Chapter 16

Clonal Propagation

16.1. INTRODUCTION

Since most fruit trees and ornamental plants are highly heterozygous their seed progeny is not true-to-type. Asexual reproduction, on the other hand, gives rise to plants which are genetically identical to the parent plant and, thus, permits the perpetuation of the unique characters of the cultivars. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation and a plant population derived from a single individual by asexual reproduction constitutes a clone. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative reproduction (regeneration of new plants from vegetative parts).

Apomixis being restricted to only a few species, horticulturists have adopted the methods of vegetative reproduction for clonally multiplying selected cultivars. For crop plants, such as some cultivars of banana, grape, fig, double petunias and chrysanthemums, which produce little or no viable seeds, vegetative multiplication is the only method for propagation. Other advantages of vegetetative propagation over sexual reproduction are: (a) in plants with long seed dormancy, vegetative propagation may be faster than seed propagation, and (b) the undesirable juvenile phase associated with seed-raised plants in some cultivars does not appear in the vegetatively propagated plants from adult material.

Tissue culture has become a popular method for vegetative propagation of plants. The most significant advantage offered by this aseptic method of clonal propagation, popularly called 'micropropagation', over the conventional methods is that in a relatively short time and space a large number of plants can be produced starting from a single individual (see also Section 16.5). For orchids, micropropagation is the only commercially viable method of clonal propagation.

General interest in the use of tissue culture for clonal propagation of plants originates from the initial success with orchids, the credit for which goes to G. Morel. Because of the historic interest, and the fact that the pattern of in vitro growth and multiplication of orchids is fairly distinct from other plants, the micropropagation of orchids is discussed before dealing with the general techniques of in vitro clonal propagation.

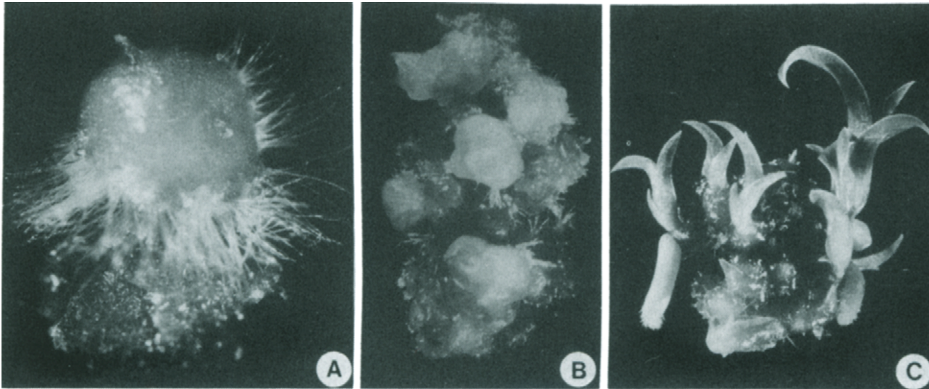

Fig. 16.1. Micropropagation of orchids. (A,C) *Cymbidium*; (B) *Cattleya*. (A) Magnified view of a protocorm with rhizoids derived from an excised shoot tip. (B) Group of protocorms developed in culture from a single shoot tip. (C) Differentiation of leafy shoots from in vitro-formed protocorms (courtesy of J. Margara, France).

16.2. ORCHID PROPAGATION

Cultivated orchids are very complex hybrids. Some of them combine the genomes of three or four different genera. *Sophrolaeliocattleya*, for example, is a triple hybrid in which *Sophronitis*, *Laelia* and *Cattleya* are involved (Morel, 1972). The only in vivo method to clonally multiply these plants is what is called 'back-bulb propagation', which involves separating the oldest pseudobulbil to force the development of dormant buds. This is an extremely slow process allowing, at best, doubling the plant number every year. Moreover, the back-bulk splitting method for clonal multiplication is applicable to only sympodial orchids; monopodial orchids do not form lateral bulbs. Traditionally, therefore, the orchid growers preferred seed propagation despite a high degree of heterogeneity in the progeny, leading to considerable economic losses. The best plants were crossed and the seedlings painstakingly raised at great expense in heated glasshouses (Morel, 1972). It takes almost 3–5 years for a plant raised from seed to reach the flowering stage.

In 1960, Morel described an in vitro method for clonally multiplying orchids at a fantastic rate. Cultured excised shoot tips of *Cymbidium*, instead of developing into a leafy shoot, formed a spherule-like body with rhizoids at the base (Fig. 16.1A). These structures, hereafter called protocorms, were morphologically similar to the protocorms formed by the embryo during seed germination. In shoot-tip cultures the protocorms originated from epidermal or sub-epidermal cells of the leaf (Champagnat et al., 1966). Some of these protocorms proliferated and formed a cluster of

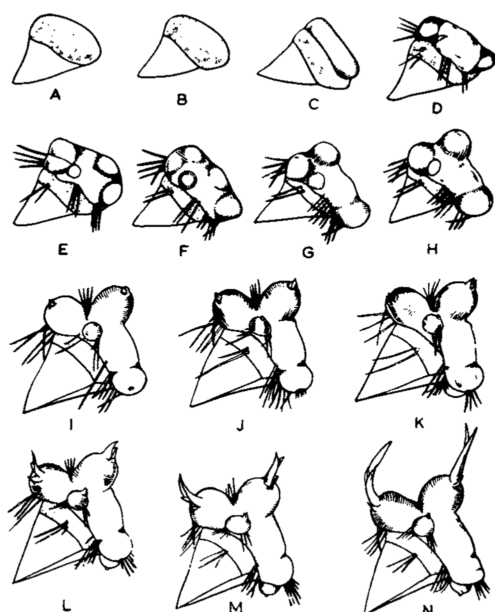

Fig. 16.2. Diagram showing the regeneration of several protocorms (B–H) followed by multiple shoot formation (I–N) from a fragment of protocorm (A) of *Cymbidium* (after Morel, 1972).

up to a dozen protocorms. Morel cut the protocorm mass into three to six pieces and planted them on a fresh Knudson C medium. Each piece formed additional protocorms (Figs. 16.1B, 16.2) by the activity of the superficial cells; the internal tissue of a protocorm lacks the regeneration potentiality. A single piece produced up to 12 protocorms in less than a month. This process of protocorm multiplication could be repeated indefinitely by regularly chopping the protocorm mass and planting the pieces on fresh medium. If chopping was stopped each protocorm developed into a full plantlet on the original medium (Figs. 16.1C, 16.2). From each shoot tip 3–5 protocorms were formed in 2 months. After this each protocorm could be cut into four to six pieces, each one of which gave rise to three to five protocorms in a month. At this rate of multiplication several million plants can be produced in a year starting from a single shoot tip measuring less than 1 mm (Morel, 1960, 1972).

The technique developed by Morel was rapidly adopted by orchid growers because of its tremendous practical applications. Today, tissue culture is the most popular method for clonal propagation of orchids. Micropropagation techniques are being applied on a commercial scale to sympodial as well as monopodial orchids (Table 16.1). Most of the economically important orchids are presently clonable in vitro (Murashige, 1978).

TABLE 16.1

List of orchids which have been clonally propagated in vitro

Plant	Explant	Reference[a]
Anacamptis pyramidalis	Shoot tip	Morel (1970)
Aranda	Shoot tip, axillary bud	Goh (1973)
	Inflorescence segment	Goh and Wong (1990)
(*Arachnis hookeriana* × *Vanda lamellata*)	Shoot tip	Cheah and Sagawa (1978)[a]
Aranthera	Shoot tip	Cheah and Sagawa (1978)[a]
Arundina bombusifolia	Shoot tip (from young seedling)	Mitra (1971)
Ascofinetia	Inflorescence segment (with flower primordia)	Intuwong and Sagawa (1973)[a]
Brassocattleya	Axillary bud	Kako (1973)
Calanthe	Shoot tip	Bertsch (1967)
Cattleya	Shoot tip	Lindemann et al. (1970)
	Axillary bud	Morel (1970)
	Lateral bud	Scully (1967)
	Leaf base	Champagnat et al. (1970)
	Leaf tip	Arditti et al. (1971, 1972), Ball et al. (1971, 1972, 1973)
Cymbidium	Shoot tip	Morel (1960, 1963, 1964a,b, 1970), Wimber (1963), Champagnat et al. (1966, 1968), Sagawa et al. (1966), Fonnesbech (1972)
Dendrobium	Shoot tip	Sagawa et al. (1966), Sagawa and Shoji (1967), Kim et al. (1970)
	Nodal segment	Arditti et al. (1973), Mosich et al. (1973, 1974)
	Flower stalk segment (with vegetative buds)	Singh and Sagawa (1972)
Epidendrum	Leaf tip	Churchill et al. (1970, 1972, 1973), Arditti et al. (1971, 1972)
Laelia	Axillary bud	Arditti (1977)
Laeliocattleya	Axillary bud	Arditti (1977)
Lycaste	Shoot tip	Arditti (1977)
Miltonia	Shoot tip	Arditti (1977)
Neostylis	Inflorescence segment (with flower primordia)	Arditti (1977)
Neottia nidus-avis	Root	Champagnat (1971)

Odontioda	Shoot tip	Arditti (1977)
Odontoglossum	Shoot tip	Arditti (1977)
Odontonia	Shoot tip	Arditti (1977)
Oncidium	Shoot tip	Bertsch (1967)
Oncidium papilio	Flower-stalk segment (with dormant apical buds)	Fast (1973)
Phajus	Shoot tip	Arditti (1977)
Phalaenopsis	Flower-stalk segment	Rotor (1949), Intuwong et al. (1972), Pieper and Zimmer (1976), Tanaka et al. (1988)[a]
	Shoot tip	Intuwong and Sagawa (1974)
	Leaf segment, stem segment, root segment	Pieper and Zimmer (1976)[a]
Pleione	Shoot tip	Weatherhead and Harberd (1980)[a]
Rhynchostylis gigantea	Shoot tip, lateral buds	Vajrabhaya and Vajrabhaya (1970)
R. retusa	Leaf segment	Vij et al. (1984)
Schomburgkia superbiens	Lateral bud	Arditti (1977)
Vanda (Terete-leaf)	Shoot tip	Kunisaki et al. (1972)
	Stem section	Sagawa and Sehgal (1967)
Vanda (Strap-leaf)	Shoot tip	Teo et al. (1973)
Vanda hybrid (*V. teres* × *V. hookeriana*)	Shoot tip, axillary bud, root segment	Goh (1970)
Vanilla planifolia	Root tips	Philips and Nainar (1986)[a]
Vascostylis	Inflorescence segment (with flower primordia)	Arditti (1977)
Vuylstekeara	Shoot tip	Arditti (1977)

[a]For references not marked thus, refer to Arditti (1977), which is the main source of information included in the table.

Cultures of *Cymbidium* and other sympodial orchids are commonly initiated from shoot tips excised from terminal or axillary buds. The size of the shoot-tip explant determines whether it would form protocorms or continue organized growth. Generally, shoot tips smaller than 1.5 cm produce a protocorm whereas larger explants develop into a plant (Kunisaki et al., 1972; Intuwong and Sagawa, 1973). However, a smaller shoot-tip piece may take 2–4 months to form the first protocorm. This should not frustrate the worker because further proliferation of protocorms is comparatively rapid (Morel, 1965b; Kunisaki et al., 1972). The commercial growers use 5–10 mm long shoot apices to initiate cultures (Murashige, 1974).

For monopodial orchids (e.g. *Vanda*, *Phalaenopsis*), where excising the terminal bud means sacrificing the selected plant, pieces of floral stalk with vegetative buds may be used to start the cultures (Intuwong and Sagawa, 1973). Such aerial explants should be good even for sympodial orchids because: (a) the procedure is relatively easy, (b) contamination of cultures is low, and (c) the percentage of cultures forming protocorms is high.

Compositions of media used for clonal propagation of some orchids are given in Appendices 16.II.1–16.II.4. For *Vanda*, liquid medium is reported to be better than semi-solid medium (Kunisaki et al., 1972). In liquid cultures shaking the medium facilitates the separation of newly developed protocorms, thus saving the manual chopping of the protocorm clumps at each subculture.

16.3. GENERAL TECHNIQUES OF MICROPROPAGATION

Micropropagation generally involves four stages: Stage 1, initiation of aseptic cultures; Stage 2, multiplication; Stage 3, rooting of in vitro formed shoots, and Stage 4, transfer of plants to greenhouse or field conditions (transplantation). Debergh and Maene (1981) introduced the Stage 0, making micropropagation a five stage process. Each stage has its special requirements.

16.3.1. Stage 0: preparative stage

This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in Stage 1. To reduce the contamination problem in Stage 1 the mother plants should be grown in a glasshouse and watered so as to avoid overhead irrigation. It would not only help minimize the incidence of infection in Stage 1 but may also reduce the need for a harsh sterilization treatment. Before the introduction of Stage 0 *Cordyline* cultures could be initiated only from apical bud; all the nodal segments got infected or did not respond favourably. With the introduction of Stage 0 both apical and all nodal segments could be used as primary explants (Debergh and Read, 1991). Senawi (1985) succeeded in obtaining responding cultures of *Theobroma cacao* only from the stock plants grown under glasshouse conditions.

Stage 0 also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. Red light-treated plants of *Petunia* provided leaf explants

which produced up to three times as many shoots as did the explants from untreated plants (Read et al., 1978). In woody and bulbous plants suitable temperature treatments should help in breaking bud dormancy and provide more responsive explants.

16.3.2. Stage 1: initiation of cultures

(i) *Explant.* The nature of the explant to be used for in vitro propagation is, to a certain extent, governed by the method of shoot multiplication to be adopted (see Section 16.3.3). For enhanced axillary branching only such explants are suitable which carry a pre-formed vegetative bud. When the objective is to produce virus-free plants from an infected individual it becomes obligatory to start with sub-millimeter shoot tips (Chapter 15). However, if the stock plant is virus-tested or, alternatively, virus eradication is not desired, the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem-tip culture may also result in the loss of certain horticultural characters which are controlled by the presence of virus, such as the clear-vein character of the geranium cv. Crocodile (Cassells et al., 1980; see Fig. 16.3).

Generally, sub-terminal and older segments withstand the toxic effects of sterilizing agents much better than the terminal cuttings. For clonal propagation of cauliflower, Crisp and Walkey (1974) used pieces of curd which provide numerous meristems capable of reverting back to vegetative buds in cultures and forming leafy shoots. For rhizomatic plants, such as strawberry and Boston fern, runner tips have been commonly used.

The physiological state of the parent plant at the time of explant excision has a definite influence on the response of the buds. Explants from actively growing shoots at the beginning of the growing season generally give best results (Seabrook et al., 1976; Yang, 1977; Anderson, 1980). The seasonal fluctuations in the response of shoot buds may be minimized by maintaining the parent plants under light and temperature conditions required for continual vegetative growth, in glasshouse or growth cabinets. Bulbs, corms, tubers and other organs should be subjected to the temperature and/or photoperiodic treatments required to break dormancy before excising the bud.

For multiplication through adventitious bud formation, with or without callusing, explants are derived from root, stem, leaf or nucellus based on their natural capacity to form adventitious buds. In monocots the meristems of leaves and scales are located at the proximal end where they are joined with the basal plate. Leaf-base and scale-base explants

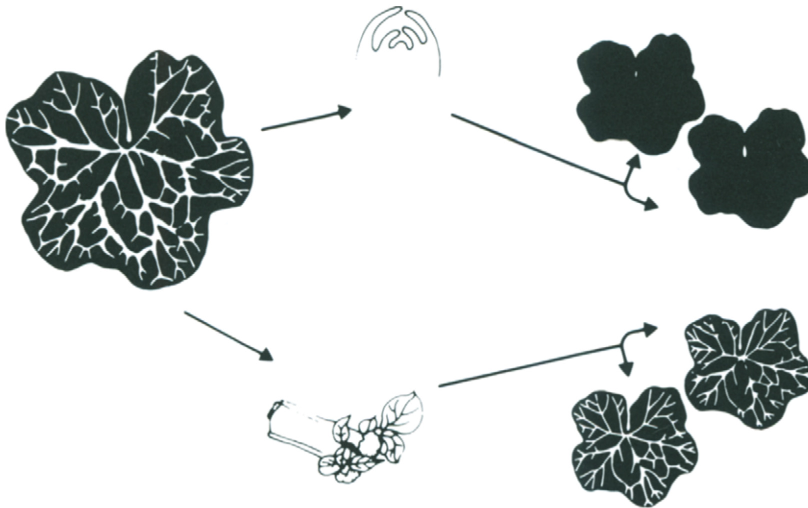

Fig. 16.3. Shoot-tip culture (top) and petiole culture (bottom) of the geranium cv. Crocodile, in which clear vein character has been attributed to infection by a virus or virus-like agent. Note that the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture (after Cassells et al., 1980).

should include a small piece of basal plate (Hussey, 1980). Embryogenic cultures of woody and other species are readily established from immature zygotic embryos. Nucellar explants of citrus and mango are highly embryogenic.

(ii) *Sterilization.* Standard methods of aseptic culture of plant tissues and organs are described in Sections 2.3.2 and 15.4.2. Special precautions need to be taken when explants are derived from field-grown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in a glasshouse. For species hard-to-root from cuttings, the growing branches on the tree may be loosely covered in a large polythene bag and the newly developed shoots, protected from wind-borne contaminants, taken to start cultures. Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination. Chances of contamination are much higher in the cultures of terminal cuttings and whole buds than that in the cultures of 0.5–1 mm shoot tips excised after removing several layers of older leaves. Shoot tips with sufficient covering of mature leaves, or

scales from the centre of the bulbs, may be dissected out sterile by wiping the buds or bulbs, respectively, with 70% ethanol and gently peeling off the outer covering (Hussey, 1980).

(iii) *Browning of medium.* A serious problem with the culture of some plant species is the oxidation of phenolic substances leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is especially common with the adult tissues from woody species (see Section 16.6.4).

16.3.3. Stage 2: multiplication

This is the most crucial stage, and is the point at which most of the failures in micropropagation occur. Broadly, three approaches have been followed to achieve in vitro multiplication (Fig. 16.4).

(i) *Through callusing*. The potentiality of plant cells to multiply indefinitely in cultures, and their totipotent nature permit a very rapid multiplication of several plant types. Differentiation of plants from cultured cells may occur via shoot-root formation (Chapter 5) or somatic embryogenesis (Chapter 6). Where applicable, this is often the fastest method of multiplication. However, there are several drawbacks in this method and, as far as possible, it should be avoided in clonal propagation of a cultivar. The most serious objection against the use of callus cultures for shoot multiplication is the genetic instability of their cells (see Chapter 9).

Between organogenesis and somatic embryogenesis the latter approach is more appealing from a commercial angle. A somatic embryogenic system once established lends itself to better control than organogenesis. Since somatic embryos are bipolar structures, with defined root and shoot meristems (see Chapter 6), the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds (see Section 6.9; Fig. 16.5), offering cost advantages from labour savings, which accounts for 60–70% of direct costs in micropropagation in developed countries (Onishi et al., 1994; Redenbaugh, 1993; Sakamoto et al., 1995). Unlike microcuttings, somatic embryos can also be stored through cold storage, cryopreservation or desiccation for prolonged periods (see Chapter 18), thus providing flexibility in scheduling production and transplantation. These characteristics make somatic embryogenesis potentially a less

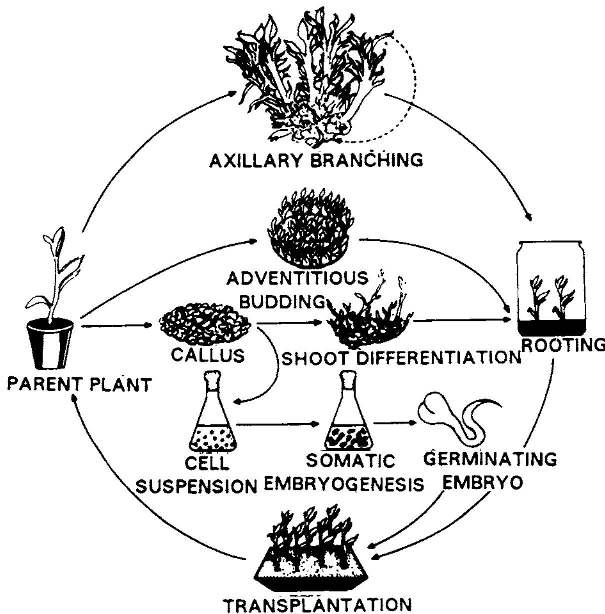

Fig. 16.4. Diagrammatic summary of steps involved in aseptic multiplication of plants. For details see text (after Dhawan and Bhojwani, 1986).

expensive and flexible system for micropropagation (Cervelli and Senaratna, 1995). For some plants, such as oil palm, somatic embryogenesis appears to be the only possible pathway to clonal propagation (Duval et al., 1995).

However, despite these advantages, somatic embryogenesis is not being used for commercial production of plants (Cervelli and Senaratna, 1995). Some of the problems currently limiting commercialization of this remarkable process are: (1) synchronization of embryogenesis is inadequate; (2) field conversion frequencies of embryos or artificial seeds are still very low (15–25%); and (3) it generally involves a callus phase. When these problems are overcome somatic embryogenesis is the method of choice for micropropagation.

(ii) *Adventitious bud formation.* Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. Strictly speaking, the shoots differentiated from calli should also be treated as adventitious buds. In this chapter, however, the use of the term adventitious buds is restricted to only those buds that arise directly from a plant organ or a piece thereof without an intervening callus phase.

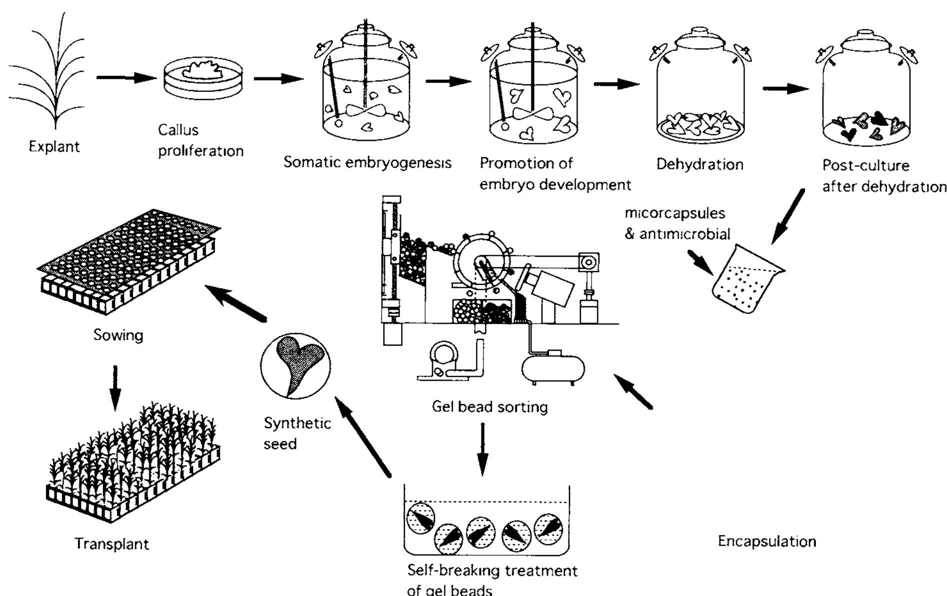

Fig. 16.5. Schematic representation of carrot synthetic seed production system. Leaf callus was initiated on gelled SH medium containing 1.0 μM 2,4-D, 0.5 μM kinetin, 3% sucrose and 0.2% gelrite and multiplied for 3 months by monthly subculture. Suspension cultures derived from this callus were transferred to liquid SH medium containing 1% sucrose and 8% mannitol, in a bioreactor, to induce embryogenesis. The somatic embryos which passed through 0.84 mm mesh were transferred to a medium containing SH basic salts, 1% sucrose and 10% mannitol for promotion of embryo development. After 7 days the embryos were put on filter papers for dehydration at 20°C, under 16 h photoperiod and 14 $\mu E\ m^{-2}\ s^{-1}$ irradiation, for 7 days. The embryos were again cultured for 14 days in a medium containing SH salts, 2% sorbitol, and 0.01 mg l^{-1} each of BAP and GA_3, in air enriched with 2% CO_2, under a 16 h photoperiod, at 20°C, so that the embryos become autotrophic. The encapsulatable embryos so obtained were suspended in 1.5% (w/v) alginate solution containing 3% sucrose microcapsules and the fungicide Topsin M (0.1%) and fed to an encapsulation machine. After the capsules had been hardened in 100 mM $CaCl_2$ solution for 10 min they were washed and the beads with green embryos were sorted out with the help of a gel-bead sorting machine. A self-breaking feature was added to the capsules (see Section 6.9.2). The synthetic seeds thus produced were sown on synthetic substrate, in trays, and placed under humid conditions to allow self-breaking of the gel beads (courtesy of T. Hirosawa, Kirin Brewery, Japan).

In many crop plants vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (*Begonia*, *Crassula*, *Peperomia*, *Saintpaulia*) cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. In *Begonia*, for example, buds normally originate only along the cut ends but in a medium containing BAP

the bud formation is so profuse that the entire surface of a cutting becomes covered with shoot buds (Reuther and Bhandari, 1981). Takayama and Misawa (1982) described an in vitro method for mass propagation of *Begonia* × *hiemalis* which allowed the production of over 10^{14} plants in a year from a single 7 × 7 mm segment of a young leaf. A liquid shake culture method for rapid multiplication of this species by adventitious bud formation has been described by Simmonds and Werry (1987). *Pinguicula moranensis* is also normally propagated from leaf pieces. By the conventional technique only one plant is produced by each leaf but in cultures it increases 15-fold (Adams et al., 1979a). In cultures, explants as small as 20–50 mg in weight, which fail to survive in nature, are able to produce adventitious buds (Hussey, 1978). Under the influence of an appropriate combination of growth regulators, in cultures, adventitious buds can be induced on the leaf and stem cuttings of even those species which are normally not propagated vegetatively (Kartha et al., 1974b; Roest and Bokelmann, 1975; Behki and Lesley, 1976; Murray et al., 1977).

Barlass and Skene (1978, 1980a,b) described a propagation method for grapes in which excised shoot tips measuring 1 mm in length (with two to three leaf primordia), were cut into nearly 20 pieces. In cultures each shoot-tip fragment formed multiple shoots adventitiously from the swollen leaf bases (Barlass and Skene, 1980a). About 8000 plants could be produced from a shoot apex in 3–4 months. All the plants multiplied by this method were normal diploid. The technique of shoot-fragment culture has also been used for the micropropagation of *Asclepias rotundifolia* (Tideman and Hawker, 1982).

A remarkable capacity to form adventitious buds in vitro is displayed by certain ferns. In a simplified method of in vitro multiplication of *Davallia* and *Platycerium*, the tissue pieces obtained by aseptically homogenizing the plants in a blender produced numerous new plants (Cooke, 1979).

For most bulbous plants adventitious bud formation is the most important mode of multiplication, and the best explants are obtained from bulb scales. Micropropagation techniques are available for many bulbous species but large scale micropropagation programmes are functional only for lilies (Capellades et al., 1991). Propagation by adventitious bulblet formation directly from aseptically cultured bulb-scale segments (see Fig. 16.6) was described by Hackett (1969) and Gupta et al. (1978). In the cultures of whole scales about 10 bulblets were formed within 15 days. The number of bulblets per bulb-scale could be increased considerably by culturing small pieces of the scales. Gupta et al. (1978) reported that within 30–45 days each of the six segments taken from the basal half of a bulb-

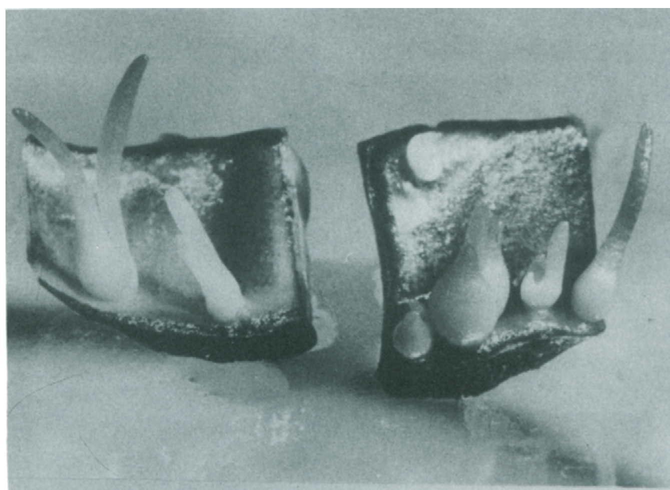

Fig. 16.6. Adventitious bulblet formation on lily bulb scales in culture (courtesy of D. Cohen, New Zealand).

scale produced, on average, 18 bulblets. Thus, almost 100 bulblets could be produced from a single scale. Maximum regeneration capacity is shown by the outer scales, and it decreases progressively among the inner scales of the bulb (Hackett, 1969). During the last decade several groups have described a fermentor technique for mass multiplication of lilies (Levin et al., 1988; Takayama et al., 1991; Takahashi et al., 1992a,b). Mitsui Petrochemical, Japan is producing virus-free bulblets of *Lilium longiflorum* in 2-l stirred drum tanks (Takahashi et al., 1992a).

Adventitious shoot formation directly from the explants is definitely a better approach than the callus method for clonal propagation of plant species. Often, where calli have produced cytologically abnormal plants, adventitious shoots have formed uniformly diploid individuals (Hussey, 1976; Krul and Myerson, 1980). This should not imply that this method always produces true-to-type plants. Westerhof et al. (1984) observed 18 types of variations after three cycles of in vitro propagation of *Begonia* × *hiemalis*. Somaclonal variants have been detected in plants regenerated directly from the explants of potato (Cassells et al., 1983) and *Brassica juncea* (Bhojwani and Arora, 1992).

A serious problem may arise when this method of propagation is applied to varieties which are genetic chimeras. Adventitious bud formation involves the risk of splitting the chimeras leading to pure type plants (Bush et al., 1976; Skirvin, 1978; Cassells et al., 1980; Johnson, 1980).

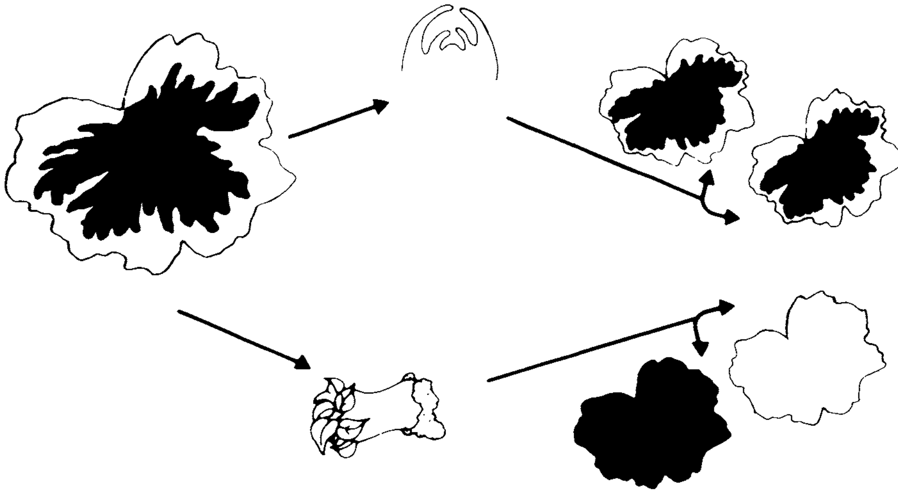

Fig. 16.7. Shoot-tip culture (top) and petiole-segment culture (bottom) of the variegated geranium cv. Mme Salleron. The chimera is perpetuated in meristem culture but broken down in petiole culture (after Cassells et al., 1980).

Some plants derived from adventitious buds induced on internodal regions of disbudded trees of apple differed from the parental clones in growth habits, fruiting characteristics and fruit pigmentation. This was ascribed to the complex chimeral nature of some of the apple clones (Dayton, 1969, 1970). Mme Salleron cultivar of geranium, with variegated leaves, is a genetic chimera. Plants derived from adventitious buds formed directly on petiole segments, without a callusing phase, never showed variegation; the plants were either green or albino. In contrast, all the plants raised from shoot-tip cultures showed the typical variegation (see Fig. 16.7) (Cassells et al., 1980). Plantlets produced in vivo or in vitro from leaf cuttings of 'Rober's Leman Rose' cultivar of geranium were of four or five different types (Skirvin and Janick, 1976b; Janick et al., 1977).

(iii) *Enhanced axillary branching*. Axillary buds are usually present in the axil of each leaf, and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods depending on the growth pattern of the plant. In species with a strong apical dominance the removal or injury of the terminal bud is necessary to stimulate the next axillary bud to grow out into a shoot. This phenomenon of apical dominance is controlled by the interplay of growth regulators. Application of a cytokinin to the axillary buds can overcome the api-

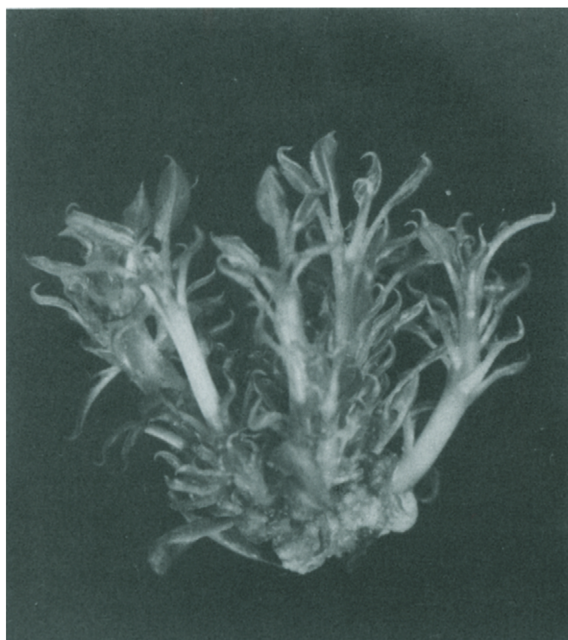

Fig. 16.8. Cluster of shoots developed from a nodal segment of *Pyrus serotina* (after Bhojwani et al., 1984b).

cal dominance effect and stimulate the lateral buds to grow in the presence of the terminal bud, but this effect is only temporary. The lateral shoot stops growing as the effect of the exogenous growth regulator diminishes (Sachs and Thimann, 1964).

The conventional method of vegetative propagation by stem cuttings utilizes the ability of axillary buds to take over the function of the main shoot in the absence of a terminal bud. The number of cuttings that can be taken from a selected plant in a year is extremely limited because, in nature, the vegetative growth is periodic, and a minimal size of the cutting (24–30 cm) is necessary in order to establish a plant from it.

In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration, with or without an auxin. Due to the continuous availability of cytokinin, the shoots formed by the bud, a priori present on the explant (nodal segment or shoot-tip cutting), develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches (Fig. 16.8). There is a limit to

which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, however, if shoots are excised and planted on a fresh medium of the same composition the shoot multiplication cycle can be repeated. This process can go on indefinitely, and can be maintained throughout the year.

In some plants, such as leguminous tree species (Dhawan and Bhojwani, 1985), *Feijoa* (Bhojwani et al., 1987), Japanese persimon (Fukui et al., 1989) and potato (McCown and Joyce, 1991), it may not be possible to break apical dominance by manipulating the hormonal composition of the medium, and the bud present a priori on the initial explant grows into an unbranched shoot. The rate of shoot multiplication in such cases would depend on the number of nodal cuttings that can be excised from the shoot at the end of each passage. Even with this alternative method of enhanced axillary branching, 3–4-fold shoot multiplication every 4–6 weeks could be achieved in the plants with strong apical dominance.

The enhanced axillary branching method of shoot multiplication may be initially slower than the other methods but with each passage the number of shoots increases logarithmically and within a year astronomical figures can be achieved. This method is currently the most popular approach to clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes under culture conditions. Chimeras, whose breakdown is common during adventitious bud development, are perpetuated in shoot-bud culture (Cassells et al., 1980; see Fig. 16.7). Moreover, the adventitious bud formation and callusing methods require de novo differentiation of shoot-buds which is not always possible. However, the plants multiplied by forced axillary branching may show some epigenetic changes. Excessive dose or wrong choice of cytokinin can be responsible for such changes. Bushiness, observed in micropropagated plants of *Gerbera jamesonii*, characterized by excessive leaves, limited number of flowers and short peduncle, is probably a consequence of the use of BAP (Debergh and Read, 1991). Unlimited subcultures in *Fragaria* result in disorders such as absence or weak rhizogenesis, excessive flowering, smaller and deformed fruits, and heterogeneous plants (Rancillac et al., 1987). Some of these abnormalities could also be due to adventitious bud formation which often occurs in older cultures on axillary shoot proliferation medium.

16.3.4. Stage 3: rooting of shoots

Somatic embryos carry a pre-formed radicle and may develop directly into plantlet. However, these embryos often show very poor conversion

into plantlets, especially under ex vitro conditions. They require an additional step of maturation to acquire the capability for normal germination (see Section 6.5).

Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt composition. The number of shoot multiplication cycles after which the rooting exercise is to be started is governed by the number of plants to be produced through micropropagation and the available nursery facilities.

For rooting, individual shoots measuring about 2 cm in length are excised and transferred to the rooting medium. However, it would not only be convenient but also economical if shoot clusters formed after a shoot multiplication cycle, can be handled as units during the rooting and transplantation stages, and individual plants separated at a later stage, as in garlic (Bhojwani, 1980b; Bhojwani et al., 1982).

It is common practice to root shoots in vitro but many commercial laboratories prefer to treat the in vitro multiplied shoots as microcuttings and root them in vivo (ex vitro), if possible. In vivo rooting offers many advantages (Debergh and Read, 1991; Preece and Sutter, 1991): (1) in vitro rooting is labour intensive and expensive, accounting for 35–75% of the total cost of micropropagation (Debergh and Maene, 1981). In vivo rooting combines the rooting and acclimatization stages and, thus, reduces aseptic handling. (2) In vivo formed roots are structurally and functionally of better quality than those developed in vitro. The in vitro formed roots are often thick and lack root hairs and good vasculature. These roots frequently die or collapse after the plantlets are removed from cultures and new, functional lateral and adventitious roots are formed during acclimatization. Under in vivo conditions, callusing at the base of the shoot occurs only rarely ensuring a continuous vascular connection between root and shoot. (3) The in vitro developed roots may get damaged during transplantation. (4) For difficult-to-root species it is easier and cheaper to create good rooting conditions in vivo than in vitro.

16.3.5. Stage 4: transplantation

The ultimate success of commercial micropropagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied in vitro are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients and growth regulators, sucrose as carbon source, high humidity,

low light and poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under in vivo conditions. The two main deficiencies of in vitro grown plants are: (i) poor control of water loss, and (ii) heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. During acclimatization the in vitro formed leaves do not recover but the plant develops normal leaves and functional roots. The major deficiencies of in vitro grown plants and the methods used or recommended for their transfer out of culture are briefly described in this section.

(i) *Deficiencies.* Scant deposition of protective epicuticular wax on the leaves developed under highly humid in vitro environment is regarded as one of the most important factors responsible for excessive loss of water by cuticular transpiration leading to poor transplantation survival (Grout and Aston, 1977; Sutter and Langhans, 1979, 1982; Fuchigami et al., 1981; Brainerd and Fuchigami, 1981, 1982; Wetzstein and Sommer, 1982; Dhawan and Bhojwani, 1987). Even the chemical nature of the wax deposited under in vitro conditions differs from that formed under natural conditions. The former has a higher proportion of polar compounds which are less hydrophobic and, therefore, allow excessive diffusion of water from in vitro formed leaves (Sutter, 1984).

Poor anatomical differentiation of leaves, greater stomatal frequency (Wetzstein and Sommer, 1982), and impaired stomatal structure and movement (Brainerd and Fuchigami, 1982; Wetzstein and Sommer, 1983; Ziv et al., 1987a,b) have also been implicated in the water imbalance exhibited by micropropagated plants after removal from culture. The stomata on in vitro developed leaves remain wide open and do not respond to the stimuli, such as dark, ABA and CO_2, that normally induce their closure (Brainerd and Fuchigami, 1982; Wardle and Short, 1983; Conner and Conner, 1984; Ziv et al., 1987a,b). Abnormally large stomata were observed in cultured plants of geranium, rose (Reuther, 1988), apple (Blanke and Belcher, 1989) and carnation (Ziv et al., 1983, 1987a,b). Structural changes in the guard cells are accompanied by lower levels of cellulose, pectins and cutin and elevated levels of callose (Ariel, 1987; Werker and Leshem, 1987; Marin et al., 1988; Ziv and Ariel, 1992, 1994).

In most of the species investigated, the in vitro developed leaves show poor mesophyll differentiation and weak vasculature. The palisade tissue is lacking or is poorly developed (Grout and Aston, 1978; Wetzstein and Sommer, 1982; Dhawan and Bhojwani, 1987), and the mesophyll tis-

sue is mainly composed of spongy parenchyma with large intercellular spaces. The chloroplasts are poorly developed, with low chlorophyll and protein contents and disorganized grana (Wetzstein and Sommer, 1983; Ziv et al., 1983; Lee et al., 1985; Capellades et al., 1990a; Ziv and Ariel, 1992).

The stem of in vitro developed plants is hypolignified. Cells are thin, and there are large intercellular spaces with a limited development of vascular tissue and considerably less collenchyma and sclerenchyma (Donnelly et al., 1985; Ziv, 1995). In carnation, the stem vascular bundles lacked normal organization (Werker and Leshem, 1987) and in cauliflower the root-stem vascular connection was incomplete (Grout and Aston, 1977a).

The low light, high humidity, lack of adequate CO_2 and fair amount of sucrose in the medium force the in vitro growing plants into heterotrophic mode of nutrition. Consequently, these plants are poor in chlorophyll content, and the enzymes responsible for photosynthesis are inactive or absent altogether (Grout and Aston, 1977a; Wetzstein and Sommer, 1982; Donnelly and Vidaver, 1984).

Thus, peculiar leaf anatomy, incomplete development of chloroplasts (Wetzstein and Sommer, 1982) and low net CO_2 uptake and fixation are some of the characteristics of in vitro raised plants.

(ii) *Acclimatization. Ex vitro:* Traditionally, transplantation follows the in vitro rooting stage but in vivo rooting is gaining popularity for obvious reasons of economy and the quality of roots (Section 16.3.4). In the latter case the rooting and acclimatization stages are combined.

While transferring out shoots/plants from culture, their lower part is gently washed to remove the medium sticking to them. The individual shoots or plantlets are then transferred to potting mix and irrigated with low concentration of inorganic nutrients (Knop's or 1/4 strength MS salt solution). A variety of potting mixes, such as peat, perlite, polystyrene beads, vermiculite, fine bark, coarse sand, etc. or their mixtures in different proportions are used for transplantation. For the initial 10–15 days, it is essential to maintain high humidity (90–100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to the ambient level over a period of 2–4 weeks. Thus, in the first phase of acclimatization the main environmental stress to the plants is the change from a substrate rich in organic nutrients to one providing only inorganic nutrients. This probably recommissions the photosynthetic machinery of the plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions.

Several methods have been used to build up high humidity around the plants during the early phase of transplantation. The most primitive approach has been to cover the plants with clean, transparent plastic bags and make small holes in them for air circulation. To reduce humidity inside the plastic bag the holes are gradually enlarged over a period of time. Another simple method to acclimatize in vitro raised plants is to gradually remove the cap of the culture vial over a period of 7–10 days (Ripley and Preece, 1986; Ziv, 1986); slightly loosen the cap for the initial 1 or 2 days, completely unscrew the cap but leave it on the vessel for the next 3–4 days, and leave the plants in the uncapped jar for about 7 days for the plants to get gradually acclimatized to low humidity. The plants are then transplanted in a suitable potting mix after removing the agar and irrigated with a suitable nutrient solution.

Acclimatization of micropropagated plants on a large scale is generally carried out in a polyhouse or glasshouse where a gradation of high-to-low humidity and low-to-high levels of irradiance are maintained. High humidity is maintained by a misting or fogging system. The latter, although expensive, is preferred because mist leaches nutrients, causes the medium to become too wet, allows the plantlets themselves to dry and creates an environment favourable for the growth of algae and some fungi and bacteria. Fogging, avoids many of these problems (see Preece and Sutter, 1991). It produces droplets less than 20 μm in diameter by forcing water through fine nozzles. This ensures that the droplets float and saturate the air rather than sink and over-wet plants and potting mix.

In tropical countries, a gradient of humidity in the hardening facility can be achieved by fixing exhaust fans at one end and cellulose or polyester pads at the opposite end. As the pads are constantly kept wet, the air sucked through them, from the outside, is fully saturated to begin with but gradually its moisture content decreases thus creating a humidity gradient from the pad end to the fan end. In summer, this system also reduces the temperature inside the glasshouse by 5–6°C, compared to the outside. The micropropagated plants are put in protrays/flats during acclimatization. Over a period of 4–6 weeks the trays are moved from a high humidity-low light zone to low humidity-high light zone. By this time the plants should be fully acclimatized and are removed to a nethouse or field for better growth. During the acclimatization phase the plants need to be fed regularly, by drenching of the potting medium, with a liquid fertilizer. A robust growth of roots during this phase would help in better survival and growth of the in vitro plants.

Some other methods recently recommended to improve the transplantation success, presumably, at lower costs are: (i) in vitro acclimatization, and (ii) in vitro formation of storage organs.

In vitro: Ex vitro acclimatization of tissue culture raised plants is labour intensive and involves high capital investment. It also restricts the market for supplying in vitro grown plants to specialized growers with the expertise and facilities to harden the micropropagated plants. A number of treatments applied during in vitro culture have been shown to improve the quality of transplants and, thus, make their transfer to greenhouse or field simple and reduce losses (Roberts et al., 1990b; Ziv., 1995). During in vitro hardening, the roots of micropropagated plants, particularly of tree species, can be infected with *Rhizobium* or other microorganisms which make symbiotic association with them, to ensure better survival and growth of the plants after transfer to field (Perinet and Lalonde, 1983; Bertrand and Lalonde, 1985; Dhawan and Bhojwani, 1987).

The high relative humidity (RH) in the headspace of the culture vial and water potential of the medium influence plant growth and development. The culture of plants at relatively low humidity reduces their wilting after transfer to soil by improving stomatal movement (Wardle et al., 1983; Short et al., 1987), and increasing the deposition of epicuticular wax on the leaves (Wardle et al., 1983; Maene and Debergh, 1986; Short et al., 1987). The methods that have been used to reduce humidity inside the culture vial include the use of desiccants (Wardle et al., 1983; Ziv et al., 1983; Short et al., 1987), use of culture vials with microporous closure to allow gaseous exchange (Short et al., 1987), and cooling the bottom of the culture vessels (Maene and Debergh, 1987). By increasing the irradiance from 20 μmol m^{-2} s^{-1} to 80 μmol m^{-2} s^{-1} and reducing the RH inside the culture vessel from 100 to 75% it was possible to induce the in vitro growing rose plants to acquire morphological and anatomical characteristics similar to those observed during ex vitro acclimatization (Capellades et al., 1990b).

Certain growth retardants have been reported to promote in vitro root and shoot growth (Chin, 1982; Khunachak et al., 1987), reduce shoot elongation and leaf area, increase chlorophyll content and improve stress resistance of in vitro grown plants by promoting epicuticular wax deposition and stomatal response and development of thick roots (Ziv, 1991a, 1992; Novello et al., 1992; Roberts et al., 1992). Addition of paclobutrazol (0.5–4 mg l^{-1}) to the rooting medium enhanced desiccation tolerance of micropropagated chrysanthemum, rose and grapevine plants (Smith et al., 1990; Roberts et al., 1992). The effect of this antigibberellin compound was further enhanced if the plants were cultured in cellulose plugs irrigated with sucrose-free nutrient medium, probably because of reduced damage to the root system during transplantation (Roberts et al., 1990b). In *Philodendron*, application of paclobutrazol or ancymidol during shoot

proliferation enhanced ex vitro survival of micropropagated plants (Ziv and Ariel, 1991).

Autotrophic micropropagation, a plant propagation method based on tissue cultures deriving all their carbon requirement from CO_2 (Kozai, 1991d), is also being explored to produce plants suitable for transfer to glasshouse or field without prolonged ex vitro hardening (Kozai, 1991a,c). Some of the advantages (realized or potential) of photoautotrophic micropropagation are: development of structurally and physiologically normal plants, simplified rooting, reduced use of growth regulators, reduced problem of microbial contamination due to lack of sucrose in the medium and, facilitation of automation, robotization and computerization of micropropagation (Kozai, 1991a,c). Fujiwara et al. (1988) and Hayashi et al. (1988b) developed a microcomputer controlled acclimatization chamber in which levels of CO_2, RH, irradiance, temperature and air flow to plants in the culture vessel could be monitored.

In several plant species an increase in plant growth and photoautotrophic behaviour could be achieved by reducing or deleting sucrose from the medium, increasing CO_2 level (to 350 ppm) around the plant and increasing irradiance (to 200 μmol m^{-1} s^{-1}). CO_2 level in vitro could be enhanced either by using gas permeable films, such as microporous polypropylene, or culture vessels of fluorocarbon polymer (Kozai, 1991c,d), which also reduces relative humidity over the plants. However, photoautotrophic micropropagation is still very much at the laboratory stage and many aspects, including its economic feasibility and practicality, have to be worked out before it can be taken up at commercial level.

In vitro formation of storage organs. The plant species which normally form storage organs, such as tubers, corms and bulbs, can be induced to form such organs under in vitro conditions for direct planting in the greenhouse or field, cutting down the tedious step of acclimatization and reducing transplantation losses. It may even eliminate the rooting step as the storage organs can be formed by unrooted shoots. Moreover, the storage organs can be stored or readily shipped and planted either manually or by machines.

In vitro storage organ formation by manipulating the sugar and/or growth regulator contents of the culture medium and light and temperature conditions has been reported in several species, including *Crocus* sp. (Plessner et al., 1990), *Dioscorea* spp. (Forsyth and van Staden, 1984), *Freesia* (Hirata et al., 1995), *Gladiolus* (Dantu and Bhojwani, 1995), lilies (Takayama et al., 1991) and potato (Dodds et al., 1992; Alchanatis et al., 1994). Bulblet formation in *Muscari armeniacum* was promoted by 1% activated charcoal (Peck and Cumming, 1986).

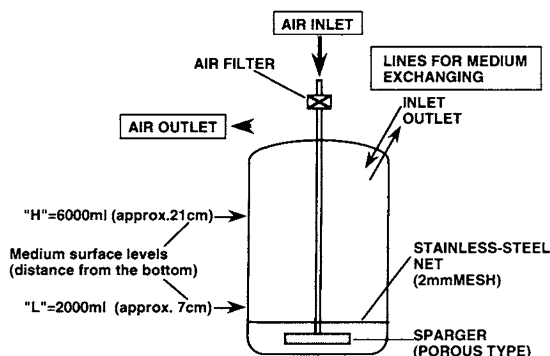

Fig. 16.9. Diagram of a jar fermentor used for mass propagation of potato tubers (after Akita and Takayama, 1993).

Most extensive work on in vitro formation of storage organs pertains to microtuber formation by nodal cuttings of potato. It is a two step process (see Appendix 16.I.1). Shoots are generally multiplied on a medium containing 3% sucrose, a cytokinin and an auxin, in light, at 25°C. Potato shoot cultures do not respond well to the classical cytokinin stimulation of axillary shoot proliferation and produce only a single multinodal shoot even in the presence of a cytokinin. Therefore, single node cuttings are used for recurrent shoot multiplication. In the second step the nodal segments are induced to form microtubers. A high concentration of sucrose (6–10%) and the presence of a cytokinin in the medium, low temperature (15–18°C) and dark are critical factors for in vitro tuberization. Addition of 500 mg l^{-1} of CCC to MS medium containing 8% sucrose and 5 mg l^{-1} BAP was shown to induce tuber formation in a broad range of genotypes (Schilde-Rentschler et al., 1984). Recently, Alchanatis et al. (1994) reported that the addition of 0.25–0.5 mg l^{-1} of ancymidol to the shoot multiplication medium (1/4 MS salts + 8% sucrose + 5 mg l^{-1} kinetin) considerably enhanced the number of nodes formed and improved the quality of shoots so that the tubers formed in the second step were in greater number and larger than those formed otherwise.

Despite the various advantages offered by microtuber formation, it has not been commercialized because the plants produced by this method are more expensive than those produced by rooted microcuttings (McCown and Joyce, 1991; Akita and Takayama, 1993). To obviate this problem attempts have been made to achieve mass production of microtubers in bioreactors. Akita and Takayama (1993) used an 8-l airlift fermentor (Fig. 16.9) and a two-step method (Fig. 16.10) to micropropagate potato via microtuber formation (Fig. 16.11). In step I, 60 single node segments from stock shoot cultures, established and multiplied on semi-solid MS (3% sucrose) medium, were transferred to the reactor containing 2 l of

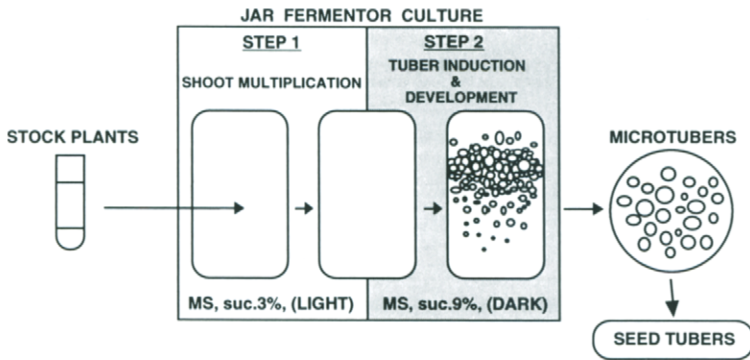

Fig. 16.10. Diagrammatic representation of the two-step culture scheme for mass propagation of potato tubers in a jar fermentor (after Akita and Takayama, 1993).

liquid MS medium with 3% sucrose. After 4 weeks of culture under continuous light (2–5 W m^{-2}), when numerous elongated shoots were formed, the culture medium was replaced by liquid MS medium containing 9% sucrose. After 5 weeks, in the dark, about 270 microtubers were formed. Small Potato Inc. (USA) has developed a cylindrical reactor that is rotated on its horizontal axis, intermittently bathing the shoots held at the outside of the reactor chamber (see McCown and Joyce, 1991). In this system the shoot multiplication is carried out under conditions which cause shoot tip necrosis resulting in the breaking down of apical dominance. As the next axillary shoot develops, it also suffers shoot tip necrosis. Thus, after some time multiple shoots are formed.

The microtubers formed in vitro are similar in colour, shape and electrophoretic pattern of storage proteins to field-produced tubers (Espinoza et al., 1986; Dodds et al., 1992), and the field grown plants derived from microtubers, in vitro produced microcuttings or field produced tubers formed similar numbers of tubers (Wang and Hu, 1982; Wattimena et al., 1983).

Micropropagated shoots of gladiolus could be induced to form corms in vitro by increasing sucrose concentration in liquid medium (Dantu and Bhojwani, 1987, 1995) or in the presence of certain growth retardants (Ziv, 1991a). The cold-treated in vitro formed corms of cvs Friendship, Gold Finch's and Her Majesty showed 100% germination in the field, and the resulting plants were morphologically and cytologically comparable to those produced by in vivo formed corms (Dantu and Bhojwani, 1995). Following in vitro formation of storage roots, in high-sucrose rooting medium, *Asparagus officinalis* plants required only 3–4 days of hardening under high humidity tents before their transfer to an open greenhouse bench (Conner et al., 1992).

Fig. 16.11. A potato plant bearing microtubers formed in the jar fermentor shown in Fig. 16.9 (after Akita and Takayama, 1993).

16.4. FACTORS AFFECTING IN VITRO STAGES OF MICROPROPAGATION

16.4.1. Culture initiation and shoot multiplication

The salt mixture of Murashige and Skoog's (1962) medium has proved satisfactory for many crop plants. It is often possible to use the same

medium for culture initiation and shoot multiplication. A basal medium containing inorganic salts of MS medium, 170 mg l^{-1} $NaH_2PO_4 \cdot H_2O$, 80 mg l^{-1} adenine sulphate dihydrate, 0.4 mg l^{-1} thiamine·HCl, 100 mg l^{-1} inositol and 3% sucrose has been successfully used for the micropropagation of a large number of plant species (Murashige, 1977). For some plants, however, the level of salts in the MS medium is either toxic or unnecessarily high (Anderson, 1975; Adams et al., 1979a). Blueberry shoots, for example, grow extremely well in a medium with MS salts reduced to one-quarter strength; higher levels are either toxic or without any beneficial effect (Cohen and Elliott, 1979). Similarly, *Dendrocalamus longispathus* showed better shoot proliferation on 1/2 or 1/4 strength MS medium than on full MS (Saxena and Bhojwani, 1993). Leaf explants of *Pinguicula moranenesis* died even on half-strength salts mixture of the LS medium (Linsmaier and Skoog, 1965) which is identical to the MS medium (Adams et al., 1979a). For their micropropagation the salt concentration had to be reduced to one-fifth.

The promoting effect of diluted mineral salt solution on shoot proliferation is probably due to reduced nitrogen level. In several Gymnosperms shoot bud differentiation was promoted by lowering nitrogen level in the MS medium (Ellis and Bilderback, 1984; Flinn et al., 1986; Perez-Bermudez and Sommer, 1987; Tuskan et al., 1990). Flinn et al. (1986) demonstrated that NH_4^+ ions accounted for much of the difference in morphogenesis in embryo cultures of *Pinus strobus* on MS and SH media. SH basal medium was better than MS medium for shoot bud differentiation. When NO_3^- level in SH medium was raised to 150% it did not affect the caulogenic response, but increase in NH_4^+ concentration to a level comparable to MS adversely affected the response.

Organic nutrients of MS or LS media are generally adequate for the micropropagation of most species.

The requirement for growth regulators varies with the system and the mode of shoot multiplication. The general concept given by Skoog and Miller (1957) that organ differentiation in plants is regulated by an interplay of auxins and cytokinins, is universally applicable and should work as a guide when developing a medium for a new plant type. According to this hypothesis the nature of organogenic differentiation is determined by the relative concentrations of the two hormones. A higher cytokinin-to-auxin ratio promotes shoot formation and a higher auxin-to-cytokinin ratio favours root differentiation. This should not imply that for adventitious shoot formation or enhanced axillary branching both the hormones must be included in the medium. The exogenous requirements of the hormones depend on their endogenous levels in the plant system which is variable with the tissues, plant type, and the phase of plant

growth. Consequently, for shoot multiplication the presence of an auxin in the medium is not obligatory. In a number of cases a cytokinin alone is enough for optimal shoot multiplication (Lane, 1979; Stoltz, 1979; Bhojwani, 1980b; Garland and Stoltz, 1981). Hormonal control of organogenesis and somatic embryogenesis in callus cultures is discussed in Chapters 5 and 6, respectively.

The qualitative and quantitative requirements for cytokinin and auxin for the maximum, but safe, rate of shoot multiplication for a new plant type should be determined by a series of experiments. A range of cytokinins (kinetin, BAP, 2-ip, and zeatin) has been used in micropropagation work. BAP, a synthetic cytokinin, is the most useful and reliable (also the cheapest) cytokinin and should be tested first for a new system. For hybrid willow (Bhojwani, 1980a) and white clover (Bhojwani, 1981) 2-ip, either autoclaved or filter-sterilized, was completely ineffective in inducing shoot multiplication whereas BAP supported the best multiplication. Anderson (1975) observed that for rhododendrons 2-ip was the best cytokinin. BAP supported a poor rate of shoot multiplication and it was toxic to the shoots; 40–70% of the rhododendron shoots died in the presence of 2.5–20 mg l^{-1} BAP. 2-ip also proved to be the best cytokinin for shoot multiplication in blueberry (Cohen, 1980) and garlic (Bhojwani, 1980b). Zeatin is not preferred because of its high cost. However, in Japanese persimmon both BAP and 2-ip were ineffective, and best shoot growth and multiplication occurred in 1/2 strength MS medium containing 1×10^{-5} M zeatin (Fukui et al., 1989). Since 1982, thidiazuron (a urea derivative) has been used as a cytokinin in several studies on shoot multiplication (see Lu, 1993). It is especially more effective than the other cytokinins (with purine ring) with recalcitrant woody species.

Cytokinins have been used in the range of 0.5–30 mg l^{-1} but, generally, 1–2 mg l^{-1} is adequate for most systems. Thidiazuron is effective at much lower concentration (0.0022–0.088 mg l^{-1} is recommended for micropropagation). At higher levels cytokinins tend to induce callusing and/or adventitious bud formation (McComb, 1978; Zimmerman and Broome, 1980) which can endanger the clonal nature of the micropropagated plants in some systems. High cytokinin concentration may also bring about morphological abnormalities and cause hyperhydration (Section 16.6.1).

With higher levels of cytokinins the number of shoots formed may be more but the growth of individual shoots remains arrested. This may require an additional in vitro step of shoot elongation on a medium with a lower concentration of cytokinin. A good growth of the shoot is especially critical when shoot multiplication through axillary branching is

achieved by taking nodal segments at each subculture. In such cases the rate of shoot multiplication is directly related to the elongation of the shoots and the number of nodal cuttings available at the end of each passage.

Of the various auxins, IAA is the least stable in the medium. Therefore, synthetic auxins such as NAA and IBA have been preferred for use in tissue culture media. For shoot multiplication their concentration ranges from 0.1 to 1 mg l^{-1}. Because of its strong tendency to induce callusing 2,4-D is avoided when shoot multiplication is attempted through axillary branching or adventitious bud development. For somatic embryogenesis, however, 2,4-D is the most effective auxin (see Chapter 6).

In some woody species GA_3 has been used in the shoot proliferation medium to improve shoot elongation (Wochok and Sluis, 1980; Brand and Lineberger, 1992). GA_3 (2.5 μM) improved the rate of multiplication, growth and quality of shoots in *Gardenia* (Economou and Spanoudaki, 1986). Debergh et al. (1993) reported stimulation of shoot multiplication in *Cordyline terminalis* by carbendazim (40–160 mg l^{-1}), a fungitoxic ingredient of some fungicides.

Since semi-solid cultures are easier to handle and maintain, the media for micropropagation are traditionally gelled with 0.6–0.8% agar. However, for several systems liquid medium has proved either critical for their survival in culture or beneficial for multiplication (Simmonds and Cummings, 1976; Snir and Erez, 1980; Molnar, 1987; Viseur, 1987; Saxena and Bhojwani, 1993). In *Cattleya* (Appendix 16.II.1) and most of the bromeliads (Hosoki and Asahira, 1980), for example, cultures could be started only in liquid medium (see Murashige, 1974). In pear the number of axillary shoots produced in liquid medium was much higher (>15 per explant) than that on semi-solid medium (1–3 per explant) but in liquid medium the shoots got vitrified (Viseur, 1987). In such cases the double-phase culture system of Maene and Debergh (1985), in which liquid medium is poured over the semi-solid medium, has been found useful (Viseur, 1987). The vitrification problem in liquid medium could also be obviated by suppressing leaf growth with the addition of paclobutrazol to the medium (Ziv, 1989; Ziv and Ariel, 1991).

Generally the pH of the medium is set at 5.8 but for some calcifuge (*Magnolia* × *soulangiana*) and acid loving (*Disanthus cercidifolius*) species, low pH is required. *Magnolia* shoot production increased from 3.7 to 7.2 by reducing pH from 4.5 to 3.5 and that of *Disanthus* from 2.3 to 7.2 with change in pH from 6.5 to 4.5 (Howard and Marks, 1989). Lowering the pH to 4, with doubling of Ca^{+2} and Mg^{+2}, promoted shoot multiplication and elongation in chestnut (Chevre et al., 1983).

Despite being green the in vitro growing shoots do not rely on photosynthesis for their food. They grow as heterotrophs, deriving all their nourishment (organic as well inorganic) from the medium. Light is required by these cultures only for certain morphogenic processes. A light intensity of 1000–5000 lx is adequate for this purpose (Hussey, 1980). Optimum light intensity for shoot multiplication in *Gerbera* and many other herbaceous species was reported by Murashige (1974) to be 1000 lx. In low light intensities, the shoots are greener and taller (Murashige, 1977). In future it may be possible to develop autotrophic cultures under high light intensity. Photoperiodism is, strictly speaking, not critical. A diurnal illumination regime of 16 h day and 8 h night is found satisfactory (Murashige, 1977).

Cultures are usually maintained at a constant temperature around 25°C. However, for tropical species a higher temperature (30°C) may be beneficial (Dhawan and Bhojwani, 1985; Rahman and Blake, 1988).

16.4.2. Rooting

For in vitro rooting, nutrient salts in the medium have a dramatic effect both on rooting percentage and root number per microcutting. Often, where shoot multiplication was induced on full-strength MS medium, the salt concentration was reduced to half (Garland and Stoltz, 1981; Zimmerman and Broome, 1981) or a quarter (Skirvin and Chu, 1979) for rooting. Bulblets and shoots of *Narcissus* rooted only when the culture medium contained MS salts at half strength (Seabrook et al., 1976). Similarly, Anderson (1984) reported that rooting of *Rhododendron* microcuttings increased from 19% on full strength medium to 77% on half strength medium. Best rooting of *Leucopogon obtectus* microcuttings occurred on agar-water medium without an auxin, and the response worsened with increasing MS salt level (Bunn et al., 1989).

As for the shoot multiplication, the promoting effect of diluted mineral salt solution on rooting is probably due to reduced nitrogen level. Driver and Suttle (1987) observed that preconditioning of walnut microcuttings on a medium with lower nitrogen and high sucrose content was beneficial for in vitro rooting. All the three apple scion cultivars (Gala, Royal Gala, Jonagold), studied by Sriskandarajah et al. (1990) showed best rooting, in response to 9.8 μM IBA, when NH_4NO_3 was deleted from MS medium. Reduction of NH_4NO_3 level to 1/2 or 1/4 strength in MS medium also significantly increased the rooting response in cvs Gala and Royal Gala but not in Jonagold. Generally, however, nitrogen must be supplied both as NO_3 and NH_4; the optimal ratio between these ions is 3 for rose cut-

tings (Hyndman et al., 1982) and 4 for *Cynara scolymus* (Moncousin, 1982).

The rooting of microcuttings requires continuous availability of free sugars (Haissig, 1974; Thorpe, 1978, 1982b, 1984; Gaspar and Coumans, 1987) which favours the differentiation of a good vascular system (Sommer and Caldas, 1981) and accelerates lignification (Thorpe, 1978; Thorpe and Biondi, 1981; Driver and Suttle, 1987). Pre-treating microcuttings of *Juglans regia* × *J. hindsii* with 3% solution of sucrose for 7 days induced 80.5% rooting compared to 55.5% with 2% sucrose (Driver and Suttle, 1987).

Kaneko et al. (1988) investigated the effect of CO_2 on adventitious root formation by lettuce cuttings. On 1/10th MS medium, without sucrose, and under 120 μmol m^{-2} s^{-1} irradiance, in an environment enriched with 0.9% CO_2 the rooting percentage was 75–90% as against 10% under non-enriched conditions.

Occasionally, as in *Gladiolus* (Hussey, 1977), *Narcissus* (Seabrook et al., 1976), and *Fragaria* (Boxus, 1974) shoots are readily rooted on a hormone-free medium. For most other species a suitable auxin is required to induce rooting. Most often IAA, IBA and NAA (0.1–1 mg l^{-1}) have been used for this purpose, but the former two seem to be more effective (George and Sherrington, 1984).

In some woody species, rooting of microcuttings may require a high concentration of auxin. Prolonged exposure to high auxin levels, however, has some undesirable effects, such as callusing, leaf chlorosis, inhibition of root elongation and quiescence or dormancy in the shoot tip which is difficult to overcome in the acclimatization stage (Maynard et al., 1991). A possible solution to this problem is to treat the cuttings with a high concentration (100–500 mg l^{-1}) of a suitable auxin for a short period (4 h–5 days) and then plant them in an auxin-free medium in vitro (Riffaud and Cornu, 1981) or ex vitro (Maynard et al., 1991; Brand and Lineberger, 1991). Microcuttings of *Prunus serotina* were rooted on 1/5th strength MS salt medium after a quick dip in 3 mM IBA (Riffaud and Cornu, 1981). Similarly, with pre-dip in 200 mg l^{-1} IBA solution mature material of *Liquidamber styraciflua* could be rooted at a rate of 70–90% (Brand and Lineberger, 1992).

Riboflavin is reported to improve the quality of the root system in *Eucalyptus ficifolia*. Several chemicals, often termed 'auxin synergists' or rooting 'co-factors', have been found to enhance the rooting response of applied auxins (Jarvis, 1986). For example, Smith and Thorpe (1977) showed that exogenously supplied aromatic amino acids and simple phenolics could enhance the formation of root primordia in *Pinus radiata*. Pythoud et al. (1986) also observed a synergistic effect between vitamin D

and IBA on adventitious root formation in *Populus tremula* (Gorst and De Fossard, 1980).

Dark treatment, particularly during the beginning (3–7 days) of the rooting stage, and raising the temperature during this period from 25 to 30°C have been reported to improve the rooting response of several apple scion cultivars (Zimmerman, 1984; Zimmerman and Fordham, 1985). In some of the hard-to-root cultivars these treatments helped achieve up to 100% rooting.

In tree species, the rootability of shoots may improve with the number of shoot multiplication cycles. For example, *Eucalyptus citriodora* shoots formed during the first two multiplication cycles failed to root but after the third passage rooting could be induced (Gupta et al., 1981). The time needed to approach maximum rooting may vary with the material. Whereas Minnesota azalea selections required four subcultures to reach over 95% rooting (Economou and Read, 1986) apple cv Delicious took 31 subcultures to reach 79% rooting (Sriskandarajah et al., 1982). The in vitro conditions seem to induce rejuvenation of the material, the effect of which may last for some time even after their transfer out of culture (Marks, 1991a,b).

The time required for in vitro rooting of shoots may vary from 10 to 15 days. It has been experienced that handling of plants during transplantation is made convenient if the roots are below 5 mm in length. Longer roots may break during transplantation and, thus, diminish the chances of survival of the plants.

For ex vitro rooting, the size of microcuttings may be critical. In the case of *Cordyline terminalis* 2.5–5 cm long shoots were best. Treatment of the microcuttings with root inducing growth regulators (auxins) or a commercial rooting powder may be necessary for their rooting in vivo. An intermediate approach between in vitro and in vivo rooting is to apply the rooting treatment in vitro and transfer cuttings out of culture before roots appear. This approach has been successful with apple (Welander, 1983; Zimmerman and Fordham, 1985). Driver and Suttle (1987) harvested 3–10 cm long microshoots of walnut and peach and placed them on a basal nutrient medium with elevated sucrose and auxin levels and reduced nitrogen concentration for 1–2 weeks and then transferred to non-sterile medium. During auxin treatment the shoots were placed under higher light intensity (66 μmol m^{-2} s^{-1}), shorter photoperiod (17 h) and lower temperature (19°C) than during shoot multiplication to promote lignification. The in vitro multiplied shoots of grape placed under 1200 ppm CO_2 during ex vitro rooting grew more rapidly than those growing continuously with 350 ppm CO_2, probably because of increased root growth (Lakso et al., 1986). When combining rooting and acclimati-

zation the same environmental considerations apply as when acclimatizing rooted plantlets (see Section 16.3.5).

16.5. APPLICATIONS OF MICROPROPAGATION

In vitro methods can be used to produce, maintain, multiply and transport pathogen-free plants safely and economically. Currently, the most popular application of micropropagation is the mass clonal multiplication of desirable genotypes of plants. Through tissue culture, over a million plants can be grown from a small, even microscopic, piece of plant tissue within 12 months. Such a prolific rate of multiplication cannot be expected by any of the in vivo methods of clonal propagation. An advantage of tissue culture propagation is that the shoot multiplication cycle is very short (2–6 weeks), each cycle resulting in a logarithmic increase in the number of shoots. Additionally, in tissue cultures plant multiplication can continue throughout the year irrespective of the season. A large number of horticultural, plantation and forest species are being propagated in vitro on commercial scale.

An enhanced rate of plant multiplication in vitro should reduce considerably the period between the selection and the release of a new cultivar. The amount of plant material released from quarantine being usually limited, micropropagation should also hasten the introduction of new crops following their passage through quarantine. In vitro conservation and propagation of endangered plants is another important application of this aseptic technique (Fay, 1992; Bhojwani and Arumugam, 1993).

Tissue culture provides reliable and economical methods for maintaining pathogen-free plants in a state that can allow rapid multiplication when needed, irrespective of the time of the year. In commercial nurseries tissue culture can be used to minimize the growing space usually provided for the maintenance of stock plants. Several thousand million plants can be maintained inside culture vials on a shelf space built into a room of about 3 × 3 × 5 m.

Vegetative propagation is extremely important in the case of dioecious species where seed progeny yields 50% males and 50% females but plants of one of the sexes are more desirable commercially. In *Asparagus officinalis*, for example, male plants are more valuable than the female plants but their clonal propagation by stem cuttings is not possible. However, it can be cloned in vitro (Desjardins, 1992). In papaya, another dioecious crop, clonal propagation of established female plants (Jordan, 1992) would save the losses suffered due to discarding a large number of male plants in a seed-raised orchard which is possible only after the plants have reached the flowering stage.

16.6. LIMITATIONS OF MICROPROPAGATION

In vitro clonal propagation of plants is a commercial technique, being practised for a wide range of herbaceous and woody species by nurserymen all over the world. However, satisfactory protocols for micropropagation of adult materials of many important hardwood and softwood tree crops, whose propagation by conventional methods is difficult, have not been developed so far.

Once a method has been evolved and aseptic cultures established it may be tempting to continue to propagate from it for many generations. This may lead to bulking up of any off-types (sport or accidental change) that might have arisen in cultures at an early stage. To offset this real problem it is suggested that in vitro multiplication should be restricted to 5–10 cycles and only a few thousand plants raised from a single explant. These and some other major problems associated with micropropagation are discussed in this section.

16.6.1. Hyperhydration

The term hyperhydration, earlier called vitrification (Debergh et al., 1992b), refers to the morphological, physiological and metabolic derangements frequently affecting herbaceous and woody plants during their in vitro culture. Hyperhydration occurs only during intensive shoot multiplication and never during the rooting stage (Gaspar, 1991). Various terms, viz. glassiness, glauciness, translucency and vitrescence have been used to describe these malformations (Gaspar, 1991; Ziv, 1991b).

Since the first description of hyperhydration by Phillips and Mathews (1964), considerable literature has accumulated on the various manifestations, causal factors and preventive measures of this phenomenon (Gaspar, 1991; Ziv, 1991b; Debergh et al., 1992a).

(i) *Characteristics*. Besides the common structural and functional abnormalities shown by in vitro growing plants (see Section 16.3.5), hyperhydrated shoots have short internodes and appear thick, brittle, glassy, and water-soaked. Leaves are the immediate organ to be affected by these abnormalities.

Hyperhydrated shoots generally show poor growth, become necrotic and, finally, die (Gaspar, 1991). On solid medium, most of the hyperhydrated shoots turn into callus after two or three subcultures (Leshem et al., 1988). Hyperhydrated shoots do not root easily either in vitro or in vivo and adversely affect the growth of new shoots (Gaspar, 1991). The plants showing hyperhydration have low survival rate during acclimati-

zation (Ziv, 1991a,b). Therefore, such shoots are discarded during multiplication. However, in some conifers the hyperhydrated shoots have shown a better rate of multiplication. For example, Bornman and Vogelmann (1984) observed that vitrified cultures of *Picea abies* produced 10 times as many buds and shoots as normal shoots. It would be worthwhile to examine if it is applicable to other tree species.

(ii) *Causes.* The physical and chemical nature of the culture medium, and the atmosphere around the cultured plant material are responsible for hyperhydration. Liquid medium causes more hyperhydration than solid medium, and in the latter case the degree of hyperhydration is influenced by the concentration and type of gelling agent. It decreases with increase in the concentration of the gelling agent. Compared to agar, gelrite or phytagel causes more hyperhydration (Zimmerman et al., 1991).

Plants are more prone to hyperhydration on media rich in mineral salts, such as MS medium (Installe et al., 1985; Paques and Boxus, 1987a,b). Reducing NH_4^+ ions in the medium increased lignification and reduced hyperhydration in willow, plum, petunia and cacti species (Beauchesne, 1981; Daguin and Letouze, 1986; Leonhardt and Kandeler, 1987; Zimmerman et al., 1991). Elevated Ca^{2+} ions reduced hyperhydration in both herbaceous (Ziv et al., 1987b) and woody (Kreutmeier et al., 1984) species.

In several cases high cytokinin concentration is reported to promote hyperhydration. In apple, normal plants transferred from solid medium to liquid medium containing BAP became hyperhydrated (Paques and Boxus, 1987b). Removal of BAP from liquid medium induced reversal to normal shoots as long as hyperhydration was not very advanced (Gaspar et al., 1987). In carnation and conifers lowering the cytokinin level led to decreased hyperhydration, which was stimulated more by high BAP than kinetin (Dencso, 1987).

The size of the culture vessel, the type of closure, and the climate parameters of the culture room all influence hyperhydration (Hakkaart and Versluijs, 1983; Debergh and Maene, 1984). High relative humidity above the cultures accelerates the hyperhydration process (Wardle et al., 1983; Ziv et al., 1983; Debergh and Maene, 1984; Bottcher et al., 1988).

In the early days of plant tissue culture, cotton plugs were used as closure for the culture tubes which allowed good aeration of the cultured material. Commercialization of this technique introduced the use of a variety of plastic caps which do not allow good gaseous exchange and, consequently, create hyperhydration-inducing conditions inside the culture vial, viz. build-up of high humidity and accumulation of various gases. To overcome this problem new plastic lids with a vent covered by a

bacteria-proof membrane have been produced. A cheaper but equally effective alternative, being practised routinely in some commercial laboratories in India, is drilling a hole in the plastic lids and closing it with a cotton plug.

(iii) *Preventive measures*. Hyperhydration can be reduced and the quality of in vitro plants improved by lowering the humidity in the head-space of the culture vial by: (1) increasing the concentration of agar (Debergh et al., 1981); (2) using an agar with higher gelling strength (Debergh, 1983); (3) improving the aeration of the container (Dillen and Buysens, 1989; Rossetto et al., 1992);(4) overlaying of medium with paraffin (Wardle et al., 1983); (5) using a desiccant such as $CaSO_4$ (Ziv et al., 1983) and silica gel (Wardel et al., 1983); and (6) bottom cooling of the culture vial (Maene and Debergh, 1987; Vanderschaeghe and Debergh, 1988). Other treatments which may help reduce hyperhydration are: lowering cytokinin levels or replacing one type of cytokinin by another (Werner and Boe, 1980; Beauchesne, 1981; Debergh et al., 1981; Wilkins and Dodds, 1983), lowering NH_4^+ concentration (Zimmerman et al., 1991), diluting MS medium or replacing it with Heller's medium (Riffaud and Cornu, 1981; Letouze and Daguin, 1983; Vieitez et al., 1985), and addition of phlorizin, phloroglucinol (Hegedus and Phan, 1983, 1987) or $CoCl_2$ (Gaspar and Kevers, 1985) to the medium.

16.6.2. Off-types

Appearance of off-types in tissue culture-raised plants (see Chapter 9) is a major limitation in wide-spread acceptance of this technology for clonal propagation of crop plants. The abnormalities induced in vitro are influenced by the choice of explant, composition of culture medium (particularly the nature and concentration of phytohormones), length of time in vitro, the level of organization during culture, and genotype of the material. Generally, the systems involving a callus phase show maximum aberrations. Therefore, the callus phase, unless unavoidable, must be kept to a bare minimum. Direct differentiation of adventitious buds or embryos from the explants may also induce abnormalities, particularly in chimeric plants by disturbing the chimeric arrangement. The abnormalities observed in systems multiplied by axillary bud proliferation mainly result due to the development of some adventitious shoots, lasting effect of in vitro environment (particularly growth regulators) and/or, in perennial species, by the appearance of juvenile traits.

Oil palm is a well known example of developmental abnormalities observed in micropropagated plants (Duval et al., 1995). The most common

abnormality shown by this crop plant is feminization (Paranjothy et al., 1990; Duval et al., 1995). Staminodes in the female flowers develop into a mantle of fleshy carpels around the fruit, leading to the formation of abnormal fruits or to partial or complete sterility, depending on the extent of abnormality. In severe cases, even the male flowers show the development of stamens into pseudocarpels. In this system, micropropagated via somatic embryogenesis, the age and nature of callus affected the degree of abnormalities; it increased with age of the callus (Corley et al., 1986). Whereas all the plants regenerated from fast growing callus exhibited severe abnormalities, the slow growing nodular callus produced a limited number (3.1%) of abnormal plants (Duval et al., 1988, 1995).

Banana is another example where several research groups have described the appearance of off-types in micropropagated plants. The most common type of abnormality is the development of dwarf phenotypes, which are not only shorter in height but also show small fruits, closer packing of the hands on the bunches and chocking of the branches (failure to emerge fully from the pseudostem when temperature remains below 15°C). This aberration ranged from 3% in Taiwan (Hwang and Ko, 1987) and Australia (Smith and Drew, 1990a,b) and 9% in Israel (Reuveni et al., 1985) to 25% in Jamaica (Stover, 1987).

Increased cytokinin level in the medium may help enhance the rate of shoot multiplication but it also induces some undesirable features, probably by promoting the differentiation of adventitious buds in systems propagated by axillary branching (see Section 16.3.3). In strawberry, frequent subculture of shoots promotes formation of adventitious buds (Zimmerman, 1991). Micropropagated plants of chrysanthemum show juvenile characteristics, such as forming basal shoots and delayed flowering. Therefore, micropropagated plants of this species are more suitable as mother plants for propagation by cuttings because this basitonic behaviour disappears after one or a few generations of in vivo propagation.

Any in vitro method adopted for commercial purpose must ensure high genetic fidelity of the propagated plants. In tree species, where several years may be required to check trueness-to-type based on morphological features, at least cytological and molecular tests (RFLP, RAPD, isozyme profiles) can be made in the initial stages.

16.6.3. Contamination

Contamination is one of the most serious problems in commercial micropropagation as it can cause a disastrous situation if it occurs at an advanced stage of the production schedule. There is a growing belief that

tissue cultures are only rarely totally free of contaminants. There are many causes of contamination of established cultures. Slow growing bacteria carried with the explant may remain latent initially and show up at a later stage. Fungal spores which occur in the air with high concentration may be carried into the laboratory on body hairs of the workers or vectors like mites and thrips, which have been occasionally observed inside culture containers (Debergh et al., 1990). It is, therefore, extremely important to maintain high standards of hygiene in and around the sterile area. Spore concentration in the laboratory can be controlled by regular vaporization of formaldehyde or a fungicide such as thiobendazole (Debergh et al., 1990). Californian thrips can be controlled by a daily treatment with Baygon™ or two treatments, at an interval of 5 days, with 10% dichloorvos (Debergh et al., 1990).

16.6.4. Oxidative browning

A problem generally faced in culturing adult tissues from woody species and some other plants is the browning/blackening of the culture medium and/or the explant. The injury caused during the excision of explants induces the cells to leach out phenolic compounds which are readily oxidized to produce quinones and cause discoloration. The oxidation products of phenols can be phytotoxic and cause necrosis and, eventually, death of the explant. Several different methods have been used to overcome this problem (Preece and Compton, 1991).

Quick transfer of explants within the same vessel or to fresh medium 2 or 3 times, at short intervals, is the simplest and a fairly successful method to protect the explants from the detrimental effects of oxidative browning (Kotomari and Murashige, 1965; Morel, 1972; Preece and Compton, 1991). During this period the cut end of the explant may become sealed up and the leaching of phenols stop. Keeping the cultures initially in the dark may also help to reduce the browning problem (Monaco et al., 1977; Adams et al., 1979b) by preventing or reducing the activity of the enzymes concerned with both biosynthesis and oxidation of phenols (George and Sherrington, 1984).

For the micropropagation of a 20-year-old tree of *Eucalyptus citriodora* it was essential to grow the shoot buds, taken directly from the parent plant, in a liquid medium (at 15°C, in continuous light of 500 lx) for 3 days before planting on a semi-solid medium (Gupta et al., 1981). Cultures raised directly on a semi-solid medium did not survive. Zimmerman (1978) followed a similar approach with apples and *Rubus* sp. The initial culture in liquid medium probably helps in getting rid of the phenolic compounds and other growth inhibitors.

When the problem of medium browning persists at each subculture the addition of antioxidants, such as cysteine·HCl (100 mg l^{-1}), ascorbic acid (50–100 mg l^{-1}) or citric acid (150 mg l^{-1}) to the culture medium is recommended (Sondahl and Sharp, 1977; Skirvin and Chu, 1979). Immersion of shoot pieces, for 24 h, in a solution of antioxidants before planting them on a medium containing activated charcoal helped establish healthy cultures of *Strelitzia reginae* which otherwise died due to oxidative browning of tissue exudates (Ziv and Halvey, 1983). Polyvinylpyrrolidon (Polycar AT or PVP), which can adsorb phenolic compounds, has also been used to save tissues from the toxic effects of the oxidized phenols (Walkey, 1972; Gupta et al., 1980). For *Tectona grandis* insoluble PVP was better than soluble PVP (Gupta et al., 1980).

16.6.5. Recalcitrance of adult-trees

During the last 10 years efficient methods have been developed for in vitro clonal propagation of several tree species, of which some have been propagated on a large scale. These include ornamentals (rhododendron, cherry), fruit trees (apple, pear), plantation crops (oil palm, date palm), and hardwood (poplars, willows, eucalyptus) and softwood (radiata pine, redwood) forest tree species (Thorpe et al., 1991). For many other tree species (sandalwood, birch, teak, loblolly pine, walnut) promising protocols have been published. In 1989, Enso-Gutzeit (Finland) produced several thousand elite birch plants through micropropagation (Anonymous, 1991).

A spectacular development has been the achievement of somatic embryogenesis in a large number of woody species. However, this success is restricted to zygotic embryos and young seedling explants (Gupta et al., 1993). In many woody species even propagation by shoot bud proliferation has been successful only with juvenile tissues (Thorpe et al., 1991). By the time trees are old enough for assessment of desirable traits they become recalcitrant for tissue culture. There are two options to overcome this problem: (1) select the most juvenile tissues (e.g. current year's basal sprouts in redwood) from adult trees, and (2) rejuvenate parts of the desired plant by special treatments prior to excision of explants, such as repeated spraying of selected branches with cytokinin (particularly BAP; Abo El-Nil, 1982), and etiolation of shoots before excising shoot tip (Ballester et al., 1989). Repeated grafting of scion from mature trees onto seedling rootstocks accentuated and prolonged juvenile behaviour in scions of *Hevea*, *Eucalyptus* and *Psudotsuga* (Franclet et al., 1987). Repeated subculture of shoot apices in cytokinin medium induced reactivation of meristem in many species, including *Prunus*, *Eucalyptus*, *Pinus*

pinaster and *Sequoia* (Thorpe et al., 1991). The degree of rejuvenation increases with the number of subcultures.

Another problem encountered with temperate woody hardwood species is the episodic growth pattern observed in culture (McCown and McCown, 1987). In the culture of mature explants the shoot developed in vitro may become quiescent for varying periods (sometimes several years) depending on the degree of maturity of the selected material.

16.6.6. High costs

Although tissue culture is being used on commercial scale for several ornamental and other horticultural crops, it is generally more expensive than other forms of clonal propagation, using cuttings or seeds (Pierik, 1991). It is labour intensive, involving manual handling at 3 or 4 stages. Therefore, micropropagation can be justified only when the conventional methods of clonal propagation do not work or the product is of high value. For some crops, micropropagation can only be economical to produce pathogen-free foundation stock which can be used to produce a number of generations in glasshouse or field. Carnations and chrysanthemums can be easily propagated in vitro and with high propagation rates but so far straight production of these crops from tissue culture is regarded as uneconomical (Debergh and Read, 1991). Similarly, large scale micropropagation protocols for strawberry are well established and have been used all over the world. However, the micropropagated plants of this fruit crop being expensive, and the fact that they produce small fruits compared to the runner propagated crop, are rarely used for fruit production. They generally serve as mother plants for runner production (Zimmerman, 1991).

Since labour charges account for 60–70% of the cost of production of tissue culture plants in developed countries there is considerable interest in automating the process partly or completely (Vasil, 1991; Aitken-Christie et al., 1995). To some extent, automation has been introduced at the media preparation stage by using dispensing machines. Bioreactors are being used to automate the multiplication stage in some cases (see Sections 16.3.3 and 16.3.5). Somatic embryogenesis is potentially the most amenable system to automation, not only at the production stage but also for mechanized planting in the field as synthetic seeds. Although considerable progress has been made in this area yet there are several problems that must be resolved before somatic embryogenesis becomes a viable method for mass clonal propagation of plants.

An alternative approach being currently followed by some of the developed countries in order to reduce the cost of tissue culture propagated

plants is to sub-contract in vitro multiplication of their materials in the developing countries where labour is comparatively very cheap. This has resulted in the establishment of several commercial tissue culture laboratories in developing countries. Whether such laboratories, with huge investments, exclusively for export purpose would be economically viable is a big question. Air freighting of highly perishable ex vitro plants for delivery at right times while meeting the strictly prescribed quality standards may result in high rejection rates. India has at least half-a-dozen such laboratories.

16.7. CONCLUDING REMARKS

From the commercial viewpoint, micropropagation is the most important aspect of plant tissue culture. Industrial application of this technique started in the late 1960s and early 1970s, first with orchids and later with other crop plants. According to one estimate, 350 million plants are produced annually through micropropagation (Tormala, 1989), mainly including ornamentals (begonia, ferns, ficus sp., gerbera, philodendron, rhododendron, saintpaulia, syngonium), and some fruit (strawberry, banana, apple, pear) and forest (poplars, eucalyptus, redwood, radiata pine) species. Protocols for several other crops have been published. However, all research reports do not provide commercially viable protocols. Often the information regarding the rates of multiplication provided in such papers is inadequate for their commercial use. Moreover, each species and, in many cases, each variety may require a different combination of hormones for successful multiplication.

The micropropagation industry is capital and labour intensive. During the 1980s many companies and entrepreneurs became fascinated by this novel technique for clonal propagation of plants and took it up as a business but only a few survived. Micropropagation should be regarded as yet another method of plant propagation and not as a business in itself. Rapid multiplication of plants under disease-free in vitro conditions is often easy but it is not enough to survive in the business. It requires the production of quality plants at competitive price and according to the demands of the market. It is extremely important in this business to plan production according to market demand rather than to have a product first and then search for a market (Cervelli and Senaratna, 1995).

Any attempt to reduce the cost of production by accelerating the rate of multiplication or automation should not be at the expense of the quality of the product. Pushing a system beyond a limit with regard to the rate of multiplication or the life-span of a culture may introduce genetic aberrations which, if not identified and discarded at the right time, would get

rapidly multiplied with normal type. For this a trained human eye is probably indispensable. Mezitt (1989), who observed several abnormalities (foliar and floral variation, abnormal flowering, increased susceptibility to disease) in his rhododendron cultivars micropropagated in other laboratories, remarked that tissue culture laboratories run by individuals unfamiliar with horticulture and with very little or no association with the plant industry run the risk of allowing off-types to go through the production line under the economic pressure to accomplish too much too quickly.

In developed countries the labour charges account for 50–70% of the cost of production of tissue culture plants. Therefore, considerable research efforts are being made to reduce costs by introducing automation at different stages of production (Vasil, 1991; Aitken-Christie, 1991; Aitken-Christie et al., 1995). Somatic embryogenesis is considered as the ultimate mode of micropropagation because it may allow automation not only at the multiplication stage but also for their field planting as synthetic seeds (Cervelli and Senaratna, 1995; Sakomoto et al., 1995). Several problems must be solved before this objective can be realized.

APPENDIX 16.I: MICROPROPAGATION PROTOCOLS FOR SOME CROP PLANTS

16.I.1. Potato (based on Dodds et al., 1992)

(a) Collect shoot pieces from 5 to 10-week-old greenhouse grown stock plants, remove the leaves, and surface sterilize in 10% household chlorine bleach with 0.05% Tween 20 as a wetting agent.

(b) If the stock plant is a certified virus-free material, cut the shoot into 2 or 3 node segments and plant on MS basal medium containing 3% sucrose and 0.8% agar. If the parent plant is virus-infected, dissect 0.1 mm shoot tips under a stereoscopic microscope and culture on the medium as above. Incubate the cultures in continuous light, at 25–30°C.

(c) After the solitary shoot derived from an axillary bud or apical bud has attained a height of 7–10 cm, subculture it on MSA medium (see Appendix 16.II.5). Discard the terminal node and place the shoot horizontally on the medium.

(d) Repeat step (c) 3 or 4 times at weekly intervals to build-up the number of shoots. By the fourth passage uniform shoot growth and multiplication should be established. In the case of uncertified material, suitable indexing of the shoots must be carried out

and only those individuals found free of the specified viruses further multiplied.

(e) Cut the shoots into single node segments and transfer about 30 segments per 250 ml Erlenmeyer flask containing M-9 medium (see Appendix 16.II.5). The flasks may be shaken at 80 rev. min^{-1} on an orbital horizontal shaker. Incubate the cultures under a 16 h photoperiod at 22°C.

(f) After 3–4 weeks, 26–30 vigorous plants develop per flask. Individual plantlets can be transferred to the greenhouse following the standard method of ex vitro acclimatization. Microcuttings 3–5 cm long can also be readily rooted in peat-perlite medium in the glasshouse.

(g) Alternative to step (f), the plantlets can be induced to form microtubers by replacing the M-9 medium with MT medium (see Appendix 16.II.5) and storing the cultures in the dark at 15–20°C. B_5 medium may be better than MS medium.

(h) Harvest the microtubers, break their dormancy by storing in sterile petri plates sealed with parafilm, in diffuse light, at 4°C, for 4 weeks.

(i) The microtubers may be planted in pots in greenhouse to produce pathogen-tested mother plants for conventional rapid multiplication programme or can be planted at high density (up to 100 tubers m^2) in nursery beds, inside an insect-proof nethouse to produce high quality seed tubers.

16.I.2. *Gladiolus* (after Dantu and Bhojwani, 1992, 1995)

(a) Dehusk the corms and scoop out individual axillary buds along with some corm tissue. Trim the corm tissue to 5 mm^3.

(b) Rinse the excised buds in 90% ethanol, air dry for 15 min, dip in a 0.5% solution of Cetavlon (or any other surfactant) for 5 min and wash thoroughly under running water for 60 min. Surface sterilize the buds in a 2.5% (w/v) solution of sodium hypochlorite for 15 min and give three quick washes in sterile distilled water under aseptic conditions.

(c) Trim the peripheries of the corm tissue and plant the bud on MS + 0.5 mg l^{-1} BAP (BAP concentration might have to be changed depending upon cultivar being used) and incubate the cultures at 25°C under continuous light (10 W m^{-2} s^{-1}).

(d) After 2 passages of 4 weeks each on the medium as in (c), when multiple shoots have developed, cut the cluster of buds into pieces, each bearing more than one bud and culture them indi-

vidually on shoot multiplication medium (see Appendix 16.II.6). At the end of 4 weeks cut the cluster of shoot buds into small propagules, each with 10–20 shoot buds, and transfer them individually to fresh medium for shoot multiplication. Incubate the cultures in continuous dark, at 25°C.

(e) Cut the shoot clusters into small pieces, each bearing *ca* 10 buds, and transfer them to liquid MS medium containing 0.5 mg l^{-1} BAP, for shoot elongation. Incubate the cultures under conditions as in (c).

(f) Separate 10 cm long shoots from 4-week-old cultures in shoot elongation medium, and individually transfer them to MS liquid medium with sucrose level raised to 6–10% (depending on cultivar). Incubate the cultures in continuous light. Corms will be ready for harvest in 10–12 weeks.

(g) Remove the corms, wipe off the medium, and store them at 5°C for 6–8 weeks.

(h) Sow the corm directly in the field following the standard planting procedures for this crop. The corms will sprout within a fortnight.

16.I.3. Banana (after Drew et al., 1991)

(a) Excise 20 × 30 mm sections containing the shoot tip from 0.4–1.0 m high sword suckers and surface sterilize them in 3.5% solution of sodium hypochlorite containing a few drops of Tween 80, for 15 min. Wash it in sterile distilled water and resterilize in 1% solution of sodium hypochlorite containing Tween 80 for 5 min.

(b) Wash the material in sterile distilled water and trim it to prepare a 5 mm^3 explant carrying the shoot tip, ensheathing leaf bases and 2–3 mm of basal stem tissue.

(c) Culture the explants on MS medium supplemented with 2.25 mg l^{-1} BAP, 2% sucrose, and 0.8% Difco Bacto agar.

(d) Multiply the shoots on the above medium by 6–8 weekly subcultures.

(e) For rooting, transfer the shoots to hormone-free MS basal medium.

(f) Harden the plantlets using standard procedures.

16.I.4. *Pyrus communis* (after Chevreau et al., 1992)

(a) Excise single node segments (2 cm) from actively growing grafted plants in the greenhouse and surface sterilize by immersion in

sodium hypochlorite (0.5–2.5%) containing 0.01% Tween-20, for 5–30 min. Rinse three times in sterile distilled water.

(b) Culture the nodal cuttings on Lepoivre mineral medium (see Appendix 16.II.8) supplemented with 1–2 mg l^{-1} BAP, 0.1 mg l^{-1} IBA or 0.01 mg l^{-1} NAA. For shoot proliferation maintain the cultures at 24°C and subculture at 30-day intervals.

(c) Multiplication and shoot elongation rates may show a progressive decline with the number of subcultures. Give 45 days of cold treatment, at 8°C, to reinitiate growth activity.

(d) For rooting, transfer individual shoots to MS medium (full or 1/2 strength) supplemented with IBA or IAA (0.025–2 mg l^{-1}). A dark treatment during the initial 7–10 days improves the rooting percentage. Liquid medium supports better rooting but such plants show poor survival after transplantation than those rooted on semi-solid medium.

(e) Transfer the rooted plants to vermiculite and maintain them under high humidity initially. Apply GA_3 spray (100–200 mg l^{-1}) every 4–5 days to improve plant growth.

16.I.5. Walnut (after Leslie and McGranahan, 1992)

(a) Collect vigorously growing softwood shoots or stump sprouts.

(b) Remove the foliage, wash the stem in soapy water and cut them into 3–4 cm nodal segments. Wash the segments in running tap water.

(c) Give a quick (5–10 s) rinse in 95% ethanol, and then surface sterilize in 10% solution of commercial bleach (0.5% sodium hypochlorite) containing 1–2 drops of detergent, with constant stirring.

(d) Rinse each explant in separate container of sterile water and plant individually in separate culture vials containing multiplication medium (see Appendix 16.II.9). Culture under continuous cool white fluorescent light, at 25°C.

(e) Transfer each explant to fresh medium daily for 1 week and thereafter at least once per week until new axillary shoot emerges.

(f) Excise the shoots when 2 cm long and culture them separately, transferring every week.

(g) Multiply shoots by cutting long shoots into segments or excising individual axillary shoots. Transfer weekly and remove basal callus every 3–4 weeks.

(h) For rooting, cut shoots 5–10 cm in length and transfer to prerooting medium (see Appendix 16.II.9) for 1 week.

(i) Remove the shoot from the pre-rooting medium, wash thoroughly to remove medium, dip the shoot bases in 2% IBA (w/w) in talc powder, plant in a well drained potting mix, and treat with a fungicide. Maintain the plants under high humidity for 2–8 weeks until new shoots emerge.

16.I.6. *Simmondsia chinensis* (after Chaturvedi and Sharma, 1989)[1]

(a) Collect single-node segments from field grown plants and wash in running tap water for 30 min, followed by 20 min of washing in 5% Teepol solution. Dip the segments in 95% ethanol for 5 s and then surface sterilize in 0.1% $HgCl_2$ for 10 min followed by a 5 min rinse in chlorine water. Despite this only about 10% aseptic cultures are obtained.

(b) Culture the nodal segments in a modified SH medium (MSH) supplemented with 1 mg l^{-1} each of BAP and IAA (MSH-I) to induce bud break and shoot proliferation. Maintain the cultures under a 15 h light regime at 27°C.

(c) Multiply the shoots on the modified SH medium containing 0.5 mg l^{-1} BAP, 1 mg l^{-1} IAA and 15 mg l^{-1} adenine sulphate (MSH-II). New shoots continue to differentiate and proliferate from the parent nodal axis.

(d) For rooting, transfer individual shoots to a modified SH medium with reduced salts and supplemented with 7 mg l^{-1} NAA and 1 mg l^{-1} caffeic acid, and pH adjusted to 5.0 (MSH-III). As soon as the shoots form roots transfer them to MSH with reduced auxin level (2 mg l^{-1} IBA and 1 mg l^{-1} caffeic acid). After 15 days about 90% shoots are expected to show rooting. At this stage transfer them to an auxin-free medium.

(e) Transfer the plantlets to pots containing coarse sand and sandy soil (1:3) and cover the plant with a glass cylinder to maintain high humidity. Gradually, over a period of 15 days, remove the glass cylinder completely.

[1] For the composition of culture media see Appendix 16.II.10.

APPENDIX 16.II: COMPOSITION OF MEDIA FOR MICRO-PROPAGATION OF SOME CROP PLANTS

16.II.1. *Cattleya*[a]

Constituents	Media (amounts in mg l^{-1})		
	Initiation[b]	Maintenance[b,c]	Rooting[d]
Inorganic nutrients			
$MgSO_4 \cdot 7H_2O$	120	120	250
KH_2PO_4	135	135	250
$Ca(NO_3)_2 \cdot 4H_2O$	500	500	1000
$(NH_4)_2SO_4$	1000	1000	500
KCl	1050	1050	–
KI	0.099	0.099	–
H_3BO_3	1.014	1.014	0.056
$MnSO_4 \cdot 4H_2O$	0.068	0.068	7.5
$ZnSO_4 \cdot 7H_2O$	0.565	0.565	0.331
MoO_3	–	–	0.016
$CuSO_4 \cdot 5H_2O$	0.019	0.019	–
$CuSO_4$	–	–	0.040
$AlCl_3$	0.031	0.031	–
$NiCl_2$	0.017	0.017	–
$FeSO_4$	–	–	25
$FeC_6H_5O_7 \cdot 3H_2O$	5.4	5.4	–
Organic nutrients			
Inositol	–	18.00	–
Nicotinic acid	–	1.22	–
Pyridoxine·HCl	–	0.21	–
Thiamine·HCl	–	0.34	–
Folic acid	–	4.4	–
Biotin	–	0.024	–
Ca-pantothenate	–	0.48	–
Glutamic acid	–	15.0	–
Asparagine	–	13.0	–
Guanylic acid	–	182.0	–
Cytidylic acid	–	162.0	–
Growth regulators			
Kinetin	0.2	0.22	–
NAA	0.1	0.18	–
GA_3	–	0.35	–
Complex nutrients			
Coconut water	150 ml l^{-1}	50–150 ml l^{-1} } either one	–
Casein hydrolysate	–	100 mg l^{-1} } either one	

Sucrose	0.5%	2%	2%
Agar	–	–	1.2–1.5%

[a]Explant: shoot tip.
[b]After Lindemann et al. (1970); taken from Arditti (1977).
[c]Maintenance medium is for protocorm multiplication and shoot development.
[d]Modified Knudson C (1946) medium; taken from Arditti (1977).

16.II.2. *Cymbidium*[a,b]

Constituents	Media (amounts in mg l^{-1})		
	Wimber[c]	Fonnesbech[d]	Modified Knudson C[e]
Inorganic nutrients			
KNO_3	525	–	–
$MgSO_4{\cdot}7H_2O$	250	250	250
KH_2PO_4	250	250	250
K_2HPO_4	–	212	–
$(NH_4)_2SO_4$	500	300	500
$Ca(NO_3)_2{\cdot}4H_2O$	–	400	1000
$CaHPO_4$	200	–	–
H_3BO_3	–	10	0.056
$MnSO_4{\cdot}4H_2O$	–	25	7.5
$ZnSO_4{\cdot}7H_2O$	–	10	0.331
$Na_2MoO_4{\cdot}2H_2O$	–	0.25	–
$CuSO_4{\cdot}5H_2O$	–	0.025	0.040
MoO_3	–	–	0.016
$Fe_2(C_4H_4O_6)_3$	300	–	–
$FeSO_4{\cdot}7H_2O$	–	27.9	25
$Na_2.EDTA$	–	37.8	–
Organic nutrients			
Inositol	–	100	–
Nicotinic acid	–	1	–
Pyridoxine·HCl	–	0.5	–
Thiamine·HCl	–	0.5	–
Glycine	–	2	–
Growth regulators			
Kinetin	–	0.215	–
NAA	–	1.86	–
Complex nutrients			
Casamino acid	–	2000–3000 } either	–
Tryptophan	2000	3000–4000 } one	–
Coconut water	–	100–150 ml l^{-1}	–
Banana, ripe	–	–	15%

Sucrose	2%	3–4%	2%
Agar	–	0.8%	1.2–1.5%

[a]Explant: shoot tip.
[b]Any one of the media is enough for all the three stages of micropropagation.
[c]After Wimber (1963); taken from Arditti (1977).
[d]After Fonnesbech (1972).
[e]Taken from Arditti (1977).

16.II.3. *Dendrobium*[a,b]

Constituents	Amount[c] ($mg\ l^{-1}$)
Inorganic nutrients	
KNO_3	525
$MgSO_4{\cdot}7H_2O$	250
KH_2PO_4	250
$(NH_4)_2SO_4$	500
$Ca_3(PO_4)_2$	200
$MnSO_4{\cdot}4H_2O$	7.5
$Fe_2(C_4H_4O_6)_3$	28
Coconut water	150 $ml\ l^{-1}$
Sucrose	2%
Agar[d]	0.8%

[a]Explant: axillary bud.
[b]After Kim et al. (1970).
[c]This medium is suitable for all the in vitro stages of micropropagation.
[d]Omitted when a liquid medium is used.

16.II.4. *Epidendrum*[a,b]

Constituents	Media (amounts in $mg\ l^{-1}$)		
	Initiation	Callus multiplication	Differentiation
Inorganic nutrients			
NH_4NO_3	1650	1650	–
KNO_3	1900	1900	–
$CaCl_2{\cdot}2H_2O$	440	440	–
$MgSO_4{\cdot}7H_2O$	370	370	250
KH_2PO_4	170	170	250
$(NH_4)_2SO_4$	–	–	500
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	1000
KI	0.83	0.83	–

H_3BO_3	6.2	6.2	0.056
$MnSO_4{\cdot}4H_2O$	22.3	22.3	–
$ZnSO_4{\cdot}7H_2O$	–	9	0.331
$ZnCl_2$	3.93	–	–
$Na_2MoO_4{\cdot}2H_2O$	–	0.25	–
MoO_3	–	–	0.016
$CuSO_4{\cdot}5H_2O$	0.025	0.025	–
$CuSO_4$	–	–	0.040
$CoCl_2{\cdot}6H_2O$	0.025	0.025	–
$FeSO_4{\cdot}7H_2O$	27.8	27.8	25
Na_2EDTA	74.6	37.3	–
Organic nutrients			
Inositol	–	100	–
Thiamine·HCl	0.4	0.4	–
Glycine	2	–	–
Growth regulators			
BAP	0.5	–	–
Kinetin	–	0.1	–
2,4-D	1	–	–
IAA	–	2	–
Banana	–	–	15%
Sucrose	3%	3%	2%
Agar	–	1%	1.2–1.5%

[a]Explant: leaf tip.
[b]After Churchill et al. (1970); taken from Arditti (1977).

16.II.5. Potato (after Dodds et al., 1992)

Constituents	Amounts (mg l^{-1})		
	MSA[a]	M–9[b]	MT[c]
Inorganic nutrients			
NH_4NO_3	1650	1650	1650
KNO_3	1900	1900	1900
$CaCl_2{\cdot}2H_2O$	440	440	440
$MgSO_4$	370	370	370
KH_2PO_4	170	170	170
H_3BO_3	6.2	6.2	6.2
$MnSO_4{\cdot}4H_2O$	16.9	16.9	16.9
$ZnSO_4{\cdot}7H_2O$	8.6	8.6	8.6
KI	0.83	0.83	0.83
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025
Na_2EDTA	37.3	37.3	37.3

$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8
Organic nutrients			
Inositol	100	100	100
Thiamine·HCl	0.4	0.4	0.4
Pyridoxine·HCl	0.5	–	–
Nicotinic acid	0.5	–	–
Glycine	2.0	–	–
Ca-pantothenate	–	2.0	–
Growth regulators			
Gibberellic acid	0.1	0.4	–
Benzylamino purine	–	0.5	5.0
Naphthalene acetic acid	–	0.01	–
Chlorocholine chloride	–	–	500
Sucrose	0.3%	2%	8%
Gelrite	1.9	–	–

[a]Semi-solid medium, for initiation of shoot cultures and initial 3–4 cycles of shoot multiplication.
[b]Liquid medium for shoot multiplication.
[c]Tuber formation medium.

16.II.6. *Gladiolus*, shoot multiplication medium (after Dantu and Bhojwani, 1992)

Constituents	Amounts (mg l^{-1})
Macronutrients	
NH_4NO_3	825
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	400
$MgSO_4 \cdot 7H_2O$	370
$NaH_2PO_4 \cdot 2H_2O$	300
Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.125
$CuSO_4 \cdot 5H_2O$	0.0125
$CoCl_2 \cdot 6H_2O$	0.0125
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
Organic nutrients	
myo-Inositol	100.0
Nicotinic acid	0.5
Pyridoxine·HCl	0.5

Thiamine·HCl	0.1
Glycine	2.0
Growth regulator	
BAP	0.5
Sugar cubes	30000

16.II.7. Papaya (after Drew et al., 1991)

Constituents	Amounts (mg l^{-1})			
	Initiation[a] of shoot culture	Shoot[b] multiplication	Rooting[c]	Single[d] shoot
Inorganic nutrients				
NH_4NO_3	1600	1600	800	1600
KNO_3	2022.20	2022.20	1011.10	2022.2
$NaH_2PO_4·2H_2O$	312.02	312.02	156.01	312.02
$CaCl_2·2H_2O$	441.06	441.06	294.04	441.06
$MgSO_4·7H_2O$	739.41	739.41	369.70	739.41
H_3BO_3	9.27	9.27	3.09	9.27
$MnSO_4·H_2O$	16.90	16.90	8.45	16.90
$ZnSO_4·7H_2O$	11.50	11.50	5.75	11.50
$CuSO_4·5H_2O$	0.37	0.37	0.02	0.37
$Na_2MoO_4·2H_2O$	0.24	0.24	0.02	0.24
$CoCl_2·6H_2O$	0.23	0.23	0.11	0.23
KI	0.83	0.83	0.41	0.83
$FeSO_4·7H_2O$	27.80	27.80	13.90	27.80
Na_2EDTA	37.22	37.22	18.61	37.22
Organic nutrients				
Inositol	108.12	108.12	54.06	108.12
Nicotinic acid	4.92	4.92	2.46	4.92
Pyridoxine·HCl	1.23	1.23	0.61	1.23
Thiamine·HCl	13.49	13.49	0.67	13.49
Biotin	0.24	0.24	0.04	0.24
Folic acid	0.88	0.88	0.44	0.88
Ca-pantothenate	2.38	2.38	0.47	2.38
Riboflavin	3.76	3.76	0.37	3.76
Ascorbic acid	1.98	1.98	0.19	1.98
l-Cysteine·HCl	37.58	37.58	18.79	37.58
Glycine	3.75	3.75	0.37	3.75
Choline chloride	1.39	1.39	0.13	1.39
Growth regulators				
BAP	0.22	0.45	–	–
NAA	0.18	0.09	–	–

IBA	–	–	2.03	–
Sucrose	20000	20000	20000	20000
Agar	8000	8000	8000	8000

[a]A good method of forcing lateral buds is to put the whole node, with lateral buds attached, onto a simple medium of high minerals, 2% sucrose and 0.8% agar. Smith and Drew (1990c) have suggested that healthy lateral shoots for culture initiation can be obtained by decapitating 30 cm plantlets growing in glasshouse and application of lanolin paste containing 225 mg l^{-1} BAP to the cut surface.
[b]Lateral shoot growth can also be promoted by pruning roots and removing the apical bud before placing the plantlets on multiplication medium. Subculture every 2–3 weeks. Alternate culture on BAP-containing and BAP-free media may help in maintaining the quality of the shoots (Smith and Drew, 1991).
[c]As soon as roots are initiated transfer the plantlets to single-shoot medium.
[d]On this medium shoots with strong apical dominance and healthy root system will be produced.

16.II.8. *Pyrus communis* (composition of Lepoivre medium)[a]

Constituents	Amount (mg l^{-1})
Inorganic nutrients	
NH_4NO_3	400
$Ca(NO_3)_2 \cdot 4H_2O$	1200
KNO_3	1800
KH_2PO_4	270
$MgSO_4 \cdot 7H_2O$	360
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.2
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
Organic nutrients	
myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine·HCl	0.5
Thiamine·HCl	0.4
Sucrose	30000
Agar	7000
pH	5.0

[a]After Quoirin et al. (1977).

16.II.9. Walnut (after Leslie and McGranahan, 1992)

Constituents	Amounts (mg l^{-1})	
	Shoot multiplication	Prerooting treatment
Inorganic nutrients		
NH_4NO_3	1416.0	456.2
$Ca(NO_3)_2{\cdot}4H_2O$	1968.0	634.0
K_2SO_4	1559.0	1559.0
$MgSO_4{\cdot}7H_2O$	740.0	740.0
$CaCl_2{\cdot}2H_2O$	149.0	149.0
KH_2PO_4	265.0	265.0
$Zn(NO_3)_2{\cdot}6H_2O$	17.0	17.0
$MnSO_4{\cdot}H_2O$	33.5	33.5
$CuSO_4{\cdot}5H_2O$	0.25	0.25
H_3BO_3	4.8	4.8
$Na_2MoO_4{\cdot}2H_2O$	0.39	0.39
$FeSO_4{\cdot}7H_2O$	33.8	33.8
Na_2EDTA	45.4	45.4
$NiSO_4{\cdot}6H_2O$	0.005	0.005
Organic nutrients		
myo-Inositol	100	100
Thiamine·HCl	2.0	2.0
Nicotinic acid	1.0	1.0
Glycine	2.0	2.0
Growth regulators		
BAP		
IBA	0.01	0.15
Sucrose	3%	3%
Gelrite	0.2%	0.24%

16.II.10. *Simmondsia chinensis* (after Chaturvedi and Sharma, 1989)

Constituents	Amounts (mg l^{-1})		
	MSH-I (Initiation)	MSH-II (Multiplication)	MSH-III (Rooting)
Inorganic nutrients			
NH_4NO_3	500	500	–
KNO_3	2500	2500	500

$MgSO_4 \cdot 7H_2O$	400	400	200
$NH_4H_2PO_4$	300	300	300
$CaCl_2 \cdot 2H_2O$	200	200	100
$MnSO_4 \cdot H_2O$	10	10	10
H_3BO_3	5	5	5
$ZnSO_4 \cdot 7H_2O$	1	1	1
KI	1	1	1
$CuSO_4 \cdot 5H_2O$	0.2	0.2	0.2
$CoCl_2 \cdot 6H_2O$	0.1	0.1	0.1
$NaMoO_4 \cdot 2H_2O$	0.1	0.1	0.1
$FeSO_4 \cdot 7H_2O$	15	15	15
Na_2EDTA	20	20	20
Organic nutrients			
Inositol	100	100	100
Thiamine·HCl	0.1	0.1	0.1
Nicotinic acid	0.5	0.5	0.5
Pyridoxine·HCl	0.1	0.1	0.1
Growth regulators			
BAP	1	0.5	–
IAA	1	1.0	–
IBA	–	–	7
NAA	–	–	1
Adenine sulphate	–	15	–
Caffeic acid	–	–	1
Sucrose	30000	30000	70000
Agar	6000	6000	6000
pH	5.8	5.8	5.0