Chapter 3
The Components of Plant Tissue Culture Media I: Macro- and Micro-Nutrients

1. INORGANIC MEDIUM COMPONENTS

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. For healthy and vigorous growth, intact plants need to take up from the soil:

- relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and,
- small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo).

According to Epstein (1971), an element can be considered to be essential for plant growth if:

1. a plant fails to complete its life cycle without it;
2. its action is specific and cannot be replaced completely by any other element;
3. its effect on the organism is direct, not indirect on the environment;
4. it is a constituent of a molecule that is known to be essential.

The elements listed above are - together with carbon (C), oxygen (O) and hydrogen (H) - the 17 essential elements. Certain others, such as cobalt (Co), aluminium (Al), sodium (Na) and iodine (I), are essential or beneficial for some species but their widespread essentiality has still to be established.

The most commonly used medium is the formulation of Murashige and Skoog (1962). This medium was developed for optimal growth of tobacco callus and the development involved a large number of dose-response curves for the various essential minerals. Table 3.1 shows the composition of MS compared to the elementary composition of normal, well-growing plants. From this table, the relatively low levels of Ca, P and Mg in MS are evident. The most striking differences are the high levels of Cl and Mo and the low level of Cu. Each plant species has its own characteristic elementary composition which can be used to adapt the medium formulation. These media result often in a much improved growth (Rugini, 1984; El Badaoui *et al.,* 1996; Pullman *et al.,* 2003; Bouman and Tiekstra, 2005; Nas and Read, 2004; Gonçalves *et al.,* 2005). A major problem in changing the mineral composition of a medium is precipitation, which may often occur only after autoclaving because of the endothermic nature of the process.

Plant tissue culture media provide not only these inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To improve growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators.

In early media, 'undefined' components such as fruit juices, yeast extracts and protein hydrolysates, were frequently used in place of defined vitamins or amino acids, or even as further supplements. As it is important that a medium should be the same each time it is prepared, materials, which can vary in their composition are best avoided if at all possible, although improved results are sometimes obtained by their addition. Coconut milk, for instance, is still frequently used, and banana homogenate has been a popular addition to media for orchid culture.

Plant tissue culture media are therefore made up from solutions of the following components:

- macronutrients (always employed);
- micronutrients (nearly always employed but occasionally just one element, iron, has been used);
- sugar (nearly always added, but omitted for some specialised purposes);
- plant growth substances (nearly always added)
- vitamins (generally incorporated, although the actual number of compounds added, varies greatly);
- a solidifying agent (used when a semi-solid medium is required. Agar or a gellan gum are the most common choices).
- amino acids and other nitrogen supplements (usually omitted, but sometimes used with advantage);

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Table 3.1 A comparison between the average concentrations of elements in plant shoots (dry weight basis) considered sufficient for adequate growth [from Epstein (1972), content of Ni is according to Brown *et al.* (1987)] and in MS. The elements that show striking differences between MS and 'plants' are indicated. For Na, no data were found, but in glycophytes grown in 1 mM Na, the endogenous level is 10 – 1000 mmol.kg^{-1} (Subbarao *et al.,* 2003)

	In tissue mmol kg^{-1}	In MS mmol l^{-1}	In tissue mol%	In MS mol%
N	1000	60	64.4	64.0
K	250	20	16.1	21.3
Ca	125	3	8.0	3.2
Mg	80	1.5	5.1	1.6
P	60	1.25	3.9	1.3
S	30	1.5	1.9	1.6
Cl	3	6	0.19	6.4
Fe	2	0.1	0.13	0.11
Mn	1	0.1	0.06	0.11
B	2	0.1	0.13	0.11
Zn	0.3	0.03	0.02	0.03
Cu	0.1	0.0001	0.0060	0.0001
Mo	0.001	0.001	0.0001	0.0011
Ni	0.001	0	0.0001	0.0000
Na		0.1	0.0000	0.1067

- undefined supplements such as coconut milk etc. (which, when used, contribute some of the five components above and also plant growth substances or regulants);
- buffers (have seldom been used, but the addition of organic acids or buffers could be beneficial in some circumstances).

Finally, it should be noted that minerals may also have a signalling role altering developmental patterns. This is most obvious in root architecture (Lopez-Bucio *et al.,* 2003) which is logical as roots have a principal function in ion uptake and the root system should be such that uptake is optimal. So growth and branching of roots should be affected by mineral concentrations in the soil. Ramage and Williams (2002) also argue that minerals appear to have an important role in the regulation of plant morphogenesis as opposed to just growth. Some reviews of whole plant mineral nutrition will be found in Grusak (2001), Leiffert *et al.,* (1995), Mengel and Kirkby (1982), Hewitt and Smith (1975) and Epstein (1971).

1.1. UPTAKE OF INORGANIC NUTRIENTS

Plants absorb the inorganic nutrients they require from soils almost entirely as ions. An ion is an atom, or a group of atoms, which has gained either a positive charge (a cation) or a negative charge (an anion). Inorganic nutrients are added to plant culture media as salts. In weak aqueous solutions, such as plant media, salts dissociate into cations and anions. Thus calcium, magnesium and potassium are absorbed by plant cells (normally those of the root) as the respective cations Ca^{2+}, Mg^{2+} and K^{+}; nitrogen is mainly absorbed in the form nitrate (the anion, NO_3^-) although uptake of ammonium (the cation, NH_4^+) may also occur, phosphorus as the phosphate ions HPO_4^{2-} and $H_2PO_4^-$; and sulphur as the sulphate ion SO_4^{2-}. In tissue culture, uptake is generally proportional to the medium concentration up to a concentration of twice MS (Williams, 1993). For specific elements this may be different. For example, Leiffert *et al,* (1995) found only a small increase in Zn uptake with increasing medium concentration indicating that the concentration of Zn in the cultured tissues was adequate, not requiring further uptake. Selective uptake also suggests active uptake.

Table 3.2 Content (mmol/kg) of elements in various agar brands.
[agar 1-7: Scholten and Pierik (1998); agar 8 and gelrite: Scherer *et al*.(1988)].
Na = not analysed, nd = not detected. It should be noted that some elements present in agar are not present in MS. This is particularly relevant for Ni which is an essential element

	Agar 1	Agar 2	Agar 3	Agar 4	Agar 5	Agar 6	Agar 7	Agar 8	gelrite
N	53	1	178	100	74	54	2	na	na
K	2	1	16	9	6	13	2	51	718
Ca	68	41	34	137	66	1	5	2.8	123
Mg	28	24	31	29	48	3	3	2.6	64
Na	202	56	552	330	427	634	114	52	296
P	1	18	1	5	1	40	1	42	68
S	184	78	232	296	204	262	66	184	6
Cl	47	33	220	113	197	95	12	na	na
Cu	0.015	0.034	0.018	0.024	0.004	0.016	nd	0.005	0.05
Mn	0.073	1.093	0.036	0.173	0.036	0.027	0.055	0.01	0.1
Fe	0.510	5.376	0.564	2.987	0.859	0.599	0.528	0.6	5
Al	0.352	12.444	1.333	4.944	0.352	0.963	0.685	0.3	6.8
Cr	0.040	0.098	0.029	0.026	0.054	0.025	0.009	0.002	0.01
Cd	0.013	0.069	0.008	0.025	0.015	nd	nd	0.0002	0.002
Zn	0.092	0.107	0.054	0.933	0.046	0.038	0.015	0.02	0.3
Sn	1.896	1.542	nd	3.572	1.862	nd	nd	0.003	0.003
Ni	0.037	0.045	nd	0.037	0.025	nd	0.007	0.005	0.004
B	na	na	na	na	na	na	na	2	0.13
Co	na	na	na	na	na	na	na	0.0005	1.0

Table 3.3 Increase of the content of Na, S and Cu relative to MS caused by adding agar (0.6%) or gelrite (0.2%) to the medium. Increases are shown as percentages. The proportional increase in other elements is maximally 20%

	Agar 1	Agar 2	Agar 3	Agar 4	Agar 5	Agar 6	Agar 7	Agar 8	gelrite
Na	1212	336	3312	1980	2562	3804	684	313	591
S	69	29	87	111	77	98	25	69	0.8
Cu	90	204	108	144	24	96	nd	28	91

In the whole plant, nutrients are either taken up passively, or through active mechanisms involving the expenditure of energy. Active uptake is generally less dependent on ionic concentration than passive uptake. Both systems are however influenced by the concentration of other elements, pH, temperature, and the biochemical or physiological status of the plant tissues. These factors can in turn be controlled by the solution presented to the roots, or they may dictate the ionic balance of an ideal solution. For example, Mg^{2+} competes with other cations for uptake. Under conditions of high K^{+} or Ca^{2+} concentrations, Mg deficiency can result, and vice versa. Active uptake of phosphate falls off if the pH of the solution should become slightly alkaline when the $(H_2PO_4)^{-}$ ion becomes changed to $(HPO_4)^{2-}$. There is some evidence that ammonium is utilised more readily than nitrate at low temperatures and that uptake may be enhanced by high carbohydrate levels within plant cells. Calcium is not absorbed efficiently and concentrations within plant tissues tend to be proportional to those in the soil. Plants are comparatively insensitive to sulphate ions, but high concentrations of dissolved phosphate can depress growth, probably through competitively reducing the uptake of the minor elements Zn, Fe and Cu. Although the biochemistry and physiology of nutrient uptake in tissue cultures may be similar, it is unlikely to be identical.

In vivo, plants take up mineral ions with their roots. No studies have been made on how uptake of nutrients occurs in shoot cultures. For IAA, it has been shown that most uptake is *via* the cut surface and that only a small fraction is taken up via the

epidermis (Guan and De Klerk, 2000). The same likely holds for minerals. It should be noted, though, that in tissue culture the stomata are always open in the portion of the explant exposed to the gaseous phase (De Klerk and Wijnhoven, 2005) and the same may apply for tissues that are exposed to semi-solid or liquid medium. Uptake via the stomata is well possible.

Once taken up, transport within the plant occurs in the mass flow via the xylem. In *in vivo* plants there are two mechanisms for driving the water flow, root pressure and water potential gradient between at one end the soil and at the other end the atmosphere. Under normal conditions, the latter is the far more important, but water flow resulting from root pressure is in itself sufficient for long-distance mineral supply (Tanner and Beevers, 2001). Plants without roots are often cultured *in vitro* where the atmosphere is very humid, and the flow driven by a difference in water potential consequently reduced. In spite of this, in tissue culture there still seems to be sufficient water flow (Beruto *et al.,* 1999) which may be favoured by the stomata being continuously open (De Klerk and Wijnhoven, 2005). There are no indications that the structure of the xylem is altered in such a way as to reduce transport of ions.

When explants are first placed onto a nutrient medium, there may be an initial leakage of ions from damaged cells, especially metallic cations (Na^+, Ca^{2+}, K^+, Mg^{2+}) for the first 1-2 days, so that the concentration in the plant tissues actually decreases (Chaillou and Chaussat, 1986). Cells then commence active absorption and the internal concentration slowly rises. Phosphate and nitrogen (particularly ammonium) are absorbed more rapidly than other ions. In liquid medium, almost all phosphorus and ammonium are taken up in the first two weeks of culture (e.g. by 5 microshoots of *Dahlia* in 50 ml stationary liquid medium; G. de Klerk, unpublished results). After uptake, phophorus is massively redistributed to tissues that are formed after the initial two weeks. Nitrate in a medium very similar to that of Wood and Braun (1961) B medium, was reduced by *Catharanthus roseus* suspensions from 24 mM to 5 mM in 15 days, while Na^+, K^+, and SO_4^{2-}, fell to only just over half the original concentrations in the same time (MacCarthy *et al.,* 1980). Carnation shoot cultures were found to use 31-75% Mg^{2+}, and 29-41% Ca^{2+} in MS medium during a 4 week period (Dencso, 1987).

1.2. UNINTENDED ALTERATIONS

Nutrients, and especially micronutrients, may also be added via impurities, and especially via agar. Such impurities may well be beneficial. This is particularly true of Ni, which has recently been shown to be an essential element (Gerendás *et al*., 1999) but was not known to be when most medium formulations were established. This element is usually not included in the inorganic constituents but can be provided by impurities. Tables 3.2 and 3.3 show impurities of various agar brands and their relative contribution to MS. Agar provides a large addition of sodium but levels of sulphur and copper are also significantly increased. Increases in the other elements in MS, are less than 20 %. Gelrite contains fewer organic impurities but inorganic ones occur at high concentrations (Table 3.2). It should be noted that the data in Table 3.2 are from determinations done more than 15 years ago and that the production process of gelrite has been improved ever since. Gelrite is being used in medicines as an ophthalmic vehicle. Furthermore, minerals are absorbed to a significant percentage by agar (Scholten and Pierik, 1998 Leiffert *et al*., 1995) and by activated charcoal (Van Winkle *et al*., 2003) but whether this has a significant effect has not been examined.

2. MACRONUTRIENTS

2.1. NITROGEN

2.1.1. Forms of nitrogen

Nitrogen is essential to plant life. It is a constituent of both proteins and nucleic acids and also occurs in chlorophyll. Most animals cannot assimilate inorganic nitrogen or synthesize many of the amino acids unless assisted by bacteria (e.g. in the rumen of cattle). Nitrogen is available in the atmosphere as N_2 but only legumes have the capacity to utilize this nitrogen using *Rhizobium* bacteria in the root nodules. In most plants, nitrate (NO_3^-) is the sole source of nitrogen. After uptake, NO_3^- is reduced to NH_4^+ prior to incorporation into organic molecules. (The removal of oxygen from a chemical compound and its replacement by hydrogen, is termed reduction.) The relevance of nitrogen is illustrated by the vast amounts of nitrogen reserves in seeds (as storage proteins).

Both growth and morphogenesis in tissue cultures are markedly influenced by the availability of nitrogen and the form in which it is presented. Compared to the nitrate ion, NO_3^- (which is a highly

oxidized form of nitrogen), the ammonium ion, NH_4^+, is the most highly 'reduced' form. Plants utilise reduced nitrogen for their metabolism and internally, nitrogen exists almost entirely in the reduced form. As a source of reduced nitrogen, plant cultures are especially able to use primary amines:

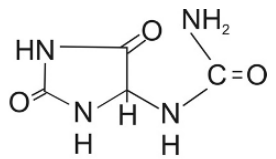
R-NH_2 and amides: R-CO-NH-R- (where R and R- are functional groups)

The primary amines which are most commonly employed in culture media are ammonia (NH_4^+) and, occasionally, amino acids.

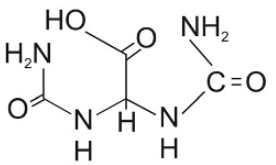
Amides are less commonly added to culture media: those which can be used by plants are particularly

NH_2-CO-NH_2 (urea)

and ureides, which include allantoin and allantoic acid (Kirby, 1982) (Fig. 3.1). Allantoin or allantoic acid are sometimes more efficient nitrogen sources than urea (Lea *et al.,* 1979).



Allantoin



Allantoic acid

Fig. 3.1 The structures of allantoin and allantoic acid.

Most media contain more nitrate than ammonium ions, but as plant tissue culture media are usually not deliberately buffered, the adopted concentrations of ammonium and nitrate ions have probably been more due to practical pH control, than to the requirement of the plant tissues for one form of nitrogen or another (see chapter 4). Uptake of nitrate only takes place effectively in an acid pH, but is accompanied by extrusion of anions from the plant, leading to the medium gradually becoming less acid. By contrast, uptake of ammonium results in the cells excreting protons (H^+) into the medium, making it more acid. The exchange of ions preserves the charge balance of the tissues and may also assist in the disposal of an excess of protons or hydroxyl (OH^-) ions generated during metabolism (Raven, 1986). Uptake of nitrogen by cell suspension cultures of *Nicotiana tabacum* is an active (energy-dependent) process (Heimer and Filner, 1971) and is dependent on a supply of oxygen (Buwalda and Greenway, 1989).

Plant culture media are usually started at pH 5.4-5.8. However, in one containing both nitrate and ammonium ions, a rapid uptake of ammonium into plant tissue causes the pH to fall to ca. 4.2-4.6. As this happens, further ammonium uptake is inhibited, but uptake of nitrate ion is stimulated, causing the pH to rise again. In unbuffered media, efficient nitrogen uptake can therefore depend on the presence of both ions. Unless otherwise stated, comments in this section on the roles of nitrate and ammonium refer to observations on unbuffered media.

There is generally a close correlation in tissue cultures between uptake of nitrogen, cell growth and the conversion of nitrogen to organic materials. A readily available supply of nitrogen seems to be important to maintain cultured cells in an undifferentiated state. The depletion of nitrogen in batch cultures, triggers an increase in the metabolism of some nitrogen-free compounds based on phenylpropanes (such as lignin), which are associated with the differentiation of secondarily-thickened cells (Hahlbrock, 1974). However, the growth in culture of differentiated cotton fibres composed largely of cellulose, is nitrate-dependent; the presence of some reduced nitrogen in the culture medium decreased the proportion of cultured embryos which produced fibres, and particularly in the absence of boron, promoted the cells of the embryos to revert to callus formation (Birnbaum *et al.,* 1974).

2.1.2. Nitrate ions (NO_3^-)

Nitrate ions are an important source of nitrogen for most plant cultures, and nearly all published media provide the majority of their available nitrogen in this form. However, once within the cell, nitrate has to be reduced to ammonium before being utilised biosynthetically. Why not simply supply nitrogen as NH_4^+ and avoid the use of NO_3^- altogether? The reason lies in the latent toxicity of the ammonium ion in high concentration, and in the need to control the pH of the medium.

Conversion of nitrate to ammonium is brought about firstly by one, or possibly two, nitrate reductase enzymes, which reduce NO_3^- to nitrite (NO_2^-). One nitrate reductase enzyme is thought to be located in the cytoplasm, while the second may be bound to membranes (Nato *et al.,* 1990). The NO_2^- produced by the action of nitrate reductase is reduced to NH_4^+ by a nitrite reductase enzyme located in plastids (Fig. 3.2). Reduction of nitrate to ammonia requires the

cell to expend energy. The ammonium ions produced are incorporated into amino acids and other nitrogen-containing compounds. Nitrate and nitrite reductase enzymes are substrate induced, and their activity is regulated directly by the level of nitrate-nitrite ions within cultured cells (Chroboczek-Kelker and Filner, 1971; Hahlbrock, 1974), but also apparently by the products of the assimilation of reduced nitrogen (see below).

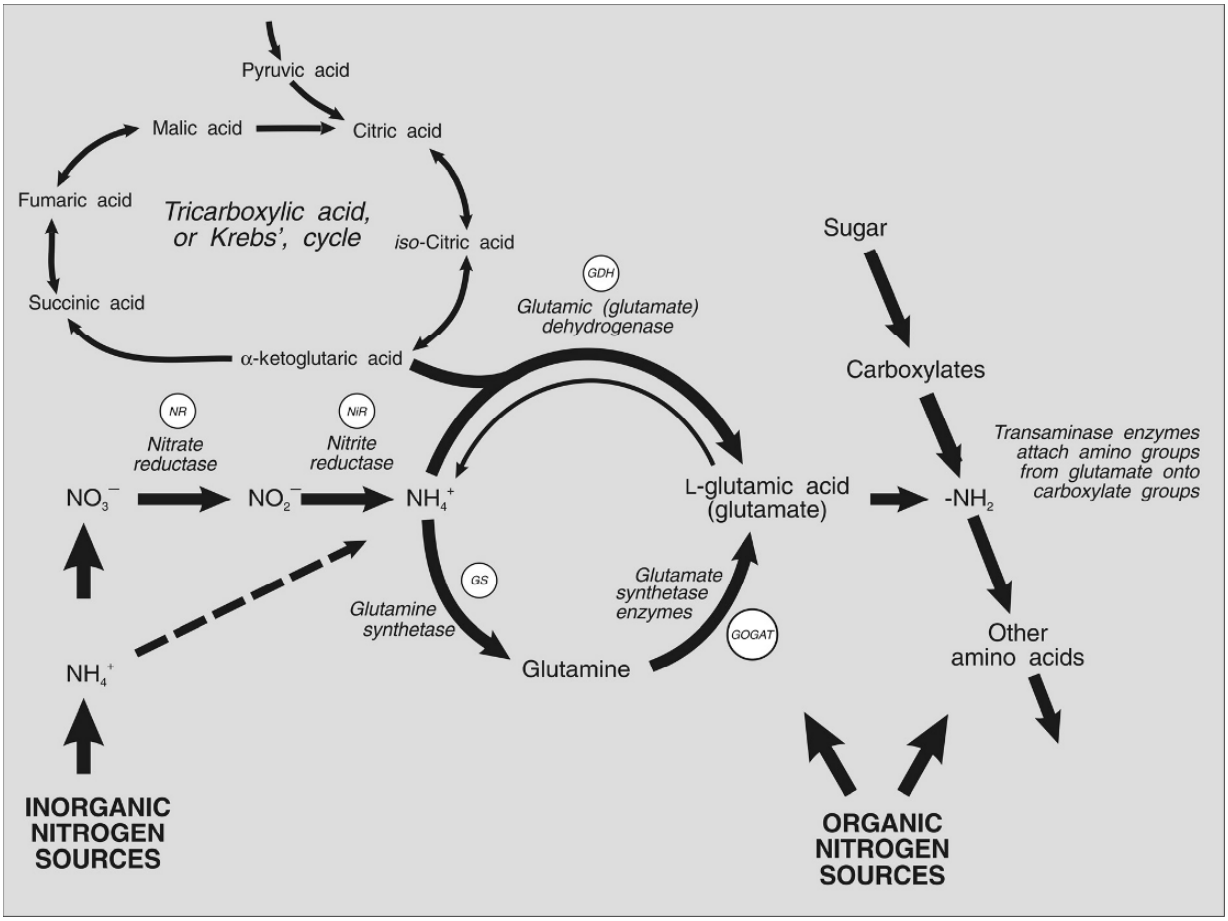


Fig. 3.2 The metabolism of nitrate and ammonium ions.

Unlike the ammonium ion, nitrate is not toxic and, in many plants, much is transported to the shoots for assimilation. On the other hand, the nitrite ion can become toxic should it accumulate within plant tissues or in the medium, for example when growth conditions are not favourable to high nitrite reductase activity and when nitrate is the only nitrogen source (Jordan and Fletcher, 1979; Grimes and Hodges, 1990). In *Pinus pinaster*, nitrate reductase is induced by the presence of KNO_3, and plants regenerated *in vitro* exhibit an ability to reduce nitrate similar to that of seedlings (Faye *et al.,* 1986).

For most types of culture, the nitrate ion needs to be presented together with a reduced form of nitrogen (usually the NH_4^+ ion), and tissues may fail to grow on a medium with NO_3^- as the only nitrogen source (Hunault, 1985).

2.1.3. Reduced nitrogen

In the natural environment and under most cropping conditions, plant roots usually encounter little reduced nitrogen, because bacteria rapidly oxidize available sources (Hiatt, 1978). An exception is forest soils in mountainous regions of the northern hemisphere, where nitrates are not usually available (Durzan, 1976). If NH_4^+, and other reduced nitrogen compounds are available, (and this is particularly the case in the aseptic *in vitro* environment), they can be taken up and effectively utilized by plants. In fact the uptake of reduced nitrogen gives a plant an

ergonomic advantage because the conversion of nitrate to ammonium ions (an energy-requiring process) is not necessary. The free ammonium ion can cause toxicity, which, at least in whole plants, can lead to an increase in ethylene evolution (Barker and Corey, 1987; Corey and Barker, 1987). Shoots grown on an unbuffered medium containing a high proportion of ammonium ions may become stunted or hyperhydric. These effects can sometimes be reversed by transfer to a medium containing a high proportion of NO_3^- or to one where NO_3^- is the only N source (Mott *et al.,* 1985). Hyperhydricity is the *in vitro* formation of abnormal organs, which are brittle and have a water-soaked appearance.

Growth of plant cultures may also be impaired in media containing high concentrations of NH_4^+ even when high concentrations of NO_3^- are present at the same time. Growth inhibition may not only be due to depressed pH (Mott *et al.,* 1985), but may reflect a toxicity induced by the accumulation of excess ammonium ions. In normal circumstances the toxic effect of ammonium is avoided by conversion of the ion into amino acids. There are two routes by which this takes place (Fig. 3.2), the most important of which, under normal circumstances, is that by which L-glutamic acid is produced from glutamine through the action of glutamine synthetase (GS) and glutamate synthetase (GOGAT) enzymes. Compounds, which block the action of GS can be used as herbicides (De Greef *et al.,* 1989). The reaction of α-ketoglutaric acid with NH_4^+ is usually less important, but seems to have increased significance when there is an excess of ammonium ions (Furuhashi and Takahashi, 1982). Detoxification and ammonium assimilation may then be limited by the availabilty of α-ketoglutaric acid, but this may be increased *in vitro* by adding to the medium one or more acids which are Krebs' (tri-carboxylic acid) cycle intermediates. Their addition can stimulate growth of some cultures on media containing high levels of NH_4^+ (Gamborg, 1970).

In comparison with media having only nitrate as the nitrogen source, the presence of the ammonium ion in media usually leads to rapid amino acid and protein synthesis, and this takes place at the expense of the synthesis of carbohydrate compounds. This diversion of cellular metabolism can be disadvantageous in some shoot cultures, and can contribute towards the formation of hyperhydric shoots. Hyperhydricity no longer occurs when NH_4^+ is eliminated from the medium or greatly reduced. It is possible that adding an organic acid to the medium might also alleviate the symptoms on some plants.

A supply of reduced nitrogen in addition to nitrate, appears to be beneficial for at least two processes involved with cell division:

- the formation of a cell wall. Without a complete cell wall, protoplasts require a factor capable of inducing wall formation. Freshly-isolated protoplasts may contain sufficient of this substance to promote wall formation for just a few divisions. The wall-forming factor is only effective when NH_4^+ is present in the medium (Meyer and Abel, 1975a,b): glutamine does not substitute for NH_4^+:
- the activity of growth regulators. (see below)

There are several reports in the literature that, with constant amounts of NO_3^-, ammonium sulphate has not provided such a good source of NH_4^+ as ammonium nitrate or ammonium chloride (De Jong *et al.,* 1974; Steward and Hsu, 1977; Singh, 1978; Kamada and Harada, 1979). Possibly the reason is that a medium containing ammonium sulphate has a greater tendency to become acid (Harris, 1956), than one containing less sulphate ions. This would result if the presence of sulphate ions accelerated the uptake of NH_4^+, or slowed the uptake of NO_3^-. Ammonium sulphate has been used as the only source of the ammonium ion in some media used for the culture of legumes, including B5 (Gamborg *et al.,* 1968).

2.1.4. Ammonium as the sole nitrogen source

pH adjustment. If plant tissues are presented with a medium containing only NH_4^+ nitrogen, the pH falls steadily as the ion is taken up (for example, a decrease of 0.9 pH units in 15 days in *Asparagus* callus – Hunault (1985) or 0.7 pH units with potato shoots – Avila *et al.,* (1998). Growth and morphogenesis is possible in suspension cultures containing only NH_4^+ ions, providing the pH of the medium is frequently adjusted by the addition of a base (Martin *et al.,* 1977), or the medium is buffered (see below). In wild carrot, the induction of embryogenesis required the medium to be adjusted to pH 5.4 at 8 hourly intervals (Dougall and Verma, 1978). Without adjustment, the pH of media containing only NH_4^+ falls rapidly to a point where cells cannot grow (Dougall, 1981).

Buffering. Ammonium can also serve as the only nitrogen source when the medium is buffered (see the section on pH, below). Tobacco cells could be grown on a medium containing NH_4^+ nitrogen if the organic acid ion, succinate, was added to the medium. Gamborg and Shyluk (1970) found that cultured cells

could be grown without frequent pH adjustment on a medium containing only NH_4^+ nitrogen, when a carboxylic acid was present. The organic acids appeared to minimize the acidification of the medium through NH_4^+ uptake. Similarly *Asparagus* internode callus grew just as well on NH_4^+ as the only nitrogen source as on a medium containing both NH_4^+ and NO_3^-, but only when organic acids (such as citrate, or malate) or MES buffer were added to the medium. When media were buffered with MES, the best callus growth occurred when the pH was 5.5 (Hunault, 1985).

The additional effect of organic acids. Although Krebs' cycle organic acids can act as buffers, they may also act as substrates for amino acid synthesis from NH_4^+. To be assimilated into amino acids via the GDH enzyme, the ammonium ion must react with α-ketoglutaric acid, which is produced by the Krebs' cycle (Fig 3.2). Its availability may govern the rate at which ammonium can be metabolised by this route. The rate of assimilation might be expected to be improved by supplying the plant with α-ketoglutarate directly, or by supplying acids which are intermediates in the Krebs' cycle (citrate, iso-citrate, succinate, fumarate or malate), for then the natural production of α-ketoglutarate should increase (Gamborg, 1970).

This hypothesis was confirmed by Behrend and Mateles (1976) who concluded that succinate, or other Krebs' cycle acids, acted mainly as a nutrient, replacing α-ketoglutarate as it was withdrawn from the cycle during NH_4^+ metabolism and amino acid synthesis. Depletion of α-ketoglutarate causes the cycle to cease unless it, or another intermediate, is replaced. The optimum molar ratio of NH_4^+ to succinate, was 1.5 (e.g. 10 mM NH_4^+: 15 mM succinate). Chaleff (1983) thought that the growth of rice callus on Chaleff (*loc. cit.*) R3 (NH_4) medium, containing 34 mM of only ammonium nitrogen, [Chaleff (1983) R3 NH_4 medium] was enabled by the presence of 20 mM succinate or a-ketoglutarate, partly by the buffering capacity of the acids, and partly by their metabolism within the plant, where they may serve as substrate for amino acid synthesis. Similar conclusions have been reached by other workers (e.g. Fukunaga *et al.,* (1978); Dougall and Weyrauch (1980); Hunault (1985); Molnar (1988b), who have found that compounds such as ammonium malate and ammonium citrate are effective nitrogen sources.

Orange juice promotes the growth of *Citrus* callus. Einset (1978) thought that this was not due to the effect of citric acid, but Erner and Reuveni (1981) showed that citric acid, particularly at concentrations above the 5.2 mM found in the juice used by Einset, does indeed promote the growth of *Citrus* callus; it had a more pronounced effect than other Krebs' cycle acids, perhaps due to the distinctive biochemistry of the genus.

Organic acids not only enhance ammonium assimilation when NH_4^+ provides the only source of nitrogen, but may sometimes also do so when nitrate ions are in attendance. The weight of rice anther callus was increased on Chaleff (1983) R3 medium, if 20 mM succinate [Chaleff (1983) R3 Succ. medium] or α-ketoglutarate was added (Chaleff, 1983). Similarly the rate of growth of *Brassica nigra* suspensions on MS medium, was improved either by adding amino acids, or 15 mM succinate. An equivalent improvement (apparently due entirely to buffering) only occurred through adding 300 mM MES buffer (Molnar, 1988b). However, the presence of organic acids may be detrimental to morphogenesis. In Chaleff's experiment, the presence of succinate in R3 medium markedly decreased the frequency of anther callus formation.

Photosynthesis. Although plants grown on nutrient solutions containing only NH_4^+ nitrogen have been found to possess abnormally high levels of PEP enzyme (Arnozis *et al.,* 1988) (the enzyme facilitating CO_2 fixation in photosynthesis), media containing high levels of NH_4^+ tend to inhibit chlorophyll synthesis (Yoshida and Kohno, 1982) and photosynthesis.

2.1.5. Urea

Plants are able to absorb urea, but like the ammonium ion, it is not a substance that is normally available in soils in the natural environment. It is however produced as a by-product of nitrogen metabolism; small quantities are found in many higher plants, which are able to utilise urea as a source of nitrogen, providing it is first converted to ammonium ions by the enzyme urease. In legumes and potato, urease requires the microelement nickel for activity (see below). In conifers, the epidermal cells of cotyledons and cotyledons are capable of urease induction and ammonium ion formation (Durzan, 1987).

Urea can be used as the sole nitrogen source for cultures, but growth is less rapid than when ammonium and nitrate ions are supplied (Kirkby *et al.,* 1987); urease enzyme increases after cultures have been maintained for several passages on a urea-based medium (King, 1977; Skokut and Filner,

1980). Although the metabolism of urea, like that of other reduced nitrogen compounds, causes the production of excess hydrogen ions, less are predicted to be secreted into the medium than during the utilisation of NH_4^+ (Raven, 1986), so that urea is less suitable than ammonium to balance the pH of media containing NO_3^-. Nitrate ions are utilized in preference to urea when both nitrogen sources are available (King, 1977). Urea is able to serve as a reduced nitrogen source during embryogenesis (Durzan, 1987), but has been used in relatively few culture media, and of these, none has been widely adopted (George *et al.,* 1987).

2.1.6. Media with nitrate and ammonium ions

Most intact plants, tissues and organs take up nitrogen more effectively, and grow more rapidly, on nutrient solutions containing both nitrate and ammonium ions, than they do on solutions containing just one of these sources. Although in most media, reduced nitrogen is present in lower concentration than nitrate, some morphogenic events depend on its presence, and it can be used in plant cultures in a regulatory role. Adventitious organs may also develop abnormally if NH_4^+ is missing (Drew, 1987).

Possible explanations, which have been put forward for the regulatory effect of NH_4^+ are:

- that the reduction and assimilation of NO_3^- is assisted by the presence of of NH_4^+ or the products of its assimilation (Bayley *et al.,* 1972a,b; Mohanty and Fletcher, 1978; 1980). When grown on a medium containing a small amount of NH_4^+ nitrogen in addition to nitrate, suspension cultured cells of 'Paul's Scarlet' rose accumulated twice as much protein as when grown on a medium containing only nitrate, even though ammonium finally accounted for only 10% of the total protein nitrogen (Mohanty and Fletcher, 1980). Dougall (1977) considered this to be an oversimplified interpretation, moreover nitrate reductase activity is effectively increased by the presence of NO_3^- (Müller and Mendel, 1982) and in some plants, a high concentration of NH_4^+ inhibits nitrate reductase activity (see below).
- that ammonium ions effectively buffer plant nutrient media in the presence of nitrate and so enhance nitrate uptake (see the section on pH).

Cultures of some plants are capable of growing with only NO_3^- nitrogen (e.g. cell cultures of *Reseda luteoli*, soybean, wheat, flax and horse radish – Gamborg (1970); callus of *Medicago sativa* - Walker and Sato (1981), although yields are generally better when the medium is supplemented with NH_4^+. Craven *et al.,* (1972) with carrot, and Mohanty and Fletcher (1978) with *Rosa* 'Paul's Scarlet', found that the presence of NH_4^+ was particularly important during the first few days of a suspension culture. After that cells increase in cell number and dry weight more rapidly on NO_3^- nitrogen alone.

The response of plant cultures to nitrate and ammonium ions depends to a large extent on the enzymes shown in Fig 3.2, and the manner in which their activities are increased or inhibited in different tissues by the presence of the ions. These factors vary according to the degree of differentiation of the tissue (Suzuki and Nato, 1982), its physiological age, and its genotype. For example, the high level of NH_4^+ in MS medium inhibited the activity of glutamate synthetase enzyme in soybean suspension cultures (Gamborg and Shyluk, 1970), while in *N. tabacum*, a peak of glutamate dehydrogenase (GDH) appeared to exist at 10 mM NH_4^+ (Lazar and Collins, 1981). The activity of GDH and NADH-dependent GOGAT developed rapidly in cultured tobacco cells, while nitrate reductase and ferridoxin-dependent GOGAT activity increased more slowly during growth. By contrast, in sunflower cultures, the specific activity of GDH and ferridoxin-dependent GOGAT only reached a maximum at the end of growth, and the presence of 15 mM NH_4^+ inhibited the activity of nitrate reductase, indicating that the cells were entirely dependent on the reduced nitrogen in the medium (Lenee and Chupeau, 1989).

In consequence of such variation, the relative concentrations of ammonium and nitrate in media may need to be altered for different cultures.

2.1.7. The correct balance of ions

When trying to find media formulations suitable for different plant species and different kinds of cultures, two important factors to be considered are:

- the total concentration of nitrogen in the medium;
- the ratio of nitrate to ammomium ions.

There is a high proportion of NH_4^+ nitrogen in MS medium [ratio of NO_3^- to NH_4^+, 66:34] and the quantity of total nitrogen is much higher than that in the majority of other media. For some cultures, the total amount of nitrogen is too high and the balance between the two forms in the medium is not optimal.

It is noticeable that in several media used for legume culture, there is a greater proportion of NO_3^- to NH_4^+ ions than in MS medium. Evans *et al.,* (1976) found that soybean leaf callus grew more rapidly and formed more adventitious roots when the ammonium nitrate in MS medium was replaced with

500 mg/l ammonium sulphate. A reduction in the ammonium level of their medium for the callus culture of red clover, was also found to be necessary by Phillips and Collins (1979, 1980) to obtain optimum growth rates of suspension cultured cells. A similar adjustment can be beneficial in other genera. Eriksson (1965) was able to enhance the growth rate of cell cultures of *Haplopappus gracilis* when he reduced the ammonium nitrate concentration to 75% of that in MS medium, and doubled the potassium dihydrogen phosphate level.

Table 3.4 lists examples of where changes to the nitrogen content of MS medium resulted in improved *in vitro* growth or morphogenesis. It will be seen that the balance between NO_3^- and NH_4^+ in these different experiments has varied widely. This implies that the ratio between the two ions either needs to be specifically adjusted for each plant species, or that the total nitrogen content of the medium is the most important determinant of growth or morphogenesis. Only occasionally is an even higher concentration of nitrate than that in MS medium beneficial. Trolinder and Goodin (1988) found that the best growth of globular somatic embryos of *Gossypium* was on MS medium with an extra 1.9 g/l KNO_3.

There are reports that adjustments to the nitrogen content and ratio of NO_3^- to NH_4^+, can be advantageous in media containing low concentrations of salts (Table 3.5). Biedermann (1987) found that even quite small adjustments could be made advantageously to the NO_3^- content of a low salt medium [that of Biedermann (1987)] for the shoot culture of different *Magnolia* species and hybrids, but too great a proportion of NO_3^- was toxic.

2.1.8. The nitrate-ammonium ratio for various purposes

Root growth. Root growth is often depressed by NH_4^+ and promoted by NO_3^-. Media for isolated root culture contain no NH_4^+, or very little. Although roots are able to take up nitrate ions from solutions, which become progressively more alkaline as assimilation proceeds, the same may not be true of cells, tissues and organs *in vitro*.

Shoot cultures. Media containing only nitrate nitrogen are used for the shoot culture of some plants, for example strawberry (Boxus, 1974), which can be cultured with 10.9 mM NO_3^- alone; supplementing the medium with 6 mM NH_4^+ causes phytotoxicity (Damiano, 1980). However, shoot cultures of strawberry grown without NH_4^+ can become chlorotic: adding a small amount of NH_4NO_3 (or another source of reduced nitrogen) to the medium at the last proliferation stage, or to the rooting medium, may then give more fully developed plants with green leaves (Zimmerman, 1981; Piagnani and Eccher, 1988). On some occasions it is necessary to eliminate or reduce NH_4^+ from the medium for shoot cultures to prevent hyperhydricity.

2.1.9. Nitrogen supply and morphogenesis

Morphogenesis is influenced by the total amount of nitrogen provided in the medium and, for most purposes, a supply of both reduced nitrogen and nitrate seems to be necessary. The requirement for both forms of nitrogen in a particular plant species can only be determined by a carefully controlled experiment: simply leaving out one component of a normal medium gives an incomplete picture. For example, cotyledons of lettuce failed to initiate buds when NH_4NO_3 was omitted from Miller (1961) salts and instead formed masses of callus (Doerschug and Miller, 1967): was this result due to the elimination of NH_4^+, or to reducing the total nitrogen content of the medium to one third of its original value?

Importance of the nitrate/ammonium balance. The importance of the relative proportions of NO_3^- and NH_4^+ has been demonstrated during indirect morphogenesis and the growth of regenerated plants. Grimes and Hodges (1990) found that although the initial cellular events which led to plant regeneration from embryo callus of indica rice, were supported in media in which total nitrogen ranged from 25 to 45 mM and the NO_3^- to NH_4^+ ratio varied from 50:50 to 85:15 (Table 3.5), differentiation and growth were affected by very small alterations to the NO_3^- to NH_4^+ ratio. Changing it from 80:20 (N6) medium, to 75:20, brought about a 3-fold increase in plant height and root growth, whereas lowering it below 75:25, resulted in short shoots with thick roots. Atypical growth, resulting from an unsuitable balance of nitrate and ammonium, has also been noted in other plants. It gave rise to abnormal leaves in *Adiantum capillus-veneris* (Pais and Casal, 1987); the absence of ammonium in the medium caused newly initiated roots of *Carica papaya* to be abnormally thickened, and to have few lateral branches (Drew, 1987). Shoot cultures may survive low temperature storage more effectively when maintained on a medium containing less NH_4NO_3 than in MS medium (Moriguchi and Yamaki, 1989).

By comparing different strengths of Heller (1953; 1955) and MS media, and varying the NH_4NO_3 and $NaNO_3$ levels in both, David (1972) was led to the conclusion that the principal ingredient in MS favouring differentiation in Maritime pine explants is

NH_4NO_3. However, in embryonic explants of *Pinus strobus*, adventitious shoot formation was better on Schenk and Hildebrandt (1972) medium than on MS (which induced more callus formation). The difference in the ammonium level of the two media was mainly responsible (Flinn and Webb, 1986).

Table 3.4 Examples of adjustments of the nitrogen content of MS medium, which resulted in improved growth or morphogenesis. In each case, only NO_3^- and NH_4^+ were changed, the rest of the medium being the same except for K^+ and Cl^-

Plant species	Type of culture	Results	NO_3^- (mM)	NH_4^+ (mM)	Ratio of NO_3^- to NH_4^+	Total N (mM)	Reference
Nicotiana tabacum	Callus	Optimum callus growth	39.4	20.61	1.91	60.01	Murashige and Skoog (1962)
Nicotiana tabacum	Callus	Equal callus growth and shoot formation	40.0	20.0	2.00	60.0	Behki and Lesley (1980)
			48.0	12.0	4.00	60.0	
			52.0	8.0	6.50	60.0	
	Callus	Callus growth and root formation	24.0	12.0	2.00	36.0	
Nicotiana tabacum	Shoot regeneration from leaf disks	Optimal number of shoots	40	20	2.3	60	Ramage and Williams (2002)
Dioscorea spp.	Callus	Callus growth and adventitious plantlets	6.25	6.25	1.00	15.0	Asokan *et al.*, (1983)
Diospyros kaki	Shoot	Shoot proliferation	19.7	20.61	0.96	40.31	Sugiura *et al.*, (1986)
Rubus ideaus	Shoot	Shoot proliferation	19.7	10.30	1.91	30.0	Welander (1987)
Prunus avium	Shoot	Shoot proliferation	29.09	10.3	2.82	39.40	Righetti *et al.*, (1988)
Castanea sativa and *Castanea* hybrids	Shoot	Shoot proliferation	18.00	3.00	6.00	21.0	Piagnani and Eccher (1988)
Peltophorum pterocarpum	Anther	Callus formation and shoot regeneration	34.7	9.99	3.47	44.6	Lakshmana Rao and De (1987)
Euphorbia esula	Suspension	Plant regeneration	18.00	8.00	2.25	27	Davis *et al.*, (1988)
Haplopappus gracillis	Suspension	Improved growth rate	33.78	14.99	2.25	8.77	Eriksson (1965)
Oryza sativa	Protoplast	Cell division	18.79	1.01	18.53	19.80	Yamada *et al.*, (1986)
Allium sativum	Shoot	Shoot proliferation	35.0	8.0	4.4	43.0	Luciani *et al.*, (2001)
Solanum tuberosum	Shoot	Shoot proliferation	41.3	17.7	2.3	59.0	Avila *et al.*, (1998)

Table 3.5 Examples of beneficial and harmful adjustments to the total nitrogen and the NO_3^- and NH_4^+ in low salt media

Basic Medium in which N modified	Plant species or variety	Type of culture and results	NO_3^- (mM)	NH_4^+ (mM)	Ratio of NO_3^- to NH_4^+	Total N (mM)	Reference
Biederman (1987)			**8.62**	**6.25**	**58:42(1.38)**	**14.87**	
Magnolia stellata		Shoot culture: maximum proliferation	10.99	6.25	1.76	17.24	Biederman (1987)
Magnolia 'Elizabeth'		Shoot culture: maximum proliferation	8.62-10.99	6.25	1.38 – 1.76	14.87 – 17.24	
Magnolia 'Yellow Bird' and '#149'		Shoot culture: maximum proliferation	7.43	6.25	1.19	13.68	
Magnolia (all vars.)		Shoot culture: death of all cultures	25.04	6.25	4.01	31.29	
Chu *et al.*, (1975) N6			**28.00**	**7.00**	**80:20(4.00)**	**35.00**	
Oryza sativa		Callus induction from immature embryos	28.00	7.00	4.00	35.00	Grimes and Hodges (1990)
		Plant regneration after callus induction with 2,4-D	12.5–38.25	22.5-3.75	1.0 - 5.66	25.00-45.00	
		Optimum number of plants per zygotic embryo	18.75	6.25	3.00	25.00	
		Poor regeneration	100 <17.5	0 >17.5	∞ <1.0	35.00	
		Optimum plantlet growth	26.25	8.75	8.75	35.00	

Morphogenesis influenced by total available nitrogen. Others have found that the total nitrogen content of culture media influences morphogenesis more than the relative ammonium concentration. Results of Margara and Leydecker (1978) indicated that adventitious shoot formation from rapeseed callus was optimal in media containing 30-45mM total nitrogen. The percentage of explants forming shoots was reduced on media containing smaller or greater amounts (e.g. on MS medium). Increasing the ratio of NH_4^+ to total N in media, from 0.20 to 0.33 was also detrimental. Similarly, Gertsson (1988) found that a small number of adventitious shoots was obtained on petiole segments of *Senecio hybridus* when the total nitrogen in MS medium was increased to 75 mM, but that an increased number of shoots was produced when the total nitrogen was reduced to 30 mM (while keeping the same ratio of NO_3^- to NH_4^+). Shoot production was more than doubled if, at the same time as the total N was reduced, the potassium ion concentration was fixed at 15 mM, instead of 20 mM.

The total amount of nitrogen in a medium was shown by Roest and Bokelmann (1975) to affect the number of adventitious shoots formed directly on *Chrysanthemum* pedicels. The combined amount of KNO_3 + NH_4NO_3 in MS medium (60 mM), was adjusted as is shown in Fig. 3.3 while the ratio of NO_3^- to NH_4^+ (66:34) was unchanged. From 30-120 mM total nitrogen was optimal. However there was clearly a strong effect of genotype, because the cultivar `Bravo' was much more sensitive to increased nitrogen than 'Super Yellow'.

Nitrogen x sugar interaction. The enhancement of morphogenesis caused by high nitrogen levels may not be apparent unless there is an adequate sucrose concentration in the medium (Margara and Rancillac, 1966; Gamborg *et al.,* 1974). In *Dendrobium*, the uptake of NO_3^- is slower than that of NH_4^+. Uptake is dependent on the nature and concentration of the sugar in the medium, being slower in the presence of fructose than when sucrose or glucose are supplied (Hew *et al.,* 1988). The rate of growth of *Rosa* 'Paul's Scarlet' suspensions was influenced by the ratio of NO_3^- to sucrose in the medium. A high ratio favoured the accumulation of reduced nitrogen, but not the most rapid rate of cell growth (Fletcher, 1980).

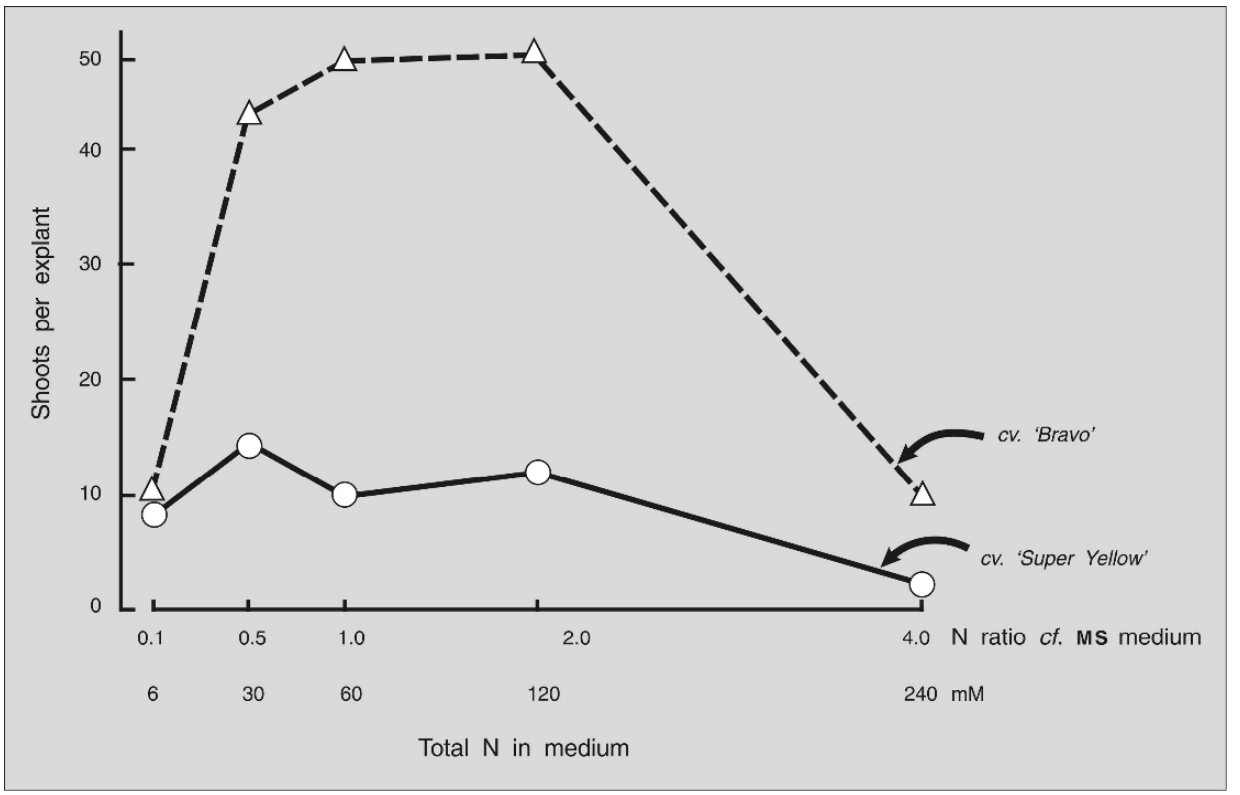


Fig. 3.3 The number of adventitious shoots formed directly on Chrysanthemum explants with increasing total nitrogen concentration in an otherwise normal **MS** medium [from data of Roest and Bokelmann, 1975]

Embryogenesis and embryo growth. It is accepted that the presence of some reduced nitrogen is necessary for somatic embryogenesis in cell and callus cultures (Halperin and Wetherell, 1965; Reinert *et al.,* 1967); but although reduced nitrogen compounds are beneficial to somatic embryo induction, apparently they are not essential until the stage of embryo development (Kamada and Harada, 1979). A relatively high level of both nitrate and ammonium ions then seems to be required. Some workers have also noted enhanced embryogenesis and/or improved embryo growth when media have been supplemented with amino acids in addition to NO_3^- and NH_4^+. Street (1979) thought that an optimum level of NH_4^+ for embryogenesis was about 10 mM (from NH_4Cl) in the presence of 12-40 mM NO_3 (from KNO_3): that is:

[NO_3^- to NH_4^+ ratio, from 55:45 to 80:20; Total N 22-50 mM]

Walker and Sato (1981) obtained no embryogenesis in alfalfa callus in the absence of either ammonium or nitrate ions. Miller's medium [Miller (1961; 1963)] (12.5 mM NH_4^+) supported a high rate of embryogenesis:

[NO_3^- to NH_4^+ ratio, 68:32; Total N 39.1 mM], but only a small number of embryos were produced on Schenk and Hildebrandt (1972) medium (SH):

[NO_3^- to NH_4^+ ratio, 90:10, Total N 27.32 mM], unless it was supplemented with NH_4^+ from either ammonium carbamate, ammonium chloride or ammonium sulphate. An optimal level of ammonium in SH medium was 12.5 mM:

[NO_3^- to NH_4^+ ratio, then 66:34, total N, 37.22], although embryogenesis was still at a high level with 100 mM ammonium ion:

[NO_3^- to NH_4^+ ratio, then, 20:80; total N, 124.72 mM].

By contrast, *Coffea arabica* leaf callus (Sondahl and Sharp, 1977), which was formed on a medium containing MS salts, was induced to become embryogenic by first being cultured on a medium with MS salts (and high auxin):

[NO_3^- to NH_4^+ ratio, 66:34; total N, 30.0 mM],

and then moved to another medium containing MS salts with an extra 2850 mg/l KNO_3:

[NO_3^- to NH_4^+ ratio, 82:18; total N, 58.2 mM] (and low auxin)

Zygotic embryos. The presence of some reduced nitrogen in the growth medium is also required for the continued growth of zygotic embryos in culture. Nitrate alone is insufficient (Mauney *et al.,* 1967; Norstog, 1967, 1973). An optimum concentration of NH_4^+ for the development of barley embryos in culture was 6.4 mM (Umbeck and Norstog, 1979). A similar provision seems to be necessary in most plants for the *in vitro* growth of somatic embryos.

Flower bud formation and growth. Nitrate was essential for the formation of adventitious buds on leaf segments of *Begonia franconis.* The greatest proportion of flower buds was obtained with 5 mM NO_3^- and 1.5 mM NH_4^+. Above this level, NH_4^+ promoted vegetative sprouts (Berghoef and Bruinsma, 1979b). The best *in vitro* growth of *Begonia franconis* flower buds detached from young inflorescences, occurred on a medium with 10-15 mM total nitrogen (NO_3^- to NH_4^+ ratio, 50:50 to 67:33) (Berghoef and Bruinsma, 1979a). Detached flower buds of *Cleome iberidella* were found to grow best *in vitro* with 25 mM total nitrogen (NO_3^- to NH_4^+ ratio, 80:20) (De Jong and Bruinsma, 1974; De Jong *et al.,* 1974), but the complete omission of NH_4NO_3 from MS medium, where the salts had been diluted to their original concentration, promoted the development (but not the initiation) of adventitious floral buds of *Torenia fournieri* (Tanimoto and Harada, 1979, 1981, 1982).

Effect on the action of growth regulants. The ratio of NO_3^- to NH_4^+ present in the culture medium has been found to affect the activity of plant growth substances and plant growth regulators. The mechanisms by which this occurs are not fully elucidated. It has been noted, for example, that cells will divide with less added cytokinin when the proportion of reduced nitrogen is reasonably high. To induce tobacco protoplasts to divide, it was necessary to add 0.5-2 mg/l BAP to a medium containing only NO_3^- nitrogen. The presence of glutamine or NH_4^+ in the medium together with NO_3^-, reduced the cytokinin requirement, and division proceeded without any added cytokinin when urea, NH_4^+, or glutamine were the sole N-sources of the medium (Meyer and Abel, 1975b). Sargent and King (1974) found that soybean cells were dependent on cytokinin when cultured in a medium containing NO_3^- nitrogen, but independent of cytokinin when NH_4^+ was present as well.

The relative proportion of nitrate and ammonium ions also affects the response of cells to auxin growth regulators in terms of both cell division and morphogenesis. It is possible that this is through the control of intracellular pH (see below). Carrot cultures that produce somatic embryos when transferred from a high- to a low-auxin medium, can also be induced into embryogenesis in a high-auxin medium, if it contains adequate reduced nitrogen. Only root initials are formed in high-auxin media which do not contain reduced nitrogen (Halperin, 1967). The number of plants regenerated from rice callus grown on Chu *et al.,* (1975) medium containing 0.5 mg/l 2,4-D, depended on the ratio of NO_3^- to NH_4^+. It was high in the unaltered medium (ratio 4:1), but considerably less if, with the same total N, the ratio of the two ions was changed to 1:1 (Grimes and Hodges, 1990).

Cells of *Antirrhinum majus* regenerated from isolated protoplasts were stimulated to divide with a reduced quantity of auxin in a medium containing 39.77 mM total nitrogen

[NO_3^- to NH_4^+ ratio, 39:77 (2.98)]

by adding further ammonium ion to give a total nitrogen content of 54.72 mM

[NO_3^- to NH_4^+ ratio, 54:46 (1.19)].

or, alternatively, 400 mg/l of casein hydrolysate (Poirier-Hamon *et al.,* 1974).

In experiments of Koetje *et al.,* (1989) and Grimes and Hodges (1990) (Table 3.5), when the NO_3^- to NH_4^+ ratio in N6 medium was 80:20, there was a strong dose response curve to the auxin 2,4-D with 0.5 mg/l being the best concentration to induce embryogenesis in *Oryza sativa* callus; if the medium was modified, so that the NO_3^- to NH_4^+ ratio was 66:34 or 50:50, 2,4-D was less effective, and there was little difference in the number of plants regenerated between 0.5 and 3 mg/l 2,4-D. The ratio of NO_3^- to NH_4^+ therefore seemed either to to alter the sensitivity of cells to the auxin, or to affect its uptake or rate of metabolism.

Walker and Sato (1981) also found that the proportion of ammonium ion in the medium can influence the way in which growth regulants control morphogenesis. Having been placed for 3 days on a medium which would normally induce root formation [Schenk and Hildebrandt (1972) medium containing 5 mM 2,4-D and 50 mM kinetin], suspension cultured cells were subsequently plated on a modification of the same medium (which contains 24.8 mM NO_3^-) without regulants, in which the concentration of NH_4^+ had been adjusted to various levels. Table 3.6 shows that the morphogenesis experienced, depended on the concentration of ammonia in the regeneration medium. Media containing high levels of ammonium ion would have tended to become acid, especially as the extra ammonium was added as ammonium sulphate. Possibly this affected the uptake or action of the regulants?

2.1.10. Addition of amino acids

Amino acids can be added to plant media to satisfy the requirement of cultures for reduced nitrogen, but as they are expensive to purchase, they will only be used in media for mass propagation where this results in improved results. For most tissue culture purposes, the addition of amino acids may be unnecessary, providing media contain adequate amounts, and correct proportions, of nitrate and ammonium ions. For example, Murashige and Skoog (1962) found that when cultures were grown on media such as Heller (1953; 1955), Nitsch and Nitsch (1956) N1, and Hildebrandt *et al.,* (1946) Tobacco, containing sub-optimal amounts of inorganic chemicals, a casein hydrolysate (consisting mainly of a mixture of amino acids, see later) substantially increased the yield of tobacco callus, whereas it gave only marginal increases in yield when added to their revised MS medium. Arginine (0.287 mM) increased the growth of sugar cane callus and suspension cultures grown on Nickell and Maretzski (1969) medium (Nickell and Maretzski, 1969) but was without effect on cultures of this plant grown on a medium based on Scowcroft and Adamson (1976) CS5 macronutrients (Larkin, 1982).

It is noticeable from the literature that organic supplements (particularly amino acids) have been especially beneficial for growth or morphogenesis when cells or tissues were cultured on media such as White (1943a), which do not contain ammonium ions. White (1937) and Bonner and Addicott (1937), for example, used known amino acids to replace the variable mixture provided by yeast extract. For the culture of *Picea glauca* callus, Reinert and White (1956) supplemented Risser and White (1964) medium with 17 supplementary amino acids, and similar, or greater numbers, were used by Torrey and Reinert (1961) and Filner (1965) in White (1943) medium for the culture of carrot, *Convolvulus arvensis*, *Haplopappus gracilis* and tobacco tissues.

Dependence on the nitrate to ammonium ratio. Grimes and Hodges (1990) found that when both NO_3^- and NH_4^+ are present in the medium, the response to organic nitrogen depends on the ratio of these two ions. Twice as many plants were regenerated from embryogenic rice callus when 1g/l CH was added to Chu *et al.,* (1975) N6 medium, providing the proportion of NO_3^- to NH_4^+ was also changed to 1 (50:50). There was little response to CH with the same amount of total N in the medium, if the NO_3^-/NH_4^+ ratio was 4 (i.e. 80%:20%, as in the original medium), or more.

2.1.11. Amino acids as the sole N source

As most of the inorganic nitrogen supplied in culture media is converted by plant tissues to amino acids, which are then assimilated into proteins, it should be possible to culture plants on media in which amino acids are the only nitrogen source. This has been demonstrated: for example, *Nicotiana tabacum* callus can be cultured on MS salts lacking NO_3^- and NH_4^+ (but with an extra 20.6 mM K^+), if 0.1 mM glycine, 1mM arginine, 2 mM aspartic acid and 6 mM glutamine are added (Muller and Grafe, 1978); wild carrot suspensions can be grown on a medium

Table 3.6 The effect of total nitrogen, and the ratio of NO_3^- to NH_4^+ on the type of organ produced by alfalfa cells which have been subcultured on a root-inducing medium (Walker and Sato, 1981)

Type of organ produced	NH_4^+ (mM)	NO_3^- (mM)	Ratio of NO_3^- to NH_4^+
Roots	<2.5	27.2	100:0 to 91:9
Roots *and* somatic embryos	12.5-37.5	37.3-62.3	66:34 to 40:60
Somatic embryos	50-100	74.7-124.7	33:67 to 20:80

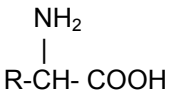
containing glutamine or casein hydrolysate as the sole nitrogen source (Anderson, 1976). Amino acids provide plant cells with an immediately available source of nitrogen, and uptake can be much more rapid than that of inorganic nitrogen in the same medium (Thom *et al.,* 1981). Only the L- form of amino acids is biologically active.

Amino acids can also provide reduced nitrogen in culture media in place of NH_4^+ and as a supplement to NO_3^-. However they are usually employed as minor additions to media containing both NH_4^+ and NO_3^-. Uptake of amino acids into cultured tissues causes a decrease in the pH of the medium, similar to that which occurs when NH_4^+ ions are absorbed.

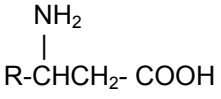
Sugar-based amines such as glucosamine and galactosamine can also serve as a source of reduced nitrogen in morphogenesis (Margara, 1969a; Margara and Leydecker, 1978).

2.1.12. Biologically-active amino acids

Amino acids are classified according to their stereoisomers and according to the relative positions of the amino group and the acidic radical. Only the L- isomers of the α-amino acids are important for plant tissue culture media. They have the general structure:



β-Amino acids are present in plants but tend to result from secondary metabolism. They have the general structure:



(where R = functional groups)

Unfortunately the particular amino acid, or mixture of amino acids, which promotes growth or morphogenesis in one species, may not do so in another. For instance, L-α-alanine, glutamine, asparagine, aspartic acid, glutamic acid, arginine and proline could serve as a source of reduced nitrogen in a medium containing 20 mM NO_3^-, and were effective in promoting embryogenesis in *Daucus carota* callus and suspensions, but lysine, valine, histidine, leucine and methionine were ineffective (Kamada and Harada, 1982).

Competitive inhibition. Some amino acids are growth inhibitory at fairly low concentrations and this is particularly observed when mixtures of two or more amino acids are added to media. Inhibition is thought to be due to the competitive interaction of one compound with another. In oat embryo cultures, phenylalanine and L-tyrosine antagonise each other, as do L-leucine and DL-valine; DL-isoleucine and DL-valine, and L-arginine and L-lysine (Harris, 1956). Lysine and threonine often exert a co-operative inhibition when present together, but do not inhibit growth when added to a medium singly (Cattoir-Reynaerts *et al.,* 1981).

Glycine. Glycine is an ingredient of many media. It has usually been added in small amounts, and has been included by some workers amongst the vitamin ingredients. Despite frequent use, it is difficult to find hard evidence that glycine is really essential for so many tissue cultures, but possibly it helps to protect cell membranes from osmotic and temperature stress (Orczyk and Malepszy, 1985).

White (1939) showed that isolated tomato roots grew better when his medium was supplemented with glycine rather than yeast extract and that glycine could replace the mixture of nine amino acids that had been used earlier. It was employed as an organic component by Skoog (1944) and continued to be used in his laboratory until the experiments of Murashige and Skoog (1962). They adopted the kinds and amounts of organic growth factors specified by White (1943a) and so retained 2 mg/l glycine in their medium without further testing.

Linsmaier and Skoog (1965), furthering the study of medium components to organic ingredients, omitted glycine from MS medium and discovered that low concentrations of it had no visible effect on the growth of tobacco callus, while at 20 mg/l it depressed growth. No doubt the success of MS medium has caused the 2 mg/l glycine of Skoog (1944) to be copied in many subsequent experiments. Many workers overlook the later Linsmaier and Skoog paper.

Casein and other protein hydrolysates. Proteins, which have been hydrolysed by acid, or enzymes, and so broken down into smaller molecules, are less costly than identified amino acids. The degree of degradation varies: some protein hydrolysates consist of mixtures of amino acids together with other nitrogenous compounds such as peptide fragments, vitamins, and elements which might (if they can form inorganic ions, or are associated with organic compounds that can be taken

up by plant tissues) be able to serve as macro- or micro-elements. Peptones are prepared from one or several proteins in a similar fashion but generally consist of low molecular weight proteins. Although protein hydrolysates are a convenient source of substances which may promote plant growth, they are by nature relatively undefined supplements. The proportion of individual amino acids in different hydrolysates depends on the nature of the source protein and the method by which the product has been prepared.

The hydrolysate most often used in culture media is that of the milk protein, casein, although lactalbumin hydrolysate has been employed (La Motte and Lersten, 1971). Peptones and tryptone have been used less frequently, but there are reports of their having been added to media with advantage (e.g. Muralidhar and Mehta, 1982; Pierik *et al.,* 1988). Casein hydrolysates can be a source of calcium, phosphate, several microelements, vitamins and, most importantly, a mixture of up to 18 amino acids. Several casein hydrolysates (CH) are available commercially but their value for plant tissue culture can vary considerably. Acid hydrolysis can denature some amino acids and so products prepared by enzymatic hydolysis are to be preferred. The best can be excellent sources of reduced nitrogen, as they can contain a relatively large amount of glutamine.

Casein hydrolysate produces an improvement in the growth of *Cardamine pratensis* and *Silene alba* suspensions, only if the medium is deficient in phosphorus. Glutamine has the same effect; it is the most common amino acid in CH, and its synthesis requires ATP. For these reasons, Bister-Miel *et al.,* (1985) concluded that CH overcomes the shortage of glutamine when there is insufficient phosphorus for adequate biosynthesis.

However several investigators have concluded that casein hydrolysate itself is more effective for plant culture than the addition of the major amino acids which it provides. This has led to speculation that CH might contain some unknown growth-promoting factor (Inoue and Maeda, 1982). In prepared mixtures of amino acids resembling those in CH, competitive inhibition between some of the constituents is often observed. For instance, the induction of embryogenesis in carrot cell suspensions on a medium containing glutamine as the only nitrogen source, was partly inhibited by the further addition of L-amino acids similar in composition to those in CH. This suppression was mainly caused by the L-tyrosine in the mixture (Anderson, 1976).

There may be a limit to the amount of CH, which can be safely added to a medium. Anstis and Northcote (1973) reported that the brand of CH known as 'N-Z-amine', can produce toxic substances if concentrated solutions are heated, or if solutions are frozen and thawed several times. Possibly these are reasons why mixtures of amino acids occasionally provide more valuable supplements than CH. *Nicotiana tabacum* callus grew better on a nitrogen-free MS medium when a mixture of the amino acids L-glutamine (6 mM), L-aspartic acid (2 mM), L-arginine (1 mM) and glycine (0.1 mM) was added, rather than 2 g/l casein hydrolysate (which would have provided about 2 mM glutamine, 0.6 mM aspartic acid, 0.2 mM arginine and 0.3 mM glycine) (Muller and Grafe, 1978).

2.1.13. Beneficial effects of amino acid additions

Improved growth. The growth rate of cell suspensions is frequently increased by the addition of casein hydrolysate or one or more amino acids (particularly glutamine) to media containing both nitrate and ammonium ions. Some workers have included a mixture of several amino acids in their medium without commenting on how they improved growth. In other cases the benefit resulting from a specific compound has been clearly shown. The lag phase of growth in suspensions of *Pseudotsuga menziesii* cultured on Cheng (1977; 1978) medium was eliminated by the addition of 50 mM glutamine, and the final dry weight of cells was 4 times that produced on the unammended medium (Kirby, 1982). Similarly the rate of growth of *Actinidia chinensis* suspensions was improved by the addition of 5mM glutamine (Suezawa *et al.,* 1988) and those of *Prunus amygdalus* cv. 'Ferragnes' could not be maintained unless 0.2% casamino acids was added to the medium (Rugini and Verma, 1982). Molnar (1988b) found that the growth of *Brassica nigra* cell suspensions was improved by adding 1-4 g/l CH or a mixture of 4 mM alanine, 4 mM glutamine and 1 mM glutamic acid. In this case the medium contained MS salts (but less iron) and B5 vitamins.

Amino acid supplements have also been used to boost the rate of growth of callus cultures. For instance, Short and Torrey (1972) added 5 amino acids and urea to a medium containing MS salts for the culture of pea root callus, and Sandstedt and Skoog (1960) found that aspartic and glutamic acids promoted the growth of tobacco callus as much as a mixture of several amino acids (such as found in yeast extract). Glutamic acid seemed to be primarily responsible for the growth promotion of sweet clover

callus caused by casein hydrolysate on a medium containing 26.6 mM NO_3^-, 12.5 mM NH_4^+ and 2.0 mM PO_4^{3-} (Taira *et al.,* 1977).

Amino acids are often added to media for protoplast culture. It was essential to add 2 mM glutamine and 2 mM asparagine to a medium containing MS salts, to obtain cell division, colony growth and plantlet differentiation from *Trigonella* protoplasts (Shekhawat and Galston, 1983).

Shoot cultures. Many shoot cultures are grown on MS medium containing glycine, although in most cases the amino acid is probably not an essential ingredient. Usually it is unnecessary to add amino acids to media supporting shoot cultures, but methionine may represent a special case. Druart (1988) found that adding 50-100 mg/l L-methionine to the medium seemed to stimulate cytokinin activity and caused cultures of *Prunus glandulosa* var. *sinensis* to have high propagation rates through several subcultures. This promotive effect of L-methionine was thought to be due to it acting as a precursor of ethylene (see Chapter 7). Glutamine inhibited the growth of apical domes excised from *Coleus blumei* shoots (Smith, 1981) and 50-100 mg/l glutamic acid inhibited shoot growth, the formation of axillary buds and shoot proliferation in cultures of woody plants (Druart, loc. cit.).

L-Citrulline is an important intermediate in nitrogen metabolism in the genus *Alnus*. The addition of 1.66 mM (4.99 mM NH_2) to WPM medium improved the growth of *A. cordata* and *A. subcordata* shoot cultures (Cremiere *et al.,* 1987).

Contaminants grow more rapidly on media containing amino acids. Casein hydrolysate is therefore sometimes added to the media for Stage I shoot cultures so that infected explants can be rejected quickly (Schulze, 1988). The health of shoots grown from seedling shoot tips of *Feijoa* (*Acca) sellowiana* was improved when 500 mg/l CH was added to Boxus (1974) medium (which does not contain ammonium ions) (Bhojwani et al., 1987).

Organogenesis. The presence of amino acids can enhance morphogenesis, either when they provide the only source of reduced nitrogen, or when they are used as a supplement to a medium containing both NO_3^- and NH_4^+. In a medium containing 25 mM nitrate, but no NH_4^+, direct adventitious shoot formation on cauliflower peduncle explants was induced by the addition of a mixture of the amino acids asparagine, proline, tyrosine and phenylalanine, each at a concentration of only 0.1 mM. (Margara, 1969b). A high rate of adventitious shoot regeneration and embryogenesis, from *Beta vulgaris* petioles or petiole callus, was achieved on a medium comprised of several amino acids and a complex vitamin mixture with MS salts (Freytag *et al.,* 1988). The addition of CH to MS medium was found to be essential for shoot formation from callus (Chand and Roy, 1981).

Adding only 1-10 mg/l of either L-leucine or L-isoleucine to Gamborg *et al.,* (1968) B5 medium, decreased callus growth of *Brassica oleracea* var *capitata*, but increased adventitious shoot formation. Basu *et al.,* (1989) thought that this might be due to these amino acids being negative effectors of threonine deaminase (TD) enzyme, the activity of which was diminished in their presence. Threonine, methionine and pyruvic acid, which increased callus growth in this species, enhanced TD activity.

There are several examples of the amino acid L-asparagine being able to stimulate morphogenesis. This may be because it too can be a precursor of ethylene (Durzan, 1982), the biosynthesis of which may be increased by greater substrate availability. Kamada and Harada (1977) found that the addition of 5 mM L-asparagine stimulated both callus and bud formation in stem segments of *Torenia fournieri*, while alanine (and, to a lesser extent, glutamic acid) increased flower bud formation from *Torenia* internode segments when both an auxin and a cytokinin were present. An increase in the number of adventitious buds formed on the cotyledons and hypocotyl of *Chamaecyparis obtusa* seedlings occurred when 1.37 mM glutamine and 1.51 mM asparagine were added together to Campbell and Durzan (1975) medium, but not when they were supplied on their own (Ishii, 1986). L-asparagine was also added to MS medium by Green and Phillips (1975), to obtain plant regeneration from tissue cultures of maize; adding it to Finer and Nagasawa (1988) 10A4ON medium caused there to be more embryogenic clumps in *Glycine max* suspension cultures (Finer and Nagasawa, 1988).

Amino acid additions do not invariably enhance morphogenesis. Supplementing Linsmaier and Skoog (1965) medium with 0.5-5 mM glutamine, caused callus of *Zamia latifolia* to show greatly decreased organogenesis (Webb and Rivera, 1981) and ammending Linsmaier and Skoog (1965) medium with 100 mg/l CH, prevented adventitious shoot formation from stem internode callus of apple and cherry rootstocks (James *et al.,* 1984).

Embryogenesis. The presence the ammonium ion is usually sufficient for the induction of

embryogenesis in callus or suspension cultures containing NO_3^-, but on media where NH_4^+ is lacking [e.g. White (1954)], casein hydrolysate, or an amino acid such as alanine, or glutamine, is often promotory (Ranga Swamy, 1958; Ammirato and Steward, 1971; Street, 1979). For embryogenesis in carrot cultures, Wetherell and Dougall (1976) have shown that in a medium containing potassium nitrate, reduced nitrogen in the form of ammonium chloride matched the effectiveness of an equivalent concentration of nitrogen from casein hydrolysate. Casein hydrolysate could be replaced by glutamine, glutamic acid, urea or alanine. Suspensions of wild carrot cells grew and produced somatic embryos on a medium containing either glutamine or CH as the sole nitrogen source (Anderson, 1976).

There have also been many reports of embryogenesis being promoted by the addition of casein hydrolysate, or one or more specific amino acids, when both NO_3^- and NH_4^+ were available in the medium. Some examples are given in Table 3.7. In many cases, embryogenic callus and/or embryo formation did not occur without the presence of the amino acid source, suggesting that without amino acid, the medium was deficient in NH_4^+ or total nitrogen. Armstrong and Green (1985) found that the frequency of friable callus and somatic embryo formation from immature embryos of *Zea mays* increased almost linearly with the addition of up to 25 mM proline to Chu *et al.,* (1975) N6 medium

[total N, 34.99 mM; NO_3/NH_4 ratio, 3.99],

but there was no benefit from adding proline to MS medium (containing 150 mg/l asparagine hydrate).

[total N, 60.01 mM; NO_3/NH_4 ratio, 1.91]

The growth of somatic embryos can also be affected by the availability of reduced nitrogen. That of *Coronilla varia* embryos was poor on Gamborg *et al.,* (1968) B5 medium (Total N 26.74 mM; NH_4^+ 2.02 mM), unless 10 mM asparagine or 20 mM NH_4Cl was added to the medium, or unless the embryos were moved to Saunders and Bingham (1972) BOi2Y medium, which has 37.81 mM total inorganic N, 12.49 mM NH_4^+ and 2000 mg/l casein hydrolysate (approx. 9.9 mM NH_4^+ equivalence) (Moyer and Gustine, 1984). However, the germination of *Triticum aestivum* somatic embryos was completely prevented by adding 800 mg/l CH to MS medium (Ozias Akins and Vasil, 1982; Carman *et al.,* 1988).

Culture of immature cotyledons. Young storage cotyledons isolated from immature zygotic embryos accumulate protein efficiently when cultured with amino acids in a medium without nitrate and ammonium ions. A medium such as that of Millerd *et al.* (1975), or of Thompson *et al.,* (1977) is normally used, but where the effect of different amino acids on protein assimilation is being studied, the amino acid content of the medium is varied. Glutamine is often found to be the most efficient nitrogen source for this purpose (Thompson *et al.,* 1977; Haga and Sodek, 1987), but protein increase from culture with asparagine and glutamate (glutamic acid) is usually also significant (Lea *et al.,* 1979).

2.1.14. Causes of the stimulatory effect of amino acids

We may conclude therefore, that for many cultural purposes, amino acids are not essential media components; but their addition as identified pure compounds, or more cheaply through casein hydrolysates, can be an easy way of ensuring against medium deficiency, or of providing a source of nitrogen that is immediately available to cultured cells or tissues. An observation by Murashige and Skoog (1962) that the presence of casein hydrolysate allowed vigorous organ development over a broader range of IAA and kinetin levels, may be of significance.

In gram moles per litre, amino acids can be a much more efficient source of reduced nitrogen than ammonium compounds. For instance, the mixture of amino acids provided by 400 mg/l of casein hydrolysate (containing at most as much reduced nitrogen as 3.3 mM NH_4^+) was as effective as 14.95 mM NH_4Cl in stimulating the division of protoplast-derived cells of *Antirrhinum* (Poirier-Hamon *et al.,* 1974).

Why should this be, and why can additions of amino acids (sometimes in comparatively small amounts) stimulate growth or morphogenesis when added to media, which already contain large amounts of NH_4^+? Some hypotheses, which have been advanced are:

- **Conservation of ATP - alleviating phosphate deficiency.** Durzan (1982) pointed out that when plant tissues take up the ammonium ion, they consume adenosine tri-phosphate (ATP) in converting it to amino acids. If suitable amino acids are available from the medium, some of ATP may be conserved. Bister-Miel *et al.,* (1985) noted that CH promoted growth in cultures where phosphate became growth-limiting. They suggested that amino acids compensated for phosphate deficiency. With the plant well supplied with amino acids, some of the phosphate, which is normally used for ATP production can be diverted to other uses. Several

Table 3.7 Some examples of the promotion of embryogenesis by amino acids in media containing NO_3^- and NH_4^+

	Type of culture	Basal medium used	Amino acid supplements	Reference
Aesculus hippocastrum	Zygotic embryo callus	MS	CH (250 mg/l) + Proline (250 mg/l)	Radojevic (1988)
Dactylis glomerata	Suspension-derived callus	Schenk and Hildebrandt (1972)	CH (1.5 g/l)	Gray *et al.* (1984)
Daucus carota	Hypocotyl callus	Gamborg *et al.* (1968) B5	Proline (100 mM) + Serine (100 mM)	Nuti Ronchi *et al.* (1984)
Dioscorea rotundata	Zygotic embryo callus	MS	CH (1 g/l)	Osifo (1988)
Glycine max	Suspension	Kartha *et al.* (1974a)	L-asparagine (5 mM)	Finer and Nagasawa (1988)
Gossypium klotzschianum	Suspension	Gamborg *et al.* (1968) B5	Glutamine (10 mM)	Price and Smith (1979a,b)
Larix decidua	Gametophyte callus	Litvay *et al.* (1981) LM	CH (1 g/l) + Glutamine (500 mg/l)	Nagmani and Bonga (1985)
Nigella sativa	Roots or leaf callus	MS	CH (100 – 500 mg/l)	Bannerjee and Gupta (1976)
Trigonella foenum-graecum	Leaf callus	MS	CH (50 mg/l) (500 mg/l was inhibitory)	Gupta *et al.* (1987)
Triticum aestivum	Anther	Chu and Hill (1988) MN6	Serine, proline, arinine, aspartic acid and alanine (each at 40 mg/l) + glutamine (400 mg/l)	Chu and Hill (1988)
Vitis vinifera	Anther	½ MS	CH (250 mg/l)	Mauro *et al.* (1986);
Zea mays	Zygotic embryo callus	Chu *et al.* (1975) N6	Proline (20-25 mM)	Kamo *et al.* (1985) Armstrong and Green (1985)

authors have pointed out that CH itself is also a source of phosphate. For example, Bridson (1978) and some chemical catalogues, show that some casein hydrolysates normally contain about 1.3g P_2O_5 per 100g. The addition of 2 g/l of CH will therefore increase the phosphate content of MS medium by 11% and that of White (1954) medium by 44% (assuming complete phosphate availability).

- **A capacity to act as chelating agents.** Some amino acids can act as chelating agents (see later in section on chelates)
- **Enhanced nitrogen assimilation.** Glutamine and glutamic acid are directly involved in the assimilation of NH_4^+. A direct supply of these amino acids should therefore enhance the utilization of both nitrate and ammonium nitrogen and its conversion into amino acids.
- **A replacement for toxic ammonium ions.** Certain plant tissues are particularly sensitive to NH_4^+. Ochatt and Caso (1986) and Ochatt and Power (1988a, b) found that protoplasts of *Pyrus* spp. would not tolerate the ion, and that to obtain sustained cell division it was necessary to eliminate it from MS medium, and use 50 mg/l casein hydrolysate as a source of reduced nitrogen. CH can however be extremely toxic to freshly isolated protoplasts of some species and varieties of plants (Ranch and Widholm, 1980; Russell and McCown, 1988). Conifer tissues too are unable to cope with high

concentrations of NH_4^+, but cultures can be supplied with equivalent levels of reduced nitrogen in the form of amino acids without the occurrence of toxicity (Durzan, 1982). In soybean suspension cultures, the high level of ammonium in MS medium has been shown to inhibit isocitrate dehydrogenase (a Krebs' cycle enzyme) and glutamine synthetase, which contribute to the conversion of NH_4^+ to glutamine (Gamborg and Shyluk, 1970).

- **Adjustment of intracellular pH.** As intracellular pH is important for the activation of sea urchin eggs, and amino acids can promote embryogenesis, Nuti Ronchi *et al.,* (1984) speculated that the uptake and assimilation of amino acids might help to regulate cellular pH in plants.

As mentioned before, there is commonly a minimum inoculation density below which growth cannot be initiated *in vitro*. This minimum varies according to both the source of the cells and the nature of the medium. It can usually be lowered by employing a 'conditioned' medium (i.e. a fresh medium into which the products of another medium in which cells are actively growing, have been added). Alternatively, initial growth at low densities can be supported by the close presence of other actively growing plant cells ('nurse cultures'). Compounds responsible for this effect must be freely diffusible from living cells and could include growth substances, reducing sugars, vitamins and amino acids. Addition of such supplements has been found to overcome the inhibited growth of some cells at low densities (Kao and Michayluk, 1975).

2.2. PHOSPHATE

Phosphorus is a vital element in plant biochemistry. It occurs in numerous macromolecules such as nucleic acids, phospholipids and co-enzymes. It functions in energy transfer via the pyrophophate bond in ATP. Phosphate groups attached to different sugars provide energy in respiration and photosynthesis and phosphate bound to proteins regulates their activity. Phosphorus is absorbed into plants in the form of the primary or secondary orthophosphate anions $H_2PO_4^-$ and HPO_4^{2-} by an active process, which requires the expenditure of respiratory energy. Phosphate, in contrast to nitrate and sulphate, is not reduced in plants, but remains in the highly oxidized form. It is used in plants as the fully oxidized orthophosphate (PO_4^{3-}) form.

In culture media the element is provided as soluble potassium mono- and di-hydrogen phosphates. The di- and mono-valent phosphate anions respectively provided by these chemicals are interconvertible in solution depending on pH. Monovalent $H_2PO_4^-$ predominates at pH values below 7, characteristic of most tissue culture media, and it is this ion, which is most readily absorbed into plants (Devlin, 1975). Conversion of $H_2PO_4^-$ into divalent HPO_4^{2-} begins to occur as solutions become more alkaline. The divalent ion is said to be only sparingly available to plants but Hagen and Hopkins (1955) and Jacobsen *et al.,* (1958) thought that its absorption could be significant, because even though the ion is normally at a relatively low concentration in nutrient solutions, its affinity with the site of absorption is greater than that of the mono-valent form. Trivalent PO_4^{3-}, which appears in alkaline solutions, is not generally absorbed by plants.

In some early tissue culture media, all (e.g. Bouharmont, 1961), or part (e.g. Vacin and Went, 1949) of the phosphorus was supplied as sparingly-soluble phosphates. A slow rate of phosphorus availability seems to be possible from such compounds. The optimum rate of uptake of phosphate (HPO_4^{2-}) into cultured *Petunia* cells occurred at pH 4 (Chin and Miller, 1982) but Zink and Veliky (1979) did not observe any decline in the absorption of phosphate by *Ipomoea* suspension cultures at pH 6.5, when HPO_4^{2-} and $H_2PO_4^-$ were present in approximately equal concentrations. Plant tissue cultures secrete phosphatase enzymes into the medium (Ciarrocchi *et al.,* 1981), which could release phosphate ions from organic phosphates.

In the cytoplasm, phosphate is maintained at a constant concentration of 5-10 mM, more or less independent of the external concentration. Phosphate in the vacuole fluctuates according to the external concentration but does not increase above 25 mM (Schachtman *et al.,* 1998). When there is a high supply of phosphate and it is taken up at rates that exceed the demand, a number of processes act to prevent toxic phosphate concentrations, among others storage into inorganic compounds such as phytic acid. High concentrations of dissolved phosphate can depress growth, possibly because calcium and some microelements are precipitated from solution and/or their uptake reduced. In *Arabidopsis thaliana*, four different phophate transporter genes have been isolated (*APT1-4*). *In vivo*, the genes are predominantly expressed in the roots and their expression is constitutive or induced by phosphate starvation. Overexpression of *APT1* gene in tobacco cell cultures increased the rate of phosphate uptake (Mitsukawa *et al.,* 1997).

Although the concentration of phosphate introduced into plant culture media has been as high as 19.8 mM, the average level is 1.7 mM and most media contain about 1.3 mM. However many reports indicate that such typical levels may be too low for some purposes. When phosphate is depleted from MS medium, there is an increase in free amino acids in *Catharanthus roseus* cells, because protein synthesis has ceased and degradation of proteins is occurring (Ukaji and Ashihara, 1987). Phosphate (starting concentration 2.64 mM) and sucrose were the only nutrients completely depleted in *Catharanthus roseus* batch suspension cultures, and the period of growth could be prolonged by increasing the levels of both (MacCarthy *et al.,* 1980). MS medium contains only 1.25 mM phosphate which may be insufficient for suspension cultures of some plants. The phosphate in MS medium is insufficient for *Cardamine pratensis* suspension cultures, all having been absorbed in 5 days: it is however adequate for *Silene alba* suspensions (Bister-Miel *et al.,* 1985).

The phosphate in MS medium is also inadequate for static cultures of some plants, or where a large amount of tissue or organs are supported on a small amount of medium (for example where many separate shoots are explanted together in a static shoot culture). The concentration of the ion is then likely to be reduced almost to zero over several weeks (Barroso *et al.,* 1985; Singha *et al.,* 1987; Lumsden *et al.,*1990). Insufficient levels of phosphate were present from MS during culture of *Hemerocallis*, *Iris* and *Delphinium* (Leiffert *et al.,* 1995). Although growth can continue for a short while after the medium is depleted of phosphate, for some purposes it has been found to be beneficial to increase the phosphate concentration of MS to 1.86 mM (Jones and Murashige, 1974), 2.48 mM (Murashige *et al.,* 1972; Murashige, 1974; Jakobek *et al.,* 1986), 3.1 mM (Miller and Murashige, 1976) or 3.71 mM (Thorpe and Murashige, 1968, 1970), for example, to induce adventitious shoot formation from callus, or to increase the rate of shoot multiplication in shoot cultures. It should be noted that there is *in vivo* a significant retranslocation of phosphate from older leaves to the growing shoot (Schachtman *et al.,* 1998). Retranslocation also occurs in tissue culture. In *Dahlia* culture in liquid medium, phosphate is almost completely taken up after 2 weeks (Fig. 3.4a). In spite of this, the concentration in tissues formed after the exhaustion is 'normal' (Fig. 3.4b). The depletion of phosphate early during culture has also a major effect on the pH of tissue culture media in which added phosphate is the major buffering component. When phosphate levels are increased to obtain a more rapid rate of growth of a culture, it can be advisable to investigate the simultaneous enhancement of the level of *myo*-inositol in the medium

2.3. POTASSIUM

Potassium is the major cation (positive ion) within plants reaching in the cytoplasm and chloroplasts concentrations of 100 – 200 mM. The biphasic uptake kinetics suggest two uptake systems: a high-affinity and a low-affinity one. K^+ is not metabolised. It contributes significantly to the osmotic potential of cells. K^+ counterbalances the negative charge of inorganic and organic anions. It functions in cell extension through the regulation of turgor, it has a major role in stomatal movements and functions in long-distance nutrient flow. Potassium ions are transported quickly across cell membranes and two of their major roles are regulating the pH and osmotic environment within cells. Potassium, calcium, sodium and chloride ions conserve their electrical charges within the plant, unlike the cation NH_4^+ and the anions NO_3^-, SO_4^{2-}, and $H_2PO_4^-$, which are rapidly incorporated into organic molecules. In intact plants, potassium ions are thought to cycle. They move, associated with cations (particularly NO_3^-), upwards from the roots in the xylem. As nitrate is reduced to ammonia and assimilated, carboxylic acid ions (RCO_3^-, malate) are produced. These become associated with the released K^+ ions and are transported in the phloem to the roots, where they are decarboxylated, releasing K^+ for further anion transport (Ben-Zioni *et al.,* 1971). Carboxylate transported to the roots gives rise to OH^- ion, which is excreted into the soil (or medium) to counterbalance NO_3^- uptake (Touraine *et al.,* 1988). Potassium ions will clearly have a similar role in cultured tissues, but obvious transport mechanisms will usually be absent.

Many proteins show a high specificity for potassium which, acting as a cofactor, alters their configuration so that they become active enzymes. Potassium ions also neutralise organic anions produced in the cytoplasm, and so stabilise the pH and osmotic potential of the cell. In whole plants, deficiency of potassium results in loss of cell turgor, flaccid tissues and an increased susceptibility to drought, salinity, frost damage and fungal attack. A high potassium to calcium ratio is said to be

characteristic of the juvenile stage in woody plants (Boulay, 1987). Potassium deficiency in plant culture media is said to lead to hyperhydricity (Pasqualetto *et al.,* 1988), and a decrease in the rate of absorption of phosphate (Chin and Miller, 1982). However quite wide variations in the potassium content of MS medium had little effect on the growth or proliferation of cultured peach shoots (Loreti *et al.,* 1988).

Lavee and Hoffman (1971) reported that the optimum rate of callus growth of two apple clones was achieved in a medium containing 3.5 mM K^+: when the concentration was much higher than this, or when it was less than 1.4 mM, the callus grew less vigorously. However, the growth rate of wild carrot suspensions was said by Brown *et al.,* (1976) to be at, or near, the maximum when K^+ concentration was 1 mM: for embryogenesis 10-50 mM K^+ was required. Uptake of potassium into plants is reduced in the absence of calcium (Devlin, 1975).

Within a large sample of different macronutrient compositions, it is found that authors have tended to relate the concentration of potassium to the level of nitrate. This is correlated with a coefficient of 0.78, $P<0.001$ (George *et al.,* 1988). The average concentration of potassium in these media was 13.6 mM and the most common value (median), 10.5 mM. Murashige and Skoog (1962) medium contains 20.04 mM K^+.

2.4. SODIUM

Sodium ions (Na^+) are taken up into plants, but in most cases they are not required for growth and development and many plants actively secrete them from their roots to maintain a low internal concentration. The element can function as an osmotic stabilizer in halophytic plants; these have become adapted so that, in saline soils with low water potential, they can accumulate abnormally high concentrations of Na^+ ion in vacuoles, and thereby maintain sufficient turgor for growth.

Sodium does appear to have a beneficial nutritional effect on some plants and is therefore considered as a functional element (Subbarao *et al.,* 2003). Small amounts of sodium chloride (e.g. 230 mg/l) can stimulate the growth of plants in the families Chenopodiaceae and Compositae even when there is no limitation on the availability of K^+ (Brownell, 1979). In other plants such as wheat, oats, cotton and cauliflower (Sharma and Singh, 1990), sodium can partially replace potassium, but is not essential.

Sodium only appears to be essential to those salt-tolerant plants, which have a C4 (crassulacean acid) metabolism. Examples are *Bryophyllum tubiflorum* (Crassulaceae) and *Mesembryanthemum crystallimum* (Aizoaceae). In these plants the element is necessary for CO_2 fixation in photosynthesis.

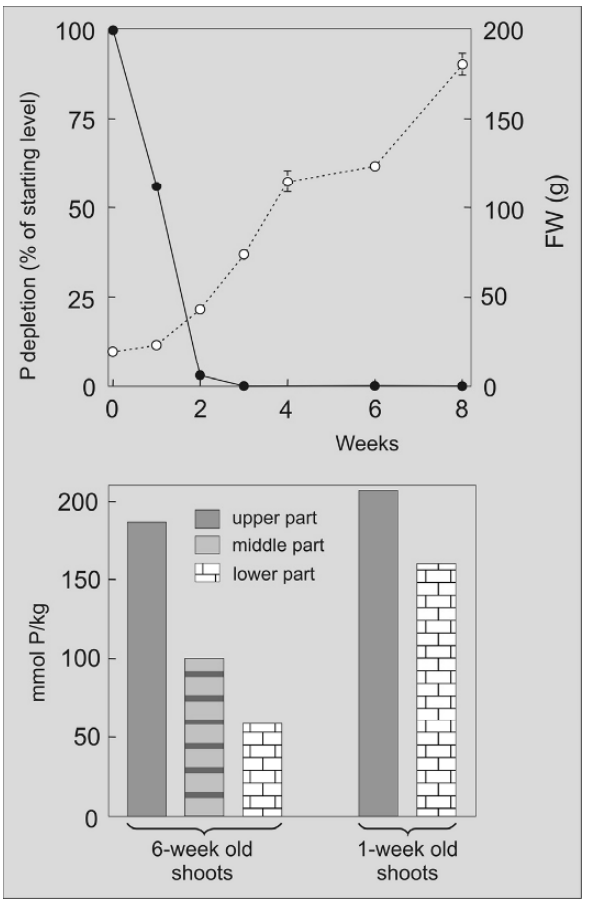


Fig. 3.4 Top: Depletion of P in the medium compared to the growth of *Dahlia* cultures.
Bottom (*Right*) P content in 1-week old *Dahlia* shoots taken from the culture after 1 week when P had not yet been exhausted. (*Left*) 6-week old shoots in which the upper part had been formed after all P had been taken up from the medium. The high content in the newly formed upper part of the shoots indicates massive retranslocation of P after uptake from older to newer tissue (G. de Klerk, unpub. data).

Most macronutrient formulations do not contain any sodium at all, and the average concentration in 615 different preparations was 1.9 mM (George *et al.,* 1988). Even if the element is not deliberately added as a macronutrient, small amounts are incorporated in most media from the salts added to provide micronutrients. Plant macronutrient preparations

containing high concentrations of both sodium and chloride ions are not well formulated.

2.5. MAGNESIUM

Magnesium is an essential component of the chlorophyll molecule and is also required non-specifically for the activity of many enzymes, especially those involved in the transfer of phosphate. ATP synthesis has an absolute requirement for magnesium and it is a bridging element in the aggregation of ribosome subunits. Magnesium is the central atom in the porphyrin structure of the chlorophyll molecule. Within plants, the magnesium ion is mobile and diffuses freely and thus, like potassium, serves as a cation balancing and neutralising anions and organic acids. Macklon and Sim (1976) estimated there to be 2.1 mM Mg^{2+} in the cytoplasm of *Allium cepa* roots while McClendon (1976) put the general cytoplasmic requirement of plants as high as 16 mM. Plant culture media invariably contain relatively low concentrations of magnesium (average 6.8 mM, median 5.3 mM). Very often $MgSO_4$ is used as the unique source of both magnesium and sulphate ions.

Walker and Sato (1981) found there to be a large reduction in the number of somatic embryos formed from *Medicago sativa* callus when Mg^{2+} was omitted from the medium. In sympathy with this finding, Kintzios *et al.,* (2004) observed in tissue culture of melon that the highest level of magnesium occurred in direct somatic embryogenic cultures and the lowest level in callus cultures.

2.6. SULPHUR

The sulphur utilised by plants is mainly absorbed as SO_4^{2-}, which is the usual source of the element in plant culture media. Uptake is coupled to nitrogen assimilation (Reuveny *et al.,* 1980), and is said to be independent of pH. It results in the excretion of OH^- ions by the plant, making the medium more alkaline. However, according to Mengel and Kirkby (1982), plants are relatively insensitive to high sulphate levels and only when the concentration is in the region of 50 mM, is growth adversely affected. Although sulphur is mainly absorbed by plants in the oxidized form, that which is incorporated into chemical compounds is mainly as reduced -SH, -S- or -S-S- groups. The sulphur-containing amino acids cysteine and methionine become incorporated into proteins. Sulphur is used by plants in lipid synthesis and in regulating the structure of proteins through the formation of S-S bridges. The element also acts as a ligand joining ions of iron, zinc and copper to metalloproteins and enzymes. The reactive sites of some enzymes are -SH groups. Sulphur is therefore an essential element and deficiency results in a lack of protein synthesis. Sulphur-deficient plants are rigid, brittle and thin-stemmed. Important sulphur compounds are glutathione, which acts in detoxification of oxygen radicals, and the proteins thioredoxin and ferrodoxin that are involved in redox chemistry.

Growth and protein synthesis in tobacco cell suspensions were reduced on a medium containing only 0.6 mM SO_4^{2-} instead of 1.73 mM (Klapheck *et al.,* 1982) and when the supply of S in the medium was used up, large amounts of soluble nitrogen accumulated in the cells. Most media contain from 2-5 meq/l SO_4^{2-} (1 – 2.5 mM).

2.7. CALCIUM

As a major cation, calcium helps to balance anions within the plant, but unlike potassium and magnesium, it is not readily mobile. Because of its capacity to link biological molecules together with coordinate bonds, the element is involved in the structure and physiological properties of cell membranes and the middle lamella of cell walls. The enzyme β-(1→3)-glucan synthase depends on calcium ions, and cellulose synthesis by cultured cells does not occur unless there are at least micro-molar quantities of Ca^{2+} in the medium. Many other plant enzymes are also calcium-dependent and calcium is a cofactor in the enzymes responsible for the hydrolysis of ATP.

Although calcium can be present in millimolar concentrations within the plant as a whole, calcium ions are pumped out of the cytoplasm of cells. Ca^{2+} is sequestered in the vacuole, complexes with calcium-binding proteins and may precipitate into calcium oxalate crystals to maintain the concentration at around only 0.1 mM. The active removal of Ca^{2+} is necessary to prevent the precipitation of phosphate (and the consequent disruption of phosphate-dependent metabolism) and interference with the function of Mg^{2+}. The uniquely low intra-cellular concentration of Ca^{2+} allows plants to use calcium as a chemical 'second messenger' (Hepler and Wayne, 1985; Sanders *et al.,* 1999). Regulatory mechanisms are initiated when Ca^{2+} binds with the protein calmodulin, which is thus enabled to modify enzyme activities. A temporary increase in Ca^{2+}concentration to 1 or 10 mM does not significantly alter the ionic environment within the cell, but is yet sufficient to

trigger fundamental cell processes such as polarized growth (for example that of embryos - Shelton *et al.,* 1981), response to gravity and plant growth substances, cytoplasmic streaming, and mitosis (Ferguson and Drbak, 1988; Poovaiah, 1988). Physiological and developmental processes, which are initiated through the action of phytochrome are also dependent on the presence of Ca^{2+} (Shacklock *et al.,* 1992). A short-term increase in cytosolic free Ca^{2+} has been observed for osmoadaptation (Taylor *et al.,* 1996), phytoalexin synthesis (Knight *et al.,* 1991), thermotolerance (Gong *et al.,* 1998) and induction of free-radical scavengers (Price *et al.,* 1994).

Large quantities of calcium can be deposited outside the protoplast, in cell vacuoles and in cell walls. Calcification strengthens plant cell walls and is thought to increase the resistance of a plant to infection. By forming insoluble salts with organic acids, calcium immobilises some potentially damaging by-products. The element gives protection against the effects of heavy metals and conveys some resistance to excessively saline conditions and low pH.

The Ca^{2+} ion is involved in *in vitro* morphogenesis and is required for many of the responses induced by plant growth substances, particularly auxins and cytokinins. In the moss *Funaria*, cytokinin causes an increase in membrane-associated Ca^{2+} specifically in those areas which are undergoing differentiation to become a bud (Saunders and Hepler, 1981). Protocorm formation from callus of *Dendrobium fibriatum* was poor on Mitra *et al.,* (1976) A medium when calcium was omitted (Mitra *et al.,* 1976) and in *Torenia* stem segments, adventitious bud formation induced by cytokinin seems to be mediated, at least in part, by an increase in the level of Ca^{2+} within cells (Tanimoto and Harada, 1986). Exogenous Ca^{2+} enhanced the formation of meristemoids and the first phases of outgrowth into organs in tobacco pith explants (Capitani and Altamura, 2004). In carrot, somatic embryogenesis coincides with a rise of free cytosolic Ca^{2+} (Timmers *et al.,* 1996) and applied Ca^{2+} increases the number of somatic embryos (Jansen *et al.,* 1990).

2.7.1. Shoot tip necrosis

Calcium deficiency in plants results in poor root growth and in the blackening and curling of the margins of apical leaves, often followed by a cessation of growth and death of the shoot tip. The latter symptoms are similar to aluminium toxicity (Wyn Jones and Hunt, 1967). Tip necrosis has been especially observed in shoot cultures, sometimes associated with hyperhydricity. It often occurs after several subcultures have been accomplished (e.g. in *Cercis canadensis* - Yusnita *et al.,* 1990). After death of the tip, shoots often produce lateral branches, and in extreme cases the tips of these will also die and branch again. The cause of tip necrosis has not always been determined [e.g. in *Pistacia* shoot cultures (Barghchi, 1986), where shoots showing symptoms may die after planting out (Martinelli, 1988)]. The occurrence of necrosis was reduced in *Pistacia* (Barghchi *loc. cit*.) and *Prunus tenella* (Alderson *et al.,* 1987) by more frequent subculturing, but this is a costly and time-consuming practice. In *Pictacia*, calcium reduced necrosis (Barghchi and Alderson, 1996).

Tip necrosis was found in *Psidium guajava* shoot cultures after prolonged subculturing, if shoots were allowed to grow longer than 3 cm, and was common in rapidly growing cultures (Amin and Jaiswal, 1988); it occurred on *Sequoiadendron giganteum* shoots only when they were grown on relatively dilute media (Monteuuis *et al.,* 1987). Necrosis of *Rosa hybrida* 'White Dream', was cured by adding 0.1 mg/l GA_3 to the medium (Valle and Boxus, 1987).

Analysis of necrotic apices has shown them to be deficient in calcium (Debergh, 1988), and a shortage of this element has been associated with tip necrosis in *Amelanchier*, *Betula*, *Populus*, *Sequoia*, *Ulmus, Cydonia* and other woody plants, although the extent of damage is variable even between genotypes within a species (Sha *et al.,* 1985; Singha *et al.,* 1990). As calcium is not remobilised within plant tissues, actively growing shoots need a constant fresh supply of ions in the transpiration stream. An inadequate supply of calcium can result from limited uptake of the ion, and inadequate transport, the latter being caused by the absence of transpiration due to the high humidity in the culture vessel. A remedy can sometimes be obtained by reducing the culture temperature so that the rate of shoot growth matches calcium supply, using vessels which promote better gas exchange (thereby increasing the transpiration and xylem transport), or by increasing the concentration of calcium in the medium (McCown and Sellmar, 1987). The last two remedies can have drawbacks: the medium will dry out if there is too free gas exchange; adding extra calcium ions to the medium is not always effective (e.g. in cultures of *Castanea sativa* - Mullins, 1987); and can introduce

undesirable anions. Chloride toxicity can result if too much calcium chloride is added to the medium (see below). To solve this difficulty, McCown *et al.,* (Zeldin and McCown, 1986; Russell and McCown, 1988) added 6 mM calcium gluconate to Lloyd and McCown (1981) WPM medium to correct Ca^{2+} deficiency, without altering the concentrations of the customary anions. There is a limit to the concentration of calcium, which can be employed in tissue culture media because several of its salts have limited solubility.

2.8. CHLORIDE

The chloride ion (Cl^-) has been found to be essential for plant growth (Broyer *et al.,* 1954; Johnson *et al.,* 1957; Ozanne *et al.,* 1957; Ozanne, 1958), but seems to be involved in few biological reactions and only very small quantities are really necessary. Rains (1976) listed chlorine as a micronutrient. Chloride is required for the water-splitting protein complex of Photosystem II, and it can function in osmoregulation in particular in stomatal guard cells. The chloride ion is freely transported and many plants can tolerate the presence of high concentrations without showing toxicity. The chief role of chloride seems to be in the maintenance of turgor and in balancing rapid changes in the level of free cations such as K^+, Mg^{2+} and Na^+. Plants deprived of Cl^- are liable to wilting (Johnson *et al.,* 1957).

In isolated chloroplasts, chloride (together with Mn^{2+}) ions are required for oxygen evolution in photosystem II of photosynthesis (Bov *et al.,* 1963; Mengel and Kirkby, 1982; Shkolnik, 1984), although there has been some doubt whether this requirement exists *in vivo* (Terry, 1977). Chloride ions are best taken into plants at slightly acid pH (Jacobson *et al.,* 1971).

The most common concentration of chloride in culture media is 3 mM, the average 6 mM. MS medium contains 6 mM Cl^-; Quoirin and Lepoivre (1977) medium, only 0.123 μM. Some species are sensitive to chloride ions. McCown and Sellmer (1987) reported that too high a concentration, seemed to cause woody species to have yellow leaves and weak stems: sometimes tissues collapsed and died. An excess of Cl^- has been thought to be one cause of the induction of hyperhydricity, and omission of the ion does seem to prevent the development of these symptoms in *Prunus* (Volume 2). Pevalek-Kozlina and Jelaska (1987) deliberately omitted chloride ions from WPM medium for the shoot culture of *Prunus avium* and obtained infrequent hyperhydricity in only one genotype. The presence of 7 mM Cl^- can be toxic to pine suspension cultures (Teasdale, 1987).

As chlorine has only a relatively small nutritional significance, steps are sometimes taken to reduce the concentration of chloride ion in culture media, but in order to adjust the concentration of other ions, it is then often necessary to make a marked increase in SO_4^{2-}. For example, using ammonium sulphate instead of ammonium chloride to supply NH_4^+ in Eeuwens (1976) Y3 medium, would increase the sulphate level from 2 to 12 meq/l (from 1 to 6 mM).

3. MICRONUTRIENTS

Plant requirements for microelements have only been elucidated over the past 50-60 years. Before the end of the last century, it had been realised that too little iron caused chlorophyll deficiency in plants, but the importance of other elements took many years to prove conclusively. Mazé, for example, used hydroponic techniques during the years 1914-1919 to show that zinc, manganese and boron improved the growth of maize plants. Sommer and Lipman (1926) also showed the essentiality of boron, and Sommer (1931) of copper, but uncertainty over which elements were really indispensible to growth still existed in 1933 when Hoagland and Snyder proposed two supplementary nutrient solutions for water culture which in total contained 26 elements. It took several further years to prove that molybdenum (Arnon and Stout, 1939) and cobalt in very small amounts, were most important for healthy plant growth. Early plant tissue culture work was to both profit from, and contribute to the findings of previous hydroponic studies. Our understanding has been enhanced by investigations into the biochemical role of minor elements.

3.1. EARLY USE IN PLANT TISSUE CULTURE MEDIA

At the time of the early plant tissue culture experiments, uncertainty still existed over the nature of the essential microelements. Many tissues were undoubtedly grown successfully because they were cultured on media prepared from impure chemicals (see below) or solidified with agar, which acted as a micronutrient source.

In the first instance, the advantage of adding various micronutrients to culture media was mainly evaluated by the capability of individual elements to improve the growth of undifferentiated callus or isolated root cultures. Knudson (1922) incorporated Fe and Mn in his very successful media for the non-symbiotic germination of orchid seeds, and, following a recommendation by Berthelot (1934), Gautheret (1939) and Nobécourt (1937) included in their media (in addition to iron) copper, cobalt, nickel, titanium and beryllium. Zinc was found to be necessary for the normal development of tomato root systems (Eltinge and Reed, 1940), and without Cu, roots ceased to grow (Glasstone, 1947). Hannay and Street (1954) showed that Mo and Mn were also essential for root growth.

An advantage adding five micronutrients to tissue culture media was perhaps first well demonstrated by Heller in 1953 who found that carrot callus could be maintained for an increased number of passages when Fe, B, Mn, Zn and Cu were present.

3.2. MICRONUTRIENTS FROM TRACE IMPURITIES

Micronutrients tend to be added to modern media by the addition of fairly standard chemicals. Street (1977) rightly emphasised that even analytical grade chemicals contain traces of impurities that will provide a hidden supply of micronutrients to a medium. An illustration of this, comes from the work of Dalton *et al.,* (1983) who found traces of silicon (Si) in a precipitate from MS medium which had been made up with analytical grade laboratory chemicals. Gelling agents contain inorganic elements but whether cultures can utilize them is unclear. Amounts of contaminating substances in chemicals would have been greater in times past, so that an early medium such as Knudson (1922; 1943) B, prepared today with highly purified chemicals, will not have quite the same composition as when it was first used by Knudson in 1922; the addition of some micronutrients might improve the results obtained from a present-day formulation of such early media.

3.3. OPTIMUM MICRO-ELEMENT CONCENTRATIONS

Most modern culture media use the microelements of Gamborg *et al.,* (1968) B5 medium, or the more concentrated mixtures in MS or Bourgin and Nitsch (1967) H media. Several research workers have continued to use Heller (1953) micro-nutrient formulation, even though higher levels are now normally recommended. Quoirin and Lepoivre (1977) showed clearly that in conjunction with MS or their Quoirin and Lepoivre (1977) B macro-elements, the concentration of Mn in Heller's salts should be increased by 100-fold to obtain the most effective growth of *Prunus* meristems.

Cell growth and morphogenesis of some species may even be promoted by increasing the level of micronutrients above that recommended by Murashige and Skoog (1962). The induction and maintenance of callus and growth of cell suspensions of juvenile and mature organs of both Douglas fir and loblolly pine, was said to be improved on Litvay *et al.,* (1981) LM medium in which Mg, B, Zn, Mo, Co and I were at 5 times the concentration of MS micro-elements, and Mn and Cu at 1.25 and 20 times respectively (Litvay *et al.,* 1981; Verma *et al.,* 1982). Other authors to have employed high micronutrient levels are Barwale *et al.,* (1986) who found that the induction of adventitious shoots from callus of 54 genotypes of *Glycine max* was assisted by adding four times the normal concentration of minor salts to MS medium.

A further example of where more concentrated micro-elements seemed to promote morphogenesis is provided by the work of Wang, *et al.,* (1980, 1981). Embryogenesis could be induced most effectively in callus derived from *Hevea brasiliensis* anthers, by doubling the concentration of microelements in MS medium, while at the same time reducing the level of macronutrients to 60-80% of the original.

Despite these reports, few research workers seem to have accepted the need for such high micronutrient levels. To diminish the occurrence of hyperhydricity in shoot culture of carnation, Dencso (1987) reduced the level of micronutrients (except iron, which was as recommended by Dalton *et al.,* 1983) to those in MS medium, but this mixture was inadequate for *Gerbera* shoot cultures and the rate of propagation was less than that with the normal MS formulation.

The need for macronutrient concentrations to be optimised as the first step in media development seems to be emphasised by results of Eeuwens (1976). In an experiment with factorial combinations of the macro- and micro-nutrient components of four media, his Eeuwens (1976) Y3 micronutrients gave a considerable improvement in the growth of coconut callus, compared with other micro-element mixtures, when they were used with Y3 and MS macronutrients, but not when used with those of White (1942) or Heller (1953; 1955).

3.4. CELLULAR DIFFERENTIATION AND MORPHOGENESIS

Welander (1977) obtained evidence, which suggested that plant cells are more demanding for minor elements when undergoing morphogenesis. Petiole explants of *Begonia hiemalis* produced callus on media without micronutrients, but would only produce adventitious shoot buds directly when micronutrients were added to the macronutrient formulation. The presence of iron is particularly important for adventitious shoot and root formation (Legrand, 1975).

That mineral nutrition can influence cellular differentiation in combination with growth hormones, was shown by Beasley *et al.,* (1974). Cotton ovules cultured on a basic medium containing 5.0 mM IAA and 0.5 mM GA_3, required 2 mM calcium (normally present in the medium) for the ovules to develop fibres. Magnesium and boron were essential for fibre elongation.

3.5. THE ROLES OF MICRONUTRIENTS

3.5.1. Manganese

The essential micronutrient metals Fe, Mn, Zn, B, Cu, Co and Mo are components of plant cell proteins of metabolic and physiological importance. At least five of these elements are, for instance, necessary for chlorophyll synthesis and chloroplast function (Sundqvist *et al.,* 1980). Micronutrients have roles in the functioning of the genetic apparatus and several are involved with the activity of growth substances.

Manganese (Mn) is one of the most important microelements and has been included in the majority of plant tissue culture media. It is generally added in similar concentrations to those of iron and boron, i.e. between 25-150 mM. Manganese has similar chemical properties to Mg^{2+} and is apparently able to replace magnesium in some enzyme systems (Hewitt, 1948). However there is normally 50- to 100-fold more Mg^{2+} than Mn^{2+} within plant tissues, and so it is unlikely that there is frequent substitution between the two elements.

The most probable role for Mn is in definition of the structure of metalloproteins involved in respiration and photosynthesis (Clarkson and Hanson, 1980). It is known to be required for the activity of several enzymes, which include decarboxylases, dehydrogenases, kinases and oxidases and superoxide dismutase enzymes. Manganese is necessary for the maintenance of chloroplast ultra-structure. Because Mn(II) can be oxidized to Mn(IV), manganese plays an important role in redox reactions. The evolution of oxygen during photosystem II of the photosynthetic process, is dependent on a Mn-containing enzyme and is proportional to Mn content (Mengel and Kirkby, 1982; Shkolnik, 1984). Mn is toxic at high concentration (Sarkar *et al.,* 2004).

In tissue cultures, omission of Mn ions from Doerschug and Miller (1967) medium reduced the number of buds initiated on lettuce cotyledons. A high level of manganese could compensate for the lack of molybdenum in the growth of excised tomato roots (and vice versa) (Hannay and Street, 1954). Natural auxin levels are thought to be reduced in the presence of Mn^{2+} because the activity of IAA-oxidase is increased. This is possibly due to Mn^{2+} or Mn-containing enzymes inactivating oxidase inhibitors, or because manganous ions are one of the cofactors for IAA oxidases in plant cells (Galston and Hillman, 1961). Manganese complexed with EDTA increased the oxidation of naturally-occurring IAA, but not the synthetic auxins NAA or 2,4-D (MacLachlan and Waygood, 1956). However, Chée (1986) has suggested that, at least in blue light, Mn^{2+} tends to cause the maintenance of, or increase in, IAA levels within tissues by inactivating a co-factor of IAA oxidase. When the Mn^{2+} level in MS medium was reduced from 100 mM to 5 mM, the production in blue light, of axillary shoots by *Vitis* shoot cultures was increased.

3.5.2. Zinc

Zinc is a component of stable metallo-enzymes with many diverse functions, making it difficult to predict the unifying chemical property of the element, which is responsible for its essentiality (Clarkson and Hanson, 1980). Zinc is required in more than 300 enzymes including alcohol dehydrogenase, carbonic anhydrase, superoxide dismutase and RNA-polymerase. Zinc forms tetrahedral complexes with N-, O-, and S-ligands. In bacteria, Zn is present in RNA and DNA polymerase enzymes, deficiency resulting in a sharp decrease in RNA levels. DNA polymerase is concerned with the repair of incorrectly formed pieces of DNA in DNA replication, and RNA polymerase locates the point on the DNA genome at which initiation of RNA synthesis is to take place. Divalent Mg^{2+}, Mn^{2+} or Co^{2+} are also required for activation of these enzymes (Eichhorn, 1980).

Zinc deficient plants suffer from reduced enzyme activities and a consequent diminution in protein, nucleic acid and chlorphyll synthesis. Molybdenum- and zinc-deficient plants have a decreased chlorophyll content and poorly developed

chloroplasts. Plants deprived of zinc often have short internodes and small leaves.

The concentration of Zn^{2+} in MS medium is 30 μM but amounts added to culture media have often varied widely between 0.1-70 μM and experimental results to demonstrate the most appropriate level are limited. When Eriksson (1965) added 15 mg/l $Na_2ZnEDTA.2H_2O$ (40μM Zn^{2+})to *Haplopappus gracilis* cell cultures, he obtained a 15% increase in cell dry weight which was thought to be due to the presence of zinc rather than the chelating agent. Zinc was also shown to increase growth of a rice suspension. The highest concentration tested, 520 μM, resulted in the fastest rate of growth and it was suggested that zinc had increased auxin activity (see below) (Hossain *et al.,* 1997). Zinc is required for adventitious root formation in *Eucalyptus* (Schwambach *et al.,* 2005). In cassava, additional zinc promotes somatic embryogenesis and rooting (C.J.J.M Raemakers, pers. commun.). However, very high concentrations of zinc are found to be inhibitory, and the microelement has been noted to prevent root growth at a concentration higher than 50 μM.

There is a close relationship between the zinc nutrition of plants and their auxin content (Skoog, 1940). It has been suggested that zinc is a component of an enzyme concerned with the synthesis of the IAA precursor, tryptophan (Tsui, 1948). The importance of Zn for tryptophan synthesis is especially noticeable in crown gall callus which normally produces sufficient endogenous auxin to maintain growth on a medium without synthetic auxins, but which becomes auxin-deficient and ceases to grow in the absence of Zn (Klein *et al.,* 1962).

3.5.3. Boron

Boron is involved in plasma membrane integrity and functioning, probably by influencing membrane proteins, and cell wall intactness. Reviews have been provided by Lewis (1980) and by Blevins and Lukaszewski (1998). The element is required for the metabolism of phenolic acids, and for lignin biosynthesis: it is probably a component, or co-factor of the enzyme which converts *p*-coumaric acid to caffeate and 5-hydroxyferulate. Boron is necessary for the maintenance of meristematic activity, most likely because it is involved in the synthesis of N-bases (uracil in particular); these are required for RNA synthesis (Mengel and Kirkby, 1982). It is also thought to be involved in the maintenance of membrane structure and function, possibly by stabilizing natural metal chelates which are important in wall and membrane structure and function (Pollard *et al.,* 1977; Clarkson and Hanson, 1980). Boron is concerned with regulating the activities of phenolase enzymes; these bring about the biosynthesis of phenylpropane compounds, which are polymerized to form lignin. Lignin biosynthesis does not take place in the absence of boron. Boron also mediates the action of phytochrome and the response of plants to gravity (Tanada, 1978).

Use in culture media. In the soil, boron occurs in the form of boric acid and it is this compound, which is generally employed as the source of the element in tissue cultures. Uptake of boric acid occurs most readily at acid pH, possibly in the undissociated form (Oertli and Grgurevic, 1974) or as $H_2BO_3^-$ (Devlin, 1975). A wide range of boron concentrations has been used in media, the most usual being between from 50 and 100 μM: MS medium contains 100 μM. Bowen (1979) found boron to be toxic to sugarcane suspensions above 2 mg/l (185 μM), but there are a few reports of higher concentrations being employed (Table 3.8). High concentrations of boron may have a regulatory function; for example, 1.6-6.5 mM have been used in simple media to stimulate pollen germination (Brewbaker and Kwack, 1963; Taylor, 1972).

Boric acid reacts with some organic compounds having two adjacent *cis*-hydroxyl groups (Greenwood, 1973). This includes *o*-diphenols, hexahydric alcohols such as mannitol and sorbitol (commonly used in plant tissue culture as osmotic agents), and several other sugars, but excludes sucrose which forms only a weak association. Once the element is complexed it appears to be unavailable to plants. This led Lewis (1980) to suggest that because boric acid was required for lignin biosynthesis, vascular plants were led, during evolution, to use sucrose exclusively for the transport of carbohydrate reserves.

Although the addition of sugar alcohols and alternative sugars to sucrose can be beneficial during plant tissue culture (see Chapter 4), it is clearly necessary to return to a sucrose-based medium for long-term culture, or boron deficiency may result.

Deficiency symptoms. Boron is thought to promote the destruction of natural auxin and increase its translocation. Endogenous IAA levels increase in the absence of boron and translocation is reduced, the compound probably being retained at the site of synthesis (Goldbach and Amberger, 1986). Plants suffering from boron deficiency have restricted root systems (Odhnoff, 1957; Whittington, 1959) and a

reduced capacity to absorb $H_2PO_4^-$ and some other ions. High levels of auxins can have the same effect on growth and ion uptake (Pollard *et al.,* 1977). Neales (1959, 1964) showed that isolated roots stopped growing unless a minimum concentration of boron was present (although the necessity for the element was not so apparent when cultures were grown in borosilicate glass vessels). Inhibition of root elongation in the absence of boron has been shown to be due to the cessation of mitosis and the inhibition of DNA synthesis (Moore and Hirsch, 1981). Boron deficiency also results in depressed cytokinin synthesis. Cell division is inhibited in the absence of boron, apparently because there is a decrease in nuclear RNA synthesis (Ali and Jarvis, 1988). However, deficiency often leads to increased cambial growth in intact dicotyledonous plants.

Table 3.8 Examples of cultures grown with unusually high concentrations of boron

Plant	Concentration of boron (μM)	Type of Culture	Reference
Antirrhinum majus	323	Embryos from protoplast colonies	Poirier-Hamon *et al.,* (1974)
Brassica napus *Capsella bursa-pastoris*	200	Embryo	Monnier (1976)
Citrus medica	646	Anther callus	Drira and Benbadis (1975)
Hevea brasiliensis	320	Anther: plant regeneration	Chen (1984)
Hordeum crosses	242	Embryo rescue	Jensen (1974)
Larix deciduas	250	Direct morphogenesis	Bonga (1984))
	250	Callus and embryogenesis	Nagmani and Bonga (1985)
Lycopersicon esculentum	242	Isolated root	Street and McGregor (1952)
Nicotiana tabacum	323	Protoplant culture	Ohyama and Nitsch (1972)
Petunia hybrida	566	Callus and root formation	Sangwan and Norreel (1975)
Prunus amygdalus	200	Shoot	Hisjima (1982a)

One of the changes seen in some plants grown under boron deficiency is the outgrowth of lateral buds resulting in plants with a bushy or rosette appearance. In pea, this was associated with a sharp decrease in IAA-export from the apex (Li *et al.,* 2001). It is generally accepted that the outgrowth of lateral buds is inhibited by polar auxin transport in the stem and that disruption of this transport by decapitation or auxin transport inhibitors results in the outgrowth of lateral buds (Tamas, 1987).

Cotton ovules which otherwise develop fibres when cultured, commence extensive callus formation when placed on a medium deficient in boron. On the other hand, the growth rate of callus cultures of *Helianthus annuus* and *Daucus carota* (Krosing, 1978), and cell cultures of sugar cane (Bowen, 1979), was much reduced when boron was not present in the growth medium. Boron influences the development of the suspensor of somatic embryos in *Larix deciduas* (Behrendt and Zoglauer, 1996). Boron had no influence on the induction of embryogenesis in *Daucus carota* but altered the development of embryos: root development was promoted at low concentrations and shoot development at high. This coincided with a high and low auxin-cytokinin ratio, respectively (Mashayekhi and Neumann, 2006).

Adventitious root formation. Boron is thought to promote the destruction of auxin. Although auxin is required for the formation of adventitious root initials, boron is necessary in light grown-plants for the growth of these primordia (Middleton *et al.,* 1978); possibly boron enhances the destruction of auxin in these circumstances, which in high concentrations is inhibitory to root growth (Jarvis, 1986). An interaction between boron and auxins in the rooting of cuttings has been noticed in several species (Hemberg, 1951; Weiser, 1959; Weiser and Blaney, 1960; Bowen *et al.,* 1975; Josten and Kutschera, 1999) and a supply of exogenous borate has been shown to be essential (Ali and Jarvis, 1988). However, excessive boron concentrations lead to a reduction in the number of roots formed (Jarvis, 1986). Boron deficiency had no observed effect

on the rooting of *Eucalyptus* microcuttings (Schwambach *et al.,* 2005).

3.6. COPPER AND MOLYBDENUM

Copper is an essential micronutrient, even though plants normally contain only a few parts per million of the element. Two kinds of copper ions exist; they are the monovalent cuprous [Cu(I)] ion, and the divalent cupric [Cu(II)] ion: the former is easily oxidized to the latter; the latter is easily reduced. The element becomes attached to enzymes, many of which bind to, and react with oxygen. They include the cytochrome oxidase enzyme system, responsible for oxidative respiration, and superoxide dismutase (an enzyme which contains both copper and zinc atoms). Detrimental superoxide radicals, which are formed from molecular oxygen during electron transfer reactions, are reacted by superoxide dismutase and thereby converted to water. Copper atoms occur in plastocyanin, a pigment participating in electron transfer.

Several copper-dependent enzymes are involved in the oxidation and hydroxylation of phenolic compounds, such as ABA and dopamine (Lerch, 1981). The hydroxylation of monophenols by copper-containing enzymes leads to the construction of important polymeric constituents of plants, such as lignin. These same enzymes can lead to the blackening of freshly isolated explants. Copper is a constituent of ascorbic acid oxidase and the characteristic growth regulatory effects of ethylene are thought to depend on its metabolism by an enzyme, which contains copper atoms.

High concentrations of copper can be toxic. Most culture media include ca. 0.1-1.0 μM Cu^{2+}. Ions are usually added through copper sulphate, although occasionally cupric chloride or cupric nitrate have been employed. In hydroponic culture of *Trifolium pratense*, uptake of copper into the plant depended on the amount of nitrate in solution. Uptake was considerably reduced when NO_3^- was depleted (Jarvis, 1984). The concentration of Cu in tissue culture media is very small relative to the level in plants (Table 3.1). It is therefore not surprising that various authors report strong increases of growth when Cu is added at 1- 5 μM (Dahleen, 1995; Nirwan and Kothari, 2003; Kintzios *et al.,* 2001; Nas and Read, 2004; Bouman and Tiekstra, 2005)

Plants utilise hexavalent molybdenum and absorb the element as the molybdate ion (MoO_4^{2-}). This is normally added to culture media as sodium molybdate at concentrations up to 1 mM. Considerably higher levels have occasionally been introduced [e.g. in the media of Abou-Mandour (1977) and Asahira and Kano (1977)] apparently without adverse effect, although Teasdale (1987) found pine suspension cultures were injured by 50 mM. Molybdenum is a component of several plant enzymes, two being nitrate reductase and nitrogenase, in which it is a cofactor together with iron: it is therefore essential for nitrogen utilisation. Tissues and organs presented with NO_3^- in a molybdenum-deficient medium can show symptoms of nitrate toxicity because the ion is not reduced to ammonia.

3.7. COBALT

Cobalt is not regarded as an essential element. Nevertheless, it was found to have been included in approximately half of a large sample of published plant culture media (George, *et al.,* 1987). Murashige and Skoog (1962) included Co in their medium because it had been shown to be required by lower plants (Holm-Hansen *et al.,* 1954) and that it might have a role in regulating morphogenesis in higher plants (Miller, 1954; Salisbury, 1959). However, no stimulatory effect on the growth of tobacco callus was observed by adding cobalt chloride to the medium at several concentrations from 0.1 μM and above, and at 80.0 and 160 μM the compound was toxic. Similarly Schenk and Hildebrandt (1972) obtained no clear evidence for a Co requirement in tests on a wide variety of plants, but retained the element in their medium because they occasionally observed an apparent stimulation to the callus growth of some monocotyledons. *Pinus* suspension cultures do not require cobalt (Teasdale, 1987). The concentration most commonly added to a medium is ca. 0.1 μM, although ten times this amount has sometimes been used. Cobalt is the metal component of Vitamin B12 which is concerned with nucleic acid synthesis (Fries, 1962), but evidence that the element has any marked stimulatory effect on growth or morphogenesis in plant tissue cultures is hard to find. Cobalt may replace nickel in urease and thereby render it inactive, e.g., in potato (Witte *et al.,* 2002).

Advantage from adding cobalt to plant culture media might be derived from the fact that the element can have a protective action against metal chelate toxicity and it is able to inhibit oxidative reactions catalysed by ions of copper and iron (Albert, 1958). The Co^{2+} ion can inhibit ethylene synthesis.

3.8. ALUMINIUM AND NICKEL

Several workers, following Heller (1953), have included aluminium and nickel in their micronutrient formulations. However, the general benefit of adding the former metal does not seem to have been adequately demonstrated.

It was believed that in most plants Ni^{2+} is not absolutely required for normal growth and development (Mishra and Kar, 1975). However, more recently, it has been found by careful experimentation that nickel is essential (Gerendás *et al.,* 1999). The ion is a component of urease enzymes (Dixon *et al.,* 1975; Polacco, 1977a), which convert urea to ammonia. It has been shown to be an essential micronutrient for some legumes and to actvate urease in potato microshoot cultures (Witte et al., 2002). In tissue cultures the presence of 0.1 mM Ni^{2+} strongly stimulates the growth of soybean cells in a medium containing only urea as a nitrogen source. Slow growth occurs on urea without the deliberate addition of nickel, possibly supported by trace amounts of the element remaining in the cells (Polacco, 1977b). Cells and tissues are not normally grown with urea as a nitrogen source, and as urease is the only enzyme, which has been shown to have a nickel component, it could be argued that nickel is not essential. However, without it soybean plants grown hydroponically, accumulate toxic concentrations of urea (2.5%) in necrotic lesions on their leaf tips, whether supplied with inorganic nitrogen, or with nitrogen compounds obtained from bacterial symbiotic nitrogen fixation. These symptoms can be alleviated in plants growing in hydroponic culture by adding 1 mg/l Ni to the nutrient solution. Absence of nickel in a hydroponic solution also results in reduced early growth and delayed nodulation (Eskew *et al.,* 1983).

Despite these findings nickel has not been added deliberately to tissue culture media. However, it should be noted that agar contains relatively high levels of nickel (Table 3.2) and the possibility of urea toxicity may have been avoided because, in tissue cultures, urea diffuses into the medium (Teasdale, 1987). Quoirin and Lepoivre (1977) showed that at the concentrations recommended by Heller, Al^{3+} and Ni^{2+} were without effect on the growth of *Prunus* meristems and were inhibitory at higher levels. If it is thought that Ni should be added to a culture medium, 0.1 mM is probably sufficient.

Aluminium has been said to be necessary for the growth of some ferns (Taubck, 1942), but is not generally added to tissue culture media for fern propagation.

3.9. IODINE

Iodine is not recognised as an essential element for the nutrition of plants (Rains, 1976), although it may be necessary for the growth of some algae, and small amounts do accumulate in higher plants (ca.12 and 3 mol/kg dry weight in terrestrial and aquatic plants respectively – Raven, 1986). However, the iodide ion has been added to many tissue culture media (e.g. to 65% of micronutrient formulations).

The practice of including iodide in plant culture media began with the report by White (1938) that it improved the growth of tomato roots cultured *in vitro*. Hannay (1956) obtained similar results and found that root growth declined in the absence of iodine which could be supplied not only from potassium iodide, but also from iodoacetate or methylene iodide, compounds which would only provide iodide ions very slowly in solution by hydrolysis. Street (1966) thought that these results indicated that iodine could be an essential nutrient element, but an alternative hypothesis is that any beneficial effect may result from the ability of iodide ions to act as a reducing agent (George *et al.,* 1988). Oxidants convert iodide ions to free iodine. Eeuwens (1976) introduced potassium iodide into his Y3 medium at 0.05 mM (ten times the level used by Murashige and Skoog), as it prevented the browning of coconut palm tissue cultures. The presence of 0.06 µM potassium iodide slightly improved the survival and growth of cultured *Prunus* meristems (Quoirin and Lepoivre, 1977).

Although Gautheret (1942) and White (1943) had recommended the addition of iodine to media for callus culture, Hildebrandt *et al.,* (1946) obtained no statistically significant benefit from adding potassium iodide to tumour callus cultures of tobacco and sunflower. However, as the average weight of tobacco callus was 11% less without it, the compound was included (at different levels) in both of the media they devised. Once again iodine also had no appreciable effect on tobacco callus yield in the experiments of Murashige and Skoog (1962), but was nevertheless included in their final medium. Other workers have omitted iodine from MS medium (e.g. Roest and Bokelmann, 1975; Périnet *et al.,* 1988; Gamborg, 1991) or from new media formulations without any apparent ill effects. However, Teasdale *et al.,* (1986); Teasdale, (1987) reported a definite requirement of *Pinus taeda* suspensions for 25 mM

KI when they were grown on Litvay *et al.,* (1981) LM medium.

There seems, at least in some plants, to be an interaction between iodine and light. Eriksson (1965) left KI out of his modification of MS medium, finding that it was toxic to *Haplopappus gracilis* cells cultured in darkness: shoot production in *Vitis* shoot cultures kept in blue light was reduced when iodine was present in the medium (Chée, 1986), but the growth of roots on rooted shoots was increased. Chée thought that these results supported the hypothesis that iodine enhanced the destruction and/or the lateral transport of IAA auxin. This seems to be inconsistent with the suggestion that I^- acts mainly as a reducing agent.

3.10. SILICON

Silicon (Si) is the second most abundant element on the surface of the earth. Si has been demonstrated to be beneficial for the growth of plants and to alleviate biotic and abiotic stress (Epstein, 1971). The silicate ion is not normally added to tissue culture media, although it is likely to be present in low concentrations. Deliberate addition to the medium might, however, improve the growth of some plants. Adatia and Besford (1986) found that cucumber plants depleted silicate from a hydroponic solution and in consequence their leaves were more rigid, had a higher fresh weight per unit area and a higher chlorophyll content than the controls. The resistance of the plants to powdery mildew was also much increased.

3.11. IRON

Chelating agents. Some organic compounds are capable of forming complexes with metal cations, in which the metal is held with fairly tight chemical bonds. The complexes formed may be linear or ring-shaped, in which case the complex is called a chelate (from the Greek word meaning a crab's claw). Metals can be bound (or sequestered) by a chelating agent and held in solution under conditions where free ions would react with anions to form insoluble compounds, and some complexes can be more chemically reactive than the metals themselves. For example, Cu^{2+} complexed with amino acids is more active biologically than the free ion (Cruickshank *et al.,* 1987). Chelating agents vary in their sequestering capacity (or avidity) according to chemical structure and their degree of ionisation, which changes with the pH of the solution. Copper is chelated by amino acids at relatively high pH, but in conditions of greater acidity, it is more liable to be complexed with organic acid ligands (White *et al.,* 1981). The higher the stability of a complex, the higher the avidity of the complexing agent. One, and in many cases, two or three molecules of a complexing agent may associate with one metal ion, depending on its valency.

Despite tight bonding, there is always an equilibrium between different chelate complexes and between ions in solution. Complexing agents also associate with some metal ions more readily than with others. In general Fe^{3+} (for agents able to complex with trivalent ions) complexes have a higher stability than those of Cu^{2+}, then (in descending order), Ni^{2+}, Al^{3+} (where possible), Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Ca^{2+} (Albert, 1958; Reilley and Schmid, 1958). For a chelated metal ion to be utilised by a plant there must be some mechanism whereby the complex can be broken. This could occur if it is absorbed directly and the ion displaced by another more avid binding agent, or if the complex is biochemically denatured. Metals in very stable complexes can be unavailable to plants; copper in EDTA chelates may be an example (Coombes *et al.,* 1977). High concentrations of avid chelating agents are phytotoxic, probably because they competitively withdraw essential elements from enzymes.

Naturally-occurring compounds act as chelating agents. Within the plant very many constituents such as proteins, peptides, porphyrins, carboxylic acids and amino acids have this property (Albert, 1958; Martin, 1979): some of those with high avidity are metal-containing enzymes. Amino acids are able to complex with divalent metals (Fig. 3.5). Grasses are thought to secrete a chelating agent from their roots to assist the uptake of iron (Römheld and Marschner, 1986). There are also synthetic chelating agents with high avidities (stability constants) for divalent and trivalent ions. Some are listed in Table 3.9, and the structure of those most commonly used in plant culture media is illustrated in Fig 3.6. The application of synthetic chelating agents and chelated micronutrients to the roots of some plants growing in alkaline soils can improve growth by supplying essential metals such as iron and zinc which are otherwise unavailable. The addition of such compounds to tissue culture media can help to make macro- and micro-nutrients more accessible to plant cells.

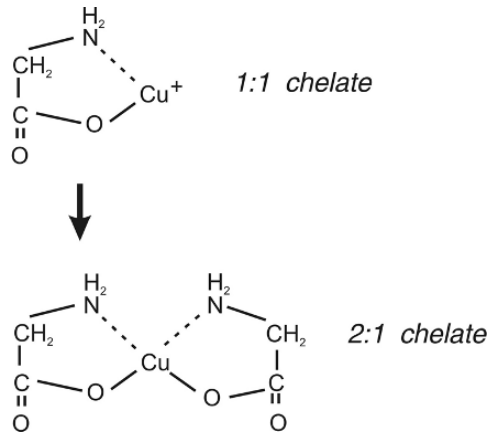


Fig. 3.5 Copper chelated with amino acid, glycine.

Iron chelates. A key property of iron is its capacity to be oxidized easily from the ferrous [Fe(II)] to the ferric [Fe(III)] state, and for ferric compounds to be readily reduced back to the ferrous form. In plants, iron is primarily used in the chloroplasts, mitochondria and peroxisomes of plants for effecting oxidation/reduction (redox) reactions. The element is required for the formation of amino laevulinic acid and protoporphyrinogen (which are respectively early and late precursors of chlorophyll) and deficiency leads to marked leaf chlorosis. Iron is also a component of ferredoxin proteins, which function as electron carriers in photosynthesis.

Iron is therefore an essential micronutrient for plant tissue culture media and can be provided from either ferrous or ferric salts. In early experiments, ferrous sulphate or ferric citrate or tartrate were used in media as a source of the element. Citric and and tartaric acids can act as chelating agents for some divalent metals (Bobtelsky and Jordan, 1945), but are not very efficient at keeping iron in solution (Fig 3.6). If Fe^{2+} and Fe^{3+} ions escape from the chelating agent, they are liable to be precipitated as iron phosphate. The iron may then not be available to plant cells, unless the pH of the medium falls sufficiently to bring free ions back into solution. The problem of precipitation is more severe in aerated media and where the pH of the medium drifts towards alkalinity. Under these conditions Fe^{2+} (ferrous) ions are oxidized to Fe^{3+} (ferric) ions and unchelated ferric ions may then also be converted to insoluble $Fe(OH)_3$. For plant hydroponic culture, the advantages of adding iron to nutrient solutions in the form of a chelate with EDTA was first recognised in the 1950's (Jacobson, 1951; Weinstein *et al.,* 1951). Street *et al.,* (1952) soon found that iron in this form was less toxic and could be utilised by *in vitro* cultures of isolated tomato roots over a wider pH range than ferric citrate. Klein and Manos (1960) showed that callus cultures of several species grew more rapidly on White (1954) medium if Fe^{3+} ions from $Fe_2(SO_4)_3$ were chelated with EDTA, rather than added to the medium from the pure compound, and Doerschug and Miller (1967), that 0.036 mM Fe from NaFeEDTA was as effective as 0.067 mM Fe as ferric citrate, in promoting shoot bud initiation on lettuce cotyledons. Iron presented as ferric sulphate (0.025 mM Fe) was much less effective than either chelated form.

Skoog and co-workers began to use EDTA in media for tobacco callus cultures in 1956 and discussed their findings in the same paper that describes MS medium (Murashige and Skoog, 1962). The addition of an iron (Fe)-EDTA chelate once again greatly improved the availability of the element. Following this publication, (Fe)-EDTA complexes were rapidly recognised to give generally improved growth of all types of plant cultures (Nitsch, 1969). EDTA has now become almost a standard medium component and is generally preferred to other alternative chelating agents (Table 3.8).

Preparation and use. (Fe)-EDTA chelates for tissue cultures are prepared in either of two ways.

- A ferric or ferrous salt is dissolved in water with EDTA and the solution is heated;
- A ready-prepared salt of iron salt of EDTA is dissolved and heated.

Heating can take place during the preparation of chelate stock solutions, or during the autoclaving of a medium.

The form of iron complexed is invariably Fe(III). If iron has been provided from ferrous salts, it is oxidised during heating in aerated solutions. The rate of oxidation of the ferrous ion is enhanced in some complexes and retarded in others (Albert, 1958). That of Fe^{2+}-EDTA is extremely rapid (Kolthoff and Auerbach (1952). Only a small proportion of Fe^{2+} is likely to remain: its chelate with EDTA is much less stable than the Fe(III) complex. Iron is however thought to be absorbed into plants in the ferrous form. Uptake of iron from EDTA probably occurs when molecules of Fe(III)-chelate bind to the outer plasma membrane (the plasmalemma) of the cytoplasm, where Fe(III) is reduced to Fe(II) and freed from the chelate (Chaney et al., 1972; Römheld and Marschner, 1983).

In most recent plant tissue culture work, EDTA has been added to media at an equimolar concentration with iron, where it will theoretically form a chelate with all the iron in solution. However, it has been found in practice that the Fe(III)-EDTA chelate, although stable at pH 2-3, is liable to lose some of its bound iron in culture media at higher pH levels; the displaced iron may form insoluble ferric hydroxides and iron phosphate (Dalton *et al.,* 1983). If this occurs, free EDTA will tend to form chelates with other metal ions in solution. Some micronutrients complexed with EDTA may then not be available to the plant tissues. Re-complexing may also happen if the EDTA to Fe ratio is increased by decreasing the amount of iron added to the medium (as has been proposed to solve the precipitation problem, see Chapter 4). It is not possible to add very much more than 0.1 mM EDTA to culture media because the chelating agent can become toxic to some plants (see below).

Hill-Cottingham and Lloyd-Jones (1961) showed that tomato plants absorbed iron from FeEDTA more rapidly than they absorbed EDTA itself, but concluded that both Fe and Fe-chelate were probably taken up. They postulated that EDTA liberated by the absorption of Fe, would chelate other metals in the nutrient solution in the order given at the beginning of Section 3.6.. Teasdale (1987) calculated that in many media, nearly all the copper and zinc, and some manganese ions might be secondarily chelated, but it is unclear whether micronutrients in this form are freely available to plant tissues. One presumes they are, for deficiency symptoms are not reported from *in vitro* cultures.

Ambiguous descriptions. In many early papers on plant tissue culture, the authors of scientific papers have failed to describe which form of EDTA was used in experiments, or have ascribed weights to EDTA, which should refer to its hydrated sodium salts. Singh and Krikorian (1980) drew attention to this lack of precision. They assumed that in papers where Na_2EDTA is described as a medium constituent, it indicates the use of the anhydrous salt (which would give 11 mol/l excess of EDTA to iron, with unknown consequences). However, the disodium salt of EDTA is generally made as the dihydrate (Beilstein's Handbuch der Organischen Chemie) and this is the form which will almost invariably have been used, Na_2EDTA merely being a shorthand way of indicating the hydrated salt without being intended as a precise chemical formula.

Further confusion has arisen through workers using ready-prepared iron-EDTA salts in media without specifying the weight or molar concentration of actual Fe used. Mono-, di-, tri-, and tetra-sodium salts of EDTA are possible, each with different (and sometimes alternative) hydrates, so that when a research report states only that a certain weight of 'FeEDTA' was used, it is impossible to calculate the concentration of iron that was employed with any certainty.

The compound 'monosodium ferric EDTA' with the formula NaFeEDTA (no water of hydration) exists, and is nowadays commonly selected as a source of chelated iron. However in some papers 'NaEDTA' has been used as an abbreviation for some other form of iron-EDTA salt. For example the paper of Eeuwens (1976) describing Y3 medium, says that to incorporate 0.05 mM iron, 32.5 mg/l 'sodium ferric EDTA' was used. The weight required using a compound with the strict molecular formula NaFeEDTA would be 18.35 mg/l. Hackett (1970) employed '$Na_4FeEDTA$'. Gamborg and Shyluk (1981) and Gamborg (1982) said that to prepare B5 or MS medium with 0.1 mM Fe, 43 mg/l of 'ferric EDTA' or 'Fe-versenate' (EDTA) should be weighed. The compound recommended in these papers was probably the $Na_2FeEDTA.2H_2O$ chelate (theoretical mol. Wt. 428.2) as was the 'FeEDTA' (13% iron) employed by Davis *et al.,* (1977). It should be noted that NaFeEDTA is the only source of Na in MS medium apart from the contamination in the gelling agents.

Alternatives to EDTA. A few other chelating agents have been used in culture media in place of EDTA. The B5 medium of Gamborg *et al.,* (1968) was originally formulated with 28 mg/l of the iron chelate 'Sequestrene 330 Fe'. According to Heberle-Bors (1980), 'Sequestrene 330 Fe' is FeDTPA (Table 3.9), containing 10% iron (Anon, 1978). This means that the concentration of Fe in B5 medium was originally 0.05 mM. Gamborg and Shyluk (1981) have proposed more recently that the level of Fe should be increased to 0.1 mM. B5 medium is now often used with 0.1 mM FeEDTA, but some researchers still prefer FeDTPA, for example (Garton and Moses, 1986) used it in place of FeEDTA in Lloyd and McCown (1981) WPM medium for shoot culture of several woody plants.

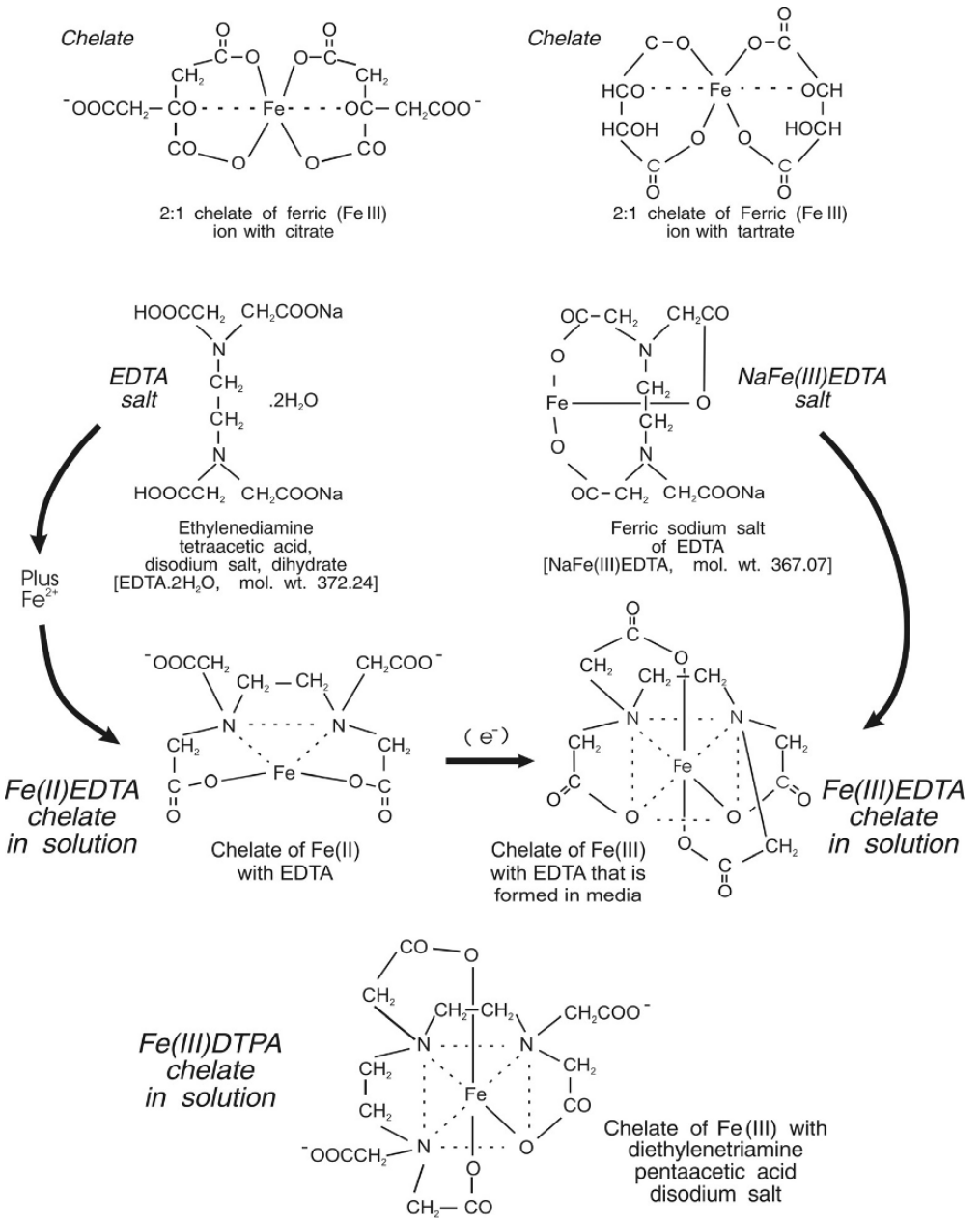


Fig. 3.6 Chemical structures of some chelating agents and iron chelates.

Growth regulatory effects of chelating agents. Although most iron, previously complexed to chelating agents such as EDTA, EDDHA and DTPA (Table 3.9) is absorbed as uncomplexed ions by plant roots, there is evidence that the chelating agents themselves can be taken up into plant tissues (Weinstein *et al.,* 1951; Tiffin *et al.,* 1960; Tiffin and Brown, 1961). Chelating compounds such as EDTA, in low concentrations, exert growth effects on plants, which are similar to those produced by auxins. The effects include elongation of oat coleoptiles (Heath and Clark, 1956a.b), and etiolated lupin hypocotyls (Weinstein *et al.,* 1956), the promotion of leaf epinasty (Weinstein *et al.,* 1956) and the inhibition of root growth (Burstrom, 1961, 1963). Hypotheses put

forward to explain these observations have included that:

- chelating agents act as auxin synergists by sequestering Ca from the cell wall (Thimann and Takahashi, 1958);
- the biological properties of the natural auxin IAA may be related to an ability to chelate ions; other chelating agents therefore mimic its action (Heath and Clark, 1960).

Burstrom (1960) noted that EDTA inhibited root growth in darkness (not in light) but that the growth inhibition could be overcome by addition of Fe^{3+} or several other metal ions (Burstrom, 1961). He recognised that reversal of EDTA action by a metal does not mean that the metal is physiologically active but that it might only release another cation, which had previously been made unavailable to the tissue by chelation.

Effects in tissue culture. Growth and morphogenesis in tissue cultures have been noted on several occasions to be influenced by chelating agents other than EDTA. It has not always been clear whether the observed effects were caused by the chelation of metal ions, or by the chelating agent *per se*.

The growth rate of potato shoot tips was increased by 0.01-0.3 mg/l 8-hydroxyquinoline (8-HQ) when cultured on a medium which also contained EDTA (Goodwin, 1966), and more callus cultures of a haploid tobacco variety formed shoots in the absence of growth regulators when DHPTA was added to Kasperbauer and Reinert (1967) medium which normally contains 22.4 mg/l EDTA. The DHPTA appears to have been used in addition to the EDTA, not as a replacement, and was not effective on callus of a diploid tobacco (Kochhar *et al.,* 1970). In the same experimental system, Fe-DHPTA and Fe-EDDHA were more effective in promoting shoot formation from the haploid-derived tissue than Fe with CDTA, citric acid or tartaric acid (Kochhar *et al.,* 1971).

The inclusion of EDTA into a liquid nutrient medium caused the small aquatic plant *Lemna perpusilla* to flower only in short day conditions whereas normally the plants were day-neutral (Hillman, 1959, 1961). In the related species *Wolffia microscopica*, plants did not flower unless EDTA was present in the medium, and then did so in response to short days (Maheshwari and Chauhan, 1963). When, however, Maheshwari and Seth (1966) substituted Fe-EDDHA for EDTA and ferric citrate, they found that plants not only flowered more freely under short days, but also did so under long days. The physiological effect of EDTA and EDDHA as chelating agents was thus clearly different. This was again shown by Chopra and Rashid (1969) who found that the moss *Anoectangium thomsonii* did not form buds as other mosses do, when grown on a simple medium containing ferric citrate or Fe-EDTA, but did so when 5-20 mg/l Fe-EDDHA was added to the medium instead. An optimum concentration was between 5 and 8 mg/l. Rashid also discovered that haploid embryoids developed more freely from *in vitro* cultures of *Atropa belladonna* pollen microspores when Fe-EDDHA was incorporated into the medium, rather than Fe-EDTA (Rashid and Street, 1973). Heberle-Bors (1980) did not obtain the same result, and found that FeEDTA was superior to FeEDDHA for the production of pollen plants from anthers of this species and of two Nicotianas. In tobacco, the production of haploid plants was greatest with FeEDTA, next best with FeDTPA, FeEGTA, FeEDDHA, and poorest with Fe citrate. Each complex was tested at or about the same iron concentration. Heberle-Bors also showed that chelating agents are differentially absorbed by activated charcoal (see Chapter 7). In tissue culture of rose (Van Der Salm, 1994), *Prunus* (Mallosiotis *et al.,* 2003), citrus (Dimassi *et al.,* 2003) and red raspberry (Zawadzka and Orlikowska, 2006), it is advantageous to use FeEDDHA rather than FeEDTA.

Toxicity caused by chelating agents. Although low concentrations of EDTA markedly stimulate the growth of whole plants in hydroponic cultures by making iron more readily available, the compound begins to be toxic at higher levels. By comparisons

Table 3.9 Some common chelating agents.

EDTA	Etyhylenediaminetetraaceticacid
EGTA	Ethyleneglycol-bis(2-aminoethylether)tetraaceticacid
EDDHA	Etyhylenediamine-di(*o*-hydroxyphenyl)acetic acid
DTPA	Dietyhylenetriaminepentaacetic acid
DHPT	1,3-diamino-2-hydroxypropane-tetraaceticacid

with observations on animal tissues, Weinstein *et al.,* (1951) suggested that toxicity arose through competition between EDTA and enzymes (and other physiologically-active complexes) in the plant, for metals essential to their activity. This will occur if the avidity of the chelating agent is greater than the metal binding capacity of proteins on the surface of cells (Albert, 1958).

Toxicity can also occur in *in vitro* cultures. Legrand (1975) found that an optimum rate of adventitious shoot initiation occurred in endive leaf segments when only 7.5 mg/l EDTA (one fifth the concentration used in MS medium) was employed. In these circumstances, higher levels of EDTA were clearly inhibitory and more than 55 mg/l prevented shoot formation. Dalton *et al.,* (1983) found that 0.3 mM EDTA (compared to the 0.1 mM in MS medium) reduced the growth rate of *Ocimum* cell suspensions.

Flower buds of *Begonia franconis* died within a few days if cultured with a high level of FeEDTA (1-1.5 mM, i.e. 10-15 times the normal level) together with 0.4-1.6 mM $H_2PO_4^-$. Berghoef and Bruinsma (1979a) thought that Fe^{3+} released from the FeEDTA complex, had precipitated the phosphate. Necrosis was avoided by increasing $H_2PO_4^-$ concentration to 6.4 mM.

Tissues may be damaged by culture in media containing synthetic chelating agents where the pH approaches neutrality, because at these pH levels, EDTA and EGTA have been shown to remove calcium ions from the membranes of mitochondria and this inhibits NAD(P)H oxidation and respiration (Moller and Palmer, 1981). Chelating agents have been found to inhibit the action of the growth substance ethylene (see Chapter 7) and are thought to do so by sequestering Cu ions within plant tissues, thereby interfering with the synthesis or action of a Cu-containing enzyme responsible for ethylene metabolism. EDTA can also inhibit the activity of plant polyphenol oxidase enzymes *in vitro* (Weinstein *et al.,* 1951) and Smith (1968) thought that this might occur because EDTA made Cu ions less available for enzyme incorporation, when he found the chelating agent was able to prevent the blackening of freshly-isolated *Carex flacca* shoot tips. Several oxidative reactions are also biochemically catalysed by ions such as Cu^{2+}, Co^{2+} and Zn^{2+}, and where this is the case [e.g. the oxidation of glutathione – Martin (1979); catechol amine oxidation – Kiss and Gergely (1979)], chelating agents such as EDTA and CDTA are inhibitory.

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