Chapter 5

Cellular Totipotency

5.1. INTRODUCTION

Unlike animals, where differentiation is generally irreversible, in plants even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus. Sieve tube elements and xylem elements whose nuclei have started to disintegrate, or fibres with cell walls thicker than 2 μm (mature tracheids have 7 μm thick walls) would, obviously, not divide any more. According to Gautheret (1966) the degree of regression a cell can undergo would depend on the cytological and physiological state it has reached in situ (see Table 5.1).

When non-dividing, quiescent cells from differentiated tissues are grown on a nutrient medium that supports their proliferation, the cells first undergo certain changes to achieve the meristematic state. These include replacement of non-functional cellular components damaged by lysosomal activity during the processes of cytoquiescence (Bornman, 1974). The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed 'dedifferentiation'. A multicellular explant generally comprises cells of diverse

TABLE 5.1

Degree to which different cell types may dedifferentiate[a]

Vegetative point	Cambium	Companion and secretory cells	Parenchyma	Thick-walled cells (collenchyma, lignified cells)	Fibre	Degenerating cells (vascular elements, sieve tubes)
•←———	•←———	•←				
•←———	•←———	•←———	•←			
	•←———			———	•	
	•←———			———	———	•
	•←———			———	———	———

[a]After Gautheret (1966).

types. As a result, the callus derived from it would be heterogeneous with respect to the ability of its component cells to form a whole plant or plant organs ('redifferentiation'). The inherent potentiality of a plant cell to give rise to a whole plant, a capacity which is often retained even after a cell has undergone final differentiation in the plant body, is described as 'cellular totipotency'. For a differentiated cell to express its totipotency it first undergoes dedifferentiation followed by redifferentiation. Mostly dedifferentiation involves embryonization of cells leading to callus formation. However, embryonic explants often exhibit differentiation of roots, shoots or embryos without an intervening callus phase.

Tissue culture techniques offer not only an excellent opportunity to study the factors that elicit the totipotentiality of cells but also allows investigation of factors controlling cytological and histological differentiation.

5.2. CYTODIFFERENTIATION

In the area of cytodifferentiation in vitro as well as in vivo the main emphasis has been on vascular differentiation, particularly the tracheary elements (TEs). Phloem has received less attention because of technological problems. Whereas TEs can be easily stained and scored in macerated preparations of the tissue, this is not possible with the small and delicate sieve tubes.

In an intact plant, tissue differentiation goes on in a fixed manner which is characteristic of the species and the organ. Torrey and coworkers have done considerable work on the control of the pattern and extent of vascular differentiation in excised organized roots (Torrey, 1966).

Kohlenbach and Schmidt (1975) observed that mechanically isolated mesophyll cells of *Zinnia elegans* differentiated into TEs without cell division when cultured on a suitable medium. The *Zinnia* system was further refined by Fukuda and Komamine (1980a,b) and Church and Galston (see Church, 1993), who achieved relatively synchronous differentiation of a high percentage (50–65%) of the cells within 72 h. Since then *Zinnia* cells have been extensively used as a model system to understand the process of TE differentiation. The special merits of this system are: (1) freshly isolated cells of *Zinnia* provide a very homogeneous single cell system composed of uniformly non-polyploid cells held in G_1 phase of the cell cycle (Fukuda and Komamine, 1981), (2) controlled differentiation can be achieved by exogenous supply of phytohormones and other chemicals into the medium, avoiding the problem of hormonal and nutritional

gradient in multicellular system, (3) since cell division is not a prerequisite for differentiation, the inductive factors influence TE differentiation directly rather than cell division, and (4) the differentiation occurs synchronously and at high frequency.

Fukuda and Komamine (1983) have shown that under inductive conditions for TE differentiation from mesophyll cells of *Zinnia*, synthesis of two proteins is shut off and two new polypeptides appear (within 48–60 h of culture) before any detectable morphological change in the mesophyll cells. These novel proteins can be regarded as biochemical markers for TE differentiation. More recently, Demura and Fukuda (1994) have isolated 3 cDNA clones for the genes (TED2, TED3, TED4) expressed preferentially in mesophyll cells of *Zinnia* during their redifferentiation into TE. For detailed reviews of the subject, see Roberts (1976), Fukuda and Komamine (1985), Fukuda (1989), Fukuda and Kobayashi (1989), Sugiyama and Komamine (1990) and Church (1993).

5.2.1. Factors affecting vascular tissue differentiation

Two substances that have a profound effect on vascular tissue differentiation are auxin and sucrose. They affect vascular differentiation qualitatively as well as quantitatively. Some evidence also points towards the involvement of cytokinins and gibberellins in the process of xylogenesis.

(i) *Growth regulators.* Camus (1949), a French botanist, grafted small vegetative buds on the upper surface of cultured root tissues of *Cichorium*, and after a few days observed the differentiation of vascular strands in the parenchymatous tissue below the bud. These strands connected the vascular tissue of the bud to the other vascular tissues in the explant. The differentiation of vascular tissue occurred even if the physical contact between the callus and the bud was broken by placing cellophane paper at the site of the graft. This suggested that the stimulus provided by the bud for vascular tissue differentiation was of the nature of diffusable chemical(s). This work was later confirmed by Wetmore and Sorokin (1955) using the undifferentiated callus (completely lacking in vascular elements) of *Syringa vulgaris* as the experimental system. Callus pieces (400 mg fresh weight) were planted on a medium which did not favour the differentiation of vascular tissue. The trimming of the callus and its orientation was such that its upper surface was fairly flat and smooth. A V-shaped incision was made on the upper surface of the callus and a vegetative bud, with its basal end cut wedge-shaped, was inserted

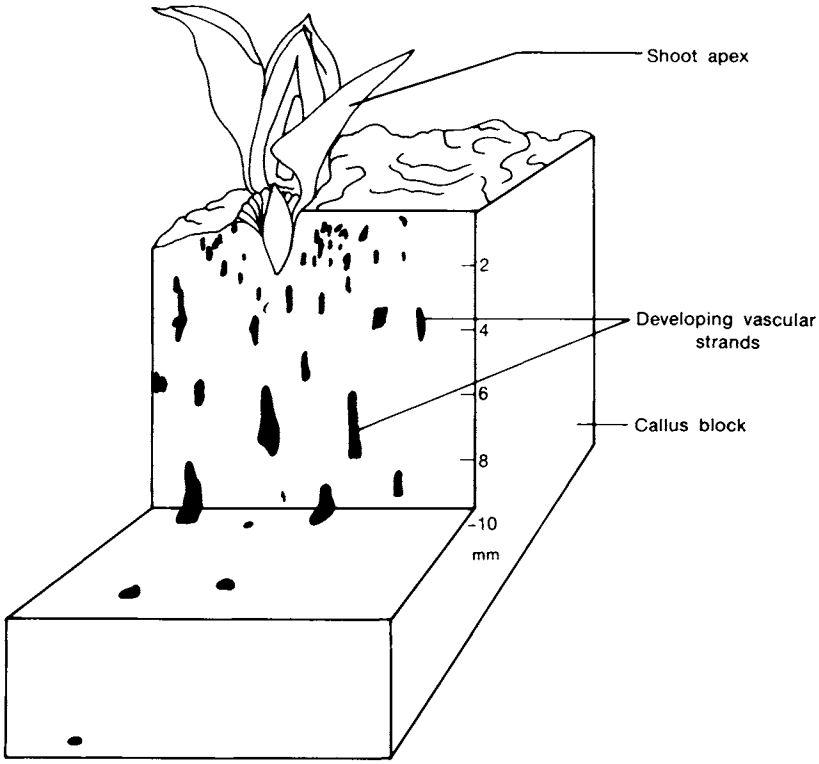

Fig. 5.1. Induction of vascularization in callus tissue of *Syringa* by grafting a stem apex bearing two or three leaf primordia. Drawing made 54 days after grafting (after Wetmore and Sorokin, 1955).

in the cavity (see Fig. 5.1). To prevent desiccation at the point of graft the cavity was filled with 1% non-nutrient agar before inserting the bud. Within 20–30 days the bud induced divisions in the cells underneath it, resulting in the appearance of short vertical columns of cells which later developed into a ring of vascularized nodules. Agar containing sucrose and auxin could effectively replace the bud for vascular differentiation (see Fig. 5.2) (Jeffs and Wetmore, 1967).

Fosket and Torrey (1969) reported a stimulatory effect of cytokinin on xylogenesis in cotyledonary callus cultures of soybean. The observations of Mizuno et al. (1971, 1973) and Mizuno and Komamine (1978) with cultured root slices of carrot also suggest the involvement of auxin and cytokinin in the differentiation of TEs. In a medium containing only auxin the carrot cvs Kuroda-gosum and Kintoki, roots of which contain zeatin, differentiated TEs in light as well as in the dark. In the

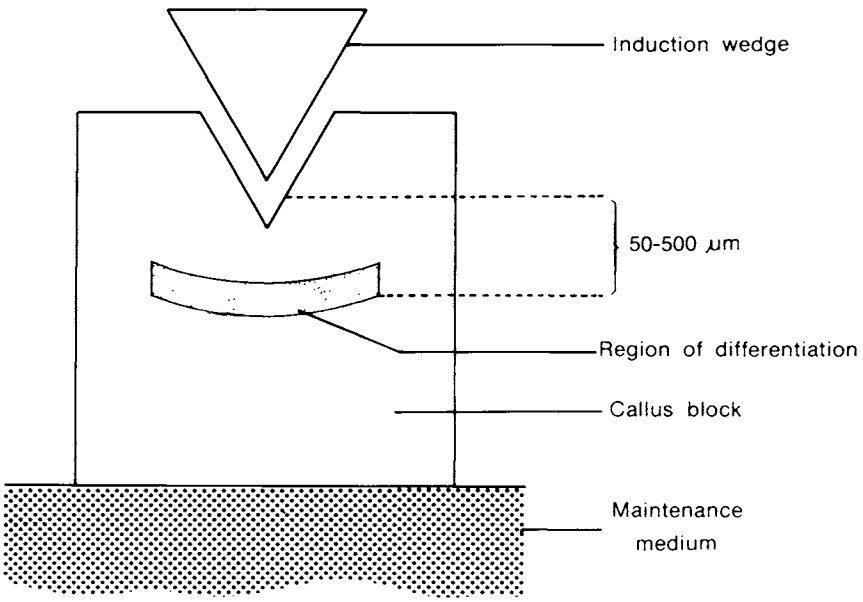

Fig. 5.2. Diagram to show the induction of vascularization in a block of *Phaseolus* callus by inserting an agar-block wedge containing auxin and sucrose (after Jeffs and Northcote, 1967).

same medium the carrot cvs Ogata-sanzun and Hakkaido-gosum, roots of which lack zeatin, formed TEs only in light. Light induces the synthesis of zeatin in Hakkaido-gosum (Mizuno and Komamine, 1978), suggesting thereby a positive role of cytokinin in xylogenesis in these systems.

In mesophyll cell cultures of *Zinnia*, maximum differentiation of TEs occurs within a narrow range of both auxin and cytokinin, suggesting that the absolute concentration of the two hormones in the culture medium is more important than auxin/cytokinin ratio (Fukuda and Komamine, 1980a; Church and Galston, 1988; Lin and Northcote, 1990). In this system the differentiation of TEs with optimum hormonal treatment (0.1 mg l^{-1} NAA and 0.1–1 mg l^{-1} BAP) is inhibited or delayed by the inhibitors of both auxin and cytokinin synthesis (Church and Galston, 1988). Whereas cytokinin is required only for a brief period in the early stage of differentiation, auxin must be present until the last stage of differentiation (Fukuda and Komamine, 1985; Church and Galston, 1988). In contrast, for TE differentiation in tuber discs of *Helianthus* exogenous cytokinin was required for 2 days while exogenous auxin was required for less than 1 day (Phillips, 1987).

A stimulatory interaction between auxin and gibberellin for xylem differentiation has been reported by Roberts and Fosket (1966), Bornman (1974), and Gautheret (1966).

(ii) *Sucrose.* The effect of auxin on vascular tissue differentiation seems to be closely dependent on the presence of sugar (Jacobs, 1952; Fosket and Roberts, 1964). The relative amounts of xylem and phloem formed in callus pieces of *Syringa* (Wetmore and Rier, 1963) and *Phaseolus vulgaris* (Jeffs and Northcote, 1967) could be changed by varying the sucrose concentration in the presence of a low concentration of auxin. In *Syringa* if the agar applied to the cavity at the top of the callus contained 0.05 mg l^{-1} IAA and 1% sucrose, only a few xylem elements appeared in the callus. Keeping the auxin concentration constant and raising the sucrose level to 2% favoured better xylem formation with little or no phloem. With 2.5–3.5% sucrose, both xylem and phloem differentiated, and with 4% sucrose, the vascular tissue formed was phloem with little or no xylem. Unlike *Syringa*, suspension cultures of *Parthenocissus tricuspidata* showed an increase in xylem elements with an increase in sucrose concentration up to 8%. It should, however, be noted that for xylogenesis in *Parthenocissus*, sucrose is hardly effective up to a concentration of 1.5% which is around optimal for *Syringa*.

Jeffs and Northcote (1967) tested a variety of sugars in the presence of an auxin and observed that besides sucrose, the disaccharides maltose and trehalose were effective in stimulating TE differentiation in *Phaseolus* callus. Glucose, fructose and other monosaccharides were non-stimulatory. The authors have expressed the view that sucrose may be acting almost like a hormone in this category of differentiation.

(iii) *Calcium.* Recent studies using *Zinnia* system have highlighted the importance of calcium in TE differentiation. Roberts and Haigler (1990) observed that calcium deprivation or application of calcium channel blockers or calmodulin antagonists inhibited TE differentiation. Whereas calmodulin antagonists were effective only when added at the beginning of culture, calcium channel blockers inhibited TE differentiation when added at any time between 0 and 48 h of culture. These results indicate the involvement of at least two calcium regulated events in TE differentiation.

Based on the fluorescence of chlortetracycline (CTC), an antibiotic that fluoresces in the presence of membrane bound Ca^{2+}, Roberts and Haigler (1990) recorded an increase in the concentration of Ca^{2+} in the mesophyll cells prior to visible wall thickening. CTC fluorescence was initially distributed evenly throughout the cytoplasm but during secon-

dary wall deposition it got localized between secondary wall thickenings and was associated with plasma membrane. Finally, the fluorescence became punctate, probably due to breakdown of the plasma membrane.

(iv) *Physical and physiological factors.* Very little attention has been paid to the effect of physical factors on vascular differentiation. In *Helianthus* there is no differentiation of vascular elements at a temperature below 17°C, and within the range of 17–31°C an increase in temperature enhances xylem formation (Gautheret, 1961). Light is reported to stimulate wound vessel differentiation in *Coleus* (Fosket, 1968).

Wound stress is reported to be another physical factor essential for the induction of TEs in *Zinnia* (Church and Galston, 1989). In leaf disc cultures very few mesophyll cells differentiate into TEs. However, peeling off the epidermis brings about considerable enhancement in the number of TEs formed. This is not due to better contact of cells with the medium because infiltration of the medium into leaf tissue did not substitute peeling (Fukuda, 1989). The stress effect could be due to ethylene production which has been implicated to play a positive role in the differentiation of TEs in lettuce explant cultures (Miller et al., 1984; Miller and Roberts, 1984). Even in *Zinnia* the inhibitors of ethylene synthesis caused blockage of TE differentiation (Fukuda, 1989).

Cells harvested from old leaves of *Zinnia* divide in culture but do not differentiate into TEs in a medium that induces the cells derived from younger leaves to differentiate into TEs (Iwasaki et al., 1986, 1988). Direct differentiation of cells into TEs in *Helianthus tuberosus* occurs only in the explants taken from immature tubers. This capacity declines with the age of tuber, and in mature tuber's TE differentiation occurs only after cell proliferation (Phillips, 1981). Another observation demonstrating the importance of the physiological condition of cells in TE differentiation is the higher frequency differentiation of TEs in the cultures of cells isolated mechanically than those obtained by enzymatic maceration of the tissue (Fukuda and Komamine, 1985).

5.2.2. Cell cycle and TE differentiation

Several workers had reported that a cell must divide before the differentiation of TE can occur (Fosket, 1968; Torrey, 1971; Torrey et al., 1971). This conclusion is based on some in vitro observations (Fosket, 1968, 1970; Fosket and Torrey, 1969; Torrey and Fosket, 1970; Tucker et al., 1986) and is supported by the in vivo situation. Procambium, from which the primary xylem and phloem are derived, usually exhibits continued

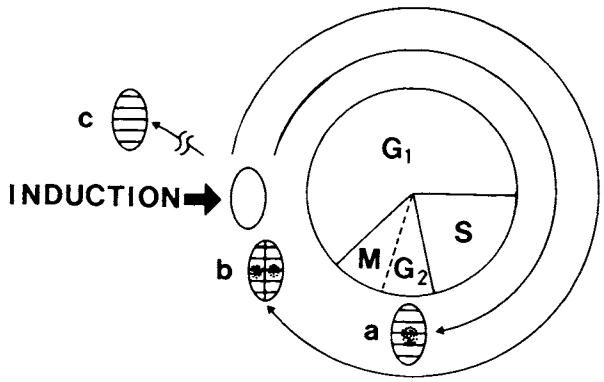

Fig. 5.3. Diagram showing relationship between Tracheary element (TE) differentiation and cell cycle in the cultures of isolated mesophyll cells of *Zinnia elegans*. Hormonal induction of TE differentiation occurs in the G_1 phase but the cell may get out of the cell cycle to differentiate at different stages in the cell cycle. The induced cell may differentiate into TE without progressing further along the cell cycle (c), it may pass through the S phase and differentiate in the G_2 phase (a), or it may undergo mitosis and both the daughter cells differentiate into TE (b) (reprinted with permission from Fukuda and Komamine, 1981, Physiol. Plant., 52: 423–430).

divisions. This is also true of the secondary vascular tissue which is contributed by the meristematic cells of vascular cambium.

The chemical factors (auxin, cytokinin, sugar, etc.) reported to be involved in xylem differentiation are generally the same as those regulating cell division. This raises the question of whether in effecting TE formation these hormones act on the differentiation process per se or the preceding cell division. A related question was asked by Dodds (1979): 'Is cell cycle activity necessary for xylem cell differentiation?'

BUdR (an inhibitor of DNA synthesis), at 10^{-5} M, completely suppressed xylem differentiation in coleus stem explants (Fosket, 1968), pea root explants (Shininger, 1975), Jerusalem artichoke tuber explants and lettuce pith tissue (Dodds, 1979). Malawer and Phillips (1979) furnished additional evidence to support the idea that differentiation of xylem elements is preceded by cell division. They added tritiated thymidine to the medium in which tissues of Jerusalem artichoke were cultured and noted that if tritiated thymidine was present throughout the culture period (48 h) all the xylem cells were labelled. Furthermore, silver-grain counting revealed that the xylem cells in culture had undergone three rounds of DNA synthesis.

Recent studies have clearly established that cell division is not always a prerequisite for TE differentiation. In cell cultures of *Centaurea cyanus*,

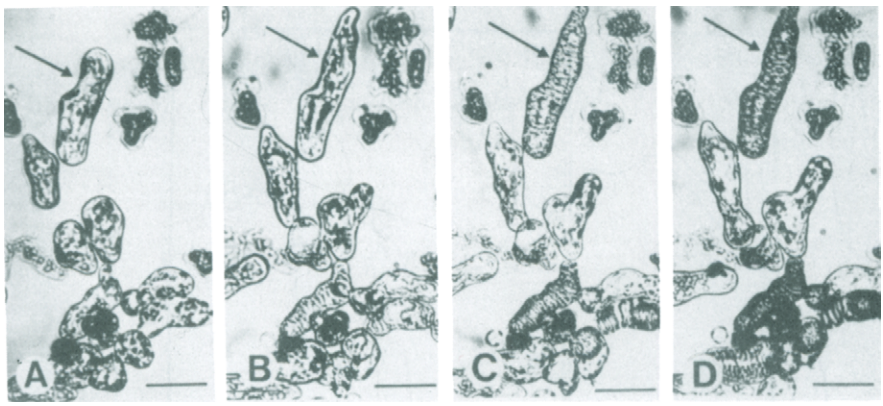

Fig. 5.4. Time-lapse pictures to show the process of tracheary element differentiation from single cells, isolated from mesophyll of *Zinnia elegans*, without cell division. The pictures taken at 48 h (A), 71 h (B), 77 h (C), and 96 h (D) after culture (from Fukuda and Komamine, 1980).

raised by Bergmann's technique of cell plating, Torrey (1975) observed that some of the single parenchymatous cells differentiated directly into TE without a preceding cell division. Since these cells were taken from fast-growing suspension cultures it could be argued that the cells directly forming TEs were derived from a recent cell division. More convincing evidence against the assumption of the need of a cell division for xylogenesis was provided by Kohlenbach and Schmidt (1975) and Fukuda and Komamine (1980b). Kohlenbach and Schmidt reported that the mechanically isolated quiescent mesophyll cells of *Zinnia* differentiated into TEs after a period of extension growth but without a cell division. Even mesophyll protoplasts of this species exhibited direct differentiation into TEs (Kohlenbach and Schopke, 1981).

Through serial observations, microdensitometry and autoradiography, Fukuda and Komamine (1980a, 1981) confirmed that in *Zinnia* the majority of TEs (60%) differentiated directly from cells in the G_1 phase (Figs. 5.3 and 5.4); the differentiation required neither the replication of total genomic DNA nor cell division. However, various inhibitors of DNA synthesis cause complete inhibition of TE differentiation (Fukuda and Komamine, 1981). It has been suggested that some kind of a minor repair type DNA synthesis is essential for cytodifferentiation but not complete genomic DNA replication during S phase of the cell cycle (Sugiyama and Komamine, 1990).

Most of the observations included above are based on gross morphology of the vascular elements. Very little, if any, is known about the structural and functional aspects of the vascular elements formed in cell and tissue

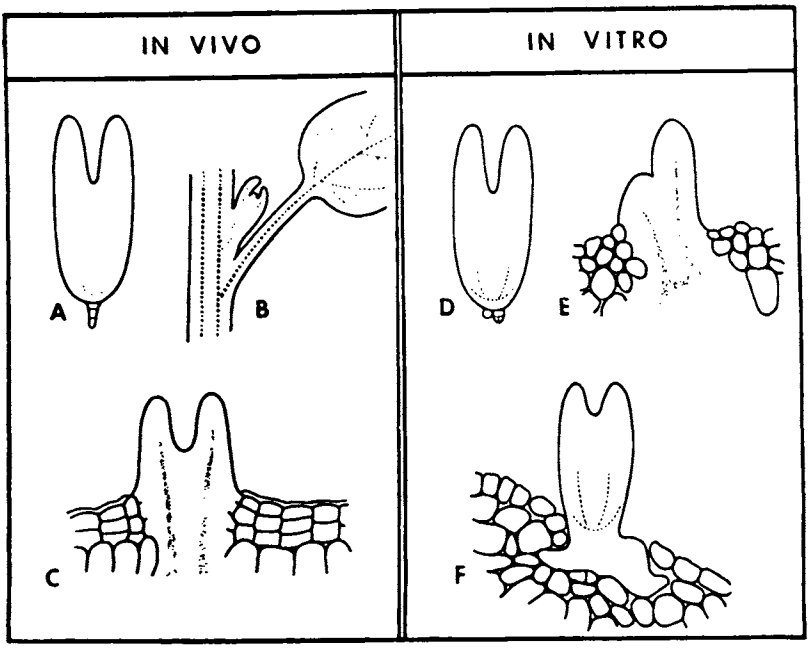

Fig. 5.5. Diagrams to show difference of anatomy in the basal ends of embryos (A,D,F) and shoot buds (B,C,E) under in vivo and in vitro conditions. The stippled areas represent the vascular traces (after Haccius, 1978).

cultures. Most probably these vascular elements are non-functional (Torrey et al., 1971).

5.3. ORGANOGENIC DIFFERENTIATION

For a considerable time the totipotency of somatic cells has been exploited in vegetative propagation of plant species. In nature, stem, leaf, and root pieces of several taxa are able to differentiate shoots and roots leading to the establishment of new individuals (Dore, 1965). In vitro studies have revealed that this potential is not restricted to only some species. Most plants provided with appropriate conditions would differentiate shoot buds and roots from somatic as well as reproductive tissues.

Whole plant regeneration from cultured cells may occur either through shoot-bud differentiation or somatic embryogenesis. A shoot bud and an embryo are distinguishable on the basis of recognizable morphological differences between the two (see Fig. 5.5) (Haccius, 1978). The former is a monopolar structure. It develops procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explant (see Fig. 5.5B,C,E). On the other hand, an

embryo is a bipolar structure with a closed radicular end (see Fig. 5.5A,D,F). It has no vascular connection with the maternal callus tissue or the cultured explant.

Plant regeneration from isolated cells, protoplasts or unorganized callus is generally more difficult than that from intact explants such as cotyledons, hypocotyl segments and immature embryos. With the advent of techniques to insert alien genes into cells of intact explants (see Sections 14.2.1 and 14.2.4) success in genetic engineering of plants no longer depends on the arduous step of plant regeneration from isolated protoplasts. The regenerants obtained through de novo differentiation of shoot buds or somatic embryos directly from the explants also exhibit genetic variability suitable for somaclonal variant selection (see Chapter 9). Therefore, during the last decade considerable attention has been paid to optimize protocols for in vitro organogenic and embryogenic differentiation directly from immature embryos and seedling explants.

Most of the recent reports of in vitro plant regeneration deal with somatic embryogenesis, as it is potentially more useful than organogenesis for plant propagation (see Sections 6.8, 6.12) and is proving to be an ideal system to investigate cellular basis of differentiation in higher plants (see Section 6.4). This chapter deals with some aspects of shoot-bud differentiation in vitro. Regeneration of plants via somatic embryogenesis is discussed in Chapter 6. Loss of morphogenic potential in long-term cultures, practical applications of cellular totipotency, and concluding remarks on the subject are considered at the end of Chapter 6.

5.3.1. Factors affecting shoot-bud differentiation

(i) *Chemical factors.* Shoot-bud differentiation in cultured tissues is known since the earliest publications in the field of plant tissue culture. White (1939b) reported the development of shoot buds in tissue cultures of tobacco maintained in liquid medium. In 1944 Skoog confirmed this observation. However, a systematic approach to shoot/root induction in vitro started after Skoog and co-workers (Skoog and Tsui, 1948; Skoog, 1954, 1955; Skoog and Miller, 1957) demonstrated that in tobacco the differentiation of the two organs can be induced more or less at will by manipulation of the balance of IAA and adenine/kinetin in the culture medium (see Fig. 5.6). The first indication of the phenomenon of chemical control of organ formation in tobacco tissue cultures was published in 1948 by Skoog and Tsui. A comprehensive summary of this and subsequent work on the subject by Skoog's group was presented in the classic paper by Skoog and Miller in 1957, and updated by Skoog in 1971. On the basis of their observations, Skoog and Miller (1957) rejected the concept

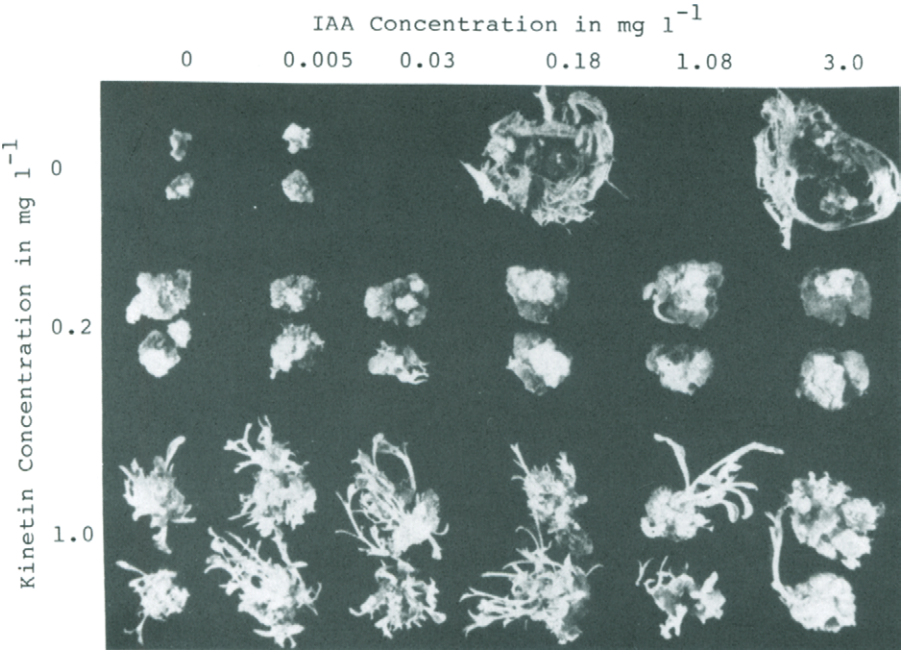

Fig. 5.6. Organogenesis in tobacco ('Wisconsin No. 38') callus. Effect of increasing IAA concentrations at different kinetin levels and in the presence of casein hydrolysate (3 mg l^{-1}) on growth and organ formation in tobacco callus cultured on semi-solid White's medium. Age of cultures: 62 days. Note root formation in the absence of kinetin and in the presence of 0.18–3.0 mg l^{-1} IAA and shoot formation in the presence of 1.0 mg l^{-1} kinetin, particularly with IAA concentration in the range of 0.005–0.18 mg l^{-1} (from Skoog and Miller, 1957).

of specific organ-forming substances (Rhizocalines and Caulocalines) proposed by Went (1938), and regarded organ formation to be determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.

In tobacco the presence of adenine or kinetin in the medium leads to the promotion of shoot bud differentiation and development. Kinetin is 30 000 times more potent than adenine (Skoog, 1971). The shoot-forming effect of adenine and kinetin is modified by other components in the medium, particularly IAA and NAA. Auxins inhibit bud formation. As low as 5 μM IAA is enough to completely suppress the spontaneous differentiation of buds in tobacco. When used in combination with kinetin or adenine, auxin counteracts their bud forming effect. Skoog and Miller (1957) and Skoog (1971) estimated that 15 000 molecules of adenine or 2 molecules of kinetin are required to offset the inhibitory effect of 1 molecule of IAA on shoot-bud differentiation. In combination, a relatively higher concentration of IAA favours cell proliferation and root differen-

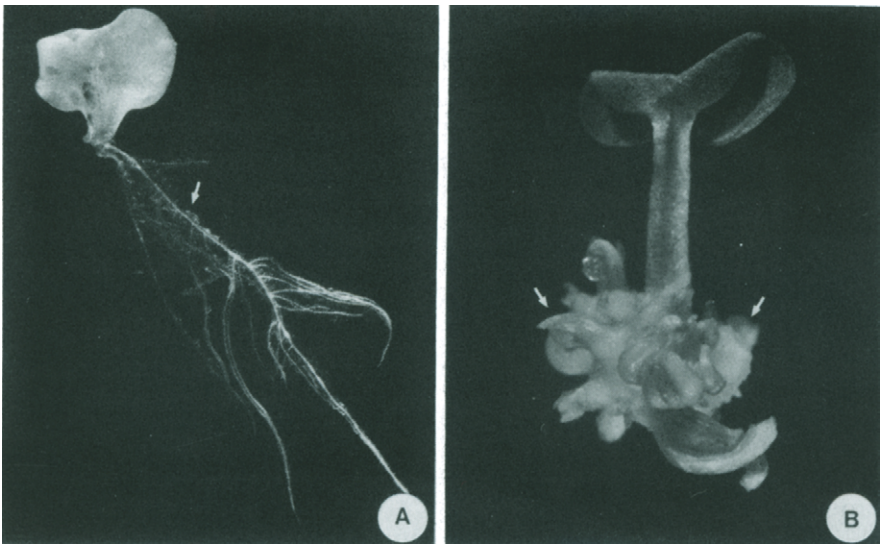

Fig. 5.7. Differentiation of roots and shoots from excised cotyledons of *Brassica juncea*. (A) On MS medium only roots (arrow marked) are formed at the cut end of the petiole. (B) On MS supplemented with 5×10^{-6} BAP multiple shoot buds differentiate from the cut end of the petiole. On this medium the petiole also elongates (after Bhojwani and Sharma, 1989).

tiation whereas relatively higher levels of adenine or kinetin promote bud differentiation. Thus, root-shoot differentiation is a function of quantitative interaction between IAA and kinetin. Despite the elegant demonstration of hormonal control of organ formation in tobacco and its applicability to several other plants, there are exceptions to the qualitative (shoot buds versus roots) differentiation based on exogenous auxin/cytokinin ratio. This may be due to: (i) the degree of cell sensitivity towards growth regulators due to the origin of the explant, (ii) the endogenous levels of active growth regulator molecules, (iii) their uptake, (iv) their degree of glycosylation and hydrolysis, (v) the type of auxin and cytokinin used, (vi) their mode of action or (vii) the activity of auxin and cytokinin oxidases (Tran Thanh Van and Trinh, 1990).

Single-cell tissue clones of *Convolvulus* differentiated shoot buds in complete absence of a growth regulator in the medium (Earle and Torrey, 1965). The addition of either IAA or kinetin promoted bud formation. Whereas IAA was promotive only at a very low level (10^{-7} M), kinetin was so up to a concentration of 10^{-5} M. The highest frequency of bud differentiation occurred with a combination of IAA (10^{-7} M) and kinetin (10^{-5} M).

Unlike tobacco and *Convolvulus*, some tissues are characterized by a complete lack of bud formation in the absence of exogenous growth regu-

lators [*Scurrula pulverulenta* (Bhojwani and Johri, 1970); *Dendrophthoe falcata* (Nag and Johri, 1971); *Taxillus vestitus* (Nag and Johri, 1971); *Lactuca sativa* (Doerschug and Miller, 1967); *Brassica juncea* (Sharma et al., 1990)]. In cotyledon cultures of *B. juncea* BAP alone induced shoot bud differentiation from the petiolar cut end; in the absence of BAP or any other hormone only roots were formed at the same site (Fig. 5.7). In some other systems a cytokinin is effective for shoot bud induction only in the presence of an auxin (Doerschug and Miller, 1967) or adenine (Nitsch and Nitsch, 1967).

Besides kinetin, several other cytokinins, viz., BAP, 2ip, SD 8339, thidiazuron and zeatin have been tested for shoot-bud induction in tissue cultures. Of these, BAP has proved most effective and has been used most widely. The number of vegetative buds per thin cell layer explants of tobacco was five times greater in the presence of CPU (a urea derivative cytokinin related to thidiazuron) than with kinetin (Tran Thanh Van and Trinh, 1990).

In most of the cereals, callus tissue exhibits organogenesis when it is transferred from a medium containing 2,4-D to a medium lacking it or having IAA or NAA in its place. Whether the tissue would form shoots or roots, however, depends on the innate capacity of the tissue; an effective exogenous control to stimulate shoot-bud formation selectively is almost unknown. Indeed, in these plants root formation is more common than shoot-bud differentiation. A two-step process of organogenic differentiation also occurs in alfalfa (Saunders and Bingham, 1972; Walker et al., 1978, 1979). Callus is initiated and multiplied on a medium containing 2,4-D and kinetin ('induction medium'). Organogenesis occurs when pieces of tissue from such calli are transferred to a hormone-free medium ('regeneration medium'). Unlike cereals, in alfalfa (*Medicago sativa*) the type of organ formed in the regeneration medium can be controlled by manipulating the ratio of the two hormones in the induction medium; a higher 2,4-D to kinetin ratio favours shoot formation, whereas a higher kinetin to 2,4-D ratio supports root differentiation (Walker et al., 1979). The hormone ratio in the induction medium during the last 4 days is critical in determining the nature of the organ formed.

Gibberellin inhibits shoot-bud differentiation in tobacco (Murashige, 1961, 1964; Thorpe and Murashige, 1970), *Plumbago indica* (Nitsch and Nitsch, 1967), *Begonia* (Heide, 1969) and rice (Maeda, 1978). In tobacco, exposure of the differentiating callus, in the dark, to GA_3 for a period as short as 30–60 min reduced shoot bud differentiation (Thorpe and Meier, 1973) and after 48 h of GA_3 treatment no meristemoids or shoot buds were left (Thorpe and Meier, 1973, 1975). Gibberellin was most effective

at the stage of meristemoid formation. Once shoot buds were formed GA_3 did not inhibit their further development. Complete inhibition by GA_3 occurred only in the dark (Thorpe and Meier, 1973).

Tobacco tissue contains gibberellin-like substances (Lance et al., 1976b) and is capable of metabolizing exogenous gibberellin (Lance et al., 1976a). Thorpe (1978) has suggested the involvement of gibberellin in bud formation in tobacco. The inhibition by exogenous gibberellin is probably because the tissue synthesizes the hormone in quantities optimal for the organogenic process. According to this theory, in *Chrysanthemum* (Earle and Langhans, 1974a) and *Arabidopsis* (Negrutiu et al., 1978a,b), where application of gibberellin promotes budding, the endogenous level of the hormone must be suboptimal. On the other hand, in sweet potato the promotion of bud formation by abscisic acid may be explained on the basis of supraoptimal quantities of endogenous gibberellins (Yamaguchi and Nakajima, 1974). At a non-toxic level (10^{-6} M) abscisic acid partially overcomes the repression of bud formation induced by GA_3 in tobacco (Thorpe and Meier, 1973).

Kumar et al. (1987) examined the role of ethylene in shoot bud differentiation in cotyledon cultures of *Pinus radiata*. The shoot forming explants produced considerable amounts of ethylene and carbon dioxide, and the frequency of shoot bud formation could be correlated with the concentration of the two gases inside the culture vial. Maximum number of buds per explant were formed when the flask contained 5–8 μl l^{-1} of C_2H_4 and 10% CO_2 in the head space, during the first 15 days of culture. Removal of these gases from the culture vessel completely stopped organogenesis. On the other hand, excessive accumulation of the gases beyond 15 days caused partial dedifferentiation of shoot buds.

In some of the recalcitrant tissues shoot formation could be induced or promoted by applying unconventional substances such as abscisic acid (Yamaguchi and Nakajima, 1974; Shepard, 1980), 2,3,5-triiodobenzoic acid (0.02 μM; Cassells, 1979), kanamycin (2.5–20 μM; Owens, 1979), and auxin synthesis inhibitors (0.01–0.1 mg l^{-1} 7-aza-indole or 5-hydroxy nitrobenzylbromide; Kochba and Spiegel-Roy, 1977a). Direct and rapid (within 2 weeks) formation of shoot buds in thin cell layer explants of *Beta vulgaris* was induced by TIBA (Tran Thanh Van and Trinh, 1990). Addition of raw powder of *Panax pseudo-ginseng* or *Panax ginseng* to culture medium, in addition to auxin and cytokinin, doubled the regeneration frequency in the cultures of cotyledons and hypocotyl of *Brassica oleracea* ssp. *italica* (Hui and Zee, 1980). Virtually all our knowledge of the role of phytohormones in differentiation is based on in vitro studies involving manipulation of exogenously applied hormones. It poses a fundamental question: Whether the exogenous phytohormones act directly

on the target cells to induce organogenic differentiation or indirectly by setting up conditions which allow some intrinsic programme to be initiated (Torrey, 1966). Transformation of plant cells with cloned T-DNA genes specific for the synthesis of auxin (*iaah* and *iaam*) or cytokinin (*ipt*) enhance the endogenous level of the respective hormone, which is associated with root/shoot differentiation in a manner similar to the effect of exogenous auxins and cytokinins (Owens and Smigocki, 1990). Furthermore, exogenous hormones can reverse the T-DNA induced morphogenesis, suggesting that the hormones play direct role in organogenesis (Inze et al., 1984).

(ii) *Electrical stimulation.* Organogenic and embryogenic (see Section 6.3.7) differentiation in tissue cultures can be markedly enhanced by the application of weak electric current. Rathore and Goldsworthy (1985a) reported 70% increase in tobacco callus growth by the application of weak electric current (1 μA) in such a way that the callus was made negative and the medium positive. This stimulation occurred only on IAA-containing medium (Rathore and Goldsworthy, 1985b). On the callusing medium, which normally does not favour any caulogenesis, some greening and shoot bud differentiation occurred after the electric treatment. On shoot differentiation medium, the application of microampere current to the callus caused 5-fold stimulation of shoot bud differentiation as compared to the control (Rathore and Goldsworthy, 1985c). The shoot buds first appeared in the most negative region of the callus irrespective of the polarity of the current. The electric stimulation of caulogenesis affected both the number of cultures forming shoot buds and the number of buds per culture.

The callus derived from mature embryos of wheat, which only formed roots, was induced to form several shoots by exposure to electrical treatments (Rathore and Goldsworthy, 1985c).

(iii) *Explant.* Whereas in some plants, such as tobacco, almost all parts are amenable to in vitro plant regeneration, in others this potential is restricted to only certain tissues. In plants where different explants respond, some may be more regenerative than the others. In *Crotalaria juncea* (Ramawat et al., 1977) and *Glycine* (Kameya and Widholm, 1981) the hypocotyl exhibits higher potentiality for shoot formation than the root segments. Similarly, in *Lactuca sativa* (Doerschug and Miller, 1967) and *B. juncea* (Sharma, 1987) cotyledon was the best explant for plant regeneration.

The regenerability of an explant is influenced by several factors, such as the organ from which it is derived, the physiological state of the ex-

plant and its size. Orientation of the explant on the medium and the inoculation density may also affect shoot bud differentiation.

The physiological status of an explant is affected by the age of the donor plant which has a direct bearing on the regenerability of the explant. The use of young and meristematic tissues has, in many cases, enabled raising of regenerative cultures when mature and differentiated explants failed to show such a response. This is especially true for cereals and tree species. Wernicke and Brettell (1980) demonstrated that in *Sorghum bicolor* the regeneration capacity is restricted to the two youngest leaves and the basal part of the third leaf. The number of shoots per culture and the percent cultures of tissue peels from hypocotyl of *Psophocarpus tetragonolobus* showed a decline with increasing age of the seedlings. In *Brassica juncea* 5-day-old seedlings provided most regenerative cotyledons (Sharma et al., 1990). The cotyledons from seedlings older than 10 days did not form shoots at all. In *Pinus radiata* the cotyledons lose the potential to form adventitious shoot buds 3 days after germination which coincides with the complete depletion of lipid in the cells of the cotyledons. In contrast, in *P. gerardiana* the cotyledons derived from ungerminated seeds show higher potential to form shoot buds (Banerji and Bhojwani, unpublished).

Preparation of explants is also important. In cotyledon cultures of *B. juncea*, shoot buds or roots, depending on the culture medium, are formed only at the cut end of the petiole (Fig. 5.7). The lamina lacks this potential. However, the presence of laminar tissue is essential for the petioler cells to exhibit totipotency. Therefore, the ideal explant to achieve regeneration is the lamina together with a short (1 mm) petiole. This is also true for *B. oleracea* (Lazzeri and Dunwell, 1986; Horeau et al., 1988).

Orientation of the explant on the medium proved to be a critical factor for organogenic differentiation in cotyledon cultures of *B. juncea* (Sharma et al., 1990). Planting the cotyledons with their abaxial surface in contact with the medium and the petiolar cut end embedded in the medium gave best response. The explants in which, due to expansion and curling of the lamina, the petiole lost contact with the medium within 3–5 days after culture failed to form roots or shoots. Similarly, the frequency of shoot formation in the cultures of thin layer explants of *B. napus* when epidermis was in contact with the medium was only 13% as against 40% regeneration when the cortical cells were touching the medium (Klimaszewska and Keller, 1985). In *Cunninghamia lanceolata* the explants plated horizontally on the medium produced three times more shoots than those planted vertically (Bigot and Engelmann, 1987).

(iv) *Genotype*: Plant regeneration was once thought to be primarily dependent on the concentration of phytohormones in the medium (Skoog and Miller, 1957). However, it is now well established that for in vitro differentiation the genotype of the plant plays an equally, if not more, critical role as the growth regulators. Indeed the success in obtaining regeneration in leguminous species, once regarded as a recalcitrant group (Bhojwani et al., 1977a), has been mainly due to shift in the emphasis from media selection to genotype selection (Bhojwani and Mukhopadhyay, 1986).

Genotype specificity to regeneration has been reported in a number of plants. Genetic variation for regeneration occurs between varieties and, in outbreeding species, even within varieties. Different cultivars of alfalfa exhibited variation in regeneration capacity when subjected to the same culture regime (Saunders and Bingham, 1972). In tomato intervarietal differences were observed regarding the percentage of rhizogenesis, ability to regenerate shoots and the number of shoots regenerated (Padmanabhan et al., 1974; Kurtz and Lineberger, 1983).

Dietert et al. (1982) reported that in *Brassica* species of the U's triangle inter-cultivar differences for organogenic potentiality were as great as inter-species variation. Intraspecific variation for regeneration in tissue cultures of *B. oleracea* was also observed by Lazzeri and Dunwell (1984), Murata and Orton (1987) and Horeau et al. (1988).

An overall survey of the literature reveals that among the three monogenomic species of the U's triangle of *Brassica* (U, 1935), *B. oleracea* (CC) is the most regenerative and *B. campestris* (AA) the least (Lazzeri and Dunwell, 1984; Chopra et al., 1986; Glimelius and Ottosson, 1983; Narasimhulu and Chopra, 1988; Jourdan and Earle, 1989). Glimelius and Ottosson (1983) cultured the protoplasts of *B. campestris*, *B. juncea*, *B. napus* and *B. oleracea* and succeeded in obtaining calli in all species but regeneration of shoots occurred only in *B. oleracea* and *B. napus*. Similar results were reported by Lu et al. (1982). It seems regeneration genes in *B. napus* have been contributed by *B. oleracea* (Murata and Orton, 1987; Narasimhulu and Chopra, 1988; Jourdan and Earle, 1989). This argument is supported by the fact that all the seedling explants of *B. carinata*, the other amphidiploid species which has received *B. oleracea* genome, have shown regeneration of shoots (Jaiswal et al., 1987), unlike *B. juncea* (George and Rao, 1980; Sharma, 1987) which is the amphidiploid species between *B. nigra* and *B. campestris*.

The studies of Jourdan et al. (1985), Robertson and Earle (1986) and Jourdan and Earle (1989) suggest that both nuclear and cytoplasmic genes affect the frequency of plant regeneration in protoplast cultures

of *B. oleracea*. The alloplasmic lines of *B. oleracea* with Ogura R_1 male sterile cytoplasm show an overall lower regeneration than those with normal *Brassica* cytoplasm (Jourdan and Earle, 1989). Narasimhulu et al. (1988) noted significant cytoplasmic influence on regenerability in *B. carinata* synthesized from reciprocal crosses between *B. oleracea* and *B. nigra*.

Several workers have independently demonstrated that regeneration in wheat is genetically controlled. Rode et al. (1988) suggested that mitochondrial genes are involved in differentiation. However, Mathias and Fukui (1986) have shown that a specific chromosome, whose substitution greatly reduces the capacity of the cultures to regenerate, controls morphogenesis in vitro. According to Galiba et al. (1986) regeneration is controlled primarily by the genes on 7B, 7D and 1D chromosomes but genes on some other chromosomes may also be involved. If the genes controlling regeneration are identified and mapped then there is the exciting possibility of the transfer of such genes to recalcitrant species.

The genotypically selected regenerating lines do not exhibit stringent culture requirements and display regeneration ability on a wide range of media (Keyes et al., 1980; Bhojwani and White, 1982; Kurtz and Lineberger, 1983).

(v) *Physical factors*. White (1939b) reported that in a solid medium the tissue cultures of *Nicotiana glauca* × *N. langsdorffii* grew in a completely unorganized state, but in the liquid medium of otherwise identical composition it formed leafy shoot buds. This report was confirmed by Skoog (1944), but Dougall and Shimbayashi (1960) observed extensive bud formation in tobacco cultures grown on medium solidified with 1% agar and negligible bud differentiation in tissues grown on the surface of liquid medium of the same composition. A striking alteration in the morphogenic pattern with change in agar concentration in the medium occurred in thin tissue peels of tobacco (Tran Thanh Van and Trinh, 1978). With 1% agar only flowers were formed. With lowering of agar concentration the frequency of flower formation dropped and vegetative bud differentiation occurred. In liquid medium the tissue exhibited only callusing and vegetative bud formation.

For shoot differentiation in callus cultures derived from mesophyll protoplasts of a dihaploid cultivar of potato it was essential to maintain the osmotic pressure between 200 and 400 mmol by adding 0.2–0.3 M mannitol. Without this, the calli did not exhibit greening which must precede shoot initiation (Shepard et al., 1980). In rice callus cultures, enhanced osmolarity of the medium, achieved by the addition of NaCl (1.5%) (Binh and Heszky, 1990) or sorbitol or mannitol (3%) (Kavi Kishore and Reddy,

1986), not only improved the frequency of regeneration in primary cultures but also helped maintaining it in long-term cultures (see Section 6.11). Even dehydration of rice callus by placing it on dry filter paper inside a sealed petriplate promoted regeneration frequency (Tsukahara and Hirosawa, 1992). Under the experimental conditions the water content of callus dropped around 50% within 1 h and remained relatively constant thereafter. However, the regeneration frequency peaked (45% as compared to 5% in the untreated control) after 24 h of dehydration treatment.

High light intensity has been shown to be inhibitory for shoot-bud formation in tobacco (Skoog, 1944; Thorpe and Murashige, 1970). Callus of *Pelargonium hortorum* differentiated shoots only under alternating light and dark periods (15–16 h day proved best). Callus maintained under continuous light remained whitish and did not exhibit organogenesis (Pillai, 1968). The quality of light also influences organogenic differentiation (Weis and Jaffe, 1969; Bagga et al., 1985). Blue light promoted shoot-bud differentiation whereas red light stimulated rooting in tobacco (Letouze and Beauchesne; cited in Narayanaswamy, 1977). The observations of Bagga et al. (1985) suggest the involvement of phytochrome in shoot induction. Calli of *Brassica oleracea* grown in dark for 20 days formed shoot buds 12 days after transfer to light while those shifted to light after 12 days of growth in dark differentiated shoots within 9 days. The calli given red light treatment for 5 min followed by 24 h of dark for 5 continuous days produced shoots within 2 days of growth in light, and the response was much more intense. Infrared radiation nullified the effect of red light.

Skoog (1944) studied the effect of a range of temperature (5–33°C) on tobacco callus growth and differentiation. Growth of the callus increased with rise in temperature up to 33°C, but for shoot-bud differentiation 18°C was optimum; no bud formation occurred at 33°C. Shoot-bud initiation in the cultures of hypocotyl segments of *Linum usitatissimum* is, however, better at higher temperatures (30°C) (Murray et al., 1977).

5.3.2. Induction of organogenic differentiation

Organogenic differentiation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, is a multistep process. A series of intracellular events, collectively called induction, occur before the appearance of a morphologically recognisable organ. The cells induced to form a specific organ in the presence of appropriate hormonal combination would continue to develop into that organ even if the inductive hormones are removed. Thus, induction leads to irreversible com-

mitment of cells to follow a particular developmental pathway. For example, in cotyledon cultures of *Brassica juncea* BAP induces shoot bud differentiation at the cut end of the petiole, and in the absence of BAP only roots are formed at the same site (Fig. 5.7) (Sharma et al., 1990). The cotyledons transferred to basal medium after 11 days of incubation on BAP-containing medium form only shoots and no roots. Similarly, the cotyledons lose the potentiality to form shoots on BAP-medium if they are pre-cultured on BAP-free medium for longer than 7 days.

Leaf explants of *Convolvulus arvensis* form only shoots on MS + 7 mg l^{-1} 2ip + 0.05 mg l^{-1} IAA (SIM), only roots on MS + 12 mg l^{-1} IBA (RIM) and only callus on MS + 0.3 mg l^{-1} kinetin + 3 mg l^{-1} IAA (CIM). Root or shoot bud formation is preceded by slight callusing. The leaf explants are induced to form shoots on SIM within 10–14 days and after this period the destiny of the cells is not changed even if the leaf pieces are transferred to RIM or CIM (Christianson and Warnick, 1983, 1984). The induction process involves two major steps. During the first 3–5 days on the induction medium the cells acquire competence to respond to the inductive conditions (Fig. 5.8). At this stage the cells are plastic in terms of their morphogenic potential and can form roots or shoots depending on the medium to which they are exposed. Under the continued action of SIM the competent cells become committed to form shoots. This irreversible commitment of the cells, which is achieved after 10–12 days on SIM is referred to as determination. The competence to respond to SIM can be acquired even on RIM or CIM. Interestingly, some of the genotypes of *C. arvensis* which exhibited poor or no shoot formation when directly cultured on SIM could be induced to form large number of shoots by pre-culture on RIM for 3–5 days followed by transfer to SIM (Christianson and Warnick, 1985). Probably these genotypes were blocked in the acquisition of competence to respond to SIM which could be achieved on RIM. Christianson and Warnick (1984) have demonstrated that the period between the acquisition of competence and the determination is composed of several sub-stages sensitive to various substances. It includes a stage sensitive to salicylate, followed by a stage sensitive to TIBA, which is followed, in turn, by a stage sensitive to sorbitol. However, the significance of these sub-stages is not clear.

5.3.3. Ontogeny of shoot buds

Under the conditions favouring unorganized growth, the meristems in a callus are random and scattered. Transfer of the tissue to conditions supporting organized growth leads first to the appearance of localized clusters of cambium-like cells. These meristemoids (also termed 'nodules'

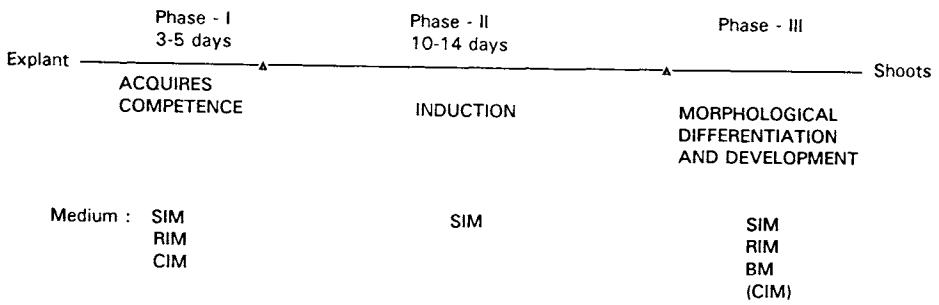

Fig. 5.8. Scheme of events in the process of in vitro shoot differentiation from leaf discs of *Convolvulus arvensis*. The cells acquire competence for shoot bud induction after 3–5 days of culture on shoot induction medium (SIM), root induction medium (RIM) or callus induction medium (CIM). The competent cells become determined to form shoot if maintained on SIM for another 10–14 days. Thereafter the cells continue to develop into shoot, irrespective of the culture medium. If SIM is replaced by RIM during the induction period the cells would form roots instead of shoots. BM, basal medium (adapted from Christianson and Warnick, 1984, 1985).

or 'growing centres'), which may become vascularized due to the appearance of tracheidal cells in the centre, are the site for organ formation in the callus (Ross et al., 1973). Initially the meristemoids are plastic, and can form roots or shoots (Torrey, 1966). Generally roots appear endogenously, whereas shoots originate exogenously. However, there are several examples of endogenous differentiation of shoots (Buvat, 1944; Bonnett and Torrey, 1966).

Steward et al. (1958) described the histological development of whole plants from carrot tissue cultures. in suspension cultures nodules with a central nest of tracheidal elements surrounded by cambium-like cells are formed. This is followed by the development of roots from the central cells of the nodules. When such rooted nodules are transferred to a semi-solid medium a shoot bud appears at another locus far removed from the root. Gradually a vascular connection develops between the root and shoot and a whole plant is formed. A somewhat different pattern of plant regeneration from cell colonies of *Convolvulus* was reported by Earle and Torrey (1965b). These authors emphasized that shoot-bud differentiation occurred independent of vascular tissue; buds often developed in the nodules completely lacking mature vascular elements (see also Torrey, 1966).

Excised cotyledons of *Pinus radiata* cultured on BAP-free medium rapidly elongate and the anticlinal divisions which occur throughout the explant at the time of culture completely cease after 2 days in culture (Villalobos et al., 1985). However, in the presence of BAP the cotyledons do not elongate and cell divisions become restricted to the epidermal and sub-epidermal cell layers on the side in contact with the medium. Six to

eight celled organised 'promeristemoids', arising from a single subepidermal cell as a result of both anticlinal and periclinal division, can be seen by day 5. The cells within each promeristemoid are tightly packed together with little or no intercellular spaces but prominent plasmodesmata are present within each promeristemoid. By day 10 the cotyledon surface becomes nodular due to the development of promeristemoids into meristemoids which give rise to shoot primordia by day 21. A similar pattern of shoot bud differentiation also occurs in cotyledon cultures of *P. gerardiana* (Banerji and Bhojwani, unpublished).

Thorpe and Murashige (1970) examined histochemically the changing status of nucleic acid, protein and carbohydrate in differentiating and non-differentiating calli of tobacco. The two tissues did not exhibit much difference in the level of DNA per cell, but RNA and protein contents were higher in the shoot-forming regions of the calli. The difference in the starch content of the two types of tissues was especially remarkable. The intracellular accumulation of starch has been ascribed a positive role in the process of shoot-bud differentiation. This conclusion is based on the following observations: (a) heavy accumulation of starch occurs only in the shoot-forming tissues; (b) no meristemoids are formed in the regions lacking heavy deposition of starch; (c) the accumulation of starch precedes any observable organized development and reaches the maximum level in 11-day-old cultures, which is 3 days before the appearance of meristemoids and shoot formation; and (d) gibberellin, which inhibits shoot formation, prevents starch accumulation reaching a threshold level required for shoot-bud differentiation by decreasing starch synthesis and increasing starch degradation (Thorpe and Meier, 1975). It has been suggested that starch, together with the free sugars in the medium, may be serving as the source of energy during meristemoid and shoot-bud differentiation, which are high energy-requiring processes. Similar conclusions are drawn from the biochemical studies of shoot bud differentiation from excised cotyledons of *Pinus radiata* (Thorpe, 1990, 1993). The shoot forming layer of the cotyledons showed elevated level of respiration, increased concentration of several enzymes, including acid phosphatase, ATPase, and succinate dehydrogenase (Patel and Thorpe, 1984), enhanced amino acid synthesis, and depletion of lipids and soluble sugars (Biondi and Thorpe, 1982).

5.3.4. Totipotency of epidermal cells

Flax (*Linum usitatissimum*) is the classic example of shoot-bud development from the intact hypocotyl (Crooke, 1933; Link and Eggers, 1946). The origin of these buds has been ascribed to single epidermal cells (Link

and Eggers, 1946). Other species reported to form shoot buds/embryos from superficial cell layers of stem in cultures are *Ranunculus sceleratus* (Konar and Nataraja, 1965), *Daucus carota* (Kato and Takeuchi, 1966; Kato, 1968), *Exocarpus cupressiformis* (Bhojwani, 1969a), *Torenia fournieri* (Bajaj, 1972; Chlyah, 1974). *Nicotiana tabacum* (Tran Thanh Van, 1973a,b) and *Brassica napus* (Thomas et al., 1976).

In very young seedlings of flax, epidermal cells all along the length of the hypocotyl are capable of forming shoot buds, but in the seedlings older than 15 days this potential is restricted to the basal half of the hypocotyl. In an intact seedling, decapitation results in the development of numerous buds from the hypocotyl, but only one of them grows into a full shoot (Link and Eggers, 1946). On the other hand, in cultures a 15 mm long hypocotyl segment may develop 160–170 potential plants (Murray et al., 1977). In cultures some shoots also develop from sub-epidermal cells.

Exclusively epidermal peels generally do not survive in culture (Chlyah et al., 1975) or give a poor response (Tran Thanh Van and Trinh, 1978). Nevertheless, in the cultures of thin superficial peels (one to seven layers) from stem and leaf the epidermal cells divide and elicit their totipotency (Kato, 1968; Tran Thanh Van and Trinh, 1990). It is interesting that in the cultures of thin surface peels from stem, epidermal cells can be induced to develop directly into a root or a shoot, or even a fertile flower (see Fig. 5.9) at will (Tran Thanh Van, 1973a,b; Tran Thanh Van et al., 1974a; Tran Thanh Van and Trinh, 1990). The advantages of such a system are: (a) it may allow direct observation of the changes in a single cell leading to different types of organogenic differentiation, and (b) since the explant lacks vascular tissue and cambium, and the amount of other parental tissues is reduced to a bare minimum, it carries very little or no influence of endogenous growth substances.

The type of buds formed in the cultures of thin tissue peels taken from the stem of a flowering plant of *Nicotiana tabacum* varies with the region of the stem from which the peel is derived (see Fig. 5.10) (Tran Thanh Van, 1973b). Under certain culture conditions the peels from floral branches produce only flower buds, while those from the basal part of the plant form only vegetative buds. The explants derived from the middle portion bear both types of buds in different proportions, depending on their distance from the base of the plant. Within the inflorescence, peels from the basal region give a better response compared to those from the terminal portion. The number of peels from basal portions of the inflorescence that form flower buds is also influenced by the physiological stage of the donor plant. For example, 100% of the tissue pieces from plants bearing green fruits exhibited flower formation. If the donor plant had reached mature fruit stage the response was slightly poor (85% explants

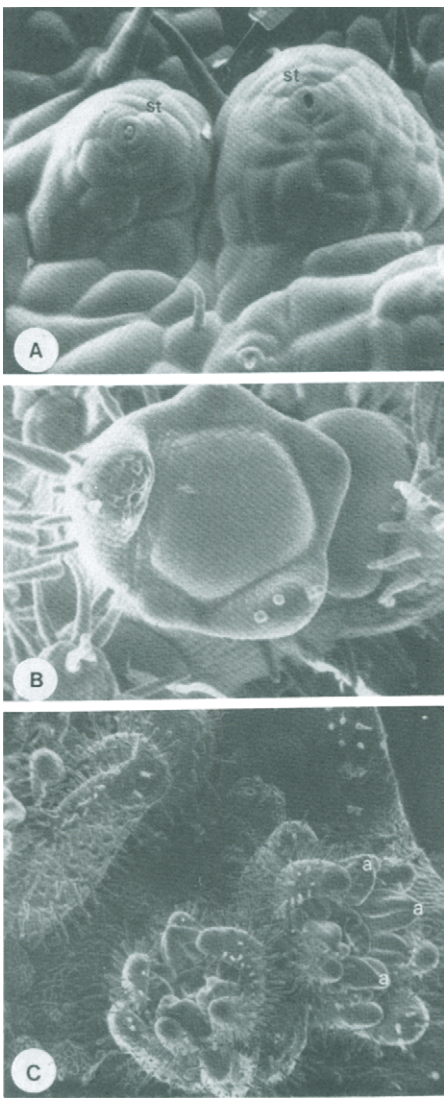

Fig. 5.9. Scanning electron micrographs to show direct differentiation of floral buds from somatic cells in the cultures of thin cell layers of *Nicotiana tabacum*. (A) Some protuberances can be seen just under stomata (st). (B) Sepal primordia have differentiated. (C) Direct and de novo formation of flowers 10–18 days after culture (a, anther) (after Tran Thanh Van, 1977).

formed buds). No flower buds were formed if the parent plant bore only flowers but no fruit.

The peels from floral branches of tobacco formed flower buds only if the medium contained kinetin and IAA at an equimolar concentration of 10^{-6} M (the absolute concentration of the hormones is important, not

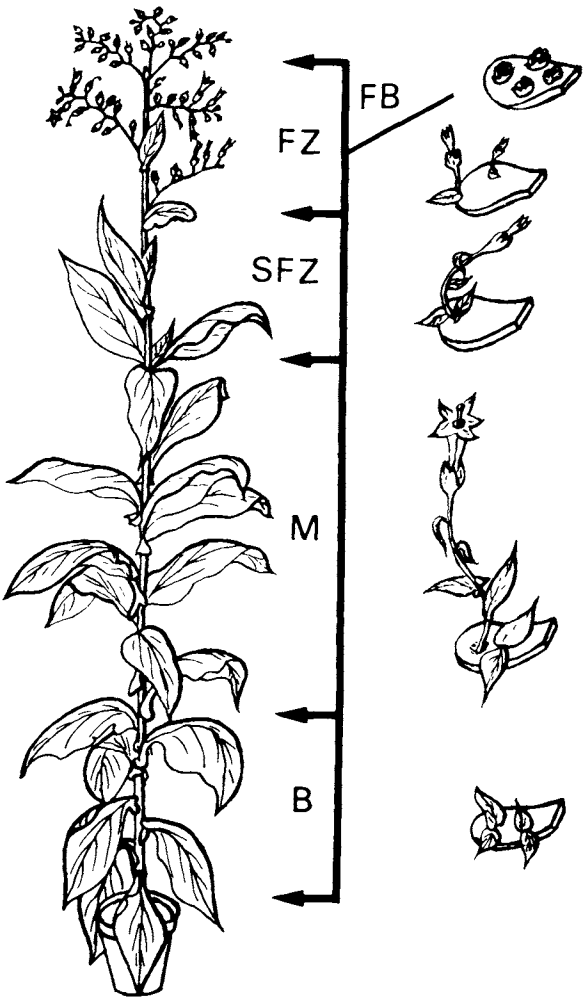

Fig. 5.10. Diagram showing capacity of thin cell layers excised from different levels of a flowering plant of tobacco to form vegetative or floral bud in culture. B, base; M, middle zone; SFZ, sub-floral zone; FZ, floral zone; FB, floral branches) (after Tran Thanh Van, 1973b).

their ratio of 1:1) together with 2–3% sucrose. An increase in kinetin concentration to 10^{-5} M without affecting the IAA concentration completely suppressed flower bud formation; instead vegetative buds appeared. With further alteration of the hormonal balance in the medium it was possible to shift the morphogenesis in favour of rooting or callusing (see Table 5.2). This is another good example of hormonal control of organ differentiation. Under ideal conditions 7–30 flower buds developed on a single explant measuring 1 × 0.4 cm.

TABLE 5.2

Optimal conditions for different types of differentiation in small explants from floral branches of tobacco[a]

Type of neoformation	Glucose ($g\ l^{-1}$)	Growth substances	Auxin/ cytokinin ratio	Other favourable conditions
Floral buds	30	IBA 10^{-6} M Kinetin 10^{-6} M	1.0	Light; terminal bud in green fruit stage
Vegetative buds	30	IBA 10^{-6} M Kinetin 10^{-5} M	0.1	Light
Roots	10	IBA 10^{-5} M Kinetin 10^{-7} M	100.0	Darkness; terminal bud in mature fruit stage
Callus	30	IBA 3×10^{-6} M Kinetin 10^{-7} M	50.0	–

[a]After Tran Thanh Van et al. (1974b) and Tran Thanh Van and Trinh (1990).

The flowers formed in cultures of thin tissue peels were normal; they formed viable gametes and set fertile seeds. Interestingly, epidermal peels from even male sterile plants of tobacco developed several fertile flowers (Tran Thanh Van and Trinh, 1978). Androgenic plants could be raised by culturing anthers from such flowers. By taking epidermal peels from these dihaploids ($2n = 24$) and culturing the anthers from flowers produced from them, and repeating this cycle once more, Tran Thanh Van (1977) could obtain some hypohaploids with less than six chromosomes (see Fig. 5.11). This could not be achieved with seed-grown plants.

To date controlled organogenesis in cultured thin-layer peels has been achieved with *Nautilocalyx lynchei* (Tran Thanh Van, 1973a), *Cichorium intybus* (Nguyen, 1975), *Nicotiana tabacum* (Tran Thanh Van, 1973a,b; Tran Thanh Van et al., 1974b), *Sesbania* (Tran Thanh Van and Trinh, 1990), *Torenia fournieri* (Chlyah, 1974), and *Bryophyllum daigremontianum* (Bigot, 1976).

5.3.5. Totipotency of crown-gall cells

A typical crown-gall tumour cell is characterized, both in the host and

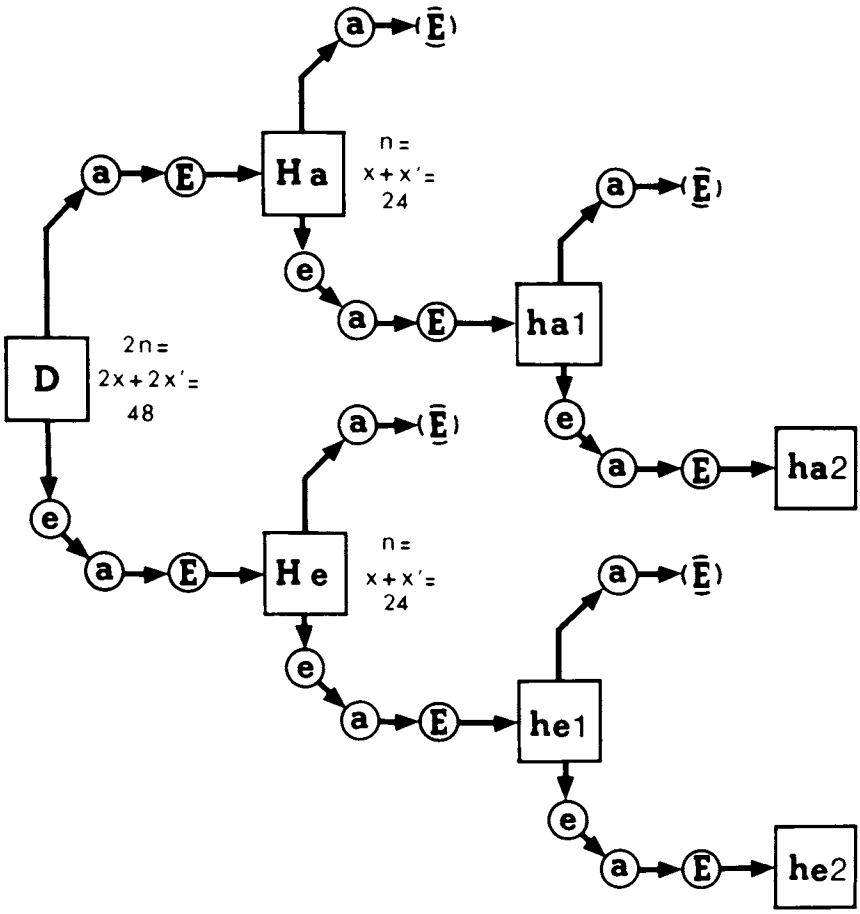

Fig. 5.11. Diagrammatic summary of androgenic embryo (E) development in the cultures of anthers (a) from flower buds formed normally on the plant and those differentiated in the cultures of thin cell layers (e). Anthers from parent plants do not form pollen embryos (E) beyond the dihaploid stage ($n = 2x = 24$) whereas those from the flowers differentiated directly from thin cell layers yield haploids (ha 1, he 1) and hypohaploids (ha 2, he 2) (after Tran Thanh Van and Trinh, 1978).

in culture, by a capacity for unlimited growth independent of exogenous hormones. It shows a complete lack of organogenic differentiation and is, therefore, considered to have permanently lost the totipotentiality of the parent cells.

In some plant species the crown-gall bacterium (*Agrobacterium tumefaciens*) induces a special type of tumour, called teratoma, the cells of which possess a pronounced capacity to differentiate shoot buds and leaves when they are in the host tissue or grown in cultures for unlimited

periods. However, the shoots formed by these cells are abnormal morphologically and in their growth form. In tobacco completely normal shoots could be recovered from teratoma shoots (Braun, 1959; Braun and Wood, 1976). Braun (1959) cultured pieces of crown-gall teratoma tissues in shake cultures, picked up individual cells from them and raised single-cell tissue clones using the nurse-culture technique of Muir et al. (1954). Before the calli started to differentiate shoot-buds pieces of tissue were taken from them and grafted onto the cut ends of stems of healthy tobacco plants from which axillary buds had been removed. In successful grafts the tumour tissue developed some highly abnormal shoot buds. Tips (3–5 mm long) of the teratoma shoots from the grafts were excised and grafted onto another healthy plant. Through a series of similar grafts of tips of teratoma shoots onto normal plants, Braun (1959) obtained actively growing shoots which appeared structurally, histologically, and functionally normal (Braun and Wood, 1976). The recovery, although complete and persistent, was not irreversible. It was a case of suppression of the neoplastic condition of the cells. If the restraints of organization were removed, and pieces of sporophytic tissues from vegetative or reproductive organs of the recovered shoots cultured, they reverted to tumour characteristics, such as auxin autonomy (Braun and Wood, 1976). Nevertheless, somatic tissues of plants raised from seeds formed by the recovered teratoma shoots did not revert back to the neoplastic growth (Turgeon et al., 1976). Even the androgenic haploids obtained by culturing anthers from the recovered shoots exhibited permanent and irreversible loss of the neoplastic condition, suggesting that the stable recovery occurred during the meiotic process (Turgeon et al., 1976). Yang et al. (1980) demonstrated that the recovered plants looking normal but still exhibiting tumorous trait when recultured carried the T-DNA, whereas the F_1 plants, which had completely lost the tumour trait, lacked it.