University of Bath

PHD

# In vitro plant regeneration studies with Capsicum annuum

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# IN VITRO PLANT REGENERATION STUDIES WITH CAPSICUM ANNUUM

Andreas G. Kanakis

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*IN VITRO* PLANT REGENERATION STUDIES

WITH *CAPSICUM ANNUUM*

submitted by *Andreas G. Kanakis*

for the degree of Ph.D.

of the University of Bath

1987

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Andreas G. Kanakis

*SUMMARY*

Cotyledon and hypocotyl explants from dry seeds, immature fruit and seedlings; young leaf, lamina discs and leaf petiole explants from expanded leaves; floral organs; petiole of cotyledon and whole immature embryo explants from various cultivars of *Capsicum annuum* were tested in various media for their morphogenic competence.
Stem segments, floral organs and lamina discs of expanded immature leaves cultured on media containing an auxin (2,4-D, NAA) or a cytokinin (BAP, zeatin), either alone or in combination, did not show any morphogenic response other than callus formation.
Nodes subjected to MS or B5 medium containing 3 and 5 mg $l^{-1}$ BAP or zeatin and 1.0 mg $l^{-1}$ GA underwent only normal development of their axillary buds to produce expanded shoots.
Cotyledon, hypocotyl, petiole of cotyledon and shoot-tip explants subjected to MS or B5 medium containing a cytokinin (BAP, zeatin, zeatin riboside and kinetin) alone at concentrations between 1.0 and 10.0 mg $l^{-1}$ or in combination with a low IAA concentration ($<1.75$ mg $l^{-1}$) formed adventitious shoots at a high frequency.
The same morphogenic response was obtained from young leaf explants cultured on a cytokinin containing medium but at a lower frequency.
The adventitious shoot-regeneration process was highly dependent upon the presence of casaminoacids sucrose concentration, developmental stage of the explant, the type of the explant and genotype.
The development of adventitious buds into shoots was strongly influenced by the GA concentration.
Adventitious shoots formed roots on full or half strength MS or B5 hormone-free medium or the same medium supplemented with IBA or NAA

at concentrations up to 0.1 mg $l^{-1}$.

*In vitro* somatic embryogenesis took place only from intact immature zygotic embryos subjected initially to MS medium containing 1.0 - 5.0 mg $l^{-1}$ 2,4-D and subcultured on MS hormone-free medium or medium supplemented with low auxin low cytokinin concentration. The process was influenced slightly by l-proline and strongly by the pH, activated charcoal and the genotype.

Anatomical and morphological observations showed that:

a) Adventitious shoot regeneration took place mostly from the abaxial surface of the cotyledon and young leaf explants.

b) Adventitious shoots were induced from the proximal regions of the cotyledon, young leaf and cotyldeon-petiole explants.

c) Somatic embryogenesis occurred only from the adaxial surface of the proximal end of the cotyledons of the immature zygotic embryos.

d) Both the adventitious shoots and the somatic embryos were produced directly from the explants or from organized tissues, without an intermediate extensive disorganized callus phase formation.

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*ABBREVIATIONS*

| | |
|---|---|
| ABA | Abscisic acid |
| AC | Activated charcoal |
| As | Adenine sulphate (6-aminopurine-sulphate) |
| BAP | 6-benzyl-aminopurine |
| B5 | B5 medium (Gamborg *et al.*, 1968) |
| B5 2 | B5 medium supplemented with 2% (w/v) sucrose |
| CH | Casein hydrolysate |
| FAA | Formalin/acetic acid/ethanol |
| fs | Foliose structures |
| GA | $GA_3$ (gibberellic acid) |
| hr | hour(s) |
| IAA | Indole-3-acetic acid |
| IBA | Indole-butyric acid |
| IEDC | Induced embryogenic determing cell(s) |
| i6Ado | Isopentenyl-adenine |
| IPA | Isopentenyl-adenosine |
| KIN | Kinetin (6-furfurylaminopurine) |
| $\mu Em^{-2} sec^{-1}$ | Microeinsteins $m^{-2}$ $sec^{-1}$ |
| MS | Murashige and Skoog basal medium (1962) |
| MS2 | MS medium supplemented with 2% (w/v) sucrose |
| MS3 | MS " " " 3% " " |
| n | Number of replicates per treatment |
| NAA | a-Napthaleneacetic acid |
| PCPA | Para-chlorophenoxyacetic acid |
| PEDC | Pre-embryonic determing cell(s) |
| SEM | Scanning electron microscope |
| SH | Schenk and Hildebrandt medium (1972) |

| | |
|---|---|
| TBA | Tertiary butyl alcohol (2-methylpropan-2-ol) |
| TIBA | 2,3,5- triiodobenzoic acid |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ZEA | Zeatin [6-(4-hydroxy-3-methylbut-2-enylo)purine] |
| ZR | Zeatin riboside |

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# C H A P T E R I

# I N T R O D U C T I O N

## 1. *Plant tissue culture*

### 1.1. *Competence, determination and totipotency*

For a cell to undergo differentiation it has to be competent. Competence of a cell is its ability to react to a stimulus which causes development in a particular direction, whereas totipotency is the stage of a cell of being potent to regenerate a whole organism. Schleiden (1838) and Schwann (1838) first propounded the "cell theory" part of which was the concept of totipotency, although they never used the term "totipotency". Schwann (1847) stated "..we can conclude that each of the elementary parts-each cell-must possess the capacity to gather new molecules to itself and to grow, and that, therefore, each cell possesses a particular force, and independent life, as a result of which it would be capable of developing identically if only there be provided the external conditions under which it exists in the organism". Among the workers who developed the "cell theory" was Vöchting who published many papers concerning the factors that played an important role during the organ formation process (Vöchting, 1878; 1884; 1892). In 1878, he concluded that "in any fragment of the organs of the plant body, rests the elements from which, by isolating the fragment, under proper external conditions the whole body can build up".
Haberlandt (1902) was the first to predict that vegetative cells could be capable of developing as "artificial embryos" given suitable conditions.

Statements like those of Schwann, Vöchting and Haberlandt incorporate all the essential elements of what is today termed totipotency. The word totipotency was first introduced by Morgan (1901), but the term was only used occasionally until 1920 when Smith and, after him,

several investigators (Allen, 1923; White, 1943; Ball, 1946) used unambiguously the term totipotent. Winkler (1903) announced that the adventitious buds which formed on leaves of *Torenia asiatica* might have originated from either a single epidermal cell or from a very small group of cells. This was subsequently confirmed with *Begonia* (Hartsema, 1926; Prevot, 1939), *Saintpaulia* (Naylor and Johnson, 1937; Sparrow, Sparrow and Schainer, 1960; Arisumi and Frazier, 1968), *Bryophyllum* (Bigot, 1975), and another species of *Torenia* (Chlyah, Tran Thanh Van and Demarly, 1975).

Levine (1950) reported *in vitro* growth of tap-root meristem from carrot and the direct proof of the totipotency of higher plant cells was made by Muir (1953) when he demonstrated the growth of isolated single cells from higher plants *in vitro* culture. Five years later, Steward, Mapes and Mears (1958) reported that carrot cells in suspensions were capable of forming unorganized cell clusters which in turn, yielded first roots and then shoots and finally whole plants. Nobècourt (1955) and Reinert (1958) also described the sporadic development of root and shoot or bipolar embryo-like structures in carrot callus masses on semi-solid media. Further and more direct proof for totipotency of cultured cells was the report of Vasil and Hildebrandt (1965) that a mature tobacco plant was regenerated from a single cell which was initially grown in a microchamber. This was the first indication that a free cultured cell was able to give rise to a whole plant.

The answer to the question of whether all plant cells are totipotent remains unknown. Evidence of the widespread phenomenon of totipotency throughout many species has come from the regeneration of somatic embryos from such highly differentiated cells, such as pollen grains, epidermal cells from cotyledon and hypocotyl, photosynthetic palisade

cells in leaves and triploid endosperm cells (Halperin, 1970; Steward, 1970; Vasil and Vasil, 1972; Stamp, 1984). Sinnott (1950) declared that "the general conclusion, with all its far-reaching implications, seems justified that every cell, fundamentally and under proper condition, is totipotent or capable of developing by regeneration into a whole organism provided that the genetic make up of the cell had remained unaltered since the time of cleavage from a meristem". Henshaw *et al.*(1982) expressed the view that diploid somatic cells in plants are totipotent, even this has been difficult to demonstrate with cells from certain species.

The concept of potency, competence and determination are intimately related to each other if we take the view that a competent system which receives a developmental signal becomes determined and as a consequence, loses potency (Henshaw *et al.*, 1982). That is a morphogenetic signal alerts a competent tissue to become determined and in turn, according to the developmental stage, this tissue either remains at this stage until it be activated by permissive conditions to produce the morphogenetic change, or becomes competent to respond to further specific signals. The stage of determination is a more stable condition and is inheritable through many cell generations, whereas competence is a transient condition, probably not cell-heritable and which might disappear. Determination and competence are both cryptic states (Waddington, 1968). Therefore, organ or embryo development may involve the sequential appearance and loss of states of competence in the tissue, with the whole operation taking place in an hierarchical sequence of processes in which sets and subsets are involved. Hence, tissues are initially competent to develop in a number of different ways according to the signals that are received and their degrees of freedom are reduced with each determinative event, until finally they become

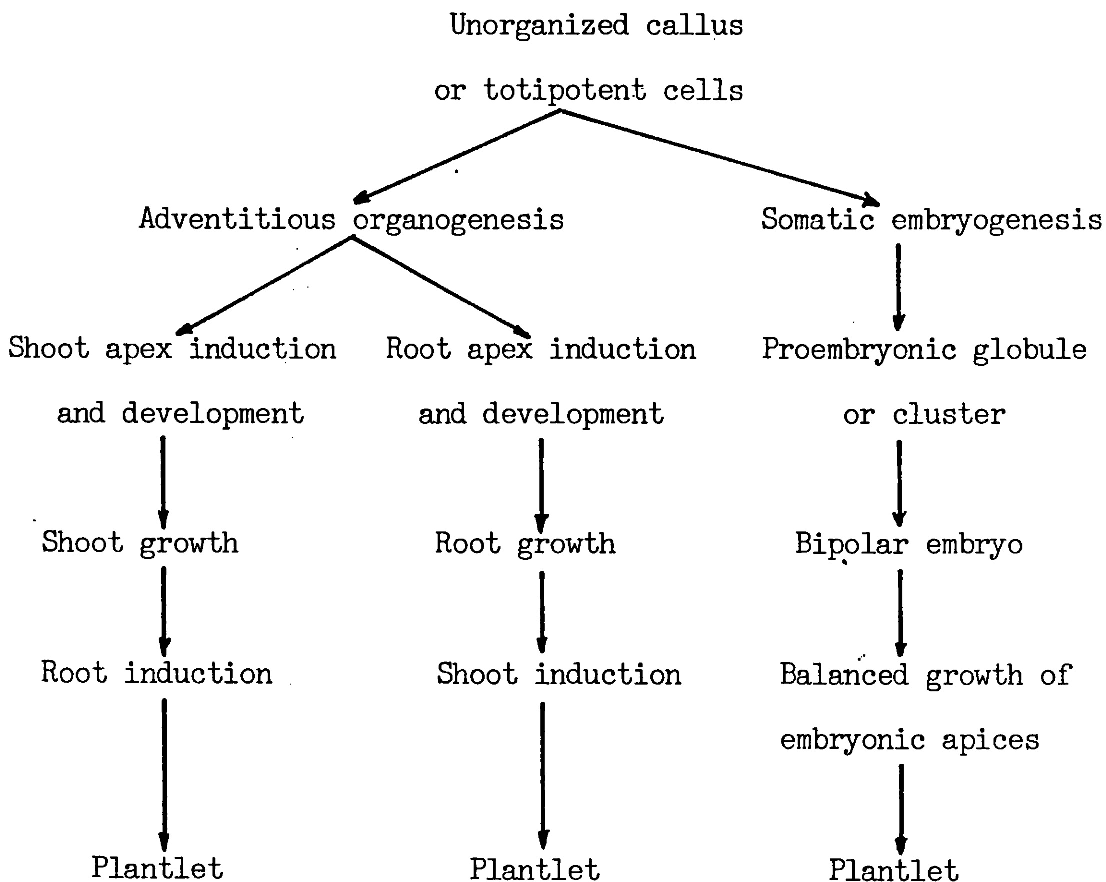
determined with respect to a single fate. Henshaw et al. (1982) pointed out "as soon as the initial cell divides, to produce a multicellular structure which behaves as an organism, determination has taken place, but not necessarily at the cellular level. The important general point is that determination can occur at any organizational level within an organism and determination at one level does not necessarily imply determination at lower levels of the organizational hierarchy. It is presumably essential that more degrees of freedom should be maintained at the lower organizational levels, otherwise the regulatory mechanisms which tend to maintain the developmental norm at any organizational level, and which are characteristic of all organisms, could not operate. A more complex organizational structure will most probably impose greater constraints on the degrees of freedom at the lower organizational levels, or conversely, a need for greater flexibility at the lower levels might impose limits on the overall organizational complexity".

The failure to demonstrate embryogenic competence in somatic cells of many plant genera under *in vitro* conditions may be due to a failure to provide the right conditions (Steward *et al.*, 1970), or according to Henshaw *et al.* (1982) to any of the following reasons: a) a permanent loss of totipotency, b) a failure to remove developmental constraints, c) a failure to induce embryogenic competence once the developmental constraints have been removed, d) a failure to provide a specific morphogenic signal and e) a failure to provide suitable conditions for embryo development.

## 1.2. *Plant regeneration*

### 1.2.1. *General*

*In vitro* plant regeneration can occur from organized and disorganized culture systems. There are two basic pathways by which plants can be regenerated *in vitro:* non-adventitious regeneration and adventitious regeneration. In adventitious regeneration we can distinguish two different avenues: somatic embryogenesis and adventitious organogenesis (Henshaw, 1979). Krikorian (1982) proposed the following modes of morphogenetic expression through adventitious regeneration:




Propagation via *in vitro* techniques may have several advantages over conventional methods for:

1. Plants that are normally propagated slowly; clonal propagation of plant via tissue culture was first commercially used with orchid (Wimber, 1963; Marston,1969).
2. Reducing the propagation time of species in high demand; *in vitro* propagation has proved useful in propagation of ornamentals and crop species that are propagated rapidly but inadequately to satisfy market demands (Takatori *et al.*, 1968).
3. The establishment of new varieties when only a few plants are initially available (Walkey and Woolfitt, 1970; Smith and Thomas,1973; Staritzky, 1970). In this case, a virus resistant or horticultural variant can be multiplied rapidly for commercial planting.
4. The production and multiplication of pest and pathogen-free plants.

*1.2.2. Non-adventitious regeneration*

Non-adventitious shoot formation is accomplished by the outgrowth of pre-existing meristems and regeneration procedures often utilize the stimulation of axillary bud development (Henshaw, 1979). Axillary meristems are initiated in the leaf axils of most plants but their development is normally inhibited by apical dominance. When the suppressing influence of the apical dominance or of the other plant parts is removed - by isolation - then the developmental potential of the axillary buds is released and the buds begin to grow. Much effort has gone into the development, utilization and promotion of micropropagation systems that capitalise on the tendency for some excised lateral buds to form precocious shoots in profusion when stimulated by exogenous

cytokinins (Krikorian, 1982). The basis for the precocious axillary shoot stimulation technique is usually explained in terms of the observations of Wickson and Thimann (1958, 1960) that the inhibiting action of indole-3-acetic acid (IAA) on lateral bud growth can frequently be overcome by exogenous cytokinins. In 1974, Boxus described the effect of synthetic cytokinin on strawberry plantlets aseptically maintained in culture and which had been generated from excised stem tips. When transferred to an otherwise standard basal medium supplemented with $N^6$-benzyl-aminopurine (6-BAP), the plantlets ceased to form roots and stopped producing characteristic triofoliate leaves. Instead, axillary buds started to appear in profusion at the base of the petioles and each of them in turn, could form a plantlet (Boxus, 1974a, 1974b). It is now well known from many studies that the method of shoot-tip or axillary bud culture has been successfully applied for micropropagation purposes to a great number of species (from herbaceous foliage plants to bulbous monocotyledons). In different species plants are produced either via the induction of multiple shoots from an "organogenic callus" developed underneath the stem tip explant or via the formation of multiple shoots due to the growth of precocious axillary branches (Westcott *et al.*, 1979; Roca *et al.*, 1979; Krikorian, 1982; and references cited there). The same method has been applied to many woody plants such as *Ficus*, *Tubidanthus*, *Nandina* (Matsuyama, 1976), *Rhododendron* (Anderson, 1978; Abbott, 1977; Zimmerman, 1980; Murashige, 1980). This method of non-adventitious regeneration is efficient enough for micropropagation especially for species where the rate of multiplication depends upon the high rate at which axillary meristems are produced *in vitro* (Hussey, 1978). When adventitious root initiation from shoots produced in this way is not easy in media supplemented

with a cytokinin, cultures can be transferred to a second medium, containing perhaps an auxin, which could stimulate rhizogenesis (Krikorian, 1982).

A combination of heat treatment of the stem tip or axillary bud with aseptic culture has been an especially useful procedure for obtaining virus-free plants (Hollings, 1965; Murashige, 1974; Raychaudhuri and Verma, 1977; Stone, 1978). Also by incorporation into the media of meristem culture some antiviral agents, such as ribavirin, it is possible to obtain virus-free plants (Long and Cassels, 1984).

It is now widely accepted that non-adventitious organogenesis is the only inherently genetically stable regeneration process in contrast to processes based on adventitious organogenesis and embryogenesis which are more likely to be genetically unstable. D'Amato (1977) suggested that in the meristematic region of the plants, there are mechanisms that prevent various nuclear abnormalities, such as polyploidy, aneuploidy and chromosomal structural changes, occurring. These mechanisms are supposed to control the sequence of DNA synthesis and mitosis as well as the continuation of cell division. Street (1976) also suggested that the high genetic stability achieved through non-adventitious meristem regeneration is a result of the maintenance of high level of cellular organization at that region. Although the mechanisms which control and ensure genetic stability in non-adventitious regeneration are not really understood, their biological significance is considerable since the shoot meristems constitute the essential germline in higher plants (D'Amato, 1975). It needs to be emphasized that mutation can arise occasionally even in non-adventitious meristems (Ben-Jaacov and Langhans, 1972) and so it is important to ensure that a mutant meristem is not selected during the multiplication process (Krikorian, 1982; Henshaw and O' Hara, 1983).

### 1.2.3. *Adventitious regeneration*

The ability of an explant to undergo adventitious regeneration *in vitro* depends upon the nature, the physiological and developmental stage of the explant and the environmental conditions of the culture. Several reports have indicated that tissues taken from early developmental stages of plants such as immature or mature zygotic embryos, seeds and seedlings or juvenile parts of plants are more capable of undergoing regeneration than more mature tissues. Sinnott (1960) stated that "...the more simple and undifferentiated a plant is, the more completely will it restore missing parts; and the more specialized and differentiated it is, the less regenerative capacity it will show... Early developmental stages are thus more likely to regenerate readily than older ones". In support of this statement come the findings that immature embryos of *Trifolium* and *Medicago* (Maheswaran and Williams, 1984) or *Theobroma cacao* (Pence *et al.*, 1980) have proved to be more morphogenically competent than mature embryos.

The adventitious regeneration process occurs also in certain conventional methods of propagation. For example, adventive plantlets develop from pre-existing meristems on leaves of genera like *Bryophyllum*, *Kalanchoe* and *Sedum* (Haccius and Larkshmanan, 1969). These adventive structures were described, incorrectly as "foliar embryos" by Naylor (1932) and Yarbrough (1932). The same happens when shoots arise from leaves of *Begonia* sp., stems and root pieces of trees such as *Salix*, *Populus*, *Pyrus* and bulb scales of *Lilium*.

Naturally occurring adventitious regeneration arises from typically non-polysomatic tissues such as cambium, procambium and pericycle. These are also the tissues which are exploited in conventional propa-

gation methods involving the formation of adventitious roots on cuttings. In all the above instances the pre-existing adventitious shoot meristems continue to grow without affecting the germline of the plant and hence a genetic stability is established.

Somatic embryogenesis occurs *in vivo* too. For example, "polyembryony" in seeds of species such as *Citrus* where embryos arise from ovule tissue surrounding the embryosac (Bacchi, 1943; Webber, 1940); or in case of bog orchid *Malaxis paludosa* where somatic embryos, known as "foliar embryos", are produced at the tip of the fully expanded leaves (Taylor, 1967). Steeves and Sussex (1972), based on the above findings, made the following statement "...this production of foliar embryos is an example of the retention of full zygotic potentialities by differentiated cells and the ability of such cells to express these potentialities within the organization of the intact plant".

The formation of callus *in vivo* at the edge of a damaged tissue, known as "wound response", is a result of the stimulation of unorganized cell division. Callus proliferation *in vivo* is usually a short-lived phenomenon (Yeoman, 1970), but *in vitro* by manipulating the environmental conditions and the culture medium one can encourage callus proliferation to be continuous.

The type and the rate of callus proliferation depends upon the nature of the tissue of which the explant is composed and the culture conditions. In most cases explants are composed of a range of tissues with cell types of varying degrees of differentiation. When the explant is heterogenous regarding to the cell type, then, to a certain degree, the callus formed in culture will consist of a heterogenous cell population too, but when the explant composed of one cell type, for instance pith or cambium, then the resulted callus will consist of one cell type only (Yeoman, 1970).

According to Aitchison *et al.*, (1977) there are three stages during the course of callus development from tissue explants. The first or "induction" stage is characterised by the activation of cell metabolism and it is controlled by the physiological state of the initial explant and the culture conditions. During the second stage, active synthesis and cell division take place. Cell division occurs at the periphery of the explant where a progressive return to meristematic stage (de-differentiation) is occurring in the outer region of the callus. The third or "differentiation" stage is characterised by increasing cellular differentiation through both maturation and expansion of the cells. The characteristic of a such developing callus, is that the induction of cell division is restricted to the peripheral layers and not throughout the tissue (Yeoman *et al.*, 1965). The limitation of cell division at the periphery of the explant may be related to interaction of several factors such as light, greater availability of oxygen, more rapid release of $CO_2$, more rapid escape of volatile inhibitors, greater nutrient availability and the wound response of the cut surface (Yeoman, 1970).
Although many plant tissues produce callus as a natural response to wounding, cell division can be further stimulated in cultures by incorporating plant growth regulators into the medium. According to Yeoman (1970) plant tissues can be divided in four groups regarding their requirement for growth regulator combinations to stimulate callus initiation: a) in hormone-free medium, b) in medium supplemented with auxin only, c) in medium supplemented with cytokinin only, and d) in medium supplemented with auxin and cytokinin.

Adventitious plant regeneration has been successfully accomplished from explants of cotyledons (Hu and Sussex, 1971; Stamp, 1984), hypo-

cotyl (Kamat and Rao, 1978), leaf (Handro, 1977; Gleddie *et al.*, 1983), shoot apex (Kartha *et al.*, 1974; Ball, 1980; Martins and Söndahl, 1984), root (Gunckel, *et al.*, 1972; Lazzeri and Dunwell, 1984a and 1984b), young inflorescences (Majumdar, 1970; Boyes and Vasil, 1984), flower petals (Heuser and Apps, 1976), ovular tissue (Kochba and Spiegel-Roy, 1977), embryos (Nag and Johri, 1969; Lu *et al.*, 1984; Maheswaran and Williams, 1984) and callus (Bajaj and Gosal, 1981).

Callus formation is often, but not always, a prerequisite of adventitious regeneration in both intact plants and *in vitro*. Yeoman (1970) noted that callus formation is an almost inevitable preliminary of the rooting of cuttings. In the case of shoot regeneration from hypocotyls of *Picea glauca* (Campbell and Durzan, 1976) or from cotyledons of *Thuja plicata* (Coleman and Thorpe, 1977) no callus formed and the shoots arose directly from the primary explant. It is also believed that in species like *Ranunculus sceleratus* (Konar *et al.*, 1972) and *Brassica napus* (Thomas *et al.*, 1976) adventitious embryos have a single cell origin in the original explant.

Hicks (1980) distinguished two types of adventitious organogenesis: "direct" and "indirect". The criterion which he used for this classification was the absence or presence, respectively, of callus formation during the sequence of organogenic process. He also proposed the following patterns of organ development during the course of the adventitious organogenesis:

a) Direct organogenesis (no callus formation)

primary explant ⟶ organ (i.e. leaf, root)

primary explant ⟶ meristemoid ⟶ organ system (i.e. shoot, flower)

b) Indirect organogenesis (callus formation)

primary explant ⟶ callus ⟶ meristemoid ⟶ organ system.

By using the term "meristemoid" he accepted the interpretation given by Bunning (1952), Torrey (1966), Thorpe and Murashige (1970), Ross and Thorpe (1973), and Thorpe (1978) that "meristemoids" are specialized groups of small cells with potentially organogenic properties.
In Hicks' view (1980) callus formation is an undesirable complication for two reasons: firstly, because in many plants the chromosome constitution of callus cells is unstable (Sheridan, 1975; D'Amato, 1977) and secondly, because it is very difficult to achieve a critical analysis of the molecular events in a meristemoid population within a callus mass, for the reason that the ratio of potentially organogenic (meristemoid) cells to the total callus mass is very small. Furthermore, according to Thorpe (1978), there is a lack of synchrony between the individual meristemoids and between meristems in a given callus. Therefore, especially for propagation purposes, the period of unorganized growth (callus) should be minimized or eliminated entirely (Street,1975).

Using the same criterion (formation or not of callus), Evans and Sharp (1981a and 1981b) distinguished two general patterns of embryogenic development: "direct embryogenesis" where embryos originate directly from tissue in the absence of callus proliferation, and "indirect embryogenesis" where callus proliferation is a prerequisite to embryo development. An understanding of these two different patterns of development depends upon the consideration of the determinative events of cytodifferentiation during the mitotic cell cycle (Sharp *et al.*, 1980). Yeoman (1970) pointed that the fate of determined daughter cells, following mitosis, occurs at least one mitotic cell cycle prior to differentiation. It has been suggested that cells which will undergo direct embryogenesis are the daughters of a prior determinative cell division and are called "pre-embryonic determined cells" - PEDC -(Kato and Takeuchi, 1963; Konar and Nataraja, 1965), and that

indirect embryogenesis requires the re-determination of differentiated cells, callus proliferation and differentiation of embryogenic determined cells (IEDC - induced embryogenic determined cell). PEDC's await either synthesis of an inducer substance or removal of an inhibitory substance, requisite to resumption of mitotic activity and embryogenic development. Cells undergoing IEDC differentiation require a mitogenic substance to re-enter the mitotic cell cycle and/or exposure to specific concentration of growth regulators (Evans *et al.*, 1981a).
Direct embryogenesis has been reported to occur in species like *Atropa belladonna* (Rashid and Street, 1973), *Daucus carota* (McWilliam *et al.*, 1974), *Datura innoxia* (Geier and Kohlenbach, 1973), *Ranunculus sceleratus* (Konar *et al.*, 1972), *Brassica napus* (Thomas *et al.*, 1976), *Trifolium repens*, *T. pratense* and *Medicago sativa* (Maheswaran and Williams, 1984). In the above instances the competence for embryogenesis is retained in cells of specialized function. This raises the possibility that differentiation, or at least some pathways of differentiation and competence to embark upon morphogenesis are not necessarily incompatible (Street, 1979).
Indirect embryogenesis or IEDC on the other hand have been reported to occur in species like *Macleaya cordata* (Kohlenbach, 1965,1966 and 1978), *Cichorium endivia* (Vasil and Hildebrandt, 1966), *Daucus carota* (Reinert *et al.*, 1967; Halperin, 1969; Kamada and Harada, 1979), *Atropa belladonna* (Konar *et al.*, 1972), *Petunia hybrida* (Rao *et al.*, 1973), *Cucurbita pepo* (Jelaska, 1974), *Corylus avellana* (Radojevic *et al.*, 1975), *Apium graveolens* (Williams and Collin 1976a and 1976b; Zee and Wu, 1979), *Nigella sativa* (Banerjee and Gupta, 1976), *Vitis vinifera* (Krul and Worley, 1977), *Carum carvi* (Ammirato, 1977; Furmanowa *et al.*, 1983),

*Theobroma cacao* (Pence *et al.*, 1979), *Solanum melongena* (Matsuoka and Hinata, 1979), *Phoenix dactylifera* (Reynolds and Murashinge, 1979), *Coffea arabica* (Söndahl *et al.*, 1979), *Echinochloa oryzicola* (Takahashi *et al.*, 1984) and *Zea mays* (Vasil *et al.*, 1984).

Cyto-differentiation and the emergence of multicellular organization are multi-step processes in which each step leads to the establishment of a particular pattern of gene activation, allowing transition to the next essential stage of development (Street, 1978), but arrest may occur at any step in the process (Evans *et al.*, 1981b). The most significant determinants for embryo initiation are the type of explant and certain of its associated physiological qualities, while the *in vitro* environment acts primarily to enhance or repress the embryonic process (Henshaw *et al.*, 1982), or to put it differently the cells that undergo initiation are predetermined, and their subsequent exposure to exogenous growth regulators simply allows embryogenesis to occur (Tisserat *et al.*, 1979). There is evidence that the auxin or auxin/cyto-kinin concentration in the primary culture or "conditioning medium" is critical not only to the onset of mitotic activity in non-mitotic differentiated cells but to the epigenetic redetermination of these cells to the embryonic state of development. So far, it is unknown if the role of growth regulators in gene expression is direct or in-direct (Sharp *et al.*, 1980). Whichever way the growth regulators control the gene expression, there is evidence that the auxin 2,4-D elicits responses at the transcriptional and translational levels during primary culture. Subsequently, additional responses at the transcriptional and translational levels occur shortly after subcul-ture onto a secondary or "induction medium" (Sengupta, 1978; Sengupta and Raghavan, 1979).

One of the most frequently mentioned theories about somatic embryogenesis is that the cell must undergo "de-differentiation" prior to embryogenesis (Halperin, 1970; Neuman, 1969). Some other investigators prefer to use the word "re-differentiation" or simply "differentiation" (Sharp *et al.*, 1980), for the reason that the term dedifferentiation implies a reversal of the sequence of states leading to the existing state of cyto-differentiation, whereas the term redifferentiation or differentiation does not. Halperin (1970) regarded the "dedifferentiation" as a prerequisite to reaching the "embryogenic state", the state of a cell in which it is specialized for embryo formation and will not differentiate in any other way. Steward *et al.*, (1958) stated that "no single parenchyma cell can "directly" recapitulate the familiar facts of embryology, but must go through the formation first of an unorganized tissue culture". Contrary to this statement, the findings that embryos were formed directly from epidermal cells of the stem of *Daucus carota*(Kato and Takeuchi, 1969) indicate that the epidermal cells need no dedifferentiation prior to embryo formation. These cells, according to Sharp *et al.*, (1980) were restricted in their cytodifferentiation options during an earlier mitotic division. These committed cells, probably a subpopulation of the epidermal cells, undergo a periclinal mitotic division at the onset of embryogenesis rather than the characteristic anticlinal mitotic division typical of epidermal cells. This change in the orientation of the spindle apparatus is indicative of a commitment to embryogenesis during a previous mitotic cell cycle (Sharp *et al.*, 1980).

Since embryos can arise from epidermal cells which are already differentiated, as well as from callus cells, which although differentiated are different from epidermal cells, it is supposed that there is more

than one state permitting embryo development. Thus it is possible that a cell without a special predisposition for embryo formation will produce an embryo if it is not influenced to follow some other path of differentiation (Caldas, 1971). Steward *et al.*, (1964) suggested that the constraints of the neighbouring cells limited the expression of the cells' potential to a small part of genome and that isolating the cell it could undergo the adventitious regeneration process. Thus, the chemical products of a cell's metabolism will influence a neighbouring cell and stimulate it to a particular type of differentiation and it is the loss of this type of communication that permits embryo formation. This theory requires that differentiation of a specialized cell type results from either particular inductive compounds (hormones) which force the differentiation, or from combinations of various conditions and compounds, such as sucrose, growth regulators, and physical factors (Sharp *et al.*, 1971; Wetmore and Rier, 1963; Brown and Sax, 1962). Evidence against the isolation theory is provided by the fact that single cells in organized tissues were competent for embryo formation, although it is difficult to rule out the possibility of physiological isolation (Konar and Nataraja, 1965; Kato and Takeuchi, 1969; Stamp, 1984).

The number of cells involved in the origins of adventitious organ primordia and somatic embryos have been the subject of considerable arguments. There are two schools of thought: either that regenerated organs or somatic embryos are the result of the division of a single cell or that several cells are involved in the initial stages of the regeneration process. It is widely believed that embryogenesis is usually initiated in a single cell (Konar and Nataraja, 1965) and this was used by Street and Withers (1974) as one of the criteria to

define the process. Similarly, Haccius (1978) suggested that the somatic embryos arise from single cells whether it be directly or indirectly. She recognized that somatic embryos arising from callus in carrot do not necessarily arise directly from single cells and that a budding process similar to cleavage polyembryony of gymnosperms is sometimes involved. Her conclusion that these budded somatic embryos must arise from single cells, seems to be based entirely upon a supposed homology between the callus from which they are derived and the gymnosperm proembryonal complex which does arise from a single cell the zygote.

Organogenesis, on the other hand, although sometimes seems to begin in a single cell it generally seems to involve further cells. For instance, bud formation in *Torenia fournieri* epidermis is a result of active cell division in single cells followed by division in two or three adjacent cells (Chlyah, 1974a and 1974b) and adventitious shoot regeneration in *Nautilocalyx lynchei* has been demonstrated to be the result of the simultaneous division of several adjacent epidermal cells (Tran Thanh Van and Drira, 1971).

Although a distinction might possibly be made between the development of embryos and organs on the basis of the number of cells from which they are derived, in both cases an important stage is the production of a morphogenetically active group of cells in which pattern-formation mechanisms become established (Henshaw *et al.*, 1982). This active group of cells referred to by Torrey (1966) as a "meristematic centre" or "meristemoid", following on Bunning's concept of the "meristemoid" as a cell in which the "character of embryonic cells" is regained as the result of an unequal division. Torrey's use of the term meristemoid rather begs the question of whether such an embryonic group of cells

is derived from one cell which has undergone an unequal division, but the term is now widely used in tissue culture literature. Whatever the origin (one or more cells) of an organ, it requires the development of polarity. This implies the development of determinative chemical and/or physical gradients within the tissue and is correlated with the sub-surface origin of most organ primordia and the superficial origin of most embryonic cells (Street, 1976). The origin of a somatic embryo may require the development of polarity within a single cell while the origin of an organ primordium more likely requires the development of polarity within a group of meristematic cells. In the case of embryo development from multicellular clumps, these clumps are already polarized because the inner cells are larger, more vacuolated, and contain more starch than the peripheral eumeristematic cells (Halperin, 1967). The inner cells appear to remain associated with the suspensor, as the root end of the embryo is always attached to this clump. This becomes clearer in cases where several embryos arise from a single clump and all have their roots attached to the clump. This uniformity extends to embryos arising from epidermal cells *in situ* on the stem of *Ranunculus* plants, in which all the embryos are attached to the plant by their roots (Konar and Nataraja, 1965).

### *1.2.4. The effect of nutrient media and culture conditions on regeneration.*

The effects of different nitrogen sources in culture medium have been conducted on primary explants, callus and developing embryos. Callus cells multiply less frequently in the presence of ammonium nitrogen and develop into multicellular clumps, while cells exposed to nitrate nitrogen seldom give rise to these structures (Halperin and

Wetherell, 1965). The requirement of ammonium or some other source of reduced nitrogen i.e. glycine, glutamine or yeast extract in carrot embryogenesis has been documented (Halperin, 1966a and 1966b; Beccari *et al.*, 1967; Kato and Takeuchi, 1966; Norreel and Nitsch, 1968; Dougall and Verma, 1978). This requirement for reduced nitrogen should be met during a critical time of development since secondary culture of callus grown on ammonium following primary culture on nitrate nitrogen fails to develop multicellular structures and embryos (Halperin and Wetherell, 1964). Strong evidence that ammonium is not required for embryogenesis and can be replaced by nitrate in the medium also exists (Reinert, 1968; Tazawa and Reinert, 1969), but in this case, it appears that a certain level of intracellular $NH_4^+$ is a prerequisite for the process (Tazawa and Reinert, 1969). The ammonium requirement is associated with the specific concentration of 2,4-D in the culture medium. Caldas (1971) reported that in presence of ammonium, concentrations of 2,4-D from 2.5 to 25 times higher than those without the ammonium were necessary for somatic embryo initiation. Additional evidence in support of this concept is found by Halperin and Wetherell (1964,1965) when they observed that ammonium was not necessary in the medium in which the embryos were developing, but only in the primary culture medium. Also, Kamada and Harada (1979) suggested that reduced nitrogen is not required for the embryogenic determination of cells cultured on 2,4-D containing medium; instead of determination, the role of reduced nitrogen is more related to the development of the embryogenic determined cells into multicellular embryos on the 2,4-D containing medium. These workers also investigated the effects of various aminoacids added to 2,4-D secondary culture medium consisting of a basal medium and 20 mM $KNO_3$ to determine the effects of amino

nitrogen on the development of embryogenically determined cells. They reported that 5 to 10 mM of a-alanine proved to be especially effective in increasing the frequency of embryo development. Stimulation of embryo development to a lesser degree occurred when glutamine, aspartic acid, glutamic acid, arginine or proline was added to the culture medium. Gamborg *et al.* (1968) demonstrated that poor growth of cell suspension cultures of soybean resulted when basic amino acids and acidic amino acids were used separately. Glutamine or acidic amino acids and amonium ions resulted in growth equivalent to that obtained when the mixture of basic and acid amino acids were used. Halperin (1964,1966b) and Nitsch and Nitsch (1969) also reported that glutamine has been partially or completely successful in replacing ammonium for embryogenesis. Gleddie *et al.* (1983) reported that the highest frequency of somatic embryogenesis in *Solanum melongena* occurred when the $NO_3/NH_4$ ratio was 2:1, and further, that when this ratio (2:1) was held constant the total nitrogen content was significant factor, with maximum embryo formation occurring in the 60 to 90 mM range.

The necessity of other inorganic (mineral) elements, such as K,P,Ca,S,Mg,Cl,Fe,B,Mn,Zn,Cu,Co,I and Na, for growth to occur in tissue culture medium, was well demonstrated by White (1943), Hildebrandt *et al.* (1946), Murashige and Skoog (1962), and Murashige (1974). The effect of potassium and sodium on embryogenesis was examined by Reinert *et al.* (1967) and Gleddie *et al.* (1983). For example, in the case of somatic embryogenesis in *Solanum melongena*, sodium ion concentrations up to 20 mM did not influence embryogenesis, while concentrations between 20 and 60 mM were slightly suppressive. Potassium ion concentrations in the range from 1.25 to 50 mM did not influence embryogenesis, while above 50 mM they were slightly suppressive (Gleddie *et al.*, 1983). A simultaneous decrease in the frequency of embryogenesis from 32%

to 20% was observed when phosphate concentration was increased from 1.25 mM to 20 mM (Tazawa and Reinert, 1969).

Carbohydrates such as sucrose or glucose, apart from being carbon sources, influence the adventitious regeneration process by affecting the osmotic potential of the medium. Glucose has only occasionally shown to be superior to sucrose (Murashige, 1974; Homés and Guillaume, 1967). The optimal sucrose level for embryogenesis in *Solanum melongena* was 0.06 M whereas 0.3 M sucrose inhibited the embryo formation as a result of the high osmotic potential of the medium (Gleddie *et al.*, 1983).

The vitamins most commonly included in the media are thiamine, inositol, nicotinic acid and pyridoxine. It has been found that thiamine is critical for adventitious regeneration but not inositol, although the latter has been shown to be beneficial in many cases (Murashige, 1974; Staudt, 1984). Biotin, choline, folic acid, pantothenic acid and riboflavin are usually used chiefly for precautionary reasons. Ascorbic acid has been used, particularly in combination with citric acid, to retard browning of freshly excised tissues (Murashige, 1974; Boyes and Vasil, 1984).

The qualitative and quantitative aspects of growth regulators in the microenvironment of embryogenic determined cells are important in the mitotic arrest or release from arrest (Tisserat and Murashige 1977a and 1977b; Caldas, 1971; Henshaw *et al.*, 1982). Furthermore, the normality of embryo development from these determined embryogenic cells as well as mitotic arrest is regulated by growth regulators in at least two instances (Ammirato, 1977, 1983). However, the answer to the question of whether growth regulators influence the organ or embryo initiation process by acting as a trigger or by re-

moving the constraints is not yet known.
The most critical organic compounds of propagation media are auxins and cytokinins. Some of these are naturally occurring hormones e.g. indoleacetic acid (IAA) and zeatin; others are synthetic growth regulators such as 2,4-D and BAP. An auxin and sometimes a cytokinin are required for inducing cell division and the formation of callus. The most frequently used auxins are 2,4-D, NAA, IAA, IBA and PCPA, while kinetin, BAP, IPA and zeatin are the most commonly used cytokinins.

Organogenesis is totally dependent upon the balance of auxin and cytokinin. In general, auxin alone or high auxin with low cytokinin initiate root regeneration and embryo formation, while cytokinin alone or high cytokinin with low auxin help the initiation of shoot regeneration. Observations on the auxin-cytokinin mediated mechanism of organogenesis have been often described, e.g. in *Daucus* (Steward *et al.*, 1958; Reinert, 1959; Halperin, 1967; McWilliam *et al.*, 1974); *Asparagus* (Harada, 1973); *Petunia* (Rao *et al.*, 1973); *Antirrhinum* (Sangwan and Harada, 1975); *Apium* (Williams and Collin 1976a and 1976b); *Limnophila* (Sangwan *et al.*, 1976); *Torenia* (Kamada and Harada, 1979; Tanimoto and Harada, 1984); *Perilla* (Tanimoto and Harada, 1980); *Solanum tuberosum* (Webb *et al.*, 1983); *Solanum melongena* (Matsuoka and Hinata, 1979; Gleddie *et al.*, 1983); *Zea mays* (Vasil *et al.*, 1984); *Echinochloa* (Takahashi *et al.*, 1984); *Sorgum* (Boyes and Vasil, 1984); *Rubus* (Street and Henshaw, 1963); *Secale* (Lu *et al.*, 1984); *Glycine* (Sargent and King,1974); *Trifolium* and *Medicago* (Maheswaran and Williams 1984); *Carum* (Ammirato, 1983; Furmanowa *et al.*, 1983); *Pinus* (Ellis and Bilderback, 1984); *Manihot* (Stamp, 1984); etc.

Embryogenesis has been found to be initiated, in most cases, by an auxin alone or by high auxin with low cytokinin. A review of the

literature shows that 2,4-D is necessary for embryo initiation, in most cases, followed by the auxins IAA and NAA. It has also been reported that while the auxin 2,4-D may be necessary during the initial culture it should be avoided or lowered in the second culture's medium for promotion of somatic embryo development (Steward *et al.*, 1958, 1964, 1970, Murashige,1974; Street, 1979; Thorpe, 1980 and references there cited). Cytokinins, apart from their ability to promote shoot formation in cultures, also may promote somatic embryogenesis. For example, kinetin is effective in maintaining the embryo-formation potential in solid cultures of carrot for a longer period (Halperin, 1966b) and allows differentiation of asparagus embryos (Wilmar and Hellendoorn, 1968); zeatin stimulates embryogenesis in carrot suspension during subculture onto an auxin-free medium, whereas BAP inhibits the process (Kato and Takeuchi, 1963); application of isopentenyladenine (i6Ado) to the proembryonic culture in anise cells, induced embryogenesis and promoted embryo development (Ernst and Oesterhelt, 1984). It is also worthwhile mentioning that embryogenesis has been reported to occur in hormone-free medium from shoot apex of *Trachymene coerulea* (Ball, 1980).

Gibberellins, while stimulating growth of organs, generally repress the organ initiation process (Bigot and Nitsch, 1968; Murashige, 1961, 1963, 1964). However, GA could substitute for light in stimulating root initiation in *Helianthus tuberosus* (Gautheret, 1969) and *Lycopersicon esculentum* (Coleman and Greyson, 1977a). This stimulatory effect of gibberellin on rooting does not indicate desirability of its general inclusion in media intended for organ multiplication, although gibberellins may be used to hasten growth of already formed organs or asexually derived embryos. GA was however found to stimulate

somatic embryogenesis from ebryogenic-competent callus of clone BC5 of *Theobroma cacao* (Kononowicz and Janick, 1984).

Abscisic acid (ABA), at the appropriate concentrations, effectively normalized development of somatic embryos derived from cell suspension cultures of *Carum carvi* (Ammirato, 1974, 1983), although there was no effect on embryo initiation.

Activated charcoal in some instances influences morphogenesis. It was shown that medium supplemented with activated charcoal induced embryogenesis in cultures of *Daucus carota* in which embryo formation could not be brought about by omitting auxin from the medium (Drew, 1979). Charcoal-medium also induced abundant root formation in older cultures of *Allium cepa*, which normally did not produce roots. The growth of cultures of *Glycine max* and *Haplopappus gracilis* was totally inhibited by charcoal (Fridborg and Eriksson, 1975). It is known that activated charcoal removes many substances from the medium including the growth regulators a-naphthylacetic acid, 6-furfuryl-aminopurine, 6-benzylaminopurine, 6($\gamma,\gamma$-dimethylallylamino)purine, the growth inhibitor 5-hydroxymethylfurfural, the phenolic compounds hydroquinone and p-hydroxybenzoic acid and the vitamins thiamine-HCl and nicotinic acid (Weatherhead *et al.*, 1978 and 1979).
Kessel *et al.* (1977) reported that embryogenesis in *Daucus* cultures occurred only below a critical level (about 1.5 ppm) of dissolved oxygen. Higher levels favoured rhizogenesis.
With some species, the success or failure of their tissue culture may depend on whether a liquid or agar nutrient is employed. As early as 1939, White observed that *Nicotiana* callus cultures produced shoots when transferred from an agar to a liquid medium. This observation was confirmed by Skoog (1944) and in 1965 Wimber noted that explants

of *Cymbidium* orchid initiated development faster in an agitated liquid medium, but further development occurred equally well in liquid or agar. Root explants of *Chichorium intybus* cultivated in a liquid nutrient and supported by a filter paper bridge produced vegetative buds, whereas explants placed on agar medium regenerated flower buds. Hence the physical form of the nutrient medium played a significant role in determining the pattern of organ differentiation (Margara and Bouniols, 1967; Bouniols and Margara, 1968).

The importance of the pH of culture medium was studied by Murashige and Skoog (1962) as well as by Gamborg *et al.* (1968). According to them, the value of 5.5 to 5.8 is suitable for maintaining all the salt in soluble form even with relatively high phosphate levels and low enough to permit rapid growth and differentiation of the tissue. Fassuliotis and Nelson (1986) observed that for embryo development in *Cucumis metuliferous* and *C. metuliferous x C. anguria* the pH should be raised to 7.0 in the medium of secondary culture, although for embryo initiation the pH value should be between 5.5 and 5.8 in the medium of the primary culture.

The light requirements of plant tissue cultures must be considered in terms of intensity, quality and exposure period. It should be noted that the light requirements are not the same as those of autotrophically developing whole plants; in tissue culture, photosynthesis is not a necessary activity, except perhaps during the late period of shoot or embryo development. It has been reported that light is important for the formation of shoots (Murashige, 1974; Nebel and Naylor,1968), the initiation of roots (Gautheret, 1969; Leroux, 1968; Ueba and Toricata, 1972), and in somatic embryogenesis (Haccius and Lakshmanan, 1965). The optimum light intensity for organ formation depends upon

the species, i.e. shoot-bud formation in the moss *Physcomitrium turbinatum* required a range of 300 to 700 lux light intensity (Nebel and Naylor, 1968); root initiation in *Helianthus tuberosum* occurred at the light intensity of 5,000 lux (Gautheret, 1969); *Asparagus*, *Gerbera* and *Saxifraga* have disclosed an optimum light intensity of 1,000 lux for the initial stage and first subculture and an optimum of 3,000 to 10.000 lux for the stage of development (Hasegawa *et al.*, 1973).

The *in vitro* requirement of periods of exposure to light each day is not the same as the photoperiod requirements *in vivo*.

The key is the total radiant energy of specified quality to which the culture is exposed. Hence it involves the combined influence of both intensity and exposure period, and for a given species it is reasonable to expect varying optima in the length of the daily exposure period, depending on the light intensity used (Murashige, 1974). For instance, for the differentiation of roots and shoots in *Nicotiana* callus and in *Asparagus* shoot tip cultures, the optimum light period each day has been 16 hr, using an intensity of 1.000 lux (Hasegawa *et al.*, 1973; Murashige and Nakano, 1967); Margara (1969) observed an optimum daily exposure period of 9 hr to 4.000 lux light for shoot induction in cauliflower cultures; sometimes a period of darkness during the first 8 to 15 days of the initial culture helped the somatic embryo initiation in cassava (Stamp, 1984), or increased rooting in apple cultures (Zimmerman, 1984). According to Weis and Jaffe (1969), the critical light spectrum for shoot induction was the blue region whereas red light was without effect. Seibert (1973) confirmed these findings and further reported that purple light (419 nm) stimulated shoot initiation. Mitra *et al.* (1965) discovered that bud formation

in *Pohlia nutant* cultures required a balanced exposure to both red and blue light. A combination of 11 hr of red and 6 hr of blue light daily gave the highest yield of shoots. Root initiation to shoot induction, is stimulated by red light but not by blue (Letouze and Beauchesne, 1969). The above observations of light quality effects, clearly indicate that key organogenetic processes in tissue cultures are photomorphogenetic phenomena, most probably regulated by phytochrome (Murashige, 1974).

Regarding the culture room temperature, as a general practice, cultures are maintained in an environment in which temperature is held almost constant in the neighbourhood of 25 °C. Skoog (1944) showed that root production in tobacco callus was highest at 18 °C, whereas temperatures of 33 °C or 12 °C were inhibitory. An increase in temperature from 25 °C to 30 °C improved rooting of shoot tips of apple cultivars such as "Delicious", "Royal Red Delicious" and "Vermont Spur Delicious"; further increase to 35 °C stimulating rooting of "Royal Red Delicious" but reduced rooting of "Vermont Spur Delicious" (Zimmerman, 1984). In *Chondrilla juncea* root cultures, maximum shoot formation was observed between 21 °C and 27 °C during the light period and between 16 °C and 22 °C during the dark period (Kefford and Caso, 1972). Gautheret (1969) suggested that the higher temperature was essential for the formation of cambia and the lower temperature during the dark period for the differentiation of the cambia into root primordia. Prior to transferring of the plants to the soil a reduction of the culture temperature is necessary to allow the plants to harden (Hildebrandt, 1971; Murashige *et al.*, 1974).

*1.2.5. Tissue culture and genetic stability*

Plants regenerated adventitiously from cultures frequently show a range of genetic variation, which is now referred to as "somaclonal variation" (Larkin and Scowcroft, 1981). The spectrum of somaclonal variation includes changes in ploidy level, chromosome number and structure as well as putative single changes in genes (Bright *et al.*, 1986; Secor and Shepard, 1981; Larkin and Scowcroft, 1981; Prased *et al.*, 1983). Such changes in cells and tissue cultures could be a product of genetic variability among cells of the explant, the mutagenetic effect of the *in vitro* culture conditions and *in vitro* selection pressures (D'Amato, 1978). Information about the nuclear cytology of cell differentiation *in vivo* is of great interest, since most of the plant explants consist of differentiated tissues or cells (Murashige, 1974; D'Amato, 1972). It is known that in gymnosperms and in many angiosperms the differentiated cell nuclei have the same DNA content which they had at the end of the mitosis, immediately before differentiation; that is, the 2C DNA content which is typical of the pre-DNA synthesis phase ($G_1$) of the diploid cell cycle (in haploids $G_1 \doteq 1C$). Only rarely, differentiated cells replicate their DNA to attain the 4C DNA content corresponding to the post-DNA synthesis phase ($G_2$) of the diploid cell cycle (D'Amato, 1978).

Another incident which might cause instability in cell and tissue culture is the nuclear fragmentation (amitosis) followed by mitosis. There is evidence that the auxin-kinetin ratio in the medium may influence the cytological mode of callus initiation, either by mitosis or by nuclear fragmentation followed by mitosis (Pätau and Das, 1961; Cionini *et al.*, 1978; Shimada and Tamata, 1967; Nuti-Ronchi *et al.*, 1973; Bennici *et al.*, 1976). In non-polysomatic species calli or

suspension cultures generally maintain a satisfactory stability at the diploid chromosome number, whereas diploid-polyploid (mixoploid) calli or suspension cultures are the rule when explants originate from polysomatic species (D'Amato, 1978). Polyploidy in tissue and cell cultures of non-polysomatic species may be due to restitution nucleus formation resulting from spindle failure or chromosome lagging at anaphase (Sunderland, 1973a). Spindle failure and chromosome lagging are also mechanisms of polyploidization *in vitro* of polysomatic species in which polyploidization by endoreduplication has been shown to occur in culture. Another mechanism of polyploidization *in vitro* seems to be nuclear fusion and/or spindle fusion in binucleate and multinucleate cells (D'Amato, 1977; Sunderland, 1973a). Nuclear or spindle fusion and probably genome segregation during multipolar anaphase are the most probable mechanisms of production of odd-ploid (triploid, pentaploid, etc.) chromosome number which have been reported to occur in calli (D'Amato, 1964). Aneuploid cells are able to survive and propagate mitotically in the culture even when provided with a chromosome number lower than haploid (Singh *et al.*, 1972). Sokolov *et al.* (1974) have presented evidence that essential condition for a cell to survive and go through mitotic cycles is the presence in its nucleus of one chromosome with a nucleolus organizer, a "locus", known to bear the genes for ribosomal RNA. In a number of species it has been observed that there is a correlation between increasing cytogenetic variability and loss of competence in long term cultures and that the most common cause of loss of regenerative capacity is aneuploidy (Sheridan, 1975; D'Amato, 1977; Karp *et al.*, 1984) and furthermore that only diploid plants were regenerated from cultures which showed different ploidy levels (Mitra *et al.*, 1960; Steward *et al.*, 1964; Nishi *et al.*, 1968). There are

also reports of cultures that have initially been mixoploid and which gradually changed to become predominantly diploid. The slow rate of chromosomal changes in these cultures suggests that a small proportion of diploid cells present initially have a selective advantage that enables them to increase in numbers relative to all other types (Bayliss, 1980).
Haploid tissue and tissue culture can be initiated from anther and pollen grains as well as from explants of somatic tissues of haploid plants (Sunderland, 1973b). Haploid cells are quite susceptible to chromosome doubling. They may be induced into chromosome endore-duplication or may diploidize by spindle failure at mitosis (Sacristan, 1971; Karp *et al.*, 1984).

Gross karyotype changes are not necessary for the appearance of somaclonal variation. The discovery that *in vitro* regenerated plants of several species including potato (Shepard *et al.*, 1980), *Sorghum* (Gamborg *et al.*, 1977), *Pelargonium* (Skirvin and Janick, 1976) and cauliflower (Grout and Crips, 1980) have normal diploid karyotypes led Larkin and Scowcroft, (1981) to suggest that genetic variation in cultured cells may result from "more cryptic chromosome rearrangements" rather than changes in chromosome number (polyploidy and aneuploidy). Examples of chromosome rearrangements include: chromosome breakage and reunion, multicentrics and translocation (Foroughi-Wehr *et al.*, 1978); reciprocal translocations, deletions and inversions (Ahloowalia, 1976) and heteromorphic pairs (Cumming *et al.*, 1976; Green and Phillips, 1977).

Not all species show inherent genetic instability *in vitro*. Cell and tissue cultures of *Lilium* (Sheridan, 1968) and *Freesia* (Davies, 1972) retained their organogenic potential for a number of years without

any sign of genetic instability. In callus cultures of *Eucalyptus canaldulensis* which contained large meristematic nodules (regions with rapid cell division) chromosome normality has been maintained for over three years (Sussex, 1965). Bayliss (1980) suggested that, because of the meristematic nodules, these cultures were not truly de-differentiated and retained the chromosome normality characteristic of organ cultures.

As a result of the genetic instability and variation *in vitro*, calli and cell suspensions are the least appropriate types of culture to be used for clonal propagation where true-to-type regenerant plants are required (Krikorian, 1982). On the other hand, because of the genetic variability, unorganized cultures (calli, cell suspension and protoplast cultures) are a potential tool in hands of breeders. In a number of species, such as potato (Shepard *et al.*, 1980), sugar-cane (Krishnamurthi and Tlaskal, 1974), tobacco (Popchristov and Zaganska, 1977) and rice (Oono, 1978), it has been observed that genetic variation *in vitro* has given rise to variant plants with desirable agronomic characteristics. The possible implication of an adventitious regeneration procedure cannot be properly determined until the degree of genetic stability inherent in the regeneration process has been assessed.

## 1.3. *Plant regeneration studies with Capsicum species*

Although, up to date, many plants of economic and horticultural value have been successfully propagated through tissue culture techniques, little work has been done with *Capsicum* species. *Capsicum* which is among the important crops worldwide is highly cross-pollinated and thus deteriorates fast. Some of the developed $F_1$ hybrid seeds are expensive as compared to open-pollinated seeds because these seeds are produced by manual pollination. The hybrid plants on the other hand are vigorous, extremely productive and produce an early crop. Thus, it is reasonable to explore the techniques of tissue and organ culture as a possible means of vegetative multiplication on a large scale.

Wang *et al.* (1973), George and Narayanaswamy (1973) and Dumas De Vaulx *et al.* (1981) reported regeneration of haploid plants from anther culture of *Capsicum*. Saxena *et al.* (1981) described a procedure for plant regeneration from mesophyll protoplasts produced from 15-day-old leaves of seedlings of cv. "California Wonder". Callus was produced from protoplasts cultured in Nagata and Takebe medium (1971) with 1 mg $l^{-1}$ each 2,4-D, NAA, and BAP, 2% sucrose and 0.5M mannitol. The protoplasts were incubated in the dark for 15 days and then returned to the standard 16 hr photoperiod for shoot regeneration. Shoots were regenerated by transferring the callus to MS medium supplemented with 4 mg $l^{-1}$ IAA, 2.56 mg $l^{-1}$ kinetin and 3% sucrose. Root regeneration occurred when shoots were finally transferred to medium with 1 mg $l^{-1}$ IAA and 0.04 mg $l^{-1}$ kinetin.

Gunay and Rao (1978) reported plant regeneration from seedling cotyledon and hypocotyl explants of *Capsicum annuum* cultured on MS medium supplemented with BA. Shoot regeneration occurred sporadically

in presence of zeatin but not at all in presence of kinetin or adenine. The combination of 1 mg $l^{-1}$ IAA and 2 mg $l^{-1}$ BAP was the best for shoot-bud formation but detailed quantitative data were not presented. Phillips and Hubstenberger (1985), working at New Mexico simultaneously with the present study, reported more detailed data from *Capsicum* tissue cultures. According to them, shoot and root regeneration in cultures of seedling explants was restricted to the primary (initial) cultures or to cultures less than three months old grown under a 12- and 16-hr photoperiod at 25 °C. Shoot organogenesis, under continuous light, was extended to 5 months at 25 °C and 8 months under continuous light at 28.5 °C. MS media supplemented with 0.05 mg $l^{-1}$ each of IAA and BAP promoted shoot elongation and rooting of some explant sources, while 0.05 - 4 mg $l^{-1}$ IAA with 10-50 mg $l^{-1}$ BAP promoted adventitious shoot bud formation. Glucose was superior to sucrose as the carbon source. Leaf discs from greenhouse-grown plants regenerated shoots for at least 2 months.

The main conclusion drawn from this literature review is that the auxin IAA and the cytokinin BAP were the most important growth regulators for adventitious plant regeneration. Also it is apparent that the most successful source of explants has been organs or tissues from seedlings. There has been no report concerning either the use of embryo tissue explants or the achievement of somatic embryogenesis.

The work described in this thesis evaluates the potential for organogenesis and embryogenesis in tissue cultures of a wide range of explant sources as well as the response of a range of *Capsicum* cultivars including bell and elongated, sweet and chili types.

Tissues or organs from mature zygotic embryos as well as the whole immature zygotic embryos at different developmental stages and juvenile parts of plants were used as explants throughout this work for the reason that such tissues, it is well known, respond better *in vitro*. Also, apart from IAA, other auxins and specially 2,4-D as well as a wide range of cytokinins were introduced to culture media to test their effect on embryogenesis and organogenesis. Gibberellins, abscisic acid as well as antiauxins such as TIBA were also tested. The effect on the morphogenic response of different basal media such as MS, B5 and SH as well as a wide range of cultural conditions such as the light intensity, the light spectrum, temperature regime were studied too.

The aim of this work was to explore the possibility of establishing a tissue culture procedure in *Capsicum* which, apart from its theoretical value, could be a useful tool in hands of breeders and horticulturists. Such a successful tissue culture procedure either by organogenesis or by somatic embryogenesis - "artificial seeds" - could be applied to produce:

1. A large number of extremely productive plants of $F_1$ hybrids or cultivars in a short period of time at a reasonable low price, contributing thus to squeeze the cost of a crop's establishment.
2. A large number of disease-free and/or virus-free plants.
3. A large number of plants furnished with some desirable agronomic characteristics such as low-temperature tolerance, salt-resistance (Dix and Street, 1975), disease-resistance. All these characteristics are of a great importance especially incrops grown in greenhouses where the energy supply contributes to the cost of

the product and the environment is almost ideal for the spread of a disease and the build-up of the soil salt concentration.

4. Desirable "clones" with high productivity due to somaclonal variation especially from cell and callus cultures.

# CHAPTER II

# MATERIAL AND METHODS

## 2.1 *Plant material*

### 2.1.1 *Seeds*

Seeds (Plate 2.1) of the following cultivars of *Capsicum annuum* L. were obtained from seed companies as indicated in Table 2.1.

Table 2.1. *Capsicum annuum* cultivars that were used; their type of fruit and the companies from which they have been obtained.

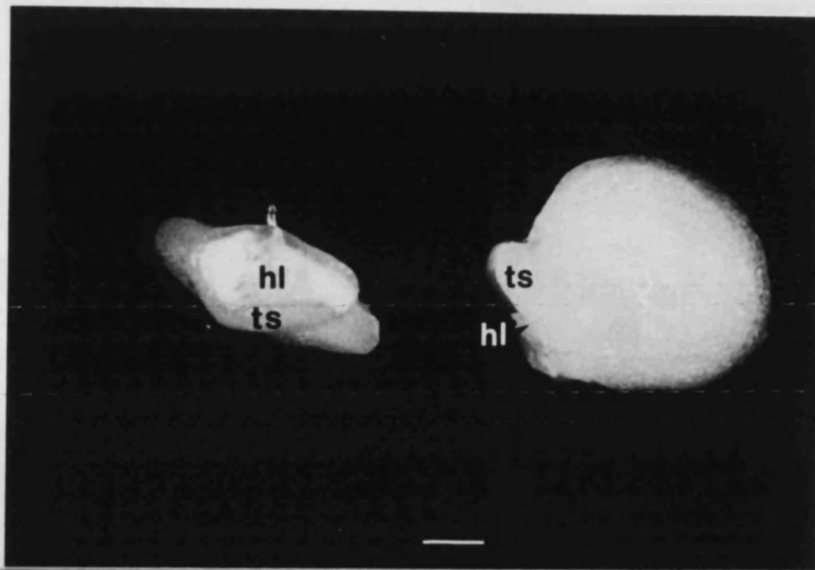
| Cultivar | Type of fruit | Company |
|---|---|---|
| Bell Boy $F_1$ hybrid | bell sweet | Elson seeds Ltd., UK. |
| California Wonder | " " | " " " " |
| Worldbeater | " " | Suttons seeds Ltd., UK. |
| Propenza | " " | Enza Zaden, Holland. |
| Hybelle $F_1$ hybrid | " " | Harris seeds Co., USA. |
| Pennbell | " " | " " " " |
| Pt-824 | " " | Bank of genetic material, Salonica, Greece. |
| Pt-452 | " " | |
| Pt-455 | " " | |
| Pt-14 | elongated sweet | Bank of genetic material, Salonica, Greece. |
| Pt-46 | " " | |
| 678 Sweet Banana | " " | Harris seeds Co., USA. |
| Pt-53/13 | elongated chili | Bank of genetic material, Salonica, Greece. |
| 655 Jalapeno | " " | Harris seeds Co., USA. |
| 675 Red Chili | " " | " " " " |
| Tomato Pimento 269A | " " | Stokes seeds Inc., USA. |

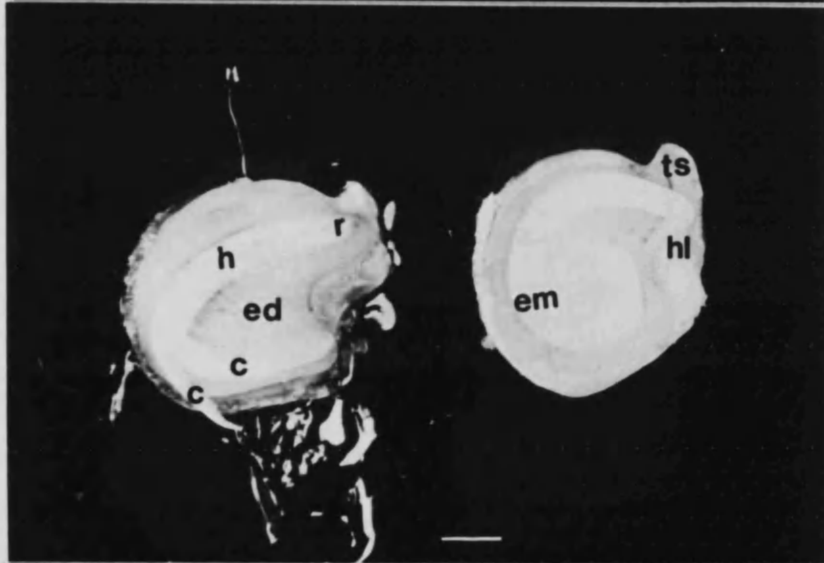
Plate 2.1. Morphology and anatomy of *Capsicum annuum* seeds.

A. Photograph of side and large surface of seed showing testa (ts) and hilum (hl). Scale bar = 1 mm.

B. Section of seed parallel to large surface showing the testa (ts), endosperm (ed), embryo (em) and the regions of embryo: cotyledon (c), hypocotyl (h) and radicle (r).
Scale bar = 1 mm.

C. As above. Scale bar = 1 mm.


hl
ts
ts
hl


r
h
ed
c
c
ts
hl
em

2.1.2. *Mature embryos*

Mature zygotic embryos (Plate 2.1. B & C and Plate 2.2. A) were excised from dry seeds of the above cultivars, under aseptic conditions and the following parts were used as explants: a) the whole embryos, b) cotyledons, c) hypocotyl, d) embryonic apex and e) radicles (Plate 2.1. B & C and Plate 2.2. A.).

2.1.3. *Plants*

The plants, while were kept in greenhouse at a minimum day temperature of 22 °C and night temperature of 20 °C, were fed once or twice a week, and subjected to a minimum 12-hour photoperiod with natural lighting, supplemented artificially with white lamps when necessary.

2.1.3.1. *Vegetative plant material*

The following vegetative parts of plants were used as explants: a) 35-50 $mm^2$ discs taken from fully expanded leaves, b) petioles from fully expanded leaves, c) 3-10 mm long young leaves, d) shoot tips, e) stem nodes with their axillary buds with or without their leaf, f) internodal segments 4-6 mm long, g) hypocotyls, cotyledons and petioles of cotyledons which have been taken from up to 30-day-old seedlings grown either under greenhouse conditions or aseptically on agar gel. (Plate 2.2. B & C).

2.1.3.2. *Flower parts*

Anthers, styles, ovaries, placentas with ovules attached

Plate 2.2. Material

A. Immature embryos at various developmental stages:

1. Immature embryo at developmental stage I
2. " " " " " II
3. " " " " " III
4. " " " " " IV
5. " " " " " V

B. lf: young leaf explants

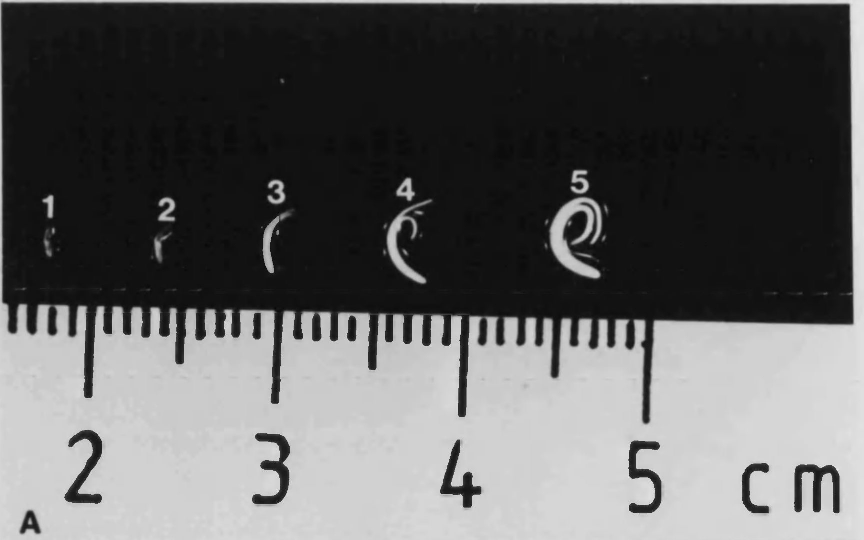
1. young leaf explant ca 1 mm long
2. " " " " 4 " "
3. " " " " 6 " "
4. " " " " 8 " "
5. " " " " 20 " "

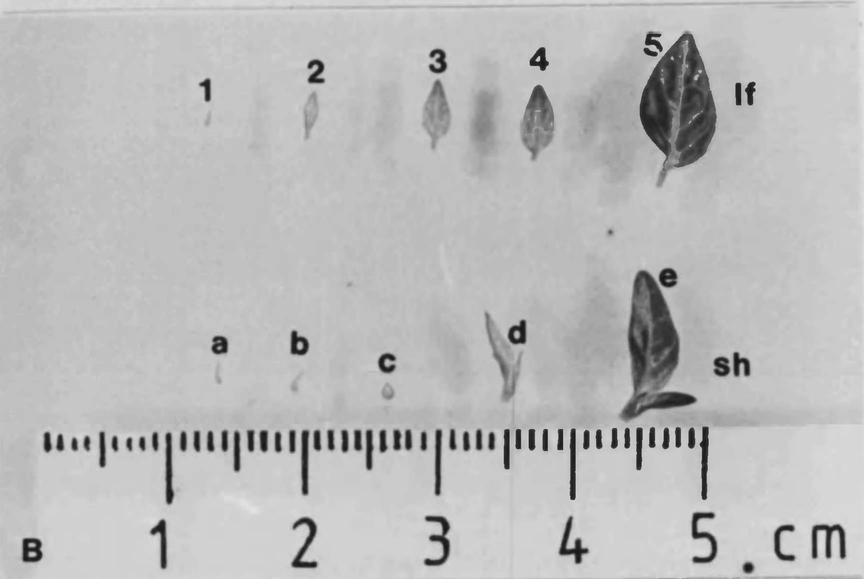
sh: shoot-tip explants

a. shoot tip explant with 1 leaf visible by naked eye
b. " " " " 2 leaves " " " "
c. " " " " 2 " " " " and possessed flower bud
d. shoot tip explant with 3 leaves visible by naked eye
e. " " " " 4 " " " " "

C. Cotyledon and hypocotyl explants

1. hypocotyl from seed-embryo
2. " " 15-day-old seedling
3. cotyledon from seed embryo
4. " " 10-day-old seedling
5. " " 18-day-old "
6. " " 28-day-old "


1
2
3
4
5
2
3
4
5 cm
A


1
2
3
4
5
lf
a
b
c
d
e
sh
B
1
2
3
4
5 cm

1
2
2
6
6
3
3
4
4
5
5
C
1
2
3
4
5 cm

and ovary walls were taken from unopened flowers and were used as explants.

2.1.3.3. *Immature embryos*

Immature fruits were harvested approximately a month after pollination and immature embryos were taken out to be used as explants as follows: a) whole immature embryos, b) cotyledons and c) hypocotyls. (Plate 2.2. A). Each fruit contained many immature seeds which possessed immature embryos of different developmental stages. The immature embryo stages were classified as follows:

| Stage | Embryo length (mm) | Embryo appearance | Endosperm appearance |
|---|---|---|---|
| I | < 0.5 | glassy | watery |
| II | 0.5 - 1.5 | glassy | milky |
| III | 1.5 - 2.5 | white-glassy | thick milk |
| IV | 2.5 - 4.5 | white | thick yogurt |
| V | > 4.5 | white | white solid soft |
| VI (mature) | > 4.5 | white | creamy solid hard |

2.2. *Sterilization*

Sterilization and all subsequent *in vitro* procedures for both plant and seed material were performed in a sterile environment provided by a laminar flow cabinet (Microflow Pathfinder Ltd., Fleet, Hampshire). Plant tissue and seeds were surface sterilized with a commercial sterilant "DOMESTOS" or sodium hypochlorite solution (available chlorine 8%), at the final concentrations indicated

Table 2.2 Preparation of explants

| Donor plant material | Explant | Sterilization procedure for explant source material | |
|---|---|---|---|
| | | Sterilant and its concentration (v/v) | Duration of sterilization (min) |
| 1 | 2 | 3 | 4 |
| Mature seeds (seeds were soaked for 24 hours before sterilization) | 1. The whole embryo<br>2. Cotyledon<br>3. Hypocotyl<br>4. Radicle<br>5. Embryo apex | Sodium hypochlorite 10%-20% | 12-20 |
| Immature seeds of various developmental stages | 1. The whole immature embryo<br>2. Cotyledon<br>3. Hypocotyl | No sterilization was necessary | |
| Mature plants | Shoot-tips (0.3-7 mm long apical meristem with 1-3 leaf primordia) | Sodium hypochlorite or "DOMESTOS" 7% | 12-15 |
| Mature plants | 35-50 $mm^2$ leaf discs from fully expanded leaves | Sodium hypochlorite or "DOMESTOS" 7% | 12-15 |
| Mature plants | 3-10 mm long young leaves | Sodium hypochlorite or "DOMESTOS" 7% | 12-15 |
| Up to 30-day-old axenic seedlings | 1. Cotyledon<br>2. Hypocotyl<br>3. Petiole of cotyledon | No sterilization was necessary | |

Cont/...

Table 2.2 continued

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Up to 30-day-old seedlings grown in greenhouse | 1. Cotyledon<br>2. Hypocotyl<br>3. Petiole of cotyledon | Sodium hypochlorite or "DOMESTOS" 7% | 12-15 |
| Unopened flowers (3-5 mm long) | 1. Anther<br>2. Style<br>3. Ovary<br>4. Placenta with ovules<br>5. Ovary wall | "DOMESTOS" 7% | 10-12 |
| Axenic root cultures | Root-tips or lateral root-tips 7-8 mm long | No sterilization was necessary | |

in Table 2.2., and supplemented with one drop per 100 ml final solution of "Tween 80" (B.D.H. Chemicals Ltd.) as wetting agent. After sterilization explants were washed four to five times with sterile distilled water. Sterilization procedures were carried out in 100 or 150 ml polystyrene containers for plant material or in 60 ml polystyrene vials (Sterilin Limited, Teddington) for seeds. Explants were removed from plant parts or seed tissues using an Olympus stereomicroscope and sterile tools (forceps, scalpel blades and hypodermic needles). Details of the type, sterilization and source of explants are shown in Table 2.2.

## 2.3. *Culture media*

Unless otherwise indicated, explants were cultured on three different basal media: a) (MS) Murashige and Skoog (1962), b) (B5) Gamborg *et al.* (1968), and c) (SH) modified Schenk and Hildebrandt (1972). Details of the composition of the above basal media are shown in Tables 2.3., 2.4. and 2.5. Basal medium supplemented with various combinations of growth regulators, including auxins (2,4-D, IAA, IBA, NAA), cytokinins (BAP, zeatin, kinetin, adenine sulphate), gibberellic acid ($GA_3$), abscisic acid (ABA), and the antiauxin TIBA. Each basal medium was prepared from stock solutions (Tables 2.3., 2.4. and 2.5.), and MS medium was also obtained in powder form from Flow Laboratories (Irvine, Scotland). The medium was made up with double distilled water. Heat-stable plant growth regulators as well as other compounds were added before autoclaving, while heat liable compounds such as IAA and $GA_3$ were filter-sterilized through a membrane filter of pore size 0.2 μm (Sartorius, U.K.) and added after autoclaving.
Unless the effect of pH was being tested all media were adjusted to pH 5.7 by using 0.1M sodium hydroxide or 0.1M hydrochloric acid. When semi-solid medium was required, 0.8% (w/v) agar (Oxoid Technical Grade 3) was added after pH adjustment. Media were autoclaved for 18-20 minutes at 15 lb $in^{-2}$ (1.87 bar) at 120 °C. Semi-solid media were stored at room temperature and used within one week of preparation, while liquid media were used within 48 hours.

## 2.4. *Culture vessels*

Explants were cultured on 20 ml semi-solid medium in 9cm diameter plastic petri dishes or 10 ml semi-solid medium in 5cm diameter plastic dishes (Sterilin Ltd.). Petri dishes were sealed with parafilm M (American Can Company).

When liquid medium was used, explants were supported by a 1x7 cm filter paper wick (Whatman No 3) folded into an inverted 'U' shape to form a bridge at the bottom of the 16 x 125 mm test tube (Corning Class Works, U.S.) into which 4 ml aliquots of liquid medium were dispensed by a glass plunger syringe and cannula (Arnold and Howell Ltd.). Test tubes were sealed with polypropylene caps (Bacti-Capell, C.L. Clark and Co.).

### 2.5. *Culture incubation conditions*

Explants in petri dishes were stacked on top of each other (up to 5) on shelves. Unless the experiment required a lower or higher temperature, cultures were kept under a 25 °C temperature regime in Gallenkamp incubators (A Gallenkamp and Co. Ltd., London). Illumination was provided by warm white 65-80 watt fluorescent side-lamps installed in pairs at three levels (Thorn EMI lighting Ltd., London) with a 16-hour photoperiod [500 lux or 10.2 microeinsteins $m^{-2}sec^{-1}$ ($\mu E\ m^{-2}sec^{-1}$) in the senter of the shelves]. The above described incubation conditions are referred to as "standard conditions". Exceptionally, mature and immature embryo explants were kept in darkness during the first 5-10 days of culture. Regenerated *in vitro* shoots were kept at 20 °C temperature until they rooted. Regenerated *in vitro* plantlets, when ready, were washed carefully to remove the agar from their roots and were transplanted into pots filled with Levington potting compost. The first 5 days after transplantation, plants were covered by transparent plastic covers for establishing high relative humidity and the whole device including the plants was kept at room temperature (18-20 °C). During this period, new roots were regenerated from the plantlets which facilitated the plant establishment in pots. Then plants were uncovered and removed to greenhouse for further development.

Table 2.3 Murashige and Skoog (1962) (MS)* medium: concentration of final complete medium, and of stock solution.

| Compound | Complete medium (mg $l^{-1}$) | | Stock solution (g $l^{-1}$) |
|---|---|---|---|
| $CaCl_2.2H_2O$ | 440 | | 17.6 |
| $KNO_3$ | 1900 | Stock A | 76.0 |
| $NH_4NO_3$ | 1650 | (x40) | 66.0 |
| $KH_2PO_4$ | 170 | | 6.8 |
| $MgSO_4.7H_2O$ | 370 | Stock B | 14.8 |
| $MnSO_4.4H_2O$ | 22.3 | (x40) | 0.89 |
| $ZnSO_4.7H_2O$ | 8.6 | | 1.720 |
| $H_3BO_3$ | 6.2 | | 1.240 |
| KI | 0.83 | Stock C | 0.166 |
| $CuSO_4.5H_2O$ | 0.025 | (x200) | 0.005 |
| $Na_2MoO_4.2H_2O$ | 0.25 | | 0.05 |
| $CoCl_2.6H_2O$ | 0.025 | | 0.005 |
| $Na_2EDTA.\ 2H_2O$ | 37.3 | Stock D | 7.46 |
| $FeSO_4.7H_2O$ | 27.8 | (x200) | 5.56 |
| Glycine | 2.0 | | 0.20 |
| Nicotinic acid | 0.5 | Stock E | 0.05 |
| Thiamine-HCl | 0.1 | (x100) | 0.01 |
| Pyridoxine-HCl | 0.5 | | 0.05 |
| Sucrose | 30,000 | | |
| myo-inositol | 100 | | |

*The above complete medium without sucrose is called "basal MS medium". The sucrose concentration of the medium is indicated by a number after the abbreviation, e.g.: MS2 means MS basal medium supplemented with 2% (w/v) sucrose.

Table 2.4 Gamborg *et al.*(1968) (B5)* medium: concentration of final complete medium, and of stock solution

| Compound | Complete medium (mg $l^{-1}$) | | Stock solution (g $l^{-1}$) |
|---|---|---|---|
| $CaCl_2.2H_2O$ | 150 | | 6.0 |
| $KNO_3$ | 2500 | Stock A | 100.0 |
| $(NH_4)_2SO_4$ | 134 | (x40) | 5.36 |
| $NaH_2PO_4$ | 150 | | 6.0 |
| $MgSO_4.7H_2O$ | 250 | Stock B | 10.0 |
| $MnSO_4.4H_2O$ | 10 | (x40) | 0.4 |
| $ZnSO_4.7H_2O$ | 2 | | 0.4 |
| $H_3BO_3$ | 3 | | 0.6 |
| KI | 0.75 | Stock C | 0.15 |
| $CuSO_4.5H_2O$ | 0.025 | (x200) | 0.005 |
| $Na_2MoO_4.2H_2O$ | 0.25 | | 0.05 |
| $CoCl_2.6H_2O$ | 0.025 | | 0.005 |
| $Na_2EDTA.2H_2O$ | 37.3 | Stock D | 7.46 |
| $FeSO_4.7H_2O$ | 27.8 | (x200) | 5.56 |
| Nicotinic acid | 1 | Stock E | 0.1 |
| Thiamine-HCl | 10 | (x100) | 1.0 |
| Pyridoxine-HCl | 1.0 | | 0.1 |
| Sucrose | 30,000 | | |
| myo-inositol | 100 | | |

*The above complete medium without sucrose is called "basal B5 medium". The sucrose concentration of the medium is indicated by a number after the abbreviation, e.g.: B5 2 means B5 basal medium supplemented with 2% (w/v) sucrose.

Table 2.5 Modified Schenk & Hildebrandt (1972) (SH)* medium: concentration of final complete medium and of stock solution.

| Compound | Complete medium ($mg\ l^{-1}$) | | Stock solution ($g\ l^{-1}$) |
|---|---|---|---|
| $CaCl_2.2H_2O$ | 200 | | 8.0 |
| $KNO_3$ | 2500 | Stock A | 100.0 |
| $NH_4H_2PO_4$ | 300 | (x40) | 12.0 |
| $MgSO_4.7H_2O$ | 400 | Stock B | 16.0 |
| $MnSO_4.4H_2O$ | 10 | (x40) | 0.4 |
| $ZnSO_4.7H_2O$ | 1 | | 0.2 |
| $H_3BO_3$ | 5 | | 1.0 |
| KI | 1 | Stock C | 0.2 |
| $CuSO_4.5H_2O$ | 0.2 | (x200) | 0.04 |
| $Na_2MoO_4.2H_2O$ | 0.1 | | 0.02 |
| $CoCl_2.6H_2O$ | 0.1 | | 0.02 |
| $Na_2EDTA.2H_2O$ | 37.3 | Stock D | 7.46 |
| $FeSO_4.7H_2O$ | 27.8 | (x200) | 5.56 |
| Nicotinic acid | 5 | Stock E | 0.5 |
| Thiamine-HCl | 5 | (x100) | 0.5 |
| Pyridoxine-HCl | 0.5 | | 0.05 |
| Sucrose | 20,000 | | |
| myo-inositol | 1.000 | | |
| Coconut milk | 10% (v/v) | | |

*The above complete medium without sucrose is called "basal SH medium". The sucrose concentration of the medium is indicated by a number after the abbreviation, e,g,: SH2 means SH basal medium supplemented with 2% (w/v) sucrose.

## 2.6. *Histology*

A modified version of Johansen's method (Johansen, 1940) was used. Specimens were fixed in FAA (formalin: acetic acid: 70% ethanol, 90:5:5, v/v/v) for at least twenty four hours and dehydrated by passing through a tertiary butyl alcohol TBA, (2-methylpropan-2-ol) series (Appendix 1). Specimens were then passed through a saturated solution of erythrosin in 100% TBA to make them more conspicuous before they were embedded in paraffin wax with ceresin (m.p. 56 °C, BDH Chemicals Ltd). 10 μm thick sections of the wax-embedded specimens were cut with a rotary microtome (Reichert, Austria).
Sections, in a ribbon form, were floated on warm water, to allow expansion, before fixation to glass slides with Haupt's adhesive where they were again encouraged to expand by the addition of a 3% (v/v) formalin solution. Sections, while on glass slides, were dewaxed by passing the slides through a xylene: ethanol series, then they were stained in a toluidine blue solution (0.05 g in 100 ml distilled water) and were dehydrated in an ethanol series as was described by Feder and O'Brien in 1968 (Appendix 1). Slides were made permanent using canada balsam (Hopkin and Williams, Essex) or D.P.X. mountant (BDH Chemicals Ltd., England) and viewed under an Olympus $BH_2$ (Japan) light microscope.

## 2.7. *Preparation for scanning electron microscope (SEM) studies*

Specimens were fixed overnight at 5 °C in 5% glutaraldehyde (one part glutaraldehyde (25%) to four parts 0.1M potassium dihydrogen orthophosphate ($KH_2PO_4$) buffer (pH 6.8). Specimens were washed three times in distilled water and passed through an acetone series (20%, 30% 40%, 50%, 70%, 90%, 95%, 100%), each step taking at least one hour.

Specimens were passed through three pure acetone stages and were dehydrated by bathing in liquid carbon dioxide for two to three hours in a critical point drying apparatus. When dehydrated, the tissues were gold plated and viewed under the scanning electron microscope.

## 2.8. *Chromosome counts*

### 2.8.1. *A version of the method of Al-Abta and Collin*

Chromosome counts were made in root tips by using a modified version of the method of Al-Abta and Collin (1978). The root tips were pretreated in 0.002M 8-hydroxy-quinoline for 3-4 hours at room temperature, fixed in a 3:1 ethanol: acetic acid mixture for at least 24 hours, washed with distilled water, hydrolized in a 1M HCl at 60 °C for 10 minutes and washed again with distilled water. Then they were immersed in Schiff's reagent (Fuchsin-sulphite reagent, Sigma Chemical Co.) for at least 90 minutes, washed with distilled water, washed with $SO_2$ water (500 mg $K_2S_2O_5$ : 5 ml 1M HCl : 95 ml water) and placed in 45% acetic acid for at least 1 hour. Root tip squashes were prepared in a drop of acetocarmine solution [45 ml glacial acetic acid + 55 ml distilled water + 0.5 g iron acetocarmine (Sharma and Sharma, 1972)] and cells were dispersed by tapping the coverslip with the end of a needle. The slide was warmed for a few seconds by passing quickly over the flame of Bunsen burner and the preparations were examined under a 100X oil immersion objective lens of an Olympus BH2 (Japan) light microscope.

### 2.8.2. *Xue's method*

Chromosome counts were made in root tips by using a method developed by Xue (personal communication). This method was more suitable for chromosome counting (better chromosome view) than the method described by Al-Abta and Collin (1978), at least concerning the genus *Capsicum*.

#### 2.8.2.1. *Preparation of Carbol fuchsin stain*

*Solution A.* 3 g of basic fuchsin was dissolved in 100 ml of 70% ethanol.

*Solution B.* 10 ml of solution A plus 90 ml of 5% phenol. This solution should be used within two or three hours.

*Solution C.* 55 ml of solution B plus 6 ml of glacial acetic acid plus 6 ml of 3% formaldehyde.

*Solution D.* 1.8 g sorbitol in 2-10 ml of solution C and 90-98 ml of 45% acetic acid.

#### 2.8.2.2. *Procedure for chromosome counts*

The root tips were pretreated at low temperature of 2-4 °C for at least 48 hours, washed three to four times with tap water, fixed in a freshly made 3:1 ethanol: acetic acid mixture at room temperature for 24-48 hours, hydrolized in a 1:1 ethanol: 35% HCl mixture for 2-4 minutes at room temperature and washed with tap water three or four times. Then the root tips squashes were prepared in a drop of solution D and cells were dispersed by tapping the coverslip with the end of a needle. Finally the preparations were examined under a 100X oil immersion objective lens of an Olympus BH.2 (Japan) light microscope.

### 2.9. *Photography*

Photographs were taken with an Olympus BH2 light microscope, a Zeiss Tessovar microscope (W. Germany) and Olympus and Miranda (Japan) 35 mm SLR cameras fitted with various lenses and extension rings. Films used included, Ilford 'Pan F' (32 ASA & 50 ASA) and 'FP4' (125 ASA) black and white (Ilford Ltd., Ilford, England) and Kodak 'Ektachrome' (50 ASA) colour-tungsten and kodachrome (64 ASA) colour-daylight (Eastman Kodak Co., Rochester, U.S.A.).

Black and white negatives were developed in "Acutol" (Paterson Products Ltd., England) and fixed in "Kodafix" (Eastman Kodak Co.) and printed on Kodak "Veribrom", 'Kodabrom', and 'Bromide' papers.

### 2.10. *Leaf area measurement*

Leaves of the 1st branch were excised and their lamina´s surface area was measured by using a SCIENTIFIC INSTRUMENTS area meter.

### 2.11. *Statistics*

For the statistical analysis of the experiments the Duncan´s new multiple range test was used (Duncan, 1955). Data within a column or a row followed by the same letter are not significantly different at the 0.05 level of probability.

# CHAPTER III

## ADVENTITIOUS SHOOT-PLANT REGENERATION FROM EMBRYONIC TISSUES OR SEEDLING COTYLEDONS

*Introduction*

Based on the results of the preliminary investigations a series of experiments was set up with the aim to find the most suitable medium and culture conditions for adventitious shoot regeneration for the development of the shoots to form stems and for their rooting to achieve complete plants. Generally a three-stage procedure was adopted: an initial stage in which the medium was supplemented with a cytokinin for bud initiation; a second stage (1st subculture) in which the medium was supplemented with GA and a cytokinin for subsequent shoot development; a third stage in which an auxin was added for rooting of the stems.

### *3.1. The effect of IAA or BAP or their combination on adventitious bud-shoot initiation.*

A series of 192 MS3 basal media supplemented with 1.75, 5.28 and 8.75 mg $l^{-1}$ IAA or 2.25, 4.5 and 9.0 mg $l^{-1}$ BAP alone or in their combinations were tested in two stage culture (Table 3.1.1). Embryonic cotyledon and hypocotyl explants of cv. "California Wonder" were inoculated onto the above media and cultured on a 25 °C temperature regime. During the period of the first eight days the cultures were kept in complete darkness and after that they were exposed to light (10.2 microeinsteins $m^{-2}sec^{-1}$) with a 16-hour photoperiod. Explants cultured initially on hormone-free medium or on that supplemented with the auxin IAA at any concentration did not form adventitious buds during their incubation time either in the first or second stage, but some explants regained their ability to initiate adventitious buds when they were subcultured on media supplemented

Table 3.1.1. Percentage of cv. "California Wonder" embryonic cotyledon explants forming adventitious buds and foliose structures in 28-day-old cultures and subjected to MS3 medium, temperature of 25 °C, complete darkness for the first 5 days and then standard light conditions.

n = 35-40.

| BAP concentration ($mg\ l^{-1}$) Stage I | BAP concentration ($mg\ l^{-1}$) Stage II | IAA concentration ($mg\ l^{-1}$) Stage I: 0 | | | | 1.75 | | | | 5.25 | | | | 8.75 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Stage II: 0 | 1.75 | 5 25 | 8.75 | 0 | 1 75 | 5.25 | 8.75 | 0 | 1.75 | 5.25 | 8.75 | 0 | 1.75 | 5.25 | 8.25 |
| 0 | 0 | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| | 2.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4.5 | 0 | 0 | 33 | 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 9.0 | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| 2.25 | 0 | 20 | 0 | - | - | 67 | 17 | - | - | 83 | 80 | - | - | 67 | 0 | - | - |
| | 2.25 | 100 | 100 | 86 | 57 | 83 | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 83 | 100 | 80 | 100 |
| | 4.5 | 100 | 100 | 80 | 71 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 67 | 33 |
| | 9.0 | 100 | 90 | - | - | 100 | 33 | - | - | 100 | 33 | - | - | 100 | 50 | - | - |
| 4.50 | 0 | 0 | 0 | - | - | 17 | 33 | - | - | 67 | 33 | - | - | 50 | 17 | - | - |
| | 2.25 | 100 | 100 | 100 | 86 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 71 | 50 |
| | 4.5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 33 | 33 |
| | 9.0 | 100 | 86 | - | - | 100 | 50 | - | - | 100 | 33 | - | - | 100 | 0 | - | - |
| 9.00 | 0 | 0 | 0 | - | - | 75 | 0 | - | - | 0 | 75 | - | - | 67 | 0 | - | - |
| | 2.25 | 100 | 100 | 86 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 80 |
| | 4.5 | 100 | 100 | 86 | 71 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 75 |
| | 9.0 | 100 | 100 | - | - | 100 | 60 | - | - | 100 | 100 | - | - | 100 | 33 | - | - |

with 2.25 or 9.0 mg $l^{-1}$ BAP in the presence or absence of 1.75 mg $l^{-1}$ IAA (Tables 3.1.1 and 3.1.2). Explants inoculated initially on medium containing 2.25, 4.5 or 9.0 mg $l^{-1}$ BAP as a sole hormone or in combination with 1.75, 5.25 or 8.75 mg $l^{-1}$ IAA formed many adventitious buds with the percentage of explants forming buds and the number of buds per each explant being reduced as the IAA concentration became higher. Also with the higher the IAA concentrations the growth rate of the buds was lower. There was no significant difference in bud initiation with various BAP concentrations (Tables 3.1.1, 3.1.2 and 3.1.3), but the best growth rates of buds and foliose structures were achieved in media supplemented with 2.25 or 4.5 mg $l^{-1}$ BAP. Foliose structures were green leaf-like structures having an abnormal epidermal cell arrangement, without stomata and petiole. Bud and foliose structure formation was a combined process.

Media containing 9.0 mg $l^{-1}$ BAP as a sole hormone or in combination with 1.75, 5.25 or 8.75 mg $l^{-1}$ IAA favoured the fast growth of callus which in a period of 20 to 25 days overgrew the buds that finally degenerated.

There were some differences among the hypocotyl and cotyledon explants concerning their response to the media. First, hypocotyl explants subjected to a medium containing 2.25, 4.5 or 9.0 mg $l^{-1}$ BAP at stage I, when they were subcultured at day 13 to a hormone-free medium or to a medium containing 1.75 mg $l^{-1}$ IAA alone, formed buds and foliose structures which were visible on 28-day-old cultures. Cotyledons, treated similarly, did not react this way (Tables 3.1.1 and 3.1.2). It can therefore be suggested that either the buds and foliose structures were initiated earlier on hypocotyl than on cotyledon explants or that the process was initiated at the same time on both types of the explants but the cotyledons need to be subjected to BAP containing

Table 3.1.2. Percentage of cv. "California Wonder" embryonic hypocotyl explants forming adventitious buds and foliose structures when they were subjected to MS3 medium and cultured for 28 days under standard conditions except the first 5 days when complete darkness was employed. n = 70-80.

| BAP concentration (mg $l^{-1}$) Stage I | BAP concentration (mg $l^{-1}$) Stage II | IAA concentration (mg $l^{-1}$) Stage I: 0 | | | | 1.75 | | | | 5.25 | | | | 8.75 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stage II: | 0 | 1.75 | 5 25 | 8.75 | 0 | 1 75 | 5.25 | 8.75 | 0 | 1.75 | 5.25 | 8.75 | 0 | 1.75 | 5.25 | 8.25 |
| 0 | 0 | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| | 2.25 | 0 | 25 | 83 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4.5 | 0 | 25 | 50 | 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 9.0 | 33 | 50 | - | - | 29 | 20 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| 2.25 | 0 | 100 | 100 | - | - | 20 | 20 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| | 2.25 | 100 | 100 | 100 | 100 | 80 | 75 | 100 | 100 | 100 | 100 | 33 | 33 | 67 | 83 | 0 | 33 |
| | 4.5 | 80 | 75 | 100 | 100 | 80 | 50 | 100 | 50 | 100 | 50 | 33 | 33 | 85 | 50 | 0 | 0 |
| | 9.0 | 100 | 100 | - | - | 100 | 0 | - | - | 100 | 0 | - | - | 60 | 80 | - | - |
| 4.50 | 0 | 100 | 80 | - | - | 20 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| | 2.25 | 100 | 100 | 100 | 100 | 83 | 100 | 100 | 100 | 75 | 75 | 29 | 0 | 100 | 75 | 14 | 0 |
| | 4.5 | 100 | 100 | 100 | 100 | 83 | 100 | 100 | 83 | 100 | 100 | 67 | 33 | 80 | 50 | 0 | 0 |
| | 9.0 | 100 | 100 | - | - | 100 | 100 | - | - | 100 | 100 | - | - | 80 | 0 | - | - |
| 9.00 | 0 | 0 | 67 | - | - | 20 | 0 | - | - | 0 | 0 | - | - | 0 | 0 | - | - |
| | 2.25 | 100 | 100 | 100 | 100 | 100 | 100 | 50 | 67 | 33 | 83 | 33 | 100 | 86 | 33 | 0 | 0 |
| | 4.5 | 100 | 100 | 100 | 100 | 80 | 50 | 100 | 100 | 83 | 100 | 33 | 40 | 100 | 83 | 0 | 0 |
| | 9.0 | 100 | 0 | - | - | 100 | 0 | - | - | 100 | 0 | - | - | 100 | 83 | - | - |

medium for a longer time to allow further growth of buds and foliose structures. Second, hypocotyl explants subjected to a medium containing high IAA concentration (5.25 or 8.75 mg $l^{-1}$ ) at stage I, when they were transferred, at day 13, to a medium containing the same high IAA concentrations in combination with 2.25, 4.5 or 9.0 mg $l^{-1}$ BAP, showed a smaller frequency of bud and foliose structure formation than did cotyledon explants treated similarly (Tables 3.1.1 and 3.1.2).

Embryonic cotyledon and hypocotyl explants of cv. "Red Chili" were subjected to MS3 basal medium containing 2.25, 4.5 and 9.0 mg $l^{-1}$ or 1.75, 5.25 and 8.75 mg $l^{-1}$ or all the possible combinations of the above BAP and IAA concentrations and cultured under the same temperature and light conditions, as previous described with cv. "California Wonder" explants, except that the cultures lasted only 8 days of which the first five days they were kept in complete darkness and then they were exposed to light (10.2 microeinsteins $m^{-2}$ $sec^{-1}$) with 16-hour photoperiod.

From the table 3.1.3 it is apparent that bud and foliose structure formation did not occur on hypocotyl or cotyledon explants subjected to hormone-free medium or to medium containing 1.75, 5.25 or 8.75 mg $l^{-1}$ IAA alone. These media favoured adventitious root formation mostly from hypocotyl and rarely from cotyledon explants. The higher the IAA concentrations the less hypocotyl explants formed roots. Bud and foliose structure formation took place only on cotyledon and hypocotyl explants subjected to media containing BAP alone at concentration of 2.25, 4.5 and 9.0 mg $l^{-1}$or in combination with IAA at concentration of 1.75, 5.25 and 8.75 mg $l^{-1}$. Here again hypocotyl explants cultured on media containing a high IAA concentration, such as 5.25 or 8.75 mg $l^{-1}$, in combination with high BAP concentration, such as 2.25,

Table 3.1.3. Percentage of embryonic cotyledon and hypocotyl explants of cv. "Red Chili" which formed adventitiously roots, buds and foliose structures (fs) in 8-day-old cultures. n = 70.

| Hormone concentration ($mg\ l^{-1}$) | | Morphogenic response | | | |
|---|---|---|---|---|---|
| | | Adventitious roots | | Adventitious buds and fs | |
| | | Explants used | | | |
| IAA | BAP | Cotyledon | Hypocotyl | Cotyledon | Hypocotyl |
| - | - | 0 | 69 | 0 | 0 |
| 1.75 | - | 7 | 25 | 0 | 0 |
| 5.25 | - | 0 | 12 | 0 | 0 |
| 8.75 | - | 7 | 7 | 0 | 0 |
| - | 2.25 | 0 | 1 | 71 | 98 |
| - | 4.50 | 0 | 0 | 65 | 95 |
| - | 9.00 | 0 | 0 | 61 | 96 |
| 1.75 | 2.25 | 0 | 0 | 96 | 86 |
| 1.75 | 4.50 | 0 | 0 | 95 | 93 |
| 1.75 | 9.00 | 0 | 0 | 94 | 83 |
| 5.25 | 2.25 | 0 | 0 | 94 | 55 |
| 5.25 | 4.50 | 0 | 0 | 98 | 66 |
| 5.25 | 9.00 | 0 | 0 | 96 | 46 |
| 8.75 | 2.25 | 0 | 0 | 86 | 31 |
| 8.75 | 4.50 | 0 | 0 | 70 | 31 |
| 8.75 | 9.00 | 0 | 0 | 83 | 32 |

Basal medium: MS3. Temperature: 25 °C.
Light: the first 5 days complete darkness, then standard light conditions.
Data scored by the aid of a stereomicroscope (magnification 10x).

4.5 or 9.0 mg $l^{-1}$, responded less frequently in forming bud-foliose structures than did cotyledon explants treated similarly (Table 3.1.3). This observation is in agreement with the results obtained from the same explants of cv. "California Wonder" exposed to the same media (see above in this Chapter).

Adventitious bud and foliose structure formation took place mostly at the abaxial surface of the cotyledon when it was in contact with the medium and often, but not exclusively, from regions close to its proximal end. In hypocotyl explants, bud regeneration occurred mostly from regions surrounding the cut surface regardless of whether it was treated in the proximal or distal region.

## 3.2. *Anatomy and morphology of shoot regeneration process.*

Cotyledon and hypocotyl explants from either mature embryos or 10-day-old seedlings were inoculated on MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP and cultured under standard conditions (25 °C temperature and 16-hour photoperiod). At regular intervals they were removed and placed in fixative in preparation for histological studies under light or scanning electron microscope (see Chapter 2). Both cotyledon and hypocotyl cultures were regularly photographed to provide a record of their development.

At the time of dissection, explants from mature embryos were pure white and 5-7 mm long. The adaxial and abaxial cotyledon surfaces were uniform and smooth and so was the surface of hypocotyl explants. Transverse sections of embryonic cotyledons indicated that freshly isolated explants consisted of uniform cells attached very closely to each other leaving extremely small or no intercellular spaces. Nuclei

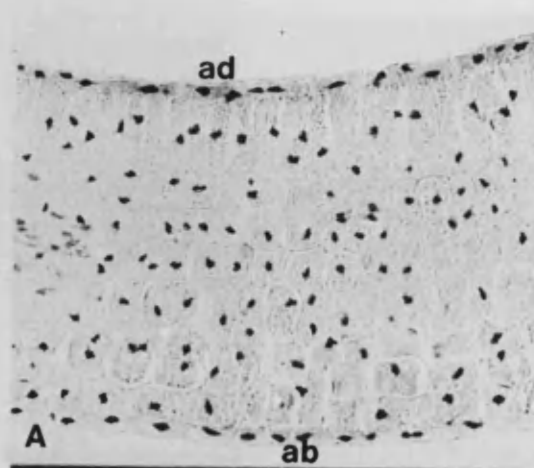
Plate 3.1. Light microscope and scanning electron microscope (SEM) studies of adventitious shoot regeneration from cotyledon explants of cv. "California Wonder".

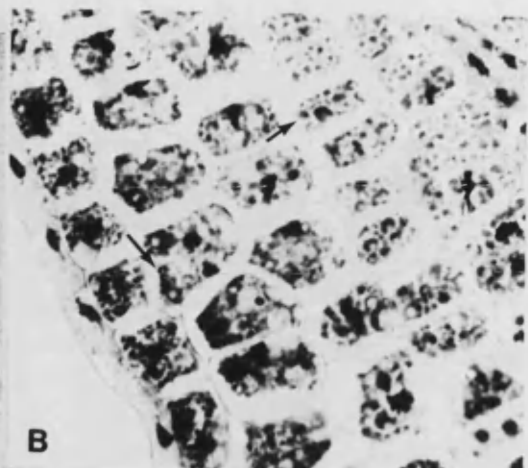
A and B. Day 0. Section parallel to cotyledon axis showing smooth abaxial (ab) and adaxial (ad) surface as well as starch grains (arrowed) in the subepidermal cells. A x200; B x400.

C. Day 0. SEM micrograph showing regular epidermal cell pattern and smooth epidermis. Scale bar = 100 μm.

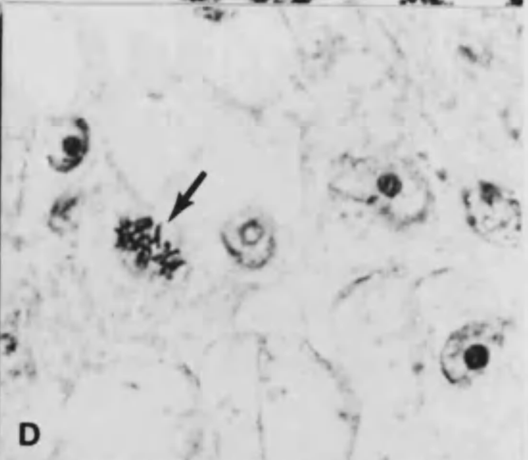
D and E. Day 2. Section parallel to cotyledon axis showing the first mitotic activities (arrowed) in subepidermal (D) and epidermal (E) cells. Both x750.

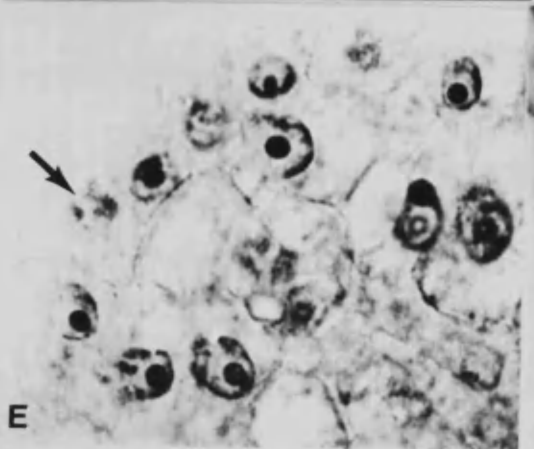
F. Day 3. Section parallel to cotyledon axis showing an unequal anticlinal cell division (arrowed) at the abaxial epidermal surface. x1000.


ad
A
ab


B

90 4206 100.0U BATHU


D


E

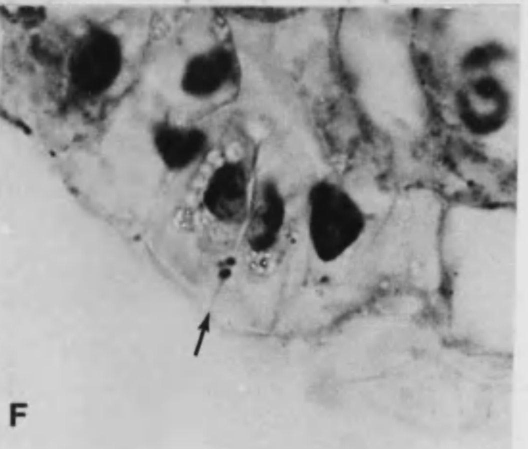

F

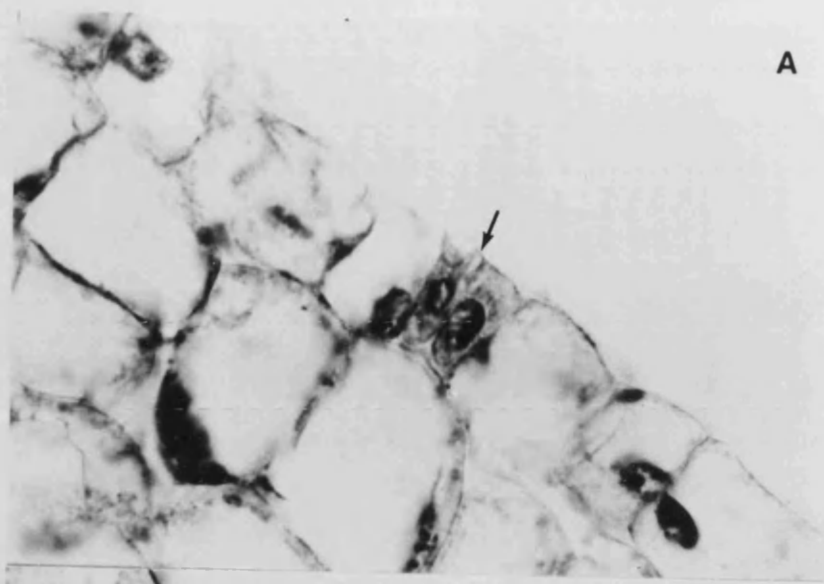
Plate 3.2. Light microscope and scanning electron microscope (SEM) studies of adventitious shoot regeneration from cotyledon explants of cv. "California Wonder".

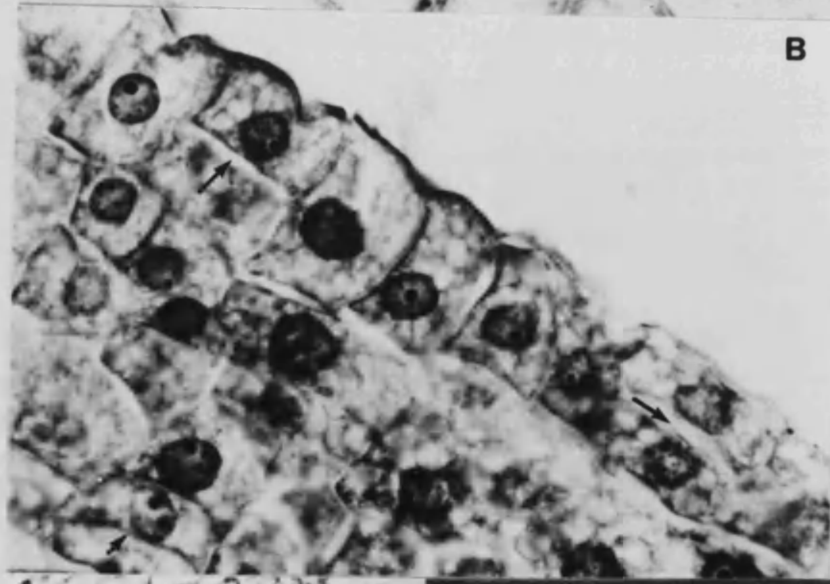
A. Day 3. Section parallel to cotyledon axis showing unequal anticlinal cell division (arrowed) at the abaxial epidermal surface. x1000.

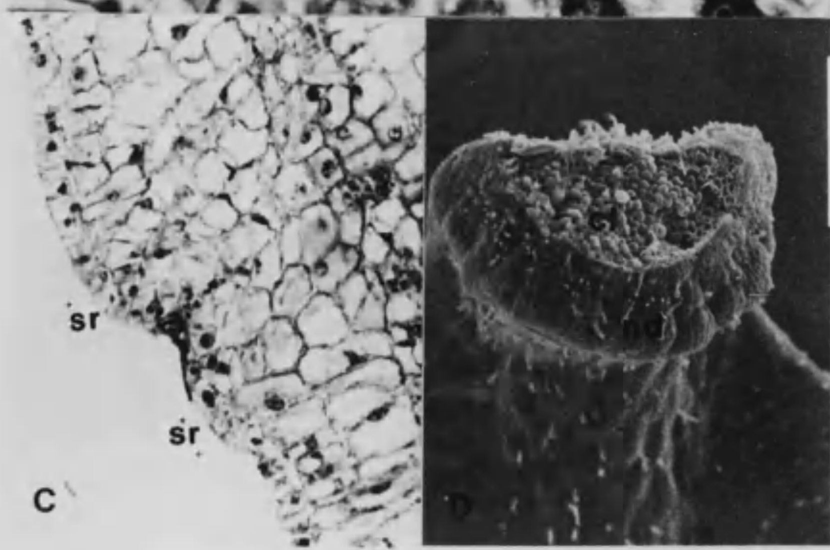
B. Day 3. Section parallel to cotyledon axis showing periclinal cell divisions (arrowed) at the epidermal and subepidermal layers. x1000.

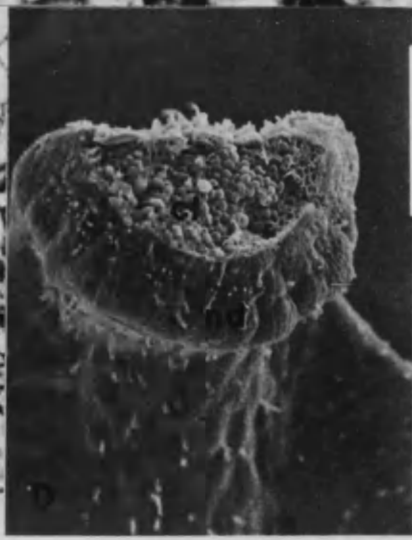
C. Day 5. Section parallel to cotyledon axis showing swollen regions (sr) at the abaxial epidermal surface. x200.

D. Day 5. SEM micrograph showing nodular structure (nd) formation at the proximal end of the cotyledon and callus (cl) proliferation at the cut surface. Scale bar = 1000 μm.


A


B


sr
sr
C



were not readily visible and the cells contained numerous starch grains (Plate 3.1 A, B). No stomata were observed on the abaxial or adaxial surfaces of freshly isolated cotyledon explants (Plate 3.1 C). After two or three days of culture, the starch grains disappeared from the cells, the nuclei became more visible occupying large portion of the entire cell and they possessed at least one nucleolus each. Mitotic activities were observed in both epidermal and subepidermal cells (Plate 3.1 D, E).

One of the most noticeable cytological events that occurred between day 2 and day 4 was that periclinal divisions started to appear in both epidermal and subepidermal cells of the cotyledon explants (Plate 3.2 B). Anticlinal divisions were observed also in the epidermal cells (Plates 3.1 F and 3.2 A). Some of these cell divisions were unequal, as judged from the location of the newly formed cell plates (Plate 3.2 A,B). Such cell division was predominant in the abaxial half of the cotyledon and particularly at the proximal end. The most responsive regions were those that were in contact with the medium. By the day 5, swollen regions ("nodules") were well distinguished at the abaxial surface, as a result of the continuous periclinal and anticlinal cell divisions. Cells of nodules were relatively small with dense cytoplasmic contents and thin cell walls. Their arrangement was ordered rather than random (Plate 3.2 C, D). In contrast, the callus cells were highly vacuolated and contained little cytoplasm; they mainly proliferated from the cut surface and randomly arranged, with large intercellular spaces. After the same period of culture, the entire explant was expanded, due to both cell division and cell expansion. Some regions of the explant, particularly those possessing nodules, turned green in colour indicating the

Plate 3.3. Light microscope and scanning electron microscope (SEM) studies of adventitious shoot regeneration from cotyledon explants of cv. "California Wonder".

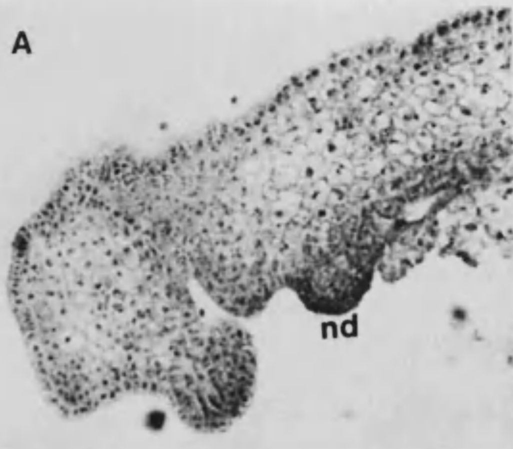
A. Day 8. Section parallel to cotyledon axis showing further development of the nodules (nd) at the abaxial surface. x112.

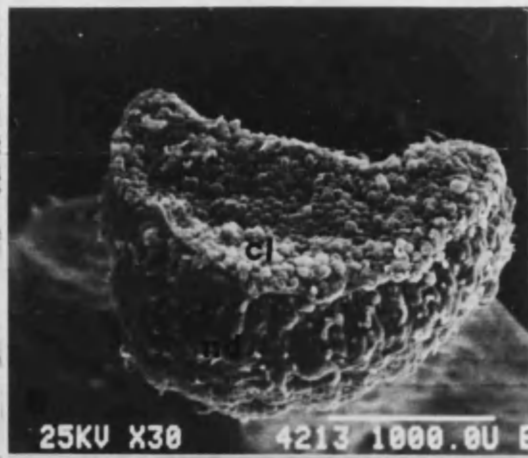
B. Day 8. SEM micrograph showing nodule (nd) formation at the proximal end of the cotyledon as well as callus (cl) proliferation at the cut surface. Scale bar = 1000 µm.

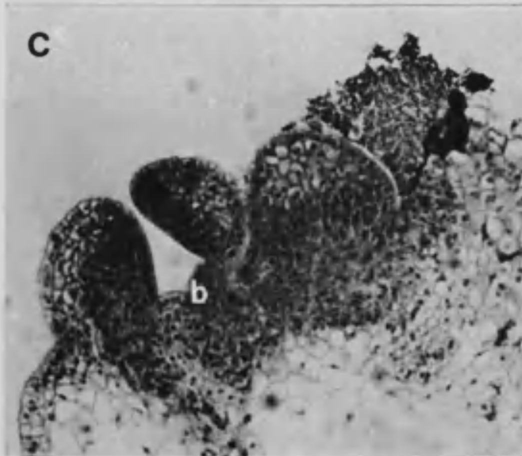
C and E. Day 12. Adventitious bud (b) and foliose (f) formation from cotyledon explants. C x90, E x36.

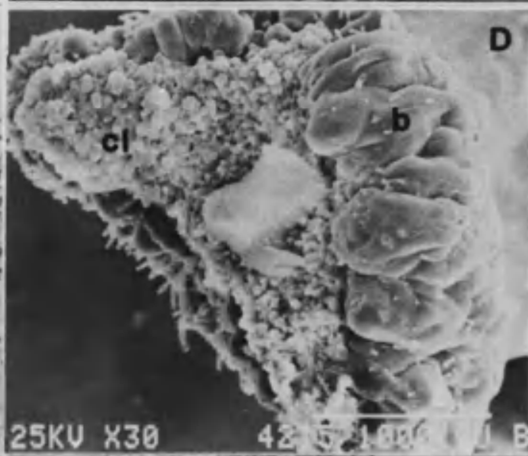
D. Day 12. SEM micrograph showing adventitious bud (b) formation and callus (cl) proliferation. Scale bar = 1000 µm.

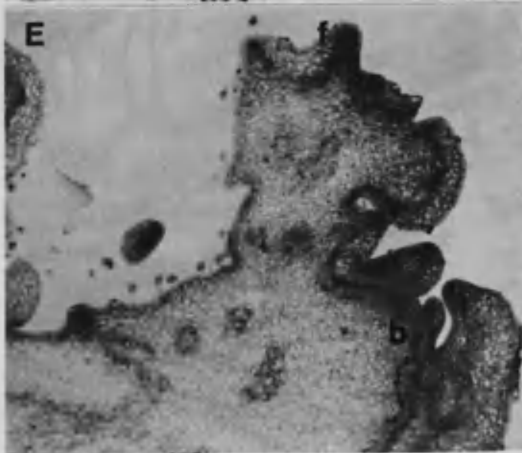
F. Day 15. Section parallel to cotyledon axis showing adventitious shoot (sh) formation. x150.

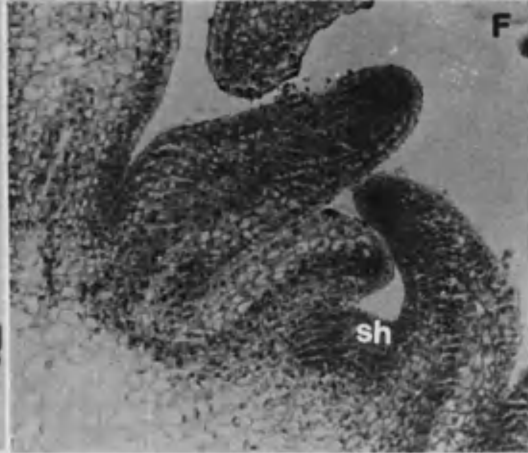

A
nd


cl
25KV X30
4213 1000.0U


C
b


D
cl
b
25KV X30


E
f
b


F
sh

presence of chlorophyll. After 8 days in culture, the nodules were further enlarged and more green coloured (Plate 3.3 A, B) and by day 12 they were recognisable as either buds or "foliose" structures (Plate 3.3 C, D, E). The cells composing the buds and particularly the domes had the characteristics of meristematic cells in general, i.e. they had large prominent nuclei and many small vacuoles. The nuclei tended to be centrally located, occupying a major proportion of the cells; starch grains were not detected in the meristematic areas. By the day 10, the original cotyledon explants turned green all over their surface and were fully expanded. Expansion and greening were observed also in those cotyledon explants which were subjected to hormone-free medium even though they did not show any morphogenic competence.

The adventitious buds and "foliose" structures developed increasingly from the cotyledon surface and by day 15 they were large enough to be seen under the stereomicroscope. The first true leaves of the adventitious shoots were well distinguished by that time (Plates 3.3 F and 3.4 C). After 38 to 40 days of culture, the adventitious shoots had grown sufficiently to produce stems with 3 to 4 true leaves (Plate 3.4 D).

The "foliose" structures were green leaf-like structures but they possessed no stomata, as judged from the SEM studies. Some of these structures possessed a very small petiole and a main vein but they lacked a normal vein network and a bud at their base.

The sequence of the cytological and histological events during the adventitious shoot regeneration process from hypocotyl explants was similar to that described for cotyledon explants but the entire process was more rapid, i.e. the size of adventitious buds on

Plate 3.4.

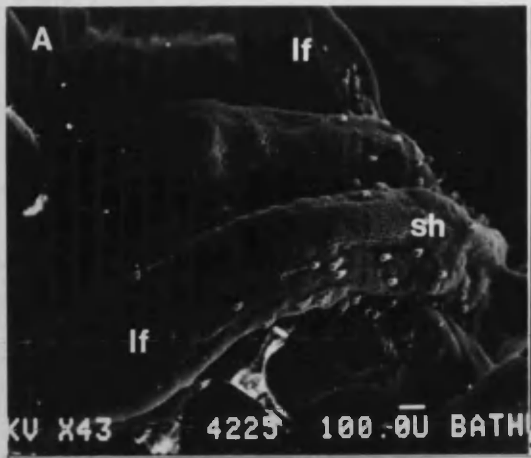
A and B. Day 15. SEM micrographs showing adventitious shoot (sh) regeneration from cotyledon explants; lf = leaf; f = foliose cl = callus. Scale bar = 100 µm.

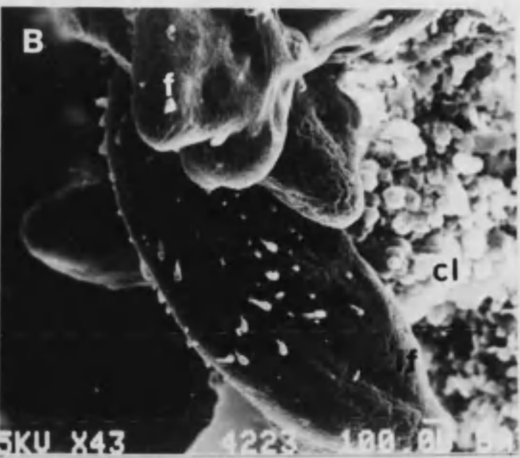
C. Day 21. SEM micrograph showing perfect leaves of adventitious shoot regenerated from cotyledon explant; ab = abaxial leaf surface, ad = adaxial leaf surface. Scale bar = 100 µm

D. Day 38. Adventitious shoot regenerated from cotyledon explant of cv. "Red Chili" cultured initially on MS3 medium supplemented with BAP (4.5 mg $l^{-1}$) for 4 weeks and subcultured to M52 medium containing 0.1 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ GA. Scale bar = 1 mm.

E. Plantlet regenerated adventitiously from cotyledon explant of cv. "California Wonder". Shoot was formed on MS - BAP medium and rooted on B5 hormone-free medium. Scale bar = 10 mm.

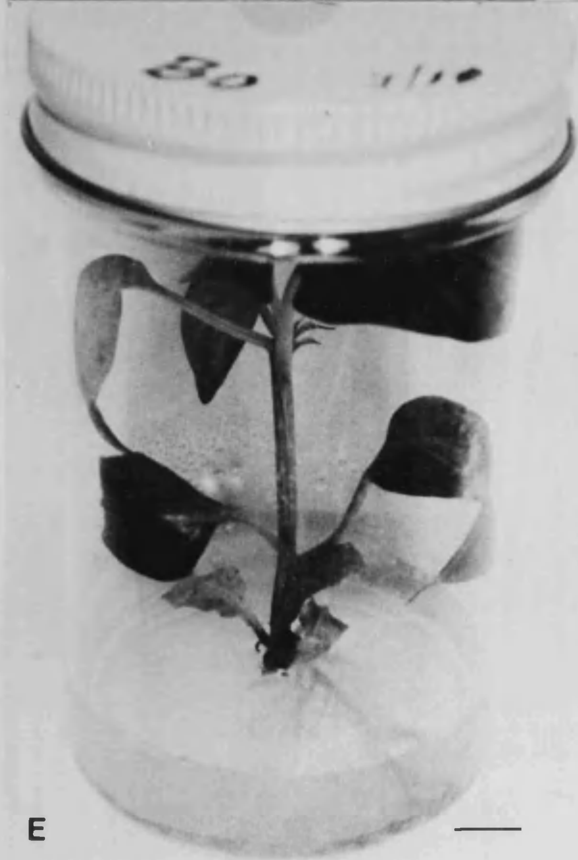
F. The above plantlet transplanted to soil.


A
lf
sh
lf
KV X43
4225
100.0U BATH


B
f
cl
f
5KV X43
4223

C
ad
ab
25KV
4284
100.0U BATH

D


E

Plant derived
adventitiously from
cotyledonous explant
F

hypocotyl explants at day 15 was the same as the bud size on cotyledon explants at day 18 or day 20.

*3.3. The effect of casaminoacids on shoot regeneration*

Embryonic cotyledons from mature embryos were inoculated on MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP with and without casaminoacids at a concentration of 100 mg $l^{-1}$. Half of the cultures were exposed to light (10.2 microeinsteins $m^{-2}sec^{-1}$) with 16-hour photoperiod from the beginning whereas the other half were kept in continuous darkness during the first 10 days and then they were exposed to the same light conditions. Cultures were subjected to a 25 °C temperature regime. Explants of two cultivars "California Wonder" and "Red Chili" were tested.

Shoot regeneration was observed at high frequency in cotyledon explants, of both cultivars, kept under continuous darkness during the first 10 days regardless of the presence or absence of casaminoacids. The light regime of 16-hour photoperiod, applied to the cultures from the beginning, suppressed shoot regeneration from explants of cv. "Red Chili" subjected to medium without casaminoacids. This negative effect was, to a large degree, removed when the medium was supplemented with 100 mg $l^{-1}$ casaminoacids (Table 3.3).

Furthermore, in all cases the presence of casaminoacids encouraged the development of buds into shoots. For that reason, casaminoacids were introduced to the MS basal medium, at the concentration of 100 mg $l^{-1}$, in all the following experiments, unless otherwise stated.

Table 3.3. The effect of casaminoacids on the formation of adventitious shoot-bud from embryonic cotyledon explants in 30-day-old cultures. n = 25-30.

| | Without casaminoacids | | 100 mg $l^{-1}$ casaminoacids | |
|---|---|---|---|---|
| Cultivar | First 10 days in darkness then light with 16-hour photoperiod | Light with 16-hour photoperiod from the beginning | First 10 days in darkness then light with 16-hour photoperiod | Light with 16-hour photoperiod from the beginning |
| | % | % | % | % |
| "California Wonder" | 73 | 62 | 76 | 93 |
| "Red Chili" | 71 | 12 | 85 | 70 |

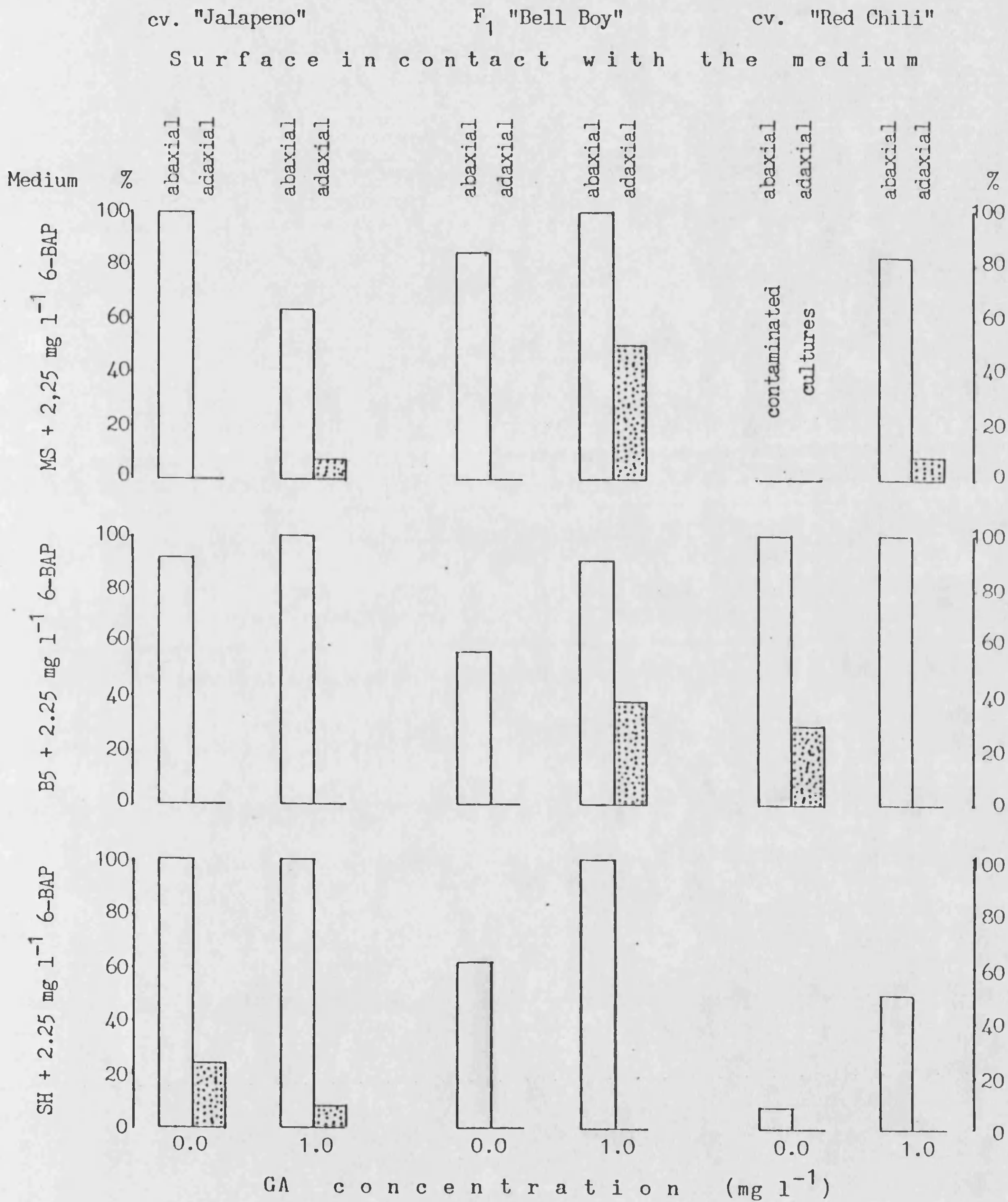
Basal medium: MS3 + 4.5 mg $l^{-1}$ BAP.
Culture conditions: standard.

### 3.4. *The effect of contact with the medium on bud-shoot regeneration from the cotyledon surface*

Cotyledons from 28-day-old seedlings of cvs "Jalapeno","Red Chili" and $F_1$ hybrid "Bell Boy" were inoculated on three different basal media with either their abaxial or adaxial surfaces in contact with the agar medium. The basal media of Murashige and Skoog (MS), Gamborg *et al.* (B5) and Schenc and Hildebrandt (SH) as modified by Liu and Chenk (1978) were supplemented with 2% sucrose and 2.25 mg $l^{-1}$ BAP. In addition to that each medium was supplemented with or without 1.0 mg $l^{-1}$ GA. Apart from the other differences, SH basal medium differed from MS and B5 by containing 10% (v/v) coconut milk.

The results of this experiment are illustrated in the Figure 3.1. It is apparent that shoot regeneration only very rarely occurred in explants with their adaxial surface in contact with the medium, regardless of the type of basal medium or the cultivar. In contrast, shoot regeneration took place at a high frequency, more than 60%, in explants with their abaxial surface in contact with the medium. MS basal medium was superior in the case of $F_1$ "Bell Boy" hybrid explants while SH medium did not encourage shoot regeneration from "Red Chili" explants. The presence of 1.0 mg $l^{-1}$ GA did not significantly affect the shoot regeneration process in "Jalapeno" and "Red Chili" explants regardless of their surface in contact with the medium and the type of the basal medium. Some positive effect of GA on the regeneration process was observed from adaxially orientated $F_1$ hybrid "Bell Boy" explants cultured on MS or B5 basal media, but not on SH medium. Also it was observed that in the presence of GA many shoots grew faster and developed stems (for details see below).

Fig. 3.1. The effect of the explant's surface in contact with the medium on the adventitious shoot regeneration from cotyledons of 28-day-old seedlings. n = 15-20




Sucrose concentration: 2% (w/v). Duration of cultures: 28 days.
Culture conditions: standard.

### 3.5. *The effect of GA on the adventitious bud regeneration and shoot growth*

Since it was apparent, from the results of the previous experiment, that the presence of GA strongly influenced the growth of the adventitiously regenerated shoots, a series of experiments was conducted to investigate the right time of GA incorporation into the basal medium. In all experiments the standard cultural conditions of 25 °C temperature and 10.2 microeinsteins $m^{-2}sec^{-1}$ light with 16-hour photoperiod were applied. Cotyledons and hypocotyls of 26 or 28-day-old seedlings of cv. "California Wonder" were inoculated onto MS3, unless otherwise stated, basal medium supplemented with 2.25 or 4.5 mg $l^{-1}$ BAP and 3.0 mg $l^{-1}$ GA. Gibberellic acid was incorporated by day 3, 6, 10 or 15 of the culture. The results of this experiment are illustrated in Tables 3.5.1 and 3.5.2.

It is well demonstrated that the incorporation of GA into the medium at any time, did not encourage the bud initiation process and its combination with BAP did not significantly influence the percentage of explants which underwent bud regeneration. In fact GA reduced the number of buds per explant by reducing either the number of buds per unit area or the number of regions of the explant from which buds were initiated. This particular effect was apparent in cultures where GA was incorporated into the medium during the first six days, while it was difficult to demonstrate it in cultures where GA was incorporated later. The remarkable positive effect was that it dramatically encouraged the bud's growth to produce shoots with extended stems. The time of GA incorporation did not significantly affect the percentage of explants formed shoot with stem (Tables 3.5.1 and 3.5.2) but the sooner the incorporation was the faster was the shoot growth. Cotyledon explants responded stronger than hypocotyl explants in producing shoots.

Table 3.5.1. The effect of the time of GA incorporation on the bud-shoot regeneration from cotyledon explants taken from 26-day-old seedlings of cv. "California Wonder". n = 15-20.

| Time of GA incorporation | Hormone concentration (mg $l^{-1}$) 1st Stage (4 weeks) BAP | 1st Stage GA | 2nd Stage (12 days) BAP | 2nd Stage GA | % explants forming foliose structures (fs) buds and shoots — Time-period in culture: 10 days fs | 10 days Buds | 15 days fs | 15 days Buds | 40 days Buds | 40 days Shoots |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st day | - | - | - | - | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | - | 50 | 0 | 50 | 0 | 60 | 40 |
| | 4.5 | - | 4.5 | - | 42 | 0 | 56 | 0 | 60 | 56 |
| | - | 3 | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | 3 | 2.25 | 3 | 40 | 10 | 50 | 10 | 60 | 60 |
| | 4.5 | 3 | 4.5 | 3 | 20 | 10 | 40 | 10 | 60 | 60 |
| 3rd day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 45 | 0 | 70 | 0 | 70 | 50 |
| | 4.5 | - | 4.5 | 3 | 30 | 0 | 50 | 0 | 60 | 60 |
| 6th day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 0 | 0 | 20 | 0 | 30 | 30 |
| | 4.5 | - | 4.5 | 3 | 40 | 0 | 50 | 0 | 60 | 60 |
| 10th day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 30 | 0 | 30 | 0 | 60 | 60 |
| | 4.5 | - | 4.5 | 3 | 30 | 0 | 40 | 0 | 60 | 60 |
| 15th day | - | - | - | 3 | - | - | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | - | - | 40 | 0 | 70 | 70 |
| | 4.5 | - | 4.5 | 3 | - | - | 40 | 0 | 60 | 60 |

Basal medium: MS3. Culture conditions: standard.

(-): treatment was not tested.

Table 3.5.2. The effect of the time of GA incorporation on the bud-shoot regeneration from hypocotyl explants taken from 26-day-old seedlings of cv. "California Wonder". n = 30.

| Time of GA incorporation | Hormone concentration ($mg\ l^{-1}$) 1st Stage (4 weeks) BAP | 1st Stage GA | 2nd Stage (12 days) BAP | 2nd Stage GA | 10 days fs* | 10 days Buds | 15 days fs | 15 days Buds | 40 days Buds | 40 days Shoots |
|---|---|---|---|---|---|---|---|---|---|---|
| 1st day | - | - | - | - | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | - | 10 | 0 | 10 | 0 | 30 | 30 |
| | 4.5 | - | 4.5 | - | 5 | 0 | 12 | 0 | 37 | 12 |
| | - | 3 | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | 3 | 2.25 | 3 | 0 | 0 | 0 | 0 | 20 | 0 |
| | 4.5 | 3 | 4.5 | 3 | 0 | 0 | 0 | 0 | - | - |
| 3rd day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 0 | 0 | 10 | 0 | 20 | 0 |
| | 4.5 | - | 4.5 | 3 | 10 | 0 | 10 | 0 | 10 | 0 |
| 6th day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 0 | 0 | 30 | 0 | 30 | 0 |
| | 4.5 | - | 4.5 | 3 | 0 | 0 | 0 | 0 | 20 | 0 |
| 10th day | - | - | - | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | 10 | 0 | 20 | 0 | 20 | 0 |
| | 4.5 | - | 4.5 | 3 | 10 | 0 | 20 | 0 | 20 | 0 |
| 15th day | - | - | - | 3 | - | - | 0 | 0 | 0 | 0 |
| | 2.25 | - | 2.25 | 3 | - | - | 10 | 0 | 50 | 40 |
| | 4.5 | - | 4.5 | 3 | - | - | 20 | 0 | 30 | 10 |

(%) of morphogenic response

Basal medium: MS3. Culture conditions: standard.

(-): treatment was not tested. fs* = foliose structures

In order to avoid an elimination of bud initiation, due to the presence of GA, this hormone was incorporated into the medium of the subcultures to which explants that had already formed adventitious buds were transferred.

To investigate the effect of GA concentration on bud growth to produce shoots with extended stems, 1.0, 3.0 or 5.0 mg $l^{-1}$ GA was incorporated to MS3 media to which cotyledon explants of 28-day-old seedlings of cv. "California Wonder" were subcultured. Explants were initially cultured for 13 days on MS3 medium supplemented with 2.25 or 4.5 mg $l^{-1}$ BAP. Temperature of 25 °C and light of 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod were applied to both initial culture and 1st subculture. The results of this experiment are illustrated in Table 3.5.3. It is obvious that explants which transferred to media containing 0, 2.25 or 4.5 mg $l^{-1}$ BAP, but where GA was omitted, did not form shoots with extended stems after 28 days in subculture although they possessed adventitiously regenerated buds. Also, explants which were subcultured to media containing 1.0, 3.0 or 5.0 mg $l^{-1}$ GA without BAP were unable to form shoots with extended stems during the same period of culture. Only media supplemented with BAP and GA favoured the growth of adventitious buds to produce shoots with extended stems. The best results were obtained when 2.25 mg $l^{-1}$ BAP in combination with 1.0 mg $l^{-1}$ GA were incorporated into MS3 basal medium where a 100% of explants, already possessed adventitious buds, produced shoots with extended stems (Table 3.5.2).

### *3.6 The morphogenic response of cotyledon and petiole explants of three different cultivars to three basal media supplemented with BAP and GA*

Based on the results obtained from the experiments of sections

Table 3.5.3. Percentage of cotyledon explants from 28-day-old seedlings of cv. "California Wonder" which formed adventitious shoots with extended stems. n = 15-20.

| Stage I BAP concentration ($mg\ l^{-1}$) | Stage II BAP concentration ($mg\ l^{-1}$) | GA 0 Bud | GA 0 Shoot | GA 1.0 Bud | GA 1.0 Shoot | GA 3.0 Bud | GA 3.0 Shoot | GA 5.0 Bud | GA 5.0 Shoot |
|---|---|---|---|---|---|---|---|---|---|
| 2.25 | 0 | 0 | 0 | 20 | 0 | 33 | 0 | 33 | 0 |
| 2.25 | 2.25 | 50 | 0 | 17 | 17 | 50 | 0 | 67 | 33 |
| 2.25 | 4.5 | 67 | 0 | 50 | 50 | 67 | 33 | 50 | 17 |
| 4.5 | 0 | 33 | 0 | 33 | 0 | 17 | 0 | 17 | 0 |
| 4.5 | 2.25 | 67 | 0 | 67 | 17 | 50 | 17 | 50 | 0 |
| 4.5 | 4.5 | 33 | 0 | 67 | 0 | 50 | 0 | 50 | 0 |

Stage II — GA concentration ($mg\ l^{-1}$) — Morphogenic response

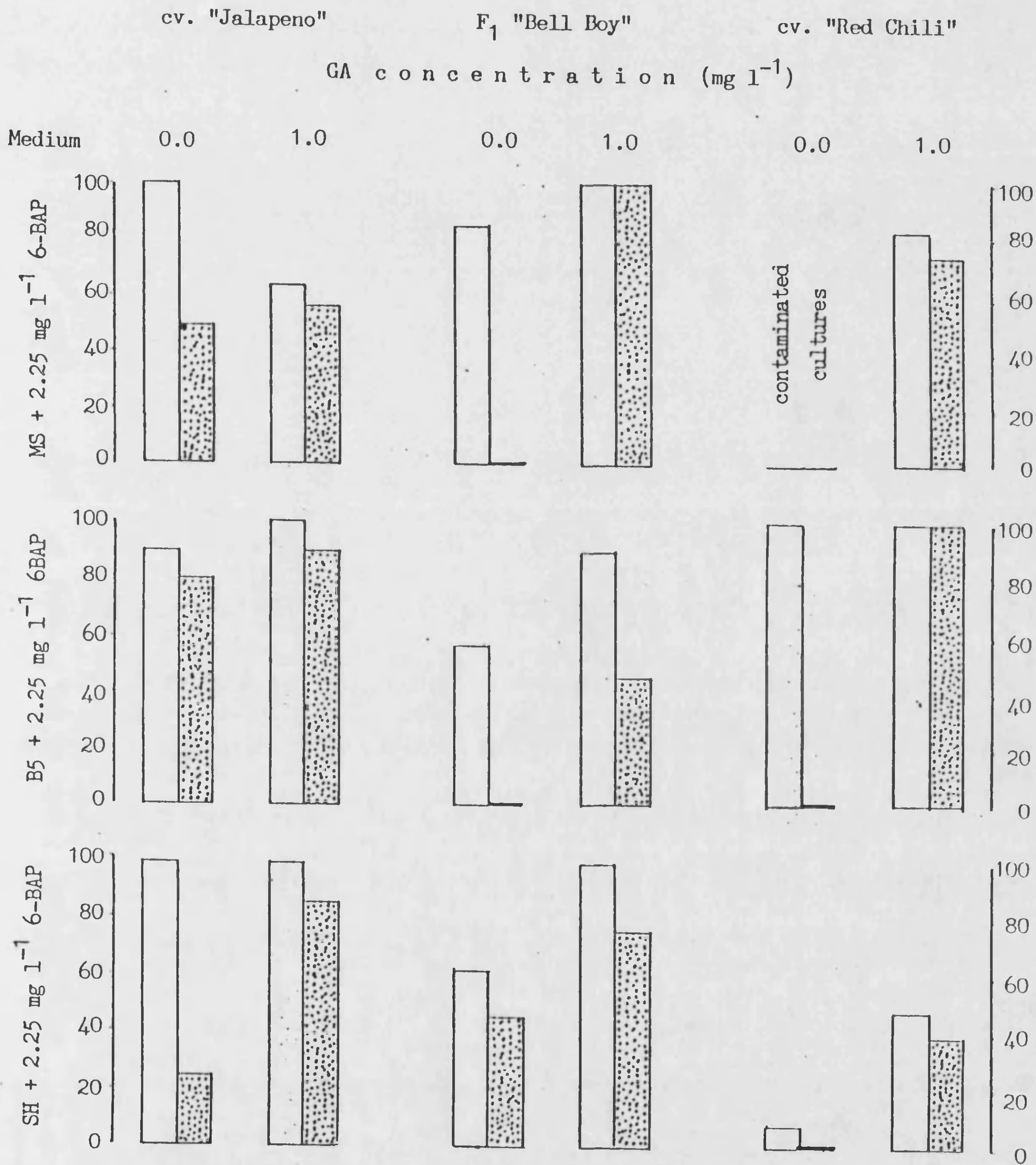
Basal medium: MS3 in both stages. Culture conditions: standard.
Duration of culture: stage I = 13 days; stage II = 28 days.

3.4 and 3.5 an experiment was conducted to clarify the effect of MS3, B5 and SH basal media supplemented with 2.25 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ GA on the morphogenic response of cotyledon explants. A two stage procedure was applied. In stage I which lasted 13 days the basal media contained only BAP at concentration 2.25 mg $l^{-1}$ and in stage II which lasted 28 days the basal media contained 2.25 mg $l^{-1}$ BAP in combination with 1.0 mg $l^{-1}$ GA. Cotyledon explants were taken from 28-day-old seedlings of cvs "Jalapeno" and "Red Chili" as well as $F_1$ hybrid "Bell Boy" and cultured under standard conditions as described in section 3.5. Data were scored by the end of the stage II, when cultures were 41 days old instead of 28 days as in the experiment described previously in section 3.4.

The results of this experiment are illustrated in Fig. 3.2. The effect of the type of basal media on the adventitious bud regeneration was similar to that obtained from the experiment illustrated in Fig 3.1 (section 3.4). The presence of 1.0 mg $l^{-1}$ GA in stage II medium significantly increased the frequency of cotyledon explants of cv. "Red Chili" and"Bell Boy" $F_1$ hybrid, regardless of the basal medium, as well as of explants of cv. "Jalapeno" subjected to SH basal medium in producing shoots with extended stems from adventitiously regenerated buds. In absence of GA, buds of cv. "Red Chili" failed to grow further to produce shoots with extended stems in all tested media; the same phenomenon was observed in buds of $F_1$ "Bell Boy" hybrid in MS and B5 media. The maximum number of shoots per each cotyledon explant was as follows: "Red Chili" 8, "Jalapeno" 6, and "Bell Boy" 3.

The effect of MS, B5 and SH basal media supplemented with 2.25 mg $l^{-1}$ BAP on morphogenic response in petiole of cotyledon explants from 28-day-old seedlings of cvs "Jalapeno" and "Red Chili" and $F_1$

Fig. 3.2. The effect of basal medium and genotype on the adventitious bud regeneration from cotyledon explants and the effect of GA on bud development in shoot with extended stem. n = 20-25.



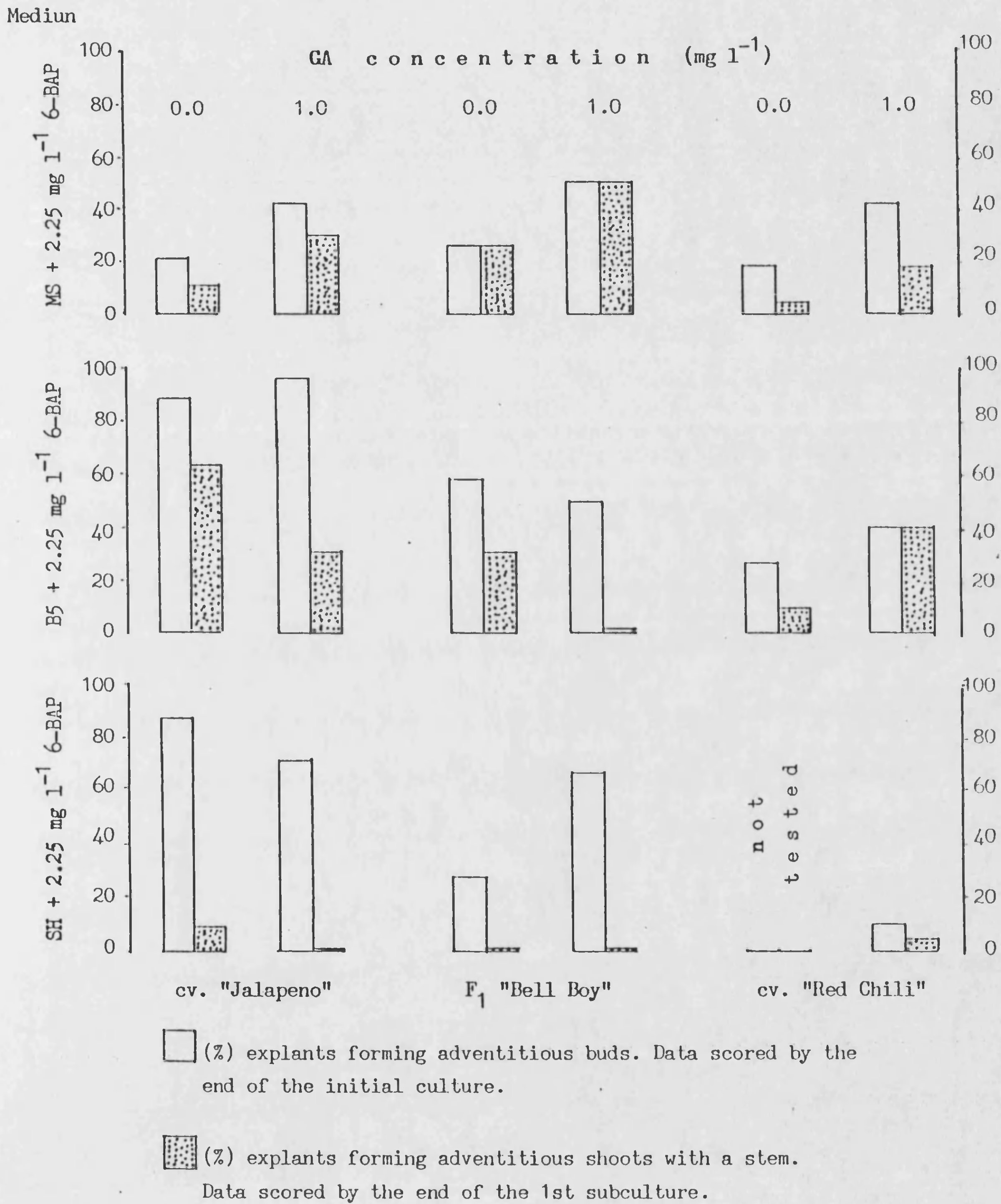

(%) of explants forming adventitious buds. Data scored by the end of the initial culture.

(%) of explants forming adventitious shoots with a stem. Data scored by the end of the 1st subculture.

Sucrose concentration: 3% (w/v) to all basal media. GA was incorporated only into the media of the 1st subculture.

Duration of culture: initial culture = 13 days; 1st subculture = 28 days. Culture conditions: standard.

Fig. 3.3. The effect of basal medium and genotype on the adventitious bud regeneration from petiole explants of cotyledons and the effect of GA on bud development in shoot with extended stem. n = 20-25.




Sucrose concentration: 3% (w/v) to all basal media. GA was incorporated only into the media of the 1st subculture. Duration of culture: initial culture = 13 days; 1st subculture = 28 days. Culture conditions: standard.

"Bell Boy" hybrid as well as the effect of 1.0 mg $l^{-1}$ GA on the growth of adventitiously regenerated buds from the same explants were tested. The experimental procedure was exactly the same as described above. The results are represented in Fig. 3.3. It is apparent that the basal media B5 and SH were better than MS in adventitious bud regeneration from petiole of cotyledon explants of cv. "Jalapeno" and $F_1$ "Bell Boy" hybrid. The presence of 1.0 mg $l^{-1}$ GA in MS basal medium positively influenced the growth of bud to produce shoots with extended stems, regardless of the explant's genotype. The same effect was observed in petiole explants of cv. "Red Chili" subjected to B5 medium. Petiole explants of any cultivar tested and cultured to SH basal medium in presence or absence of GA did not form shoots with extended stems although they possessed adventitiously regenerated buds. The maximum number of shoots with stem per each explant was as follows: "Jalapeno" 4, "Red Chili" 3 and "Bell Boy" 3.

### 3.7. *The effect of the age of explant on the adventitious shoot regeneration process*

In order to investigate the influence of the developmental stage (age) of the explant on the adventitious-shoot regeneration process, cotyledons from non-emerged seeds (white explants) and from 5, 10, 15, 20 or 25-day-old seedlings (green explants) were cultured on MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP and incubated for the first 5 days under complete darkness and then exposed to light of 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod. Temperature was kept at 25 °C. By the end of the initial culture period (28 days), the explants which formed adventitious shoots were scored.
The results are presented in Table 3.7.

Table 3.7. The effect of explant age on the adventitious-shoot regeneration process from cotyledon explants of cv. "California Wonder". n = 20.

| Percentage of responded explants | |
|---|---|
| Source of cotyledon | Adventitious shoots |
| Non-emerged seeds | 96 (ex) |
| 5-day-old seedlings | 100 (ex) |
| 10-day-old seedlings | 90 (vg) |
| 15-day-old seedlings | 55 (s ) |
| 20-day-old seedlings | 80 (sg) |
| 25-day-old seedlings | 70 (sg) |

Basal medium: MS3 + 4.5 mg $l^{-1}$ BAP. Culture conditions: standard.
Duration of culture: 28 days.

(ex): shoots with 2 true leaves visible at magnification x4
(vg): " " 1 true leaf " " x4
(sg) " " 1 true leaf " " x10
(s ): " " smaller leaves

Cotyledon explants from non-emerged seeds as well as those from 5 or 10-day-old seedlings responded better than the ones taken from 15, 20 or 25-day-old seedlings. The shoot growth rate was greater in the former explants than it was in the latter ones. Furthermore, the number of shoots per explant was also greater in cotyledons of non-emerged seeds and 5-day-old seedlings than in those from older seedlings. The worst results were obtained from cotyledons from 15-day-old seedlings while the situation improved to some degree when cotyledons originated from 20 or 25-day-old seedlings (Table 3.7).

### *3.8. The effect of temperature, BAP and GA on adventitious-shoot regeneration from embryonic tissues*

Cotyledon and hypocotyl explants from mature embryos of cv. "California Wonder" were inoculated on MS3 basal medium supplemented with either 2.25 or 4.5 mg $l^{-1}$ BAP alone or in combination with 0.1 or 1.0 mg $l^{-1}$ GA and were incubated under three different temperature regimes of 20 °C, 25 °C or 30 °C. Cultures were kept under complete darkness during the first 5 days and then exposed to standard light conditions. Observations were taken from 18 as well as from 28-day-old cultures. From Table 3.8.1 it is obvious that the 25 °C or 30 °C temperature regime encouraged a higher number of cotyledon or hypocotyl explants to undergo bud regeneration than did the 20 °C temperature regime, but they did not influence the number of adventitious buds formed on each explant. The above beneficial effect of the higher temperature regimes lasted approximately until the twentieth day of culture. After this critical period, rapid callus proliferation took place and a number of the adventitious buds, which were covered by the callus degenerated; this was particularly applied to cotyledon explants whereas hypocotyl explants seemed to suffer less.

Table 3.8.1. The effect of temperature, BAP and GA on adventitious bud regeneration from embryonic cotyledon and hypocotyl explants of cv. "California Wonder". n = 20.

a. Data from 18-day-old cultures

| Hormone Concentration ($mg\ l^{-1}$) | | % explants forming adventitious buds | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cotyledon explants | | | | Hypocotyl explants | | | |
| | | Temperature | | | | Temperature | | | |
| BAP | GA | 20 °C | 25 °C | 30 °C | Mean | 20 °C | 25 °C | 30 °C | Mean |
| 2.25 | - | 50 | 75 | 75 | 67 a | 60 | 85 | 80 | 75 a |
| 4.5 | - | 40 | 80 | 80 | 67 a | 65 | 90 | 85 | 80 a |
| 2.25 | 0.1 | 65 | 75 | 80 | 73 a | 65 | 90 | 95 | 83 a |
| 2.25 | 1.0 | 45 | 80 | 85 | 70 a | 55 | 70 | 80 | 68 a |
| 4.5 | 0.1 | 45 | 75 | 85 | 68 a | 70 | 95 | 95 | 87 a |
| 4.5 | 1.0 | 55 | 80 | 85 | 67 a | 67 | 95 | 95 | 86 a |
| | Mean | 50 a | 78 b | 82 b | | 64 a | 87 b | 88 b | |

b. Data from 28-day-old cultures

| Hormone Concentration ($mg\ l^{-1}$) | | % explants forming adventitious buds | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cotyledon explants | | | | Hypocotyl explants | | | |
| | | Temperature | | | | Temperature | | | |
| BAP | GA | 20 °C | 25 °C | 30 °C | Mean | 20 °C | 25 °C | 30 °C | Mean |
| 2.25 | - | 50 | 68 | 55 | 56 a | 100 | 90 | 80 | 90 a |
| 4.5 | - | 40 | 75 | 60 | 58 a | 90 | 100 | 90 | 93 a |
| 2.25 | 0.1 | 70 | 65 | 35 | 57 a | 100 | 90 | 100 | 97 a |
| 2.25 | 1.0 | 45 | 45 | 60 | 50 a | 70 | 70 | 80 | 73 a |
| 4.5 | 0.1 | 45 | 50 | 90 | 62 a | 100 | 100 | 100 | 100 a |
| 4.5 | 1.0 | 55 | 50 | 90 | 65 a | 70 | 100 | 100 | 90 a |
| | Mean | 51 a | 59 a | 65 a | | 88 a | 92 a | 92 a | |

Basal medium: MS3. Temperature: 25 °C.

Light: the first 5 days complete darkness, then standard light conditions.

In both cotyledon and hypocotyl explants, during the initial period of twenty days, a high temperature regime (25 °C or 30 °C) encouraged the rapid growth of the adventitious buds which were initiated the early days of culture. [It should be mentioned here that the initiation of the first batch of the adventitious buds occurred before callus formation. The growth of buds, initiated after the appearance of the callus, was suppressed by the excessive callus growth, especially when they stayed on the same medium for longer than twenty days period under 25 °C or 30 °C temperature regime (Table 3.8.1)].

The presence of 0.1 mg $l^{-1}$ GA in the medium influenced the adventitious bud regeneration process not by affecting the frequency of the explants which underwent bud initiation but rather by reducing the number of buds per explant (Table 3.8.2). Furthermore, in the presence of GA, adventitious buds developed rapidly in shoots, escaping the danger of being covered by the callus mass which proliferated during the second fortnight of the initial culture period.

The level of BAP concentration (2.25 or 4.5 mg $l^{-1}$) affected equally well both the percentage of explants that formed adventitious buds and the number of buds per explant.

The most dramatic effect of the temperature regime was observed during the culture period between 5th and 8th week when buds developed into shoots. High temperature regime of 30 °C seemed to be unfavourable for shoot development whereas the lower temperature regimes (25 °C and 20 °C) encouraged the process (Fig.3.4). Under the 20 °C temperature regime, the incorporation of 1.0 mg $l^{-1}$ GA seemed to be more effective than 0.1 mg $l^{-1}$ GA on shoot development whereas at 25 °C both GA concentrations appeared to affect equally well the

Table 3.8.2. The effect of temperature, BAP and GA on the number of adventitious buds regenerated from cotyledon explants of cv. "California Wonder". n = 20.

a. Average number of adventitious buds per explant. Included are also the explants which did not form adventitious buds.

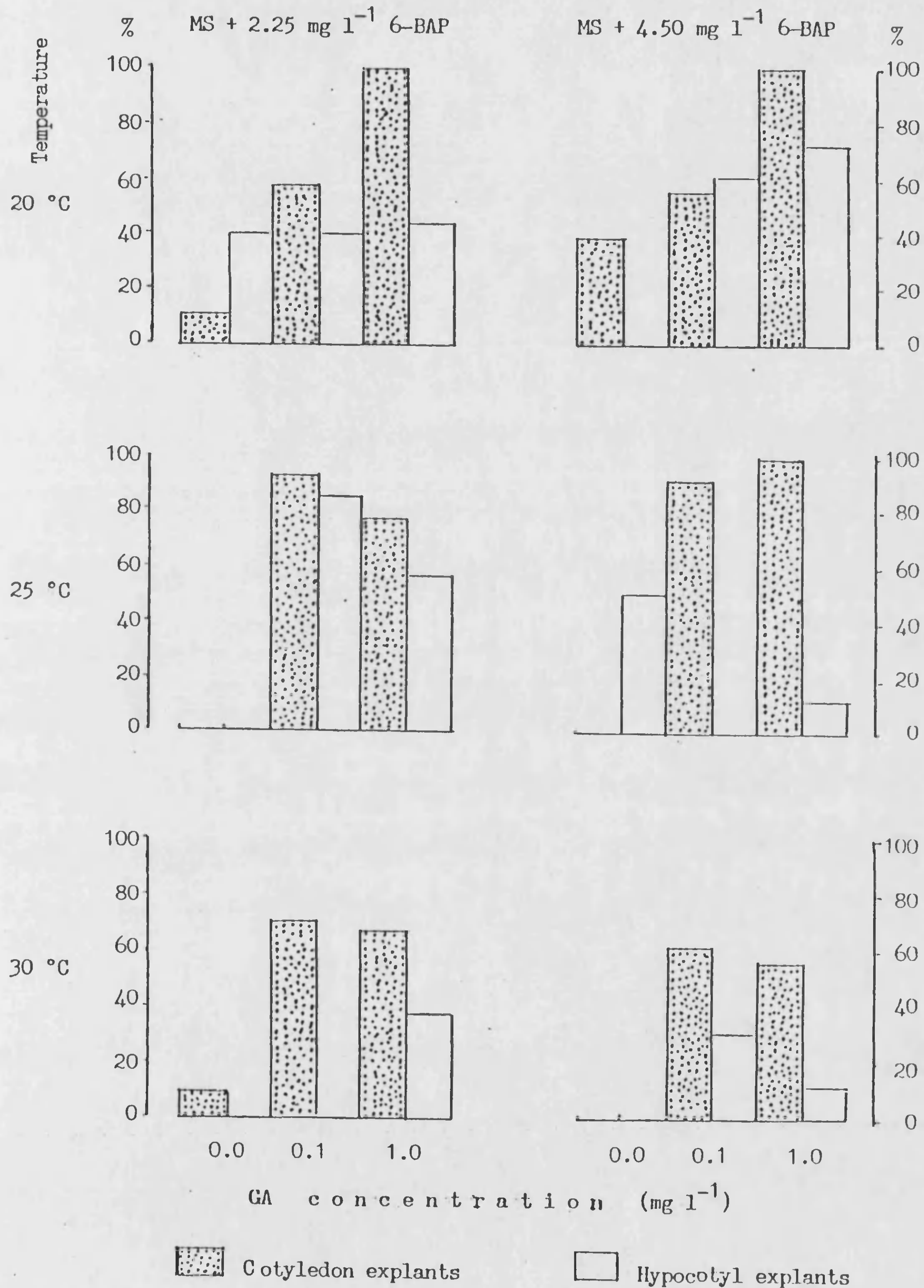
| Hormone concentration ($mg\ l^{-1}$) | | Temperature regime | | | |
|---|---|---|---|---|---|
| BAP | GA | 20 °C | 25 °C | 30 °C | Mean |
| 2.25 | - | 1.05 a | 2.10 b | 2.00 b | 1.72 b |
| 4.5 | - | 0.90 a | 2.40 b | 2.15 b | 1.82 b |
| 2.25 | 0.1 | 1.15 a | 1.50 ab | 1.55 ab | 1.40 ab |
| 2.25 | 1.0 | 0.90 a | 1.50 ab | 1.50 ab | 1.30 ab |
| 4.5 | 0.1 | 0.85 a | 1.50 ab | 1.85 b | 1.47 ab |
| 4.5 | 1.0 | 0.80 a | 1.70 ab | 1.65 ab | 1.32 ab |
| | Mean | 0.94 a | 1.78 b | 1.78 b | |

b. Average number of adventitious buds per explant. Excluded are the explants which did not form adventitious buds.

| Hormone concentration ($mg\ l^{-1}$) | | Temperature regime | | | |
|---|---|---|---|---|---|
| BAP | GA | 20 °C | 25 °C | 30 °C | Mean |
| 2.25 | - | 2.10 ab | 2.80 b | 2.67 b | 2.58 b |
| 4.5 | - | 2.25 ab | 3.00 b | 2.69 b | 2.73 b |
| 2.25 | 0.1 | 1.77 a | 2.00 a | 1.94 a | 1.91 a |
| 2.25 | 1.0 | 2.00 a | 1.88 a | 1.76 a | 1.86 a |
| 4.5 | 0.1 | 1.88 a | 2.27 ab | 2.18 a | 2.14 ab |
| 4.5 | 1.0 | 1.45 a | 1.88 a | 1.94 a | 1.79 a |
| | Mean | 1.91 a | 2.32 a | 2.20 a | |

Basal medium: MS3. Temperature: 25 °C. Duration of culture: 18 days.
Light: the figst 5 days complete darkness, then standard light conditions.

Fig. 3.4. The effect of temperature on the development of adventitiously regenerated buds inshoots with extended stems, in 56-day-old cultures. n = 20.




Light: the first 5 days complete darkness, then standard light condions. Sucrose concentration: 3% (w/v).

development of shoots regenerated from cotyledons. GA-free medium did not encourage shoot development and growth from either the cotyledon or the hypocotyl explants (Fig. 3.4 ). Although, during the period of the first 4 weeks of culture, the frequency of hypocotyl explants that formed adventitious buds was greater than that of cotyledon explants, more shoots with extended stems were obtaind from the latter explants by the end of 8th week of culture (compare the results given in Table 3.8.1 and Fig. 3.4).

*3.9. The morphogenic response of embryonic cotyledon explants to media supplemented with various sucrose concentrations*

The effect of sucrose concentration was tested on embryonic cotyledon explants excised either from immature fruit and dry seeds of cv. "Jalapeno" or from dry seeds of cv. "California Wonder". Explants were inoculated on MS basal medium supplemented with either 4.5 mg $l^{-1}$ BAP alone or in combination with 1.0 mg $l^{-1}$ GA. To those media 3%, 6% or 9% (w/v) sucrose was added. The cultures were kept under a 25 °C temperature regime and exposed to light of 10.2 microeinsteins $m^{-2}sec^{-1}$ with a 16-hour photoperiod from day 5 onwards (the first 5 days, cultures were kept under complete darkness).
The data of this experiment, which scored on 28-day-old cultures, are summarized in Table 3.9.
In all cases, a concentration of 9% sucrose reduced the proportion of the explants which formed adventitious buds and more dramatically reduced the number of shoots regenerated from each explant. Media supplemented with 3% sucrose were slightly better than those supplemented with 6% sucrose concerning the frequency of explants forming adventitious buds, with the exception the cotyledon explants that were

Table 3.9. The morphogenic responce of embryonic cotyledon explants to media supplemented with various sucrose concentrations. n = 20-25.

| Cultivar | Explant source | Sucrose concentration (w/v) | Hormone concentr. ($mg\ l^{-1}$) BAP | GA | % explants forming adventitious organs buds | shoots | Average shoot number per explant |
|---|---|---|---|---|---|---|---|
| Jalapeno | Embryos from immature fruit | 3 % | 4.5 | - | 24 | 24 | 1.83 |
| | | | 4.5 | 1.0 | 24 | 24 | 1.83 |
| | | 6 % | 4.5 | - | 96 | 96 | 1.38 |
| | | | 4.5 | 1.0 | 63 | 56 | 1.18 |
| | | 9 % | 4.5 | - | 81 | 56 | 0.82 |
| | | | 4.5 | 1.0 | - | - | - |
| | Embryos from dry seeds | 3 % | 4.5 | - | 92 | 76 | 1.13 |
| | | | 4.5 | 1.0 | 48 | 41 | 1.23 |
| | | 6 % | 4.5 | - | 74 | 30 | 0.45 |
| | | | 4.5 | 1.0 | 62 | 35 | 0.63 |
| | | 9 % | 4.5 | - | 54 | 15 | 0.29 |
| | | | 4.5 | 1.0 | 58 | 31 | 0.53 |
| California Wonder | Embryos from dry seeds | 3 % | 4.5 | - | 88 | 36 | 0.50 |
| | | | 4.5 | 1.0 | 54 | 54 | 1.64 |
| | | 6 % | 4.5 | - | 73 | 50 | 0.74 |
| | | | 4.5 | 1.0 | 69 | 27 | 0.56 |
| | | 9 % | 4.5 | - | 34 | 15 | 0.44 |
| | | | 4.5 | 1.0 | 44 | 0 | 0 |

Basal medium: MS. Temperature: 25 °C.
Light: the first 5 days complete darkness, then standard light conditions.
Duration of culture: 28 days.

excised from immature embryos of cv. "Jalapeno". The most striking effect of 3% sucrose was that it stimulated most of the explants to produce adventitious buds and shoots. The number of the regenerated shoots per explant at this sucrose concentration was significantly higher than it was in media supplemented with 6% or 9% sucrose. The developmental stage of the explant seemed to play a significant role; for instance, the cotyledon explants from immature embryos, which produced adventitious buds, went on to produce a higher proportion of shoots with stems than did the explants from dry seeds. Similar results were obtained concerning the number of shoots per each explant (Table 3.9). Although the percentage of explants forming adventitious buds was not significantly different between the two cultivars, it seemed that explants of cv. "Jalapeno", subjected to medium supplemented with 3% sucrose, produced more shoots per experimental unit than did explants from cv. "California Wonder". The growth rate of shoots regenerated from explants subjected to 3% sucrose was much higher than that in media containing 6% or 9% sucrose.
Callus growth in media supplemented with 9% sucrose was faster than that in media containing 6% sucrose, whereas relatively small and rather slowly growing callus was produced in media with 3% sucrose. In 28-day-old cultures the callus mass covered a considerably larger number of buds regenerated from cotyledons subjected to media supplemented with 9% or 6% sucrose.

### *3.10. The effect of genotype on adventitious shoot regeneration*

The effect of genotype was tested by examining the morphogenic response of embryonic cotyledon explants from dry seeds of ten cultivars (most of them Greek) subjected to MS3 basal medium supplemented

with 4.5 mg $l^{-1}$ BAP. Cultures were kept the first 5 days under complete darkness and then exposed to light of 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod. The temperature regime was that of 25 °C. A procedure of two stages, each of 28 days, was applied. Table 3.10 illustrates the results that obtained by the end of each stage.

A considerable range of morphogenic responces was observed in cotyledon explants with different genetic backgrounds. For instance, cultivars were classified in three well distinguishable groups according to their capability to initiating bud and foliose structures: a) explants of cvs Pt-46, Pt-14, Pt-455, Pt-824 and Pt-53/13 initiated bud and foliose structures at a frequency higher than 79%; b) explants of cvs Hybelle, Pennbell and Pt-452 responded at a frequency between 69% and 79% and c) explants of cvs Propenza and Tomato-pimento responded at a frequency lower that 50%.

According to the cotyledon's potential for producing extended shoots the cultivars could be divided in two groups: a) Pt-46, Pt-14, Pt-455, Pt-824 and Hybelle in which more than 68% of the explants produced shoots and b) Pt-452, Tomato-pimento, Pt-53/13 and Propenza in which less than 50% of the explants responded.

Concerning the number of shoots per explant, cultivars were classified in three groups: a) Pt-46 with an average shoot number of 3, b) Pt-14, Hybelle, Pt-455 and Pt-824 with an average number between 1.67 and 2.00 and c) Pennbell, Pt-452, Tomato-pimento, Pt-53/13 and Propenza with an average shoot number smaller than 1.50. By any measure, it seemed that cvs Pt-46, Pt-14, Hybelle, Pt-455 and Pt-824 were superior to the others in terms of the morphogenic response. The response of cvs Propenza and Tomato-pimento were extremely limited,

Table 3.10. The effect of genotype on the adventitious bud-shoot regeneration from embryonic cotyledon explants.

| | Stage I | | | Stage II | | | |
|---|---|---|---|---|---|---|---|
| | Number of explants | Adventitious bud formation | | Adventitious shoot development | | Number of shoots per explant | |
| Cultivar | used | Number | % | Number | % | Maximum | Average |
| Pt-46 | 22 | 22 | 100 c | 22 | 100 d | 5 | 3.09 c |
| Pt-14 | 24 | 24 | 100 c | 21 | 88 cd | 7 | 2.00 b |
| Hybelle | 32 | 22 | 69 bc | 22 | 100 d | 4 | 1.95 b |
| Pt-455 | 23 | 19 | 83 c | 15 | 79 cd | 3 | 1.93 b |
| Pt-824 | 7 | 7 | 100 c | 6 | 86 cd | 3 | 1.67 b |
| Pennbell | 24 | 19 | 79 bc | 13 | 68 c | 2 | 1.23 a |
| Pt-452 | 24 | 17 | 71 bc | 7 | 41 b | 1 | 1.14 a |
| Tomato-pimento | 24 | 7 | 29 a | 2 | 28 ab | 1 | 1.00 a |
| Pt-53/13 | 25 | 23 | 92 c | 2 | 10 a | 2 | 1.50 ab |
| Propenza | 29 | 13 | 49 ab | 2 | 15 a | 1 | 1.00 a |

Basal medium: MS3 + 4.5 mg $l^{-1}$ BAP. Temperature: 25 °C in both stages.

Light: the first 5 days complete darkness, then standard light conditions in both stages.

Duration of culture: 56 days.

whereas explants of Pt-53/13, although they initially formed many bud and foliose structures, failed to support the development of buds into shoots with extended stems (Table 3.10).

Creamy and compact callus was produced from explants of all tested cultivars, but it was a predominant process in explants of cvs "Propenza", "Tomato-pimento" and Pt-53/13, where callus proliferation was rapid, exceeding the growth rate of buds.

### *3.11. The morphogenic response of embryonic tissues on media supplemented with kinetin or zeatin*

The response of the embryonic tissues to kinetin or zeatin has been investigated by culturing embryonic cotyledon and hypocotyl explants of cv. "California Wonder" on MS3 medium supplemented with either kinetin or zeatin at concentration of 0.1, 1.0, 5.0 and 10.0 mg $l^{-1}$. The light and temperature regimes that applied were those as described in the section 3.9.

Table 3.11 shows that zeatin at any of the concentrations tested was more suitable for adventitious bud regeneration from both cotyledon and hypocotyl explants than was kinetin. Almost all of the explants exposed to 5 or 10 mg $l^{-1}$ zeatin formed adventitious buds, whereas only 58% and 79% of the hypocotyls exposed to 5 or 10 mg $l^{-1}$ kinetin respectively and 50% of the cotyledons exposed to either 5 or 10 mg $l^{-1}$ kinetin underwent the process. The presence of 0.1 or 1.0 mg $l^{-1}$ kinetin did not promote bud regeneration from cotyledon explants or encouraged the process in hypocotyl explants. In contrast to kinetin, the application of 1.0 mg $l^{-1}$ zeatin promoted the bud initiation process in a high number (35%) of cotyledons or even higher number (55%) of hypocotyls. Incorporation of 0.1 mg $l^{-1}$ zeatin either did not promote or slightly encouraged the process in cotyledon or hypocotyl explants respectively (Table 3.11.)

Table 3.11. The morphogenic response of embryonic cotyledon and hypocotyl explants of cv. "California Wonder" to media supplemented with kinetin or zeatin. n =20

| Hormone concentration (mg $l^{-1}$) | | Percentage of cotyledon explants forming | | Percentage of hypocotyl explants forming | |
|---|---|---|---|---|---|
| | | buds | roots | buds | roots |
| kinetin | 0.1 | 0 | 0 | 20 | 85 |
| " | 1.0 | 0 | 0 | 0 | 84 |
| " | 5.0 | 50 | 0 | 58 | 37 |
| " | 10.0 | 50 | 0 | 79 | 10 |
| zeatin | 0.1 | 0 | 0 | 16 | 44 |
| " | 1.0 | 35 | 0 | 55 | 20 |
| " | 5.0 | 95 | 0 | 90 | 0 |
| " | 10.0 | 95 | 0 | 90 | 0 |

Basal medium: MS3. Temperature: 25 °C.
Light conditions: The first 5 days complete darkness; then standard light conditions.
Duration of culture: 28 days.

Rhizogenesis did not take place at all in cotyledons cultured on media containing kinetin or zeatin at any level of concentration tested. But rhizogenesis indeed occurred in hypocotyl explants. Incorporation of 0.1 or 1.0 mg $l^{-1}$ kinetin strongly promoted the root-formation process in hypocotyl explants, whereas higher concentrations reduced the frequency of the explants which underwent the process. Zeatin at concentration of 5.0 or 10.0 mg $l^{-1}$ inhibited rhizogenesis in hypocotyl explants.

Callus formation occurred in cotyledon and hypocotyl explants exposed to any concentration of kinetin or zeatin that was tested. The callus was creamy and compact in the presence of either cytokinin but a little harder under the influence of kinetin.

### *3.12. Response of young leaves to media supplemented with BAP, kinetin, zeatin, zeatin riboside and 2,4-D*

Whole 2 to 7 mm long young leaves of cv. "Red Chili" were inoculated on MS3 basal medium supplemented with 2.25 and 4.5 mg $l^{-1}$ BAP or 5 and 10 mg $l^{-1}$ zeatin or zeatin riboside or 5 mg $l^{-1}$ kinetin or 3 and 5 mg $l^{-1}$ 2,4-D. Callus proliferation occurred on almost all of the explants exposed to any tested medium. It started at the proximal cut edge or at the main vein of the lower (abaxial) epidermis, which was in contact with the solid medium. White friable callus production was favoured by the presence of 2,4-D, whereas cytokinins (BAP, kinetin, zeatin and zeatin riboside) encouraged the formation of cream-white and compact callus. None of the media favoured the initiation of adventitious roots.

Adventitious shoots regenerated from 7% of explants subjected to medium supplemented with 4.5 mg $l^{-1}$ BAP, 13% and 22% of those on media

Table 3.12. The effect of 2,4-D, BAP, zeatin, zeatin riboside (ZR) and kinetin on shoot regeneration from the entire young leaf explants of cv. "Red Chili". n = 17-25.

| Hormone concentration ($mg\ l^{-1}$) | Adventitious shoot regeneration |
|---|---|
| 3.0 2,4-D | 0 |
| 5.0 2,4-D | 0 |
| 2.25 BAP | 0 |
| 4.5 BAP | 7 |
| 5.0 zeatin | 13 |
| 10.0 zeatin | 22 |
| 5.0 ZR | 0 |
| 10.0 ZR | 4 |
| 5.0 kinetin | 0 |

Data from 28-day-old cultures represent the percentage of explants forming adventitious shoots.

Incubation conditions: standard.

Entire young leaf explants: 2-7 mm long.

containing 5 or 10 mg $l^{-1}$ zeatin respectively and 4% of those on medium with 10 mg $l^{-1}$ zeatin riboside. The introduction of 5 mg $l^{-1}$ zeatin riboside or kinetin did not encourage the adventitious shoot regeneration process (Table 3.12). The most responsive explants to BAP or zeatin for shoot regeneration appeared to be those explants of 3 to 5 mm length. The initiation of adventitious shoots took place at the proximal parts of young leaves and particularly from the abaxial surface attached to the agar medium.

# C H A P T E R IV

## SHOOT-TIP AND AXILLARY BUD CULTURE

## *4.1. Shoot-tip culture*

### *4.1.1. The response of shoot-tip cultures to media supplemented with GA, BAP or 2,4-D*

Shoot-tips, consisting of the apical meristem and four leaf primordia, were excised from 10 to 15-day-old seedlings of cvs "Jalapeno", "Red Chili" and "Sweet Banana" and inoculated onto MS or B5 basal media containing 3% (w/v) sucrose. These media were hormone-free or supplemented with 1.0 mg $l^{-1}$ GA, 0.1 mg $l^{-1}$ BAP or 3.0 mg $l^{-1}$ 2,4-D. Cultures were grown in the standard conditions.
The aim of this experiment was either to encourage the growth of the original apical meristem ("normal developmment") or to initiate any adventitious morphogenic response.

A high proportion (more than 90%) of the apical meristems from all cultivars developed into shoots when they were cultured on MS or B5 hormone-free media or on those supplemented with 1.0 mg $l^{-1}$ GA. The addition of 0.1 mg $l^{-1}$ BAP to both MS and B5 media reduced significantly the number of shoot-tips from cv. "Jalapeno" which grew, as it did in "Red Chili" shoot-tips cultured on B5 basal medium. In contrast, the presence of BAP did not alter the frequency of the shoot-tips of cv. "Sweet Banana" cultured on MS or B5 basal media. These explants underwent "normal development (Table 4.1.1).
The presence of 3.0 mg $l^{-1}$ 2,4-D, in both MS and B5 basal media, completely suppressed the growth of the apical meristems with all of the cultivars tested.
A considerable variation in shoot growth rate was observed according to the cultivar on either basal media or on media containing hormone. For instance, "Jalapeno" shoots grew faster on MS medium supplemented

Table 4.1.1. The response of shoot-tip explants to media supplemented with GA, BAP or 2,4-D. n =15

| Medium | | Percentage of shoot-tip explants in which the apical meristem developed into plantlet | | |
|---|---|---|---|---|
| | Hormone concentration | Cultivar | | |
| Basal | $(mg\ l^{-1})$ | Jalapeno | Red Chili | Sweet Banana |
| MS3 | hormone-free | 100 c | 100 c | 100 c |
| | 1.0 GA | 100 c | 100 c | 100 c |
| | 0.1 BAP | 55 b | 100 c | 80 c |
| | 3.0 2,4-D | 0 a | 0 a | 0 a |
| B5 3 | hormone-free | 90 c | 60 c | 100 c |
| | 1.0 GA | 100 c | 100 c | 100 c |
| | 0.1 BAP | 30 b | 0 a | 86 c |
| | 3.0 2,4-D | 0 a | 28 a | 0 a |

Culture conditions: standard. Duration of culture: 60 days.

with 1,0 mg $l^{-1}$ GA than on MS or B5 hormone-free medium or B5 medium supplemented with 1.0 mg $l^{-1}$ GA. "Red Chili" shoots grew faster on MS medium supplemented with 1.0 mg $l^{-1}$ GA than on MS hormone-free medium or that containing 0.1 mg $l^{-1}$ BAP. The B5 medium seemed to favour less the shoot growth than did MS medium. Concerning the growth rate of the "Sweet Banana" shoots, it was mostly influenced by the type of the growth regulator regardless of the basal medium. Thus, faster growth was observed in medium supplemented with 1.0 mg $l^{-1}$ GA followed by the hormone-free medium and that containing 0.1 mg $l^{-1}$ BAP.

Callogenesis occurred on almost all explants cultured in any tested medium. Callus from explants inoculated onto hormone-free medium was confined mostly to the healing process at the cut surface. The presence of BAP resulted in a white or creamy or white-creamy callus that was compact and hard, whereas 2,4-D resulted in the formation of a white or glassy large callus mass that was friable. Shoot-tip explants cultured on medium containing GA produced callus with characteristics graded between the above described ones, that is white-creamy friable and/or compact callus.

### *4.1.2. The response of cv. "California Wonder" shoot-tips to media supplemented with BAP, NAA, IBA alone or in combination*

Shoot-tips consisting of the apical meristem and up to four leaf primordia were excised from 15-day-old seedlings of cv. "California Wonder" and were inoculated onto MS2 basal medium supplemented with

BAP (2.25 or 4.5 mg $l^{-1}$) or NAA (0.1, 1.0, 3.0 or 10.0 mg $l^{-1}$) or IBA (0.1, 1.0, 3.0 or 10.0 mg $l^{-1}$) as a sole hormone or in combination with 0.1 mg $l^{-1}$ BAP, as indicated in Table 4.1.2. The cultural conditions were the same as those described in section 4.1.1.

Primary leaf expansion, but not shoot growth, occurred with high frequency (73% to 100%) in explants subjected to media supplemented with 4.5 mg $l^{-1}$ BAP, 0.1 and 1.0 mg $l^{-1}$ NAA or 0.1, 1.0 and 3.0 mg $l^{-1}$ IBA. The number of explants showing primary leaf expansion was reduced in media containing 2.25 mg $l^{-1}$ BAP alone, 3.0 and 10.0 mg $l^{-1}$ NAA alone or IBA at any tested concentration in combination with 0.1 mg $l^{-1}$ BAP. The process was completely inhibited in media containing 0.1 mg $l^{-1}$ BAP in combination with NAA (at any level) or in a medium supplemented with only 10.0 mg $l^{-1}$ NAA (Table 4.1.2).

Adventitious root regeneration occurred from shoot-tip explants cultured on media supplemented with either 0.1, 1.0 and 3.0 mg $l^{-1}$ NAA alone or 1.0, 3.0 and 10.0 mg $l^{-1}$ IBA alone. The presence of 0.1 mg $l^{-1}$ BAP in media containing 0.1, 1.0, 3.0 and 10.0 mg $l^{-1}$ IBA or in those containing 0.1 or 1.0 mg $l^{-1}$ NAA inhibited rhizogenesis completely, whereas it did not significantly affect the frequency of the explants forming adventitious roots in media supplemented with 3.0 or 10.0 mg $l^{-1}$ NAA (Table 4.1.2). It seemed that IBA was required at a higher concentration than NAA to achieve similar results concerning the proportion of explants which rooted, but that the IBA was more effective than NAA in producing roots capable of rapid elongation and branching. The presence of 0.1 mg $l^{-1}$ BAP not only inhibited the rhizogenesis or reduced the frequency of rooting explants in some cases, but also reduced the number of roots per explant, inhibited root branching and forced the unbranched roots to become

Table 4.1.2. The effect of BAP, NAA and IBA on shoot-tip cultures of cv. "California Wonder". n = 15-20.

| Hormone concentration ($mg\ l^{-1}$) | | Explant response (%) | | |
|---|---|---|---|---|
| | | Primary leaf | Production of adventitive organs | |
| Auxin | BAP | expansion | Roots | Shoots |
| - | 2.25 | 30 | 0 | 60 |
| - | 4.5 | 100 | 0 | 0 |
| 0.1 NAA | - | 94 | 19 | 0 |
| 1.0 " | - | 81 | 25 | 0 |
| 3.0 " | - | 19 | 6 | 0 |
| 10.0 " | - | 0 | 0 | 0 |
| 0.1 NAA | 0.1 | 0 | 0 | 0 |
| 1.0 " | 0.1 | 0 | 0 | 0 |
| 3.0 " | 0.1 | 0 | 13 | 0 |
| 10.0 " | 0.1 | 0 | 6 | 0 |
| 0.1 IBA | - | 73 | 0 | 0 |
| 1.0 " | - | 78 | 11 | 0 |
| 3.0 " | - | 80 | 10 | 0 |
| 10.0 " | - | 20 | 30 | 0 |
| 0.1 IBA | 0.1 | 20 | 0 | 0 |
| 1.0 " | 0.1 | 18 | 0 | 0 |
| 3.0 " | 0.1 | 10 | 0 | 0 |
| 10.0 " | 0.1 | 20 | 0 | 0 |

Basal medium: MS2. Culture conditions: standard.
Duration of culture: 28 days.

"tuberous" at the later stages of the cultures.

Adventitious shoot regeneration from shoot-tip explants occurred only in medium supplemented with 2.25 mg $l^{-1}$ BAP (Table 4.1.2).

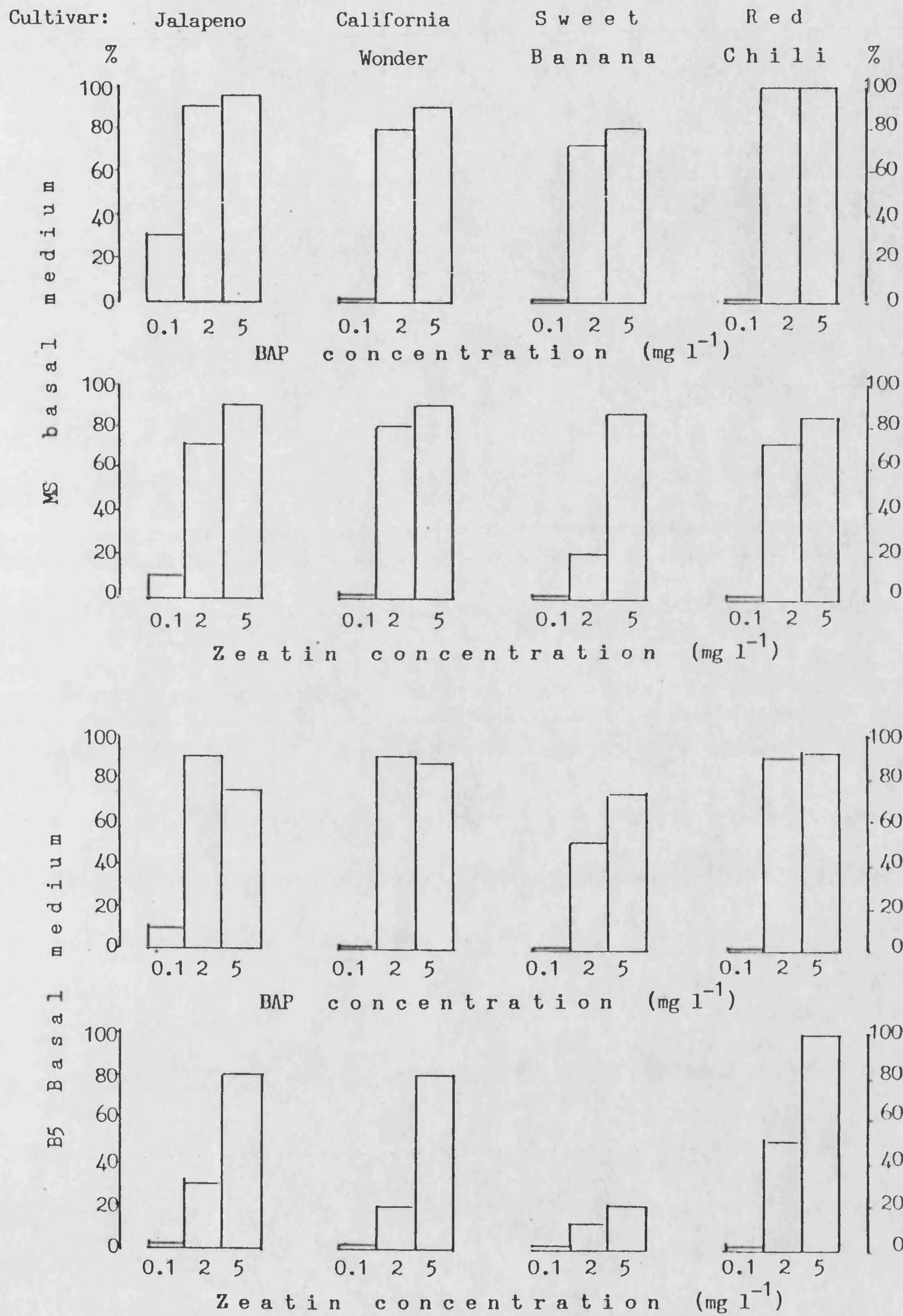
*4.1.3. Adventitious shoot regeneration from shoot-tip cultures*

The results concerning the adventitious shoot regeneration from shoot-tip cultures suggested that shoot-tip culture could be used as a rapid method for micropropagation purposes especially for high value hybrids.

For this reason, shoot-tips, 1 to 3 mm long, consisting of the apical meristem and up to three leaf primordia were excised from 10-day-old seedlings of cvs "Jalapeno", "California Wonder", "Sweet Banana" and "Red Chili" and cultured on MS or B5 basal media supplemented with 2% (w/v) sucrose and 0.1, 2.0 or 5.0 mg $l^{-1}$ BAP or zeatin. Incubation conditions were standard throughout the course of the culture.

Adventitious bud or foliose-structure regeneration occurred at a high frequency from explants of all cultivars in media supplemented with 2,0 or 5.0 mg $l^{-1}$ BAP or zeatin. A lower concentration of 0.1 mg $l^{-1}$ BAP or zeatin induced only foliose structures on some explants of cv. "Jalapeno". Zeatin at 2.0 mg $l^{-1}$ slightly promoted bud regeneration in B5 basal medium, but strongly promoted the process in MS basal medium. Zeatin at concentration of 5.0 mg $l^{-1}$ and BAP at 2.0 or 5.0 mg $l^{-1}$ greatly encouraged the adventitious bud regeneration process from explants of all cultivars subjected to MS

Fig. 4.1. The effect of various media on the adventitious bud regeneration from shoot-tip cultures. n = 17-20.




Culture conditions: standard. Duration of culture: 28 days.
Sucrose concentration: 2% (w/v).

or B5 media where more than 80% of the explants responded vigorously (Fig. 4.1).

Concerning the frequency of adventitious shoots per explant, some differences were observed due to either the basal medium, or the genotype or the hormone kind. Among the cultivars, "Jalapeno" and "Red Chili" responded better than "California Wonder" and "Sweet Banana" in forming more adventitious shoots per explant, regardless the basal medium or the hormone presence (Table 4.1.3). It should be noted that plants of "California Wonder" and "Sweet Banana" produce sweet pepper fruit, whereas those of "Red Chili" and "Jalapeno" produce hot (chili) fruit. Between basal media, MS was more suitable than B5 to the "Jalapeno" explants, but there was no significant difference between them concerning the response of the other cultivars' explants. Application of 2.0 or 5.0 mg $l^{-1}$ BAP as well as 5.0 mg $l^{-1}$ zeatin resulted in no differences in the number of adventitious shoots per explant of all cultivars (except "Red Chili"). Regarding the response of "Red Chili" explants it seemed that those cultured on MS medium containing 5.0 mg $l^{-1}$ BAP or zeatin produced more shoots than did explants cultured on MS supplemented with 2.0 mg $l^{-1}$ BAP. But no significant difference was observed when explants of the same cultivar were cultured on B5 basal medium supplemented with 5.0 or 2.0 mg $l^{-1}$ BAP or zeatin. No shoots were produced from explants of any cultivar cultured on either MS or B5 basal medium supplemented with 0.1 mg $l^{-1}$ BAP or zeatin, although some of the explants produced foliose structures.

The same results were observed on explants of cv. "Jalapeno" or "California Wonder" cultured on B5 medium containing 2.0 mg $l^{-1}$ zeatin (Table 4.1.3).

Table 4.1.3. The effect of various media on adventitious shoot regeneration from shoot-tip cultures. n = 17-20.

| Medium | | | Number of adventitious buds per explant | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Hormone | Cultivar | | | | | | | |
| | | concentration | Jalapeno | | California Wonder | | Sweet Banana | | Red Chili | |
| Basal | Hormone | (mg $l^{-1}$) | Average | Maximum | Average | Maximum | Average | Maximum | Average | Maximum |
| MS 2 | BAP | 0.1 | 0 a | 0 | 0 a | 0 | 0 a | 0 | 0 a | 0 |
| | | 2 | 2.94 efg | 4 | 1.50 bc | 2 | 1.20 bc | 2 | 1.25 bc | 2 |
| | | 5 | 3.50 gh | 5 | 2.00 cd | 4 | 1.33 bc | 3 | 3.63 gh | 7 |
| | zeatin | 0.1 | 0 a | 0 | 0 a | 0 | 0 a | 0 | 0 a | 0 |
| | | 2 | 1.60 bc | 4 | 1.17 bc | 2 | 1.00 b | 2 | 1.50 bc | 2 |
| | | 5 | 2.63 gh | 5 | 1.56 bc | 3 | 1.00 b | 2 | 4.00 h | 7 |
| B5 2 | BAP | 0.1 | 0 a | 0 | 0 a | 0 | 0 a | 0 | 0 a | 0 |
| | | 2 | 1.50 bc | 4 | 1.00 b | 2 | 1.00 b | 2 | 3.43 fgh | 4 |
| | | 5 | 1.00 b | 4 | 1.40 bc | 2 | 1.29 bc | 3 | 3.67 h | 6 |
| | zeatin | 0.1 | 0 a | 0 | 0 a | 0 | 0 a | 0 | 0 a | 0 |
| | | 2 | 0 a | 0 | 0 a | 0 | 1.00 b | 2 | 1.50 bc | 2 |
| | | 5 | 1.50 bc | 2 | 1.09 b | 2 | 1.20 bc | 3 | 2.53 de | 5 |

Culture conditions: standard. Duration of culture: 56 days.

Referring to the growth rates of the adventitious shoots as well as their health, the following observations were made:

a) "Red Chili" shoots grew very well on MS2 medium supplemented with 5 mg $l^{-1}$ zeatin followed by the MS2 medium containing 2 mg $l^{-1}$ zeatin or 2 mg $l^{-1}$ BAP and slightly less well on B52 medium with 2 mg $l^{-1}$ zeatin.

b) "Jalapeno" shoots grew very well on MS2 medium supplemented with 2 or 5 mg $l^{-1}$ zeatin, followed by the same basal medium containing 5 or 2 mg $l^{-1}$ BAP. Shoots grew very slowly on B52 basal medium supplemented with either BAP or zeatin.

c) Good growth of shoots of "California Wonder" was established on MS2 basal medium containing 5 mg $l^{-1}$ BAP, followed by B5 medium supplemented with 5 mg $l^{-1}$ BAP or zeatin.

d) "Sweet Banana" shoots grew better on MS2 medium supplemented with 5 mg $l^{-1}$ BAP than on the same basal medium containing 5 mg $l^{-1}$ zeatin.

In conclusion, BAP seemed to be better than zeatin for both bud induction and shoot growth from explants of cv "California Wonder", whereas zeatin was better than BAP for explants of the other cultivars.

#### *4.1.4. Anatomy and morphology of the adventitious regeneration process from shoot-tip explants*

Shoot-tip explants were subjected to MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP and cultured under standard conditions (25 °C temperature and 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod). During the course of culture, explants were removed at regular intervals and placed in fixative in preparation for histological studies under light or scanning electron microscope

Plate 4.1. Anatomy and morphology of shoot-tip explants subjected to MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP for regeneration studies.

A. Day 0. Section of shoot-tip, parallel to the axis, showing no sign of meristematic tissue around the cut edges. Nucleus of the cells of the same regions is not visible. x40

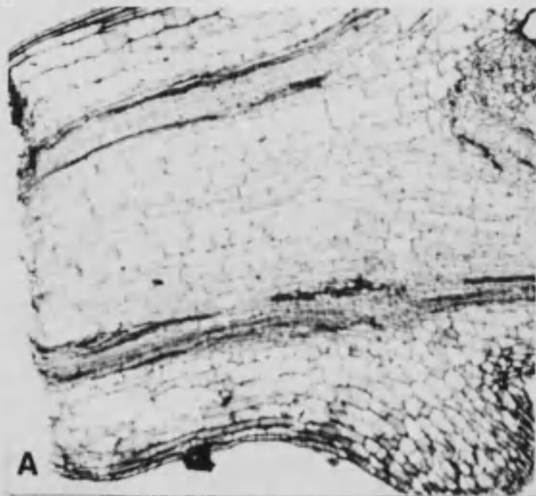
B. Day 0. SEM micrograph of the distal end of the shoot-tip explant showing no sign of meristems. Scale bar = 100 μm.

C. Day 1. Section of shoot-tip explant, parallel to the axis, showing the appearance of nuclei in the cells around the cut edges (arrowed). x 100

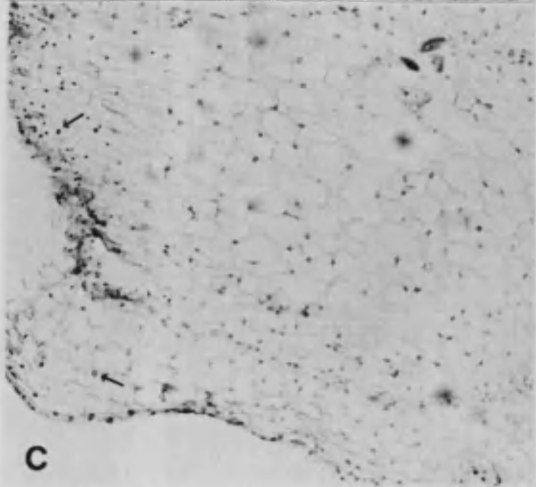
D. Day 2. SEM micrograph showing no change around the cut edges. The epidermal surface is still smooth. Scale bar = 100 μm.

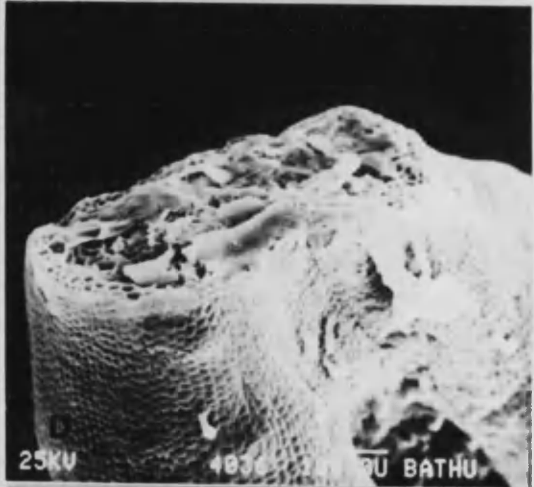
E. Day 3. Transverse section showing mitotic activities (arrowed) in cells close to the distal end of the shoot-tip. x 750

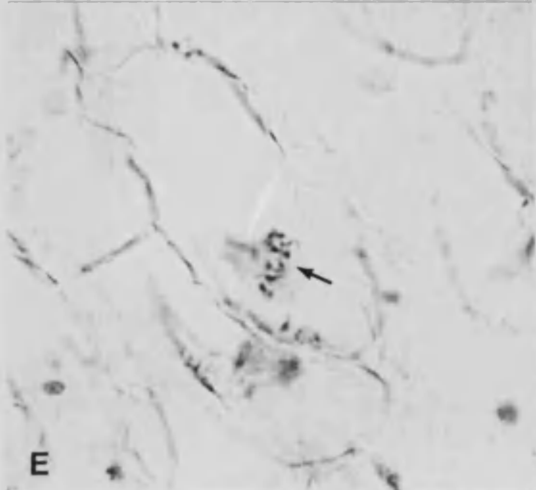
F. Day 4. SEM micrographs showing callus proliferation (arrowed) at the cut surface and swollen regions (sr) from the epidermis around the cut edges. Scale bar = 100 μm.

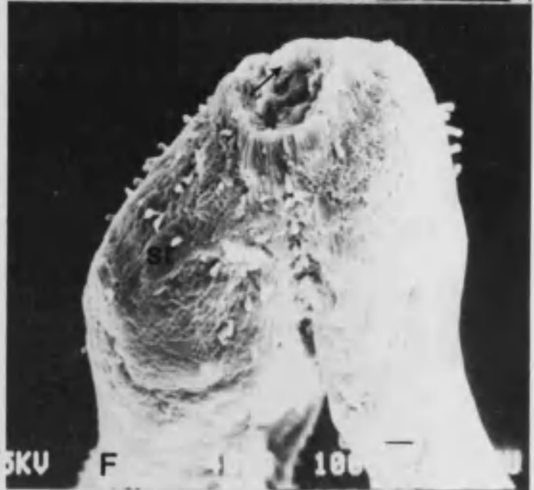

A

B
25KV X50
100.0U BA


C


25KV
BATHU


E


F

(sections 2.6 and 2.7).
At the time of dissection, explants were from 2 to 5 mm long and the light microscope and SEM micrographs showed that the epidermal layer around the cut edges was smooth and did not possess any trace of meristematic tissue (Plate 4.1 A and B). After one day in culture, no mitotic activities were observed either at the epidermal layer or in the vascular elements (Plate 4.1 C). By the second day of culture, no cell division was observed and the epidermal layer was still smooth with a regular arrangement of the cells (Plate 4.1 D). After three days in culture, mitotic activities were observed at the epidermal layer as well as in the vascular elements (Plate 4.1 E). One day later, cell division was extensive at the cut surface and the healing process was well advanced while in the epidermal layer small swollen regions had began to form (Plate 4.1 F). By the sixth and seventh day of culture, the swollen regions were enlarged and "nodule" structures were well established (Plate 4.2 A).
These "nodules" consisted of many uniform meristematic cells with densely stained cytoplasm, thin walls and without intercellular spaces (Plate 4.2 B and C). They were produced from the epidermal or sub-epidermal cells proximal to the cut surface regions.
After 10 days in culture, "bud-initials" or "bud-primordia" were easily recognized by either the light microscope (Plate 4.2 D) or the scanning electron microscope (Plate 4.2 E and F). The "bud-primordia" were derived from the "nodule" structures. Parallel to "nodule" and "bud-primordia" development, callus proliferation was occurring at the cut surface but well away of the regions which produced "nodules" (Plate 4.3 A). After twelve to sixteen days of culture, adventitious buds were very well organized (Plate 4.3 A and B) and their first true

Plate 4.2. Anatomy and morphology of shoot-tip explants subjected to MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP for regeneration studies.

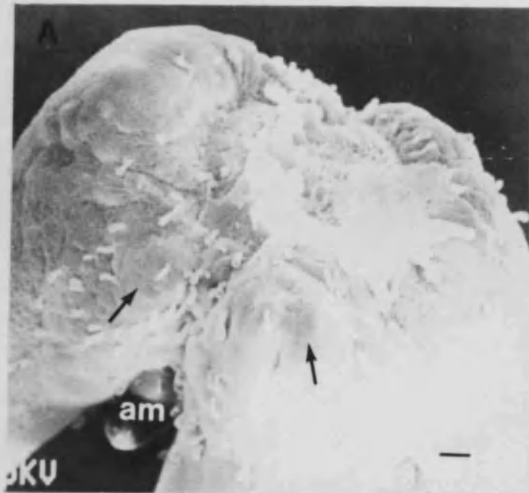
A. Day 6. SEM micrograph showing numerous swollen regions -nodules (arrowed) and the original shoot apical meristem (am). Scale bar = 100 μ m.

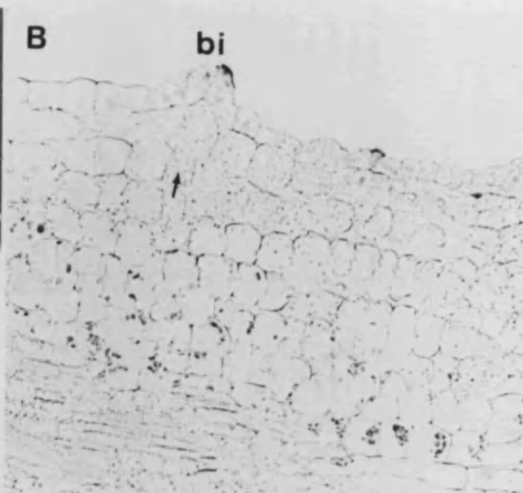
B. Day 7. Section parallel to shoot axis showing bud initial (bi) formation as a result of the epidermal cell division and periclinal division in subepidermal cell (arrowed). x 100

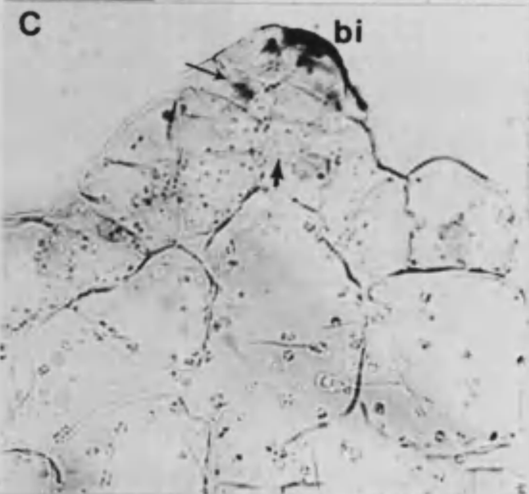
C. Day 7. As above. Note the mitotic activity (thin arrow) in one of the cells of the bud initial as well as periclinal division in subepidermal cell (thick arrow). x 400

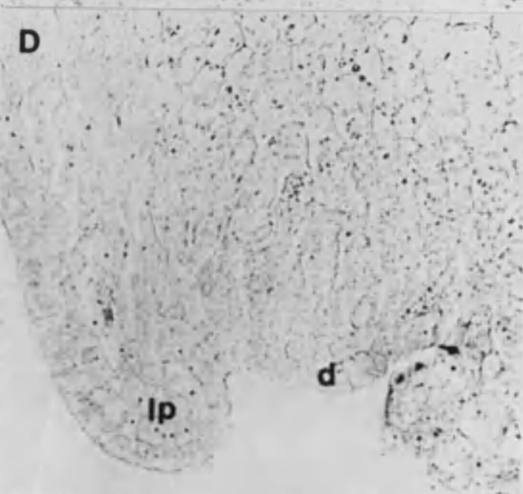
D. Day 10. Section parallel to the shoot axis showing adventitious bud formation at the edge of the cut surface. d = dome, lp = leaf primordium. x 400

E and F. Day 10. SEM micrograph showing adventitious bud formation (arrowed) from epidermal regions close to the cut edges, as well as callus (cl) proliferation at the cut surface. lp = leaf primordium. Scale bar: E = 1.000 m., F = 100 μ m.


A
am
KV


B
bi


C
bi


D
lp
d

cl
E
25KV
4048 1000.0U BA

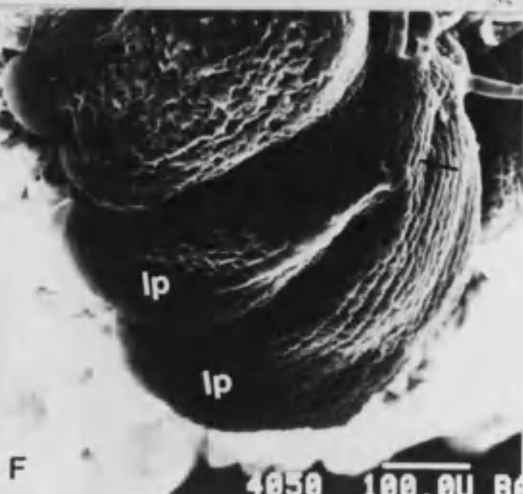

lp
lp
F
4050 100.0U BA

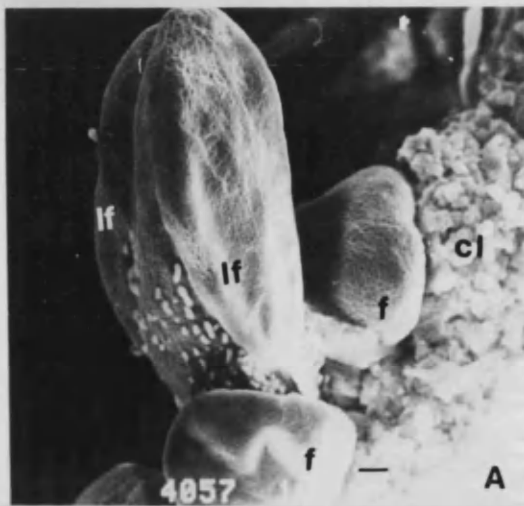
Plate 4.3. Anatomy and morphology of shoot-tip explants subjected to MS3 basal medium supplemented with 4.5 mg $l^{-1}$ BAP for regeneration studies.

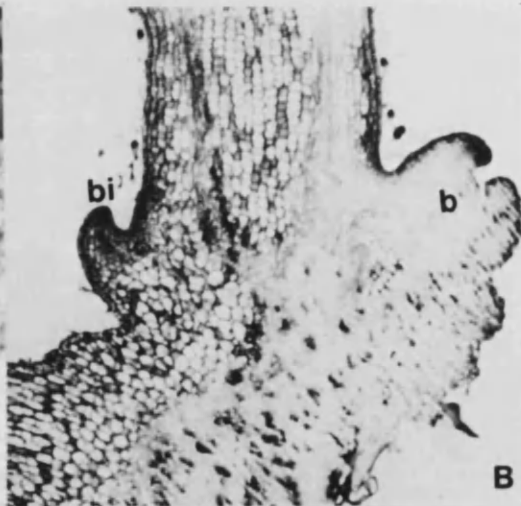
A. Day 12. SEM micrograph showing adventitious shoot. Note the callus (cl), foliose structure (f) and the leaf (lf). Scale bar = 100 μm.

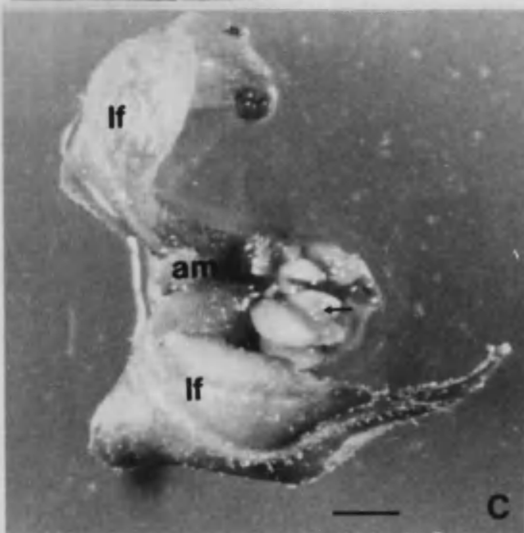
B. Day 13. Section parallel to the shoot-tip axis showing adventitious bud (b) and bud initial (bi). x 40

C. Day 18. Photograph showing adventitious shoots (arrowed) regenerated around the cut edges of the shoot-tip explant. lf = original leaf of the explant, am = original shoot apex of the explant. Scale bar = 1 mm.

D. Day 22. As above. Scale bar = 1mm.


lf
lf
f
cl
f
4057
A


bi
b
B


lf
am
lf
C

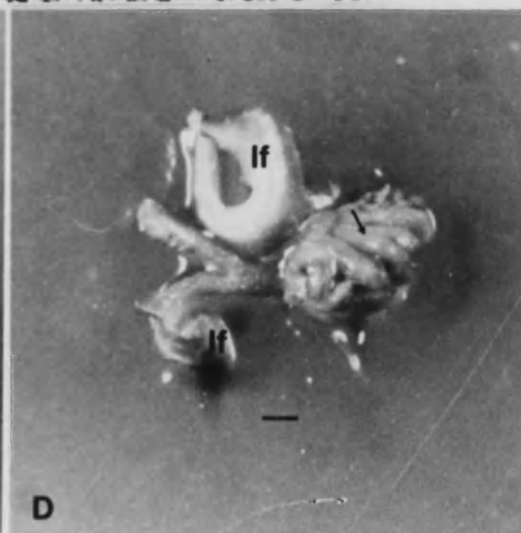

lf
lf
D

Plate 4.4. Plantlets derived adventitiously from tissue culture.

A. Shoots regenerated adventitiously from embryonic cotyledon cultured on MS medium supplemented with 4.5 mg $l^{-1}$ BAP and rooted on B5 medium supplemented with o.1 mg $l^{-1}$ IBA. x 1.5

B. Plants of cv. "Red Chili" regenerated adventitiously from shoot-tip cultures.

C. Plants of cv. "Red Chili" regenerated adventitiously from petiole cotyledon.

A

B

C
Plants regenerated
from tissue Culture
cv. "Red Chili"

leaf was visible by scanning electron microscope. By the eighteenth day, adventitious shoots were recognizable by stereo microscope (Plate 4.3 C) or even by the naked eye.

In general, the process of adventitious shoot regeneration from shoot-tip explants was similar to that described in section 3.2 for adventitious shoot regeneration from cotyledon and hypocotyl explants.

## 4.2. *The response of axillary buds in culture*

Nodes were excised from 20 to 25-day-old seedlings of cvs "California Wonder", "Jalapeno", "Red Chili" and "Sweet Banana" and inoculated, with or without their associated leaves, onto MS3 or B53 hormone-free media or the same media supplemented with 1.0 mg $l^{-1}$ GA or 5.0 mg $l^{-1}$ zeatin or 3.0 mg $l^{-1}$ BAP. Cultures were incubated at 25 °C and 10.2 microeinsteins $m^{-2}sec^{-1}$ light with 16-hour photoperiod.

Adventitious organogenesis did not occur from any explant cultured on any of the media. The only responses that were observed were either callus formation or normal growth of the axillary bud to form a stem. The latter process was influenced by the genotype, the culture medium and the presence or absence of the node′s leaf. The frequency of the event is indicated in Table 4.2. In general, the proportion of the axillary bud explants associated with their leaves of cvs "California Wonder" and "Jalapeno" which developed normally was lower than explants without their leaves regardless the type of the medium onto which they were inoculated. This difference did not occur in explants of cvs "Red Chili" and "Sweet Banana" except the case of "Sweet Banana" explants inoculated onto B5 medium where the axillary buds without their associated leaves grew better than those with their associated leaves.

Table 4.2. The response of axillary buds of various cultivars to media supplemented with GA, zeatin or BAP. n = 18-20.

| Medium | | | Percentage of explants producing a shoot | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cultivar | | | | | | | |
| | | California Wonder | | Jalapeno | | Red Chili | | Sweet Banana | |
| | Hormone concentration | Type of explant | | Type of explant | | Type of explant | | Type of explant | |
| Basal | $(mg\ l^{-1})$ | + leaf | - leaf | + leaf | - leaf | + leaf | - leaf | + leaf | - leaf |
| MS 3 | hormone-free | 25 | 100 | 60 | 100 | 60 | 69 | 0 | 0 |
| | GA 1.0 | 43 | 90 | 100 | 85 | 73 | 58 | 0 | 0 |
| | zeatin 5.0 | 40 | 79 | 33 | 100 | 100 | 100 | 88 | 100 |
| | BAP 3.0 | 0 | 0 | 36 | 100 | 100 | 100 | 38 | 30 |
| B5 3 | hormone-free | 0 | 33 | 100 | 71 | 100 | 71 | 0 | 0 |
| | GA 1.0 | 60 | 58 | 13 | 82 | 55 | 73 | 0 | 33 |
| | zeatin 5.0 | 73 | 75 | 50 | 83 | 100 | 90 | 50 | 100 |
| | BAP 3.0. | 0 | 10 | 21 | 100 | 88 | 100 | 100 | 20 |

Culture conditions: standard. Duration of culture: 45 days.
Explants were consisted of a node with the axillary bud with or without their associated leaf.

Referring to the effect of the kind of the hormone it seemed that zeatin and GA were equally suitable for axillary bud growth of cvs "California Wonder", "Jalapeno" and "Red Chili" inoculated onto MS basal medium, whereas zeatin and BAP were equally suitable for explants of cvs "Jalapeno", "Red Chili" and "Sweet Banana" cultured on B5 medium. Hormone-free media were also suitable for bud development of cvs "Jalapeno" and "Red Chili" explants regardless of the type of the basal medium or the presence or absence of their associated leaves.

With reference to the rate of the shoot growth the following observations were made:

a) "Jalapeno" shoots grew very well on MS or B5 media either hormone-free or supplemented with GA.

b) "California Wonder" shoots grew very well on MS medium supplemented with GA.

c) "Red Chili" shoots grew very well on MS medium supplemented with zeatin.

d) "Sweet Banana" shoots grew best on MS or B5 media supplemented with zeatin and on B5 medium supplemented with BAP.

Shoots grown on media containing GA possessed leaves with a pale-green colour, whereas those on hormone-free medium or supplemented with a cytokinin possessed leaves with dark-green colour.

The proportion of the axillary buds which developed into stems as well as their growth rate were greater in explants of cvs "Jalapeno" and "Red Chili" than in explants of cvs "California Wonder" and "Sweet Banana".

The presence of node´s leaf in most of the cases affected negatively the bud development. This was mainly due to the fact that sooner or

later they abscised and from the abscission zone callus rapidly proliferated to the extent that it covered the bud and eventually prevented its further development.

### *4.3. Root formation on shoot cultures*

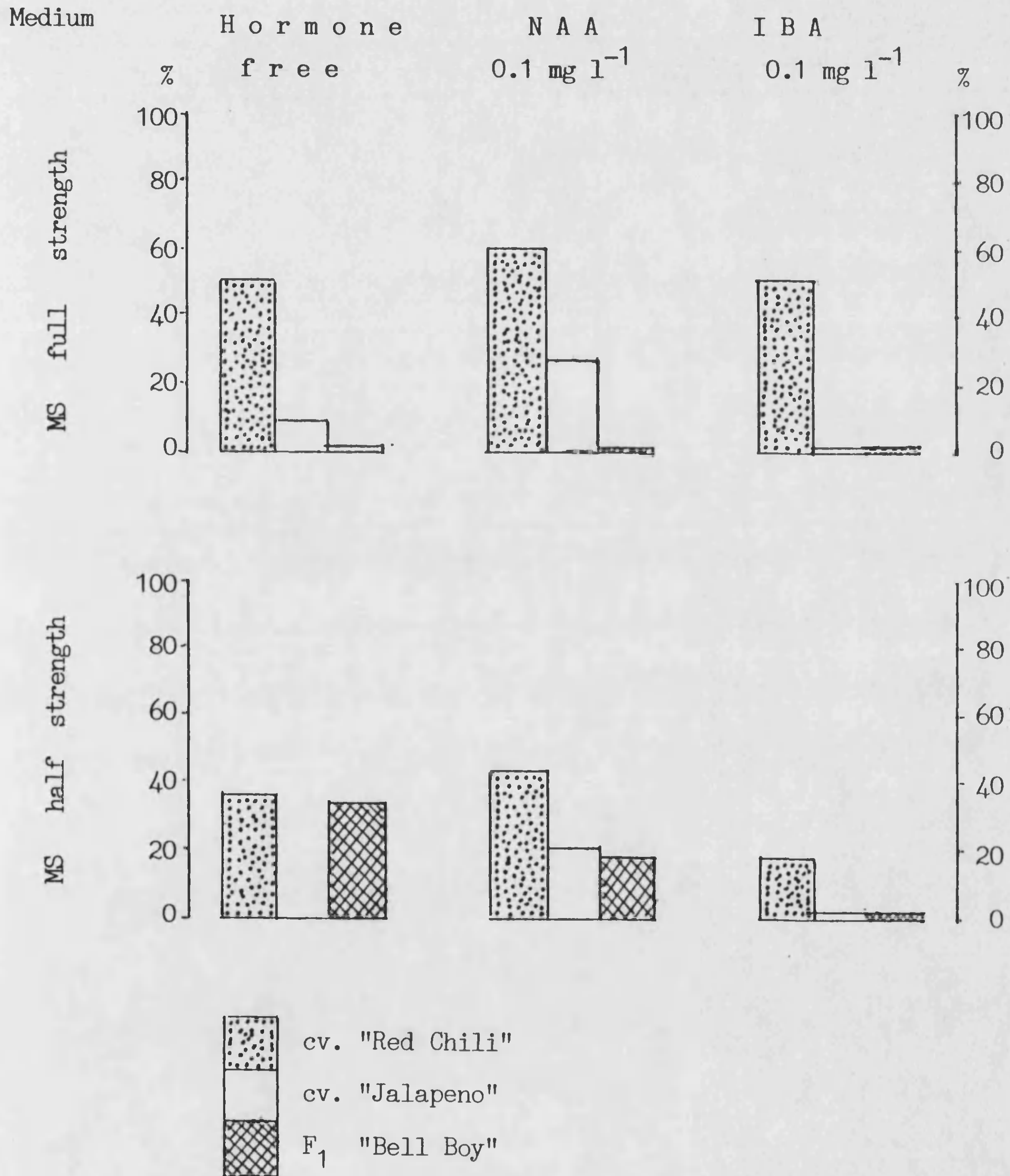
For obtaining complete plants, shoots derived adventitiously or non-adventitiously from tissue cultures or from seedlings were subjected to a series of media supplemented with an auxin with the aim of inducing root formation. Incubation conditions were 20 °C and light of 20.40 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod.

#### *4.3.1. Root formation on shoots from cotyledon culture*

Adventitiously-regenerated shoots up to 5 mm long from cotyledon culture of cvs "Red Chili", "Jalapeno" and "Bell Boy" $F_1$ hybrid, were subjected to full or half strength MS2 medium either free of hormones or supplemented with 0.1 mg $l^{-1}$ NAA or IBA and incubated at temperature and light conditions as described above.

Concerning the frequency of shoots which rooted the followed remarks were observed: Full strength MS2 medium was more suitable than half strength for the induction of roots in shoots of cv. "Red Chili", while the opposite results were obtained with the shoots of "Bell Boy" $F_1$ hybrid. Among the cultivars, shoots of "Red Chili" rooted more easily than those of "Jalapeno" or "Bell Boy", but neither the presence of IBA nor NAA in the medium improved on the effectiveness of hormone-free medium (Fig. 4.2).

Fig. 4.2. The effect of NAA and IBA on rooting response of adventitiously regenerated shoots in 40-day-old cultures. n = 15-20.




Sucrose concentration: 2% (w/v). Temperature: 20 °C.

Light: 20,40 microeinsteins m$^{-2}$sec$^{-1}$ with 16-hour photoperiod.

*4.3.2. Root formation on shoots from shoot-tip cultures*

Shoots, up to 8 mm long, adventitiously regenerated from shoot-tip cultures of cvs "Red Chili", "Jalapeno" and "Sweet Banana" were cultured on B53 basal medium supplemented with either 0.2 or 0.9 mg $l^{-1}$ IBA under temperature and light conditions as described earlier.

Shoot-tips from all cultivars underwent rhizogenesis at a higher frequency when subjected to 0.2 mg $l^{-1}$ IBA rather than 0.9 mg $l^{-1}$ IBA, although this difference was not statistically significant. Also shoot-tips of cvs "Red Chili" and "Jalapeno" responded slightly better than those of cv. "Sweet Banana" irrespectively of the IBA concentration (Table 4.3).

*4.3.3. Root formation on shoots from seedlings*

Shoot-tips, 8-10 mm long, excised from 25-day-old seedlings of cvs "Jalapeno", "California Wonder", "Sweet Banana" and "Red Chili" and cultured on B52 basal medium supplemented with 0.01, 0.1, 0.5 or 1.0 mg $l^{-1}$ IBA under temperature and light conditions described in section 4.3.1.

Shoots of cv. "Red Chili" rooted more easily and more frequently than those of the other cultivars (Table 4.4). During the first 26 days of culture, rhizogenesis at a comparable high (more than 50%) frequency occurred only from shoot-tips of cv. "Jalapeno" subjected to 0.1, 0.5 or 1.0 mg $l^{-1}$ IBA. In all other cases, the rhizogenesis process took longer period of time to reach a frequency of more than 50%. After 60 days of culture a high percentage (73.3% and more) of "Red Chili" shoots rooted under any tested medium. Similar results were achieved from shoot-tips of cvs "Jalapeno" and "California Wonder"

Table 4.3. The effect of IBA on rooting of adventitiously regenerated shoots from shoot-tip cultured for 40 days.

| | $0.2\ mg\ l^{-1}$ IBA | | | $0.9\ mg\ l^{-1}$ IBA | | |
|---|---|---|---|---|---|---|
| | Total number of shoot-tips | Shoot-tips forming roots | | Total number of shoot-tips | Shoot-tips forming roots | |
| Cultivar | | Number | % | | Number | % |
| Red Chili | 17 | 14 | 82.4 | 74 | 45 | 60.8 |
| Jalapeno | 23 | 17 | 73.9 | 21 | 14 | 66.7 |
| Sweet Banana | 45 | 29 | 64.4 | 61 | 35 | 57.4 |

Basal medium: B5 3. Temperature: 20 °C.
Light: 20.4 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod.

Table 4.4. The effect of IBA concentration on the root induction in shoot-tip excised from 25-day-old seedlings. n = 16-20.

| IBA concentration | Percentage of shoot-tips forming adventitious roots | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cultivar | | | | | | | |
| | Jalapeno | | California Wonder | | Sweet Banana | | Red Chili | |
| | Period of culture (days) | | | | | | | |
| (mg $l^{-1}$) | 26 | 60 | 26 | 60 | 26 | 60 | 26 | 60 |
| 0 | 25 a | 50 b | 25 a | 55 b | 0 a | 33 ab | 35 ab | 86 b |
| 0.01 | 25 a | 30 ab | 31 ab | 42 ab | 18 a | 48 b | 35 ab | 77 b |
| 0.1 | 56 b | 63 b | 31 ab | 75 b | 13 a | 50 b | 50 b | 81 b |
| 0.5 | 13 a | 40 ab | 22 a | 27 a | 38 ab | 50 b | 67 b | 88 b |
| 1.0 | 22 a | 22 a | 13 a | 13 a | 27 a | 64 b | 60 b | 73 b |

Basal medium: B5 2. Temperature: 20 °C.
Light: 20.40 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod.

cultured only on medium supplemented with 0.1 mg $l^{-1}$ IBA or from those of cv. "Sweet Banana" subjected to 1.0 mg $l^{-1}$ IBA. In all other cases, the frequency of the explants which rooted was lower than 50%.

Comparing the results described in sections 4.3.1, 4.3.2 and 4.3.3 it seems that shoot-tips excised from seedlings rooted more easily and quickly than those derived adventitiously from shoot-tip or cotyledon cultures. This was due mostly to the shoot-tip length at the time of inoculation. It was observed that explants longer than 10 mm rooted quicker and easier than the smaller ones.

### *4.3.4. Establishment of rooted plantlets in soil*

For plant establishment in the soil, plantlets were moved from culture containers, washed carefully with tap water to remove the agar from their roots and finally they were transplanted into pots filled with Levington potting compost. Acclimation of the plants was aided by placing the pots in a tray covered with a transparent plastic dome fitted with adjustable ventilators. During the first two days the ventilators were completely closed for establishing very high levels of humidity in the microenvironment. The ventilators were gradually opened and after a week plants were uncovered and exposed to the greenhouse conditions.

During this period of time, the plants produced new roots and gradually a balance of water uptake and water loss was established. By the use of this procedure, all of the tested plants survived and grew rapidly.

# CHAPTER V

# SOMATIC EMBRYOGENESIS

*Introduction*

A series of experiments was set up to investigate the most suitable media and culture conditions as well as the most appropriate explants for the induction of somatic embryogenesis. With exception of the preliminary investigations, a three-stage procedure was adopted: stage A in which the medium was supplemented with a high 2,4-D concentration for embryogenic tissue induction; stage B in which a hormone-free medium or one supplemented with a low auxin to cytokinin ratio was applied for proembryo formation; stage C in which a hormone-free medium or one supplemented with a low level of zeatin alone or in combination with a low GA concentration was applied to encourage the somatic embryos to develop into complete plantlets. Immature embryo tissues were used as explants and the cultures were kept initially in full darkness for 8 to 10 days, to allow adaptation to the new environmental conditions; otherwise the change from darkness (fruit environment) to light (culture), in addition to other changes, might have caused some damage. After this period, cultures were exposed to 10.2 microeinsteins $m^{-2}sec^{-1}$ light with a 16-hour photoperiod and 25 °C temperature. In all experiments, unless otherwise stated, an MS basal medium supplemented with 3% (w/v) sucrose, 100 mg $l^{-1}$ casaminoacids and 0.8% agar was used (MS3 medium).

*5.1. Preliminary investigations*

*5.1.1. The response of immature embryos to media supplemented with NAA, IBA and BAP*

Whole immature embryos or cotyledons and hypocotyls from immature embryos were used as explants. The immature embryos at various

developmental stages (section 2) were excised from immature pepper fruits of cv. "California Wonder" and the explants were cultured on MS3 basal medium supplemented with either NAA and IBA, each one applied alone at concentrations of 0.1, 1.0, 3.0 and 10.0 mg $l^{-1}$ or in combination with 1.0 or 4.5 mg $l^{-1}$ BAP. Cultures were subjected to light and temperature conditions described previously.

The results are presented in Table 5.1.1.. In medium supplemented with a high BAP to NAA or IAA portion, a number of cotyledon explants produced adventitious shoots in a manner similar to that described in section 4 concerning the response of cotyledon explants from mature seeds or from young seedlings. The frequency of the explants which responded, and as it is shown in Table 5.1.1 was not very high (only 20% to 30%). This was due to the fact that only cotyledons from immature embryos close to maturation (stages IV and V) responded whereas cotyledons from embryos at early developmental stages (I, II and III) did not show any morphogenic response; in fact they did not expand at all, remaining in the same states as initially. Hypocotyl explants did not show any sign of adventitious shoot regeneration.

Adventitious root regeneration occurred, at a low frequency, from cotyledon explants subjected to media supplemented with 1.0, 3.0 and 10.0 mg $l^{-1}$ IBA alone. A high proportion of the hypocotyl explants underwent adventitious root regeneration when exposed to media supplemented with either IBA alone at any level between 0.1 and 10.0 mg $l^{-1}$ or a high IBA to BAP rates. Adventitious rhizogenesis from both cotyledon and hypocotyl explants did not occur in media supplemented with any tested level of NAA alone or in combination with BAP. Roots produced in media with 0.1 or 1.0 mg $l^{-1}$ IBA were long and well branched, while those in media with 3.0 or 10.0 mg $l^{-1}$ IBA alone or in combination with BAP were smaller and poorly branched.

Table 5.1.1. The effect of NAA and IAA alone or in combination with BAP on the morphogenic response of the whole immature embryo, immature cotyledon and immature hypocotyl explants of cv. "California Wonder".

| Hormone concentration (mg $l^{-1}$) | | Frequency of morphogenic response (%) | | | | |
|---|---|---|---|---|---|---|
| | | Cotyledon explants forming adventitious organs | | Hypocotyl explants forming adventitious organs | | Embryos developing normally into complete |
| Auxin | BAP | Buds | Roots | Buds | Roots | plantlets |
| 0.1 NAA | - | 0 | 0 | 0 | 0 | 100 |
| 1.0 " | - | 0 | 0 | 0 | 0 | 0 |
| 3.0 " | - | 0 | 0 | 0 | 0 | 0 |
| 10.0 " | - | 0 | 0 | 0 | 0 | 0 |
| 0.1 NAA | 1.0 | 20 | 0 | 0 | 0 | 50 |
| 0.1 " | 4.5 | 30 | 0 | 0 | 0 | 0 |
| 1.0 " | 0.1 | 0 | 0 | 0 | 0 | 0 |
| 3.0 " | 0.1 | 0 | 0 | 0 | 0 | 0 |
| 0.1 IBA | - | 0 | 0 | 0 | 88 | 100 |
| 1.0 " | - | 0 | 10 | 0 | 30 | 100 |
| 3.0 " | - | 0 | 20 | 0 | 80 | 80 |
| 1.0 " | - | 0 | 10 | 0 | 40 | 80 |
| 0.1 IBA | 1.0 | 0 | 0 | 0 | 0 | 100 |
| 0.1 " | 4.5 | 19 | 0 | 0 | 0 | 0 |
| 1.0 " | 0.1 | 0 | 0 | 0 | 30 | 40 |
| 3.0 " | 0.1 | 0 | 0 | 0 | 60 | 100 |

Basal medium: MS3. Temperature: 25 °C.

Light: The first 8 days complete darkness; then standard light conditions.

Number of explants per treatment: embryos 10, hypocotyls 10 and cotyledons 20.

The immature embryos continued to develop normally, producing complete plants, in media supplemented with 0.1 mg $l^{-1}$ NAA alone or in combination with 1.0 mg $l^{-1}$ BAP. Similar, even better, results were obtained in media supplemented with IBA at any level between 0.1 and 10.0 mg $l^{-1}$ as well as in media containing a high IBA to BAP ratio or when 0.1 mg $l^{-1}$ IBA was combined with 1.0 mg $l^{-1}$ BAP (Table 5.1.1). The combination of 0.1 mg $l^{-1}$ IBA and 4.5 mg $l^{-1}$ BAP inhibited the development of the immature embryos. The growth rate of the immature embryos was greater in media containing 0.1 mg $^{-1}$ NAA alone, or 0.1 and 1.0 mg $l^{-1}$ IBA. In all other cases, where embryo development occurred, the growth rate was lower than that in the above media.

Somatic embryogenesis did not occur in any of the tested media.

### *5.1.2. The response of whole-immature embryo, immature cotyledon and hypocotyl explants to media containing 2,4-D and BAP*

Whole immature embryos, immature cotyledons and hypocotyls, at their II or III developmental stage, were excised from immature fruit of cv. "California Wonder" and were inoculated onto MS3 medium supplemented with 1.0 and 4.5 mg $l^{-1}$ BAP alone, or 0.1, 1.0, 3.0 and 10.0 mg $l^{-1}$ 2,4-D alone, or combinations of these hormones. Cultures were kept under light and temperature regimes described previously in this section.

Table 5.1.2 details the results. Cotyledon and hypocotyl explants did not respond to any of the media tested. They remained alive more or less in their initial state without any expansion. Callus formation occurred, at a high frequency, from immature embryo explants exposed to any 2,4-D concentration between 0.1 and 3.0 mg $l^{-1}$ or to 1.0 mg $l^{-1}$ BAP.

Table 5.1.2. The effect of 2,4-D and BAP on the morphogenic response of the whole immature embryo, immature cotyledon and immature hypocotyl explants of cv. "California Wonder".

| Hormone concentration (mg $l^{-1}$) | | Response of explants (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Callus formation | | | Adventitious bud formation | | | Expansion | | |
| 2,4-D | BAP | Cotyledon explants | Hypocotyl explants | Whole embryo explants | Cotyledon explant | Hypocotyl explant | Whole embryo explants | Cotyledon explant | Hypocotyl explant | Whole embryo explants |
| - | 1.0 | 0 | 0 | 50 | 0 | 0 | 40 | 0 | 0 | 10 |
| - | 4.5 | 0 | 0 | 10 | 0 | 0 | 20 | 0 | 0 | 0 |
| 0.1 | - | 0 | 0 | 90 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | - | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.0 | - | 0 | 0 | 67 | 0 | 0 | (*) | 0 | 0 | 0 |
| 10.0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1.0 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 4.5 | 0 | 0 | 25 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Data from 30-day-old cultures. Basal medium: MS3. Temperature: 25 °C.
Light: The first 10 days complete darkness; then standard light conditions.
Number of explants per treatment: 10 whole embryos, 10 hypocotyls and 20 cotyledons.
(*): explants forming adventitious embryo-like structures at a frequency of 30 %.

The number of immature embryos which formed callus was reduced in the presence of 4.5 mg $l^{-1}$ BAP or 0.1 mg $l^{-1}$ 2,4-D in combination with 1.0 or 4.5 mg $l^{-1}$ BAP. In all cases callus formation started from the hypocotyl region and spread downwards to the radical and/or upwards to the embryonic apex. Media supplemented with 10.0 mg $l^{-1}$ 2,4-D alone, or with 0.1 and 3.0 mg $l^{-1}$ 2,4-D in combination with 0.1 mg $l^{-1}$ BAP did not encourage callus formation.

Adventitious shoot regeneration occurred only from the whole immature embryo explants exposed to 1.0 or 4.5 mg $l^{-1}$ BAP in absence of 2,4-D. The process took place on the cotyledon regions and its course was similar to that described in section 3, when excised cotyledons from mature seeds were used as explants.

Normal development of the immature embryos occasionally occurred in media supplemented with 1.0 mg $l^{-1}$ BAP.

Unlike IBA and NAA, the auxin 2,4-D completely inhibited the normal development of immature embryos.

A number of immature embryo explants formed globular structures. These structures started as epidermal protuberances on to the proximal adaxial cotyledon regions. When the explants were subcultured on a hormone-free medium or medium containing very low 2,4-D concentration in combination with low cytokinin level they formed bipolar structures which in a very low frequency produced complete plantlets (for details see section 5.2.2).

## 5.2. *Embryogenesis from immature embryos*

### 5.2.1. *The response of immature embryos to media containing 2,4-D*

Based on the preliminary observations that some immature embryos

produced embryogenic tissues in media containing 3.0 mg $l^{-1}$ 2,4-D, an experiment was set up to examine in more detail the effect of various 2,4-D concentrations incorporated into the stage A medium as well as the effect of various media applied at stage B and C. Immature embryos, at various developmental stages, were exposed to stage A media supplemented with 1.0, 3.0, 5.0 and 10.0 mg $l^{-1}$ 2,4-D. Stage B media were supplemented with either 0.1 mg $l^{-1}$ kinetin alone or in combination with 0.1 and 1.0 mg $l^{-1}$ NAA. Stage C media contained either 0.03 and 0.3 mg $l^{-1}$ ABA alone or in combination with 0.002 mg $l^{-1}$ zeatin and 0.1 mg $l^{-1}$ GA. The cultural conditions during stage A were the same as described previously while during stage B and C the temperature was kept at 25 °C and applied a light regime of 10.2 microeinsteins $m^{-2}sec^{-1}$ with a 16-hour photoperiod.

During the stage A culture period, a small proportion of immature embryos (exclusively those at development stage II and III) formed protuberances on the proximal adaxial surface of the cotyledons, when they were exposed to media containing 1.0, 3.0 or 5.0 mg $l^{-1}$ 2,4-D. In the presence of 10.0 mg $l^{-1}$ 2,4-D the above phenomenon was inhibited and callus formation was encouraged. These protuberances (swollen regions) gradually enlarged and, by the end of the stage B, globular structures(embryogenic tissue) were produced.
The media of stage B encouraged the globular structures to grow further and to form bipolar structures, that is, structures capable of producing simultaneously roots from the one end and green leaf-like structures from the other end.

Since it has been reported that ABA affected positively the development of somatic embryos (Ammirato, 1974), 0.3 and 0.03 mg $l^{-1}$ ABA alone or in combination with 0.002 mg $l^{-1}$ zeatin and 0.1 mg $l^{-1}$

Table 5.2.1. The response of the whole immature embryo explants of cv. "California Wonder" to various culture media.

| Frequency of explants responded (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stage A | | Stage B | | | Stage C | | | |
| | | Hormone concentration (mg $l^{-1}$) | | | Hormone concentration (mg $l^{-1}$) | | | |
| 2,4-D concentration (mg $l^{-1}$) | Globular structure formation | NAA | Kinetin | Bipolar structure formation | ABA | Zeatin | GA | Abnormal somatic embryos |
| 1.0 | 13 | 0.1 | 1.0 | 80 | 0.03 | - | - | 80 |
| | | | | | 0.03 | 0.002 | 0.1 | 0 |
| | | | | | 0.3 | - | - | 0 |
| | | | | | 0.3 | 0.002 | 0.1 | 0 |
| 3.0 | 13 | - | 1.0 | 100 | 0.03 | - | - | 50 |
| | | | | | 0.03 | 0.002 | 0.1 | 67 |
| | | | | | 0.3 | - | - | 25 |
| | | | | | 0.3 | 0.002 | 0.1 | 0 |
| 5.0 | 23 | - | 0.1 | 100 | 0.03 | - | - | 40 |
| | | | | | 0.03 | 0.002 | 0.1 | 50 |
| | | | | | 0.3 | - | - | 33 |
| | | | | | 0.3 | 0.002 | 0.1 | 40 |
| 5.0 | 23 | 1.0 | 0.1 | 100 | 0.03 | - | - | 60 |
| | | | | | 0.03 | 0.002 | 0.1 | 80 |
| | | | | | 0.3 | - | - | 55 |
| | | | | | 0.3 | 0.002 | 0.1 | 38 |

Basal medium: MS3. Temperature: 25 °C throughout the culture.

Light: the first 10 days of stage A complete darkness, then standard Light conditions throughout the culture.

Duration of culture: stage A, stage B and stage C 28 days each.

GA were incorporated into the stage C media. When bipolar structures were subcultured to above media, they grew gradually to form abnormal embryos. The development of the bipolar structures to abnormal embryos was not a synchronous process and by the end of the stage C a considerable variation in performance was observed among explants or even on the same explant. Thus, on the same culture abnormal embryos of different size as well as bipolar structures coexisted. Abnormal embryos consisted of a root system (predominantly tap root system), hypocotyl and cotyledons but without shoot tip. In most of the cases two or more embryos were fused together. No complete plantlets were obtained by the end of the stage C. The results of this experiment are listed in Table 5.2.1.

### 5.2.2. *Anatomy and morphology of somatic embryogenesis process*

Immature embryos of cv. "Sweet Banana", at developmental stage III, were subjected to the two-stage culture procedure. Stage A MS3 medium was supplemented with 3.0 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ casaminoacids and stage B MS3 medium with a high cytokinin to auxin ratio (1.0 mg $l^{-1}$ BAP to 0.01 mg $l^{-1}$ 2,4-D). Explants were removed at regular intervals and placed in a fixative in preparation for either histological studies under the light microscope or morphological studies under the scanning electron microscope (SEM). Explants stayed in initial medium for at least thirty days, during which embryogenic tissue was induced. The second stage, lasted another 30 to 35 days, encouraging the establishment of somatic proembryos and in some cases the development of the complete embryos. Cultures in both stages were kept under a temperature regime of 25 °C and 10.2 microeinsteins $m^{-2}sec^{-1}$ light with 16-hour photoperiod.

Plate 5.1. Anatomy and morphology of immature embryos subjected to MS3 basal medium supplemented with 3 $mgl^{-1}$ 2,4-D for somatic embryo regeneration.

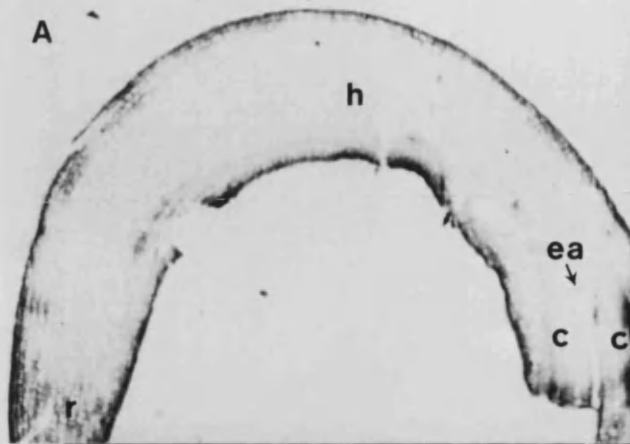
A. Day 0. Transverse section of immature embryo showing the smooth epidermal surfaces all over the embryo and particularly at the proximal regions of the cotyledons (c) and around the embryonic apex (ea); h = hypocotyl, r = radical. x 13

B. Day 0. SEM micrograph of immature embryo showing the same characteristics as above. Scale bar = 1000 μm.

C and D. Day 3. SEM micrographs of immature embryo showing the swollen regions (sr) at the cotyledon base. Scale bar : C = 1000 μm., D = 100 μm.

E. Day 6. Section-parallel to embryonic axis of immature embryo, showing the enlargement of the swollen regions (sr) around the cotyledon base, while there is no morphological changes of the embryonic apex (ea). x 40

F. Day 13. Section of immature embryo showing the formation of nodule (nd) structures. x 70


A
h
ea
c
c
r

ea
h
c
c
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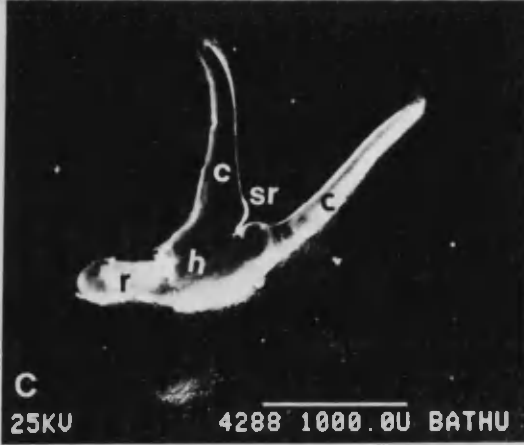
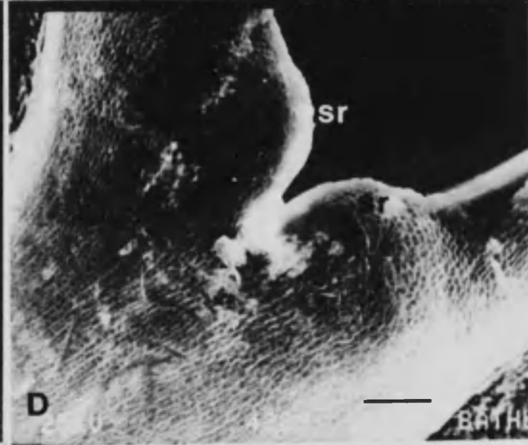
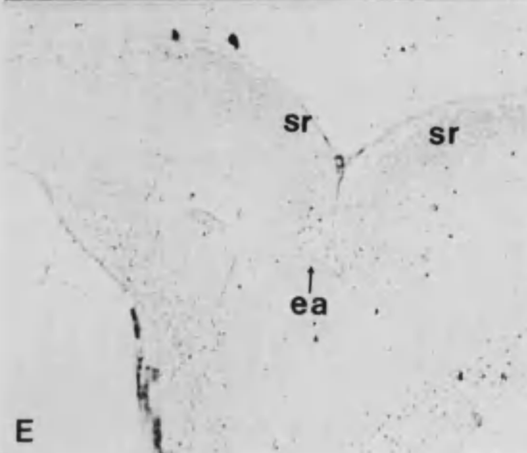
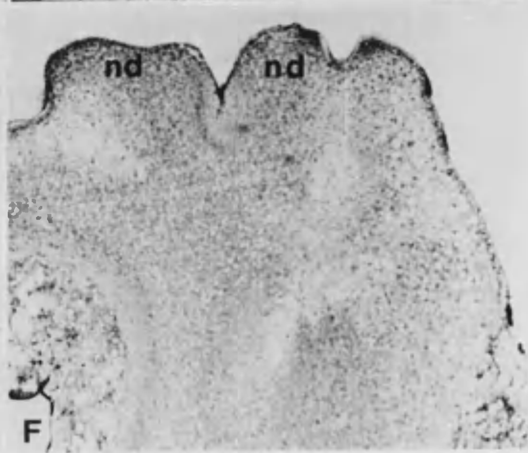
B
4637 1000.0U BATHU

c
sr
c
h
r
C
25KV
4288 1000.0U BATHU

sr
D

sr
sr
ea
E

nd
nd
F

The freshly isolated immature embryos were composed of uniform cells and their surfaces were smooth (Plate 5.1 A, B, C). By the third day of culture, swollen regions had appeared at the base of cotyledons and particularly at the adaxial surface around the embryonic apex (Plate 5.1 D and E). That was due to the rapid cell division mainly in the epidermal and sub-epidermal layers. After six days of culture, these swollen regions were enlarged enough to be seen under the light microscope, while the embryonic apex ceased to grow showing no sign of differentiation (Plate 5.1 E). By the day thirteen of culture, the swollen regions appeared to be more organized forming nodular structures, the surface of which were very smooth (Plate 5.1 F and Plate 5.2 A, B). Three days later, the nodular structures, or "embryonic tissues" had become bigger forming globular structures, or "embryonic tissues", enlarged further and in most of the cases the appearance of their upper surface was "crater-like" (Plate 5.3 A and B) with the peripheral cells following a regular pattern. The arrangement as well as the shape of the surface cells of the embryonic tissues were completely different from those of the epidermal cells of the original cotyledons. That is, they were shorter and wider than those of the epidermal cells of the original cotyledon. Furthermore, their external walls were swollen in contrast to those of the original cotyledon which were flat or deepened (Plate 5.3 C, D and Plate 5.4 A and B). From day 23 up to day 30, the globular structures continued to enlarge keeping their initial white or very pale creamy colour. At the end of stage A, the globular structures were dissected singly or in groups of two to three and then inoculated onto the stage B medium. There, they grew furthermore and after twenty to twenty five days each one produced one or more adventitious embryos (Plate 5.4 C and D).

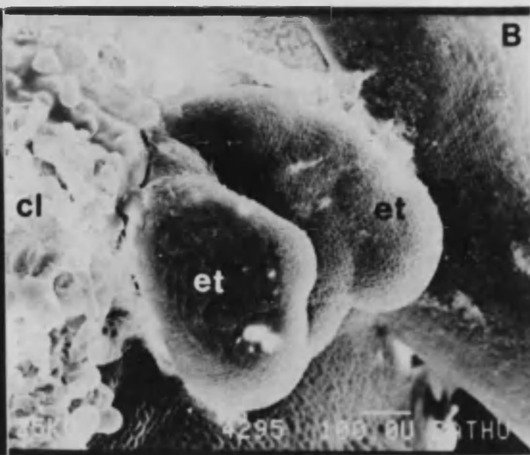
Plate 5.2. Anatomy and morphology of immature embryos subjected to MS3 basal medium supplemented with 3 $mgl^{-1}$ 2,4-D for somatic embryo regeneration.

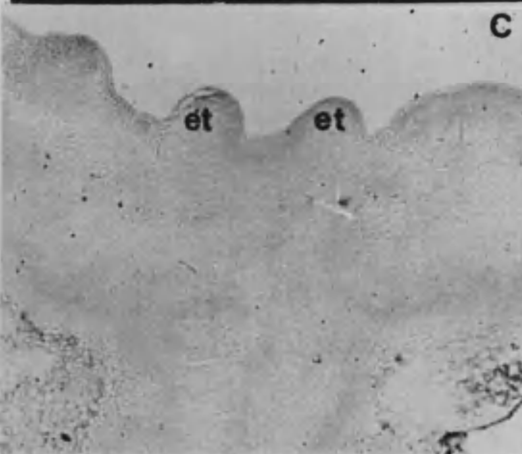
A and B. Day 13. SEM micrographs showing the nodular structures, or "embryogenic tissue" (et) at the cotyledon base; cl = callus, c = cotyledon, h = hypocotyl. Scale bar: A = 1000 μm., B = 100 μm.

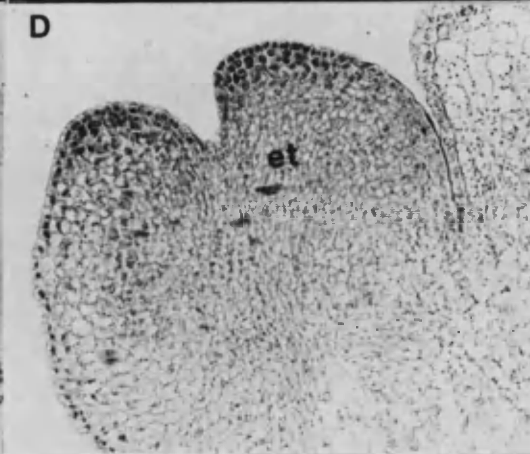
C and D. Day 16. Section of immature embryo showing the globular structures, or "embryogenic tissue" (et). C x 40, D x 100

E and F. Day 16. SEM micrographs. As above; et = embryogenic tissue, cl = callus proliferation, c = cotyledon, h = hypocotyl. Scale bar : E = 1000 μm., F = 1000 μm.

A
c
cl
h
25KV 4294 1000.0U BATHU


B
cl
et
et


C
et
et


D
et

E
et
c
25KV 4296 1000.0U BATHU

F
cl
c
et
h
c

These embryos were bipolar individuals (Plate 5.4 E, F and Plate 5.5 A), which in ten to fifteen more days formed either "fused embryos" (Plate 5.5 B) or abnormal embryos or normal germinated embryos. "Fused embryos" were joined mainly at their hypocotyl regions and they also failed to produce complete plants. Abnormal embryos, although possessing root(s), hypocotyl and cotyledons, failed to form shoots (Plate 5.6 B, C and Plate 5.7 A, B). Normal embryos produced minute complete plantlets (Plate 5.5 C and Plate 5.6 A). From the cotyledons of the abnormal embryos, secondary shoots were occasionally regenerated, especially when they were transferred to medium containing a cytokinin (Plate 5.8 A).

Somatic embryogenesis seemed to be a direct process from the cotyledon's adaxial surface surrounding the embryonic apex since no callus formation appeared to occur from the same regions simultaneously with the globular structure initiation. In some explants, especially those of cv. "Jalapeno", when callus formation took place, no sign of embryogenesis observed. These two processes, somatic embryogenesis and callogenesis, seemed to be mutually exclusive.

Plate 5.3. Anatomy and morphology of immature embryos subjected to MS3 basal medium supplemented with 3 $mgl^{-1}$ 2,4-D for somatic embryogenesis.

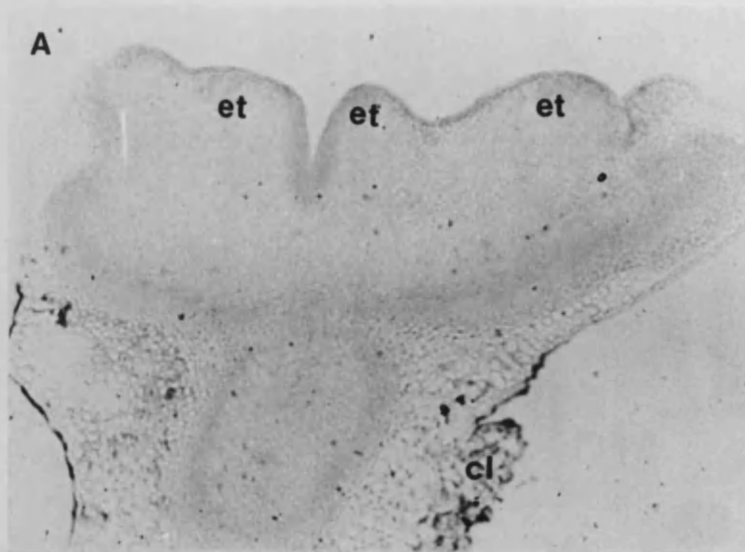
A. Day 23. Section showing enlargement of "embryogenic tissue" (et) as well as callus (cl) proliferation. x 40

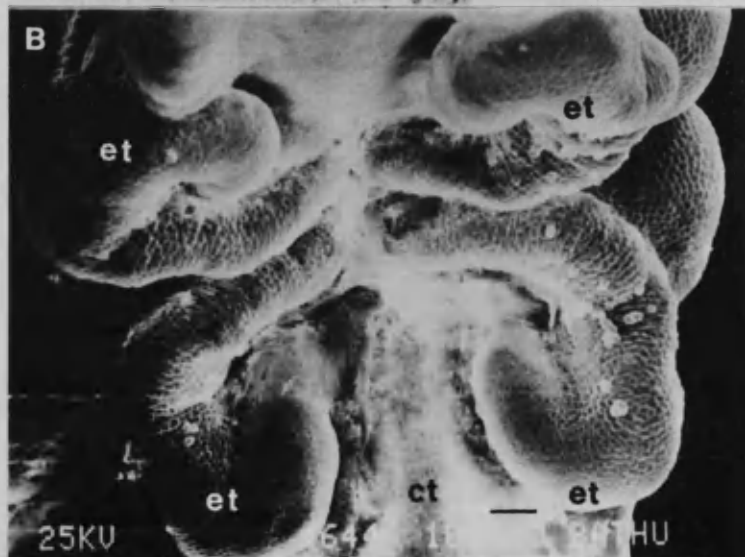
B. Day 23. SEM micrograph showing globular structure or "embryogenic tissue" (et) formation from the cotyledon (ct) of the immature embryo. Scale bar = 100 μm.

C. Day 23. SEM micrograph showing the arrangement and the shape of the surface cells of the embryogenic tissue. Scale bar = 10 μm.

D. Day 23. SEM micrograph showing the arrangement and the shape of the epidermal cells of the original cotyledon. Scale bar = 10 μm.

Note: C and D are magnifications of the regions (et) and (ct) respectively of the micrograph B.


A
et
et
et
cl


B
et
et
ct
et
et
25KV

C

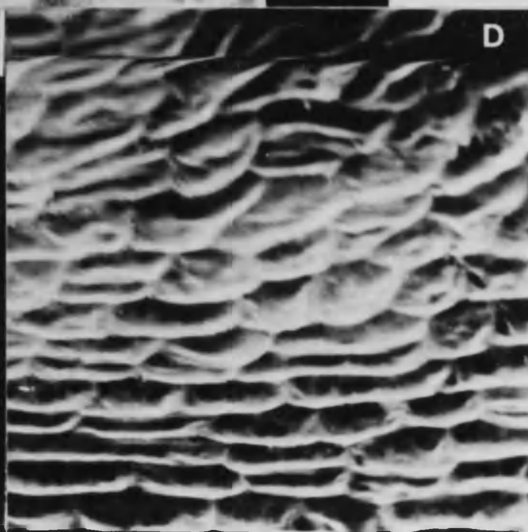

D

Plate 5.4. Anatomy and morphology of immature embryos subjected to MS3 basal medium supplemented with 3 mg $l^{-1}$ 2,4-D for somatic embryogenesis.

A. Transverse section showing the arrangement of the epidermal cells of cotyledon region which did not undergo embryogenesis. The large axis of the cells is parallel to the surface. x 200. This section corresponds to the micrograph D of Plate 6.3.

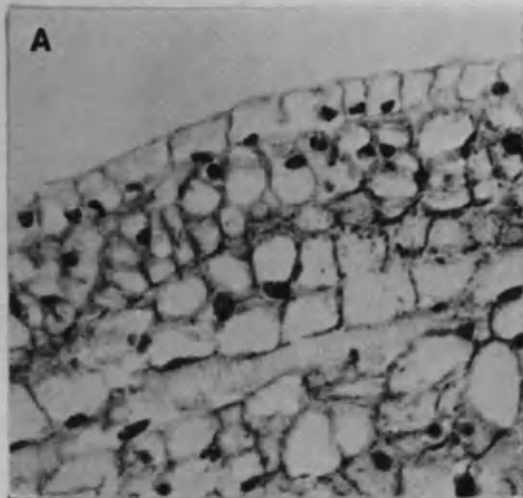
B. Transverse section showing the arrangement of the cells of the embryogenic tissue surface. The large axis of the cells is vertical to the surface. x 85. This section corresponds to the micrograph C of Plate 6.3.

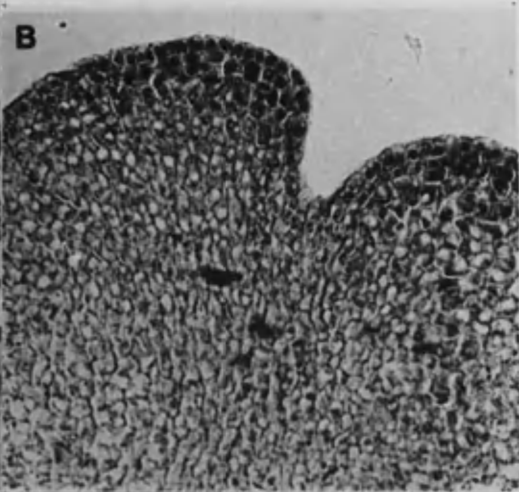
C. Day 45. Embryogenic tissue (et) or globular structure formation from immature embryos. Explants were initially subjected to MS3 medium, supplemented with 3 mg $l^{-1}$ 2,4-D, for 4 weeks and then subcultured on MS3 medium supplemented with 0.1 mg $l^{-1}$ kinetin. x 13.5

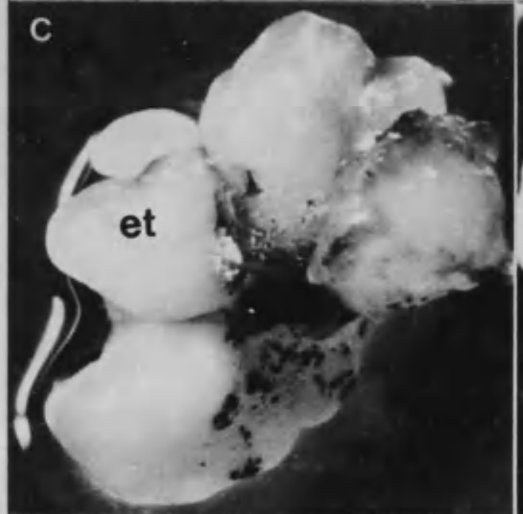
D. Day 45. Embryogenic tissue (et) formation from immature embryos. Explants were initially subjected to MS3 medium, supplemented with 3 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ l-proline, for 4 weeks, then subcultured to MS3 medium supplemented with 1 mg $l^{-1}$ NAA and 0.1 mg $l^{-1}$ kinetin. x 12

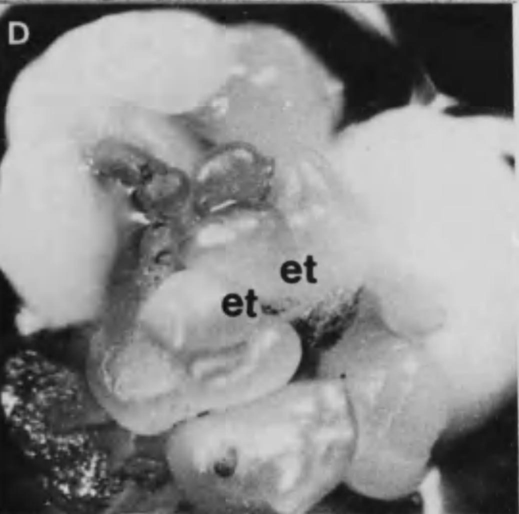
E. Day 65. SEM micrograph showing bipolar (bp) structures derived from immature embryo explants. Scale bar = 1000 µm.

F. Day 70. SEM micpograph showing a somatic embryo regenerated from immature embryo explant; c = cotyledon, h = hypocotyl. Scale bar = 100 µm.


A


B


C
et


D
et
et

E
bp
bp
25KV
BATHU

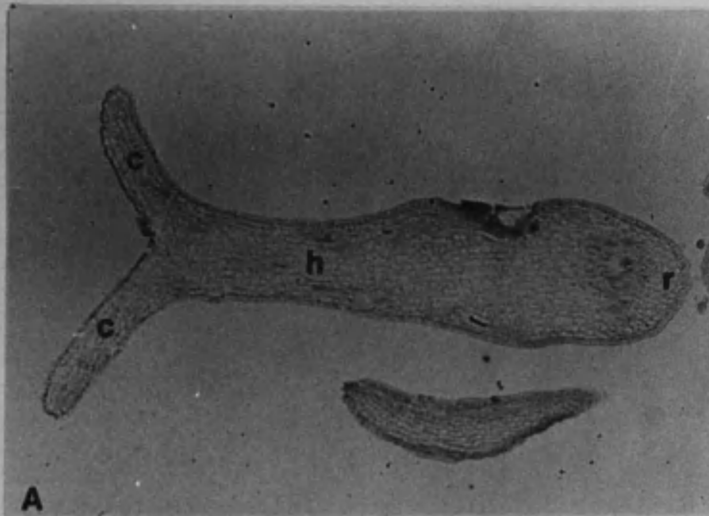
F
c
c
h
BATHU

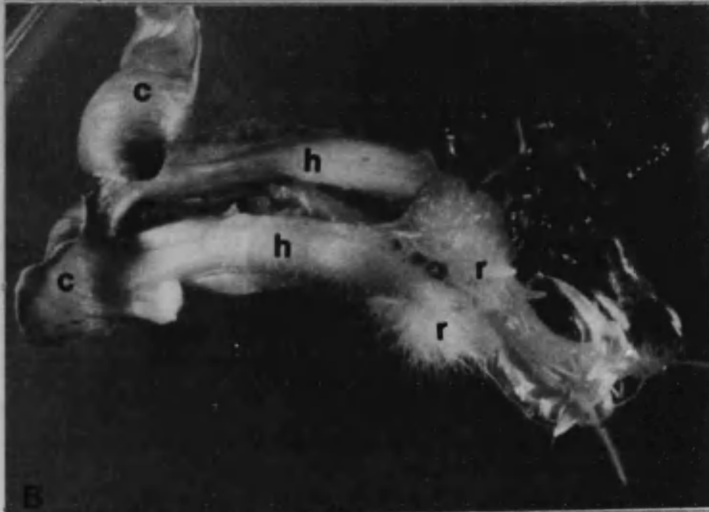
Plate 5.5. Anatomy and morphology of "embryogenic tissues" subjected to MS3 medium supplemented with 0.01 mg $l^{-1}$ 2,4-D and 0.1 mg $l^{-1}$ BAP for somatic embryogenesis.

A. Day 75. Longitudinal section of somatic embryo; c = cotyledon, h = hypocotyl, r = radical. x 10

B. Day 90. "Fused" somatic embryos; c = cotyledon, h = hypocotyl and r = root. x 3.4

C. Day 110. Plantlet regenerated through somatic embryogenesis. x 1.5


c
h
c
r
A


c
h
h
c
r
r
B

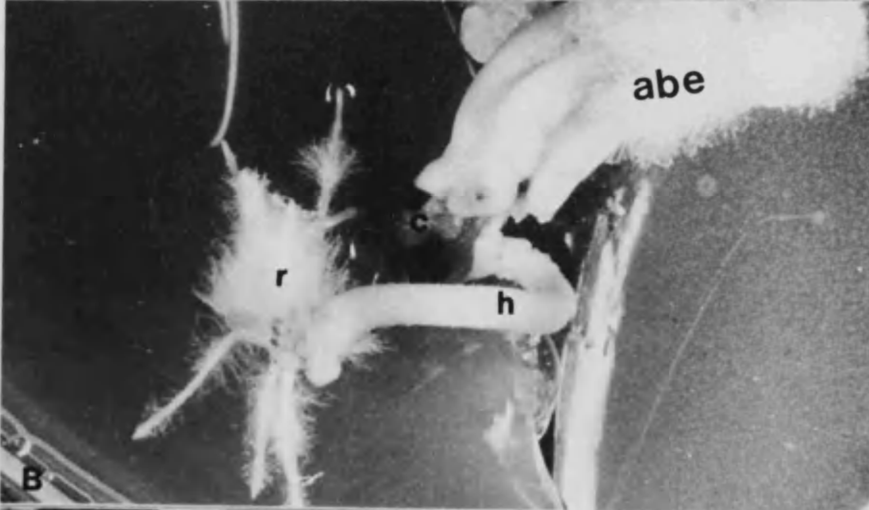
C

Plate 5.6.

A. Plantlet regenerated through somatic embryogenesis from 115-day-old culture. x3.6

B and C. Somatic embryos from 120-day-old cultures; c = cotyledon h = hypocotyl, r = root, abe = abnormal embryo. B: x 3.6 C: x3.6

c
c
A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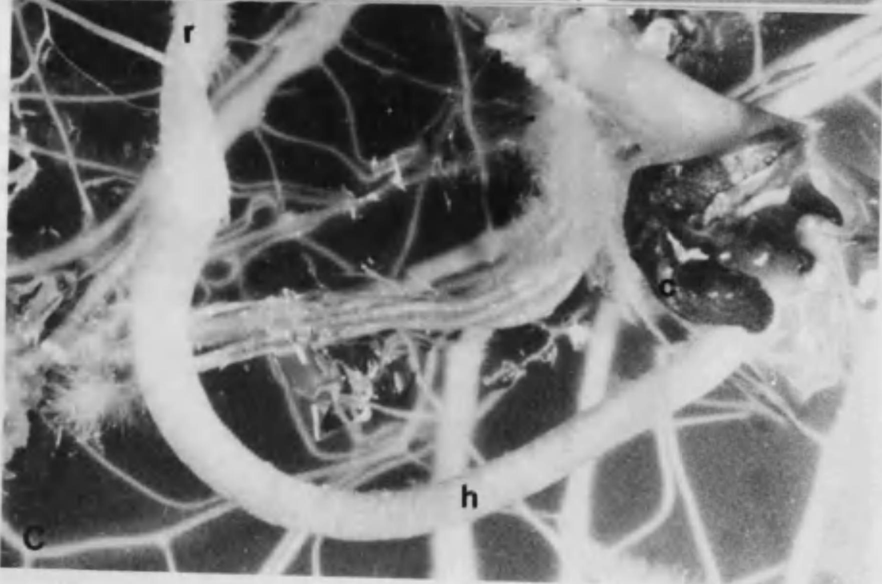
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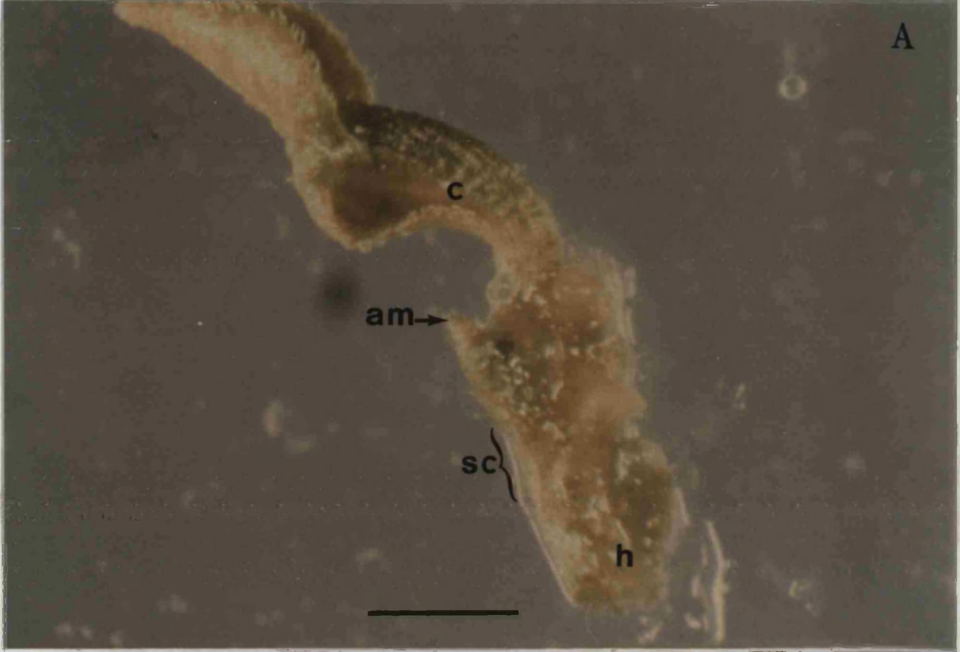
Plate 5.7.

A and B. Day 130. Somatic embryos, the apical meristem (am) of which failed to develop into shoot with stem; c = cotyledon, h = hypocotyl and sc = scar of the cotyledon which was moved for revealing the apical meristem. Scale bar = 1 mm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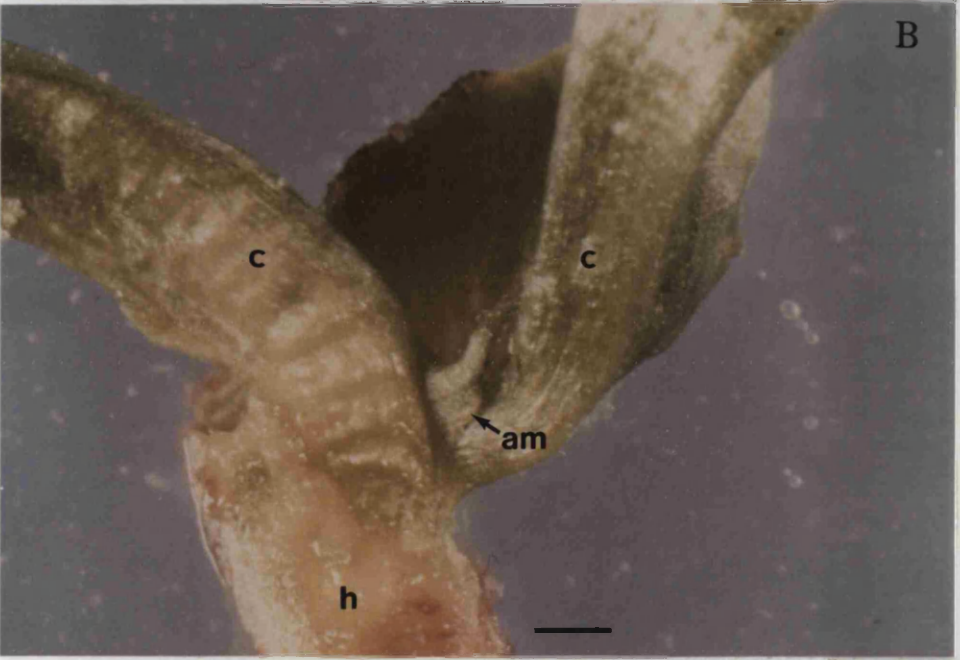
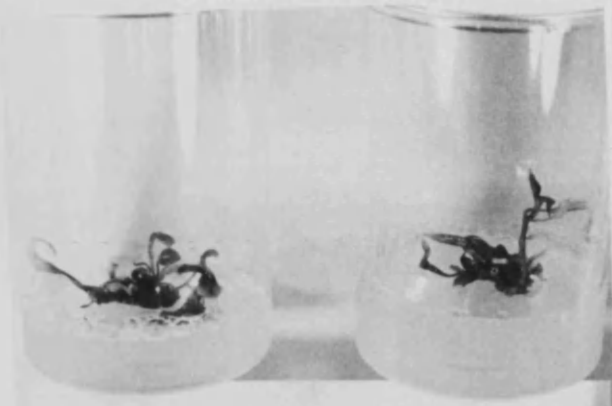

B
c
c
am
h

Plate 5.8.

A. Secondary shoots adventitiously regenerated from cotyledons of somatic embryos when they cultured on MS3 medium supplemented wirh 5 mg $l^{-1}$ zeatin. Scale bar = 10 mm

B and C. Plantlets derived from somatic embryos of 130-day-old cultures. B: from MS3 initial medium supplemented with 3 mg $l^{-1}$ 2,4-D; C: from MS3 initial medium supplemented with 3.0 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ proline; c = cotyledon of somatic embryo. B: x 1, C: x 0.8

D. Plant of cv. "Sweet Banana" derived from somatic embryo, after 145 days of the immature embryo's inoculation.



C

D

### 5.2.3. *The effect of proline, ABA and pH on the somatic emryogenesis process*

Since it has been reported that proline stimulated embryogenesis in some genera (Green *et al.*, 1983; Stuart and Strickland, 1984), a three stage experiment was set up to test the effect of this compound on the embryogenesis process in *Capsicum annuum*. For that immature embryos at their II and III developmental stage were excised from immature fruits of cv. "Sweet Banana" and were cultured on MS3 medium supplemented with 100 or 250 mg $l^{-1}$ l-proline in combination with 3 or 5 mg $l^{-1}$ 2,4-D. Cultures were kept under standard temperature and light regimes, as described previously in this section.

A high percentage of the explants formed globular structures ("embryogenic tissue") by the end of stage A. Concerning the frequency of the explants forming globular structures, no difference was found as a result of the influence of either the proline or 2,4-D concentration (Table 5.2.2). Regarding the performance of the globular structures, it seemed that the presence of either 100 or 250 mg $l^{-1}$ proline resulted in more uniform and organized structures (Plate 5.4 D). By the end of stage A, globular structures were transferred to the stage B media either attached to their original explant or separated if they were at an advanced developmental stage and hence sufficiently well organized to survive. The stage B media were either hormone-free or supplemented with various combinations of 2,4-D, BAP, zeatin, ABA, IBA or adenine sulphate as indicated in Table 5.2.3. All tested media encouraged equally well the formation of bipolar structures (embryos), but those containing ABA encouraged the formation of more uniform and advanced embryos. Embryos at that stage were formed in groups of two to four together, and very rarely were simple.

Table 5.2.2. The effect of 2,4-D and proline on immature embryo explants of cv. "Sweet Banana" in 28-day-old cultures. n = 30.

| 2.4-D and proline concentration (mg $l^{-1}$) | | Percentage explants producing globular structures (embryogenic tissues) |
|---|---|---|
| 2,4-D | Proline | |
| 3.0 | - | 76.7 |
| 3.0 | 100 | 73.3 |
| 3.0 | 250 | 66.7 |
| 5.0 | - | 43.3 |
| 5.0 | 100 | 73.3 |
| 5.0 | 250 | 66.7 |

Basal medium: MS3. Temperature: 25 °C.

Light conditions: The first 10 days complete darkness, then standard light conditions.

Table 5.2.3. The effect of various media of stage B on the morphogenic response of globular structures, which have been produced in stage A media from immature embryo explants of cv. "Sweet Banana".

| Stage A | | Stage B | | | |
|---|---|---|---|---|---|
| MS3 + 2,4-D + proline concentration (mg $l^{-1}$) pH: 5.7 | | MS3 + hormone concentration (mg $l^{-1}$) pH: 5.7 | Globular-structure explants forming bipolar clubs* | | |
| | | | % of globulars forming bipolars | Number of bipolar clubs per globular | |
| 2,4-D | Proline | | | Average | Maximum |
| 3.0 | - | 2,4-D 0.01 BAP 0.1 | 100 | 2.6 | 5 |
| 3.0 | - | " 0.01 " 1.0 | 100 | 2.5 | 6 |
| 3.0 | 100 | ABA 0.01 zeatin 0.1 | 100 | 3.2 | 5 |
| 3.0 | 100 | " 0.01 " 1.0 | 100 | 2.9 | 5 |
| 3.0 | 250 | " 0.1 - - | 100 | 3.4 | 6 |
| 3.0 | 250 | " 0.1 zeatin 0.1 | 100 | 1.9 | 3 |
| 5.0 | - | ABA 0.01 - - | 100 | 2.4 | 4 |
| 5.0 | 100 | hormone-free | 100 | 1.7 | 4 |
| 5.0 | 100 | ABA 0.1 zeatin 1.0 | 100 | 3.1 | 5 |
| 5.0 | 250 | hormone-free | 100 | 3.5 | 5 |
| 5.0 | 250 | IBA 0.5 As** 10.0 | 100 | 2.8 | 4 |

(*): each club concisted of 2-4 bipolar structures.

(**): As = adenine sulphate (6-aminopurine-sulphate).

Temperature: 25 °C. Light: the first 10 days of stage A complete darkness, then standard light conditions to both stages.

Duration of culture: stage A and stage B = 28 days each.

Number of explants per treatment: stage A = 30; stage B = 15.

The average number as well as the maximum number of embryo clubs per each globular structure did not significantly differ among the media (Table 5.2.3).

These groups of embryos were subcultured onto the stage C media with the aim of facilitating their development into complete plantlets. B53 media were supplemented with 0.1 mg $l^{-1}$ zeatin alone or in combination with 0.1 mg $l^{-1}$ GA, or they were hormone-free. Their pH was adjusted to 5.7 or 7.0. This was done because it has been reported by Fassuliotis and Nelson (1986), that a change from low to high pH encouraged the development of embryos into complete plants in some species. The results are listed in Table 5.2.4. Concerning the effect of the media alone, there was no significant difference among them in reference to the complete plant production. The pH of the media seemed to affect slightly the process, since media with pH 7.0 encouraged, in most of the cases, more embryos to produce plantlets than did the same media with pH 5.7 (Table 5.2.4). But it is very hard to say that generally a real improvement has been made due to higher pH value. The main difference was that at pH 7.0 the embryos developed more rapidly than they did at pH 5.7. Most of the embryos, that did not form plantlets, were abnormal and few of them changed course to produce callus.

### 5.2.4. *The response of immature embryo cultures to pH and cobaltous ions*

Since there was evidence, from the previous experiments, that pH value of 7.0 was beneficial for embryo development, especially when employed with the stage C medium, an experiment was set up to examine the effect of this pH value as well as the effect of high

Table 5.2.4. The effect of various hormone combinations on the induction and development of the adventitious embryos. Explants were subjected to three-stage culture (each stage lasted 28 days).

Percentage of explans responded

| Stage A | | Stage B | Stage C | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Response of globular structures | | | |
| 2,4-D concentration (mg $l^{-1}$) | Proline concentration (mg $l^{-1}$) | Hormone concentration (mg $l^{-1}$) | Hormone concentration (mg $l^{-1}$) | | Producing abnormal embryos | | Producing plants | |
| | | | Zeatin | GA | pH: 5.7 | pH: 7.0 | pH: 5.7 | pH: 7.0 |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 3.0 | - | 2,4-D 0.01 + BAP 0.1 | - | - | 40 | 40 | 0 | 0 |
| | | | 0.1 | - | 50 | 0 | 0 | 25 |
| | | | 0.1 | 0.1 | 25 | 75 | 25 | 25 |
| | | 2,4-D 0.01 + BAP 1.0 | - | - | 17 | 71 | 0 | 14 |
| | | | 0.1 | - | 75 | 33 | 25 | 0 |
| 3.0 | 100 | ABA 0.01 | - | - | 43 | - | 0 | - |
| | | | 0.1 | - | 57 | 29 | 0 | 14 |
| | | | 0.1 | 0.1 | 100 | - | 0 | - |
| | | ABA 0.01 zeatin 1.0 | 0.1 | - | 33 | 14 | 0 | 14 |
| | | | 0.1 | 0.1 | 50 | 14 | 0 | 14 |
| 3.0 | 250 | ABA 0.1 | - | - | 100 | 83 | 0 | 0 |
| | | | 0.1 | - | 83 | 83 | 0 | 14* |
| | | | 0.1 | 0.1 | 100 | 17 | 17* | 0 |
| | | ABA 0,1 + zeatin 0.1 | - | - | 71 | 20 | 0 | 0 |
| | | | 0.1 | - | - | 100 | - | 0 |
| 5.0 | - | ABA 0.01 | - | - | 50 | 50 | 0 | 0 |
| | | | 0.1 | - | 83 | 50 | 17* | 17 |
| 5.0 | 100 | ABA 0.1 + zeatin 0.1 | - | - | - | 78 | - | 0 |
| | | | 0.1 | - | 38 | 71 | 0 | 0 |
| | | | 0.1 | 0.1 | 29 | - | 0 | - |
| | | NAA 0.01 + BAP 0.1 | - | - | 20 | 17 | 0 | 17* |
| | | | 0.1 | 0.1 | - | 50 | 0 | 0 |
| 5.0 | 250 | As 0.5 | - | - | - | 57 | - | 0 |
| | | | 0.1 | - | 38 | - | 13* | - |
| | | | 0.1 | 0.1 | 71 | 43 | 0 | 0 |
| | | - | - | - | 50 | 50 | 0 | 0 |
| | | - | 0.1 | - | 14 | 29 | 0 | 0 |

Basal medium: stages A and B = MS3; stage C = B5 3. Temperature: 25 °C. Light: the first 10 days complete darkness, then standard light conditions. Number of explants per treatment: stage A = 30; stage B = 15; stage C = 8-10. (*): plants from somatic embryos appeared later. (-): non tested treatment.

cobaltous ion concentration on the somatic embryogenesis initiation. Cobaltous was used because it stimulated the growth of embryos from almond explants (Rugini, personal communication). For that, immature embryos of cv. "Sweet Banana" and "Bell Boy" $F_1$ hybrid were inoculated onto MS3 medium supplemented with 3.0 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ l-proline, to which either 0.025 (standard level) or 0.25 mg $l^{-1}$ $CoCl_2.6H_2O$ were added and the medium was adjusted to a pH value of 5.7 or 7.0.

The results are presented in Table 5.2.5. High cobaltous ion concentration inhibited the embryogenesis process from immature embryos of "Bell Boy" $F_1$ hybrid, whereas it did not have any negative effect on "Sweet Banana" explants, and it seemed to encourage slightly the process in media adjusted to 5.7 pH value. The high pH value (7.0) significantly reduced the frequency of explants undergoing embryogenic tissue initiation from both "Sweet Banana" and "Bell Boy" explants. The medium of stage B at pH 7.0 was significantly better than that at pH 5.7 concerning the frequency of explants undergoing embryogenesis from "Sweet Banana" immature embryos, but not from "Bell Boy" $F_1$ hybrid ones. Also, high pH value (7.0) was slightly better than low pH value (5.7) for complete plant production in stage C media (Table 5.2.5).

### 5.2.5. *The effect of activated charcoal on immature embryo cultures*

In a preliminary experiment, immature embryos of cv. "Sweet Banana" were cultured on MS3 medium supplemented with 100 mg $l^{-1}$ l-proline, 3.0 mg $l^{-1}$ 2,4-D, 1% (w/v) activated charcoal (AC), 0.025 or 0.25 mg $l^{-1}$ $CoCl_2.6H_2O$ and at a pH value of 5.7 or 7.0. The temperature and light conditions were the same as previously described.

Table 5.2.5. The effect of cobaltous ions and pH on the adventitious embryogenesis from immature zygotic embryos of cv. "Sweet Banana" and "Bell Boy" $F_1$ hybrid in three-stage culture (each stage lasted 28 days).

| | Stage A: MS3 medium + 3.0 mg $l^{-1}$ 2,4-D + 100 " proline | | | Stage B: MS3 medium + 0.1 mg $l^{-1}$ BAP + 0.01 " 2,4-D | | Stage C: MS3 medium + 0.1 mg $l^{-1}$ GA + 0.1 " zeatin, pH: 7.0 |
|---|---|---|---|---|---|---|
| Cultivar | $CoCl_2.6H_2O$ concentration (mg $l^{-1}$) | pH | Explants forming globular structures (%) | pH | Globular structures forming bipolar structures (%) | Bipolar structures producing clonal plants (%) |
| Sweet Banana | 0.025 | 5.7 | 35 | 5.7 | 30 | 40 |
| | 0.25 | 5.7 | 60 | 5.7 | 50 | 20 |
| | 0.025 | 7.0 | 15 | 7.0 | 60 | 50 |
| | 0.25 | 7.0 | 15 | 7,0 | 100 | 40 |
| Bell Boy | 0.025 | 5.7 | 75 | 5.7 | 100 | 44 |
| | 0.25 | 5.7 | 0 | 5.7 | 0 | 0 |
| | 0.025 | 7.0 | 33 | 7.0 | 100 | 50 |
| | 0.25 | 7.0 | 0 | 7.0 | 0 | 0 |

Temperature: 25 °C. Light: the first 10 days complete darkness, then standard light conditions. Number of explants per treatment: stage A = 10-15; stage B = 20-25: stage C = 15-20.

During the stage A period, no explants showed signs of somatic embryogenesis and instead each immature embryo developed normally producing the original complete plant (Table 5.2.6). It was obvious that activated charcoal (AC) counteracted the effect of the auxin 2,4-D probably by absorbing it. Based on this observation and on reports that AC normalized the embryogenic process (Fridborg and Eriksson, 1975), an experiment was set up in which, to otherwise standard medium for embryogenesis (MS3 + 3 mg $l^{-1}$ 2,4-D + 100 mg $l^{-1}$ l-proline, pH: 5.7 or 7.0), activated charcoal was introduced on either the 1st or 15th day of stage A, or the 1st day of the stage B; a control medium was kept free of AC during the stage A.

The results are presented in Table 5.2.7. It is apparent that the AC incorporation from day 1 completely prohibited embryogenesis. When AC incorporated on the 15th day of the stage A, explants underwent somatic embryogenesis at a high frequency, regardless of the medium pH value. But the most significant role of AC was demonstrated during stage B. In presence of AC, better organized and more advanced globular structures were formed, regardless of the presence or absence of AC during stage A, and they had a greater chance to develop into complete plants when they were subcultured to media of stage C. Again, better results were obtained from the media with the higher pH value.

In completing the picture of the AC effect, immature embryos of cv. "Sweet Banana", at their II-III or IV developmental stage, were cultured on MS3 basal medium supplemented with 100 mg $l^{-1}$ l-proline, 1% (w/v) activated charcoal and 0, 10, 20, 30, 40, or 100 mg $l^{-1}$ 2,4-D. The incubation conditions were the same as previously described.

The results are presented in Table 5.2.8. It is apparent that

Table 5.2.6. The effect of cobaltous ions and pH in combination with AC on the somatic embryogenesis process from immature zygotic embryo explants of cv. "Sweet Banana". n = 20.

| Stage A | | Response of immature embryos | |
|---|---|---|---|
| $CoCl_2.6H_2O$ concentration (mg $l^{-1}$) | pH | Explants producing globular structures (%) | Explants which developed normally into complete plants (%) |
| 0.025 | 5.7 | 0 | 80 |
| 0.025 | 7.0 | 0 | 85 |
| 0.25 | 5.7 | 0 | 100 |
| 0.25 | 7.0 | 0 | 80 |

Basal medium: MS3 + 3.0 mg $l^{-1}$ 2.4-D + 100 mg $l^{-1}$ l-proline + 1% (w/v) AC.

Duration of culture: 28 days. Temperature: 25 °C.

Light conditions: The first 10 days complete darkness; then standard light conditions.

Table 5.2.7. The effect of activated charcoal (AC) on the somatic embryogenesis process from immature embryos of "Bell Boy" $F_1$ hybrid.

| Stage A | | | Stage B | | | Stage C |
|---|---|---|---|---|---|---|
| MS3 basal medium + 3.0 mg $l^{-1}$ 2,4-D + 100 " l-proline | | | MS3 basal medium + 0.1 mg $l^{-1}$ BAP + 0.01 " 2,4-D | | | MS3 basal medium + 0.1 mg $l^{-1}$ zeatin + 0.1 " GA; pH: 7.0 |
| Day of 1% (w/v) AC incorporation | pH | Explants forming globular structures (%) | AC 1% (w/v) | pH | Globular structures producing embryos (%) | Adventitious embryos producing complete plantlets (%) |
| no AC | 5.7 | 50 | Yes | 5.7 | 100* | 50 |
| no AC | 7.0 | 80 | Yes | 7.0 | 100* | 42 |
| 1 | 5.7 | 0 | - | - | - | - |
| 1 | 7.0 | 0 | - | - | - | - |
| 15 | 5.7 | 75 | Yes | 5.7 | 100 | 0 |
| 15 | 7.0 | 75 | Yes | 7.0 | 100* | 53** |
| 15 | 5.7 | 65 | No | 5.7 | 100 | 0 |
| 15 | 7.0 | 70 | No | 7.0 | 100 | (?) |

Light conditions: The first 10 days of stage A complete darkness; then standard light conditions.

Temperature: 25 °C to all stages.

AC incorporated into stage B media the 1st day.

Duration of cultures: stage A, stage B and stage C = 28 days each.

Number of explants per treatment: 8-10 to each stage.

(*): explants forming many and well organized adventitious embryos.

(**): more than 5 complete plantlets were obtained from one explant.

(?): contaminated cultures.

immature embryos at II - III developmental stage, exposed to media containing 1% AC and 2,4-D at concentration up to 100 mg $l^{-1}$ underwent normal development and produced complete plants; the extent of the plant growth was better than that on hormone-free, AC-free medium. Immature embryos, at developmental stage IV only when cultured on hormone-free medium supplemented with AC grew better than on hormone-free, AC-free medium. But when the same explants were exposed to medium supplemented with AC and 2,4-D at concentrations up to 30 mg $l^{-1}$ they continued to grow normally but with a gradually reduced rate of growth as the 2,4-D concentration was increased, and ceased to grow at 2,4-D concentration higher than 40 mg $l^{-1}$ (Table 5.2.8, Plate 5.9 and Plate 5.10). All these observations lead to the conclusion that activated charcoal absorbed or inactivated the exogenous auxin and perhaps some other endogenous substance(s) which may play a role in the development -(germination)- of the immature embryos.

Because the effects of AC and 2,4-D are not quite similar in immature embryos at different developmental stages, it is clear that some other factors might be involved too. Such a factor could be the embryo's dormancy. To clarify the influence of dormancy, immature embryos, at their IV and V developmental stages, were subjected to various treatments for germination. They germinated more easily and rapidly than those at their early developmental stages, when subjected to hormone-free, AC-free media. But exactly the opposite occurred when they were cultured on media supplemented with 2,4-D in combination with activated charcoal.

Table 5.2.8. The effect of activated charcoal (AC) and 2,4-D on the germination of the immature embryos of cv. "Sweet Banana" cultured for 20 days. n = 15.

| Supplement | | Explants | | | |
|---|---|---|---|---|---|
| | | Stage II-III embryos | | Stage IV embryos | |
| 2,4-D concen-tration ($mg\ l^{-1}$) | AC 1% (w/v) | Embryos germination (%) | Length of hypocotyl (mm) | Embryos germination (%) | Length of hypocotyl (mm) |
| 0 | No | 100 | 10.9 bc | 72 | 12.0 bc |
| 0 | Yes | 93 | 18.3 cde | 93 | 22.9 e |
| 10 | Yes | 100 | 22.7 e | 80 | 10.5 bc |
| 20 | Yes | 100 | 22.3 e | 60 | 5.7 ab |
| 30 | Yes | 100 | 28.8 e | 70 | 5.0 ab |
| 40 | Yes | 100 | 20.1 de | 20 | 1,5 a |
| 100 | Yes | 100 | 16.4 cde | 20 | 0.9 a |

Basal medium: MS3. Incubation conditions: standard.

Plate 5.9. The effect of activated charcoal (AC) on the immature embryo cultures of cv. "Sweet Banana".

A. Immature embryos at their developmental stage II-III (left) and IV (right) cultured on MS3 basal medium supplemented with 1% (w/v) activated charcoal and 10.0 mg $l^{-1}$ 2,4-D. Embryos continued to develop normally.

B. As above with the exception that 20 mg $l^{-1}$ 2,4-D was incorporated into the medium. Immature embryos at developmental stage II- III grew normally, but those at developmental stage IV ceased to grow.

C. As in A except that 30 mg $l^{-1}$ 2,4-D was added into the medium. Immature embryos at developmental stage II-III continued to develop but not those at developmental stage IV.

A, B and C were taken from 8-day-old cultures.

Temperature: 25 °C

Light: 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod.

stage II-III　　　　stage IV

A

stage II-III　　　　stage IV

B

C　　stage II-III　　　　stage IV

Plate 5.10. The effect of activated charchoal (AC) on the immature embryo cultures of cv. "Sweet Banana".

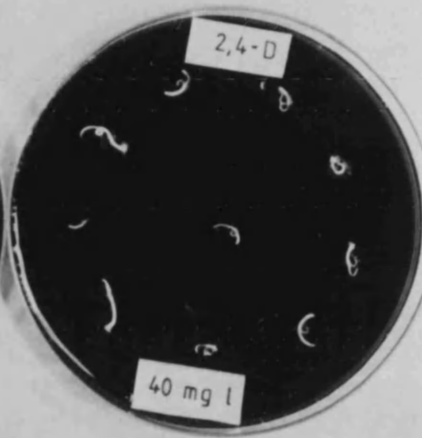
A. Immature embryos, at their developmental stage II-III (left) and IV (right), cultured on MS3 basal medium supplemented with 1% (w/v) activated charcoal and 40 mg $l^{-1}$ 2,4-D. Immature embryos at stage II-III continued to develop normally but not those at stage IV.

B. As above except that 100 mg $l^{-1}$ 2,4-D were added into the basal medium. Immature embryos at stage II-III continued to develop whereas those at stage IV ceased to do so.

A and B were taken from 8-day-old cultures.

Temperature: 25 °C.
Light: 10.2 microeinsteins $m^{-2}sec^{-1}$ with 16-hour photoperiod.

A




stage II-III stage IV

stage II-III stage IV

B

### 5.3. *The response of mature embryos to media supplemented with 2,4-D, BAP, kinetin, NAA and GA*

For comparison with the response of immature embryos, mature embryos were excised from seeds of cv. "California Wonder" and were subjected to stage A MS3 medium supplemented with either 3.0 or 5.0 mg $l^{-1}$ 2,4-D. The pH was adjusted to 7.0. After 28 days of culture on the stage A medium, the explants were transferred to the stage B MS3 media. The latter were supplemented with various concentrations of BAP, kinetin, GA or NAA as indicated in Table 5.3. The temperature and light regimes were the same as described in immature embryo cultures.

The only response that was observed by the end of the stage A was the formation of white and friable callus, irrespectively of the 2,4-D concentration. No signs of adventitious organs were found in any explant. When explants were subcultured to stage B media, they continued to produce further friable callus in all cases except in the media supplemented with 2.25 or 4.5 mg $l^{-1}$ BAP where the callus proliferation changed to the production of compact callus. The performance and the quantity of callus produced by the end of the stage B are indicated in Table 5.3. The callus formation process started from the radical and hypocotyl regions of the embryo explants and spread all over the surface later, to produce large callus mass by the end of the stage B.

Table 5.3. The response of mature embryo explants of cv. "California Wonder" cultured onto MS3 basal medium supplemented with 2,4-D during the stage A and various hormones during the stage B.

| Stage B | | | | Stage A | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Hormone concentration ($mg\ l^{-1}$) pH: 7.0 | | | | $3.0\ mg\ l^{-1}$ 2,4-D pH: 7.0 | | | $5.0\ mg\ l^{-1}$ 2,4,-D pH: 7.0 | | |
| | | | | Response | | | Response | | |
| BAP | 2.25 | | | Callus | l-vl | fr-c | Callus | l | fr |
| " | 4.5 | | | " | l-vl | fr-c | " | l-vl | fr |
| BAP | 2.25 | + GA 0.1 | | " | l | fr-c | " | l-vl | gr |
| " | 4.5 | + GA 0.1 | | " | g-l | fr | " | vl | fr |
| " | 2.25 | + GA 1.0 | | " | l | fr | " | l-vl | fr |
| " | 4.5 | + GA 1.0 | | " | l-vl | fr | " | l | fr |
| Kinetin | 0.1 | + GA 0.1 | + NAA 0.01 | " | g-l | fr | " | l-vl | fr |
| " | 3.0 | + GA 0.1 | + NAA 0.01 | " | l-vl | fr | " | l-vl | fr |

Basal medium: MS3 + 100 mg $l^{-1}$ casamino acids.
Temperature: 25 °C to both stages.
Light conditions: The first 10 days complete darkness; then light of 10.2 microeinsteins $m^{-2}sec^{-1}$ throughout the experiment.
Number of replicates per treatment: 8-10
A hundred per cent of the explants produced callus.
Characteristics of callus: a. Size: g-l callus weight 40-100 mg
l " " 100-250 mg
l-vl " " 250-500 mg
vl " " > 500 mg
b. Type: fr = friable callus
fr-c = friable-compact callus.

# CHAPTER VI

# GENETIC STABILITY OF REGENERATED PLANTS

*6. Genetic stability of regenerated plants.*

Plants regenerated adventitiously through either organogenesis or embryogenesis appeared to be phenotypically uniform among themselves and in comparison with their mother plants (Plate 6.1 A, B and C). To support this conclusion the leaves of the 1st branch of eight plants of cv. "Red Chili", adventitiously regenerated from cotyledon cultures, were excised and their lamina's maximum length and width as well as their surface area were measured (section 2.10). Data are presented in Table .1. It is apparent that there was no significant difference among the values obtained with the various plants.

The chromosome number of plants regenerated through either organogenesis or embryogenesis as well as from seedlings was also determined (Table 6.2). Chromosomes counted in cells of root-tips which were appropriately prepared (see section 2.8). It is obvious that chromosome number was constant (24) for all plants. In some cases, 23 chromosomes were found in some cells and it was assumed that a chromosome was missed during the preparation process, since other cells from the same root-tip possessed 24 chromosomes. Only in one particular root-tip of cv. "Red Chili" (Table 6.2) the chromosome number of 4 cells was 18, but in one cell of the same root-tip as well as in 7 more root-tips, of the same plant, the chromosome number was constant, at 24.

Twelve chromosomes were found in one cell of root-tip from cv. "Jalapeno" seedling (Plate 6.2 B), whereas such a chromosome number was never detected in any root-tip of plants adventitiously regenerated through either organogenesis or somatic embryogenesis.
The germinability of seeds collected from adventitiously regenerated plants was examined in comparison with seeds from plants propagated

Plate 6.1. Comparison of plants derived from tissue culture to seedlings of cv. "Red Chili".

A. Plantlets derived adventitiously from cotyledon culture (aged 90 days) and 45-day-old seedlings.

B. Plantlets derived adventitiously from shoot-tip culture (aged 135 days).

C. 30-day-old seedlings.

Plants regenerated
from tissue Culture
cv. "Red Chili"
Seedlings
cv. "Red Chili"
A

Plants regenerated
from tissue Culture
cv. "Red Chili"
B

Seedlings
cv. "Red Chili"
C

by conventional methods (Table 6.3).

There was no significant difference among seeds of cv. "Jalapeno" regardless their origin. But concerning the cv. "Red Chili", seeds from plants adventitiously-regenerated germinated in a significantly higher frequency than those collected from plants propagated by conventional methods. This different response was expected since the commercial seeds (traditional method of propagation) were older than one year.

Again, plants produced from the seeds were phenotypically uniform regardless of the seed origin (tissue culture or conventional method).

Table 6.1. Dimensions of leaves from the first branch of cv. "Red Chili" plants regenerated adventitiously from cotyledon cultures.

| Plant number | Average length of lamina (mm) | Average width of lamina (mm) | Average area of lamina ($cm^{-2}$) |
|---|---|---|---|
| 1 | 69.9 a | 33.7 a | 14.7 a |
| 2 | 61.7 a | 29.4 a | 11.1 a |
| 3 | 63.3 a | 30.2 a | 11.8 a |
| 4 | 65.7 a | 31.5 a | 11.9 a |
| 5 | 62.4 a | 29.7 a | 11.4 a |
| 6 | 63.7 a | 28.5 a | 10.2 a |
| 7 | 67.3 a | 31.4 a | 12.8 a |
| 8 | 66.1 a | 30.3 a | 11.5 a |

Each datum is the average value of 10 to 18 leaves.

Table 6.2. Chromosome number in root-tip cells of regenerants.

| Cultivar | Original explant | No. of plants examined | Total No. of root tip squashes | No. of chromosomes at late anaphase | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 24 | 23 | 22 | 21 | 18 |
| "Red Chili" | Cotyledons | 17 | 56 | 96 | 9 | 2 | 1 | 4 |
| "Jalapeno" | " | 10 | 28 | 39 | - | - | - | - |
| "Red Chili" | Shoot-tips | 10 | 30 | 50 | 1 | - | - | - |
| "Sweet Banana" | Immature embryos | 3 | 10 | 12 | - | - | - | - |

Table 6.3. Percentage of seeds which germinated in 23 days after their sowing date.

| Source of seeds | Cultivar | |
|---|---|---|
| | Jalapeno | Red Chili |
| Plants propagated by the conventional method | 93.3 b | 79.8 a |
| Plants regenerated adventitiously from cotyledon culture | 93.8 b | 93.7 b |

Number of seeds per treatment: 196.

Plate 6.2

A. Twenty two chromosomes from root-tips of cv. "Jalapeno" seedlings. x 1594

B. Twelve chromosomes from root-tip of cv. "Jalapeno" seedlings. x 2325

C. Twenty four chromosomes from root-tip of cv. "Jalapeno" seedlings. x 1204

D. Twenty four chromosomes from root-tip of cv. "Jalapeno" plant derived adventitiously from cotyledon culture. x 1260

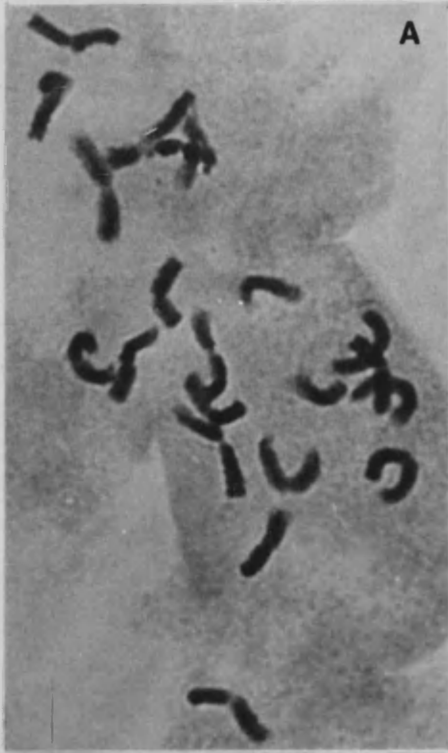
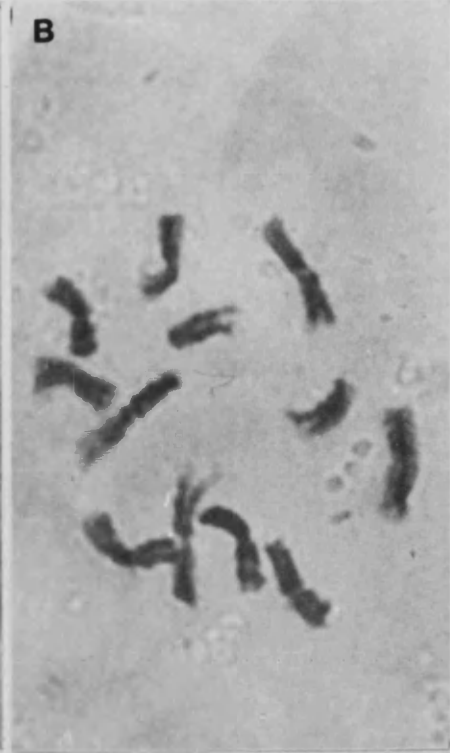
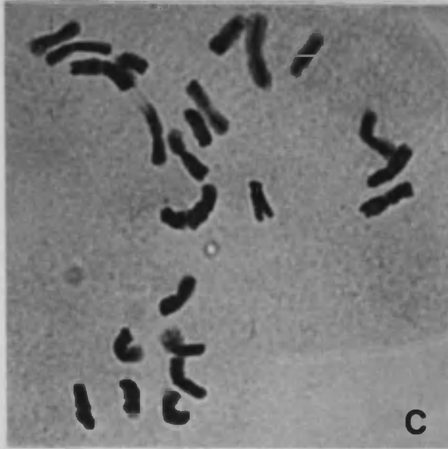
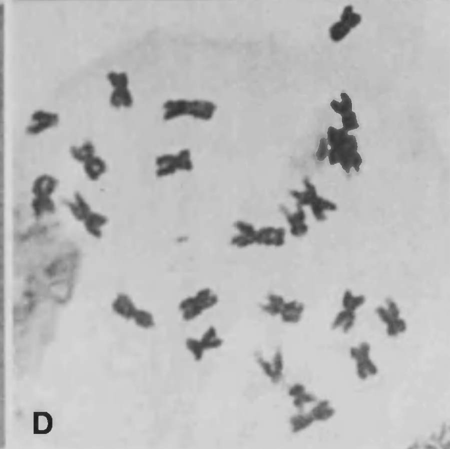

A

B

C

D

Plate 6.3

A. Twenty-four chromosomes from root-tip of cv. "California Wonder " seedling. x 1695

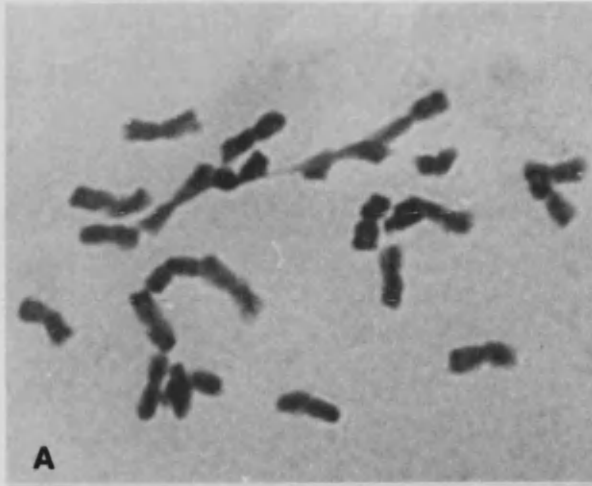
B. Twenty-four chromosomes from root-tip of cv. "California Wonder" plant derived adventitiously from tissue culture. x 1309

C. Twenty-four chromosomes from root-tip of cv. "Red Chili" seedling. x 1068

D. Twenty-four chromosomes from root-tip of cv. "Red Chili" plant derived adventitiously from tissue culture. x 1390

E. Twenty-four chromosomes from root-tip of cv. "Sweet Banana" seedling. x 2346

F. Twenty-four chromosomes from root-tip of cv. "Sweet Banana" plant derived from somatic embryo. x 800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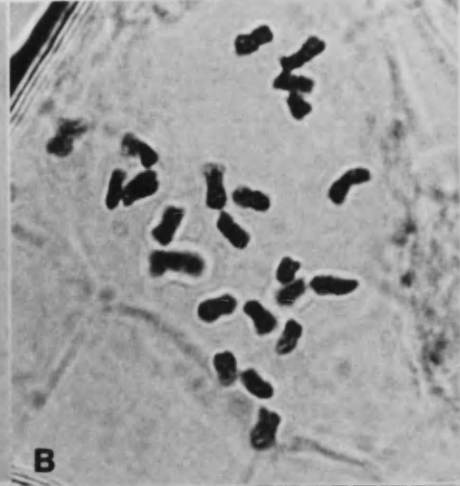
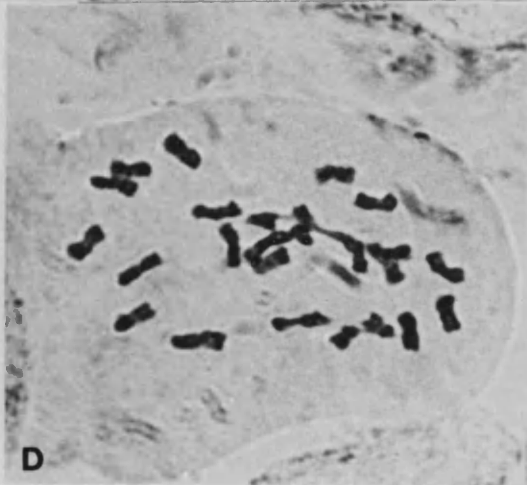
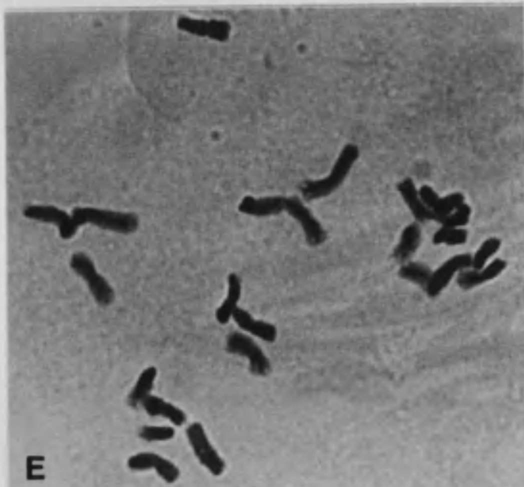
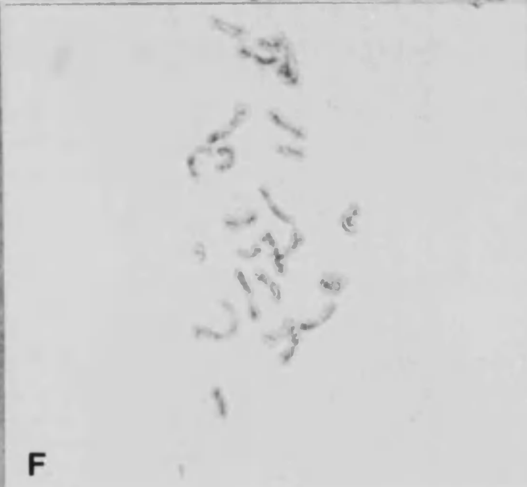

B

C


D


E


F

# CHAPTER VII

# DISCUSSION

## *7.1. Non-adventitious regeneration*

### *7.1.1. Callogenesis*

Callus proliferation took place with any type of explant (cotyledon, hypocotyl, root, intact embryo, leaf disc, leaf petiole, internodal segments, shoot-tips etc.) subjected to media supplemented with any growth regulator tested, i.e. 2,4-D, IAA, NAA, IBA, BAP, kinetin, zeatin, zeatin riboside and GA.

Callus formation occurred first from the cut edges or from the damaged regions of the explant. It was apparent that in these circumstances callus formation was associated with the wound response. Also callus proliferation occurred in regions of the explants well away from the cut edges or wounded regions and particularly those in contact with the agar medium. This might be the result of cell activation which is thought to be influenced by physiological gradients of materials moving out of the medium into the tissues (Ross and Thorpe, 1973). The nature of callus was determined by the plant growth regulator used. Explants subjected to media containing 2,4-D, IBA, NAA or GA formed white friable callus, while on media containing IAA, BAP, kinetin or zeatin-riboside they formed hard compact white or yellowish callus (sections 3.1, 3.9 and 3.11).

Explants of floral parts (anthers, styles, ovaries, ovules, placenta and ovary walls) favoured the formation of compact callus regardless the nature of the plant growth regulator (data are reserved in the hands of the writer). Callus was obtained in all experiments under most treatment conditions, unless the concentration of the tested growth regulator was so high that it killed the explants.

The growth rate of the callus depended upon the nature and the concentration of the plant growth regulator in the medium (data are reserved).

Shoot organogenesis or embryogenesis was never observed from either short-term or long-term callus cultures. Root organogenesis was observed rarely and always from short-term callus attached to the original explant but never from isolated callus pieces in culture. Root formation took place only from compact callus but never from friable callus.
No organogenesis was observed in suspension cultures established from the callus produced on cotyledon explants and grown on media containing a range of concentrations of 2,4-D or NAA alone or in combination with either of two concentrations of BAP ( data not presented).

### *7.1.2. Rhizogenesis*

Non-adventitious rhizogenesis occurred in embryonic root-tip explants cultured on hormone-free medium or medium supplemented with an auxin (NAA, IBA and 2,4-D). The process was encouraged more in lower than 3.0 mg $l^{-1}$ auxin concentrations. Among the auxins, IBA and NAA were superior to 2,4-D in relation to both root induction and root growth (data are reserved). The presence of the cytokinin BAP in the medium resulted in a reduction of the frequency of root induction and in root growth regardless of the presence of an auxin in the medium. The above results were more or less expected since the explants possessed the growing point (root meristem) and the media tested contained the essential mineral elements, the energy source (sucrose) and in some cases low auxin concentration.

### *7.1.3. Non-adventitious shoots*

Non-adventitious shoots were obtained from shoot-tip and node explants of cvs "California Wonder", "Jalapeno", "Red Chili" and "Sweet

Banana". Shoots with a stem were obtained by the normal development of either the apical meristem of the shoot-tip explants or the axillary bud of the node explants (sections 4.1.1 and 4.2). A high proportion (higher than 90%) of the apical meristems from all cultivars tested, developed into shoots when they were cultured in MS or B5 hormone-free medium or on those media supplemented with 1.0 mg $l^{-1}$ GA. It seemed that there was an interaction between the basal medium, the genotype and BAP in producing non-adventitious shoots. This was concluded from the results showing that the addition of 0.1 mg $l^{-1}$ BAP significantly reduced the frequency of shoots from explants of cv. "Jalapeno" in both MS and B5 medium but it so did with cv. "Red Chili" in B5 medium only. In contrast, the presence of this hormone did not alter the frequency of non-adventitious shoot production from cv. "Red Chili" explants cultured on MS medium or those from cv. "Sweet Banana" cultured on MS as well as on B5 medium. The normal growth of axillary buds to form shoots with a stem was influenced by the genotype, the culture medium and the presence or absence of the nodal leaf. Hormone-free media as well as those supplemented with GA and zeatin were equally suitable for axillary bud growth of cvs "Red Chili" and "Jalapeno" regardless the type of basal medium. MS medium was better than B5 for "California Wonder" explants and exactly the opposite occurred with "Sweet Banana" explants. In general, the proportion of leafless axillary-bud explants of cvs "California Wonder" and "Jalapeno" which developed normally was higher than that of explants with leaves, regardless of the type of the medium onto which they were inoculated, and so it was with "Sweet Banana" explants inoculated onto B5 medium.

The negative effect of the presence of the nodal leaf on bud development was mainly due to the fact that sooner or later this abscised

and from the abscission zone callus rapidly proliferated to the extent that it covered the bud and eventually prevented its further development (section 4.2).
Such non-adventitious shoot regeneration from either the apical meristem or from axillary bud cultures could be the basis for a micropropagation scheme relating especially to the high value *Capsicum annuum* $F_1$ hybrids.

### 7.2. *Adventitious regeneration*

#### 7.2.1. *Shoot regeneration*

##### 7.2.1.1. *Factors affecting the initiation and development of shoots*

Adventitious-shoot regeneration occurred at a high frequency from dry-seed cotyledon and hypocotyl (section 3.1), immature embryo tissues (section 3.9), petiole of cotyledon (section 3.6), shoot-tip (section 4.3) explants and at a relatively low frequency from young leaf explants (section 3.12) when they were subjected to a cytokinin-containing medium at a concentration range between 1.0 and 10.0 mg $l^{-1}$. All of the cytokinins tested (BAP, zeatin riboside, zeatin, kinetin) induced shoot regeneration, but BAP and zeatin at concentrations between 1.0 and 5.0 mg $l^{-1}$ appeared to be optimal (sections 3.1 and 3.11). None of the auxins (2,4-D, IAA, NAA, IBA) used alone elicited shoot regeneration (preliminary investigations). Only IAA at a concentration lower than 0.175 mg $l^{-1}$ in combination with BAP elicited higher number of adventitious buds per explant than BAP alone (data are reserved) but this led subsequently to a slow shoot growth. The above investigations are in full agreement

with the findings of Phillips and Hubstenberger (1985) who also reported that IAA in combination with BAP was the best treatment for shoot regeneration in *Capsicum annuum* cotyledon explants. According to the present investigations, the best treatment for rapid shoot regeneration was that of BAP or zeatin in combination with GA rather than BAP and IAA (section 3.5). It should be emphasized that although the low IAA concentrations (lower than 0.175 mg $l^{-1}$) in combination with BAP elicited high number of adventitious buds per explant, these buds rarely developed into shoot with extended stem. In an attempt to overcome this problem, TIBA and GA were incorporated into the stage II medium. TIBA was used because it has been reported that it stimulated the development of adventitious shoots from green nodules and bud-like organs found in petiole-derived callus of *Pelargonium* hybrids (Cassels, 1979) and it slightly stimulated somatic embryo formation in carrot tissue cultures (Kamada and Harada, 1979). In the present work TIBA failed to elicit any organogenesis from cotyledon explants in stage I media or to facilitate shoot growth in stage II media.
Similar results were reported by Phillips and Hubstenberger (1985) in relation to meristematic or non-meristematic explants of *Capsicum annuum*. In the present work the incorporation of GA into the medium of stage I in combination with BAP enhanced the development of buds into shoots and hence shortened the time of producing shoots, suitable for rooting, from 50-60 days to 30-40 days. That means that by the end of stage I, the first "harvest" of shoots (ready for transfer to rooting medium) can be produced. The only disadvantage resulting from the incorporation of GA in the medium from the 1st day of culture was the reduction in the number of shoots

per explant, although the frequency of the explants which produced shoots did not change (section 3.5).
From the observations described above two important conclusions can be drawn: a) if the main aim is the production of a greater number of shoots, even in longer period of time, the incorporation of GA should be in the stage II medium, b) if the time of shoot "harvesting" is of a great concern then GA should be empoloyed from the first day of the stage I.
It should be emphasized that GA alone has not elicited any bud-shoot organogenesis. This is in full agreement with the findings of Jarret *et. al.* (1981) that GA inhibited shoot meristem initiation from tuber discs of potato cv. "Superior", but it was required for continuous shoot development once the meristems were initiated.
There are also reports that GA can be a necessary growth regulator for shoot initiation; for instance, Grout *et al.* (1977) reported that callus derived from meristem-tip cultures of the potato *Solanum x curtilobum* initiated primordia and shoot development on medium supplemented with GA as the only plant growth regulator.
Similarly, Webb *et al.* (1983) described adventitious shoot initiation and development in *Solanum tuberosum* ssp *tuberosum* leaf discs and noted that, in a two-stage culture procedure, GA stimulated maximum shoot formation when present in both stages.
Thorpe (1980) suggested that the stimulation of organogenesis *in vitro* by GA is indicative of low endogenous GA levels in the callus, whereas inhibition of organ formation by exogenous GA may result from supra-optimal levels on the cells.

That cytokinins alone or in combination with low levels of an auxin stimulate shoot regeneration has been reported in many *in vitro*

investigations (for more details, see Chapter I).
The consistent observation, throughout the course of this study, was that the frequencies of shoot production from cotyledon, hypocotyl or shoot-tip explants declined rapidly with time. So, more than 70% of the shoots were harvested by the end of the stage I, a further 20-25% of the shoots were harvested by the end of the stage II and the remaining 5-10% of the shoots were harvested later. If shoots were not harvested by the end of the stage I and the entire cotyledon or hypocotyl explant, which was associated with them, was transferred intact to the same BAP-medium of the stage II, then the number of shoots harvested by the end of stage II did not exceed the number of shoots present at stage I. It seemed that the first developed shoots displayed a dominance over the rest. Similar results were reported by Phillips and Hubstenberger (1985) when *Capsicum* explants were cultured under the same incubation conditions (25 °C and 16-hour photoperiod). They further reported that at 28.5 °C and under continuous light conditions the explants maintained shoot production for 2 months, but they did not indicate whether the explants formed any new buds during the second passage. According to the present investigations, shoot harvested during the second and third passages developed from the buds already initiated during the first 20 days of the initial culture, providing that the dominance of the first developed shoots was removed by their removal.
The decline of the frequency in shoot production, apart from the incapability of the explants to regenerate new buds, was due also to rapid proliferation of hard-compact callus which eventually covered the buds.

It was apparent from the observations throughout this work that the proximal parts of the cotyledon explants responded more rapidly

and more strongly than the distal parts to produce adventitious shoots *in vitro* (sections 3.1 and 3.12). Evidence of the influence of proximal-distal polarity on shoot regeneration was found particularly when more "aged" explants were used. For instance, cotyledon as well as cotyledon-petiole explants excised from 28-day-old seedlings displayed a greater polarity than was found with cotyledon explants excised from embryos. Similar observations have been reported to occur in cotyledon explants of Douglas-fir, where 81% of proximal, 69% of medium and 52% of distal explants exhibited adventitious shoots (Cheng, 1977) and in *Solanum brevidens* cotyledon explants, where proximal parts showed a greater capacity to form callus and adventitious shoot-buds than the distal explants (Osifo, 1983). Stamp (1984) found a similar response in cotyledon explants of cassava. Proximal halves of immature leaflets of *Arachis hypogaea* initiated more adventitious buds than distal ones (Mroginski *et al.*, 1981) and the same response was observed in leaf explants of *Echeveria elegans* by Raju and Mann (1971), who suggested that the difference in generation potential might be due to the differences in anatomy of proximal and distal parts. The tendency for shoot regeneration to occur preferentially at the proximal regions, according to Vardjan and Nitsch (1961), might be due to the establishment of polarity in the explants resulting from endogenous transport of growth substances and/or nutrient.

Evidence concerning the site of regeneration is provided by reports relating to *in vitro* regeneration with several plant species. For instance, in tobacco stem segments adventitious shoots arose mostly from elements of phloem near the surface (Sterling, 1951), while the cells near the protoxylem of root explants of *Convolvulus*

*arvensis* were the site for adventitious shoot regeneration (Bonnet and Torrey, 1966). Also, in *Annona squamosa* maximum shoot-bud formation was observed from explants taken from the basal portion of the leaf containing the petiole, followed by the region of the leaf containing the leaf base without the petiole and by the lamina with midvein respectively (Nair *et al.*, 1984).

It was also apparent that shoot regeneration occurred mostly from the abaxial cotyledon surface and exclusively from the abaxial surface of the young leaves (sections 3.1 and 3.12). The orientation of the cotyledon and leaf explants also strongly influenced this organogenic response. Placing the adaxial surface of cotyledon in contact with the agar medium increased slightly the shoot induction from this surface but reduced dramatically the response from the abaxial surface. When leaf explants were placed with their adaxial surface in contact with the medium then no organogenesis observed in either abaxial or adaxial surface. The adaxial-abaxial polarity of regeneration observed in *Capsicum* is not unusual since there are reports concerning a number of plant species. For instance, Haydu and Vasil (1981) observed that somatic embryos were initiated from the abaxial surface of leaf tissues of *Pennisetum purureum* and similar results have been reported for leaf tissues of *Pennisetum maximum* (Lu and Vasil, 1981) and scutellar tissues of *Pennisetum americanum* (Vasil and Vasil, 1982). On the other hand, adventitious shoot regeneration occurred preferentially from the adaxial leaf surface of *Echeveria elegans* (Raju and Mann, 1970 and 1971) and from the adaxial leaf surface of *Solanum melongena* (Gleddie *et al.*, 1983). Also, somatic embryos were initiated from the adaxial surface of cassava cotyledon explants (Stamp, 1984).

The age of the explant played a significant role in the morphogenic competence. Lamina discs of fully expanded leaves did not respond at all, while younger leaves formed adventitious shoots (section 3.12). Similarly, cotyledon explants excised from dry seeds or from 10-day-old seedlings produced adventitious shoots at a higher frequency than cotyledon explants excised from 15, 20 or 25-day-old seedlings (section 3.7). The most dramatic reduction in organogenic competence was found in cotyledon explants from 15-day-old seedlings. This reduction could be due to insufficient nutrient supply for regeneration, since it was observed that starch grains in the cotyledon gradually disappeared with time and most of the endogenous carbohydrates were used to support the seed germination and the very young seedling. Fifteen days after germination the true leaves had not yet appeared and the cotyledons were pale green; by day 20 and afterwards, the true leaves were enlarged and photosynthetically active. The carbohydrates thus provided by the leaves re-established the balance of nutrient and energy in cotyledons which returned to dark green colour. This could be a reason for the higher frequencies of shoot regeneration from cotyledon explants of 25-day-old seedlings than from those of 15-day-old seedlings. However, these changes in the cotyledons, accompanied the increased photosynthetic activity, were not accompanied by a full re-establishment of the morphogenic competence and, further, one would expect a full complement of nutrients would have been available from the medium.

The effect of the age of the explant on morphogenic competence has been studied in other species. For instance, Biondi and Thorpe (1982) working with cotyledon explants of *Pinus radiata*

found that the cells in the epidermis and subepidermis of older cotyledons had differentiated to such an extent that they were no longer capable of responding to BAP as did those of 5 to 7-day-old cotyledons. Yeung *et al.* (1981) suggested that young cotyledons, of the same species, were physiologically suitable explants, whose epidermal and subepidermal cells were not fully "determined" and were capable of being channelled into an alternative morphogenic pathway expressing a high shoot-forming competence. High morphogenic competence from young organs or tissues was reported to occur in immature tissues of *Lolium multiflorum* (Dale, 1980), *Theobroma cacao* (Pence *et al.*, 1980), *Gerbera* (Murashige et al., 1974) and *Sorghum bicolor* (Wernicke and Brettell, 1980; Brettell *et al.*, 1980).

The present study demonstrated that organogenic competence is greatly dependent upon the genotype (sections 3.4 and 3.10). Generally, chili-type cultivars such as "Red Chili" and "Jalapeno" and long sweet-type such as Pt-46, Pt-14 responded better than bell-type cultivars such as Pt-455, Pt-824, Pt-452, "California Wonder", "Worldbeater", "Hybelle", "Pennbell" and "Bell Boy" $F_1$ hybrid. The less responsive cultivars were "Sweet Banana", Pt-53/13, "Tomato-pimento" and "Propenza". The importance of the genotype in shoot regeneration *in vitro* was also reported by Gunay and Rao (1978) in *Capsicum annuum*, by Matsuoka and Hinata (1979) and Gleddie *et al.* (1983) in *Solanum melongena*, by Webb *et al.* (1983) in *Solanum tuberosum*, by Oelek and Schieder (1983) in *Arachis*, *Glysine*, *Melilotus* and *Vicia*, by Campell and Tomes (1984) in *Trifolium*, by Kumar and Reddy (1983) in *Cajanus*, by Mariotti and Arcioni (1983) in *Cornilla*, by Meijer (1982) in *Phaseolus* and *Stylosanthes*, by Davery (1983) in *Lotus* and by Brown and Atanassov (1985) in *Medicago*.

The effect of temperature on the adventitious shoots regeneration was tested on cotyledon and hypocotyl explants (section 3.8). A high temperature level such as 25 °C and 30°C elicited a higher frequency of bud-shoot initiation than 20 °C, but subsequent shoot development was better under the 20 °C temperature regime. The latter was probably the result of an indirect effect of the temperature, since a temperature of 20 °C slowed down the speed of callus proliferation and hence removed the constraints that callus exercised on the shoot development. Phillips and Hubstenberger (1985) reported similar results and furthermore they achieved the best shooting response at 28.5 °C under continuous light; under these conditions meristematic explants (shoot-tips and cotyledonary nodes) of *Capsicum annuum* responded for 8 months (8 passages) and non-meristematic explants (hypocotyls and cotyledons) for 4 months (4 passages). They also found that shooting occurred only, at a low level, during the first passage in meristematic explants exposed to 25 °C and 12-hour photoperiod. Patel and Thorpe (1984) reported that bud initiation from cotyledon explants of *Pinus contora* was influenced by the temperature and it was better at 27 °C than at 20 °C.

The effect of light on the shoot initiation process was tested in combination with the effect of casaminoacids on cotyledon explants which were kept either under complete darkness or exposed to light (10.2 microeinsteins $m^{-2}sec^{-1}$) with 16-hour photoperiod, during the first 10 days of the culture. Adventitious shoot regeneration occurred at significantly higher frequencies in explants kept under complete darkness than in explants exposed to light. The negative effect of the light during the first 10 days could be overcome by the

presence of 100 mg $l^{-1}$ casaminoacids in the medium (section 3.3). Casaminoacids were not essential for shoot regeneration from explants kept in darkness for the first 10 days of the culture, but were beneficial to those kept under 16-hour photoperiod from the first day and they removed the negative effect imposed by the light. Casein hydrolysate has been found to be beneficial to the regeneration process in many plant species; for instance, it increased the growth rate of shoot-tips of carnation (Davis and Hanan, 1977), it was beneficial for induction of somatic embryos especially from the most immature zygotic embryos of *picea abies* (Hakman *et al.*, 1985) and of *Dactylis glomerata* (Gray and Gonger, 1985) and stimulated adventitious shoot regeneration in *Cuscuta reflexa* (Maheshwari and Baldev, 1961), in *Oryza sativa* (Nakamo and Maeda, 1979) and *Arachis hypogaea* (Bejaj *et al.*, 1981).

The concentration of sucrose played a significant role in the shoot regeneration process. A 2% or 3% sucrose concentration was more suitable for high frequency shoot initiation and shoot growth than was 6% or 9% (section 3.9). High sucrose levels might suppress the process by increasing the osmotic potential of the basal medium as has been suggested by Thorpe (1982) and Stamp (1984), but, in cotyledon explants excised from immature embryos of cv. "Jalapeno", 6% sucrose promoted the shoot initiation process better than either 3% or 9%. This might have been because at that early developmental stage cotyledons had not stored sufficient carbohydrates to meet the needs of energy necessary for full expression of the organogenic capacity and hence higher levels of exogenous carbohydrates were advantageous.

Concerning the role of the basal medium it was found that there was an interaction between it and genotype, the explant type and the cytokinin on the shoot regeneration process. In general, the SH basal medium was less suitable than MS and B5 medium for shoot regeneration with cotyledon explants of cv. "Red Chili" while it was as effective as MS and B5 with cotyledon explants of cv. "Jalapeno" and "Bell Boy" $F_1$ hybrid (section 3.6). The B5 and MS media were equally effective in forming adventitious shoots from shoot-tip explants of cvs "Jalapeno", "California Wonder", "Red Chili" and "Sweet Banana" (section 4.1.3).

7.2.1.2. *Anatomy and morphology*

The pattern of adventitious shoot regeneration seemed to be similar in cotyledon, cotyledon-petiole, hypocotyl, young leaf and shoot-tip explants inoculated onto medium supplemented with a cytokinin alone or in combination with the auxin IAA. The sequence of histological and morphological changes which took place during the course of the process was studied in detail in the cotyledon and shoot-tip explants (section 3.2 and 4.1.4). The formation of organized structures occurred in the epidermal and subepidermal cells of cotyledon explants. That the most responsive sites for direct organogenesis are those at the periphery (epidermal surface) of the explant has been demonstrated in many *in vitro* investigations, as for instance in cotyledon explants of *Pinus radiata* (Yeung *et al.*, 1981) and Douglas-fir (Cheah and Cheng, 1978) and in stem cultures of *Torenia fournieri* (Chlyah, 1974a). Such a limitation of cell division to the periphery may be related to interaction of several factors, i.e. the light shedding, the greater availability of oxygen, more rapid release of $CO_2$,

more rapid escape of volatile inhibitors, greater nutrient availability and the wounding response of the cut surface (Yeoman, 1970) or to the establishment of a physiological gradient of materials moving out of the medium into the tissues (Ross and Thorpe, 1973). In the present work, as it has already been noticed, organogenesis occurred at the periphery of the cut edges as well as on the epidermis of cotyledon and hypocotyl explants away from the cut surface. The process leading to shoot formation involved localized cell division to produce a meristematic tissue. Within the first few days of the culture, the disappearance of starch grains from the cotyledon pieces and the appearance of large nuclei occurred (section 3.2). Soon after the degradation of starch grains, cell division took place in the epidermal and subepidermal regions along the entire length of the cotyledon abaxial surface in contact with the agar medium. The utilization of starch grains during that period suggests that they might provide a source of energy for the regeneration process , or at least for the cell divisions.

Mobilization of starch reserves within the first days of culture has been reported to occur in cotyledon explants of *Pinus radiata* (Yeung *et al.*, 1981) and cassava (Stamp.1984). Maeda and Thorpe (1979) and Thorpe and Murashige (1968), working with tobacco callus, observed a heavy accumulation of starch in the tissues, particularly in the shoot-forming regions, before initiation of zones of cell division. In these zones, as well as the surrounding tissues, the starch content declined as the meristemoids and shoot primordia formed. In cotyledon explants, the mitotic figures were observed during the first 2 days of cultures. One of the most noticeable cytological events, concerning the pattern of cell division, was that the initial

cell divisions were random in their distribution but by the day 5 of culture they were mostly restricted to the proximal part of the abaxial half. The most significant feature was that periclinal as well as anticlinal cell divisions were frequently observed in both epidermal and subepidermal layers. Furthermore, quite a high number of these divisions seem to be asymmetrical, as judged by the cell plate positions (Plates 3.1 F and 3.2 A, B). This asymmetry might be due to physical or biochemical differences in the two daughter cells after the first division, as was suggested by Chlyah (1974b). Such asymmetrical (unequal) cell divisions have been observed in several developmental processes *in vivo*, e.g. formation of stomata guard cell (Kaufman *et al.*, 1970), root hair formation (Cutter and Hung, 1972), pollen grain formation (Angold, 1968) and zygotic embryo formation (Jensen, 1964). In all the above cases, it was the smaller daughter cell that developed into the specialized structures. Bunning (1952) has defined one of the two daughter cells from such an asymmetrical divisions which generally involved in further meristematic activity as a "meristemoid"; it was generally the smaller, more densely cytoplasmic cell. This study on *Capsicum* cotyledon explants does not however provide any evidence whether the smaller or both cells were involved in meristematic tissue formation.

By day 5 of culture, *de novo* meristematic regions appeared on the epidermis of cotyledon explants and they consisted of typical meristematic cells. These meristematic regions developed to produce bud or leaf-like ("foliose") structures (Plates 3.3 and 3.4 A, B). Again, it was not possible to determine whether each meristematic region developed from the division of a single cell or from more than one cell, since the formation of meristematic tissue took place along

the entire surface of the proximal part of the cotyledon explants. There was no evidence to suggest that the foliose structures initiated from *Capsicum* explants on cytokinin-medium could develop into adventitious leaves and they were not seen to be associated with the meristems or shoot-primordia which were initiated simultaneously. They originated independently from the meristems even if the regions of origin were very close to each other. The development of the foliose-structures was faster than the development of the adventitious shoots, particularly during the first 20 days of culture, and hence foliose structures were seen by the naked eye earlier than were shoots. Similar results concerning the origin and characteristics of the foliose structures were reported by Stamp (1984) on cassava cotyledon explants.

Concerning the initiation and development of the buds and foliose-structures similar histological and morphological changes were also observed on shoot-tip cultures (section 4.1.4) with the following exceptions: starch grains have not been detected in shoot-tip explants prior to cell division and it is possible that the carbohydrates, necessary for the process, could be provided in a soluble form by the medium. Thorpe (1980) suggested that the degradation of starch, observed in many explants, might fulfill an osmotic requirement for adventitious regeneration. Rathbone *et al*. (1976), working with tobacco pith callus, concluded that starch hydrolysis was not necessary for meristem formation but rather for a subsequent step in adventitious shoot formation.

In the present work, the initial cell divisions were restricted to the epidermis of the proximal end of the shoot-tip explants surrounding the cut edges (Plates 4.2 and 4.3). Referring to the

foliose-structures arising from the shoot-tip explants, there have been no differences observed in comparison with those of cotyledon or hypocotyl explants.

### 7.2.2. *Somatic embryogenesis*

#### 7.2.2.1. *Factors affecting embryogenesis*

Somatic embryogenesis *in vitro* is of considerable interest from several points of view; e.g. to understand the physical and chemical requirements of embryo induction and development and possibly to produce propagules on a large scale. Somatic embryogenesis has been reported to occur in various types of explants from a considerable number of plant species when they were subjected to media supplemented with an auxin alone or in combination with a cytokinin at a high auxin to cytokinin ratio. For instance, somatic embryogenesis has been achieved from *Nigella sativa* leaf callus (Banerjiee and Gupta, 1976), *Perilla frutences* leaf explants (Tanimoto and Harada, 1980), cauliflower leaf callus (Pareek and Chandra, 1978), *Brassica campestris* leaf petioles (Bhattacharya and Sen, 1980), *Daucus carota* cell suspension cultures (Smith and Street, 1974), celery petiole callus (Williams and Collin, 1976a and 1976b), *Solanum melongena* leaf explants and cell suspension cultures (Gleddie *et al.*, 1983), cassava cotyledon and leaf lobe explants (Stamp, 1984). For more extensive list see reviews by Tran Thanh Van (1981) and Ammirato (1983).

There have been no reports concerning the occurrence of somatic embryogenesis in *Capsicum annuum*. During the course of this study, somatic embryogenesis was observed to occur only with immature zygotic

embryo explants out of thirteen different types of meristematic or non-meristematic explants that were investigated. Immature zygotic embryos, at stages II and III of development, subjected to MS medium supplemented with 2,4-D at concentrations between 1 and 5 mg $l^{-1}$ initiated only embryogenic tissue during the stage A of the culture. Somatic embryo formation occurred during the stage B of culture on MS medium from which 2,4-D was either omitted or applied at a low concentration in combination with a low level of a cytokinin (section 5.2.1).

Immature zygotic embryos have also proved to have a high embryogenic potential in other plants species such as *Secale sereale* (Lu *et al.*, 1984), *Picea abies* (Hakman *et al.*, 1985), *Trifolium repens*, *T. pratence* and *Medicago sativa* (Maheswaran and Williams, 1984), *Dendrophthoe falcata* (Johri and Bajaj, 1962), *Cuscuta reflexa* (Maheshwari and Baldev, 1961), *Phoenix dactylifera* (Reynolds and Murashige, 1979), *Lolium multiflorum* (Dale, 1980) etc. Green and Phillips (1975) working with *Zea mays* and Pence *et al.* (1980) working with *Panicum maximum* commented that the high embryogenic potential of immature zygotic embryo was associated with their phase of most rapid growth. In *Capsicum*, the optimum concentration of 2,4-D for the induction of somatic embryogenesis was between 3 and 5 mg $l^{-1}$. Although the frequency of embryogenic tissue formation was very high as also was the frequency of somatic embryo formation, the number of plantlets derived from these somatic embryos was very low because many of the embryos did not seem to possess a shoot tip. In an attempt to reduce the number of the abnormal somatic embryos, ABA was introduced into the stage B medium. This was not succesful however, although the appearance of the globular and bipolar structures was

improved. In contrast, Ammirato (1983) was able to normalize abnormal somatic embryos in *Carum carvi* cell suspension cultures by the use of ABA.

It has been reported that l-proline stimulated embryogenesis in *Zea mays* (Green *et al.*, 1983) and *Medicago sativa* cultures (Stuart and Strickland, 1984). In *Capsicum* immature zygotic embryo cultures, however, l-proline did not significantly stimulate embryogenesis, although again this treatment improved the appearance of the globular and bipolar structures (section 5.2.3 and Plate 5.4. D).

Rugini (personal communication) found that 0.25 mg $l^{-1}$ cobaltous chloride (ten times the standard concentration in MS medium) increased the frequency of shoot regeneration and stimulated the growth of embryos from almond explants. In *Capsicum* cultures involving immature zygotic embryos of cv. "Sweet Banana", however, the data showed that 0.25 mg $l^{-1}$ cobaltous chloride reduced the frequency of the plantlets derived from somatic embryos in media with pH values of 5.7 or 7.0. The same concentration of cobaltous ions completely inhibited embryogenesis in immature zygotic embryo explants of "Bell Boy" $F_1$ hybrid (section 5.2.4).

Fassuliotis and Nelson (1986) found that the transfer of *Cucumis metuliferus* embryos from medium at pH 5.7 to medium at pH 7.0 further stimulated their development into complete plants. By applying the same procedure to *Capsicum* immature zygotic embryo cultures, the frequency of normal somatic embryo formation was apparently increased but not to the extent that the procedure could be regarded as being satisfactory. The important observation was that higher pH value (7.0) was more beneficial to somatic embryogenesis only when the stage B medium was adjusted to that level (sections 5.2.3 and 5.2.4).

Activated charcoal (AC) seemed to play a significant role in

the somatic embryogenesis process (section 5.2.5). Incorporation of AC into the stage A medium from the first day of culture of immature zygotic embryos inhibited completely the embryogenic process, allowing the normal development of the zygotic embryos into complete plants, even in the presence in the medium of 100 mg $l^{-1}$ 2,4-D. It was likely that the AC absorbed the auxin from the medium as demonstrated by Weatherhead *et al.* (1978). Incorporation of 1% AC into the stage A medium on day 15 of culture, or on day 1 of the stage B, increased dramatically the frequency of normal somatic embryos and hence the number of plantlets that were produced. AC has been reported to restore the ability to produce somatic embryos in carrot cultures which had previously lost embryogenic competence (Fridborg and Eriksson, 1975; Fridborg *et al.*, 1978; Drew, 1979). Fridborg *et al.* (1978) suggested that the likely cause of this was the removal of inhibitors of embryogenesis, in particular phenylacetic acid and benzoic acid derivatives, by absorption on to the charcoal. Weatherhead *et al.* (1978) demonstrated that AC had absorbed the compound HMF (5-hydroxymethylfurfural) which inhibited the growth of *Nicotiana tabacum* anther cultures and which was produced by sucrose dehydration during autoclaving. They also demonstrated that AC absorbed the phenolic compounds hydroquinone and p-hydroxybenzoic acid which also inhibited completely the growth of *Nicotiana tabacum* anther cultures.

In summary, the best procedure for high frequency somatic embryogenesis from immature zygotic embryos of *Capsicum* could be described as follows: a) stage A: MS3 medium with 3 mg $l^{-1}$ 2,4-D and 100 mg $l^{-1}$ l-proline at pH value of 5.7, b) stage B: MS3 medium with 0.01 mg $l^{-1}$ 2,4-D or NAA, 0.1 mg $l^{-1}$ cytokinin and 1% AC at pH value of 7.0 and c) stage C: MS or B5 medium hormone-free or supplemented with

0.1 mg $l^{-1}$ zeatin at pH 5.7 or 7.0.

The embryogenic competence of immature zygotic *Capsicum* embryos was tested with four cultivars - "Sweet Banana", "Jalapeno", "California Wonder", "Red Chili" - and the $F_1$ hybrid "Bell Boy". The cultivars "Jalapeno" and "Red Chili", which displayed a high shoot-regeneration capacity, failed even to form embryogenic tissue. In contrast, genotypes which showed low shoot-regeneration capacity such as "Sweet Banana", "California Wonder" and "Bell Boy" $F_1$ hybrid, displayed a higher frequency of embryogenic tissue formation and a reasonably high frequency of somatic embryo production (sections 5.2.1, 5.2.3 and 5.2.4).

By the end of stage C, globular structures, bipolar structures, abnormal embryos and normal embryos as well as plantlets were coexisting. A number of normal embryos failed to grow into plantlets, although underwent elongation of the root and hypocotyl before becoming arrested at this stage.

#### 7.2.2.2. *Origin of somatic embryos*

Reviewing the literature it would seem that somatic embryos have either a single or a multiple-cell origin. The term "multicellular origin" has the meaning that embryos are risen at the periphery of a cell clump which may or may not be derived from a single cell; while "single-cell origin" means that each embryo derived directly from a single cell. Somatic embryos of a multicellular origin developed from leaf cultures of *Sorghum bicolor* and particularly from massive outgrowths of embryogenic tissues close to the wound caused during the excision (Wernicke *et al.* , 1982). Somatic embryos initiated on immature zygotic embryos of the same species arose directly

from cells of the primary scutellum without an intervening callus phase, either by folding of the scutellum or from single cells (Dunstan *et al.*, 1978). A similar observation was reported for somatic embryos initiated on immature zygotic embryos of *Juglans regia* by Tulecke and McGranahan (1985). For more details about the origin of somatic embryos see Chapter I.

*Capsicum annuum* immature zygotic embryos subjected to the medium containing 2,4-D showed that protuberances of meristematic cells were initiated only from the proximal-adaxial cotyledon surface of the intact zygotic embryos (section 5.2.2 and plates 5.1. C, D and E). Excised immature cotyledons cultured on the same medium did not respond at all. These protuberances grew to form globular structures which consisted of cells with large nuclei and dense cytoplasm and they were highly organized. The orientation of the surface cells differed between the globular structures and the original cotyledon epidermis. The cells at the surface of the globular structures had their larger axis vertical to the surface in contrast to the epidermal cells of the original cotyledon where the larger axis was parallel to the surface (Plate 5.3 C and D and Plate 5.4 A and B). Although some of the globular structures had the appearance of adventitious embryos they did not develop directly into plantlets but they always were transformed into bipolar structures from which four or more fused abnormal or normal somatic embryos emerged (Plate 5.5 B). These globular structures at their early stage resembled the embryogenic structures described by Springer *et al.* (1979) in immature-embryo cultures of *Zea mays*.

The sequence of the developmental changes from the protuberances to globular structures and later to bipolar structures and somatic embryos was completely different from those already described for

shoot organogenesis.

An important feature was that during the shoot-organogenesis process callus was formed close to the bud-regeneration regions, while during the somatic embryogenesis process callus did not forme at all close to the responsive regions even if the explants remained in culture for more that 50 days. When callus formation took place during the first period of culture of immature zygotic embryo explants (stage A) then no signs of embryogenic tissue were observed. That leads to the conclusion that callogenesis and embryogenesis are completely incompatible processes in *Capsicum* cultures of immature zygotic embryos and that the process can be defined as being closer to "direct embryogenesis" as described by Evans *et al.* (1981a and 1981b). Another difference between the processes of shoot-organogenesis and somatic embryogenesis in *Capsicum* is that the former took place mostly from the abaxial surface of cotyledon explants whereas the latter occurred exclusively from the adaxial cotyledon surface of the intact immature zygotic embryos.

*Capsicum* somatic embryos were defined as such on evidence provided by their final morphology and nature rather than the sequence of events which led to their formation since the characteristic "heart", "cotyledonary" and "torpedo" stages (McWilliam *et al.*, 1974) were not detected. Haccius and Lakshmanan (1969) and Haccius (1978) observed that adventitious embryo's vascular system did not have any connection with the parent tissues. Similar observation has been reported in cassava somatic embryos (Stamp,1984) and this was the case in this work for *Capsicum* somatic embryos (Plate 5.5 A in section 5.2.2);

Protuberances, globular and bipolar structures as well as somatic embryos during their early developmental stages were colourless,

whereas protuberances and shoot primordia, observed during the shoot-regeneration process, were green from their earliest stages. Furthermore, extension of somatic embryos took place only when they were moved from their parental explant, whereas in contrast, adventitious shoots regenerated from various explants subjected to a cytokinin containing medium extended while they were still attached to the parental explant. The somatic embryos, however, had no potential to undergo secondary somatic embryogenesis when cultured on 2,4-D containing medium. This was probably due to their maturity when tested, since it was clearly demonstrated that mature zygotic embryos were incapable of undergoing somatic embryogenesis when they inoculated on to the same medium (section 5.3).

Globular structures cultured on MS medium supplemented with low 2,4-D concentrations were able to produce more globular structures but this ability lasted for only one passage, after which they started to produce friable colourless callus.

Cotyledons of somatic embryos when cultured on cytokinin containing medium underwent shoot organogenesis (Plate 5.8 A) as did cotyledon explants from zygotic embryos.

### *7.2.3. Adventitious rhizogenesis*

Adventitious rhizogenesis was investigated in a series of preliminary experiments. It was found that the frequency of adventitious root formation was strongly influenced by the nature of the explant, the genotype, the incubation conditions and the nature of the plant growth regulator employed. Adventitious-root regeneration took place from root segments cultured on hormone-free medium or medium supplemented with an auxin (NAA, IBA and 2,4-D), from cotyledon and hypo-

cotyl explants subjected to media containing an auxin (2,4-D, IAA, NAA, and IBA) or the cytokinin BAP at a low concentration. Referring to root-segment explants, among the auxins , IBA and NAA were superior to 2,4-D in relation to both root induction and root growth (data are reserved by the author). The presence of the cytokinin BAP in medium resulted in a reduction of the frequency of root induction and in root growth, regardless of the presence of an auxin in the medium.

Adventitious root formation was more likely to occur from root-segments since they possessed pericycle, the main region from which the lateral roots are usually originated (Esau, 1977).

Referring to cotyledon and hypocotyl explants, in general, the frequency of rhizogenesis was higher in hypocotyls than in cotyledons. Roots were formed either directly from the vascular tissues and particularly from the proximal end of the midvein, or indirectly from the callus of the cotyledon explants. Direct or indirect rhizogenesis observed also in hypocotyl explants. Adventitious rhizogenesis from hypocotyl and cotyledon explants occurred not only on media containing an auxin but also on hormone-free medium. In this case, the frequency of direct rooting was very low in cotyledon but very high in hypocotyl explants.

The exact seat of the origin of the directly (on the explant itself) regenerated adventitious roots as well as the sequence of the events during the process was not investigated, but according to Satoo (1955) adventitious roots commonly arise in the interfascicular parenchyma and thus the new root appears close to both xylem and phloem.

Rooting from cotyledon and hypocotyl depended upon the nature and the concentration of the plant growth regulator employed. BAP at concentration lower than 1 mg $l^{-1}$ encouraged rhizogenesis in hypocotyl explants,while it inhibited completely the process in cotyledon

explants. The same hormone at concentration higher than 1 mg $l^{-1}$ not only suppressed the process but also reduced the branching capacity and finally induced normal roots to become "tuberous" or swollen in appearance (data are reserved).

Among the auxins, IAA seemed to be superior to 2,4-D and NAA in relation to the induction of adventitious rhizogenesis. Concentrations of auxin between 0.1 and 1.0 mg $l^{-1}$ appeared to be optimal for rooting. Cotyledon explants rooted with difficulty on 2,4-D medium, but very easily on IAA medium. The same explants did not root at all on NAA-containing medium. The low BAP to low IAA treatment elicited high levels of rooting from both cotyledon and hypocotyl explants, whereas media with a high BAP to IAA ratio reduced dramatically the rooting response from cotyledon explants (data are reserved). Similar results were reported by Phillips and Hubstenberger (1985) for cotyledon and hypocotyl explants of *Capsicum*. In contrast, to their report, these investigations showed that rooting from cv. "California Wonder" hypocotyl explants was slightly but not severely reduced on media with a high BAP to IAA ratio.

In a preliminary experiment, it was found that a low light intensity (500 lux) was more favourable than a high light intensity (2000 lux) for the induction of adventitious rhizogenesis from hypocotyl but not from cotyledon explants of cv. "California Wonder".

The genetic background of the explants played a significant role in the rooting response; for instance, "Worldbeater" cotyledon explants rooted more easily and better than those of "California Wonder" or "Bell Boy" $F_1$ hybrid and exactly the opposite was found with hypocotyl explants. Furthermore, chili type cultivars such as "Red Chili" and "Jalapeno" responded better than bell type cultivars such as "California Wonder", "Worldbeater" and "Bell Boy" $F_1$ hybrid.

Adventitious root regeneration occurred from lamina discs of immature leaves subjected to NAA-medium (data are reserved). The frequency of rooting was greatly influenced by the hormone level and the leaf position on the plant. Lamina discs from leaves of the second node produced root earlier and more frequently than those of the first node. This is probably due to the fact that the leaf of the second node is more juvenile than that of the first node. Unlike embryonic tissues (cotyledons, hypocotyls) leaf discs required higher concentration of NAA to root; optimal concentrations were being between 10 and 15 mg $l^{-1}$. Adventitious roots originated mostly from the compact callus which were formed on the surface of the lamina while it was still attached to the explant.

In attempting to producing complete plants, the adventitiously regenerated shoots were subjected to different media (sections 4.3.1 and 4.3.2). The rooting response was influenced by the genotype, the type of the medium, and the nature and concentration of the auxin employed. The optimal concentrations of IBA for shoot rooting were those between 2 and 5 mg $l^{-1}$, whereas the optimal concentrations of NAA for the same response were those between 1 and 5 mg $l^{-1}$. Adventitiously regenerated shoots of cv. "Red Chili" and "Bell Boy" $F_1$ hybrid were also rooted at a considerable high frequency when they were subjected to a hormone-free medium. That the addition of an auxin to the basal medium induced or encouraged rhizogenesis has been reported for many species, e.g. in *Daucus* (Steward and Shantz, 1958; Reinert, 1959; McWilliam *et al.*, 1974), *Solanum tuberosum* (Webb *et al.*, 1983), *Zea mays* (Vasil *et al.*, 1984). For more references see section 1.2.4.

Concerning the effect of the basal medium it was found that MS medium was slightly better than B5 for rooting. Among the cultivars, it was found that "Red Chili" shoots rooted more easily than shoots of "Jalapeno", "Sweet Banana" and "Bell Boy" $F_1$ hybrid. In general, adventitious shoots from chili type cultivars rooted better and more easily than those from bell type cultivars.

### 7.3. *Establishment of plants in soil*

Plantlets derived through either organogenesis or embryogenesis were established in soil in order to study their behaviour under the greenhouse conditions in comparison with the seed-derived plants and to examine their chromosomal status. Acclimation of the plantlets was not difficult to achieve and it was aided by placing the pots in a tray covered with a transparent plastic dome fitted with adjustable ventilators. By this device, very high levels of humidity were established during the first days after transplantation into the pots. This induced a good performance of plants necessary for new root initiation. The ventilators were gradually opened and after a week plants were uncovered and exposed to the greenhouse conditions. By that time, the plants produced new roots and gradually a balance of water uptake and water loss was established. By using this technique the leaves of the plantlets, which had been produced in culture, remained green. All of the transplanted plants survived, grew rapidly, bloomed satisfactorily and set many and probably more fruits than plants derived from seeds.

7.4. *Genetic stability*

Plantlets which developed *in vitro* from somatic embryos and adventitious shoots were normal, healthy and relatively uniform among themselves and in comparison with those obtained from seeds. Features such as stem and leaf colour; leaf area and dimensions; flower shape, size and colour; fruit setting, size and colour; the whole-plant appearance and growth habit were checked, together with the chromosome numbers in root-tips of both seedlings and plants, derived *in vitro*. Furthermore, seedlings of the second generation were also examined in the same ways.

Data concerning leaf area and dimensions (section 6) from plants derived through organogenesis as well as the germinability of their seeds from the same plants (section 6) showed that there were no significant differences among the plants. Only the germination ability of seeds from adventitious plants was greater than of commercially supplied seeds of cv. "Red Chili". This was to a degree expected since the commercial seeds were older than one year. The above difference in germinability was not observed with cv. "Jalapeno" seeds since they were collected from freshly mature fruits either from adventitious plants or from seedlings.

No differences were observed concerning the form (Plates 4.4 B, C and 6.1 A, B, C), the habit and the appearance of the whole plants, the flower shape and colour, fruit shape and colour between adventitiously-derived plants and those produced from seeds.

No differences were detected concerning the chromosome number between seedlings and plants raised *in vitro*. These observations indicated that the regeneration systems employed have a high degree of genetic stability at least at the level of chromosome number

(section 6 and Plates 6.2 and 6.3). Studies for detecting changes in chromosome structure, "cryptic chromosome rearrangements" as referred to by Larkin and Scowcroft (1981) were not conducted. Such genetic stability at the level of chromosome number was perhaps not surprising since both regeneration pathways (shoot organogenesis and somatic embryogenesis) occurred directly from the parental explants without an extensive intermediate callus phase, and according to Krikorian (1982) such a regenerative system is characterized by high genetic stability.

### 7.5. *Further in vitro studies with Capsicum*

According to the findings of the present study, *Capsicum annuum* explants (meristematic or non-meristematic) showed a high competence for adventitious organogenesis (rhizogenesis and shoot regeneration). Two specific problems, however, must attract attention: firstly to improve the procedure for bud growth into shoots and secondly to increase the frequency of rooting on shoots. This would result in high rates of multiplication and the *in vitro* techniques could be adequate for propagation purposes.

The first problem might be solved by establishing a procedure which could eliminate callus proliferation after the shoot initiation process has started. With the media tested and the incubation conditions employed throughout this work, it was impossible for the callus formation to be eliminated. The large callus mass, friable or compact, which was always produced, seemed to impose constraints on the ability of the adventitious buds to develop into shoots. Also the light regime, concerning either its photoperiod, as was reported by Phillips and Hubstenberger (1985) or its intensity and quality as some

preliminary investigations have indicated, could also help the adventitious bud initiation. Furthermore, the removal of some inhibitors of the shoot growth, which might be present in the cultures, could also encourage the growth of buds (Weatherhead *et al.*, 1979).

Another problem which needs further investigation concerns somatic embryogenesis. It has been shown that immature embryo explants possessed a strong competence to initiate embryogenic tissue and that a high frequency of abnormal embryo production occurred. Also a high number of the normal embryos failed to grow into plantlets. Further investigation has to be conducted to minimize the frequency of abnormal embryos and overcome the resting phase of the normal embryos.

It has been found that *Capsicum* cell suspension cultures can be easily established. Studies should be conducted to identify the conditions which would enable such cultures to be established from embryogenic tissues so that somatic embryos might be produced on a large scale. This type of system might produce plants which will not be true-to-type since they would be products of callus cells. On the other hand it might be possible or even essential to control the conditions so that an adequate level of organization is retained in the suspension culture, thus avoiding the problems apparently caused by extreme disorganization.

Both regenerative systems (organogenesis and embryogenesis) proved to be stable at the level of chromosome number, at least with the numbers tested. If it could be confirmed that they are stable at the sub-chromosomal level, then the process could be used for rapid multiplication and propagation provided also that the multiplication rates could be raised above those achieved by the procedures used during this study. Then the greater application could be to

facilitate and shorten the time for the introduction of selected new hybrids, or to enable the multiplication in very large numbers of individual plants considered to have desirable characteristics such as high productivity, a particular habit or a significant degree of disease resistance (Sagawa, 1976; Krikorian, 1982). On the other hand, if sub-chromosomal changes took place so that the plants so produced could not be used for propagation purposes, it is possible that some of the mutants would possess good agronomic properties such as resistance to extreme environmental conditions (Dix and Street, 1976), tolerance to salinity (Dix and Street, 1975) etc. These mutants could then be multiplied by traditional or *in vitro* techniques or incorporated into conventional breeding programmes.

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# A P P E N D I C E S

Appendix 1. Details of the procedure for fixing, embedding, sectioning and staining small specimens (after Johansen, 1940; see Section 2.6).

1. Fix specimens in FAA (formalin: acetic acid: 70% ethanol, 90:5:5 v/v/v) for at least twenty hours at 5 °C.
2. Pass specimens through TBA series (see below). One hour in each solution except TBA2, overnight, at room temperature. The tertiary butyl alcohol series, for dehydrating specimens before embedding in was as follow:

| TBA | Water | : 95% ethanol | : TBA | : 100% ethanol |
|---|---|---|---|---|
| 1 | 50 | 40 | 10 | 0 |
| 2 | 30 | 50 | 20 | 0 |
| 3 | 15 | 50 | 35 | 0 |
| 4 | 0 | 50 | 50 | 0 |
| 5 | 0 | 0 | 75 | 25 |

3. Transfer specimens to saturated solution of erythrosin B made up in pure TBA and leave overnight on top of oven at 70-80 °C.
4. Change to pure TBA and leave overnight on top of oven.
5. Add wax chips to specimens in TBA. Put specimens in vials inside oven at 70-80 °C and let TBA evaporate. Leave for 4-5 hours.
6. Remove wax and replace with fresh molten wax three times. Leave for 3-4 hours per wax change.
7. Pour specimens and molten wax into prepared mould on hot plate. Orientate specimens with forceps. Allow wax blocks to cool and immerce in cold water.

8. Section specimens at 8-10 μm with microtome.

9. Float section on warm water to allow expansion and place on slide smeared with Haupt's adhesive (see below). Add a few drops of 3% formalin to allow further expansion.

Haupt's Adhesive: Disolve 1g gelatin in 100 ml distilled water at 60 °C. Add 2g phenol crystals and 15 ml of glycerol. Stir and filter.

10. Immerse slide in xylene baths (5 and 3 minutes each) and then in xylene: ethanol (50:50) bath for 3 minutes.

11. Rehydrate sections in ethanol series (100%, 95%, 70%, 50%, 30%) for 2 minute each and then distilled water (1 minute).

12. Stain in filtered toluidine blue (0.05 g in 100 ml distilled water) for 3 minutes.

13. Dehydrate sections-pass quickly through distilled water and ethanol series, xylene and ethanol (50:50), two washes of xylene.

14. Attach cover slip to specimens on slide with canada balsam or D.P.X. mountant.