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Chapter 4
The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems

1. ORGANIC SUPPLEMENTS

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins (including some substances that are not strictly animal vitamins), amino acids and certain undefined supplements. The amount of these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant.

1.1. VITAMINS

Vitamins are compounds required by animals in very small amounts as necessary ancillary food factors. Absence from the diet leads to abnormal growth and development and an unhealthy condition. Many of the same substances are also needed by plant cells as essential intermediates or metabolic catalysts, but intact plants, unlike animals, are able to produce their own requirements. Cultured plant cells and tissues can however become deficient in some factors; growth and survival is then improved by their addition to the culture medium.

In early work, the requirements of tissue cultures for trace amounts of certain organic substances were satisfied by "undefined" supplements such as fruit juices, coconut milk, yeast or malt extracts and hydrolysed casein. These supplements can contribute vitamins, amino acids and growth regulants to a culture medium. The use of undefined supplements has declined as the need for specific organic compounds has been defined, and these have become listed in catalogues as pure chemicals.

1.2. THE DEVELOPMENT OF VITAMIN MIXTURES

The vitamins most frequently used in plant tissue culture media are thiamine (Vit. B_1), nicotinic acid (niacin) and pyridoxine (Vit. B_6) and apart from these three compounds, and *myo*-inositol, there is little common agreement about which other vitamins are really essential.

The advantage of adding thiamine was discovered almost simultaneously by Bonner (1937, 1938), Robbins and Bartley (1937) and White (1937). Nicotinic acid and pyridoxine appear, in addition to thiamine, in media published by Bonner (1940), Gautheret (1942) and White (1943b); this was following the findings of Bonner and Devirian (1939) that nicotinic acid improved the growth of isolated roots of tomato, pea and radish; and the papers of Robbins and Schmidt (1939a,b) which indicated that pyridoxine was also required for tomato root culture. These four vitamins; *myo*-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige and Skoog (1962) medium and have been used in varying proportions for the culture of tissues of many plant species (Chapter 3). However, unless there has been research on the requirements of a particular plant tissue or organ, it is not possible to conclude that all the vitamins which have been used in a particular experiment were essential.

The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture. Welander (1977) found that Nitsch and Nitsch (1965) vitamins were not necessary, or were even inhibitory to direct shoot formation on petiole explants of *Begonia* x *hiemalis*. Roest and Bokelmann (1975) on the other hand, obtained increased shoot formation on *Chrysanthemum* pedicels when MS vitamins were present. Callus of *Pinus strobus* grew best when the level of inositol in MS medium was reduced to 50 mg/l whereas that of *P. echinata.* proliferated most rapidly when no inositol was present (Kaul and Kochbar, 1985).

Research workers often tend to adopt a 'belt and braces' attitude to minor media components, and add unusual supplements just to ensure that there is no missing factor which will limit the success of their experiment. Sometimes complex mixtures of as many as nine or ten vitamins have been employed.

Experimentation often shows that some vitamins can be omitted from recommended media. Although four vitamins were used in MS medium, later work at Professor Skoog's laboratory showed that the optimum rate of growth of tobacco callus tissue on MS salts required the addition of only *myo*-inositol and thiamine. The level of thiamine was increased four-fold over that used by Murashige and Skoog (1962), but nicotinic acid, pyridoxine and glycine

E. F. George et al. (eds.), Plant Propagation by Tissue Culture 3rd Edition, 115–173.

(amino acid) were unnecessary (Linsmaier and Skoog, 1965). A similar simplification of the MS vitamins was made by Earle and Torrey (1965) for the culture of *Convolvulus* callus.

Soczck and Hempel (1988) found that in the medium of Murashige *et al.* (1974) devised for the shoot culture of *Gerbera jamesonii*, thiamine, pyridoxine and inositol could be omitted without any reduction in the rate of shoot multiplication of their local cultivars. Ishihara and Katano (1982) found that *Malus* shoot cultures could be grown on MS salts alone, and that inositol and thiamine were largely unnecessary.

1.3. SPECIFIC COMPOUNDS

***Myo*-inositol.** *Myo*-inositol (also sometimes described as *meso*-inositol or i-inositol) is the only one of the nine theoretical stereoisomers of inositol which has significant biological importance. Medically it has been classed as a member of the Vitamin B complex and is required for the growth of yeast and many mammalian cells in tissue culture. Rats and mice require it for hair growth and can develop dermatitis when it is not in the diet. *Myo*-inositol has been classed as a plant 'vitamin', but note that some authors think that it should be regarded as a supplementary carbohydrate, although it does not contribute to carbohydrate utilization as an energy source or as an osmoticum.

Historical use in tissue cultures. *Myo*-inositol was first shown by Jacquiot (1951) to favour bud formation by elm cambial tissue when supplied at 20-1000 mg/l. Necrosis was retarded, though the proliferation of the callus was not promoted. *Myo*-inositol at 100 mg/1 was also used by Morel and Wetmore (1951) in combination with six other vitamins for the culture of callus from the monocotyledon *Amorphophallus rivieri* (Araceae). Bud initials appeared on some cultures and both roots and buds on others according to the concentration of auxin employed. The vitamin was adopted by both Wood and Braun (1961) and Murashige and Skoog (1962) in combination with thiamine, nicotinic acid and pyridoxine in their preferred media fur the culture of *Catharanthus roseus* and *Nicotiana tabacum* respectively. Many other workers have since included it in culture media with favourable results on the rate of callus growth or the induction of morphogenesis. Letham (1966) found that *myo*-inositol interacted with cytokinin to promote cell division in carrot phloem explants.

Occurrence and biochemistry. Part of the growth promoting property of coconut milk is due to its *myo*-inositol content (Pollard *et al.,* 1961). Coconut milk also contains *scyllo*-inositol (Table 4.1). This can also promote growth but to a smaller extent than the *myo*-isomer (Pollard *et al.,* 1961). Inositol is a constituent of yeast extract (Steiner *et al.,* 1969; Steiner and Lester, 1972) and small quantities may also be contained in commercial agar (Wolter and Skoog, 1966). *Myo*-inositol is a natural constituent of plants and much of it is often incorporated into phosphatidyl-inositol which may be an important factor in the functioning of membranes (Jung *et al.,* 1972; Harran and Dickinson, 1978). The phosphatidylinositol cycle controls various cellular responses in animal cells and yeasts, but evidence of it playing a similar role in plants is only just being accumulated. Enzymes which are thought to be involved in the cycle have been observed to have activities in plants and lithium chloride (which inhibits *myo*-inositol-1-phosphatase and decreases the cycle) inhibits callus formation in *Brassica oleracea* (Bagga *et al.,* 1987), and callus growth in *Amaranthus paniculatus* (Das *et al.,* 1987). In both plants the inhibition is reversed by *myo*-inositol.

As the *myo*-inositol molecule has six hydroxyl units, it can react with up to six acid molecules forming various esters. It appears that inositol phosphates act as second messengers to the primary action of auxin in plants: phytic acid (inositol hexaphosphate) is one of these. Added to culture media it can promote tissue growth if it can serve as a source of inositol (Watanabe *et al.,* 1971). In some species, auxin can be stored and may be transported as IAA-*myo*-inositol ester (Chapter 5). *o*-Methyl-inositol is present in quite large quantities in legumes; inositol methyl ethers are known to occur in plants of several other families, although their function is unknown (Phillips and Smith, 1974).

The stimulatory effect of *myo*-inositol in plant cultures probably arises partly from the participation of the compound in biosynthetic pathways leading to the formation of the pectin and hemicelluloses needed in cell walls (Loewus *et al.,* 1962*;* Loewus, 1974; Loewus and Loewus, 1980; Harran and Dickinson, 1978; Verma and Dougall, 1979; Loewus and Loewus, 1980) and may have a role in the uptake and utilization of ions (Wood and Braun, 1961). In the experiments of Staudt (1984) mentioned below, when the $P0_4^{3-}$ content of the medium was raised to 4.41 mM, the rate of callus growth of cv. 'Aris' was

progressively enhanced as the *myo*- inositol in the medium was put up to 4000 mg/l. This result seems to stress the importance of inositol-containing phospholipids for growth.

Table 4.1. Substances identified as components of coconut milk (water) from mature green fruits and market-purchased fruits.

SUBSTANCE	QUANTITY/REFERENCE		SUBSTANCE	QUANTITY/REFERENCE	
	Mature green fruits	**Mature fresh fruits**		**Mature green fruits**	**Mature fresh fruits**
Amino acids (mg/l)			**Sugars (g/l)**		
Alanine	127.3 (14)	312 (13), 177.1 (14)	Sucrose	9.2 (14)	8.9 (14)
Arginine	25.6 (14)	133 (13), 16.8 (14)	Glucose	7.3 (14)	2.5 (14)
Aspartic acid	35.9 (14)	65 (13), 5.4 (14)	Fructose	5.3 (14)	2.5 (14)
Asparagine	10.1 (14)	ca.60 (13), 10.1 (14)	**Sugar alcohols (g/l)**		
γ-Aminobutyric acid	34.6 (14)	820 (13), 168.8 (14)	Mannitol		(1)
Glutamine acid	70.8 (14)	240 (13), 78.7 (14)	Sorbitol		15.0 (12), (17)
Glutamine	45.4 (14)	ca.60 (13), 13.4 (14)	*myo*-Inositol		0.1 (12), (17)
Glycine	9.7 (14)	13.9 (14)	*scyllo*-Inositol		0.5 (12), (17)
Histidine	6.3 (14)	Trace (13,14)	**Vitamins (mg/l)**		
Homoserine	-- (14)	5.2 (14)	Nicotinic acid		0.64 (4)
Hydroxyproline		Trace (13,14)	Pantolhenic acid		0.52 (4)
Lysine	21.4	65.8 (14)	Biotin, Riboflavin		0.02 (4)
Methionine	16.9 (14)	8 (13), Trace (14)	Riboflavin		0.01 (4)
Phenylalanine	-- (14)	12 (13), 10.2 (14)	Folic acid		0.003 (4)
Proline	31.9	97 (13), 21.6 (14)	Thiamine, pyridoxine		Trace (4)
Serine	45.3 (14)		**Growth substances (mg/l)**		
Typtophan		39 (13)	Auxin		0.07 (7), (28)
Threonine	16.2 (2)	44 (13), 26.3 (14)	Gibberellin		Yes (10,28)
Tyrosine	6.4 (14)	16 (13), 3.1 (14)	1,3-Diphenylurea		5.8 (8), (6,17)
Valine	20.6 (14)	27 (13), 15.1 (14)	Zeatin		(22,26)
Other nitrogenous compounds			Zeatin glucoside		(26)
Ammonium		(19)	Zeatin riboside		(20), (24), (25)
Ethanolamine		(19)	6-Oxypurine growth promoter		(27)
Dihydroxyphenyl alanine		(19)	Unknown cytokinin/s		6, (18) (22)
Inorganic elements (mg/100g dry wt.)			**Other (mg/l)**		
Potassium		312.0 (3)	RNA-polymerase		(23)
Sodium		105 (3)	RNA-phosphorus	20.0 (14)	35.4 (14)
Phosphorus		37.0 (3)	DNA-phosphorus	0.1 (14)	3.5 (14)
Magnesium		30.0 (3)	Uracil, Adenine		21
Organic acids (meq/ml)			Leucoanthocyanins		(11) (15,17)
Malic acid	34.3 (14)	12.0 (14)	Phyllococosine		(16)
Shikimic, Quinic and 2 unknowns	0.6 (14)	0.41 (2)	Acid Phosphatase		(5,9)
Pyrrolidone carboxylic acid	0.4 (14)	0.2 (14)	Diastase		(2)
Citric acid	0.4 (14)	0.3 (14)	Dehydrogenase		(5)
Succinic acid	-- (14)	0.3 (14)	Peroxidase		(5)
			Catalase		(5)

Numbered references (within brackets) in the above table are listed in Section 1.11 of this Chapter.

Activity in tissue cultures. Cultured plant tissues vary in their capacity for *myo*-inositol biosynthesis. Intact shoots are usually able to produce their own requirements, but although many unorganised tissues are able to grow slowly without the vitamin being added to the medium (Murashige, 1974) the addition of a small quantity is frequently found to stimulate cell division. The compound has been discovered to be essential to some plants. In the opinion of Kaul and Sabharwal (1975) this includes all monocotyledons, the media for which, if they do not contain inositol, need to be complemented with coconut milk, or yeast extract.

Fraxinus pennsylvanica callus had an absolute requirement for 10 mg/l *myo*-inositol to achieve maximum growth; higher levels, up to 250 mg/l had no further effect on fresh or dry weight yields (Wolter and Skoog, 1966). The formation of shoot buds on callus of *Haworthia* spp was shown to be dependent on the availability of *myo*-inositol (Kaul and Sabharwal, 1972, 1975). In a revised Linsmaier and Skoog (1965) medium [Staudt (1984) containing 1.84 mM PO_4^{3-}], callus tissue of *Vitis vinifera* cv 'Müller-Thurgau' did not require *myo*-inositol for growth, but that of *Vitis vinifera* x *V. riparia* cv. 'Aris' was dependent on it and the rate of growth increased as the level of *myo*-inositol was increased up to 250 mg/l (Staudt, 1984).

Gupta *et al.* (1988) found that it was essential to add 5 g/l *myo*-inositol to Gupta and Durzan (1985) DCR-1 medium to induce embryogenesis (embryonal suspensor masses) from female gametophyte tissue of *Pseudotsuga menziesii* and *Pinus taeda*. The concentration necessary seems insufficient to have acted as an osmotic stimulus (see section 3). *myo*-Inositol reduced the rate of proliferation in shoot cultures of *Euphorbia fulgens* (Zhang *et al.,* 1986).

Thiamine. Thiamine (Vit. B_1, aneurine) in the form of thiamine pyrophosphate, is an essential co-factor in carbohydrate metabolism and is directly involved in the biosynthesis of some amino acids. It has been added to plant culture media more frequently than any other vitamin. Tissues of most plants seem to require it for growth, the need becoming more apparent with consecutive passages, but some cultured cells are self sufficient. The maize suspension cultures of Polikarpochkina *et al.* (1979) showed much less growth in passage 2, and died in the third passage when thiamine was omitted from the medium.

MS medium contains 0.3 μM thiamine. That this may not be sufficient to obtain optimum results from some cultures is illustrated by the results of Barwale *et al.* (1986): increasing the concentration of thiamine-HCI in MS medium to 5 μM, increased the frequency with which zygotic embryos of *Glycine max* formed somatic embryos from 33% to 58%. Adding 30 μM nicotinic acid (normally 4 μM) improved the occurrence of embryogenesis even further to 76%. Thiamine was found to be essential for stimulating embryogenic callus induction in *Zoysia japonica*, a warm season turf grass from Japan (Asano *et al.,* 1996). It has also been shown to stimulate adventitious rooting of *Taxus* spp. (Chée, 1995).

There can be an interaction between thiamine and cytokinin growth regulators. Digby and Skoog (1966) discovered that normal callus cultures of tobacco produced an adequate level of thiamine to support growth providing a relatively high level of kinetin (ca. 1 mg/l) was added to the medium, but the tissue failed to grow when moved to a medium with less added kinetin unless thiamine was provided.

Sometimes a change from a thiamine-requiring to a thiamine-sufficient state occurs during culture (see habituation – Chapter 7). In rice callus, thiamine influenced morphogenesis in a way that depended on which state the cells were in. Presence of the vitamin in a pre-culture (Stage I) medium caused thiamine-sufficient callus to form root primordia on an induction (Stage II) medium, but suppressed the stimulating effect of kinetin on Stage II shoot formation in thiamine-requiring callus. It was essential to omit thiamine from the Stage I medium to induce thiamine-sufficient callus to produce shoots at Stage II (Inoue and Maeda, 1982).

1.4. OTHER VITAMINS

Pantothenic acid. Pantothenic acid plays an important role in the growth of certain tissues. It favoured callus production by hawthorn stem fragments (Morel, 1946) and stimulated tissue proliferation in willow and black henbane (Telle and Gautheret, 1947; Gautheret, 1948). However, pantothenic acid showed no effects with carrot, vine and Virginia creeper tissues which synthesize it in significant amounts (ca. 1 μg/ml).

Vitamin C. The effect of Vitamin C (L-ascorbic acid) as a component of culture media will be discussed in Chapter 12. The compound is also used during explant isolation and to prevent blackening.

Besides, its role as an antioxidant, ascorbic acid is involved in cell division and elongation, e.g., in tobacco cells (de Pinto *et al.,* 1999). Ascorbic acid (4-8 x 10^{-4} M) also enhanced shoot formation in both young and old tobacco callus. (Joy *et al.,* 1988). It speeded up the shoot-forming process, and completely reversed the inhibition of shoot formation by gibberellic acid in young callus, but was less effective in old callus. Clearly its action here was not as a vitamin.

Vitamin D. Some vitamins in the D group, notably vitamin D_2 and D_3 can have a growth regulatory effect on plant tissue cultures. Their effect is discussed in Chapter 7.

Vitamin E. The antioxidant activity of vitamin E (α-tocopherol) will be discussed in Chapter 12.

Other vitamins. Evidence has been obtained that folic acid slows tissue proliferation in the dark, while enhancing it in the light. This is probably because it is hydrolysed in the light to *p*-aminobenzoic acid (PAB). In the presence of auxin, PAB has been shown to have a weak growth-stimulatory effect on cultured plant tissues (de Capite, 1952a,b).

Riboflavin which is a component of some vitamin mixtures, has been found to inhibit callus formation but it may improve the growth and quality of shoots (Drew and Smith, 1986). Suppression of callus growth can mean that the vitamin may either inhibit or stimulate root formation on cuttings. Riboflavin has been shown to stimulate adventitious rooting on shoots of *Carica papaya* (Drew *et al.,* 1993), apple shoots (van der Krieken *et al.,* 1992) and *Eucalyptus globulus* (Trindade and Pais, 1997). It also enhances embryogenic callus induction in *Zoysia japonica* in association with cytokinins and thiamine (Asano *et al.,* 1996).

Glycine is occasionally described as a vitamin in plant tissue cultures: its use has been described in the section on amino acids.

Adenine. Adenine (or adenine sulphate) has been widely used in tissue culture media, but because it mainly gives rise to effects which are similar to those produced by cytokinins, it is considered in the chapter on cytokinins (Chapter 6).

Stability. Some vitamins are heat-labile; see the section on medium preparation in Volume 2.

1.5. UNDEFINED SUPPLEMENTS

Many undefined supplements were employed in early tissue culture media. Their use has slowly declined as the balance between inorganic salts has been improved, and as the effect of amino acids and growth substances has become better understood. Nevertheless several supplements of uncertain and variable composition are still in common use.

The first successful cultures of plant tissue involved the use of yeast extract (Robbins, 1922; White, 1934). Other undefined additions made to plant tissue culture media have been:

- meat, malt and yeast extracts and fibrin digest;
- juices, pulps and extracts from various fruits (Steward and Shantz, 1959; Ranga Swamy, 1963; Guha and Maheshwari, 1964, 1967), including those from bananas and tomatoes (La Rue, 1949);
- the fluids which nourish immature zygotic embryos;
- extracts of seedlings (Saalbach and Koblitz, 1978) or plant leaves (Borkird and Sink, 1983);
- the extract of boiled potatoes and corn steep liquor (Fox and Miller, 1959);
- plant sap or the extract of roots or rhizomes. Plant roots are thought to be the main site of cytokinin synthesis in plants (Chapter 6);
- protein (usually casein) hydrolysates (containing a mixture of all the amino acids present in the original protein). Casein hydrolysates are sometimes termed casamino acids: they are discussed in Chapter 3).

Many of these amendments can be a source of amino acids, peptides, fatty acids, carbohydrates, vitamins and plant growth substances in different concentrations. Those which have been most widely used are described below.

1.6. YEAST EXTRACT.

Yeast extract (YE) is used less as an ingredient of plant media nowadays than in former times, when it was added as a source of amino acids and vitamins, especially inositol and thiamine (Vitamin B_1) (Bonner and Addicott, l937; Robbins and Bartley, 1937). In a medium consisting only of macro- and micro-nutrients, the provision of yeast extract was often found to be essential for tissue growth (White, 1934; Robbins and Bartley, 1937). The vitamin content of yeast extract distinguishes it from casein hydrolysate (CH) so that in such media CH or amino acids alone, could not be substituted for YE (Straus and La Rue, 1954; Nickell and Maretzki, 1969). It was soon found that amino acids such as glycine, lysine and arginine, and vitamins such as thiamine and nicotinic acid, could serve as replacements for YE, for example in the growth of tomato roots (Skinner and Street, 1954), or sugar cane cell suspensions (Nickell and Maretzki, 1969).

The percentage of amino acids in a typical yeast extract is high (e.g. 7% amino nitrogen - Nickell and Maretzki, 1969; Bridson, 1978; Thom *et al.,* 1981), but there is less glutamic acid than in casein or other protein hydrolysate. Malt extract contains little nitrogen (ca. 0.5% in total).

Yeast extract has been typically added to media in concentrations of 0.1-1 g/l; occasionally 5, 10 and even 20 g/l (Morel and Muller, 1964) have been included. It normally only enhances growth in media containing relatively low concentrations of nitrogen, or where vitamins are lacking. Addition of 125-5000 mg/l YE to MS medium completely inhibited the growth of green callus of 5 different plants whereas small quantities added to Vasil and Hildebrandt (1966) THS medium (which contained 0.6 times the quantity of NO_3^- and NH_4^+ ions and unlike MS did not contain nicotinic acid or pyridoxine) gave more vigorous growth of carrot, endive and lettuce callus than occurred on MS. There was still no growth of parsley and tomato callus on THS medium: these tissues only grew well on unmodified MS (Vasil and Hildebrandt, 1966a,b,c).

Stage I media are sometimes fortified with yeast extract to reveal the presence of micro-organisms which may have escaped decontamination procedures: it is then omitted at later stages of culture.

Yeast extract has been shown to have some unusual properties which may relate to its amino acid content. It elicits phytoalexin accumulation in several plant species and in *Glycyrrhiza echinata* suspensions it stimulated chalcone synthase activity leading to the formation of narengin (Ayabe *et al.,* 1988). It also stimulated furomocoumarin production in *Glehnia littoralis* cell suspensions (Kitamura *et al.,* 1998). On Monnier (1976, 1978) medium 1 g/l yeast extract was found to inhibit the growth of immature zygotic embryos of *Linum*, an effect which, when 0.05 mg/1 BAP and 400 mg/l glutamine were added, induced the direct formation of adventitious embryos (Pretova and Williams, 1986).

Yeast extract is now purchased directly from chemical suppliers. In the 1930s and 1940s it was prepared in the laboratory. Brink *et al.* (1944) macerated yeast in water which was then boiled for 30 minutes and, after cooling, the starchy material was removed by centrifugation. However, Robbins and Bartley (1937) found that the active components of yeast could be extracted with 80% ethanol.

1.7. POTATO EXTRACT

Workers in China found that there was a sharp increase in the number of pollen plants produced from wheat anthers when they were cultured on an agar solidified medium containing only an extract of boiled potatoes, 0.1 mM FeEDTA, 9% sucrose and growth regulators. Potato extract alone or potato extract combined with components of conventional culture media (Chuang *et al.,* 1978) has since been found to provide a useful medium for the anther culture of wheat and some other cereal plants. For example, the potato medium was found to be better for the anther culture of spring wheat than the synthetic (N6) medium (McGregor and McHughen, 1990). Sopory *et al.* (1978) obtained the initiation of embryogenesis from potato anthers on potato extract alone and Lichter (1981) found it beneficial to add 2.5 g/l Difco potato extract to a medium for *Brassica napus* anther culture, but it was omitted by Chuong and Beversdorf (1985) when they repeated this work. We are not aware of potato extract being added to media for micropropagation, apart from occasional reports of its use for orchid propagation. Sagawa and Kunisaki (1982) supplemented 1 litre of Vacin and Went (1949) medium with the extract from 100g potatoes boiled for 5 minutes, and Harvais (1982) added 5% of an extract from 200g potatoes boiled in 1 litre water to his orchid medium. Of interest was the finding that potato juice treatment enabled *in vitro* cultures of *Doritaenopsis* (Orchidaceae) to recover from hyperhydricity (Zou, 1995).

1.8. MALT EXTRACT

Although no longer commonly used, malt extract seems to play a specific role in cultures of *Citrus*. Malt extract, mainly a source of carbohydrates, was shown to initiate embryogenesis in nucellar explants (Rangan *et al.,* 1968; Rangan, 1984). Several recent studies showed a role for the extract in the multiplication of *Citrus sinensis* somatic embryos (Das *et al.,* 1995), and in other *Citrus* spp. (Jumin, 1995), in the promotion of plantlet formation from somatic embryos derived from styles of different *Citrus* cultivars (De Pasquale *et al.,* 1994), and in somatic embryogenesis and plantlet regeneration from pistil thin cell layers of *Citrus* (Carimi *et al.,* 1999). Malt extract also promoted germination of early cotyledonary stage embryos arising from the *in vitro* rescue of zygotic embryos of sour orange (Carimi *et al.,* 1998). The extract is commercially available and used at a level of 0.5 – 1 g/l.

1.9. BANANA HOMOGENATE

Homogenised banana fruit is sometimes added to media for the culture of orchids and is often reported to promote growth. The reason for its stimulatory effect has not been explained. One suggestion mentioned earlier is that it might help to stabilise the pH of the medium. Pierik *et al.* (1988) found that it was slightly inhibitory to the germination of *Paphiopedilum ciliolare* seedlings but promoted the growth of seedlings once germination had taken place.

1.10. FLUIDS WHICH NOURISH EMBRYOS

The liquid which is present in the embryo sac of immature fruits of *Aesculus* (e.g. *A. woerlitzensis*) (Shantz and Steward, 1956, 1964; Steward and Shantz, 1959; Steward and Rao, 1970) and *Juglans regia* (Steward and Caplin, 1952) has been found to have a strong growth-promoting effect on some plant tissues cultured on simple media, although growth inhibition has occasionally been reported (Fonnesbech, 1972). Fluid from the immature female gametophyte of *Ginkgo biloba* (Steward and Caplin, 1952) and extracts from the female gametophyte of *Pseudotsuga menziesii* (Mapes and Zaerr, 1981) and immature *Zea mays* grains (less than two weeks after pollination) can have a similar effect. The most readily obtained fluid with this kind of activity is coconut milk (water).

1.11. COCONUT MILK/WATER

When added to a medium containing auxin, the liquid endosperm of *Cocos nucifera* fruits can induce plant cells to divide and grow rapidly. The fluid is most commonly referred to as coconut milk, although Tulecke *et al.* (1961) maintained that the correct English term is 'coconut water', because the term coconut milk also describes the white liquid obtained by grating the solid white coconut endosperm (the 'meat') in water and this is *not* generally used in tissue culture media. However, in this section, both terms are used.

Coconut milk was first used in tissue cultures by Van Overbeek *et al.* (1941, 1942) who found that its addition to a culture medium was necessary for the development of very young embryos of *Datura stramonium*. Gautheret (1942) found that coconut milk could be used to initiate and maintain growth in tissue cultures of several plants, and Caplin and Steward (1948) showed that callus derived from phloem tissue explants of *Daucus carota* roots grew much more rapidly when 15% coconut milk was added to a medium containing IAA. Unlike other undefined supplements to culture media (such as yeast extract, malt extract and casein hydrolysate) coconut milk has proved harder to replace by fully defined media. The liquid has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis. Although commercial plant tissue culture laboratories (particularly those in temperate countries) would endeavour not to use this ingredient on account of its cost, it is still frequently employed for special purposes in research.

It is possible to get callus growth on coconut milk alone (Steward *et al.,* 1952), but normally it is added to a recognised medium. Effective stimulation only occurs when relatively large quantities are added to a medium; the incorporation of 10-15 percent by volume is quite usual. For instance, Burnet and Ibrahim (1973) found that 20% coconut milk (i.e. one-fifth of the final volume of the medium) was required for the initiation and continued growth of callus tissue of various *Citrus* species in MS medium; Rangan (1974) has obtained improved growth of *Panicum miliaceum* in MS medium using 2,4-D in the presence of 15% coconut milk. By contrast, Vasil and co-workers (e.g. Vasil and Vasil, 1981a,b) needed to add only 5% coconut milk to MS medium to obtain somatic embryogenesis from cereal callus and suspension cultures.

Many workers try to avoid having to use coconut milk in their protocols. It is an undefined supplement whose composition can vary considerably (Swedlund and Locy, 1988). However, adding coconut milk to media often provides a simple way to obtain satisfactory growth or morphogenesis without the need to work out a suitably defined formulation. Suggestions that coconut milk is essential for a particular purpose need to be treated with some caution. For instance, in the culture of embryogenic callus from root and petiole explants of *Daucus carota*, coconut milk could be replaced satisfactorily either by adenine or kinetin, showing that it did not contribute any unique substances required for embryogenesis (Halperin and Wetherell, 1964).

Preparation. Ready prepared coconut water (milk) can be purchased from some chemical suppliers, but the liquid from fresh nuts (obtained from the greengrocer) is usually perfectly adequate. One nut will usually yield at least 100 ml. The water is most simply drained from dehusked coconuts by drilling holes through two of the micropyles. Only normal uncontaminated water should be used and so

nuts should be extracted one by one, and the liquid endosperm from each examined to ascertain that it is unfermented before addition to a bulk supply. Water from green but mature coconuts may contain slightly different quantities of substances to that in the nuts purchased in the local market (Table 4.1) and has been said to be a more effective stimulant in plant media than that from ripe fruits, but Morel and Wetmore (1951) found to the contrary. Tulecke *et al.* (1961) discovered that the water from highly immature coconuts contained smaller quantities of the substances normally present in mature nuts.

References to composition of coconut water (numbers refer to citations in Table 4.1). (1) Dunstan (1906), (2) DeKruijff (1906), (3) McCance and Widdowson (1940), (4) Vandenbelt (1945), (5) Sadasivan (1951), (6) Shantz and Steward (1952), (7) Paris and Duhamet (1953), (8) Shantz and Steward (1955), (9) Wilson and Cutter (1955), (10) Radley and Dear (1958), (11) Steward and Shantz (1959), (12) Pollard *et al.* (1961), (13) Figures of Steward *et al.* (1961), given by Raghavan (1977), (14) Tulecke *et al.* (1961), (15) Steward and Mohan Ram (1961), (16) Kuraishi and Okumura (1961), (17) Steward (1963), (18) Zwar *et al.* (1963), (19) Steward *et al.* (1964), (20) Letham (1968), (21) Steward *et al.* (1969), (22) Zwar and Bruce (1970), (23) Mondal *et al.* (1972), (24) Letham (1974), (25) Van Staden and Drewes (1975), (26) Van Staden (1976), (27) Letham (1982), (28) Dix and Van Staden (1982).

Use of Coconut water. Coconut water is usually strained through cloth and deproteinized by being heated to 80-100°C for about 10 minutes while being stirred. It is then allowed to settle and the supernatant is separated from the coagulated proteins by filtration through paper. The liquid is stored frozen at -20°C. Borkird and Sink (1983) did not boil the water from fresh ripe coconuts, but having filtered it through several layers of cheesecloth, adjusted the pH to 10 with 2 N NaOH and then kept it overnight at 4°C. The following day the pH was re-adjusted to 7.0 with 5 N HCl, and the preparation was refiltered before being stored frozen at –20°C.

Some workers autoclave media containing coconut milk; others filter-sterilise coconut milk and add it to a medium after autoclaving has been carried out. Morel and Wetmore (1951) used filter sterilisation, but found that the milk lost its potency if stored sterile (but presumably unfrozen) for 3 months. Street (1977) advocated autoclaving coconut milk after it had been boiled and filtered; it was then stored at -20°C until required.

Active ingredients. The remarkable growth stimulating property of coconut milk has led to attempts to isolate and identify the active principles. This has proved to be difficult because the fractions into which coconut milk has been separated each possess only a small proportion of the total activity and the different components appear to act synergistically. Substances so far identified include amino acids, organic acids, nucleic acids, purines, sugars, sugar alcohols, vitamins, growth substances and minerals (Table 4.1). The variable nature of the product is illustrated in the table by the analytical results obtained by different authors.

Auxin activity. The liquid has been found to have some auxin activity which is increased by autoclaving, probably because any such growth substances exist in a bound form and are released by hydrolysis. But although coconut milk can stimulate the growth of some *in vitro* cultures in the absence of exogenous auxin, it normally contains little of this kind of growth regulator and an additional exogenous supply is generally required. In modern media, where organic compounds are often added in defined amounts, the main benefit from using coconut milk is almost certainly due to its providing highly active natural cytokinin growth substances.

Cytokinin activity. Coconut milk was shown to have cytokinin activity by Kuraishi and Okumura (1961) and recognised natural cytokinin substances have since been isolated [9-β-D-ribo-furanosyl zeatin (Letham, 1968); zeatin and several unidentified ones (Zwar and Bruce, 1970); N, N′-diphenyl urea (Shantz and Steward, 1955)] but the levels of these compounds in various samples of coconut milk have not been published. An unusual cytokinin-like growth promoter, 2-(3-methylbut-2-enylamino)-purin-6-one was isolated by Letham (1982).

Because coconut milk contains natural cytokinins, adding it to media often has the same effect as adding a recognised cytokinin. This means that a beneficial effect on growth or morphogenesis is often dependent on the presence of an auxin. Steward and Caplin (1951) showed that there was a synergistic action between 2,4-D and coconut milk in stimulating the growth of potato tuber tissue. Lin and Staba (1961) similarly found that coconut milk gave significantly improved callus growth on seedling explants of peppermint and spearmint initiated by 2,4-D, but only slightly improved the growth initiated by the auxin 2-BTOA (2-benziothiazoleoxyacetic acid). The occurrence of gibberellin-like substances in coconut milk has also been reported (Radley and Dear, 1958).

Suboptimum stimulation and inhibition. In cases where optimal concentrations of growth adjuvants have been determined, it has been found that the level of the same or analogous substances in coconut milk may be suboptimal. La Motte (1960) noted that 150 mg/l of tyrosine most effectively induced morphogenesis in tobacco callus cultures, but coconut milk added at 15% would provide only 0.96 mg/l of this substance (Tulecke *et al.,* 1961). Fresh and autoclaved coconut milk from mature nuts has proved inhibitory to growth or morphogenesis (Noh *et al.,* 1988) in some instances. It is not known which ingredients cause the inhibition but the growth of cultured embryos seems particularly liable to be prevented, suggesting that the compound responsible might be a natural dormancy-inducing factor such as abscisic acid. Van Overbeck *et al.* (1942, 1944) found that a factor was present in coconut milk which was essential for the growth of *Datura stramonium* embryos, but that heating the milk or allowing it to stand could lead to the release of toxic substances. These could be removed by shaking with alcohols or ether or lead acetate precipitation. Duhamet and Mentzer (1955) isolated nine fractions of coconut milk by chromatography, and found one of these to be inhibitory to cultured crown gall tissues of black salsify when more than 10-20% coconut milk was incorporated into the medium. Norstog (1965) showed that autoclaved coconut milk could inhibit the growth of barley embryos but that filter-sterilised milk was stimulatory. Coconut water inhibited somatic embryo induction in *Pinus taeda* (Li and Huang, 1996) and both autoclaved or filter-sterilized coconut milk inhibited the growth of wheat embryo-shoot apices (Smith, 1967).

2. ORGANIC ACIDS

Organic acids can have three roles in plant culture media:

- they may act as chelating agents, improving the availability of some micronutrients,
- they can buffer the medium against pH change,
- they may act as nutrients.

A beneficial effect is largely restricted to the acids of the Krebs' cycle. Dougall *et al.* (1979) found that 20 mM succinate, malate or fumarate supported maximum growth of wild carrot cells when the medium was initially adjusted to pH 4.5. Although 1 mM glutarate, adipate, pimelate, suberate, azelate or phthalate controlled the pH of the medium, little or no cell growth took place.

2.1. USE AS BUFFERS

The addition of organic acids to plant media is not a recent development. Various authors have found that some organic acids and their sodium or potassium salts stabilise the pH of hydroponic solutions (Trelease and Trelease, 1933) or *in vitro* media (Van Overbeek *et al.,* 1941, 1942; Arnow *et al.,* 1953), although it must be admitted that they are not as effective as synthetic biological buffers in this respect (see Section 5). Norstog and Smith (1963) discovered that 100 mg/l malic acid acted as an effective buffering agent in their medium for barley embryo culture and also appeared to enhance growth in the presence of glutamine and alanine. Malic acid, now at 1000 mg/l was retained in the improved Norstog (1973) Barley II medium. In the experiments of Schenk and Hildebrandt (1972) low levels of citrate and succinate ions did not impede callus growth of a wide variety of plants and appeared to be stimulatory in some species. The acids were also effective buffers between pH 5 and pH 6, but autoclaving a medium containing sodium citrate or citric acid caused a substantial pH increase..

2.1.1. Complexing with metals

Divalent organic acids such is citric, maleic, malic and malonic (depending on species) are found in the xylem sap of plants, where together with amino acids they can complex with metal ions and assist their transport (White *et al.,* 1981). These acids can also be secreted from cultured cells and tissues into the growth medium and will contribute to the conditioning effect. Ojima and Ohira (1980) discovered that malic and citric acids, released into the medium by rice cells during the latter half of a passage, were able to make unchelated ferric iron available, so correcting an iron deficiency.

2.1.2. Nutritional role

As explained in Chapter 3, adding Krebs' cycle organic acids to the medium can enhance the metabolism of NH_4^+. Gamborg and Shyluk (1970) found that some organic acids could promote ammonium utilisation and the incorporation of small quantities of sodium pyruvate, citric, malic and fumaric acids into the medium, was one factor which enabled Kao and Michayluk (1975) to culture *Vicia hajastana* cells at low density. Their mixture of

organic acid ions has been copied into many other media designed for protoplast culture. Cultures may not tolerate the addition of a large quantity of a free acid which will acidify the medium. For example, *Triticale* anther callus grew well on Chu *et al.* (1975) N6 medium supplemented with 35 mg/l of a mixture of sodium pyruvate, malic acid, fumaric acid, citric acid, but not when 100 mg/l was added (Chien and Kao, 1983). When organic anions are added to the medium from the sodium or potassium salts of an acid there are metallic cations to counterbalance the organic anions, and it seems to be possible to add larger quantities without toxicity. Five mM (1240 mg/l $.3H_2O$) potassium succinate enhanced the growth of cultured peach embryos (Ramming, 1990), and adding 15 mM (4052 mg/1 $.4H_2O$) sodium succinate to MS medium (while also increasing the sucrose content from 3% to 6%) increased the cell volume and dry weight of *Brassica nigra* suspensions by 2.7 times (Molnar, 1988).

Some plants seem to derive nutritional benefit from the presence of one particular organic acid. Murashige and Tucker (1969) showed that orange juice added to a medium containing MS salts promoted the growth of *Citrus* albedo callus. Malic and other Krebs' cycle acids also have a similar effect; of these, citric acid produces the most pronounced growth stimulation. A concentration of up to 10.4 mM can be effective (Goldschmidt, 1976; Einset, l978; Erner and Reuveni, 1981). Succulent plants, in particular those in the family Crassulaccae, such as *Bryophyllum* and *Kalanchoe* fix relatively large amounts of carbon dioxide during darkness, converting it into organic acids, of which malic acid is particularly important. The organic acids are metabolised during daylight hours. In such plants, malic acid might be expected to prove especially efficient in enhancing growth if added to a culture medium. Lassocinski (1985) has shown this to be the case in chlorophyll-deficient cacti of three genera. The addition of L-malic acid to the medium of Savage *et al.* (1979) markedly improved the rate of survival and vigour of small cacti or areoles.

Organic acid (citrate, lactate, succinate, tartrate, and oxalate) pretreatment of alfalfa callus dramatically decreased the growth of callus, but increased the subsequent yield of somatic embryos and embryo development, as well as conversion to plantlets (Nichol *et al.,* 1991). They suggested that the acids may act in the physiological selection for embryogenic callus, by inducing preferential growth of slower-growing-compact cell aggregates compared to the faster growing friable callus.

3. SUGARS -NUTRITIONAL AND REGULATORY EFFECTS

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is known (Koch, 1996). Plant gene responses to changing carbohydrate status can vary markedly. Some genes are induced, some are repressed, and others minimally affected. As in microorganisms, sugar-sensitive plant genes are part of an ancient system of cellular adjustment to critical nutrient availability. However, there is no evidence that this role of carbohydrate is important in normal growth and organized development in cell cultures.3.1. Sugars as energy sources

3.1.1. Carbohydrate autotrophy.

Only a limited number of plant cell lines have been isolated which are autotrophic when cultured *in vitro*. Autotrophic cells are capable of fully supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis (Bergmann, 1967; Tandeau de Marsac and Peaud-Lenoel, 1972a,b; Chandler *et al.,* 1972; Anon, 1980; Larosa *et al.,* 1981). Many autotrophic cultures have only been capable of relatively slow growth (e.g. Fukami and Hildebrandt, 1967), especially in the ambient atmosphere where the concentration of carbon dioxide is low (see Chapter 12). However, since these early trials, very good progess is being made with photoautrophic shoot cultures and photo-autotrophic micropropagation is now possible (Kozai, 1991). Success is dependent on enriching the CO_2 concentrations in the vessels during the photoperiod, reducing or eliminating sugar from the medium, and optimising the *in vitro* environment.

Nevertheless, for the normal culture of either cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used for micropropagation purposes as it is so generally utilisable by tissue cultures. Refined white domestic sugar is sufficiently pure for most practical purposes. The presence of sucrose in tissue culture media specifically inhibits chlorophyll formation and photosynthesis (see below) making autotrophic growth less feasible.

3.2 ALTERNATIVES TO SUCROSE

3.2.1. Other Sugars.

The selection of sucrose as the most suitable energy source for cultures follows many comparisons between possible alternatives. Some of the first work of this kind on the carbohydrate nutrition of plant tissue was done by Gautheret (1945) using normal carrot tissue. Sucrose was found to be the best source of carbon followed by glucose, maltose and raffinose; fructose was less effective and mannose and lactose were the least suitable. The findings of this and other work is summarized in Table 4.2. Sucrose has almost invariably been found to be the best carbohydrate; glucose is generally found to support growth equally well, and in a few plants it may result in better *in vitro* growth than sucrose, or promote organogenesis where sucrose will not; but being more expensive than sucrose, glucose will only be preferred for micropropagation where it produces clearly advantageous results.

Multiplication of *Alnus crispa*, *A. cordata* and *A. rubra* shoot cultures was best on glucose, while that of *A. glutinosa* was best on sucrose (Tremblay and Lalonde, 1984; Tremblay *et al.,* 1984; Barghchi, 1988). Direct shoot formation from *Capsicum annum* leaf discs in a 16 h day required the presence of glucose (Phillips and Hubstenberger, 1985). Glucose is required for the culture of isolated roots of wheat (Furguson, 1967) and some other monocotyledons (Bhojwani and Razdan, 1983).

Table 4.2. The main sugars which can utilized by plants.
The value of as sugar for carbon nutrition is indicated by the size of the type.

SUGAR	Reducing Capacity	Products of hydrolytic/enzymatic breakdown
Monosaccharides		
Hexoses		
Glucose	Reducing sugar	None
Fructose	Reducing sugar	None
Galactose	Reducing sugar	None
Mannose	Reducing sugar	None
Pentoses		
Arabinose	Slow reduction	None
Ribose	Slow reduction	None
Xylose	Slow reduction	None
Disaccharides		
Sucrose	Non-reducing	Glucose, fructose
Maltose	Reducing sugar	Glucose
Cellobiose	Reducing sugar	Glucose
Trehalose	Non-reducing	Glucose
Lactose	Reducing sugar	Glucose, fructose
Trisaccharides		
Raffinose	Non-reducing	Glucose, galactose, fructose

Some other monosaccharides such as arabinose and xylose; disaccharides such as cellobiose, maltose and trehalose; and some polysaccharides; all of which are capable of being broken down to glucose and fructose (Table 4.2), can also sometimes be used as partial replacements for sucrose (Straus and LaRue, 1954; Sievert and Hildebrandt, 1965; Yatazawa *et al.,* 1967; Smith and Stone, 1973; Minocha and Halperin, 1974; Zaghmout and Torres, 1985). In *Phaseolus* callus, Jeffs and Northcote (1967) found that sucrose could be replaced by maltose and trehalose (all three sugars have an alpha-glucosyl radical at the non-reducing end), but not by glucose or fructose alone or in combination, or by several other different sugars. Galactose has been said to be toxic to most plant tissues; it inhibits the growth of orchids and other plants in concentrations as low as 0.01% (0.9 mM) (Ernst *et al.,* 1971; Arditti and Ernst, 1984). However, cells can become adapted and grown on galactose, e.g., sugar cane cells (Maretzski and Thom, 1978). The key was the induction of the enzyme galactose kinase, which converts galactose to galactose-1-phosphate. More recently, other reports on galactose use have appeared. It promoted callus growth in rugosa rose, but inhibited somatic embryogenesis (Kunitake *et al.,* 1993). Galactose

promoted early somatic embryo maturation stages in European silver fir (Schuller and Reuther, 1993). When used instead of sucrose, it improved rooting of *Annona squamosa* microshoots (Lemos and Blake, 1996). In addition, galactose has been found to reduce or overcome hyperhydricity in shoot cultures (Druart, 1988; see Volume 2). Fructose has also been reported to be effective in preventing hyperhydricity (Rugini *et al.,* 1987).

There are some situations where fructose supports growth just as well as sucrose or glucose (Steffen *et al.,* 1988) and occasionally it gives better results. Some orchid species have been reported to grow better on fructose than glucose (Ernst, 1967; Ernst *et al.,* 1971; Arditti, 1979). Fructose was the best sugar for the production of adventitious shoots from *Glycine max* cotyledonary nodes, especially if the concentration of nutrient salts supplied was inadequate (Wright *et al.,* 1986). Shoot and leaf growth and axillary shoot formation in *Castanea* shoot cultures was stimulated when sucrose was replaced by 30 g/l fructose. The growth of basal callus was reduced and it was possible to propagate from mature explants of *C. crenata*, although this was not possible on the same medium supplemented with sucrose (Chauvin and Salesses, 1988). However, fructose was reported to be toxic to carrot tissue if, as the sole source of carbon, it was autoclaved with White (1943a) A medium. When filter sterilized, fructose supported the growth of callus cultures which had a final weight 70% of those grown on sucrose (Pollard *et al.,* 1961).

Sucrose in culture media is usually hydrolysed totally, or partially, into the component monosaccharides glucose and fructose (see below) and so it is logical to compare the efficacy of combinations of these two sugars with that of sucrose. Kromer and Kukulczanka (1985) found that meristem tips of *Canna indica* survived better on a mixture of 25 g/l glucose plus 5 g/l fructose, than on 30 g/l sucrose. Germination of *Paphiopedilum* orchid seeds was best on a medium containing 5g/l fructose plus 5 g/l glucose; a mixture of 7.5 g/l of each sugar was optimal for further growth of the seedlings (Pierik *et al.,* 1988). In spite of its rapid hydrolysis to glucose and fructose, sucrose appears to have a specific stimulatory effect on embryo development in Douglas fir, that was not observed when it was replaced by the monosaccharides (Taber *et al.,* 1998).

The general superiority of sucrose over glucose for the culture of organised plant tissues such as isolated roots may be on account of the more effective translocation of sucrose to apical meristems (Butcher and Street, 1964). In addition, there could be an osmotic effect, because, from an equal weight of compound, a solution of glucose has almost twice the molarity of a sucrose solution, and will thus, in the absence of inversion of the disaccharide, induce a more negative water potential (see below).

Maltose. Plant species vary in their ability to utilise unusual sugars. For instance, although Gautheret (1945) could grow carrot callus on maltose, Mathes *et al.* (1973) obtained only minimal growth of *Acer* tissue on media supplemented with this sugar. Similarly, growth of soybean tissue on maltose is normally very slow, but variant strains of cells have been selected which can utilise it (Limberg *et al.,* 1979), perhaps because the new genotypes possessed an improved capacity for its active transport. Later studies have given a more prominent role to maltose as a component of tissue culture media. Maltose serves as both a carbon source and as an osmoticum. Compared to sucrose there is a slower rate of extracellular hydrolysis, it is taken up more slowly, and hydrolysed intracellularly more slowly. Maltose led to a substantial increase in somatic embryos from *Petunia* anthers (Raquin, 1983). It also led to an increase in callus induction and plantlet regeneration during *in vitro* androgenesis of hexaploid winter triticale and wheat (Karsai *et al.,* 1994). Maltose also increased callus induction in rice microspore culture, with an acceleration of initial cell divisions (Xie *et al.,* 1995). For barley microspore culture, the inclusion of maltose led to a higher frequency of green plants (Finnie *et al.,* 1989). Maltose has been reported to equal or surpass sucrose in supporting embryogenesis in a number of species, including carrot (Verma and Dougall, 1977; Kinnersley and Henderson, 1988), alfalfa (Strickland *et al.,* 1987), wild cherry (Reidiboym-Talleux *et al.,* 1999), *Malus* (Daigny *et al.,* 1996), *Abies* (Norgaard 1997) and loblolly pine (Li *et al.,* 1998). The number of plants regenerated from *indica* (Biswas and Zapata, 1993), and *japonica* (Jain *et al.,* 1997) rice varieties was also greater when protoplasts were cultured with maltose rather than sucrose. Transfer from a medium containing sucrose or glucose to one supplemented with maltose has been used by Stuart *et al.* (1986) and Redenbaugh *et al.* (1987) to enhance the conversion of alfalfa embryos. Similarly, maltose led to a much higher germination rate from asparagus somatic embryos than sucrose (Kunitake *et al.,* 1997).

Lactose. The disaccharide lactose has been detected in only a few plants. When added to tissue

culture media it has been found to induce the activity of β-galactosidase enzyme which can be secreted into the medium. The hydrolysis of lactose to galactose and glucose then permits the growth of *Nemesia strumosa* and *Petunia hybrida* callus, cucumber suspensions (Hess *et al.,* 1979; Callebaut and Motte, 1988), cotton callus and cell suspensions (Mitchell *et al.,* 1980), and Japanese morning glory callus (Hisajima and Thorpe, 1981). The key to lactose utilization in Japanese morning glory was not only the extracellular hydrolysis of this disaccharide, but the induction of galactose kinase, which prevented the accumulation of toxic galactose (Hisajima and Thorpe, 1985). Rodriguez and Lorenzo Martin (1987) found that adding 30 g/l lactose to MS medium instead of sucrose increased the number of shoots produced by a *Musa accuminata* shoot culture, but no new shoots were produced on subsequent subculture, although they were when sucrose was present.

In addition to lactose, plant cells have been shown to become adapted and then to grow on other galactose-containing oligosaccharides, including melibiose (Nickell and Maretzki, 1970; Gross *et al.,* 1981), raffinose (Wright and Northcote, 1972; Thorpe and Laishley, 1974; Gross *et al.,* 1981), and stachyose (Verma and Dougall, 1977; Gross *et al.,* 1981).

Corn syrups. Kinnersley and Henderson (1988) have shown that certain corn syrups can be used as carbon sources in plant culture media and that they may induce morphogenesis which is not provoked by supplementing with sucrose. Embryogenesis was induced in a 10-year old non-embryogenic cell line of *Daucus carota* and plantlets were obtained from *Nicotiana tabacum* anthers by using syrups. Those used contained a mixture of glucose, maltose, maltotriose and higher polysaccharides. Their stimulatory effect was reproduced by mixtures of maltose and glucose.

3.2.2. Sugar alcohols.

Sugar alcohols were thought not usually to be metabolised by plant tissues and therefore unavailable as carbon sources. For this reason, mannitol and sorbitol have been frequently employed as osmotica to modify the water potential of a culture medium. In these circumstances, sufficient sucrose must also be present to supply the energy requirement of the tissues. Adding either mannitol or sorbitol to the medium may make boron unavailable (See Chapter 3).

Mannitol was found to be metabolised by *Fraxinus* tissues (Wolter and Skoog, 1966). Later, studies with carrot and tobacco suspensions and cotyledon cultures of radiata pine showed that although mannitol was taken up very slowly, it was readily metabolized (Thompson *et al.,* 1986). Thus, this sugat alcohol is only of value as a short-term osmotic agent. In contrast, sorbitol is readily taken up and metabolized in some species. It has been found to support the growth of apple callus (Chong and Taper, 1972, 1974a,b) and that of other rosaceous plants (Coffin *et al.,* 1976), occasionally giving rise to more vigorous growth than can be obtained on sucrose. The ability of Rosaceae to use sorbitol as a carbon source is reported to be variety dependent. Albrecht (1986) found that shoot cultures of one crabapple variety required sorbitol for growth and would not grow on sucrose; another benefited from being grown on a mixture of sorbitol and sucrose and the growth of a third suffered if any sucrose was replaced by sorbitol. The apple rootstock 'Ottawa 3' produced abnormal shoots on sorbitol (Chong and Pua, 1985). Evidence is accumulating to show that sugar alcohols generally exhibit non-osmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products (Steinitz, 1999). In addition to being metabolised to varying degrees in heterotrophic cultures, such as tobacco, maize, rice, citrus and chichory, sugar alcohols stimulate specific molecular and physiological responses, where they apparently act as chemical signals.

The cyclic hexahydric alcohol *myo*-inositol does not seem to provide a source of energy (Smith and Stone, 1973) and its beneficial effect on the growth of cultured tissues when used as a supplementary nutrient must depend on its participation in biosynthetic pathways (see vitamins above).

3.2.3. Starch.

Cultured cells of a few plants are able to utilise starch in the growth medium and appear to do so by release of extracellular amylases (Nickell and Burkholder, 1950). Growth rates of these cultures are increased by the addition of gibberellic acid, probably because it increases the synthesis or secretion of amylase enzymes (Maretzki *et al.,* 1971, 1974).

3.3. HYDROLYSIS OF SUCROSE.

The remainder of this section on sugars is devoted to the apparent effects of sucrose concentration on cell differentiation and morphogenesis. Reports on

the subject should be tempered with the knowledge that some or all of the sucrose in the medium is liable to be broken down into its constituent hexose sugars, and that such inversion will also occur within plant tissues, where reducing sugar levels of at least 0.5 per cent are likely to occur (Helgeson *et al.,* 1972).

3.3.1. Autoclaving.

A partial hydrolysis of sucrose takes place during the autoclaving of media (Ball, 1953; Wolter and Skoog, 1966) the extent being greater when the compound is dissolved together with other medium constituents than when it is autoclaved in pure aqueous solution (Ferguson *et al.,* 1958). In a fungal medium, not dissimilar to a plant culture medium, Bretzloff (1954) found that sucrose inversion during autoclaving (15 min at 15 lbs/in^2) was dependent on pH in the following way:

pH 3.0	100%
pH 3.4	75%
pH 3.8	40%
pH 4.2	25%
pH 4.7	12.5%
pH 5.0	10%
pH 6.0	0%

These results suggest that the proportion of sucrose hydrolysed by autoclaving media at conventional pH levels (5.5-5.8) should be negligible. Most evidence suggests that this is not the case and that 10-15% sucrose can be converted into glucose and fructose. Cultures of some plants grow better in media containing autoclaved (rather than filter-sterilised) sucrose (Ball, 1953; Guha and Johri, 1966; Johri and Guha, 1963; Verma and Van Huystee, 1971; White, 1932) suggesting that the cells benefit from the availability of glucose and/or fructose. However, Nitsch and Nitsch (1956) noted that glucose only supported the growth of *Helianthus* callus if it had been autoclaved, and Romberger and Tabor (1971) that the growth of *Picea* shoot apices was less when the medium contained sucrose autoclaved separately in water (or sucrose autoclaved with only the organic constituents of the medium) than when all the constituents had been autoclaved together. They suggested that a stimulatory substance might be released when sugars are autoclaved with agar.

In other species there has been no difference between the growth of cultures supplied with autoclaved or filter sterilised sucrose (Mathes *et al.,* 1973) or growth has been less on a medium containing autoclaved instead of filter sterilised sucrose (Stehsel and Caplin, 1969).

3.3.2. Enzymatic breakdown.

Sucrose in the medium is also inverted into monosaccharides during the *in vitro* culture of plant material. This occurs by the action of invertase located in the plant cell walls (Burstrom, 1957; Yoshida *et al.,* 1973) or by the release of extracellular enzyme (King and Street, 1977). In most cultures, inversion of sucrose into glucose and fructose takes place in the medium; but, because the secretion of invertase enzymes varies, the degree to which it occurs differs from one kind of plant to another. After 28 days on a medium containing either 3 or 4% sucrose, single explants of *Hemerocallis* and *Delphinium* had used 20-30% of the sugar. Of that which remained in the medium which had supported *Hemerocallis*, about 45% was sucrose, while only 5% was sucrose in the media in which *Delphinium* had been grown. In both cases the rest of the sucrose had been inverted (Lumsden *et al.,* 1990).

The sucrose-inverting capacity of tomato root cultures was greatest in media of pH 3.6-4.7. Activity sharply declined in less acid media (Weston and Street, 1968). Helgeson *et al.* (1972) found that omission of IAA auxin from the medium in which tobacco callus was cultured, caused there to be a marked rise in reducing sugar due to the progressive hydrolysis of sucrose, both in the medium and in the tissue. A temporary increase in reducing sugars also occurred at the end of the lag phase when newly transferred callus pieces started to grow rapidly. It is interesting to note that cell wall invertase possessed catalytic activity *in situ*, whether or not tobacco tissue was grown on sucrose (Obata-Sasamoto and Thorpe, 1983).

In cultures of some species, uptake of sugar may depend on the prior extracellular hydrolysis of sugar. This is the case in sugar cane (Komor *et al.,* 1981); and possibly also in *Dendrobium* orchids (Hew *et al.,* 1988) and carrot (Kanabus *et al.,* 1986). Nearly all the sucrose in suspension cultures of sugar cane and sugar beet was hydrolysed in 3 days (Zamski and Wyse, 1985) and *Daucus carota* suspensions have been reported to hydrolyse all of the sucrose in the medium (Thorpe, 1982) into the constituent hexoses within 3 days (Kanabus *et al.,* 1986) or within 24 h (Dijkema *et al.,* 1990). However, most species can take up sucrose directly as was shown through studies with asymetrically labeled ^{14}C-sucrose (Parr and Edelman, 1975), and metabolise it intracellularly. Within the cell, soluble invertase, sucrose phosphate synthetase and sucrose synthetase serve to hydrolyse sucrose (Thorpe, 1982). Thus, in *Acer* (Copping and

Street, 1972) the soluble invertase activity paralleled growth rate, while in tobacco (Thorpe and Meier, 1973) and Japanese morning glory (Hisajima *et al.,* 1978) sucrose synthetase was more important. In the last species, the change in activity of sucrose synthetase was greater than that of sucrose phosphate synthetase, an enzyme not extensively examined in cultured cells.

The growth of shoots from non-dormant buds of mulberry is not promoted by sucrose, only by maltose, glucose or fructose. Even though mulberry tissue hydrolysed sucrose into component monosaccharides, shoots did not develop. In the presence of 3% fructose, sucrose was actually inhibitory to shoot development at concentrations as low as 0.2% (Oka and Ohyama, 1982).

3.4. UPTAKE.

The uptake of sugar molecules into plant tissues appears to be partly through passive permeation and partly through active transport. The extent of the two mechanisms may vary. Active uptake is associated with the withdrawal of protons (H^+) from the medium. Charge compensation is effected by the excretion of a cation (H^+ or K^+) (Komor *et al.,* 1977, 1981). Glucose was taken up preferentially by carrot suspensions during the first 7 days of a 14 day passage; fructose uptake followed during days 7-9 (Dijkema *et al.,* 1990). At concentrations below 200 mM, glucose was taken up more rapidly into strawberry fruit discs and protoplasts than either sucrose or fructose (Scott and Breen, 1988).

3.5. EFFECTIVE CONCENTRATIONS.

In most of the comparisons between the nutritional capabilities of sugars discussed above, the criterion of excellence has been the most rapid growth of unorganised callus or suspension-cultured cells. For this purpose 2-4% sucrose w/v is usually optimal. Similar concentrations are also used in media employed for micropropagation, but laboratories probably pay insufficient attention to the effects of sucrose on morphogenesis (see below) and plantlet development. Sucrose levels in culture media which result in good callus growth may not be optimal for morphogenesis, and either lower or higher levels may be more effective.

The optimum concentration of sucrose to induce morphogenesis or growth differs between different genotypes, sometimes even between those which are closely related. For instance, Damiano *et al.* (1987) found that the concentration of sucrose necessary to produce the best rate of shoot proliferation in *Eucalyptus gunnii* shoot cultures varied between clones. The influence of sucrose concentration on direct shoot formation from *Chrysanthemum* explants varied with plant cultivar (Fig 4.1).

Experiments of Molnar (1988) have shown that the optimum level of sucrose may depend upon the other amendments added to a culture medium. The most rapid growth of *Brassica nigra* suspensions on one containing MS salts (but less iron and B5 vitamins) occurred when 2% sucrose was added. However, when it was supplemented with 1-4 g/l casein hydrolysate or a mixture of 3 defined amino acids, growth was increased on up to 6% sucrose. A similar result was obtained if, instead of the amino acids, 15 mM sodium succinate was added. There was an extended growth period and the harvested dry weight of the culture was 2.8 times that on the original medium with 2% sucrose.

The level of sucrose in the medium may have a direct effect on the type of morphogenesis. Thus, sucrose (87 mM) favored organogenesis, while a higher level (350 mM) favoured somatic embryogenesis from immature zygotic embryos of sunflower (Jeannin *et al.,* 1995). *In vitro* minicrowns of asparagus developed short, thickened storage roots at high frequencies when the sucrose concentration in the medium was increased to 6% (Conner and Falloon, 1993). Lower sucrose concentrations, even with the addition of non- or poorly metabolised carbohydrates, such as cellobiose, maltose, mannose, melibiose and sorbitol produced thin fibrous roots, indicating that the additional sucrose was nutritional rather than osmotic.

The respiration rate of cultured plant tissues rises as the concentration of added sucrose or glucose is increased. In wheat callus, it was found to reach a maximum when 90 g/1 (0.263 M) was added to the medium, even though 20 g/l produced the highest rate of growth and number of adventitious shoots (Galiba and Erdei, 1986). The uptake of inorganic ions can be dependent on sugar concentration and the benefit of adding increased quantities of nutrients to a medium may not be apparent unless the amount of sugar is increased at the same time (Gamborg *et al.,* 1974).

3.5.1. Cell differentiation

Formation of vascular elements. Although sugars are clearly involved in the differentiation of xylem and phloem elements in cultured cells, it is still uncertain whether they have a regulatory role apart from providing a carbon energy source necessary for

active cell metabolism. Sucrose is generally required to be present in addition to IAA before tracheid elements are differentiated in tissue cultures. The number of both sieve and xylem elements formed [and possibly the proportion of each kind - Wetmore and Rier (1963); Rier and Beslow (1967)] depends on sucrose concentration (Aloni, 1980). In *Helianthus tuberosus* tuber slices, although sucrose, glucose and trehalose were best for supporting cell division and tracheid formation, maltose was only a moderately effective carbon source (Minocha and Halperin, 1974). Shininger (1979) has concluded that only carbohydrates which enable significant cell division are capable of promoting tracheary element formation. The occurrence of lignin in cultured cells is not invariably associated with thickened cell walls. Sycamore suspension cultures produced large amounts of lignin when grown on a medium with abnormally high sucrose (more than 6%) and 2,4-D levels. It was deposited within the cells and released into the medium (Carceller *et al.,* 1971).

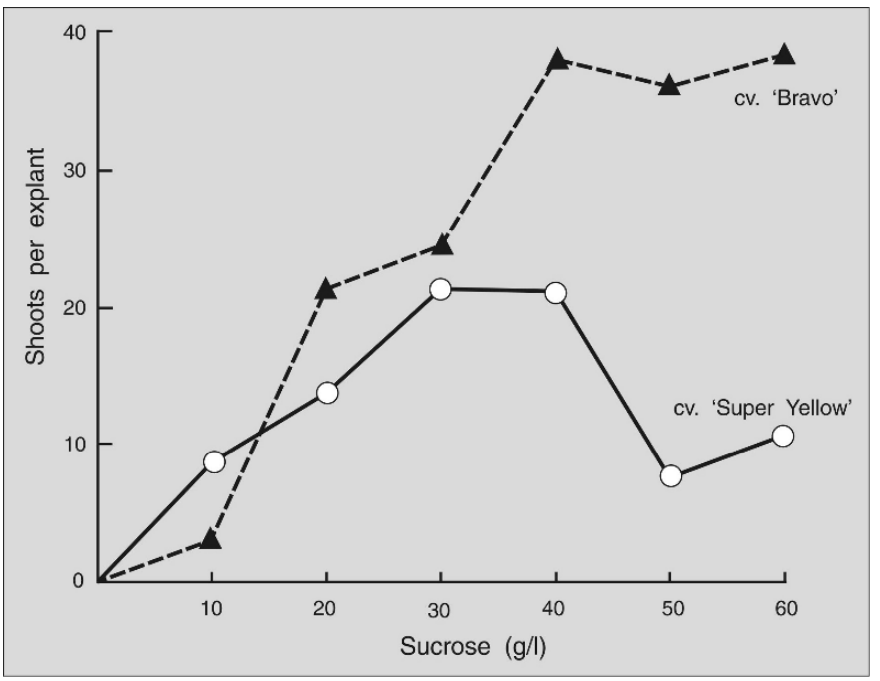

Fig. 4.1 The effect of sucrose concentration on direct adventitious shoot formation from flower pedicels of two chrysanthemum cultivars [from data of Roest and Bokelmann, 1975].

Chlorophyll formation. Levels of sucrose normally used to support the growth of tissue cultures are often inhibitory to chlorophyll synthesis (Rier and Chen, 1964; Edelman and Hanson, 1972) but the degree of inhibition does vary according to the species of plant from which the tissue was derived. In experiments of Hildebrandt *et al.* (1963) and Fukami and Hildebrandt (1967) for example, carrot and rose callus had a high chlorophyll content on Hildebrandt *et al.* (1946) tobacco medium with 2-8% sucrose; but tissue of endive, lettuce and spinach only produced large amounts of the pigment on a medium with no added sucrose (although a small amount of sugar was probably supplied by 15-16% coconut milk).

Cymbidium protocorms contain high chlorophyll levels only if they are cultured on media containing 0.2-0.5% sucrose. Their degree of greening declines rapidly when they are grown on sucrose concentrations higher than this (Vanséveren-Van Espen, 1973). Likewise, orchid protocorm-like bodies will not become green and cannot develop into plantlets if sucrose is present in the medium beyond the stage of their differentiation from the explants. Where added sucrose does reduce chlorophyll formation, it is thought that the synthesis of 5-aminolaevulinic acid (ALA - a precursor of the porphyrin molecules of which chlorophyll is composed) is reduced due to an inhibition of the activity of the enzyme ALA synthase (Pamplin and

Chapman, 1975). Sugars apart from sucrose are not inhibitory (Edelman and Hanson, 1972; El Hinnawiy, 1974). It has been said that cells grown on sucrose for prolonged periods may permanently lose the ability to synthesise chlorophyll (Van Huystee, 1977). Plastids become converted to amyloplasts packed with starch and this may change the expression of plastid DNA (Gunning and Steer, 1975) or result in a reduction of plastid RNA (Rosner *et al.,* 1977).

A small amount of photosynthesis may be carried out by cultured shoots providing they are not maintained on media containing a high concentration of sucrose. Photosynthesis increased in *Rosa* shoot cultures when they were grown initially on 20 or 40 g/l sucrose which was decreased to 10 g/l in successive subcultures (Langford and Wainwright, 1987). An increase in photosynthesis occurs when sucrose is omitted from the medium in which rooted plantlets are growing (Short *et al.,* 1987), but these treatments are not successful in ensuring a greater survival of plantlets when they are transferred *extra vitrum*. A more recent study also showed the relative contribution of autotrophic and heterotrophic carbon metabolism in cultured potato plants (Wolf *et al.,* 1998). With 8% sucrose in the medium 90% of the tissue carbon was of heterotrophic origin in light-grown plants; while on 3% sucrose, only 50% was of heterotrophic origin.

3.6 STARCH ACCUMULATION AND MORPHOGSNESIS

3.6.1. Starch deposition preceeding morphogenesis.

Cells of callus and suspension cultures commonly accumulate starch in their plastids and it is particularly prevalent in cells at the stationary phase. Starch in cells of rice suspensions had different chemical properties to that in the endosperm of seeds (Landry and Smyth, 1988). In searching for features which might be related to later morphogenetic events in *Nicotiana* callus, Murashige and co-workers (Murashige and Nakano, 1968; Thorpe and Murashige, 1968a, b) noticed that starch accumulated preferentially in cells sited where shoot primordia ultimately formed. The starch is produced from sucrose supplied in the culture medium (Thorpe *et al.,* 1986). As a result of this work, it was suggested that starch accumulation might be a prerequisite of morphogenesis. In tobacco, starch presumably acts as a direct cellular reserve of the energy required for morphogenesis, because it disappears rapidly as meristenoids and shoot primordia are formed (Thorpe and Meier, 1974, 1975). Morphogenesis is an energy-demanding process and callus maintained on a shoot-inducing medium does have a greater respiration rate than similar tissue kept on a non-inductive medium (Thorpe and Murashige, 1970; Thorpe and Meier, 1972). Organ-forming callus of tobacco has been found to accumulate starch prior to shoot or root formation, whereas callus not capable of morphogenesis did not do so (Kavi Kishor and Mehta, 1982).

3.6.2. Morphogenesis without starch deposition

Cells of other plants which become committed to initiate organs do not necessarily accumulate starch as a preliminary to morphogenesis and it seems likely that the occurrence of this phenomenon is species-related. The deposition of starch was observed as an early manifestation of organogenesis in *Pinus coulteri* embryos (Patel and Berlyn, 1983), but not in those of *Picea abies* (Von Arnold, 1987). Although zygotic embryos of the latter species immediately began to accumulate starch (particularly in the chloroplasts of cells in the cortex) when they were placed on a medium containing sucrose. It was never observed in meristematic cells from which adventitious buds developed (Von Arnold, 1987). However, if a major role for the accumulation of starch prior to the initiation of organized development is for energy production, this role would be satisfied by the lipid reserves in zygotic embryos of conifers (Thorpe, 1982). Indeed, the rapid and nearly linear degradation of triglycerides during the period of high respiration during shoot initiation in excised cotyledons of radiata pine (Biondi and Thorpe, 1982; Douglas *et al.,* 1982) would support this view.

Meristematic centres in bulb scales of *Nerine bowdenii* can be detected as groups of cells from which starch is absent (Grootaarts *et al.,* 1981). Starch was not accumulated in caulogenic callus of *Rosa persica* x *R. xanthina* initiated from recently-initiated shoot cultures, but cells did accumulate starch when the shoot forming capacity of the callus was lost after more than three passages (Lloyd *et al.,* 1988). Callus derived from barley embryos was noted to accumulate starch very rapidly and this was accompanied by a reduction in osmotic pressure within the cells (Granatek and Cockerline, 1978). Gibberellic acid, which in this plant could be used to induce shoot formation, brought about an increase in cell osmolarity.

There are some further examples where a diminution of the amount of stored carbohydrate in cultured tissues has restored or improved their

organogenetic capacity. A salt-tolerant line of alfalfa cells which showed no ability for shoot regeneration after three and a half years in culture on 3% sucrose was induced to form shoots and plantlets by being cultured for one passage of 24 days on 1% sucrose, before being returned to a medium containing 3% sucrose and a high 2,4-D level (Rains *et al.,* 1980, and personal communication). Cells which in 3% sucrose were full of starch became starch-depleted during culture on a lower sucrose level. The number of somatic embryos formed by embryogenic 'Shamouti' orange callus, was increased when sucrose was omitted from the medium for one passage, before being returned to Murashige and Tucker (1969) medium with 5-6% sucrose (Kochba and Button, 1974).

3.6.3. Unusual sugars

In some plants, unusual sugars are able to regulate morphogenesis and differentiation. Galactose stimulates embryogenesis in *Citrus* cultures (Kochba *et al.,* 1978) and can enhance the maturation of alfalfa embryos. Callus of *Cucumis sativus* grew most rapidly on raffinose and was capable of forming roots when grown on this sugar; somatic embryos were only differentiated when the callus was cultured on sucrose (88-175 mM), but if a small amount of stachyose (0.3 mM) was added to 88 mM sucrose the callus produced adventitious shoots instead (Kim and Janick, 1989). Stachyose is the major translocated carbohydrate in cucurbits.

4. OSMOTIC EFFECT OF MEDIA INGREDIENTS

Besides having a purely nutritive effect, solutions of inorganic salts and sugars, which compose tissue culture media, influence plant cell growth through their osmotic properties. A discussion is most conveniently accommodated at this point, as many of the papers published on the subject stress the osmotic effects of added sugars.

4.1. OSMOTIC AND WATER POTENTIALS: A GENERAL INTRODUCTION

Water movement into and out of a plant cell is governed by the relative concentrations of dissolved substances in the external and internal solutions, and by the pressure exerted by its restraining cell wall. The manner of defining the respective forces has changed in recent years, and as both old and new terminology are found in the tissue culture literature, the following brief description may assist the reader. More detailed explanations can be found in many text books on plant physiology.

In the older concept, cells were considered to take up water by suction (i.e. by exerting a negative pressure) induced by the osmotically active concentration of dissolved substances within the cell. The suction force or suction pressure (SP) was defined as that resulting from the osmotic pressure of the cell sap (OP_{cs}) minus the osmotic pressure of the external solution (OP_{ext}), and the pressure exerted on, and stretching the cell wall, turgor pressure (TP) - so called because it is at a maximum when the cells are turgid. This may be represented by the equations:

$SP = (OP_{cs} - OP_{ext}) – TP$ or

$SP = OP_{cs} - (OP_{ext} + TP)$.

These definitions devised by botanists, are not satisfactory thermodynamically because water should be considered to move down an energy gradient, losing energy as it does so. The term water potential (Ψ - Greek capital letter psi) is now used, and solutions of compounds in water are said to exert an osmotic potential. As the potential of pure water is defined as being zero, and dissolved substances cause it to be reduced, solutions have osmotic potentials, Ψs (or $\Psi\pi$ - Greek small letter pi) which are negative in value. Water is said to move from a region of high potential (having a less negative value) to one that is lower (having a more negative value). Both osmotic pressure and osmotic potential are used as terms in tissue culture literature and are equivalent except that they are opposite in sign (i.e. an OP of +6 bar equals a Ψs of -6 bar). Thermodynamically, the cell's turgor pressure is defined as a positive pressure potential (Ψp). The water potential of a cell (Ψcell) is then equal to the sum of its osmotic and pressure potentials plus the force holding water in microcapillaries or bound to the cell wall matrix (Ψm):

Ψ cell = $\Psi s + \Psi p + \Psi m$

Modern statements of the older suction pressure concept are therefore that the difference in water potential (i.e. the direction of water movement) between a cell and solution outside is given by:

$\Delta\Psi = (\Psi s_{\text{inside cell}} – \Psi s_{\text{outside cell}}) – \Psi p$ or

$\Delta\Psi = \Psi$ cell $– \Psi\pi_{\text{outside}}$

and when $\Delta\Psi = 0$ (at equilibrium)

$\Psi\pi_{\text{outside}} = \Psi$ cell

The force of water movement between two cells, `

'A' and 'B', of different water potential, is given by:

$\Delta\Psi = \Psi$ cellA – ΨcellB

the direction of movement being towards the more negative water potential.

The osmotic potential (pressure) of solutions is determined by their molar concentration and by temperature. The water potential of a plant tissue culture medium (Ψtcm) is equivalent to the osmotic potential of the dissolved compounds (Ψs). There is no pressure potential but, if they are added, substances such as agar and Gelrite contribute a matric potential (Ψm):

$\Psi tcm = \Psi s + \Psi m$

Osmotic pressure and water potential are measured in standard pressure units thus:

1 bar = 0.987 atm

$= 10^6$ dynes cm^{-2}

$= 10^5$ Pa (0.1 MPa = 1 bar).

Whereas molarity is defined as number of gram moles of a substance in one litre of a solution (i.e. one litre of solution requires less than one litre of solvent), molality is the number of gram moles of solute per kilogram of solvent, and thus, unlike osmotic potential (osmolality, measured in pressure units) is independent of temperature. It is therefore more convenient to give measurements of osmotic pressure in osmolality units. The osmole (Osm) is defined as:

The unit of the osmolality of a solution exerting an osmotic pressure equal to that of an ideal non-dissociating substance which has a concentration of one mole of solute per kilogram of solvent.

The osmolality of a very dilute solution of a substance which does not dissociate into ions, will be the same as its molality (i.e. g moles per kilogram of solvent). The osmolality of a weak solution of a salt, or salts, which has completely dissociated into ions, will equal that of the total molality of the ions.

The osmotic potential of dilute solutions approximates to Van't Hoff's equation: $\Psi = -cRT$; where

c = concentration of solutes in mol/litre;

R = the gas constant and

T = temperature in °K

From the above equation, at 0°C one litre of a solution containing 1 mole of an undissociated compound, or 1 mole of ions, could be expected to have an osmolality of 1 Osm/kg, and an osmotic (water) potential of:

Ψ= -1 (mole) x 0.082054 (atm /mole/°C)
x 273.16 (°K) = - 22.414 atm

Thus, although in practice the Van't Hoff equation must be corrected by the osmotic coefficient, Φ:

$\Psi = -\Phi cRT$ (Lang, 1967),

it is possible to give approximate figures for converting osmolality into osmotic potential pressure units. These are shown in Table 4.3. This table can also be used to estimate the osmotic potential of non-dissociating molecules such as sugars or mannitol. Thus at 25°C, 30 g/l sucrose (molecular wt. 342.3) should exert an osmotic potential of:

$$-2.4789 \times \frac{30}{342.3} = -0.217 \text{ MPa}$$

The observed potential is -0.223 Mpa (Table 4.4).

A definition. The osmotic properties of solutions can be difficult to describe without confusion. In this book, the addition of solutes to a solvent (which makes the osmotic or water potential more negative, but makes the osmolality of the solution increase to a larger positive value), has been said to reduce

Table 4.3 Factors by which osmolality (Osm/kg) should be multiplied to estimate an equivalent osmotic potential in pressure units.

For conversion to	Multiply Osm/kg by the factor shown for an equivalent osmotic potential in pressure units[1]				
	15 ºC	20 ºC	0 ºC	25 ºC	30 ºC
Atm	-22.414	-23.645	-24.055	-24.465	-24.875
Bar	-22.711	-23.958	-24.374	-24.789	-25.205
Dyne/cm^2	-22.711 x 10^6	-23.958 x 10^6	-24.374 x 10^6	-24.789 x 10^6	-25.205 x 10^6
Mpa	-2.2711	-2.3958	-2.4374	-2.4789	-2.5205

[*] Divide pressure units by the figures shown to find approximate osmolality,

$$\text{e.g. } -223 \text{ kPa} = \frac{-223}{1000} \times \frac{1}{-2.4789} = 0.090 \text{ Osm/kg.}$$

Table 4.4 The osmolality and osmotic potential of sucrose solutions at different concentrations.

Sucrose concentration		Osmolality	Osmotic potential at
(% , w/v)	(mM)	(Osm/kg)	25 ºC (MPa)
0.5	14.61	0.015	-0.037
1.0	29.21	0.030	-0.074
1.5	43.82	0.045	-0.112
2.0	58.43	0.060	-0.149
2.5	73.04	0.075	-0.186
3.0	87.64	0.090	-0.223
4.0	116.86	0.121	-0.300
5.0	175.28	0.186	-0.461
6.0	233.71	0.253	-0.627
8.0	292.14	0.324	-0.803
10.0	350.57	0.396	-0.982

(decrease) the osmotic potential of solutions. MS medium containing 3% sucrose (osmolality, ca. 186 mOsm/kg; ca. -461 kPa at 25°C) is thus described as having a lower water potential than the medium of White (1954) supplemented with 2% sucrose (osmolality, ca. 78 mOsm/kg; ca. −193 kPa at 25°C).

4.2. THE OSMOTIC POTENTIAL OF TISSUE CULTURE MEDIA

4.2.1. The total osmotic potential of solutes

The approximate total osmotic potential of a medium due to dissolved substances, can be estimated from: $\Psi s = \Psi s_{macronutrients} + \Psi s_{sugars}$

When 3% w/v sucrose is added to Murashige and Skoog (1962) medium, the osmolality of a filter sterilised preparation rises from 0.096 to 0.186 Osm/kg and at 25°C, the osmotic potential of the medium decreases from -0.237 to -0.460 MPa. Sugars are thus responsible for much of the osmotic potential of normal plant culture media. Even without any inversion to monosaccharides, the addition of 3% w/v sucrose is responsible for over four fifths of the total osmotic potential of White (1954) medium, 60% of that of Schenk and Hildebrandt (1972), and for just under one half that of MS.

The contribution of the gelling agent. The water potential of media solidified with gels is more negative than that of a liquid medium, due to their matric potential, but this component is probably relatively small (Amador and Stewart, 1987). In the following sections, the matric potential of semi-solid media containing ca. 6 g/l agar has been assumed to be -0.01 MPa at 25°C, but as adding extra agar to media helps to prevent hyperhydricity, it is possible that this is an underestimate.

The contribution of nutrient salts. Inorganic salts dissociate into ions when they are dissolved in water, so that the water potential of their solutions (especially weak solutions) does not depend on the molality (or molarity) of undissociated compounds, but on the molality (or molarity) of their ions. Thus a solution of KCl with a molality of 0.1, will have a theoretical osmolality of 0.2, because in solution it dissociates into 0.1 mole K^+ and 0.1 mole Cl^-. Osmolality of a solution of mixed salts is dependent on the total molality of ions in solution.

Dissociation may not be complete, especially when several different compounds are dissolved together as in plant culture media, which is a further reason why calculated predictions of water potential may be imprecise. In practice, osmotic potentials should be determined by actual measurement with an osmometer. Clearly though, osmotic potential of a culture medium is related to the concentration of solutes, particularly that of the macronutrients and sugar.

Of the inorganic salts in nutrient media, the macronutrients contribute most to the final osmotic (water) potential because of their greater concentration. The osmolality of these relatively dilute solutions is very similar to the total osmolarity of the constituent ions at 0°C, and can therefore be estimated from the total molarity of the macronutrient ions. Thus based on its macronutrient composition, a liquid Murashige and Skoog (1962) medium (without sugar) with a total macronutrient ion concentration of 95.75 mM, will have an osmolality of ca. 0.0958 osmoles (Osm) per kilogram of water solvent (95.8 mOsm/kg), at 25°C, an osmotic potential of ca. -0.237 MPa (237 kPa). Estimates of osmolality

derived in this way agree closely to actual measurements of osmolality or osmolarity for named media given in the papers of Yoshida *et al.* (1973), Kavi Kishor and Reddy (1986) and Lazzeri *et al.* (1988) (see Table 4.5).

The contribution of sugars. The osmolality and osmotic potential of sucrose solutions can be read from Table 4.6. Those of mannitol and sorbitol solutions of equivalent molarities will be approximately comparable. At concentrations up to 3% w/v, the osmolality of sucrose is close to molarity. It will be seen that the osmotic potential (in MPa) of sucrose solutions at 25°C can be roughly estimated by multiplying the % weight/volume concentration by -0.075: that of the monosaccharides fructose, glucose, mannitol and sorbitol (which have a molecular weight approximately 0.52 times that of sucrose), by multiplying by -0.14.

If any of the sucrose in a medium becomes hydrolysed into monosaccharides, the osmotic potential of the combined sugar components (sucrose + glucose + fructose), (Ψs_{sugars}), will be lower (more negative) than would be estimated from Table 4.5. The effect can be seen in Table 4.6. From this data it seems that 40-50% of the sucrose added to MS medium by Lazzeri *et al.* (1988) was broken down into monosaccharides during autoclaving. Hydrolysis of sucrose by plant-derived invertase enzymes will also have a similar effect on osmotic potential. In some suspension cultures, all sucrose remaining in the medium is inverted within 24 h. A fully inverted sucrose solution would have almost double the negative potential of the original solution, but as the appearance of glucose and fructose by enzymatic hydrolysis usually occurs concurrently with the uptake of sugars by the tissues, it will tend to have a stabilizing effect on osmotic potential during the passage of a culture. Reported measurements of the osmolality of MS medium containing 3% sucrose, after autoclaving, are:

230 mOsm/kg (0.65% Phytagar; Lazzeri *et al.,* 1988)

240 ± 20 mOsm/kg (0.6-0.925% agar; Scherer *et al.,* 1988)

230 ± 50 mOsm/kg (0.2-0.4% Gelrite; Scherer *et al.,* 1988)

4.2.2. Decreasing osmotic potential with other osmotica

By adding soluble substances in place of some of the sugar in a medium, it can be shown that sugars not only act as a carbohydrate source, but also as osmoregulants. Osmotica employed for the deliberate modification of osmotic potential, should be largely lacking other biological effects. Those most frequently selected are the sugar alcohols mannitol and sorbitol. It is assumed that plants that do not have a native pathway for sugar alcohol biosynthesis are also deficient in pathways to assimilate them. Sugar alcohols, though, are usually translocated, and may be metabolised and utilized to various degrees (Steinitz, 1999; for mannitol Lipavska and Vreugedenhil, 1996 Tian and Russell, 1999; for sorbitol Pua *et al.,* 1984). Polyethylene glycol may be more helpful as an inert nonpenetrating osmolyte although it may contain toxic contaminants (Chazen *et al.,* 1995). Mannitol can easily penetrate cell walls, but the plasmalemma is considered to be relatively impermeable to it (Rains, 1989), whereas high-molecular-weight polyethylene glycol 4000 is too large to penetrate cell walls (Carpita *et al.,* 1979; Rains, 1989). Thus, a nonpenetrating osmolyte cannot penetrate into the plant cells, but inhibits water uptake. Sodium sulphate and sodium chloride have also been used in some experiments.

Table 4.5. Predicted osmolality and the osmolality actually observed after autoclaving (data from Lazzeri *et al.,* 1988).

	Predicted osmolality (no sucrose hydrolysis)				Oberved total osmolality (mOsm/kg)
	Salts (mOsm/kg)	Agar (mOsm/kg)	Sucrose (mOsm/kg)	Total (mOsm/kg)	
MS + agar	96	4	-	100	-
MS + agar + 0.5% sucrose	96	4	15	115	115
MS + agar + 1.0% sucrose	96	4	30	130	140
MS + agar + 2.0% sucrose	96	4	60	160	184†
MS + agar + 4.0% sucrose	96	4	121	221	276

† 161 mOsm/kg by Brown *et al.* (1989)

Table 4.6. The osmolality and osmotic potential of sucrose solutions of different concentrations.

Sucrose Concentration		Osmolality	Osmotic potential at 25°C
(% w/v)	mM	Osm/Kg	MPa
0.5	14.61	0.015	-0.037
1.0	29.21	0.030	-0.074
1.5	43.82	0.045	-0.112
2.0	58.43	0.060	-0.149
2.5	73.04	0.075	-0.186
3.0	87.64	0.090	-0.223
4.0	116.86	0.121	-0.300
6.0	175.28	0.186	-0.461
8.0	233.71	0.253	-0.627
10.0	292.14	0.324	-0.803
12.0	350.57	0.396	-0.982

4.3. EFFECTS AND USES OF OSMOLYTES IN TISSUE CULTURE MEDIA

4.3.1. Protoplast isolation and culture

The osmotic potential of a plant cell is counter-balanced by the pressure potential exerted by the cell wall. To safely remove the cell wall during protoplast isolation without damaging the plasma membrane, it has been found necessary to plasmolyse cells before wall-degrading enzymes are used. This is done by placing the cells in a solution of lower water potential than that of the cell.

Glucose, sucrose and especially mannitol and sorbitol, are usually added to protoplast isolation media for this purpose, either singly or in combination, at a total concentration of 0.35-0.7 M. These addenda are then retained in the subsequent protoplast culture medium, their concentration being progressively reduced as cell colonies start to grow. Tobacco cell suspensions take up only a very small amount of mannitol from solution (Thompson and Thorpe, 1981) and its effect as an osmotic agent appears to be exerted outside the cell (Thorpe, 1982). When protoplasts were isolated from *Pseudotsuga* and *Pinus* suspensions, which required a high concentration of inositol to induce embryogenesis , it was found to be essential to add 60 g/l (0.33 M) myo-inositol (plus 30 g/l sucrose, 20 g/l glucose and 10 g/l sorbitol) to the isolation and culture media (Gupta *et al.,* 1988). Mannitol and further amounts of sorbitol could not serve as substitutes.

4.3.2. Osmotic effects on growth

Solutions of different concentrations partly exert their effect on growth and morphogenesis by their nutritional value, and partly through their varying osmotic potential. Lapeña *et al* (1988) estimated that three quarters of the sucrose necessary to promote the optimum rate of direct adventitious shoot formation from *Digitalis obscura* hypocotyls, was required to supply energy, while the surplus regulated morphogenesis osmotically.

How osmotic potential influences cellular processes is still far from clear. Cells maintained in an environment with low (highly negative) osmotic potential, lose water and in consequence the water potential of the cell decreases. This brings about changes in metabolism and cells accumulate high levels of proline (Rabe, 1990). The activity of the main respiratory pathway of cells (the cytochrome pathway) is reduced in conditions of osmotic stress, in favour of an alternative oxidase system (De Klerk-Kiebert and Van der Plas, 1985). The increase of the concentration of osmolytes, may also result in high levels of the plant hormone abscisic acid, both *extra vitrum* and *in vitro* (recent reviews Zhu, 2002; Riera *et al.,* 2005).

Equilibrium between the water potential of the medium and that of *Echinopsis* callus, only occurred when the callus was dead. Normally the water potential of the medium was greater so that water flowed into the callus (Kirkham and Holder, 1981). Clearly this situation could not occur in media which were too concentrated, and Cleland (1977) proposed that a critical water potential needs to be established within a cell before cell expansion and cell division, can occur. The osmotic concentration of culture media could therefore be expected to influence the rate of cell division or the success of morphogenesis of the cells or tissues they support. Both the inorganic and organic components will be

contributory. The cells of many plants which are natives of sea-shores or deserts (e.g. many cacti) characteristically have a low water potential (Ψcell) and in consequence may need to be cultured in media of relatively low (highly negative) osmotic potentials (Lassocinski, 1985). Sucrose concentrations of 4.5-6% have sometimes been found to be beneficial for such plants (Sachar and Iyer, 1959; Johnson and Emino, 1979; Mauseth, 1979; Lassocinski, 1985).

Above normal sucrose concentrations can often be beneficial in media for anther culture [e.g 13% sucrose in Gamborg *et al.* (1968) B5 medium - Chuong and Beversdorf, 1985], and for the culture of immature embryos [e.g. 10% sucose in MS medium - Stafford and Davies (1979); 12.5% in Phillips and Collins (1979) L2. medium - Phillips *et al.* (1982)].

If the osmotic potential of the medium does indeed influence the growth of tissue cultures, one might expect the sucrose concentration, which is optimal for growth, to vary from one medium to another, more sucrose being required in dilute media than in more concentrated ones. Evans *et al.* (1976) found this was so with cultures of soybean tissue. Maximum rates of callus growth were obtained in media containing either:

1. 50-75% of MS basal salts + 3-4% sucrose, or,
2. 75-100% of MS basal salts + 2% sucrose.

Similarly Yoshida *et al.* (1973) obtained equally good growth rates of *Nicotiana glutinosa* callus with nutrient media in which

1. the salts exerted -0.274 MPa and sucrose -0.223 MPa, or
2. the salts exerted -0.365 MPa and sucrose -0.091 MPa.

It was essential to add 60 g/l sucrose (*i.e.* $\Psi s_{sucrose}$= -0.461 MPa at 25°C) to the 'MEDIUM' salts of de Fossard *et al.* (1974) (Ψ = -0.135 MPa) to obtain germination and seedling growth from immature zygotic embryos of tomato. However, if the 'HIGH' salts (Ψs_{medium} = -0.260 MPa) were used, 60 g/l sucrose gave only slightly better growth than 2.1 g/l ($\Psi s_{sucrose}$ = -0.156 MPa) (Neal and Topoleski, 1983).

A detailed examination of osmotic effects of culture media on callus cultures was conducted by Kimball *et al.* (1975). Various organic substances were added to a modified Miller (1961) medium (which included 2% sucrose), to decrease the osmotic potential (Ψs) from -0.290 MPa. Surprisingly, the greatest callus growth was said to occur at -1.290 to -1.490 MPa (unusually low potentials which normally inhibit growth — see below) in the presence of mannitol or sorbitol, and between -1.090 to -1.290 MPa when extra sucrose or glucose were added. On the standard medium, many cells of the callus were irregularly shaped; as the osmotic potential of the solution was decreased there were fewer irregularities and at about Ψs = -1.090 MPa all the cells were spherical. The percentage dry matter of cultures also increased as Ψs was decreased.

Doley and Leyton (1970) found that decreasing the water (osmotic) potential of half White (1963) medium by -0.100 or -0.200 MPa through adding more sucrose (and/or polyethylene glycol), caused the rate of callus growth from the cut ends of *Fraxinus* stem sections to be lower than on a standard medium. At the reduced water potential, callus had suberised surfaces and grew through the activity of a vascular cambium. It also contained more lignified xylem and sclereids. At each potential there was an optimal IAA concentration for xylem differentiation.

When the concentration of sucrose in a high salt medium such as MS is increased above 4-5 per cent, there begins to be a progressive inhibition of cell growth in many types of culture. This appears to be an osmotic effect because addition of other osmotically-active substances (such as mannitol and polyethyleneglycol) to the medium causes a similar response (Maretzki *et al.,* 1972). Usually, high concentrations of sucrose are not toxic, at least not in the short term, and cell growth resumes when tissues or organs are transferred to media containing normal levels of sugar. Increase of Ψs is one method of extending the shelf life of cultures. Pech and Romani (1979) found that the addition of 0.4 M mannitol to MS medium (modified organics), was able to prevent the rapid cell lysis and death which occurred when 2,4-D was withdrawn from pear suspension cultures.

Decreasing the osmotic potential (usually by adding mannitol) together with lowering of the temperature has been used to reduce the growth rate for preservation of valuable genotypes *in vitro*. This has been reported, among others, for potato (Gopal *et al.,* 2002; Harding *et al.,* 1997), *Dioscorea alata* (Borges *et al.,* 2003) and enset (Negash *et al.,* 2001)

4.3.3. Tissue water content

The water content of cultured tissues decreases as the level of sucrose in the medium is increased. Isolated embryos of barley were grown by Dunwell (1981) on MS medium in sucrose concentrations up to 12 per cent. Dry weight increased as the sucrose concentration was raised to 6 or 9 per cent and shoot length of some varieties was also greater than on 3 per cent sucrose. Water content of the developing

plantlets was inversely proportional to the sucrose level.

The dry weight of *Lilium auratum* bulbs and roots on MS medium increased as the sucrose concentration was augmented to 90 g/l but decreased dramatically with 150 g/l because growth was inhibited. The fresh weight/dry weight ratio of both kinds of tissue once again declined progressively as sucrose was added to the medium (0 - 150 g/l). In a medium containing 30 mg/l sucrose, the number and fresh weight of bulblets increased as MS salt strength was raised from one eighth to two times its normal concentration. An interaction between salt and sucrose concentrations was demonstrated in that optimum dry weight of bulbs could be obtained in either single strength MS + 120 g/l sucrose (osmolality, without sucrose inversion = ca. 492 mOsm/kg; Ψs = ca. -1.22 MPa), or double strength MS + 60 g/l sucrose (osmolality, without sucrose inversion = ca. 378 mOsm/kg; Ψs = ca. -0.94 MPa) (Takayama and Misawa, 1979).

4.3.4. Morphogenesis

The osmotic effect of sucrose in culture solutions was well demonstrated by a series of experiments on tobacco callus by Brown *et al.,* (1979): rates of callus growth and shoot regeneration which were optimal on culture media containing 3 per cent sucrose, could be maintained when the sugar was replaced partially by osmotically equivalent levels of mannitol. The optimal (medium plus sucrose) here was between -0.4 and -0.6 MPa, and increasing sucrose levels above 3 per cent brought a progressive decrease in shoot regeneration. Similar results were obtained by Barg and Umiel (1977), but when they kept the osmotic potential of the culture solution roughly constant by additions of mannitol, the sucrose concentrations optimal for tobacco callus growth or morphogenesis were not the same (Fig. 4.2)

Brown and Thorpe (1980) subsequently found that callus of *Nicotiana* capable of forming shoots, had a water potential (Ψ) of -0.8 MPa, while non-shoot-forming callus had a Ψ of -0.4 MPa. The two relationships were:

Shoot forming callus
Ψcell = Ψs +Ψp + Ψm
–0.8 =–1 + 0.4 + 0 MPa
Non-shoot-forming callus
Ψcell = Ψs +Ψp + Ψm
–0.4 = –0 + 0.3 + 0 MPa

Correct water potential. An optimum rate of growth and adventitious shoot formation of wheat callus occurred on MS medium containing 2% sucrose. Only a small number of shoots were produced on the medium supplemented with 1% sucrose, but if mannitol was added so that the total Ψcell = Ψs was the same as when 2% sucrose was present, the formation of adventitious shoots was stimulated (Galiba and Erdei, 1986). Very similar results were obtained by Lapeña *et al.* (1988). A small number of shoot buds were produced from *Digitalis obscura* hypocotyls on MS medium containing 1% sucrose: more than twice as many if the medium contained 2% sucrose (total Ψs given as -0.336 MPa), or 1% sucrose plus mannitol to again give a total Ψs equal to -0.336 MPa.

Water potential can modify commitment. Morphogenesis can also be regulated by altering the water potential of media. Shepard and Totten (1977) found that very small (ca. 1-2 mm) calluses formed from potato mesophyll protoplasts were unable to survive in 1 or 2% sucrose, and the base of larger (5-10 mm) ones turned brown, while the upper portions turned green but formed roots and no shoots. The calli became fully green only on 0.2-0.5% sucrose. At these levels shoots were formed in the presence of 0.2-0.3 M mannitol. When the level of mannitol was reduced to 0.05 M, the proportion of calli differentiating shoots fell from 61% to 2%. The possibility of an osmotic affect was suggested because equimolar concentrations of myo-inositol were just as effective in promoting shoot regeneration.

Another way to modify morphogenesis is to increase the ionic concentration of the medium. Pith phloem callus of tobacco proliferates on Zapata *et al.* (1983) MY1 medium supplemented with 10^{-5} M IAA and 2.5 x 10^{-6} M kinetin, but forms shoots on Murashige *et al.* (1972) medium containing 10^{-5} M IAA and 10^{-5} M kinetin. These two media contain very similar macronutrients (total ionic concentrations, respectively 96 and 101mM), yet adding 0.5-1.0% sodium sulphate (additional osmolality 89-130 mOsm/kg) decreased the shoot formation of callus grown on the shoot-forming medium, but increased it on the medium which previously only supported callus proliferation (Pua *et al.,* 1985a). Callus cultured in the presence of sodium sulphate retained its shoot-producing capacity over a long period, although the effect was not permanent (Pua *et al.,* 1985b; Chandler *et al.,* 1987). In these experiments shoot formation was also enhanced by sodium chloride and mannitol.

Increasing the level of sucrose from 1 to 3 per cent in MS medium containing 0.3 mg/l IAA,

induced tobacco callus to form shoots, while further increasing it to 6 per cent resulted in root differentiation (Rawal and Mehta, 1982; Mehta, 1982). The formation of adventitious shoots from *Nicotiana tabacum* pith callus is inhibited on a medium with MS salts if 10-15% sucrose is added. Preferential zones of cell division and meristemoids produced in 3% sucrose then become disorganised into parenchymatous tissue (Hammersley-Straw and Thorpe, 1988).

It should be noted that auxin which has a very important influence on the growth and morphogenesis of cultured plant cells, causes their osmotic potential to be altered (Van Overbeek, 1942; Hackett, 1952; Ketellapper, 1953). When tobacco callus is grown on a medium which promotes shoot regeneration, the cells have a greater osmotic pressure (or more negative water potential) than callus grown on a non-inductive medium (Brown and Thorpe, 1980; Brown, 1982).

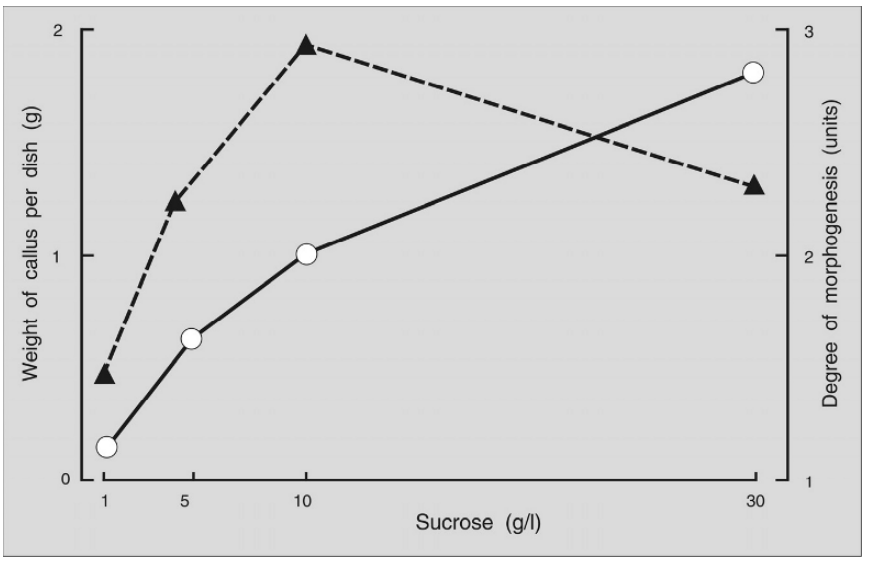

Fig. 4.2 The effect of sucrose concentration on the growth and morphogenesis of tobacco callus.
[Drawn from data for four lines of tobacco callus in Barg and Umiel, 1977]. Callus growth = solid line. Morphogenesis = line of dashes.
Scale was, 1 = No differentiation, 2 = Dark green callus with meristemoids, 3 = leafy shoots

Apogamous buds on ferns. Whittier and Steeves (1960) found a very clear effect of glucose concentration on the formation of apogamous buds on prothalli of the fern *Pteridium*. (Apogamous buds give rise to the leafy and spore-producing generation of the plant which has the haploid genetic constitution of the prothallus). Bud formation was greatest between 2-3% glucose (optimum 2.5%). Results which confirm this observation were obtained by Menon and Lal (1972) in the moss *Physcomitrium pyriforme*. Here apogamous sporophytes were formed most freely in low sucrose concentrations (0.5-2%) and low light conditions (50-100 lux), and were not produced at all when prothalli were cultured in 6% sucrose or high light (5000-6000 lux). Whittier and Steeves (loc. cit.) noted that they could not obtain the same rate of apogamous bud production by using 0.25% glucose plus mannitol or polyethyleneglycol; on the other hand, adding these osmotica to 2.5% glucose (so that the osmotic potential of the solution was equivalent to one with 8% sucrose) did reduce bud formation. It therefore appeared that the stimulatory effect of glucose on morphogenesis was mainly due to its action as a respiratory substrate, but that inhibition might be caused by an excessively depressed osmotic potential.

Differentiation of floral buds. Pieces of cold-stored chicory root were found by Margara and Rancillac (1966) to require more sucrose (up to 68 g/l; 199 mM) to form floral shoots than to produce vegetative shoots (as little as 17 g/l; 50 mM). Tran Thanh Van and co-workers (Tran Thanh Van and Trinh, 1978) have similarly shown that the specific formation of vegetative buds, flower buds, callus or roots by thin cell layers excised from tobacco stems, could be controlled by selecting appropriate concentrations of sugars and of auxin and cytokinin growth regulators.

Root formation and root growth. Media of small osmotic potential are usually employed for the

induction and growth of roots on micropropagated shoots. High salt levels are frequently inhibitory to root initiation. Where such levels have been used for Stage II of shoot cultures, it is common to select a low salts medium (e.g. ¼ or ½ MS), when detached shoots are required to be rooted at Stage III. By testing four concentrations of MS salts (quarter, half, three quarters and full strength) against four levels of sucrose (1, 2, 3 and 4%), Harris and Stevenson (1979) found that correct salt concentration (½ or ¼ MS) was more important than sucrose concentration for root induction on grapevine cuttings *in vitro*. The benefit of low salt levels for root initiation may be due more to the need for a low nitrogen level, than for an increased osmotic potential. Dunstan (1982) showed that microcuttings of several tree-fruit rootstocks rooted best on MS salts, but that in media of these concentrations, the amount of added sugar was not critical, although it was essential for there to be some present. For the best rooting of *Castanea*, it was important to place shoots in Lloyd and McCown (1981) WPM medium containing 4% sucrose (Serres, 1988).

There are reports that an excessive sugar concentration can inhibit root formation. Green cotyledons of *Sinapis alba* and *Raphanus sativus* were found by Lovell *et al.* (1972) to form roots in 2% sucrose in the dark, but not in light of 5500 lux luminous intensity. In the light, rooting did occur if the explants were kept in water, or (to a lesser extent) if they were treated with DCMU (a chemical inhibitor of photosynthesis) before culture in 2% sucrose. The authors of this paper suggested that sugars were produced within the plant tissues during photosynthesis, which, added to the sucrose absorbed from the medium, provided too great a total sugar concentration for rooting. Rahman and Blake (1988) reached the same conclusion in experiments on *Artocarpus heterophyllus*. When shoots of this plant were kept on a rooting medium in the dark, the number and weight of roots formed on shoots, increased with the inclusion of up to 80 g/l sucrose. The optimum sucrose concentration was 40 g/l if the shoots were grown in the light.

Root formation on avocado cuttings in 0.3 MS salts [plus Linsmaier and Skoog (1965) vitamins] was satisfactory with 1.5, 3 or 6% sucrose, and only reduced when 9% sucrose was added (Pliego-Alfaro, 1988).

Although 4% (and occasionally 8%) sucrose has been used in media for isolated root culture, 2% has been used in the great majority of cases (Butcher and Street, 1964). In an investigation into the effects of sucrose concentration on the growth of tomato roots, Street and McGregor (1952) found that although sucrose concentrations of between 1.5 and 2.5% caused the same rate of increase of root fresh weight, 1.5% sucrose was optimal. It produced the best rate of growth of the main root axis, and the greatest number and total length of lateral roots.

Somatic embryogenesis. The osmotic potential of a medium can influence whether somatic embryogenesis can occur and can regulate the proper development of embryos. As will be shown below, a low osmotic potential is often favourable, but this is not always the case. For instance immature cotyledons of *Glycine max* produced somatic embryos on Phillips and Collins (1979) L2 medium containing less than 2% sucrose, but not if the concentration of sugar was increased above this level (Lippmann and Lippmann, 1984).

Placing tissues in solutions with high osmotic potential will cause cells to become plasmolysed, leading to the breaking of cytoplasmic interconnections between adjacent cells (plasmodesmata). Wetherell (1984) has suggested that when cells and cell groups of higher plants are isolated by this process, they become enabled to develop independently, and express their totipotency. He pointed out that the isolation of cells of lower plants induces regeneration, and plasmolysis has long been known to initiate regeneration in multicellular algae, the leaves of mosses, fern prothallia and the gemmae of liverworts (Narayanaswami and LaRue, 1955; Miller, 1968). Carrot cell cultures pre-plasmolysed for 45 min in 0.5-1.0 M sucrose or 1.0 M sorbitol gave rise to many more somatic embryos when incubated in Wetherell (1969) medium with 0.5 mg/l 2,4-D than if they had not been pre-treated in this way. Moreover embryo formation was more closely synchronized. Ikeda-Iwai *et al.* (2003) found that in *Arabidopsis* a 6-12 hour treatment with 0.7 M sucrose, sorbitol or mannitol resulted in somatic embryogenesis.

Callus derived from hypocotyls of *Albizia richardiana*, produced the greatest numbers of adventitious shoots on B5 medium containing 4% sucrose, but somatic embryos grew most readily when 2% sucrose was added. At least 1% sucrose was necessary for any kind of morphogenesis to take place (Tomar and Gupta, 1988). A similar result was obtained by Ćulafić *et al.* (1987) with callus from axillary buds of *Rumex acetosella*: adventitious shoots were produced on a medium containing MS

salts and 2% sucrose (Ψs = ca. -0.39 MPa at 25°C), but embryogenesis occurred when the sucrose concentration was increased to 6% ($\Psi s_{sucrose}$ = -0.46 MPa, Ψs = ca. -0.70 MPa at 25°C) or if the medium was supplemented with 2% sucrose plus 21.3 g/l mannitol or sorbitol (which together have the same osmolality as 6% sucrose).

A low (highly negative) osmotic potential helps to induce somatic embryogenesis in some other plants. Adding 10-30 g/l sorbitol to Kumar *et al.* (1988) L-6 medium (total macronutient ions 64.26 mM; 20 g/l sucrose), caused there to be a high level of embryogenesis in *Vigna aconitifolia* suspensions and the capacity for embryogenesis to be retained in long-term cultures. The formation of somatic embryos in ovary callus of *Fuchsia hybrida* was accelerated by adding 5% sucrose to B5 medium (Dabin and Beguin, 1987), and the induction of embryogenic callus of *Euphorbia longan* required the culture of young leaflets on B5 medium with 6% sucrose (Litz, 1988).

There are exceptions, particularly with regard to embryo growth. The proportion of *Ipomoea batatas* somatic embryos forming shoots was greatest when a medium containing MS inorganics contained 1.6%, rather than 3% sucrose (Chée *et al.,* 1990). Protocorm proliferation of orchids is most rapid when tissue is cultured in high concentrations of sucrose, but for plantlet growth, the level of sucrose must be reduced (Homès and Vanseveran-Van Espen, 1973).

The induction of embryogenic callus from immature seed embryos of *Zea mays* was best on MS medium with 12% sucrose (Lu *et al.,* 1982), and Ho and Vasil (1983) used 6-10% sucrose in MS medium to promote the formation of pro-embryoids from young leaves of *Saccharum officinarum*. However, in the experiments of Ahloowalia and Maretski (1983), somatic embryo formation from callus of this plant was best on MS medium with 3% sucrose, but growth of the embryos into complete plantlets required that the embryos should be cultured first on MS medium with 6% sucrose and then on MS with 3% sucrose.

Polyethylene glycol 4000 (PEG 4000) improves root and shoot emergence without limiting embryo histodifferentiation in soybean somatic embryos (Walker and Parrott, 2001). Likewise in spruce, it was reported that polyethylene glycol might improve the quality of somatic embryos by promoting normal differentiation of the embryonic shoot and root (e.g. Stasolla *et al.,* 2003). Non-penetrating osmotica like polyethylene glycol cannot enter plant cells, but restrict water uptake and provide a simulated drought stress during embryo development. A combination of ABA and an osmoticum prevents precocious germination in white spruce (Attree *et al.,* 1991) and allows embryo development to proceed. Advantageous effects of polyethylene glycol and ABA have been reported in a number of species (*Hevea brasiliensis*, Linossier *et al.,* 1997; *Picia abies*, Bozhkov and Von Arnold, 1998; white spruce, Stasolla *et al.,* 2003, *Corydalis yanhusuo*, Sagare *et al.,* 2000; and *Panax ginseng*, Langhansová *et al.,* 2004).

When cultured on Sears and Deckard (1982) medium, embryogenesis in callus initiated from immature embryos of 'Chinese Spring' and some other varieties of wheat was incomplete, because shoot apices germinated and grew before embryos had properly formed. More typical somatic embryos could be obtained by adding 40 mM sodium or potassium chloride to the medium. The salts had to be removed to allow plantlets to develop normally (Galiba and Yamada, 1988). Transferring somatic embryos to a medium of lower (more negative) water potential is often necessary to ensure their further growth and/or germination. High sucrose levels are often required in media for the culture of zygotic embryos if they are isolated when immature. The use of 50-120 g/l sucrose in media is then reported, the higher concentrations usually being added to very weak salt mixtures. Embryos which are more fully developed when excised, grow satisfactorily in a medium with 10-30 g/l sugar.

Storage organ formation. At high concentration, sucrose promotes the formation of tubers, bulbs and corms (e.g. Xu *et a*l., 1998; Vreugdenhil *et al*., 1998; Ziv 2005, Gerrits and de Klerk 1992). This promotion might be mediated by ABA since osmotic stress induces ABA synthesis (Riera *et al*., 2005) and ABA promotes bulb (Kim *et al.*, 1994) and tuber (Xu *et al*., 1998) formation. The situation is, however, more complex. Suttle and Hultstrand (1994) did not find a reduction of tuber formation in potato by adding fluridone, an ABA-synthesis inhibitor, and Xu *et al.* (1998) did not observe an increased ABA-level at high sucrose concentration. So in potato, the effect of sucrose is not likely to be mediated by ABA. Exogenous ABA does not promote *Gladiolus* corm formation (Dantu and Bhojwani, 1995) but bulb formation of lily was completely inhibited by fluridone and restored by simultaneous addition of ABA (Kim *et al*., 1994). To establish the effect of osmoticum directly, experiments have been carried out with addition of

mannitol instead of sucrose. Mannitol did not promote corm production of *Gladiolus* (De Bruyn and Ferreira, 1992) or bulb production in onion (Kahane and Rancillac, 1996), but data for lily suggested that, although it had a toxic effect, in this plant mannitol did stimulate bulb formation (Gerrits and De Klerk, 1992).

Anther culture. The use of high concentrations of sucrose is commonly reported in papers on anther culture where the addition of 5-20% sucrose to the culture medium is found to assist the development of somatic embryos from pollen microspores. This appears to be due to an osmotic regulation of morphogenesis (Sunderland and Dunwell, 1977), for once embryoid development has commenced, such high levels of sucrose are no longer required, or may be inhibitory. A high concentration of mannitol has been used for pretreatment before the culture of barley anthers (Roberts-Oehlschlager and Dunwell 1990) and pollen (Wei *et al*., 1986); tobacco anthers (Imamura and Harada 1980) and pollen (Imamura *et al.,* 1982); and before wheat microspore culture (Hu *et al.,* 1995). A high concentration of mannitol has also been used to induce osmotic stress in microspore derived embryos of *Brassica napus* (Huang *et al.,* 1991) and before anther culture of *Brassica campestris* (Hamaoka *et al.,* 1991). Isolated microspores of *Brassica napus* cultured on a high concentration of mannitol and at a low concentration of sucrose (0.08–0.1%) yield no embryos whereas on high polyethyleneglycol 4000 the embryo yield is comparable to that of the sucrose control (Ikeda-Iwai, 2003). These results demonstrate that in microspore embryogenesis of *Brassica napus* the level of metabolizable carbohydrate required for microspore embryo induction and formation may be very low and that an appropriate osmoticum (polyethylene glycol 4000 or sucrose) is required.

The temporary presence of high sucrose concentrations is said to prevent the proliferation of callus from diploid cells of the anther that would otherwise swamp the growth of the pollen-derived embryoids. The concentration of macronutrient ions generally used in anther culture media is not especially low. In a sample of reports it was found to be 68.7 mM (George *et al.*, 1987), and so the total osmotic potential, Ψ_s, (salts plus sucrose) of many anther culture media is in the range -0.55 to -1.15 MPa.

4.3.5. Relative humidity.

The vapour pressure of water is reduced by dissolving substances in it. This means that the relative humidity of the air within closed culture vessels is dependent on the water potential of the medium according to the equation:

$$\Psi = \frac{1000RT}{W_0} \ln_e\left(\frac{p}{p_0}\right). \left(p - c\frac{dp}{dc}\right)$$

(after Glasstone, 1947)

where: Ψ, R and T are as in the Van't Hoff equation, W_0 is the molecular weight of water, c is the concentration of the solution in moles per litre, and p_0 and p are respectively the vapour pressures of water and the solution.

If the change in vapour pressure dependent on the density of the solution, $p - c$ (dp/dc), is treated as unity (legitimate perhaps for very dilute solutions, or when the equation is expressed in molality, rather than molarity), it is possible to estimate relative humidity ($100 \times p/p_0$) above tissue culture media of known water potentials, from:

$$\Psi = \frac{1000RT}{18.016} \ln_e\left(\frac{p}{p_0}\right), \text{ (Lang, 1967)}$$

The relative humidity above most plant tissue cultures in closed vessels is thus calculated to be in the range 99.25-99.75% (Table 4.7), the osmolality of some typical media being

- White (1963), liquid, 2% sucrose, 106 mOsm/kg
- MS, agar, 3% sucrose, 230 mOsm/kg
- MS, agar, 6% sucrose, 359 mOsm/kg
- MS, agar, 12% sucrose (unusual), 659 mOsm/kg

(in these cases, 50% hydrolysis of sucrose into monosaccharides is assumed to have taken place during autoclaving)

Relative humidity can be reduced below the levels indicated above by, for example, covering vessels with gas-permeable closures while using non-gelatinous support systems (see Section 6.3.1).

Table 4.7 The relative humidity within culture vessels which would be expected to result from the use of plant culture media of various osmolalities or water potentials

Relative humidity in vessel (%)	Osmolality (mOsm/kg)	Water potential (kPa) at temperature:			
		15 ºC	20 ºC	25 ºC	30 ºC
98	1121	-2686	-2733	-2780	-2826
99	558	-1337	-1360	-1383	-1406
99.5	278	-667	-678	-690	-701
99.75	139	-333	-339	-344	-350

5. pH OF TISSUE CULTURE MEDIA

The relative acidity or alkalinity of a solution is assessed by its pH. This is a measure of the hydrogen ion concentration; the greater the concentration of H^+ ions (actually H_3O^+ ions), the more acid the solution. As pH is defined as the negative logarithm of hydrogen ion concentration, acid solutions have low pH values (0-7) and alkaline solutions, high values (7-14). Solutions of pH 4 (the concentration of H^+ is 10^{-4} mol.l^{-1}) are therefore more acid than those of pH 5 (where the concentration of H^+ is 10^{-5} mol.l^{-1}); solutions of pH 9 are more alkaline than those of pH 8. Pure water, without any dissolved gases such as CO_2, has a neutral pH of 7. To judge the effect of medium pH, it is essential to discriminate between the various sites where the pH might have an effect: (1) in the explant, (2) in the medium and (3) at the interface between explant and medium.

The pH of a culture medium must be such that it does not disrupt the plant tissue. Within the acceptable limits the pH also:

- governs whether salts will remain in a soluble form;
- influences the uptake of medium ingredients and plant growth regulator additives;
- has an effect on chemical reactions (especially those catalysed by enzymes); and
- affects the gelling efficiency of agar.

This means that the effective range of pH for media is restricted. As will be explained, medium pH is altered during culture, but as a rule of thumb, the initial pH is set at 5.5 – 6.0. In culture media, detrimental effects of an adverse pH are generally related to ion availability and nutrient uptake rather than cell damage.

5.1. THE pH OF MEDIA

5.1.1. Buffering

The components of common tissue culture media have only little buffering capacity. Vacin and Went (1949) investigated the effect on pH of each compound in their medium. The chemicals which seemed to be most instrumental in changing pH were $FeSO_4.7H_2O$ and $Ca(NO_3)_2.4H_2O$. Replacing the former with ferric tartrate at a weight which maintained the original molar concentration of iron, and substituting $Ca_3(PO_4)_2$ and KNO_3 for $Ca(NO_3)_2.4H_2O$, they found that the solution was more effectively buffered. While amino acids also showed promise as buffering agents, KH_2PO_4 was ineffective unless it was at high concentration. In some early experiments attempts were made to stabilise pH by incorporating a mixture of KH_2PO_4 and K_2HPO_4 into a medium (see Kordan, 1959, for example), but Street and Henshaw (1966) found that significant buffering was only achieved by soluble phosphates at levels inhibitory to plant growth. For this reason Sheat *et al.* (1959) proposed the buffering of plant root culture media with sparingly-soluble calcium phosphates, but unfortunately if these compounds are autoclaved with other medium constituents, they absorb micronutrients which then become unavailable.

A buffer is a compound which can poise the pH level at a selected level: effective buffers should maintain the pH with little change as culture proceeds. As noted before, plant tissue culture media are normally poorly buffered. However, pH is stabilised to a certain extent when tissues are cultured in media containing both nitrate and ammonium ions. Agar and Gelrite gelling agents may have a slight buffering capacity (Scherer, 1988).

Organic acids: Many organic acids can act as buffers in plant culture media. By stabilizing pH at ca. pH 5.5, they can facilitate the uptake of NH_4^+ when this is the only source of nitrogen, and by their own metabolism, assist the conversion of NH_4^+ into amino acids. There can be improvement to growth from adding organic acids to media containing both NH_4^+ and NO_3^-, but this is not always the case. Norstog and Smith (1963) noted that 0.75 mM malic

acid was an effective buffer and appeared to enhance the effect of the glutamine and alanine which they added to their medium. Vyskot and Bezdek (1984) found that the buffering capacity of MS medium was increased by adding either 1.25 mM sodium citrate or 1.97 mM citric acid plus 6.07 mM dibasic sodium phosphate. Citric acid and some other organic acids have been noted to enhance the growth of *Citrus* callus when added to the medium (presumably that of Murashige and Tucker, 1969) (Erner and Reuveni, 1981). For the propagation of various cacti from axillary buds, Vyskot and Jara (1984) added sodium citrate to MS medium to increase its buffering capacity.

Recognized biological buffers: Unlike organic acids, conventional buffers are not metabolised by the plant, but can poise pH levels very effectively. Compounds which have been used in plant culture media for critical purposes such as protoplast isolation and culture, and culture of cells at very low inoculation densities, include:

- TRIS, Tris(hydroxymethyl)aminomethane;
- Tricine, N-tris(hydroxymethyl)methylglycine;
- MES, 2-(N-morpholino)ethanesulphonic acid;
- HEPES, 4-(2-hydroxyethyl)-1-piperazine(2-ethanesulphonic acid); and
- CAPS, 3-cyclohexylamino-1-propanesulphonic acid.

Such compounds may have biological effects which are unrelated to their buffering capacity. Depending on the plant species, they have been known to kill protoplasts or greatly increase the rate of cell division and/or plant regeneration (Conrad *et al.,* 1981). The 'biological' buffers MES and HEPES have been developed for biological research (Good *et al.,* 1966).

MES: MES is one of the few highly effective and commercially available buffers with significant buffering capacity in the pH range 5-6 to which plant culture media are usually adjusted and has only a low capacity to complex with micronutrients. It is not toxic to most plants, although there are some which are sensitive. Ramage and Williams (2002) report that shoot regeneration from tobacco leaf discs was not affected by MES when increasing the concentration up to 100 mM. De Klerk et al (2007), though, observed a decrease of rooting from apple stem slices with increasing MES concentration (see below; Fig. 4.4). This effect of MES was not understood. It only occurred during the first days of the rooting process and was not observed during the outgrowth phase after the meristems had been formed.

During the culture of thin cell layers of *Nicotiana*, Tiburcio *et al.* (1989) found that the pH of LS medium could be kept close to 5.8 for 28 days by adding 50 mM MES, whereas without the buffer pH gradually decreased to 5.25. Regulating pH with MES alters the type of morphogenesis which occur in this (and other) tissues (see below).

Parfitt *et al.* (1988) found 10 mM MES to be an effective buffer in four different media used for tobacco, carrot and tomato callus cultures, and peach and carnation shoot cultures, although stabilizing pH did not result in superior growth. The tobacco, peach and carnation cultures were damaged by 50 mM MES. Tris was toxic at all concentrations tested, although Klein and Manos (1960) had found that the addition of only 0.5 mM Tris effectively increased the fresh weight of callus which could be grown on White (1954) medium when iron was chelated with EDTA.

MES has also been used successfully to buffer many cultures initiated from single protoplasts (Müller *et al.,* 1983) and its inclusion in the culture medium can be essential for the survival of individual cells and their division to form callus colonies (e.g. those of *Datura innoxia* - Koop *et al.,* 1983). MES was found to be somewhat toxic to single protoplasts of *Brassica napus*, but 'Polybuffer 74' (PB-74, a mixture of polyaminosulphonates), allowed excellent microcolony growth in the pH range 5.5-7.0 (Spangenberg *et al.,* 1986). Used at $^1/_{100}$ of the commercially available solution, it has a buffering capacity of a 1.3 mM buffer at its pK value (Koop *et al.,* 1983).

Banana homogenate is widely used in orchid micropropagation media. Ernst (1974) noted that it appeared to buffer the medium in which slipper orchid seedlings were being grown.

5.1.2. The uptake of ions and molecules

The pH of the medium has an effect on the availability of many minerals (Scholten and Pierik, 1998). In general, the uptake of negatively charged ions (anions) is favoured at acid pH, while that of cations (positively charged) is best when the pH is increased. As mentioned before, the relative uptake of nutrient cations and anions will alter the pH of the medium. The release of hydroxyl ions from the plant in exchange for nitrate ions results in media becoming more alkaline; when ammonium ions are

Table 4.8 The uptake of ions and its consequences in plant culture media

	Uptake anion (e.g. NH_4^+)	**Uptake cation** (e.g. NO_3^-)
Rate	Best in alkaline or weakly acid solutions	Best in relatively acid solutions
Consequence	Protons (H^+) extruded by plant Medium becomes more ACID	Hydroxyl (OH^-) ions extruded by plant Medium becomes more ALKALINE

taken up in exchange for protons, media become more acid (Table 4.8).

Nitrate and ammonium ions: The uptake of ammonium and nitrate ions is markedly affected by pH. Excised plant roots can be grown with NH_4^+ as the sole source of nitrogen providing the pH is maintained within the range 6.8 to 7.2 and iron is available in a chelated form. At pH levels below 6.4 the roots grow slowly on ammonium alone and have an abnormal appearance (Sheat et al.,1959). This accords with experiments on intact plants where ammonium as the only source of nitrogen is found to be poorly taken up at low pH. It was most effectively utilised in *Asparagus* in a medium buffered at pH 5.5. At low pH (ca. 4 or less) the ability of the roots of most plants to take up ions of any kind may be impaired, there may be a loss of soluble cell constituents and growth of both the main axis and that of laterals is depressed (Asher, 1978).

Nitrate on the other hand, is not readily absorbed by plant cells at neutral pH or above (Martin and Rose, 1976). Growth of tumour callus of *Rumex acetosa* on a nitrate-containing medium was greater at pH 3.5 than pH 5.0 (Nickell and Burkholder, 1950), while Chevre *et al.* (1983) reported that axillary bud multiplication in shoot cultures of *Castanea* was most satisfactory when the pH of MS was reduced to 4 and the Ca^{2+} and Mg^{2+} concentrations were doubled.

pH Stabilization: One of the chief advantages of having both NO_3^- and NH_4^+ ions in the medium is that uptake of one provides a better pH environment for the uptake of the other. The pH of the medium is thereby stabilized. Uptake of nitrate ions by plant cells leads to a drift towards an alkaline pH, while NH_4^+ uptake results in a more rapid shift towards acidity (Street, 1969; Behrend and Mateles, 1975; Hyndman *et al.,* 1982). For each equivalent of ammonium incorporated into organic matter, about 0.8-1 H^+ (proton) equivalents are released into the external medium; for each equivalent of nitrate assimilated, 1-1.2 proton equivalents are removed from the medium (Fuggi *et al.,* 1981). Raven (1986) calculated that there should be no change of pH resulting from NO_3^- or NH_4^+ uptake, when the ratio of the two is 2 to 1.

The pH shifts caused by uptake of nitrate and ammonium during culture (see above) can lead to a situation of nitrogen deficiency if either is used as the sole nitrogen source without the addition of a buffer (see later) (Hyndman *et al.,* 1982). The uptake of NH_4^+, when this is the sole source of nitrogen is only efficient when a buffer or an organic acid is also present in the medium. In media containing both NO_3^- and NH_4^+ with an initial pH of 5-6, preferential uptake of NH_4^+ causes the pH to drop during the early growth of the culture. This results in increased NO_3^- utilisation (Martin and Rose, 1976) and a gradual pH rise. The final pH of the medium depends on the relative proportions of NO_3^- and NH_4^+ which are provided (Gamborg *et al.,* 1968). After 7 days of root culture, White (1943a) medium (containing only nitrate), adjusted to pH 4.8-4.9, had a pH of 5.8-6.0 (Street *et al.,* 1951, 1952), but Sheat *et al.* (1959) could stabilize the medium at pH 5.8 by having one fiftieth of the total amount of nitrogen as ammonium ion, the rest as nitrate. Changes in the pH of a medium do however vary from one kind of plant to another. Ramage and Williams (2002) observed a decrease in pH when tobacco leaf discs were cultured with only NH_4^+ nitrogen whereas no such decrease was observed on medium with both NH_4^+ and NO_3^-. No organogenesis occurred when the medium with only NH_4^+ was unbuffered but the inclusion of MES resulted in the formation of meristems (but no shoots).

Media differing in total nitrogen levels (but all having the same ratio of nitrate to ammonium as MS medium), had a final pH of ca. 4.5 after being used for Stage III root initiation on rose shoots, whereas those containing ammonium alone had a final pH of ca. 4.1 (Hyndman *et al.,* 1982). A similar observation with MS medium itself was made by Delfel and Smith (1980). No matter what the starting value in the range 4.5-8.0, the final pH after culture of *Cephalotaxus* callus was always 4.2. The medium of De Jong *et al.* (1974) always had a pH of 4.8-5.0 after *Begonia* buds had been cultured, whatever the

starting pH in the range 4.0-6.5. This is not to say that the initial pH was unimportant, because there was an optimum for growth and development (see later) (Berghoef and Bruinsma, 1979).

Other Compounds: The availability and uptake of other inorganic ions and organic molecules is also affected by pH. As explained above, the uptake of phosphate is most efficient from acid solutions. Petunia cells took up phosphate most rapidly at pH 4 and its uptake declined as the pH was raised (Chin and Miller, 1982). Vacin and Went (1949) noted the formation of iron phosphate complexes in their medium by pH changes. Insoluble iron phosphates can also be formed in MS medium at pH 6.2 or above unless the proportion of EDTA to iron is increased (Dalton *et al.,* 1983).

The uptake of Cl^- ions into barley roots is favoured by low pH, but Jacobson *et al.* (1971) noticed that it was only notably less at high pH in solutions strongly stirred by high aeration. They therefore suggested that H^+ ions secreted from plant roots as a result of the uptake of anions, can maintain a zone of reduced pH in the Nernst layer, a stationary film of water immediately adjacent to cultured plant tissue (Nernst, 1904). In plant tissue culture, uptake of ions and molecules may therefore more liable to be affected by adverse pH in agitated liquid media, than in media solidified by agar.

Lysine uptake into tobacco cells was found by Harrington and Henke (1981) to be stimulated by low pH. The effect of pH on uptake is especially relevant for auxins (e.g., Edwards and Goldsmith 1980). Depending on the pH and their pKa, auxins are either present as an undissociated molecule or as an anion. For influx, the undissociated auxin molecule may pass through the membrane by diffusion whereas the anion is taken up by a carrier (Delbarre *et al.,* 1996; Morris 2000). Dissociation dependens upon the pH. In the apoplast, the pH is low, ca. 5. When taken up, the auxin enters the cytoplasm with pH 7. At this pH, most auxin is present as anion and cannot diffuse out. Efflux of the anion is brought about by an efflux carrier system. Thus, the net uptake into cells of plant growth regulators which are weak lipophilic acids (such as IAA, NAA, 2,4-D and abscisic acid) will be greater, the more acid the medium and the greater the difference between its pH and that of the cell cytoplasm (Rubery, 1980). Shvetsov and Gamburg (1981) did in fact find that the rate of 2,4-D uptake into cultured corn cells increased as the pH of the medium fell from 5.5 to 4. Increased uptake of auxin at low pH was also found in apple microcuttings, both for IBA (Harbage *et al.,* 1998) and IAA (De Klerk *et al.,* 2007). As previously mentioned, auxins can themselves modify intra- and extra-cellular pH. Adding 2,4-D to a medium increased the uptake of nicotine into culture of *Acer pseudoplatanus* cells (Kurkdjian *et al.,* 1982).

5.1.3. Choosing the pH of culture media: Starting pH

Many plant cells and tissues *in vitro*, will tolerate pH in the range of about 4.0-7.2; those inoculated

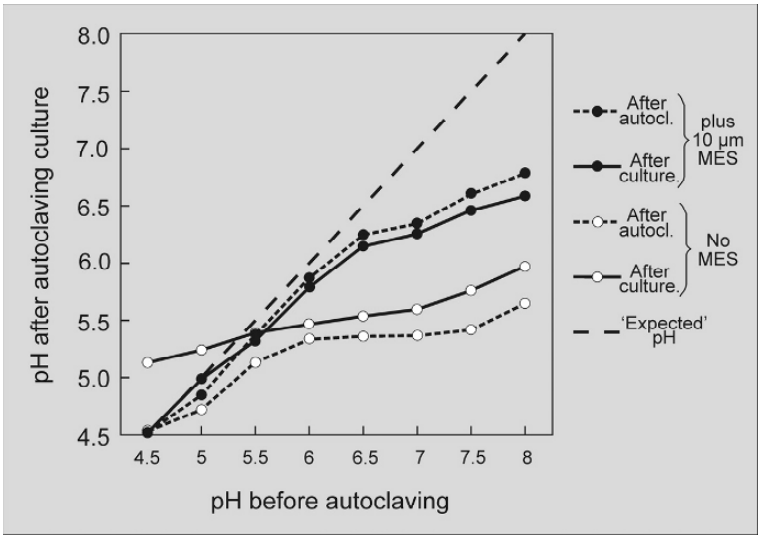

Fig. 4.3 Development of pH during tissue culture. The pH was set before autoclaving as is usually done, and measured directly after autoclaving and after 5 days of culture with 1-mm stem-slices cut from apple microshoots. The medium was in Petri dishes with BBL agar and modified MS medium. Per Petri dish, 30 ml of medium was added and 30 slices were cultured. (Data from de Klerk *et al.,* 2007).

into media adjusted to pH 2.5-3.0 or 8.0 will probably die (Butenko *et al.,* 1984). Best results are usually obtained in slightly acid conditions. In a random sample of papers on micropropagation, the average initial pH adopted for several different media was found to be 5.6 (mode 5.7) but adjustments to as low as 3.5 and as high as 7.1 had been made.

The pH for most plant cultures is thus lower than that which is optimum for hydroponic cultures, where intact plants with their roots in aerated solution usually grow most rapidly when the pH of the solution is in the range 6.0-7.3 (De Capite, 1948; Sholto Douglas, 1976; Cooper, 1979).

Suspension cultured cells of *Ipomoea*, grew satisfactorily in Rose and Martin (1975) P2 medium adjusted initially to pH 5.6 or pH 6.3, but the yield of dry cells was less at two extremes (pH 4.8 and pH 7.1). Martin and Rose (1976) supposed that a low yield of cells from a culture started at pH 7.1 was due to the inability of the culture to utilise NO_3^-, but the cause of a reduction in growth at pH 4.8 was less obvious. It might have resulted from the plant having to expend energy to maintain an appropriate physiological pH internally.

Kartha (1981) found that pH 5.6-5.8 supported the growth of most meristem tips in culture and that cassava meristems did not grow for a prolonged period on a medium adjusted to pH 4.8. The optimum pH (before autoclaving) for the growth of carnation shoot tips on Linsmaier and Skoog (1965) medium, was 5.5-6.5. When the medium was supplemented with 4 mg/l adenine sulphate and 2 g/l casein hydrolysate, the optimum pH was 5, and on media adjusted to 6.0 and 6.5, there was significantly less growth (Davis *et al.,* 1977). Shoot proliferation in *Camellia sasanqua* shoot cultures was best when the pH of a medium with MS salts was adjusted to 5-5.5. Only in these flasks was the capacity of juvenile explants to produce more shoots than adult ones really pronounced (Torres and Carlisi, 1986).

Norstog and Smith (1963) suggested that the pH of the medium used for the culture of isolated zygotic embryos, should not be greater than 5.2.

5.1.4. pH adjustment

Because there is then no need to take special aseptic precautions, and it is impractical to adjust pH once medium has been dispensed into small lots, the pH of a medium is usually adjusted with acid or alkali before autoclaving. According to Krieg and Gerhardt (1981), agar is partially hydrolysed if autoclaved at pH 6 or less and will not solidify so effectively when cooled. They recommend that agar media for bacteriological purposes should be sterilised at a pH greater than 6 and, if necessary, should be adjusted to acid conditions with sterile acid after autoclaving, when they have cooled to 45-50°C. The degree of hydrolysis in plant culture media autoclaved at pH 5.6-5.7, is presumably small.

The effect of autoclaving: Autoclaving changes the pH of media (Fig. 4.3). In media without sugars, the change is usually small unless the phosphate concentration is low, when more significant fluctuations occur. Media autoclaved with sucrose generally have a slightly lower pH than those autoclaved without it, but if maltose, glucose, or fructose have been added instead of sucrose, the post-autoclave pH is significantly reduced (Owen *et al.,* 1991).

The pH of liquid media containing MS salts [e.g. Linsmaier and Skoog (1965) or Skirvin and Chu (1979) media] containing 3-3.4% sucrose, has been found to fall during autoclaving from an adjusted level of 5.7, to pH 5.17 (Singha, 1982), to pH 5.5 (Owen *et al.,* 1991), or to pH 4.6 (Skirvin *et al.,* 1986). The drop in pH may vary according to the pH to which the medium was initially adjusted. In the experiments of Skirvin *et al.* (loc. cit.), the pH of a medium adjusted to 5.0, fell to 4.2; one adjusted to 6.4, fell to 5.1; that set at pH 8.5, fell to 8.1.

Most agars cause the pH of media to increase immediately they are dissolved. Knudson (1946) noticed that the pH of his medium shifted from 4.6-4.7 to 5.4-5.5 once agar had been added and dissolved; and Singha (1982) discovered that the unadjusted pH of MS medium rose from pH 4 to pH 5.2, depending on the amount of agar added. However if a medium containing agar was adjusted to pH 5.7, and then autoclaved, the medium became more acid than if agar had not been added, the fall in pH being generally in proportion to the amount of agar present.

The effect of storage. The pH of autoclaved plant media tends to fall if they are stored. Vacin and Went (1949) noted that autoclaving just accelerated a drop in the pH of Knudson (1946) C medium, as solutions left to stand showed similar changes. Sterilisation by filtration (see below) was not a satisfactory alternative, as it too effected pH changes. The compounds particularly responsible were thought to be unchelated ferrous sulphate (when the pH had initially been set between 3 and 6), and calcium nitrate (when the original pH was 6 to 9). Complex iron phosphates, unavailable to plants, were produced from the ferrous sulphate, but if iron was added as

ferric citrate or ferric tartrate (chelates), no significant pH changes resulted from autoclaving, and plants showed no iron-deficiency symptoms.

Skirvin *et al.* (1986) found that both with and without agar, autoclaved MS medium tended to become more acid after 6 weeks storage, for example:

Time	MS medium	
	Liquid	With 0.6% Difco Bacto Agar
Starting pH	5.7	5.7
After autoclaving	4.6	4.6
6 weeks later	4.1	4.4

To minimise a change in the pH of stored media, it is suggested that they are kept in the dark: Owen *et al.,* (1991) reported that the pH of MS containing 0.1M sucrose or 0.8% Phytagar, remained relatively stable after autoclaving if kept in the dark at 4°C, but fell by up to 0.8 units if stored in the light at 25°C. De Klerk *et al.* (2007) using BBL agar, also observed a shift of pH, but this was negligible when 10 mM MES was added (Fig. 4.3).

Hydrolysis: Some organic components of culture media are liable to be hydrolysed by autoclaving in acid media. The degree of hydrolysis of different brands of agar may be one factor influencing the incidence of hyperhydricity in plant cultures. Agar media may not solidify satisfactorily when the initial pH has been adjusted to 4-4.5. The reduction in pH which occurs in most media during autoclaving may also cause unsatisfactory gelling of agar which has been added in low concentrations. Part of the sucrose added to plant culture media adjusted to pH 5.5 is also hydrolysed during autoclaving: the proportion degraded increases if the pH of the medium is much less than this. Hydrolysis of sucrose is, however, not necessarily detrimental.

Activated charcoal: The presence of activated charcoal can alter the pH of a medium. As in the production of activated charcoal it is, at one stage washed with HCl, the pH of a medium can be lowered by acid residues (Owen *et al.,* 1995; Wann *et al.,* 1997).

4.1.5. pH changes during culture

Due to the differential uptake of anions and cations into plant tissues, the pH of culture media does not usually stay constant, but changes as ions and compounds are absorbed by the plant. It is usual for media containing nitrate and ammonium ions to decline slowly in pH during a passage, after being adjusted initially to pH 5.4-5.7, Sometimes after a preliminary decrease, the pH may begin to rise and return to a value close to, or even well above that at which the culture was initiated. The pH of White (1954) medium, which contains only NO_3^- nitrogen, drifted from an initial 5.0 to 5.5 towards neutrality as callus was cultured on it (Klein and Manos, 1960).

With some cultures, the initial pH of the medium may have little effect. Cell suspension cultures of *Dioscorea deltoides* in the medium of Kaul and Staba (1968) [containing MS salts], adjusted to a pH either 3.5, 4.3, 5.8 or 6.3, all had a pH of 4.6-4.7 within 10 h of inoculation. There was a further fall to pH 4.0-4.2 in the next 2-3 days, but during the following 15-17 days the pH was 4.7-5.0, finally rising to pH 6.0-6.3 on about day 19 (Butenko *et al.,* 1984). Similarly, Skirvin *et al.* (1986) found that MS medium adjusted to pH 3.33, 5.11, 6.63, or 7.98 before autoclaving, and then used for the culture of *Cucumis melo* callus, had a pH after 48 h in the range 4.6-5.0. Visseur (1987) also reported that although the pH of his medium (similar macronutrients to MS but more Ca^{2+} and PO_4^{3-}) decreased if it was solidified with agar, but on a 2-phase medium, the final pH was 6.9 ± 0.4 irrespective of whether it was initially 4.8, 5.5 or 6.2.

Despite the above remarks, it should be noted that the nature of the pH drift which occurs in any one medium, differs widely, according to the species of plant grown upon it. The pH of the medium supporting shoot cultures of *Disanthus cercidifolius* changed from 5.5 to 6.5 over a 6 week period, necessitating frequent subculturing to prevent the onset of senescence, whereas in the medium in which shoots of the calcifuge *Lapageria rosea* were grown, the pH, initially set to 3.5-5.0, only changed to 3.8-4.1 (Howard and Marks, 1987). Note also that the reversion of media to a homeostatic pH, may be due to the presence of both NO_3^- and NH_4^+ ions (Dougall, 1980). Adjustment of media containing only one of these nitrogen sources to a range of pH levels, would be expected to result in a more variable set of final values.

As the pH of media deviate from the original titration level, simple unmonitored cultures may not provide the most favourable pH for different phases of growth and differentiation. In *Rosa* 'Paul's Scarlet' suspensions, the optimum pH for the cell division phase was 5.2-5.4: pH 5.5-6.0 was best for the cell expansion phase (Nesius and Fletcher, 1973). The maximum growth rate of *Daucus carota* habituated callus on White (1954) medium with iron as Fe-EDTA, occurred at pH 6.0 (Klein and Manos, 1960).

It has been suggested that acidification of media is partly due to the accumulation of carbon dioxide in tightly closed culture flasks (Leva *et al.,* 1984), but the decrease in pH associated with incubating anther cultures with 5% CO_2 was found by Johansson and Eriksson (1984) to be only ca. 0.1 units. Removal of CO_2 from an aerated cell suspension culture of *Poinsettia* resulted in an increase of about 0.2 pH-units (Preil, 1991).

Auxin plant growth regulators promote cell growth by inducing the efflux of H^+ ions through the cell wall. Hydrogen ion efflux from the cell is accompanied by potassium ion influx. When cultures are incubated in a medium containing an auxin, the medium will therefore become more acid while the pH of the cell sap will rise. The extent of these changes was found by Kurkdjian *et al.* (1982) to be proportional to auxin (2,4-D) concentration.

5.2. pH CONTROL WITHIN THE PLANT

The various compartments of cells have a different pH and this pH is maintained (Felle, 2001). In the symplasm, the pH of the cytoplasm is ca. 7 and of the vacuole ca. 5. The apoplasm has a pH of ca. 5. Plant cells typically generate an excess of acidic compounds during metabolism which have to be neutralised (Felle, 1998). One of the most important ways by which this is accomplished is for H^+, or K^+ to be pumped out of the cell, in exchange for anions (e.g. OH^-), thereby decreasing the extracellular pH. Plant cells also compensate for an excess of H^+ by the degradation of organic acids. Synthesis of organic acids, such as malate, from neutral precursors is used to increase H^+ concentration when the cytoplasmic pH rises, for instance if plants are grown in alkaline soils (Raven and Smith, 1976; Findenegg *et al.,* 1986). In intact plants, there is usually a downwards gradient from the low pH external to the cell, to higher pH levels in more mature parts, and this enables the upwards transport of non-electrolytic compounds such as sugars and amino acids (Böttger and Luthen, 1986).

Altering the pH of the external solution surrounding roots or cells can alter the pH of the cell (Smith and Raven, 1979). Because of necessary controls, the pH of the cytoplasm may be only slightly altered, that of vacuoles may show a more marked change. Changing the pH of the medium in which photo-autotrophic *Chenopodium rubrum* suspensions were cultured from 4.5 to 6.3, caused the pH of the cytoplasm to rise from 7.4 to 7.6 and that of the cell vacuoles to increase from 5.3 to 6.6. The increase in cytoplasmic pH caused there to be a marked diversion of carbon metabolism, away from sugar and starch, into the production of lipids, amino acids and proteins (Hüsemann *et al.,* 1990). The maintenance of the pH is also illustrated in an experiment with detached leaves of *Vicia faba* (Felle and Hanstein, 2002). When the leaves were placed in a 10 mM MES-TRIS buffer and transferred to buffer with another pH, changes in the pH of the apoplasm were small: with an initial buffer pH = 4.1 and transfer to buffer pH = 6.8, the apoplastic pH of substomatal cavities increased from 4.71 to 5.13 and in the reverse transfer decreased from 5.13 to 4.70. This indicates that the pH of the apoplasm is not strongly influenced by the medium but stays close to the 'natural' pH of ca. 5.0. No exact details are given but in this experiment the distance between the site of the pH measurements and the MES-TRIS solution in which the leaves had been placed was probably large. The symplasm has a much larger capacity to buffer (Felle, 2001) so that its pH will be even less influenced by the medium pH. Thus, within the explant the pH of both apoplasm and symplasm will be affected only little by the medium pH. The situation may be different at the interface between explant and medium. The influence of medium pH will extend towards inner tissues of the explant as the buffering capacity of the medium is increased (and thus overcomes buffering by the tissue). Inside the explant, the pH will also greatly influence movement

Table 4.9. The influence of the initial pH of Linsmaier Skoog (1965) medium on morphogenesis in thin cell layers of *Nicotiana* (data of Mutaftschiev *et al.,* 1987)

Initial pH of the medium	Mean number of organs per explant	Mean percentage of explants forming:			
		Callus	Roots	Vegetative buds	Flowers
3.8	4 ± 2	60 ± 10	40 ± 10	0	0
5.0	2 ± 1	90 ± 10	10 ± 10	0	0
6.1	20 ± 10	0	0	100	0
6.8	6 ± 2	0	0	20 ± 10	80 ± 10

through membranes, i.e. uptake in cells, as this often depends on the dissociation of compounds which is pH dependent.

Changing the pH of the medium can thus have a regulatory role on plant cultures which is similar to that of plant growth regulating chemicals, one of the actions of which is to modify intracellular pH and the quantity of free calcium ions. Auxins can modify cytoplasmic pH by triggering the release of H^+ from cells. In plant tissue culture these ions can acidify the medium (Kurkdjian *et al.,* 1982). Proton release is thought to be the first step in acid-triggered and turgor-triggered growth (Schubert and Matzke, 1985). It should be noted that pH changes themselves may act as a signal (Felle, 2001).

5.3. THE EFFECT OF pH ON CULTURES

5.3.1. Initiating cultures at low pH

Plants of the family Ericaceae which only grow well on acid soils (e.g. rhododendrons and blueberries), have been said to grow best on media such as Anderson (1975), Anderson (1978; 1980) and Lloyd and McCown (1981) WPM, when the pH is first set to ca. 4.5 (Anderson, 1975; Skirvin, 1981), but for highly calcifuge species such as *Magnolia soulangiana*, a starting pH of 3.5 can result in the highest rate of shoot proliferation in shoot cultures (Howard and Marks, 1987). Chevre *et al.* (1983) state that chestnut shoot cultures grew and proliferated best at pH 4 provided MS medium was modified by doubling the usual levels of calcium and magnesium.

De Jong *et al.* (1974), using a specially developed medium, found that a low pH value favoured the growth of floral organs. A similar result was seen by Berghoef and Bruinsma (1979c) with *Begonia* buds. Growth was greatest when the pH of the medium was initially adjusted to acid, 4.5-5.0 being optimal. At pH 4.0 the buds became glassy.

Before the discovery of effective chelating agents for plant cultures, root cultures were grown on media with a low pH. Tomato roots were, for instance, unable to grow on media similar to those of White (1943a), when the pH rose to 5.2 (Street *et al.,* 1951). Boll and Street (1951) were able to show that the depression of growth at high pH was due to the loss of Fe from the medium and that it could be overcome by adding a chelated form of iron (see chapter 3). Using FeEDTA, Torrey (1956) discovered that isolated pea roots [grown on a medium containing Bonner and Devirian (1939) A macronutrients, which do not contain NH_4^+], grew optimally at pH 6.0-6.4 but were clearly inhibited at pH 7.0 or greater; and Street (1969) reported that growth of tomato roots could be obtained between pH 4.0 and pH 7.2, if EDTA was present in the medium. Because agar does not gel properly when the initial pH of the medium is adjusted to 4, it is necessary to use liquid media for low pH cultures; or employ another gelling agent, or a mechanical support.

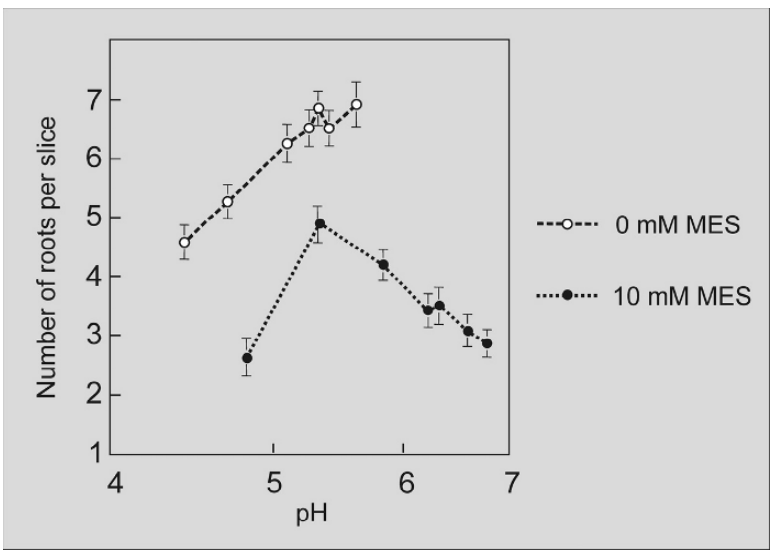

Fig. 4.4 Effect of medium pH on adventitious rooting from apple stem disks. The pH at the x-axis is the pH as measured at the start of culture after autoclaving (cf. Fig. 4.3). (from de Klerk *et al.,* 2007).

Some other cultures may also be beneficially started at low pH, which may indicate that the tissues have an initial requirement for NO_3^-. Embryogenesis of *Pelargonium* was induced more effectively if MS, or other media, were adjusted to pH 4.5-5.0 before autoclaving (rather than pH 5.5 and above) (Marsolais *et al.,* 1991).

5.3.2. Differentiation and Morphogenesis

Differentiation and morphogenesis are frequently found to be pH-dependent. Xylogenesis depends on the medium pH (Khan *et al.,* 1986). The growth of callus and the formation of adventitious organs from thin cell layers excised from superficial tissues of the inflorescence rachis of *Nicotiana*, depended on the initial pH of Linsmaier and Skoog (1965) medium containing 0.5 μM IBA and 3 μM kinetin (Table 4.9) (Mutaftschiev *et al.,* 1987). Pasqua *et al.* (2002) reported many quantitative effects of pH during regeneration from tobacco thin cell layers. The types of callus produced from the plumules of *Hevea* seedlings differed according to the pH of the medium devised by Chua (1966). Soft and spongy callus formed at acid (5.4) or alkaline (8.0) pH. A compact callus was obtained between pH 6.2 and 6.8.

5.3.3. Adventitious root formation

There are several reports in the literature which show that the pH of the medium can influence root formation of some plants *in vitro*. A slightly acid pH seems to be preferred by most species. Zatkyo and Molnar (1986), who showed a close correlation between the acidity of the medium (pH 7.0 to 3.0) and the rooting of *Vitis*, *Ribes nigrum* and *Aronia melancarpa* shoots, suggested that this was because acidity is necessary for auxin action.

Sharma *et al.* (1981) found it advantageous to reduce the pH of the medium to 4.5 to induce rooting of *Bougainvillea* shoots and a reduction of the pH of MS medium to 4.0 (accompanied by incubation in the dark) was required for reliable root formation of two *Santalum* species (Barlass *et al.,* 1980) and *Correa decumbens* and *Prostanthera striatifolia* (Williams *et al.,* 1984; 1985). Other Australian woody species rooted satisfactorily at pH 5.5 and pH 4 was inhibitory (Williams *et al.,* loc. cit.). Shoots from carnation meristem tips rooted more readily at pH 5.5 than pH 6.0 (Stone, 1963), and rooting of excised potato buds was best at pH 5.7, root formation being inhibited at pH 4.8 and at pH 6.2 or above (Mellor and Stace-Smith, 1969). Direct root formation on *Nautilocalyx* leaf segments was retarded if a modified MS medium containing IAA was adjusted initially to an acid pH (3.5 or 4.0) or a neutral pH (6.5). Good and rapid root formation occurred when the medium was adjusted to between pH 5.0 to 6.3 (Venverloo, 1976). De Klerk *et al* (2007), working with apple stem slices, found only a small effect of pH on rooting (Fig. 4.4): when the pH was set before autoclaving at 4.5 (after autoclaving the pH was 4.54), the number of roots was 4.5, and with the pH set at 8.0 (after autoclaving the pH had dropped to 5.65), the number of roots per slice increased to 7. In medium buffered with MES, the maximum number of roots was formed at pH 4.4 (measured after autoclaving). In these experiments, the dose-response curve for root number did not correspond with the effect of pH on IAA uptake. Such discrepancy between the effects of the pH on uptake and root number, was also reported by Harbage, Stimart and Auer (1998).

Direct formation of roots from *Lilium auratum* bulb scales occurred when MS medium was adjusted within the range 4-7 but was optimal at pH 6. The pH range for adventitious bulblet formation in this plant was from 4 to 8, but most bulblets were produced when the initial pH was between 5 and 7 (Takayama and Misawa, 1979).

Substrates which are to acid or too alkaline can adversely affect rooting *ex vitro*.

5.3.4. Embryogenesis

Smith and Krikorian (1989) discovered that pre-globular stage pro-embryos (PGSP) of carrot could be made to proliferate from tissues capable of direct embryo formation, with no auxin, on a medium containing 1-5 mM NH_4^+ (and no nitrate). Somatic embryos were formed when this tissue was moved to MS medium. The pH of the 'ammonium-nitrogen' medium fell from 5.5 to 4.0 in each subculture period, and it was later found (Smith and Krikorian, 1990a,b) that culture on a medium of low pH was essential for the maintenance of PGSP cultures. Sustained culture at a pH equal or greater than 5.7, with no auxin, allowed somatic embryo development. A similar observation to that of Smith and Krikorian had been made by (Stuart *et al.,* 1987). Although the pH of a suspension culture of alfalfa 'Regen-S' cells in a modified Schenk and Hildebrandt (1972) medium with 15 mM NH_4^+ was adjusted to 5.8, it quickly fell back to pH 4.4-5.0 in a few hours. The pH then gradually increased as somatic embryos were produced, until at day 14 it was 5.0. In certain suspension cultures, the pH was titrated daily to 5.5, but on each occasion soon returned to nearly the same pH as that in flasks which were untouched. Even so,

the pH-adjusted suspensions produced more embryos than the controls.

The ammonium ion has been found to be essential for embryogenesis. Is one of its functions to reduce the pH of the medium through rapid uptake and metabolism, thereby facilitating the uptake of nitrate, upon which embryogenesis is really dependent?

Embryogenesis from leaf explants of *Ostericum koreanum*, was found to depend strongly on pH (Cho *et al* 2003). As the explants were cultured continuously with NAA, it is possible that the observed relationship was caused by differential NAA uptake. This is also suggested by the slower rate of embryo development seen at low pH, because this would be expected where there is a high internal NAA concentration.

6. LIQUID MEDIA AND SUPPORT SYSTEMS

The nutritional requirements of plant cultures can be supplied by liquid media but growth in liquid medium may be retarded and development affected by oxygen deprivation and hyperhydration. The oxygen concentration of liquid media is often insufficient to meet the respiratory requirements of submerged cells and tissues. It can be increased either by raising the oxygen concentration of the medium or placing cells or tissues in direct contact with air. If the water potential of the medium is greater (less negative) than that of a cell, water flows into the cell and the vacuole becomes distended. Cells and tissues affected in this way are described as hyperhydric. Shoots often show physiological disturbances with symptoms that can be recognised visually (Chapter 13) (Debergh *et al.,* 1992; Gaspar *et al.,* 1987; Preece and Sutter, 1991; Ziv, 1991). The term hyperhydric is preferable to the previously used term 'vitrified' for reasons explained by Debergh *et al.* (1992). Water potential is determined by osmotic potential of the solutes in the medium and, in the case of a gelled medium, by the matric potential of the gel (Section 4.1) (Beruto *et al.,* 1995; Fujiwara and Kozai, 1995; Owens and Wozniak, 1991). Reductions in hyperhydricity can be achieved by increasing the concentrations of the solutes and the gel. Hyperhydricity may also be reduced through evaporation of water from tissues if they are placed in contact with air.

Contact of cultured tissues with air, to alleviate problems of hyperhydricity and hypoxia, can be achieved by the use of either porous or semi-solid (gelled) supports. The advantage of supports, as opposed to thin layers of liquid medium, is that tissues can be placed in a sufficient volume of medium to prevent depletion of nutrients and allow for the dispersion of any toxins that might be produced by the plant tissues. The relative advantages of liquid medium, solid supports or gelled media, varies with the type of material being cultured, the purpose of the culture and the scale of culture, as discussed below.

6.1. LIQUID MEDIA

Liquid medium, without supporting structures, is used for the culture of protoplasts, cells or root systems for the production of secondary metabolites, and the propagation of somatic embryos, meristematic nodules, microtubers and shoot clusters. In liquid medium, these cultures often give faster growth rates than on agar-solidifed medium. Cultures may be fully or only partially immersed in the medium.

Aeration of liquid medium in stationary Petri dishes is sometimes adequate for the culture of protoplasts and cells because of the shallow depth of the medium, but may still be suboptimal. Anthony *et al.* (1995) cultured protoplasts of cassava, in liquid medium in Petri dishes with an underlying layer of agarose in which glass rods were embedded vertically. Sustained protoplast division was observed in the cultures with glass rods but not in the controls without glass rods. The authors suggested that the glass rods extended the liquid meniscus, where the cell colonies were clustered thus causing gaseous exchange between the liquid and the atmosphere above to be facilitated.

Anthony *et al.* (1997) cultured protoplasts of *Passiflora* and *Petunia* in 30 ml glass bottles containing protoplast suspensions in 2 ml aliquots, either alone or in the presence of the oxygen carriers Erythrogen™ or oxygenated Perfluorodecalin. Cell division in each of the two species was stimulated by both oxygen carriers.

Laboratory-scale experimentation on immersed cultures of cells, tissues and organs, may be carried out in 125 ml or 250 ml Erlenmeyer flasks. Large-scale cultures are usually carried out in bioreactors with a capacity of 1 litre or more. The concentration of oxygen in the medium is raised by oxygen in the gas phase above and air bubbles inside the liquid. Increasing the oxygen concentration and circulation of the medium is facilitated in flasks by the use of gyratory shakers and in bioreactors by stirring and/or bubbling air through the medium (Chapter 1). The

use of bioreactors often involves the automated adjustment of the culture medium. The design of bioreactors for plant cells and organs was reviewed by Doran (1993) and the use of shake-flasks and bioreactors for the scale-up of embryogenic plant suspension cultures has been reviewed by Tautorus and Dunstan (1995). The importance of oxygen concentration in bioreactors can be illustrated by an investigation into the growth of hairy roots of *Atropa belladonna* (Yu and Doran, 1994). They found that no growth occurred at oxygen tensions of 50% air saturation but between 70% and 100% air saturation, total root length and the number of root tips increased exponentially. Hyperhydricity in liquid cultures may be avoided by adding to the medium osmoregulators, such as mannitol, maltose and sorbitol, and inhibitors of gibberellin biosynthesis including ancymidol and paclobutrazol (Ziv, 1989).

Plantlets and microtubers can be cultured by partial submersion in liquid medium. One method of aerating tissues is by the automated flooding and evacuation of tissues by liquid medium. This method has been used to produce microtubers of potato from single node cuttings (Teisson and Alvard, 1999). An alternative approach to aeration is to apply the liquid medium over the plant tissues as a nutrient mist. For example, Kurata *et al.* (1991) found that nodes of potato grew better in nutrient mist than on agar-based cultures.

6.2. SUPPORT BY SEMI-SOLID MATRICES

Gelled media provide semi-solid, supporting matrices that are widely used for protoplast, cell, tissue and organ culture. Agar, agarose, gellan gums and various other products have been used as gelling agents.

6.2.1. Agar

Agar is very widely employed for the preparation of semi-solid culture media. It has the advantages that have made it so widely used for the culture of bacteria, namely :-

- it forms gels with water that melt at approx. 100°C and solidify at approx. 45°C, and are thus stable at all feasible incubation temperatures;
- gels are not digested by plant enzymes;
- agar does not strongly react with media constituents.

To ensure adequate contact between tissue and medium, a lower concentration of agar is generally used for plant cultures than for the culture of bacteria. Plant media are not firmly gelled, but only rendered semi-solid. Depending on brand, concentrations of between 0.5-1.0% agar are generally used for this purpose. Agar is thought to be composed of a complex mixture of related polysaccharides built up from galactose. These range from an uncharged neutral polymer fraction, agarose, that has the capacity to form strong gels, to highly charged anionic polysaccharides, sometimes called agaropectins, which give agar its viscosity. Agar is extracted from species of *Gelidium* and other red algae, collected from the sea in several different countries. It varies in nature according to country of origin, the year of collection and the way in which it has been extracted and processed. The proportion of agarose to total polysaccharides can vary from 50 to 90% (Adrian and Assoumani, 1983). Agars contain small amounts of macro- and micro-elements; particularly calcium, sodium, potassium, and phosphate (Beruto *et al.,* 1995; Debergh, 1983; Scherer *et al.,* 1988), carbohydrates, traces of amino acids and vitamins (Day, 1942) that affect the osmotic and nutrient characteristics of a gel. They also contain phenolic substances (Scherer *et al.,* 1988) and less pure grades may contain long chain fatty acids, inhibitory to the growth of some bacteria.

As agar can be the most expensive component of plant media, there is interest in minimising its concentration. Concentrations of agar can be considered inadequate if they do not support explants or lead to hyperhydricity. Hyperhydricity decreases as the agar concentration is raised but there may be an accompanying reduction in the rate of growth. For example, Debergh *et al.* (1981) found that shoot cultures of *Cynara scolymus* were hyperhydric on medium containing 0.6% Difco 'Bacto' agar. No hyperhydricity occurred on medium containing 1.1% agar but shoot proliferation was reduced. Likewise, Hakkaart and Versluijs (1983) found that shoots of carnation were hyperhydric on medium containing 0.6% agar whereas growth was severely reduced on medium containing 1.2%. During studies to optimise the production of morphogenic callus from leaf discs of sugarbeet, Owens and Wozniak (1991) found large differences in the numbers of somatic embryos and shoots according to the gelling agent employed. They found that water availability, determined by gel matric potential, was the dominant factor involved. When they adjusted the concentrations of the gelling agents to give media of equal gel matric potential, somatic embryos and shoots were found in similar numbers on Bacto agar (0.7%), HGT agarose (0.46%), Phytagar (0.62%) and Gelrite (0.12%).

Various brands and grades of agar are available commercially. These differ in the amounts of impurities they contain and their gelling capabilities. The gelling capacity of Difco brands of agar increased with increasing purity i.e. 'Noble' > 'Purified' > 'Bacto' (Debergh, 1983). After impurities of agar were removed by sealing agar in a semi-permeable bags and washing in deionized water, the water potentials of gels of three brands were substantially lower than unwashed gels (Beruto *et al.,* 1995). A ten-fold difference in the regeneration rate of sugar cane was observed by Anders *et al.* (1988) on media gelled with the best and least effective of seven brands of agar. Scholten and Pierik (1998) investigated the different growth characteristics of seven different agar brands on the growth of axillary shoots, adventitious shoots and adventitious roots of rose, lily and cactus. They concluded that no single bioassay could identify 'good or bad' agars for a large group of plant species but Merk 1614, Daishin, MC29, and BD Purified gave the best results in most experiments. 'Daishin' showed no batch-to-batch variations. They found no relationship between price and quality of the brands of agar.

Agarose. Agarose is the high gel strength moiety of agar. It consists of β-D(1→3) galactopyranose and 3,6-anhydro-α-L(1 → 4) galactopyranose polymer chains of 20-160 monosaccharide units alternatively linked to form double helices. There are also several different agaro-pectin products available, which have been extracted from agar and treated to remove most of the residual sulphate side groupings (Shillito *et al.,* 1983). Because preparation involves additional processes, agarose is much more expensive than agar and its use is only warranted for valuable cultures, including protoplast and anther culture. Brands may differ widely in their suitability for these applications. Concentrations of 0.4-1.0% are used. Agarose derivatives are available which melt and gel at temperatures below 30°C, making them especially suitable for testing media ingredients that are heat-labile, or for embedding protoplasts. Low melting-point agarose is prepared by introducing hydroxyethyl groups into the agarose molecules (Shillito *et al.,* 1983). Another advantage of agarose over agar lies in the removal of toxic components of agar during its preparation. Bolandi *et al.* (1999) preferred to embed protoplasts in agarose, rather than use liquid medium, because in agarose the semi-solid matrix applies a direct pressure on the plasma membrane of the protoplasts. They mixed protoplasts of sunflower with 0.5% agarose, pipetted 50 µl droplets of the mixture into Petri dishes and covered them with a thin layer of culture medium. A similar method was used to culture protoplasts of *Dioscorea* by Tor *et al.* (1999). The use of droplets has the advantage that a high plating density can be achieved in the droplets, while exposing the protoplasts to a larger reservoir of culture medium in the liquid phase. Bishoi *et al.* (2000) initially cultured anthers of Basmati rice on liquid medium, then used 1.0% agarose to culture calli derived from microspores.

6.2.2. Gellan gum

Gellan gum is a widely used gelling agent in plant tissue culture, that is marketed under various trade names including Gelrite, Phytagel and Kelcogel. It is an exopolysaccharide that encapsulates cells of the bacterium *Sphingomonas paucimobilis* (= *Auromonas elodea* = *Pseudomonas elodea*), from which it is obtained by industrial fermentation. The structure, physico-chemical properties and the rheology of solutions of gellan gum and related polysaccharides has been reviewed by Banik *et al.* (2000). Gellan gum consists of a linear repeating tetrasaccharide of D-glucose, D-glucuronic acid, D-glucose and L-rhamnose. Heating solutions of gellan gum in solutions that contain cations, such as K^+, Ca^{2+}, Mg^{2+}, causes the polysaccharide to form a gel in which the polymers form a half-staggered parallel double helix. The commercial deacetylated and purified polysaccharide forms a firm non-elastic gel. The gel sets rapidly at a temperature, determined by the concentrations of the polysaccharide and the cation, which varies between 35-50 °C (Kang *et al.,* 1982). The commercial product contains significant quantities of potassium, sodium, calcium and magnesium (Pasqualetto *et al.,* 1988a,b; Scherer *et al.,* 1988) but is said to be free of the organic impurities found in agar. It is unclear whether or not these cations remain fully available to plant cultures. Some researchers (Gawel *et al.,* 1986; 1990; Trolinder and Goodin, 1987; Umbeck *et al.,* 1987) add an extra 750 mg l^{-1} $MgCl_2$ to a medium containing MS salts to aid the gelling of 1.6% Gelrite. In most other reports on the use of Gelrite, cations in the medium have been sufficient to produce a gel.

Beruto *et al.* (1995) found that 0.12 % Gelrite and 0.7% Bacto agar have equivalent matric potential and support equivalent adventitious regeneration in leaf discs of sugarbeet. As gellan gum is used in lower concentration than agar, the cost per litre of medium is less. It produces a clear gel in which plant tissues can be more easily seen and microbial

contamination more easily detected than in agar gels. It has proved to be a suitable gelling agent for tissue cultures of many herbaceous plants and there are reports of its successful use for callus culture, the direct and indirect formation of adventitious organs and somatic embryos, shoot culture of herbaceous and semi-woody species and the rooting of plantlets. In most cases the results have been as good as, and sometimes superior to, those obtainable on agar-solidified media. Anders *et al.* (1988) described the regeneration of greater numbers of plants from of sugar cane on Gelrite than on the most productive brand of agar, and Koetje *et al.* (1989) obtained more somatic embryos from callus cultures of rice on media solidified with Gelrite than with Bacto-agar. Shoot cultures, particularly of some woody species, are liable to become hyperhydric if Gelrite, like agar, is used at too low a concentration. There are sharp differences in the response of different species to concentration of Gelrite. Turner and Singha (1990) found the highest rate of shoot proliferation in *Geum* occurred on 0.2% and in *Malus* on 0.4%. Garin *et al.* (2000) obtained more mature somatic embryos of *Pinus strobus* on gellan gum at 1% concentration of than at 0.6%. Pasqualetto *et al.* (1986a,b) used mixtures of Gelrite (0.1-0.15%) and Sigma @ agar (0.2-0.3%) to prevent the hyperhydricity that occurred in shoot cultures of *Malus domestica* 'Gala' on media solidified with Gelrite alone. Nairn (1988) used a mixture of Gelrite (0.194%) and Difco 'Bacto' agar (0.024%) to prevent the hyperhydricity that occurred in shoot cultures of *Pinus radiata* on medium gelled with 0.2% Gelrite alone.

6.2.3. Alginates

Alginic acid is a binary linear heteropolymer 1,4-β-D-mannuronic acid and 1,4-α-L-guluronic acid (Larkin *et al.,* 1988). It is extracted from various species of brown algae. When the sodium salt is exposed to calcium ions, gelation occurs. Alginates are widely used for protoplast culture and to encapsulate artificial seed.

Protoplasts embedded in beads or thin films of alginate can plated densely while yet exposed to a large pool of medium that dilutes inhibitors and toxic substances. Embedded protoplasts can be surrounded by nurse cells, either free in the surrounding medium or separated by filters or membranes. Alginate has the advantages over agarose that protoplasts do not have to be exposed to elevated temperatures when they are mixed with the gelling agent and the gel can be liquified by adding sodium citrate, releasing protoplasts or cell colonies for transfer to other media. The method of embedding protoplasts in beads as employed by Larkin *et al.* (1988) involved mixing the protoplast suspensions with an aqueous solution of sodium alginate and dropping it, through a needle, into a solution of calcium chloride. Beads containing the protoplasts were formed when the alginate made contact with the calcium ions. Beads with a final concentration of 1.5% sodium alginate were washed and cultured in liquid medium on an orbital shaker. Protoplasts may also be captured in thin layers of alginate (Dovzhenko *et al.,* 1998).

Synthetic seeds (synseeds, somatic seeds) encapsulated in alginate (Fig. 4.4), can be prepared from somatic embryos (Timbert *et al.,* 1995), shoot tips (Maruyama *et al.,* 1998), apical and axillary buds (Piccioni and Standardi, 1995), single nodes (Piccioni, 1997), and cell aggregates from hairy roots (Repunte *et al.,*1995). The uses of sythetic seeds include direct planting into soil, storage of tissues and transfer of materials between laboratories under sterile conditions. The methods of encapsulation of somatic embryos of carrot were described by Timbert *et al.* (1995). Torpedo-shaped embryos were mixed with a 1% sodium alginate solution. The mixture was dropped into a solution 100 mM calcium chloride in 10% sucrose. The beads (3-3.5 mm in diameter) were then rinsed in a 10% sucrose solution.

6.2.4. Starch

Sorvari (1986a,c) found that plantlets formed in higher frequencies in anther cultures of barley on a medium solidified with 5% corn starch or barley starch rather than with agar. Corn starch only formed a weak gel and it was necessary to place a polyester net on its surface to prevent the explants from sinking. Sorvari (1986b) found that it took 5-14 weeks for adventitious shoots to form on potato discs on agar-solidified medium but only 3 weeks on medium containing barley starch. Henderson and Kinnersley (1988) found that embryogenic carrot callus cultures grew slightly better on media gelled with 12% corn starch than on 0.9% Difco `Bacto' agar.

6.2.5. 'Kappa'--carrageenan

Carrageenan is a product of sea weeds of the genus *Euchema* and the kappa form has strong gelling properties. Like gellan gum, kappa-carrageenan requires the presence of cations for gelation. In Linsmaier and Skoog (1965) medium at 0.6% w/v, the gel strength was slightly less than that of 0.2% Gelrite or 0.8% of extra pure agar (Ichi *et al.,*

1986). Chauvin *et al.* (1999) found that regeneration from cultures of tulip, gladiolus and tobacco shoots was possible in the presence of 200 mg l^{-1} kanamycin, whereas in several other gelling agents a concentration of 100 mg l^{-1} inhibited regeneration.

6.2.6. Pectins

A mixture of pectin and agar can be a less expensive substitute for agar. For example, a semi-solid medium consisting of 0.2% agar plus 0.8-1.0% pectin, was employed for shoot culture of strawberry and some other plants (Zimmerman, 1979).

6.2.7. Other gelling agents

Battachary *et al.* (1994) found sago (from *Metroxylon sagu*) and isubgol (from *Plantago ovata*) were satisfactory substitutes for agar at, respectively, one eighth and one tenth of the cost of Sigma purified agar A7921.

6.3. POROUS SUPPORTS

Aeration of the tissues on a porous substrate is usually better than it would be in agar or static liquid.

Chin *et al.* (1988) used a buoyant polypropylene membrane floated on top of a liquid medium to culture cells and protoplasts of *Asparagus*. The membrane (Celgard 3500, Questar Corp., Charlotte, N.C.) has a pore size of 0.04 mm and is autoclavable. Conner and Meredith (1984) found that cells grew more rapidly on filter papers laid over polyurethane foam pads saturated with medium than on agar. Young *et al.* (1991) supported shoots of tomato over liquid culture medium on a floating microporous polypropylene membrane and entrained the growing shoots through polypropylene netting. They reported opportunities for the development of this method for mechanisation by mass handling. Membrane rafts were also used by Teng (1997) and Watad *et al.* (1995,1996).

Cheng and Voqui (1977) and Cheng (1978) used polyester fleece to support cultures of Douglas fir that were irrigated with liquid media in Petri dishes. Plantlets that were regenerated from cotyledon explants were cultured on 3 mm-thick fabric. When a protoplast suspension was dispersed over 0.5 mm-thick fabric, numerous colonies were produced in 12 days, whereas in the absence of the support, cell colonies failed to proliferate beyond the 20 cell stage. A major advantage in using this type of fabric support is that media can be changed simply and quickly without disturbing the tissues. The system has also been used for protoplast culture of other plants (e.g. by Russell and McCown, 1986).

Heller and Gautheret (1949) found that tissues could be satisfactorily cultured on pieces of ashless filter paper moistened by contact with liquid medium. Very small explants, such as meristem tips, that might be lost if placed in a rotated or agitated liquid medium, can be successfully cultured if placed on an M-shaped strip of filter paper (sometimes called a 'Heller' support). When the folded paper is placed in a tube of liquid medium, the side arms act as wicks (Goodwin, 1966). This method of support ensures excellent tissue aeration but the extra time required for preparation and insertion has meant that paper wicks are only used for special purposes such as the initial cultural stages of single small explants which are otherwise difficult to establish. Whether explants grow best on agar or on filter paper supports, varies from one species of plant to another. Davis *et al.* (1977) found that carnation shoot tips grew less well on filter paper bridges than on 0.6% agar but axillary bud explants of *Leucospermum* survived on bridges but not on agar.

Paper was also used in the construction of plugs (marketed by Ilacon Ltd, Tonbridge, UK TN9 1NR) known as Sorbarods (Roberts and Smith, 1990). These are cylindrical (20 mm in length and 18 mm in diameter) and consist of cold-crimped cellulose, longitudinally folded, wrapped in cellulose paper. The plug has a porosity (total volume – volume of cellulose) of 94.2% and high capillarity, so that the culture medium is efficiently drawn up into the plug, leaving the sides of the plug in direct contact with air. Roots permeate the plugs and are protected by the cellulose during transfer to soil. Plantlets of chrysanthemum in Sorbarods formed longer stems, larger leaves, more roots, and developed greater fresh mass, dry weights and fresh to dry mass ratios than plantlets in agar-solidified medium (Roberts and Smith, 1990). The greater fresh to dry mass ratio indicates that contact with liquid medium led to greater hydration of tissues. This was subsequently controlled by the inclusion of a growth retardant, paclobutrazol (1 mg l^{-1}), in the culture medium (Smith *et al.,* 1990a). Other porous materials that have been used to support plant growth include rockwool (Woodward *et al.,* 1991; Tanaka *et al.,* 1991), polyurethane foam (Gutman and Shiryaeva, 1980; Scherer *et al.,* 1988), vermiculite (Kirdmanee *et al.,* 1995), a mixture of vermiculite and Gelrite (Jay-Allemand *et al.,* 1992).

Afreen-Zobayed *et al.* (2000) cultured sweet potato, on sugar-free medium in autotrophic conditions, on mixtures of paper pulp and vermiculite in various proportions. Optimal growth was obtained on a mixture containing 70% paper pulp. On this

mixture, the fresh mass of plantlets was greater by a factor of 2.7 than on agar-solidified medium. Mixtures of paper pulp and vermiculite, in unspecified proportions, are prepared in a commercial product known as Florialite (Nisshinbo Industries, Inc., Tokyo). Afreen-Zobayed *et al.* (1999) found that growth rates of plantlets of sweet potato grown autotrophically on Florialite were greater, in ascending order, on agar, gellan gum, vermiculite, Sorbarods and Florialite (best). The dry mass of leaves and roots were greater by factors of 2.9 and 2.8, respectively, on Florialite than on an agar matrix. These authors observed that roots spread better in Florialite than in Sorbarods. They attributed this to the net-like orientation of fibers in Florialite that contrasted with the vertical orientation of fibres in Sorbarods. Ichimura and Oda (1995) found three substances that stimulated plant growth in extracts of paper pulp. Each was characterised by low molecular weight and high polarity. It is possible that these substances contribute to the superior growth observed on substrates containing paper pulp.

6.3.1. Opportunities for improved ventilation and photoautotrophy

When plantlets are cultured in vessels containing air at a relative humidity (RH) of less than 100%, transpiration occurs which is an important factor in reducing hyperhydricity (Gribble, 1999). Relative humidity in culture vessels can be reduced through ventilation, but gelled substrates are then unsuitable because the absorbance of water by the roots of a transpiring plant is impeded by the gel's low hydraulic conductivity (Fujiwara and Kozai, 1995) and this increases as the gel dries. Thus a common feature of studies using ventilated vessels has been the use of liquid medium supported by porous materials.

For example, when plantlets of chrysanthemum were grown in Sorbarods in a culture vessel with air 94% RH, a reduction in the tissue hydration was indicated by a significantly lower fresh to dry mass ratio than at 100% RH (Smith *et al.,* 1990b) and increases in stem length and leaf area. In this investigation, the RH was reduced to 94% by gaseous diffusion through a gas-permeable membrane that covered holes drilled in the lid of the culture vessel. The use of such ventilated culture vessels can significantly improve plant growth by reducing hyperhydration and facilitating the movement of solutes to the leaves in the transpiration stream. It also provides opportunities for photoautotrophic growth in sugar-free media. When plantlets are cultured in closed vessels, carbon dioxide concentrations fall to low levels in the light period, as Kozai and Sekimoto (1988) demonstrated in cultures of strawberry plants. Photosynthesis requires an adequate supply of carbon dioxide and suitable lighting. Adequate concentrations of carbon dioxide for photoautotrophy can be maintained in ventilated culture vessels with (Afreen-Zobayed *et al.,* 1999, 2000) or without (Horan *et al.,* 1995) elevated levels of carbon dioxide in the atmosphere outside the culture vessel. Adequate lighting can be achieved under lights delivering a photosynthetic photon flux of 150 μmol m^{-2} s^{-1} in a culture room (Afreen-Zobayed *et al.,* 1999, 2000) or in day-light in a greenhouse (Horan *et al.,* 1995). The environmental requirements of photoautotrophy *in vitro* have been reviewed by Jeong *et al.* (1995) and its advantages have been described by Kozai *et al.* (1995).

6.4. IMMOBILISED CELLS

Yields of secondary metabolites are usually greater in differentiated, slow-growing cells than in fast growing, undifferentiated cells. By immobilising cells in a suitable matrix, their rate of growth can be slowed and the production of secondary products enhanced. Several ingenious methods of immobilisation have been employed. Examples include immobilization in spirally wound cotton (Choi *et al.* (1995), glass fibre fabric reinforced with a gelling solution of hybrid SiO_2 precursors (Campostrini *et al.,* 1996), loofa sponge and polyurethane foam (Liu *et al.,* 1999) and alginate beads (Serp *et al.,* 2000).

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