A further reason for transfer, or subculture, is that the growth of plant material in a closed vessel eventually leads to the accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus, even to maintain the culture, all or part of it must be transferred onto fresh medium. Callus subcultures are usually initiated by moving a fragment of the initial callus (an inoculum) to fresh medium in another vessel. Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. The interval between subcultures depends on the rate at which a culture has grown: at 25°C, subculturing is typically required every 4-6 weeks. In the early stages of callus growth it may be convenient to transfer the whole piece of tissue to fresh medium, but a more established culture will need to be divided and only small selected portions used as inocula. Regrowth depends on the transfer of healthy tissues.

Decontamination procedures are theoretically no longer necessary during subculturing, although sterile transfer procedures must still be used. However, when using shoot or node cultures for micropropagation, some laboratories do re-sterilise plant material at this stage as a precaution against the spread of contaminants (see Volume 2). Cultures which are obviously infected with micro-organisms should not be used for subculturing and should be autoclaved before disposal.

2.8. SUBCULTURING HAZARDS

There are several hazards in subculturing which are discussed more fully in other chapters of this book. Several kinds of callus may arise from the initial explant, each with different morphogenic potential. Strains of callus tissue capable of giving rise to somatic embryos and others without this capability can, for instance, arise simultaneously from the culture of grass and cereal seed embryos. Careful selection of the correct strain is therefore necessary if cultures capable of producing somatic embryos are ultimately required. Timing of the transfer may also be important, because if left alone for some while, non-embryogenic callus may grow from the original explant at the expense of the competent tissue, which will then be obscured or lost.

Although subculturing can often be continued over many months without adverse effects becoming apparent, cultures of most unorganised cells and of some organised structures can accumulate cells that are genetically changed. This may cause the characteristics of the culture to be altered and may mean that some of the plants regenerated from the culture will not be the same as the parent plant. This subject is discussed further in Chapter 2. Cultures may also inexplicably decline in vigour after a number of passages, so that further subculture becomes impossible.

3. TYPES OF TISSUE CULTURE

3.1. ORGAN CULTURES

Differentiated plant organs can usually be grown in culture without loss of integrity. They can be of two types:

- Determinate organs which are destined to have only a defined size and shape (e.g. leaves, flowers and fruits);
- Indeterminate organs, where growth is potentially unlimited (apical meristems of roots and non-flowering shoots).

In the past, it has been thought that the meristematic cells within root or shoot apices were not committed to a particular kind of development. It is now accepted that, like the primordia of determinate organs such as leaves, apical meristems also become inherently programmed (or determined) into either root or shoot pathways (see Chapter 8). The eventual pattern of development of both indeterminate and determinate organs is often established at a very early stage. For example, the meristematic protrusions in a shoot apex become programmed to develop as either lateral buds or leaves after only a few cell divisions have taken place (see Chapter 10).

3.1.1. Culture of determinate organs

An organ arises from a group of meristematic cells. In an indeterminate organ, such cells are theoretically able to continue in the same pattern of growth indefinitely. The situation is different in the primordium of a determinate organ. Here, as meristematic cells receive instructions on how to differentiate, their capacity for further division becomes limited.

If the primordium of a determinate organ is excised and transferred to culture, it will sometimes continue to grow to maturity. The organ obtained *in vitro* may be smaller than that which would have developed on the original plant *in vivo*, but otherwise is likely to be normal. The growth of determinate organs cannot be extended by subculture as growth ceases when they have reached their maximum size.

Organs of limited growth potential, which have been cultured, include leaves (Caponetti and Steeves, 1963; Caponetti, 1972); fruits (Nitsch, 1951, 1963; Street, 1969); stamens (Rastogi and Sawhney, 1988); ovaries and ovules (which develop and grow into embryos) and flower buds of several dicotyledonous plant species (Table 1.1).

Until recently, a completely normal development was obtained in only a few cases. This was probably due to the use of media of sub-optimum composition. By experimenting with media constituents, Berghoef and Bruinsma (1979a) obtained normal growth of *Begonia franconis* buds and were thus able to study the effect of plant growth substances and nutritional factors on flower development and sexual expression (Berghoef and Bruinsma, 1979b). Similarly, by culturing dormant buds of *Salix*, Angrish and Nanda (1982a,b) could study the effect of bud position and the progressive influence of a resting period on the determination of meristems to become catkins and fertile flowers. In several species, flowers have been pollinated *in vitro* and have then given rise to mature fruits (e.g. Ruddat *et al.,* 1979)

Table 1.1 Some species in which flower buds have been cultured

Cucumis sativus	Galun *et al.* (1962)
Viscaria spp.	Blake (1966, 1969)
Nicotiana tabacum	Hicks and Sussex (1970)
Aquilegia formosa	Bilderback (1971)
Cleome iberidella	De Jong and Bruinsma (1974)
Nicotiana offinis	Deaton *et al.* (1980)

Plants cannot be propagated by culturing meristems already committed to produce determinate organs, but providing development has not proceeded too far, flower meristems can often be induced to revert to vegetative meristems *in vitro*. In some plants the production of vegetative shoots from the flower meristems on a large inflorescence can provide a convenient method of micropropagation (see Chapter 2).

3.1.2. Culture of indeterminate organs

Meristem and shoot culture. The growing points of shoots can be cultured in such a way that they continue uninterrupted and organised growth. As these shoot initials ultimately give rise to small organised shoots which can then be rooted, their culture has great practical significance for plant propagation. Two important uses have emerged:

Meristem culture. Culture of the extreme tip of the shoot, is used as a technique to free plants from virus infections. Explants are dissected from either apical or lateral buds. They comprise a very small stem apex (0.2-1.0 mm in length) consisting of just the apical meristem and one or two leaf primordia;

Shoot culture or shoot tip culture. Culture of larger stem apices or lateral buds (ranging from 5 or 10 mm in length to undissected buds) is used as a very successful method of propagating plants.

The size and relative positions of the two kinds of explant in a shoot apex of a typical dicotyledon is shown in Fig. 1.5. Node culture is an adaptation of shoot culture.

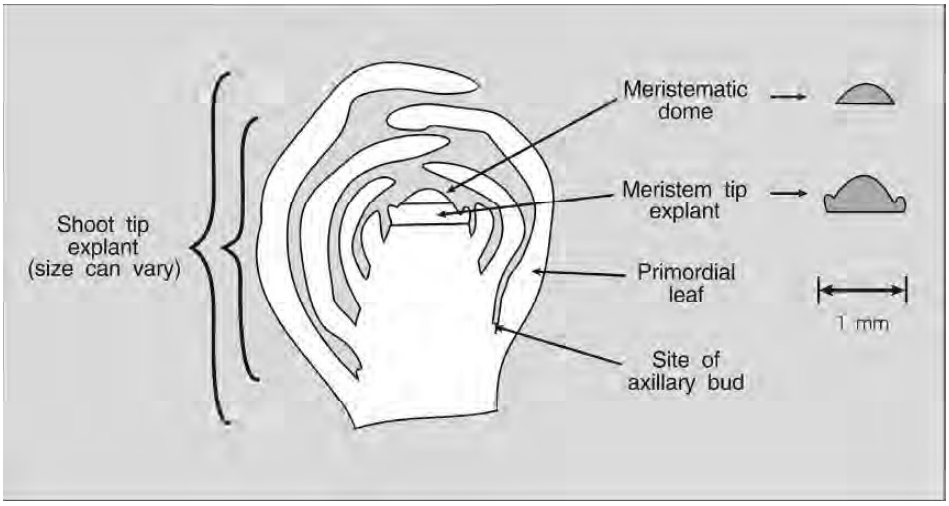

Fig. 1.5 A diagrammatic section through a bud showing the locations and approximate relative sizes of a meristematic dome, the meristem tip and shoot tip explants.

If successful, meristem culture, shoot culture and node culture can ultimately result in the growth of small shoots. With appropriate treatments, these original shoots can either be rooted to produce small plants or 'plantlets', or their axillary buds can be induced to grow to form a cluster of shoots. Plants are propagated by dividing and reculturing the shoot clusters, or by growing individual shoots for subdivision. At a chosen stage, individual shoots or shoot clusters are rooted. Tissue cultured shoots are removed from aseptic conditions at or just before the rooting stage, and rooted plantlets are hardened off and grown normally. Shoot culture, node culture and meristem tip culture are discussed in greater detail in Chapter 2.

Embryo culture. Zygotic or seed embryos are often used advantageously as explants in plant tissue culture, for example, to initiate callus cultures. In embryo culture however, embryos are dissected from seeds, individually isolated and 'germinated' *in vitro* to provide one plant per explant. Isolated embryo culture can assist in the rapid production of seedlings from seeds that have a protracted dormancy period, and it enables seedlings to be produced when the genotype (e.g. that resulting from some interspecific crosses) conveys a low embryo or seed viability.

During the course of evolution, natural incompatibility systems have developed which limit the types of possible sexual crosses (see De Nettancourt and Devreux, 1977). Two kinds of infertility occur:

- Pre-zygotic incompatibility, preventing pollen germination and/or pollen tube growth so that a zygote is never formed;
- Post-zygotic incompatibility, in which a zygote is produced but not accepted by the endosperm. The embryo, not receiving sufficient nutrition, disintegrates or aborts.

Pre-zygotic incompatibility can sometimes be overcome in the laboratory using a technique developed by Kanta *et al.* (1962) called *in vitro* pollination (or *in vitro* fertilisation). For a description of this technique see review articles by Ranga Swamy (1977), Zenkteler (1980) and Yeung *et al.* (1981). Reviews of embryo culture have been provided by Torrey (1973), Norstog (1979) and Raghavan (1967, 1977a, 1980).

Embryo culture has been used successfully in a large number of plant genera to overcome post-zygotic incompatibility which otherwise hampers the production of desirable hybrid seedlings. For example, in trying to transfer insect resistance from a wild *Solanum* species into the aubergine, Sharma *et al.* (1980a) obtained a few hybrid plants (*Solanum melongena* x *S. khasianum*) by embryo culture. Embryo culture in these circumstances is more aptly termed embryo rescue. Success rates are usually quite low and the new hybrids, particularly if they arise from remote crosses, are sometimes sterile. However, this does not matter if the plants can afterwards be propagated asexually. Hybrids between incompatible varieties of tree and soft fruits (Tukey, 1934; Skirm, 1942) and *Iris* (in Reuther, 1977) have been obtained by culturing fairly mature embryos.

Fruits or seeds are surface sterilised before embryo removal. Providing aseptic techniques are strictly adhered to during excision and transfer to a culture medium, the embryo itself needs no further sterilisation. To ease the dissection of the embryo, hard seeds are soaked in water to soften them, but if softening takes more than a few hours it is advisable to re-sterilise the seed afterwards. A dissecting microscope may be necessary to excise the embryos from small seeds as it is particularly important that the embryo should not be damaged.

Culture of immature embryos (pro-embryos) a few days after pollination frequently results in a greater proportion of seedlings being obtained than if more mature embryos are used as explants, because incompatibility mechanisms have less time to take effect. Unfortunately dissection of very small embryos requires much skill and cannot be done rapidly: it also frequently results in damage which prevents growth *in vitro*. In soybean, Hu and Sussex (1986) obtained the best *in vitro* growth of immature embryos if they were isolated with their suspensors intact. Excised embryos usually develop into seedlings precociously (i.e. before they have reached the size they would have attained in a normal seed).

As an alternative to embryo culture, in some plants it has been possible to excise and culture pollinated ovaries and immature ovules. Ovule culture, sometimes called '*in ovulo* embryo culture', can be more successful than the culture of young embryos. Pro-embryos generally require a complex medium for growth, but embryos contained within the ovule require less complicated media. They are also easily removed from the plant and relatively insensitive to the physical conditions of culture (Thengane *et al.,* 1986). The difference

between embryo and ovule culture is shown diagrammatically in Fig. 1.6.

Because seedlings, which resulted from ovule culture of a *Nicotiana* interspecific cross all died after they had developed some true leaves, Iwai *et al.* (1985) used leaves of the immature seedlings as explants for the initiation of callus cultures. Most shoots regenerated from the callus also died at an early stage, but one gave rise to a plant, which was discovered later to be a sterile hybrid. Plants were also regenerated from callus of a *Pelargonium* hybrid by Kato and Tokumasu (1983). The callus in this case arose directly from globular or heart-shaped zygotic embryos which were not able to grow into seedlings.

The seeds of orchids have neither functional storage organs, nor a true seed coat, so dissection of the embryo would not be possible. In fact, for commercial purposes, orchid seeds are now almost always germinated *in vitro*, and growth is often facilitated by taking immature seeds from green pods (see Volume 2).

Many media have been especially developed for embryo culture and some were the forerunners of the media now used for general tissue culture. Commonly, mature embryos require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes coconut milk or some other endosperm extract. Raghavan (1977b) encouraged the incorporation of mannitol to replace the high osmotic pressure exerted on proembryos by ovular sap. Seedlings obtained from embryos grown *in vitro* are planted out and hardened off in the same manner as other plantlets raised by tissue culture (Chapter 2 and Volume 2).

Although embryo culture is especially useful for plant breeders, it does not lead to the rapid and large scale rates of propagation characteristic of other micropropagation techniques, and so it is not considered further in this book. More details can be found in papers by: Sanders and Ziebur (1958); Raghavan (1967, 1980); Torrey (1973); Zilis and Meyer (1976); Collins and Grosser (1984), Monnier (1990) and Ramming (1990). Yeung *et al.* (1981) have suggested a basic protocol, which with modifications, should be applicable to any species.

The induction of multiple shoots from seeds is described in Chapter 2.

Isolated root culture. Root cultures can be established from root tips taken from primary or lateral roots of many plants. Suitable explants are small sections of roots bearing a primary or lateral root meristem. These explants may be obtained, for example, from surface sterilised seeds germinated in aseptic conditions. If the small root meristems continue normal growth on a suitable medium, they produce a root system consisting only of primary and lateral roots (Fig. 1.7.). No organised shoot buds will be formed.

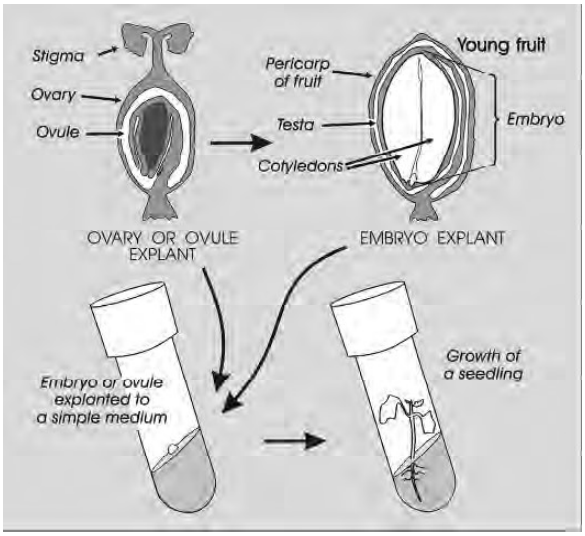

Fig. 1.6 Ovule and embryo culture.

The discovery that roots could be grown apart from shoot tissue was one of the first significant developments of modern tissue culture science. Root culture initially attracted a great deal of attention from research workers and the roots of many different species of plants were cultured successfully (see the comprehensive reviews of Street, 1954, 1957, 1969; and Butcher and Street, 1964).

Plants fall generally into three categories with regard to the ease with which their roots can be cultured. There are some species such as clover, *Datura*, tomato and *Citrus*, where isolated roots can be grown for long periods of time, some seemingly, indefinitely (Said and Murashige, 1979) providing regular subcultures are made. In many woody species, roots have not been grown at all successfully in isolated cultures. In other species such as pea, flax and wheat, roots can be cultured for long periods but ultimately growth declines or insufficient lateral roots are produced to provide explants for subculture.

The inability to maintain isolated root cultures is due to an induced meristematic dormancy or 'senescence', related to the length of time that the