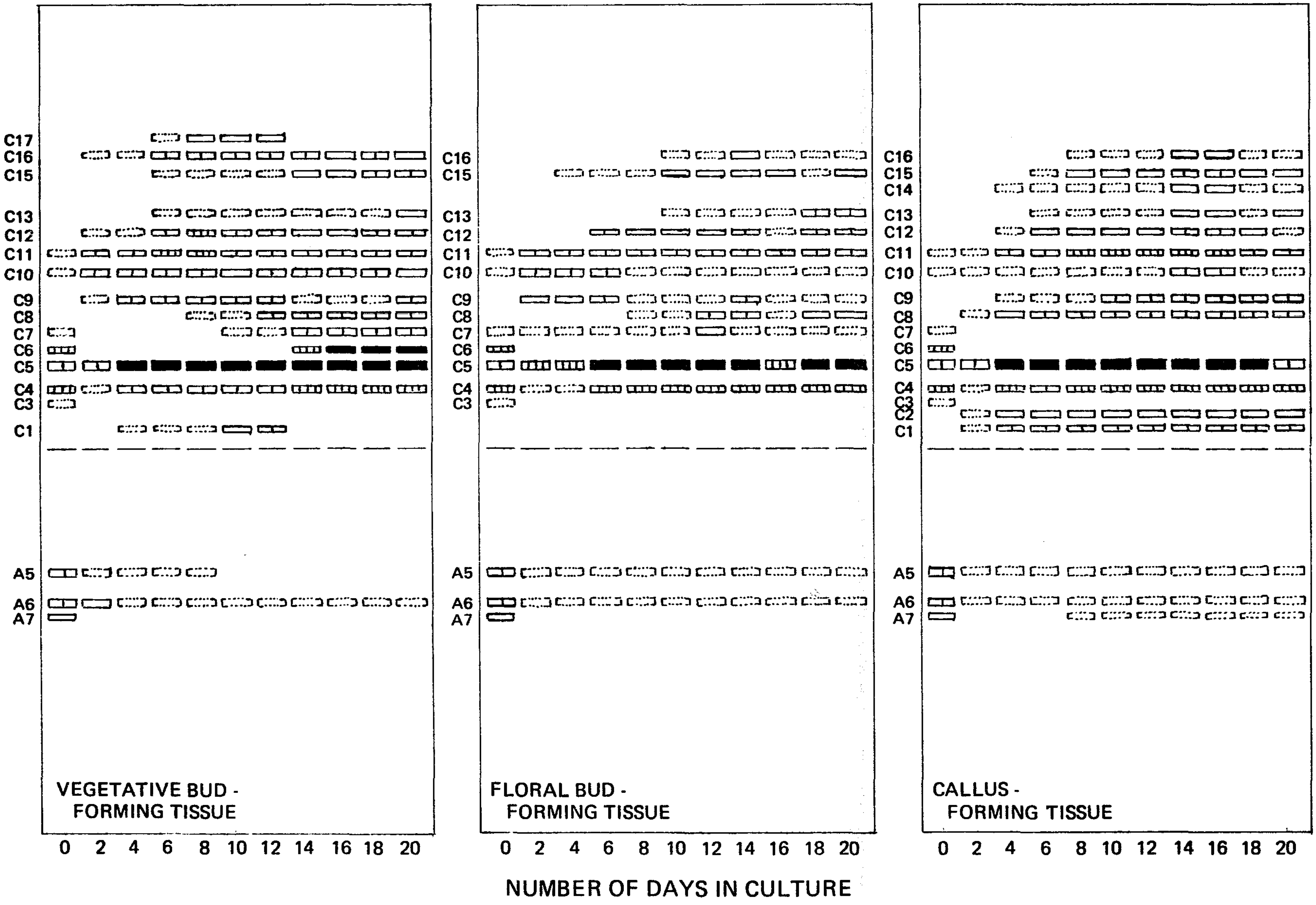

C17
C16
C15
C13
C12
C11
C10
C9
C8
C7
C6
C5
C4
C3
C1
A5
A6
A7
VEGETATIVE BUD - FORMING TISSUE
C16
C15
C13
C12
C11
C10
C9
C8
C7
C6
C5
C4
C3
A5
A6
A7
FLORAL BUD - FORMING TISSUE
C16
C15
C14
C13
C12
C11
C10
C9
C8
C7
C6
C5
C4
C3
C2
C1
A5
A6
A7
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

Figure 13. Isoperoxidase banding patterns in acetate gels of the covalently bound extraction fraction. Note the unique presence of C9 and C1 in the vegetative bud-forming tissue, and of A5 in the fresh thin layers and during development only in callus tissue. A6 was found continuously in the developing callus tissue and in fresh thin layers, and only very briefly in floral bud-forming tissue. C4 appeared only in the floral and vegetative bud-forming tissues, although earlier in floral.

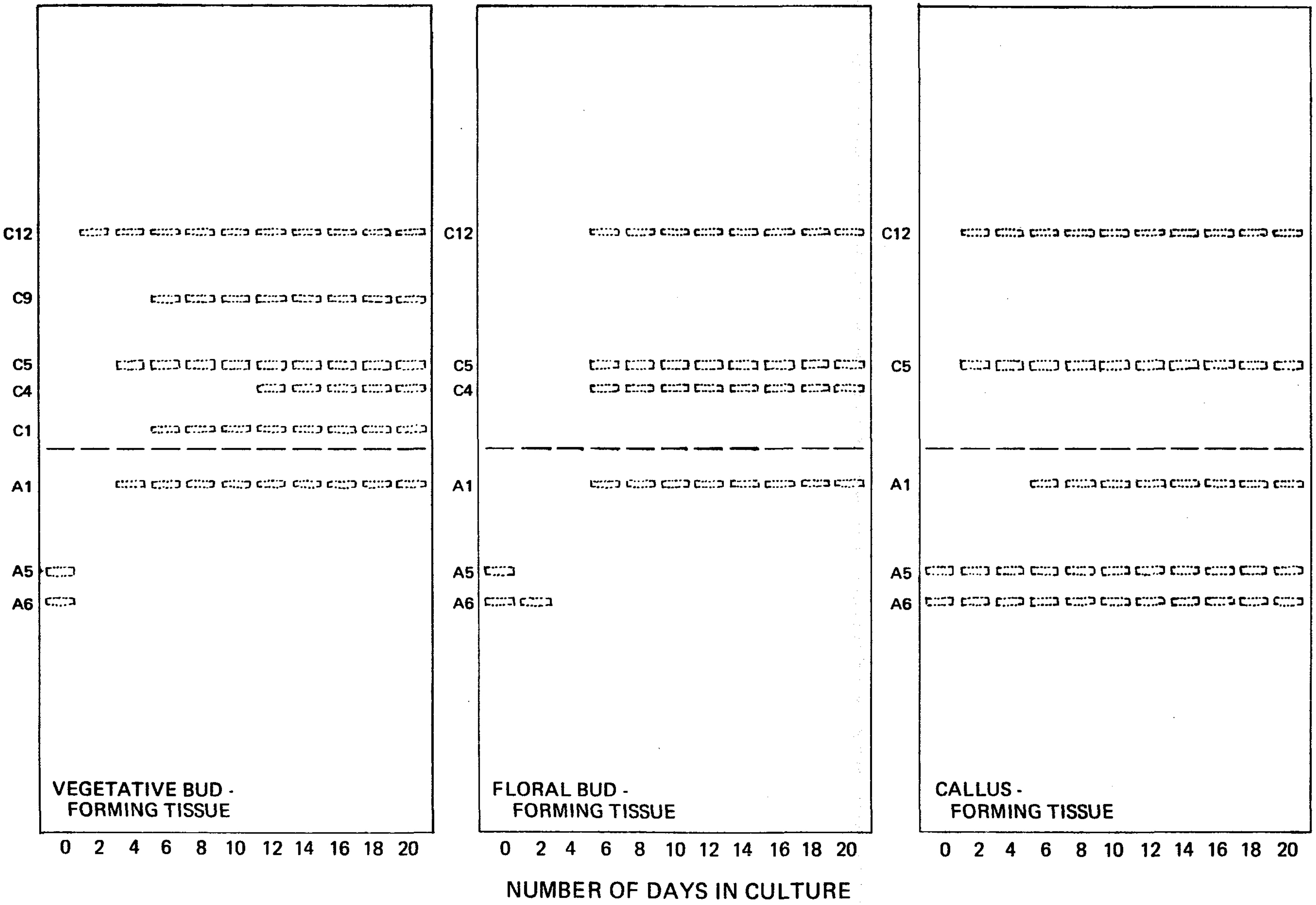

C12
C9
C5
C4
C1
A1
A5
A6
VEGETATIVE BUD - FORMING TISSUE
C12
C5
C4
A1
A5
A6
FLORAL BUD - FORMING TISSUE
C12
C5
A1
A5
A6
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

Figure 14. Isoperoxidase banding patterns in borate gels of the soluble extraction fraction. The band A'7 was unique in its appearing only in floral bud-forming tissue. A'2 and A'5 both eventually disappeared in vegetative and floral bud-forming tissues. A'4 appeared only in floral bud- and callus-forming tissues.

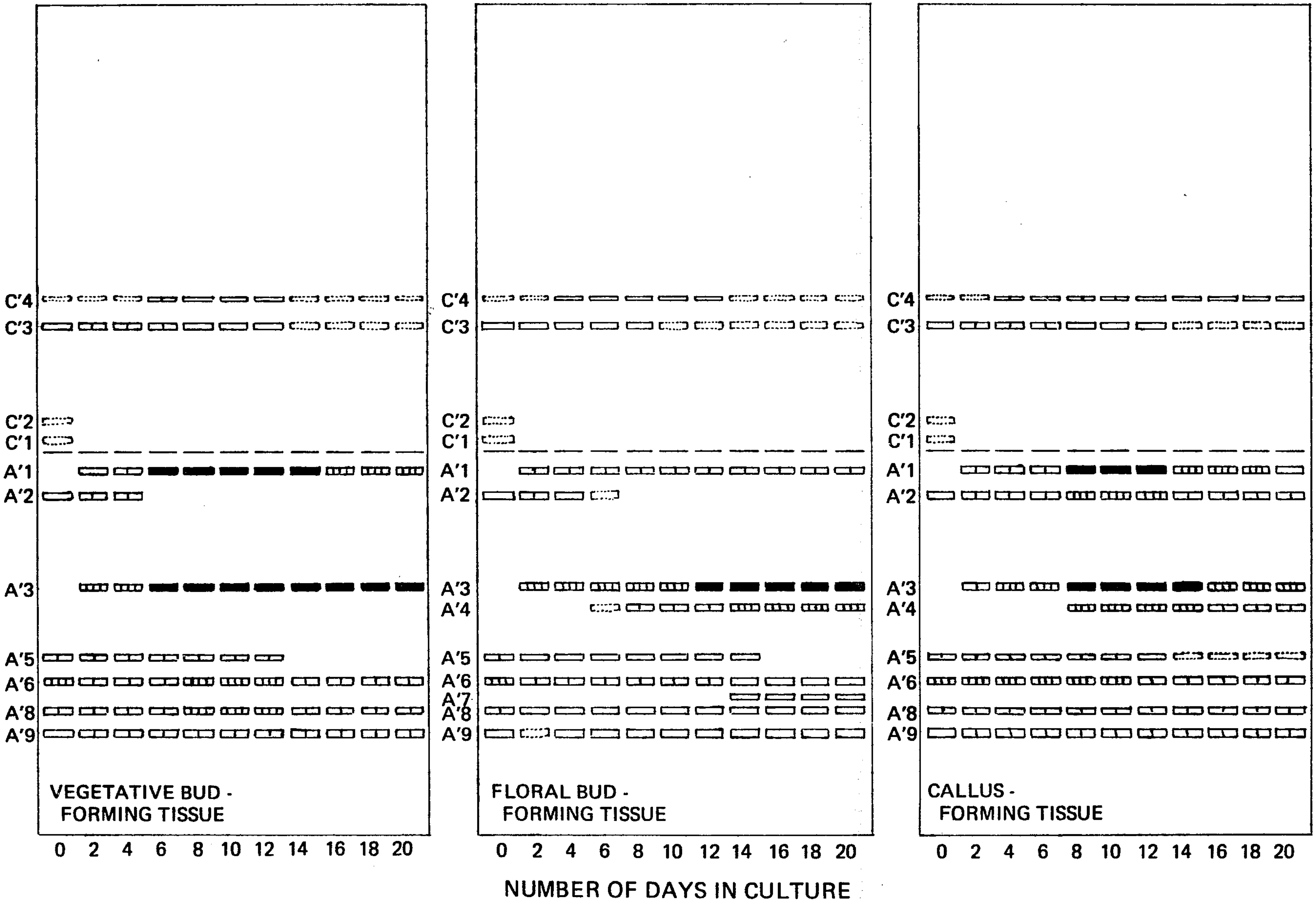

C'4
C'3
C'2
C'1
A'1
A'2
A'3
A'4
A'5
A'6
A'7
A'8
A'9
VEGETATIVE BUD - FORMING TISSUE
FLORAL BUD - FORMING TISSUE
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

Figure 15. Isoperoxidase banding patterns in borate gels of the ionically bound extraction fraction. Band C'1 was unique in its appearing only in callus-forming tissue. A'5 disappeared in vegetative bud-forming tissue while remaining in floral bud- and callus-forming tissues.

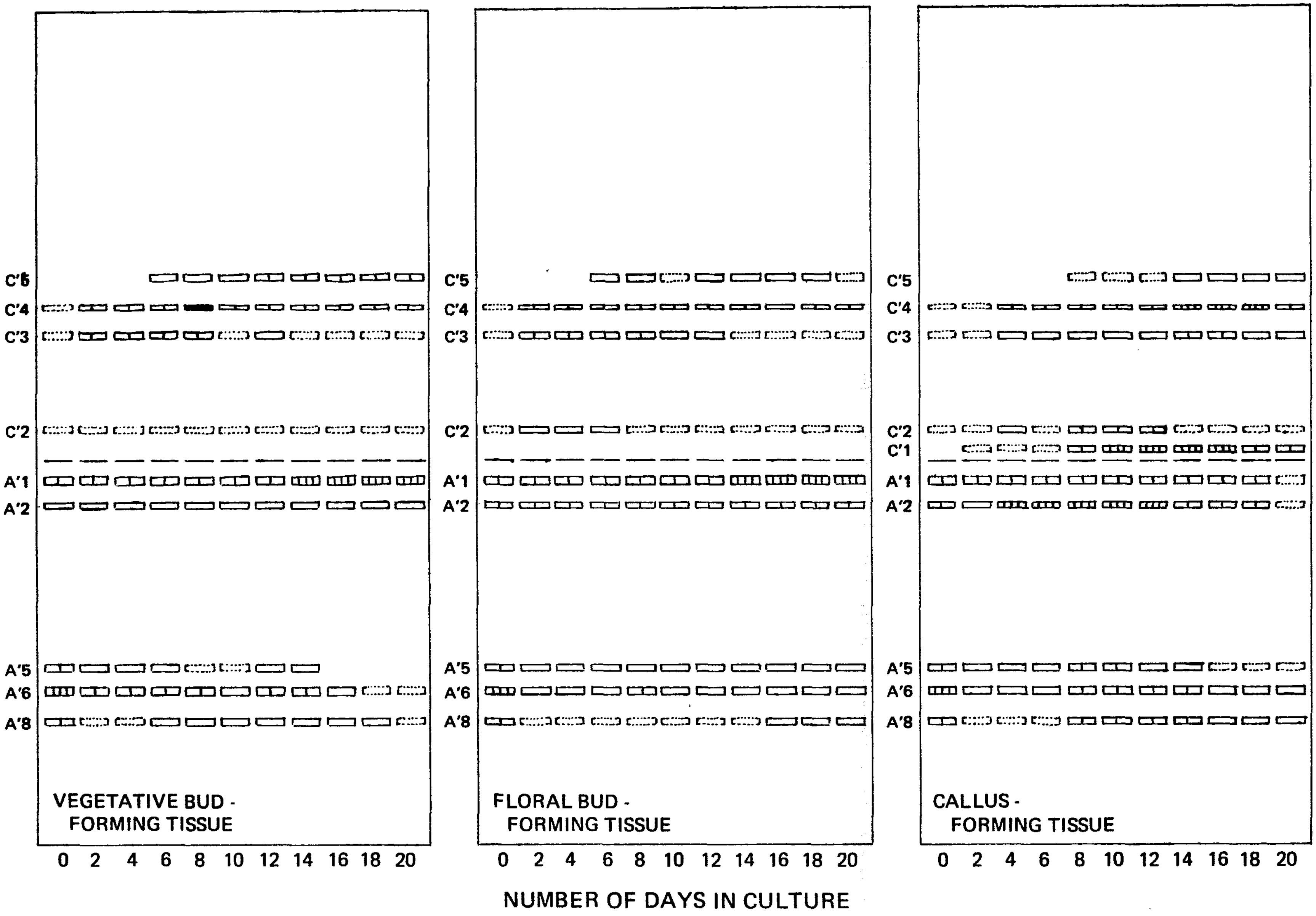
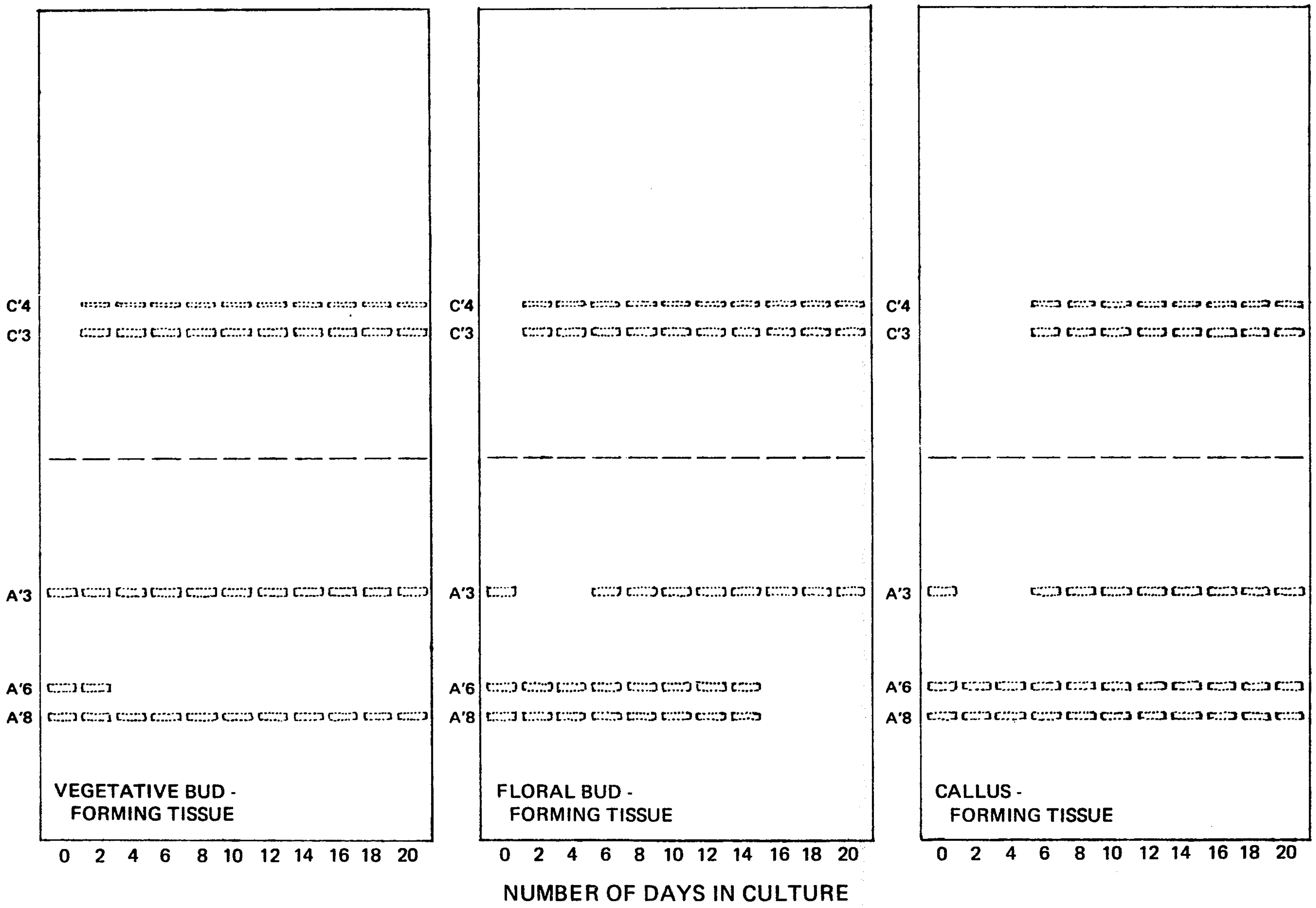
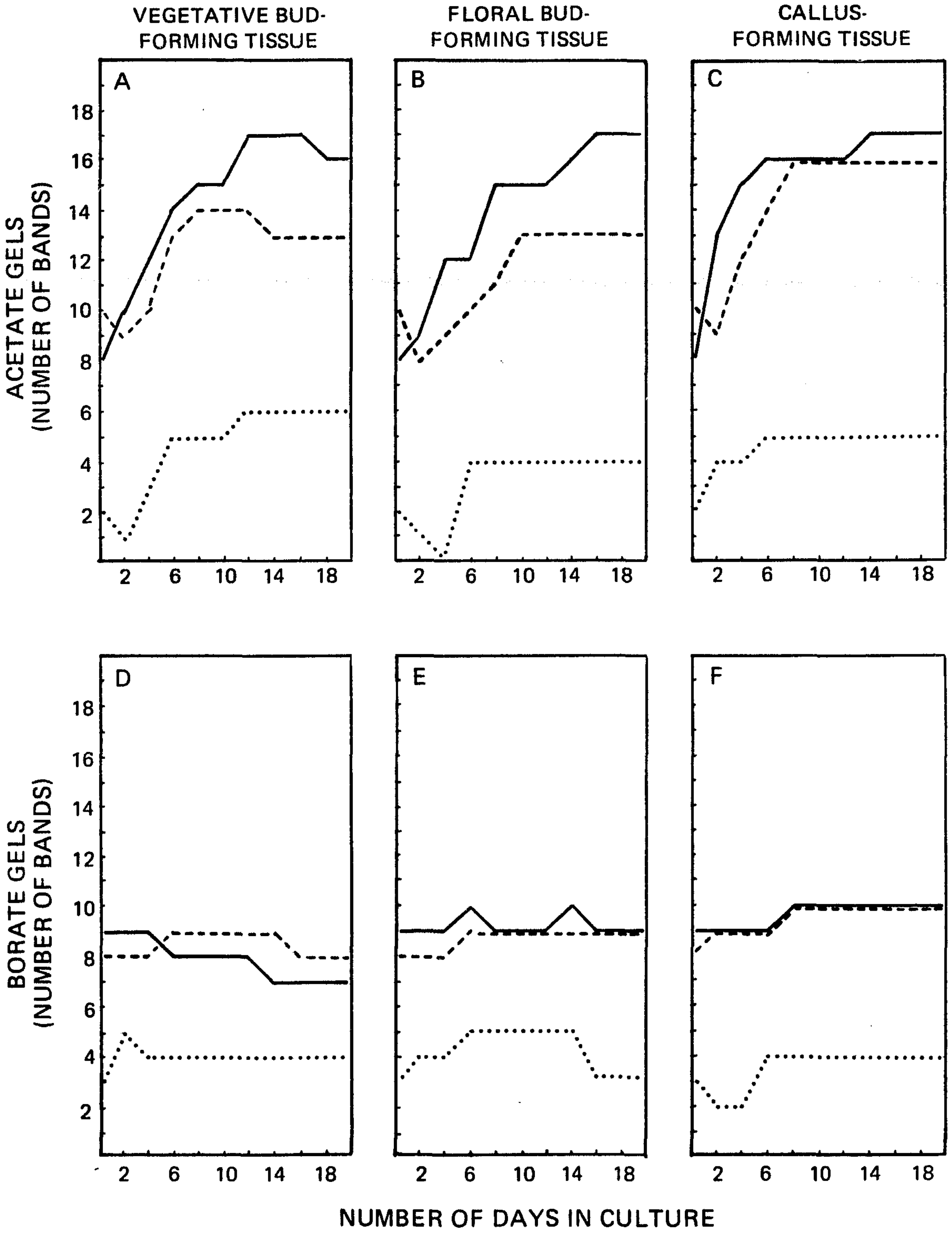

C'5
C'4
C'3
C'2
C'1
A'1
A'2
A'5
A'6
A'8
VEGETATIVE BUD - FORMING TISSUE
FLORAL BUD - FORMING TISSUE
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

Figure 16. Isoperoxidase banding patterns in borate gels of the covalently bound extraction fraction. Bands C'4 and C'3 appeared later in callus-forming tissue than in the others. A'3 disappeared for a short time in callus- and floral bud-forming tissues. A'6 disappeared totally in vegetative bud-forming tissue, and after buds were visible in floral bud-forming tissue. A'8 also disappeared after floral buds had been formed although it was present continuously in the other tissues.


C'4
C'3
A'3
A'6
A'8
VEGETATIVE BUD - FORMING TISSUE
C'4
C'3
A'3
A'6
A'8
FLORAL BUD - FORMING TISSUE
C'4
C'3
A'3
A'6
A'8
CALLUS - FORMING TISSUE
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
0 2 4 6 8 10 12 14 16 18 20
NUMBER OF DAYS IN CULTURE

we might first look at the number of isoperoxidase bands present at any given time (Figure 17). The acetate and borate gels gave striking differences in this aspect. Far more bands were observed in the acetate gels, and there was also greater variation in their number than in the borate gels. In the soluble extraction fraction of the vegetative bud-forming tissue there was an increase in the number of isoperoxidase bands until day twelve, as revealed using acetate gels. Borate gels, on the other hand, indicated a gradual decrease in the number of bands. The soluble fractions of the floral bud- and callus-forming tissues had similar, although less striking, differences between the results of acetate and borate gels. In both tissues, in acetate gels, there was an increase in the number of bands; in floral bud-forming tissue the rise was over a sixteen-day-period while in callus-forming tissue the bulk of the increase was in the first six days. In borate gels the increase in band number was almost nonexistent. The ionically bound fraction in all three organogenetic regimes, as revealed in acetate gels, had an initial decrease in the number of isoperoxidases, and then an increase until day eight or ten. Only in the vegetative bud-forming tissue was there a subsequent decrease in the number of bands. This latter decrease was also observed in the borate gels. In the covalently bound enzyme fraction, as seen in acetate gels, the vegetative and floral bud-forming tissues had an initial decrease in the number of isoperoxidase bands, followed by a sharp increase until day six. The callus-forming tissue had no initial decrease, but here also a sharp increase existed until day six. Interestingly, in borate gels the pattern of band number for the covalently bound fraction was somewhat reversed. The callus-forming tissue had an initial decline and the bud-forming tissues did not.

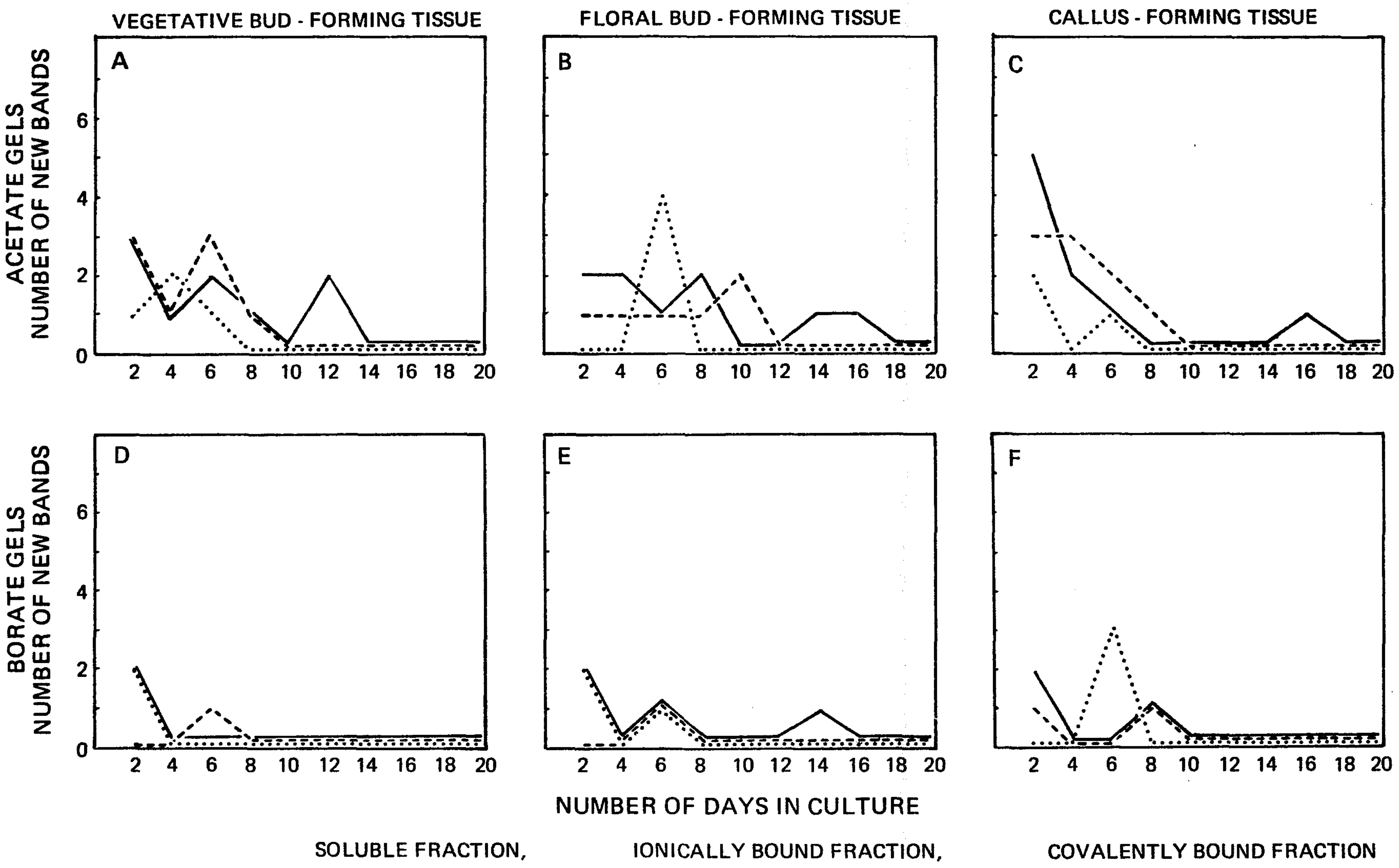
Figure 17. The number of isoperoxidase bands present vs time.
—— Soluble fraction, --- ionically bound fraction,
....Covalently bound fraction.



On the days when new isoperoxidase bands appeared (Figure 18), the acetate and borate gels both revealed a similar pattern. The majority of new bands appeared on days two and six. The data from the acetate gels exposed some interesting differences between the organogenetic regimes. The vegetative bud-forming tissue had the most new bands, seven in total, on day two, and a smaller peak of six new bands on day six. The floral bud-forming tissue had only one major peak, with six new bands, on day six. Callus-forming tissue had one very substantial peak of ten new bands on day two.

Turning from isoperoxidase bands as generalities to specific isoperoxidases, a listing of the bands found (Tables 2 and 3) illustrates that there were distinct differences between the three extraction fractions. In the acetate gels (Table 2) the soluble fraction had nineteen bands, four were unique to that fraction. There were more bands (twenty) in the ionically bound fraction, and six of them were unique to that fraction. There were only eight bands in the covalent fraction and none of them were unique to that fraction. Of the twenty-five bands in total, only seven were found in all three fractions. The data from the borate gels were different. The soluble fraction had thirteen bands, three of which were unique. The ionically bound fraction had ten bands, but only one was unique (a much lower ratio than found in acetate gels). The covalently bound fraction had five bands, and as in acetate gels, none of them were unique to this fraction. Of the fourteen bands total in borate gels, four were found in all three extraction fractions; this was about the same percentage as in acetate gels. Interestingly, in both acetate and borate gels, all of the bands that were unique to the

Figure 18. Days when new isoperoxidase bands appeared.



soluble fraction were anodic (negatively charged) and the ones unique to the ionically bound fraction were cathodic (positively charged).

Table 2. Isoperoxidase bands present in acetate gels, 25 bands total
*Indicates this band unique to its fraction.

Soluble Fraction (19 Bands)	Ionically Bound Fraction (20 Bands)	Covalently Bound Fraction (8 Bands)
	C17*	
C16	C16	
C15	C15	
	C14*	
	C13*	
C12	C12	C12
C11	C11	
C10	C10	
C9	C9	C9
	C8*	
C7	C7	
	C6*	
C5	C5	C5
C4	C4	C4
	C3*	
C2	C2	
C1	C1	C1
A1		A1
A2*		
A3*		
A4*		
A5	A5	A5
A6	A6	A6
A7	A7	
A8*		

McLellan and Robinson (1983) found similarly interesting data; in cabbage and Brussels sprouts all the ionically bound isoperoxidases were cathodic.

Table 3. Isoperoxidase bands present in borate gels, 14 bands total
*Indicates this band unique to its fraction.

Soluble Fraction (13 Bands)	Ionically Bound Fraction (10 Bands)	Covalently Bound Fraction (5 Bands)
	C'5*	
C'4	C'4	C'4
C'3	C'3	C'3
C'2	C'2	
C'1	C'1	
A'1	A'1	
A'2	A'2	
A'3		A'3
A'4*		
A'5	A'5	
A'6	A'6	A'6
A'7*		
A'8	A'8	A'8
A'9*		

The time of appearance of specific isoperoxidases is presented in Tables 4 and 5. It became evident that most isoperoxidase bands that appeared on day two in any extraction fraction tended to appear in the other regimes (in the same extraction fraction) by day six. Most bands that appeared in one extraction fraction, however, rarely arose in the other extraction fractons. Only one isoperoxidase, C12, found using acetate gels, occurred in all three extraction fractions.

An examination of the days when isoperoxidase bands disappeared (Tables 6 and 7) revealed that acetate and borate gels gave different results. In the acetate gels no bands disappeared during the first sixteen days in the soluble extraction fraction, while four bands were lost during this period using borate gels. The situation was reversed in the ionically and covalently bound fractions. Here far more bands disappeared in acetate gels than in borate gels. The one noteworthy feature

Table 4. Days when new isoperoxidase bands appeared in acetate gels.

	SOLUBLE FRACTION			IONICALLY BOUND FRACTION			COVALENTLY BOUND FRACTION		
	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE
2d	C15, C12 A2	C12 A2	C15, C12, C1 A2, A8	C16, C12, C9	C9	C8, C2, C1	C12		C12, C5
4d	A3	C15 A3	C9 A3	C1	C15	C14, C12, C9	C5 A1		
6d	C9 A8	A8	A1	C17, C15, C13	C12	C15, C13	C9	C12, C5, C4 A1	A1
8d	A1	C9 A1		C8	C8	C16			
10d					C16, C13				
12d	C16 A4						C4		
14d		A4	C16						
16d		C2							
18d									
20d									

Table 5. Days when new isoperoxidase bands appeared in borate gels.

	SOLUBLE FRACTION			IONICALLY BOUND FRACTION			COVALENTLY BOUND FRACTION		
	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE
2d	A'3, A'1	A'3, A'1	A'3, A'1			C'1	C'4, C'3	C'4, C'3	
4d									
6d		A'4		C'5	C'5				C'4, C'3
8d			A'4			C'5			
10d									
12d									
14d		A'7							
16d									
18d									
20d									

Table 6. Days when isoperoxidase bands disappeared in acetate gels.

	SOLUBLE FRACTION			IONICALLY BOUND FRACTION			COVALENTLY BOUND FRACTION		
	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE
2d				C3 A7	C6, C3 A7	C7, C6, C3	A6, A5	A5	
4d								A6	
6d									
8d									
10d				A5					
12d									
14d				C17, C1					
16d									
18d	A6								
20d									

Table 7. Days when isoperoxidase bands disappeared in borate gels.

	SOLUBLE FRACTION			IONICALLY BOUND FRACTION			COVALENTLY BOUND FRACTION		
	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE	VEGETATIVE BUD - FORMING TISSUE	FLORAL BUD - FORMING TISSUE	CALLUS - FORMING TISSUE
2d	C'2, C'1	C'2, C'1	C'2, C'1						
4d							A'6		
6d	A'2								
8d		A'2							
10d									
12d									
14d	A'5								
16d		A'5		A'5				A'6, A'8	
18d									
20d									

in both was that the callus-forming tissue never lost any isoperoxidase bands after day two.

There was a set of isoperoxidases that attracted attention because of their occurrence at specific times in specific organ-forming tissues (see Tables 8 and 9) e.g., those found in Figure 11 (acetate gels, soluble extraction fraction). Band C16 was found in vegetative bud-forming tissue starting at day twelve and in callus-forming tissue starting at day fourteen. Band C9 appeared earlier in callus-forming tissue than in the others, and band C7 remained continuously in callus-forming tissue while it disappeared for the first few days in the bud-forming tissues. Band C2 occurred only in floral bud-forming tissue from day sixteen; perhaps it was associated with a specific floral organ. Band C1 arose only in callus-forming tissue, starting at day two, corresponding to the period of cell division. Band A4 appeared in vegetative and floral bud-forming tissues after the buds had appeared, while they were developing. Band A6 was the only soluble isoperoxidase seen in acetate gels that disappeared after organ initiation. Its disappearance came after vegetative buds had appeared and started to develop. Band A8 appeared in callus-forming tissue starting at day two, but did not appear in the bud-forming tissues until day six. In the ionically bound extraction fraction (Figure 12), band C17 appeared in vegetative bud-forming tissue from days six to twelve, while the vegetative bud meristems were forming. The band C16 eventually appeared in all three tissue regimes, but it appeared before organ initiation in vegetative bud-forming tissue and after initiation in the other two tissues. Band C14 was found only in callus-forming tissue starting at day four. Bands C13 and C12 both appeared first in callus- and vegetative bud-forming tissues, and then a few days later in floral bud-forming tissue. The band C8 became apparent on day two in callus-forming tissue, but not until after organ initiation was complete (day eight) in the bud-forming tissues. Band C7's occurrence was intriguing; it was found in the fresh thin layers, in floral bud-forming tissue the entire time, and in vegetative bud-forming tissue starting at day ten. The band C6 was also found in the fresh thin layers, then it disappeared and reappeared only in vegetative bud-forming tissue starting at day fourteen. Band C2 was seen only in callus-forming tissue, starting from day two, thus it seemed to be another band associated with unregulated cell division. Band C1 may also be related to cell division; it occurred in callus-forming tissue during the entire time assayed and in vegetative bud-forming tissue from days four through twelve. Band A5 was found in all three tissue regimes but it disappeared in vegetative bud-forming tissue after day eight. Band A7 was present in the fresh thin layers and in callus-forming explants from day eight on, when the callus tissue was visible. The covalently bound fraction, as observed in acetate gels (Figure 13), also had some interesting isoperoxidase bands. Band C9 was found exclusively in vegetative bud-forming tissue starting when organ induction was complete (day six) and continuing during bud development. Band C5 varied in its time of appearance, from day two in callus-forming tissue to day six in floral bud-forming tissue. Band C4 was present in floral bud-forming tissue starting at day six, and in vegetative bud-forming tissue starting when the buds were visible, day twelve. Band C1 was present only in vegetative bud-forming tissue, and then only from the time organ induction was complete. Bands A5 and A6 were both found in the fresh thin layers and in callus-forming tissue; A6 was also found in floral bud-forming tissue on day two.

Table 8. Isoperoxidase bands present in acetate gels that varied among different organogenetic regimes.

Soluble Fraction	Ionically Bound Fraction	Covalently Bound Fraction
	C17 (vegetative only)	
C16	C16	
	C14 (callus only)	
	C13	
	C12	
C9		C9 (vegetative only)
	C8	
C7	C7	
	C6 (d0 and veg. only)	
		C5
		C4
C2 (floral only)	C2 (callus only)	
C1 (callus only)	C1	C1 (vegetative only)
A4		
	A5	A5 (d0 and callus only)
A6		A6 (d0, floral d2, and callus only)
	A7 (d0 and callus only)	
A8		
8 of 19 Bands ~ 42%	12 of 20 Bands ~ 60%	6 of 8 Bands ~ 75%

The borate gels also revealed some isoperoxidase bands of interest. In the soluble extraction fraction (Figure 14), band A'2 was observed in fresh thin layer tissue, in callus-forming tissue the entire time, and

Table 9. Isoperoxidase bands present in borate gels that varied among different organogenetic regimes.

Soluble Fraction	Ionically Bound Fraction	Covalently Bound Fraction
		C'4
		C'3
	C'1 (callus only)	
A'2		
		A'3
A'4		
A'5	A'5	
		A'6
A'7 (floral only)		
		A'8
4 of 13 Bands ~ 30%	2 of 10 Bands ~ 20%	5 of 5 Bands ~ 100%

in both bud-forming tissues the first several days. It was not found in bud-forming tissues after organ induction was completed. Band A'4 was seen in callus-forming tissue starting on day eight and in floral bud-forming tissue starting on day six. Band A'5 was found in all three tissue regimes as well as fresh thin layers, but it disappeared in floral and vegetative bud-forming tissues after the buds appeared. Band A'7 was found only in floral bud-forming tissue starting on day fourteen; perhaps this band was identical to band C2 that was found in acetate gels. The ionically bound fraction (Figure 15) yielded two bands of interest. Band C'1 was found only in callus-forming tissue starting on day two. Band A'5 was found in all the tissues but disappeared in vegetative bud-forming tissue after day fourteen. This was similar to band A5 in acetate gels, but there the band disappeared after day eight. The covalently bound extraction fraction (Figure 16) also revealed some noteworthy bands. Two bands, C'3 and C'4 showed the same pattern of occurrence. They appeared on day two in both bud-forming tissues, but not until day six in callus-forming tissue. Band A'3 was found in the fresh thin layer tissue; it disappeared in callus- and floral bud-forming tissues but then reappeared at day six. It was always evident in the vegetative bud-forming tissue. Since all the bands in the covalently bound fraction were faint it is possible that this isoperoxidase might still have been present, but in very low amounts. Band A'6 was found in the fresh thin layers; it continued to exist in tissue induced to form vegetative buds only a couple of days, in floral bud-forming tissue until after the buds had become visible (day fourteen), and in callus-forming tissue the entire time. Band A'8 was present in all the tissues but it was lost in floral bud-forming tissue after day fourteen.

It is both interesting and problematic that there seemed to be almost no correlation between bands of interest in acetate gels and bands of interest in borate gels, even though samples of the exact same extracts were applied to both. It is also distressing that the ionically bound fraction, which gave such interesting results in the acetate gels, gave so little of interest in the borate gels.

DISCUSSION

In the pioneering study of the isoperoxidases of the thin layer organogenesis system, Tran Thanh Van and her colleagues (Gaspar, Thorpe, and Tran Thanh Van, 1977; Thorpe, Tran Thanh Van, and Gaspar, 1978) found changes in the isoperoxidase pattern that were characteristic for each of the organ-forming regimes. The patterns were distinguished by the times that specific isoperoxidases appeared and when their activity peaked. There were, however, no isoperoxidase bands that were found in only one organ-forming tissue. While this was also the case in most other studies of isoperoxidases, there have been several reports that found isoperoxidases that were specific to one organ or tissue phase. Arnison and Boll (1976b) in a study of bush bean suspension culture found one isoperoxidase, D2, that was only present when the culture was in the division phase. Wochok and Burleson (1974) found an isoperoxidase in eighteen-day-old wild carrot suspension embryos that was not present in proembryos or in older embryos. Siegel and Galston (1967) found when examining pea seedlings that some isoperoxidase bands were present only in one organ. Kahlem (1975) discovered a stamen-specific isoperoxidase in twenty-six plant species. Sawhney, Basra, and Kohli (1981) noted the appearance of two new isoperoxidases in the shoot apex of <u>Amaranthus viridus</u> L. upon the induction of flowering. Koul and Bhargava (1983) reported even more dramatic results. They found seven isoperoxidase bands that were present only during specific stages of flowering in <u>Scandix pecten-veneris</u> L.

In light of the above reports, the lack of specific isoperoxidases limited to specific organ-forming tissues in the thin layer organogenesis system seemed a paradox. It was the hope, at the beginning of this study, that by the use of a more extensive extraction procedure, and the utilization of a more sensitive electrophoresis system that allowed the simultaneous separation of anodic and cathodic isoperoxidases over a wide range of pHs, any striking changes in isoperoxidase composition, if they existed in this organogenesis system, would become evident.

The extraction technique used in this study gave three distinct fractions: soluble, ionically bound, and covalently bound. Electrophoresis was performed using buffers of two different pHs, 4.5 and 8.0. The difference between the results of the two pHs was quite unexpected, but informative, especially since most studies of isoperoxidase have been done at high pHs, which in this study gave far fewer isoperoxidase bands. Not only were there fewer isoperoxidase bands seen in the borate gels (pH 8.0) than in the acetate gels (pH 4.5) (14 bands in borate gels, 25 bands in acetate gels), but their pattern of occurrence was vastly different (Figure 17). In all three regimes, as seen in acetate gels, the number of isoperoxidase rose sharply in the soluble and ionically bound fractions during the first six days. In contrast, in the borate gels there was an increase of only one isoperoxidase per extraction fraction at best. Of additional interest, the fraction of isoperoxidase bands that varied in their pattern of occurrence (between the organogenetic regimes) within one extraction fraction was quite different between the two buffers. In the soluble extraction fraction the acetate gels revealed eight bands out of nineteen that varied, while the borate gels revealed only four out of thirteen. The covalently bound fraction had six bands that varied out of eight seen in the acetate gels, while all five seen in the borate gel varied between the regimes. Of most interest here was the ionically bound fraction. In the acetate gels twelve of the twenty observed varied while in the borate a mere two, out of ten, varied. This last situation was of special concern since the ionically bound fraction had never been examined before in respect to organogenesis. If the borate buffer alone had been used the potential significance of this extraction fraction might not have been recognized.

In searching for a reason why there were such differences between the two buffers several possibilities came to mind. The borate ion is known to form complexes with glycopeptides (Weitzman, Scott, and Keegstra, 1979) that alter the relative migration rates during electrophoresis. There is no reason to believe, however, that it would destroy the actual peroxidase activity, or alter the migration rates to such an extent that 40% of the isoperoxidases would comigrate with other isoperoxidase forms. The isoperoxidase bands observed using borate gels in this study tended to be poorly resolved. The same was found to be true in isoelectric focusing (Rücker and Radola, 1971; Benvenuto et al., 1983; McLellan and Robinson, 1983). In all these cases the "basic" isoperoxidases were poorly resolved while the "acidic" ones showed good resolution. The only apparent reason for the lowered number of isoperoxidase bands in the borate gels was Lee's finding (1973) that pH effects staining. He found pH 4.5 to be optimal for peroxidase staining. This suggested that those isoperoxidases in the borate gel that had a narrow range of pH tolerance for activity simply were not active and produced no colored reaction product.

Since the isoperoxidase bands of interest in borate gels (pH 8.0) were fewer in number than those in the acetate gels (pH 4.5), and since they showed patterns that were also seen in the bands found in the acetate gels, only the data from the acetate gels will be discussed in this section.

The results of this study have revealed several very interesting isoperoxidase bands (Table 10). Twelve bands were found in essentially only one organ-forming regime (and only one extraction fraction). Another four were found in only two organ-forming regimes. An additional two bands were interesting because they were present in all three regimes initially, but disappeared in the vegetative bud-forming tissue. Eight more bands were found in all three regimes, but varied in their time of appearance. The occurrence of many of the above isoperoxidase bands, especially the unique ones, could be correlated with the induction of specific organ-forming regimes or stages of organogenesis as seen in the histological findings. This allowed speculation about possible correlations of function with specific isoperoxidases. While these correlations do not prove the functions of specific isoperoxidases, they are significant and indicate the possible importance of isoperoxidases in organogenesis. Many of the findings here agreed with findings of other researchers, as will be discussed below.

The eight bands whose time of appearance varied from one tissue-forming regime to another showed no consistent pattern among themselves, and the reasons for their differing times of appearance were not clear. No other researchers with similar findings have been able to make correlations either.

Of the twelve isoperoxidase bands that were unique in their appearance in essentially only one tissue (and only one extraction fraction)

Table 10. Correlations of physiological and histological phenomena with isoperoxidase band occurrence.

Correlated With:	Band No.	Occurrence
Unregulated cell division and/or high auxin levels	C1 sol	Callus d2→
	C14 ion	Callus d4→
	C2 ion	Callus d2→
	C1 ion	Callus d2→ Veg bd d4→d12
	A7 ion	Callus d8→ d0
	A5 cov	Callus d2→ d0
	A6 cov	Callus d2→ Floral bd d2 only d0
Wound healing and cell division	A2 sol	Callus Veg bd Floral bd d2→
	A3 sol	Callus Veg bd Floral bd d4→
Wound healing and lignification	C12 all	Callus d2-4→ Veg bd d2→ Floral bd d2-6→
	C15 sol + ion	Callus d2-6→ Veg bd d2-6→ Floral bd d4→
Cessation of cell division and differentiation in zone three	C16 sol	Callus d14→ Veg bd d12→

Correlated With:	Band No.	Occurrence
The disappearance of these two bands was correlated with repression of non-bud growth during vegetative bud formation	A6 sol	Callus d0→ Veg bd d0→d16 Floral bd d0→
	A5 ion	Callus d0→ Veg bd d0→d8 Floral bd d0→
Bud development, organized apical meristems	A4 sol	Veg bd d12→ Floral bd d14→
	C7 ion	Veg bd d10→ Floral bd d2→ d0
	C4 cov	Veg bd d12→ Floral bd d6→
Vegetative bud formation and development	C9 cov	Veg bd d6→
	C1 cov	Veg bd d6→
Vegetative bud formation	C17 ion	Veg bd d6→d12
Vegetative bud development perhaps specifically the chlorenchyma	C6 ion	Veg bd d14→ d0
Development of the stamens	C2 sol	Floral bd d16→

seven were found in callus forming tissues (soluble-C1, ionically bound-C14, C2, C1, and A7, and covalently bound-A5 and A6). The occurrence of these bands could be correlated with one or both of two factors. The auxin levels in the callus culture medium were five to ten times higher than those in the culture media of the bud-forming tissues. It was supplied as indolebutyric acid, an artificial auxin. These isoperoxidases may have been formed in response to the higher auxin levels in an attempt on the tissues part to regulate the auxin levels. On the other hand, these isoperoxidases may have been correlated with continuous unorganized cell division. This increase in number of isoperoxidases agreed with the results of Goff (1975), Vanden Born (1963), and Ramaiah, Durzan, and Mia (1971) that showed an increase in peroxidase activity associated with rapidly dividing cells or cells that were going to divide. There were, additionally, three other reports of isoperoxidase bands that were unique to callus tissue. Rawal and Mehta (1982) found haploid tobacco callus had one unique isoperoxidase. Rücker and Radola (1971) observed two unique isoperoxidases in non-differentiating tobacco callus. Bassiri and Carlson (1979) obtained callus tissue from several different plant parts of tobacco, and found all had three isoperoxidase bands that were not present in any of the parent tissues.

Gordon and Alldridge (1967) found that during wound healing in tomato one isoperoxidase appeared one to two days after wounding in the cytoplasm of cells adjacent to the wound. After three or four days the activity of this isoperoxidase was localized in the cell wall, rather than in the cytoplasm. Another isoperoxidase appeared three to four days after wounding, in the cortical cells that had begun to divide. The development of this isoperoxidase took place only after several cell divisions had occurred. In the present organogenetic system, in which there was wound healing in all three regimes, two soluble bands, A2 and A3, appeared after a few days in culture in all three regimes. These bands might have been associated with wound healing and cell division. There were two additional bands, C12 and C15, that might also have been implicated in wound healing, especially lignification, which was important both in the sealing over of the wounded surface and later in differentiation of tracheary elements. Band C12 was present in all of the extraction fractions of all three regimes. Band C15 was present in the soluble and ionically bound fractions in all three regimes. Fukuda and Komamine's (1982) study showing that the activity of the ionically bound peroxidase peaked just before lignification, and the activity of the covalently bound peroxidase peaked at the time of active lignin synthesis, implied that any isoperoxidase involved in lignification should at least be found in the ionically and covalently bound fractions. Fleuriet and Delcire (1982) found histochemical evidence that lignification in wounded tomato fruits was linked to a covalently bound isoperoxidase. There was always a possibility, in the current study, that other isoperoxidases were involved in lignification. There were no others, however, that seemed as likely based on their pattern of occurrence. I assumed that isoperoxidases involved in lignification would probably not have been present in the fresh thin layers.

Band C16 of the soluble fraction was present in vegetative bud-forming tissue starting on day twelve, and in callus-forming tissue starting on day fourteen. In both of these tissues, rapid division and differentiation in the third zone had ceased, whereas tracheary element differention in the floral bud-forming tissue continued. The occurrence of this isoperoxidase appeared to be correlated with the cessation of cell division and differentiation.

Mäder (1975) found that shoot differentiation in tobacco callus consisted of two processes that were correlated with changes in isoperoxidases. Meristemoid formation was accompanied by a sharp rise in the activity of several isoperoxidases. There was also an inhibition of growth in the non-differentiating cells that was correlated with a reduction of activity in the fast migrating anodic isoperoxidases. In the current study, there were two isoperoxidase bands in the vegetative bud-forming tissue that paralleled the repression that Mäder found. Band A6 in the soluble fraction was found in all three regimes. It disappeared in the vegetative bud-forming tissue after day sixteen. Band A5 of the ionically bound fraction had the same essential pattern, but it disappeared from the vegetative bud-forming tissue after day eight. Wochok and Burleson (1974) obtained similar results with wild carrot suspension cultures. As the embryoids matured into plantlets the number of isoperoxidases decreased.

There were three bands that were found in both bud-forming regimes. Band A4 of the soluble extraction fraction was found after the buds had appeared, and might have been involved in some phase of bud development. The same might be true of C7 of the ionically bound fraction, although it was present in the floral bud-forming tissue before organ initiation was completed. Band C4 of the covalently bound fraction was also found earlier in the floral bud-forming tissue, but it did not appear until day six, when induction was completed. In the vegetative bud-forming tissue, it (C4) appeared on day twelve, when the buds became visible.

There are four isoperoxidases that were correlated with various stages of vegetative bud development and formation. Band C17 of the ionically bound fraction was found only from days six to twelve, after the tissue was committed to form vegetative buds, but only as long as the meristems were forming. It was not found after the buds had developed. Band C6 of the ionically bound fraction was found only in the fresh thin layers and in vegetative bud-forming tissue starting from day fourteen. Perhaps it was in the epidermis or chlorenchyma of the young leaves. Two bands in the covalently bound fraction (C9 and C1) appeared at day six and remained during vegetative bud formation and development.

Rawal and Mehta (1982) observed similar bands in their study of shoot regeneration from haploid tobacco callus. They reported that the shoot buds appeared after nine days in inductive conditions. Although they reported no histological data, some assumptions as to the physiclogical state could be made based on the histological data of the current report and others. There were seven isoperoxidases that were unique to the shoot-forming tissue. Three isoperoxidases were present only at day three, when shoot initiation was probably occurring. Two other bands appeared at day three and remained present during shoot formation and development. A sixth isoperoxidase became evident at day six when the meristems should have been forming. The last of the seven was seen starting at day nine, when the shoot buds were visible. Mäder, Münch, and Bopp (1975) in another study using tobacco (starting with shoot tissue, going through callus, and then regenerating shoots), determined that there were three isoperoxidase bands that typified shoot tissue. There are two additional pertinent studies. Chen, Towill, and Loewenberg (1970) and Braber (1980) found that there were isoperoxidases whose appearance was correlated with specific stages during leaf development.

One band was present during a very specific stage of floral bud development. Band C2 of the soluble fraction was found only in floral bud-forming tissue starting at day sixteen, shortly after the stamens had started to develop. Kahlem (1975) found that three-fourths of the plant species he examined had a specific isoperoxidase that occurred in the stamens or "male" flowers, but not in the leaves. When he used histoimmunology (Kahlem, 1976) he was able to locate the "stamen specific" isoperoxidase of _Mercurialis annua_ L. in the microspore and tapetum tissues of the stamen. The data of Koul and Bhargava (1983) was similar, although more extensive. They observed seven isoperoxidase bands that were found only during specific stages of flowering in _Scandix pecten-veneris_ L. Less dramatic changes were found by two other groups of investigators. Jaiswal and Kumar (1980) discovered two isoperoxidases in the floral primordia of _Coccinia indica_ that were not present in the vegetative primordia. Sawhney, Basra, and Kohli (1981) found that upon induction of flowering two new isoperoxidases appeared in the shoot apex of _Amaranthus viridis_ L.

Because of the use of a different electrophoretic technique the study reported here was able to add some further findings, of organ specific isoperoxidase bands present in the soluble fraction at specific times during initiation and development, to a preexisting classical study. By the further extraction and separation of the ionically and covalently bound fractions this study added totally new evidence that the isoperoxidases of these fractions may also play a role in organogenesis.

Conclusions

This investigation has shown that there are specific isoperoxidases, the presence of which be correlated with specific periods and events during organogenesis. These correlations indicate that isoperoxidases may well be an important component in regulating necessary functions in organ initiation and development. Many other studies have strongly indicated that this may be so, but due to the use of different techniques and examination of two additional extraction fractions (ionically bound and covalently bound), the results of this study are far more extensive than any published to date. To prove the correlations between isoperoxidase functions and organogenetic involvement further research needs to be undertaken. A study using histoimmunology would be very helpful in locating the specific isoperoxidases to see if they are located in the suspected tissues. Additionally, determining the substrate specificities of the isoperoxidases of interest could possibly help elucidate their functions in the tissues. For example, are the isoperoxidases that are found exclusively in the callus-forming tissue acting as auxinoxidases, or are they involved in cell wall rigidification in the rapidly proliferating cells? It would also be of interest to have further studies that reveal what the factors are that determine where in a cell, particularly with regard to the cell wall, the isoperoxidases are located. The literature reviewed in this dissertation suggests that for such processes as lignification and cell wall rigidification the isoperoxidases must be in the cell wall.

Additionally, the results of this study indicated that the method used to extract and separate isoperoxidases may be far more critical than suspected. This study used a procedure that allowed simultaneous resolution of both anodic and cathodic isoperoxidases, so that all could be seen at once. Even with this care to resolve all isoperoxidases simultaneously, the pH of the electrophoresis buffer seemed to be critical. The basic buffer permitted detection of only two-thirds as many isoperoxidases as seen in the acidic buffered gel. This is particularly noteworthy in view of the great number of peroxidase studies done using basic buffers. One suspects that important results have been undetected in these studies because of this factor. Furthermore, because of this the results of most studies of isoperoxidases are probably not truly comparable. The extraction procedures are generally not the same and the electrophoretic methods vary as to pH, buffer strength and composition, gel medium (starch vs. polyacrylamide), and gel concentration. In many cases these aspects of technique are not even specified in the paper. With a view toward allowing greater correlations to be made between reports in the literature it is suggested that uniform extraction and electrophoretic procedures be adopted.

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