inside a clear plastic enclosure and misted by hand. With some plants, an *in vitro* Stage III can be omitted; shoots from Stage II are rooted directly in high humidity, and, at the same time, gradually hardened to the exterior environment.

The rooting of shoots using these methods is discussed fully in Volume 2.

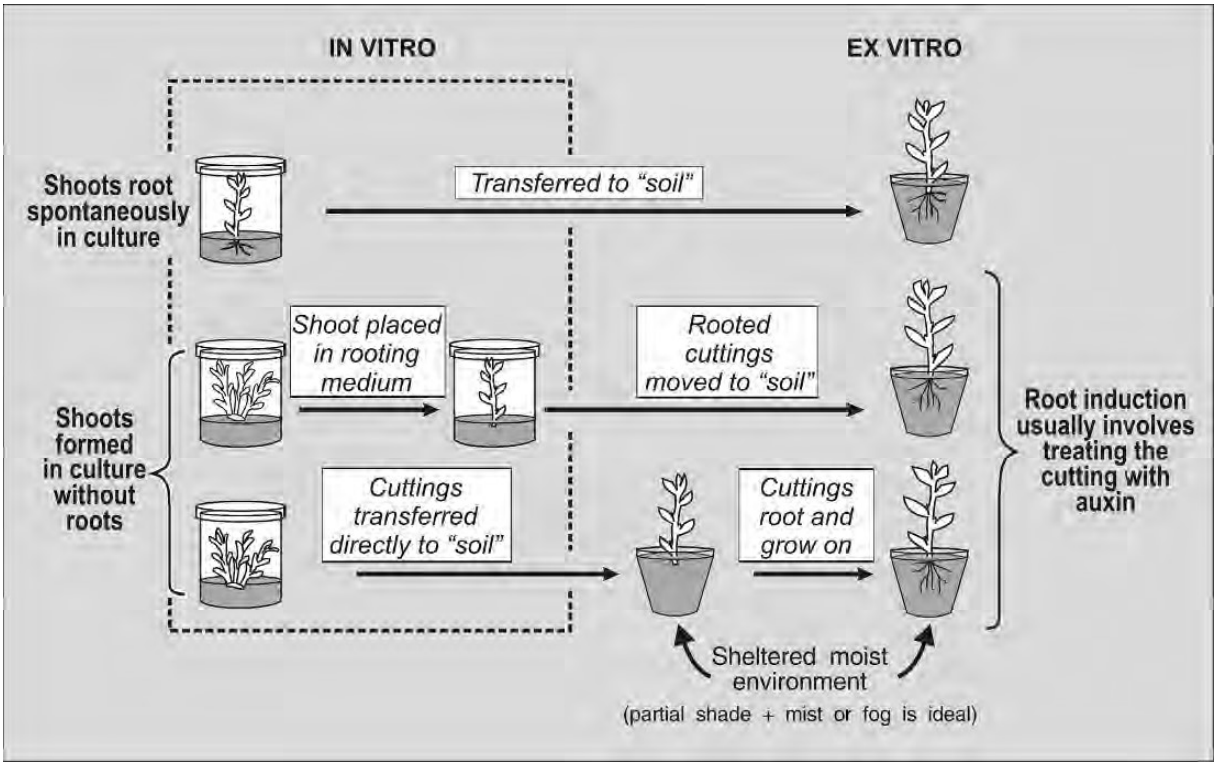

Fig. 2.2 Alternative methods of rooting micropropagated shoots.

3. MICROPROPAGATION METHODS

3.1. THE PROPAGATION OF PLANTS FROM AXILLARY BUDS OR SHOOTS

The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation. Two methods are commonly used:

- Shoot culture
- Single, or multiple, node culture.

Both depend on stimulating precocious axillary shoot growth by overcoming the dominance of shoot apical meristems.

3.1.1. Shoot (or shoot tip) culture

The term shoot culture is now preferred for cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by the repeated formation of axillary branches. In this technique, newly formed shoots or shoot bases serve as explants for repeated proliferation; severed shoots (or shoot clumps) are finally rooted to form plantlets which can be grown *in vivo*. This is the most widely used method of micropropagation.

Explant size. Shoot cultures are conventionally started from the apices of lateral or main shoots, up to 20 mm in length, dissected from actively-growing shoots or dormant buds. Larger explants are also sometimes used with advantage: they may consist of a larger part of the shoot apex or be stem segments bearing one or more lateral buds; sometimes shoots from other *in vitro* cultures are employed. When apical or lateral buds were used almost exclusively as explants, the name 'shoot tip culture' came to be widely used for cultures of this kind. As the use of larger explants has become more common, the term shoot culture has become more appropriate.

Large explants have advantages over smaller ones for initiating shoot cultures in that they:

- better survive the transfer to *in vitro* conditions
- more rapidly commence growth
- contain more axillary buds

However, the greater the size of the explant, the more difficult it may be to decontaminate from micro-organisms; in practice the size used will be the largest that can be gained in aseptic conditions. Shoot cultures are also frequently started directly from the shoots obtained from meristem tip cultures. Virus eradication then proceeds the shoot multiplication phase. Occasionally fragmented or macerated shoot tips are used (see elsewhere in this Chapter). Meristem tip or meristem cultures are used for virus and bacteria elimination. Meristem cultures are initiated from much smaller explants and a single plantlet is usually produced from each. This terminology is very often abused.

Regulating shoot proliferation

The growth and proliferation of axillary shoots in shoot cultures is usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Most often such a treatment effectively removes the dominance of apical meristems so that axillary shoots are produced, often in large numbers. These shoots are used as miniature cuttings for plant multiplication.

Removing the apex. In some plants, pinching out the main shoot axis is used as an alternative, or an adjunct, to the use of growth regulators for decreasing apical dominance. Pinching was found to be effective for some kinds of rose (Bressan *et al.,* 1982) and for some apple cultivars (Yae *et al.,* 1987). Pinching or 'tipping' is usually done when plant material is removed for subculturing, for example removing the apical bud at the first subculture increased the branching of *Pistacia* shoot cultures (Barghchi, 1986). An effective kind of shoot tipping occurs when shoots are cropped as microcuttings. Standardi (1982) and Shen and Mullins (1984) obtained effective shoot proliferation of kiwi and pear varieties by transferring the basal shoot clump that is left at this stage, to fresh medium for further proliferation. (Note however that this practice can increase the likelihood of obtaining deviant plants - see below). In just a few plants neither cytokinins nor pinching effectively remove apical dominance. Geneve *et al.* (1990) reported that seedling shoots of *Gymnocladus dioicus* produced 1–5 shoots, but only one grew to any appreciable length. If this shoot was removed, another took over.

Placing explants horizontally. In pear, pinching out the tips of shoots resulted in the growth of larger axillary shoots than in the controls, but the number of shoots was less. The most effective physical check to apical dominance was achieved by pinching the tips, and/or placing shoot explants horizontally on the medium (Lane, 1979; MacKay and Kitto, 1988). The treatment can be effective with many other woody plants: horizontal placement of shoot sections, consisting of 2-3 nodes, resulted in more axillary shoots being produced in cultures of *Acer rubrum, Amelanchier spicata*, *Betula nigra, Forsythia intermedia* and *Malus domestica*, than when explants were upright (McClelland and Smith, 1990). Favourable results have also been reported with lilac (Hildebrandt and Harney, 1983) and some apple cultivars (Yae *et al.,* 1987).

The origin of shoots

Unfortunately not all the shoots arising in shoot cultures may originate from axillary buds. Frequently, adventitious shoots also arise, either directly from cultured shoot material, or indirectly from callus at the base of the subcultured shoot mass. For example, Nasir and Miles (1981) observed that in subcultures of an apple rootstock, some new shoots arose from callus at the base of the shoot clump; both adventitious and axillary shoots were produced in *Hosta* cultures (Papachatzi *et al.,* 1981); and shoot proliferation from some kinds of potato shoot tips was exclusively from organogenic callus (Roca *et al.,* 1978).

The precise origin of shoots can sometimes only be determined from a careful anatomical examination. Hussey (1983) has termed cultures providing both adventitious and axillary shoots, 'mixed cultures'. Adventitious shoots, particularly those arising indirectly from callus, are not desirable. For reasons described in Volume 2, shoots of axillary origin will normally be genetically identical to the parent plant, whereas there is a probability that those regenerated from callus may differ in one or more characters. Genetically deviant plants may not occur with high frequency from newly initiated callus, but could begin to appear in significant number if shoot masses incorporating basal callus are simply chopped up to provide explants for subculture. The use of a strict protocol, using only axillary shoots, may present problems with some plants where the rate of shoot multiplication is comparatively slow. This has led to attempts by some workers to use a more relaxed regime and accept a proportion of

adventitious shoots (e.g. with *Kalanchoe blossfeldiana* - Schwaiger and Horn, 1988). The usual consequence is a degree of variation amongst ramets which may, or may not, be acceptable. The formation of callus and the subsequent development of adventitious shoots can often be controlled by modifying the growth regulators in the medium.

Fragmentation of a meristem tip, or its culture in a certain way, can lead to the formation of multiple adventitious shoots which can be used for plant propagation. These modifications of conventional shoot culture are described on in Chapter 10.

History

Although shoot culture has proved to be a widely applicable method of micropropagation, the appreciation of its potential value developed only slowly, and utilisation largely depended on improvements in tissue culture technology. Robbins (1922) seems to have been the first person to have successfully cultured excised shoot tips on a medium containing sugar. Tip explants of between 1.75 and 3.75 mm were taken from pea, corn and cotton, and placed in a liquid medium. For some reason the cultures were maintained in the dark where they only produced shoots with small chlorotic leaves and numerous roots. Although it is tempting to suppose that the potential of shoot culture for plant propagation might have been appreciated at a much earlier date had the cultures been transferred to the light, the rapid rate of shoot multiplication achieved in modern use of this technique depends on later developments in plant science.

Only very slow progress in shoot culture was made during the next 20 years. As part of his pioneering work on plant tissue culture, White (1933) experimented with small meristem tips (0.1 mm or less) of chickweed (*Stellaria media*), but they were only maintained in hanging drops of nutrient solution. Leaf or flower primordia were observed to develop over a six-week period. Shoot culture of a kind was also carried out by La Rue (1936). His explants largely consisted of the basal and upper halves of seed embryos. Nevertheless, the apical plumular meristems of several plants were grown to produce entire plants. Whole plants were also obtained from axillary buds of the aquatic plant *Radicula aquatica*.

Significant shoot growth from vegetative shoot tip explants was first achieved by Loo, and reported in 1945 and 1946 a, b. *Asparagus* shoot tips 5-10 mm in length were supported on glass wool over a liquid medium and later grown on a solidified substrate. Loo (1945, 1946a, 1946b) made several significant observations showing that:

- growth depended on sucrose concentration, higher levels being necessary in the dark than in the light;
- explants, instead of being supported, could be grown satisfactorily on 0.5% agar;
- *in vitro* shoot growth could apparently be continued indefinitely (35 transfers were made over 22 months);
- shoot tip culture afforded a way to propagate plant material (clones were established from several excised shoot apices).

This work failed to progress further because no roots were formed on the *Asparagus* shoots in culture. Honours for establishing the principles of modern shoot culture must therefore be shared between Loo and Ball. Ball (1946) was the first person to produce rooted shoots from cultured shoot apices. His explants consisted of an apical meristem and 2–3 leaf primordia. There was no shoot multiplication but plantlets of nasturtium (*Tropaeolum majus*) and white lupine (*Lupinus alba*) were transferred to soil and grown successfully.

During several subsequent years, shoot apex (or meristem tip) culture was of interest only to plant pathologists who recognised its value for producing virus-tested plants. It was during studies of this kind that Morel made the significant discovery of protocorm formation from *Cymbidium* orchid shoot tips. Although they may be started from the same explants, cultures giving rise to protocorms are not typical shoot cultures (see later in this chapter).

The two major developments which made shoot culture feasible were the development of improved media for plant tissue culture (Murashige and Skoog, 1962) and the discovery of the cytokinins as a class of plant growth regulators (Miller, 1961b; Skoog *et al.,* 1965), with an ability to release lateral buds from dormancy (Wickson and Thimann, 1958; Sachs and Thimann, 1964). These developments were not immediately applied to shoot culture, and some years elapsed before it was appreciated that multiple shoots could be induced to form by appropriate growth regulator treatments.

Hackett and Anderson (1967) got either single shoots from carnation shoot apices, or else a proliferative tissue from which shoots were later regenerated. Walkey and Woolfitt (1968) reported a similar kind of direct or indirect shoot proliferation from *Nicotiana rustica* shoot tips. Vine and Jones (1969) were able to transfer large shoot tips of hop (*Humulus*) to culture, but shoots only rooted, and

showed a high propensity for callus formation. Reports of plant multiplication using conventional shoot culture methods began to appear in the next decade.

Haramaki (1971) described the rapid multiplication of *Gloxinia* by shoot culture and by 1972 several reports of successful micropropagation by this method had appeared (Adams, 1972; Haramaki and Murashige, 1972). Since then the number of papers on shoot culture published annually has increased dramatically and the method has been utilised increasingly for commercial plant propagation. Factors which have influenced the choice of shoot culture for practical micropropagation have been:

- the way in which the method can be applied to a wide range of different plant species, using the same principles and basic methods;
- the possibility of obtaining simultaneous virus control;
- a general uniformity and 'trueness to type' of the regenerated plants;
- the relatively high rates of propagation which is possible in many species.

Methods

Primary explants In most herbaceous plants, shoot tip explants may be derived from either apical or lateral buds of an intact plant, and consist of a meristematic stem apex with a subtended rudimentary stem bearing several leaf initials (Fig 2.3). In the axils of the more developed leaf primordia there will be axillary bud meristems. In some species (e.g. *Eucalyptus*) it is an advantage to commence shoot cultures with a piece of the stem of the mother plant bearing one or more buds (stem nodes). Shoot growth from the bud, and treatment of the culture, is thereafter the same as in conventional shoot tip culture. The use of nodal explants should not be confused with node culture in which a method of shoot multiplication is used that is different to that in shoot culture (see later).

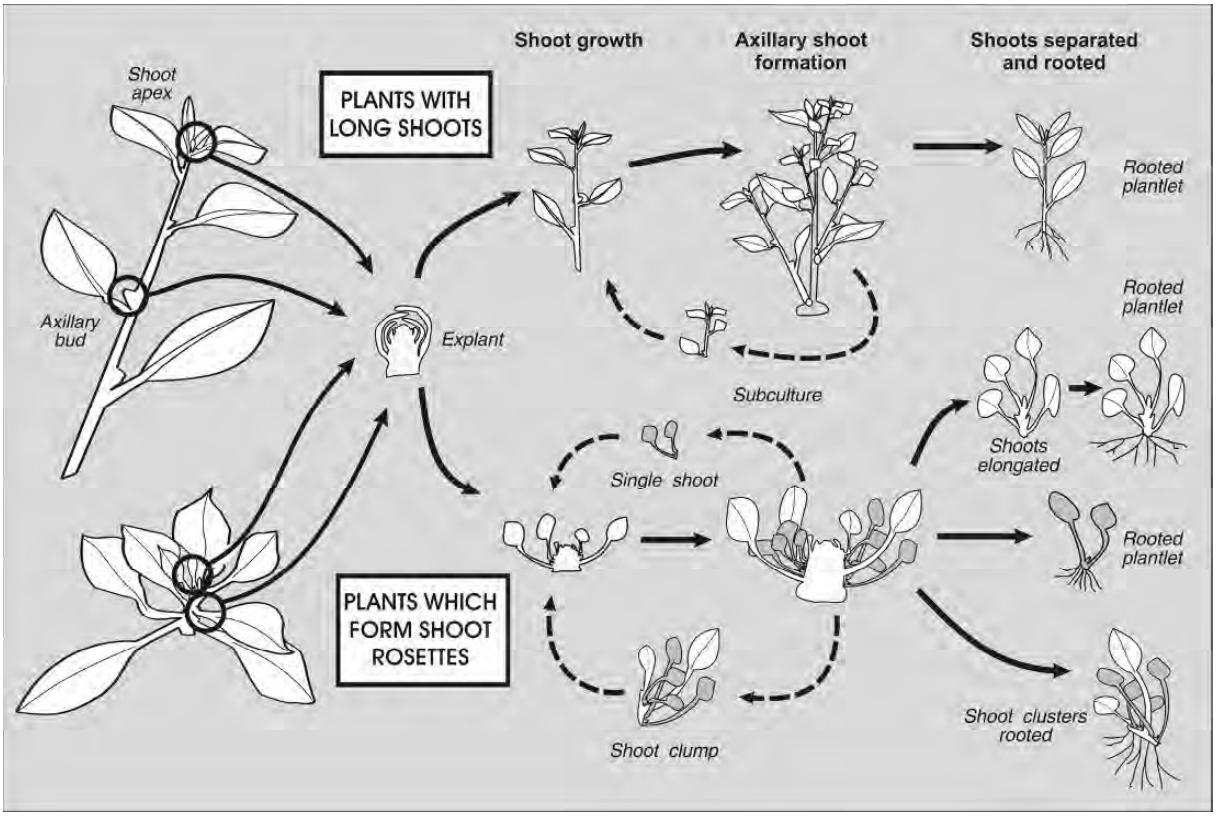

Fig. 2.3 Shoot tip culture.
Separation of axillary shoots for rooting (or subculture) is easier in species which naturally produce long shoots.

Shoot tips from trees, or other woody perennials, can be difficult to decontaminate. Because of this, Standardi and Catalano (1985) preferred to initiate shoot cultures of *Actinidia chinensis* from meristem

tips which could be sterilised more easily. Shoot tips of woody plants are more liable than those of herbaceous species to release undesirable phenolic substances when first placed onto a growth medium. Buds taken from mature parts of the shrub or tree can also be reluctant to grow *in vitro* and seasonal factors may reinforce natural dormancy in buds from any source, so that cultures can only be readily initiated at certain times of the year (see Chapter 11). Shoot tip or lateral bud explants are usually most readily induced into growth if taken from juvenile shoots (see Chapter 11) such as those of seedlings or young plants. The juvenile shoots which sometimes emerge from the base of mature plants or which arise form heavily pruned or coppiced bushes and trees, are alternative sources. However, developing techniques have made it possible to propagate some woody ornamentals, forest trees and fruit trees, using explants derived from mature shoots (see Chapter 11). De Fossard *et al.* (1977) could initiate cultures of *Eucalyptus ficifolia* with shoot tips from 36 year-old trees, but forest-gathered material was very difficult to decontaminate unless covered and protected for some period before excision (stage 0).

Secondary explants. Stage II subcultures are initiated from axillary shoots separated from primary shoot clusters. The place of the secondary explant within the primary shoot (cluster) can have a remarkable influence on the subsequent performance of the subcultures. A higher rate of shoot proliferation is often obtained from nodal explants or by subdivision of the basal shoot mass. Shoot tips were the best secondary explant for *Rosa* 'Fraser McClay', but with cherry ('F12/1') nodal explants gave more than twice as many shoots, and basal masses, three times as many as shoot tips (Hutchinson, 1985). In Sitka spruce, cultures that had been apices in the previous subculture were able to proliferate buds at higher rates than those that had been axillary buds (John and Murray, 1981). The origin of an explant can also have a tremendous influence on the subsequent behaviour of the plant when established under field conditions. This was illustrated by Marks and Meyers (1994) for *Daphne odorata.*

To minimise the risk of genetic change in ramets, explants for subculture and shoots to be transferred to Stage III, should, as far as possible, be chosen from new shoots of axillary origin. It may be advisable to adjust the growth regulator content of the medium so that adventitious shoots are not formed, even though the rate of overall shoot multiplication is thereby reduced. In some circumstances callus arising at the base of an explant may be semi-organised and therefore capable of producing genetically-stable plants (Vol. 2).

Stage II cultures are typically without roots, and shoots need to be detached and treated as miniature cuttings which, when rooted, will provide the new plants that are required. An alternative is to allow shoot clusters to elongate and to root singulated shoots under *ex vitro* conditions (Fig 2.2).

Media and growth regulators Advice on the selection of appropriate media for shoot cultures is given in Volume 2. A notable feature of shoot cultures of most plant species is the need for high cytokinin levels at Stage II to promote the growth of multiple axillary shoots. A description of the compounds which can be employed and effective rates of treatment are given in Chapters 3–7.

Cytokinin growth regulators are usually extremely effective in removing the apical dominance of shoots. Their use can be combined with pinching the apex of shoots, or placing explants in an horizontal position (Chapter 6). A cytokinin treatment can not only promote the formation of multiple shoots (axillary and/or adventitious), but also (if the compound used is unsuitable, or the concentration used is too high), cause the shoots formed to be too short for rooting and transfer.

Because or their nature, or the absence of an adequate method of culture, plants of some kinds fail to produce multiple shoots at Stage II and retain their apical dominance. In shoot cultures of *Gymnocladus dioicus*, for example, despite the formation of several axillary shoots in the presence of BA cytokinin, one shoot nearly always became dominant over the others (Geneve *et al.,* 1990). Most plants of this kind are best propagated by node culture (see below).

Elongation

The length of the axillary shoots produced in shoot cultures varies considerably from one kind of plant to another. Species which have an elongated shoot system *in vivo* will produce axillary shoots which can be easily separated as microcuttings and then individually rooted. Apically dominant shoots which have not branched can be treated in the same way.

At the other extreme are plants with a natural rosette habit of growth, which tend to produce shoot clusters in culture (Fig 2.3). When these are micropropagated, it is difficult to separate individual shoots for use as secondary explants. It may then only be practical to divide the shoot mass into pieces and re-culture the fragments. Such shoot clusters can be

induced to form roots when plants with a bushy habit are required (e.g. many species sold in pots for their attractive foliage). Otherwise it is necessary to specially elongate shoots before they are rooted (Stage IIIa). Shoot clusters are treated in such a way that axillary shoot formation is reduced, and shoot growth promoted. Individual shoots are then more readily handled and can be rooted as microcuttings. Methods for elongating shoots are discussed in Volume 2.

Rooting and transfer The cytokinin growth regulators added to shoot culture media at Stage II to promote axillary shoot growth, usually inhibit root formation. Single shoots or shoot clusters must therefore be moved to a different medium for rooting *in vitro* before being transferred as plantlets to the external environment. An alternative strategy for some plants is to root the plant material *ex vitro*. The methods employed are described in Volume 2. Treatments need to be varied according to the type of growth; the nature of the shoot proliferation produced during Stage II culture; and the plant habit required by the customer.

Current applications Conventional shoot culture continues to be the most important method of micropropagation, although node culture is gaining in importance. It is very widely used by commercial tissue culture laboratories for the propagation of many herbaceous ornamentals and woody plants (see Volume 2 for further details). The large numbers of manipulations required do, however, make the cost of each plantlet produced by this method comparatively expensive. Some success has been achieved in automating some stages of the process, in applying techniques for large-scale multiplication and in the use of robotics for plant separation and planting (Vol. 2).

3.1.2. Shoot proliferation from meristem tips

Barlass and Skene (1978; 1980a,b; 1982a,b) have shown that new shoots can be formed adventitiously when shoot tips of grapevine or *Citrus* are cut into several pieces before culture. Tideman and Hawker (1982) also had success using fragmented apices with *Asclepias rotundifolia* but not with *Euphorbia peplus*. Usually leaf-like structures first develop from the individual fragments; these enlarge and shoots form from basal swellings. Axillary shoots often arise from the initial adventitious shoots.

Shoot cultures transferred to agitated liquid culture may form a proliferating mass of shoots. Although high rates of multiplication are possible, leafy shoots usually become hyperhydric (see Volume 2). However, in some species at least, shoots can be reduced in size to little more than proliferating shoot initials (by adding plant growth retardants, Ziv *et al*,, 1994) which are then suitable for large-scale multiplication (Volume 2). A somewhat similar kind of culture consisting of superficial shoot meristems on a basal callus can sometimes be initiated from shoot tip explants or from the base of shoot cultures (see later in this chapter).

3.1.3. Single and multiple node culture (in vitro layering)

Single node culture is another *in vitro* technique which can be used for propagating some species of plants from axillary buds. As with shoot culture, the primary explant for single node culture is a shoot apex, a lateral bud or a piece of shoot bearing one or more buds (i.e. having one or more nodes). When shoot apices are used, it can be advantageous to initiate cultures with large explants (up to 20 mm), unless virus-tested cultures are required, and small meristem-tips will be employed. Unbranched shoots are grown at Stage I until they are 5–10 cm in length and have several discrete and separated nodes. An environment that promotes etiolated shoot growth may be an advantage. Then at Stage II, instead of inducing axillary shoot growth with growth regulators (as in shoot culture), one of two manipulative methods is used to overcome apical dominance and promote lateral bud break (Fig 2.2):

- intact individual shoots may be placed on a fresh medium in an horizontal position. This method has been used by Wang (1977) to propagate potatoes, and has been termed '*in vitro* layering';
- each shoot may be cut into single-, or several-node pieces which are sub-cultured. Leaves are usually trimmed so that each second stage explant consists of a piece of stem bearing one or more lateral buds.
- each approach can be reiterated to propagate during stage II.

Unfortunately, *in vitro* layering seldom results in several axillary shoots of equal length, as shown in Fig 2.4; apical dominance usually causes the leading shoot, or shoots, to grow more rapidly than the rest. El Hasan and Debergh (1987) found that, even in potato, node culture was preferable. Node culture is therefore the simplest method of *in vitro* propagation, as it requires only that shoot growth should occur. Methods of rooting are the same as those employed for the microcuttings derived from shoot culture, except that prior elongation of shoots is unnecessary.

Note that "node culture" is distinct from shoot cultures started from the nodes of seedlings or mature plants.

Media and growth regulators.

Media for single node culture are intrinsically the same as those suitable for shoot culture. As in shoot culture, optimum growth rate may depend on the selection of a medium particularly suited to the species being propagated, but adequate results can usually be produced from well-known formulations. It is often unnecessary to add growth regulators to the medium; for example, the shoots of some plants (e.g. *Chrysanthemum morifolium*) elongate satisfactorily without any being provided. If they are required, regulants at both Stages I and II will usually comprise an auxin and a cytokinin at rates sufficient to support active shoot growth, but not tissue proliferation or lateral bud growth. Sometimes gibberellic acid is advantageously added to the medium to make shoots longer and thus facilitate single node separation.

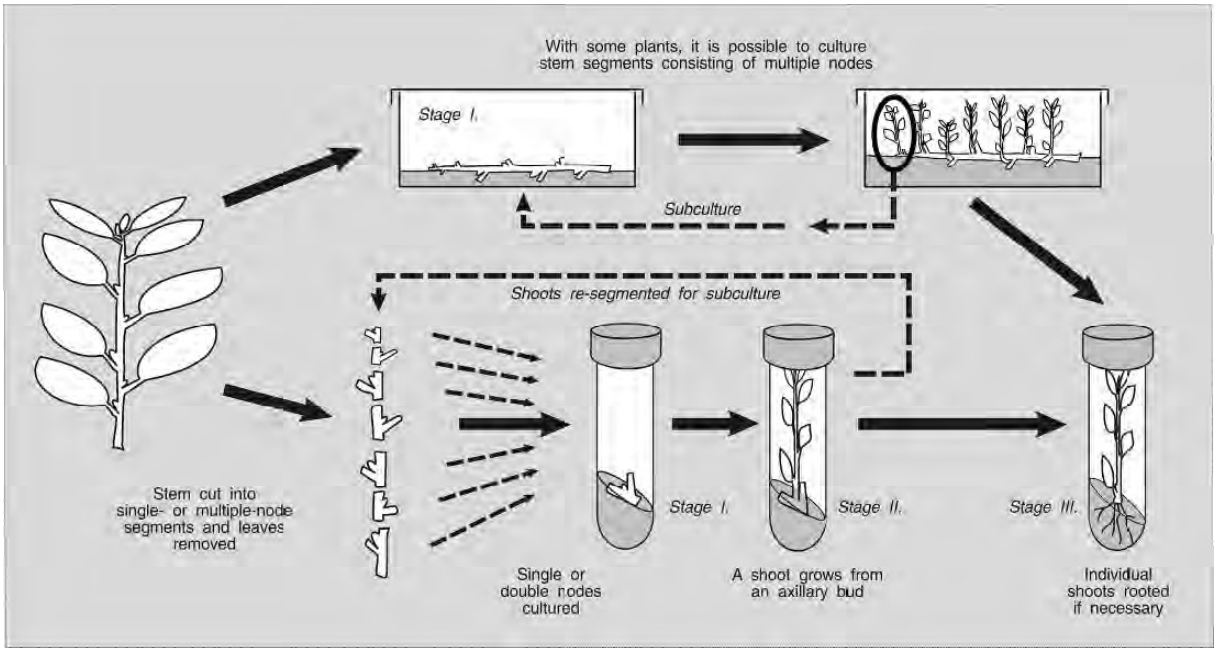

Fig. 2.4 Single and multiple node culture
Stage one cultures can be initiated also from meristem or shoot tips.

Current applications

Node culture is of value for propagating species that produce elongated shoots in culture (e.g. potato and *Alstroemeria*), especially if stimulation of lateral bud break is difficult to bring about with available cytokinins. Nowadays the technique becomes more and more popular in commercial micropropagation. The main reason is that it gives more guarantee for clonal stability. Indeed, although the rate of multiplication is generally less than that which can be brought about through shoot culture, there is less likelihood of associated callus development and the formation of adventitious shoots, so that Stage II subculture carries very little risk of induced genetic irregularity. For this reason, node culture has been increasingly recommended by research workers as the micropropagation method least likely to induces somaclonal variation.. Some of the plants for which node culture has been described are listed in Table 2.2.

3.1.4. Multiple shoots from seeds (MSS)

During the early 1980's it was discovered that it was possible to initiate multiple shoot cultures directly from seeds. Seeds are sterilised and then placed onto a basal medium containing a cytokinin. As germination occurs, clusters of axillary and/or adventitious shoots ('multiple shoots') grow out, and may be split up and serially subcultured on the same medium. High rates of shoot multiplication are possible. For instance, Hisajima (1982a) estimated that 10 million shoots of almond could be derived theoretically from one seed in a year.

It is likely that multiple shoots can be initiated from the seeds of many species, particularly dicotyledons. The technique is effective in both herbaceous and woody species: soybean (Cheng *et al.,* 1980; Hisajima, 1981; Hisajima and Church, 1981): sugar beet (Powling and Hussey, 1981): almond (Hisajima, 1981; 1982a,b,c): walnut (Rodriguez, 1982): pumpkin and melon (Hisajima, 1981): cucumber and pumpkin (Hisajima, 1981, 1982c): pea, peanut, mung bean, radish, *Zea mays* and rice (Hisajima, 1982c). This technique does only make sense when elite seed is used or to gain preliminary information on the behaviour of a plant species under *in vitro* conditions.

Table 2.2 Examples of the use of node culture in micropropagation

Monocotyledons	
Alstroemeria	Hussey *et al*. (1980)
Cymbopogon spp.	Jagadish Chandra and Sreenath (1982)
Poa pratensis	Pieper and Smith (1988)
Asparagus officinalis	Yang and Clore (1973, 1974a)
Dioscorea spp.	Ammirato (1976, 1982), Chaturvedi and sinha (1979b)
Zea mays	King and Shimamoto (1984)
Orchid monocots	
Dendrobium spp.	Ball and Arditti (1976)
Phalaenopsis spp.	Tanaka and Sakanishi (1978)
Thunia alba	Singh and Prakash (1984)
Vanilla planifolia	Kononowicz and Janick (1984a)
Herbaceous dicotyledons	
Angelonia salicariaefolia	Datta and Datta (1984)
Cucumis sativus	Handley and Chambliss (1979)
Glycyrrhiza glabra	Shah and Dalal (1980, 1982)
Rosmarinus officinalis	Misra and Chaturvedi (1984)
Rorippa nasturtium	Wainwright and Marsh (1986)
Solanum tuberosum	Hussey and Stacey (1981a) Kristensen (1984)
Solanum spp.	Haberlach *et al.* (1985), Levy (1988), Sihachakr *et al.* (1988)
Woody dicotyledons	
Carpinus betulus	Chalupa (1981a)
Castanea sativa *Castanea mollissima*	Vieitez and Vieitez (1980b) Yang *et al.* (1986)
Eucalyptus grandis	Cresswell and Nitsch (1975)
Forsythia ovata	Einset and Alexander (1985)
Fraxinus pennsylvanica	Einset and Alexander (1985)
Jugans regia	Dandekar *et al.* (*1988)*
Hevea brasiliensis	Chen Z. (1984)
Leucaena obtusifoliun	Einset and Alexander (1985)
Lonicera periclymemum	Boonnour *et al.* (1988)
Olea europea	Rugini and Fontenazza (1981)
Paulownia tomentosa	Burger *et al.* (1985)
Poncirus trifoliate	Barlass and Skene (1982b)
Prosopis julflora	Wainwright and England (1987)
Prunus armeniaca	Snir (1984)
Quercus robur	Chalupa (1984a,b)
Salix spp.	Chalupa (1981a, 1983)
Syringa spp.	Einset and Alexander (1985)
Syringa x *chinensis*	Welander N.T. (1987)

3.1.5. Shoots from floral meristems

Meristems that would normally produce flowers or floral parts can sometimes be induced to give vegetative shoots *in vitro*. Success depends on the use of young inflorescences where the determination of individual flower meristems is not canalized. Meristems in older inflorescences are likely to give rise to floral structures. Culture of immature inflorescence segments has, for example, resulted in shoot formation in:

- Bamboo - Gielis & Debergh (1998)
- Broccoli - Anderson and Carstens (1977)
- Cauliflower - Pow (1969), Margara (1969a,b,c; 1977a), Crisp and Walkey (1974), Grout and Crisp (1977), Trimboli et al. (1977)
- Coconut - Eeuwens and Blake (1977)
- *Dendranthema* - Shu O Wang and Su Shien Ma (1978)
- Date palm - Drira and Benbadis (1985)
- *Gerbera* - Topoonyanont and Dillen (1988)
- *Limonium* - Topoonyannt *et al*. (1999)
- Onion - Dunstan and Short (1977b; 1979a)
- Sugar beet - Coumans-Gilles *et a*l. (1981)

The exact origin of the shoots produced has not always been determined. In cauliflower and coconut they were thought to originate from actual flower meristems, but in sugar beet, from floral axillary buds. Some shoots formed from onion flower heads arose from various parts of the flower buds, but they were accompanied by other shoots which arose adventitiously over the entire receptacle surface. Shoots formed from young flower buds may therefore

not always result from the reversion of floral meristems. In fact the direct formation of adventitious shoots is more widely reported.

3.2. PROPAGATION BY DIRECT ORGANOGENESIS

3.2.1. Direct adventitious shoot initiation

In certain species, adventitious shoots which arise directly from the tissues of the explant (and not within previously-formed callus) can provide a reliable method for micropropagation. However, the induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived, and is highly dependent on plant genotype. In responsive plants, adventitious shoots can be formed *in vitro* on pieces of tissue derived from various organs (e.g. leaves, stems, flower petals or roots); in others species, they occur on only a limited range of tissues such as bulb scales, seed embryos or seedling tissues. Direct morphogenesis is observed rarely, or is unknown, in many plant genera.

Direct shoot formation is sometimes accompanied by proliferation of unorganised cells, and a regenerative tissue that could be classed as callus, may ultimately appear. Its formation can usually be reduced by adjustment of the growth regulators in the medium. Because there is a risk of regenerating plants with a different genetic identity (see Vol. 2), use of the callus for further propagation is not recommended unless it has a highly organised nature (see later). In some instances, the growth regulators used to initiate shoot buds directly on explants may not be conducive to continued bud growth. A closely packed mass of shoot primordia may then be mistaken for organised callus.

In those species where adult tissues have a high regenerative capacity, the main advantages of micropropagation by direct adventitious shoot regeneration are that:

- Initiation of Stage I cultures and Stage II shoot multiplication, are more easily achieved than by shoot culture. It is, for example, simpler to transfer aseptically several pieces of *Saintpaulia* leaf petiole to culture medium, than to isolate an equivalent number of shoot meristems.
- Rates of propagation can be high, particularly if numerous small shoots arise rapidly from each explant.

Stage I

Stage I consists of the establishment *in vitro* of suitable pieces of tissue, free from obvious contamination. As adventitious shoots are usually initiated on the tissue without transfer, Stages I and II are not generally discrete.

Stage II

Initially Stage II of this micropropagation method is recognised by the formation, growth and proliferation of adventitious shoots from the primary explant. Subsequently Stage II subcultures might, theoretically, be established from individual shoots by the techniques familiar in shoot culture. In practice, in plants such as *Saintpaulia*, both further adventitious and axillary shoots may develop in later stages of propagation. The result is a highly proliferative shoot mass and a very rapid rate of propagation. Subcultures are made by transferring shoot clumps (avoiding basal callus) to fresh media. In most commercial laboratories the micro-propagation of *Anthurium* species is initiated by adventitious shoot formation on leaf explants, followed by only axillary shoot development during the succeeding subcultures (Debergh *et al*,, 1990). Adventitious shoots sometimes arise directly from the leaves of plants during shoot culture. This often happens when leaves bend down to touch the semi-solid medium. Adventitious shoot formation of certain plants will take place in large vessels of aerated liquid medium, allowing the scale of propagation to be much increased (see the discussion on liquid media in Chapter 12).

Stage III

This is similar to the Stage III of most propagation systems. Individual shoots or shoot clumps are transferred to a nutrient medium with added growth regulators and ingredients that do not encourage further shoot proliferation and which promote rooting; alternatively shoots may be removed from culture and rooted *ex vitro*.

Some current applications

Several ornamental plants are at present propagated *in vitro* by direct shoot regeneration. Chief among these are plants of the family Gesneriaceae, (including *Achimenes, Saintpaulia, Sinningia* and *Streptocarpus*), where shoot buds can be freely regenerated directly on leaf explants without the formation of any intervening callus phase. Many other ornamentals and crop plants either are (or could be) propagated efficiently by this means, for example, begonias, *Epiphyllum*, cacti, *Gerbera, Hosta* and *Lilium*. Further examples are quoted in the tables of Volume 2. Remember that this technique is more prone to yield off-types than shoot and node cultures,

and that the technology is not applicable for the propagation of chimeras.

Regeneration from root pieces

In vitro shoot regeneration from root pieces is mainly reported from plants that possess thick fleshy roots such as those of the genera *Cichorium, Armoracia, Convolvulus*, and *Taraxacum*. It is, however, a method of propagation that is potentially applicable to a wide range of species (Browse, 1980; Hodge, 1986). Shoots have, for instance, been induced to form directly on segments and apices of the roots of *Citrus* and *Poncirus* seedlings (Sauton *et al.,* 1982). Shoot regeneration from root pieces does not offer a continuous method of micropropagation unless there is a ready supply of aseptic root material (e.g. from isolated root cultures). Roots grown in soil *in vivo* are usually heavily contaminated and can be difficult to sterilize to provide an adequate number of uncontaminated cultures. They can however be used as an initial source of shoots which can be multiplied afterwards by shoot culture [e.g. *Robinia* (Chalupa, 1992)].

Tissue maceration or fragmentation

The capacity of young fern tissue to regenerate adventitious shoots can be very high. Fern prothallus tissue (the gametophyte generation produced from germinating spores) has a high capacity for regeneration; a new prothallus can usually be grown from small isolated pieces of tissue (Whittier and Steeves, 1962), or even from single cells produced by maceration (Miller J.H., 1968; De Fossard, 1976; Knauss, 1976). Plants can also be regenerated from homogenised sporophyte tissue of some fern genera, and homogenisation has been incorporated into tissue culture, or partial tissue culture techniques for the propagation of plants of this class (see Volume 2).

Because a high proportion of the direct cost of micropropagation is attributable to the manual separation and transfer of explants and cultured material between media, the ability to regenerate plants from macerated or fragmented tissue would be extremely advantageous. Unfortunately there seem to be only a limited number of publications describing the formation of shoots directly from machine-macerated tissue of higher plants. One of them is the patent of Lindemann (1984), the claims of which may have been somewhat optimistic. Also Levin *et al.* (1997) reported on the regeneration of different plant species using a homogenisation technology. However, shoot regeneration from fragmented shoot tips, or micropropagation of some plants by culturing shoot material or tissue fragments in fermentors (Vol. 2), are somewhat comparable.

3.2.2. Organised calluses

In most callus cultures, shoots are produced from meristems which arise irregularly and may therefore be genetically altered. By contrast, so-called 'organised' or 'semi-organised' calluses are occasionally isolated in which there is a superficial layer of proliferating shoot meristems, overlaying an inner core of vacuolated cells acting as a mechanical and nutritional support. Calluses of this kind were termed organoid colonies by Hunault (1979): the names *meristemoids* and *nodules* have also been proposed. A meristemoid is defined as a cluster of isodiametric cells within a meristem or cultured tissue, with the potential for developmental (totipotential) growth. Meristemoids may give rise to plant organs (shoots, roots) or entire plants in culture (Donnelly and Vidaver, 1988). Nodules also comprise meristematic cells, but they are distinct from meristemoids because they are independent spherical, dense cell clusters which form cohesive units, with analogy to both mineral nodules in geology and root nodules of legumes (McCown *et al.,* 1988). Nodule culture has been extensively used for the propagation of *Cichorium intybus* (Pieron *et al.,* 1993).

The presence, in meristemoids, of an outer layer of shoot meristems seems to inhibit the unbridled proliferation of the unorganised central tissue (Hussey, 1983). Geier (1988) has suggested that the control mechanisms which ensure the genetic stability of shoot meristems are still fully, or partly, active. Maintenance of a semi-organised tissue system depends on a suitable method of subculture and upon the use of growth regulator levels which do not promote excessive unorganised cell growth. Repeated selective transfer of unorganised portions of an organised *Anthurium scherzerianum* callus eventually resulted in the loss of caulogenesis (Geier, 1986). Conversely, by consistently removing the unorganised tissue when subculturing took place, shoot formation from the callus was increased.

Cultures consisting of superficial shoot meristems above a basal callus, seem to occur with high frequency amongst those initiated from meristem tip, or shoot tip, explants. Hackett and Anderson (1967) induced the formation of tissue of this type from carnation shoot tips by mutilating them with a razor blade before culture. Similar cultures were also obtained from seedling plumular tip explants of two (out of five tested) varieties of *Pisum sativum* placed

on an agar medium (Hussey and Gunn, 1983; 1984). The calli were highly regenerative for 2-3 years by regular subculture to agar or shaken liquid medium. Maintenance was best achieved with an inoculum prepared by removing larger shoots and chopping the remainder of the callus and small shoots into a slurry. A callus, formed at the base of *Solanum curtilobum* meristem tips on filter paper bridges, gave rise to multiple adventitious shoots from its surface when transferred to shake culture in a liquid medium (Grout *et al.,* 1977).

Callus with superficial proliferative meristems has also been induced by culture of shoot or meristem tips on a rotated liquid medium, in:

- *Nicotiana rustica* (Walkey and Woolfitt, 1968);
- *Chrysanthemum morifolium* (Earle and Langhans 1974c);
- *Stevia rebaudiana* (Miyagawa *et al.,* 1986).

In *Stevia rebaudiana* (above), a slow rotation speed (2 rpm) was essential for initiation of an organised callus. A small callus formed upon the explant and in 2-3 weeks came to possess primary superficial shoot primordia which were globular and light green. Dark green aggregates of shoot primordia (termed 'secondary shoot primordia'' by Miyagawa *et al.,* 1986) were developed within 6 weeks. If divided, the aggregations of shoot initials in both *Nicotiana* and *Stevia* could be increased by subculture or, if treated to a different cultural regime, could be made to develop into shoots with roots. Shoots were produced from *Chrysanthemum* callus upon subculture to an agar medium. A spherical green dome-like structure was produced from meristem tips dissected from germinated *Eleusine coracana* (Gramineae) caryopses. When cut into four and subcultured, a green nodular structure was formed which grew to 5-10 mm in diameter. It was similar in appearance to a shoot dome, but much larger (a natural shoot dome is only 70-80 μm wide). The nodular structures were termed 'supradomes' by Wazizuka and Yamaguchi (1987) because, unlike normal callus, superficial cells were arranged in an anticlinal plane and those beneath had a periclinal arrangement. Numerous multiple buds could be induced to form when the organised tissue was subcultured to a less complex medium.

Although proliferative meristematic tissue formed from shoot tips always appears to be accompanied by a basal callus, the superficial meristematic cells may well be derived directly from the cells of the apical shoot meristem of the explant, for they preserve the same commitment to immediate shoot formation. The presence of the apical meristem in the explant seems to be essential and culture of tissue immediately beneath it does not produce a callus with the same characteristics (Hussey and Gunn, 1984). Similar semi-organised callus can appear at the base of conventional shoot cultures. In the green granular callus mass which formed at the base of *Rhododendron* shoot tips, each granule represented a potential shoot (Kyte and Briggs, 1979). Organised caulogenic callus is thus closely comparable to embryogenic callus formed from pre-embryogenically determined cells.

Organised callus can be produced from explants other than shoot tips; in *Anthurium*, it has been derived from young leaf tissue (above, Geier, 1986) and from spadix pieces (Geier, 1987).Organised callus has two characteristics which distinguish it from normal unorganised callus: the plants produced from it show very little genetic variation, and it can be subcultured for a very long period without losing its regenerative capacity. The callus of *Nicotiana* (above) was able to produce plantlets over a ten-year period, while that of *Chrysanthemum* gave rise to plants continuously during four years.

The use of cultures with superficial proliferative meristems has not yet been widely used for micropropagation. There are three possible reasons:

- the genetic variation which is almost invariably induced by shoot regeneration from normal callus, has cautioned against the use of any sort of callus culture for this purpose;
- organised callus may not always be readily distinguished from its unorganised counterpart;
- methods of initiating organised callus in a predictable fashion have not yet been fully elucidated.

There are examples of the initiation of organised callus from a sufficiently wide range of plant species (particularly from meristem tip explants) to suggest that it could be a method of general applicability. Multiplication may well be amenable to large-scale culture in fermentors (Vol. 2).

3.2.3 Direct embryogenesis

Somatic embryos are often initiated directly upon explanted tissues. Of the occurrences mentioned in Chapter 1, one of the most common is during the *in vitro* culture of explants associated with, or immediately derived from, the female gametophyte. The tendency for these tissues to give rise to adventitious somatic embryos is especially high in plants where sporophytic polyembryony occurs

naturally, for example, some varieties of *Citrus* and other closely related genera.

Ovules, nucellar embryos, nucellus tissues and other somatic embryos are particularly liable to display direct embryogenesis. In *Carica* somatic embryos originated from the inner integument of ovules (Litz and Conover, 1981a,b) and in carrot tissue of the mericarp seed coat can give rise to somatic embryos directly (Smith and Krikorian, 1988).

The nucellus tissue of many plants has the capacity for direct embryogenesis *in vitro* (Haccius and Hausner, 1976; Eichholtz *et al.,* 1979; Rangaswamy, 1982; Litz, 1987). As explained in Chapter 1, explants may also give rise to a proliferative tissue capable of embryogenesis. The high embryogenic competence of the nucellus is usually retained during subsequent cell generations *in vitro*, should the tissue be induced to form 'callus' (or cell suspensions). It is not clear whether all cells of the nucellus are embryogenically committed. In *Citrus,* somatic embryos are formed from the nucellus even in cultivars that are normally monoembryonic (i.e. the seeds contain just one embryo derived from the zygote), whether the ovules have been fertilised or not. It has been suggested that only those cells destined to become zygotic proembryos can become somatic proembryos or give rise to embryogenic callus (Sabharwal, 1963); somatic embryos have been shown to arise particularly from the micropylar end of *Citrus* nucellus.

Adventitious (adventive) embryos are commonly formed *in vitro* directly upon the zygotic embryos of monocotyledons, dicotyledons and gymnosperms, upon parts of young seedlings (especially hypocotyls and cotyledons) and upon somatic embryos at various stages of development (especially if their growth has been arrested). The stage of growth at which zygotic embryos may undergo adventive embryogenesis is species-dependent: in many plants it is only immature zygotic embryos which have this capacity. Unfortunately, as the phenotypic potential of seedlings is rarely known, using them as a source of clonal material is of limited value.

Embryogenic determination can be retained through a phase of protoplast culture. Protoplasts isolated from embryogenic suspensions, may give rise to somatic embryos directly, without any intervening callus phase (Miura and Tabata, 1986; Sim *et al.,* 1988). Treating protoplasts derived from leaf tissue of *Medicago sativa* with an electric field, induced them to produce somatic embryos directly upon culture (Dijak *et al.,* 1986).

Adventitious embryos arising on seedlings are sometimes produced from single epidermal cells (Konar *et al.,* 1972a; Thomas *et al.,* 1976 - see Chapter 1). Zee and Wu (1979) described the formation of proembryoids within petiole tissue of Chinese celery seedlings, and Zee *et al.* (1979) showed that they arose from cortical cells adjacent to the vascular bundles which first became meristematic. Hypocotyl explants from seedlings of the leguminous tree *Albizia lebbek* showed signs of cracking after two weeks of culture and frequently young embryoids emerged (Gharyal and Maheshwari, 1981). Stamp and Henshaw (1982) found that primary and secondary embryogenesis occurred in morphogenically active ridges produced on the surface of cotyledon pieces taken from mature cassava seeds.

Somatic embryos have been observed on the roots and shoots of *Hosta* cultures (Zilis and Zwagerman, 1980) and on the needles and cultured shoots of various gymnosperm trees (Bonga, 1976; McCown and Amos, 1982).

Protocorm formation in orchids

The seeds of orchids (like those of some other saprophytic or semi-parasitic plants) contain a small embryo of only about 0.1 mm diameter, without any associated endosperm storage tissue. Upon germination, the embryo enlarges to form a small, corm-like structure, called a protocorm, which possesses a quiescent shoot and root meristem at opposite poles. In nature, a protocorm becomes green and accumulates carbohydrate reserves through photosynthesis. Only when it has grown and has sufficient stored organic matter does it give rise to a shoot and a root. Normal seedling growth then continues utilising the stored protocorm food reserves.

Bodies which, in their structure and growth into plantlets, appear to be identical with seedling protocorms (except that on synthetic media they may not be green), are formed during *in vitro* culture of different types of orchid organs and tissues. These somatic protocorms can appear to be dissimilar to seedling protocorms, and many workers on orchid propagation, have used terms such as 'protocorm-like bodies' (PLBs) to describe them.

When a shoot tip of an orchid is transferred to culture on a suitable medium, it ceases to grow and to develop as a mature shoot apex; instead it behaves as though it were the apex of an embryo, i.e. it gives rise

to a protocorm (Vol. 2). Protocorm-like bodies also arise directly on some other orchid explants and proliferate from other PLBs in a fashion which is exactly comparable to the direct formation of somatic embryos.

Champagnat and Morel (1972) and Norstog (1979) considered the appearance of protocorms to be a manifestation of embryogenesis because they represent a specialised stage in embryo development and are normally derived directly from zygotic embryos. We think that this is the correct interpretation: in a previous edition of this book, protocorms were described under 'storage organs'.

Other protocorm-like structures. *In vitro* culture of small immature proembryos from developing barley seeds (Norstog, 1961, 1965a, 1970) or from the fern *Todea barbara* (De Maggio and Wetmore, 1961) has been noted to result in the formation of protocorm-like tissue masses from which root and shoots are regenerated after a period of irregular growth. Mapes (1973) recorded the appearance of such protocorm-like structures on shoot tips of pineapple, and Abo El-Nil and Zettler (1976) describe their direct formation on shoot tip explants of the yam *Colocasia esculenta*, or indirectly in subsequent callus cultures.

Embryogenesis from microspores or anther culture

Somatic embryos can be initiated directly from microspores. Usually it is necessary to culture the microspores within anthers, but occasionally it has been possible to induce embryogenesis from isolated microspores. Anther and microspore culture are described in Chapter 1, but because the plants produced by anther culture are likely to be dissimilar to their parents, we shall not consider the method in any detail in this book, and reports of anther culture have been largely omitted from the tables in Volume 2. Good references to this topic are available in the series of books published by Jain *et al.* (1996 and 1997).

Anther culture can result in callus formation; the callus may then give rise to plants through indirect embryogenesis or adventitious shoot formation.

Embryo proliferation

Accessory embryos on zygotic embryos. Occasionally new somatic embryos are formed directly on zygotic embryos that have been transferred to *in vitro* culture. Such adventitious embryos have been reported, for example, in: *Cuscuta reflexa* (Maheshwari and Baldev, 1961); barley (Norstog, 1970); *Ilex aquifolium* (Hu and Sussex, 1972; Hu, 1977; Hu *et al.,* 1978); *Thuja orientalis* (Konar and Oberoi, 1965); *Trifolium repens* (Maheswaran and Williams, 1985); *Zamia integrifolia* (Norstog, 1965b; Norstog and Rhamstine, 1967); *Theobroma cacao* (Pence *et al.,* 1980a,b); *Linum usitatissimum* (Pretova and Williams, 1986); *Vitis vinifera* (Stamp and Meredith (1988).

When direct embryogenesis occurs on pre-formed embryonic tissue, the newly formed embryos are sometimes termed direct secondary embryos or accessory embryos.

Accessory embryos on somatic embryos The *in vitro* induction of somatic embryogenesis starts a highly repetitive process, lacking some of the controls which must exist in nature during the formation of zygotic embryos. This results in the frequent development of small additional embryos on somatic embryos which have arisen directly on explants, or indirectly in callus and suspension cultures. Accessory embryos can occur along the whole axis of the original embryo, or grow preferentially from certain sites (e.g. the hypocotyl region or the scutellum of monocot. embryoids). In walnut, accessory embryos appear to arise from single cells of the epidermis of somatic embryos (McGranahan *et al.,* 1988).

Sometimes the term polyembryony is used to describe the formation of accessory, or secondary, embryos (Radojevic, 1988) (c.f. the term polyembryogenesis in Chapter 1). The process has also been called repetitive embryogenesis (Tulecke and McGranahan, 1985) or recurrent somatic embryogenesis (Lupotto, 1986). Such additional embryos are liable to be developed during all kinds of *in vitro* embryogenesis. They have been noted for example, on the somatic embryos formed in:

- Anther cultures: *Atropa belladonna* (Rashid and Street, 1973); *Brassica napus* (Thomas *et al.,* 1976); *Carica papaya* (Tsay and Su, 1985); *Citrus aurantifolia* (Chaturvedi and Sharma, 1985)*; Datura innoxia* (Geier and Kohlenbach, 1973)*; Vitis hybrids* (Rajasekaran and Mullins, 1979*)*;
- Suspension cultures: *Daucus carota* (Ammirato and Steward, 1971; McWilliam *et al.,* 1974); *Ranunculus scleratus* (Konar and Nataraja, 1965b; Konar *et al.,* 1972a);
- Callus cultures: *Aesculus hippocastanum* (Radojevic, 1988); alfalfa (when individual embryoids were transferred to a fresh medium) (Saunders and Bingham, 1972); carrot (Petrù, 1970); Citrus (Button and Kochba, 1977); parsley (Vasil and

Hildebrandt, 1966b); *Pennisetum purpureum* (Wang and Vasil, 1982); *Ranunculus sceleratus* (Konar and Nataraja, 1965a,b), *Theobroma cacao* (Li *et al.,* 1998).

Protocorms arising directly on explanted shoot tips or leaf pieces of orchids, frequently produce other adventive 'daughter' protocorms in culture, in a fashion that is similar to the adventive formation of somatic embryos. Somatic embryos formed in callus of oil palm have been reported to give rise to protocorm-like bodies, which regenerated shoots repeatedly as subculture was continued (Paranjothy and Rohani, 1982).

Practical uses in propagation

From a quantitative point of view, indirect embryogenesis does provide an efficient method of micropropagation; the same is not true of direct embryogenesis when it is unaccompanied by the proliferation of embryogenic tissue. Although plants can be regenerated from embryos directly initiated *in vitro*, and may be present in sufficient numbers for limited plant production in breeding programmes, the numbers of primary embryos per explant will usually be inadequate for large scale cloning. To increase the number of somatic embryos formed directly on immature zygotic embryos of sunflower, Freyssinet and Freyssinet (1988) cut larger zygotic embryos into four equal pieces.

Additional embryos are generally unwanted: they are frequently joined one to another as twins or larger groups so that abnormal seedlings with multiple shoots develop from them. The presence of accessory embryos can also impede the growth of the primary somatic embryo. Growth then becomes asynchronous and normal seedlings may not be obtained unless the adventive embryos are removed.

However it has been suggested that accessory embryos might be used for micropropagating some species. Perhaps this is a method of micropropagation which will be developed more in the future? Examples of where it has been successful are:

- *Helianthus annuus* (Plissier *et al*.. 1990);
- *Juglans regia*. (McGranahan *et al*.1988b);
- *Medicago sativa* (Lupotto 1986).

As mentioned earlier embryogenesis has a great potential for mass propagation, however, all adventitious techniques do still have the associated problem of the lack of clonal stability. Therefore the commercial application of this technology remains limited except, perhaps, where embryos arise directly from parental tissue.

The propagation of orchids

Morel (1960) noticed that when protocorms of *Cymbidium* were divided new protocorms were formed from the pieces, whereas if they were not divided, original and regenerated protocorms developed into new plantlets. Morel (1960, 1964) suggested that meristem or shoot tip explants could be used to establish cultures for the clonal propagation of orchids, providing thereby the basis of the method which is now used for many orchid genera. Rates of propagation are improved through the use of slightly more complex media than used by Morel, and by including growth regulators. However, many commercial micropropagation laboratories do not favour the use of protocorms for micropropagation because of the lack of clonal fidelity.

Some orchids not only form protocorms on apical meristems, but also directly on explants such as leaves (Churchill *et al.,* 1971; 1973; Tanaka *et al.,* 1975), or flower stalks (Flamée and Boesman, 1977; Arditti *et al.,* 1977), or they may be formed from callus or callus via suspension cultures (see the section on orchids in Volume 2).

3.3. PROPAGATION BY INDIRECT ORGANOGENESIS

Propagation by all methods of indirect organogenesis carries a risk that the regenerated plants will differ genetically from each other and from the stock plant. Propagation by indirect organogenesis is described here for the sake of completeness; because of its potential as a propagation method, if the occurrence of genetic variation can be controlled; and because it is necessary for the regeneration and propagation of plants, which have been genetically transformed.

3.3.1. Indirect adventitious shoots from callus

Because they are not formed on tissues of the original mother plant, shoots (or other organs) are said to be regenerated indirectly when they are formed on previously unorganised callus, or in cell cultures. Separate root and shoot initials are characteristically formed in callus cultures and are only observed occasionally in suspensions where they are typically produced in large cell aggregates. Somatic embryogenesis occurs in both callus and suspension cultures. Adjustment of the growth regulators in the culture medium can bring about shoot or root formation in callus from a very large number of species. Inception of roots and shoots is most frequent in tissues that have been recently

isolated, and morphogenic capacity generally declines with time as the tissues are subcultured. Nevertheless, some callus cultures maintain their regenerative ability over long periods.

As explained in Chapter 10, callus cultures vary in their morphogenic potential or competence. Because of this, the callus which originates from some plants, or from some kinds of explant, may not be responsive to techniques and media which frequently result in morphogenesis. The tissue may be non-morphogenic, or may only produce roots, from which plants cannot be regenerated. In some cases callus lines with different appearances (texture, colour, etc) and/or morphogenic capacities can be isolated from the same explant (Fig 2.5). These differences may reflect the epigenetic potential of the cells, or be caused by the appearance of genetic variability amongst the cells of the culture (Volume 2) and support Street's (1979) suggestion that primary explants may be composed of cells or tissues capable of morphogenesis (competent cells) and others that are incapable (non-competent cells). Another possibility is that the operator is not able to create the appropriate conditions to express the full potential of the plant material he is working with.

Morphogenic and non-morphogenic callus lines, selected from primary callus, can retain their characteristics over many years (Reuther, 1990). Special treatments, such as, a change of medium, an altered cultural environment, or an adjustment of the growth regulators added to the medium, may induce shoots or roots to form in some apparently non-morphogenic calluses; but generally, treatments to reverse a non-regenerative condition are unsuccessful.

In practice, the speed and efficiency with which plantlets can be regenerated from callus depends upon:

- the interval between culture initiation and the onset of morphogenesis;
- the choice of the appropriate type of callus;
- the frequency and rate of shoot bud initiation;
- whether shoot regeneration can be readily re-induced when the callus is subcultured;
- the number of subcultures that are possible without loss of morphogenesis;
- whether newly-initiated buds can be grown into shoots capable of being isolated and subsequently rooted.

Normal callus cultures produce shoots relatively slowly, but from some plants, and certain explants, under conditions that are not yet fully understood, callus can be initiated which has an especially high ability to regenerate shoots or somatic embryos.

Stage I.

In most herbaceous broad leafed plants, it is possible to initiate morphogenically competent callus cultures from explants derived from many different tissues. Leaf, stem or root segments, pieces of storage tissue (e.g. tubers), seed embryos, shoot tips and seedling tissues have been used at various times. In monocotyledons there is a narrower range of suitable organs; embryos, very young leaf tissue, stem nodes and immature inflorescences being the most common sources. Initiation of callus cultures of many tree species, including gymnosperms, is frequently limited to explants derived from tissues near the vascular bundles or the cambium of stem or root sections. Explants containing actively dividing cells may be necessary if callus possessing a high level of morphogenic competence is to be isolated.

Callus growth is usually initiated by placing the chosen explant on a semi-solid medium into which auxin has been incorporated at a relatively high level, with or without a cytokinin. Details of the compounds used are given in Chapter 5. One or more transfers on the same medium may be necessary before the callus is separated from the parental tissue for subculture. Because more than one kind of callus may arise from a single explant, successful propagation can depend on being able to recognise and subculture only the type (or types) which will eventually be able to give rise to shoots or somatic embryos. In the absence of previous experience, samples of each type of callus may have to be carried forward for testing on inductive media. Translucent, watery callus is seldom morphogenic, whereas nodular callus frequently is.

Organised adventitious shoots are usually induced to form in callus or suspension cultures by reducing the auxin level in the medium and/or increasing the concentration of cytokinin. To grow callus-derived shoots into plantlets capable of survival in the soil, they must be rooted as micro-cuttings. Root production by callus is of little consequence for micropropagation purposes; even if roots are formed concurrently with adventitious shoots, the vascular connections between roots and shoots, through the callus tissue, are almost invariably insufficient for the development of a functional plantlet.

Stage II.

Once a morphogenic callus has been isolated, propagation is carried out either by callus subdivision, or by the preparation of cell suspensions.

The success of each technique depends on the subcultured tissues or cells continuing to regenerate shoots.

Callus subdivision. Callus is cut into smaller pieces which increase in size when subcultured in a liquid or an agar-solidified medium. The callus can either be subdivided further, or shoot regeneration allowed to occur. This may take place on the same medium, or the callus may need to be transferred to another shoot-inducing medium. The organogenic capacity of callus is easily lost on repeated subculture. Use of high growth regulator levels can encourage the proliferation of non-regenerative callus which will displace tissues having the competence to form new shoots (e.g. in *Pelargonium*; Holdgate, 1977).

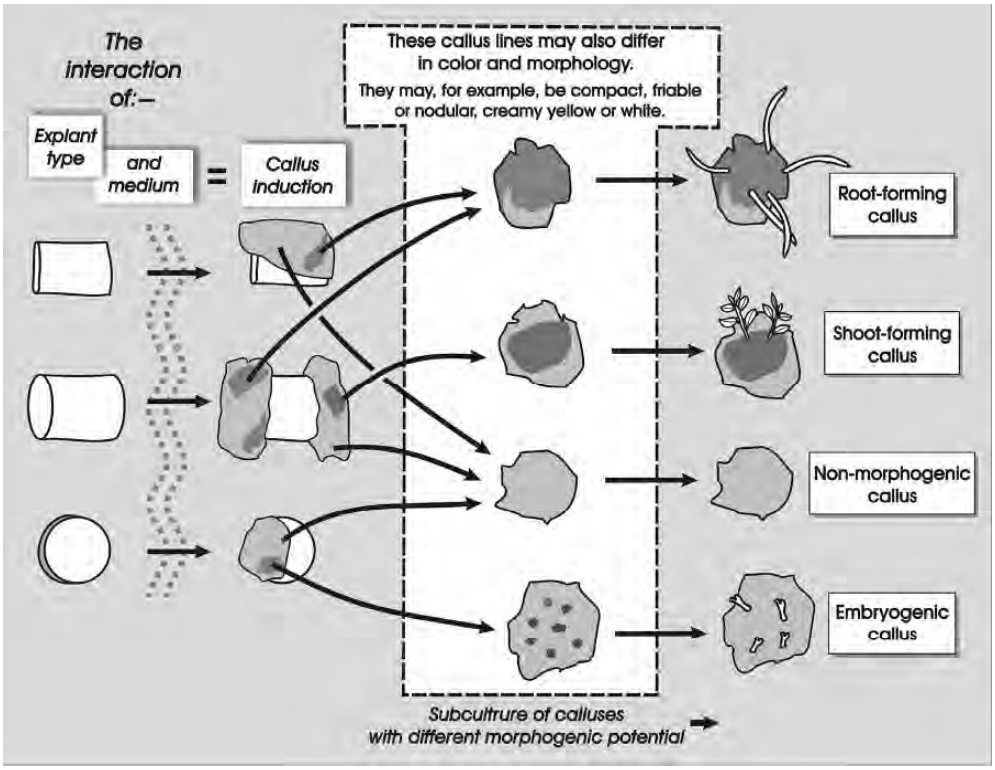

Fig. 2.5 Callus with different morphogenic potential is often isolated from a single explant.

The preparation of cell suspensions. Compared to the relatively rapid rates of propagation that are possible with shoot culture of some kinds of plants, propagation from morphogenically competent callus can be slow initially. Krikorian and Kann (1979) quoted a minimum of 135 days from the excision of daylily explants to the potting of plantlets. The rate at which propagation can proceed after that depends on the rate at which callus can be grown and subdivided. Providing a shoot-forming capacity is retained, a much faster rate of multiplication can be achieved by initiating a suspension culture from competent callus. After being increased by culture, the cells or cell aggregates can then be plated to produce new regenerative callus colonies. This is not an easy operation, as growth regulators favouring the formation of a dispersed cell suspension can cause the cells to lose their morphogenic capacity (see Chapter 10). There is also the problem that, by prolonging the period before shoot regeneration, genetic variability within the cell line will be increased.

Genetic stability

In some crop plants, the genetic differences between plants derived from callus and suspension cultures (discussed in Volume 2) are considerable, and are sufficient to have attracted the interest of plant breeders as a new source of selectable variability. However, plants obtained from callus lines with a high degree of morphogenic competence, appear to be much more uniform genetically. Care must be taken though to see that primary explants are

not taken from plant tissue likely to be endopolyploid. Subsequent exposure to high levels of growth substances such as 2,4-D should also be avoided as far as possible. Genetic stability of plants from highly competent callus cultures may be assisted by the continual presence of superficial meristems. As mentioned previously in Section 3.2.2, these probably repress shoot formation from cells within the callus mass (Hussey, 1983).

Morphogenic cereal cultures

The shoot forming capacity of some callus cultures has been attributed to the proliferation of meristematic centres derived from the tissues of the explant. King et al. (1978) have suggested that the small number of shoots produced by certain cereal tissue cultures arises in this way (e.g. in wheat, rice, oat and maize). Cure and Mott (1978) noticed that aberrant root-like structures existed within cereal cultures from which shoots arose. Such primordia, whether of root or shoot origin, are thought to proliferate adventitiously *in vitro*, surrounded by less organised tissues. Regenerative capacity is usually lost rapidly when the shoot primordia are diluted during subculture. Cereal callus of this kind does not have the same kind of inherent morphogenic capacity found in other types of callus cultures. Despite these observations, experience shows that morphogenesis can occur from previously unorganised cereal callus.

Current applications

In the past, several ornamental plants [e.g. *Freesia* (Hussey and Hargreaves, 1974) and *Pelargonium* (Holdgate, 1977)] have been micropropagated from adventitious shoots produced indirectly from callus. It had been hoped to extend the technique to other species possessing a strong natural tendency towards diploidy (e.g. some forest trees) where plantlets produced *in vitro* might have a normal karyotype (Mott, 1981), but it is now realised that the genetic changes which are almost universally induced in the genotype of cells during callus and cell culture make cloning by this technique inadvisable except where new genotypes are required for selection or further plant breeding. Another possibility is that mutated somatic cells, already present in the mother plant, are given the opportunity to develop into a plantlet.

3.3.2 Indirectly-initiated somatic embryos

Indirect formation of somatic embryos (or adventitious somatic embryogenesis) from callus or suspension cultures is observed more frequently than direct embryogenesis. Frequently callus which is wholly or partly embryogenic can be induced during the initial culture of explants derived from young meristematic tissues (see below), but induction is less common in cultures which have been kept and transferred for some period without organogenesis.

There are important requirements for the successful induction of embryogenic callus and suspension cultures:

- The plant genotype must be capable of embryogenesis on the chosen system of induction (medium plus added growth regulators). In some genera most genotypes are competent, but in others there may be a wide variation in competence even between different varieties or cultivars within a species.
- In most practical situations, cultures should be grown in the presence of an auxin for the induction (and initiation) of embryogenesis (Stage I).
- The level of sugar (e.g. sucrose or glucose) in the medium may need to be within critical concentrations, and no embryos may be formed at all if the sugar concentration is too high (Lippman and Lippmann, 1984).
- After the beginning of embryogenesis, it is usually (but not invariably) necessary for Stage I tissues or cells to be subcultured to a medium containing a reduced auxin concentration, or containing no auxin at all (Stage II) (Chapter 9).
- There may be an optimum length of time during which the Stage I routine should be maintained. An extended period before subculture can result in the failure to obtain embryogenesis at Stage II (e.g. Dos Santos *et al.*1980). Maintenance of the cultures on high auxin usually causes embryo development to be arrested or a loss of embryogenic capability.
- A supply of reduced nitrogen is required. This may be supplied in the form of NH_4^+ ion and/or as an amino acid such as glutamine or alanine (see Chapter 3).

Embryogenesis in primary callus cultures

Callus capable of producing somatic embryos (embryogenic callus) is most reliably obtained from an explant during the initial phase of culture, and is frequently produced in conjunction with non-morphogenic tissue. Embryogenic callus can usually be distinguished by its nodular appearance, and is frequently produced preferentially from one part of an explant (e.g. the scutellum of a monocotyledon embryo), probably because only the cells of that part of the explant were embryogenically pre-determined. These may be the same tissues, which in another

cultural environment are capable of producing embryos directly (Sharp *et al.,* 1980). According to this hypothesis, although competent and non-competent cells may produce callus, only that which grows from competent cells will give rise to somatic embryos.

The expression of competence depends on the use of a suitable medium for the culture, containing requisite growth regulators at the correct concentration. The formation of somatic embryos in *Lolium multiflorum*, for example, was medium dependent (Dale *et al.,* 1981). On the most suitable medium, immature inflorescence explants produced three types of callus, only one of which spontaneously formed embryo-like structures. Unless such different callus types are separated, cells of different regenerative capabilities may become mixed. Morphogenically competent cells could then be lost by competition in the combined callus tissue that results.

Stage I. Selection of an appropriate explant is most important. Embryogenic callus has been commonly gained from seed embryos, nucelli or other highly meristematic tissues such as parts of seedlings, the youngest parts of newly initiated leaves and inflorescence primordia. Within an inflorescence, staminoids [*Theobroma cacao* (Li *et al.,* 1998)] and filaments [*Aesculus hippocastanum* (Radojevic, 1995)] have been reported to be adequate sources of explants. The initiation of embryogenic callus from root tissue is rare but has been reported in some monocotyledons e.g. rice (Inoue and Maeda, 1982; Toshinari and Futsuhara, 1985); oil palm (Paranjothy and Rohani, 1982), Italian ryegrass (Jackson and Dale, 1988) and *Allium carinatum* (Havel and Novak, 1988). Callus is usually commenced on a semi-solid medium incorporating a relatively high level of an auxin; compounds commonly used for this purpose are described in Chapters 11 and 12. Only a few tissues with a high natural embryogenic capacity do not require the addition of endogenous auxin for the development of embryogenic callus. Occasionally, primary callus arising from an explant may show no morphogenic capacity, but can be induced to give rise to new embryogenic tissue during later (secondary) subcultures by transfer to an inductive medium. Ahee *et al.* (1981) have used this method to propagate oil palms. On the medium used, calluses arising on the veins of young leaf fragments had no morphogenic capability. However, when primary calluses were subcultured onto appropriate media (unspecified), some of them gave rise to tissue that was different in structure and form, and grew at a much faster rate. These 'fast-growing calluses' could be induced to produce structures resembling embryoids, and afterwards plantlets, upon further subculture to other media.

One highly embryogenic tissue that has been extensively studied is that of the nucellus of the polyembryonic 'Shamouti' orange (Spiegel-Roy and Kochba, 1980). Here it seems that cells at just one end of the embryo sac (the micropylar end) are embryogenically predetermined and retain this capacity in subsequent cell generations. On subculture, proliferation of the nucellus cells proceeds without the addition of growth regulators to the medium, and results in the formation of an habituated callus. A tissue is said to have become habituated when it will grow without a growth regulator, or some other organic substance which is normally necessary, being added to the medium (see Chapter 7). Addition of auxins to the growth medium is inhibitory to the growth of auxin-habituated 'Shamouti' orange tissue, which has been thought to be composed (at least initially) of numerous pro-embryos and not of undifferentiated cells (Button *et al.,* 1974). Embryogenic callus has also been obtained from the nucellus tissue of other plants, mainly tropical fruit species (Litz and Jaiswal, 1991).

Stage II. As a general rule, somatic embryos formed on a medium containing a relatively high concentration of an auxin, will only develop further if the callus culture is transferred to a second medium from which auxin has been omitted, another 'less active'' auxin has been substituted, or the level of the original auxin much reduced. This treatment is occasionally ineffective (Handley and Sink, 1985) and sometimes adding a cytokinin helps to ensure embryo growth. A further essential requirement is the need for a supply of reduced nitrogen in the form of an ammonium salt or amino acid. No change of nitrogen source is required if MS medium was used for Stage I, but if, for example, White's medium were used for Stage I, it would need to be supplemented with reduced nitrogen, or the culture transferred to MS.

Callus subculture. Once obtained, embryogenic callus can continue to give rise to somatic embryos during many subcultures over long periods. The continued production of somatic embryos in these circumstances depends either on the continued proliferation of pro-embryogenic nodules, and/or the *de novo* formation of embryogenic tissue from young somatic embryos during each subculture. Inocula for

subcultures must be carefully selected. In wheat, callus with continued embryogenesis was only re-initiated from inocula taken close to somatic embryos; tissue from the same culture which did not contain embryos was not embryogenic in the next passage (Chu *et al.,* 1987).

Sometimes the number of embryos produced per unit weight of callus rises during a few passages and then slowly falls, the capacity to form somatic embryos eventually being irrevocably lost. Callus derived from the nucellus of 'Shamouti' oranges increased in its capacity to form somatic embryos when subcultured at 10-15 week intervals, while transfer at 4-5 week intervals, reduced embryogenesis (Kochba and Button, 1974).

Somatic embryos can be formed relatively freely in callus tissue, but where they are to be used for large scale propagation, their numbers can often be increased more rapidly and conveniently by initiating an embryogenic suspension culture from the primary callus (see below).

Embryogenesis in suspension cultures

Cultures from embryogenic callus (Stage I). Suspension cultures can sometimes be initiated from embryogenic callus tissue, and the cells still retain the capacity to regenerate somatic embryos freely. Obtaining such cultures is not always a simple matter, for the auxin levels that are often used to promote cell dispersion may result in the loss of morphogenic capability. Embryogenic cell suspensions are most commonly initiated from embryogenic callus that is placed in liquid medium on a shaker. Vasil and Vasil (1981a,b) and Lu and Vasil (1981a,b) have reported producing cultures of this type from pearl millet and guinea grass respectively. Suspensions were initiated and subcultured in MS medium containing 1-2.5 mg/l 2.4-D and 2.5-5% coconut milk, and came to be composed of a mixture of embryogenic cells (small, highly cytoplasmic and often containing starch) and non-embryogenic cells (large and vacuolated). Embryoids were induced to develop into somatic seedlings when plated onto an agar medium without growth regulators, or with lower levels of auxin than used at the previous stage.

Cultures from non-embryogenic sources. Embryogenesis can be induced in cell suspensions of some plants when the cultures are produced from non-morphogenic callus and have been maintained without morphogenesis for one or more transfers. Induction occurs most readily in recently isolated suspensions and usually becomes much less probable with increasing culture age. Loss of regenerative ability is often associated with the appearance of some cells with abnormal chromosome numbers, but it can also be due to culture on an inappropriate medium.

Embryogenesis in suspension cultures seems to require media at Stage I and Stage II with similar compositions to those necessary for somatic embryo formation in callus cultures. Somatic embryos can be formed in suspension cultures in very large numbers. Reinert *et al.* (1971) demonstrated that the continued capacity of carrot cell suspensions to form embryos depended on an adequate supply of nitrogen. Embryogenesis ceased on a medium containing little nitrogen, but it was re-induced for several transfers after the culture was returned to a high-nitrogen medium.

In a few kinds of plants it is possible to induce embryogenesis in previously unorganised suspension cultures. Success is so far recorded only in members of the families Apiaceae (Umbelliferae), Cruciferae and Scrophulariaceae. This is not therefore a method of propagation which can be readily utilised. There is a greater chance of obtaining an embryogenic suspension culture from embryogenic callus.

Abnormal embryos and plantlets

Unfortunately embryogenesis in both callus and suspension cultures is seldom synchronous so that embryoids at different stages of development are usually present in a Stage II culture from the onset. This presents a major drawback for plant propagation which could otherwise be very rapid, especially from suspensions. A proportion of the seedlings developing from somatic embryos can also be atypical: abnormalities include the possession of multiple or malformed cotyledons, more than one shoot or root axis, and the presence of secondary adventive embryos. Embryos with three cotyledons have been observed to give rise to well-formed plantlets (Smith and Krikorian, 1990). Abnormal somatic embryos do however produce secondary embryos, which are usually of normal morphology.

Pretova and Williams (1986) suggested that embryo proliferation, or 'cleavage' (see earlier in chapter), and the formation of accessory cotyledons and root poles, to be homologous with the production of discrete complete embryoids. They suggested that production of accessory cotyledons and somatic embryos on the hypocotyl, and additional root poles near the base of existing embryos, may represent either a gradient along these organs in the early stages of determination, or, be caused by a factor affecting cell to cell co-ordination.

Differential filtering and sedimentation to separate embryos at different stages of development (Giuliano et al., 1983) can improve the uniformity of embryo populations in suspension cultures. More recently image analysis has been used to select embryos in specific developmental stages (Kurata, 1995). In addition cultures can be maintained on media containing high levels of sucrose (Ammirato and Steward, 1971), and/or low levels of abscisic acid (Ammirato, 1973; 1974). Both approaches, as well as the addition of imazalil to the culture medium (Werbrouck *et al.,* 2000), limit the number of abnormalities and give a higher degree of synchronisation. High levels of sucrose and abscisic acid induce reversible dormancy in somatic embryos and thus might be used to temporarily suspend growth should this be advantageous in a planned micropropagation programme.

Dormancy is however not always reversible. Indeed somatic embryos can remain dormant, and conversion to plantlets can be problematic. Different types of approaches can be used to overcome this problem, i.e. desiccation, supplementing the medium with osmotic agents [e.g. polyethylene glycol (PEG), mannitol] (Capuana and Debergh, 1997).

Genetic stability

Plants regenerated through somatic embryogenesis are usually morphologically and cytologically normal, but sometimes a proportion of aberrant plants is obtained. Genetically abnormal plants are more likely to occur where embryogenesis is initiated in callus or suspension cultures after a period of unorganised growth or when embryogenic cultures are maintained for several months (Orton, 1985).

A proportion of albino plants lacking chlorophyll is characteristically produced in anther culture of cereals and grasses (Sunderland and Dunwell, 1977) and during embryogenesis from other monocot explants. Dale *et al.* (1980) found that plants produced from embryogenic callus cultures of Italian ryegrass were more likely to be devoid of chlorophyll the longer the cultures were maintained. After one year, some cultures produced only albinos. Embryo-like structures (although still present on the surface of the callus) tended to be distorted.

Current applications

Few plant species are at present propagated on a large scale via embryogenesis *in vitro*. This method of morphogenesis does however offer advantages which suggest that it will be used increasingly for plant cloning in the future:

- In some monocotyledons (e.g. cereals, date palm and oil palm) it provides a method of micropropagation where shoot culture has not been successful (but note however that in some attempts to clone oil palms through embryogenesis, the resulting plants have been very variable);
- Providing embryogenic cell suspensions can be established, plantlets can theoretically be produced in large numbers and at much lower cost because plantlets do not have to be handled and subcultured individually;
- Somatic embryos probably provide the only way for tissue culture methods of plant propagation to be economically deployed on extensively planted field crops and forest trees.

Techniques for the conversion (germination) and field planting of somatic embryos are discussed in Volume 2.

4. STORAGE ORGAN FORMATION

Many ornamental and crop species are normally propagated, stored and planted in the form of vegetative storage organs. It is therefore not surprising that, where such organs are produced *in vitro*, they often provide a convenient means of micropropagation and/or genotype storage. Characteristic, though small, storage structures can be induced to form in cultures of several plant species, for example:

Bulbils	*Amaryllis*, hyacinth, lily, onion *Narcissus*
Cormlets	*Gladiolus*
Miniature tubers	Potato, yams.

Protocorm formation as a method of propagation has been considered under Direct Embryogenesis.

Methods of obtaining storage organs vary according to the kind of tissue being cultured. Some storage organs formed *in vitro* can be planted *ex vitro* directly into the soil.

4.1. THE PRODUCTION OF BULBILS AND CORMLETS

Species that naturally produce bulbs can be induced to form small bulbs (bulbils or bulblets) in culture. Bulbils can be produced from axillary buds, but frequently they are formed from adventitious buds developed on pieces of leaf, on inflorescence